Lipopolysaccharide (LPS) core biosynthesis in *Proteus mirabilis*

Estudio de la biosíntesis del núcleo de lipopolisacarido (LPS) en *Proteus mirabilis*

Eleonora Aquilini

Departamento de Microbiología y Parasitología Clínica

Facultad de Farmacia

Universidad de Barcelona

Lipopolysaccharide (LPS) core biosynthesis in *Proteus mirabilis*
Estudio de la biosíntesis del núcleo de lipopolisacarido (LPS) en *Proteus mirabilis*

Memoria presentada por Eleonora Aquilini

para optar al grado de Doctora por la Universidad de Barcelona

Programa de Doctorado

Microbiología Ambiental y Biotecnología

VºBº del director   La doctoranda

Dr. Miguel Regué Queralt   Eleonora Aquilini

Barcelona, Septiembre 2012

Por encima de todo, me gustaría expresar el más sincero y profundo agradecimiento a mi director de tesis, Dr. Miguel Regué Queralt. Quiero darle las gracias por creer en mí desde el principio, cuando tener una pipeta en la mano no me resultaba tan natural y las estructuras químicas me parecían incomprensible jeroglíficos. Gracias por haberme dado no sólo la inmensa oportunidad, sino también los espacios y las herramientas para crecer como investigadora y científica. Quiero ademas agradecerle por haberme guiado paso a paso sin oprimir, por la constante y inconmensurable ayuda, por inspirarme con su pasión sin igual para la investigación, por ser paciente y con visión de futuro, y por último, por compartir conmigo su inmenso conocimiento en todos los momentos de investigación y de escritura de esta tesis.

Un agradecimiento muy sentido a todos y cada uno de los estudiantes, doctorandos y profesores que participan a la vida diaria del laboratorio de Microbiología de la Facultad de Farmacia, es un placer por la mañana entrar por la puerta del Departamento y andar por nuestro largo pasillo. Gracias por hacer que sea un lugar agradable donde investigar.

Algunas personas son irremplazables en un laboratorio, y es gracias a ellas que es un lugar ordenado, limpio y acogedor donde llegar. Gracias a Lucía para cuidar todos los aspectos burocráticos con la amabilidad, la precisión y la eficiencia que sólo ella sabe poner en ello. Gracias a M. Jesús y Carmen por el apoyo técnico inestimable, por la paciencia infinita en hacer más fácil y más rápido el trabajo de los demás, y sin duda... por saber siempre dónde está todo!

Quiero agradecer el Dr. Juan Tomas por haberme dado la oportunidad de colaborar con su grupo de investigación, por aportar a este trabajo visión critica y intuición científica, por toda la ayuda a este proyecto y por la confianza en mi trabajo. Un sincero agradecimiento a la Dra. Susana Merino por su gran disponibilidad y los tremendamente valiosos consejos experimentales. Gracias en fin a todo el laboratorio 7 del Departamento de Microbiología de la Facultad de Biología: en particular gracias a Silvia y Natalia por ayudarme a dar los primeros pasos en el mágico mundo de la biología molecular y a Maite por el precioso soporte técnico.

I would like to express my heartfelt gratitude to Professor Herry Mobley and to all members of the Mobley Research Group at University of Michigan. I am grateful for the chance to visit your lab and proud to have been part of your group. Thank you for your friendly welcome and for helping me to develop and realize part of the ideas of this thesis. Thanks

"Happiness only real when shared"
Christopher McCandless

1.Introduction

GENUS *PROTEUS*

Taxonomy

Proteus belongs to the *Enterobacteriaceae* family within the Proteobacteria. They are Gram-negative rods with peritrichous flagella, polymorphic, with a diameter ranging between 0.4 – 0.8 μm, and characterized by rapid motility and by production of the urease enzyme. The genus *Proteus* currently consists of five named species (*P. mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxofaciens* and *P. hauseri*) and three unnamed genomospecies (*Proteus* genomospecies 4, 5, and 6). Proteus from Homer's *Odyssey* was pursued by mortals and gods alike for his ability to foretell the future, but he evaded pursuers by taking the shape of animals, plants, water, or even fire. The term Proteus therefore refers to readily changing appearance: "and has the gift of endless transformation" [Wenner and Rettger, 1919]. The genus originally had two species: *P. mirabilis* and *P. vulgaris*, both first described by Hauser in 1885 [Hauser, 1885]. He noted the swarming nature of the organisms and divided the strains into the two species based on the speed of their ability to liquify gelatin: *P. vulgaris* liquefies gelatin "rapidly," and *P. mirabilis* does so "more slowly".

In 1919, Wenner and Rettger were studying the biochemical characteristics of a larger group of *Proteus* strains [Wenner and Rettger, 1919]. Consistent with Hauser's work of 1885, their strains also swarmed and liquefied gelatin. All of these strains also produced hydrogen sulfide and were lactose non fermenters. The ability to ferment glucose, sucrose, and maltose served as a means to further subdivide the strains into two groups, as Hauser had done. *P. vulgaris* fermented glucose, sucrose, and maltose readily, while *P. mirabilis* fermented glucose readily and sucrose slowly and did not ferment maltose.

In 1966, Cosenza and Podgwaite described another new species, *P. myxofaciens*, that they isolated from the larvae of the gypsy moth [Cosenza and Podgwaite, 1966]. It produces slime and is not known to have been isolated from a human. The name derives from "myxo" (Greek for slime) and "faciens" (Latin for producing).

Until the early 1960s, bacterial classification had been based primarily on cultural observations and phenotypic analysis. After the advent of techniques such as DNA-DNA hybridization and guanine-plus-cytosine (G+C) determination, which enabled scientists to place new species into their correct genera, biochemical testing remained important but only secondarily to the results of experiments in genetic relationships.

In a 1978 study of 122 strains, Brenner and colleagues, utilizing this technology, showed for the first time the genetic heterogeneity of *P. vulgaris* [Brenner D. J. et al., 1978]. One group of strains was indole, salicin, and esculin negative and was designated *P. vulgaris* biogroup 1. In 1982, Hickman and colleagues proposed that this group be renamed *P. penneri* in honor of John Penner, the Canadian microbiologist who made many contributions to studies of the three genera of *Proteeae* [Hickman et al., 1982].

The mid-1980s saw the advent of high-resolution polyacrylamide gel electrophoresis of proteins with computerized analysis of patterns as another taxonomic tool to identify and type bacteria. In 1993, Costas used this technique to further subdivide *P. vulgaris* into biogroups 2, 3a, and 3b, with the type strain (NCTC 4175/ATCC 13315) being included in biogroup 3a with only one other strain [Costas et al., 1993]. During the same time, O'Hara in United States, in collaboration with Grimont in France, studied many of the same strains by DNA-DNA hybridization, phenotypic characterization, and carbon source utilization. Their results paralleled those of Costas revealed that the type strain of *P. vulgaris* was genetically uncharacteristic of the hundreds of strains considered typical members of *P. vulgaris* [O'Hara et al., 2000]. In 1995, Brenner requested that a neotype strain be designated for *P. vulgaris* [Brenner et al., 1995]. In 1999, this request was approved by the Judicial Commission. The name did not change, but a new phenotypically accurate type strain, ATCC 29905, was designated.

The studies of O'Hara, cited above, confirmed the existence of four genomospecies within biogroup 3, which were called *Proteus* genomospecies 3, 4, 5, and 6. Genomospecies 3 contains only the original type strain of *P. vulgaris* (ATCC 13315) and one other strain. These authors have proposed that genomospecies 3 be named *P. hauseri* and that genomospecies 4, 5, and 6 remain unnamed as *Proteus* genomospecies 4, 5, and 6, respectively [O'Hara et al., 2000]. *P. hauseri* would honor Gustav Hauser, the German microbiologist, who first described the genus.

In the early 1980s, sequencing of 16S rRNA genes with subsequent comparison of these sequences and phylogenetic analysis became another promising taxonomic tool. Now a days 16S rRNA gene sequence analysis is by far the most widely used method for the molecular identification and differentiation of bacterial species [Woese et al., 1990]. However, members of the family *Enterobacteriaceae* have not been subjected to extensive phylogenetic 16S rRNA gene sequence analysis because the high degree of conservation in closely related species leaves many taxonomic problems unresolved. In particular, the genetic relationships between closely related species *P. vulgaris* and *P. penneri* could not be clearly resolved by this method [Cao et al., 2009].

rpoB gene, encoding the RNA polymerase β-subunit, has been proposed as a powerful tool for universal bacterial genetic identification [Adékambi et al., 2009; Mollet et al., 1997]. Giammanco and colleagues used phylogenetic analysis based on *rpoB* gene sequences, to classify the type strain *P. myxofaciens* to a separate genus: *Cosenzaea* gen., as *Cosenzaea myxofaciens,* in honor to Cosenza, the microbiologist who first described this micro-organism as *P. myxofaciens* in 1966 [Giammanco et al., 2011].

A summary of the evolution of the genus *Proteus* is summarized in a timeline in the Table 1 [adapted from O 'Hara et al., 2000].

The respective *Proteus* type strains are listed in Table 2 [O 'Hara et al., 2000].

TABLE 1. Timeline of the genus *Proteus**

Date	Author	Event
1885	Hauser	Described the genus *Proteus* and species *P. mirabilis, P. vulgaris*, and *P. zenkeri*
1919	Wenner and Rettger	Separated *P. vulgaris* and *P. mirabilis* on the basis of sugar fermentations
1966	Cosenza and Podgwaite	Described *P. myxofaciens*
1978	Brenner et al.	Defined *P. vulgaris* biogroup 1
1982	Hickman et al.	Described *P. penneri*; established two additional biogroups of *P. vulgaris*
1993	Costas et al.	Used SDS-PAGE to separate *P. vulgaris* biogroup 2 and further subdivide *P. vulgaris* biogroup 3 into two separate "taxa"
1995	Brenner et al.	Requested replacement of *P. vulgaris* type strain NCTC 4175 with ATCC 29905
2000	O'Hara et al.	Proposed that genomospecies 3 be named *P. hauseri* and that genomospecies 4, 5, and 6 remain unnamed until better phenotypic differentiation
2011	Giammanco et al.	Used phylogenetic analysis of partial nucleotide sequences of the *rpoB* gene to advance the reclassification of *P. myxofaciens* in a new genus, *Cosenzaea*, as *Cosenzaea myxofaciens.*

* adapted from O 'Hara et al., 2000

<p align="center">TABLE 2. Type strains for *Proteus**</p>

Species	Type strain[a]						
	ATCC	CCUG	CDC	CIP	DSM	JCM	NCTC
Proteus hauseri	700826	35386	1732-80				
Proteus mirabilis	29906	26767	PR 14; 9165-79	103181	4479	1669	11938
Proteus myxofaciens	19692	18769	9338-76		4482	1670	
Proteus penneri	33519	15722	1808-73	103030	4544	3948	12737
Proteus vulgaris	29905	35382	9166-79	104989			13145

[a] ATCC, American Type Culture Collection; CCUG, Culture Collection, University of Göteberg, Göteborg, Sweden; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; CIP, Collection d'Institut Pasteur, Paris, France; DSM, Deutsche Sämmlung von Mikroorganismem und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms; NCTC, National Collection of Type Cultures, London, United Kingdom.

* O 'Hara et al., 2000

Genome Sequence

In 2008, a *P. mirabilis* isolate from the urine of a long-term catheterized patient became the first agent of catheter-associated urinary tract infections (CaUTI) to be sequenced. *P. mirabilis* HI4320 has a smaller genome than most *Enterobacteriaceae*, with a 4.06-Mb chromosome containing 3,685 protein coding sequences (CDS). While not all strains of *P. mirabilis* carry plasmids, HI4320 contains one plasmid (pHI4320) with 55 additional CDS that may contribute to virulence. The genome of HI4320 also contains three seemingly complete and three degenerate prophages, a conjugative transposon, and an integrative and conjugative element (ICEPm1). Strain HI4320 also has 18 small noncoding RNAs that may have regulatory roles, including two copies of *ryhB*, known in *Escherichia coli* to down-regulate iron storage and iron-containing proteins under iron-limited conditions [Pearson et al., 2008; Armbruster et al., 2012].

Compared to 12 other *Enteobacteriaceae* family members, *P. mirabilis* HI4320 contains 784 unique CDS, many of which are thought to contribute to virulence. *P. mirabilis* also has fewer redundant virulence factors than most other *Enterobacteriaceae* as single mutations result in attenuation of this species more frequently than other uropathogenic species [Pearson et al., 2008; Burall et al., 2004; Bahrani-Mougeot et al., 2002; Zhao et al., 1999].

<p align="center">6</p>

Habitat

Both *P. mirabilis* and *P. vulgaris* have been isolated from the intestinal tract of mammals, birds and reptiles. They also are distributed widely in the environment, with reservoirs in soil, polluted water, and sewage, where they play an important role in decomposing organic matter of animal origin. [Guentzel 1996]. Besides the saprophytic mode of life in the natural environment and in the intestines of humans and wild and domestic animals, *P. mirabilis*, also may colonize, under certain circumstances, the urinary tract where it is considered an opportunistic pathogen and one of the principal causes of urinary tract infections (UTIs) in hospital patients with indwelling urinary catheters. *P. vulgaris* is also a common inhabitant of the human gut and a urinary tract pathogen; however, it is associated much less commonly with UTIs than *P. mirabilis*.

Other species of *Proteus* are less widely distributed. *P. penneri,* however, has been also isolated from a number of diverse clinical sites, including abdominal wounds, urine samples, bladder calculi, epidural ulcers and bronchoalveolar lavage fluid [Krajden et al., 1984; Krajden et al., 1987; Li Z. et al., 1992; Latuszynski et al., 1998].

Swarming
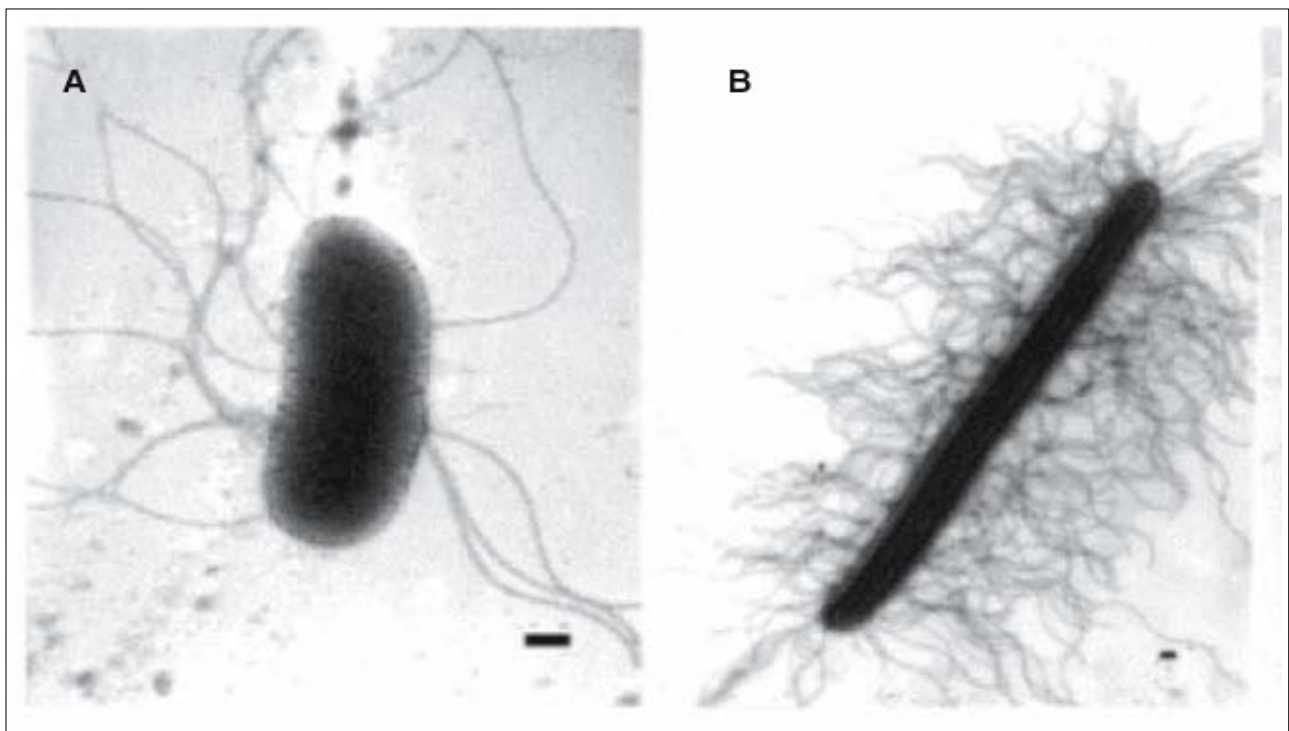
Figure 1: Electron micrographs of *P. mirabilis*. (A) *P. mirabilis* swimmer cell; (B) *P. mirabilis* swarmer cell; The bar represents 200 nm [Manos and Belas, 2006].

One significant phenotypic characteristic shared by members of the genus *Proteus* is the ability to transform into a distinctive "swarmer" cell when cultured on a solid agar-containing medium. Differentiation of *P. mirabilis* to the swarmer stage has been studied extensively [Williams and Schwarzhoff, 1978; Allison and Hughes, 1991; Belas, 1992]. When grown in liquid media, the cells exist as 1.5–2.0 μm rods with 6–10 peritrichous flagella. These so-called "swimmer" cells (Figure 1A) exhibit characteristic swimming and chemotactic behavior, moving away from repellents and towards attractants [Lominski and Lendrum, 1947; Allison et al., 1993]. Transfer of swimmer cells onto a solid growth medium, such as that containing agar, results in a remarkable physiological and morphological transformation of the bacteria. Shortly after contact with the surface, the swimmer cells begin to differentiate into a morphologically and biochemically unique cell known as "the swarmer cell" (Figure 1B).

The characteristics of *P. mirabilis* swimmer and swarmer cells are summarized in Figure 2.

Environmental Signals

Flagellar Rotation
Glutamine
Peptide Signals (?)
Others (??)

Swimmer Cell	Characteristic	Swarmer Cell
1.5 to 2.0 μm	*Length*	10 to >80 μm
4 to 10	*Flagella*	10^3 to 10^4
1 to 2	*Genomes*	Polyploid
Swimming & Chemotaxis	*Motile Behavior*	Swarming, Chemotaxis & Coordinated Cell-to-Cell Communication
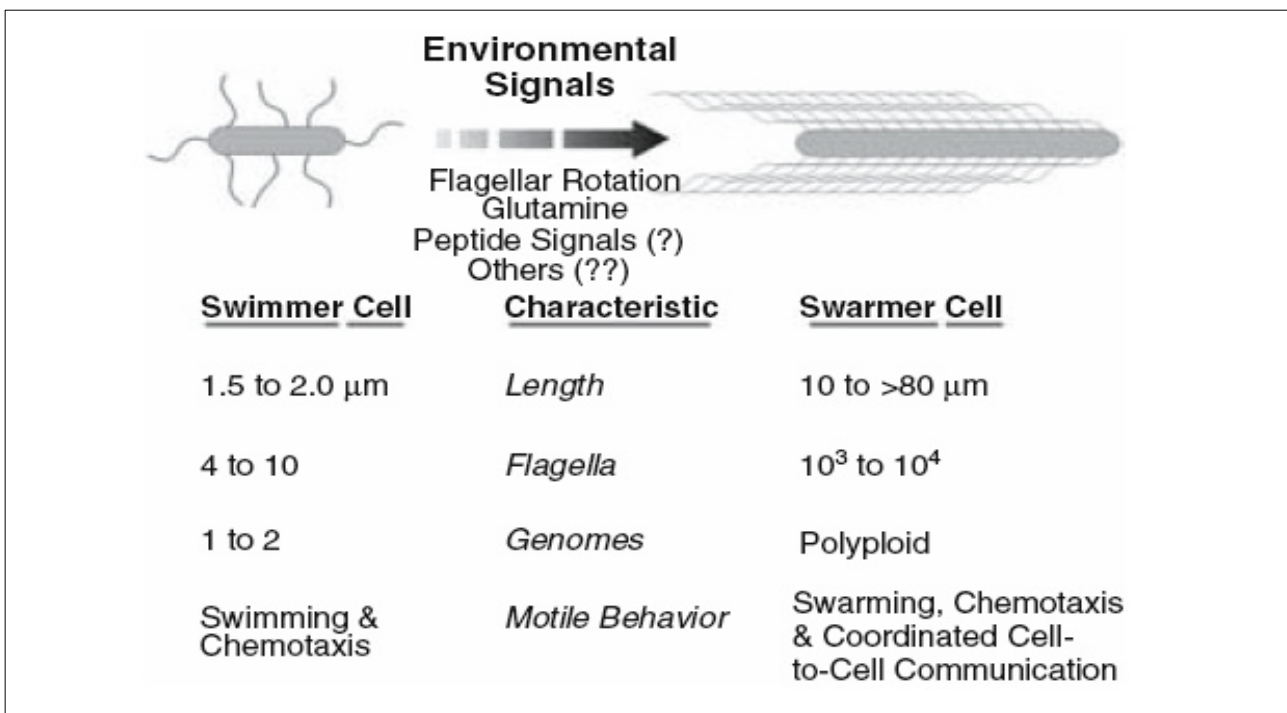
Figure 2: The characteristics of *P. mirabilis* swimmer and swarmer cells. The swarmer cell is characterized by an elongated polyploid cell that synthesizes numerous flagella in response to the aforementioned signals [Manos and Belas, 2006].

Swarmer cell differentiation and swarming behavior may be broken down into discrete steps. The first step in swarmer cell morphogenesis is cellular elongation: in this process the cell continues to grow, above all in length, because septum formation and cell division are inhibited [Armitage et al., 1974; Armitage et al., 1979]. Elongated swarmer cells are typically 60-80 μm in length and are polyploid, with the number of chromosomes per cell being roughly proportional to the increase in

length. Concurrent with this, over expression of the flagellin protein leads to the synthesis of hundreds to thousands of new flagella required for movement across the solid surface [Houwink and Van Iterson, 1950; Leifson et al., 1955; Hoeniger, 1964; Hoeniger, 1965; Hoeniger, 1966; Armitage and Smith, 1978]. Bacteria synthesize also an acid polysaccharide 'slime' which is tough to assist the movement of swarm cell across the agar surface and virulence factors such as urease, haemolysin and protease [Allison et al., 1993].

Swarmer cell differentiation and swarming behavior are inextricably linked, but a differentiated swarmer cell by itself is unable to swarm across a nutrient agar surface. Rather, swarming behavior is a cell-cell contact event that requires intimate contact and interaction between groups of swarmer cells to coordinate their movements. Recent work allowed the visualization of the helical connections formed in swarm rafts during migration, consisting of interwoven flagellar filaments from adjacent swarm cells as shown in Figure 4 (panel B) and at higher magnification in the same figure (panel C) [Jones et al., 2004]. It is not yet clear how *P. mirabilis* coordinates formation of these structures, but they appear to be required for normal swarming motility.

Another important aspect of the *P. mirabilis* swarming colony pattern is its cyclic nature, as shown in Figure 3.
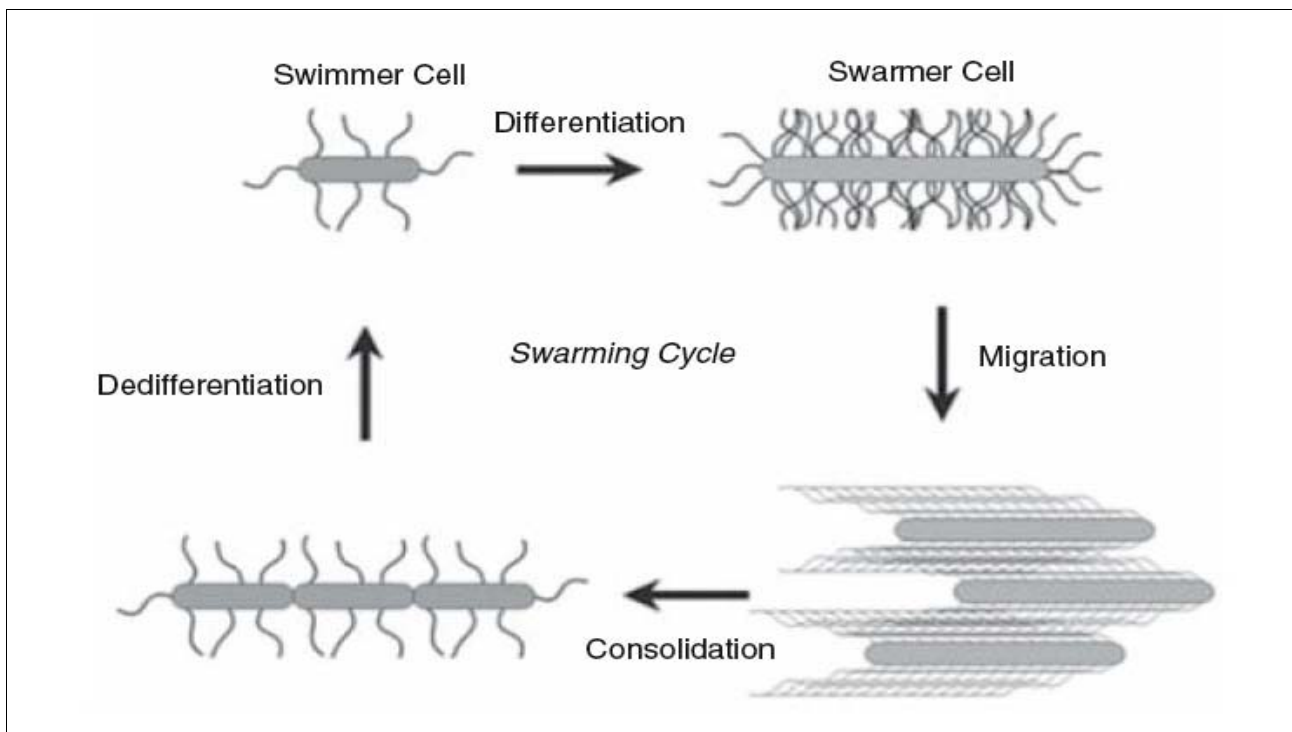
Figure 3: The cyclic nature of *P. mirabilis* swarming behavior. Inoculation of *P. mirabilis* swimmer cells onto a solid nutrient surface such as Luria-agar induces the expression of swarmer-cell specific genes, leading to differentiation into the swarmer cell and migration away from the site of inoculation. This process is interspersed with periods of consolidation and dedifferentiation back into the swimmer cell morphology [Manos and Belas, 2006].

Each cycle can be broken down into four parts: swarmer cell differentiation, the lag period prior to active movement, swarming colony migration and consolidation (where the cells stop moving and dedifferentiate back to swimmer cell morphology). During the migration phase, the fully differentiated swarmer cells move outward in unison in all directions from the original site of inoculation. The duration and extent of this swarming period is highly variable according to the strain and the cultural conditions. Movement then ceases and process referred to as consolidation takes place: the swarmer cells dedifferentiate back into swimmer cells. After a period in this stage, the swarming phase recommences and proceeds until the next consolidation phase [Hoeniger, 1964; Hoeniger, 1965; Hoeniger, 1966; Hoeniger and Cinitis, 1969; Bisset, 1973a; Bisset, 1973b; Williams and Schwarzhoff, 1978].

The cycle of swarming and consolidation is then repeated several times, until concentric rings, formed by the swarming bacteria and delineating the phase changes, cover the agar surface. Although consolidation was once considered a resting stage, P. mirabilis is considerably more metabolically active during consolidation than swarming and overall gene expression is also higher [Armitage, 1981; Pearson et al., 2010]. Genes most highly up-regulated during consolidation include those involved in amino acid import and synthesis, peptide uptake, central metabolic pathways, peptidoglycan remodeling and cell wall synthesis, stress response proteins, proteases, and many flagellar genes. In contrast, few genes are up-regulated in swarm cells compared to consolidate and swarming can occur in the absence of protein synthesis, indicating that swarming is almost entirely devoted to flagellar-mediated motility while consolidation appears to be the active stage in which P. mirabilis prepares for the next round of motility [Pearson et al., 2010].

P. mirabilis swarming motility occurs on 1.5% agar at both 30° and 37°C, resulting in a characteristic bull's eye pattern shown in Figure 4 (panel A). The bull's eye pattern is due to sequential rounds of swarm cell differentiation, swarming colony migration is designated with "S", and consolidation with de-differentiation back to swimmer cell morphology is designated with a "C" in Figure 4 (panel A).

Many ways of inhibiting swarming in cultural plates have been described: these include the physical restriction of movement of *Proteus* cells by increasing the agar concentration to 3-4 %, the prevention of the formation of flagella through the incorporation into the media of ethanol 5.5%, detergents, bile salts or other surface-active agents and the retardation of the cell growth rate by incorporating into the media growth inhibitors. Swarming is inhibited in MacConkey agar by the bile salts, so on this kind of medium *Proteus* strains form discrete colonies.
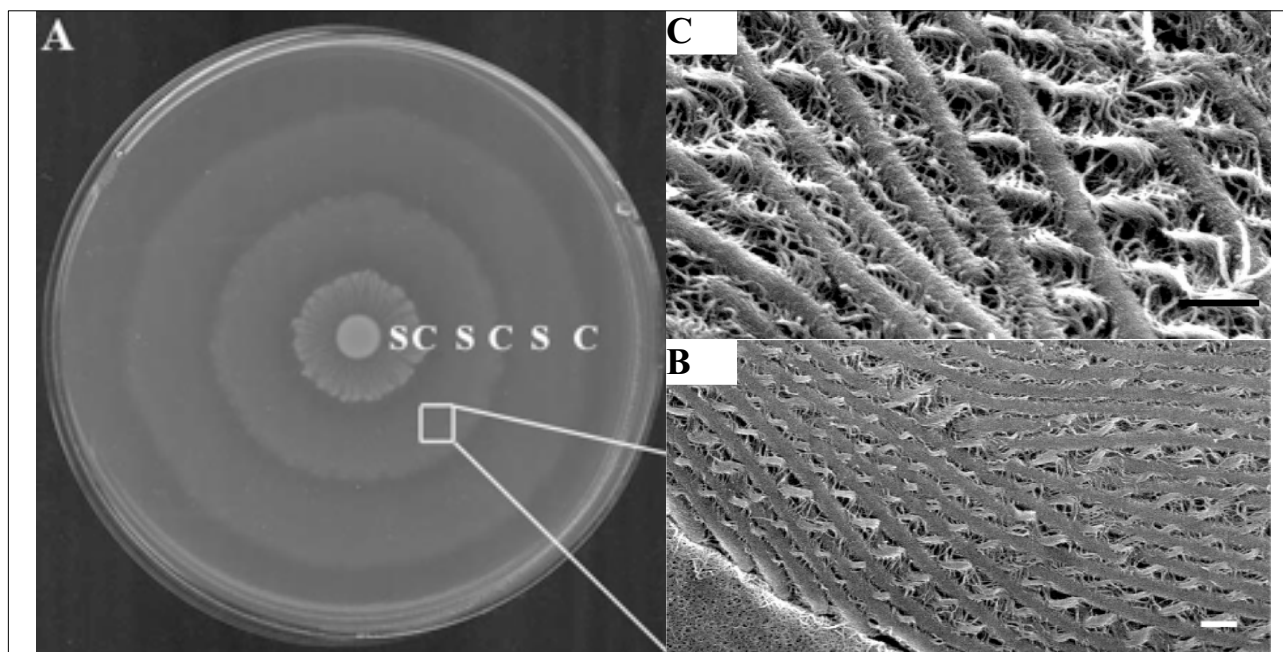
Figure 4:(A) Characteristic bull's eye pattern shown by *P. mirabilis* during swarming; S is for swarming colony migration and C is for consolidation phase (B) Scanning Electron Microscope (SEM) image of in situ vapor-fixed swarm fronts of wild-type *P. mirabilis* swarmer cell rafts, and (C) at higher magnification. Bars ≅ 1 μm. [Jones et al., 2004].

This unique type of motility is notable as it allows *P. mirabilis* to migrate across catheter surfaces, thus gaining entry to the urinary tract [Stickler and Hughes, 1999; Sabbuba et al., 2002; Jacobsen et al., 2008]. Swarming appears to be required for migration across most catheter types as non-swarming mutants can only traverse hydrogel-coated latex catheters [Jones et al., 2004]. In addition to directly swarming across the catheter surface, *P. mirabilis* catheter biofilms contain protruding swarm cells. The role of these biofilm-associated swarm cells is unclear, but they may represent a mechanism for seeding dispersal from the catheter to the urinary tract [Jones et al., 2007]. Interestingly, a pattern similar to *P. mirabilis* swarming can be generated in *E. coli* by placing motility under control of a cell-density-dependent signal [Liu et al., 2011].

While there is a cell-density component to *P. mirabilis* swarming, many additional signals also appear to be integrated. Several factors implicated in the regulation of swarm cell differentiation and swarming motility have been identified and recently reviewed [Rather, 2005; Morgenstein et al., 2010; Armbruster et al., 2012] (Figure 5). They include the Umo proteins, Rcs phosphorelay, RppAB two-component system, the CsrA homolog RsmA, Lon protease, DisA, and the leucine-responsive regulatory protein Lrp. Lrp is part of a family of transcription factors that link gene regulation to metabolism [Calvo and Matthews, 1994; Ren et al., 2007], as Lrp activity is regulated by leucine and other amino acids [Shrivastava and Ramachandran, 2007; Hart and Blumenthal, 2011]. Thus, the decision to swarm is influenced by metabolic status and the presence or absence of

specific amino acids, including glutamine [Allison et al., 1993]. In addition, it is thought that surface contact also triggers swarm cell differentiation. Together, these studies revealed a complex regulatory network for swarm cell differentiation. The Rcs phosphorelay, composed of a response regulator (RcsB), sensor kinase (RcsC), outer membrane activator protein (RcsF), and phosphotransferase protein (RcsD), ultimately results in phosphorylated RcsB, which represses the master regulator of flagellar genes *flhDC* [Belas et al., 1998; Majdalani and Gottesman, 2005; Majdalani et al., 2005; Clemmer and Rather , 2007]. When *P. mirabilis* contacts a surface, this signal is sensed and propagated by some combination of FliL, WosA, and changes in the cell wall likely involving the O antigen [Alavi and Belas, 2001; Belas and Suvanasuthi, 2005; Hatt and Rather, 2008; Cusick et al., 2012; Morgenstein and Rather, 2012]. The signal is relayed through the Rcs phosphorelay by inhibition of RcsF and increased UmoD activity, leading to activation of UmoB and a reduction in phosphorylated RcsB, alleviating *flhDC* repression [Dufour et al., 1998; Morgenstein and Rather, 2012]. The Lon protease negatively regulates *flhDC* and swarming motility by degrading FlhD and possibly Lrp [Clemmer and Rather, 2008]. RppA also negatively regulates flagellin synthesis by decreasing expression of *flhDC* in addition to modulating lipopolysaccharide synthesis [Wang et al., 2008; Jiang et al., 2010a; Jiang et al., 2010b], and the putative amino acid decarboxylase DisA negatively regulates swarming by decreasing expression of the class 2 and class 3 flagellar genes [Stevenson and Rather, 2006].
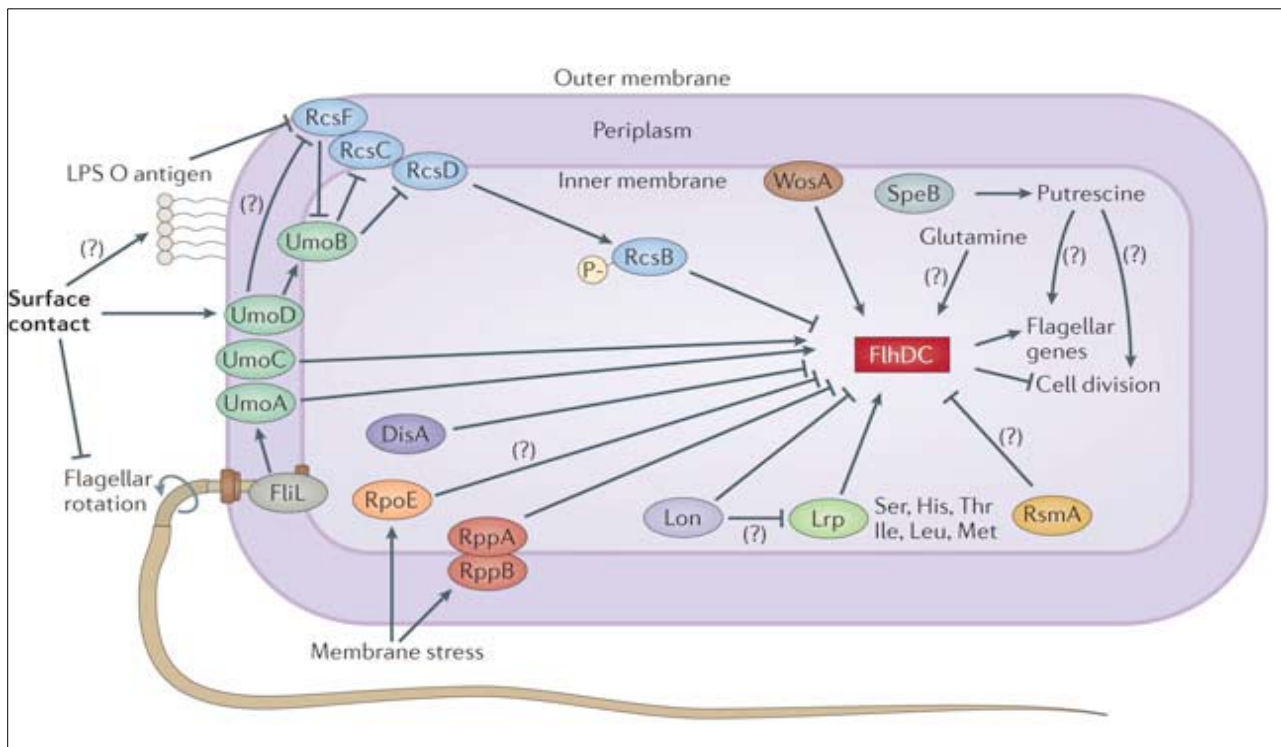
Figure 5: Swarm cell differentiation in *Proteus mirabilis.* [Armbruster and Mobley, 2012]

Several virulence factors are coordinately expressed with swarm cell differentiation, such as hemolysin (HpmA) [Allison et al., 1992a]. Three putative promoters exist upstream of *hpmA* but the promoter upstream of *hpmB* appears to be responsible for swarming-coupled expression [Fraser et al., 2002]. The region upstream of *hpmB* is also necessary for full up-regulation during swarming and contains at least four binding sites for Lrp, indicating that expression of hemolysin is likely coupled to the metabolic and nutritional cues to which Lrp is responsive. Whether production of hemolysin *in vivo* occurs via this same swarming-regulated promoter is unknown.

Another fascinating aspect of swarming involving inter-bacterial interactions is the Dienes phenomenon, in which two swarming colonies of a single strain merge with each other while swarms of different strains form a distinct boundary where they meet, known as a Dienes line [Dienes, 1946]. Formation of the Dienes line requires direct cell-cell contact by living bacteria, and is thought to involve killing of one strain at the boundary. Interestingly, competitive killing is only observed during swarming as strains sensitive to killing on swarm agar are not outcompeted in broth culture or on agar that is not swarming-permissive [Budding et al., 2009]. One explanation for the formation of the Dienes line involves the production of proticine capable of killing sensitive strains. Indeed, boundaries form between *P. mirabilis* strains that differ in proticine production and sensitivity [Senior, 1977]. However, some strains deficient in proticine production still form boundaries, even with other strains lacking proticine production, indicating that another underlying mechanism mediates Dienes line formation [Gibbs et al., 2008]. In the search for this mechanism, a transposon mutant was identified that formed a boundary with its parent strain rather than merging, and the disrupted locus was named *ids* for identification of self [Gibbs et al., 2008]. Further work involving the *ids* locus determined that *idsABCDEF* constitutes an operon, and that *idsD* and *idsE* appear to encode strain-specific factors essential for self-recognition while *idsB, idsC,* and *idsF* encode factors essential for self-recognition that can be complemented by *ids* genes from other strains [Gibbs et al., 2008; Gibbs et al., 2011]. As two swarm fronts merge, only a subset of cells in the advancing swarm express the *ids* genes and can traverse the boundary of the approaching swarm, and this subset is sufficient to somehow propagate the signal of self versus non-self [Gibbs et al., 2011]. Furthermore, *ids* expression decreases as an advancing swarm approaches another swarm of the same strain. The *ids* system alone, however, does not fully explain the differences in strain interactions that occur within the Dienes line as mutations within this locus allow for boundary formation but without the formation of rounded cells or any apparent competitive killing [Budding et al., 2009]. As the *ids* locus encodes putative type six secretion system (T6SS) effector

proteins Hcp and VgrG, it was hypothesized that T6SS may be involved in Dienes line formation [Gibbs et al., 2008]. In agreement with this hypothesis, Mobley's laboratory has identified additional loci encoding T6SS effector and structural proteins involved in Dienes line formation [Alteri, personal communication]. T6SS is thought to be involved in maintenance of a balanced relationship between bacterium and host as well as mediating competitive inter-bacterial interactions, and the T6SS of *Vibrio cholerae* was recently shown to be involved in inter-species competition and killing [MacIntyre et al., 2010]. The T6SS of *P. mirabilis* along with proticine and the *ids* genes may therefore mediate a combination of inter-bacterial killing during swarming and Dienes line formation, competition on the catheter surface, or even some form of interaction with the host during UTI. In addition to the T6SS, *P. mirabilis* HI4320 encodes a two-partner secretion system (PMI0592-PMI0593) similar to the *cdiAB* system of *E. coli* [Aoki, 2005; Pearson, 2008]. The *cdiAB* system allows for cell-cell contact-dependent inhibition of growth without killing the target cell [Aoki et al., 2005]. It is therefore intriguing to speculate that this secretion system may also be involved in strain recognition and Dienes line formation in situations where the susceptible strain is not killed.

Epidemiology

P. mirabilis is a documented cause of UTIs in the complicated urinary tract (functional, metabolic, or structural abnormalities) [Mobley and Warren, 1987; Chen et al., 2012].

The urinary tract is a epithelial-lined tube with an opening to the body surface. It is susceptible to infections by exogenous organisms including bacterial species with specific virulence factors that allow colonization of the urinary tract. Such organisms must be able to colonize the urinary tract epithelium, avoid the host response and usually cause overt damage to the host with accompanying symptoms. Most UTIs are thought to occur by the ascending route. Organisms of fecal origin gain access to the urethra and enter the bladder where they multiply. Lower UTIs, such as cystitis, are typically characterized by symptoms including frequency, urgency, and dysuria [Guay, 2008]. If left untreated, these infections can ascend the ureters to the kidneys, and they can be associated with additional symptoms such as fever, nausea, vomiting, and flank pain. The most virulent and invasive pathogens can breach the single cell-thick barrier afforded by the proximal tubules and enter the bloodstream possibly causing a systemic infection [Nielubowicz and Mobley, 2010].

An estimated 40% of women and 12% of men will experience a symptomatic UTI during their

lifetime, and approximately a quarter of affected women will suffer recurrent UTIs within 6–12 months [O'Hanley, 1996; Foxman, 2002].

P. mirabilis is not a common cause of UTIs in the normal host. Rather it infects the urinary tract, one with functional or anatomical abnormalities, or chronic instrumentation such as catheterization, making it the most common nosocomial infection [Warren, 2001]. *P. mirabilis* is most often associated with urinary stones and catheter encrustation and is rarely a cause of acute cystitis, hospital-acquired UTIs or recurrent UTIs, but is more often associated with complicated UTIs, intermittent catheterization, condom catheter, and ileal loop [Li X., et al., 2002]. The organism is especially prominent in long-term catheterized patients, being isolated in one study, in the urine at 10^5 CFU from 44% of the cases [Warren, 2001].

CaUTIs is the most common health care-associated infection worldwide, accounting for up to 40% of all hospital-acquired infections [Hooton et al., 2010]. Length of catheterization is the most important risk factor associated, as 10-50% of patients undergoing short-term catheterization (1-7 days) develop bacteriuria while essentially all patients who are catheterized for 28 days or longer will develop UTI [Morris et al., 1999]. CaUTI is generally thought to be caused by self-inoculation of the catheter, and indeed for *P. mirabilis* the strains causing bacteriuria are identical to strains isolated from fecal samples [Mathur et al., 2005]. Following colonization of the catheter, bacteria can migrate to the bladder in as little as 1-3 days [Sabbuba et al., 2003; Hooton et al., 2010].

P. mirabilis is generally not one of the first organisms to colonize the catheter surface and it is rarely isolated from patients undergoing short-term catheterization, but the longer a patient is catheterized the more likely it is for *P. mirabilis* to be present [Wazait et al., 2003; Stickler and Feneley, 2010]. The biofilms initially formed on catheters tend to be monomicrobial, but rapidly become polymicrobial during long-term catheterization, with up to 72% of catheters being colonized by two or more species [Macleod and Stickler, 2007]. Biofilms taken from patients with long-term catheterization frequently contain combinations of *P. mirabilis, Morganella morganii, Providencia stuartii, E. coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae,* and these are also the organisms most frequently isolated from polymicrobial UTIs [Breitenbucher, 1984; Rahav et al., 1994; Siegman-Igra et al., 1994; Nicolle, 2005; Hooton et al., 2010; Nicolle, 2012]. Depending on study parameters and the specific sample population, data complied over the last 30 years have revealed that up to 77% of CaUTIs are polymicrobial, and *P. mirabilis* is generally the most common organism isolated from these infections [Breitenbucher, 1984; Kunin, 1989; Rahav et al., 1994; Siegman-Igra et al., 1994; Ronald, 2003].

Pathogenesis and Virulence factors

Like many other pathogens, *P. mirabilis* has evolved numerous virulence factors that are important for causing UTIs, and several of these factors appear to be more important for establishing infection in different areas of the urinary tract. These factors include adherence to host mucosal surfaces, damage and invasion of host tissues, evasion of host immune systems, and iron acquisition. There are, as well, virulence factors that are crucial for successful colonization of the urinary tract (for example urease for hydrolyzing urea and flagella for ascending to kidneys through ureters). Expression of these virulence factors is most likely spatially and temporally regulated. The bacterium must gain entry to the urinary tract via the urethra (or indwelling catheter), travel to and colonize the bladder, ascend the ureters to the kidneys, colonize the kidneys and maintain infection. In some cases the organism may gain access to capillaries and establish bacteremia. These events require the organism to express different adhesins and flagella concordant with morphological changes from vegetative swimmer cell to hyper-flagellated swarmer cell, express toxins, and avoid the host immune response. While some of these factors may be constitutively expressed, it is clear that many are inducible.

It is hypothesized that *P. mirabilis* gains access to the human urethra by self-contamination of the periurethral area [Chow et al., 1979]. When at the site of colonization, *P. mirabilis* may undergo swarmer cell differentiation, which is accompanied by the production of hundreds of flagella per cell. Flagella-mediated motility is required to move from the urethra to target tissues in the bladder and allow close contact with bladder epithelial cells. *P. mirabilis* causes pyelonephritis (infection of the kidney parenchyma) by an ascending route of infection [Coker et al., 2000]. From the bladder, the bacteria ascends the ureters and gain access to the kidneys, in this process MR/P fimbriae are crucial. Once in the kidneys, the bacteria are able to replicate and colonize the tissue. Tissue cell invasion has been observed *in vitro* using cultured human proximal tubular epithelial cells [Bahrani et al., 1991], but it is unclear if *Proteus* is able to invade kidney epithelial cells *in vivo*. Cell invasiveness, also termed cell penetration, in relation to *Proteus* rods, is an important step in infection and has been investigated by several groups of authors [Peerbooms et al., 1984; Finlay and Falkow, 1989; Finlay, 1990]. It was found that the invasion of mammalian cells by *P. mirabilis* rods *in vivo* and *in vitro* was also stimulated by urea [Braude and Siemienski, 1960]. A correlation of cell-associated hemolytic activity with penetration was also observed [Peerbooms et al., 1984].

Flagella and swarming motility

In general, the presence of flagella on the surface of pathogenic and opportunistic bacteria has been thought to facilitate the colonization and dissemination from the initial site. The association of motility with the virulence of other flagellated Gram-negative bacilli like *V. cholerae* [Guentzel and Berry, 1975] and *P. aeruginosa* [McManus et al., 1980] has already been demonstrated. It is accepted that the flagella and swarming behavior play a role in the pathogenicity of *P. mirabilis*. *P. mirabilis* mutants lacking flagella are non-invasive to uroepithelial cells and motile but non swarming cells are twenty-five-fold less invasive than wild-type motile, swarming cells [Allison et al., 1992b].

Swarming as a form of bacterial translocation across the solid surface of artificial or natural media is characteristic not only for *Proteus* spp. but also for the Gram-negative bacteria *Vibrio* spp. and *Serratia* spp. as well as the Gram-positive microorganisms *Bacillus* spp. and *Clostridium* spp. [Henriksen, 1972; McCarter and Silverman, 1989; Alberti and Harshey, 1990].

The genetic basis of the *P. mirabilis* swarming phenomenon was studied by transposon mutagenesis. The transposon Tn*5* was used to obtain mutants defective in swarming motility. Analysis of mutants defective in swarm cell differentiation and swarming motility provided results which suggested that approximately 40 to 60 genes were involved in *P. mirabilis* multicellular swarming behavior [Belas et al., 1991].

The swarming growth can be simply described as a differentiation of short rods into non-septate, multinucleate swarmers, which is accompanied by an increase in the number of flagella. The newly synthesized flagella in swarmers are composed of the same protein (36.7-kDa flagellin) as are the flagella in the swimmer cells [Belas et al., 1991] .

Another aspect is the important role of flagellin as a bacterial surface antigen (H antigen). Since flagellin is strongly immunogenic, it can be assumed that at least part of the immunoresponse of the host during the infection is directed against this antigen. Thus, the possible changes in flagellin antigenicity (it has been reported the occurrence of multiple genes encoding flagellin in *P. mirabilis*) may enable bacteria to escape the immunoresponse of the infected macroorganisms. *P. mirabilis* flagellin variation has been demonstrated *in vitro* [Belas, 1994].

Fimbriae and adherence ability

Bacterial adhesion to epithelial surfaces is thought to be one of the most important virulence factors, playing a significant role in the initiation of UTIs [Schoolnik et al., 1980; Reid and Sobel, 1987]. The data obtained by several authors suggest that adhesion of bacterial cells to uroepithelial cells is very important in this process in infections caused by *Proteus* spp. [Eden et al., 1980; Savoia et al., 1983; Cellini et al., 1987].

The bacterial adhesion capacity is most frequently associated with the presence of fimbriae on bacterial cells. It has been already shown that fimbriae are indeed responsible for the attachment of *Proteus* bacilli to uroepithelial cells. It was observed in an experimental ascending infection that a heavily fimbriated *P. mirabilis* strain caused pyelonephritis with higher efficacy than a lightly fimbriated one did [Silverblatt and Ofek, 1978]. By electron microscopy, it was possible to see bacteria with fimbriae bound to renal pelvic mucosa [Silverblatt, 1974]. Also, studies *in vitro* have shown that fimbriae enhance the binding of bacterial cells to uroepithelial cells even if they render the pathogen more susceptible to phagocytosis [Rozalski et al., 1997].

P. mirabilis encodes 17 putative fimbrial operons, the greatest number of fimbriae of any sequenced organism [Pearson et al., 2008]. Of these, only a handful have been characterized and recently reviewed: Mannose-resistant/*Proteus*-like fimbriae (MR/P), Mannose-resistant/*Klebsiella*-like fimbriae (MR/K), uroepithelial cell adhesin (UCA; also called NAF for non-agglutinating fimbriae), *Proteus mirabilis* fimbriae (PMF), ambient temperature fimbriae (ATF), and *Proteus mirabilis* P-like fimbriae (PMP) [Rocha et al., 2007].

Recent work elucidated binding specificity for several fimbriae and their contribution to UTI (Table 3). MR/P fimbriae mediate adherence to the lumin and cytoplasm of tubular cells in the kidney and to epithelial cells present in urine [Saraneva et al., 1990], MR/K fimbriae mediate adherence to Bowman's capsules of glomeruli in the kidney [Saraneva et al., 1990], UCA/NAF fimbriae bind to uroepithelial cells lines [Saraneva et al., 1990]. PMF fimbriae are also thought to contribute to colonization of the bladder but are not necessary for kidney colonization [Massad et al., 1994; Zunino, 2003]. However, the exact contribution of PMF fimbriae to infection remains unclear as expression of the major structural subunit (PmfA) is decreased during infection [Pearson et al., 2011]. *Proteus* has furthermore, 13 additional orphan fimbrial genes not associated with complete operons [Pearson et al., 2008].

Bacteria generally do not express adherence factors at the same time as flagella, suggesting an

underlying mechanism for reciprocal regulation of these factors [Hoeniger, 1965; Latta et al., 1999]. For instance, *P. mirabilis* swarm cells express thousands of flagella but few fimbriae, and expression of the MR/P operon is highest when expression of flagellar genes is reduced [Pearson et al., 2011; Mobley et al., 1996]. It is suggested that expression of MR/P fimbriae is crucial for early stages of infection, but decreases over time to allow for flagellar-mediated motility. This is noteworthy as swarm cells appear to be rare during early stages of UTI but may play a critical role in later stages of infection, particularly as individual swarm cells have been identified within urinary stones [Allison et al., 1994; Li X. and Mobley, 2002; Jansen et al., 2003; Pearson et al., 2011].

TABLE 3. Fimbriae of *Proteus* rods and their importance in pathogenicity

Type of fimbriae	Contribution to the pathogenicity
MR/P	Adherence to the lumin and cytoplasm of tubular cells in the kidney and colonization of the upper part of the urinary tract.
MR/K	Adherence to Bowman's capsules of glomeruli in the kidney and associacion with the adhesion of strains to cathaters.
PMF	Colonization of bladder but not kidneys
UCA/NAF	Adherence to uroepithelial cells lines
ATF	Not clear, they may play a role in the growth in the natural environment.
PMP	Not studied

Outer membrane proteins

In general, outer membrane (OM) proteins (OMP) possess immunogenic properties and mitogenic activity for B cells [Melchers et al., 1975; Bessler and Henning U., 1979; Chen et al., 1980] furthermore, OM lipoproteins and their synthetic analogs function as adjuvants [Bessler, 1986] and can also activate macrophages to produce tumor necrosis factor (TNF) [Hoffmann et al., 1988]. The OM of *P. mirabilis* contains three major proteins of 39.0, 36.0, and 17.0 kDa [Nixdorff et al., 1977; Bub et al., 1980]. The 39-kDa protein was identified as the OmpA protein [Karch and Nixdorff,1983], and the 36-kDa protein appeared to be a peptidoglycan-associated matrix protein [Lugtenberg et al., 1977].

Lipopolysaccharide

This subject, as main object of study in this work, is described in an extensive and detailed form, in chapter 1.2.

Capsule

Polysaccharide capsules are ubiquitous structures found on the cell surface of a broad range of bacterial species. Capsular polysaccharides are linked to the cell surface of the bacterium via covalent attachments to either phospholipid or lipid-A molecules [Whitfield and Valvano, 1993]. Capsular polysaccharides are highly hydrated molecules that are over 95% water [Costerton et al., 1981]. They are composed of repeating single units (monosaccharides) joined by glycosidic linkages. They can be homo- or heteropolymers and may be substituted by both organic and inorganic molecules. Any two monosaccharides may be joined in a number of configurations as a consequence of the multiple hydroxyl groups within each monosaccharide unit that may be involved in the formation of a glycosidic bond. As a result of this, capsular polysaccharides are an incredibly diverse range of molecules that may differ not only by monosaccharide units but also in how these units are joined together. The introduction of branches into the polysaccharide chain and substitution of both organic and inorganic molecules yield additional structural complexity. In the case of human pathogens, a large number of different capsule serotypes have been identified. Over 80 different capsular polysaccharides or K antigens have been described for *E. coli*, of which a small fraction are associated with invasive infections [Orskov and Orskov, 1992]. The expression of particular K antigens can be associated with specific infections. Chemically identical capsular polysaccharides may also be synthesized by different bacterial species [Roberts, 1996].

A number of possible functions have been suggested for polysaccharide capsules: the formation by capsules of a hydrated gel around the surface of the bacteria may protect the bacteria from the harmful effects of desiccation [Roberson and Firestone, 1992], and this may be particularly relevant in aiding the transmission of encapsulated pathogens from one host to the next. Capsular polysaccharides may also promote the adherence of bacteria to both surfaces and to each other, and they thereby facilitate the formation of a biofilm and the colonization of various ecological niches [Costerton et al., 1987]. During colonization of oral surfaces and the temporal development of bacterial plaque, specific colonizing bacteria may provide bridges for the subsequent attachment of

other bacterial species [Kolenbrander, 1993]. This intergeneric interaction that establishes microbial consortia within the biofilm is mediated in part through lectin-ligand interactions that involve cell-surface polysaccharide molecules [Jenkinson, 1994]. The formation of biofilms may offer the individual bacteria protection from phagocytic protozoa and infection by bacteriophages as well as nutritional advantages. The ability of bacteria to attach to surfaces and establish a biofilm can have far-reaching consequences. The colonization of indwelling catheters in hospitalized patients can lead, in fact, to serious nosocomial infections. Other than these adherent properties, capsular polysaccharides may have lubricant properties that facilitate the swarming of *P. mirabilis* over solid substrata by reducing friction [Stahl et al., 1983]. Furthermore, during invasive bacterial infections, interactions between the capsular polysaccharide and the host's immune system can decide the outcome of the infection [Roberts et al., 1989]: in absence of specific antibody, the presence of a capsule is thought to confer resistance to nonspecific host defense mechanisms.

Capsule gene clusters have been cloned from a number of Gram-negative bacteria, including *E. coli* [Roberts et al., 1986; Silver et al., 1981], *Haemophilus influenzae* [Kroll et al., 1989], *Neisseria meningitidis* [Frosch et al., 1989], *Salmonella typhi* [Hashimoto et al., 1993], *Pseudomonas solanacearum* [Huang and Schell, 1995], *K. pneumoniae* [Arakawa et al., 1995], *Erwinia stewartii* [Dolph et al., 1988], and *E. amylovora* [Bugert and Geider, 1995]. In all these cases the capsule genes are clustered at a single chromosomal locus, which allows the coordinate regulation of a large number of genes that may be involved in the biosynthesis and export of capsular polysaccharides.

Proteus bacilli have both capsular polysaccharide (CPS) and lipopolysaccharide (LPS, endotoxin) on their surfaces. CPS is the most external surface component of these bacteria, but detailed studies have shown that only a few strains can synthesize a capsule antigen, and its structure is identical to the O-specific chain of their LPS [Beynon et al., 1992; Perry and MacLean, 1994; Rozalski et al., 1997]. The role of *Proteus* capsule structure, also termed as slime material or glycocalyx (highly hydrated polymers present on the surface of bacteria), in the pathogenicity of *Proteus*, especially in urinary tract infections, is controversial. The negatively charged polysaccharides are important barriers against the bactericidal action of the complement system [Kaca et al., 2000]. They also play an important role in the migration of swarm cells by the reduction of surface friction [Gygi et al., 1995]. The acidic character of *Proteus* extracellular polysaccharides may play a crucial role in stone formation within the urinary tract. It has been speculated that the structure and anionic character of *P. mirabilis* O6 CPS enhances struvite formation by weakly concentrating Mg^{2+} ions during struvite crystal formation [Dumanski et al., 1994].

Urease

One of the hallmarks of UTI caused by *P. mirabilis* is urolithiasis, the production of stones in the urinary tract.

The formation of these stones is the result of activity of the bacterial enzyme urease. Urea represents the main nitrogenous excretory product in humans and the majority of animals. Urease catalyzes the hydrolysis of this compound to yield ammonia and carbon dioxide, which results in an increase in the urine pH [McLean et al., 1988; Mobley and Hausinger, 1989; Clapham et al., 1990; Mobley et al., 1995] resulting in the precipitation of ions (as Mg^{2+} and Ca^{2+}) that are normally soluble in urine. This process leads to the formation of stones, such as magnesium ammonium phosphate (struvite), that can block the flow of urine through catheters and result in renal scarring owing to calculus formation within the renal pelvis. Stone formation provides a number of benefits for the microorganism including, but not limited to, protection from the host immune system (bacteria become trapped in the interstices of the stones and are able to replicate; antibiotics and immunoglobulins are unable to reach the bacteria sequestered in the stone cavities), blockage of urine drainage into the ureters, ammonia toxicity to host cells, and direct tissue damage. All of these events lead to a protective and nutrient-rich environmental niche for the bacteria. This phenomenon does not take place during UTI due to urease-negative *E. coli* [Mobley and Hausinger, 1989].

Urease activity has been found in over 200 species of Gram-negative and Gram-positive bacteria [Griffith et al., 1976; McLean et al., 1988; Mobley et al., 1995; Mobley and Belas, 1995]. This enzyme has also been implicated as a factor contributing to the pathogenicity of many bacteria including *Proteus*, *Providencia*, and *Morganella* species [Guo and Liu, 1965; Magana-Plaza and Ruiz-Herrera, 1967; Magana-Plaza et al., 1971; Penner et al., 1976; Rosenstein et al., 1981; Senior, et al., 1980; Jones and Mobley, 1987]. The urease activity of these bacteria is used to distinguish them from other *Enterobacteriaceae* family members. This activity is constitutive in most *P. mirabilis* strains [Mobley and Hausinger, 1989], plasmid mediated in others species as *P. stuartii* [McLean et al., 1988; Mobley and Hausinger, 1989], and inducible in some *P. mirabilis*, *P. vulgaris*, and *P. penneri* strains [Mobley et al., 1987], as well as in *Providencia rettgeri* [Mobley and Hausinger, 1989].

Damage to host tissue : Hemolysin

Hemolysin is a pore-forming toxin that inserts into target eukaryotic cell membranes, causing the efflux of Na+ ions and subsequent cell damage [Braun and Focareta, 1991]. The hemolysin genes of *P. mirabilis*, *hpmA* and *hpmB*, code for 166-kDa and 63-kDa polypeptides, respectively [Lukomski et al., 1991]. HpmA is secreted from the bacterium (in a process mediated by HpmB) and has calcium independent hemolytic and cytotoxic activities [Welch, 1987; Uphoff and Welch, 1990]. HpmA is found in the periplasm while HpmB is hypothesized to be located in the outer membrane, aiding in the secretion of HpmA [Lukomski et al., 1991]. It is further hypothesized that *P. mirabilis* is able to spread into the kidney via the tissue damage invoked by HpmA. *P. mirabilis* may not use hemolysin as a means for colonization; however, hemolysin may play a role in the spread of infection into the kidneys, initiating acute pyelonephritis.

Iron sequestration: amino acid deaminase

For a long time, it has been known that virtually all bacteria require soluble iron as an important nutritive compound. It is indispensable for growth and metabolism, mainly for most redox processes in all ecological systems: in the natural environment (soil, water), in artificial media, and in such niches as living organisms. In the presence of a deficiency of iron, bacteria produce iron chelators, named siderophores, which are excreted to the surroundings; they bind iron and transport it into the bacterial cells by using suitable receptor proteins and appropriate transport mechanisms. The synthesis of siderophores is under the control of chromosomal or plasmid genes. In all kinds of the host-bacterium relationships (commensals and conventional and opportunistic pathogens), the bacteria are in competition with their host for iron. Eukaryotic proteins like transferrin and lactoferrin, with high iron affinity, render prokaryotic cells iron deficient. An efficient production of siderophores may seal the fate of an invader. From this point of view, they can be considered one of the virulence (invasiveness) factors [Rozalski et al., 1997].

In the family *Enterobacteriaceae*, most intensively studied for the production of siderophores, the situation is very differentiated. Certain species produce nearly exclusively the catecholate type (enterobactins) [O'Brien et al., 1970; Pollack and Neilands, 1970], some produce the hydroxamate type (aerobactins) [Gibson and Magrath, 1969; Payne, 1988], and others produce the described ferrioxamine type [Berner et al, 1988; Reissbrodt et al., 1990] siderophores. In contrast to other

members of the family *Enterobacteriaceae*, none of the *Proteus-Morganella-Providencia* (PPM) group produced the siderophores enumerated above.

Because *P. mirabilis* infects the iron-limited environment of the urinary tract, a siderophore is necessary to capture iron for its survival. *P. mirabilis* produces an amino acid deaminase that generates α-keto-acids, a less common form of siderophore [Drechsel et al., 1993]. The amino acid deaminase catalyzes the deamination of amino acids to α-keto acids, which are capable of binding iron. The single gene encoding *P. mirabilis* amino acid deaminase, *aad*, was isolated and sequenced. The *aad* open reading frame codes for a 473 amino acid protein that has a molecular mass of 51 kDa. Attempts to construct a deaminase-deficient mutant were unsuccessful, suggesting that the deaminase gene is critical for the survival of the bacteria. Surprisingly, the *aad* gene is not affected by iron concentrations, leading to speculation that perhaps iron acquisition is not the sole function of *aad* [Massad et al., 1995].

Immune system avoidance: IgA-degrading protease

Part of the survival strategy for mucosal pathogens is the evasion of host defenses. *P. mirabilis* strains have been shown to have immunoglobulin A (IgA) - degrading protease activity; the activity has been detected in the urine of infected patients [Loomes et al., 1992; Senior et al., 1991]. The secretory immunoglobulins A (sIgA) is the predominant Ig in mucus secretion. Its function is to protect mucous membrane and underlying tissue from bacteria and their products. sIgA is markedly resistant to degradation by proteolytic enzymes of many microorganisms examined; only a few microbes have been found to synthesize extracellular proteolytic enzymes capable of degrading IgA. These include *N. meningitidis*, *Neisseria gonorrhoeae*, *H. influenzae*, and *Streptococcus pneumoniae*, which are associated with diseases at mucosal surfaces, as well as some periodontal pathogens [Plaut, 1983; Mulks, 1985;]. Because other related but not pathogenic species of the same bacterial genera do not synthesize IgA proteases, production of these enzymes may be associated with the virulence. The IgA-degrading protease of *P. mirabilis*, or ZapA metalloprotease (488 amino acids, 55 kDa), was cloned and sequenced [Wassif et al., 1995]. Zap A metalloprotease is very similar to the IgA and IgG proteases of *P. aeruginosa*, *Serratia marcescens*, and *Erwinia chrysanthemi*. All these proteins belong to the serralysin family of zinc metalloproteases, which are members of the ABC superfamily of prokaryotic and eukaryotic transporters. The serralysin family is a branch of a larger group of virulence proteins from pathogenic *Actinobacillus* spp., *Bordetella*

pertussis, enterohemorrhagic *E. coli*, *N. meningitidis*, and *Pseudomonas fluorescens*. The optimum pH for the action of *Proteus* proteases is 8, which is not surprising because of the alkaline surroundings in which these enzymes "work" *in vivo* [Loomes et al., 1992]. It seems that during infection, *P. mirabilis* strains synthesize urease, which degrades urea, resulting in the production of alkaline conditions optimal for the action of IgA (and IgG) proteases. Is has been demonstrated that the differentiation of *P. mirabilis* short vegetative rods into filamentous, multinucleate, and hyperflagellate swarmer cells is accompanied by substantial increases in the activities of virulence factors, including proteases [Allison et al., 1992a; Allison et al., 1994]. Since the ability of *P. mirabilis* to invade human urothelial cells is primarily characteristic of swarmer cells but not vegetative bacilli, the protease activity has been hypothesized to be relevant to differentiation and possibly to the pathogenicity of *P. mirabilis*. The role of *P. mirabilis* IgA and IgG proteases as virulence factors in UTI seems to be important. However, the absolute amount of specific antibody isotypes in the urinary tract is not known. Since complement is not secreted in large amounts, it is possible that host defense against the infection process occurring in the urinary tract includes antibody-mediated opsonization of bacteria and, afterward, binding of the Fc fragment of antibodies to phagocytic cells. This defense mechanism may play a major role, since IgA and IgG antibodies can be opsonic and the Fc receptor of IgA has been identified on human polymorphonuclear cells. Thus, the synthesis and action of the above-described proteinases *in vivo* in the urinary tract might diminish or even eliminate phagocytosis due to hydrolysis of opsonic antibodies to ineffective fragments [Loomes et al., 1992].

All the virulence factors are summarized in table 4.

TABLE 4. Virulence factors of *Proteus* bacilli *

Virulence factor	Contribution to the pathogenicity
Fimbriae	Adherence of bacteria to the epithelial tissue
Flagella (swarming phenomenon)	Ascension of bacteria from ureter to the kidneys
Urease	Elevation of pH in the surrounding of bacterial growth resulting in stone formation; cytotoxicity against HRPTEC
IgA proteases	Disruption of IgA
Amino acid deaminases	Production of α-keto acids acting as siderophores
Invasiveness	Penetration and internalization of bacteria into host cells
Hemolysins (HpmA and HlyA)	Cytotoxicity
CPS	Formation of biofilm and stones
LPS (endotoxin)	Endotoxicity; resistance to the human serum

*[Rozalski et al. 1997]

<u>Identification of novel pathogenicity factors</u>

Signature-tagged mutagenesis (STM) has been utilized to investigate P. mirabilis UTI in three separate studies to identify key determinants of pathogenicity [Zhao et al., 1999; Burall et al., 2004; Himpsl et al., 2008]. Combined, these studies screened enough transposon mutants for 70% theoretical coverage of the genome and a total of 76 mutants were significantly attenuated in vivo. While several of these mutants also had defects in expression of fimbriae, flagella, LPS, urease, or swarming motility, this approach successfully identified previously unknown virulence factors that do not have obvious links to pathogenicity and warrant further investigation (see Table 5). Eight STM mutants had transposon insertions in genes that were also found to be up-regulated during experimental UTI, further validating the importance of these virulence factors.

Treatment of Proteus mirabilis infections

Once *P. mirabilis* gains access to the urinary tract it has a remarkable ability to persist despite antibiotic treatment and catheter changes [Sabbuba et al., 2004; Stickler, 2008;]. *P. mirabilis* is intrinsically resistant to tetracycline and nitrofurantoin with intermediate resistance to chloramphenicol. *Proteus* species isolated from CaUTI are fairly resistant to amoxicillin and trimethoprim, and an increasing number of isolates exhibit multi-drug resistance against cephalosporins, aminoglycosides, fluoroquinolones, amoxicillin clavulonate, and cotrimoxazole [Wazait et al., 2003; Cohen-Nahum et al., 2010]. This complicates antibiotic efficacy since CaUTI from long-term catheterization is commonly treated with trimethoprim-sulfamethoxazole or ampicillin in combination with another antibiotic [Cravens and Zweig, 2000; Jacobsen et al., 2008]. The polymicrobial nature of CaUTI further complicates antibiotic efficacy, making it difficult to eliminate infection with antibiotics while the catheter remains in place [Clayton et al., 1982]. In many cases, normally-susceptible bacteria colonizing the catheter surface survive the concentration of antibiotics in the urine following conventional treatment [Ganderton et al., 1992; Morris et al., 1999]. This is likely due to the protection that biofilms provide against antimicrobials, and possibly to persistence within urinary stones [Coker, 2000 et al.; Li X. et al., 2002; Mathoera et al., 2002]. As biofilms provide an environment ideal for exchange of genetic material, including antimicrobial resistance genes, the increased rate of genetic exchange in catheter biofilms also likely contributes to resistance.

TABLE 5. Contribution of virulence determinants to UTI**

| | Entry to the Urinary Tract | | Ascending UTI | | |
	Catheter Colonization	Swarming	Urine	Bladder	Kidneys
Fimbriae	ATFs and/or MRK fimbriae?	MrpJ represses expression of fimbriae	MRP fimbriae, PMI3001	MRP fimbriae, PMFs	MRK and MRP fimbriae
Adhesins	ND	ND	ND	PMI2575	AipA
Motility	Not relevant	*FlgE, *FliF, FliL	*CheW, FlaD	*CheW, *FliF, FlaD	*CheW, *FlgE, *FliF, FlaD
Regulators of gene expression	ND	DisA, HexA, Lrp, Rcs proteins, RppAB, RsmA, Umo proteins, WosA	*AsnC, *HdfR, HexA, *NhaR, UreR	*AsnC, HexA, *HdfR, UreR	*AsnC, HexA, *NhaR, UreR
Urease	Promotes colonization	Coordinately expressed	*UreF	UreC, *UreF	UreC, *UreF
Proteases and Toxins	ND	Lon, HpmA and ZapA (coordinately expressed)	ZapA	Pta, ZapA,	Pta, ZapA, *U32 peptidase family protease, HpmA?
Metabolic Pathways	ND	*AceE, *CyaA, *SdhC (Upregulated)	*CarA,*CbbC, *CyaA, *Edd, *GuaB, *SdaA, *SdhC	*AceE, *CarA, *CyaA, GhdA, *GuaB, *SdhC,	*AceE, *CarA, *CbbC, *CyaA, *Edd, GhdA, *GuaB, *SdhC,
Methal acquisition	ND	ZnuC	ZnuC	HmuR2, Nrp, Pbt, ZnuC, PMI0842	HmuR2, ZnuC,PMI0842, PMI2596
Other	ND	*CpsF, CysJ, DppA, WaaL	DppA, *DsbA, *ExbD, *HemY, *MetN, *MrcA, *NrpG, *ParE, *PpiA, *PstC, *PstS, *SerC, *SurA, *YidA, *PMI1000, *PMI1193, *PMI1448, *PMI3359, *PMI3705	*CpsF,DppA, *DsbA, *ExbD, *MrcA, *ParE, *PstC, *PstS, *SurA, TaaP, *PMI1193, *PMI2014	*CpsF, CysJ, DppA, *DsbA, *ExbD, *MetN, *MrcA, *NrpG, *ParE, *PpiA, *PstC, *PstS, *SufI, *SurA, *PMI0283, *PMI1184, *PMI1193, *PMI3705

ATFs, ambient-temperature fimbriae; GdhA, glutamate dehydrogenase; HpmA, haemolysin; HmuR2, haemin receptor; Lrp, leucine-responsive regulator; MRK, mannose-resistant Klebsiella-like; MRP, mannose-resistant Proteus-like; ND, no data; Pbt, proteobactin; PMFs, Proteus mirabilis fimbriae; Pta, Proteus toxic agglutinin; Rcs, regulator of colanic acid capsule synthesis; Umo, upregulator of flagellar master operon; Ure, urease operon; ZapA, serralysin; ZnuC, zinc uptake protein C. *Identified by signature-tagged mutagenesis. ND, no data.

** [Armbruster and Mobley, 2012]

Natural resistance of Proteus to Polymyxins

Proteus spp. are naturally resistant to polycationic, cyclic antibiotics, like polymyxins. These antibiotics, due to the positive charges in the structure, bind negatively charged surfaces of bacterial cell envelopes (LPS, capsule antigens, and phospholipids). This results in a disorganization of the outer and inner membranes of Gram-negative bacteria [Vaara and Viljanen, 1985; Vaara 1992]. Polymyxin B (PmxB) interacts with the negatively charged 3-Deoxy-D-manno-oct-2-ulosonic acid (Kdo)-lipid A region of LPS [Morrison and Jacobs, 1976; Schindler and Osborn, 1979]. The relationship between the composition of LPS and the ability to bind polycationic antibiotics by *Enterobacteriaceae* was demonstrated [Vaara and Viljanen, 1985]. Complete substitution of the ester-linked phosphate group of lipid A by L-arabinoso-4-amine (L-Ara4N) led to the resistance of bacteria to PmxB action. This modification results in a less negatively charged bacterial outer membrane, decreasing the electrostatic binding of generic cationic antimicrobial peptides (CAPs) to the bacterial cell wall. This also explains the natural resistance to this antibiotic of several *P. mirabilis*, *M. morganii*, *P. rettgeri*, and *S. marcescens* strains, since LPS of these bacteria contain L-Ara4N [Basu et al., 1986]. In most Gram-negative bacteria synthesis of this sugar is dispensable under most growth conditions and the sugar is not normally found in the lipid A molecule. A recent study [Vinogradov, 2011] has shown that *Proteus mirabilis* HI4320 produce L-Ara4N constitutively as part of their LPS molecule, placing the sugar in both the lipid A and core oligosaccharide Kdo portions of the molecule. The constitutive presence of this sugar in the LPS molecule could be a major determinant of antimicrobial peptide resistance in *P. mirabilis* HI4320. Moreover, it was shown that a *P. mirabilis* mutant, which lacked L-Ara4N residues, appeared to be sensitive to PmxB activity [Kaca et al., 1990]. It has been demonstrated that the L-Ara4N present in the inner core region of LPS (attached to Kdo residue) can play a decisive role in the decreasing of binding of PmxB, which results in the resistance of bacteria to this antibiotic [Boll et al., 1994].

Vaccine strategies

Due to the severe complications of *P. mirabilis* UTI and the finding that infection does not protect against subsequent UTI [Li X. et al., 2004a], attention has been focused on vaccine development. Most vaccine studies have utilized fimbrial subunits as fimbriae contribute to infection and elicit high antibody titers, and initial research determined that intranasal immunization provided greater

protection than other immunization routes [Scavone et al., 2004; Li X. et al., 2004b].

As none of the vaccine candidates provide complete protection against UTI, the ideal *P. mirabilis* vaccine may need to incorporate several virulence determinants in a live attenuated vaccine. Furthermore, a vaccine that impacts colonization and encrustation of the catheter surface or motility may be ideal to limit entry of both *P. mirabilis* and other non-motile species to the urinary tract, thereby limiting persistence of urease-negative species by removing a major cause of catheter encrustation and blockage.

LIPOPOLYSACCHARIDE (LPS)

Biological significance

LPS is the major component of the outer leaflet of Gram-negative bacteria (Figure 6). One bacterial cell contains approximately 3.5×10^6 LPS molecules occupying an area of $4.9 \ \mu m^2$. As the surface of an *E. coli* cell amounts to $6.7 \ \mu m^2$ it appears that three-quarters of the bacterial surface consists of LPS, the remaining area being filled by proteins [Nikaido and Vaara, 1987].

The outer membrane reacts to changes of the environment, inhibits the entrance of toxic compounds (such as antibiotics), plays an important role in nutrient transport, and mediates the physiological and pathophysiological interaction of bacteria with host organisms. Because of the fundamental role in the organization and maintenance of the structure of the external membrane, the presence of the LPS is necessary for the structural integrity, and therefore for the survival of the Gram-negative bacteria. In fact, mutants unable to form LPS are not viable.
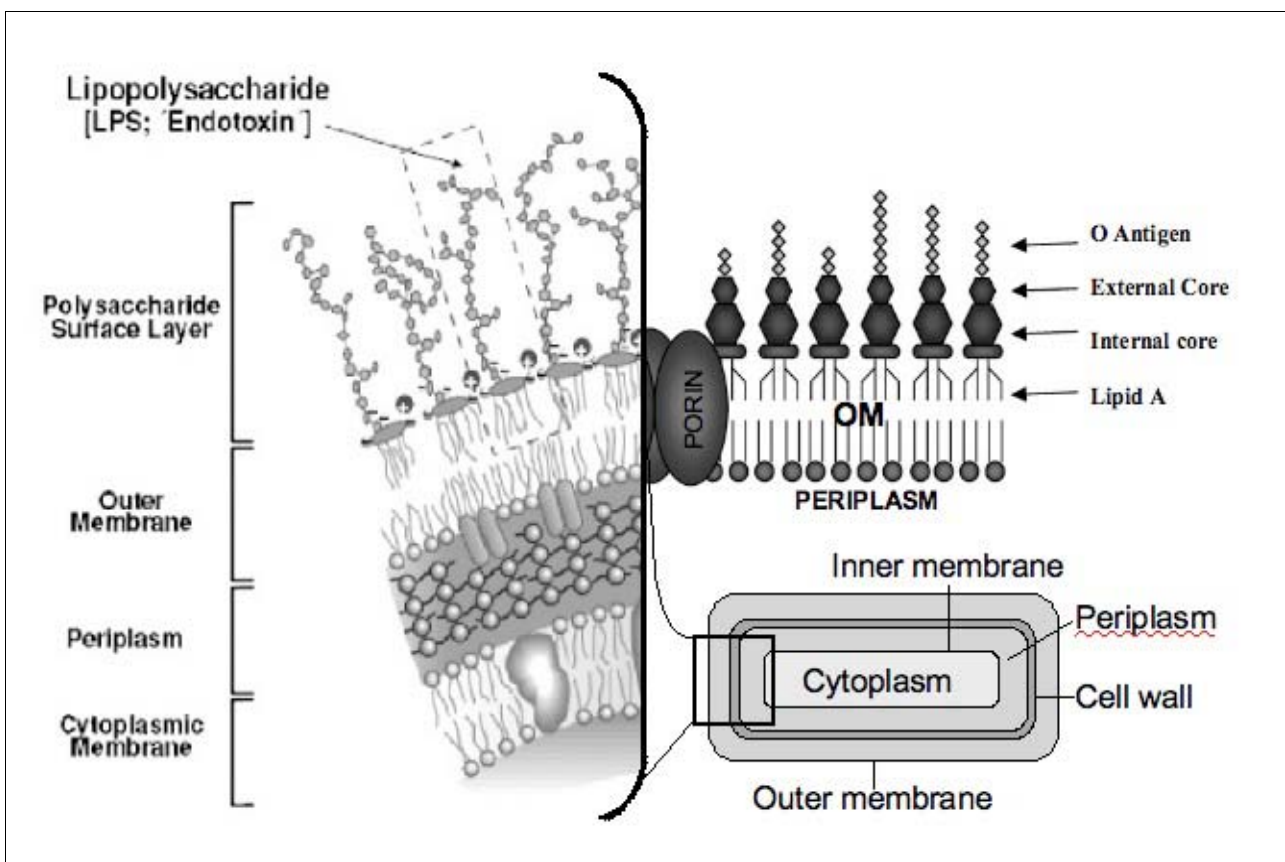
Figure 6: The composition of a Gram negative membrane. The inner or cytoplasmic membrane surrounds the bacterial cell, the periplasm, which contains peptidoglycan, is surrounded by the OM. LPS is embedded in the outer leaflet of the OM and is composed of three distinct regions: Lipid A, oligosaccharide core, and O antigen.

The outer membrane is an asymmetric lipidic bilayer, with phospholipids on its inner surface, and LPS on the outside (Figure 6). As LPS is in direct contact with the medium, it is the most important surface antigen in Gram-negative bacteria. Because of this crucial role and its exposed position, LPS represents also an ideal target both for the attack of disease-producing bacteria by antibodies and other immunological or pharmacological agents.

LSPs are large molecules consisting of a lipid and a polysaccharide domain, joined by a covalent bond. LPS is oriented such that his hydrophobic component (lipid A) forms the outside of the membrane and serves as anchor for LPS, and, at the same time, it remains hidden by polysaccharide components of the molecule [Raetz and Whitfield, 2002].

LPS are endotoxins, well-known pathogenic factors of Gram-negative bacteria, which cause a broad spectrum of pathophysiological effects such as fever, hypotension, disseminated intravascular coagulation, and lethal shock [Rietschel et al., 1994]. Endotoxin can be released from cell surfaces of bacteria during their multiplication, lysis, and death. Such a free LPS is a bioactive molecule and acts through its biological center (lipid A component) on various cell types, of which macrophages and monocytes are the most important. Low doses of endotoxins are thought to be beneficial for the host, causing immunostimulation and enhanced resistance to infections and malignancies [Vogel and Hogan, 1990]. On the other hand, the presence of a large amount of endotoxin in the bloodstream, as observed during severe Gram-negative bacterial infections leads to pathophysiological reactions such as fever, leukopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation, and multi-organ failure; the resulting septic shock syndrome has a mortality rate of about 20-50% [Dal Nogare, 1991]. The harmful as well as the beneficial host responses to endotoxin are not induced directly, but are rather mediated by production of immune modulator molecules. In fact, endotoxin exerts his profound biological effects stimulating his target cells in the host to produce and release endogenous mediators. Among the immune system's humoral components that interact directly with the LPS it is possible to highlight the high density lipoprotein (HDL), the protein sCD14 (soluble form of the receptor CD14), and, above all, the LPS binding protein (LBP), that increases dramatically the activity of LPS and lipid A. The HDL acts by attenuating the effects caused by the LPS at a systemic level, exerting a detoxifying function, while sCD14 binds to LPS and activates the production of cytokines in endothelial cells, which do not express the CD14 receptor. LBP protein transports LPS to various targets cell, acting as a biological amplifier that allows the host to detect small amounts of LPS, to activate its defense system and at last to face the invasion of microorganism [Rietschel et

al., 1996].

The cellular targets that can be affected by the presence of LPS are mainly monocytes and macrophages, in which the LPS is bound to CD14 protein on macrophage surface by protein LBP. This leads to LTR4 receptor (toll like receptor 4) activation, resulting in its turn in the production of biologically active lipids (prostaglandins, thromboxane A_2, and platelet-activating factor), oxygenfree radicals (O_2-, H_2O_2, and NO), and peptide mediators (TNFα, IL-1, IL-6, IL-8, and IL-10). These mediators act independently or in concert, and, depending on their level in the macroorganisms, they elicit beneficial (adjuvant activity) or detrimental (shock syndrome) effects [Rietschel and Brade, 1992]. Moreover, LPS stimulates the phagocytic ability of polymorphonuclear leukocytes (important in the early steps nonspecific host defense system), actives proliferation, differentiation and antibody secretion in B lymphocytes. All these LPS-induced effects are desirable to eliminate localized infections.

LPS molecules are also responsible for the resistance of Gram-negative bacteria to certain kind of compounds. The O-antigen and the core form a hydrophilic barrier, hardly permeable for hydrophobic compounds and antibiotics. This barrier weakens the effectiveness of the polycationic peptides, avoiding the access to the cell across the membrane. Also, in general, the chain length of LPS plays an important role in the interaction of external compounds with the cell, in such way that long polysaccharide chains inhibit this interaction [Caprodici et al., 1994]. Also, from bacterial perspective, endotoxin plays an important role in virulence as it can confer bacterial resistance against bactericidal action of serum and intracellular killing by phagocytes [Rietschel et al., 1992].

The biological properties of endotoxin described above also refer to *Proteus* LPS.

The obvious medical significance of endotoxin and its wide range of immunological and biological properties, explains the still expanding scientific interest in this molecule, from the standpoint of their structure, in terms of biosynthesis, genetic organization and biological action.

General structure

With regard to structure, all LPSs possess the same general chemical architecture independent to the bacterial activity (pathogenic, symbiotic, commensal), ecological niches (human, animal, soil, plant, water), or growth conditions [Raetz and Whitfield, 2002; Holst and Müller-Loennies, 2007] (Figure 7).
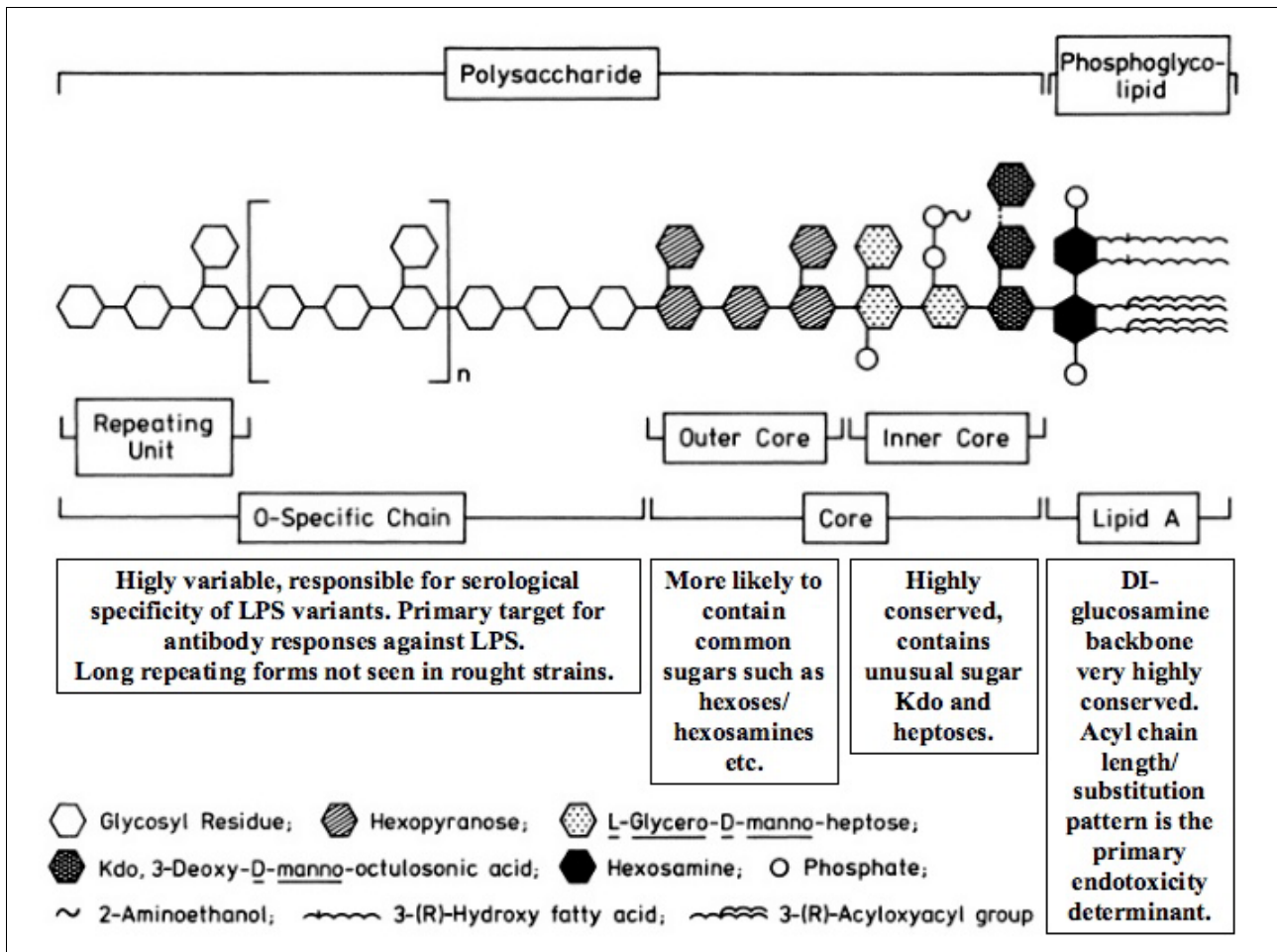
Figure 7: General structure of Gram-negative LPS.

Endotoxins are amphiphilic molecules consisting of a hydrophilic polysaccharide part, and a covalently bound hydrophobic and highly conserved lipid component, termed lipid A. The polysaccharide part can be conceptually divided into two sub-domains: one more internal and conserved, the core region, and one more external and highly variable, the O-specific chain, named also O-antigen for its immunogenic properties [Lüderitz et al., 1982]. The LPS core represents the point of attachment for the O antigen, and can be subdivided further into an inner and outer core (Figures 6 and 7). These three regions can be differentiated by their chemical structure, degree of conservation, biosynthetic pathways and genetic determination. LPS structural variability gradually decreases from the side chain O, surface exposed, to the lipid A region, covered by the polysaccharide and immersed in the outer membrane. The reason for this range can be the evolutionary pressure performed by phagocytic cells and antibodies on Gram-negative bacteria [Nikaido, 1970]. It is plausible that microorganisms try to avoid this pressure through the variation of the exposed surface structure, that is the O antigen and to a lesser extent, the outer core [Rietschel et al., 1996]. On the other hand, the high conservation of the overall characteristics of

33

lipid A and the inner core may reflect the restrictions imposed by its essential role in maintaining the integrity of the outer membrane [Heinrichs et al., 1998].

Complete LPS molecules that present lipid A, core and O antigen are known as S forms of LPS, by the Smooth phenotype showed by the colonies that synthesize the complete LPS on agar plates. However, bacteria that have lost the ability to synthesize the O antigen or in which the polysaccharide fraction only comprises the core, complete or truncated, form colonies with Rough edges on agar plates, so these incomplete LPS molecules are termed R [Whitfield et al., 1997].

Lipid A

Lipid A main features

Lipid A is the highly immunoreactive endotoxic center of LPS. It is also the innermost of the three regions of the LPS molecule, and the most conserved region of LPS, it may exist, however, small variations from species to species that can affect their biological properties, as variations in in the length, position, and number of the fatty acids. Lipid A is essential for the viability of most Gram-negative bacteria: it anchors the LPS in the outer leaflet of the membrane, and has a fundamental role in the integrity of the whole outer membrane. Also, there is a specific interaction between Lipid A and some membrane proteins, so that it contributes probably to the membrane correct folding [de Cock et al., 1999]. The interaction between the negative charges of the lipid A with divalent cations (Mg^{2+} and Ca^{2+}) is indispensable for the stabilization of the membrane. In fact, the neutralization and bridging of negative charges by divalent cations is a major factor in determining the lateral interaction between neighboring lipids and hence the fluidity and melting behavior of the bilayer [Nikaido, 2003].

The indispensability of this region of LPS, its high conservation and its role as endotoxin have attracted considerable interest, in the perspective of possible new bactericidal agents, and drugs against septic shock [Onishi et al., 1996; Rietschel et al., 1994].

Lipid A chemical structure

The lipid A basic structure is a backbone made of a disaccharide of D-glucosamine (GlcN) β-(1→6) inter-linked. Two phosphate groups are attached to the backbone at the 1 and 4' positions. The disaccharide is substituted with fatty acids (up to seven residues) ester-linked at positions 3 and 3'

and amide-linked at positions 2 and 2'. The amino linked fatty acids are exclusively R-3-hydroxymyristate, while the ester linked can vary their length (myristate, laurate or palmitate). The residues of R-3-hydroxyacyl of positions 2' and 3' are esterified with other two acyl groups of 12 or 14 carbon atoms (laurate or myristate) [Raetz, 1990]. The grade of acylation and the fatty acid distribution determines the three-dimensional structure, that is the conical or cylindrical molecular shape, of lipid A [Seydel et al., 1993; Seydel et al., 2000; Brandenburg et al., 1996], which is correlated to its biological activity. The lipid A molecule is glycosylated in position 6' by 3-Kdo), that is already a part of the LPS internal core (Figure 8).
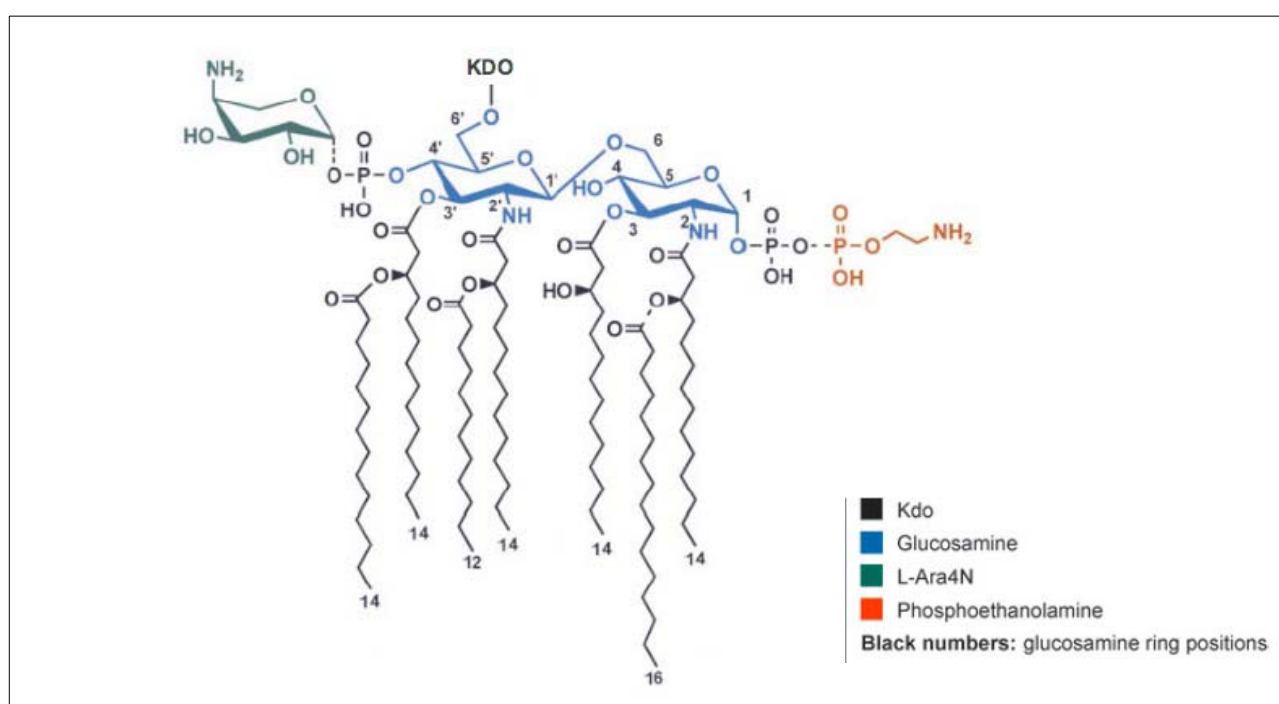
Figure 8: Chemical structure and most frequent covalent modifications of Lipid A in *E. coli* K-12 and *Salmonella* [Raetz C.R., 1990]. The known covalent modifications of lipid A are indicated by the substituents with the dashed bonds. Under some conditions, the positions of the PEtN and L-Ara4N substituents are reversed (not shown). Lipid A species with two PEtN units or two L-Ara4N moieties may also be present [Gibbons et al., 2005]. When grown with high concentrations of divalent cations, both *E. coli* and *Salmonella* make a subset of lipid A species with a diphosphate group at the 1-position (not shown). Adapted from Raetz et al., 2007.

Although the structure of lipid A has a high degree of conservation, it may be modified in response to environmental changes. Most of the enzymes necessary for the covalent modification of lipid A are located in the periplasm or outer membrane. In *E. coli* and *Salmonella enterica* sv. Typhimurium (*S. typhimurium*), several modifications have been described: phosphoethanolamine (PEtN), L-Ara4N, diphosphate and palmitate (16 carbon atoms) addiction, laurate replacement with palmitoleate (in *E. coli*) and hydroxylation or removal of a myristate chain (in *Salmonella*) [Raetz et al., 2007].

Lipid A biosynthesis

The biosynthetic pathway of lipid A is a process that takes place in the cytoplasm and in the cytoplasmic side of the inner membrane.

The enzymology and molecular genetics of the conserved steps of lipid A biosynthesis are best characterized in *E. coli* [Raetz, 1990; Raetz, 1996; Wyckoff et al., 1998], but the nine constitutive enzymes involved are highly conserved in most Gram-negative bacteria [Raetz and Whitfield, 2002]. The precursors required for the synthesis of lipid A are: uridine diphosphate (UDP)-N-acetylglucosamine (UDP-GlcNAc), R-3-hydroxymyristoyl coupled with acyl carrier protein (ACP), myrystoyl-ACP, lauroyl-ACP, adenosine-5'-triphosphate (ATP) and CMP-Kdo. The precursors UDP-GlcNAc and R-3-hydroxymyristoyl are also involved in other biosynthetic pathways of important bacterial compounds. As a matter of fact, the UDP-GlcNAc is involved in the biosynthesis of peptidoglycan [Morrison and Ryan, 1992], of *Enterobacteriaceae* common antigen (ECA) [Meier-Dieter et al., 1992] and of the O antigen (Figure 9), while R-3-hydroxymyristoyl is necessary for the synthesis of membrane phospholipids [Raetz, 1996].

The formation of the UDP-GlcNAc precursor is catalyzed by a bifunctional enzyme encoded by the gene *glmU*. This enzyme, GlmU, catalyzes the acetylation of glucosamine-1-phosphate (GlcN-1-P), resulting in the formation of GlcNAc. The same enzyme activates GlcNAc to form UDP-GlcNAc [Mengin-Lecreulx and Van Heijenoort, 1994].

The first reaction of lipid A biosynthesis is the acylation of the sugar nucleotide UDP-GlcNAc. LpxA catalyses the first step transferring a 3-hydroxytetradecanoic acid (β-hydroxymyristate) [14:0(3-OH)] chain to UDP-GlcNAc [Galloway and Raetz, 1990], a precursor of both peptidoglycan and lipid A. The second and first committed step of the pathway is catalyzed by the zinc metalloenzyme LpxC [Young et al., 1995; Jackman et al., 1999]. The enzyme LpxC catalyses the irreversible hydrolysis of the amide linkage at position 2 of the backbone glucosamine by removing an acetyl group. LpxC is highly conserved in all Gram-negative bacteria and, since the enzyme has no homology to mammalian deacetylases or amidases, it has been an attractive target for the development of novel antibacterial compounds [Kline et al., 2002; McClerren et al., 2005]. Once deacetylated, a second 14:0(3-OH) chain is added by LpxD to form UDP-2,3-diacylglucosamine [Kelly et al., 1993].
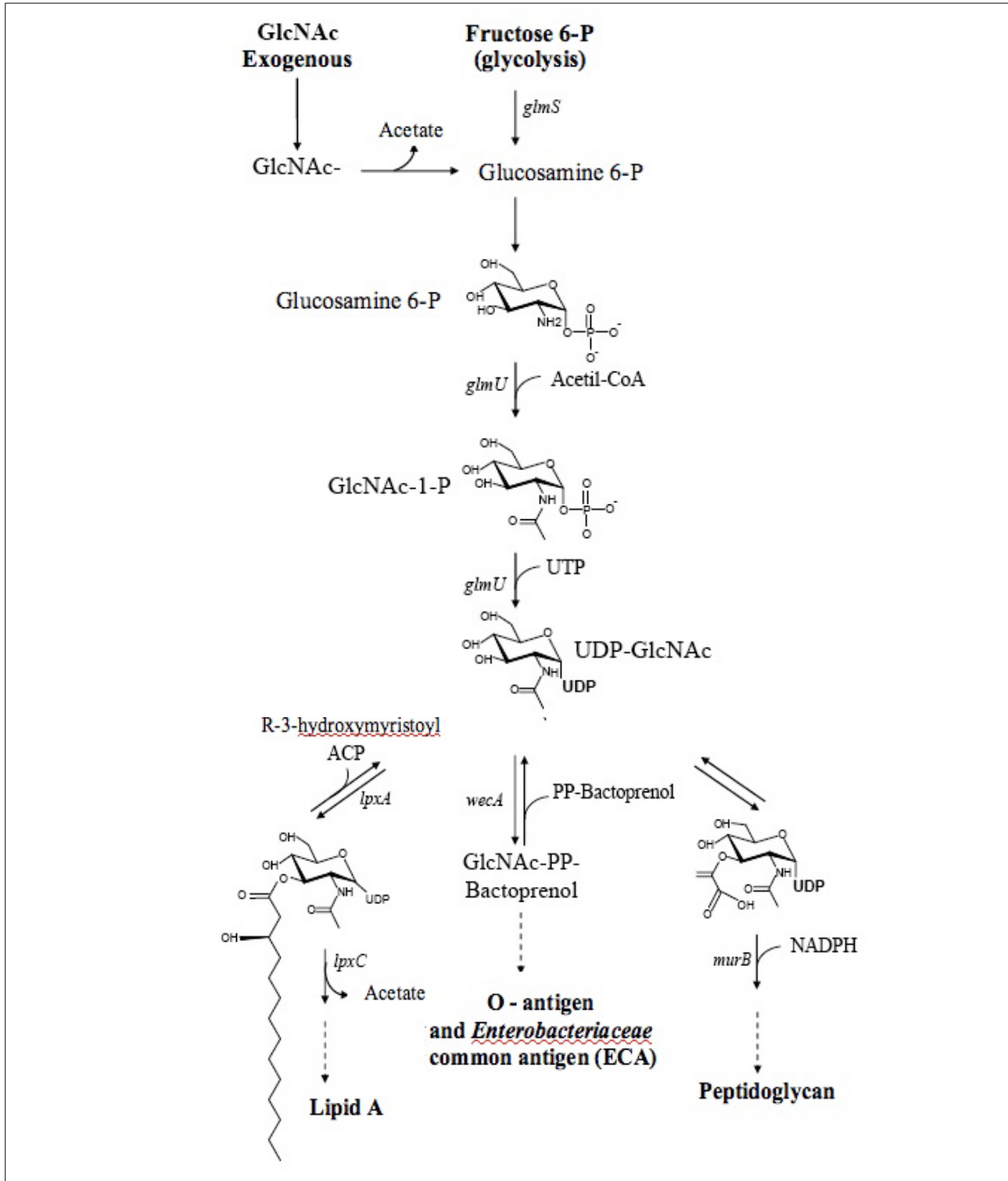
Figure 9: Link between lipid A biosynthetic pathway and the biosynthesis of other structures

Both LpxA and LpxD utilize acyl-ACP as the obligate acyl donor [Galloway and Raetz, 1990; Kelly et al., 1993]. Unlike acyltransferases involved in phospholipid biosynthesis, these enzymes usually have a high degree of specificity for their acyl donor with regards to fatty acyl chain length and

extent of saturation. The fourth step is cleavage of the pyrophosphate linkage of UDP-2,3-diacylglucosamine by LpxH to form uridine monophosphate (UMP) and 2,3-diacylglucosamine 1-phosphate (lipid X) [Babinski et al., 2002] (see Figure 10).

Next, LpxB condenses one molecule of UDP-2,3-diacylglucoasmine with one molecule of lipid X and releases UDP resulting in the formation of the characteristic β-(1′→6)-glycosidic linkage present in lipid A molecules [Crowell et al., 1986]. The early steps of the pathway occur in the cytoplasm, however, both LpxB and LpxH are peripherally associated with the inner membrane. The final steps of the constitutive pathway are catalyzed by four integral membrane proteins: LpxK, WaaA (KdtA), LpxL (HtrB) and LpxM (MsbB). Since these reactions all require cytolosic factors, the active sites of these enzymes are presumably in the cytoplasm. A specific kinase, LpxK, catalyses the phosphorylation of the 4′-OH group of the tetra-acyl-disaccharide-1-phosphate intermediate thereby producing lipid IV_A, a key lipid A precursor [Garrett et al., 1997], which is the first intermediate of the route featuring some of the biological properties of endotoxin, and also the smallest lipopolysaccharide molecule that can support viability.

It seems that the presence of this phosphate group at position 4' is essential for subsequent transfer to the growing molecule of two residues of Kdo, that are already part of the inner core. This indicates a close relationship between the LPS lipid A and the inner core synthesis. The transfer of two Kdo residues onto position-6′ of the disaccharide backbone is catalyzed by a bifunctional glycosyltransferase (coded by *waaA* gene) and utilizes cytidine monophosphate (CMP)-Kdo as the nucleotide sugar donor [Clementz and Raetz, 1991]. Kdo is present in nearly all Gram-negative bacteria, even if interestingly, the number of Kdo residues transferred differs in various bacterial species. For example, the Kdo transferase of *H. influenzae* [White et al., 1997] transfers a single Kdo residue, whereas in *Chlamydia* and *Chlamydophila* spp. it can transfer three or even four residues to the disaccharide backbone [Brabetz et al., 2000]. The final steps of the *E. coli* biosynthetic pathway involve the addition of two secondary acyl chains by LpxL and LpxM. The protein LpxL (HtrB) transfers a dodecanoic acid (laurate) (12:0) to the 2′-position [Clementz et al., 1996] and LpxM (MsbB) a tetradecanoic acid (myristate) (14:0) to the 3′-position [Clementz et al., 1997]. These enzymes utilize acyl-ACPs as their substrate and function only after the addition of the Kdo residues. Like the early acyltransferases (LpxA and LpxD), LpxL and LpxM are very selective for a specific acyl chain length and degree of saturation [Clementz et al., 1996; Clementz et al., 1997; Vorachek-Warren, 2002]. In some bacteria, a third acyl transferase, termed LpxP, is expressed during cold shock and replaces the function of LpxL in the constitutive pathway [Carty et

al., 1999]. The LpxP protein incorporates a 9-hexadecenoic acid (palmitoleate) (16:1) chain which is thought to aid in adjusting the fluidity of the outer membrane when shifted to lower growth temperatures. Finally, it is important to note that not all Gram-negative bacteria contain two late acyltransferases. For example, *Chlamydia trachomatis* contains a single LpxL orthologue and produces a lipid A with a single secondary acyl chain at the 2′-position [Rund et al., 1999]. Interestingly, some Gram-negative organisms, like *Campylobacter jejuni*, synthesize a lipid A bearing two secondary acyl chains [Moran et al., 1991], but contain a single late acyltransferase orthologue. The differences in the acyl chain specificity and number of late acyltransferases contributes greatly to the diversity of lipid A structures found in Gram-negative bacteria. Since the secondary acyl chains of lipid A are critical for the endotoxicity of the molecule [Golenbock et al.,1991; Somerville et al., 1996; Nichols et al., 1997] these differences are also of significant biological importance.
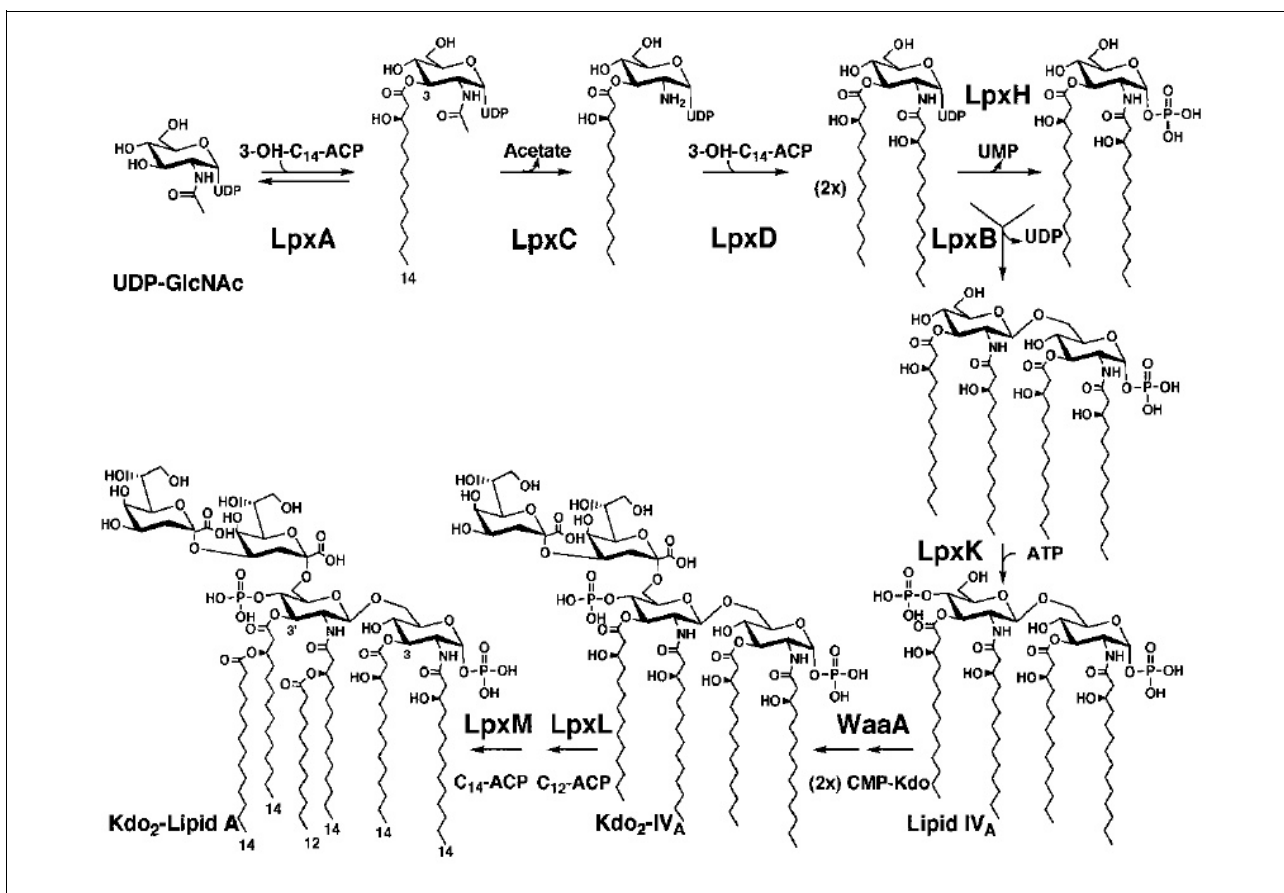
Figure 10: The constitutive biosynthetic pathway of the Kdo$_2$-lipid A domain of LPS. The structures shown are for the biosynthesis of Kdo$_2$-lipid A in *E. coli* K-12 and *S. enterica* sv. Typhimurium. The names of the major intermediates are indicated and the nine enzymes are listed below and above reaction arrows. Acyl-acyl carrier protein serves as the obligate acyl donor for the various acyltransferases. The pathway is also referred to as the Raetz pathway, named after its discoverer by Christian R.H. Raetz.

Covalent Modification of the KDO-lipid A domain of LPS

Following the conserved pathway, the Kdo-lipid A domain of LPS can be modified by latent enzymes that make up the variable component of lipid A biosynthesis [Trent et al. 2006]. In most cases, these modifications occur at the periplasmic face of the inner membrane or in the outer membrane, separating the constitutive and variable pathways. How these modifications are regulated and the number and type of modifications vary for each bacterial species contributing towards the diversity of microbial cell surfaces [Trent et al., 2006; Raetz et al., 2007].

Modification to the disaccharide backbone of lipid A centers on the removal or decoration of the lipid A phosphate groups. The lipid A disaccharide of *E. coli* K-12 typically contains a mono-phosphate group at positions 1 and 4', however, approximately 20–30% of the lipid A contains an unsubstituted diphosphate group at the 1-position [Raetz and Whitfield, 2002]. *E. coli* LpxT phosphorylates the 1-position of Kdo_2-lipid A forming a 1-diphosphate lipid A species [Touzé et al., 2008]. The reaction occurs on the periplasmic side of the inner membrane and undecaprenyl pyrophosphate (C55-PP) serves as the phosphate donor releasing undecaprenyl-phosphate (C55-P). The latter is an essential carrier lipid required for the synthesis of various bacterial polymers [Bouhss et al., 2008], such as peptidoglycan and the O-antigen domain of LPS.

Two additional enzymes, ArnT and EptA, modify the phosphate groups of *E. coli* and *S. enterica* lipid A catalysing the addition of amine-containing residues. The protein ArnT transfers the sugar, 4-amino-4-deoxy-l-arabinopyranose (l-Ara*p*4N) [Trent et al., 2001a] and EptA serves as a PEtN transferase [Trent and Raetz, 2002; Lee et al., 2004]. Expression of ArnT and EptA is under the control of the transcription factor PmrA that is induced during growth under conditions of low pH and high Fe3+ concentration [Groisman et al, 1997; Wosten et al., 2000]. Attachment of these positively charged residues to lipid A can provide resistance to some cationic antimicrobial peptides including polymyxin B [Helander et al., 1994; Nummila et al., 1995; Gunn et al., 1998; Cox et al., 2003; Tzeng et al., 2005]. It is important to note that formation of the 1-diphosphate species by the action of LpxT does not occur under growth conditions that promote the addition of l-Ara*p*4N and PEtN [Guo et al., 1997; Zhou et al., 2000]. Like LpxT, both ArnT and EptA contain multiple transmembrane domains and modify lipid A on the outer surface of the inner membrane [Doerrler et al., 2004].

ArnT utilizes the sugar donor undecaprenyl-phosphate-l-Ara*p*4N [Trent et al., 2001a] that is first synthesized within the cytoplasm and transported across the inner membrane [Raetz et al., 2007].

The l-Ara*p*4N residue is transferred primarily to the 4-phosphate group of *E. coli* and *S. enterica* sv. Typhimurium lipid A [Trent et al., 2001a]. In pathogens lacking the phosphoethanolamine modification, for example *Yersinia pestis* and *P. aeruginosa*, l-Ara*p*4N can be found attached to both phosphate groups [Trent M.S et al., 2006]. Interestingly, most Gram-negative bacteria produce either phosphoethanolamine-modified or l-Ara*p*4N-modified lipid A, whereas *E. coli* and *S. enterica* sv. Typhimurium contain both residues. In different organisms, l-Ara*p*4N addition to lipid A has been correlated with increased resistance to cationic antimicrobial peptides and l-Ara*p*4N-deficient mutants of *S. enterica* sv. Typhimurium show reduced virulence [Gunn et al., 1998; McCoy et al., 2001; Moskowitz et al., 2004; Winfield et al., 2005]. Most of *Proteus* strains have 4-amino-4- deoxy-L-arabinosyl residues (AraN) both Kdo and lipid A regions [Sidorczyk et al., 1983; Vinogradov et al., 1994; Boll et al., 1994], which may provide them with their resistance to polymyxin B.

The addition of phosphoethanolamine groups to lipid A occurs in a large number of pathogenic bacteria. Phosphatidylethanolamine serves as the phosphoethanolamine donor substrate [Trent et al., 2001b; Trent and Raetz, 2002]. In *E. coli* and *S. enterica*, EptA adds phosphoethanolamine predominantly to the 1-phosphate group [Zhou et al., 2000]. *S. enterica eptA* mutants show only slight increases in susceptibility to polymyxin B suggesting that phosphoethanolamine addition is not critical for resistance to cationic antimicrobial peptides [Lee et al., 2004]. On the other hand, loss of phosphoethanolamine addition in *N. meningitidis* [Tzeng et al., 2005] leads to significant polymxyin sensitivity. *N. meningitidis* lack the enzymatic machinery to produce l-Ara*p*4N-modified lipid A and these bacteria modify both lipid A phosphate groups with phosphoethanolamine. Further investigation will be required to determine the roles of phosphoethanolamine modification in cationic antimicrobial peptide resistance and pathogenesis.

In *S. enterica* sv. Typhimurium, two different virulence-associated two-component regulatory systems are involved in adding these modifications. The first is the PhoP/PhoQ system encoded by genes located in the 25-min region of the *S. Typhimurium* map [Sanderson and Demerec, 1965], which encodes resistance to a number of CAMPs [Miller et al., 1989]. The second is the PmrA/PmrB system [Roland et al., 1993] encoded by genes in the 93.5-min region, which is involved in resistance to polymyxin and adds PEtN and Ara4N to lipid A. PmrA/PmrB is activeted by PhoP/PhoQ, but it is also activated independently by growth in mildly acidic conditions [Soncini et al., 1996].

Several enzymes that alter the acylation pattern of the lipid A moiety of LPS have also been

described [Trent et al., 2006].

Lipid A genetic organization

As may be expected for such an important process, most of the genes involved in the synthesis of lipid A disaccharide and its acylation, initial phosphorylation and export are located in growth-related gene clusters and macromolecular synthesis operons. This is an arrangement which presumably allows them to share growth-related regulatory signals. The basic lipid A pathway and the genes which determine it are strongly conserved across strain and species lines, and at this level of LPS synthesis there is almost no biochemical or genetic polymorphism. In contrast the genes involved in some of the late steps of lipid A modification are located in special environmentally regulated gene clusters termed virulence clusters.
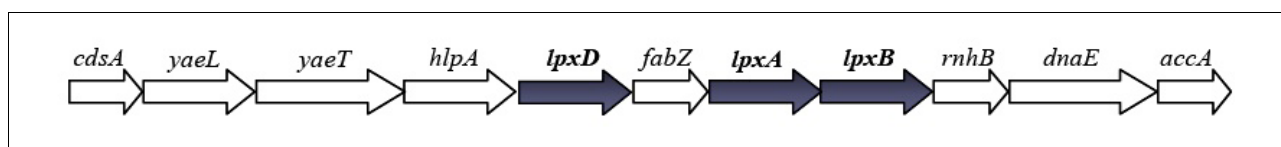
Figure 11: Schematic Macromolecular Synthesis Operon II operon in *E. coli* K-12. Genes involved in lipid A biosynthesis are shaded. Figure adapted from Genevrois, 2003.

Most of the genes necessary for production are scattered throughout the bacterial genome due to the intermediary molecules sharing with other macromolecules biosynthetic pathways. Only some of them (*lpxA*, *lpxD* and *lpxB*) are grouped in a complex operon called Macromolecular Synthesis Operon II (OSMII) (see figure 11). In this operon it is possible to find, among its eleven genes, also genes for DNA replication, glycerophospholipids biosynthesis, genes required for the biogenesis of the outer membrane, genes involved in the transport and assembly of OMP and genes involved in the regulation of heat shock response [Tomasiewicz, 1990; Genevrois et al., 2003; Bos et al., 2007]. *lpxD* and l*pxA* genes, which encode acyltransferases, are separated by the gene *fabz,* encoding an R-3-hydroxymyrystoil dehydratase related to the biosynthesis of palmitate. Thus, three genes encoding enzymes that use the same substrate are grouped and presumably co-regulated [Schnaitman and Klena, 1993]. *lpxB* gene, which encodes the disaccharide synthase, is located immediately below *lpxA* gene, so that in *E. coli* exists an overlap between the termination codon of the first and the start of transcription of the other, suggesting that they are transcribed and translated in a coupled way [Crowell et al., 1987].

The remaining genes are located in different regions of the chromosome. *lpxC* gene, which encodes

the deacetylase, is in the 3 'end of a large group of genes associated with cell division and protein secretion [Klena and Schnaitman, 1993]. On the other hand the gene *lpxK*, which encodes the kinase that allows the formation of lipid IV_A, is located together with the gene *msbA*, involved in the initial stages of the export of lipid A and phospholipids [Garrett et al., 1997].

The bifunctional enzyme Kdo-transferase is the product of the *waaA* gene, which is located in the *rfa* (*waa*) cluster. The *waaA* gene is adjacent to a long operon, which contains genes for biosynthesis of the lipopolysaccharide core, and is the first gene in a two-gene operon. The second gene is the *coaD* gene, which encodes the rate-limiting enzyme in coenzyme A biosynthesis [Geerlof et al., 1999] (Figure 16). In *E. coli* the *waaA* operon is transcribed divergently from the 10-gene operon, beginning with *wabQ*, wich encodes enzymes involved in synthesis and modification of the LPS core oligosaccharide (see Figure 16). A short non-coding region between *wabQ* and *waaA* is almost identical between *S. typhimurium* and *E. coli* K-12, and presumably contains transcription information for both operons [Schnaitman and Klena, 1993].

In Table 6 are resumed the enzymes required for the biosynthesis of lipid A.

TABLE 6: Enzymes required for the biosynthesis of lipid A.

Enzymes	Function	Genes	Map Location
LpxA	LpxA catalyzes the fatty acylation of UDP-GlcNAc. It requires the thioester R-3-hydroxymyristoyl acyl carrier protein as its donor.	*lpxA*	4.4 (OSMII)
LpxC	LpxC catalyzes the deacetylation of UDP-3-O-(acyl)-GlcNAc which is the actual committed step of lipid A biosynthesis.	*lpxC*	2.3
LpxD	LpxD adds a second R-3-hydroxymyristate chain to make UDP-2,3-diacyl-GlcN.	*lpxD*	4.3 (OSMII)
LpxH	LpxH cleaves the pyrophosphate linkage of UDP-2,3-diacyl-GlcN to make lipid X.	*lpxH*	-
LpxB	LpxB condenses UDP-2,3-diacyl-GlcN with lipid X to form the 10-6-linkage in lipid A.	*lpxB*	4.4 (OSMII)
LpxK	LpxK phosphorylates the 40-position of the disaccharide 1-phosphate generated by LpxB to form lipid IV_A.	*lpxK*	20.9
WaaA	WaaA incorporates two Kdo residues to the 60-position of lipid IV_A	*waaA*	82.0
LpxL	LpxL adds a secondary lauroyl residue to the fatty acid chain at 20-position of lipid A. It prefers acyl-ACP donors	*lpxL*	24.0
LpxM	LpxM adds a secondary lauroyl residue to the fatty acid chain at 30-position of lipid A. It prefers acyl-ACP donors	*lpxM*	41.8

Core

<u>Core main features</u>

As with lipid A, much of our understanding of the structure and biosynthesis of the core oligosaccharide is founded on work in *E. coli* and *Salmonella*, but detailed structures are now available from a variety of organisms with different lifestyles.

In bacteria that produce smooth LPS (S-LPS), the core oligosaccharides are conceptually divided into two regions: inner core (lipid A proximal) and outer core, which provides an attachment site for O polysaccharide (O antigen). Mucosal pathogens often lack O polysaccharides. Instead they produce lipooligosaccharides (LOSs) that contain a recognizable inner core from which extend one or more mono- or oligosaccharide branches (equivalent to the outer core). These extensions determine the serological specificity of LOS.

Within a genus or family, the structure of the inner core tends to be well conserved, and the fact that the core oligosaccharides from distantly related bacteria share structural features in the inner core is a reflection of the importance of the core in outer-membrane integrity. The base structure of inner core is often decorated with non-stoichiometric additions. The varying extent of these modifications contributes to the heterogeneity of LPS molecules extracted from a culture. In many cases, only the structure of the predominant carbohydrate backbone is known for a given core oligosaccharide, and the extent of phosphorylation and non stoichiometric additions are unknown. This is a reflection of the difficulties encountered in structural analyses.

The outer core shows more structural diversity, as might be expected for a region with more exposure to the selective pressures of host responses, bacteriophages, and environmental stresses. However, the extent of structural variation in core oligosaccharides within a given species, or even a genus, is still limited. For example, in *E. coli* there are five known core types (R1, R2, R3, R4, and K-12). All are found among commensal isolates [Amor et al., 2000], whereas the R1 type predominates among strains that cause extraintestinal infections [Gibb et al., 1992] and the R3 type is found in most verotoxigenic isolates such as O157:H7 [Amor et al., 2000; Currie and Poxton, 1999]. These cores differ in the nonstoichiometric inner-core substituents found in a minor fraction of isolated LPS molecules, but the most substantial changes are evident in the outer core. The two known cores from *Salmonella* are quite similar to those of *E. coli*, and genetic data indicate that the structure found in serovar Typhimurium is common to isolates from routine human infections

(subspecies I), whereas the structure found in serovar Arizonae (subspecies IIIA) predominates in other subspecies [Kaniuk et al., 2000].

All the common structural elements tipical of the core-lipid A region, are found to be present also in the LPS's of all *Proteus* strains studied to date. The core residues form the characteristic heptasaccharide fragment, which is present in the LPS of all *Enterobacteriaceae*. The LPS's of *Proteus* are characterized by a structural variability of the core region from strain to strain, and by the occurrence of multiple structural variants within one strain. Structural diversity of the core region is provided by substitution of the common structural element with various mono- and oligo-saccharides and non-sugar components.

The limited structural variation in the core oligosaccharide within a genus is in striking contrast to the hypervariable O polysaccharides and has stimulated interest in the possibility of targeting the core oligosaccharides to generate immunotherapeutic antibodies [Di Padova et al., 1993; Di Padova et al., 1994; Bennett-Guerrero et al., 2000; Stanislavsky and Lam, 1997; Currie et al., 2001]. Similarly, the enzymes responsible for the conserved structural features in inner cores provide potential targets for novel therapeutics.

Core chemical structure

The inner core typically contains residues of Kdo and L-glycero-D-mannoheptose (L,D-Hep) (Figures 7, 12 -13). The Kdo residue, which is the particular residue that links the core region to the lipid A, is the only structural element found in all known core regions, although in some cases a derivative, D-glycero-D-talo-oct-ulosonic acid (Ko), is also present [Isshiki et al., 2003]. Some bacteria contain D-glycero-D-mannoheptose (D,D-Hep) alone or in combination with the more prevalent L,D-Hep, whereas others, like *Rhizobium*, lack heptose entirely [Kadrmas et al., 1996; Kadrmas et al., 1998; Forsberg and Carlson, 1998]. The base structure of inner core is often decorated with nonstoichiometric additions of other sugars and with phosphate (P), pyrophosphorylethanolamine (2-aminoethanol; PPEtN), or phosphorylcholine (PCho) residues.

For its part, the outer core is made up of hexoses, among which there are, mainly, glucose, galactose and N-acetylglucosamine.

Internal and external core are also named sometimes as heptose and hexose region respectively (see Figures 12).
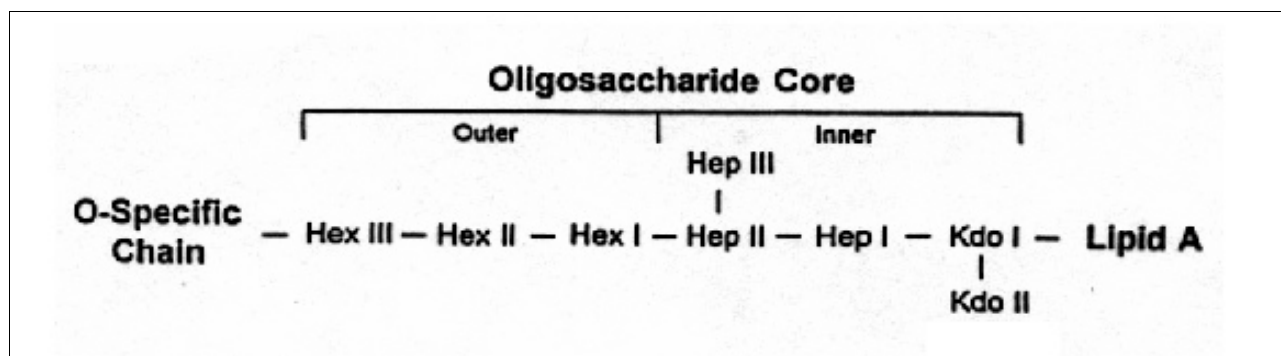
Figure 12: General structure of smooth LPS showing the O-specific chain, inner and outer core, and lipid A. (Kdo =2-keto-2-deoxyoctonate; Hep = heptose; Hex = hexose)

As has been said previously, the core structure tends to be quite conserved, especially inside in a bacterial family. So, it's possible to describe characteristics features common to all *Enterobacteriaceae,* and for this, also shared by *Proteus* spp. They include the lipid A backbone, the disaccharide of Kdo, and the trisaccharide of L,D-Hep. In *P. mirabilis* inner core LPS was found a glucose (Glc) residue linked by a β-1,4 bond to the HepI. This feature is shared by two species of the *Enterobacteriaceae* family, *K. pneumoniae* and *S. marcescens*, and some non enterobacterial Gram-negative bacterial pathogens, such as *V. cholerae, Aeromonas hydrophila, A. salmonicida, H. influenzae, N. meningitidis* and *N. gonorrhoeae.* [Regué et al., 2001; Izquierdo et al., 2002; Coderch et al., 2004; Regué et al., 2005a]. The second Hep (HepII) residue is substituted at O(3) by an α-galacturonic acid (α-GalA) residue. The nature of the substituents at these heptose sites is the basis for subdivision of the core oligosaccharides of *Enterobacteriaceae* into two groups [Holst, 1999].

In those of the *S. enterica* type, position 4 of HepI is not glycosylated and is often phosphorylated, and position 3 of HepII is substituted by an α-Glc residue. Together with *S. enterica*, this group includes *E. coli*, *Shigella*, *Citrobacter*, *Hafnia alvei*, and *Erwinia carotovora*. In the core oligosaccharides of the other type, which occur in *Proteus, K. pneumoniae, S. marcescens*, and *Yersinia enetrocolitica*, the substituent at HepI is β-Glc and the substituent at HepII is different from α-Glc. Substitution of HepII by α-GalA is shared by *Proteus* and *Klebsiella* [Holst, 1999].

In all *Proteus* strains, except for *P. mirabilis* O27, a 4-amino-4-deoxy-β-L-arabinose (β-L-Ara4N) residue is linked to the first Kdo residue at O(8) [Vinogradov et al., 1994; Sidorczyk et al., 1987]. Ara4N also substitutes one or both phosphate groups in lipid A of *Proteus*, [McCoy et al., 2001; Vinogradov et al., 1994] that at position 4' of GlcN. While Ara4N is common in lipid A of various bacteria [Zähringer et al., 1994], it occurs in the LPS core beyond the genus *Proteus* only in *Burkholderia cepacia* and *S. marcescens* [Isshiki et al., 1998].

Substitution of the third Hep (HepIII) residue at O(7) by a β-GalA residue is common in the core of *Proteus*. In strains of *P. mirabilis*, the substitution is stoichiometric, and in some strains β-galacturonic acid (GalA) is partially amidated by aliphatic polyamines. In strains of *P. vulgaris* and *P. penneri*, β-GalA is present in minor structural variants and is completely amidated by residues of spermidine, putrescine or, in *P. penneri* by 14, 4-azaheptane-1,7-diamine. A similar amide of propane-1,3-diamine with GalA has been found in the core part of *Acinetobacter* LPS [Vinogradov et al., 1997]. In *P. mirabilis* O27, one of the structural core variants has neither β-GalA nor HepIII, but the second α-GalA residue is attached to the β-Glc residue of HepI at O(6). Glycosylation of the α-GalA residue at HepII O(2) is characteristic of most *Proteus* strains studied. The substituent is a residue of α-DDHep or a α-Hep-(1→2)-α-DD-Hep disaccharide consisting of two 6-epimeric heptose residues. In a structural variant of the core of *P. mirabilis* O27, α-GalA residue carries a β-Gal-(1→7)-α-DD-Hep disaccharide at O(2). The core of *P. mirabilis* O28, and *P. penneri* 42 and some structural core variants in *P. mirabilis* O3 and *P. penneri* 13 and 14 had no substituent at this position [Vinogradov, 2002]. The HepII residue is phosphorylated stoichiometrically or, in a minority of strains, non-stoichiometrically at O(6) by PEtN. The phosphorylation depends on the amidation of β-GalA: in most cases when the β-GalA residue is amidated, PEtN is absent. The only exception is a structural variant of *P. penneri* 13 and 14, which contain both amidated β-GalA and PEtN at HepII. Remarkably, this variant lacks the α-Hep- (1→2)-α-DD-Hep substituent at O(2) of α-GalA, which is present in all other variants. A mandatory substituent at O(4) of the α-GalA residue is a hexosamine (HexN), which is either α-GlcN or α-GalN.

The reducing end of the *P. mirabilis* outer-core contains a HexN-GalA disaccharide. This feature is shared by *S. marcescens* and *K. pneumoniae* where the HexN is a GlcN [Vinogradov, 2002]. In *P. mirabilis* some strains contain GlcN and others galactosamine (GalN) (Figure 17). The presence of D-galacturonic acid allows to distinguish the *Proteus* core region from *E. coli* and *Salmonella* cores [Kotelko et al., 1983; Radziejewska-Lebrecht et al., 1990]. In *Proteus*, the amino group of HexN may be free, or acylated by a glycine residue (completely in *P. mirabilis* O28, or by 10–20% in *P. penneri* 7, 8, and 14). In some *Proteus* strains the HexN residue is *N*-acetylated [Vinogradov, 2002]. In LPS of most *Proteus* strains studied, HexN carries different saccharides, from mono- to tri-saccharides. These saccharides have diverse composition and include both widespread monosaccharides (Glc, Gal, GlcNAc, and GalNAc) and less common monosaccharides, like D,D-Hep, Kdo, amide of GalA with L-lysine (GalALys), 3-amino-3,6-dideoxy-D-galactose and 4-amino-4,6-dideoxy-D-glucose *N*-acylated by *(R)*-3-hydroxybutyryl (Fuc3NHb) and L-alanyl-L-alanyl

(Qui4NAlaAla) groups, respectively, as well as phosphoethanolamine [Vinogradov, 2002]. Some components are present in nonstoichiometric amounts.

Another, striking structural feature of the *Proteus* LPS core is the occurrence of a new linkage type between monosaccharides, which is formed by an *N*-acetylgalactosamine residue in the open-chain form (Gal*o*NAc) linked to O(4) and O(6) of the neighboring GalN residue to form a cyclic acetal [Vinogradov and Bock, 1999]. Cyclic ketals of pyruvic acid are rather common in bacterial exopolysaccharides [Lindberg, 1998], and a cyclic acetal of arabinose with hederagenin has been found in a triterpene glycoside from *Anemoclema glaucifolium* [Li X. et al., 1995]. However, this type of linkage has not previously been reported between monosaccharide residues in natural glycopolymers and not even been discussed as a theoretical possibility. The Gal*o*NAc residue was found in 11 *Proteus* strains, in all of which it is glycosylated itself at O(4) or O(5) [Vinogradov, 2002].

The high structural heterogeneity of the outer core is associated with the substituents present in the molecule, and the presence of some sugar components in the core is mutually dependent, being presumably a result of a peculiar specificity of glycosyl transferases involved in the stepwise assembly of the core oligosaccharide. These substitutions, arising on the bacterial surface, may influence the surface charge of the cell and may play a role in the outer membrane permeability.

Also, in many cases, the structure of the outermost core region is O-serogroup-dependent. Together with the O-polysaccharide, which has a structurally different repeating unit, this region is likely to contribute to the immunospecificity of *Proteus* strains, further enhancing their antigenic diversity.

The general structure of the core-lipid A region of the lipopolysaccharide of most *Proteus* strains is reported in figure 13.

A

Serotype or strain	R	R1	R2	R3
P. mirabilis O3	α-DD-Hep-(1→6)-α-GlcN	PEtN	H	β-GalA
		PEtN	α-DD-Hep	β-GalA
		H	α-DD-Hep	β-GalAN
P. mirabilis OXK	β-Gal-(1→3)-α-GalNAc-(1→2)-α-DD-Hep-(1→6)-α-GlcN	PEtN	α-DD-Hep	β-GalA
P. mirabilis O6	β-Qui4NAlaAla-(1→3)-α-Gal-(1→6)-α-GlcNAc-(1→4)-α-GalN	PEtN	α-DD-Hep	β-GalA
		H	α-DD-Hep	β-GalAN
P. mirabilis O28	β-GalALys4*P*Etn-(1→3)-β-GlcNAc-(1→6)-α-GlcNGly	PEtN	H	β-GalA
		PEtN	H	H
P. mirabilis O57	β-Qui4NAlaAla-(1→3)-α-GalNAc-(1→6)-α-Glc-(1→4)-α-GalN	H	α-DD-Hep	β-GalA

B

Serotype or strain	R	R1	R2	R3
P. mirabilis O27	β-Glc-(1→5)-(1*S*)-Gal*o*NAc-(1→4,6)-α-GalN	PEtN	α-DD-Hep	H
		PEtN	α-DD-Hep	β-GalA
		H	α-DD-Hep	β-GalAN

Figure 13: General structure of the core-lipid A region of the lipopolysaccharide of most *Proteus* strains (A) and of the LPS of *P. mirabilis* O27 (B). Saccharide substituent R at O(4) of the α-GalA residue H in the core-lipid A regions, and substituents R1–R3. Adapted from Vinogradov, 2002.

Core biosynthesis

The final step of the lipid A pathway can be thought of as overlapping the first steps of the core oligosaccharide pathway in the nature of the reactions. As a consequence of this concept, the Kdo transferase, WaaA is discussed above because, although Kdo is a core component, its assembly in the nascent LPS molecule precedes completion of lipid A.

The general conservation, between different bacteria, of the inner core has facilitated the recognition of the enzymes involved in the transference of the residues that make up this region. In the majority of the cases the heptosyl-transferase WaaC and WaaF genes are implicated in the transfer of L-D-HepI and L-D-HepII to the principal chain of the LPS core, it has also been confirmed that the configuration of L,D-Hep residues in the core is established by the specificity of these heptosyltransferases. These enzymes exhibit preference for ADP-L,D-heptose as their substrate, with ADP-D,D-heptose being used at low efficiency [Zamyatina et al., 2000; Gronow et al., 2000]. ADP-D-mannose can also serve as a surrogate substrate for WaaC [Kadrmas and Raetz, 1998]. WaaC and WaaF homologs have been identified in a variety of bacteria, and in most cases, convincing assignments have been made by complementation of the corresponding mutations in *Salmonella* and *E. coli* [Raetz and Whitfield, 2002]. The pathway for ADP-L,D-heptose synthesis starts with the conversion of sedheptulose-7-phosphate to D,D-heptose-7-phosphate by the isomerase, GmhA. Later a bifunctional enzyme, named HldE adds, by a kinase reaction, a phosphate group to the residue to generate a D,D-heptose-1,7-bisphosphate intermediate. This intermediate is submitted to an additional phosphatase step, carried out by GmhB, to generate D,D-heptose-1-phosphate. Subsequently, the enzyme HldE works once again, to generate ADP-D,D-Hep. The terminal epimerase enzyme (HldD) converts this compound to the preferred heptosyltransferase substrate ADP-L-*glycero*-D-*manno*-heptose. The modification of the inner core region of *E. coli* and *Salmonella* LPS requires the action of three enzymes, WaaP (an LPS kinase), WaaY (an enzyme required for a second phosphorylation), and WaaQ (a transferase that adds the side-branch heptose) [Raetz and Whitfield, 2002]. The WaaP enzyme is the most important of these activities since the modifications must proceed in the strict order WaaPQY [Yethon et al., 1998], and as indicated, mutants lacking WaaP activity exhibit the deep-rough phenotype and are avirulent. The outer core of LPS is synthesized through the sequential transference of the different residues from nucleotides-activated precursors. The process is due to the action of several glycosyl-transferase that act on the cytoplasmatic surface of the internal membrane, where is available not

only the acceptor but also the activated precursors.

All of the *E. coli* and *Salmonella* cores have a glucose residue as the first sugar in the outer core. Biochemical data, LPS structure, and heterologous gene complementation experiments identify WaaG as the 1,3-glucosyltransferase in *E. coli* K-12 [Creeger and Rothfield, 1979] and *Salmonella* [Heinrichs et al., 1998]. As expected, the homologs of WaaG from each of the core types are highly conserved. The subsequent two transferases form 1,3- and 1,2-linkages, respectively, and depending on the resulting structure, the enzymes transfer glucose or galactose to an acceptor of either glucose or galactose. The corresponding transferases are all members of family 8 in the glycosyltransferases classification system and share several highly conserved motifs [Campbell et al., 1997].

These enzymes are best characterised in the core biosynthesis pathway of *E.coli*, *S. enterica*, *K. pneumoniae, S. marcescens* and *A. hydrophila* AH-3 (See Figure 14). With respect to the mechanism leading to the incorporation of the outer core GlcN residue, in *K. pneumoniae* has been described a mechanism involving two different enzymes. First, the glycosyltransferase WabH embeds a GlcNAc residue, from the activated precursor UDP- GlcNAc, and later on the deacetylase WabN removes the acetyl group from the growing core. This mechanism, together with the fact that UDP-GlcNAc is at a much higher concentration than the UDPGlcN, virtually non-existent in the bacterial cell [Gabriel, 1982], indicates that the initial incorporation of GlcNAc and its subsequent deacetylation procedure could be the general mechanism of core biosynthesis in the *Enterobacteriaceae* family [Regué et al., 2005b].

Once synthesized the core, the covalent attachment of O antigen in the periplasmic face of the inner membrane can be carried out. MsbA is an essential ATP-binding cassette (ABC) transporter, closely related to eukaryotic MDR proteins [Karow and Georgopoulos C., 1993]. MsbA is an homodimer with a transmembrane domain, constituted by six *trans*-membrane helices [Doerrler et al., 2001], and a large cavity between the subunits that can accommodate lipid A molecules, and a ATPase domain. Exactly how MsbA would mediate lipid flip-flop by binding and hydrolyzing ATP in the nucleotide binding domains is unclear. It is speculated that the nucleotide binding domains may come together following lipid entry into the putative transport chamber present in the *trans* membrane region, forcing the lipid to move to the periplasmic side of the inner membrane. MsbA has also been associated with the transport of phospholipids through the inner membrane in *E. coli* [Doerrler et al., 2004] and on the other hand, it is capable to recognize and transport a wide range of toxic molecules [Woebking et al., 2005].
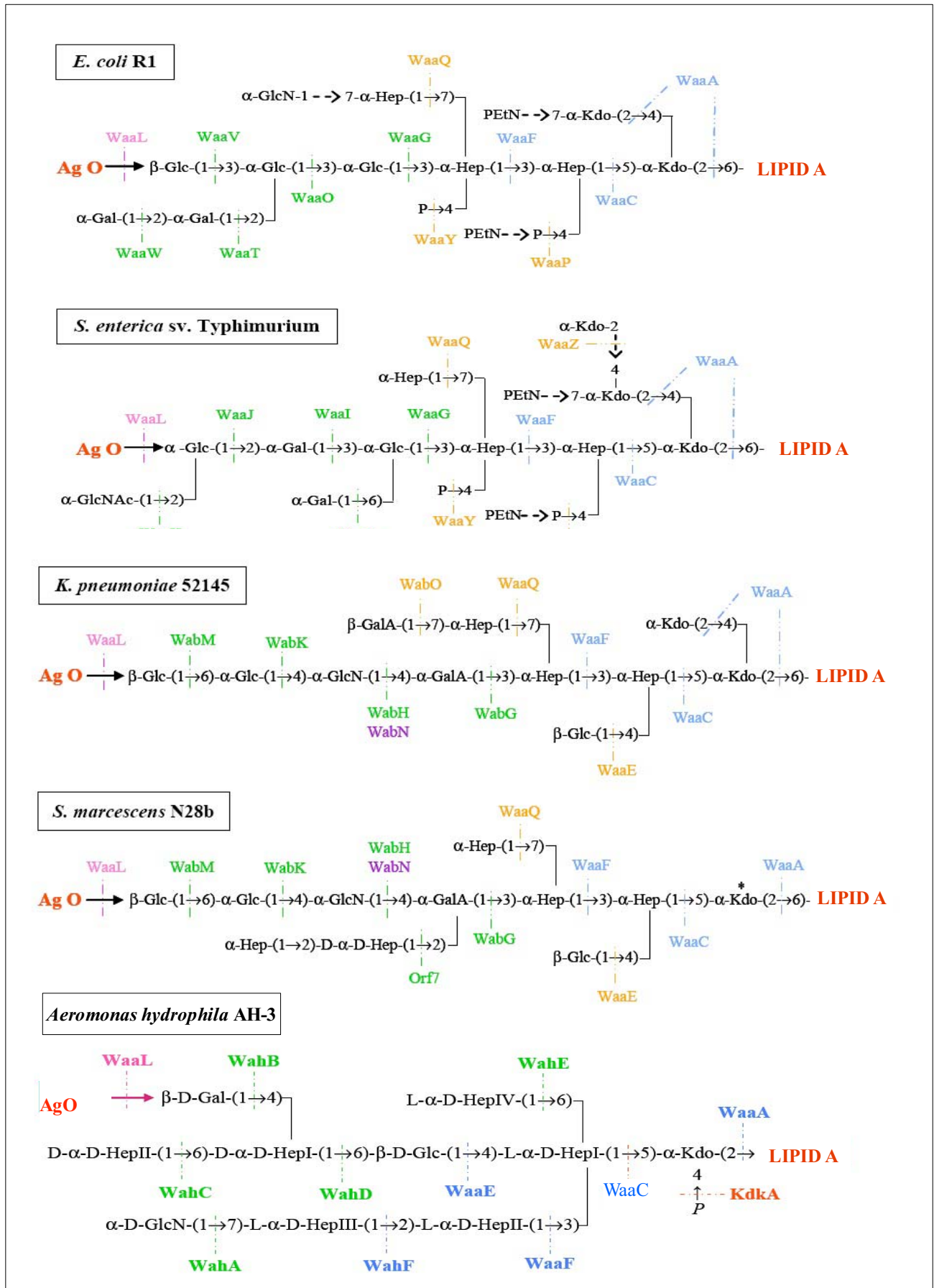
Figure 14: Outline of the chemical structure and known transferases involved in the biosynthesis of the cores of *E. coli* R1 [Raetz and Whitfield, 2002], *S. enterica* sv. Typhimurium [Heinrichs et al., 1998; Kaniuk et al., 2002], *K. pneumoniae* 52145 [Regué et al., 2005a; Regué et al., 2005b; Fresno et al., 2007], *S. marcescens* N28b [Coderch et al., 2004; Regué et al., 2005a; Regué et al., 2005b] and *Aeromonas salmonicida* [Jimenez et al., 2009]. In the figure are marked the enzymes that form: the inner core (blue), the outer core (green), the ones introduced changes (orange), the GlcNAc deacetylase (violet), and ligase (pink). Non-stoichiometric substitutions are indicated by dashed arrows. The hexoses are D-pyranoses and heptoses have the configuration L-D-Hep, unless otherwise indicated.

Core genetic organization

Most of the genes involved in the biosynthesis of the core are contained within a chromosomal gene cluster called *waa*. Other conserved genes, related to the biosynthesis and activation of Kdo and involved in the biosynthesis and activation of heptoses, are located outside the *waa* cluster. In *E. coli* and *Salmonella*, the *waa* locus consists of three operons (Figure 15) mapping between *cysE* and *pyrE* on the chromosome. The operons are defined by the first gene in each transcriptional unit: *gmhD-*, *waaQ-*, and *waaA*. The *gmhD, waaF* and *waaC* genes are required for biosynthesis and transfer of L,D-heptose [Raetz and Whitfield, 2002]. Transcription of the *gmhD* operon in *E. coli* K-12 is regulated by a heat-shock promoter, perhaps indicating a requirement for the heptose domain of LPS, at least in *E. coli* K-12, for growth at elevated temperatures [Raetz, 1996; Schnaitman and Klena, 1993]. The long central *waaQ* operon contains genes necessary for the biosynthesis of the outer core and for modification and/or decoration of core. This operon also contains the "ligase" structural gene (*waaL*), whose product is required to link O polysaccharide to the completed core. The *waaQ* operon is preceded by a JUMPStart (*J*ust *U*pstream of *M*any *P*olysaccharide- associated gene *Start*s) sequence that includes the conserved 8 bp region known as *ops* (*o*peron *p*olarity *s*uppressor) that, together with RfaH, is required for operon polarity suppression [Bailey et al., 1997, Marolda and Valvano, 1998]. This region is also found in operons involved in the biosynthesis of other extra-cytoplasmatic macromolecules representing virulence factors targeted to the cell surface [Bailey et al., 1997]. The *waaA* transcript contains the structural gene (*waaA*) for the bifunctional Kdo transferase [Clementz and Raetz, 1991] and a "non-LPS" gene encoding phosphopantetheine adenylyltransferase (*coaD*) [Geerlof et al., 1999]. Between these genes, in *K. pneumoniae* and *S. marcescens*, is located the gene that encodes the glycosyltransferase WaaE, that links, by a β(1-4) link a glucose residue to the HepI in the inner core. This gene is also found in other members of the *Enterobacteriaceae* family such as *P. mirabilis, Y. enterocolitica*, and *Enterobacter aerogenes* [Izquierdo et al., 2002] (Figure 15).

Genes involved in the biosynthesis of the LPS core have been identified in a large number of

bacteria, but the function allocation for the encoded proteins was carried out in very few cases. However thanks to the chemical characterization of mutants in LPS-specific genes and complementation studies performed on some bacteria, it has been possible to assign specific functions to several genes. The gene clusters of *E. coli* [Heinrichs et al., 1998; Yethon et al., 1998], *S. enterica* [Heinrichs et al., 1998; Kaniuk et al., 2002), *K. pneumoniae* [Regué et al., 2001, Izquierdo et al., 2003; Frirdich et al., 2004; Regué et al., 2005a; Regué et al., 2005b; Fresno et al., 2007], *S. marcescens* [Coderch et al., 2004; Regué et al., 2005a; Regué et al., 2005b] and *Aeromonas salmonicida* [Jimenez et al., 2009] are the best characterized (Figure 15).
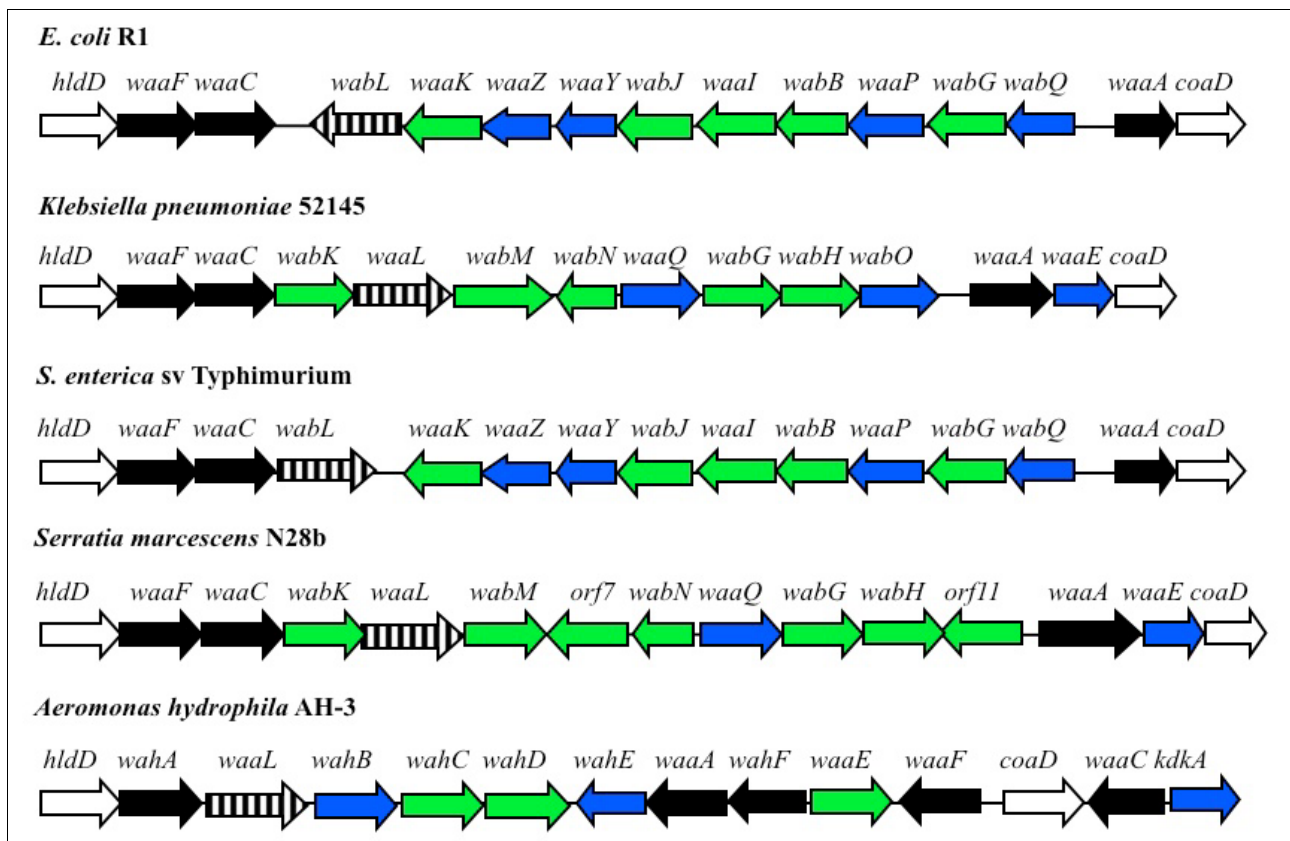
Figure 15: Outline of the *waa* gene cluster of *E. coli* R1, *S. enterica* sv. Typhimurium, *K. pneumoniae* 52145, *S. marcescens* N28b and *Aeromonas hydrophila* AH-3. Genes for glycosiltransferases of the internal core are marked with black arrows, with blue are highlighted genes for enzymes that modify the basic structure, genes for enzymes involved in the outer core biosynthesis are marked with green, the O ántigen ligase is the stripped arrow. White indicates genes involved in the biosynthesis of heptoses (*hldD*) and *coaD*, not related to the biosynthesis of the LPS core.

O-antigen

O-antigen main features

The O-specific polysaccharide (O-PS) or O-antigen represents the polymer chain of LPS, it is exposed on the bacterial surface bound, frequently, to the terminal residue of the outer core.

The structural diversity of O antigens is remarkable; more than 60 monosaccharides and 30 different non-carbohydrate components have been recognized [Knirel and Kochetkov, 1994]. It consists of a number of repetitions, often between 10 and 30, of a basic oligosaccharide subunit called O-unit or subunit, which generally contains from 2 to 6 sugar residues [Reeves et al., 1996]. The O-polysaccharide repeat unit structures can differ in the monomer glycoses, the position and stereochemistry of the *O* glycosidic linkages, and the presence or absence of non-carbohydrate substituents. O-repeat units from different structures may comprise varying numbers of monosaccharides, they may be linear or branched, and they can form homopolymers or, more frequently, heteropolymers. In some cases, nonstoichiometric modifications (*O*-acetylation or glycosylation) complicate the identification of a precise O-repeat unit. When the LPS molecules extracted from any S-LPS-containing strain are separated by SDS-PAGE, there is extensive heterogeneity in the sizes of the molecules due to variations in the chain length of the O polysaccharides. This is evident in the classical "ladder" patter in SDS-PAGE, where each "rung" up the ladder represents a lipid A-core molecule substituted with an increment of one additional O unit. The spacing between the rungs is determined by the size of the O unit. The S-LPS from most isolates exhibits a preferred and strain-specific size distribution pattern (modal distribution).

The structure of the O polysaccharide defines the O-antigen serological specificity in an organism, but the numbers of unique O antigens within a species vary considerably. *E. coli,* for example, produces ∼170 O serotypes. O-antigen specificity is only relevant when discussed within a species because there are several examples where all, or part, of an O-repeat unit structure is found in taxonomically distant species. A given isolate generally expresses one O antigen, but some bacteria contain multiple lipid A-core linked polymers, complicating both structural analysis and O-antigen terminology [Raetz and Whitfield, 2002].

The location of O polysaccharide at the cell surface places it at the interface between the bacterium and its environment. The primary role of the O polysaccharides appears to be protective. In animal pathogens, O polysaccharides may contribute to bacterial evasion of host immune responses, particularly the alternative complement cascade. O-antigen is an important virulence factor, which provides a hydrophilic surface layer which may mask important and conserved antigenic epitopes on the outer membrane. Assembly of the membrane attack complex is in fact affected by the chemistry of O polysaccharide, its chain length [Burns and Hull, 1998], and the relative amounts of

long chain S-LPS [Joiner, 1988]. On the other hand, the O antigen may play the role of adhesin, as has been described in many pathogenic bacteria, consequently, this makes re-evaluate its importance in the colonization of the mucous membranes of the host organism [Merino et al., 1996a; Merino et al., 1996b].The exact role played by O polysaccharide in different bacteria is therefore variable, and this is potentially magnified in bacteria that can produce more than one lipid A-core-linked polymer.

O-antigen chemical structure in *P. mirabilis*

Proteus is an antigenically heterogeneous genus, principally because of structural differences of its O-specific polysaccharide chain of LPS (O antigen).

With the aim of organizing the variability of the structures various schemes have been proposed. The serological classification scheme includes 49 different *P. mirabilis* and *P. vulgaris* O serogroups and 19 serologically distinct H antigens [Perch, 1948; Kauffmann, 1966; Kotelko, 1986]. Also, it has been introduced a separate O-grouping scheme to classify clinical isolates of *P. vulgaris* and *P. mirabilis* [Penner and Hennessy, 1980]. The chemical classification of *P. mirabilis* and *P. vulgaris* LPS into 16 chemotypes [Sidorczyk et al., 1975] and *P. penneri* into 7 chemotypes [Zych and Sidorczyk, 1989] remains to be completed. Results of immunochemical and structural studies on all three regions of the *P. mirabilis* and *P. vulgaris* LPS (O-specific part, core, and lipid A) have been reviewed [Kotelko, 1986.; Knirel and Kochetkov, 1994; Knirel and Kochetkov, 1993a/b].

The composition and structure of several O-specific polysaccharides of *P. mirabilis* strains were reported [Kaca et al., 1987; Vinogradov et al., 1988; Vinogradov et al., 1989a; Vinogradov et al., 1989b; Vinogradov et al., 1990; Beynon et al.,1992; Cedzynski et al., 1993; Cedzynski et al., 1995; Sidorczyk et al., 1995; Shashkov et al., 1996; Vinogradov and Bock, 1999; Shashkov et al., 2000; Vinogradov and Radziejewska-Lebrecht, 2000; Vinogradov and Perry, 2000; Vinogradov et al. 2000; Bartodziejska et al., 2000; Vinogradov, 2011].

Besides the typical sugar constituents widespread in nature, like hexoses, hexosoamines, and uronic acids, *Proteus* spp. also contain 6-deoxyamino sugars like L-fucosamine, L-quinovosamine, D-quinovoso-3-amine, and D-fucoso-3-amine. In addition, amino acids (L- and D-alanine, L-serine, L threonine, and L-lysine) are attached to the carboxyl group of uronic acids. In *Proteus* O antigens, other unusual acidic components like (R)- and (S)-lactic acid ethers and (R)-hydroxybutyryl, pyruvic, and phosphate groups were found.

Using of polyclonal rabbit antisera, degraded polysaccharides and their partial structures, and synthetic antigens corresponding to *Proteus* O antigens, it has been possible to investigate the epitopes that play an important role in immunogenicity [Chernyak et al., 1992; Chernyak et al., 1994]. It is worth noting that the unusual LPS components mentioned above do not always play an immunodominant role. The immunodeterminant oligosaccharide characteristic for *P. mirabilis* R110 (O3 serogroup) was found to be D-galacturonyl-1,4-D-galactosamine disaccharide substituted by lysine [Gromska and Mayer, 1976; Gromska et al., 1978; Kaca et al., 1987] (Figure 13). The immunodominant role of the lateral *N*-acetyl-D-glucosamine linked to the β-D-GlcA-L-Lys, as well as phosphoethanolamine linked to the additional residue of β-D-Glc-NAc in the O-specific polysaccharide from *P. mirabilis* O27, was also described [Krajewska-Pietrasik et al., 1995; Vinogradov et al., 1989b] (Figure 13). Serological studies with the synthetic antigens showed the importance of α-D-GalA-(L-Lys) and α-D-GalA-(L-Thr) in the specificity of *P. mirabilis* O28 [Radziejewska-Lebrecht et al., 1995].

Epidemiological investigations showed that among *Proteus* spp., strains classified into the O3, O27, O10, and O28 serogroups occurred most often in clinical isolates [Larsson, 1984].

O-antigen biosynthesis

The biogenesis of entire LPS is a complex process involving various steps that occur at the cytoplasmic membrane followed by the translocation of LPS molecules to the bacterial cell surface. The core OS is assembled on pre-formed lipid A by sequential glycosyl transfer of monosaccharides, while the O antigen is assembled on undecaprenolphosphate (Und-P), a polyisoprenoid lipid to which O antigen is linked via a phosphodiester bond [Whitfield and Valvano, 1993].

These pathways converge by the ligation of the O antigen onto outer core domain of the lipid A-core OS acceptor, with the concomitant release of Und-PP [Whitfield and Valvano, 1993; Whitfield, 1995; Raetz, 1996; Heinrichs et al., 1998; Heinrichs et al., 1999]. Und-P is also required as a lipid intermediate for the biosynthesis of other cell surface structures including peptidoglycan and the ECA [Whitfield and Valvano, 1993; Whitfield, 1995; Rick and Silver, 1996].

The biogenesis of O antigens can be mechanistically conceived into three interconnected stages: the first, the initiation reaction, involves the biosynthesis of nucleotide sugar precursors in the cytoplasm. The second step, located on the cytoplasmic face of the inner membrane, is the

sequential processing of the O antigen, which involves the elongation, polymerization, and membrane translocation of O repeating subunits. Polymerization is mediated by different glycosyltransferases, which, from nucleotide precursors, form oligos or polysaccharides on a the lipid carrier Und-P. The final step is the ligation reaction between the O-antigen and the lipid A-core OS. Also, one additional step that would be taken into consideration, is the recycling of the Und-PP polyisoprenoid carrier into Und-P to reinitiate biosynthesis (Figure 16).
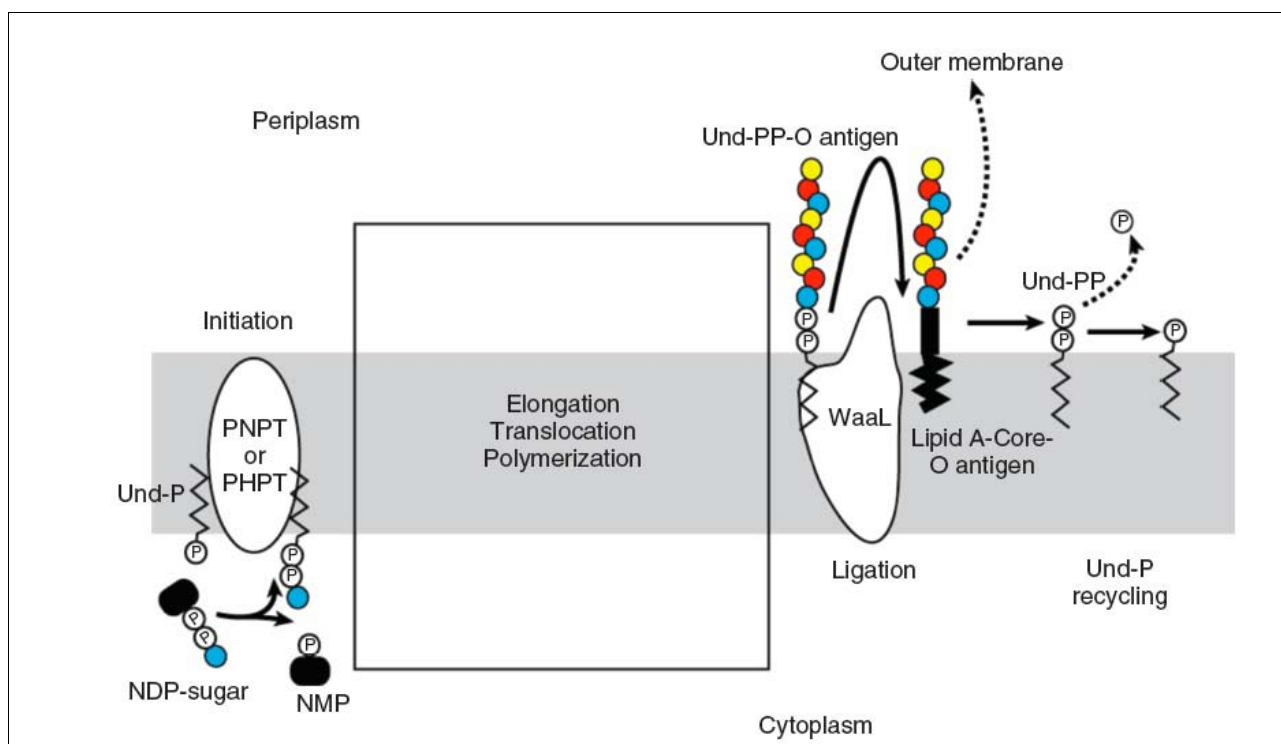
Figure 16: General steps in the biogenesis of O antigens. The gray rectangle denotes the inner membrane of the bacterial cell. The initiation reaction requires a nucleotide-diphosphate sugar and Und-P and is catalyzed by PHPT or PNPT enzymes. This reaction results in the formation of a Und-PP-linked sugar and the release of nucleotide-monophosphate. The large box denotes a number of different reactions that involve the elongation/ translocation/ polymerization of O repeating subunits, which result in the production of a polymeric O antigen linked to Und-PP (Und-PP-O antigen) that is localized to the periplasmic side of the inner membrane. The ligation reaction, catalyzed by WaaL, results in the formation of a complete LPS molecule (lipid A-core oligosaccharide-O antigen), which is further translocated to the outer membrane and becomes surface exposed. The remaining Und-PP is recycled to Und-P by dephosphorylation reactions and reused in the synthesis of Und-PP-linked polymers.

The precursors of several common sugars of the O antigen or LPS core part of other metabolic pathways of bacteria, such as UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal) and UDP-N-acetylglucosamine (UDP-GlcNAc). Genes involved in the biosynthesis of these nucleotide sugars are not normally present in the gene cluster responsible for the biosynthesis of the antigen O. In contrast, it is possible to find in the antigen O cluster, those genes involved in the biosynthesis of sugars which only form part of the O antigens, and are not essential to the bacterium. Precursors

allow direct incorporation of sugar and/or serve as intermediates for the synthesis of other activated monosaccharides. Several of the biosynthetic pathways of sugars precursors present in O antigens have already been described, and they appear conserved in a wide range of species [Samuel and Reeves, 2003].

The following step in O-polysaccharide biosynthesis involves the formation of an Und-PP-linked sugar by transfer of a cytosolic sugar-1-P residue to the membrane-associated lipid carrier Und-P, forming a phosphodiester bond and releasing UMP. The energy of the sugar-phosphate linkage in the donor molecule is conserved in the resulting Und-PP-linked intermediate and is available to drive the postpolymerization lipid A-core ligation reaction. Depending on the specific microorganism, this reaction is catalyzed by two different families of proteins. One of these families corresponds to the polyisoprenyl-phosphate *N*-acetylhexosamine- 1-phosphate transferases (PNPT family) [Anderson et al. 2000; Dal Nogare et al., 1998; Lehrman, 1994] comprising proteins that are present both in prokaryotes and in eukaryotes. The other family corresponds to the polyisoprenyl-phosphate hexose-1-phosphate transferases (PHPT family), and its prototype member is WbaP from *S. enterica*. This family of proteins has no known homologues in eukaryotic cells. The best-characterized in the PNPT family is WecA from *E. coli* [Meier-Dieter et al., 1992], that is a GlcNAc-1-phosphate transferase that initiates the polymerization by formation of Und-PP-GalNAc. In the *Enterobacteriaceae* family, WecA is located in the cluster for the biosynthesis of ECA, because of its participation in that process. The example of glycosyltransferase belonging to PHPT family, the enzyme WbaP from *S. enterica* [Osborn et al., 1972; Wang et al., 1996], catalyzes the reversible transfer of galactose-1-P (Gal-1-P) from UDP-Gal to und-P.

The three currently known pathways for O-polysaccharide biosynthesis are distinguished by their respective export mechanisms: they are called Wzy-dependent, ABC-transporter dependent, and synthase dependent. The first two processes are widespread, but the synthase pathway has very limited distribution in O-polysaccharide biosynthesis. Despite the export differences, the pathways have similar initiation reactions and are completed by the same ligation process.

In the <u>Wzy-dependent system</u>, exlusive for heteropolysaccharides often with side-branch residues, the synthesis starts with the binding of a first carbohydrate residue at Und-P through a galactosyl-transferase, WbaP, or an N-acetylglucosamine-1-phosphotransferase, WecA. Once initiated, the process continues with the sequential addition of different monosaccharides that form a subunit O.

These reactions occur on the cytoplasmic face of the inner membrane, and are catalyzed by different transferases specific for type of sugar and glycosidic bond. Following their assembly, the individual Und-PP-linked O units are exported to the site of polymerization at the periplasmic face of the plasma membrane , by a transmembrane protein, with "flippase" function, called Wzx (Figure 17). Wzx proteins were classified within a family of integral membrane proteins with 12 predicted transmembrane helices [Paulsen et al., 1997]. Wzx proteins share very little amino acid sequence similarity, and their genes have very poor nucleotide sequence homology, to the point that they can be used as genetic markers for distinguishing among specific O antigens [Wang and Reeves, 1998; Marolda et al., 1999]. One possible explanation for the abundant differences among Wzx proteins could be the requirement for the recognition of specific O subunits, which are highly variable in terms of structure and sugar composition. The exact mechanism of subunits transport through the lipid bilayer is at present unknown.

The second component of the Wzy/Wzx pathway is the Wzy protein. This protein is proposed to be involved in the polymerization of Und-PP-linked O subunits at the periplasmic face of the cytoplasmic membrane (Figure 17). Wzy proteins appear to be integral membrane proteins with 11–13 predicted transmembrane domains, and they exhibit little primary sequence similarity [Morona et al., 1994; Daniels et al., 1998]. However, Wzy proteins are specific for the O unit or for structures containing the same linkage between O-repeat units [Schnaitman and Klena, 1993; Whitfield, 1995; Whitfield and Valvano, 1993].

The reaction involves transfer of nascent polymer from its Und-PP carrier to the non-reducing end of the new Und-PP-linked O repeat [Bray and Robbins, 1967; Robbins et al., 1967]. The net effect is chain-length increase by one new repeat unit added to the reducing end of the nascent polymer. Mutants affected in the *wzy* gene produce LPS consisting of lipid A-core capped with a single O unit (SR-LPS) [Collins and Hackett, 1991]. This is a defining feature of the pathway. The released Und-PP must be recycled to the active monophosphoryl form.

The final component of the Wzy-dependent pathway is the Wzz protein. More than one *wzz* gene has been observed in some microorganisms like *P. aeruginosa* [Rocchetta et al., 1999] and *S. flexneri* [Stevenson et al., 1995]. It is not clear if the presence of additional Wzz activities would have an additive effect in the overall length of the O-polysaccharides or alternatively, they would be differentially required under varying physiological conditions. Wzz proteins are located in the plasma membrane, and all have two transmembrane helices flanking a periplasmic loop with a predicted coiled-coil structure [Morona et al., 2000]. The coiled-coil domains may be important for

interactions of Wzz with Wzy, WaaL, or both. Wzz generates the strain-specific modal distribution of O-polysaccharide chain lengths that is reflected in characteristic clusters of bands in SDS-PAGE [Whitfield et al., 1997; Morona et al., 2000]. Wzz was hypothesized to act as a timing clock, interacting with the Wzy polymerase and modulating its activity between two states that favor either elongation or transfer to the ligase (chain termination). A role for WaaL in this process is also inferred from the observation that Wzz can only regulate chain length of polymers that are linked to lipid A-core [Whitfield et al., 1997]. The ability of Wzz to somehow modulate the kinetics of the polymerization and/or ligation reactions suggests that the protein must recognize and form a complex with either Wzy, or WaaL, or both. However, there is currently no definitive evidence of cross-linking of Wzz to either Wzy or WaaL. The function of Wzz homologs is not restricted by a specific O-repeat unit structure, even if to date, no clear picture has emerged concerning the regions of the protein that confer specificity.
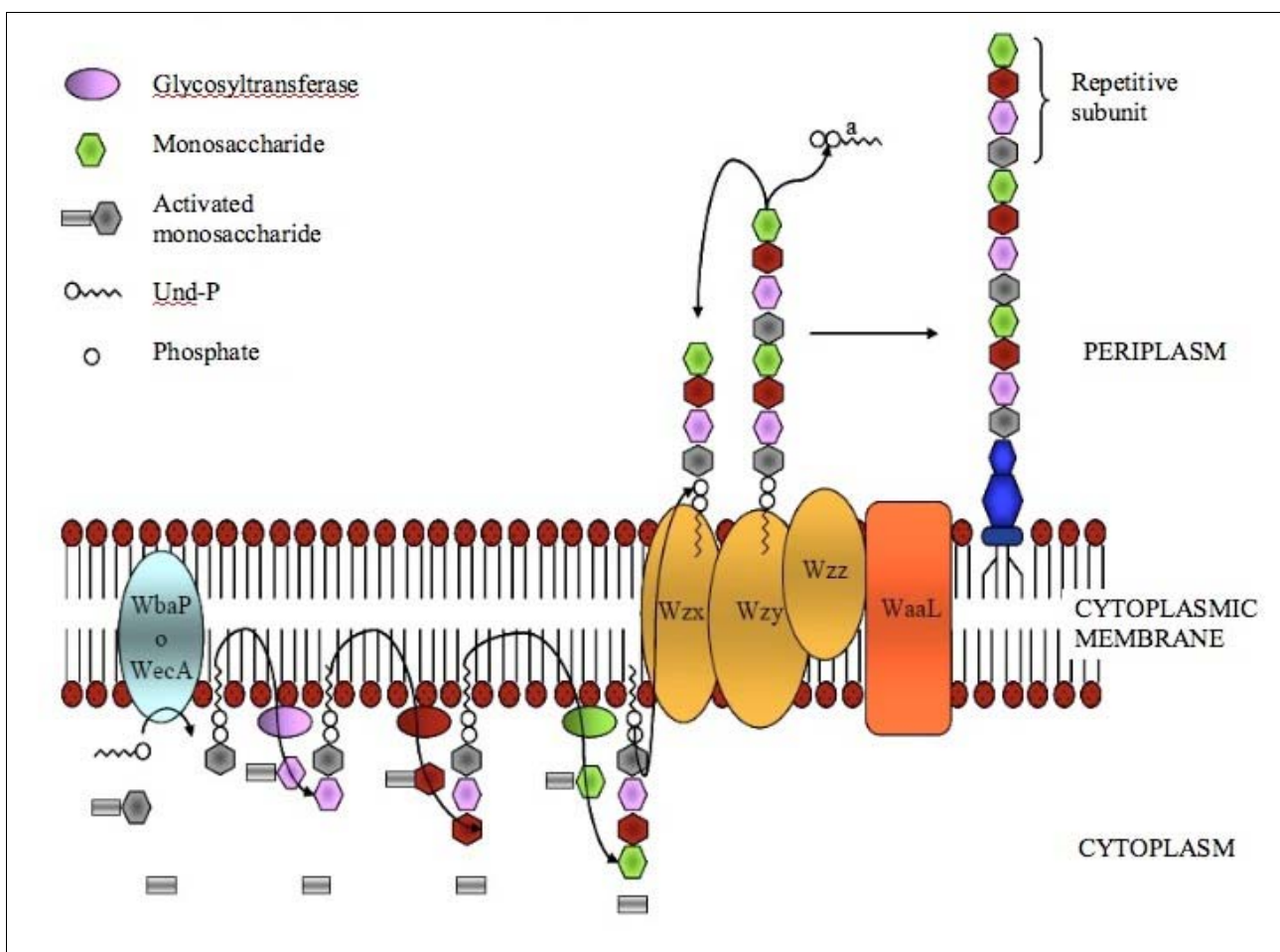
Figure 17: Outline of the Wzy-dependent system for the biosynthesis of the O-antigen.

The <u>ABC transporter-dependent</u> pathway is currently confined to linear (unbranched) O-polysaccharide structures and involves chain extension by processive addition of glycosyl residues to the non-reducing terminus of the Und-PP-linked growing chain.

There is no requirement for a specific polymerase enzyme, and the polymer is completed at the inner face of the cytoplasmic membrane. Export of the polymer to the outer face for ligation requires an ABC transporter, precluding the involvement of Wzx. One other salient feature of the O polysaccharides formed by this pathway is the participation of a primer Und-PPGlcNAc intermediate followed by the addition of a sugar adaptor molecule (Figure 18). Although these O polysaccharides are all initiated by the activity of WecA, they differ from Wzy/Wzx O polysaccharides in that the GlcNAc residue transferred to lipid A-core during ligation occurs only once per chain, and thus it is not found within the repeat unit structure itself [Rick et al., 1994; Süsskind et al., 1998]. Next, an O polysaccharide- specific glycosyltransferase adds an adaptor sugar residue between the und-PP-GlcNAc primer and the repeat subunit domain, and this reaction also occurs only once per chain. Different enzymes are involved in adding the adaptor. The O polysaccharide is then assembled in the chain extension phase, by the processive transfer of residues to the non-reducing terminus of the Und-PP-linked acceptor [Kido et al., 1995; Guan et al., 2001; Weisgerber and Jann, 1982], which may be mediated by either monofunctional or multifunctional transferases [Kido et al., 1995; Kido et al., 1998; Kido and Kobayashi, 2000]. Following polymerization, the nascent Und-PPlinked O polysaccharide is exported across the plasma membrane by an ABC-2 subfamily of transporters [Reizer et al., 1992]. ABC-2 transporters consist of an integral membrane protein, Wzm, with an average of six transmembrane domains, and a hydrophilic protein containing an ATP-binding motif, Wzt. Genes encoding these two components are present within the O-polysaccharide biosynthesis clusters. As with other ABC transporters involved in transmembrane export, Wzm homologues for O-polysaccharide biosynthesis display very little primary sequence identity, but Wzt homologues are much more highly conserved, especially in the nucleotide binding region. No structural data are available on the ABC transporters for O polysaccharides and little is known about their organization and exact mode of action. Presumably, the export process, driven by ATP hydrolysis, may involve an ATP-binding-induced conformational change of the Wzt protein, resulting in its insertion into the membrane via an interaction with the Wzm, introducing polymer into the channel [Raetz and Whitfield, 2002]. Due to the processive nature of the polymerization, an intriguing aspect of the polymers assembled by the ABC transport dependent pathway resides in their mode of termination.

In the case of the Wzy/Wzx-exported O polysaccharides, this process results from the interactions that involve the Wzy and Wzz proteins. Despite that the ABC-transport dependent pathway does not involve a Wzz protein, the O-specific polysaccharides formed by this pathway display strain specific modal distributions [Whitfield et al., 1997]. A current model favors a competition between "termination-export" and "chain-extension" activities [Bronner et al., 1994]. The presence in several O polysaccharides from ABC-transporter-dependent pathways of novel non-reducing terminal residues on the glycan chains, suggest that these residues may play a role as termination signals.
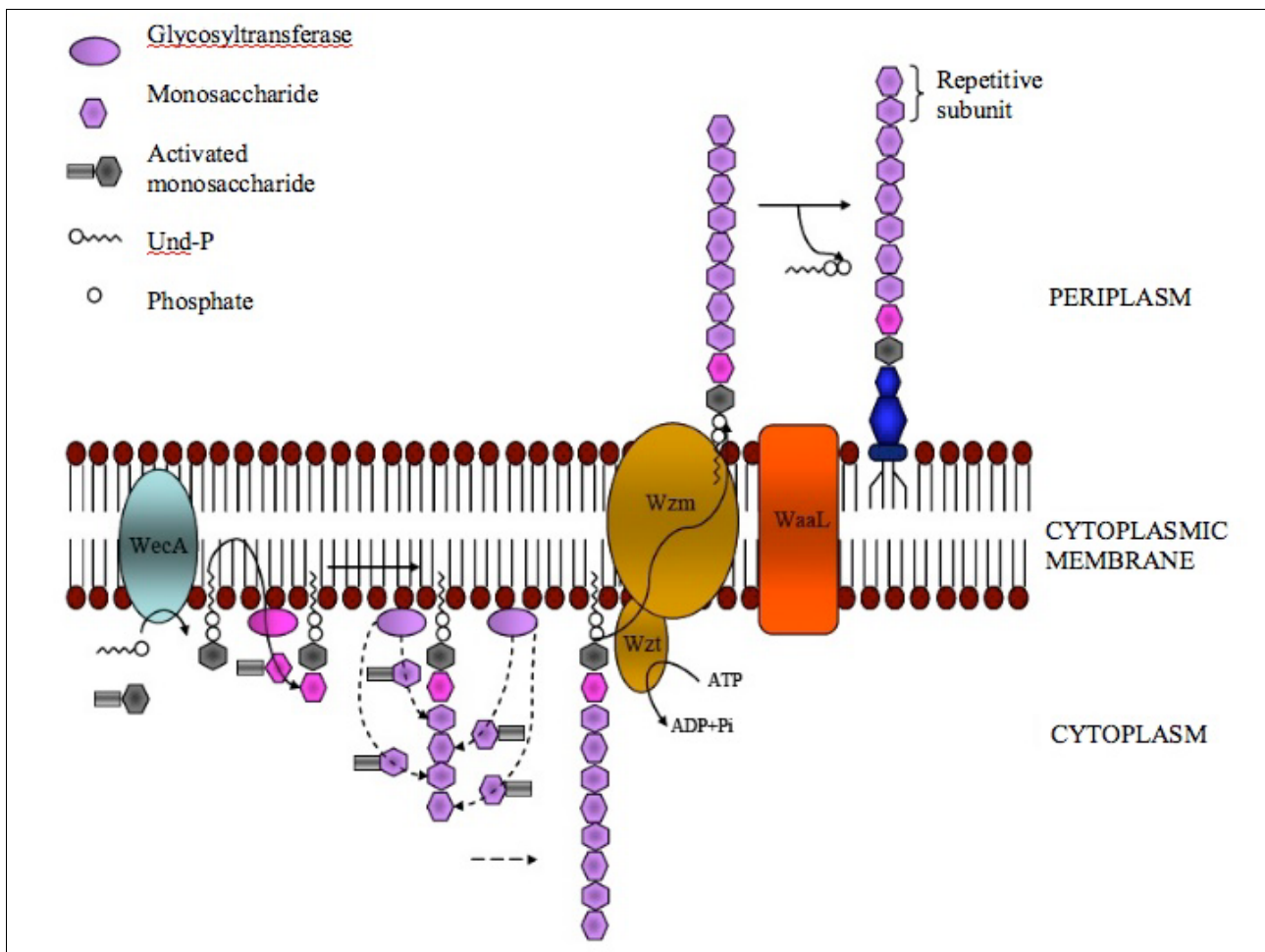
Fig. 18: Outline of the ABC transporter-dependent system for the biosynthesis of the O-antigen.

The only known example of synthase-dependent pathway for a O polysaccharide is the plasmid-encoded O:54 antigen of *S. enterica* serovar Borreze [Keenleyside et al., 1994; Keenleyside and Whitfield, 1995; Keenleyside and Whitfield, 1996].

The O:54-specific polysaccharide is a homopolymer made of N-acetylmannosamine (ManNAc). In a similar fashion to the ABC transport-dependent pathway, the synthesis of the O:54 subunit is

initiated by WecA [Keenleyside et al., 1994] and the first ManNAc residue is transferred to the Und-PP-GlcNAc primer by the non-processive ManNAc transferase WbbE [Keenleyside et al., 2001]. The second transferase, WbbF, belongs to the HasA (hyaluronan synthase) family of glycosaminoglycan glycosyltransferases [De Angelis, 2002], and it is proposed that this enzyme performs the chain-extension steps (Figure 19). Synthases are integral membrane proteins [De Angelis, 1999; De Angelis, 2002], which appear to catalyze a vectorial polymerization reaction by a processive mechanism resulting in the extension of the polysaccharide chain with the simultaneous extrusion of the nascent polymer across the plasma membrane [De Angelis, 1999]. Although the exported the polymer is presumably Und-PP-linked, there is very little information on the exact mechanism of export mediated by WbbF as well as in the process leading to chain termination. These enzymes are characterized by two conserved domains, one likely involved in the glycosyl transfer reaction and the other implicated in the translocation of the nascent polymer.
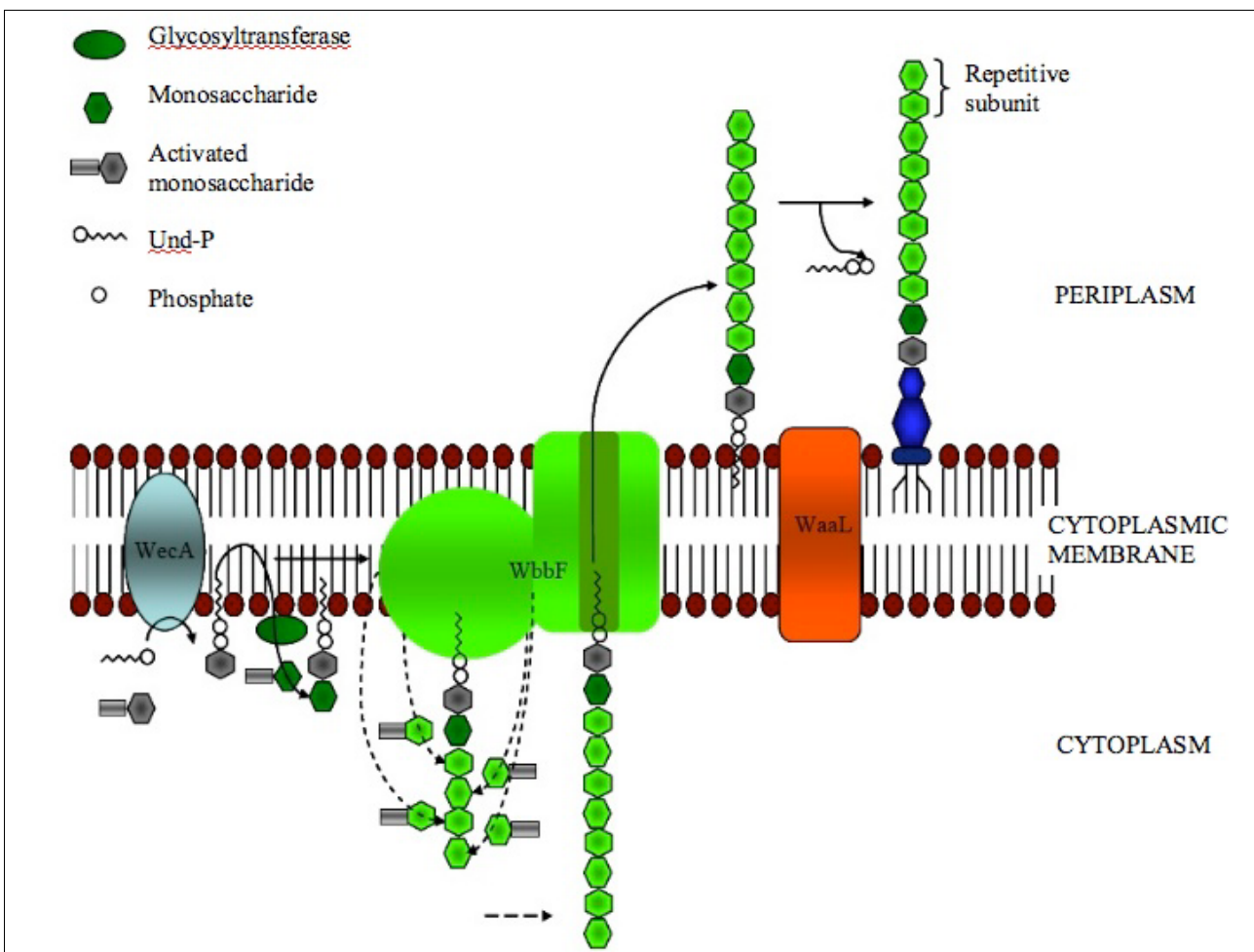
Figure 19: Outline of the synthase-dependent system for the biosynthesis of the O-antigen.

The <u>ligation reaction</u> resulting in the completion of the LPS molecule involves the addition of O polysaccharide to the lipid A-core OS. This reaction occurs at the periplasmic face of the cytoplasmic membrane [McGrath and Osborn, 1991], but its mechanism is currently unknown. The *waaL* gene product is currently the only protein presumed to be required for ligation. The WaaL homologues do not have any obvious distinguishing features. Although these proteins are presumably capable of forming glycosidic linkages, they share no similarity with any of the families of glycosyltransferases that utilize sugar nucleotide donors. Collectively, WaaL homologues exhibit low levels of similarity in their amino acid sequences, and they are all predicted to be integral membrane proteins with 8 or more membrane-spanning domains. WaaL presumably functions as part of a complex that involves highly specific interactions with the lipid-linked O-polysaccharide intermediates and lipid A-core acceptor. In the case of *E. coli* K-12, WaaL can link to its lipid A-core O polysaccharides with diverse structures, arising from any of the known biosynthesis pathways. The substrates for thus reaction may be Und-PP-linked oligosaccharides, polysaccharides, and even a single Und-PP-GlcNAc. Thus, the lack of discrimination for donor structures suggests that WaaL may recognize the undecaprenol pyrophosphate carrier rather than the saccharide attached to it. The variations in ligase sequences may then reflect specificity for the structure of the lipid A-core OS acceptor. This is supported by genetic and structural evidence demonstrating acceptor specificity that not only involves the core residue providing the attachment site, but also other proximal residues in the core OS [Heinrichs et al., 1998; Klena et al., 1992].

The <u>recycling of Und-PP</u> is very important, in fact the availability of Und-P is a limiting factor in the biosynthesis of O polysaccharides, since this isoprenoid lipid carrier is made in very small amounts, and it is also required for the biosynthesis of cell wall peptidoglycan, teichoic acids, and other carbohydrate polymers. Isopentenyl diphosphate and its isomer dimethylallyl diphosphate are, together, the universal precursors of all isoprenoids [Boucher and Doolittle, 2000], and they are synthesized *de novo* by the mevalonate and the 1-deoxy-D-xylulose 5-phosphate pathways [White, 1996]. Und-PP is a precursor for Und-P, and is synthesized by the UppS synthetase from isopentenyl pyrophosphate units [Shimizu et al., 1998; Apfel et al., 1999]. To participate in polymer biosynthesis as lipid carrier, newly synthesized Und-PP is dephosphorylated by a membrane bound pyrophosphatase to Und-P [Goldman and Strominger, 1972]. Since the UppS protein lacks features indicative on an integral membrane protein these biosynthetic steps probable occur at the cytosolic interface with the plasma membrane. Following initiation and the subsequent processing and

translocation reactions, the Und-PP-linked polymers are transferred to the appropriate acceptor on the periplasmic side of the plasma membrane. This process results in the release of Und-PP, which is recycled back to Und-P by a pyrophosphatase.

Bacitracin is peptide antibiotic produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* which, by binding to Und-PP in the presence of divalent cations, prevents the Und-PP dephosphorylation and thus interrupts the recycling of Und-PP to Und-P [Stone and Strominger, 1971; Storm and Strominger, 1973]; thus it functions as an inhibitor of cell wall and O antigen biosynthesis. The biosynthesis of O polysaccharides that utilize the Wzy/Wzz-dependent pathway is severely affected by bacitracin. This may be reflected on the fact that this pathway involves one molecule of Und-PP per O repeating subunit. In contrast, the biosynthesis of ABC transport- and synthase-dependent O antigens requires the participation of only one Und-P molecule per polymer chain. Consequently, Und-PP recycling is not a limiting factor in these cases and the process is more resistant to bacitracin. The enzyme or enzymes targeted by bacitracin, which carry out the Und- PP dephosphorylation reaction, have not been identified. Conceivably, these enzymes must be located in the plasma membrane and they may also have an active site facing the periplasmic space.

O-antigen genetic organization

The enzymes involved in O-polysaccharide assembly are encoded by genes at the locus known as *wb*. The majority of systems are chromosomally encoded, but notable exceptions include the plasmid-encoded O:54 polysaccharide of *S. enterica* [Popoff and Le Minor 1985; Keenleyside et al., 1994]. These loci encode enzymes for the synthesis of novel sugar nucleotide precursors, glycosyltransferases that synthesize the O polysaccharide, and enzymes required for export processes.

Throughout the cluster it is possible to find highly strains or species conserved genes, and also genes much more variable. Thus, in general, genes involved in the biosynthesis of the monosaccharides are the most conserved. Genes encoding glycosyltransferases are described as semi-conserved, first because the proteins encoded by these genes are clearly related, but they exhibit a lower degree of similarity to the previous ones, and also because they are enzymes which catalyze reactions that recognize similar but different substrates. Finally, genes encoding proteins related to specific O antigen structure, polymerization or transport are less conserved. The proteins encoded by these last genes are structurally similar but have a very low amino acid identity

percentage [Klena and Schnaitman, 1993].

As expected, the diversity of O polysaccharide structures is reflected in highly polymorphic *wb* loci. There are significant studies on the effects of lateral gene transfer, and deletion or insertion events in the evolution and diversification of the various types of antigens O [Schnaitman and Klena, 1993]. One feature of particular interest regarding the group *wb* is the content of guanine and cytosine (G + C), that is 10% lower than the rest of the genome, and becomes even lower in the less conserved regions within the group. This observation suggests that these clusters may have been formed from a series of genetic exchanges involving widely separated organisms.

Most of the *wb* operons appear to be constitutively expressed, and they are often preceded by a JUMPStart/*ops* sequence indicating that an active anti-termination process. One exception, the O-polysaccharide biosynthesis locus of *Y. enterocolitica* O:3, is transcriptionally regulated by temperature [al-Hendy et al., 1991; Skurnik and Toivanen, 1993], and regulation is mediated by a repressor encoded outside of the cluster, so that, at 20ºC the transtripcion is optimal and at 37ºC a very little amount of antigen O is produced. It should be noted that in this organism the core biosynthesis genes are not thermo-regulated.
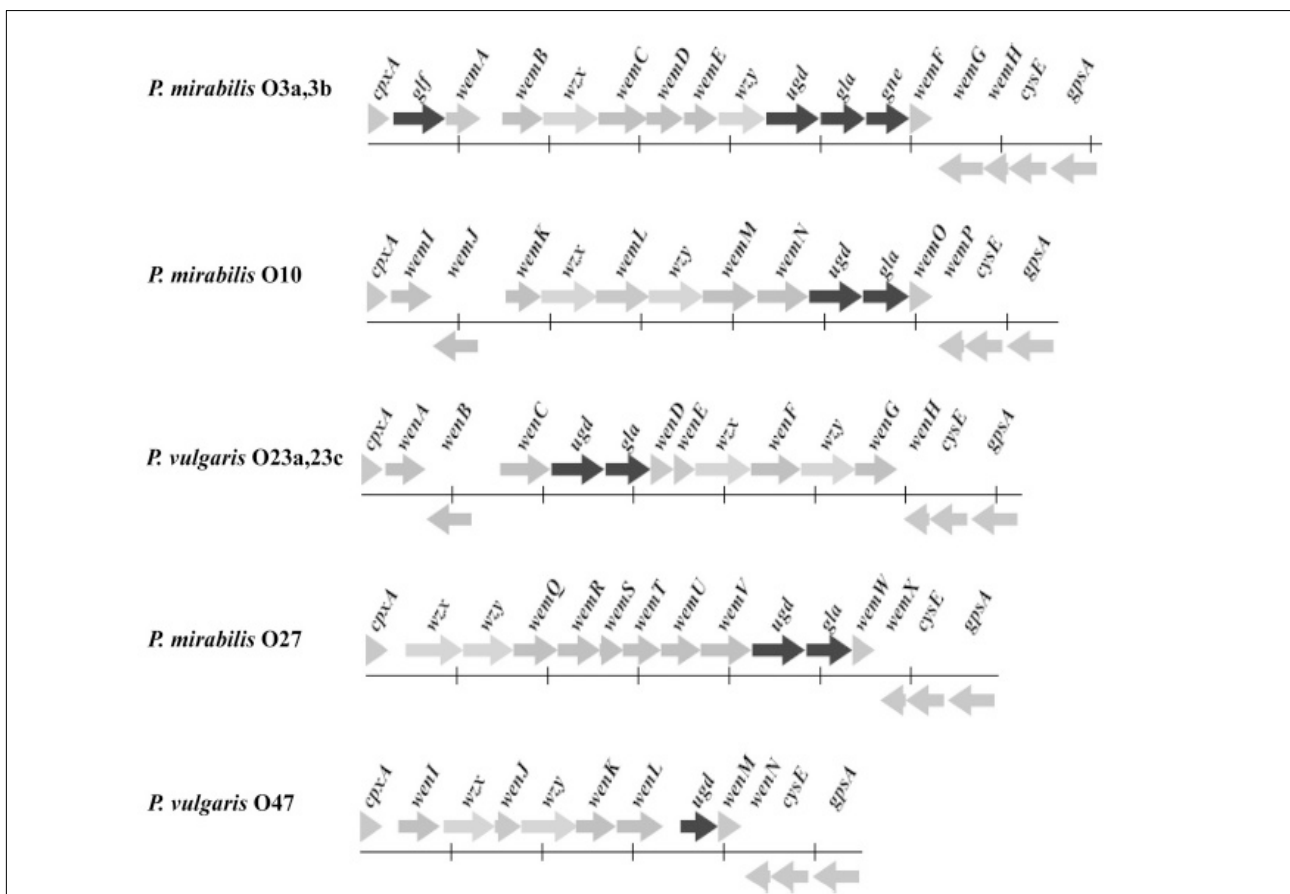
Figure 20: Structural organization of O antigen gene clusters of *P. mirabilis* O3a,3b, *P. mirabilis* O10, *P. vulgaris* O23a,23c, *P. mirabilis* O27, and *P. vulgaris* O47 [Wang et al., 2010].

In *Proteus*, the O antigen gene cluster is located between the two housekeeping genes, *cpxA* (encoding two-component system sensor kinase) and *secB* (encoding a pre´protein translocase subunit)(Figure 20). Similar to the *E. coli* and *Salmonella* O antigen gene cluster, the *Proteus* cluster also contains three major gene classes: sugar biosynthetic pathway genes, sugar transferase genes, and O antigen-processing genes. All of the O antigen-processing genes analyzed were *wzx* and *wzy*, indicating that the assembly of *Proteus* O antigen was likely to be Wzx/Wzy dependent [Wang et al., 2010].

The molecular characterisation of the putative gene cluster responsible for *Proteus* O antigen biosynthesis in five different serogroups is reported in Figure 20 [Wang et al., 2010].

2. Aims

The present work is part of a research project focused on virulence factors in Gram negative bacteria. Study of the roles played by the macromolecules of the bacterial surface has, in this context, aroused particular interest. Previous works in our lab, focused on the study of the LPS core biosynthesis in members of the *Enterobacteriaceae* and *Aeromonadaceae* families, allowed the identification of most of the genes implicated in the biosynthesis of their core LPS, and to resolve their function in *S. marcescens*, *K. pneumoniae*, *A. salmonicida,* and *A. hydrophila*.

Study of LPS core is particularly relevant for essentially four reasons: (1) it is a conserved region, although it is increasingly clear that there is some variability at the genus or groups of similar genera, (2) its chemical structure modulates the endotoxic activity of lipid A, (3) alteration of the LPS core, which generates less virulent bacteria, more susceptible to the defense mechanisms of the immune system, encourages the search of substances that interfere with the biosynthesis of this region, and (4) conserved regions of the core LPS could be useful as antigens in preventing diseases caused by pathogens that contain these conserved regions.

P. mirabilis is not a common cause of Urinary Tract Infection (UTI) in the normal host. Surveys of uncomplicated cystitis or acute pyelonephritis show that *P. mirabilis* comprises only a few percent of cases. Even in patients with recurrent UTI, the incidence of infections by this organism is only a few percentage points higher. So, why to propose intensive studies of the pathogenesis of *P. mirabilis*? The answer lies in the fact that this organism infects much higher proportions (up to 44%) of patients with complicated urinary tracts; that is, those with functional or anatomic abnormalities or with chronic instrumentation such as long-term catheterization, making it the most common nosocomial infection. While infecting the urinary tract, *P. mirabilis* has a predilection for the kidney. Finally and importantly, not only does this bacterium cause cystitis and acute pyelonephritis, but the production of urinary stones, a hallmark of infection with this organism, adds another dimension to these already complicated urinary tracts. In addition, *P. mirabilis* presents several important and unique peculiarities in its core LPS structure when compared to other enteropathogenic bacteria.

Aims

On the basis of the mentioned argumentations the specific aims of this project have been to:

– Identify genes involved in core LPS biosynthesis.

   This part of the work has been realized using six model strains producing different serotype O-PS: *P. mirabilis* strains R110 (O3), 51/57 (O28), 50/57 (O27), TG83 (O57), and genome strain HI4320 (O10).

– Functionally characterize genes involved in the biosynthesis of the *P. mirabilis* LPS core.

   This goal was achieved by using mutant strains from *E. coli* and *K. pneumoniae*, producing surrogate acceptor LPS , and analysis of generated core structures.

– Elucidate the mechanism of incorporation of GalN to the *P. mirabilis* core LPS.

– Identify the genes coding for PEtN modifications in *P. mirabilis* LPS.

– Characterize the gene encoding the PEtN transferase involved in the modification of L,D-HepII and study of its biological effects.

3. Impact factor and contribution to the papers of publications included

The PhD thesis supervisor certifies that the following publications are the result of Eleonora Aquilini PhD thesis research. Her contribution to the results is specified for each publication, as well as the corresponding impact factor:

1. **Aquilini E**, Azevedo J, Jimenez N, Bouamama L, Tomás JM and Regué M. (2010). Functional identification of the *Proteus mirabilis* core lipopolysaccharide biosynthesis genes. *J. Bacteriol.* 192(17):4413-24.

   E. Aquilini performed the most of the research of this work, done in collaboration with Dr. Tomás group at Microbiology Department, Biology Faculty. The 2010 impact factor of the J*ournal of Bacteriology* was 3.825, corresponding to the first quartile among the Microbiology indexed publications. The *Eigenfactor* score was 0.129 and the Article Influence score was 1.41 according to the 2010 Journal Citation Reports. It was the # 3 out of 107 journals in Microbiology ranked by *Eigenfactor* score. It was also the #2 cited journal in Microbiology, receiving more than more than 62.000 citations.

2. **Aquilini E**, Azevedo J, Merino S, Jimenez N, Tomás JM and Regué M. (2010) Three enzymatic steps required for the galactosamine incorporation into core lipopolysaccharide. *J. Biol. Chem.* 17;285(51):39739-49.

   E. Aquilini performed most of the research of this work, done in collaboration with Dr. Tomás group at Microbiology Department, Biology Faculty. The 2010 impact factor of the Journal of Biological Chemistry was 5.328, corresponding to the first quartile among the Biochemistry & Molecular Biology indexed publications. The *Eigenfactor* score was 0.885 and the Article Influence score was 2.191, according to the 2010 Journal Citation Reports. It was the #1 out of 286 journals in Biochemistry & Molecular Biology ranked by *Eigenfactor* score. It was also the most cited journal in Biochemistry & Molecular Biology, receiving more than 412,003 citations.

3. **Aquilini E**, Azevedo J, Merino S, Knirel Y A, Alteri C, Mobley H, Juan M. Tomás JM, Regué M. Functional identification of a *Proteus mirabilis eptC* gene encoding a core lipopolysaccharide phosphoethanolamine transferase. Work intended for *J. Bacteriol*, 2013.

    E. Aquilini performed most of the research of this work, done in collaboration with Dr. Tomás group at Microbiology Department, Biology Faculty and Dr. Mobley group at Departament of Microbiology and Immunology, University of Michigan Medical School. The current impact factor of the J*ournal of Bacteriology* is 3.825, corresponding to the first quartile among the Microbiology indexed publications. The *Eigenfactor* score is 0.111 and the Article Influence score is 1.358 according to the 2011 Journal Citation Reports. It is the # 4 out of 107 journals in Microbiology ranked by *Eigenfactor* score. It is also the #2 cited journal in Microbiology, receiving more than more than 66.000 citations.

Barcelona, September 3rd 2012

Signed:

Dr. M. Regué

4. Publications

Functional Identification of the *Proteus mirabilis* Core Lipopolysaccharide Biosynthesis Genes[▽]

Eleonora Aquilini,[1] Joana Azevedo,[1] Natalia Jimenez,[2] Lamiaa Bouamama,[2]
Juan M. Tomás,[2]* and Miguel Regué[1]

*Departamento de Microbiología y Parasitología Sanitarias, Facultad de Farmacia, Universidad de Barcelona, Av. Joan XXIII s/n,
08028 Barcelona, Spain,[1] and Departamento de Microbiología, Facultad de Biología, Universidad de
Barcelona, Diagonal 645, 08071 Barcelona, Spain[2]*

In this study, we report the identification of genes required for the biosynthesis of the core lipopolysaccha-rides (LPSs) of two strains of *Proteus mirabilis*. Since *P. mirabilis* and *Klebsiella pneumoniae* share a core LPS carbohydrate backbone extending up to the second outer-core residue, the functions of the common *P. mirabilis* genes was elucidated by genetic complementation studies using well-defined mutants of *K. pneumoniae*. The functions of strain-specific outer-core genes were identified by using as surrogate acceptors LPSs from two well-defined *K. pneumoniae* core LPS mutants. This approach allowed the identification of two new heptosyl-transferases (WamA and WamC), a galactosyltransferase (WamB), and an *N*-acetylglucosaminyltransferase (WamD). In both strains, most of these genes were found in the so-called *waa* gene cluster, although one common core biosynthetic gene (*wabO*) was found outside this cluster.

Gram-negative motile and frequently swarming bacteria of the genus *Proteus* and the family *Enterobacteriaceae* are oppor-tunistic human pathogens (33). Currently, the genus consists of five species (*Proteus mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxo-faciens*, and *P. hauseri*) and three genomospecies (4, 5, and 6) (33, 35). *P. mirabilis* is a common uropathogen that causes urinary tract infections especially in individuals with functional or anatomical abnormalities of the urinary tract (52) and el-derly persons undergoing long-term catheterization (53) but less frequently in normal hosts (43). Potentially serious com-plications arising from *P. mirabilis* infections include bladder and kidney stone formation, catheter obstruction due to the formation of encrusting biofilms, and bacteremia (reviewed in reference 2). This bacterium is found more frequently than *Escherichia coli* in kidney infections (14) and may be associated with rheumatoid arthritis (38). Studies aimed at the identifi-cation of *P. mirabilis* virulence factors showed that flagella and fimbriae (MR/P and PMF) are required for entry into and colonization of the bladder, respectively (reviewed in reference 12). Other important virulence factors are urease, hemolysin, and iron acquisition (12). More recently, an extracellular met-alloprotease (37) and several putative DNA binding regula-tory, cell-envelope related, and plasmid-encoded proteins have been identified by signature-tagged mutagenesis (8, 21).

The lipopolysaccharide (LPS), as in other members of the family *Enterobacteriaceae*, consists of three domains, an endo-toxic glycolipid (lipid A), an O-polysaccharide (O-PS) chain or O-antigen, and an intervening core oligosaccharide (OS) re-gion. The O-antigen is the major surface antigen, and its se-rological O specificity, in contrast to that of other Gram-neg-ative bacteria (31), is defined by the structure of the O-PS chain and that of the core OS (51). On the basis of immuno-specificity, 60 O serogroups (28, 36) have been recognized in *P. mirabilis* and *P. vulgaris*, and several new *Proteus* O serogroups have been proposed for *P. penneri* (27, 55). The LPS is a potential *Proteus* virulence factor (42), and recently two mu-tants deficient in a glycosyltransferase and with attenuated virulence have been isolated and it has been speculated that this glycosyltransferase could be involved in LPS biosynthesis (21). LPS plays a significant role in the resistance of *P. mirabilis* to antimicrobial peptides (32), and LPS charge alterations may influence the swarming motility of the bacterium (3, 32). In addition, the core LPS is a charged OS which plays an impor-tant role in the biological activities of the LPS and the function of the bacterial outer membrane (10). In *Proteus*, the core OS structures of up to 34 strains of different O serogroups have been determined (51). These structures revealed that *Proteus* core OSs share a heptasaccharide fragment that includes a 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) disaccharide, an L-*glycero*-α-D-*manno*-heptose (L,D-Hep) trisaccharide, and one residue each of D-glucose (D-Glc), D-galacturonic acid (D-GalA), and either D-glucosamine (D-GlcN) or D-galactosamine (D-GalN) (51). This common fragment is also found in the core LPSs of *Klebsiella pneumoniae* and *Serratia marcescens* (11, 41, 50). The rest of the *Proteus* core OS is quite variable, and it is possible to recognize up to 37 and 11 different structures in the genus and *P. mirabilis*, respectively (51). Some *P. mirabilis* core OS structures are characterized by the presence of unusual residues, such as, for instance, quinovosamine; an open-chain form of *N*-acetylgalactosamine (GalNAc); or unusual amino acids (51). In contrast, little is known about the genes encoding enzymes involved in core LPS biosynthesis in *P. mirabilis*, which makes detailed genetic analysis of the role of LPS in *P. mirabilis* pathogenesis difficult. Thus, we decided to identify these genes by using *P. mirabilis* strains R110 and 51/57, the whole structures of whose core LPSs are known (Fig. 1).

---

* Corresponding author. Mailing address: Departamento Microbi-ología, Facultad Biología, Universidad Barcelona, Diagonal 645, 08071 Barcelona, Spain. Phone: 34-93-4021486. Fax: 34-93-4039047. E-mail: jtomas @ub.edu.
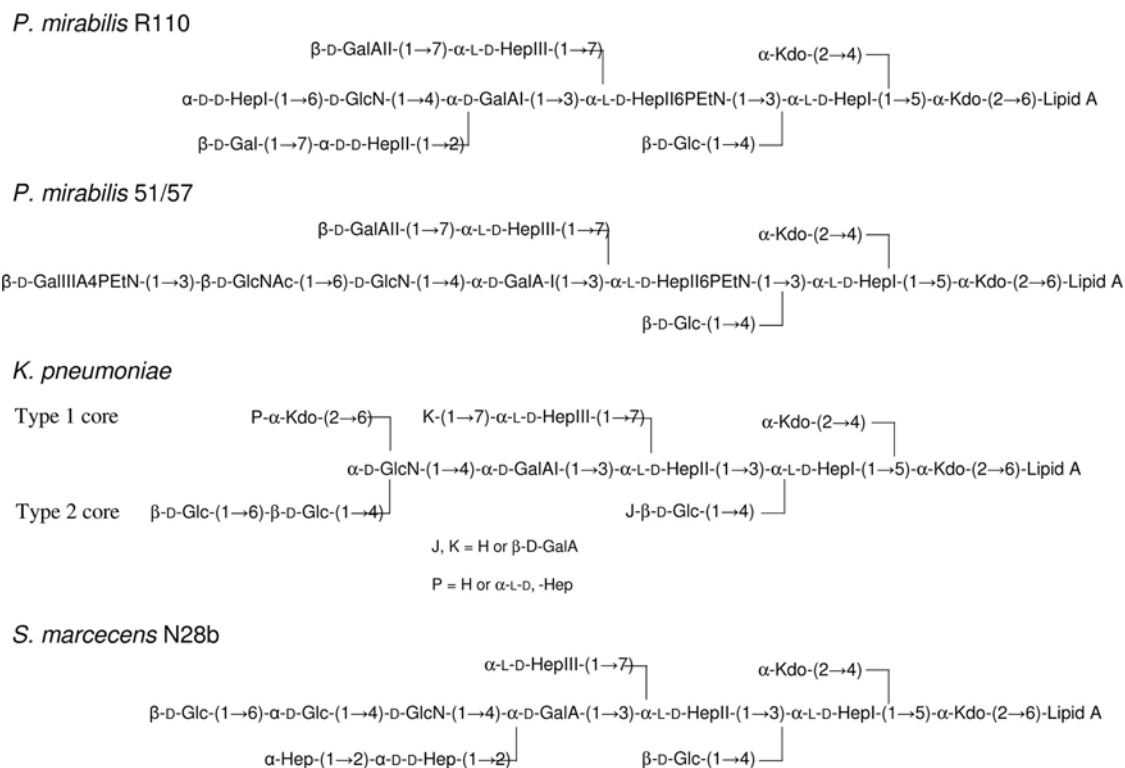
FIG. 1. Chemical structures of the core LPSs of *P. mirabilis* strains R110 and 51/57 (51), *K. pneumoniae* types 1 (50) and 2 (41), and *S. marcescens* N28b (11).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were routinely grown in Luria-Bertani (LB) broth and LB agar (44) at 37°C unless stated otherwise. Ampicillin (100 and 150 μg ml^{-1} for *E. coli* and *K. pneumoniae* strains, respectively), chloramphenicol (25 μg ml^{-1}), and polymyxin B (16 μg ml^{-1}) were added to the different media when required.

General DNA methods. General DNA manipulations were done essentially as previously described (44). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

DNA sequencing and computer analysis of sequence data. Double-stranded DNA sequencing was performed by using the dideoxy-chain termination method (45) with the ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer). Oligonucleotides used for genomic DNA amplifications and DNA sequencing were purchased from Pharmacia LKB Biotechnology. The DNA sequence was translated in all six frames, and all open reading frames (ORFs) were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST (1) network service at the National Center for Biotechnology Information and the European Biotechnology Information. ClustalW was used for multiple-sequence alignments (48).

Plasmid constructions and mutant complementation studies. For complementation studies, the *P. mirabilis* R110 genes *waaA*, *waaC*, *waaF*, *waaE*, *wabG*, *wabH*, *wabN*, *wabO*, *wamA*, *wamC*, and *wamB* and strain 51/57 *wamD* were PCR amplified by using specific primer pairs (Table 2) and chromosomal DNA as the template, ligated to plasmid pGEMT (Promega), and transformed into *E. coli* DH5α. Transformants were selected on LB plates containing ampicillin. Once checked, plasmids with the amplified genes were independently transformed into *K. pneumoniae* core LPS mutants.

Recombinant plasmid pBAD18-Cm-WamB was obtained by PCR amplification of this gene, subcloning in pBAD18-Cm, and transformation into *E. coli* DH5α. This construct was transformed into *K. pneumoniae* core LPS mutants, and *wamB* was expressed from the arabinose-inducible and glucose-repressible pBAD18-Cm promoter. Repression from the *araC* promoter was achieved by growth in medium containing 0.2% (wt/vol) D-glucose, and induction was ob-

tained by adding L-arabinose to a final concentration of 0.2% (wt/vol). The cultures were grown for 18 h at 37°C in LB medium supplemented with chloramphenicol and 0.2% glucose, diluted 1:100 in fresh medium (without glucose), and grown until they reached an A_{600} of about 0.2. Then, L-arabinose was added and the cultures were grown for another 8 h. Repressed controls were maintained in glucose-containing medium.

Mutant construction. The chromosomal in-frame mutation-containing strain *P. mirabilis* S1959Δ*waaL* was constructed by allelic exchange as described by Link et al. (30). The four primers used to obtain this mutant were MutA (5′-TCCCCCGGGTACGGAGCTGGTGGCTAGAT-3′), MutB (5′-CCCATCCACTAAACTTAAACATCACGCACCAGATACCAAAG-3′), MutCII (5′-TGTTTAAGTTTAGTGGATGGGATGGTACCCAAGGTTCA-3′), and MutDII (5′-TCCCCCGGGTTGTGCTGACCTCGCTGTTA-3′). Double underlining denotes SmaI sites, and single underlining denotes complementary bases. Using strain S1959 DNA, two asymmetric PCRs were carried out to obtain two DNA fragments (MutA-MutB and MutCII-MutDII) that were annealed at their overlapping regions and PCR amplified as a single DNA fragment using primers MutA and MutDII. The amplified in-frame deletion was purified, SmaI digested, ligated into SmaI-digested and phosphatase-treated temperature-sensitive suicide vector pKO3, electroporated into *E. coli* S-17, and plated on chloramphenicol LB agar plates at 30°C to obtain pKO3Δ*waaL*$_{S1959}$. This plasmid was transferred by mating between *E. coli* S-17(pKO3Δ*waaL*$_{S1959}$) and *P. mirabilis* S1959. Conjugants were selected on polymyxin B and chloramphenicol LB plates at 30°C. To recombine the suicide plasmid into the chromosome of *P. mirabilis* S1959, conjugant colonies were grown at 42°C. To complete the allelic exchange, the integrated suicide plasmid was forced to recombine out of the chromosome by growth on agar plates containing 15% sucrose. Colonies were screened for the loss of the chloramphenicol-resistant marker of plasmid pKO3Δ*waaL*$_{S1959}$. DNA from chloramphenicol-sensitive colonies was amplified using primers MutE (5′-ATATTGCCAACACCCACCAC-3′) and MutF (5′-TGCTATCTGGCTGAGAACCA-3′) flanking the *waaL* gene to identify the candidate *P. mirabilis* S1959Δ*waaL* mutant. The mutation was confirmed by nucleotide sequencing.

LPS isolation and SDS-PAGE. For screening purposes, LPS was obtained after proteinase K digestion of whole cells (13). LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
P. mirabilis strains		
S1959	Wild-type, serovar O3	Z. Sydorckzyk
R110	Rough mutant of strain S1959	Z. Sydorckzyk
S1959Δ*waaL*	Nonpolar *waaL* mutant	This study
51/57	Serovar O28	Z. Sydorckzyk
50/57	Serovar O27	Z. Sydorckzyk
14/57	Serovar O6	Z. Sydorckzyk
TG83	Serovar O57	Z. Sydorckzyk
OXK	Serovar O3	Z. Sydorckzyk
CECT170		CECT[a]
K. pneumoniae strains		
52145Δ*waaC*	Nonpolar *waaF* mutant	26
52145Δ*waaF*	Nonpolar *waaC* mutant	26
52145Δ*waaQ* (NC19)	Nonpolar *waaQ* mutant	39
52145Δ*waaE* (NC16)	Nonpolar *waaE* mutant	39
52145Δ*wabO*	Nonpolar *wabO* mutant	15
52145Δ*wabH*	Nonpolar *wabH* mutant	40
52145Δ*wabN*	Nonpolar *wabN* mutant	40
52145Δ*wabK*	Nonpolar *wabK* mutant	41
52145Δ *waaL*	Nonpolar *waaL* mutant	26
E. coli strains		
DH5α	F⁻ *endA hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyr-A96* ϕ80*lacZ*	19
CJB26	*waaA*::*kan recA* harboring plasmid pJSC2	4
S17-1	*hsdR pro recA* RP4-2 in chromosome Km::Tn7 (Tc::Mu)	47
Plasmids		
pKO3	Cm^r *sacB* temperature-sensitive replication suicide vector	30
pKO3ΔWaaL	pKO3 containing engineered *waaL* deletion from strain S1959	This study
pJSC2	Cm^r temperature sensitive for replication, containing *E. coli waaA*	4
pGEMT easy	PCR-generated DNA fragment cloning vector, Amp^r	Promega
pGEMT-WaaA$_{R110}$	pGEM-T with *waaA* from strain R110, Ap^r	This study
pGEMT-WaaC$_{R110}$	pGEM-T with *waaC* from strain R110, Ap^r	This study
pGEMT-WaaF$_{R110}$	pGEM-T with *waaF* from strain R110, Ap^r	This study
pGEMT-WaaQ$_{R110}$	pGEM-T with *waaQ* from strain R110, Ap^r	This study
pGEMT-WaaE$_{R110}$	pGEM-T with *waaE* from strain R110, Ap^r	This study
pGEMT-WabO$_{R110}$	pGEM-T with *wabO* from strain R110, Ap^r	This study
pGEMT-WabG$_{R110}$	pGEM-T with *wabG* from strain R110, Ap^r	This study
pGEMT-WabH$_{R110}$	pGEM-T with *wabH* from strain R110, Ap^r	This study
pGEMT-WabN$_{R110}$	pGEM-T with *wabN* from strain R110, Ap^r	This study
pGEMT-WamA$_{R110}$	pGEM-T with *wamA* from strain R110, Ap^r	This study
pGEMT-WamB$_{R110}$	pGEM-T with *wamB* from strain R110, Ap^r	This study
pGEMT-WamC$_{R110}$	pGEM-T with *wamC* from strain R110, Ap^r	This study
pGEMT-WamD$_{51/57}$	pGEM-T with *wamD* from strain 51/57, Ap^r	This study
pGEMT-WaaL$_{R110}$	pGEM-T with *waaL* from strain R110, Ap^r	This study
pBAD18-Cm	Arabinose-inducible expression vector, Cm^r	18
pBAD18-Cm-WamB	Arabinose-inducible *wamB*	This study

[a] CECT, Spanish Type Culture Collection.

SDS-Tricine-PAGE and visualized by silver staining as previously described (13, 22).

Large-scale isolation and mild acid degradation of LPS. Dry bacterial cells of each strain in 25 mM Tris-HCl buffer containing 2 mM CaCl₂, pH 7.63 (10 ml g^{-1}), were treated at 37°C with RNase and DNase (24 h, 1 mg g^{-1} each) and then with proteinase K (36 h, 1 mg g^{-1}). The suspension was dialyzed and lyophilized, and the LPS was extracted by either the phenol-water procedure (54) or the method of Galanos et al. (16). A portion of the LPS (50 mg) from each strain was heated with aqueous 2% acetic acid (6 ml) at 100°C for 45 min. The precipitate was removed by centrifugation (13,000 × *g*, 20 min), and the supernatant was fractionated on a column (56 by 2.6 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer, pH 4.5, with monitoring by using a differential refractometer (Knauer, Berlin, Germany).

Mass spectrometry studies. Positive-ion reflectron matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were acquired on a Voyager DE-PR instrument (Applied Biosystems) equipped with a delayed-extraction ion source. The ion acceleration voltage was 20 kV, the grid voltage

was 14 kV, the mirror voltage ratio was 1.12, and the delay time was 100 ns. Samples were irradiated at a frequency of 5 Hz by 337-nm photons from a pulsed nitrogen laser. Mass calibration was obtained with a malto-OS mixture derived from corn syrup (Sigma). A solution of 2,5-dihydroxybenzoic acid in 20% CH₃CN in water at a concentration of 25 mg/ml was used as the MALDI matrix. One microliter of matrix solution and 1 μl of the sample were premixed and than deposited on the target. The droplet was allowed to dry at room temperature. Spectra were calibrated and processed under computer control using the Applied Biosystems Data Explorer software.

Dot blot hybridization. The 13 DNA probes used consisted of the digoxigenin (DIG)-labeled amplification products of strain R110 obtained with the DIG DNA labeling and detection kit (Boehringer, Mannheim, Germany). These probes were obtained using primer pair MutE-MutF and those in Table 2. Cell lysates were obtained by resuspending the cells of 500-μl overnight cultures in LB in 100 μl of 0.4 M NaOH and heating them for 30 min at 80°C. One microliter of each cell lysate was blotted on Hybond membranes and bound by UV cross-linking. Hybridization was done by following the standard protocol (44) with

TABLE 2. Primers used to amplify and subclone individual genes in pGEMT

Amplified gene	Primer	Sequence
waaA	AAf	5′-TACTCATAACGCTCCAAAAGCA-3′
	AAr	5′-TTTCATAAAACGGCCCATAAAC-3′
waaC	ACf	5′-GTCTTTAGCGAACTCGAGCAAT-3′
	ACr	5′-AGCACTTTCGATGGATTTGATT-3′
waaF	AFf	5′-TCCAAGCCGTGGCTGATGCAG-3′
	AFr	5′-GAGTGGTGGGAAGTGGGTAA-3′
wabN	BNf	5′-GTGCACGAATTGCTCTGATG-3′
	BNr	5′-ATGGGTGGCAAGATAATGCT-3′
wabH	BHf	5′-TGGCGATGGCAAATTTTACT-3′
	BHr	5′-ATTCCGGCCGATAACTTAGG-3′
wabG	BGf	5′-ACGCAAACGCGTTATTTAAGTT-3′
	BGr	5′-GCCATGGTAACTATCTGCATCA-3′
waaQ	AQf	5′-CACTGAAACGGAGTGCAATAAC-3′
	AQr	5′-TCCAAGAGCGTGTAATCACATT-3′
waaE	AEf	5′-TTTTAGTTCCCCGCCATC-3′
	AEr	5′-AAATGGTCGCTTGCTGTT-3′
wabO	BOf	5′-GATGCGGCTGATATTGGTTT-3′
	BOr	5′-TCCATCGGATCAAGACTTCC-3′
wamA	MAf	5′-AATGCATGCGGTAGAGCGTATC-3′
	Mar	5′-GAGTTTATGCCTGGTGGAAG-3′
wamB	MBf	5′-GTTGCTGAAAACGGGGTAAA-3′
	MBr	5′-TGCATGTTGCTACTGCTTTTG-3′
wamC	MCf	5′-CCATACCTCCTAAGCCTTGC-3′
	MCr	5′-ACGTAAGCCTTTCGCTTTGA-3′
wamD	MDf	5′-GTGGGGATATTGGGGAGATT-3′
	MDr	5′-TTCGGAAGGCCTACTTTTGA-3′
	MDfx	MDf with XabI tail
	MDfr	MDr with XbaI tail

stringent washing at 65°C in 0.2× SSC (20× SSC is 3 M NaCl plus 0.3 M sodium citrate, pH 7.0). Probes that remained bound to homologous sequences were detected with the DIG DNA labeling and detection kit in accordance with the supplier's instructions.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the *P. mirabilis* R110 and 51/57 gene clusters described here have been submitted to GenBank and assigned accession numbers HM146785 and HM146786.

RESULTS

Organization of the *P. mirabilis* waa gene cluster. In most of the *Enterobacteriaceae* studied so far, the genes involved in core LPS biosynthesis are found clustered (*waa* gene cluster) (11, 20, 39, 41). Usually, the first gene of the cluster is *hldD* (ADP-D-*glycero*-D-*manno*-heptose epimerase), and at the 3′ end of the cluster are found the genes not related to core biosynthesis, i.e., *coaD* (phosphopantetheine adenylyltransferase) (17), *fpg* (formamidopyrimidine-DNA glycosylase) (5), *rpmB* (ribosomal protein L28), *rpmG* (ribosomal protein L33) (29), and *radC* (DNA repair protein) (46). An alignment of the nucleotide sequences of these four genes allowed the identifi-

cation of highly conserved nucleotide regions in *hldD* and *radC*. From these regions, primer pairs HLDC1-HLDC2 (5′-AGTATTGTAGCCGGTGATGATT-3′ and 5′-GCTTGATA ACGACCTTTGAGTT-3′) and RADC3-RADC4 (5′-TGGA AAACGCAACCATAGAGA-3′ and 5′-ACCCCGTTCAGC AAAAGAA-3′) were designed. PCR amplification using genomic DNA from *P. mirabilis* strain R110 was successful, and the nucleotide sequence of the amplified fragment confirmed that inner regions of the *hldD* and *radC* genes of this strain were indeed amplified. Similarly, other primer pairs were designed from other conserved genes in the *waa* gene cluster, such as, for instance, RADC1-RADC-2 (5′-CATCCG CAGAAACCAAAAG-3′ and 5′-GTGGCGTAATGAAGCA CAAG-3′), FPG1-FPG2 (5-ATCTTAGTCCGCTATTGGTTT G-3′ and 5′-TCTTGCTCGGTTAAGCTACTG-3′), WABGQ1-WABGQ2 (5′-TGACATACAGCCATCAAGACAA-3′ and 5′-TC AATAAATTCTGCTCCACCAC-3′), and WAACF1-WAACF2 (5′-CTTGGGTTGGGGATATGATG-3′ and 5′-GAGTGGTGG GAAGTGGGTAA-3′). From the nucleotide sequences of these new amplified fragments, it was possible to design further primers allowing the amplification of DNA fragments encompassing the already sequenced ones. This strategy allowed the determination of the full nucleotide sequences of the *waa* gene clusters from *P. mirabilis* strains R110 (21,279 bp) and 51/57 (19,980 bp). Comparison of the *waa* gene clusters of strains R110 and 51/57 and the equivalent clusters of *E. coli*, *K. pneumoniae*, and *S. marcescens* (Fig. 2) shows that the organization of the 5′ end of the *waa* cluster is similar, with the presence of *hldD*, *waaF*, and *waaC* gene homologues in the same order. The 3′ end contains the *waaA* and *waaE* gene homologues, as found in *K. pneumoniae* and *S. marcescens*. In contrast to other known *Enterobacteriaceae*, the *waaL* gene homologue is located downstream from the *fpg* gene. In addition, between the *waaL* gene homologue and *rpmB*, four genes (*walM*, *walN*, *walO*, and *walR*) encoding putative glycosyltransferases are found (Fig. 2).

Comparison of the *waa* gene clusters from *P. mirabilis* R110 and 51/57, as well as that of strain HI4320 (Fig. 2), whose whole genome sequence has been determined (34) but whose core LPS structure is unknown, showed that the *wabG*, *wabH*, and *wabN* homologues are transcribed in the same direction in all three strains, but in strain R110, two additional genes that we named *wamB* and *wamC* (*wam* stands for <u>wa</u> genes from *P. mirabilis*) are inserted between *wabH* and *wabN* homologues. In strain 51/57, two contiguous genes, *wamD* and *mig-14*-like, were found between *waaC* and *wabN* instead of *wamA*. These results suggest the existence of gene insertion phenomena in the different *P. mirabilis waa* gene clusters. In agreement with this hypothesis, analysis of the guanine-plus-cytosine percentages along these *waa* gene clusters revealed significantly low G+C percentages in the regions containing the gene pairs *wamB-wamC* and *wamD–mig-14*-like (Fig. 2).

Proteus inner-core genes. The pentasaccharide L-α-D-Hep*p*III-(1→7)-L-α-D-Hep*p*II-(1→3)-L-α-D-Hep*p*I-(1→5)-[α-Kdo*p*II-(2→4)-]-α-Kdo*p*I (23, 24) has been found in the inner-core regions of all of the *Enterobacteriaceae* studied. This pentasaccharide is biosynthesized by the sequential transfer to lipid A of one to two residues of Kdo by the CMP-Kdo:lipid A Kdo bifunctional transferase (WaaA) and three residues of L,D-heptose by ADP-heptose-heptosyltransferases I, II, and III
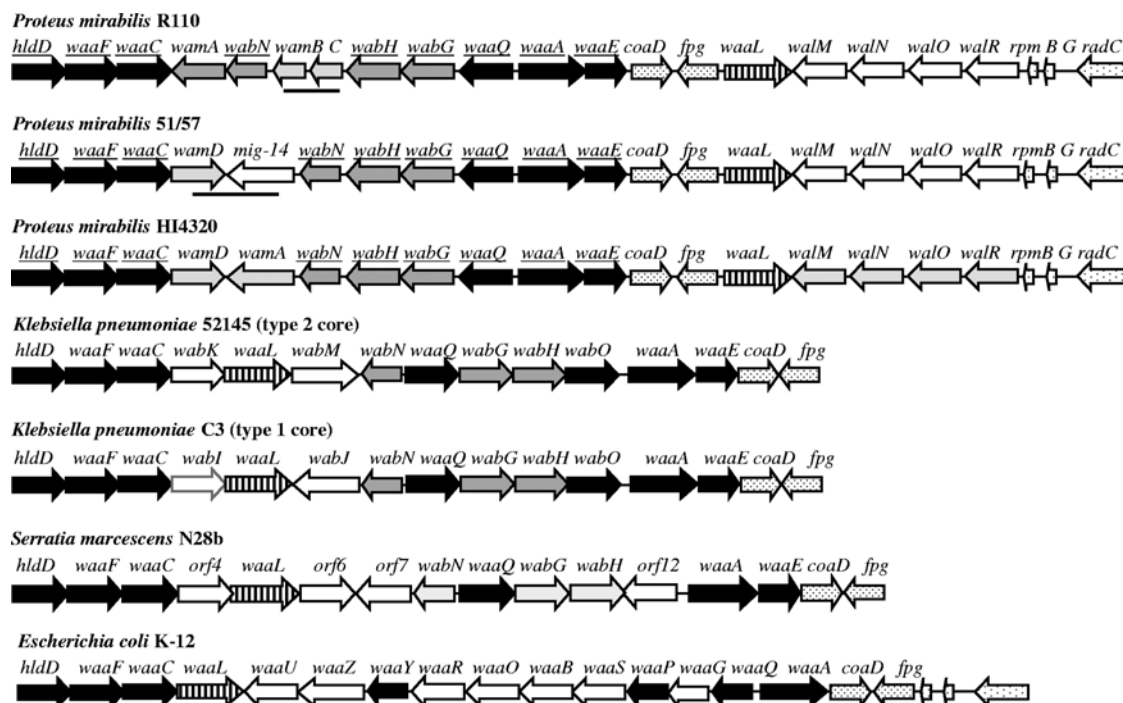
FIG. 2. Genetic organization of the chromosomal region (*waa* gene cluster) containing the core LPS biosynthesis genes of *P. mirabilis* strains R110, 51/57, and HI4320 (34), *K. pneumoniae* strains 52145 (41) and C3 (39), *S. marcescens* N28b (11), and *E. coli* K-12 (20). Common inner (black arrows)- and outer (gray arrows)-core genes, specific outer-core genes (light gray arrows), O-PS ligase genes (stripped arrows), genes with unknown functions (white arrows), and genes unrelated to core LPS (dotted arrows) are illustrated. The horizontal bars indicate regions with a significantly lower C+G percentage than the whole *wa* gene cluster. Genes common to the seven *P. mirabilis* strains studied (R110, 51/57, 14/57, 50/57, TG83, OXK, and CECT170) are underlined.

(WaaC, WaaF, and WaaQ). The *P. mirabilis* homologue WaaA showed high levels of amino acid identity and similarity to *E. coli* MG1655 WaaA (75 and 85%) (Table 3). The WaaC, WaaF, and WaaQ homologues showed high levels of identity and similarity to *K. pneumoniae* 52145 homologues WaaC (68 and 80%), WaaF (75 and 83%), and WaaQ (54 and 69%) (Table 3).

Proper identification of the functions of these four inner-core genes was performed as previously described (11), by complementation studies of known inner-core mutants. A plasmid containing the *waaA* gene from strain R110(pGEMT-WaaA$_{R110}$) was introduced into *E. coli* CJB26, a strain with a kanamycin resistance gene inserted in the chromosomal *waaA* gene and harboring a wild-type *waaA* gene in a temperature-sensitive plasmid (pJSC2). The pGEMT-WaaA$_{R110}$ plasmid restored the growth at 44°C of the CJB26 mutant. Analysis of LPSs by SDS-Tricine-PAGE showed that *K. pneumoniae* 52145 mutant strains 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*waaQ* (41) were complemented by plasmids pGEMT-WaaC$_{R110}$, pGEMT-WaaF$_{R110}$, and pGEMT-WaaQ$_{R110}$, respectively (Fig. 3).

We have previously shown that the *waaE* gene from *K. pneumoniae* encodes a glucosyltransferase responsible for the transfer of β-D-Glc to the O-4 position of L,D-Hep I and the presence of *waaE* gene homologues able to complement the *K. pneumoniae* 889Δ*waaE* mutant in both *S. marcescens* N28b and *P. mirabilis* CECT170 (25). The *waaE* gene from *P. mirabilis* R110 was also able to complement *waaE* mutants of both *K.*

pneumoniae 889 (data not shown) and 52145 (Fig. 3), confirming the function attributed to it.

The presence of a β-D-GalA II residue substitution at the O-7 position of L,D-Hep III is a common feature of type 1 and 2 core LPSs from *K. pneumoniae* (41, 50) and *P. mirabilis* strains R110 and 51/57 (51). We have shown that *K. pneumoniae* WabO catalyzes the transfer of this D-GalA residue from UDP-GalA to L,D-Hep III (15), but no *wabO* homologue was found in the two *P. mirabilis waa* gene clusters reported here. A search for highly similar WabO proteins in the whole genome of *P. mirabilis* strain HI4320 revealed that PMI2517 could be a WabO homologue. The putative HI4320 *wabO* homologue is located between *yntA* and *hycI*, encoding putative a nickel/dioligopeptide substrate-binding protein and a hydrogenase maturation protease, respectively (34). A primer pair, BOf-BOr (Table 2), was designed to amplify the *wabO* homologue, and PCR amplifications using these primers and genomic DNAs from strains R110 and 51/57 as the templates allowed the amplification of DNA fragments of about 3.3 kb (Fig. 4A). Determination of the nucleotide sequences of these fragments confirmed the presence of putative *wabO* homologues in *P. mirabilis* R110 and 51/57. The strain R110 *wabO* homologue was subcloned into pGEMT to obtain pGEMT-WabO$_{R110}$ and transformed into *K. pneumoniae* 52145Δ*wabO*. Analysis of the LPSs from these two strains showed that the R110 *wabO* homologue is able to complement the *K. pneumoniae* mutant (Fig. 4B and C). The LPSs were extracted from strains 52145Δ*wabO* and 52145Δ*wabO*(pGEMT-WabO$_{R110}$)

TABLE 3. Characteristics of *P. mirabilis* proteins involved in core LPS biosynthesis

Strain(s) and *P. mirabilis* protein	Protein size (amino acids)	Homologous protein	Organism (accession no.)	% Identity, similarity
R110, 51/57				
HldD	312[a]	ADP-L-*glycero*-D-*manno*-heptose 6-epimerase	*P. mirabilis* HI4320 (YP_002152859.1)	100, 100
			P. mirabilis ATCC 29906 (EEI49918.1)	99, 100
			P. penneri ATCC 35198 (EEG87050.1)	94, 97
			E. coli MG1655 (P37691)	80, 89
WaaF	350	Heptosyltransferase II	*P. mirabilis* HI4320 (YP_002152861.1)	100, 100
			P. mirabilis ATCC 29906 (EEI49920.1)	99, 99
			P. penneri ATCC 35198 (EEG87051.1)	89, 93
			K. pneumoniae 52145 (AAX20098.1)	75, 83
WaaC	320	Heptosyltransferase I (P37693)	*P. mirabilis* ATCC 29906 (EEI49920.1)	96, 97
			P. mirabilis HI4320 (YP_002152860.1)	94, 96
			K. pneumoniae 52145 (AAX20099.1)	66, 78
WabN	320	LPS:GlcNAc deacetylase	*P. mirabilis* ATCC 29906 (EEI49922.1)	100, 100
			P. mirabilis HI4320 (YP_002152857.1)	98, 99
			K. pneumoniae 52145 (YP_001337619.1)	65, 81
			S. marcescens N28b (YP_001481052.1)	70, 83
WabH	378	GlcNAc transferase	*P. mirabilis* HI4320 (YP_002152856.1)	99, 99
			P. mirabilis ATCC 29906 (EEI49925.1)	99, 99
			P. penneri ATCC 35198 (EEG87063.1)	87, 92
			K. pneumoniae 52145 (AAX20105.1)	58, 76
			S. marcescens N28b (AAD28802.2)	49, 66
WabG	376	GalA I transferase	*P. mirabilis* HI4320 (YP_002152855.1)	100, 100
			P. mirabilis ATCC 29906 (EEI49926.1)	98, 99
			K. pneumoniae 52145 (AAX20104.1)	64, 77
			S. marcescens N28b (AAD28801.1)	65, 80
WaaQ	354	Heptosyltransferase III	*P. mirabilis* HI4320 (YP_002152854.1)	100, 100
			P. mirabilis ATCC 29906 (EEI49927.1)	99, 100
			Proteus penneri ATCC 35198 (EEG87067.1)	82, 91
			E. coli MG1665 (P37704)	42, 62
			K. pneumoniae 52145 (AAX20103.1)	54, 69
WaaA	425	Kdo transferase	*P. mirabilis* HI4320 (YP_002152853.1)	100, 100
			P. mirabilis ATCC 29906 (EEI 49928.1)	99, 99
			S. marcescens N28b (AAC44432.1)	79, 88
			K. pneumoniae 52145 (AAX20107.1)	76, 87
			E. coli MG1665 (P37705)	75, 85
WaaE	259	Inner-core glucosyltransferase	*P. mirabilis* HI4320 (YP_002152852.1)	100, 100
			P. mirabilis ATCC 29906 (EEI 49929.1)	99, 99
			P. penneri ATCC 35198 (EEG87070.1)	84, 90
			K. pneumoniae 52145 (AAX20108.1)	64, 76
			S. marcescens N28b (AAC44433.1)	64, 77
WaaL	422	O-antigen ligase	*P. mirabilis* HI4320 (YP_002152849.1)	100, 100
			P. mirabilis ATCC 29906 (EEI49933.1)	98, 98
			S. enterica serovar Typhimurium LT2 (NP_462613.1)	39, 60
WabO	330	GalA transferase II	*P. mirabilis* HI4320 (YP_002152236.1)	100, 100
			P. mirabilis ATCC 29906 (EEI 47376.1)	99, 100
			P. penneri ATCC 35198 (EEG86878.1)	76, 87
			K. pneumoniae 52145 (AAX20106.1)	59, 76
R110				
WamA	357	Heptosyltransferase	*P. mirabilis* HI4320 (YP_002152858.1)	85, 90
			P. mirabilis ATCC 29906 (EEI 49921.1)	98, 98
			S. proteamaculans 568 (YP_001481051.1)	43, 58
			S. marcescens N28b ORF7? (AAL23759.1)	43, 64

TABLE 3—*Continued*

Strain(s) and *P. mirabilis* protein	Protein size (amino acids)	Homologous protein	Organism (accession no.)	% Identity, similarity
WamB	330	Glycosyltransferase	*P. mirabilis* ATCC 29906 (EEI49923.1)	100, 100
			P. penneri ATCC 35198 (EEG87061.1)	82, 90
WamC	295	Heptosyltransferase	*P. mirabilis* ATCC 29906 (EEI49924.1)	91, 93
			P. penneri ATCC 35198 (EEG87062.1)	75, 82
51/57 HI4320, WamD	298	Glycosyltransferase	*P. mirabilis* HI4320 (YP_002152859.1)	83, 90
			P. luminescens subsp. *laumondii* TT01 (NP_930250.1)	46, 64
51/57, Mig-14	293	Mig-14 family	*P. penneri* ATCC 35198 (EEG87057.1)	76, 88
			Erwinia pyrifoliae Ep1/96 (YP_002649034.1)	39, 59
			S. enterica serovar Typhimurium LT2 (NP_461708.1)	33, 54

a C-terminal fragment.

and purified, and the corresponding OS fractions were obtained by mild acid hydrolysis (see Materials and Methods). Gas chromatography (GC) of the alditol acetates derived after full acid hydrolysis of the OS fractions from strains 52145Δ*wabO* and 52145Δ*wabO*(pGEMT-WabO$_{R110}$) showed an increase in the GalA/GlcN ratios from 1 to 1.7, in agreement with the hypothesized function of WabO$_{R110}$.

Common outer-core genes. The outer-core disaccharide α-D-GlcN-(1→4)-D-GalA I is another feature common to *K. pneumoniae* (11, 41, 50) and *P. mirabilis* R110 and 51/57 (51). We have previously shown that *K. pneumoniae* WabG is responsible for the transfer of D-GalA to the O-3 position of L,D-Hep II (26). Similarly, we have shown that two *K. pneumoniae* enzymes (WabH and WabN) are required for the incorporation of the GlcN residue. WabH transfers a D-GlcNAc residue from UDP-GlcNAc to the first outer-core residue (D-GalA), and WabN deacetylates the D-GlcNAc residue to D-GlcN (40). *K. pneumoniae* mutants 52145Δ*wabG*, 52145Δ*wabH*, and 52145Δ*wabN* produce shorter core LPSs than wild-type strain

52145, and their LPSs are devoid of O-PS. As expected, pGEMT-WabG$_{R110}$, pGEMT-WabH$_{R110}$, and pGEMT-WabN$_{R110}$ were able to restore wild-type core LPS mobility in SDS-Tricine-PAGE and O-PS production when introduced into strains 52145Δ*wabG*, 52145Δ*wabH*, and 52145Δ*wabN*, respectively (Fig. 5). Compositional analysis of the core OS fractions by GC showed the presence of GlcNAc and GlcN in strains 52145Δ*wabH*(pGEMT-WabH$_{R110}$) and 52145Δ*wabN*-(pGEMT-WabN$_{R110}$), respectively. In contrast, the OS fraction from strain 52145Δ*wabH* lacks either GlcNAc or GlcN and strain 52145Δ*wabN* shows GlcNAc instead of GlcN, as previously reported (26, 40). A similar analysis of the OS fractions from strain 52145Δ*wabG*(pGEMT-WabG$_{R110}$) showed the presence of GalA, while this residue was absent from strain 52145Δ*wabG*. These results show that these three genes are functional homologues of the *K. pneumoniae* genes.

Specific *P. mirabilis* R110 outer-core genes. The remaining genes in the R110 *waa* gene cluster (*wamA*, *wamB*, and *wamC*) were expected to be involved in outer-core completion. A BLAST search of the putative proteins encoded by *wamA* and *wamC* showed high similarity and identity to heptosyltransferases of unknown function from *P. mirabilis* HI4320 and ATCC 29906 (Table 3), and they are candidates for the transfer of the two outer-core D,D-Hep residues. A similar search with the putative protein encoded by *wamB* showed high levels of similarity and identity to glycosyltransferases from the same two *P. mirabilis* strains (Table 3). To determine the functions of these three genes, we introduced them into the *K. pneumoniae* 52145Δ*wabH* and 52145Δ*wabK* mutants because they produce truncated core LPSs extending up to the outer-core D-GalA and D-GlcN residues, respectively. We expected that these mutant LPSs could be good acceptors for residues transferred by some of the proteins encoded by these three *P. mirabilis* R110 genes.

LPS from *K. pneumoniae* 52145*wabH* analyzed by SDS-Tricine-PAGE showed an increase in mobility in comparison to that of wild-type 52145 LPS (Fig. 6A). Introduction of pGEMT-WamA into mutant 52145Δ*wabH* resulted in LPS with a decrease in mobility compared to that of 52145Δ*wabH*

FIG. 3. SDS-Tricine-PAGE analysis of LPS samples from *K. pneumoniae* 52145, 52145Δ*waaC*, 52145Δ*waaC*(pGEMT-WaaC$_{R110}$), 52145Δ*waaF*, 52145Δ*waaF*(pGEMT-WaaF$_{R110}$), 52145Δ*waaE*, 52145Δ*waaE*(pGEMT-WaaE$_{R110}$), 52145Δ*waaQ*, and 52145Δ*waaQ*(pGEMT-WaaQ$_{R110}$).
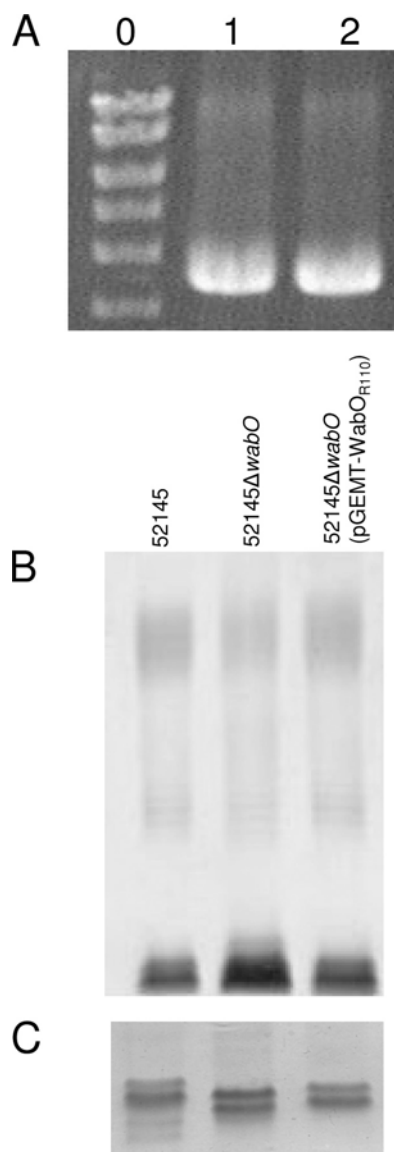
FIG. 4. (A) PCR-amplified DNA products obtained using oligonucleotides BOf and BOr and genomic DNAs from *P. mirabilis* R110 (lane 1) and 51/57 (lane 2). Lane 0, molecular mass marker. SDS-PAGE (B) and SDS-Tricine-PAGE (C) analyses of LPSs from *K. pneumoniae* 52145, 52145Δ*wabO*, and 52145Δ*wabO*(pGEMT-WabO$_{R110}$) are also shown.

FIG. 5. SDS-PAGE (A) and SDS-Tricine-PAGE (B) analyses of LPSs from *K. pneumoniae* 52145, 52145Δ*wabG*, 52145Δ*wabG* (pGEMT-WabG$_{R110}$), 52145Δ*wabH*, 52145Δ*wabH*(pGEMT-WabH$_{R110}$), 52145Δ*wabN*, and 52145Δ*wabN*(pGEMT-WabN$_{R110}$).

harboring vector pGEMT (Fig. 6A). Chemical analysis of LPS isolated from strain 52145Δ*wabH*(pGEMT-WamA) showed the presence of small amounts of D,D-Hep in addition to L,D-Hep, suggesting that this gene encodes an outer-core heptosyltransferase. Similar results were obtained when the same experiment was performed in the genetic background of mutant 52145Δ*wabK* (Fig. 7A). The LPSs were extracted from strains 52145Δ*wabH* and 52145Δ*wabH*(pGEMT-WamA) and purified, the corresponding OS fractions were obtained by mild acid hydrolysis (see Materials and Methods), and MALDI-TOF spectra were obtained in the positive mode. Major signals at *m/z* 1,327.09 and 1,309.10 were obtained from 52145Δ*wabH* corresponding to Kdo-Hep$_3$-Hex-HexA$_2$ and its anhydrous

form, respectively (Fig. 6B). In agreement with the presence of a D,D-Hep residue, the OS fraction of LPSfrom 52145Δ*wabH* (pGEMT-WamA) showed major signals at *m/z* 1,519.32 and 1,501.41, about 192.67 Da higher than those obtained from 52145Δ*wabH* (Fig. 6C).

pGEMT-WamC introduced into mutant 52145Δ*wabH* did not modify the migration of LPS in SDS-Tricine-PAGE (Fig. 6A), suggesting that core LPS extending up to the first outer GalA residue could not act as an acceptor of WamC transferase. In contrast, the LPS from mutant 52145Δ*wabK* harboring pGEMT-WamC showed a decrease in gel migration compared to that from 52145Δ*wabK* (Fig. 7A). Chemical analysis of the purified LPS from 52145Δ*wabK*(pGEMT-WamC) showed again the presence of D,D-Hep. MALDI-TOF analysis of the OS from 52145Δ*wabK*(pGEMT-WamC) showed major signals at *m/z* 1,680.67 and 1,662.54, corresponding to Kdo-Hep$_4$-Hex-HexN-HexA$_2$ and its anhydrous form, respectively (Fig. 7C). These major signals are approximately 192 Da higher than those of OS from 52145Δ*wabK* (Fig. 7B). These results strongly suggest that WamA and WamC are heptosyltransferases involved in the transfer of D,D-Hep to outer-core residues D-GalA I and D-GlcN, respectively.

According to the outer-core structure of *P. mirabilis* R110, the *wamB*-encoded product could be involved in the transfer of D-Gal to the D,D-Hep-1,2-D-GalA I disaccharide. In agreement
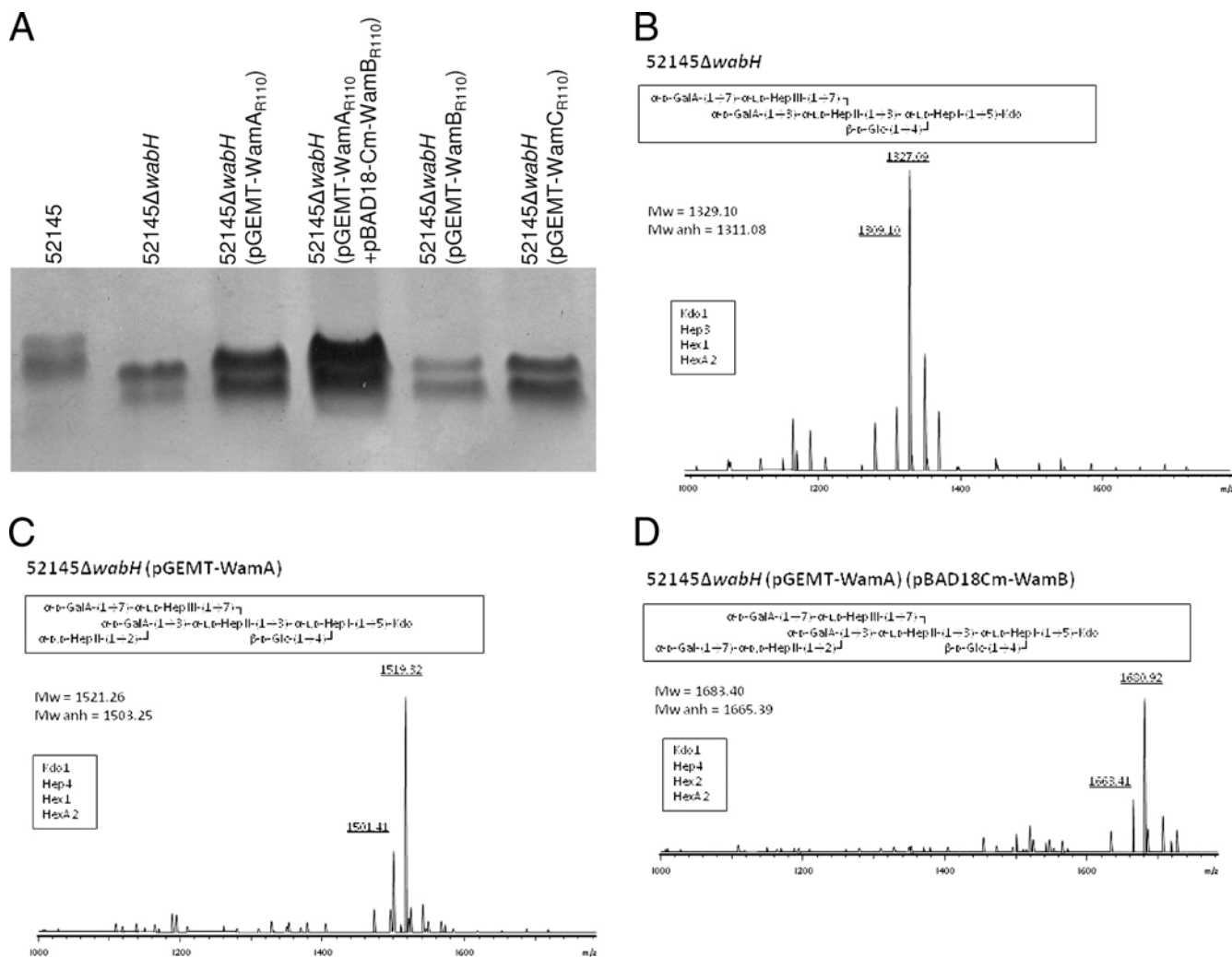
FIG. 6. (A) SDS-Tricine-PAGE analysis of LPS samples from *K. pneumoniae* 52145, 52145Δ*wabH*, 52145Δ*wabH*(pGEMT-WamA$_{R110}$), 52145Δ*wabH*(pGEMT-WamA$_{R110}$)(pBAD18-Cm-WamB), 52145Δ*wabH*(pGEMT-WamB$_{R110}$), and 52145Δ*wabH*(pGEMT-WamC$_{R110}$). Positive-ion MALDI-TOF analyses of acid-released core OSs from the LPSs of *K. pneumoniae* 52145Δ*wabH* (B), 52145Δ*wabH*(pGEMT-WamA$_{R110}$) (C), and 52145Δ*wabH*(pGEMT-WamA$_{R110}$)(pBAD-WamB) (D) are also shown.

with this hypothesis, pGEMT-WamB did not modify the mobility of LPS when introduced into mutant 52145Δ*wabH* or 52145Δ*wabK* (Fig. 6A and 7A). In contrast, LPS from mutant 52145Δ*wabH* harboring both *wamA*(pGEMT-WamA) and *wamB*(pBAD18-Cm-WamB) showed less mobility in SDS-Tricine-PAGE than LPS from 52145Δ*wabH*(pGEMT-WamA) (Fig. 6A). Chemical analysis of LPS from 52145Δ*wabH* harboring both pGEMT-WamA and pBAD18-Cm-WamB showed the presence of Gal in addition to D,D-Hep. MALDI-TOF analysis of the OS from 52145Δ*wabH*(pGEMT-WamA) (pBAD18-Cm-WamB) showed major signals at *m/z* 1,680.92 and 1,663.41, corresponding to Kdo-Hep$_4$-Hex$_2$-HexA$_2$ and its anhydrous form, respectively (Fig. 6D). These major signals are 162 Da higher than those from the 52145Δ*wabH*(pGEMT-WamA) OS. These results strongly suggest that *wamB* encodes the outer-core galactosyltransferase.

Specific *P. mirabilis* 51/57 outer-core gene. The *waa* gene cluster from *P. mirabilis* 51/57 contains two genes without attributed functions, *mig-14* and *wamD* (Fig. 2), and the disac-

charide β-D-GalA III-(1→3)-D-GlcNAc constitutes the nonreducing end of its core OS (Fig. 1). The *mig-14*-like-encoded product showed similarities to a family of proteins of unknown function, with that encoded by *mig-14* from *Salmonella enterica* serovar Typhimurium being the best studied. Mig-14 from *S. enterica* has been shown to be involved in virulence and to have a role in resistance to cationic antimicrobial peptides (6, 7, 49). The LPS from an *S. enterica mig-14* mutant did not show apparent changes compared to that of the wild-type strain; thus, it seems unlikely that the *P. mirabilis mig-14*-like gene would play a role in core OS biosynthesis.

The *wamD*-encoded product showed a high level of identity to a putative glycosyltransferase from *P. mirabilis* HI4320; it also showed similarity to a glycosyltransferase from *Photorhabdus luminescens laumondii* (Table 3). Thus, *wamD* is hypothesized to be involved in the transfer of either D-GlcNAc or D-GalA III to the nonreducing end disaccharide. To test this hypothesis, *wamD* was introduced into 52145Δ*wabK* and the LPS of the transformed strain was analyzed by SDS-Tricine-
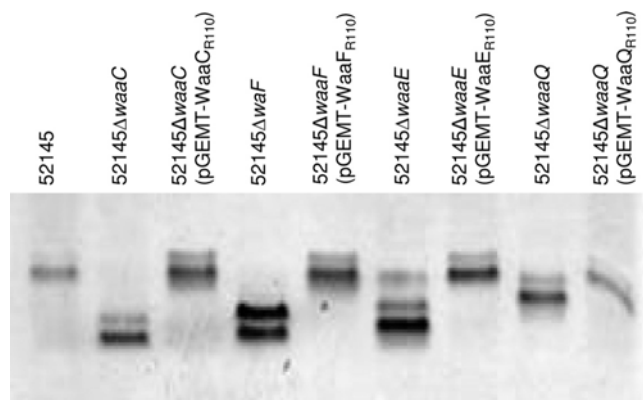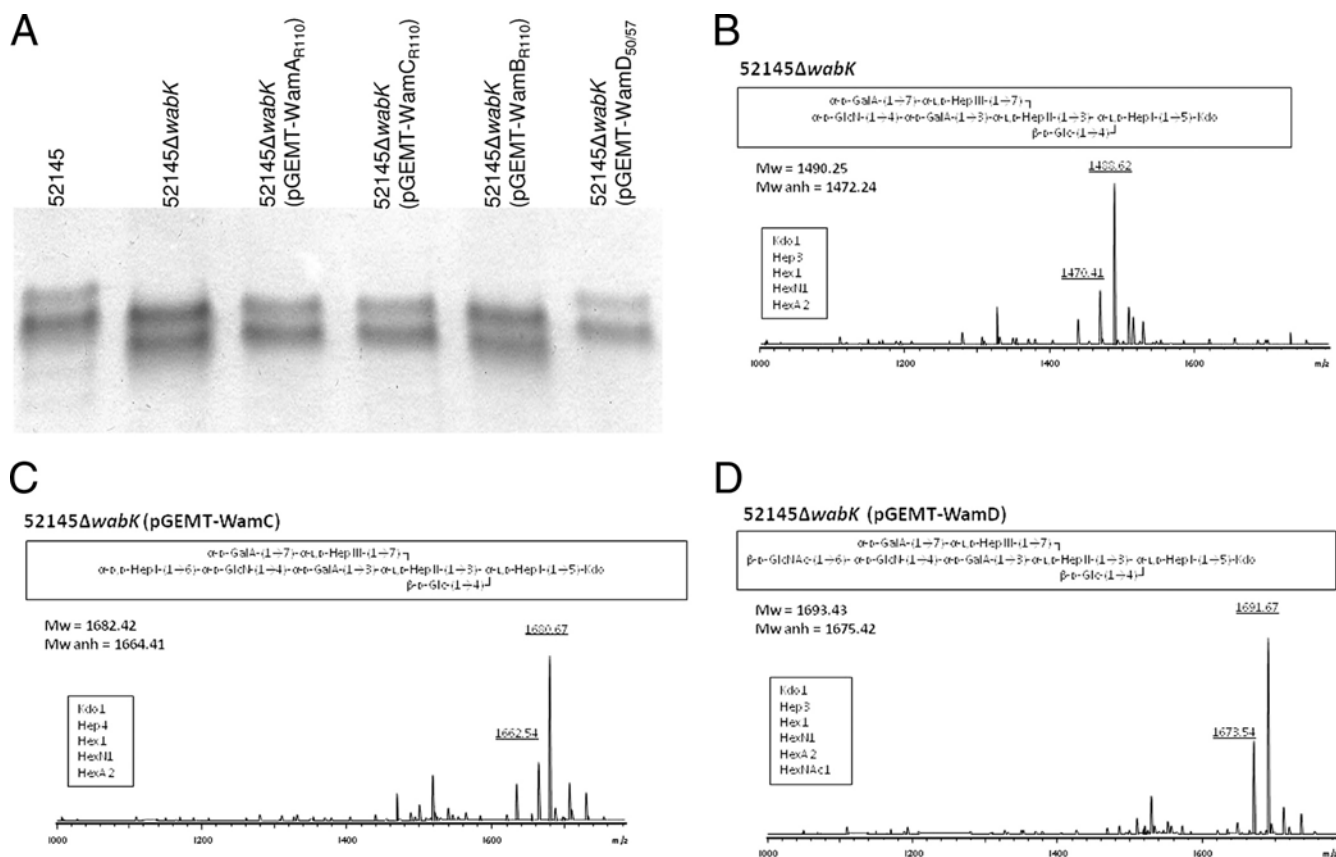
FIG. 7. (A) SDS-Tricine-PAGE analysis of LPS samples from *K. pneumoniae* 52145, 52145Δ*wabK*, 52145Δ*wabK*(pGEMT-WamA$_{R110}$), 52145Δ*wabK*(pGEMT-WamC$_{R110}$), 52145Δ*wabK*(pGEMT-WamB$_{R110}$), and 52145Δ*wabK*(pGEMT-WamD$_{51/57}$. Positive-ion MALDI-TOF analyses of acid-released core OSs from the LPSs of *K. pneumoniae* 52145Δ*wabK* (B), 52145Δ*wabK*(pGEMT-WamC$_{R110}$) (C), and 52145Δ*wabK* (pGEMT-WamD$_{51/57}$ (D) are also shown.

PAGE. As shown in Fig. 7A, *wamD* induces a decrease in LPS mobility in comparison to that of LPS from 52145Δ*wabK*, suggesting that the *wamD*-encoded product is able to add an additional residue to the acceptor LPS. Chemical analysis of the LPS from 52145Δ*wabK*(pGEMT-WamD) showed the presence GlcNAc, while this sugar was absent from that of 52145Δ*wabK*. MALDI-TOF analysis of the OS from 52145Δ*wabK*(pGEMT-WamD) was also in agreement with the presence of a GlcNAc residue, since major signals (*m/z* 1,691.67 and 1,673.54) were approximately 203 Da higher than those of the OS fraction from 52145Δ*wabK* (Fig. 7B and D). These results are in agreement with an *N*-acetylglucosaminyl-transferase function for WamD.

O-antigen polymerase ligase. The putative O-PS ligases encoded by the *waaL* gene homologues from strains HI4320, R110, and 51/57 showed identical deduced amino acid sequences, despite the fact WaaL$_{R110}$ and WaaL$_{51/57}$ ligate O-PS O3 and O28 to core LPS, respectively. As in other O-PS ligases, analysis of *P. mirabilis* WaaL showed the presence of 10 putative transmembrane helices and a match with the Pfam PF04932 protein family. To prove its function, a nonpolar deletion mutant of wild-type strain S1959 was constructed by replacing the wild-type gene with an in-frame internal deletion (see Materials and Methods). The 1959Δ*waaL* mutation in frame was checked by amplification of the chromosomal *waaL*

deletion using primers MutE and MutF and determination of the nucleotide sequence of the amplified product. Analysis of the LPS from S1959Δ*waaL* showed that it was devoid of O-PS, confirming its O-PS ligase function. Furthermore, chemical and mass spectral analyses of the core OS from this mutant (data not shown) revealed that both R110 and S1959Δ*waaL* had the same core OS. This result suggests that the O-PS deficiency in strain R110 arises from a mutation in O-PS O3 biosynthesis and not in the *waa* cluster.

Distribution of core biosynthetic genes in *P. mirabilis*. To determine the degree of conservation of the genes putatively involved in core LPS biosynthesis in *P. mirabilis*, specific DIG-labeled PCR amplification probes were used in dot blot hybridization experiments. Thirteen probes, one for each of the genes putatively involved in core LPS biosynthesis by strain R110, were synthesized using primer pair MutE-MutF (for *waaL*) and those shown in Table 2 for *waaA*, *waaC*, *waaF*, *waaQ*, *waaE*, *waaL*, *wabN*, *wabH*, *wabG*, *wabO*, *wamA*, *wamB*, and *wamC*. These probes were used in dot blot assays to screen genomic DNAs from five additional *P. mirabilis* strains (14/57, 50/57, TG83, OXK, and CECT170). As controls, genomic DNAs from strains R110 and 51/57 were used. All of the *P. mirabilis* genomic DNAs reacted with probes for the genes *waaA*, *waaC*, *waaF*, *waaQ*, *waaE*, *waaL*, *wabN*, *wabH*, *wabG*, and *wabO* (Fig. 2, underlined genes), suggesting that genes

involved in the biosynthesis of the core LPS up to the second outer residue are conserved in this bacterial species. In contrast, the dot blot assay indicates that *wamA* was only missing in strain 51/57, *wamB* was R110 specific, and *wamC* was found only in strains R110 and OXK. These *wam* genes appear to be strain specific and are involved in the biosynthesis of the variable region of the outer-core LPS.

DISCUSSION

In this work, we have been able to identify the functions of the genes found in the *waa* gene cluster from three *P. mirabilis* strains (R110, 51/57, and HI4320). The approach used for their identification was based on complementation studies of genes with homologues of known function. For the remaining genes, we took advantage of the fact that sugar residues and bonds between core LPSs from *K. pneumoniae* and *P. mirabilis* are identical up to the second outer-core residue. Thus, we have used LPS molecules extending up to the first or second outer-core LPS residues from *K. pneumoniae* 52145 mutants as surrogates of *P. mirabilis* acceptor molecules to identify the functions of two heptosyltransferases (WamA and WamC), a galactosyltransferase (WamB), and an *N*-acetylglucosaminyl-transferase (WamD). This identification was facilitated by the absence of D,D-Hep, D-Gal, and D-GlcNAc residues in the core LPS of *K. pneumoniae* 52145 and was confirmed by mass spectrometry analysis of OS from LPS molecules modified *in vivo* by the action of the corresponding enzymes. This approach allowed the identification all of the genes required for the biosynthesis of the sugar components of the core LPS of strain R110. Although the structure of the core LPS of strain HI4320 has not been determined, the presence of WamA and WamD homologues strongly suggests the presence a D,D-Hep residue linked to D-GalAI and a D-GlcNAc residue linked to D-GlcN.

Our results show that a gene(s) located outside the *waa* gene cluster is required for core LPS biosynthesis, such as, for instance, *wabO*, encoding the branched inner-core residue D-GalA II transferase and found in the three *P. mirabilis* strains. In strain 51/57, an additional gene encoding the transfer of outer-core residue D-GalA III (Fig. 1) should be also located outside the *waa* gene cluster since no candidate for this function was found. Some residues are modified with phosphoethanolamine (L,D-Hep II in R110 and 51/57 and D-GalA III in 51/57) (Fig. 1), and again no genes putatively encoding these modifications were found in the *waa* gene cluster. Finally, additional modifications of some residues with amino acids have been reported for the core LPSs from strains R110 and 51/57 (51) and the genes encoding these functions should also be located outside the *waa* gene cluster.

The four genes located downstream from *waaL* putatively encode enzymes belonging to glycosyltransferase families 4 (*walM*, *walN*, and *walR*) and 9 (*walO*) according to the Carbohydrate-Active EnZymes database classification (9). Members of these two families were shown to be involved in the biosynthesis of core LPSs in several Gram-negative bacteria (http://www.cazy.org/). Since these four genes are found in the two strains studied here (R110 and 51/57) and in strain HI4320, one should expect the presence of four unique and specific common residues in the *P. mirabilis* core LPS if these genes are involved in core OS biosynthesis. Nine different

genes (*waa* and *wab*) have been identified in this work as responsible for the transfer of the eight common core OS residues. Thus, the putative glycosyltransferases encoded by these four *wal* genes do not appear to be involved in the biosynthesis of the reported core LPS structures of strains R110 and 51/57.

In *P. mirabilis* strain 51/57, a gene encoding a protein of the Mig-14 family was identified. Inspection of the available whole genome of *P. mirabilis* HI4320 did not allow the identification of a gene similar to *mig-14*. A BLAST search revealed that genes encoding Mig-14 family members are found inside the *Gammaproteobacteria* families *Pseudomonadaceae* and *Enterobacteriaceae*. In members of the family *Pseudomonadaceae* such as *P. aeruginosa* PAO1 (accession no. AE004091) and *Azotobacter vinelandii* DJ (accession no. CP001157), the *mig-14*-like gene is found inside the *waa* gene cluster, suggesting that they could have some unknown function in core LPS biosynthesis. In contrast, in *Enterobacteriaceae*, *mig-14*-like genes are found away from the *waa* gene cluster, as in *S. enterica* subsp. *enterica* serovar Typhimurium LT2 (accession no. AE006471), and even sometimes in plasmids, as in *E. coli* APECO1 (accession no. NC 009837.1). The only well-studied member of this family is Mig-14 from strain LT2, where it has been shown to be an inner-membrane protein involved in virulence and protection from antimicrobial cationic peptides (6, 7, 49). Although in some of these studies the LPS of a *mig-14* mutant was analyzed, no changes in its structure were detected (7). Thus, the localization of the *mig-14*-like gene in strain 51/57 appears to be an exception in the family *Enterobacteriaceae* and it is unlikely that this gene would be involved in core LPS biosynthesis.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Belas, R.** 1996. *Proteus mirabilis* swarmer cell differentiation and urinary tract infection, p. 271–298. *In* H. L. T. Mobley and J. W. Warren (ed.), Urinary tract infections: molecular pathogenesis and clinical management. ASM Press, Washington, DC.
3. **Belas, R., M. Goldman, and K. Ashliman.** 1995. Genetic analysis of *Proteus mirabilis* defective in swarmer cell elongation. J. Bacteriol. **177:**823–828.
4. **Belunis, C. J., T. Clementz, S. M. Carty, and C. H. R. Raetz.** 1995. Inhibition of lipopolysaccharide biosynthesis and cell growth following inactivation of the *kdtA* gene of *Escherichia coli.* J. Biol. Chem. **270:**27646–27652.
5. **Boiteux, S., T. R. O'Connor, and J. Laval.** 1987. Formamidopyrimidine-DNA glycosylase of *Escherichia coli*: cloning and sequencing of the *fpg* structural gene and overproduction of the protein. EMBO J. **6:**3177–3183.
6. **Brodsky, I. E., R. K. Ernst, S. I. Miller, and S. Falkow.** 2002. *mig-14* is a *Salmonella* gene that plays a role in bacterial resistance to antimicrobial peptides. J. Bacteriol. **184:**3203–3213.
7. **Brodsky, I. E., N. Ghori, S. Falkow, and D. Monack.** 2005. Mig-14 is an inner-membrane-associated protein that promotes *Salmonella typhimurium* resistance to CRAMP, survival within activated macrophages and persistent infection. Mol. Microbiol. **55:**954–972.
8. **Burall, L. S., J. M. Harro, X. Li, C. V. Lockatell, S. T. Himpsl, J. R. Hebel, D. E. Johnson, and H. L. T. Mobley.** 2004. *Proteus mirabilis* genes that contribute to pathogenesis of urinary tract infections: identification of 25

signature-tagged mutants attenuated at least 100-fold. Infect. Immun. **72:**2922–2938.

9. **Cantarel, B. L., P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat.** 2008. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. **37:**D233–D238.

10. **Cavaillon, J. M., and N. Haeffner-Cavaillon.** 1992. Structure-function relationships of core oligosaccharide, p. 205–224. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol. 1: molecular biochemistry and cellular biology. CRC Press, Inc., Boca Raton, FL.

11. **Coderch, N., N. Piqué, B. Lindner, N. Abitiu, S. Merino, L. Izquierdo, N. Jimenez, J. M. Tomás, O. Holst, and M. Regué.** 2004. Genetic and structural characterization of the core region of the lipopolysaccharide from *Serratia marcescens* N28b (serovar O4). J. Bacteriol. **186:**978–988.

12. **Coker, C., C. A. Poore, X. Li, and H. L. T. Mobley.** 2000. Pathogenesis of *Proteus mirabilis* urinary tract infection. Microbes Infect. **2:**1497–1505.

13. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.

14. **Fairley, K. F., et al.** 1971. Site of infection in acute urinary-tract infection in general practice. Lancet **2:**615–618.

15. **Fresno, N., Jiménez, R. Canals, S. Merino, M. M. Corsaro, R. Lanzetta, M. Parrilli, G. Pieretti, M. Regué, and J. M. Tomás.** 2007. A second galacturonic acid transferase is required for core lipopolysaccharide biosynthesis and complete capsule association with the cell surface in *Klebsiella pneumoniae*. J. Bacteriol. **189:**1128–1137.

16. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9:**245–249.

17. **Geerlof, A., A. Lexendon, and V. Shaw.** 1999. Purification and characterization of phosphopantheine adenyltransferase from *Escherichia coli*. J. Biol. Chem. **274:**27105–27111.

18. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.

19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.

20. **Heinrichs, D. E., J. A. Yethon, and C. Whitfield.** 1998. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. Mol. Microbiol. **30:**221–232.

21. **Himpsl, S. D., C. V. Lockatell, J. R. Hebel, D. E. Jhonson, and H. L. T. Mobley.** 2008. Identification of virulence determinants in uropathogenic *Proteus mirabilis* using signature-tagged mutagenesis. J. Med. Microbiol. **57:**1068–1078.

22. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.

23. **Holst, O.** 1999. Chemical structure of the core region of lipopolysaccharides, p. 115–154. *In* H. Brade, S. M. Opal, S. N. Vogel, and D. C. Morrison (ed.), Endotoxins in health and disease. Marcel Dekker Inc., New York, NY.

24. **Holst, O.** 2002. Chemical structure of the core region of lipopolysaccharides. An update. Trends Glycosci. Glycotechnol. **14:**87–103.

25. **Izquierdo, L., N. Abitiu, N. Coderch, B. Hita, S. Merino, R. Gavin, J. M. Tomás, and M. Regué.** 2002. The inner-core lipopolysaccharide biosynthetic *waaE* gene: function and genetic distribution among some *Enterobacteriaceae*. Microbiology **148:**3485–3496.

26. **Izquierdo, L., N. Coderch, N. Piqué, E. Bedini, M. Corsaro, S. Merino, S. Fresno, J. M. Tomás, and M. Regué.** 2003. The *Klebsiella pneumoniae wabG* gene: its role in the biosynthesis of the core lipopolysaccharide and virulence. J. Bacteriol. **185:**7213–7221.

27. **Knirel, Y. A., W. Kaca, A. Rozalsky, and Z. Sydorczyk.** 1999. Structure of the O-antigenic polysaccharide of Proteus bacteria. Pol. J. Chem. **73:**895–907.

28. **Larsson, P.** 1984. Serology of *Proteus mirabilis* and *Proteus vulgaris*, p. 187–214. *In* T. Bergan (ed.), Methods in microbiology, volume 14. Academic Press Inc., London, United Kingdom.

29. **Lee, J. S., G. An, J. D. Friesen, and K. Isono.** 1981. Cloning and the nucleotide sequence of the genes for *Escherichia coli* ribosomal proteins L28 (*rpmB*) and L33 (*rpmG*). Mol. Gen. Genet. **184:**218–223.

30. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179:**6228–6237.

31. **Mamat, U., U. Seydel, D. Grimmecke, O. Holst, and E. T. Rietschel.** 1999. Lipopolysaccharides, p. 179–239. *In* B. M. Pinto (ed.), Carbohydrates and their derivatives including tannins, cellulose, and related lignins. Elsevier Science, Amsterdam, Netherlands.

32. **McCoy, A. J., H. Liu, T. J. Falla, and J. S. Gunn.** 2001. Identifications of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. Antimicrob. Agents Chemother. **45:**2030–2037.

33. **O'Hara, C. M., F. W. Brenner, and J. M. Miller.** 2000. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clin. Microbiol. Rev. **13:**534–546.

34. **Pearson, M. M., M. Sebaihia, C. Churcher, M. A. Quail, A. S. Seshasayee, N. M. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. R. Thomson, P. N. Rather, J. Parkhill, and H. L. T. Mobley.** 2008. Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. J. Bacteriol. **190:**4027–4037.

35. **Penner, J. L.** 1981. The tribe Proteeae, p. 1204–1224. *In* M. P. Start, H. Stolp, H. G. Trüper, A. Bellows, and H. G. Schlegel (ed.), The prokaryotes, a handbook of habitats, isolation and identification of bacteria. Springer-Verlag, Berlin, Germany.

36. **Penner, J. L., and J. N. Hennessy.** 1980. Separate O-grouping schemes for serotyping isolates of *Proteus vulgaris* and *Proteus mirabilis*. J. Clin. Microbiol. **12:**304–309.

37. **Phan, V., R. Belas, B. F. Gilmore, and H. Ceri.** 2008. ZapA, a virulence factor in a rat model of *Proteus mirabilis*-induced acute and chronic prostatitis. Infect. Immun. **76:**4859–4864.

38. **Rashid, T., and A. Ebringer.** 2007. Rheumatoid arthritis is linked to *Proteus*—the evidence. Clin. Rheumatol **26:**1036–1043.

39. **Regué, M., N. Climent, N. Abitiu, N. Coderch, S. Merino, L. Izquierdo, M. Altarriba, and J. M. Tomás.** 2001. Genetic characterization of the *Klebsiella pneumoniae waa* gene cluster, involved in core lipopolysaccharide biosynthesis. J. Bacteriol. **183:**3564–3573.

40. **Regué, M., L. Izquierdo, S. Fresno, N. Jimenez, N. Piqué, M. M. Corsaro, M. Parrilli, T. Naldi, S. Merino, and J. M. Tomás.** 2005. The incorporation of glucosamine into enterobacterial core lipopolysaccharide. Two enzymatic steps are required. J. Biol. Chem. **280:**26648–36656.

41. **Regué, M., L. Izquierdo, S. Fresno, N. Piqué, M. M. Corsaro, T. Naldi, C. De Castro, D. Waidelich, S. Merino, and J. M. Tomás.** 2005. A second outer-core region in *Klebsiella pneumoniae* lipopolysaccharide. J. Bacteriol. **187:**4198–4206.

42. **Rózalski, A., Z. Siidorczyk, and K. Kotelko.** 1997. Potential virulence factors of *Proteus* bacilli. Microbiol. Mol. Biol. Rev. **61:**65–89.

43. **Rubin, R. H., N. E. Tolkoff-Rubin, and R. S. Cotran.** 1986. Urinary tract infection, pyelonephritis, and reflux nephropathy, p. 1085–1141. *In* B. M. Brenner and F. C. Rector (ed.), The kidney. W. B. Saunders Co., Philadelphia, PA.

44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. **74:**5463–5467.

46. **Saveson, C. J., and S. T. Lovett.** 1999. Tandem repeat recombination induced by replication fork defects in *Escherichia coli* requires a novel factor, RadC. Genetics **152:**5–13.

47. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Nat. Biotechnol. **1:**784–791.

48. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.

49. **Valdivia, R. H., D. M. Cirillo, A. K. Lee, D. M. Bouley, and S. Falkow.** 2000. *mig-14* is a horizontally acquired, host-induced gene required for *Salmonella enterica* lethal infection in the murine model of typhoid fever. Infect. Immun. **68:**7126–7131.

50. **Vinogradov, E. V., and M. B. Perry.** 2001. Structural analysis of the core region of the lipopolysaccharides from eight serotypes of *Klebsiella pneumoniae*. Carbohydr. Res. **335:**291–296.

51. **Vinogradov, E., Z. Sidorczyk, and Y. A. Knirel.** 2002. Structure of the lipopolysaccharide core region of the bacteria of the genus *Proteus*. Aust. J. Chem. **55:**61–67.

52. **Warren, J. W.** 1991. The catheter and urinary tract infection. Med. Clin. North Am. **75:**481–493.

53. **Warren, J. W., H. L. Muncie, Jr., and M. Hall-Craggs.** 1988. Acute pyelonephritis associated with bacteriuria during long-term catheterization: a prospective clinicopathological study. J. Infect. Dis. **158:**1341–1346.

54. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem. **5:**83–89.

55. **Zych, K., M. Kowalczyk, Y. A. Knirel, and Z. Sydorczyk.** 2000. New serogroups of the genus *Proteus* consisting of *Proteus penneri* strains only, p. 339–344. *In* L. Emody, T. Pal, J. Hacker, and G. Blum-Oehler (ed.), Genes and proteins underlying microbial urinary tract virulence, basic aspects and applications. Kluwer Academic/Plenum Publishers, New York, NY.

Identificación funcional de los genes involucrados en la biosíntesis del núcleo de lipopolisacárido en *Proteus mirabilis*.

Eleonora Aquilini, Joana Azevedo, Natalia Jimenez, Lamiaa Bouamama, Juan M. Tomás y Miguel Regué

En este trabajo, se han identificado y caracterizado los genes que codifican las enzimas implicadas en la biosíntesis del núcleo del lipopolisacárido (LPS) en dos cepas de *Proteus mirabilis*. En estas cepas (escogidas por tener una estructura química definida y peculiar), al igual que en la mayoría de las *Enterobacteriaceae*, se ha podido identificar un agrupamiento génico (*waa*) que contiene la mayor parte de los genes implicados en la biosíntesis del oligosacárido que compone el núcleo del LPS. Para caracterizar esta agrupación génica *waa*, se ha estudiado en primer lugar la conservación de los genes pertenecientes a agrupaciones génicas ya descritas y implicadas en la biosíntesis del núcleo de LPS en otras bacterias. Se han utilizado, además, los datos del proyecto genoma de la cepa HI4320 de *P. mirabilis* (http://www.ebi.ac.uk/InterProScan/index.html) y las secuencias de las regiones homologas de *Klebsiella pneumoniae* y *Serratia marcescens*. De esta forma se ha podido completar la secuencia del entero agrupamiento genico *waa*, desde los dos extremos delimitados por los genes *hldD* y *radC*. Las secuencias de ADN obtenidas han sido traducidas siguiendo las pautas de lectura posibles. Cada uno de los ORF identificados se han comparado con los bancos de datos de proteínas de origen bacteriano GenBank y EMBL, utilizando los programas BLAST y FASTA del *National Center for Biotechnology Information* y del *European Biotechnology Information*, respectivamente.

Estos resultados han permitido determinar los genes potencialmente presentes en la agrupación génica *waa*, su organización y la homología de los mismos con otras especies de bacterias Gram negativas. Finalmente, la organización general de este grupo de genes es similar a la encontrada en la cepa de *P. mirabilis* HI4320.

Gracias a la elevada similitud del esqueleto olisacaridico del núcleo de LPS entre *P. mirabilis* y *K. pneumoniae* (hasta el segundo residuo del núcleo externo), se han podido realizar experimentos in vivo de complementación heterologa utilizando cada vez específicos mutantes de *K. pneumoniae*. Mediante extracción y análisis con geles de SDS y Tricina y espectrometría de masa de los oligosacáridos de LPS obtenidos como moléculas modificadas *in vivo* por la acción de las enzimas correspondientes, se ha podido confirmar el papel de cada gen en la formación del núcleo de LPS. Gracias a este enfoque se ha podido confirmar la ruta biosintetica, en *P. mirabilis*, del pentasacarido

común a todas las *Enterobacteriaceae* (Hept III - Hept II – Hept I – Kdo I – Kdo II): este es biosintetizado mediante la actividad secuencial de enzimas conservadas codificadas por los genes *waaA*, *waaC*, *waaF* y *waaQ*. Ademas, esta estrategia ha permitido la identificación de dos nuevas eptosyltransferasas (WamA y WamC), una galactosiltransferasa (WamB), y una N-acetil-glucosaminyltransferasa (WamD). Todas las cepas de *P. mirabilis* estudiadas contienen ademas un homologo del gen *waaE*. Este gen se encuentra localizado entre el gen *waaA* y el gen *coaD*, como en *K. penumoniae* y *S. marcescens*, donde se ha demostrado estar involucrado en la transferencia de un residuo de glucosa a la eptosa I con un enlace β1,4.

En ambas cepas incluidas en este estudio, la mayoría de los genes para la biosíntesis del núcleo de LPS han sido encontrados en el agrupamiento genico *waa*, aunque el gen *wabO*, que codifica por la D-GalA transferasa II, ha sido encontrado a fuera de este agrupamiento genico.

El producto codificado por el gen *mig14*, encontrado en la cepa PrK 51/57, posee similitudes con genes que codifican por una familia de proteínas de función desconocida: en este grupo el gen mejor estudiado es el *mig-14* de *Salmonella enterica* serovar Typhimurium. Se ha demostrado que el gen *mig-14* de *S. enterica* participa en la virulencia de la bacteria y que juega un papel en la resistencia a los péptidos antimicrobianos catiónicos. No obstante, parece poco probable que el gen *mig-14* de *P. mirabilis* juegue un papel central y constitutivo en la biosíntesis del LPS.

Se han descrito modificaciones aminoacídicas adicionales en algunos residuos del núcleo de LPS en las cepas R110 y 51/57 y los genes que codifican estas funciones tampoco se han encontrado en la agrupación genica *waa*, y por tanto deberían encontrarse a fuera de este ultimo.

Los cuatro genes localizados después del gen *waaL* (que codifica la ligasa del antigeno O) codifican supuestamente enzimas que pertenecen a las familias de glicosiltransferasas 4 (*walM*, w*alN* y *walR*) y 9 (*walO*), según la clasificación de Carbohydrate-Active enZYmes Database (http://www.cazy.org/). Los miembros de estas dos familias se han relacionado con la biosíntesis del núcleo de LPS en varias bacterias Gram-negativas. En este trabajo se han identificado nueve genes como responsables de la transferencia de los ocho residuos comunes identificados en la estructura química de los núcleos de LPS. Así pues, las supuestas glicosiltransferasas codificadas por estos cuatro genes *wal*, no parecen estar implicadas el la biosíntesis de la estructura del núcleo de LPS de las cepas incluidas en el estudio.

Three Enzymatic Steps Required for the Galactosamine Incorporation into Core Lipopolysaccharide*⒮

Eleonora Aquilini‡1, **Joana Azevedo**‡, **Susana Merino**§, **Natalia Jimenez**§, **Juan M. Tomás**§2, and **Miguel Regué**‡

From the ‡Departamento de Microbiología y Parasitología Sanitarias, Facultad de Farmacia, Universidad de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain, and the §Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona, Spain

The core lipopolysaccharides (LPS) of *Proteus mirabilis* as well as those of *Klebsiella pneumoniae* and *Serratia marcescens* are characterized by the presence of a hexosamine-galacturonic acid disaccharide (αHexN-(1,4)-αGalA) attached by an α1,3 linkage to L-*glycero*-D-*manno*-heptopyranose II (L-*glycero*-α-D-*manno*-heptosepyranose II). In *K. pneumoniae*, *S. marcescens*, and some *P. mirabilis* strains, HexN is D-glucosamine, whereas in other *P. mirabilis* strains, it corresponds to D-galactosamine. Previously, we have shown that two enzymes are required for the incorporation of D-glucosamine into the core LPS of *K. pneumoniae*; the WabH enzyme catalyzes the incorporation of GlcNAc from UDP-GlcNAc to outer core LPS, and WabN catalyzes the deacetylation of the incorporated GlcNAc. Here we report the presence of two different HexNAc transferases depending on the nature of the HexN in *P. mirabilis* core LPS. *In vivo* and *in vitro* assays using LPS truncated at the level of galacturonic acid as acceptor show that these two enzymes differ in their specificity for the transfer of GlcNAc or GalNAc. By contrast, only one WabN homologue was found in the studied *P. mirabilis* strains. Similar assays suggest that the *P. mirabilis* WabN homologue is able to deacetylate both GlcNAc and GalNAc. We conclude that incorporation of D-galactosamine requires three enzymes: Gne epimerase for the generation of UDP-GalNAc from UDP-GlcNAc, *N*-acetylgalactosaminyltransferase (WabP), and LPS:HexNAc deacetylase.

Proteus mirabilis is a common uropathogen that causes urinary tract infections, especially in individuals with functional or anatomical abnormalities of the urinary tract (1) and elderly ones undergoing long term catheterization (2), but less frequently in normal hosts (3). Potentially serious complications arising from *P. mirabilis* infections include bladder and kidney stone formation, catheter obstruction by formation of encrusting biofilms, and bacteremia (reviewed in Ref. 4). This bacterium is found more frequently than *Escherichia coli* in kidney infection (5) and may be associated to rheumatoid arthritis (6).

As in other *Enterobacteriaceae*, in the genus *Proteus* LPS, three domains are recognized: the highly conserved and hydrophobic lipid A; the hydrophilic and highly variable O-antigen polysaccharide (O-PS)3 with more than 60 serogroups recognized (7–10); and the core oligosaccharide (OS), connecting lipid A and O-antigen. The core domain is usually divided into inner and outer core on the basis of sugar composition.

The core OS structure of 34 *Proteus* strains of different O-serogroups has been determined (11). The core OS of these strains share a common heptasaccharide fragment that includes a 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) disaccharide, a L-*glycero*-α-D-*manno*-heptosepyranose trisaccharide, and one residue each of Glc, GalA, and either D-glucosamine (GlcN) or D-galactosamine (GalN) (Fig. 1) (reviewed in Ref. 11). This common fragment is also found in the core LPS of *Klebsiella pneumoniae* and *Serratia marcescens* (12–14), and we have shown that two enzymes are required for the incorporation of GlcN into the core LPS of *K. pneumoniae*: the WabH enzyme catalyzing the incorporation of GlcNAc from UDP-GlcNAc to outer core LPS and WabN catalyzing the deacetylation of the incorporated GlcNAc (15). The rest of the *Proteus* core OS is quite variable, with up to 37 different structures recognized in the genus *Proteus* and 11 in *P. mirabilis* (11).

Recently, we have identified the genes involved in the biosynthesis of the *P. mirabilis* common core heptasaccharide for strains containing GlcN (R110 and 51/57) (16). As in other *Enterobacteriaceae*, these genes were clustered in the so-called *wa* region, and among these genes, *K. pneumoniae* *wabH* and *wabN* homologues were found able to complement the corresponding non-polar mutants (16). In this work, we characterize the *wa* region for two *P. mirabilis* strains containing GalN instead of GlcN and present evidence for a specific GalNAc transferase in these strains.

3 The abbreviations used are: O-PS, O-antigen polysaccharide; GalA, galacturonic acid; GalN, D-galactosamine; GlcN, D-glucosamine; HexN, D-hexosamine; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; OS, oligosaccharide(s); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; contig, group of overlapping clones.

TABLE 1
Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
P. mirabilis		
50/57	Serogroup O27	J. Sidorczyk
TG83	Serogroup O57	J. Sidorczyk
R110	Serogroup O3ab	J. Sidorczyk
OXK	Serogroup O3ab	J. Sidorczyk
51/57	Serogroup O28	J. Sidorczyk
14/57	Serogroup O6	J. Sidorczyk
ATCC 29906	Type strain	CECT[a]
CECT 170		CECT[a]
K. pneumoniae		
52145	Serogroup O1:K2 (core type 2)	Ref. 37
52145Δ*wabN*	Non-polar *wabN* mutant	Ref. 15
52145Δ*wabH*	Non-polar *wabH* mutant	Ref. 15
52145Δ*waaL wabN*	Double non-polar *waaL wabN* mutant	Ref. 15
52145Δ*waaL wabH*	Double non-polar *waaL wabH* mutant	Ref. 15
E. coli		
DH5α	F$^-$ *endA hsdR17* (r$_k^-$ m$_k^-$) *supE44 thi-1 recA1 gyr-A96* ϕ80*lacZ*	Ref. 38
BL21(λD3)	F$^-$ *ompT hsdS$_B$* (r$_B^-$ m$_B^-$) *gal dcm*(λD3)	Novagen
Plasmids		
pGEM-T Easy	PCR-generated DNA fragment cloning vector Amp^r	Promega
pGEM-T- *wabH$_{52145}$*	pGEM-T containing the PCR-amplified *waaN$_{52145}$* gene	
pGEM-T-*wabH$_{R110}$*	pGEM-T containing the PCR-amplified *wabH$_{R110}$* gene	Ref. 16
pGEM-T-wabP$_{50/57}$	pGEM-T containing the PCR-amplified *wabP$_{50/57}$* gene	This study
pGEM-T-*wabN$_{R110}$*	pGEM-T containing the PCR-amplified *wabN$_{R110}$* gene	Ref. 16
pGEM-T-*wabN$_{50/57}$*	pGEM-T containing the PCR-amplified *wabN$_{50/57}$* gene	This study
pET28a(+)	T4-inducible expression vector, K_m^R	Novagen
pET28a-*wabH$_{R110}$*	pET28a expressing His$_6$-WabH$_{R110}$	This study
pET28a-*wabP$_{50/57}$*	pET28a expressing His$_6$-WabP$_{50/57}$	This study
pET28a-*wabN$_{R110}$*	pET28a expressing His$_6$-WabN$_{R110}$	This study
pET28a-*wabN$_{50/57}$*	pET28a expressing His$_6$-WabN$_{50/57}$	This study
pACYC-GNE$_{A. hydrophila}$	pACYC184 with *A. hydrophila* AH-3 *gne*	Ref. 31
pACYC-GNE$_{P. mirabilis}$	pACYC184 with *P. mirabilis* ATCC 29906 *gne*	This study

[a] Colección Española de Cultivos Tipo (Spanish Collection of Culture Types).

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are shown in Table 1. Bacterial strains were grown in LB broth and LB agar (17). LB medium was supplemented with kanamycin (50 μg/ml), tetracycline (20 μg/ml), and ampicillin (100 μg/ml) when needed.

General DNA Methods—Standard DNA manipulations were done essentially as described (18). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

DNA Sequencing and Computer Analysis of Sequence Data—Double-stranded DNA sequencing was performed by using the dideoxy-chain termination method (19) with the ABI Prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences). Oligonucleotides used for genomic DNA amplifications and DNA sequencing were purchased from Amersham Biosciences. The DNA sequence was translated in all six frames, and all open reading frames (ORFs) were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from nonredundant GenBank^{TM} and EMBL databases by using the BLAST (20) network service at the National Center for Biotechnology Information and the European Bioinformatics Institute. ClustalW was used for multiple-sequence alignments (21).

Plasmid Constructions for Mutant Complementation Studies—The *wabH$_{R110}$* (1137-bp) and *wabN$_{R110}$* (963-bp) genes from *P. mirabilis* R110 were PCR-amplified from strain R110 genomic DNA with oligonucleotide pairs BHf (5′-TGG-

CGATGGCAAATTTTACT-3′)-BHr (5′-ATTCCGGCCGA-TAACTTAGG-3′) and BNf (5′-GTGCACGAATTGCTCTG-ATG-3′)-BNr (5′-ATGGGTGGCAAGATAATGCT-3′), obtaining fragments of 1310 and 1508 bp, respectively. Similarly, the *wabP$_{50/57}$* and *wabN$_{50/57}$* were amplified from strain 50/57 genomic DNA with oligonucleotides 50-7 (5′-TAGCT-GCAGCTATTTCAGCC-3′)-50-4 (5′-GGGATAATGGTGG-CGTAATG-3′) and BNf-BBNr (5′-GGCTTTCCATTGGTC-AGCTA-3′), obtaining fragments of 1881 and 1621 bp, respectively. These amplicons were ligated to vector pGEM-T (Promega) and transformed into *E. coli* DH5α to generate plasmids pGEM-T-*wabH$_{R110}$*, pGEM-T-*wabN$_{R110}$*, pGEM-T-*wabP$_{50/57}$*, and pGEM-T- *wabN$_{50/57}$*. A DNA fragment (1400 bp) containing the *gne* from *P. mirabilis* ATCC 29906 was amplified with oligonucleotides GneExtFw (5′-<u>GAGCTC</u>CC-ATGGTGAAATGAAACGTG-3′) and GneExtRv (5′-<u>TCTA-GA</u>ACCCGTATTCGGTGGAATTT-3′), where underlined letters denote sequences cut by SacI and XbaI, respectively. The amplified fragment was cloned in pGEM-T to obtain pGEM-T-GNE$_{P. mirabilis}$. By SacI digestion, a 1454-bp fragment was obtained, gel-purified, and blunt-ended with T4 DNA polymerase. This fragment was ligated to SalI-digested and blunt-ended pACYC184 to obtain pACYC-GNE$_{P. mirabilis}$.

Gene Distribution—To determine the distribution among several *P. mirabilis* strains of the *gne* gene an internal fragment of this gene was amplified using oligonucleotides GneIntFw (5′-ACTCGCCTATCAGGCCAATT-3′) and GneIntRev (5′-TGACTATGGCGTTTGTCGAG-3′). Oligonucleotide pairs WabHIntFw (5′-TGCCAACTCGATGGAT-AAGA-3′)-WabHIntRv (5′-GCGTAATTTTAGGCGGGTTA-

3′) and WabPIntFw (5′-ACAACCTAACCCGTTTGCAG-3′)-WabPIntRv (5′-TCTGCGAGTGAGTCTGCATC-3′) were used to determine the distribution of *wabH* and *wabP* genes, respectively.

LPS Isolation and Electrophoresis—LPS was extracted from dry cells grown in LB. The phenol/chloroform/light petroleum ether method (22) was used for strains producing rough LPS, whereas the phenol/water procedure (23) was used for the strains producing the O antigen domain (smooth LPS). For screening purposes, LPS was obtained after proteinase K digestion of whole cells (24). LPS samples were separated by SDS-PAGE or Tricine-SDS-PAGE and visualized by silver staining as described previously (25, 26).

Preparation of Oligosaccharides—The LPS preparations (20 mg) were hydrolyzed in 1% acetic acid (100 °C, 120 min), and the precipitate was removed by centrifugation ($8000 \times g$, 30 min) and lyophilized to give Lipid A. The supernatants were fractionated on a column (56×2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer, pH 4.5, with monitoring using a differential refractometer to obtain the oligosaccharide fractions.

GC-MS Analysis—Partially methylated alditol acetates and methyl glycoside acetates were analyzed on a Agilent Technologies 5973N MS instrument equipped with a 6850A gas chromatograph and an RTX-5 capillary column (Restek, 30 m × 0.25-mm inner diameter, flow rate 1 ml/min, helium as carrier gas). Acetylated methyl glycosides analysis was performed with the following temperature program: 150 °C for 5 min, $150 \rightarrow 250$ °C at 3 °C/min, 250 °C for 10 min. For partially methylated alditol acetates, the temperature program was as follows: 90 °C for 1 min, $90 \rightarrow 140$ °C at 25 °C/min, $140 \rightarrow 200$ °C at 5 °C/min, $200 \rightarrow 280$ °C at 10 °C/min, 280 °C for 10 min.

Mass Spectrometry Studies—Positive and negative ion reflectron time-of-flight mass spectra (MALDI-TOF) were acquired on a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed extraction ion source. Ion acceleration voltage was 25 kV, grid voltage was 17 kV, mirror voltage ratio was 1.12, and delay time was 150 ns. Samples were irradiated at a frequency of 5 Hz by 337-nm photons from a pulsed nitrogen laser. A solution of 2,5-dihydroxybenoic acid in 20% CH_3CN in water at a concentration of 25 mg/ml was used as the MALDI matrix. One μl of matrix solution was deposited on the target, followed by loading of 1 μl of the sample. The droplets were allowed to dry at room temperature. Spectra were calibrated and processed under computer control using the Applied Biosystems Data Explorer software.

Plasmid Constructions for Histidine-tagged Protein Overexpression—Constructs allowing the expression of N-terminal histidine-tagged $WabH_{R110}$ (His_6-$WabH_{R110}$), $WabP_{50/57}$ (His_6-$WabP_{50/57}$), $WabN_{R110}$ (His_6-$WabN_{R110}$), and $WabN_{50/57}$ (His_6-$WabN_{50/57}$) were based in pET28a. The $wabH_{R110}$ and $wabP_{50/57}$ genes were amplified from pGEM-T-$wabH_{R110}$ and pGEM-T-$wabP_{50/57}$ with primer pairs HR1 (5′-ACGCCATATGAAGGAACATATACTTTTTATT-3′)-HR2 (5′-ACGCGGATCCTAAAACGTGTTCTGACTCATT-ATT-3′) and PR1 (5′-ACGCCATATGAATCAACGACATA-ATT-3′)

TACTTTTT-3′)-PR2 (5′-ACGCGGATCCAACATGCTATC-AAATTCACTTATC), respectively. The $wabN_{R110}$ and $wabN_{50/57}$ genes were amplified from pGEM-T-$wabN_{R110}$ and pGEM-T-$wabN_{50/57}$ with primer pairs NR1 (5′-ACGCC-ATATGAAAAAACCAGCATTTATTATCAC-3′)-NR2 (5′-ACGCGGATCCAAAGAGTGGGGTTATAGAGATTTA-3′) and NRR1 (5′-ACGCCATATGAAAAAACCAGCATTT-ATTATC-3′)-NRR2 (5′-ACGCGGATCCATAGTGCACCC-ATTTTAATTTC), respectively. The four amplified DNA fragments were digested with NdeI (double underlined nucleotides in HR1, PR1, NR1, and NRR1) and HindIII (double underlined nucleotides in HR2, PR2, NR2, and NRR2) and ligated to pET28a digested with the same enzymes to obtain plasmids pET28a-$wabH_{R110}$, pET28a-$wabP_{50/57}$, pET28a-$wabN_{R110}$, and pET28a-$wabN_{50/57}$.

Preparation of Cell-free Extracts Containing Core LPS Biosynthetic Enzymes—E. coli BL21(λD3) was used to overexpress genes from the T7 promoter in pET28a-based plasmids. The cultures were grown in LB supplemented with kanamycin for 18 h at 37 °C, diluted 1:100 in fresh medium, grown until they reached an $A_{600 \, nm}$ of about 0.5, induced by adding isopropyl-1-thio-β-D-galactopyranoside, and grown for an additional 2 h.

The cells from induced cultures were harvested, washed once with 50 mM Tris-HCl (pH 8.0), and frozen until used. To prepare the lysate cell pellets, they were resuspended in 50 mM Tris-HCl (pH 8.0) and sonicated on ice (for a total of 2 min using 10-s bursts followed by 10-s cooling periods). Unbroken cells, cell debris, and membrane fraction were removed by ultracentrifugation at $100,000 \times g$ for 60 min. Protein expression was monitored by SDS-PAGE, and protein contents of cell-free extracts were determined using the Bio-Rad Bradford assay.

His-tagged Protein Purification—Cell-free lysates from *E. coli* BL21(λD3) harboring pET28a-$wabH_{R110}$, pET28a-$wabP_{50/57}$, pET28a-$wabN_{R110}$, and pET28a-$wabN_{50/57}$ were prepared as above using a phosphate-saline buffer (20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl). His_6-tagged proteins were purified by affinity chromatography on an FPLC system (Amersham Biosciences) using 1-ml HiTrap chelating HP columns (Amersham Biosciences) previously loaded with nickel sulfate and equilibrated with the phosphate-saline buffer. The columns were washed with phosphate-saline buffer containing 5 mM imidazole (10 column volumes), and the His-tagged proteins were eluted by a continuous gradient of 50–500 mM imidazole in phosphate-saline buffer. The buffer in the eluted proteins was exchanged into 50 mM Tris-HCl (pH 8.0), and the proteins were concentrated.

GlcNAc and GalNAc Transferase Assay—The transferase activity of His_6-$WabH_{R110}$ and His_6-$WabP_{50/57}$ were determined essentially as described (27). Briefly, reactions were performed in a total 0.1-ml volume at a final concentration of 50 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. 1 mM UDP-GlcNAc or UDP-GalNAc was used as donor, and 0.001 mg of LPS from strain 52145Δ*wabH* was used as acceptor. The reactions were started by the addition of 0.02 mg of either His_6-$WabH_{R110}$ or His_6-$WabP_{50/57}$. After

2 h at 37 °C, the reactions were stopped by adding SDS-PAGE sample buffer and boiling for 10 min. The reaction products were separated in Tricine-SDS-PAGE.

Identical reactions using 0.25 μCi of UDP-[^{14}C]GlcNAc or UDP-[^{14}C]GalNAc (NEN Life Science; with specific activities of 51.2 and 55.5 mCi/mmol, respectively) were performed. Unincorporated radioactivity was removed by minigel filtration in 3 ml of Sephadex G-100, and the pooled and concentrated LPS fractions were separated as above by Tricine-SDS-PAGE. The dried gel was autoradiographed to visualize the incorporation of GlcNAc and GalNAc.

For quantitative analysis, the reactions were stopped by adding an equal volume of stop solution (27) and incubated on ice for 1 h. The LPS was recovered by filtration (0.45 μm) and washed, and the amount of radioactivity incorporated into LPS was measured in a scintillation counter.

Quantitative experiments using different concentrations of UDP-GlcNAc or UDP-GalNAc (1–800 μM) and acceptor LPS (1–800 μM) were performed. These reactions were stopped at different times (expressed in minutes), and the data from three independent experiments were used to determine the apparent K_m for acceptor LPS and enzyme substrate.

HexNAc-Core LPS-Deacetylase Assay—The ability of His$_6$-WabN$_{R110}$ and His$_6$-WabN$_{50/57}$ to catalyze the deacetylation of GlcNAc or GalNAc residue in LPS was assayed by using LPS from mutant 52145Δ*wabH* in reactions containing 0.4 mg each of His$_6$-WabH and His$_6$-WabN or His$_6$-WabP and His$_6$-WabN. Assay reactions using UDP-[^{14}C]GlcNAc or UDP-[^{14}C]GalNAc were carried out in a total of 0.1 ml at a final concentration of 50 mM Tris-HCl (pH 8.0) containing 10 mM MgCl$_2$, 1 mM dithiothreitol, 0.03 mg of 52145Δ*wabH* acceptor LPS, and 0.02 mg of each histidine-tagged protein. The reactions were started by the addition of 0.25 μCi of UDP-[^{14}C]GlaNAc or UDP-[^{14}C]GalNAc. Assays were performed at 37 °C for 2 h and were stopped by adding 2 volumes of 0.375 M MgCl$_2$ in 95% ethanol and cooling at −20 °C for 2 h. The LPS was recovered by centrifugation at 12,000 × g for 15 min and suspended in 100 μl of water. The LPS was precipitated with 2 volumes of 0.375 M MgCl$_2$ in 95% ethanol; this step was repeated three times to eliminate the unincorporated UDP-[^{14}C]GlcNAc or UDP-[^{14}C]GalNAc. The LPS was hydrolyzed by resuspension in 100 μl of 0.1 M HCl and heating to 100 °C for 48 h. The labeled residues from the hydrolyzed LPS samples were separated by thin layer chromatography (TLC) (Kieselgel 60; Merck) with *n*-butanol, methanol, 25% ammonia solution, water (5:4:2:1, v/v/v/v). The labeled residues were detected by autoradiography, using as standards [^{14}C]GlcNAc, [^{14}C]GalNAc, [^{14}C]GlcN, and [^{14}C]GalN.

RESULTS

P. mirabilis wa Gene Cluster—Similarly to the majority of *Enterobacteriaceae* in the *P. mirabilis* strains R110 and 51/57 a gene cluster (*wa*) was identified containing most of the genes involved in the biosynthesis of the core OS (16). The overall structure of these gene clusters was similar to the one found in strain HI4320 for which the whole genome sequence is available (28). Because strains R110 and 51/57 contain GlcN in the outer core LPS, we determined the organization of this

cluster for strains 50/57 and TG83 containing GalN instead of GlcN (Fig. 1). Oligonucleotide pairs previously used in the amplification of DNA fragments of the cluster in strains R110 and 51/57 were used to amplify fragments from genomic DNA of strains 50/57 and TG83 and to determine its nucleotide sequence. Some of these oligonucleotide pairs did work, whereas others did not when using genomic DNA from strains 50/57 and TG83, suggesting that there are conserved and unconserved regions in the *wa* cluster among these four *P. mirabilis* strains. The nucleotide sequence of the amplified fragments was used to design further primers, allowing the amplification of DNA fragments encompassing the already sequenced ones. This strategy allowed the determination of the full nucleotide sequence of the *wa* gene cluster from *P. mirabilis* strains 50/57 and TG83 (19,155 bp). These two clusters showed more than 95% identity.

The comparative analysis of these sequences revealed that in all four strains the 5′-end of the cluster contains the *hldD* (ADP-D-*glycero*-D-*manno*-heptose epimerase), *waaF* (ADP-L-*glycero*-α-D-*manno*-heptosepyranose transferase II), and *waaC* (ADP-L-*glycero*-α-D-*manno*-heptosepyranose transferase I) genes. The 3′-end of the cluster contains the *waaA* (CMP-Kdo:lipidA Kdo bifunctional transferase and *waaE* (glucosyltransferase) genes adjacent to core OS-unrelated genes *coaD* (phosphopantetheine adenylyltransferase) (29) and *fpg* (formamidopyrimidine-DNA glycosylase) (30). In the middle of the cluster, genes *waaQ* (ADP-L-*glycero*-α-D-*manno*-heptosepyranose transferase III), *wamA* (ADP-D-*glycero*-D-*manno*-heptopyranose transferase), *wabG* (UDP-GalA transferase), *wabH*-like (UDP-GlcNAc transferase), and *wabN* (LPS-GlcNAc deacetylase) were identified (Fig. 1). The *wa* clusters of these two *P. mirabilis* strains shared additional characteristic features with those of previously studied ones (16, 28), such as the location of *waaL* (O-PS ligase) downstream from *fpg* and the presence of four additional genes (*walM, -N, -O,* and *-R*) encoding putative glycosyltransferases of unknown function (Fig. 1). The main difference between the 50/57 and TG83 *wa* clusters and those of R110, 51/57, and HI4320 is the inversion of the three-gene *waaQ-wabH* region (Fig. 1), explaining why some oligonucleotide pairs derived from strain R110 sequence failed in PCR amplification experiments using genomic DNA from either 50/57 or TG83 strains.

Comparison of the *wa* cluster with the known core OS structures for these four strains (Fig. 1) shows that additional genes located outside of the cluster should be necessary to complete the biosynthesis of this structure. Only one of these genes has been identified (*wabO*) (16). For strains 50/57 and TG83, it can be postulated that four and three additional genes should be located outside of the *wa* cluster, respectively.

Two Different WabH Homologues—Gene-deduced amino acid alignment among homologue proteins shared by the five *P. mirabilis* strains studied so far showed amino acid identities and similarities over 95 and 98%, respectively. The only exception was for WabH and WabH-like proteins, where two groups were found, WabH-like$_{50/57}$-WabH-like$_{TG83}$ and WabH$_{R110}$-WabH$_{51/57}$, with 100 and 99% identities, respectively. Both groups shared 60 and 89% levels of identity and
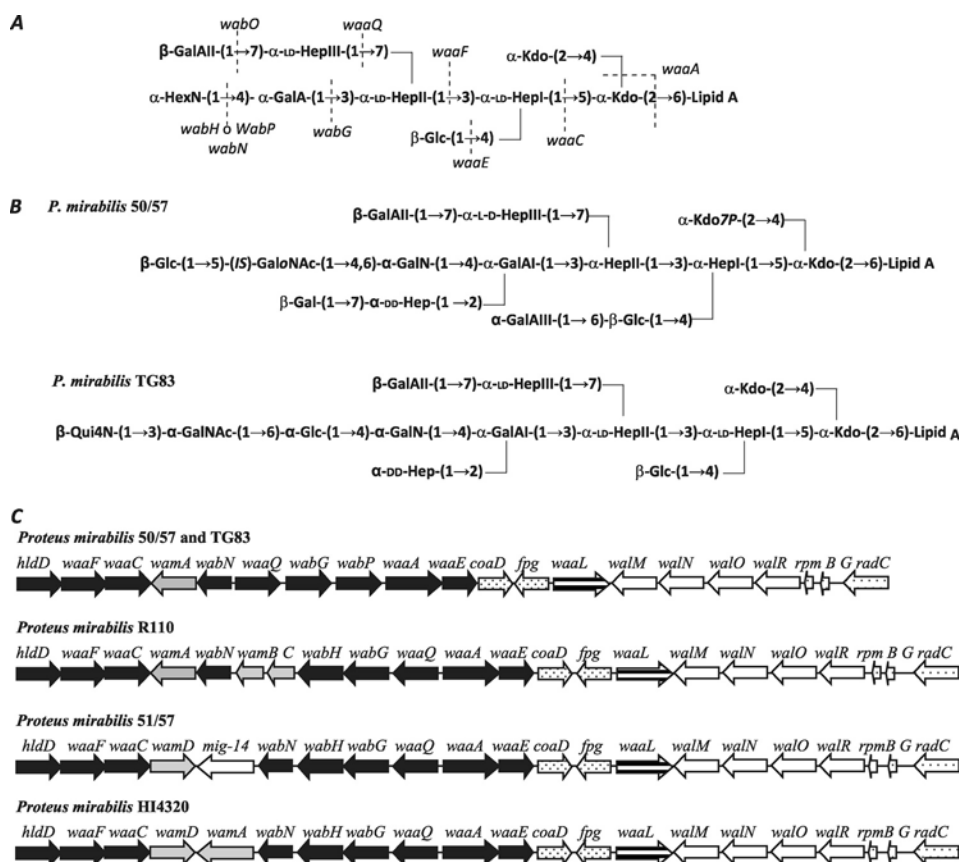
FIGURE 1. **P. mirabilis core OS structures and genetic organization of the core OS biosynthetic clusters.** *A*, the common part of core OS shared by all of the studied strains of *P. mirabilis* (11), *K. pneumoniae*, and *S. marcescens* (11, 13, 14). *Broken lines* denote the truncation level for the different core biosynthetic gene mutations (13, 14, 15, 16, 35). *B*, the core OS structures of *P. mirabilis* strains 50/57 and TG83 (11). *LD-Hep*, L-*glycero*-D-*manno*-heptopyranose *DD-Hep*, D-*glycero*-D-*manno*-heptopyranose; *GalNAc*, *N*-acetylgalactosamine open chain form; *Qui*, quinovosamine. *C*, a diagram of the *wa* gene cluster from *P. mirabilis* strains 50/57 and TG83 (this work), R110 and 51/57 (16), and HI4320 (28). Common core genes (*black arrows*), other outer core genes (*gray arrows*), *waaL* (*O*-antigen:lipidA-core ligase) (*striped arrows*), non-core-related (*stippled arrows*), and genes of unknown function (*white arrows*) are shown.

similarity. An alignment between these proteins and those of *K. pneumoniae* strain C3 and 52145, where it has been shown that this protein transfers the GlcNAc from UDP-GlcNAc to core OS (27), was done to identify residues specific for the WabH-like$_{50/57}$-WabH-like$_{TG83}$ pair (Fig. 2). This analysis suggested that these two types of proteins could recognize different HexNAc substrates, GlcNAc or GalNAc, and thus we propose to name them WabH and WabP, respectively.

WabH Complementation Assays—To test the above hypothesis, we took advantage of the fact that the saccharide structure of the core OS from *K. pneumoniae* and that of *P. mirabilis* is identical up to the first outer core OS residue (GalA) (Fig. 1) to express independently *wabH*$_{R110}$ and *wabP*$_{50/57}$ in the non-polar mutant *K. pneumoniae* 52145Δ*wabH*. Oligonucleotide pairs BNf-BNr and 50/7-50/4 were used to amplify the 1137-bp *wabH*$_{R110}$ and 1104-bp *wabP*$_{50/57}$ genes, respectively. These amplicons were cloned in vector pGEM-T, and plasmids pGEM-T-*wabH*$_{R110}$ and pGEM-T-*wabP*$_{50/57}$ were electroporated into mutant 52145Δ*wabH*.

LPS was isolated from the transformed strains and analyzed in both SDS-PAGE and Tricine-SDS-PAGE. The core lipid A region of an LPS preparation from 52145Δ*wabH* migrated faster than that of wild type 52145, in agreement with a core OS missing the outer core GlcN (Fig. 3). LPS from strain

52145Δ*wabH* (pGEM-T-*wabH*$_{R110}$) migrated to the same level as that of wild type strain 52145, strongly suggesting that WabH$_{R110}$ is a functional homologue of WabH$_{52145}$ catalyzing the transfer of GlcNAc from UDP-GlcNAc to core OS. LPS was extracted from strain 52145Δ*wabH* (pGEM-T-WabH$_{R110}$), and the OS fraction was obtained by mild acid hydrolysis (see "Experimental Procedures"). The GC-MS analysis of acetylated methyl glycosides from the OS fraction revealed the presence of Kdo, L-*glycero*-D-*manno*-heptopyranose, GalA, Glc, and GlcN. By contrast, WabP$_{50/57}$ do not appear to compensate for the deficiency in WabH$_{52145}$ because LPS from strain 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$) migrated to the same level as that of mutant 52145Δ*wabH* (Fig. 3).

Role of Gne in WabP Complementation—One possible reason for the absence of core OS modification induced by WabP$_{50/57}$ could be the absence of UDP-GalNAc in the 52145 strain. Analysis of the whole genome of *K. pneumoniae* strains 342 (GenBank™ CP000964) and MGH 78578 (GenBank™ CP000647) reveals the presence of one *galE* (UDP-Gal 4-epimerase) homologue for each genome. Generation of UDP-GalNAc from UDP-GlcNAc will require the presence of a *gne* (UDP-GalNAc 4-epimerase) homologue. Thus, we introduced the *Aeromonas hydrophila gne* containing plasmid pACYC-GNE$_{A.\ hydrophila}$ (31) into strain 52145Δ*wabH* (pGEM-T-

```
Pm 50/57    1   MNQRHILFIIDGLPGGGAENVTLRLANGFHQQGYQVTLLSIHNKLAYELPDFIDYIVDHDEYRGIFRKLTEISRRAKSLDKVLTRLFAQK   90
Pm TG83     1   MNQRHILFIIDGLPGGGAENVTLRLANGFHQQGYQVTLLSIHNKLAYELPDFIDYIVDHDEYRGIFRKLTEISRRAKSLDKVLTRLFAQK   90
Pm R110     1   MKEHILFIIDGLPGGGAENVTIRLCHGLSQRGYGVTLLSIAEKCDYAIPADIELLIDADSYHGLFYRQTELKRRANSMDKTLQALFARK    89
Pm 51/57    1   MKEHILFIIDGLPGGGAENVTIRLCHGLSQRGYGVTLLSIAEKCDYAIPADIELLIDADSYHGLFYRQTELKRRANSMDKTLQALFARK    89
Pm HI4320   1   MKEHILFIIDGLPGGGAENVTIRLCHGLSQRGYGVTLLSIAEKCDYAIPADIELLIDADSYHGLFYRQTELKRRANSMDKTLQALFARK    89
Kp C3       1   MRILEVIDGLPGGGAEKVVILTLAAQELRDGDRVSLISISIRDVCEYPLPEGLDYQVVADRCRKPWRKLTELSRRARQLDAAVVR-AEQQ   86
Kp 52145    1   MRILEVIDGLPGGGAEKVVILTLAAQELRDGDRVSLISISIRDVCEYPLPEGLDYQVVADRCRKPWRKLTELSRRARQLDAAVVR-AEQQ   86
                  :***:*********:*.: *. : : *:*:*:** :  * :* :: : * : : :**:.***..:* .:  :: ::

Pm 50/57   91   GRPALVLSNLHKTDRIVCRAKQLRHCNVWYCIHGIYSQSYLGNKSGFSYWLKKKKIQRVYKDKNLICVSNAVKDDLIHAIGVDAQQLKTI  180
Pm TG83    91   GRPALVLSNLHKTDRIVCRAKQLRHCNVWYCIHGIYSQSYLGNKSGFSYWLKKKKIQRVYKDKNLICVSNAVKDDLIHAIGVDAQQLKTI  180
Pm R110    90   GIPALIVSNLHKTDRIVALSKQILADKNVWYCIHGIFSQSYLGNKSGFSRWLKQKKIQKVYQGKNIITVSNAAGQDLIENKIVPNQLKTI  179
Pm 51/57   90   GIPALIVSNLHKTDRIVALSKQILADKNIWYCIHGIFSQSYLGNKKGFSRWLKQKKIQKVYQGKNIITVSNAAGQDLIENKIVPNQLKTI  179
Pm HI432   90   GIPALIVSNLHKTDRIVALSKQILADKNVWYCIHGIFSQSYLGNKKGFSRWLKQKKIQKVYQGKNIITVSNAAGQDLIENKIVPNQLKTI  179
Kp C3      87   GQFDLVLSNLHKTDRIVARSRALRERNVWFCLHGVFSASYLGHRTGFDRWMKQQKIKRIYQGRNVVTVSDAVGRDLVEEFALRPAQLKTI  176
Kp 52145   87   GQFDLVLSNLHKTDRIVARSRALRERNVWFCLHGVFSASYLGHRTGFDRWMKQQKIKRIYQGRNAVTVSDAVGRDLVEEFALRPAQLKTI  176
                *  *:::**********.:: * . *:*:*:**:* ****::.**. *:*::**:::*::.* : **:*.  **:. :  :  *****

Pm 50/57  181   YNPFNIMEIQHKASQPNPFAGKAYILHVGRFHEVKRQDRLINAFAKANIPCQLILLGEGTPVIKQQLQQQIAQLNINEKVILAGFIANPL  270
Pm TG83   181   YNPFNIMEIQHKASQPNPFAGKAYILHVGRFHEVKRQDRLINAFAKANIPCQLILLGEGTPVIKQQLQQQIAQLNINEKVILAGFIANPL  270
Pm R110   180   YNPFDIQEIRTLASENNPYQKQDYLLHIGRFHQVKRHDRLLEAFALANIPCKLLIAGQGSSEVTSNIKNKIVALNIENKVSLIGFLANPF  269
Pm 51/57  180   YNPFDIQEIRTLASENNPYQKQDYLLHIGRFHQVKRHDRLLEAFALADIPCKLLIAGQGSSEVTSNIKNKIVALNIENKVSLIGFLANPF  269
Pm HI4320 180   YNPFDIQEIRTLASENNPYQKQDYLLHIGRFHQVKRHDRLLEAFALANIPCKLLIAGQGSSEVTSNIKNKIVALNIENKVSLIGFLANPF  269
Kp C3     177   YNPFDITALRAEAEADSERPDGDYLIHVGRFHPGKRHDRLLEAYAQSGIDAPLVLLGQGKPEQEQRLRQLAKTLHIDDRVWFKGFQKNPL  266
Kp 52145  177   YNPFDITALRAEAEADSERPDGDYLIHVGRFHPGKRHDRLLEAYAQSGIDAPLVLLGQGKPEQEQRLRQLAKTLHIDDRVWFKGFQKNPI  266
                ****:*  ::  *.  *   *:*:**** **:***::*:* ::. *:: *:*.. .::::  *::::*:*  :  ** **:

Pm 50/57  271   PIIQGAKVVALSSDSEGLPTVLIEALICGTPIVSTCPGGVSEIMTDELANYLSDMDADSLADKLRLAYYAPPQILPSSYAKEEINHILK  360
Pm TG83   271   PIIQGAKVVALSSDSEGLPTVLIEALICGTPIVSTCPGGVSEIMTDELANYLSDMDADSLADKLRLAYYAPPQILPSSYAKEEINHILK  360
Pm R110   270   PVIKDAKAVVLSSDSEGLGNVLVESLICNTPIVSTNCLGGISEIMEGELADYKSEFNSASLAEKMRLAYFNPPKITPNMYQKFDIDEVIR  359
Pm 51/57  270   PVIKDAKAVVLSSDSEGLGNVLVESLICNTPIVSTNCLGGISEIMEGELAGYKSEINSASLAEKMRLAYFNPPKITPNMYQKFDIDEVIR  359
Pm HI4320 270   PVIKDAKAVVLSSDSEGLGNVLVESLICNTPIVSTNCLGGISEIMEGELADYKSEINSASLAEKMRLAYFNPPKITPNMYQKFDIDEVIR  359
Kp C3     267   PWIKGARMLVLSSDSEGFGNVVEALLLHTPVASTRCPGGVTEILTGELARGLADITSPALAQTMQSIYHNPPAIDDAALEKFSVVSTCQ   356
Kp 52145  267   PWIKGARMLVLSSDSEGFGNVVEALLLHTPVASTRCPGGVTEILTGELARGLADITSPALAQTMQSIYHNPPAIDDAALEKFSVVSTCQ   356
                * *:.*: :.*******: .:::*:*:  *:.** * **::**:.*** ::: : :**::: *. ** *    **.: ::

Pm 50/57  361   QYIALIP        367
Pm TG83   361   QYIALIP        367
Pm R110   360   QYIELIYSQRISIMSQNTF  378
Pm 51/57  360   QYISLI         365
Pm HI4320 360   QYISLI         365
Kp C3     357   QYRQLQRT       364
Kp 52145  357   QYRQLQRT       364
                ** *
```

FIGURE 2. **Amino acid alignment of WabH and WabP proteins.** Shown are WabH homologues from *P. mirabilis* strains R110 (*Pm R110*), 51/57 (*Pm 51/57*), and HI4320 (*Pm HI4320*); and *K. pneumoniae* strains 52145 (*Kp 52145*) and C3 (*Kp C3*); and WabP homologues from *P. mirabilis* 50/57 (*Pm 50/57*) and TG83 (*Pm TG83*). *White letters* on *black background* indicate identical amino acid residues, *black letters* on *gray background* indicate similar amino acid residues, and *arrows* denote specific amino acid residues for WabP from strains 50/57 and TG83. *, identical residues; :, conserved residues; ·, semi-conserved residues.

wabP₅₀/₅₇). LPS from this strain migrated into an intermediate position between those of 52145 and 52145Δ*wabH* (supplemental Fig. S1).

A search for *gne* homologues in *P. mirabilis* revealed such gene in the species type strain ATCC 29906, whose whole genome sequence is being completed. This *gne* (in contig GG668582, GenBank™ ACLE01000000) was amplified and cloned to obtain pACYC-GNE$_{P. mirabilis}$. The LPS isolated from 52145Δ*wabH* (pGEM-T-*wabP₅₀/₅₇* pACYC-GNE$_{P. mirabilis}$) also migrated between those of 52145 and 52145Δ*wabH* (Fig. 3).

To determine the core LPS changes produced by expression of both *gne* and *wabP₅₀/₅₇* in the 52145Δ*wabH* background, the LPS was extracted from strain 52145Δ*waaL wabH* (pGEM-T-*wabP₅₀/₅₇* pACYC-GNE$_{P. mirabilis}$) by the phenol, chloroform, and petroleum ether method (22), and the core OS fraction was isolated by mild acid hydrolysis. Compositional analysis of this fraction by GC-MS of acetylated methyl glycosides revealed the presence of Kdo, L-*glycero*-D-*manno*-heptopyranose, GalA, Glc, and GalN. Compara-

tive MALDI-TOF analysis of this core OS fraction with that of strain 52145Δ*waaL wabH* (15) revealed major signals at 1488.19 and 1470.27 *m/z*, corresponding to a Kdo-Hep3-Hex-HexA2-HexN and its anhydro form. This is in agreement with a core extending up to the second outer OS residue (GalN) (Fig. 4*A*). Analysis of the core OS fraction from strain 52145Δ*waaL wabH* (pGEM-T-*wabP₅₀/₅₇* pACYC-GNE$_{A. hydrophila}$) resulted in the same chemical composition and major *m/z* signals (supplemental Fig. S2). A similar analysis of the OS fraction from strain 52145Δ*waaL wabH* (pGEM-T-*wabH$_{R110}$*) showed major signals at 1812.56 and 1650.54 corresponding to Kdo-Hep3-Hex3-HexA2-HexN and Kdo-Hep3-Hex2-HexA2-HexN (Fig. 4*B*). These structures are in agreement with a full type 2 *K. pneumoniae* core OS (13). Other signals attributable to OS with terminal Kdo modifications or sodium abducts are shown in Fig. 4.

A search for *gne* homologues in *P. mirabilis* revealed such a gene in the species type strain ATCC 29906, whose whole genome sequence is being completed. This *gne* (in contig GG668582, GenBank™ ACLE01000000) was amplified and
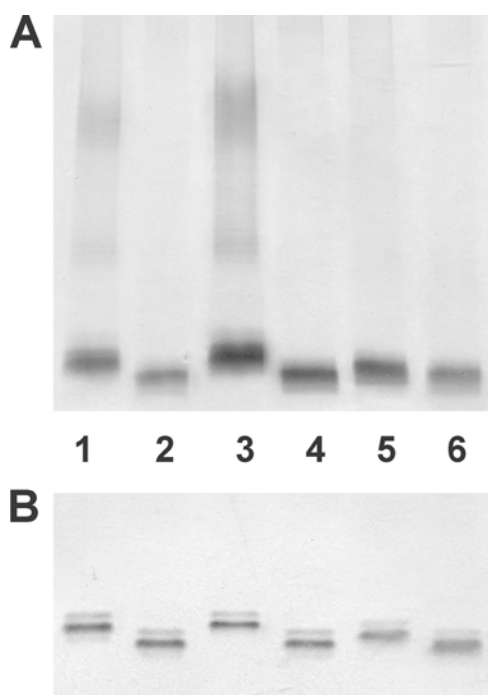
FIGURE 3. **Polyacrylamide gels showing the migration of LPS from 52145Δ*wabH* mutant and its complementation.** The LPS samples were separated on SDS-PAGE (*A*) and SDS-Tricine-PAGE (*B*) and visualized by silver staining. Shown are LPS samples from *K. pneumoniae* 52145 (*lane 1*), 52145Δ*wabH* (*lane 2*), 52145Δ*wabH* (pGEM-T-*wabH*$_{R110}$) (*lane 3*), 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$) (*lane 4*), 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$ pACYC-GNE$_{P. mirabilis}$) (*lane 5*), and 52145Δ*wabH* (pACYC-GNE$_{P. mirabilis}$) (*lane 6*).

cloned to obtain pACYC-GNE$_{P. mirabilis}$. The LPS isolated from 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$ pACYC-GNE$_{P. mirabilis}$) behaved as that from 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$ pACYC-GNE) in Tricine-SDS-PAGE. In addition, no differences in chemical composition or in major *m/z* signals could be found between these two core LPS samples (data not shown).

WabH and WabP in Vitro Activity—*In vitro* enzymatic assays were performed (27) to test if the WabH$_{R110}$ and WabP$_{50/57}$ are specific for the transfer of GlcNAc and GalNAc, respectively. This assay measured the amount of radiolabeled GlcNAc or GalNAc incorporated into acceptor LPS from UDP-[^{14}C]GlcNAc or UDP-[^{14}C]GalNAc. LPS from mutant *K. pneumoniae* 52145Δ*wabH* was used as acceptor. Recombinant plasmids expressing N-terminal histidine-tagged WabH$_{R110}$ (His$_6$-WabH$_{R110}$) and His$_6$-WabP$_{50/57}$ were constructed to facilitate the purification of the enzymes used in the assays by affinity chromatography.

Qualitative assays based on autoradiography of LPS reaction samples after Tricine-SDS-PAGE showed that His$_6$-WabH$_{R110}$ was able to transfer radiolabeled GlcNAc from UDP-GlcNAc to acceptor LPS, whereas little radioactivity was incorporated into acceptor LPS when using UDP-GalNAc as donor. By contrast, His$_6$-WabP$_{50/57}$ directed the incorporation of mainly GalNAc and little GlcNAc (Fig. 5).

Quantitative experiments were used to determine the apparent kinetic parameters of WabH$_{R110}$ and WabP$_{50/57}$. In one set of experiments, the concentration of UDP-GlcNAc or UDP-GalNAc was maintained constant at 200 μM, and a

range of acceptor LPS from mutant 52145Δ*wabH* was used (1–800 μM). In the other set, the acceptor LPS was held constant (200 μM), and different levels of UDP-GlcNAc or UDP-GalNAc were used (1–800 μM). The data allowed us to determine the apparent K_m of His$_6$-WabH$_{R110}$ and His$_6$-WabP$_{50/57}$ for acceptor LPS, UDP-GlcNAc, and UDP-GalNAc (Table 2). In reactions containing 100 nM enzyme preparations, the apparent K_m for acceptor LPS was essentially the same for both enzymes. By contrast, the apparent K_m of His$_6$-WabP$_{50/57}$ for UDP-GalNAc was 10-fold lower than that for UDP-GlcNAc, whereas the His$_6$-WabH$_{R110}$ behaved in a reverse way (Table 2).

WabN Complementation Studies—The compositional analysis of the OS fraction from strain 52145Δ*wabH* with pGEM-T-*wabP*$_{50/57}$ and pACYC-GNE or pACYC-GNE$_{P. mirabilis}$ plasmids revealed the presence of GalN (see above), suggesting that the WabN deacetylase from strain 52145 is able to deacetylate both GlcNAc- and GalNAc-containing OS. The comparative analysis of the known WabN homologues from *K. pneumoniae* and *P. mirabilis* revealed that they share around 65% amino acid identity, whereas among those of *P. mirabilis*, identity is more than 95%. The WabN proteins from strains 50/57 and TG83 were identical and shared levels of residue identity of 99.7, 98.4, and 95,9% with those of strains R110, HI4320, and 51/57, respectively. These levels of identity suggest that they will have the same role.

Complementation experiments confirmed this hypothesis. The LPS from *K. pneumoniae* 52145Δ*wabN* is devoid of the last two outer core residues and O-PS and presents GlcNAc instead of GlcN (15). Introduction of pGEM-T-*wabN*$_{R110}$ or pGEM-T-*wabN*$_{50/57}$ restores wild type 52145 LPS migration and O-PS production (Fig. 6). In addition, an R110Δ*wabN* mutant was constructed, and LPS from this mutant was devoid of O-PS and migrated faster than that of wild-type R110. This mutant was fully complemented by *wabN* homologues from strain 50/57 and *K. pneumoniae* 52145 as judged by LPS migration in Tricine-SDS-PAGE (data not shown). These results suggest that both genes codify for enzymes able to deacetylate GlcNAc containing core OS.

WabN Deacetylates Both GlcNAc and GalNAc LPS in Vitro—An *in vitro* enzymatic assay measuring the amount of radiolabeled GlcNAc or GlcN incorporated into acceptor LPS from UDP-[^{14}C]GlcNAc after acid hydrolysis and TLC separation of the radiolabeled residues was performed (15). LPS from mutant 52145Δ*wabH* was used as an acceptor with combinations of histidine-tagged enzymes WabH, WabP, and WabN. In reactions using UDP-[^{14}C]GlcNAc as donor and both His$_6$-WabH$_{R110}$ and His$_6$-WabN$_{R110}$ or His$_6$-WabN$_{50/57}$, radiolabeled GlcN was detected (Table 3). Replacing His$_6$-WabH$_{R110}$ by His$_6$-WabP$_{50/57}$ did not allow a substantial detection of GlcN. In reactions using UDP-[^{14}C]GalNAc as donor, His$_6$-WabP$_{50/57}$ and His$_6$-WabN$_{50/57}$ or His$_6$-WabN$_{R110}$ radiolabeled GalN was detected (Table 3). In these reactions, replacement of His$_6$-WabP$_{50/57}$ by His$_6$-WabH$_{R110}$ resulted again in substantial GalN reduction detection (Table 3). These results strongly suggest that in *P. mirabilis*, there are two specific HexNAc transferases (WabH and WabP), whereas WabN enzymes are able to deacetylate both GlcNAc- and GalNAc-containing OS.
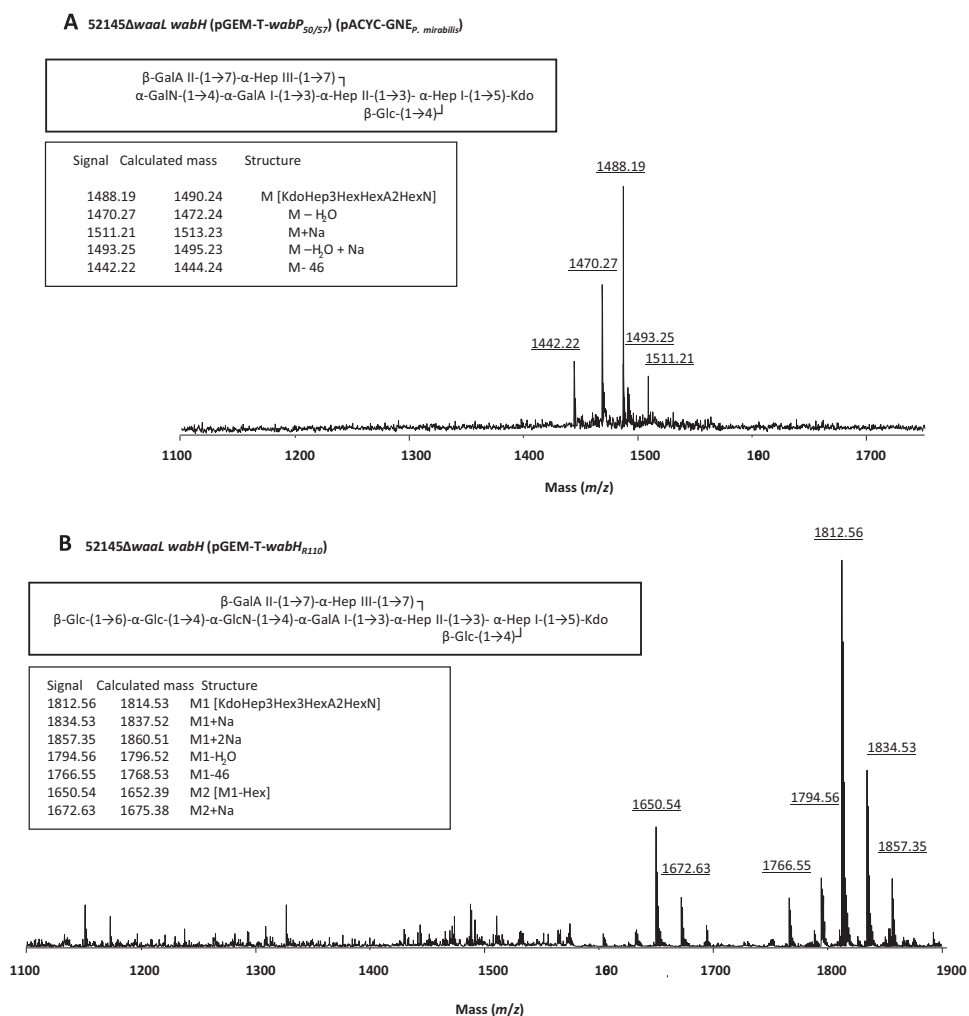
FIGURE 4. **Positive ion MALDI-TOF of acid-released core LPS oligosaccharides.** The spectrum of OS from *K. pneumoniae* 52145Δ*wabH* was previously reported (15). Shown are spectra of OS isolated from *K. pneumoniae* 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$ pACYC-GNE) (*A*) and *K. pneumoniae* 52145Δ*wabH* (pGEM-T-*wabH*$_{R110}$) (*B*). Schematic structures of the most representative compounds are shown in the *insets*. Signals that are 18, 46, and 88 Da, respectively, below the described pseudomolecular ion are attributable to OS with terminal Kdo containing a ring double bond (−18 Da), a ketone at C-1 (−44 Da) or ring fragmentation (−88 Da). These artifacts have been described for LPS samples that are hydrolyzed in presence of acetic acid (36).
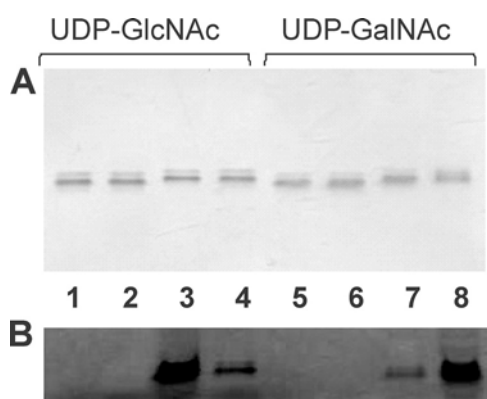
FIGURE 5. **HexNAc transferase qualitative *in vitro* analysis by Tricine-SDS-PAGE of product reactions.** Reactions contained LPS from 52145Δ*wabH*, 0.25 μCi of UDP-[^{14}C]GlcNAc (*lanes 1–4*) or UDP-[^{14}C]GalNAc (*lanes 5–8*), and the indicated histidine-tagged proteins. After a 2-h reaction at 37 °C, LPS was recovered, washed, and separated on Tricine-SDS-PAGE. The gel was stained (*A*), dried, and autoradiographed (*B*). *Lanes 1* and *5*, control reactions without the addition of the enzyme; *lane 2*, reactions with His$_6$-WabN$_{R110}$; *lanes 3* and *7*, reactions with His$_6$-WabH$_{R110}$; *lane 6*, reactions with His$_6$-WabN$_{50/57}$; *lanes 4* and *8*, reactions with His$_6$-WabP$_{50/57}$.

gne, wabH, and wabP Distribution—In order to determine the distribution of the genes determining the incorporation of GlcN/GalN into the core LPS, three pairs of oligonucleotides were designed to amplify internal fragments of *gne* (694 bp), *wabH* (815 bp), and *wabP* (417 bp). Internal *gne* fragments were amplified from strains containing GalN in their core LPS, such as 50/57, TG83, and 15/57, as expected, but also from two strains, R110 and OXK, containing GlcN instead of GalN (Fig. 7*A*). The core LPS of strain OXK contains a GalNAc residue in addition to GlcN (11), and the O-PS of strains R110 and OXK also contain GalNAc (9). Among strains of unknown LPS structure, *gne* was found in strain ATCC 29066 but not in CECT 170 (Fig. 7*A*). Thus, *gne* will be present in strains requiring the synthesis of UDP-GalNAc to be incorporated into either core or O-antigen LPS.

A *wabH* internal fragment was amplified from GlcN-containing core LPS strains, such as R110, 51/57, and OXK (11), as well as from strains CECT 170 and ATCC 29906 of unknown core LPS structure (Fig. 7*B*). A *wabP* internal fragment was amplified from GalN-containing core LPS strains, such as 50/57, TG83, and 14/57 (11) (Fig. 7*C*). After testing 25 differ-

TABLE 2

Kinetics of His$_6$-WabH$_{R110}$ and His$_6$-WabP$_{50/57}$

The Prism GraphPad program was used to calculate the apparent Michaelis-Menten parameters from three replicate experiments.

Donor	Enzyme	K_{cat}	K'_m UDP-HexNAc	K'_m LPS	K_{cat}/K'_m UDP-HexNAc	K_{cat}/K'_m LPS
		min^{-1}	μM	μM		
UDP-GlcNAc	His$_6$-WabH$_{R110}$	26 ± 5	34 ± 3	13 ± 3	0.76	2.0
	His$_6$-WabP$_{50/57}$	4 ± 2	120 ± 15	14 ± 2	0.03	0,28
UDP-GalNAc	His$_6$-WabH$_{R110}$	3 ± 1	130 ± 21	13 ± 5	0.02	0,23
	His$_6$-WabP$_{50/57}$	27 ± 6	38 ± 4	12 ± 4	0.71	2.2
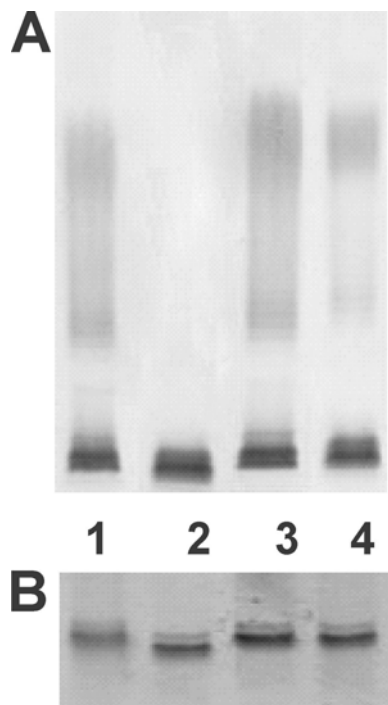
FIGURE 6. **Polyacrylamide gels showing the migration of LPS from 52145Δ*wabN* mutant and its complementation.** The LPS samples were separated on SDS-PAGE (*A*) and SDS-Tricine-PAGE (*B*) and visualized by silver staining. Shown are of LPS samples from *K. pneumoniae* 52145 (*lane 1*), 52145Δ*wabN* (*lane 2*), 52145Δ*wabN* (pGEM-T-*wabN*$_{R110}$) (*lane 3*), and 52145Δ*wabN* (pGEM-T-*wabN*$_{50/57}$) (*lane 4*).

TABLE 3

In vitro analysis of LPS-HexNAc deacetylation

Reactions contained LPS from *K. pneumoniae* 52145Δ*wabH*, 0.25 μCi of UDP-[^{14}C]GlcNAc or UDP-[^{14}C]GalNAc, and the indicated histidine-tagged proteins. After a 2-h reaction at 37 °C, LPS was recovered by centrifugation, precipitated, washed, and hydrolyzed in 100 μl of 0.1 M HCl and applied to a TLC plate. Non-radioactive controls were used to localize the positions of GlcN, GalN, GlcNAc, and GalNAc. Radioactive spots were scraped and suspended in scintillating liquid, and the radioactivity was measured.

Protein	UDP-GlcNAc radioactivity		UDP-GalNAc radioactivity	
	GlcNAc	GlcN	GalNAc	GalN
	cpm		*cpm*	
WabH$_{R110}$ + WabN$_{R110}$	<100	2670	<100	<100
WabH$_{R110}$ + WabN$_{51/57}$	<100	2345	<100	<100
WabP$_{50/57}$ + WabN$_{R110}$	<100	<100	<100	2565
WabP$_{50/57}$ + WabN$_{50/57}$	<100	<100	<100	2479

ent *P. mirabilis* strains, in no case was it possible to detect the simultaneous presence of *wabH* and *wabP*.

DISCUSSION

Sequence similarity suggested that, depending on the nature of the HexN in outer core OS, two different types of HexNAc transferases are present in *P. mirabilis* strains. Tak-
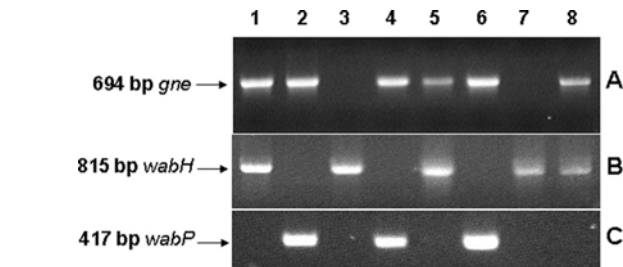
FIGURE 7. **PCR-based analysis of the distribution of *gne*, *wabH*, and *wabP* genes among *P. mirabilis* strains.** Shown is agarose gel electrophoresis of PCR-amplified products generated from template DNA obtained from strains R110 (*lane 1*), 50/57 (*lane 2*), 51/57 (*lane 3*), TG83 (*lane 4*), OXK (*lane 5*), 14/57 (*lane 6*), CECT170 (*lane 7*), and ATCC 29066 (*lane 8*). Amplification products obtained with oligonucleotide pairs designed to amplify internal regions of *gne* (GneIntFw-GneInt-Rev) (*A*), *wabH* (wabHIntFw-WabHIntRev) (*B*), and *wabP* (WabpIntFw-WabPIntRv) (*C*) are shown.

ing advantage of the identity between *P. mirabilis* and *K. pneumoniae* core OS sugar backbone up to the first outer core residue, we performed *in vivo* experiments using *K. pneumoniae* non-polar *wabH* mutant LPS as acceptor substrate. This new approach has the advantage that we look for a positive trait as the addition to the mutant LPS of a particular residue instead of the mutagenesis studies to determine the function of core LPS biosynthetic genes usually employed. This is only possible, as in our case, if proper surrogate LPS acceptor is available. By this methodology, we were able to identify a GlcNAc transferase (WabH$_{R110}$) in strain R110, containing GlcN as the second outer core residue. By contrast, the WabP$_{50/57}$ protein from a strain containing GalN as the second outer core residue was able to transfer GalNAc to *K. pneumoniae* core LPS only when the *gne* (UDP-GalNAc 4-epimerase) gene was also provided. An R110Δ*wabH* mutant was complemented (presence of O-antigen LPS) by *wabH* from strain 51/57 but not by *wabP* from strain 50/57 (absence of O-antigen LPS) (data not shown).

In vitro experiments using the purified enzymes and UDP-GlcNAc or UDP-GalNAc confirmed the GlcNAc and GalNAc transferase nature for WabH$_{R110}$ and WabP$_{50/57}$, respectively. As inferred from their functions and sequence similarity, both glycosyltransferases belong to the GT-B motif-containing glycosyltransferase family 4 according to the Carbohydrate Active Enzyme (CAZy) classification. This family contains also WabH homologues from *K. pneumoniae* and *S. marcescens* (32) (see the CAZy Web site).

The core OS structures of *E. coli* share a common substructure up to the first outer core residue (GlcI) (33). In *E. coli* strains with core OS types K-12, R1, R2, and R4, the WaaO glucosyltransferase links a second Glc residue (GlcII) to GlcI by an α1,2 linkage (33). In strains with R3 core type, the WaaI galactosyltransferase links a Gal residue to GlcI with the same

$\alpha 1,2$ linkage (33). Both WaaO and WaaI belong to the GT-A fold containing CAZy glycosyltransferase family 8. An alignment (data not shown) between these two glycosyltransferases (WaaO, Q8KMW9; WaaI, Q9ZIT4) revealed that they share 51 and 80% amino acid residue identity and similarity, respectively. These levels of identity/similarity are similar to those shared by the *P. mirabilis* N-acetylglucosaminyl (WabH$_{R110}$) and N-acetylgalactosaminyl (WabP$_{50/57}$) transferases. Thus, it appears that glycosyltransferases involved in the transfer of sugar epimer residues to the same LPS acceptor can be differentiated by their levels of identity/similarity.

Once GlcNAc is incorporated from UDP-GlcNAc into LPS, the WabN deacetylase converts this residue to GlcN, as previously shown in *K. pneumoniae* (15). Both complementation experiments and *in vitro* assays with purified WabN proteins show that in *P. mirabilis*, these proteins can deacetylate both GlcNAc and GalNAc containing nearly identical core LPS. Furthermore, an R110Δ*wabN* mutant was fully complemented by WabN homologues from *K. pneumoniae* and *P. mirabilis* 50/57. The fact that the WabN deacetylases are able to act on carbohydrate backbones nearly identical but with terminal GlcNAc or GalNAc residues could represent an efficient system to reduce unnecessary biodiversity.

From the known core OS structures from the genus *Proteus* (11), we can predict that *wabH* and *wabP* homologues should be present in *P. mirabilis* strains containing GlcN and GalN in their core LPS, respectively. Two pairs of oligonucleotides specific for *wabH* and *wabP* confirmed this prediction (Fig. 7, *B* and *C*). These results suggest that a simple amplification test can be used to predict the HexN nature in *P. mirabilis* core LPS. This diagnostic amplification indicates the presence of GlcN in the core LPS of strains for which the chemical structures have not been determined, such as CECT 170, HI4320, and ATCC 29906. In agreement with the test, the analysis of the genome sequence available for strains HI4320 (whole genome sequence determined) (28) and ATCC 29906 (genome sequencing in progress) (ACLE01000000) confirmed the presence of *wabH* but not *wabP*.

By contrast, *gne* required for UDP-GalNAc generation from UDP-GlcNAc is present in strains containing GalN in their core LPS but also in strains containing GlcN when these strains present GalNAc in their O-PS. In *P. mirabilis* ATCC 29906, the *gne* is located in a gene cluster similar to those putatively involved in O-PS (contig GG668582, GenBank^{TM} ACLE01000000) (34). However, there are examples of *P. mirabilis* strains that should require *gne* for its O-PS biosynthesis where this gene was not found inside the O-PS biosynthetic cluster (34). Thus, *gne* cannot be used to predict the presence of GalN or GlcN in the core LPS. Nevertheless, the absence of *gne* is fully correlated with presence of *wabH* and thus of GlcN in the LPS core of the strains tested.

We conclude that although the incorporation of GlcN into core LPS requires *N*-acetylglucosaminyltransferase (WabH) and LPS:GlcNAc deacetylase (WabN), the incorporation of GalN requires three enzymes: Gne epimerase for the generation of UDP-GalNAc from UDP-GlcNAc, *N*-acetylgalactosaminyltransferase (WabP), and LPS:GalNAc deacetylase. Of these three enzymes, only the LPS:HexNAc deacetylase

(WabN) is common to the incorporation of GlcN and GalN into core LPS.

REFERENCES

1. Warren, J. W. (1991) *Med. Clin. North Am.* **75,** 481–493
2. Warren, J. W., Muncie, H. L., Jr., and Hall-Craggs, M. (1988) *J. Infect. Dis.* **158,** 1341–1346
3. Rubin, R. H., Tolkoff-Rubin, N. E., and Cotran, R. S. (1986) in *The Kidney* (Brenner, B. M., and Rector, F. C., eds) pp. 1085–1141, W.B. Saunders Co., Philadelphia, PA
4. Belas, R. (1996) in *Urinary Tract Infections: Molecular Pathogenesis and Clinical Management* (Mobley, H. L. T., and Warren, J. W., eds) pp. 271–298, American Society for Microbiology Press, Washington, D. C.
5. Fairley, K. F., Carson, N. E., Gutch, R. C., Leighton, P., Grounds, A. D., Laird, E. C., McCallum, P. H., Sleeman, R. L., and O'Keefe, C. M. (1971) *Lancet* **2,** 615–618
6. Rashid, T., and Ebringer, A. (2007) *Clin. Rheumatol.* **26,** 1036–1043
7. Larsson, P. (1984) *Methods Microbiol.* **14,** 187–214
8. Penner, J. L., and Hennessy, J. N. (1980) *J. Clin. Microbiol.* **12,** 304–309
9. Knirel, Y. A., Kaca, W., Rozalsky, A., and Sydorczyk, Z. (1999) *Pol. J. Chem.* **73,** 895–907
10. Zych, K., Kowalczyk, M., Knirel, Y. A., and Sydorczyk, Z. (2000) in *Genes and Proteins Underlying Microbial Urinary Tract Virulence: Basic Aspects and Applications* (Emody, L., Pal, T., Hacker, J., and Blum-Oehler, G., ed) pp. 339–344, Kluwer Academic/Plenum Publishers, New York
11. Vinogradov, E., Sidorczyk, Z., and Knirel, Y. A. (2002) *Aust. J. Chem.* **55,** 61–67
12. Vinogradov, E., and Perry, M. B. (2001) *Carbohydr. Res.* **335,** 291–296
13. Regué, M., Izquierdo, L., Fresno, S., Piqué, N., Corsaro, M. M., Naldi, T., De Castro, C., Waidelich, D., Merino, S., and Tomás, J. M. (2005) *J. Bacteriol.* **187,** 4198–4206
14. Coderch, N., Piqué, N., Lindner, B., Abitiu, N., Merino, S., Izquierdo, L., Jimenez, N., Tomás, J. M., Holst, O., and Regué, M. (2004) *J. Bacteriol.* **186,** 978–988
15. Regué, M., Izquierdo, L., Fresno, S., Jimenez, N., Piqué, N., Corsaro, M. M., Parrilli, M., Naldi, T., Merino, S., and Tomás, J. M. (2005) *J. Biol. Chem.* **280,** 36648–36656
16. Aquilini, E., Azevedo, J., Jimenez, N., Bouamama, L., Tomás, J. M., and Regué, M. (2010) *J. Bacteriol.* **192,** 4413–4424
17. Miller, J. H. (1972) *Experiments in Molecular Genetics*, p. 433, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5463–5467
20. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
21. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
22. Galanos, C., Lüderitz, O., and Westphal, O. (1969) *Eur. J. Biochem.* **9,** 245–249
23. Westphal, O., and Jann, K. (1965) *Methods Carbohydr. Chem.* **5,** 83–91
24. Hitchcock, P. J., and Brown, T. M. (1983) *J. Bacteriol.* **154,** 269–277
25. Pradel, E., and Schnaitman, C. A. (1991) *J. Bacteriol.* **173,** 6428–6431
26. Tsai, C. M., and Frasch, C. E. (1982) *Anal. Biochem.* **119,** 115–119
27. Frirdich, E., Vinogradov, E., and Whitfield, C. (2004) *J. Biol. Chem.* **279,** 27928–27940
28. Pearson, M. M., Sebaihia, M., Churcher, C., Quail, M. A., Seshasayee, A. S., Luscombe, N. M., Abdellah, Z., Arrosmith, C., Atkin, B., Chillingworth, T., Hauser, H., Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Walker, D., Whithead, S., Thomson, N. R., Rather, P. N., Parkhill, J., and Mobley, H. L. T. (2008) *J. Bacteriol.* **190,**

4027–4037

29. Geerlof, A., Lewendon, A., and Shaw, W. V. (1999) *J. Biol. Chem.* **274,** 27105–27111

30. Boiteux, S., O'Connor, T. R., and Laval, J. (1987) *EMBO J.* **6,** 3177–3183

31. Canals, R., Jiménez, N., Vilches, S., Regué, M., Merino, S., and Tomás, J. M. (2007) *J. Bacteriol.* **189,** 540–550

32. Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008) *Annu. Rev. Biochem.* **77,** 521–555

33. Amor, K., Heinrichs, D. E., Frirdich, E., Ziebell, K., Johnson, R. P., and Whitfield, C. (2000) *Infect. Immun.* **68,** 1116–1124

34. Wang, Q., Torzewska, A., Ruan, X., Wang, X., Rozalski, A., Shao, Z., Guo, X., Zhou, H., Feng, L., and Wang, L. (2010) *Appl. Environ. Microbiol.* **76,** 5471–5478

35. Regué, M., Climent, N., Abitiu, N., Coderch, N., Merino, S., Izquierdo, L., Altarriba, M., and Tomás, J. M. (2001) *J. Bacteriol.* **183,** 3564–3573

36. Olsthoorn, M. M., Haverkamp, J., and Thomas-Oates, J. E. (1999) *J. Mass Spectrom.* **34,** 622–636

37. Nassif, X., Fournier, J. M., Arondel, J., and Sansonetti, P. J. (1989) *Infect. Immun.* **57,** 546–552

38. Hanahan, D. (1983) *J. Mol. Biol.* **166,** 557–580

FIGURE S1. **Polyacrylamide gels showing the migration of LPS from 52145Δ*wabH* mutant and its complementation.** The LPS samples were separated on SDS-PAGE (**A**) and SDS-Tricine-PAGE (**B**) and visualized by silver staining. Shown are LPS samples from *K. pneumoniae* 52145 (Lane 1), 52145Δ*wabH* (Lane 2), 52145Δ*wabH* (pGEM-T-*wabH*$_{R110}$) (Lane 3), 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$) (Lane 4), 52145Δ*wabH* (pGEM-T-*wabP*$_{50/57}$ pACYC-GNE $_{A.\ hydrophila}$) (Lane 5), and 52145Δ*wabH* (pACYC-GNE) (Lane 6).
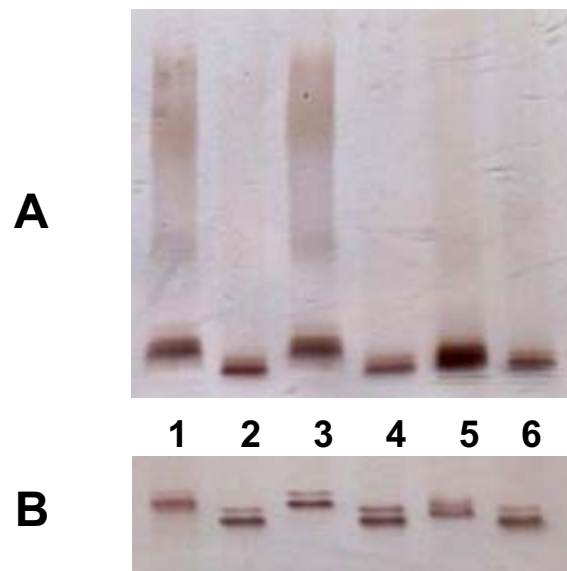
FIGURE S2.   **Positive ions MALDI-TOF of acid-released core LPS oligosaccharides.** Spectrum of OS from *K. pneumoniae* 52145Δ*wabH* was previously reported (15). Shown are spectra of OS isolated from *K. pneumoniae* 52145Δ*wabH* (pGEM-T-*wabP$_{50/57}$* pACYC-GNE $_{A. hydrophila}$) (**A**), and *K. pneumoniae* 52145Δ*wabH* (pGEM-T-*wabH$_{R110}$*) (**B**). Schematic structures of the most representative compounds are shown in the insets. Signals which are 18, 46 and 88 Da, respectively below the described pseudomolecular ion are attributable to OS with terminal Kdo containing a ring double bond (- 18), a ketone at C-1 (- 44) or ring fragmentation (- 88). These artifacts have been described for LPS samples that are hydrolyzed in presence of acetic acid (37).

A   52145Δ*waaL wabH* (pGEM-T-*wabP$_{50/57}$*) (pACYC-GNE$_{A. hydrophila}$)

β-GalA II-(1→7)-α-Hep III-(1→7) ⌐
α-GalN-(1→4)-α-GalA I-(1→3)-α-Hep II-(1→3)- α-Hep I-(1→5)-Kdo
β-Glc-(1→4)⌐

Signal	Calculated mass	Structure
1488.19	1490.24	M [KdoHep3HexHexA2HexN]
1470.27	1472.24	M – H$_2$O
1511.20	1513.23	M+Na
1493.25	1495.23	M –H$_2$O + Na
1442.23	1444.24	M- 46

B   52145Δ*waaL wabH* (pGEM-T-*wabH$_{R110}$*)

β-GalA II-(1→7)-α-Hep III-(1→7) ⌐
β-Glc-(1→6)-α-Glc-(1→4)-α-GlcN-(1→4)-α-GalA I-(1→3)-α-Hep II-(1→3)- α-Hep I-(1→5)-Kdo
β-Glc-(1→4)⌐

Signal	Calculated mass	Structure
1812,56	1814.53	M1 [KdoHep3Hex3HexA2HexN]
1834.53	1837.52	M1+Na
1857.35	1860.51	M1+2Na
1794.56	1796.52	M1-H$_2$O
1766,55	1768.53	M1-46
1650.54	1652.39	M2 [M1-Hex]
1672.63	1675.38	M2+Na

Para la incorporación de la galactosamina en el núcleo del lipopolisacárido son necesarios tres pasos enzimáticos.

Eleonora Aquilini, Joana Azevedo, Susana Merino, Natalia Jimenez, Juan M. Tomás y Miguel Regué

El núcleo del lipopolisacárido (LPS) de *Proteus mirabilis*, así como el de *Klebsiella pneumoniae* y el de *Serratia marcescens*, es caracterizado por la presencia de un disacárido de αHexN-1,4-GalA en su núcleo externo. En *K. pneumoniae*, *S. marcescens* y algunas cepas de *P. mirabilis* este residuo corresponde a D-glucosamina (GlcN), mientras que, en otras cepas de *P. mirabilis* este es una D-galactosamina (GalN). Se ha demostrado previamente que la proteína WabG es la responsable de la transferencia del residuo de GalA. Ademas, se había demostrado que se necesitaban dos enzimas para la incorporación de GlcN en el núcleo de *K. pneumoniae*: el enzima WabH, identificado como la GlcNAc transferasa que permite la incorporación de GlcNAc en el núcleo creciente del LPS, y la proteína WabN que desacetila el residuo de GlcNAc a GlcN.

En este trabajo se demuestra la presencia de dos genes homólogos, que codifican para HexNAc transferasas diferentes y especificas para cada HexN incluida en los núcleos de *P. mirabilis.*

Mediante experimentos *in vitro*, utilizando UDP-GlcNAc, UDP-GalNAc y enzimas purificadas se han podido determinar las diferentes funciones de transferencia de las dos proteínas WabH y WabP con los respectivos substratos. *In vivo*, gracias a la elevada similitud del esqueleto olisacaridico del núcleo de LPS entre *P. mirabilis* y *K. pneumoniae*, y teniendo en cuenta que los residuos de azúcar y los enlaces dentro del núcleo de LPS en estas dos especies son idénticos hasta el segundo residuo externo del núcleo se han podido realizar experimentos de complementación heteróloga con mutantes de *K. pneumoniae* defectivos en el gen *wabH*; estos han permitido confirmar la especificidad de estos dos enzimas en la transferencia de GlcNAc o GalNAc.

En cambio se ha encontrado, en las cepas de *P. mirabilis* estudiadas, un solo gen homologo para la proteína WabN. La misma estrategia ha permitido estudiar el rol de la proteína WabN, en la biosíntesis del núcleo del LPS en *P. mirabilis*. Los resultados han sugerido que WabN sea capaz de desacetilar tantos núcleos que contienen GlcNAc como aquellos con GalNAc.

Finalmente se ha podido concluir que, mientras la incorporación de GlcN en el núcleo de LPS necesita solo dos enzimas, una N-acetylglucosaminyltransferasa (WabH) y una GlcNAc deacetilasa (WabN), la incorporación de GalN requiere tres: Gne epimerasa, para la generación de UDP-GalNAc de UDP-GlcNAc, una N-acetylgalactosaminyltransferasa (WabP) y una LPS:GalNAc deacetilasa (WabN). Solamente WabN es común a la incorporación tanto de GlcN como de GalN.

Functional identification of a *Proteus mirabilis eptC* gene encoding a core lipopolysaccharide phosphoethanolamine transferase

Running title: ***Proteus mirabilis* core LPS phosphoethanolamine transferase**

Eleonora Aquilini[1], Joana Azevedo[1], Susana Merino[2], Yuriy A Knirel[3], Christopher J. Alteri[4], Harry L. T. Mobley[4], Juan M. Tomás[2*] and Miguel Regué[1]

[1]Departamento de Microbiología y Parasitología Sanitarias, Facultad de Farmacia, Universidad de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain.

[2] Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona, Spain.

[3]N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia.

[4]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

* Corresponding author. Mailing address: Departamento Microbiología. Facultad Biología. Universidad Barcelona.Diagonal 645, 08071 Barcelona. SPAIN.

Phone: 34-93-4021486; Fax: 34-93-4039047; E-mail: jtomas @ub.edu

ABSTRACT

By comparison of the genome of *P. mirabilis* HI4320 with known lipopolysaccharide (LPS) phosphoethanolamine transferases three putative candidates (PMI3040, PMI3576, and PMI3104) were identified. One of them, *eptC* (PMI3104) was able to modify the LPS of two defined non-polar core LPS mutants of *Klebsiella pneumoniae*. Mass spectrometry and nuclear magnetic resonance showed that *eptC* directs the incorporation of phosphoethanolamine to the O-6 of L-*glycero*-D-*mano*-heptose II. An *eptC* insertion mutant was constructed and showed outer membrane destabilization, as indicated by increased sensitivity to anionic detergent sodium dodecyl sulfate. This destabilization does not lead to a decrease in a UTI model, but their moderate sensitivity to complement could compromise their ability to perform a systemic infection. Putative *eptC* homologues were found for only two additional genera, *Photobacterium* and *Providencia*, of the *Enterobacteriaceae* family.

KEYWORDS: *Proteus mirabilis*, core lipopolysaccharide, phosphoethanolamine

Abbreviations: LPS, lipopolysaccharide; OS, oligosaccharide; O-PS, O-polysaccharide or O-antigen; PEtN, phosphoethanolamine; L,D-Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid or 3-deoxyoctulosonic acid; cationic antimicrobial peptides (CAMPs); L-Ara4N, 4-amino-4-deoxy-L-arabinose.

INTRODUCTION

Gram-negative motile and frequently swarming bacteria of the genus *Proteus* from the family *Enterobacteriaceae* are usually found in soil, water, and the intestines of human and animals. The genus includes five species (*P. mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxofaciens*, and *P. hauseri*) and three genomospecies (4, 5, and 6) (35, 38). Among these species, *P. mirabilis*, *P. vulgaris*, and *P. penneri* are the most common opportunistic pathogens (43). *P. mirabilis* is frequently associated with urinary tract infections (UTI) in individuals with functional or anatomical abnormalities of the urinary tract (56) or long-term catheterized patients (57). Complications arising from *P. mirabilis* infections include bladder and kidney stone formation, catheter obstruction by encrusting biofilms, and bacteremia (4). Identified virulence factors include swarming, fimbriae, urease, hemolysin, and iron acquisition systems (9). Signature-tagged mutagenesis (7, 16) has allowed the additional identification of an extracellular metalloprotease (40), several putative DNA binding regulatory, cell-envelope related, and plasmid encoded proteins.

In the lipopolysaccharide (LPS) of *Proteus mirabilis,* as in other members of the family *Enterobacteriaceae,* three domains can be recognized: lipid A, an endotoxic glycolipid; an O-polysaccharide chain or O-antigen (O-PS); and a core oligosaccharide (OS) domain linking lipid A and O-PS. The chemical structure of the *P. mirabilis* lipid A has been determined for one strain showing the presence of a residue of 4-amino-4-deoxy-L-arabinose (L-Ara4N) substituting the phosphate at position 1 of lipid A (46), this L-AraN modification has been related to the high intrinsic *P. mirabilis* resistance to polymyxin B and related cationic antimicrobial peptides (CAMPs) (54).

Among *P. mirabilis* and *P. vulgaris* 60 O-serogroups have been recognized (23, 39) and the gene clusters involved in the biosynthesis of *P. mirabilis* serogroups O3,

O10, and O27 have been reported (55). In most *P. mirabilis* O-serogroups studied so far, the O-PSs contain acidic or both acidic and basic components, such as uronic acids, their amides with amino acids, including lysine, and phosphate groups (reviewed in 21).

The core OS structure of the *P. mirabilis* genome strain HI4320 has been recently reported (50) (Figure 1). This structure up to the first outer-core galacturonic acid residue (D-GalA I) is shared by 11 *P. mirabilis* strains and also by several *P. vulgaris* and *P. penneri* serogroups (53). This common fragment is also found in the core LPS of *Klebsiella pneumoniae* and *Serratia marcescens*, but without the Ara4N and phosphoethanolamine (PEtN) residues (8, 41, 52) (Figure 1). Some *P. mirabilis* core-OS structures contain unusual residues such as quinovosamine, an open-chain form of *N*-acetyl-galatosamine, or amide linked amino acids (53). Recently, most of the genes involved in the biosynthesis of the sugar backbone of the core LPS from several *P. mirabilis* strains have been identified and characterized (2, 3).

The LPS is a potential *Proteus* virulence factor (43), two mutants in a putative LPS glycosyltranferase (16) show attenuated virulence, LPS plays a significant role in *P. mirabilis* CAMPs resistance (29), and LPS charge alterations may influence the swarming motility of the bacterium (6, 29). In addition, the acidic character of *P. mirabillis* O-PSs enables bacteria to bind metal cations, Mg^{2+} and Ca^{2+}, via electrostatic interaction that may enhance the formation of struvite and carbonate apatite stones in the urinary tract (11). By contrast little is known about the role of the non-sugar charged residues or groups in the core OS. In this study we identify a gene, *eptC*, essential for core LPS modification with PEtN and characterize an *eptC::kan* mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were routinely grown in in LB medium (per liter, 0.5 g NaCl, 5.0 g yeast extract, 10.0 g tryptone) or on LB agar (15.0 g l^{-1} agar). For *P. mirabilis* minimal A medium (per liter, 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.47 g sodium citrate and 1.0 g $(NH_4)_2SO_4$) was prepared and autoclaved. After cooling, 1 ml 1 M $MgSO_4$, 10 ml 20% glycerol and 1 ml 1% nicotinic acid were added per liter (5). Growth curves were performed using a Bioscreen growth curve analyzer (Growth Curves) following culture of bacterial samples in triplicate. Ampicillin (100 µg ml^{-1}), chloramphenicol (20 µg ml^{-1}), and kanamycin (25 µg ml^{-1}) were added to the different media when required.

General DNA methods. General DNA manipulations were done essentially as previously described (44). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

DNA sequencing and computer analysis of sequence data. Double-stranded DNA sequencing was performed by using the dideoxy-chain termination method (45) from PCR amplified DNA fragments with the ABI Prism dye terminator cycle sequencing kit (Perkin Elmer). Oligonucleotides used for genomic DNA amplifications and DNA sequencing were purchased from Sigma-Aldrich. Deduced amino acid sequences were compared with those of DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST (1) network service at the National Center for Biotechnology Information and the European Biotechnology Information. ClustalW was used for multiple-sequence alignments (48).

Plasmid constructions and mutant complementation studies. For complementation studies, the *P. mirabilis* R110 gene *eptC* (PMI3104) was PCR amplified by using two specific oligonucleotides (see the list of primers in Table 2) and chromosomal strain R110 DNA as template, ligated to plasmid pGEMT (Promega), and transformed into *E. coli* DH5α. Transformants were selected on LB plates containing ampicillin. Once checked, the plasmid with the amplified *eptC* gene (pGEMT-*eptC*) was transformed into *K. pneumoniae* core LPS mutants.

Recombinant plasmid pBAD18-Cm-*eptC* was obtained by pGEMT-*eptC* double digestion with SalI and PvuII and subcloning into pBAD18-Cm doubly digested with SalI and SmaI. This construct was transformed into *K. pneumoniae* core LPS mutants and *eptC* was expressed from the arabinose-inducible and glucose-repressible pBAD18-Cm promoter. Repression from the *araC* promoter was achieved by growth in medium containing 0.2% (wt/vol) D-glucose, and induction was obtained by adding L-arabinose to a final concentration of 0.2% (wt/vol). The cultures were grown for 18 h at 37°C in LB medium supplemented with chloramphenicol and 0.2% glucose, diluted 1:100 in fresh medium (without glucose), and grown until they reached an A_{600} of about 0.2. Then, L-arabinose was added, and the cultures were grown for another 8 h. Repressed controls were maintained in glucose-containing medium.

Mutant construction. To test the biological effects of absence of PEtN in core LPS, a mutation of PMI3104 (*eptC*) was constructed using the TargeTron Gene Knockout System (Sigma) following a modified protocol (36). Oligonucleotides for mutant design were created using the TargeTron Design Site (Sigma) and are listed in Table 2. PCR confirmation of the mutants was performed using oligonucleotides designed with the PrimerQuest program as previously described and are listed in Table 2. Mutants constructed are identified as *eptC::kan* and *eptC* referring to the *eptC* insertion mutant with the kanamycin marker retained and deleted, respectively. The kanamycin resistance

cassette was removed from the intron by IPTG induction of the cre recombinase in pQL123 (26).

LPS isolation and SDS-PAGE. For screening purposes, LPS was obtained after proteinase K digestion of whole cells (10). LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Tricine (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine)-SDS-PAGE and visualized by silver staining as previously described (10, 17).

Large-scale isolation and mild-acid degradation of LPS. Dried bacterial cells in 25 mM Tris-HCl buffer containing 2 mM $CaCl_2$, pH 7.63 (10 ml g^{-1}), were treated at 37°C with RNase and DNase (24 h, 1 mg g^{-1} each), and then with proteinase K (36 h, 1 mg g^{-1}). The suspension was dialyzed and lyophilized, and the LPS was extracted with aqueous phenol (59). After dialysis of combined water and phenol layers, contaminants were precipitated by adding 50% aqueous CCl_3CO_2H at 4 °C, the supernatant was dialyzed and lyophilized to give the LPS. A portion of the LPS was degraded with 2% acetic acid for 2 h at 100 °C, a precipitate was removed by centrifugation (13,000 x g, 20 min), and the supernatant was fractionated on a column (50 2.5 cm) of Sephadex G-50 Superfine in 0.05 M pyridinium acetate buffer (4 mL pyridine and 10 mL acetic acid in 1 l of water) with monitoring using a differential refractometer (Knauer, Germany).

Mass spectrometry and NMR studies. High-resolution electrospray ionization mass spectrometry was performed in the negative ion mode using a micrOTOF II instrument (Bruker Daltonics). A sample of the OS (~50 ng μl^{-1}) was dissolved in a 1:1 (v/v) water–acetonitrile mixture and sprayed at a flow rate of 3 μl min^{-1}. Capillary entrance voltage was set to 4.5 kV and exit voltage to -150 V; drying gas temperature was 180 °C.

NMR spectra were obtained on a Bruker DRX-500 spectrometer using standard Bruker software at 30 °C in 99.95% D_2O. Prior to the measurements, samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O. A 150-ms duration of

MLEV–17 spin–lock was used in a two-dimensional TOCSY experiments. Chemical shifts are referenced to internal sodium 3-trimethylsilylpropanoate-2,2,3,3-d$_4$ (δ_H 0) or acetone (δ_C 31.45).

MIC determination. MICs were determined by the standard broth twofold microdilution method using 96-well flat-bottom polystyrene plates (60). Briefly, starter bacterial cultures were grown in LB, diluted to 5 x 10^5 CFU ml^{-1} in LB and inoculated into rows of wells. Twofold serial dilutions of the tested antimicrobial compound were added to each row of wells. For FeSO$_4$ MIC determination overnight LB cultures were diluted in minimal medium A supplemented with 10 μM MgCl$_2$ and 25 μM FeSO$_4$. The plates were incubated at 37°C for 24 h. The MIC was determined as the lowest concentration of the antimicrobial compound that inhibit any visible bacterial growth, as measured turbidimetrically at OD600 as a value < 0.5 (turbidity visual detection limit).

CBA/J mouse model of ascending UTI. The CBA mouse model of ascending UTI (14, 20) was used to assess the contribution to fitness of the PEtN deficient mutant. For cochallenge competition experiments, 6–8 week old female CBA/J mice (20–22 g; Jackson Laboratories) were transurethrally infected with a 50 ml bacterial suspension of 5 x 10^7 CFU containing a 1:1 ratio of *P. mirabilis* HI4320 and *P. mirabilis eptC::kan* mutant. Mice were euthanized 7 days post infection. Urine, bladder and kidneys were collected, homogenized and plated on plain LB agar and/or LB agar with kanamycin (Autoplate 4000, Spiral Biotech). Viable colony counts were enumerated using a QCount (Spiral Biotech) and expressed as CFU g^{-1} of tissue. Wild type infection was determined by subtracting the number of colonies on LB agar containing antibiotic from the number of colonies on plain LB agar. Competitive indices (CI) was calculated by dividing the ratio of the CFU of wild type to mutant recovered from mice following infection by the ratio of the CFU of wild type HI4320 to the CFU of mutant present in the input. A CI > 1 indicates the wild type out-competes the mutant strain and a CI < 1

indicates the wild type is outcompeted by the mutant. The cochallenge competition experiment was analyzed using the Wilcoxon signed-rank test.

Bacterial survival in human serum. Bacterial cells (10^7 CFU) in the logarithmic phase were suspended in 90% serum–PBS and incubated at 37°C. Viable counts were made at different times until 3 h by dilution and plating as previously described (30). A pool of nonimmune human sera (NHS) was obtained from healthy volunteers. Control experiments using heat-decomplemented NHS were also performed (30).

RESULTS

Identification of putative LPS PEtN transferases. Previous work done in *S. enterica* LT2 allowed the identification of several LPS PEtN transferases by similarity to *lpt-3* gene encoded protein Lpt3 (NMB2010) of *Neisseria meningitidis* (47). This protein of *N. meningitdis* is responsible for the transfer of a PEtN residue to the O-2 of L,D-Hep II (28, 34, 58). A similar search was performed for *P. mirabilis* genome strain HI4320 (37) leading to the identification of PMI3040 (e value 1.e^{-19}, 28% identity, 48% similarity) and PMI3576 (e value 6.e^{-19}, 25% identity, 42% similarity). PMI340 and PMI3576 shared with other known PEtN transferases the presence of a sulfatase domain. PMI3040 showed significant levels of amino acid identity and similarity to *E. coli* MG1665 YhbX (e value 1.e^{-84}, 33% identity, 52% similarity, 94% coverage), YbiP (e value 3.e^{-37}, 30% identity, 48% similarity, 57% coverage) and CptA (e value 6.e^{-27}, 23% identity, 43% similarity, 89% coverage) (Figure 2). Similar results were found with *S. enterica* Typhimurium LT2 CptA (STM4118) and YbiP (STM08354) (Figure 2). While no function has been established for YhbX and YbiP, CptA is a PEtN transferase responsible for the linkage of PEtN to phosphorylated L,D-Hep I residue of the inner core in *S. enterica*, (47). PMI3576 shared significant levels of amino acid identity and

similarity to EptB proteins from *E. coli* MG1665 (e value 0, 51% identity, 70% similarity, 98% coverage) and *S. enterica* LT2 (e value 0, 50% identity, 71% similarity, 96% coverage), and *Citrobacter rodentium* ICC168. PMI3576 also showed similarity to EptA proteins from *E. coli* and *S. enterica* with e values of $2.e^{-43}$ and $6.e^{-47}$, respectively (Figure 2). In *E. coli* EptB has been shown to transfer a PEtN moiety to the 3-deoxy-D-*manno*-oct-2-ulosonic II (Kdo II) residue of inner core LPS (42), and in *S. enterica* EptA, also known as PmrC, transfers PEtN to the phosphate at the 1 and/or 4' positions of lipid A (24).

In *Neisseria meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenza*, another PEtN residue is found substituting the O-6 position of L,D-Hep II (32). A highly conserved Lpt6 protein, *N. meningitides* (NMA0408)*, N. gonorrhoeae* (NGO2071), and *H. influenza* (HI0275), is required for the transfer of this PEtN (58). The whole genome of *P. mirabilis* HI4320 (37) was analyzed by BLAST search for putative proteins being similar to NMA0408 allowing the identification of PMI3104 (e value $2.e^{-99}$, 33% identity, 53% similarity). Similar levels of similarity were found for the NMA0408 homologues NGO2071 and HI0275 (Figure 2). The PMI3104 shared with these proteins the presence of several predicted transmembrane regions before a sulfatase domain(Figure 3). PMI3104 showed high levels of identity (75 to 77%) along the whole protein with putative homologues from *Photorhabdus asymbiotica*, *Pho. luminescens*, *Providencia rettgeri*, *Prov. alcalifaciens*, and *Prov. rustigianii* (Figure 2, Figure 3), but no to others members of the *Enterobacteriaceae* family. The three genera containing PMI3104 or its homologues are closely related at the phylogenetic level.

Identification of a core LPS PEtN transferase. Since the core LPS isolated from *P. mirabilis* strains present a moiety of PEtN substituting the O-6 position of L,D-Hep II (HepII6-PEtN) (50, 53), it was hypothesized that PMI3104 would be the enzyme

involved in this PEtN transfer and hence named *eptC*, for ethanolamine phosphate transferase C. On the basis of carbohydrate backbone composition and structure identity between the core LPS of *K. pneumoniae* and *P. mirabilis* up to the first residue of the outer core (50, 53) (Figure 1), we decide to use the LPS from non-polar mutants *K. pneumoniae* 52145Δ*wabH* and *K. pneumoniae* 52145Δ*wabG* as surrogate LPS acceptors to test the *eptC* activity. Plasmid pGEMT-*eptC* was introduced into *K. pneumoniae* 52145Δ*wabH* and *K. pneumoniae* 52145Δ*wabG*. LPS from these strains were analyzed on Tricine-SDS-PAGE to check for electrophoretic patterns attributable to the *eptC* gene activity, but no changes were observed (data not shown). To guaranty the inducible expression of the *eptC* gene plasmid pBAD18-Cm-*eptC* was constructed and electroporated into the two *K. pneumoniae* mutants. LPS from *K. pneumoniae* 52145Δ*wabG* (pBAD18-Cm-*eptC*) migration was slower than that of LPS from *K. pneumoniae* 52145Δ*wabG* under arabinose promoter inducing conditions (Figure 4, lanes 2 and 3). Similar results were obtained when comparing LPS from *K. pneumoniae* 52145Δ*wabH* (pBAD18-Cm-*eptC*) and *K. pneumoniae* 52145Δ*wabH* (Figure 4, lanes 4 and 5).

To unambiguously test the *eptC* requirement for PEtN transfer, LPS from 52145Δ*wabG* (pBAD18-Cm-*eptC*) was isolated from dried cells by phenol-water extraction and purified (see Materials and Methods). Mild acid degradation of this LPS resulted in a core fraction purified by gel-permeation chromatography on Sephadex G-50. High-resolution negative ion electrospray ionization mass spectrum of this fraction showed a major [M-H]$^-$ ion peak at *m/z* 906.2499 for a compound corresponding to an OS composed of two heptose residues and one residue each of hexose, Kdo and PEtN ($Hex_1Hep_2Kdo_1P_1EtN_1$) (calculated ion mass 906.2497 amu). In addition, a number of lower-molecular mass ions were observed in the spectrum at *m/z* 888.2416, 862.2590, and 818.2335 which were assigned to various Kdo artifacts

generated by loss of H_2O, CO_2, and $C_3H_4O_3$, respectively (Figure 5). No non-phosphorylated OS was detected.

The ^{13}C-NMR spectrum of the OS (Figure 6) showed signals for four anomeric carbons at δ 97.0-103.7, one C-CH_2-C group (C-3 of Kdo) at δ 35.4, four $HOCH_2$-C groups (C-6 of Hex, C-7 of Hep, C-8 of Kdo) at δ 62.5-64.4, other sugar carbons in the region of δ 67.0-77.5, and one PEtN group at δ 41.2 (CH_2N) and 63.3 (CH_2O). The ^1H-NMR spectrum contained, *inter alia,* signals for three anomeric protons at δ 4.55-5.34 and a PEtN group at 3.31 (CH_2N). The ^{31}P-NMR spectrum showed one signal for a monophosphate group at δ 0.5.

The ^1H-NMR spectrum of the OS was assigned using two-dimensional COSY and TOCSY experiments, and then the ^{13}C-NMR spectrum was assigned using a two-dimensional ^1H,^{13}C HSQC experiment. The assigned ^1H-and ^{13}C-NMR chemical shifts (Table 3) were in full agreement with the carbohydrate backbone composition and structure expected for the core OS derived from *K. pneumoniae wabG* deletion mutant (18). The signals for H-6 and C-6 of the terminal heptose residue (L,D-Hep II) were shifted downfield to δ 4.57 and 74.6, as compared with their positions in the non-substituted heptose at δ 4.04 and 70.4, respectively (52). These data defined the site of phosphorylation at position O-6 of L,D-Hep II and, hence, the OS has the following structure:

$$\beta\text{-Glc}p\text{-}(1{\rightarrow}4)\rceil$$
$$\alpha\text{-}{\text{LD}}\text{Hep}p6\text{PEtn-}(1{\rightarrow}3)\text{-}\alpha\text{-}{\text{LD}}\text{Hep}p\text{-}(1{\rightarrow}5)\text{-Kdo}$$
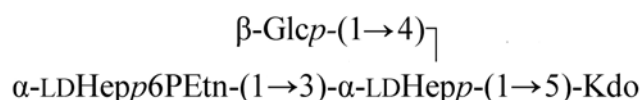
***eptC* mutant construction and analysis.** To determine the role of PEtN substitution in *P. mirabilis* biology an *eptC::kan* insertion mutant was constructed in the genome strain

HI4320. The mutation did not affect the growth kinetics in lab conditions in LB or minimal A medium (data not shown). Comparison of LPS extracted from the mutant and the wild type strain by SDS-PAGE and Tricine-SDS-PGE showed that the mutant is still able to produce O-PS (Figure 7A, lanes 1 and 2) and that its LPS core-lipidA fraction mobility is increased when compared to wild type HI4320 (Figure 7B, lanes 1 and 2). Introduction of plasmid pBAD18-Cm-*eptC* restored wild type electrophoretic mobility (Figure 7B, lane 3). In order to test for a possible change in the permeability and/or stability of the mutant OM, the Minimal Inhibitory Concentration (MIC) for different antibiotics and detergents, including cationic, anionic and zwitterionic ones, was determined for the mutant and the wild type strain HI4320. The only difference observed was a higher sensitivity of the *eptC::kan* mutant to SDS than wild type HI4320 (Table 4). When confronting the mutant and parent strains with 0.5% SDS for 45 minutes, a decrease in cell viability of about 80% was observed for the mutant while the parent strain viability was essentially unchanged (Figure 8). The difference in SDS sensitivity strongly suggests a moderate destabilization of the outer-membrane (OM) in the absence of *eptC*.

In order to investigate the effect of the PEtN mutation in *P. mirabilis* fitness during UTI, an *in vivo* co-challenge competition assay was set up. In this assay a 1:1 mixture of overnight cultures of wild type HI4320 and *eptC::kan* mutant was administered transurethrally into CBA/J mice. At seven days postinoculation the mice were euthanized and the levels of wild type and mutant were determined in the urine, bladder, and kidneys (Figure 9). The results indicate no statistically significant differences between the wild type and the mutant strain in this infection assay.

Wild-type *P. mirabilis* HI4320 strain was resistant to the bactericidal activity of NHS (98% survival after 3h of incubation with NHS), while the *eptC::kan* or *eptC*

mutants showed a moderate sensitivity (less than 20% survival after 3h of incubation with NHS). The introduction of the *etpC* (pBAD18-Cm-*etpC*) renders both mutants resistant to the bactericidal activity of NHS (96% survival after 3h of incubation with NHS), as found for wild type strain. No changes were observed by the introduction of the plasmid vector alone (pBAD18-Cm). Control experiments using heat-decomplemented NHS render more than 95% survival for all the strains.

***eptC* gene distribution.** Since the presence of a PEtN moiety linked to the O-6 of the L,D-Hep is a common feature of the core LPS of the studied *P. mirabilis* strains (53) the *eptC* gene should be present in these strains. To confirm the above hypothesis a collection of *P. mirabilis* strains obtained from Z. Sydorckzyk were used. Genomic DNA from these strains was used as template for amplification with oligonucleotides F1*eptC* and R1*eptC* (Table 2). Fragments of about 2000 bp were amplified for all the strains studied, as exemplified by the results for the four strains shown in Figure 10. Sequencing of the amplified fragments confirmed the presence of the *eptC* gene. In addition, in all the amplified fragments the presence of a sequence coding for the C-terminal amino acid residues of *celY* (PMI3103) gene, encoding a putative cellulase, were found adjacent to *eptC*, suggesting that in the analyzed strains the *eptC* location in their genome is the same as that found in genome strain HI4320 (Figure 1).

DISCUSSION

In this work we have identified two putative PEtN transferases PMI3040 and PMI3576. A phylogenetic tree constructed from an alignment between PMI3040, PMI3576, PMI3104 (*eptC*), and representative known and putative PEtN transferases shows that PMI3040 clusters with putative PEtN transferases YhbX (*E. coli*), YbiP (*E. coli* and *S. enterica*) and ROD 08171 (*C. rodentium*) (Figure 2). PMI3576 appears phylogenetically related to EptB and to a lesser degree to EptA of several

Enterobacteriaceae species (Figure 2). No *P. mirabilis* HI4320 encoded protein appears closely related to CptA. CptA is involved in the transfer of PEtN to phosphorylated L,D-Hep I (46), but no phosphorylated L,D-Hep I has been found in the core OS of *P. mirabilis* (50, 53), and in agreement with this no homologue of the WaaP kinase, required for addition of a phosphate group to the O-4 of L,D-Hep I (61), was detected in the genome strain HI4320.

EptA, also known as PmrC, transfers a PEtN moiety to the phosphate at the 1 and/or 4' positions of lipid A (24). Usually*, eptA* is found in a transcriptional unit with *pmrA-B*, also known as *basS-R*, two component system, but no *eptA* homologue is found adjacent to the *pmrA-B* A putative homologues *rppA* (PMI1696)-*rppB*(PMI1697) in strain HI4320 (37). PMI3576 is found between PMI3575, a putative oxidase, and *fdhF*, formate dehydrogenase-H subunit (Figure 1). In addition no PEtN substituting lipid A was found in *P. mirabilis* (46) grown in standard conditions. EptB transfers PEtN to the Kdo II residue of the inner core in *E. coli* (42), but no such Kdo II modification has been described in *P. mirabilis* core LPS (50, 53). It cannot be ruled out the presence of a moiety of PEtN linked to Kdo II in minor *P. mirabilis*core molecules or when these strains are grown in non-standard conditions. The above considerations suggest that PMI3576 could be a PEtN transferase more likely involved in Kdo than in lipid A modification, while no function can be hypothesized for PMI3040.

The chemical data obtained in this work (Figures 5 and 6, and Table 3) clearly establish the role of *eptC* (PMI3104) product in the transfer of PEtN to the O-6 of L,D-Hep II. The measure of the sensitization to hydrophobic probe compounds is an indirect way to study OM stability and permeability. The sensitization can be quantified as the decrease of the MIC of the probe compound. In addition, when bactericidal compounds are used their effect on bacterial viability can also be measured. When these methods were applied to an *eptC::kan* the results obtained indicate that the absence of the PEtN

group leads to an increase in SDS susceptibility and, thus, destabilization of the OM. This destabilization does not lead to a decrease in polymyxin B resistance or fitness in a UTI model, but the serum killing results indicate a moderate sensitivity to complement that compromise their ability to perform a systemic infection. It could be expected that the absence of a positive charge due to PEtN would lead to an increase in (CAMP) resistance such as, polymyxin B, but this is not the case in the *eptC::kan* mutant. Changes in polymyxin B sensitivity have been found in *eptA* or *eptA*-like mutants (24, 25), while minor or no changes have been seen in *cptA* (25, 47) and *eptB* mutants (42), leading to the notion of a major role for PEtN modification of lipid A versus PEtN modification of the inner core residues in polymyxin B and CAMP resistance. In addition, it should be noted that in *E. coli* and *S. enterica* CptA PEtN modification act on phosphorylated L,D-Hep II-P compensating the phosphate negative charge, while in *P. mirabilis* the core OS negative charges are contributed by the D-GalA residues.

In *P. mirabilis* most of its LPS molecules are modified with L-Ara4N residues at the 4' position of lipid A and at the Kdo I residue of the inner core (46, 50, 53). These modifications with L-Ara4N explain the high innate resistance of *P. mirabilis* to polymyxin B and other CAMPs. In agreement with these L-Ara4N modifications in the genome of strain HI4320 two genes encoding homologues of *Salmonella enterica arnT* gene (for a review see 12) can be detected. The *arnT* gene encodes a lipid A L-Ara4N transferase. The *arnT* homologue PMI1047 from strain HI4329 is located, as well as *arnT* from *S. enterica*, in the *arn* operon containing the genes required for L-Ara4N synthesis, activation, and transfer to lipid A (12). The *arnT* homologue PMI0275 is located between *mtrF*, coding for a putative efflux pump component, and *zapD*, coding for a type I secretion OM protein (37). Thus, from this analysis it can be hypothesized that PMI1047 and PMI0275 will correspond to L-Ara4N transferases responsible for lipid A and Kdo I modifications in strain HI4320, respectively. The importance of these

two L-AraN modifications in *P. mirabilis* polymyxin B resistance was recently shown by a mutation in one of the genes, *arnA*, of the *arn* cluster involved in the biosynthesis of undecaprenyl-phosphate-α-L-Ara4N in *P. mirabilis* (19). In addition in *C. rodemtium*, lacking the *arn* operon, *cptA* deletion causes a minor decrease in polymyxin B resistance (49).

In agreement with the EptC function as the transferase responsible for the linkage of a PEtN moiety to the O-6 position of L,D-Hep II, the *eptC* gene is found in all the *P. mirabilis* strains analyzed in this study. Within the *Enterobacteriaceae eptC* homologues appear to be limited to species of the *Proteus* phylogenetic related genus *Photobacterium* and *Providencia* (Figure 2), and recently the presence of a PEtN moiety linked to the O-6 position of L,D-Hep II was shown in *Prov. alcalifaciens* O8 and O35, and *Prov. stuartii* O49 (22). Further work will be necessary to understand the reasons for the presence of this common feature in these three closely related genera.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.

2. **Aquilini, E., J. Azevedo, N. Jimenez, L. Bouamama, J. M. Tomás, and M. Regué.** 2010. Functional identification of the *Proteus mirabilis* core lipopolysaccharide biosynthesis genes. J. Bacteriol. **192:** 4413-4424.

3. **Aquilini, E., J. Azevedo, S. Merino, N. Jimenez, J. M. Tomás, and M. Regué.** 2010. Three enzymatic steps required for the galactosamine incorporation into core lipopolysaccharide. J. Biol. Chem. **285:** 39739-39749.

4. **Belas, R.** 1996. *Proteus mirabilis* swarmer cell differentiation and urinary tract infection, p. 271-298. *In* H.L.T. Mobley and J.W. Warren (ed.), Urinary Tract Infections: Molecular Pathogenesis and Clinical Management. ASM Press, Washington DC.

5. **Belas, R., D. Erskine, and D. Flaherty.** 1991. Transposon mutagenesis in *Proteus mirabilis*. J. Bacteriol. **173:** 6289–6293.

6. **Belas, R., M. Goldman, and K. Ashliman.** 1995. Genetic analysis of *Proteus mirabilis* defective in swarmer cell elongation. J. Bacteriol. 177: 823-828.

7. **Burall, L. S., J. M. Harro, X. Li, C. V. Lockatell, S. T. Himpsl, J. R. Hebel, D. E. Jhonson, and H. L. T. Mobley.** 2004. *Proteus mirabilis* genes that contribute to pathogenesis of the urinary tract infections: identification of 25 signature-tagged mutants attenuated at least 100-fold. Infect. Immun. **72:** 2922-2938.

8. **Coderch, N., N. Piqué, B. Lindner, N. Abitiu, S. Merino, L. Izquierdo, N. Jimenez, J. M. Tomás, O. Holst, and M. Regué.** 2004. Genetic and structural characterization of the core region of the lipopolysaccharide from *Serratia marcescens* N28b (serovar O4). J. Bacteriol. **186:**978–988.

9. **Coker, C., C. A. Poore, X. Li, H. L. T. Mobley.** 2000. Pathogenesis of *Proteus mirabilis* urinary tract infection. Microbes and Infection **2:** 1497-1505

10. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.

11. **Dumanski, A.J., H. Hedelin, A. Edin-Lijegren, D. Beauchemin, and R. J. C. McLean.** 1994. Unique ability of *Proteus mirabilis* capsule to enhance mineral growth in infectious urinary calculi. Infect. Immun.**62:** 2998–3003.

12. **Gunn, J. S.** 2008. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol. **16:**284-290.

13. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter.J. Bacteriol. **177:**4121–4130.

14. **Hagberg, L., I. Engberg, R. Freter, J. Lam, S. Olling, and C. S. Eden.** 1983. Ascending, unobstructed urinary tract infection in mice caused by pyelonephritogenic *Escherichia coli* of human origin. Infect Immun **40:** 273–283.

15. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

16. **Himpsl, S. D., C. V. Lockatell, J. R. Hebel, D. E. Jhonson, and H. L. T. Mobley.** 2008. Identification of virulence determinants in uropathogenic *Proteus mirabilis* using signature-tagged mutagenesis. J. Medical Microbiol. **57:** 1068-1078.

17. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.

18. **Izquierdo, L., N. Coderch, N. Piqué, E. Bedini, M. Corsaro, S. Merino, S. Fresno, J. M. Tomás, and M. Regué.** 2003. The *Klebsiella pneumoniae wabG* gene: its role in the biosynthesis of the core lipopolysaccharide and virulence. J. Bacteriol. **185:**7213–7221.

19. **Jiang, S. S., M. C. Liu, L. J. Teng, W. B. Wang, P. R. Hsueh, and S. J. Liaw.** 2010. *Proteus mirabilis pmrI*, a RppA-regulated gene necessary for polymyxin B resistance, biofilm formation, and urothelial cell invasion. Antimicrob. Agents Chemother. **54:** 1564-1571.

20. **Johnson, D.E., C. V.Lockatell, M. Hall-Craigs, H. L. Mobley, and J. W. Warren.** 1987. Uropathogenicity in rats and mice of *Providencia stuartii* from long-term catheterized patients. J. Urol. **138:** 632–635.

21. **Knirel, Y. A., A. V. Perepelov, A. N. Kondakova, S. N. Senchenkova, Z. Sydorczyk, A. Rozalsky, and W. Kaca** 2011. Structure and serology of O-antigens as the basis for classification of *Proteus* strains. Innate Immun. **17:** 70-96.

22. **Kondakova, A.N., E. Vinogradov, B. Lindner, N. A. Kocharova, A. Rozalski, and Y. A. Knirel.** 2006. Elucidation of the lipopolysaccharide core structures of bacteria of the genus *Providencia*. J. Carbohydr. Chem. **25:** 499-520.

23. **Larsson, P.** 1984. Serology of *Proteus mirabilis* and *Proteus vulgaris*, p. 187-214. *In* T. Bergan (ed.), Methods in Microbioly Volume 14**.** Academic Press Inc., London.

24. **Lee, H., F. F. Hsu, J. Turk, and E. A. Groisman.** 2004. The PmrA-regulated *pmrC* mediates phosphoethanolamine modifications of lipid A and polymyxin resistance in *Salmonella enterica*. J. Bacteriol. **146:** 4124-4133.

25. **Lewis, L. A., B. Choudhury, J. T. Balthazar, L. E. Martin, S. Ram, P. A. Rice, D. S. Stephens, R. Carlson, and W. M. Shafer.** 2009. Phosphoethanolamine substitution of lipid A and resistance of *Neisseria gonorrhoeae* to cationic

antimicrobial peptides and complement-mediated killing by normal human serum. Infect. Immun. **77:** 1112-1120.

26. **Liu, Q., M. Z Li, D. Leibham, D. Cortez, and S. J. Elledge.** 1998. The univector plasmid-fusion system, a method for rapid construction of recombinant DNA without restriction enzymes. Curr Biol **8:** 1300–1309.

27. **Loh, B., C. Grant, and R. E. Hancock.** 1984. Use of the fluorescent probe 1-*N*-phenylnaphthylamide to study the interaction of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:** 546-551.

28. **MacKinnon, F. G., A. D. Cox, J. S. Plested, C. M. Tang, K. Makepeace, P. A. Coull, J. C. Wright, R. Chalmers, D. W. Hood, J. C. Richards, and E. R. Moxon.** 2002. Identifications of a gene (*lpt-3*) required for the addition of phosphoethanolamine to the lipopolysaccharide inner core of *Neisseria meningitidis* and its role in mediating susceptibility to bactericidal killing and opsonophagocytosis. Mol. Microbiol. **43:** 931-943.

29. **McCoy, A.J.; H. Liu; T.J. Falla, and J. S. Gunn.** 2001. Identifications of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. Antimicrob. Agents Chemother. **45:** 2030-2037

30. **Merino, S.; S. Camprubí, and J.M. Tomás** 1992. Effect of growth temperature on outer-membrane components and virulence of *Aeromonas hydrophila* strains of serotype O:34. Infect. Immun. **60**: 4343-4349

31. **Michael, F. S., E. Vinogradov, C. Q. Wenzel-Brendan, J. Li, J. C. Hoe, J. C. Richards, and A. D. Cox.** 2009. Phosphoethanolamine is located at the 6-position and not at the 7-position of the distal heptose residue in the lipopolysaccharide from *Neisseria meningitidis*. Glycobiology **19:** 1436-1445.

32. **Murata, T., W. Tseng, T. Guina, S. I. Miller, and H. Nikaido.** 2007. PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **189:** 7213-7222.

33. **Nassif, X., J. M. Fournier, J. Arondel, and P. J. Sansonetti.** 1989. Mucoid phenotype of *Klebsiella pneumoniae* is a plasmid-encoded virulence factor. Infect. Immun. **57:** 546–552.

34. **O'Connor, E. T., A. Piekarowicz, K. V. Swanson, J. M. Griffiss, and D. C. Stein.** 2006. Biochemical analysis of Lpt3, a protein responsible for phosphoethanolamine addition to lipooligosaccharide of pathogenic *Neisseria*. J. Bacteriol. **188:** 1039-1048.

35. **O'Hara, C. M., F. W. Brenner, and J. M. Miller.** 2000. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Moganella*. Clin. Microbiol. Rev. **13:** 534-546.

36. **Pearson, M. M. and H. L. and Mobley.** 2007. The type III secretion system of *Proteus mirabilis* HI4320 does not contribute to virulence in the mouse model of ascending urinary tract infection. J Med Microbiol **56:** 1277–1283.

37. **Pearson, M. M., M. Sebaihia, C. Churcher, M. A. Quail, A. S. Seshasayee, N. M. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. R. Thomson, P. N. Rather, J. Parkhill, and H. L. T. Mobley.** 2008. Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. J. Bacteriol. **190:** 4027-4037.

38. **Penner, J.L.** 1981. The tribe Proteeae, p. 1204-1224. *In* M.P. Start, H. Stolp, H.G. Trüper, A. Bellows, and H.G. Schlegel (ed.), The Prokaryotes, a Handbook of Habitats, Isolation and Identification of Bacteria Springer-Verlag, Berlin.

39. **Penner, J. L. and J. N. Hennessy.** 1980. Separate O-grouping schemes for serotyping isolates of *Proteus vulgaris* and *Proteus mirabilis*. J. Clin. Microbiol. **12:** 304-309.

40. **Phan, V., R. Belas, B. F. Gilmore, and H. Ceri.** 2008. ZapA, a virulence factor in a rat model of *Proteus mirabilis*-induced acute and chronic prostatitis. Infect. Immun. **76:** 4859-4864.

41. **Regué, M., L. Izquierdo, S. Fresno, N. Piqué, M. M. Corsaro, T. Naldi, C. De Castro, D. Waidelich, S. Merino, and J. M. Tomás.** 2005. A second outer-core region in *Klebsiella pneumoniae* lipopolysaccharide. J. Bacteriol. **187:**4198-4206.

42. **Reynolds, C. M., S. R. Kalb, R. J. Cotter, and C. R. H. Raetz.** 2005. A phosphoethanolamine transferase specific for outer 3-deoxy-D-*manno*-octulosonic acid residue of *Escherichia coli* lipopolysaccharide. J. Biol. Chem. **280:** 21202-21211.

43. **Rozalski, A., Z. Sidorczyk, and K. Kotelko.** 1997. Potential virulence factors of *Proteus* bacilli. Microbiol. Mol. Biol. Rev. **61:** 65-89.

44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

46. **Sidorczyk, Z., U. Zahringer, and E. T. Reitschel.** 1983. Chemical structure of the lipid A component of lipopolysaccharide from a *Proteus mirabilis* Re mutant. Eur. J. Biochem. **137:** 15–22.

47. **Tamayo, R., B. Choudhury, A. Septer, M. Merighi, R. Carlson, and J. S. Gunn.** 2005. Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar Typhimurium lipopolysaccharide core. J. Bacteriol. **187:** 3391-3399.

48. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-80.

49. **Viau, C., V. L. Sage, D. K. Ting, J. Gross, and H. L. Moual.** 2011. Absence of PmrAB-mediated phosphoethanolamine modifications of *Citrobacter rodentium* lipopolysaccharide affects outer membrane integrity. J. Bacteriol. **193:** 2168-2176.

50. **Vinogradov, V.** 2010. Structure of the core part of the lipopolysaccharide from *Proteus mirabilis* HI4320. Biochemistry (Moscow) **76:** 803-87.

51. **Vinogradov, E, M. Cedzynski, A. Ziolkowski, A. Swierzko.** 2001. The structure of the core region of the lipopolysaccharide from *Klebsiella pneumoniae* O3. 3-Deoxy-α-D-manno-octulosonic acid (α-Kdo) residue in the outer part of the core, a common structural element of *Klebsiella pneumoniae* O1, O2, O3, O4, O5, O8, and O12 lipopolysaccharides. Eur. J. Biochem. 268:1722-1729.

52. **Vinogradov, E. V., and M. B. Perry.** 2001. Structural analysis of the core region of the lipopolysaccharides from eight serotypes of *Klebsiella pneumoniae.* Carbohydr. Res. **335:**291–296.

53. **Vinogradov, E.; Z. Sidorczyk; Y.A. Knirel.** 2002. Structure of the lipopolysaccharide core region of the bacteria of the genus *Proteus*. Aust. J. Chem. **55:** 61-67.

54. **Wang, W.B., I. C. Chen, S. S. Jiang, H. R. Chen, C. Y. Hsu, P. R. Hsueh, W. B. Hsu, and S. J. Liaw.** 2008. Role of RppA in the regulation of polymyxin B susceptibility, swarming, and virulence factor expression in *Proteus mirabilis*. Infect. Immun. **76:** 2051-2062.

55. **Wang, Q., A. Torzewska, X. Ruan, X. Wang, A. Rozalski, Z. Shao, X. Guo, H. Zhou, L. Feng, and L. Wang.** 2010. Molecular and genetic analyses of the putative *Proteus* O antigen gen locus. J. Bacteriol. **76:** 5471-5478.

56. **Warren, J. W.** 1991. The catheter and urinary tract infection. Med. Clin. North Am. **75:** 481-493.

57. **Warren, J. W., H. L. Muncie Jr, M. Hall-Craggs.** 1988. Acute pyelonephritis associated with bacteriuria during long-term catheterization: a prospective clinicopathological study. J. Infect. Dis. **158:** 1341-1346.

58. **Wenzel, C. Q., F. S. Michael, J. Stupak, J. Li, A. D. Cox, and J. C. Richards.** 2010. Functional characterization of Lpt3 and Lpt6, the inner-core lipooligosaccharide phosphoethanolamine transferases from *Neisseria meningitidis*. J. Bacteriol. **192:** 208-216.

59. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem. **5:** 83–89.

60. **Wiegand, I., K. Hilpert, and R. E. Hancock.** 2008. Agar and broth dilution methods to determine minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. **3:** 163-175.

61. **Yethon, J. A. and C. Whitfield.** 2001. Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. J. Biol. Chem. **276:** 5498-5504.

TABLE 1. Bacterial strains and plasmids used.

Strain or plasmid	Relevant characteristics	Reference or source
P. mirabilis		
HI4320	Wild type	H.L.T. Mobley
eptc::kan	HI4320 derived *eptC* insertion mutant, Km^R	This study
eptC	Derived from *etC::kan* by deletion of Km^R gene	This study
S 1959	Wild type, serovar O3	Z. Sydorckzyk
R110	Rough mutant of strain S 1959	Z. Sydorckzyk
51/57	Serovar O28	Z. Sydorckzyk
50/57	Serovar O27	Z. Sydorckzyk
14/57	Serovar O6	Z. Sydorckzyk
TG83	Serovar O57	Z. Sydorckzyk
K. pneumoniae		
52145	Wild type	33
52145*wabH*	Non-polar *wabH* mutant	41
52145*wabG*	Non-polar *wabG* mutant	18
E. coli		
DH5	F⁻*endA hsdR17* (r$_k$⁻ m$_k$⁻) *supE44 thi-1 recA1 gyr-A96* ϕ80*lacZ*	15
Plasmid		
pGEMT easy	PCR generated DNA fragment cloning vector Amp^R	Promega
pGEMT-*eptC*	pGEMT with *eptC* from strain HI4320, Ap^R	This study
pBAD18-Cm	Arabinose-inducible expression vector, Cm^R	13
pBAD18-Cm-*eptC*	Arabinose-inducible *eptC*, Cm^R	This study
pQL123	IPTG-inducible cre recombinase, Amp^R	26

TABLE 2. Oligonucleotides used in this study**.**

Experiment	Gene or mutant	Primer name[1]	Sequence (5'→3')
pGEMT-*eptC* construction	*eptC*	F1*eptC*	TGGCTGGATATGAGCATTCA
		R1*eptC*	CCAGGTATGATGGCGGTAAG
Mutant Design & confirmation	*eptc::kan*	*eptC*IBS	AAAAAAGCTTATAATTATCCTTAGTGGACTTGCC CGTGCGCCCAGATAGGGTG
		*eptC*EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTC TTGCCCGATAACTTACCTTTCTTTGT
		*eptC*EBS2	TGAACGCAAGTTTCTAATTTCGATTTCCACTCGA TAGAGGAAAGTGTCT
		F*eptC::kan*	TGGCTGGATATGAGCATTCA
		R*eptC::kan*	CCAGGTATGATGGCGGTAAG

[1] F and R denote forward and reverse, respectively.

TABLE 3.. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the modified core OS from *K. pneumoniae* 52145$\Delta wabG$ plus pBAD18-Cm-*eptC*.

Sugar residue	C-1 / H-1	C-2 / H-2	C-3 / H-3 (H-3a,3e)	C-4 / H-4	C-5 / H-5	C-6 / H-6 (H-6a,6b)	C-7 / H-7 (H-7a,7b)	C-8 / H-8a,8b
β-Glc*p*-(1→	103.7	74.9	76.7	71.2	77.5	62.7		
	4.55	*3.32*	*3.52*	*3.37*	*3.47*	*3.97, 3.74*		
α-LDHep*p*6P-(1→	102.5	70.9	71.5	67.2	72.4	74.6	62.5	
	5.34	*4.15*	*3.91*	*3.91*	*3.79*	*4.57*	*3.76, 3.85*	
→3,4)-α-LDHep*p*-(1→	102.2	71.4	74.8	74.9	72.5	69.5	64.4	
	5.08	*4.17*	*4.15*	*4.26*	*4.15*	*4.13*	*3.72, 3.74*	
→5)-α-Kdo*p*	n.f.	97.0	35.4	67.0	75.9	72.3	70.3	64.4
			2.11, 1.94	*4.12*	*4.12*	*3.88*	*3.70*	*3.81, 3.65*
NH$_2$CH$_2$CH$_2$O-	63.3	41.2						
	4.17	*3.31*						

Assignment was performed using a two-dimensional ^{1}H,^{13}C-HSQC experiment. The ^{31}P NMR signal is at δ 0.5.

n.f., not found

TABLE 4. MICs for *P. mirabilis* HI4320 and mutant *eptC::kan*.

Antimicrobial Compound	MIC (mg/ml)[a]	
	HI4320	*eptC::kan*
Polymyxin B	> 0,64	> 0,64
Novobiocin	0,004	0,004
SDS	12,5	3,1
Triton X100	> 100	> 100
Laurylsarcosine	25	25
Zwittergent	25	25

[a] MIC was defined as the lowest concentration of antimicrobial compound that completely inhibited any visible bacterial growth (OD600 < 0.5).

LEGENDS TO THE FIGURES

Figure 1. (A) *P. mirabilis* HI4320 core OS structure (50) and genes involved in core biosynthesis (2, 3). 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), L-*glycero*-D-*manno*-heptose (L,D-Hep), D-*glycero*-D-*manno*-heptose (D,D-Hep), glucosamine (GlcN) galactunonic acid (GalA), *N*-acetylglucosamine (GlcNAc), glucose (Glc), 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (PEtN), and lysine (Lys). (B) Location in the genome of strain HI4320 of *eptC* (PMI3104) and putative PEtN transferases PMI3040 and PMI3576.

Figure 2. Phylogenetic tree of selected known and putative PEtN transferases. Shown are proteins from *P. mirabilis* HI4320 (PMI3040, PMI3576, PMI3104 [*eptC*]), *E. coli* MG1655 (YbiP-Ec, YhbX-Ec, CptA-Ec, EptA-Ec, EptB-Ec), *S. enterica* LT2 (YbiP-Se, CptA-Se, EptA-Se, EptB-Se), *Citrobacter rodentium* ICC168 (ROD 08171, CptA-Cr, EptA-Cr), *Photorhabdus asymbiotica asymbiotica* ATCC 43949 (PAU 04383), *Pho. luminescnes laumondii* TT01 (Plu4899), *Providencia stuartii* ATCC 25827 (PROSTU 00437), *Providencia rettgeri* DSM 1131 (PROVRETT 01897), *Providencia alcalifaciens* DSM 30120 (PROVALCAL 03689), *Providencia rustigianii* DSM 4541 (PROVRUST 00071), *Haemophilus influenzae* Rd KW20 (HI0275), *Neisseria meningitidis* Z2491 (NMA0408), and *N. gonorrhoeae* FA 1090 (NGO2071).

Figure 3. Amino acid alignment among known and putative proteins responsible for the transfer of PEtN to the O-6 of L,D-HepII. *P. mirabilis* (PMI3104 [EptC]), *Photorhabdus asymbiotica asymbiotica* ATCC 43949 (PAU 04383), *Pho. luminescnes laumondii* TT01 (Plu4899), *Providencia stuartii* ATCC 25827 (PROSTU 00437), *Prov. rettgeri* DSM 1131 (PROVRETT 01897), *Prov. alcalifaciens* DSM 30120 (PROVALCAL 03689), *Prov. rustigianii* DSM 4541 (PROVRUST 00071), *Haemophilus influenzae* Rd KW20 (HI0275), *Neisseria meningitidis* Z2491 (NMA0408), and *N. gonorrhoeae* FA 1090 (NGO2071). Lines denote putative transmembrane regions and the box denote amino acid residues similar to the sulfatase domain in PMI3104 (EptC)

Figure 4. LPS electrophoretic pattern in Tricine-SDS-PAGE. LPS samples isolated from *K. pneumoniae* 52145 (lane 1), *K. pneumoniae* 52145Δ*wabG* (lane 2), *K. pneumoniae* 52145Δ*wabG* plus pBAD18-Cm-*eptC* (lane 3), *K. pneumoniae*

52145Δ*wabH* (lane 4), and *K. pneumoniae* 52145Δ*wabH* plus pBAD18-Cm-*eptC* (lane 5). Samples were obtained from strains grown under inducing conditions.

Figure 5. High-resolution electrospray ionization mass spectrum of core OS obtained by mild acid hydrolysis from LPS isolated from *K. pneumoniae* 52145Δ*wabG* harboring plasmid pBAD18-Cm-*eptC*.

Figure 6. ^{13}C NMR of core OS obtained by mild acid hydrolysis from LPS isolated from *K. pneumoniae* 52145Δ*wabG* harboring plasmid pBAD18-Cm-*eptC*.

Figure 7. LPS electrophoretic pattern in SDS-PAGE (A) and Tricine-SDS-PAGE (B). LPS samples isolated from *P. mirabilis*HI4320 (lane 1), *eptC::kan* (lane 2), and *eptC::kan* pluspBAD18-Cm-*eptC* (lane 3). Samples were obtained from strains grown under inducing conditions.

Figure 8. Percentage of cell survival of *P. mirabilis* HI4320 and mutant *eptC::kan* after exposure to 0.5% SDS for 45 minutes.

Figure 9. CBA/J mouse model of ascending UTI cochallenge assay, with *P. mirabilis* HI4320 and mutant *eptC::kan*. Competitive indices (CI) were calculated by dividing the ratio of the CFU of wild type HI4320 to mutant *eptC::kan* recovered from mice following infection by the ratio of the CFU of wild type HI4320 to the CFU of mutant present in the mixture before inoculation.

Figure 10. PCR DNA amplification using oligonucleotide pair F1*eptC*-R1*eptC* and DNA template from *P. mirabilis* strains R110, 50/57, 51/57, and TG83.

Figure 1
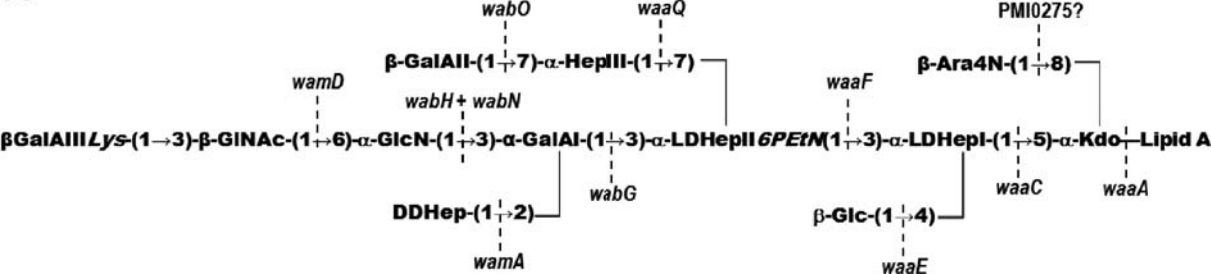
Figure 2

Figure 3

```
PMI3104          -MKKLSGRIYLAUVLIASLFIVFEKASLIYTALLSVSVYFIHFTLCFVLSRALFSAITTGTLFIIIKFNQLKVHYYKESLLFSDFNUAFDSSNLGTLT   99
PAU_04383         MKKKIISSVVYLGULFIATLILVFEKSDAIYPALIALGVFGVIFGLVFFMSARLFSVAFTGTLFIIIKFUNQIKVHYYKEQLMFSDLNLMFDPANQETUR 100
Plu4899           MKKKIISGVVYLGULFIATLILVFEKSEDIYPALIAVGYFGVIFGLVFFASARLFSTAFTGTLFIIIKFUNQIKVHYYKEQLMFSDLNLMFDPANQETUR 100
PROVRETT_01897    MKKKLFFGFYLALIFIGSLFLVFEKGSVIYPALISVSVYAVLFGILMVLTARWLFSAIITSTLFIIIKFUNQLKVHYYKEQLFFSDINIKYDTSNLGTLG 100
PROVALCAL_03689   MKKKLIASIYLALIFIGSLFLVFEKSGVIYPGLISICVYAVIFGALFTLTARWLFSAIITSTLFIIIKFNQLKVHYYKEQLFFTDINIKYDTSNLGTLG  100
PROSTU_00437      MKKRVFSAVYLTLIFIASLFLVFEKTSVIYPGLISVSVYAVIFGVLYLVSARWLFSAVVTNTLFIIIKFUNQLKVHYYKEQLFYSDINIKYDTSNLGTLG 100
PROVRUST_00071    MNKKIIASIYLALIFIGSLFLVFEKSSVIYPALISVSVPAIIFGVLFTLTARWLFSVIVTSTLFIIIKFUNQLKVHYYKBQLFFDINLKYDTSNLGTLG  100
HI0275           ----MIAYIELAUFTIAAVIFIVN-SHYRWTYFFAITLFTFUGGMLMVSSQWQRALNFSSVLFVVLMLFHRLKFHYYKQFLLISDFFLVVDWRNWETLI   95
NMA0408          ----MVAYVELFFFVTFAALVFIIIR-SHYRWTYFFASALFVFLAGGMLMLTAQWQRALNFASVWFVVLILFHRLKFHYYKQFLLISDFLLIADWRNWETLF  95
NGO2071          ----MVAYAFLFFFVTFAAVLFIIVR-SHYRWTYFFASALFVFLAGGMLMLTAQWQRALNFASVWFVVLILFHRLKFHYYKQFLLISDFLLIADWRNWETLF  95

PMI3104           QYWEAGVAILAMAIWLIINWTAWRFSRKSP-IATRLSSLLLIIIELTTIHFTVNKWQSENEGTLPG-GRGTVTNLIMSGYQTAWHPFYYAQSADYFIEC 197
PAU_04383         HYWLAGLAVIAMLIWLIENWVLSWKKSSPSRGFKFRTVGLYLVVVVCVWEVSVTANAFREQMESSLPK-GRGTVTNLVMSAKDAKYHAPQFGQSSDYFLQQ 199
Plu4899           HYWLAGLGVVAMFIWLIENMTLSWKKSSSLRGFKFRTVGLVUAAFCTWEVSATADAFREQMESSLPK-GRGTVTNLVMSAKDAKYHAPQFGQSSDYFLQQ 199
PROVRETT_01897    HYWLAGVALYGLLILLVIINGVISWRLLKFSKSKMTRVIGVVLIVAGYFGINFTSSHYYDRMSQTLPK-GRGTVTNIIMSAQNASYQSPEEAGSADYFMEK 199
PROVALCAL_03689   HYWLAGVAVLGLFILLIINGVISWRLSTPCKRWISRVVSVLMIVVGYFGADWASTHYFDKMSQTLPK-GRGTVTNLVMSAQNAKYKSPHQVGDSSYFEQK 199
PROSTU_00437      HYWLAGVALIGLLILLIINAVLSWRLLRFTKTFSWRLIGILLGVGGYFGASFAASHYYNQWFETLPK-GRGTVTNLVMSAKNSNYHSPQIKGSSDYFIQK 199
PROVRUST_00071    HYWLAGLAVIGLFIVLIINGIISWRLSFPCKRWISRVVSILMVVAGYFGADWASAHYYDRMSQTLPK-GRGTVTNLVMSANNAKYKSPHQVGDSSYFEKK 199
HI0275            HYKGALFGVIGLLALLGYAIFGFNDVESLGVLGNSIGALLFIVSFSLMWHYSKNPSAVQVMLDSLPDDGRDVFLNLPMSCRGIFFKVPMQDGNSQMEIEK 195
NMA0408           HYKEAVIGMAGLLALAAYAVFGWSGADSLDVPWRMAGAVLFAAAFVSMRHFSKHPGAVKTMLDSLPDDGRDVFLNLPMSCRAVFFQVPVFGEGDGEABARQ 195
NGO2071           HYKEAVIGMAGLLALAGYAVFGWSGADSLGMPWRMAGAVLFAAAFVSVRHFSKHPGAVKTMLDSLPDDGRDVFLNLPMSCRAVFFQVPVFGEGDGEABARQ 195

PMI3104           ANRVVLPET-QTEVKPDIVVLLQESTVNFSIYCFDKNVDLPDLFMFKKDEGVSAQSPLRVQTFGGGTWLSEFSVLTGLNTDDFGARKNSVFYFVVDKLNE 296
PAU_04383         AKRVTLPVP-QSEIKPDVVVLLQESTVTPHIYCLPDDSRLPNLYMFQRNTGVSAQSPMRVQTFGGGTWLSEFSALTGLNTDDFGSRKNAVFYFIVDHLKY 298
Plu4899           AKNVVLPVP-QSAIKPDVVVLLQESTVTPHIYCLPDDTRLPHLYMFORDSGVSAQSPMRVQTFGGGTWLSEFSALTGLNTDDFGSRKNAVFYFIVDHLKY 298
PROVRETT_01897    AAAISLENN-AASEKPDIVLLLLQESTVDPHIYDLPEGTKLPDLFMFQODSGVSAHSQMRVQTFGGGTWLSEFSALTGLNSDDFGACKSGVFYFIVDHLKN 298
PROVALCAL_03689   AANISLANN-AQTEKPDIVLLLLQESTVNPTVYDFPAGTKLPDLFMFQODANLVAHSPMRVQTFGGGTWLSEFSALTGLNSDDFGSQKSGVFYFVVDHLNN 298
PROSTU_00437      AATVSLPAS-DITEKPDILLLLQESTVNFSIYKLPDGTKLPNLFMFQODNELIAHSHMRVQTFGGGTWLSEFEALTGLNSDDFGSQKSGVFYFIVDHLNN 298
PROVRUST_00071    AADVVLNNQ-GESEKPDIVLLLQESSVDPKVYDLPEGVKLPBLFMFQODANVVAHSPMRVQTFGGGTWLSEFSALTGLNSDDFGSQKSGVFYFVVDHLNH 298
HI0275            MTALSSDANNLSETKPDIVVTLMESTLNPHQFAPSQ-QSIPPLSMDEPQNDTVFASPLRVHTPAGATWKSEBAFLAGVPSTDFGALASGVFYSVVPHLQS 294
NMA0408           MPSETRPCG-MSDEKPDIVVTLMESTLDPHCFDFAA-AKIPDLKMFGRQEDTVFSSPLRVFTPGGATWKSEBAFLAGVPSTDFGALASGVFYSVVPHLQT 293
NGO2071           MPSETRPYG-MSDEKPDIVVTLMESTLDPHCFDFAA-AKIPDLKMFGRQEDTVFSSPLRVFTPGGATWKSEBAFLAGVPSTDFGALASGVFYSVVPHLQT 293

PMI3104           SLFRQLKSCGYYTVLLTPFNRMAYHAGYAYEKLGVDEIIQPQELGYPGSLEENLWTIPTQDKINYLKEILKTHT------DKPLFIFSLTMYEHGPYKGS 390
PAU_04383         SLFREMKQNGYYTVVLTPFNKSSYHSGHAYQTLGVDRIIQPQELGYPASLDENLWTIPTQSKINYVKEILGQET------DKPLFIFSLTMYEHGPYDBD 392
Plu4899           SLFREMKQNGYYTVVLTPFNKSSYHSGHAYQTLGVDRIIQPQELGYPASLDRNLWTISTQEMLGVVKEILAQET------DKPLFIFSLTMYEHGPYDBD 392
PROVRETT_01897    SLFKEMKANGYYTVVLTPFNKGAYHSGHAYETLGVDRIIQPQELGYPGKLQDNLWTISTDDMLKYVKEILAKET------DKPVFIFSLTMYEHGPYKES 392
PROVALCAL_03689   SLFKEMKANGYYTVVLTPFNKGAYHSGHAYQTLGVDRIIQPQELGYPGKLQDNLWTISTEDMLKYVKEILAQET------DKPVFIFSLTMYEHGPYKEG 392
PROSTU_00437      SLFKEMKANGYYTVVLTPFNKGAYHSGHAYQTLGVDRIIQPQELGYPGKLQDNLWTISTEDMLKYVKEILBKET------DKPVFIFSLTMYEHGPYKEG 392
PROVRUST_00071    GLFKEMKANGYYTVVLTPFNKGAYHSGHAYETLGVDRIIQPQELGYPGKLQDNLWTISTEDMLKYVKEILDBKET------DKPVFIFSLTMYEHGPYKEG 392
HI0275            GLVKNLKACGYFCVALSPPTKGNYNAKSAYDHFGFDLMLQPQDLGYPAPISKNLWDISSBBMKYTRMILBKQHPALENVDQFMBVYVLTFREHGPYELG 394
NMA0408           GFVRNLREHGYFCVALSPPTKGNYNAKAAYDHFGFNLMFQPQDLGYPAPMGKNLWHISSBBMMQYARMILBKRHPDLENVRQFMBVYVLTFKEHGPYRTD 393
NGO2071           GFVRNLREHGYFCVALSPPTKGNYNAKAAYDHFGFNLMFQPQDLGYPAPMGKNLWHISSBBMMQYARMILBKRHPDLENVRQFMBVYVLTFKEHGPYRTD 393

PMI3104           HSDDYQLAGQTESPDAPCKFSHYMEKIIASDPAIEDFSHFVANRERPTLFLYFGDHQPNID--UKNYQSPFSDPAHITQFTLRDNLTQGATLSTGELTDI 488
PAU_04383         HSDDYGLSGHVKNLQAVGKFSHYMEKIKASDPAIKDFSEFVAKREKPTIFLYFGDHQPNIN--LDQYDSVFSMPGYITQFTMRDNLKQGEFVQTDELTDI 490
Plu4899           HADDYRLTGHVKNSQAVGKFSHYMEKIKASDPAIKDFSEFVAKREKPTMFLYFGDHQPNIS--LNQYNGVFSMPGHITQFTMRDNLQQGESIQTDELTDI 490
PROVRETT_01897    HSDDYGIKDKIKNSDAAGEFSHYMDKIVTSDASIRDEAEFIEKRQRPLMFLYFGDHQPGIS--LNQYNSSFSSPAYLTQFTLRDNLKSGTAITTGCLTDI 490
PROVALCAL_03689   HSDDYGIKDKLDNKDSAGEFSHYMEKIVHSDDAIRDFVAFMEKRQRPLMFLYFGDHQPGIS--LNKYQSFFSSPAYYTQFTLRDNLKSGTSIKSGELTDI 490
PROSTU_00437      HHDDYGLKGKVKNADSVGEFSHYMEKIVASDNAIRDFVEFMAKRBRPLMFLYFGDHQPGIS--LNQYQSBFSNPAFITQFTLRDNLKSGNQIKTGFLTDI 490
PROVRUST_00071    HSDDYGIKDKLDNKEAAGEFSHYMEKIVHSDASIRDFVSFMDKRQRPLMFLYFGDHQPGIS--LNQYKSTFPTPAYLTQFTLRDNLKAATPINVGELTDI 490
HI0275            MENTFNLQMPNLGAKSISALNDYTQRIVALNDAIEGINNYLHERKKPFVLGYFGDHQVAFDNAIPPKKGDYAQPDVYTQBFVRSNCASQ-FKQEQKFLDL 493
NMA0408           TDNVFDLDAPDLNAKTVSALNDYIGRIADLDKMVESDDRYLHERGKPFVFGYFGDHQVPFEGVSVRKKWDYAQPDVYTQBAVRSNIAGG-FVQRQNFLDL 492
NGO2071           TDNVFDLDAPDLNAKTVSALNDYIGRIADLDKMVESDDRYLHERGKPFVFGYFGDHQVPFEGVSVRKKWDYAQPDVYTQBAVRSNIAGG-FVQRQDFLDL 492

PMI3104           NFLGGYLLERARUPLSP-YYKANIQMRHLCQGKLNDCEDEKLVNSYKQNYIYDKLNVAGGKDN--  549
PAU_04383         SFLGGMIMERAKLNVSP-FYBANMTMRHLCRGALDDCQDKBLVNSYKHYIYQQLEVADKK----  549
Plu4899           SFLGGMIMERAKLDASS-FYBANMTMRHLCRGALDCQDKALVESYKHYIYQQLDVAGKK----  549
PROVRETT_01897    SFLGGYLLERANLNISP-FYBANIKMRHLCDGKLNDCEDQKLLDSYRNIYVYGTLEAFNANKGDM  553
PROVALCAL_03689   SFLGGYLLERANLKVSP-FYBANIKMRHLCDGKLNDCEDKQLLDGYRHYVYVLGVANKDEAK-  552
PROSTU_00437      AFLGGYIILERANLHVSP-FYBANIKMRHLCDGKLNDCEDKBLLESYKHYIYHTLQAFNKNVEE-  552
PROVRUST_00071    SFLGGYLLERANLUKISP-FYBANIKMRHLCDGKLNDCEDQKLLDGYRHYIYNVLNVANKDAAK-  552
HI0275            ABAGGVLMNVAGUSAEDEFMFGNNHAYCKLSDGKLBDSSDIQLLNNYRHYLYQTLAIAR------  551
NMA0408           ABAGGVLMBAAGUEAKDGFMFANNHAMRGLCGGGLBDCPNWBLVGNYRNYLYDVLKIAR------  550
NGO2071           ABAGGVLMBAAGUEAKDGFMFANNHAMRGLCGGGLBDCPNRBLVGNYRNYLYDVLKIAR------  550
```
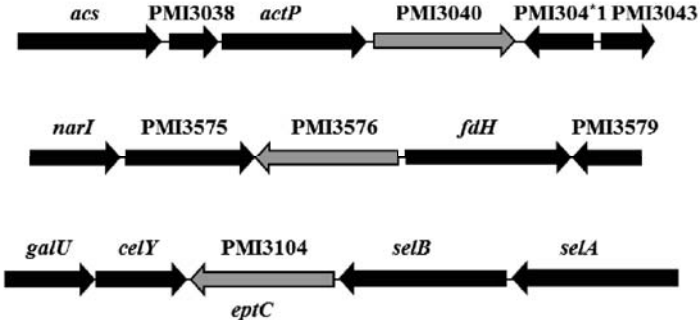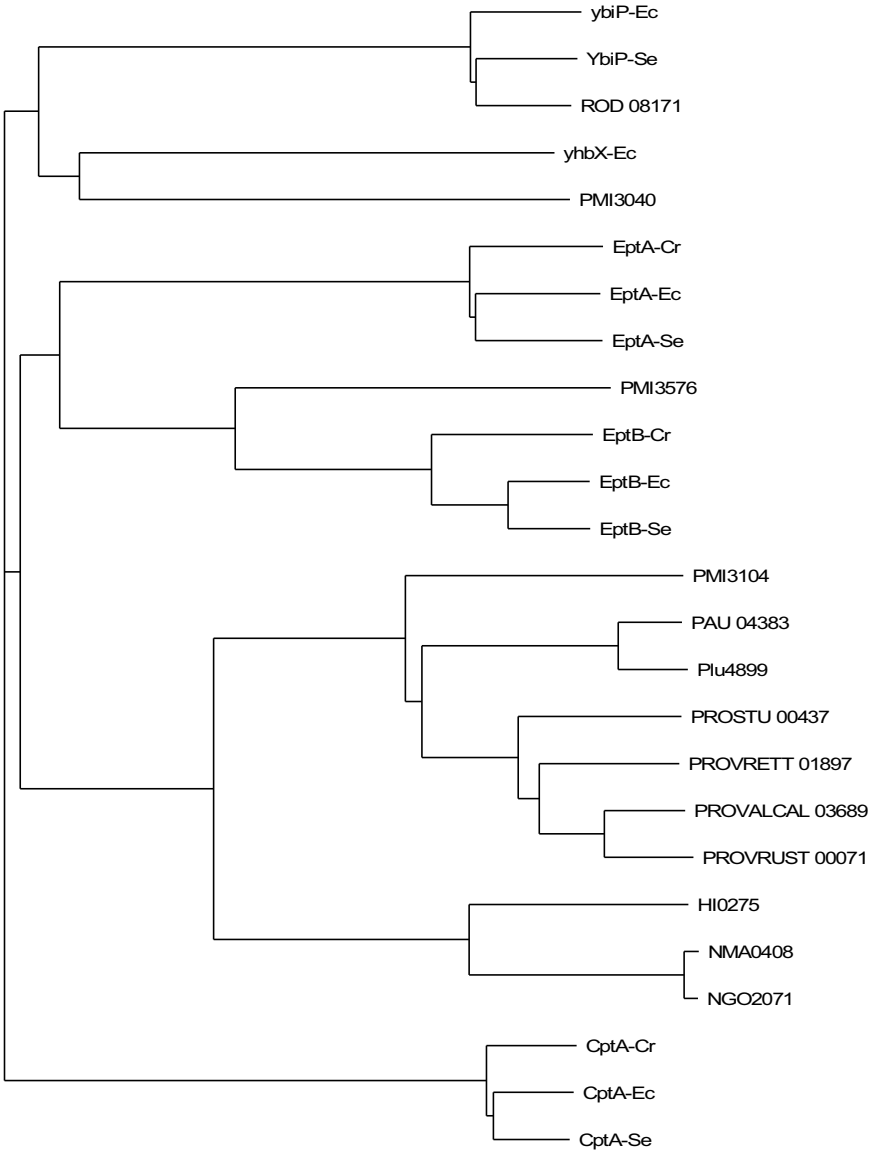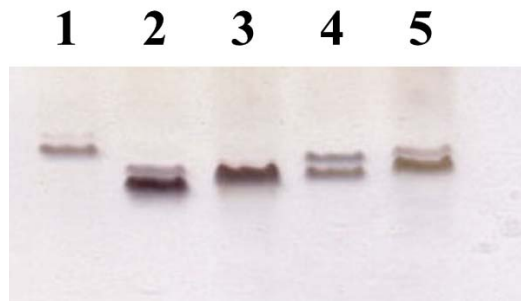
Figure 4

Figure 5
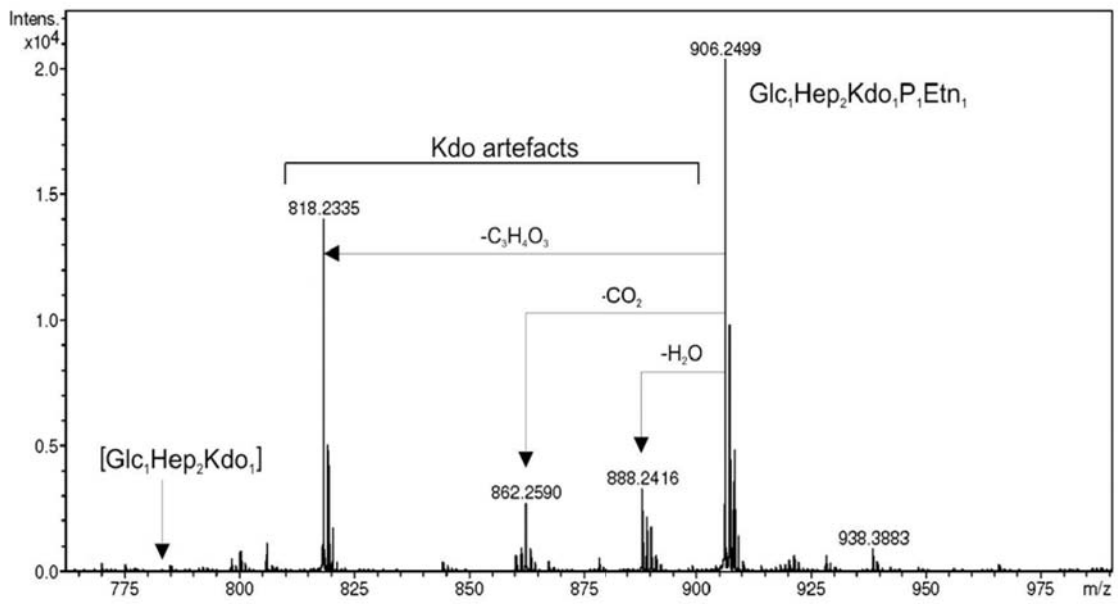
Figure 6
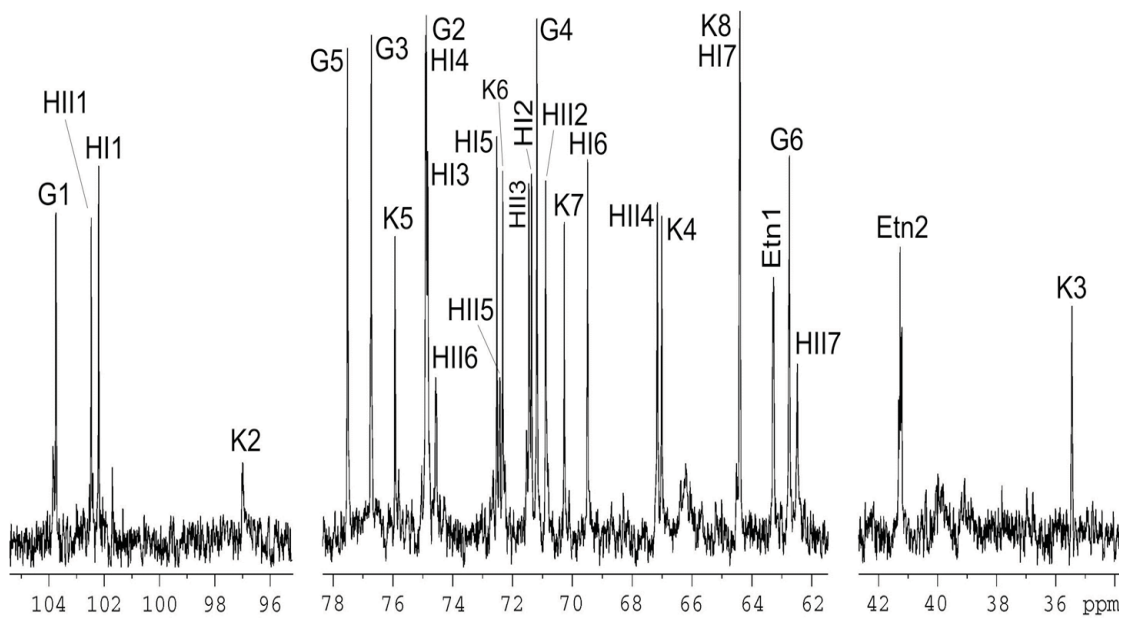
Figure 7
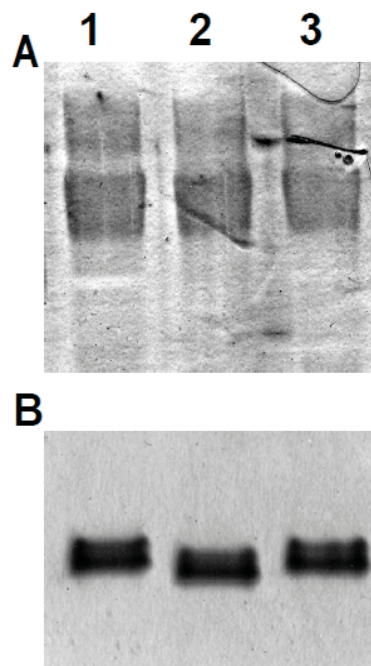
Figure 8
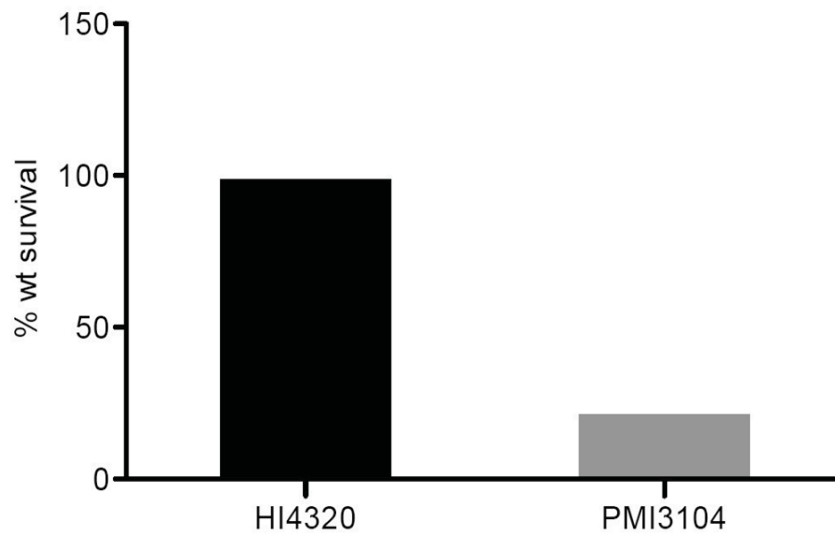
Figure 9
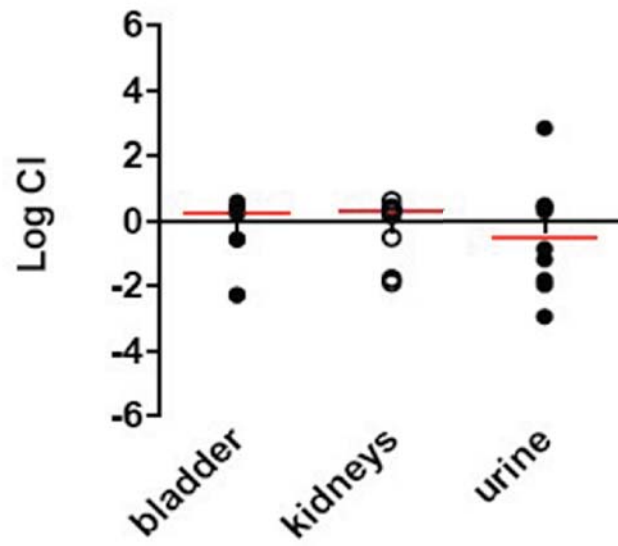
Figure 10
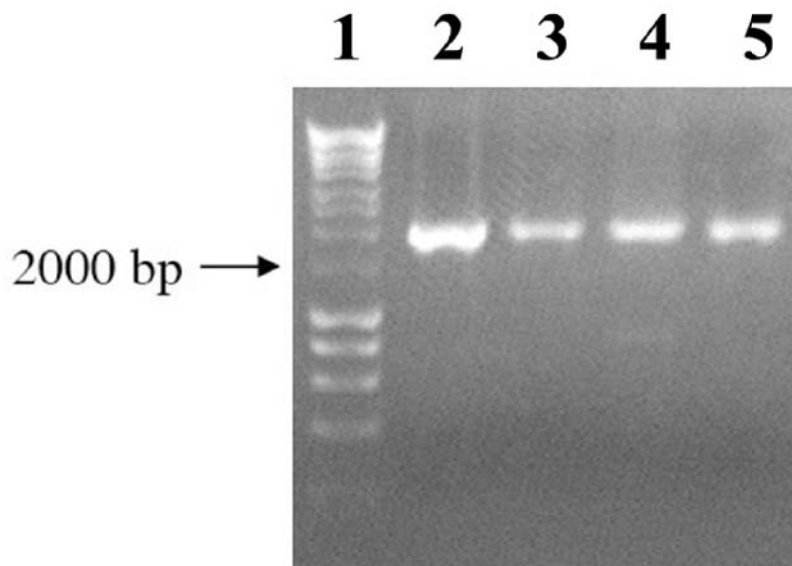
Identificación funcional de una fosfoetanolamina transferasa de *P. mirabilis*

Eleonora Aquilini, Joana Azevedo, Susana Merino, Yuriy A Knirel, Christopher J. Alteri, Harry L. T.Mobley, Juan M. Tomás and Miguel Regué

La presencia de un grupo fosfoetanolamina (PEtN) unido al O-6 de la L,D-Hep II es una característica compartida por todos los núcleos de LPS de *P. mirabilis* descritos químicamente hasta ahora. La proteína codificada por *lpt-3* (NMB2010) en *Neisseria meningitidis* transfiere PEtN al O-2 del residuo de L,D-Hep II. En *S. entérica* LT2 se identificaron cuatro posibles PEtN transferasas por similitud al gen *lpt-3* de *Neisseria meningitidis*. Con el objetivo de identificar al gen que codifica la transferasa necesaria para colocar el grupo PEtN en *P. mirabilis*, se procedió a la búsqueda de genes que codificaran proteínas similares a *lpt3* en el genoma de *P. mirabilis* HI4320 mediante el programa BLAST. Esta estrategia permitió identificar las ORF PMI3040 (e = 1.e^{-19}, 28% de identidad y 48% de similitud) y PMI3576 (e = 6.e^{-19}, 25% de identidad y 42% de similitud) localizadas fuera del agrupamiento *waa*. Ambas ORFs comparten con otras transferasas de PEtN la presencia de un dominio sulfatasa. PMI3040 presenta niveles significativos de similitud con las proteínas de *E. coli* MG1665 YhbX (e = 1.e^{-84}, 33% de identidad y 52% de similitud) y CptA (e = 6.e^{-27}, 23% de identidad y 43% de similitud). Mientras que no se conoce la función de YhbX, la proteína CptA transfiere PEtN al residuo de L,D-Hep I fosforilado en posición 4, tanto en *E. coli* como en *S. enterica* LT2 (STM4118). PMI3576 es similar tanto a las proteínas EptB presentes en *E. coli* MG1665 (e = 1, 51% de identidad y 70% de similitud, 98% de superposición) y *S. enterica* LT2 (e = 0, 50% de identidad y 71% de similitud, 96% de superposición), como a las proteínas EptA de *E. coli* y *S. enterica,* aunque con valores e inferiores (2.e^{-43} y 6.e^{-47}). Las proteínas EptB y EptA, también conocida como PmrC, transfieren PEtN al residuo de Kdo II del núcleo interno y al fosfato en posición 1 y/o 4' del lípido A, respectivamente.

En *Neisseria meningitidis*, *N. gonorrhoeae* y *Haemophilus influenza* se encuentra un grupo PEtN unido al O-6 del residuo de L,D-Hep II. Una proteína conservada en las tres especies (NMA0408, NGO2071 y HI0275) es la responsable de la transferencia de esta

PEtN. Mediante el programa BLAST se identificó el ORF PMI3104 por similitud a NMA0408 (e = $2.e^{-99}$, 33% de identidad y 53% de similitud). PMI3104, NMA0408, NGO2071 y HI0275 comparten la presencia de varias secuencias transmembrana y la presencia de un dominio sulfatasa. Además, dentro de la familia *Enterobacteriaceae* solo se encuentran proteínas con altos niveles de similitud a PMI3104 en *Photorhabdus asymbiotica*, *Pho. luminescens*, *Providencia rettgeri*, *Prov. alcalifaciens* y *Prov. rustigianii*.

Dado que la substitución del O-6 de la L,D-Hep II con PEtNes un hecho compartido por *P. mirabilis* y las especies mencionadas de los géneros *Neisseria* y *Haemophilus* parecía lógico pensar que PMI3104 podía ser la PEtN transferasaresponsable de este detalle estructural. De acuerdo con esta hipótesis se renombro el ORF PMI3401 como gen *eptC*. Aprovechando, a nivel experimental, el hecho que los núcleos del LPS de *K. pneumoniae* y *P. mirabilis* HI4320 comparten la misma estructura sacarídica hasta el segundo residuo (D-GalA I) del núcleo externo, se utilizaron los dos mutantes *K. pneumoniae* 52145Δ*wabH* y *K. pneumoniae* 52145Δ*wabG*, como aceptores subrogados de la posible PEtN transferasa. Se introdujo el plásmido pBAD18-Cm-*eptC* en ambos mutantes y se comparó su patrón de bandeo de LPS, en Tricina-SDS PAGE, con el de los mutantes sin el gen *eptC*. La disminución de la migración electroforética observada en ambos mutantes sugería cambios en el LPS debidos a la presencia y acción del gen *eptC* y compatibles con la adición de un grupo PEtN.

El espectro obtenido, mediante espectrofotometría de masas, de la fracción del núcleo del LPS obtenido de la cepa 52145Δ*wabG* (pBAD18-*eptC*), ha confirmado los resultados obtenidos, con una señal mayoritaria (*m/z* 906.2499) correspondiente a un ion [M-H]$^-$ con una composición: Kdo_1-P_1-EtN_1-Hep_2-Hex_1. Se observaron otros iones de menor masa molecular atribuibles a diferentes artefactos del Kdo.

Los análisis por NMR demostraron que el grupo PEtN se encuentra unido al O-6 de la L,D-Hep II. Esta conclusión se basó en los espectros de ^{13}C-NMR y^{1}H-NMR. La asignación de las señales del espectro de ^{1}H-NMR del OS se realizo mediante experimentos COSY y TOCSY, y las del espectro de ^{13}C-NMR mediante experimentos ^{1}H,^{13}C HSQC. La asignación de las señales de los diversos espectros estaba de acuerdo con la siguiente estructura:

145

$$\beta\text{-Glc}p\text{-}(1\rightarrow4)\urcorner$$
$$\alpha\text{-LDHep}p6\text{PEtn-}(1\rightarrow3)\text{-}\alpha\text{-LDHep}p\text{-}(1\rightarrow5)\text{-Kdo}$$
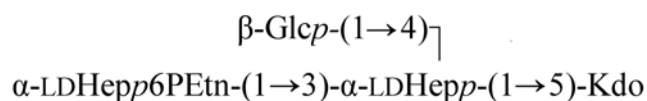
Para analizar los efectos biológicos de la presencia del grupo PEtN en el núcleo de *P. mirabilis* se construyó un mutante, *eptC::kan*, en la cepa de *P. mirabilis* HI4320. Se ha observado como la mutación no afecta al crecimiento ni en medio LB ni en medio mínimo. El LPS del mutante presenta un patrón de O-PS indistinguible del de la cepa parental, en cambio presenta una mayor migración en Tricina-SDS PAGE. Determinando las MICs para una serie de compuestos hidrofóbicos y detergentes, se ha observado una mayor sensibilidad del mutante frente al detergente aniónico SDS. También se ha observado un mayor grado de mortalidad en el mutante si tratado con 0,5% de SDS durante 45 minutos. Estos datos sugieren una desestabilización de la membrana externa (OM) del mutante *eptC::kan*.

Para establecer el efecto de la ausencia del residuo de PEtN durante una infección urinaria, se ha utilizado un ensayo de competitividad *en vivo*, utilizando el raton como modelo animal. El análisis estadístico de los resultados indicó que no hay diferencias significativas entre la cepa salvaje y la cepa mutada. Ademas, para testar el posible efecto de la misma modificación en una infección sistémica, se ha ensayado la resistencia de la bacteria en suero humano. Mientras la bacteria salvaje se ha demostrado resistente a la actividad bactericida del suero humano, los dos mutantes *eptC*::kan y *eptC* han mostraron una moderada sensibilidad. Estos resultados sugieren que la ausencia de esta modificación con PEtN pueda comprometer la capacidad de realizar una infección sistémica.

Finalmente, se analizó la distribución del gen *eptC*, en una colección de cepas de *P. mirabilis*. Los resultados indican la presencia del gen *eptC* en todas las cepas analizas. Este resultado es coherente con la presencia de la modificación con PEtN del residuo L,D-Hep II en todas las cepas de *P. mirabilis* la cuya estructura sea químicamente descrita.

5. Global discussion of results

In this work, first the genetic data of the *waa* cluster for four strains of *P. mirabilis*: R110 (O3), 51/57, (O28), 50/57 (O27) and TG83 (O57) have been obtained. These strains have been included in the study because of the chemical structure of the LPS core was already known and showed significant differences between them and compared with other strains [Vinogradov, 2002]. These four genetic clusters were compared to that of genome strain HI4320. In second place the functions of the genes in these clusters were identified and finally two genes involved in core LPS biosynthesis located outside these clusters were also identified.

The *P. mirabilis waa* gene clusters. In the best studied core LPS genetics in members of the *Enterobacteriaceae* family, most of the genes involved in this domain biosynthesis have been found clustered in a region denoted *waa*. An inspection of the available whole genomes and/or published work allows including in this group strains of *E. coli*, *Shigella*, *Salmonella* spp., *K. pneumoniae*, *S. marcescens*, and many others. *Y. enterocolitica* and *Y. pestis* are an exception in the sense that genes involved in core LPS biosynthesis are found in at least three different localizations in their genome.

With the aim to determine the genes present in the *waa* cluster of the four strains of *P. mirabilis* studied here, a strategy based in amplification and nucleotide sequenced determination was used, to obtain the data for the whole four clusters. Oligonucleotides for the initial amplifications were obtained from alignments of highly conserved genes in these *waa* clusters and adjacent genes in *Enterobacteriacea*. Thus alignments from genes such as *hldD* (ADP-D-*glycero*-D-*manno*-heptose epimerase) usually found at the beginning of the *waa* gene clusters and from *coaD* (phosphopantetheine adenylyltransferase) and *fpg* (formamidopyrimidine-DNA glycosylase) usually found just downstream of these clusters were used to obtain initial oligonuccleotide pairs. From the sequences obtained from these initial amplified fragments new sequence derived oligonucleotides were designed until completion of the sequence projects.

From the raw sequence the relevant genes were tentatively identified on the basis of their putative encoding products of glycosyltransferase nature, since these types of enzymes are the ones involved in core LPS biosynthesis as a consequence of its oligosaccharide (OS) nature and thus, found in the *waa* clusters of other studied *Enterobacteriaceae*. In addition, bioinformatic analyses (BLASTP and BLAST-P-T) and comparison with the equivalent region of the *P. mirabilis* genome strain HI4320 [Pearson et al., 2008], and with those of other *Enterobacteriaceae* species, has allowed to

hypothesize the possible functions for most of the *P. mirabilis* genes.

The data obtained from the sequence projects and the above analysis revealed several interesting features. First, in all the studied *P. mirabilis* strains the classical cluster begins with a putative three gene transcriptional unit or operon (*hldD* operon including *hldD*, *waaF* and *waaC*) and finishes with a two gene operon (*waaA* operon including *waaA* and *waaE*). The nature of these operons is deduced from the fact that the start and stop codons of the mentioned adjacent genes are either overlapped or are only separated by 9 nucleotides. Between these two more external operons we found five (in strains 50/57, 51/57 and TG83) and seven genes (in strain R110). They are located and organized in different transcriptional units on a strain basis.

In second place, based on an analysis of the percentage of Guanine plus Cytosine (% C+G), there is some evidence of lateral gene acquisition in at least three of these clusters. Thus, while a 38.9% is found in the genome of strain HI4320 this value falls to 22.5 to 18.3 % from the 3'end to the 5' end of the *wamD* gene. Similar low levels of % of C+G were detected in the *waa* gene clusters from strains R110 and 51/57. Thus, in the first case % of 22.5 to 18.3 were found between the 3'end of *wamB* and the 5'end of *wamC*, and in the second case % of 18.33 to 26.66 were found in the sequence including *wamD* and *mig-14* genes. In addition, there is an inversion of the DNA fragment including the *wabQ wabG wabP* in strains 50/57 and TG83 compared to the equivalent region of strains R110, 51/57, and HI4320.

In third place, the *waa* gene cluster from the studied *E. coli* K-12 and *S. enterica* LT2 begins with a similar *hldD* operon and ends with *waaA* gene, with a 10 and 9 gene operon in between, respectively. To avoid premature termination of these long operons the RfaH protein is used in *E. coli* and *S. enterica*. The RfaH protein is thought to abolish operon polarity by recognizing a JUMPstart sequnece (Just Upstream of Many Polysaccharides) containing a partial double repeat and including an *ops* (operon suppression sequence) 5'-GGCGGTAGC-3'. The RfaH also is involved in avoiding operon polarity in gene clusters involved in O-PS and capsule but also in operons not involved in polysaccharide or oligosaccharide biosynthesis such as the *tra* operon in F factor and hemolysin production in *E. coli* [Bailey et al. 1997]. In the studied strains of *P. mirabilis* strains no such JUMPstart sequence was found in the *waa* gene clusters, despite the presence of a homologue of the *rafH* gene in the genome of strain HI4320 suggesting that either this homologue

recognizes a JUMPstart sequence substantially different from that described in *E. coli* and *S. enterica*, or that the shorter nature of the longest operons involved in core LPS biosynthesis does not require this mechanism of operon antitermination, or that some alternative unknown mechanism for preventing operon polarity is used in these *P. mirabilis waa* clusters.

Finally the *waaL* gene homologue (encoding O-PS ligase) is located downstream from the *fpg* gene and thus outside of the classical *waa* cluster, while in most *Enterobacteriaceae* the *waaL* gene is found just inside the cluster and flanked by genes encoding glycosyltransferases. An analogous localization for the *waaL* gene as found in *P. mirabilis* can be also detected from the analysis of the genome strains of the close phylogenetic species *Photorhabdus luminescens laumondii* TT01 (GenBank™ BX470251.1), *Phot. asymbiotica* ATCC 43949 (GenBank™ FM162591.1), *Xenorhabdus nematophila* ATCC 19061 (GenBank™ FN667742.1) and *Xeno. bovierii* SS-2004 (GenBank™ FN667741). In addition the *P. mirabilis* strains studied here and these species of *Photorhabdus* and *Xenorhabdus* share another common feature, the presence of four genes found downstream of the respective *waaL* homologues and transcribed in the opposite direction encoding putative glycosyltransferases of the GT4 (*walM*, *walN*, and *walR*) and GT9 (*walO*) families of glycosyltransferases and adjacent to *rpmB* (ribosomal protein L2) gene. The glycosyltransferase family nomenclature and classification is the one described in the CAZy (Carbohydrate-Active enZymes) web page based on amino acid sequence similarities [Campbell et al., 1997; Coutinho et al., 2003; Lairson et al., 2008]. Interestingly, these similarities suggests that there can be a link between *waa* gene organization and species phylogeny in this subgroup of *Enterobacteriaceae*.

Functional identification of common core LPS genes. The experimental identification of the functions of the gene encoded proteins in the core LPS biosynthesis is fundamental to understand the biosynthesis of this LPS domain. Two approaches are possible; one is based on the construction of individual mutations and analysis of the core LPS defects; the second one, uses surrogate LPS molecules to test the function of individual glycosyltransferases. The first approach can be difficult in some bacterial species and has the problem that the conclusions are based on a negative feature represented by the shortage of the wild type core, in addition sometimes the interpretation of data is complicated by the presence of different core OS in the mutant strains. The second approach requires having surrogate LPS molecules highly similar to the predicted glycosyltransferase LPS acceptor molecule. The LPS surrogate acceptors would be provided by previously obtained mutants

151

from other bacteria constructed in such a way to avoid undesired effects on downstream genes. The use of surrogate LPS molecules has as advantage the addition of a new residue to surrogate core LPS: analysis is facilitated because the modified surrogate core LPS will be visualized by a modification of LPS mobility in either SDS-PAGE or Tricine-SDS-PAGE, even leading to regain O-PS production in some cases, and further confirmed by OS chemical composition and MS analysis. This second strategy was used in this work. Characterization of genes with homologues of known function was based on the use of surrogate LPS from *E. coli waaA* mutant, and *K. pneumoniae* 52145 in-frame non-polar mutations in the genes *waaC*, *waaF*, *waaQ*, *wabH*, *wabG*, and *wabN*. For the genes with no homologues of known function we took advantage of the fact that sugar residues and bonds between core LPSs from *K. pneumoniae* and *P. mirabilis* are identical up to the second outer core residue. Thus, LPS molecules extending up to the first or second outer core LPS residues from *K. pneumoniae* 52145 mutants were used as surrogates of *P. mirabilis* acceptor molecules to identify the functions of these genes.

From the genetic data of the strains analyzed and the characterization studies it was deduced that there are 9 common genes involved in *P. mirabilis* core LPS biosynthesis that are responsible for transfering the eight common core residues. The common pentasaccharide L-α-D-HeppIII-(1→7)-L-α-D-HeppII-(1→3)-L-α-D-HeppI-(1→5)-[α-KdopII-(2→4)-]-α-KdopI is present in all *Enterobacteriaceae* LPS core studied, and here it is demonstrate that in *P. mirabilis*, as in the other *Enterobacteriaceae*, this pentasaccharide is biosynthesized by the sequential transfer of sugars residues to lipid A. As mentioned, the results are based in the use of truncated core LPS from strains of *E. coli* and *K. pneumoniae* with defined mutations, as acceptor molecules. The complementation of these mutants with genes of the four strains of *P. mirabilis*, allowed to unambiguously identify the genes *waaA* (bifunctional Kdo transferase), *waaC* (heptosiltransferase I), *waaF* (heptosiltransferase II), and *waaQ* (heptosiltransferase III). In strains R110, 51/57, and HI4320 genes *waaQ* and *waaA* are divergently transcribed, as in *Escherichia coli* and *Salmonella enterica*. In contrast, in *P. mirabilis* 50/57 and TG83 the *waaQ* gene lies adjacent to the gene *wabP* and is transcribed in the same direction as in *K. pneumoniae* and *S. marcescens*.

In addition, all the *P. mirabilis* strains studied here contain a *waaE* gene. This gene is positioned between the genes *waaA* and the *coaD*, as in *K. pneumoniae* and *S. marcescens* [Izquierdo et al., 2002], and it has been identified by complementation of a *K. pneumoniae* 52145Δ*waaE* mutant and

thus shown to be involved in transferring the D-Glc residue to the L,D-HepI by a β 1, 4 glycosidic bond. Similarly, all the strains of *P. mirabilis* for which the core LPS structure is known, share a common D-GalA I residue linked to L,D-Hep II by an α 1,3 bond. This feature is also shared by the LPS core from *K. pneumoniae* and *S. marcescens*, where it was shown that the protein WabG is responsible for the transfer of the GalA residue to the L,D-Hep II [Fresno et al., 2007]. Protein homologues of *wabG* are present in all the strains of *P. mirabilis* studied here and the *wabG* from strains R110 and 51/57 were identified as the mentioned galacturosyltransferase again by genetic complementation of the corresponding *K. pneumoniae* 52145 mutant Δ*wabG*.

Molecular basis for the transfer of HexN. According to a review of the *Proteus* spp. core LPS structures [Vinogradov, 2002] a major difference among strains is the nature of the HexN linked to GalA I by an α 1,6 bond. In strains R110 and 51/57 this HexN is a D-GlcN while in strains 50/57 and TG83 corresponds to a D-GalN. This D-GlcN residue is also common to *K. pneumoniae* and *Serratia marcescens* where the HexN corresponds to a GlcN and where it was shown that the WabH protein encodes a GlcNAc transferase leading to the incorporation of GlcNAc to core LPS from UDP-GlcNAc, a second enzyme (WabN) deacetylates the GlcNAc residue to GlcN [Regue et al, 2005b].

The *wabH*$_{R110}$ and *wabN*$_{R110}$ genes complemented *K. pneumoniae* 52145Δ*wabH* and *K. pneumoniae* 52145Δ*wabN* mutants, respectively. The same results were obtained with *wabH*$_{51/57}$ and *wabN*$_{51/57}$. By contrast, initial studies showed that *wabH*$_{50/57}$ and *wabH*$_{TG83}$ were unable to complement *K. pneumoniae* 52145Δ*wabH*, while *wabN,* irrespective of its origin (R110, 51/57, 50/57 or TG83), always complemented *K. pneumoniae* 52145Δ*wabN*.

The alignment of the corresponding *P. mirabilis* WabH and WabN homologues suggested, by identity and similarity levels, the presence of two types of WabH similar proteins, the ones from strains R110, 51/57, and HI4320, and those from strains 50/57 and TG83. For the firsts group, sharing levels of amino acid identity above 95%, the WabH name was maintained and the second group, sharing levels of amino acid identity of 60% with the first group, was named WabP. As mentioned above complementation experiments *in vivo*, using a *K. pneumoniae* 52145 non-polar *wabH* mutant as LPS acceptor substrate, allowed the identification of two HexN transferase genes: *wabH*, in strains containing GlcN as the second outer core residue and *wabP* from strains

containing GalN in the same position. This GalNAc transferase was able to transfer GalNAc to *K. pneumoniae* 52145 core LPS only when the *gne* (UDP-GalNAc 4-epimerase) gene from *Aeromonas hydrophila* was also provided. A reason for the *gne* requirement could be the absence of UDP-GalNAc in *K. pneumoniae* 52145. The analysis of the *K. pneumoniae* genome strains 342 (GenBank™ CP000964) and MGH 78578 (GenBank™ CP000647) showed the presence of one *galE* (UDP-Gal 4-epimerase) homologue for each genome, but generation of UDP-GalNAc from UDP-GlcNAc will require the presence of a *gne* (UDP-GalNAc 4-epimerase) homologue that was not found in this analysis. The role of WabH and WabP enzymes was further confirmed by chemical composition and MS studies of the core LPS fraction from strain 52145Δ*wabH* expressing *wabH*$_{R110}$ or *wabP*$_{50/57}$ and *gne*, respectively.

In addition, an *in vitro* assay was developed to study the activities of purified WabH$_{R110}$ (His$_6$-WabH$_{R110}$) and WabP$_{50/57}$ (His$_6$-WabP$_{50/57}$). The results obtained showed the specificity of WabH$_{R110}$ and WabP$_{50/57}$ for UDP-GlcNAc and UDP-GalNAc as donors, respectively. In addition, *in vitro* quantitative experiments allowed determining the apparent kinetic parameters for the two enzymes. The K'_m values confirmed that the basis for the selectivity of these enzymes is the affinity for UDP-HexNAc rather than their affinity for acceptor LPS, as expected since both enzymes use the same acceptor LPS.

The core OS structures of *E. coli* share with *P. mirabilis* a common substructure up to the first outer core residue (Glc I) [Amor et al., 2000]. In *E. coli* strains with core OS types K-12, R1, R2, and R4, the WaaO glucosyltransferase links a second Glc residue (GlcII) to GlcI by an α1,2 bond. In strains with R3 core type, the WaaI galactosyltransferase links a Gal residue to Glc I with the same 1,2 linkage [Amor et al., 2000]. Both WaaO and WaaI belong to the GT-A fold containing CAZy glycosyltransferase family GT-8. An alignment between these two glycosyltransferases (WaaO, Q8KMW9; WaaI, Q9ZIT4) revealed that they share 51 and 80% amino acid residue identity and similarity, respectively. These levels of identity/similarity are similar to those shared by the *P. mirabilis N*-acetylglucosaminyl (WabH) and *N*-acetylgalactosaminyl (WabP) transferases belonging to CAyZ family GT4. Thus, it appears that glycosyltransferases involved in the transfer of sugar epimer residues to the same LPS acceptor can be differentiated by their levels of identity/similarity.

In addition, from the known core OS structures from the genus *Proteus*, it can be predicted that

wabH and *wabP* homologues should be present in *P. mirabilis* strains containing GlcN and GalN in their core LPS, respectively. Two pairs of oligonucleotides specific for *wabH* and *wabP* confirmed this prediction. These results suggest that a simple amplification test can be used to predict the HexN nature in *P. mirabilis* core LPS. This diagnostic amplification indicates the presence of GlcN in the core LPS of strains for which the chemical structures have not been determined at the time the present study was performed, such as CECT 170, HI4320, and ATCC 29906. In agreement with the test, the analysis of the genome sequence available for strains HI4320 [Pearson et al., 2008] and ATCC 29906 (genome sequencing in progress) confirmed the presence of *wabH* but not *wabP*. This prediction was recently confirmed for the genome strain HI4320 [Vinogradov, 2011].

An *in vitro* assay was developed to assay the deacetylase activities of His_6-WabN$_{R110}$ and His_6-WabN$_{50/57}$ based on the concomitant use of either His_6-WabH$_{R110}$ or His_6-WabP$_{50/57}$ and LPS from mutant *K. pneumonia* 52145Δ*wabH* as acceptor. The results obtained showed that *in vitro* both WabN enzymes are able to deacetylate LPS-GlcNAc and LPS-GalNAc to LPS-GlcN and LP-GalN. These results are in agreement with the high level of amino acid identity, above 95%, among the WabN proteins from strains R110, 51/57, HI4320, 50/57, and TG83. The fact that *P. mirabilis* WabN deacetylases, independently from the origin strain, are able to act on carbohydrate backbones nearly identical, but with GlcNAc or GalNAc terminal residues, could represent an efficient system to reduce unnecessary biodiversity.

The *gne* required for UDP-GalNAc generation from UDP-GlcNAc is present in strains containing GalN in their core LPS but also in strains containing GlcN, when these strains present GalNAc in their O-PS. In *P. mirabilis* HI4320 and ATCC 29906, the *gne* is located in a gene cluster similar to those putatively involved in O-PS (contig GG668582, GenBank™ ACLE01000000) (Wang et al., 2010). However, there are examples of *P. mirabilis* strains that should require *gne* for its O-PS biosynthesis, where this gene was not found inside the O-PS biosynthetic cluster (Wang et al., 2010). Thus, *gne* cannot be used to predict the presence of GalN or GlcN in the core LPS. Nevertheless, the absence of *gne* is fully correlated with presence of *wabH* and thus of GlcN in the LPS core of the strains tested.

The requirement for two (*wabH* and *wabN*) and three genes (*wabP*, *wabN*, and *gne*) appears to be related to the lack of a reported direct biosynthetic route for UDP-GlcN in bacterial cells [Gabriel

O., 1982]. The results obtained in the present study, and those reported for *K. pneumoniae* and *S. marcescens,* led to the conclusion that this (a HexNAc residue first incorporated, and later deacetylated to HexN), could be a general mechanism in the biosynthesis of HexN-containing enterobacterial core OS. Interestingly, in both the lipid A, and nodulation factor biosynthesis, a GlcNAc residue is first incorporated from UDP-GlcNAc and later deacetylated to GlcN [for reviews, see Long, 1996; Raetz and Whitfield, 2002]. In addition, the limited similarities shown by the WabN homologues from *P. mirabilis* studied here, and those of *K. pneumoniae* and *S. marcescens,* to members of the hydrolase/deacetylase superfamily probably reflect the specificity of these enzymes for their natural substrates (chitin, lipid A, and core LPS). Finally, it should be noted that this double enzymatic requirement has been also shown in bacteria no belonging to *Enterobacteriaceae* family. Not always the HexNAc transferase and deacetylase activities reside in independent polypeptides. Recently, work in the lab showed that in *A. hydrophila* and *A. salmonicida* these two enzymatic activities are located in two domains of the same enzymatic protein [Jimenez et al., 2009].

Functional identification of other strain specific core LPS genes. These genes were identified first by introduction into specific *K. pneumoniae* 52145 mutants, and then selection of the ones modifying LPS mobility in Tricine-SDS-PAGE, followed by chemical analysis, and mass determination by MS. In this way it was possible to identify the *wamA* gene as the one encoding the heptosyltransferase IV responsible for the transfer of D,D-Hep to GalAI by an α1,2 linkage. This conclusion was inferred from the study of the chemical composition and MS of the LPS core of a *K. pneumoniae* 52145 strain with a deletion in the gene *wabH* in which the gene *wamA* was expressed. The chemical composition of LPS of this mutant and MS of the corresponding OSs from the core LPS are in agreement with the proposed function for the WamA enzyme. Furthermore, it should be noted that *wamA* homologues were found in three (R110, 50/57 and TG83) of the four strains studied and in genome strain HI4320, but not in strain 51/57. This is agreement with the presence of α-D,D-Hep 1→2 α-GalA disaccharide in strains R110, 50/57, and TG83 but not in strain 51/57 [Vinogradov, 2002]. It was also inferred that this D,D-Hep residue should be present also in the core LPS of genome strain HI4320 that at the time the study was performed remained unknown. The recent elucidation of the core LPS structure of strain HI4320 [Vinogradov, 2011] confirmed the above hypothesis.

Similarly, through the analysis of the chemical composition and MS of specific mutants of *K.*

pneumoniae 52145 that produces LPS molecules that act as acceptors of residues transferred by *wam* gene products, it was possible to identify the functions of enzymes encoded by genes *wamB* (galactosyltransferase), and *wamC* (heptosyltransferase V) of the R110 strain, responsible for the transfer of residues D-Gal [β-D-Gal 1→7 α-D,D-HepI] and D,D-HepII [α-D,D-HepII 1→6 α-D-GlcN], respectively. With the identification of the functions of *wamA*, *wamB*, and *wamC* all the functions of the genes of the *waa* cluster from strain R110 were known. But, at least an additional glycosyltransferase, a galacturonyltransferase, responsible for the transfer of β-D-GalAII to L,D-Hep III was necessary to complete the core LPS structure of strain R110. Thus, it was concluded that this additional galacturonosyltransferase gene was located outside of the *waa* gene cluster.

The situation is even worse for strain 51/57 were in addition to the common glycosyltranferase genes there is only one specific glycosyltranferase, the *wamD* gene. The study of the *wamD* function leads to the conclusion that it encodes an enzyme responsible for the transfer of D-GlcNAc to GlcN. Thus, at least two additional glycosyltransferases are required for the transfer of D-GlcNAc to GlcN and β-D-GalAII to L,D-HepIII, and both should be located outside the *waa* gene cluster of strain 51/57. It is interesting to note that a *wamD* gene was also found in the genome strain HI4320 indicating the presence in this strain of the same GlcNAc residue found in strain 51/57, this has been recently confirmed with the publication of the core LPS structure of strain HI4320 [Vinogradov, 2011].

The requirement for additional glycosyltranferases encoded by genes located outside the *waa* gene cluster is even more important for strains 50/57 and TG83 where in addition to the common *P. mirabilis* glycosyltranferase genes there is only a *wamA* homologue inside the *waa* gene cluster.

Thus, the comparison of the glycosyltransferases encoded in the *waa* gene cluster of each strain with the chemical structure of the corresponding core LPS structures indicates that additional glycosyltransferases encoding genes for core LPS biosynthesis should localize outside the sequenced *waa* gene clusters. In the strains included in this work and in most strains of *Proteus* spp. studied from the chemical point of view, there is a second residue of GalA (GalA II) linked to L,D-Hep III. The bioinformatic analysis of all glycosyltransferases present in the genome of strain HI4320 allowed the selection of the Open Reading Frame (ORF) PMI2517 as a candidate for the transfer of this second residue of GalA. This hypothesis was confirmed by complementation with

PMI2517 of a mutant of *K. pneumoniae* 52145 with a deletion of the gene *wabO*. Therefore, the gene *wabO* of *P. mirabilis* corresponds to the ORF PMI2517 and is homologous to the *wabO* of *K. pneumoniae,* where it was shown to be a galacturonosyltransferase responsible for the transfer of GalA II to the L,D-Hep III [Fresno et al., 2007]. The HI4320 *wabO* homologue is located between *yntA* and *hycI*, encoding putative a nickel/dioligopeptide substrate-binding protein and a hydrogenase maturation protease, respectively [Pearson et al., 2008]. A primer pair designed to amplify the *wabO* homologue, and PCR amplifications using these primers and genomic DNAs from strains R110, 51/57, 50/57 and TG83 as the templates allowed the amplification of DNA fragments of about 3.3 kb that were shown by nucleotide sequence determination to contain the *P. mirabilis wabO* gene, thus confirming that this gene is present in all the *P. mirabilis* strains studied as expected from its function.

In *P. mirabilis* strain 51/57, a gene coding a protein of the Mig-14 family was identified inside the *waa* gene cluster. Inspection of the available whole genome of *P. mirabilis* HI4320 did not allow the identification of a gene similar to *mig-14*. In *P. aeruginosa* PAO1 a *mig-14*-like gene is found inside the *waa* gene cluster, suggesting that it could have some unknown function in core LPS biosynthesis. In contrast, in *Enterobacteriaceae*, *mig-14*-like genes are found away from the *waa* gene cluster, as in *S. enterica* subsp. *enterica* serovar Typhimurium LT2. Thus, the localization of the *mig-14*-like gene in strain 51/57 appears to be an exception in the family *Enterobacteriaceae*. The *mig-14* is a gene, best studied in *S. enterica* subsp. *enterica* serovar Typhimurium LT2 [Brodsky et al., 2002; Brodsky et al., 2005], where it is regulated by the PhoP-PhoQ two component regulatory system. It is reported that it is required for bacterial virulence during later stages of an acute mouse infection model; also, more recent studies indicate that *mig-14* expression is up-regulated in the macrophage vacuole and in the presence of antimicrobial peptides and also that *mig-14* is necessary for resistance to the antimicrobial peptides polymyxin B and protegrin-1 *in vitro* [Brodsky et al., 2002]. Further data suggests that IFN-γ signaling limits the replication of *mig-14* mutant bacteria in mouse infection, that *mig-14* contributes to long-term persistent infection, and promotes *Salmonella* replication in activated macrophages. In the same work they identified Mig-14 as a 35 kDa inner membrane associate protein. The predicted sequence of Mig-14 in fact, does not contain an obvious hydrophobic trans-membrane domain or a signal sequence which would be associated with an integral inner membrane protein. It may therefore be a periplasmatic protein that associates with the inner membrane fraction. Thus, the 51/57 *mig-14* homologue do not appear to be

a candidate glycosyltransferase, or at least not one belonging to the already described families of these enzymes. There is also the possibility that the *mig-14* homologue could be the sensor of a two-component regulatory system that could induce the expression of relevant glycosyltransferase.

Phosphoethanolamine (PEtN) transferases. Previous work done in *S. enterica* LT2 allowed the identification of several LPS PEtN transferases by similarity to *lpt-3* gene encoded protein (NMB2010) of *N. meningitidis* [Tamayo et al., 2005]. This protein of *N. meningitdis* is responsible for the transfer of a PEtN residue to the O-2 of L,D-Hep II [St Michael et al., 2009; O'Connor et al. 2006]. A similar search was performed for strain HI4320 leading to the identification of PMI3040 and PMI3576 located outside of the *waa* gene cluster. PMI3040 and PMI3576 shared with other known and putative PEtN transferases the presence of a sulfatase domain. In addition PMI3040 shared significant levels of amino acid identity and similarity, overlapping alignments of 70 to 94% of the whole sequence, to CptA and a putative PEtN transferase of unknown function of *E. coli* (YhbX). PMI3040 also showed a lesser degree of similarity (e values from $2.e^{-20}$ to $1.e^{-27}$) to EptA, and EptC from *E. coli* and *S. enterica*. EptA (also known as PmrC) has been shown to transfer PEtN to the phosphate at the 1 and/or 4' positions of lipid A [Lee et al., 2004] and EptB to the Kdo II residue of inner core in *E. coli* [Reynolds et al., 2005]. In *E. coli* and *S. enterica* a third PEtN transferase, CptA, is involved in the transfer of PEtN to the phosphorylated L,D-Hep I residue of the inner core [Tamayo et al., 2005]. A functional homologue of CptA is not possible in *P. mirabilis* since the L,D-Hep I residue of its inner core is unphosphorylated [Vinogradov, 2011]. In agreement with the reported core OS structure of *P. mirabilis* HI4320 no homologue of the WaaP kinase [Yethon and Whitfield, 2001] required for addition of a phosphate group to the O-4 of L,D-Hep I was found in its genome. On the other hand, none of the core LPS structures elucidated for *P. mirabilis* strains grown on standard conditions [Vinogradov, 2002; Vinogradov, 2011] present a PEtN linked to Kdo II. Nevertheless, it cannot be ruled out the presence this PEtN moiety in minor *P. mirabilis* core molecules or when these strains are grown in non-standard conditions. Finally, EptA (PmrC) is found in a transcriptional unit with PmrAB, also known as BasSR, two component system in *E. coli* and *S. enterica*. But in the genome strain HI4320, PMI3576 is found between PMI3575, a putative oxidase, and *fdhF*, formate dehydrogenase-H subunit. In addition no PEtN substituting lipid A was found in *P. mirabilis* [Sidorczyk et al., 1983] grown in standard conditions. The above considerations suggest that PMI3576 could be a PEtN transferase more likely involved in Kdo than in lipid A modification, while no suggestions can be made for PMI3040.

A common feature shared by all the studied *P. mirabilis* core LPS is the presence of a PEtN moiety substituting the O-6 position of L,D-Hep II (HepII6-PEtN). In several non-enteric mucosal pathogens, such as *Neisseria meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenza*, an identical PEtN residue is found in the same position. A highly conserved protein at the amino acid level (Lpt-6, corresponding to NMA0408 in *N. meningitidis*) is required for the transfer of this PEtN residue [Cox, et al., 2003; Wright et al., 2004]. Thus, the whole genome of *P. mirabilis* HI4320 was analyzed by BLASTX and BLASTP searching for proteins similar to NMB2010. This search allowed the identification of PMI3104 as the best candidate PEtN transferase putatively involved in the biosynthesis of the L,D-Hep II6-PEtN. The PMI3104 shared with NMB2010 homologues from *Neisseria* and *H. influenza* the presence of several predicted transmembrane regions before a sulfatase domain. PMI3104 did not show any significant similarity to *E. coli* and *S. enterica* proteins. On the basis of the above analyses it was hypothesized that PMI3104 would be involved in PEtN transfer to the O-6 position of the L,D-Hep II. Accordingly PMI3104 was named *eptC* (for ethanolamine phosphate transferase C).

This hypothesis was initially supported by the LPS mobility shift caused in Tricine-SDS-PAGE by *eptC* introduced either in mutant *K. pneumoniae* 52145Δ*wabG* or in *K. pneumoniae* 52145Δ*wabH*. This mobility shift can be attributed to the increase in mass by 123 Da, to the increase in positive charge, or both due to PEtN. The presence of a PEtN residue linked to the O-6 position of L,D-Hep II residue was confirmed by a detailed analysis of core LPS of strain *K. pneumoniae* 52145Δ*wabG* expressing *eptC*. MS analysis confirmed an increase in core LPS mass by 123 Da. ^{13}C-NMR analysis of the major OS from this core LPS fraction showed signals attributed to PEtN group at δ 41.2 (CH_2N) and 63.3 (CH_2O). The ^{1}H-NMR spectrum contained a PEtN group at 3.31 (CH_2N) and the ^{31}P-NMR spectrum showed one signal for a monophosphate group at δ 0.5. The ^{1}H-NMR spectrum was assigned by two-dimensional COSY and TOCSY experiments, and then the ^{13}C-NMR spectrum was assigned using a two-dimensional ^{1}H,^{13}C HSQC experiment. These experiments allowed to define unambiguously the O-6 position of L,D-Hep II as the site of the PEtN modification.

To determine the role of PEtN substitution in *P. mirabilis* biology a *eptC* insertion mutant, *eptC::kan*, was constructed in the genome strain HI4320. The mutation did not affect the growth kinetics in lab conditions in rich or minimal media. Comparison of LPS extracted from the mutant

and the wild type strain by SDS-PAGE and Tricine-SDS-PGE showed that the mutant is still able to produce O-PS, and that its LPS core-lipid A fraction mobility is increase. In order to test a possible change in the permeability and/or stability of the OM the Minimal Inhibitory Concentration (MIC) for different antibiotics and detergents, including cationic, anionic and zwitterionic ones, were determined for the mutant and the parent strain HI4320. The measure of the sensitization to hydrophobic probe compounds is an indirect way to study OM permeability. The sensitization can be quantified as the decrease of the MIC for the probe compound. In addition, when bactericidal compounds are used their effect on bacterial viability can also be measured. The only difference observed was a higher sensitivity of the *eptC::kan* mutant to SDS that parent HI4320. This difference was confirmed by determining the % of cells retaining viability when confronted with 0,5%% SDS for 45 minutes. The difference in SDS sensitivity strongly suggests a moderate destabilization of the OM in the absence of PEtN.

In order to investigate the effect of the PEtN mutation in *P. mirabilis* fitness during UTI, an *in vivo* co-challenge competition assay was set up. In this assay a 1:1 mixture of overnight cultures of parent HI4320 and *eptc::kan* mutant was administered transurethrally into CBA/J mice. At seven days post-inoculation the mice were euthanized and the levels of wild type and mutant were determined in the urine, bladder, and kidneys. The results indicate no statistically significant differences between the wild type and the mutant strain fitness in this infection assay.

To investigate the effect of the PEtN mutation in *P. mirabilis* fitness during a systemic infection, we tested the bacterial survival in human serum. Wild-type *P. mirabilis* HI4320 strain was resistant to the bactericidal activity of NHS, while the *eptC::kan* or *eptC* mutants showed a moderate sensitivity. At the same time, the introduction of the *etpC* renders both mutants resistant to the bactericidal activity of NHS, as found for wild type strain. These results indicate that the absence of this PEtN modification can compromise the ability to perform a systemic infection.

It could expect that the absence of a positive charge due to PEtN should lead to an increase in CAMP (cationic antimicrobial peptides) resistance such as polymyxin B but this is not the case in the *eptC::kan* mutant. Major changes in polymyxin B sensitivity have been found in *eptA* or *eptA*-like mutants [Lee et al., 2004; Lewis et al., 2009], while minor or no changes have been seen in *cptA* [Lewis et al., 2009; Tamayo et al., 2005] and *eptB* mutants [Reynolds, 2005], leading to the notion

of a major role for PEtN modification of lipid A versus PEtN modification of the inner core residues in polymyxinand CAMP resistance. In addition, it should be noted that in *E. coli* and *S. enterica* CptA PEtN modification act on phosphorylated L,D-Hep II-P compensating the phosphate negative charge, while in *P. mirabilis* the core OS negative charges are contributed by the D-GalA residues.

In *P. mirabilis* most of its LPS molecules are modified with L-Ara4N residues at the 4' position of lipid A and at the Kdo I residue of the inner core [Sidorczyk et al., 1983; Vinogradov, 2002; Vinogradov, 2011]. These modifications with L-Ara4N explain the high innate resistance of *P. mirabilis* to polymyxin B and other CAMPs. In agreement with these L-Ara4N modifications, in the genome of strain HI4320 two genes encoding homologues of *S. enterica arnT* gene [for a review see Gunn, 2008] can be detected. The *arnT* gene encodes a lipid A L-Ara4N transferase. The *arnT* homologue PMI1047 from strain HI4329 is located, as well as *arnT* from *S. enterica*, in the *arn* operon containing the genes required for L-Ara4N synthesis, activation, and transfer to lipid A [Gunn, 2008]. The *arnT* homologue PMI0275 is located between *mtrF*, coding for a putative efflux pump component, and *zapD*, coding for a type I secretion outer-membrane protein [Pearson et al., 2008]. Thus, from this analysis it can be hypothesized that PMI1047 and PMI0275 will correspond to L-Ara4N transferases responsible for lipid A and Kdo modifications in strain HI4320, respectively. The importance of these two L-AraN modifications in *P. mirabilis* polymyxin B resistance was recently shown by a mutation in one of the genes, *arnA*, of the *arn* cluster involved in the biosynthesis of undecaprenyl-Phosphate-α-L-Ara4N in *P. mirabilis* [Jiang et al., 2010b]. In addition in *Citrobacter rodemtium*, lacking the *arn* operon, *cptA* deletion causes only a minor decrease in polymyxin B resistance [Viau et al., 2011].

In agreement with the EptC function as transferase responsible for the linkage of a PEtN moiety to the O-6 position of L,D-Hep II, the *eptC* gene is found in all the *P. mirabilis* strains analyzed in this study. Within the *Enterobacteriaceae*, *eptC* homologues appear to be limited to species of the *Proteus* phylogenetic related genus *Photobacterium* and *Providencia*, and recently the presence of a PEtN moiety linked to the O-6 position of L,D-Hep II was shown in *Providencia alcalifaciens* O8 and O35, and *Prov. stuartii* O49 [Kondakova, 2006]. Further work will be necessary to understand the reasons for the presence of this common feature in these three closely related genera.

Future perspectives. The results reported in this work allowed the identification of all the genes required for the biosynthesis of the sugar core LPS structure for strain R110. Nevertheless, for *P. mirabilis* genome strain HI4320 a gene required for the transfer of a third D-GalA residue [Vinogradov, 2011] remains unknown. Thus future efforts will be directed to its identification. This terminal D-Gal III residue is linked by a β 1, 3 bond to D-GlcNAc and since usually the source of GalA residues is UDP-α-D-GalA the enzyme required should be of the inverting type (see CAZy home page: http://www.cazy.org/). A study of all the glycosyltransferases of the inverting type in the genome of strain HI4320 will help in selecting candidates for completion of the core OS structure of strain HI4320 to be tested biologically.

In several of the studied core LPS OS from *P. mirabilis* amino acid/s, such as Gly, Ala, and Lys, covalently linked to sugar core OS residues have been found [Vinogradov, 2002]. In the genome strain HI4320 a Lys covalently linked to the D-Gal III residue has been reported [Vinogradov, 2011]. A better understanding of the biosynthesis of core LPS in *P. mirabilis* will require and effort to identify the enzyme required for the Lys modification. An initial screen for candidate genes based in conserved features of amidoligases is expected to help in the identification of possible candidates for this Lys modification In addition, mutant/s devoid of this Lys modification will be necessary to determine the biological significance of this core LPS modification.

In all the *P. mirabilis* strains studied here and in genome strain HI4320 the same four glycosyltransferases encoding genes were found downstream from the *waaL* gene and outside the classical *waa* gene cluster. They belong to the glycosyltransferase family GT4 (*walM*, *walN* y *walR*) and GT9 (*walO*) according to the CAZy classification. Since the common sugar residues found in P. mirabilis core LPS have been identified in the present study it appears unlikely that these genes (*wal*) are required for the biosynthesis of the LPS core, at least in standard laboratory culture conditions, but they could be involved in the biosynthesis of variants of the core LPS during specific times of the complex vital cycle of this bacterium, or in the biosynthesis of other relevant OS or PS such as capsule. Thus do to its degree of conservation it seems worth the effort to try to obtain mutant/s for some of them to elucidate its function in *P. mirabilis*.

6. Conclusions

1. *Proteus mirabilis* has most of the genes for the biosynthesis of LPS core grouped in the *waa* cluster in the chromosome. Despite this, additional genes required for core LPS biosynthesis are found outside the *waa* cluster. Such as *wabO* (PMI2517), present in all strains studied and encoding for the transferase that places the D-GalA II residue to the L,D-HepIII.

2. Nine genes involved in *P. mirabilis* core LPS biosynthesis were found in the *waa* cluster. They are responsible for the transfer of eight common *P. mirabilis* core residues.
The pentasaccharide of the inner core, shared by all *Enterobacteriaceae*, is biosynthesized in *P. mirabilis*, by the sequential activity of a bifunctional transferase (WaaA) and three heptosyltransferases (WaaC, WaaF, and WaaQ). These enzymes are conserved in *P. mirabilis* strains analyzed and show a high identity and similarity level to homologues proteins of *E. coli*, *K. penumoniae* and *S. marcescens*. Glycotransferase functions have been assigned to the chemical structure of *P. mirabilis* according to the following scheme:
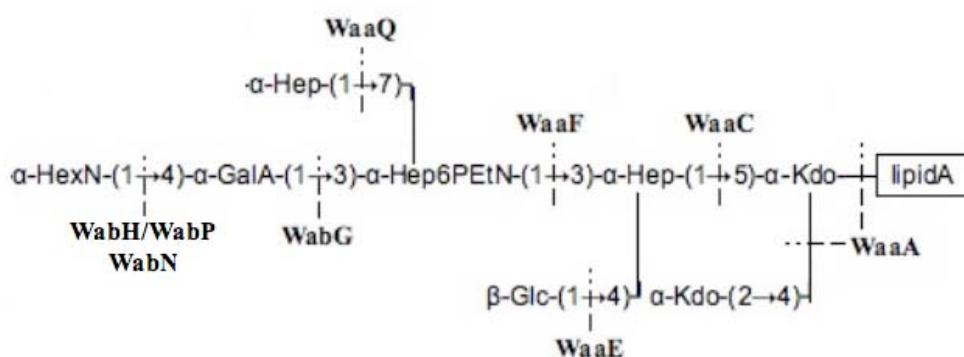
3. The *waaL* gene, coding for the O-antigen polymerase ligase, is found adjacent to the classic *waa* cluster. Downstream this gene, four genes encoding enzymes belonging to the 4 (*walM*, *walN*, and *WalR*), and 9 (*walO*) glycosyltransferase family were found. Even if members of these families were related to LPS core biosynthesis in several Gram-negative bacteria, in *P. mirabilis* they do not appear to be involved in the biosynthesis of the reported core LPS structures.

4. The presence of the disaccharide HexN-1,4-GalA is a feature of *P. mirabilis* LPS outer core. Depending on the nature of the HexN outer core residue, two different homologues for HexNAc transferases are present in the *waa* cluster: *wabH* or *wabP*. Altought the incorporation of GlcN into LPS core requires an acetylglucosaminyltransferase (WabH) and a deacetilase (WabN), the incorporation of GalN requires three enzymes: an acetylgalactosaminyltransferase (WabP), a deacetilase (WabN) and an epimerase (gne). An amplification test with specific primers for this two different homologues can be used to predict the HexN nature in *P. mirabilis* LPS cores.

5. The strain-specific genes *wamB* and *wamC* code for a galactosyltransferase and a heptosyl transferase respectively in strain of *P. mirabilis* R110. The enzyme encoded by gene *wamD* is a N-acetylglucosaminyltransferase, and it is found in strain of *P. mirabilis* 51/57. WamA, coded by *wamA* gene in the *waa* cluster of strains R110, 50/57, TG83 and HI4320, is a heptosyltransferase responsible for the incorporation of a quarter residue of Hep, in DD configuration, to the GalA II of the outer core.

6. In *P. mirabilis* strain 51/57, a gene coding a protein of the Mig-14 family was identified inside the *waa* gene cluster, this localization appears to be an exception in the family *Enterobacteriaceae*. Inspection of the available whole genome of *P. mirabilis* HI4320 did not allow the identification of a gene similar to *mig-14*.

7. There are three putative phosphoethanolamine transferases in the genome of *P. mirabilis*: PMI3040, PMI3576, and PMI3104. The gene identified as *eptC* (PMI3104) transfers the moiety of phosphoethanolamine to the O-6 position of L,D-Hep II (HepII6PEtN), in *P. mirabilis* LPS core.

8. The absence of the positive charge due to phosphoethanolamine residue doesn't affect the bacterial growth kinetics in lab conditions in rich or defined media. The absence of the phosphoethanolamine residue, causes a moderate destabilization of the outer-membrane.

9. Despite the lack of the phosphoethanolamine residue on the Hep II in *P. mirabilis* LPS core, has no statistically effects during urinary tract infection assays in mouse model, the absence of this modification causes an increase sensitivity to complement in nonimmune human sera.

7. Summary/Resumen

INTRODUCCIÓN

El género *Proteus* forma parte de la familia *Enterobacteriaceae*, dentro del grupo de las Proteobacterias. Los miembros de este género se describen como bacilos gram-negativos polimórficos y con flagelos peritricos. Se caracterizan por su rápida movilidad y por la producción del enzima ureasa. El género *Proteus* se compone en la actualidad de cinco especies (*P. mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxofaciens* y *P. hauseri*) y tres genomoespecies (*Proteus* genomoespecies 4, 5, y 6) que se han diferenciado mediante técnicas de biología molecular y que aún carecen de nombre científico [O 'Hara, 2000; Giammanco, 2011]. El género *Proteus* es ampliamente difundido en la naturaleza y también forma parte de la microbiota intestinal. Se ha aislado de muestras ambientales, incluyendo tierras, abonos y aguas contaminadas, y de una gran variedad de muestras de animales [Guentzel, 1996]. En el año 2008, un aislado de *P. mirabilis* (HI4320) proveniente de la orina de un paciente cateterizado a largo plazo fue la primera cepa del genero para la que se ha determinado la secuencia de todo su genoma [Pearson, 2008].

Una característica fenotípica significativa del género *Proteus* es la capacidad de movimiento "swarming". Este proceso es un comportamiento grupal de las bacterias y consiste en cambios en los procesos de elongación durante la división celular, que llevan a la formación de células alargadas y a la hiperexpresión de la síntesis de flagelina, que determina un recubrimiento profuso por flagelos de las células de estos microorganismos (Figura 2) y un movimiento celular coordinado. Este fenómeno ocurre en tres fases: diferenciación, migración y consolidación (Figuras 3 y 4) [Hoeniger, 1964; Hoeniger, 1965; Hoeniger, 1966; Hoeniger and Cinitis, 1969; Bisset, 1973 a; Bisset, 1973 b; Williams, 1978].

Proteus mirabilis es un patógeno oportunista que causa infecciones de las vías urinarias (UTI), sobretodo en el caso de anomalías funcionales, metabólicas o estructurales de las mismas [Mobley, 1987a; Chen, 2012]. La mayoría de las infecciones urinarias se producen por vía ascendente [Coker, 2000]: organismos de origen fecal, después de colonizar la región perineal, ganan acceso a la uretra, y desde allí, migran y colonizan el epitelio del tracto urinario y, evitando la respuesta inmune del huésped, suben a la vejiga urinaria. Desde la vejiga pueden llegar a alcanzar los riñones donde pueden multiplicarse y dañar sus tejidos. Los patógenos más virulentos e invasivos pueden romper la barrera celular que ofrecen los túbulos proximales y entrar al torrente sanguíneo causando una infección sistémica [Nielubowicz, 2010]. Las infecciones de las bajas vías urinarias y la cistitis se

caracterizan por síntomas como micción frecuente y urgente, y disuria. Sin tratamiento, estas infecciones, una vez en los riñones, pueden estar asociadas con otros síntomas como fiebre, náuseas, vómitos y dolor en el costado [Guay, 2008].

P. mirabilis no es una causa frecuente de infecciones urinarias en huéspedes sano, más bien infecta el tracto urinario con alteraciones funcionales o anatómicas, o instrumentación crónica como el cateterismo [Mobley, 1987a; Chen, 2012]. *P. mirabilis* es a menudo asociado con cálculos urinarios e incrustaciones de los catéteres y es particularmente importante en pacientes con cateterización prolongada. Las infecciones del tracto urinario asociadas a cateterización son mundialmente reconocidas como la causa mas común de infección asociada a tratamientos en ambiente hospitalario [Warren, 2001].

La patogenicidad de *P. mirabilis* se asocia a la presencia de numerosos factores de virulencia: fimbrias, flagelos, proteínas de la membrana externa, lipopolisacárido (LPS), capsula, ureasa, hemolisinas, enzimas proteolíticos y aminoácido deaminasas que actúan como sideróforos.

Uno de los aspectos distintivos de las infecciones del tracto urinario causadas por *P. mirabilis* es la producción de cálculos como resultado de la actividad del enzima ureasa. La producción de ureasa por parte de las especies del género *Proteus* se considera de gran importancia para su patogenicidad. La ureasa es capaz de hidrolizar eficazmente la urea presente en la orina y producir la alcalinización de la misma por producción de hidróxido amónico. Como resultado de dicha alcalinización el Mg^{2+} y el Ca^{2+}, que habitualmente son solubles al pH fisiológico urinario, precipitan y, como consecuencia de este proceso, se producen los mencionados cálculos de estruvita [McLean, 1988; Mobley, 1989; Clapham, 1990; Mobley et al., 1995].

El LPS es un factor de virulencia importante. También conocido como endotoxina, es una molécula glicolipídica que constituye la estructura mayoritaria de la cara externa de la membrana externa (OM) de las bacterias Gram negativas. Dicha membrana está formada por una bicapa lipídica asimétrica, la cara interna de la cual está constituida por fosfolípidos, mientras que la cara externa está formada, principalmente, por el LPS, cuyo componente hidrófobo, el lípido A, forma la parte exterior de esta bicapa y queda oculto por los componentes sacarídicos (Figura 6) [Raetz, 2002]. El LPS es una molécula anfipática constituida por una región sacarídica (polar) unida covalentemente a una estructura lipídica (apolar), altamente conservada, denominada lípido A, que le permite el

anclaje a la membrana. El lípido A, la única región del LPS reconocida por el sistema inmune innato, es el responsable de la actividad endotóxica del LPS [Rietschel et al., 1996] y puede llegar a provocar una importante inflamación sistémica conocida como shock séptico o endotoxemia [Rietschel, 1994]. La fracción sacarídica suele constar de dos partes: un oligosacárido (OS) más interno y conservado, unida al lípido A, conocido como núcleo del LPS, que se puede subdividir en núcleo interno y núcleo externo; y otra polisacarídica más externa y variable, anclada al núcleo y denominada polisacárido O, cadena lateral O u antígeno O (O-PS) por sus características inmunógenas (Figura 7) [Lüderitz, 1982]. El antígeno O consiste en la repetición de una subunidad oligosacarídica básica que varía en función de las diversas especies y serotipos, y que puede estar formada por uno o más monosacáridos constituyendo subunidades lineales o ramificadas. Su contacto directo con el medio lo convierten en un importante antígeno de superficie y en una de las principales dianas de bacteriófagos, así como del sistema de defensa del organismo huésped.

Estas tres regiones (lípido A, núcleo del LPS y O-PS), diferenciadas en base a su estructura química, también se diferencian en sus rutas de biosíntesis, en las agrupaciones génicas implicadas en su biosíntesis y en el grado de conservación. La variabilidad estructural del LPS disminuye gradualmente desde el antígeno O hasta el lípido A. La elevada conservación de las características generales del lípido A y del núcleo interno puede ser reflejo de las restricciones impuestas por su papel en el mantenimiento de la integridad de la membrana externa [Heinrichs et al., 1998], mientras que las estructuras más superficiales están sometidas a una mayor presión evolutiva ejercida tanto por el ambiente como por la interacción con el sistema inmune del huesped [Nikaido, 1970]. Esta variabilidad química, en el núcleo externo y en el antígeno O, se correlaciona con la amplia diversidad de los genes implicados sus rutas biosínteticas.

El LPS también es responsable de la resistencia de las bacterias Gram negativas a determinadas moléculas. El núcleo y el O-PS crean una barrera hidrofílica difícilmente penetrable por compuestos o antibióticos hidrofóbicos. Además, esta misma barrera hidrofílica debilita la efectividad de los péptidos policatiónicos: estos quedan atrapados mediante interacciones electrostáticas con las cargas negativas del LPS y forman autoagregados que difícilmente consiguen acceder a la membrana. La estructura del LPS de las especies de *Proteus* spp., posee una gran cantidad de grupos 4-amino-L-arabinosa (L-Ara4N), que reducen la afinidad de la membrana para los antibióticos policatiónicos.

Summary/Resumen

OBJETIVOS

El estudio del núcleo del LPS es de especial relevancia por básicamente cuatro razones: (1) se trata de una región más conservada que el dominio más externo o antígeno O, aunque cada vez es más evidente que existe una cierta variabilidad a nivel de género o grupos de géneros similares; (2) su estructura química modula la actividad endotóxica del lípido A; (3) la alteración del núcleo del LPS genera bacterias menos virulentas y más sensibles a los mecanismos de defensa del sistema inmune, lo que plantea la búsqueda de substancias que interfieran con la biosíntesis de esta región del LPS; y (4) regiones conservadas del núcleo del LPS, comunes a diversos géneros, podrían ser útiles como antígenos en la prevención de enfermedades causadas por patógenos que contengan estas regiones conservadas. Además, el conocimiento en profundidad de la interacción de la molécula con los mecanismos de defensa del huésped permitiría el desarrollo de nuevos agentes anti-bacterianos, vacunas, y, en definitiva, ayudaría a facilitar la eliminación de las bacterias por parte del sistema defensivo. Por otro lado, los estudios estructurales que se están llevando a cabo con una gran cantidad de moléculas de LPS diferentes tienen también una gran importancia, puesto que permitirán establecer una relación entre la conformación química del LPS y su actividad, de forma que se puedan determinar las estructuras particulares que juegan un papel crítico en la virulencia bacteriana y como causan esta actividad.

Estudios previos del grupo de investigación han permitido caracterizar las peculiaridades del núcleo del LPS de *Serratia marcescens*, *Klebsiella pneumoniae*, *Aeromonas hydrophila* y *A. salmonicida* que han puesto de manifiesto la importancia de estos elementos como factores de virulencia y también diferencias importantes tanto a nivel estructural como funcional respecto de los núcleos del LPS previamente estudiados. Estos estudios han contribuido a poner de manifiesto la existencia, dentro de los miembros de la familia *Enterobacteriaceae*, de dos grandes grupos de núcleos del LPS: por una parte aquellos que contienen ácido galacturónico (D-GalA) como primer residuo del núcleo externo y presentan, además, un residuo de glucosa (D-Glc) unida a la L,D-Hep I, caso de *K. pneumoniae* y *S. marcescens*, y por otra parte aquellos que contienen D-Glc como primer residuo del núcleo externo y carecen de D-Glc unida a la L,D-Hep I, como en el caso de *Escherichia coli* y *Salmonella enterica*.

P. mirabilis es una de las enterobacterias patógenas que presenta mayores peculiaridades en la estructura química del núcleo del LPS. Todas las cepas presentan un residuo de hexosamina

(glucosamina [D-GlcN] o galactosamina [D-GalN]) en su núcleo externo unido por enlace α-1,4 al residuo de D-GalA. En algunas cepas de *P. mirabilis* se encuentra un residuo de *N*-acetil galactosamina en configuración abierta (GaloNAc), y por tanto unido mediante dos enlaces al residuo adyacente del núcleo: este hecho es excepcional y solo descrito en esta especie. Finalmente, es frecuente encontrar en el núcleo del LPS de *P. mirabilis* residuos sacarídicos covalentemente unidos a aminoácidos, como por ejemplo GlcNGly [Vinogradov, 2002].

A partir de las consideraciones anteriores los objetivos específicos planteados al inicio de esta tesis fueron:

- Detectar, identificar y secuenciar los genes que codifican los enzimas necesarios para la biosíntesis del núcleo de LPS.

  Esta parte del trabajo se ha realizado mediante la determinación de la secuencia completa de los genes de la biosíntesis del núcleo del LPS de las cepas de *P. mirabilis* R110 (O3), 51/57 (O28), 50/57 (O27) y TG83 (O57).

- Caracterizar funcionalmente los genes implicados en la biosíntesis del núcleo de LPS en *P. mirabilis*.

  Este objetivo se ha logrado mediante el uso de moléculas de LPS subrogadas o bien, en algún caso, la construcción de mutantes en *P. mirabilis* HI4320. En ambas aproximaciones las funciones se establecieron mediante el análisis de las estructuras oligosacarídicas generadas.

- Determinar la importancia del núcleo de LPS, especialmente sus características y peculiaridades, en la biología de *P. mirabilis* incluyendo su efecto en la patogenicidad de ésta especie bacteriana. Este objetivo ha sido dirigido específicamente al estudio de la función biológica del residuo de fosfoetanolamina (PEtN) unido a la L,D-Hep II en el núcleo interno de *P. mirabilis* HI4320.

DISCUSIÓN

Los agrupamientos génicos *waa* de *P. mirabilis*. En este trabajo se han identificado y caracterizado los genes que codifican para las enzimas implicadas en la biosíntesis del núcleo LPS en cuatro cepas de *P. mirabilis*: R110 (O3), 51/57 (O28), 50/57 (O27) y TG83 (O57). Estas cepas se han incluido en el proyecto porque las estructuras químicas de sus núcleos de LPS se habían descrito previamente y muestran peculiaridades y diferencias significativas entre ellas y con respecto a otras cepas de ésta especie [Vinogradov, 2002]. En estas cepas de *P. mirabilis* se ha podido identificar un agrupamiento génico (*waa*) que contiene la mayor parte de los genes implicados en la biosíntesis de los OSs que componen el núcleo del LPS. Agrupaciones *waa* se encuentran en la mayoría de las *Enterobacteriaceae* como, entre otras, *E. coli*, *Shigella*, *Salmonella* spp., *Klebsiella pneumoniae* y *Serratia marcescens*. *Yersinia enterocolitica* y *Y. pestis* son una excepción ya que los genes involucrados en la biosíntesis del núcleo de LPS se hallan distribuidos en al menos tres localizaciones diferentes en sus genomas. La organización general de los genes involucrados en la biosíntesis del núcleo de LPS es similar, en las cepas de *Proteus* estudiadas, a la encontrada en la cepa de *P. mirabilis* HI4320, la única cepa de *P. mirabilis* cuyo genoma ha sido completamente secuenciado [Pearson et al., 2008].

La estrategia utilizada para determinar los genes presentes en el agrupamiento génico *waa* de las cepas incluidas en el estudio se ha basado en la amplificación y secuenciacion del ADN de éstos agrupamientos. En la mayoría de las *Enterobacteriaceae* el gen *kbl* se encuentra justo antes del primer gen dedicado a la biosíntesis del núcleo del LPS (*hldD*), además, los genes *coaD* (biosíntesis del coenzima A), *fpg* (enzima de reparación del ADN), *rpmB* (proteína ribosomal L33) y *radC* (enzima de reparación del ADN) se encuentran al final del agrupamiento *waa*. Mediante el alineamiento de las secuencias nucleotídicas de estos genes en varias *Enterobacteriaceae* ya estudiadas se ha podido establecer que los genes más conservados eran *hldD* y *radC*. Estos dos genes se han utilizado para obtener las primeras parejas de oligonucleótidos en estas regiones conservadas. Estas parejas de oligonucleótidos se han utilizado para la amplificación de las regiones equivalentes de *P. mirabilis* y para determinar la secuencia de las regiones amplificadas. Las siguientes amplificaciones y secuencias se han obtenido mediante el uso de nuevos cebadores diseñados a partir las secuencias obtenidas inicialmente. Se han utilizado, además, los datos del proyecto genoma de la cepa HI4320 de *P. mirabilis* y las secuencias de las regiones homólogas de

K. pneumoniae y *S. marcescens* como referencia. De esta forma las secuencias nucleotídicas de los agrupamientos *waa* se han podido completar, progresando desde los dos extremos delimitados por los genes *hldD* y *radC*. Las secuencias obtenidas fueron traducidas virtualmente en las seis pautas de lectura posibles. Cada una de las ORF identificadas se ha sometido a una análisis bioinformático de semejanza con los bancos de datos de proteínas de origen bacteriano de GenBank y EMBL utilizando los programas BLAST (Basic Local Alignment Search Tool) y FASTA del National Center for Biotechnology Information y del European Biotechnology Information, respectivamente. Además, las secuencias de ADN obtenidas se han comparado con las de la regiones equivalentes de *P. mirabilis* HI4320 y de otras especies de *Enterobacteriaceae*.

Los datos y los análisis mencionados han permitido hacer hipótesis sobre la posible función de la mayoría de los genes.

En primer lugar, en todas las cepas de *P. mirabilis* estudiadas el agrupamiento clásico empieza con el operón *hldD*, que incluye los genes *hldD*, *waaF* y *waaC*, y acaba con el operón *waaA*, compuesto por la pareja de genes *waaA* y *waaE*. Entre estas dos unidades transcripcionales se encuentran cinco (para las cepas 50/57 y TG83) y siete genes (en la cepa R110) respectivamente, organizados en distintas unidades transcripcionales dependiendo de la cepa.

En segundo lugar, el análisis del porcentaje de Guanina más Citosina (% G+C) indica la existencia de regiones con un % G+C muy inferior a la media de los agrupamientos *waa* y al del genoma de la cepa HI4320, lo que sugiere la adquisición genética lateral en por lo menos tres de estos agrupamientos. Además, hay una inversión del fragmento de ADN que comprende los genes *waaQ*, *wabG* y *wabP* en las cepas 50/57 y TG83 en comparación con las cepas R110, 51/57 y HI4320.

En tercer lugar, en *E. coli* K12 y en *S. enterica* LT2 el agrupamiento génico *waa* empieza con el operón *hldD* y termina con el gen *waaA* con otro óperon entre ellos que contiene 9 y 10 genes, respectivamente. Para evitar la terminación precoz de este gran operón estas especies utilizan la proteína RfaH. Esta proteína reconoce una secuencia conservada "JUMPstart" (Just Upstream of Many Polysaccharides) que contiene una doble repetición parcial y el elemento *ops* (operon suppression sequence) a unas 120 pb del inicio de la traducción. No obstante la presencia en la cepa HI4320 de un gen homologo a *rfaH*, ni en la cepa HI4320 ni en las cepas estudiadas de *P. mirabilis* se ha encontrado ninguna secuencia parecida a la JUMPstart en los agrupamientos génicos *waa*. La

proteína RfaH de *P. mirabilis* podría reconocer una secuencia JUMPstart substancialmente diferente de la descrita en *E. coli* y *S. enteric*a. Otra alternativa podría ser que el contenido inferior en número de genes de los operones involucrados en la biosíntesis del núcleo de LPS in *P. mirabilis* no requiera este tipo de control.

Finalmente, el gen homologo a *waaL* (antigeno O u O-PS ligasa) se encuentra en *P. mirabilis* después del gen *fpg* y, por tanto, fuera del clásico agrupamiento *waa*. En la mayoría de las *Enterobacteriaceae* este gen *waaL* se encuentra dentro del agrupamiento *waa* flanqueado por genes que codifican para glicosiltransferasas. Una localización análoga a la encontrada en *P. mirabilis* para el gen *waaL* se encuentra en especies filogenéticamente cercanas como *Photorhabdus luminescens laumondii* TT01, *Phot. asymbiotica* ATCC 43949, *Xenorhabdus nematophila* ATCC 19061 y *Xeno. bovierii* SS-2004. Además de la localización del gen *waaL*, las especies mencionadas comparten con las cepas de *P. mirabilis* estudiadas la presencia de cuatro genes localizados después del *waaL* y transcritos en dirección opuesta. Estos cuatro genes codifican para proteínas, cuyas características de secuencia sugieren, que correspondan a enzimas de tipo glicosiltransferasa de la familia 4 (*walM*, *WalN* y *walR*) y 9 (*walO*) según la clasificación de la Carbohydrate-Active enZymes Database (CAZy). Los miembros de estas dos familias se han relacionado con la biosíntesis del núcleo de LPS en varias bacterias Gram-negativas. Dado que estos cuatro genes se encuentran en las cepas de *P. mirabilis* estudiadas aquí, además de en la cepa HI4320 sería lógico esperar que, si estos genes estuvieran involucrados en la biosíntesis de los OSs del núcleo, deberían ser necesarios para la transferencia de cuatro residuos específicos y comunes en núcleo de LPS de estas bacterias. Como se discute más adelante en este trabajo se han identificado, entre otros, todos los genes involucrados en la transferencia de los residuos oligosacáridicos comunes del núcleo del LPS de *P. mirabilis*. Por tanto, las glicosiltransferasas putativas codificadas por los cuatro genes *wal* no parecen estar implicados el la biosíntesis de las estructuras núcleo de LPS de las cepas incluidas en el estudio. Sin embargo, no se puede descartar que estas glicosiltransferasas pudiesen estar implicadas en modificaciones comunes del núcleo del LPS durante momentos específicos del ciclo biológico de *P. mirabilis*, por ejemplo durante la fase "swarming". Por otra parte, las semejanzas entre *P. mirabilis* y especies filogenéticamente próximas en la localización de los genes *waaL* y *walM N R* y *O* sugieren una conexión entre la organización genética y la filogenia en este subgrupo de *Enterobacteriaceae*.

Identificación de los genes comunes en la biosíntesis del núcleo del LPS. La identificación experimental de las funciones de las proteínas codificadas por los genes presentes en los agrupamientos génicos *waa* e involucrados en la biosíntesis del núcleo del LPS se ha basado en una estrategia de complementación heteróloga. Estos experimentos han sido posibles gracias a la elevada similitud del esqueleto OS del núcleo del LPS entre *P. mirabilis* y *K. pneumoniae*, lo que permite utilizar OSs del núcleo de *K. pneumoniae* como aceptores subrogados de residuos transferidos por glicosiltransferasas de *P. mirabilis*. Estos experimentos se han realizado aprovechando mutantes de *K. pneumoniae* defectivos en los genes de interés. Para los genes sin homólogos de función conocida se ha aprovechado la total concordancia de la estructura oligosacarídica entre *P. mirabilis* y *K. pneumoniae* hasta el primer residuo del núcleo externo, utilizándose esta estructura como molécula aceptora. Los análisis de complementación se han estudiado mediante visualización del perfil electroforético en geles de poliacrilamida con dodecilsulfato sódico (SDS) y en geles de poliacrilamida con Tricina y SDS (SDS-PAGE y Tricina-SDS-PAGE). Las separaciones en Tricina-SDS-PAGE son las más apropiadas para la visualización de las posibles alteraciones del núcleo del LPS, puesto que dan lugar a una mejor definición en las fracciones de baja masa molecular del LPS. Esta estrategia de adición génica tiene la ventaja, respecto a la construcción de mutantes utilizada normalmente, de permitir deducir informaciones de una función adquirida y no eliminada. Este enfoque ha permitido la identificación de las funciones de las proteínas codificadas tanto por los genes comunes como por los específicos presentes en los agrupamientos *waa* de las cepas *P. mirabilis* estudiadas.

Se ha podido demostrar que en *P. mirabilis* la biosíntesis del pentasacárido común a todas las *Enterobacteriaceae* (L-α-D-HeppIII-(1→7)-L-α-D-HeppII-(1→3)-L-α-D-HeppI-(1→5)-[α-KdopII-(2→4)-]-α-KdopI) se lleva a cabo mediante la actividad secuencial de enzimas conservados, codificados por los genes *waaA*, *waaC*, *waaF* y *waaQ*. Como era de esperar, hay un elevado grado de identidad entre los genes homólogos y sus productos (95%) en las cuatro cepas de *P. mirabilis* estudiadas. En las cepas R110, 51/57 y HI4320 el gen *waaQ* (L,D-Hep III transferasa) y *waaA* (transferasa bifuncional de Kdo) son transcritos de forma divergente, como ocurre en *E. coli* y *S. enterica*. Al contrario, en *P. mirabilis* 51/57 y TG83 el gen *waaQ* se encuentra adyacente al gen *wabH* y se transcribe en la misma dirección, como ocurre en *K. pneumoniae* o *S. marcescens*. Todas las cepas de *P. mirabilis* estudiadas contienen un homologo del gen *waaE*. Este gen se encuentra localizado entre el gen *waaA* y el gen *coaD*, como en *K. penumoniae* y *S. marcescens*, donde se ha

181

demostrado que el enzima WaaE esta involucrado en la transferencia de un residuo de D-Glc a la L,D-Hep I mediante un enlace β1,4 [Izquierdo et al., 2002].

Bases moleculares de la transferencia de GlcN. Una característica común del núcleo del LPS de *P. mirabilis* es la presencia del disacárido αHexN-1,4-α-D-GalA de su núcleo externo. El residuo de HexN es diferente en las cepas estudiadas: este residuo corresponde a D-GlcN en las cepas R110, 51/57 y HI4320; y a una D-GalN en las cepas 50 /57 y TG83. Un disacárido α-D-GlcN-1,4-α-D-GalA se encuentra también en la misma posición en *K. pneumoniae* y *S. marcescens*, donde se ha demostrado previamente que la proteína WabG es la responsable de la transferencia del residuo de D-GalA. En estas mismas especies se ha identificado el enzima WabH como responsable de la transferencia de D-GlcNAc al núcleo del LPS, mientras que la proteína WabN desacetila el residuo de D-GlcNAc a D-GlcN [Fresno et al., 2007; Regué et al, 2005b].

El alineamiento de las secuencias y análisis de similitud de las proteínas de *P. mirabilis* similares a WabH ha revelado la presencia de dos tipos diferentes de homólogos de WabH en *P. mirabilis*. Los que presentan un mayor grado de similitud con WabH se encuentran en las cepas R110, 51/57 y HI4320. Los que presentan un grado de similitud inferior con WabH se han nombrado WabP y se encuentran en las cepas 50/57 y TG83. Los genes $wabH_{R110}$ y $wabH_{51/57}$ son capaces de complementar el mutante *K. pneumoniae* 52145Δ*wabH* (contiene una mutación por deleción en pauta de lectura y sin efectos polares) y, por tanto, codifican para una transferasa de D-GlcNAc. La presencia de *wabH* en las cepas R110, 51/57 y HI4320 es lógica ya que todas ellas presentan D-GlcN como segundo residuo del núcleo externo. Por el contrario, el gen $wabP_{50/57}$ no complementa el mutante *K. pneumoniae* 52145Δ*wabH*. Dicha complementación solo es posible en presencia del gen *gne* de *A. hydrophila*. El gen *gne* codifica una epimerasa necesaria para generar UDP-D-GalNAc a partir de UDP-D-GlcNAc, lo que sugiere que el gen *wabP* codifica una transferasa de D-GalNAc. De acuerdo con esta hipótesis el gen *wabP* se encuentra en las cepas 50/57 y TG83 que presentan D-GalN como segundo residuo del núcleo externo. Mediante experimentos *in vitro* utilizando los enzimas purificados $WabH_{R110}$ y $WabP_{50/57}$, UDP-D-GlcNAc o UDP-D-GalNAc y LPS de *K. pneumoniae*Δ*wabH* se ha podido demostrar la función de estos enzimas como transferasas específicas de D-GlcNAc (WabH) y D-GalNAc (WabP).

Los genes de *P. mirabilis* homólogos al gen *wabN* de *K. pneumoniae* presentan un grado de

identidad muy elevado entre si y, independientemente de la cepa de que proceden, pueden complementar el mutante *K. pneumoniae* Δ*wabN*, lo que sugiere que las desacetilasas codificadas por estos genes sean capaces de desacetilar tanto núcleos de LPS que contienen D-GlcNAc como D-GalNAc. Un ensayo *in vitro* ha permitido demostrar esta hipótesis.

El gen *gne* debería estar presente en las cepas de *P. mirabilis* que contienen D-GalN en el núcleo del LPS y también en aquellas cepas que contienen D-GlcN en la estructura del núcleo cuando éstas posean D-GalNAc en el antígeno O. Por tanto, la presencia de *gne* no sirve como diagnóstico para determinar si una cepa presenta D-GlcN o D-GalN como segundo residuo del núcleo externo del LPS en *P. mirabilis*. Sin embargo la ausencia del gen *gne* esta estrictamente relacionada con la presencia del gen *wabH* y del residuo de D-GlcN en el núcleo de LPS de las cepas ensayadas. Una forma fácil de predecir la presencia de D-GlcN o D-GalN es mediante la amplificación con parejas de oligonucleótidos específicos para *wabH* o *wabP*.

La presencia necesaria de dos (*wabH* and *wabN*) o tres (*wabP*, *wabN* y *gne*) genes para la incorporación de HexN en el núcleo del LPS parece relacionada con la falta de una vía de biosíntesis que permita formar directamente UDP-D-GlcN en las células bacterianas [Gabriel, 1982]. Parece pues, que el mecanismo descrito aquí podría ser un mecanismo general para la incorporación de residuos de HexN al LPS, con la incorporación primero de un residuo de HexNAc y su posterior desacetilación a HexN. También hay evidencias que esta doble actividad enzimática no sea solamente confinada a la familia de las *Enterobacteriaceae* y que no tenga que residir necesariamente en polipéptidos independientes. Recientemente se ha demostrado la presencia, en *A. hydrophila* y *A. salmonicida*, de estas dos actividades enzimaticas (HexNAc transferasa y desacetilasa) en dos dominios diferentes del mismo enzima [Jimenez et al., 2009].

Identificación de los genes específicos en la biosíntesis del núcleo del LPS. La identificación de otros genes involucrados en la biosíntesis del núcleo del LPS se ha llevado a cabo mediante su introducción en mutantes específicos de *K. pneumoniae* 52145, seguida por la selección de aquellos que produjeron un cambio en la movilidad electroforética en Tricina-SDS-PAGE y la determinación de la composición química y de la/s masa/s molecular/es de los correspondientes OSs mediante espectrometría de masas.

Así se han podido identificar y caracterizar dos heptosiltransferasas (WamA y WamC), una galactosiltransferasa (WamB), y una *N*-acetil-glucosaminiltransferase (WamD). El gen *wamA* codifica para la heptosiltransferasa responsable de la transferencia del residuo de D,D-Hep al D-GalA I mediante un enlace α1,2. Este gen se ha encontrado en las cepas de *P. mirabilis* R110, 50/57, TG83 y HI4320, pero no en la cepa 51/57. Esto concuerda con la presencia del disacárido α-D,D-Hep 1→2 α-D-GalA en la estructura del núcleo de LPS en las cepas R110, 50/57 y TG83, pero no en la cepa 51/57. La presencia del gen *wamA* en la cepa HI4320 indicaba que este mismo disacárido debería estar presente en el núcleo del LPS de dicha cepa, cuya estructura química era desconocida en el momento en que se realizó el estudio. La elucidación reciente de la estructura del núcleo LPS de la cepa de *P. mirabilis* HI4320 [Vinogradov, 2011] ha confirmado la hipótesis anterior. Los genes *wamB* y *wamC*, que codifican respectivamente para una galactosiltransferasa y una heptosiltransferasa, se han encontrado en la cepa R110. Los enzimas WamB y WamC son responsables de la transferencia de un residuo de D-Gal a la D,D-Hep I y de un residuo de D,D-Hep II a la D-GlcN, respectivamente. Con la identificación de los genes *wamA*, *wamB* y *wamC* se completó la caracterización de todos los genes presentes en la agrupación *waa* de la cepa R110. Sin embargo, para completar la estructura del núcleo del LPS de la cepa R110 es necesaria una glicosiltransferasa adicional, y mas precisamente, una galacturonosiltransferasa, responsable de la transferencia del residuo GalA II a la L,D-Hep III. El gen responsable de codificar este enzima no se encuentra en el agrupamiento génico *waa*.

En la cepa 51/57 se ha estudiado el gen *wamD* y los resultados obtenidos indican que codifica para un enzima responsable de la transferencia de un residuo de D-GlcNAc al residuo de D-GlcN. El gen *wamD* se ha encontrado también el el genoma de la cepa HI4320, sugiriendo la presencia en esta cepa del mismo residuo de GlcNAc. Esta hipótesis inicial ha sido recientemente confirmada gracias a la publicación de la estructura del núcleo de LPS de la cepa HI4320 [Vinogradov, 2011].

La comparación de la estructura química del núcleo de LPS de cada cepa con las glicosiltransferasas codificadas por genes presentes en los agrupamientos génicos *waa* indica, que para la biosíntesis del núcleo de LPS son necesarias glicosiltransferasas adicionales, codificadas por genes localizados fuera de los mencionados agrupamientos *waa*. Esta situación se presenta, en grados diferentes, en todas las cepas de *P. mirabilis* incluidas en este estudio.

En las cepas estudiadas en este trabajo, y en la mayoría de las cepas de *Proteus* spp. caracterizadas desde el punto de vista químico, hay un segundo residuo de GalA (D-GalA II) unido al residuo de L,D-Hep III. El análisis bioinformático de todas las glicosiltransferasas presentes en el genoma de la cepa HI4320de *P. mirabilis* permitió seleccionar la ORF PMI2517 como candidata para la transferencia de este segundo residuo de D-GalA. Esta hipótesis ha sido confirmada mediante la complementación del mutante *K. pneumoniae* 52145Δ*wabO* con PMI2517. Por lo tanto, la ORF PMI2517 de *P. mirabilis* corresponde a un gen funcionalmente homólogo al gen *wabO* de *K. pneumoniae* donde se ha demostrado que codifica una galacturonosiltransferasa responsable de la transferencia del D-GalA II a la L,D-Hep III [Fresno et al., 2007]. El uso de una pareja de oligonucleótidos diseñados al efecto ha permitido amplificar fragmentos de DNA que contienen el gen *wabO* a partir del genoma de cada una de las cepas de *P. mirabilis* estudiadas en este trabajo.

En la cepa de *P. mirabilis* 51/57, se ha identificado, en el agrupamiento genico *waa*, un gen que codifica una proteína de la familia Mig-14. El análisis del genoma de la cepa HI4320 de *P. mirabilis* no ha evidenciado la presencia de ningún gen parecido. En *P. aeruginosa* PAO1 un gen similar, denominado *mig-14*, se encuentra dentro del agrupamiento génico *waa*, lo que sugiere que podría tener alguna función desconocida en la biosíntesis del núcleo de LPS. En contraste, en las enterobacterias, los genes parecidos a *mig-14* se encuentran lejos de la agrupación génica *waa*, como en *S. enterica enterica* LT2. Por lo tanto, la localización del gen *mig-14* en la cepa de *P. mirabilis* 51/57 parece ser una excepción en la familia *Enterobacteriac*eae. En este grupo el gen mejor estudiado es el *mig-14* de *S. enterica* LT2 [Brodsky et al, 2002; Brodsky et al, 2005], en esta especie *mig-14* participa en la virulencia y juega un papel en la resistencia a los péptidos antimicrobianos catiónicos [Brodsky et al., 2002]. Datos recientes sugieren que *mig-14* contribuye a las infecciones persistentes, y promueve la replicación de *Salmonella* en los macrófagos activados. Por lo tanto, el homologo *mig-14* de *P. mirabilis* 51/57 no parece ser un candidato a codificar una glicosiltransferasa, o al menos no una pertenecientes a las familias ya descritas de estos enzimas. También existe la posibilidad de que el homólogo *mig-14* pueda ser el sensor de un sistema regulador que podría inducir la expresión de determinados genes y entre ellos quizás glicosiltransferasas.

Transferasas de fosfoetanolamina. Una característica común de los núcleos del LPS de las cepas de *P. mirabilis* incluidas en este estudio, y en la mayoría de las cepas de *Proteus* spp. estudiadas

desde el punto de vista químico, es la presencia de un grupo de fosfoetanolamina (PEtN) sustituyendo el O-6 de la L,D-Hep II (HepII6-PEtN). No hay genes que codifiquen enzimas supuestamente responsables para esta modificación en el grupo de genes *waa*. En varios patógenos de las mucosas no entéricos, tales como *Neisseria meningitidis*, *N. gonorrhoeae* y *Haemophilus influenzae*, se encuentra un residuo de PEtN en la misma posición. La transferencia de este residuo de PEtN en *N. meningitidis*, *N. gonorrhoeae* y en *H. influenzae* la lleva a cabo una proteína, Lpt-6, altamente conservada a nivel de secuencia de aminoácidos en estas tres especies [Cox, et al., 2003; Wright et al., 2004]. El análisis del genoma de *P. mirabilis* HI4320 mediante los programas BLASTX y BLASTP ha permitido la identificación de la ORF PMI3104, en base a su similitud a Lpt-6, como la mejor candidata a PEtN transferasa, supuestamente implicada en la biosíntesis de la L,D-Hep-II6PEtN. Inicialmente, esta hipótesis fue respaldada por el cambio de movilidad en Tricina-SDS PAGE inducido por gen PMI3104 introducido en los mutantes *K. pneumoniae* 52145Δ*wabG* y 52145Δ*wabH*. Posteriormente, la presencia de PEtN en la posición O-6 de la L,D-Hep II ha sido confirmada mediante el análisis de la composición química, espectrometría de masas (MS), y resonancia magnética nuclear (NMR) del OS del núcleo de la cepa *K. pneumoniae* 52145Δ*wabG* transformada con pBAD18-PMI3104.

Para determinar la función de la PEtN unida a la L,D-Hep II en la biología de *P. mirabilis* se ha construido un mutante de *P. mirabilis* HI4320 defectivo en PMI3104. Se ha verificado que esta mutación no afecta a la cinética de crecimiento en condiciones estándar de laboratorio sea en medios ricos o definidos. El análisis y la comparación de la movilidad en geles de SDS PAGE y Tricina-SDS PAGE de los LPS extraídos de las cepas salvaje y mutada, demuestra que el mutante es todavía capaz de producir antígeno O y que su núcleo de LPS tiene una menor movilidad. Con el fin de determinar un posible cambio en la permeabilidad y/o estabilidad de la membrana externa causada por la mutación, se ha determinado la concentración mínima inhibitoria (MIC) para diferentes antibióticos y detergentes (catiónicos, aniónicos y zwitteriónicos) para el mutante PMI3104 y la cepa salvaje HI4320. El aumento de la sensibilidad a compuestos hidrofóbicos y detergentes es una forma, indirecta de estudio de la permeabilidad de la membrana externa, cuantificable mediante la disminución de la MIC del compuesto ensayado. Además, en el caso de compuestos bactericidas se puede cuantificar el efecto sobre la viabilidad bacteriana. La única diferencia observada ha sido una mayor sensibilidad del mutante PMI3104, respecto a la cepa salvaje, frente a SDS. Esta diferencia se ha confirmado determinando el porcentaje de células

viables después de una corta exposición a SDS. La diferencia en la sensibilidad a SDS sugiere una desestabilización moderada de la membrana externa en ausencia de PEtN unida a L,D-Hep II.

Con el fin de investigar el efecto de la ausencia de la PEtN unida a la L,D-Hep II del núcleo de LPS en la patogénesis de *P. mirabilis* durante la infección del tracto urinario, se ha utilizado un ensayo de co-infección en el cual la cepa salvaje y la mutada compiten para generar infección. En este ensayo se ha administrado por vía transuretral una mezcla 1:1 de cultivos de la cepa salvaje y del mutante PMI3104 a ratones CBA/J. Siete días después de la inoculación, se han sacrificado los ratones y se ha determinado y cuantificado la presencia de la cepa salvaje y de la mutante en la orina, en la vejiga y en los riñones. Los resultados indican que no hay diferencias estadísticamente significativas entre la capacidad infecciosa de la cepa salvaje y la de la cepa mutante, al menos en este ensayo.

Con el fin de testar el efecto de la misma mutación en una infección sistémica, se ha ensayado la resistencia de la bacteria en suero humano. Para este experimento se han recolectado varios sueros humanos non inmunes (NHS). Las bacterias, crecidas en fase logarítmica, se han resuspendido en una solución de suero humano al 90%, e incubadas a 37ºC. El recuento de viabilidad se ha hecho diluyendo y placando aliquotas a distintos tiempos hasta las tres horas. La cepa salvaje de *P. mirabilis* ha demostrado ser resistente a la actividad bactericida del NHS (98% de supervivencia después de 3 horas), al contrario, las cepas mutadas, *eptC::kan* y *eptC*, han evidenciado una moderada sensibilidad (menos que el 20% de supervivencia después de 3 horas). Estos resultados sugieren que la ausencia de esta modificación con PEtN pueda comprometer la capacidad de realizar una infección sistémica.

La ausencia de PEtN, y por lo tanto la ausencia de una carga positiva en el núcleo de LPS en la cepa mutante podía sugerir un aumento de la resistencia a péptidos antimicrobianos (AMP) como la polimixina B, pero no se han observado cambios en la sensibilidad frente a polimixina B entre el mutante y la cepa salvaje. Una posible razón para este resultado puede ser la modificación de las moléculas de LPS con un residuo de L-Ara4N en la posición 4 del lípido A y en el primer residuo de Kdo del núcleo interno [Vinogradov et al., 2000; Vinogradov, 2011]. Estas modificaciones con L-Ara4N explican además la alta resistencia innata de *P. mirabilis* a la polimixina B y a otros AMP. De acuerdo con estas modificaciones con L-Ara4N, se han detectado en el genoma de la cepa

HI4320 de *P. mirabilis* dos genes que codifican homólogos del gen *arnT* de *Salmonella enterica* [Gunn, 2008]. El gen *arnT* codifica para una transferasa de L-Ara4N. El homólogo de *arnT* PMI1047 de la cepa HI4320 se encuentra, al igual que en *S. entérica*, en el operón *arn*, que contiene los genes necesarios para la síntesis, activación y transferencia de la L-Ara4N al lípido A [Gunn, 2008]. El homólogo de *arnT* PMI0275 está situado entre *mtrf*, que codifica para un componente de una bomba de eflujo, y *zapD*, que codifica para una proteína de secreción de tipo I en la membrana externa [Pearson et al., 2008]. Por lo tanto, a partir de este análisis se puede especular que PMI1047 y PMI0275 corresponden respectivamente a la transferasas responsables de las modificaciones con L-Ara4N del lípido A y del primer Kdo del núcleo interno. La importancia de estas dos modificaciones con L-Ara4N en la resistencia a la polimixina B se ha demostrado recientemente por mutación en uno de los genes de la agrupación génica *arn*, *arnA*, implicado en la biosíntesis de undecaprenol-fosfato-α-L-Ara4N en *P. mirabilis* [Jiang et al., 2010a].

CONCLUSIONES

1. En *Proteus mirabilis* la mayoría de los genes responsables de la biosíntesis de núcleo de LPS están localizados en el cromosoma, en el agrupamiento genico *waa*. A pesar de esto, algunos genes adicionales, necesarios para la biosíntesis del núcleo de LPS, se encuentran ubicados fuera del agrupamiento genico *waa*. Así como el gen *wabO* (PMI2517), presente en todas las cepas estudiadas, que codifica para la enzima que transfiere el residuo de D-GalA II a la L,D-Hep II del núcleo interno.

2. En el agrupamiento genico *waa* se han localizado nueve genes implicados en la biosíntesis del núcleo de LPS en *P. mirabilis*. Estos genes son responsables de la transferencia de ocho residuos comunes. El pentasacárido del núcleo interno, común a todas las *Enterobacteriáceae*, se biosintetiza en *P. mirabilis*, por la actividad secuencial de una transferasa bifunciona (WaaA) y tres heptosiltransferasas (WaaC, WaaF, y WaaQ). Estas enzimas están conservadas en todas las cepas de *P. mirabilis* analizadas y muestran un elevado nivel de identidad y de similitud con las secuencias de proteinas homologas de *E. coli*, *K. pneumoniae* y *S. marcescens*.

La función de las diferentes glicosiltransferasas se ha asignado a la estructura química del núcleo de LPS de *P. mirabilis* de acuerdo con el siguiente esquema:
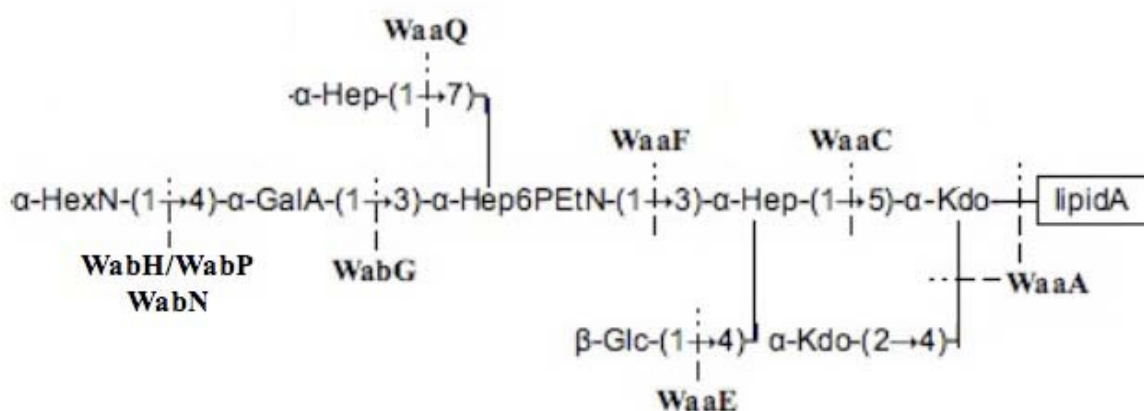
3. El gen *waaL*, que codifica para la ligasa del antígeno-O, se encuentra adyacente agrupamiento genico *waa* clásico. Tras este gen, se han encontrado cuatro genes que codifican enzimas que pertenecen a las familias 4 (*walM*, *walN*, y *walR*), y 9 (*walO*) de glicosiltransferasas. Aun que los miembros de estas familias estén relacionados con la biosíntesis del núcleo de LPS en varias bacterias Gram-negativas, en *P. mirabilis* no parecen estar implicados en la biosíntesis de las estructuras reportadas del núcleo de LPS.

4. La presencia del disacárido HexN-1,4-GalA es característica del núcleo externo de LPS en *P. mirabilis*. Dependiendo de la naturaleza del residuo de HexN, se encuentran, en el agrupamiento genico *waa*, dos HexNAc transferasas diferentes: *wabH* o *wabP*. Se ha demostrado que si la incorporación de GlcN en núcleo de LPS requiere una acetilglucosaminiltransferasa (WabH) y una deacetilasa (WabN); la incorporación de GalN requiere tres enzimas: una acetilgalactosaminiltransferasa (WabP), la deacetilasa (WabN) y una epimerasa (Gne). Una prueba de amplificación con cebadores específicos para *wabH* y *wabP* puede utilizarse para predecir la naturaleza de la HexN del núcleo en *P. mirabilis*.

5. Los genes *wamB* y *wamC* de la cepa de *P. mirabilis* R110 codifican para una galactosiltransferasa y una heptosiltransferasa respectivamente. El enzima codificado por el gen *wamD* es una N-acetilglucosaminiltransferasa, y se encuentra en la cepa de *P. mirabilis* 51/57. WamA, codificada por el gen *wamA,* que se encuentra en el agrupamiento genico *waa* de las cepas R110, 50/57, TG83 y HI4320, es la heptosiltransferasa responsable de la incorporación del cuarto residuo de D,D-Hep a la GalA II del núcleo externo.

6. En *P. mirabilis* 51/57 se ha identificado, en la agrupación *waa*, un gen que codifica una proteína de la familia Mig-14; esta localización parece ser una excepción en la familia *Enterobacteriaceae*. En el genoma de *P. mirabilis* HI4320, no se ha encontrado ningún gen similar a *mig-14*.

190

7. En el genoma de *P. mirabilis* se ha evidenciado la presencia de tres posibles fosfoetanolamina transferasas: PMI3040, PMI3576 y PMI3104. El gen *eptC* (PMI3104) codifica para la enzima que transfiere el residuo de fosfoetanolamina a la posición O-6 de la L,D-Hep II (HepII6PEtN), en el núcleo de LPS de *P. mirabilis*.

8. La ausencia de la carga positiva del residuo de fosfoetanolamina no afecta a la cinética de crecimiento de las bacterias en condiciones standard de laboratorio sea en medios ricos o definidos. La ausencia del residuo fosfoetanolamina provoca una desestabilización moderada de la membrana externa que se traduce en una disminución de la MIC para SDS.

9. La falta del residuo de fosfoetanolamina en la Heptosa II en el núcleo de LPS en *P. mirabilis* no tiene efectos estadísticamente significativos en la infección del tracto urinario en ratones. La ausencia de la misma modificación causa un incremento de la sensibilidad frente al complemento en el suero inmune.

PERSPECTIVAS FUTURAS

Los resultados obtenidos en este trabajo han permitido identificar todos los genes necesarios para la biosíntesis de la estructura del núcleo del LPS en la cepa de *P. mirabilis* R110. Sin embargo, en la cepa de *P. mirabilis* HI4320 falta todavía identificar un gen necesario para la transferencia de un tercer residuo de D-GalA descrito en la estructura química de esta cepa y que sigue siendo desconocido [Vinogradov, 2011]. Así, en el futuro se intentara identificar dicho gen en base a las glicosiltransferasas de función desconocida presentes en el genoma de la cepa HI4320.

En varios de los LPS estudiados de *P. mirabilis* se han encontrado aminoácidos, como Gly, Ala y Lys, unidos covalentemente a los azúcares del núcleo [Vinogradov, 2002]. En la cepa de *P. mirabilis* HI4320 en particular se ha encontrado una Lys covalentemente unida al residuo de D-GalA III del núcleo externo [Vinogradov, 2011]. Un análisis inicial de las proteínas codificadas por el genoma de la cepa HI4320, basada en las características conservadas de las amidoligasas, podría ayudar en la identificación de los posibles genes candidatos a codificar un enzima que coloque este residuo de Lys. Además, será necesario generar un mutante que carezca de esta modificación con Lys para determinar la importancia biológica de esta modificación del núcleo del LPS.

Los cuatro genes que supuestamente codifican para glicosiltransferasas pertenecientes a las familias GT4 (*walM*, *walN* y *walR*) y GT9 (*walO*), y que se encuentran después del gen *waaL* (antígeno O u O-PS ligasa) en todas las cepas de *P. mirabilis* analizadas en este trabajo y en la cepa HI4320, presentan una función todavía desconocida. Parece poco probable que estas glicosiltransferasas estén involucradas en la biosíntesis del núcleo estándar del LPS, aunque podrían estar involucradas en la biosíntesis de variantes de LPS específicas para determinados momentos del complejo ciclo vital de esta bacteria, o en la biosíntesis de otros oligo o polisacáridos, como por ejemplo la cápsula. Para aclarar la función de estos genes será necesario obtener mutante/s defectivo en uno o varios de estos genes.

8. References

Adekambi, T., M. Drancourt, and D. Raoult. 2009. The *rpoB* gene as a tool for clinical microbiologists. *Trends in Microbiology* 17 (1) (Jan): 37-45.

Al-Hendy, A., P. Toivanen, and M. Skurnik. 1991. The effect of growth temperature on the biosynthesis of *Yersinia enterocolitica* O:3 lipopolysaccharide: Temperature regulates the transcription of the *rfb* but not of the *rfa* region. *Microbial Pathogenesis* 10 (1)(Jan):81-6.

Alavi, M., and R. Belas. 2001. Surface sensing, swarmer cell differentiation, and biofilm development. *Methods in Enzymology* 336 : 29-40.

Alberti, L., and R. M. Harshey. 1990. Differentiation of *Serratia marcescens* 274 into swimmer and swarmer cells. *Journal of Bacteriology* 172 (8) (Aug): 4322-8.

Allison, C., and C. Hughes. 1991. Bacterial swarming: an example of prokaryotic differentiation and multicellular behavior. *Science Progress* 75 (298 Pt 3-4): 403-22.

Allison, C., H. C. Lai, and C. Hughes. 1992a. Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. *Molecular Microbiology* 6 (12) (Jun): 1583-91.

Allison, C., H. C. Lai, D. Gygi, and C. Hughes. 1993. Cell differentiation of *Proteus mirabilis* is initiated by glutamine, a specific chemoattractant for swarming cells. *Molecular Microbiology* 8 (1) (Apr): 53-60.

Allison, C., L. Emody, N. Coleman, and C. Hughes. 1994. The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. *The Journal of Infectious Diseases* 169 (5) (May): 1155-8.

Allison, C., N. Coleman, P. L. Jones, and C. Hughes. 1992b. Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infection and Immunity* 60 (11) (Nov): 4740-6.

Amor, K., D. E. Heinrichs, E. Frirdich, K. Ziebell, R. P. Johnson, and C. Whitfield. 2000. Distribution of core oligosaccharide types in lipopolysaccharides from *Escherichia coli*. *Infection and Immunity* 68 (3) (Mar): 1116-24.

Anderson, M. S., S. S. Eveland, and N. P. Price. 2000. Conserved cytoplasmic motifs that distinguish sub-groups of the polyprenol phosphate:N-acetylhexosamine-1-phosphate transferase family. *FEMS Microbiology Letters* 191 (2) (Oct 15): 169-75.

References

Aoki, S. K., R. Pamma, A. D. Hernday, J. E. Bickham, B. A. Braaten, and D. A. Low. 2005. Contact-dependent inhibition of growth in *Escherichia coli*. *Science (New York, N.Y.)* 309 (5738) (Aug 19): 1245-8.

Apfel, C. M., B. Takacs, M. Fountoulakis, M. Stieger, and W. Keck. 1999. Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: Cloning, expression, and characterization of the essential *uppS* gene. *Journal of Bacteriology* 181 (2) (Jan): 483-92.

Arakawa, Y., R. Wacharotayankun, T. Nagatsuka, H. Ito, N. Kato, and M. Ohta. 1995. Genomic organization of the *Klebsiella pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *Journal of Bacteriology* 177 (7) (Apr): 1788-96.

Armbruster C. and H. L. Mobley. 2012. Merging mythology and morphology: The multifaceted lifestyle of *Proteus mirabilis*. *Nature Reviews Microbiology* 10 (Nov): 743-754.

Armitage, J. P. 1981. Changes in metabolic activity of *Proteus mirabilis* during swarming. *Journal of General Microbiology* 125 (2) (Aug): 445-50.

Armitage, J. P., and D. G. Smith. 1978. Flagella development during swarmer differentiation in *Proteus mirabilis*. *FEMS Microbiol. Lett.* 4 : 163-5.

Armitage, J. P., D. G. Smith, and R. J. Rowbury. 1979. Alterations in the cell envelope composition of *Proteus mirabilis* during the development of swarmer cells. *Biochimica Et Biophysica Acta* 584 (3) (May 16): 389-97.

Armitage, J. P., R. J. Rowbury, and D. G. Smith. 1974. The effects of chloramphenicol, nalidixic acid and penicillin on the growth and division of swarming cells of *Proteus mirabilis*. *Journal of Medical Microbiology* 7 (4) (Nov): 459-64.

Babinski, K. J., S. J. Kanjilal, and C. R. Raetz. 2002. Accumulation of the lipid A precursor UDP-2,3-diacylglucosamine in an *escherichia coli* mutant lacking the *lpxH* gene. *The Journal of Biological Chemistry* 277 (29) (Jul 19): 25947-56.

Bahrani-Mougeot, F. K., E. L. Buckles, C. V. Lockatell, J. R. Hebel, D. E. Johnson, C. M. Tang, and M. S. Donnenberg. 2002. Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract. *Molecular Microbiology* 45 (4) (Aug): 1079-93.

Bahrani, F. K., D. E. Johnson, D. Robbins, and H. L. Mobley. 1991. *Proteus mirabilis* flagella and MR/P fimbriae: Isolation, purification, N-terminal analysis, and serum antibody response

following experimental urinary tract infection. *Infection and Immunity* 59 (10) (Oct): 3574-80.

Bailey, M. J., Colin Hughes, and Vassilis Koronakis. 1997. RfaH and the *ops* element, components of a novel system controlling bacterial transcription elongation. *Molecular Microbiology* 26 (5): 845-51

Bartodziejska, B., F. V. Toukach, E. V. Vinogradov, S. N. Senchenkova, A. S. Shashkov, A. Ziolkowski, J. Czaja, M. B. Perry, Y. A. Knirel, and A. Rozalski. 2000. Structural and serological studies of the related O-specific polysaccharides of *Proteus vulgaris* O21 and *Proteus mirabilis* O48 having oligosaccharide-phosphate repeating units. *European Journal of Biochemistry / FEBS* 267 (23) (Dec): 6888-96.

Basu, S., J. Radziejewska-Lebrecht, and H. Mayer. 1986. Lipopolysaccharide of *Providencia rettgeri*. chemical studies and taxonomical implications. *Archives of Microbiology* 144 (3) (Apr): 213-8.

Belas, R. 1992. The swarming phenomenon of *Proteus mirabilis*. *Am. Soc. Microbiol. News* 58 : 15-22.

Belas, R. 1994. Expression of multiple flagellin-encoding genes of *Proteus mirabilis*. *Journal of Bacteriology* 176 (23) (Dec): 7169-81.

Belas, R., and R. Suvanasuthi. 2005. The ability of *Proteus mirabilis* to sense surfaces and regulate virulence gene expression involves FliL, a flagellar basal body protein. *Journal of Bacteriology* 187 (19) (Oct): 6789-803.

Belas, R., D. Erskine, and D. Flaherty. 1991. Transposon mutagenesis in *Proteus mirabilis*. *Journal of Bacteriology* 173 (19) (Oct): 6289-93.

Belas, R., R. Schneider, and M. Melch. 1998. Characterization of *Proteus mirabilis* precocious swarming mutants: Identification of *rsbA*, encoding a regulator of swarming behavior. *Journal of Bacteriology* 180 (23) (Dec): 6126-39.

Bennett-Guerrero, E., T. J. McIntosh, G. R. Barclay, D. S. Snyder, R. J. Gibbs, M. G. Mythen, and I. R. Poxton. 2000. Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine. *Infection and Immunity* 68 (11) (Nov): 6202-8.

Berner, I., S. Konetschny-Rapp, G. Jung, and G. Winkelmann. 1988. Characterization of ferrioxamine E as the principal siderophore of *Erwinia herbicola* (*Enterobacter agglomerans*). *Biology of Metals* 1 (1): 51-6.

References

Bessler, W. G., A. Lex, B. Suhr, A. Ortmann, S. Schlecht, H. Bühring, C. Müller, MetzgerJ., K. H. Wiesmüller, and G. Jung. 1986. The synthetic analogs of bacterial lipoprotein are protect immunoadjuvants in combination with or covalently linked to antigen. In J. Oppenheim and D. Jacobs (ed.), *Leukocytes and host defense*, vol. 5. Alan R. Liss, Inc., New York, N.Y. : 337-48.

Bessler, W. G., and U. Henning. 1979. Protein I and protein II from the outer membrane of *Escherichia coli* are mouse B-lymphocyte mitogens. *Zeitschrift Fur Immunitatsforschung. Immunobiology* 155 (5) (Jun): 387-98.

Beynon, L. M., A. J. Dumanski, R. J. McLean, L. L. MacLean, J. C. Richards, and M. B. Perry. 1992. Capsule structure of *Proteus mirabilis* (ATCC 49565). *Journal of Bacteriology* 174 (7) (Apr): 2172-7.

Bisset, K. A. 1973 a. The motion of the swarm in *Proteus mirabilis*. *Journal of Medical Microbiology* 6 (1) (Feb): 33-5.

Bisset, K. A. 1973 b. The zonation phenomenon and structure of the swarm colony in *Proteus mirabilis*. *Journal of Medical Microbiology* 6 (4) (Nov): 429-33.

Boll, M., J. Radziejewska-Lebrecht, C. Warth, D. Krajewska-Pietrasik, and H. Mayer. 1994. 4-amino-4-deoxy-L-arabinose in LPS of enterobacterial R-mutants and its possible role for their polymyxin reactivity. *FEMS Immunology and Medical Microbiology* 8 (4) (May): 329-41.

Bos, M. P., V. Robert, and J. Tommassen. 2007. Biogenesis of the gram-negative bacterial outer membrane. *Annual Review of Microbiology* 61 : 191-214.

Boucher, Y., and W. F. Doolittle. 2000. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Molecular Microbiology* 37 (4) (Aug): 703-16.

Bouhss, A., A. E. Trunkfield, T. D. Bugg, and D. Mengin-Lecreulx. 2008. The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiology Reviews* 32 (2) (Mar): 208-33.

Brabetz, W., B. Lindner, and H. Brade. 2000. Comparative analyses of secondary gene products of 3-deoxy-D-manno-oct-2-ulosonic acid transferases from *Chlamydiaceae* in *Escherichia coli* K-12. *European Journal of Biochemistry / FEBS* 267 (17) (Sep): 5458-65.

Brandenburg, K., U. Seydel, A. B. Schromm, H. Loppnow, M. H. J. Koch, and E. T. Rietschel. 1996. Conformation of lipid A, the endotoxic center of bacterial lipopolysaccharide. *J. Endotoxin Res.* 3 : 173.

Braude, A. I., and J. Siemienski. 1960. Role of bacterial urease in experimental pyelonephritis. *Journal of Bacteriology* 80 (Aug): 171-9.

Braun, V., and T. Focareta. 1991. Pore-forming bacterial protein hemolysins (cytolysins). *Critical Reviews in Microbiology* 18 (2): 115-58.

Bray, D., and P. W. Robbins. 1967. The direction of chain growth in *Salmonella anatum* O-antigen biosynthesis. *Biochemical and Biophysical Research Communications* 28 (3) (Aug 7): 334-9.

Breitenbucher, R. B. 1984. Bacterial changes in the urine samples of patients with long-term indwelling catheters. *Archives of Internal Medicine* 144 (8) (Aug): 1585-8.

Brenner, D. J., F. W. Hickman-Brenner, B. Holmes, P. M. Hawkey, J. L. Penner, P. A. Grimont, and C. M. O'Hara. 1995. Replacement of NCTC 4175, the current type strain of *Proteus vulgaris*, with ATCC 29905. request for an opinion. *International Journal of Systematic Bacteriology* 45 (4) (Oct): 870-1.

Brenner, D. J., J. J. Farmer J. J., Fanning G. R., Steigerwalt A. G., Klykken P., Wathen H. G., Hickman F. W., and and Ewing W. H. 1978. Deoxyribonucleic acid relatedness of *Proteus providencia* species. *Int. J. Syst. Bacteriol.* 28 : 269-82.

Brodsky, I. E., N. Ghori, S. Falkow, and D. Monack. 2005. Mig-14 is an inner membrane-associated protein that promotes S*almonella typhimurium* resistance to CRAMP, survival within activated macrophages and persistent infection. *Molecular Microbiology* 55 (3) (Feb): 954-72

Brodsky, I. E., R. K. Ernst, S. I. Miller, and S. Falkow. 2002. Mig-14 is a *Salmonella* gene that plays a role in bacterial resistance to antimicrobial peptides. *Journal of Bacteriology* 184 (12) (Jun): 3203-13.

Bronner, D., B. R. Clarke, and C. Whitfield. 1994. Identification of an ATP-binding cassette transport system required for translocation of lipopolysaccharide O-antigen side-chains across the cytoplasmic membrane of *Klebsiella pneumoniae* serotype O1. *Molecular Microbiology* 14 (3) (Nov): 505-19.

References

Bub, F., P. Bieker, H. H. Martin, and K. Nixdorff. 1980. Immunological characterization of two major proteins isolated from the outer membrane of *Proteus mirabilis*. *Infection and Immunity* 27 (2) (Feb): 315-21.

Budding, A. E., C. J. Ingham, W. Bitter, C. M. Vandenbroucke-Grauls, and P. M. Schneeberger. 2009. The dienes phenomenon: Competition and territoriality in swarming *Proteus mirabilis*. *Journal of Bacteriology* 191 (12) (Jun): 3892-900.

Bugert, P., and K. Geider. 1995. Molecular analysis of the ams operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Molecular Microbiology* 15 (5) (Mar): 917-33.

Burall, L. S., J. M. Harro, X. Li, C. V. Lockatell, S. D. Himpsl, J. R. Hebel, D. E. Johnson, and H. L. Mobley. 2004. *Proteus mirabilis* genes that contribute to pathogenesis of urinary tract infection: Identification of 25 signature-tagged mutants attenuated at least 100-fold. *Infection and Immunity* 72 (5) (May): 2922-38.

Burns, S. M., and S. I. Hull. 1998. Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5. *Infection and Immunity* 66 (9) (Sep): 4244-53.

Calvo, J. M., and R. G. Matthews. 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. *Microbiol.Rev.* 58 : 466-90.

Campbell, J. A., G. J. Davies, V. Bulone, and B. Henrissat. 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *The Biochemical Journal* 326 (Pt 3) (Pt 3) (Sep 15): 929-39.

Cao, B., M. Wang, L. Liu, Z. Zhou, S. Wen, A. Rozalski, and L. Wang. 2009. 16S-23S rDNA internal transcribed spacer regions in four *Proteus* species. *Journal of Microbiological Methods* 77 (1) (Apr): 109-18.

Caprodici, C., S. Chen, Z. Sidorczyk, P. Elsbach, and J. Weiss. 1994. Effects of lipopolysaccharide (LPS) chain length on interactions of bactericidal/permeability-increasing protein and its bioactive 23-kilodalton NH2-terminal fragment with isolated LPS and intact *Proteus mirabilis* and *Escherichia coli*. *Infect. Immun.* 62 : 259-65.

Carty, S. M., K. R. Sreekumar, and C. R. Raetz. 1999. Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. induction at 12 degrees C of an acyltransferase specific for

palmitoleoyl-acyl carrier protein. *The Journal of Biological Chemistry* 274 (14) (Apr 2): 9677-85.

Cedzynski, M., A. Rozalski, K. Kotelko, W. Kaca, E. V. Vinogradov, Y. A. Knirel, and N. K. Kochetkov. 1993. Structural and immunologic studies of *Proteus mirabilis* 033 O-specific polysaccharide. *Medycyna Doswiadczalna i Mikrobiologia* 45 (1): 93-7.

Cedzynski, M., Y. A. Knirel, A. Rozalski, A. S. Shashkov, E. V. Vinogradov, and W. Kaca. 1995. The structure and serological specificity of *Proteus mirabilis* O43 O antigen. *European Journal of Biochemistry / FEBS* 232 (2) (Sep 1): 558-62.

Cellini, L., R. Piccolomini, N. Allocati, and G. Ravagnan. 1987. Adhesive properties of *Proteus* genus related to antimicrobial agents resistance. *Microbiologica* 10 (3) (Jul): 291-9.

Chen, C. Y., Y. H. Chen, P. L. Lu, W. R. Lin, T. C. Chen, and C. Y. Lin. 2012. *Proteus mirabilis* urinary tract infection and bacteremia: Risk factors, clinical presentation, and outcomes. *Journal of Microbiology, Immunology, and Infection* 45 (3) (Jun): 228-36.

Chen, Y. H., R. E. Hancock, and R. I. Mishell. 1980. Mitogenic effects of purified outer membrane proteins from *Pseudomonas aeruginosa*. *Infection and Immunity* 28 (1) (Apr): 178-84.

Chernyak, A. Y., L. O. Kononov, and N. K. Kochetkov. 1994. Glycopolymers from synthetic fragments (amides of a-D-galacturonic acid with amino acids) of *Proteus* O-antigens. *J. Carbohydr. Chem.* 13 : 386-96.

Chernyak, A. Ya, L. O. Kononov, P. R. Krishna, N. K. Kochetkov, and A. V. Rao. 1992. Synthesis of lysine-containing fragments of the *Proteus mirabilis* O27 O-specific polysaccharide and neoglycoconjugates therefrom. *Carbohydrate Research* 225 (2) (Mar 2): 279-89.

Chow, A. W., P. R. Taylor, T. T. Yoshikawa, and L. B. Guze. 1979. A nosocomial outbreak of infections due to multiply resistant *Proteus mirabilis*: Role of intestinal colonization as a major reservoir. *The Journal of Infectious Diseases* 139 (6) (Jun): 621-7.

Clapham, L., R. J. C. McLean, J. C. Nickel, J. Downey, and J. W. Costerton. 1990. The influence of bacteria on struvite crystal habit and its importance in urinary stone formation. *J. Crystal Growth* 104 : 475-84.

Clayton, C. L., J. C. Chawla, and D. J. Stickler. 1982. Some observations on urinary tract infections in patients undergoing long-term bladder catheterization. *The Journal of Hospital Infection* 3 (1) (Mar): 39-47.

Clementz, T., and C. R. Raetz. 1991. A gene coding for 3-deoxy-D-manno-octulosonic-acid transferase in *Escherichia coli.* identification, mapping, cloning, and sequencing. *The Journal of Biological Chemistry* 266 (15) (May 25): 9687-96.

Clementz, T., J. J. Bednarski, and C. R. Raetz. 1996. Function of the *htrB* high temperature requirement gene of *Escherchia coli* in the acylation of lipid A: HtrB catalyzed incorporation of laurate. *The Journal of Biological Chemistry* 271 (20) (May 17): 12095-102.

Clementz, T., Z. Zhou, and C. R. Raetz. 1997. Function of the *Escherichia coli msbB* gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipid A. acylation by MsbB follows laurate incorporation by HtrB. *The Journal of Biological Chemistry* 272 (16) (Apr 18): 10353-60.

Clemmer, K. M., and P. N. Rather. 2007. Regulation of *flhDC* expression in *Proteus mirabilis*. *Research in Microbiology* 158 (3) (Apr): 295-302.

Clemmer, K. M., and P. N. Rather. 2008. The Lon protease regulates swarming motility and virulence gene expression in *Proteus mirabilis*. *Journal of Medical Microbiology* 57 (Pt 8) (Aug): 931-7.

Coderch, N., N. Pique, B. Lindner, N. Abitiu, S. Merino, L. Izquierdo, N. Jimenez, J. M. Tomas, O. Holst, and M. Regue. 2004. Genetic and structural characterization of the core region of the lipopolysaccharide from *Serratia marcescens* N28b (serovar O4). *Journal of Bacteriology* 186 (4) (Feb): 978-88.

Cohen-Nahum, K., L. Saidel-Odes, K. Riesenberg, F. Schlaeffer, and A. Borer. 2010. Urinary tract infections caused by multi-drug resistant *Proteus mirabilis*: Risk factors and clinical outcomes. *Infection* 38 (1) (Feb): 41-6.

Coker, C., C. A. Poore, X. Li, and H. L. Mobley. 2000. Pathogenesis of *Proteus mirabilis* urinary tract infection. *Microbes and Infection / Institut Pasteur* 2 (12) (Oct): 1497-505.

Collins, L. V., and J. Hackett. 1991. Molecular cloning, characterization, and nucleotide sequence of the *rfc* gene, which encodes an O-antigen polymerase of *Salmonella typhimurium*. *Journal of Bacteriology* 173 (8) (Apr): 2521-9.

Cosenza, B. J., and J. D. Podgwaite. 1966. A new species of *Proteus* isolated from larvae of the gypsy moth *Porthetria dispar* (L.). *Antonie Van Leeuwenhoek* 32 (2): 187-91.

Costas, M., B. Holmes, K. A. Frith, C. Riddle, and P. M. Hawkey. 1993. Identification and typing of *Proteus penneri* and *Proteus vulgaris* biogroups 2 and 3, from clinical sources, by computerized analysis of electrophoretic protein patterns. *The Journal of Applied Bacteriology* 75 (5) (Nov): 489-98.

Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie. 1987. Bacterial biofilms in nature and disease. *Annual Review of Microbiology* 41 : 435-64.

Costerton, J. W., R. T. Irvin, and K. J. Cheng. 1981. The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology* 35 : 299-324.

Coutinho, P. M., E. Deleury, G. J. Davies, and B. Henrissat. 2003. An evolving hierarchical family classification for glycosyltransferases. *Journal of Molecular Biology* 328 (2) (Apr 25): 307-17.

Cox, A. D., J. C. Wright, J. Li, D. W. Hood, E. R. Moxon, and J. C. Richards. 2003. Phosphorylation of the lipid A region of meningococcal lipopolysaccharide: Identification of a family of transferases that add phosphoethanolamine to lipopolysaccharide. *Journal of Bacteriology* 185 (11) (Jun): 3270-7.

Cravens, D. D., and S. Zweig. 2000. Urinary catheter management. *American Family Physician* 61 (2) (Jan 15): 369-76.

Creeger, E. S., and L. I. Rothfield. 1979. Cloning of genes for bacterial glycosyltransferases. I. selection of hybrid plasmids carrying genes for two glucosyltransferases. *The Journal of Biological Chemistry* 254 (3) (Feb 10): 804-10.

Crowell, D. N., M. S. Anderson, and C. R. Raetz. 1986. Molecular cloning of the genes for lipid A disaccharide synthase and UDP-N-acetylglucosamine acyltransferase in *Escherichia coli*. *Journal of Bacteriology* 168 (1) (Oct): 152-9.

Crowell, D. N., W. S. Reznikoff, and C. R. Raetz. 1987. Nucleotide sequence of the *Escherichia coli* gene for lipid A disaccharide synthase. *Journal of Bacteriology* 169 (12) (Dec): 5727-34.

Currie, C. G., and I. R. Poxton. 1999. The lipopolysaccharide core type of *Escherichia coli* O157:H7 and other non-O157 verotoxin-producing *E. coli*. *FEMS Immunology and Medical Microbiology* 24 (1) (May): 57-62.

References

Currie, C. G., K. McCallum, and I. R. Poxton. 2001. Mucosal and systemic antibody responses to the lipopolysaccharide of *Escherichia coli* O157 in health and disease. *Journal of Medical Microbiology* 50 (4) (Apr): 345-54.

Cusick, K., Y. Y. Lee, B. Youchak, and R. Belas. 2012. Perturbation of FliL interferes with *Proteus mirabilis* swarmer cell gene expression and differentiation. *Journal of Bacteriology* 194 (2) (Jan): 437-47.

Dal Nogare, A. R. 1991. Septic shock. *The American Journal of the Medical Sciences* 302 (1) (Jul): 50-65.

Dal Nogare, A. R., N. Dan, and M. A. Lehrman. 1998. Conserved sequences in enzymes of the UDP-GlcNAc/MurNAc family are essential in hamster UDP-GlcNAc:Dolichol-P GlcNAc-1-P transferase. *Glycobiology* 8 (6) (Jun): 625-32.

Daniels, C., C. Vindurampulle, and R. Morona. 1998. Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy). *Molecular Microbiology* 28 (6) (Jun): 1211-22.

De Angelis, P. L. 1999. Hyaluronan synthases: Fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. *Cellular and Molecular Life Sciences : CMLS* 56 (7-8) (Nov 15): 670-82.

De Angelis, P. L. 2002. Microbial glycosaminoglycan glycosyltransferases. *Glycobiology* 12 (1) (Jan): 9R-16R.

De Cock, H., K. Brandenburg, A. Wiese, O. Holst, and U. Seydel. 1999. Non-lamellar structure and negative charges of lipopolysaccharides required for efficient folding of outer membrane protein PhoE of *Escherichia coli*. *The Journal of Biological Chemistry* 274 (8) (Feb 19): 5114-9.

Di Padova, F. E., H. Brade, G. R. Barclay, I. R. Poxton, E. Liehl, E. Schuetze, H. P. Kocher, G. Ramsay, M. H. Schreier, and D. B. McClelland. 1993. A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides. *Infection and Immunity* 61 (9) (Sep): 3863-72.

Di Padova, F. E., V. Mikol, G. R. Barclay, I. R. Poxton, H. Brade, and E. T. Rietschel. 1994. Anti-lipopolysaccharide core antibodies. *Progress in Clinical and Biological Research* 388 : 85-94.

Dienes, L. 1946. Reproductive processes in *Proteus* cultures. *Society for Experimental Biology and Medicine (New York, N.Y.)* 63 (2) (Nov): 265-70.

Doerrler, W. T., H. S. Gibbons, and C. R. Raetz. 2004. MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*. *The Journal of Biological Chemistry* 279 (43) (Oct 22): 45102-9.

Doerrler, W. T., M. C. Reedy, and C. R. Raetz. 2001. An *Escherichia coli* mutant defective in lipid export. *The Journal of Biological Chemistry* 276 (15) (Apr 13): 11461-4.

Dolph, P. J., D. R. Majerczak, and D. L. Coplin. 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. *Journal of Bacteriology* 170 (2) (Feb): 865-71.

Drechsel, H., A. Thieken, R. Reissbrodt, G. Jung, and G. Winkelmann. 1993. Alpha-keto acids are novel siderophores in the genera *Proteus*, *Providencia*, and *Morganella* and are produced by amino acid deaminases. *Journal of Bacteriology* 175 (9) (May): 2727-33.

Dufour, A., R. B. Furness, and C. Hughes. 1998. Novel genes that upregulate the *Proteus mirabilis flhDC* master operon controlling flagellar biogenesis and swarming. *Molecular Microbiology* 29 (3) (Aug): 741-51.

Dumanski, A. J., H. Hedelin, A. Edin-Liljegren, D. Beauchemin, and R. J. McLean. 1994. Unique ability of the *Proteus mirabilis* capsule to enhance mineral growth in infectious urinary calculi. *Infection and Immunity* 62 (7) (Jul): 2998-3003.

Eden, C. S., P. Larsson, and H. Lomberg. 1980. Attachment of *Proteus mirabilis* to human urinary sediment epithelial cells in vitro is different from that of *Escherichia coli*. *Infection and Immunity* 27 (3) (Mar): 804-7.

Finlay, B. B. 1990. Cell adhesion and invasion mechanisms in microbial pathogenesis. *Current Opinion in Cell Biology* 2 (5) (Oct): 815-20.

Finlay, B. B., and S. Falkow. 1989. Common themes in microbial pathogenicity. *Microbiological Reviews* 53 (2) (Jun): 210-30.

Forsberg, L. S., and R. W. Carlson. 1998. The structures of the lipopolysaccharides from *Rhizobium etli* strains CE358 and CE359. the complete structure of the core region of *R. etli* lipopolysaccharides. *The Journal of Biological Chemistry* 273 (5) (Jan 30): 2747-57.

Foxman, B. 2002. Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *The American Journal of Medicine* 113 Suppl 1A (Jul 8): 5S-13S.

References

Fraser, G. M., L. Claret, R. Furness, S. Gupta, and C. Hughes. 2002. Swarming-coupled expression of the *Proteus mirabilis hpmBA* haemolysin operon. *Microbiology (Reading, England)* 148 (Pt 7) (Jul): 2191-201.

Fresno, S., N. Jimenez, R. Canals, S. Merino, M. M. Corsaro, R. Lanzetta, M. Parrilli, G. Pieretti, M. Regue, and J. M. Tomas. 2007. A second galacturonic acid transferase is required for core lipopolysaccharide biosynthesis and complete capsule association with the cell surface in *Klebsiella pneumoniae*. *Journal of Bacteriology* 189 (3) (Feb): 1128-37.

Frirdich, E., E. Vinogradov, and C. Whitfield. 2004. Biosynthesis of a novel 3-deoxy-D-manno-oct-2-ulosonic acid-containing outer core oligosaccharide in the lipopolysaccharide of *Klebsiella pneumoniae*. *The Journal of Biological Chemistry* 279 (27) (Jul 2): 27928-40.

Frosch, M., C. Weisgerber, and T. F. Meyer. 1989. Molecular characterization and expression in *Escherichia coli* of the gene complex encoding the polysaccharide capsule of *Neisseria meningitidis* group B. *Proceedings of the National Academy of Sciences of the United States of America* 86 (5) (Mar): 1669-73.

Gabriel, O. 1982. Isolation and synthesis of sugar nucleotides. *Methods in Enzymology* 83 : 332-53.

Galloway, S. M., and C. R. Raetz. 1990. A mutant of *Escherichia coli* defective in the first step of endotoxin biosynthesis. *The Journal of Biological Chemistry* 265 (11) (Apr 15): 6394-402.

Ganderton, L., J. Chawla, C. Winters, J. Wimpenny, and D. Stickler. 1992. Scanning electron microscopy of bacterial biofilms on indwelling bladder catheters. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology* 11 (9) (Sep): 789-96.

Garrett, T. A., J. L. Kadrmas, and C. R. Raetz. 1997. Identification of the gene encoding the *Escherichia coli* lipid A 4'-kinase. facile phosphorylation of endotoxin analogs with recombinant LpxK. *The Journal of Biological Chemistry* 272 (35) (Aug 29): 21855-64.

Geerlof, A., A. Lewendon, and W. V. Shaw. 1999. Purification and characterization of phosphopantetheine adenylyltransferase from *Escherichia coli*. *The Journal of Biological Chemistry* 274 (38) (Sep 17): 27105-11.

Genevrois, S., L. Steeghs, P. Roholl, J. J. Letesson, and P. van der Ley. 2003. The Omp85 protein of *Neisseria meningitidis* is required for lipid export to the outer membrane. *The EMBO Journal* 22 (8) (Apr 15): 1780-9.

Giammanco, G. M., P. A. Grimont, F. Grimont, M. Lefevre, G. Giammanco, and S. Pignato. 2011. Phylogenetic analysis of the genera *Proteus*, *Morganella* and *Providencia* by comparison of *rpoB* gene sequences of type and clinical strains suggests the reclassification of *Proteus myxofaciens* in a new genus, C*osenzaea* gen. nov., as C*osenzaea myxofaciens* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 61 (Pt 7) (Jul): 1638-44.

Gibb, A. P., G. R. Barclay, I. R. Poxton, and F. di Padova. 1992. Frequencies of lipopolysaccharide core types among clinical isolates of *Escherichia coli* defined with monoclonal antibodies. *The Journal of Infectious Diseases* 166 (5) (Nov): 1051-7.

Gibbons, H. S., S. R. Kalb, R. J. Cotter, and C. R. Raetz. 2005. Role of Mg2+ and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumor cells. *Molecular Microbiology* 55 (2) (Jan): 425-40.

Gibbs, K. A., L. M. Wenren, and E. P. Greenberg. 2011. Identity gene expression in *Proteus mirabilis*. *Journal of Bacteriology* 193 (13) (Jul): 3286-92.

Gibbs, K. A., M. L. Urbanowski, and E. P. Greenberg. 2008. Genetic determinants of self identity and social recognition in bacteria. *Science (New York, N.Y.)* 321 (5886) (Jul 11): 256-9.

Gibson, F., and D. I. Magrath. 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-I. *Biochimica Et Biophysica Acta* 192 (2) (Nov 18): 175-84.

Goldman, R., and J. L. Strominger. 1972. Purification and properties of C 55 -isoprenylpyrophosphate phosphatase from *Micrococcus lysodeikticus*. *The Journal of Biological Chemistry* 247 (16) (Aug 25): 5116-22.

Golenbock, D. T., R. Y. Hampton, N. Qureshi, K. Takayama, and C. R. Raetz. 1991. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *The Journal of Biological Chemistry* 266 (29) (Oct 15): 19490-8.

Griffith, D. P., D. M. Musher, and C. Itin. 1976. Urease, the primary cause of infection-induced urinary stones. *Investigative Urology* 13 (5) (Mar): 346-50.

Groisman, E. A., J. Kayser, and F. C. Soncini. 1997. Regulation of polymyxin resistance and adaptation to low-Mg2+ environments. *Journal of Bacteriology* 179 (22) (Nov): 7040-5.

Gromska, W., and H. Mayer. 1976. The linkage of lysine in the O-specific chains of *Proteus mirabilis* 1959. *European Journal of Biochemistry / FEBS* 62 (2) (Feb 16): 391-9.

References

Gromska, W., W. Kaca, and K. Kotelko. 1978. The role of lysine in the serological specificity of some *Proteus mirabilis* lipopolysaccharides. *Archivum Immunologiae Et Therapiae Experimentalis* 26 (1-6): 237.

Gronow, S., W. Brabetz, and H. Brade. 2000. Comparative functional characterization in vitro of heptosyltransferase I (WaaC) and II (WaaF) from *Escherichia coli. European Journal of Biochemistry / FEBS* 267 (22) (Nov): 6602-11.

Guan, S., A. J. Clarke, and C. Whitfield. 2001. Functional analysis of the galactosyltransferases required for biosynthesis of D-galactan I, a component of the lipopolysaccharide O1 antigen of *Klebsiella pneumoniae. Journal of Bacteriology* 183 (11) (Jun): 3318-27.

Guay, D. R. 2008. Contemporary management of uncomplicated urinary tract infections. *Drugs* 68 (9): 1169-205.

Guentzel, M. N. 1996. *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter,* and *Proteus*. In *Medical microbiology.*, ed. S. Baron. 4th ed. Galveston (TX): The University of Texas Medical Branch at Galveston.

Guentzel, M. N., and L. J. Berry. 1975. Motility as a virulence factor for *Vibrio cholerae. Infection and Immunity* 11 (5) (May): 890-7.

Gunn, J. S. 2008. The *Salmonella* PmrAB regulon: Lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends in Microbiology* 16 (6) (Jun): 284-90.

Gunn, John S., Kheng B. Lim, Jackie Krueger, Kevin Kim, Lin Guo, Murray Hackett, and Samuel I. Miller. 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Molecular Microbiology* 27 (6): 1171-82

Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller. 1997. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ. Science (New York, N.Y.)* 276 (5310) (Apr 11): 250-3.

Guo, M. M., and P. V. LIU. 1965. Serological specificities of ureases of *Proteus* species. *Journal of General Microbiology* 38 (Mar): 417-22.

Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes. 1995. A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis. Molecular Microbiology* 17 (6) (Sep): 1167-75.

Hart, B. R., and R. M. Blumenthal. 2011. Unexpected coregulator range for the global regulator *lrp* of *Escherichia coli* and *Proteus mirabilis*. *Journal of Bacteriology* 193 (5) (Mar): 1054-64.

Hashimoto, Y., N. Li, H. Yokoyama, and T. Ezaki. 1993. Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. *Journal of Bacteriology* 175 (14) (Jul): 4456-65.

Hatt, J. K., and P. N. Rather. 2008. Characterization of a novel gene, *wosA*, regulating FlhDC expression in *proteus mirabilis*. *Journal of Bacteriology* 190 (6) (Mar): 1946-55.

Hauser G. 1885. Über fäulnissbacterien und deren beziehungen zur septicämie. ein betrag zur morphologie der spaltpilze. *Vogel, Leipzig, Germany.*

Heinrichs, D. E., J. A. Yethon, and C. Whitfield. 1998. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. *Molecular Microbiology* 30 (2) (Oct): 221-32.

Heinrichs, D. E., M. A. Valvano, and C. Whitfield. 1999. Biosynthesis and genetics of lipopolysaccharide core. in: Endotoxin in health and disease. eds: Brade H, morrison D C, vogel S & opal S, marcel dekker, inc., new york, N.Y. : 305-30.

Helander, Ilkka M., Ilkka Kilpeläinen, and Martti Vaara. 1994. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: A 31P-NMR study. *Molecular Microbiology* 11 (3): 481-7

Henrichsen, J. 1972. Bacterial surface translocation: A survey and a classification. *Bacteriological Reviews* 36 (4) (Dec): 478-503.

Hickman, F. W., A. G. Steigerwalt, J. J. Farmer 3rd, and D. J. Brenner. 1982. Identification of *Proteus penneri* sp. nov., formerly known as *Proteus vulgaris* indole negative or as *Proteus vulgaris* biogroup 1. *Journal of Clinical Microbiology* 15 (6) (Jun): 1097-102.

Himpsl, S. D., C. V. Lockatell, J. R. Hebel, D. E. Johnson, and H. L. Mobley. 2008. Identification of virulence determinants in uropathogenic *Proteus mirabilis* using signature-tagged mutagenesis. *Journal of Medical Microbiology* 57 (Pt 9) (Sep): 1068-78.

Hoeniger, J. F. 1964. Cellular changes accompanying the swarming of *Proteus mirabilis*. I. observations of living cultures. *Canadian Journal of Microbiology* 10 (Feb): 1-9.

Hoeniger, J. F. 1965. Development of flagella by *Proteus mirabilis*. *Microbiology* 40 (1): 29-42.

References

Hoeniger, J. F. 1966. Cellular changes accompanying the swarming of *Proteus mirabilis*. II. observations of stained organisms. *Canadian Journal of Microbiology* 12 (1) (Feb): 113-23.

Hoeniger, J. F., and E. A. Cinits. 1969. Cell wall growth during differentiation of *Proteus* swarmers. *Journal of Bacteriology* 98 (1) (Apr): 226-31.

Hoffmann, P., S. Heinle, U. F. Schade, H. Loppnow, A. J. Ulmer, H. D. Flad, G. Jung, and W. G. Bessler. 1988. Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. *Immunobiology* 177 (2) (May): 158-70

Holst, O. 1999. *Endotoxin in health and disease* H. Brade, S. M. Opal, S. N. Vogel, D. C. Morrison.

Holst, O., and S. Müller-Loennies. 2007. Microbial polysaccharide structures. In *Comprehensive glycoscience.* Vol. 1, 123-179 Kamerling JP, Boons G-J, Lee YC, Suzuki A, Taniguchi N, Voragen AGJ (eds).

Hooton, T. M., S. F. Bradley, D. D. Cardenas, R. Colgan, S. E. Geerlings, J. C. Rice, S. Saint, et al. 2010. Diagnosis, prevention, and treatment of catheter-associated urinary tract infection in adults: 2009 international clinical practice guidelines from the infectious diseases society of america. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 50 (5) (Mar 1): 625-63.

Houwink, A. L., and W. van Iterson. 1950. Electron microscopical observations on bacterial cytology; a study on flagellation. *Biochimica Et Biophysica Acta* 5 (1) (Mar): 10-44.

Huang, J., and M. Schell. 1995. Molecular characterization of the *eps* gene cluster of *Pseudomonas solanacearum* and its transcriptional regulation at a single promoter. *Molecular Microbiology* 16 (5) (Jun): 977-89.

Isshiki, Y., K. Kawahara, and U. Zahringer. 1998. Isolation and characterisation of disodium (4-amino-4-deoxy-beta-L-arabinopyranosyl)-(1-->8)-(D-glycero-alpha-D-talo-oct-2ulopyranosylona te)- (2-->4)-(methyl 3-deoxy-D-manno-oct-2-ulopyranosid)onate from the lipopolysaccharide of *Burkholderia cepacia*. *Carbohydrate Research* 313 (1) (Nov): 21-7.

Isshiki, Y., U. Zahringer, and K. Kawahara. 2003. Structure of the core-oligosaccharide with a characteristic D-glycero-alpha-D-talo-oct-2-ulosylonate-(2-->4)-3-deoxy-D-manno-oct-2-ulosonate [alpha-ko-(2-->4)-kdo] disaccharide in the lipopolysaccharide from *Burkholderia cepacia*. *Carbohydrate Research* 338 (23) (Nov 14): 2659-66.

Izquierdo, L., N. Coderch, N. Pique, E. Bedini, M. M. Corsaro, S. Merino, S. Fresno, J. M. Tomas, and M. Regue. 2003. The *Klebsiella pneumoniae wabG* gene: Role in biosynthesis of the core lipopolysaccharide and virulence. *Journal of Bacteriology* 185 (24) (Dec): 7213-21.

Izquierdo, L., S. Merino, N. Coderch, M. Regue, and J. M. Tomas. 2002. The *wavB* gene of *Vibrio cholerae* and the *waaE* of *Klebsiella pneumoniae* codify for a beta-1,4-glucosyltransferase involved in the transfer of a glucose residue to the L-glycero-D-manno-heptose I in the lipopolysaccharide inner core. *FEMS Microbiology Letters* 216 (2) (Nov 5): 211-6.

Jackman, J. E., C. R. Raetz, and C. A. Fierke. 1999. UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase of *Escherichia coli* is a zinc metalloenzyme. *Biochemistry* 38 (6) (Feb 9): 1902-11.

Jacobsen, S. M., D. J. Stickler, H. L. Mobley, and M. E. Shirtliff. 2008. Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. *Clinical Microbiology Reviews* 21 (1) (Jan): 26-59.

Jansen, A. M., C. V. Lockatell, D. E. Johnson, and H. L. Mobley. 2003. Visualization of *Proteus mirabilis* morphotypes in the urinary tract: The elongated swarmer cell is rarely observed in ascending urinary tract infection. *Infection and Immunity* 71 (6) (Jun): 3607-13.

Jenkinson, H. F. 1994. Adherence and accumulation of oral streptococci. *Trends in Microbiology* 2 (6) (Jun): 209-12.

Jiang, S. S., M. C. Liu, L. J. Teng, W. B. Wang, P. R. Hsueh, and S. J. Liaw. 2010a. *Proteus mirabilis pmrI*, a RppA-regulated gene necessary for polymyxin B resistance, biofilm formation, and urothelial cell invasion. *Antimicrobial Agents and Chemotherapy* 54 (4) (Apr): 1564-71.

Jiang, S. S., T. Y. Lin, W. B. Wang, M. C. Liu, P. R. Hsueh, and S. J. Liaw. 2010b. Characterization of UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase mutants of *Proteus mirabilis*: Defectiveness in polymyxin B resistance, swarming, and virulence. *Antimicrobial Agents and Chemotherapy* 54 (5) (May): 2000-9.

Jimenez, N., A. Lacasta, S. Vilches, M. Reyes, J. Vazquez, E. Aquillini, S. Merino, M. Regue, and J. M. Tomas. 2009. Genetics and proteomics of *Aeromonas salmonicida* lipopolysaccharide core biosynthesis. *Journal of Bacteriology* 191 (7) (Apr): 2228-36.

References

Joiner, K. A. 1988. Complement evasion by bacteria and parasites. *Annual Review of Microbiology* 42 : 201-30.

Jones, B. D., and H. L. Mobley. 1987. Genetic and biochemical diversity of ureases of *Proteus*, *Providencia*, and *Morganella* species isolated from urinary tract infection. *Infection and Immunity* 55 (9) (Sep): 2198-203.

Jones, B. V., R. Young, E. Mahenthiralingam, and D. J. Stickler. 2004. Ultrastructure of *Proteus mirabilis* swarmer cell rafts and role of swarming in catheter-associated urinary tract infection. *Infection and Immunity* 72 (7) (Jul): 3941-50.

Jones, S. M., J. Yerly, Y. Hu, H. Ceri, and R. Martinuzzi. 2007. Structure of *Proteus mirabilis* biofilms grown in artificial urine and standard laboratory media. *FEMS Microbiology Letters* 268 (1) (Mar): 16-21.

Kaca, W., E. Literacka, A. G. Sjoholm, and A. Weintraub. 2000. Complement activation by *Proteus mirabilis* negatively charged lipopolysaccharides. *Journal of Endotoxin Research* 6 (3): 223-34.

Kaca, W., J. Radziejewska-Lebrecht, and U. R. Bhat. 1990. Effect of polymyxins on the lipopolysaccharide-defective mutants of *Proteus mirabilis*. *Microbios* 61 (246): 23-32.

Kaca, W., Y. A. Knirel, E. V. Vinogradov, and K. Kotelko. 1987. Structure of the 0-specific polysaccharide of *Proteus mirabilis* S 1959. *Archivum Immunologiae Et Therapiae Experimentalis* 35 (4): 431-7.

Kadrmas, J. L., and C. R. Raetz. 1998b. Enzymatic synthesis of lipopolysaccharide in *Escherichia coli*. Purification and properties of heptosyltransferase I. *The Journal of Biological Chemistry* 273 (5) (Jan 30): 2799-807.

Kadrmas, J. L., D. Allaway, R. E. Studholme, J. T. Sullivan, C. W. Ronson, P. S. Poole, and C. R. Raetz. 1998. Cloning and overexpression of glycosyltransferases that generate the lipopolysaccharide core of *Rhizobium leguminosarum*. *The Journal of Biological Chemistry* 273 (41) (Oct 9): 26432-40.

Kadrmas, J. L., K. A. Brozek, and C. R. Raetz. 1996. Lipopolysaccharide core glycosylation in *Rhizobium leguminosarum*. an unusual mannosyl transferase resembling the heptosyl transferase I of *Escherichia coli*. *The Journal of Biological Chemistry* 271 (50) (Dec 13): 32119-25.

Kaniuk, N. A., M. A. Monteiro, C. T. Parker, and C. Whitfield. 2002. Molecular diversity of the genetic loci responsible for lipopolysaccharide core oligosaccharide assembly within the genus *Salmonella*. *Molecular Microbiology* 46 (5) (Dec): 1305-18.

Karch, H., and K. Nixdorff. 1983. Modulation of the IgG subclass responses to lipopolysaccharide by bacterial membrane components: differential adjuvant effects produced by primary and secondary stimulation. *Journal of Immunology (Baltimore, Md.: 1950)* 131 (1) (Jul): 6-8.

Karow, M., and C. Georgopoulos. 1993. The essential *Escherichia coli msbA* gene, a multicopy suppressor of null mutations in the *htrB* gene, is related to the universally conserved family of ATP-dependent translocators. *Molecular Microbiology* 7 (1) (Jan): 69-79.

Kauffmann, F. 1966. The bacteriology of *Enterobacteriaceae*. collected studies of the author and his co-workers. In *Collected studies of the author and his co-workers.* The Williams & Wilkins, Co. ed. Vol. 3rd ed., 333. Baltimore: .

Keenleyside, W. J., A. J. Clarke, and C. Whitfield. 2001. Identification of residues involved in catalytic activity of the inverting glycosyl transferase WbbE from *Salmonella enterica* serovar borreze. *Journal of Bacteriology* 183 (1) (Jan): 77-85.

Keenleyside, W. J., and C. Whitfield. 1995. Lateral transfer of *rfb* genes: A mobilizable ColE1-type plasmid carries the $rfb_{O:54}$ (O:54 antigen biosynthesis) gene cluster from *Salmonella enterica* serovar borreze. *Journal of Bacteriology* 177 (18) (Sep): 5247-53.

Keenleyside, W. J., and C. Whitfield. 1996. A novel pathway for O-polysaccharide biosynthesis in *Salmonella enterica* serovar borreze. *The Journal of Biological Chemistry* 271 (45) (Nov 8): 28581-92.

Keenleyside, W. J., M. Perry, L. Maclean, C. Poppe, and C. Whitfield. 1994. A plasmid-encoded $rfb_{O:54}$ gene cluster is required for biosynthesis of the O:54 antigen in *Salmonella enterica* serovar borreze. *Molecular Microbiology* 11 (3) (Feb): 437-48.

Kelly, T. M., S. A. Stachula, C. R. Raetz, and M. S. Anderson. 1993. The *firA* gene of *Escherichia coli* encodes UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-acyltransferase. the third step of endotoxin biosynthesis. *The Journal of Biological Chemistry* 268 (26) (Sep 15): 19866-74.

References

Kido, N., and H. Kobayashi. 2000. A single amino acid substitution in a mannosyltransferase, WbdA, converts the *Escherichia coli* O9 polysaccharide into O9a: Generation of a new O-serotype group. *Journal of Bacteriology* 182 (9) (May): 2567-73.

Kido, N., T. Sugiyama, T. Yokochi, H. Kobayashi, and Y. Okawa. 1998. Synthesis of *Escherichia coli* O9a polysaccharide requires the participation of two domains of WbdA, a mannosyltransferase encoded within the *wb* gene cluster. *Molecular Microbiology* 27 (6) (Mar): 1213-21.

Kido, N., V. I. Torgov, T. Sugiyama, K. Uchiya, H. Sugihara, T. Komatsu, N. Kato, and K. Jann. 1995. Expression of the O9 polysaccharide of *Escherichia coli*: Sequencing of the *E. coli* O9 *rfb* gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. *Journal of Bacteriology* 177 (8) (Apr): 2178-87.

Klena, J. D., and C. A. Schnaitman. 1993. Function of the *rfb* gene cluster and the *rfe* gene in the synthesis of O antigen by *Shigella dysenteriae* 1. *Molecular Microbiology* 9 (2) (Jul): 393-402.

Klena, J. D., R. S. Ashford 2nd, and C. A. Schnaitman. 1992. Role of *Escherichia coli* K-12 *rfa* genes and the *rfp* gene of *Shigella dysenteriae* 1 in generation of lipopolysaccharide core heterogeneity and attachment of O antigen. *Journal of Bacteriology* 174 (22) (Nov): 7297-307.

Kline, T., N. H. Andersen, E. A. Harwood, J. Bowman, A. Malanda, S. Endsley, A. L. Erwin, et al. 2002. Potent, novel in vitro inhibitors of the *Pseudomonas aeruginosa* deacetylase LpxC. *Journal of Medicinal Chemistry* 45 (14) (Jul 4): 3112-29.

Knirel, IuA, and N. K. Kochetkov. 1993a. Structure of lipopolysaccharides from gram-negative bacteria. I. common characteristics of lipopolysaccharides and lipid A structure (review). *Biokhimiia (Moscow, Russia)* 58 (2) (Feb): 166-81.

Knirel, IuA, and N. K. Kochetkov. 1993b. Structure of lipopolysaccharides from gram-negative bacteria. II. core structure (review). *Biokhimiia (Moscow, Russia)* 58 (2) (Feb): 182-201.

Knirel, IuA, and N. K. Kochetkov. 1994. Structure of lipopolysaccharides from gram-negative bacteria. III. structure of O-specific polysaccharides. *Biokhimiia (Moscow, Russia)* 59 (12) (Dec): 1784-851.

Kolenbrander, P. E. 1993. Coaggregation of human oral bacteria: Potential role in the accretion of dental plaque. *The Journal of Applied Bacteriology* 74 Suppl : 79S-86S.

Kondakova, A. N., E. Vinogradov, B. Lindner, N. A. Kocharova, A. Rozalski, Y. A. Knirel. 2006. Elucidation of the lipopolysaccharide core structures of bacteria of the genus *Providencia*. *J. Carbohydr. Chem. Vol*. 25, Iss. 6

Kotelko, K. 1986. *Proteus mirabilis*: Taxonomic position, peculiarities of growth, components of the cell envelope. *Current Topics in Microbiology and Immunology* 129 : 181-215.

Kotelko, K., M. Deka, W. Gromska, W. Kaca, J. Radziejewska-Lebrecht, and A. Rozalski. 1983. Galacturonic acid as the terminal constituent in the R core polysaccharide of *Proteus* R110 (ra) mutant. *Archivum Immunologiae Et Therapiae Experimentalis* 31 (6): 833-8.

Krajden, S., M. Fuksa, C. Petrea, L. J. Crisp, and J. L. Penner. 1987. Expanded clinical spectrum of infections caused by P*roteus penneri*. *Journal of Clinical Microbiology* 25 (3)(Mar):578-9

Krajden, S., M. Fuksa, W. Lizewski, L. Barton, and A. Lee. 1984. *Proteus penneri* and urinary calculi formation. *Journal of Clinical Microbiology* 19 (4) (Apr): 541-2.

Krajewska-Pietrasik, D., A. Y. Cherniak, and A. Rozalski. 1995. Use of synthetic antigens in studies of antigenic specificity of *Proteus mirabilis* 027 lipopolysaccharide. *Medycyna Doswiadczalna i Mikrobiologia* 47 (3-4): 169-76.

Kroll, J. S., S. Zamze, B. Loynds, and E. R. Moxon. 1989. Common organization of chromosomal loci for production of different capsular polysaccharides in H*aemophilus influenzae*. *Journal of Bacteriology* 171 (6) (Jun): 3343-7.

Kunin, C. M. 1989. Blockage of urinary catheters: Role of microorganisms and constituents of the urine on formation of encrustations. *Journal of Clinical Epidemiology* 42 (9): 835-42.

Lairson, L. L., B. Henrissat, G. J. Davies, and S. G. Withers. 2008. Glycosyltransferases: Structures, functions, and mechanisms. *Annual Review of Biochemistry* 77 : 521-55.

Larsson, P. 1984. Serology of *Proteus mirabilis* and *Proteus vulgaris*. *Methods Microbiol.* 14 : 187-214.

Latta, R. K., A. Grondin, H. C. Jarrell, G. R. Nicholls, and L. R. Berube. 1999. Differential expression of nonagglutinating fimbriae and MR/P pili in swarming colonies of *Proteus mirabilis*. *Journal of Bacteriology* 181 (10) (May): 3220-5.

Latuszynski, D. K., P. Schoch, M. T. Qadir, and B. A. Cunha. 1998. *Proteus penneri* urosepsis in a patient with diabetes mellitus. *Heart & Lung : The Journal of Critical Care* 27 (2) (Mar-Apr): 146-8.

References

Lee, H., F. F. Hsu, J. Turk, and E. A. Groisman. 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *Journal of Bacteriology* 186 (13) (Jul): 4124-33.

Lehrman, M. A. 1994. A family of UDP-GlcNAc/MurNAc: Polyisoprenol-P GlcNAc/MurNAc-1-P transferases. *Glycobiology* 4 (6) (Dec): 768-71.

Leifson, E., S. R. Carhart, and M. Fulton. 1955. Morphological characteristics of flagella of *Proteus* and related bacteria. *Journal of Bacteriology* 69 (1) (Jan): 73-82.

Lewis, L. A., B. Choudhury, J. T. Balthazar, L. E. Martin, S. Ram, P. A. Rice, D. S. Stephens, R. Carlson, and W. M. Shafer. 2009. Phosphoethanolamine substitution of lipid A and resistance of *Neisseria gonorrhoeae* to cationic antimicrobial peptides and complement-mediated killing by normal human serum. *Infection and Immunity* 77 (3) (Mar): 1112-20

Li, X. C., C. R. Yanga, Y. Q. Liua, R. Kasai, K. Ohtani, K. Yamasaki, K. Miyahara, and K. Shingu. 1995. Triterpenoidglycosides from *Anemoclema glaucifolium*. *Phytochemistry* 39 (5): 1175-9.

Li, X., and H. L. Mobley. 2002. Vaccines for *Proteus mirabilis* in urinary tract infection. *International Journal of Antimicrobial Agents* 19 (6) (Jun): 461-5.

Li, X., C. V. Lockatell, D. E. Johnson, M. C. Lane, J. W. Warren, and H. L. Mobley. 2004a. Development of an intranasal vaccine to prevent urinary tract infection by *Proteus mirabilis*. *Infection and Immunity* 72 (1) (Jan): 66-75.

Li, X., H. Zhao, C. V. Lockatell, C. B. Drachenberg, D. E. Johnson, and H. L. Mobley. 2002. Visualization of *Proteus mirabilis* within the matrix of urease-induced bladder stones during experimental urinary tract infection. *Infection and Immunity* 70 (1) (Jan): 389-94.

Li, X., J. L. Erbe, C. V. Lockatell, D. E. Johnson, M. G. Jobling, R. K. Holmes, and H. L. Mobley. 2004b. Use of translational fusion of the MrpH fimbrial adhesin-binding domain with the cholera toxin A2 domain, coexpressed with the cholera toxin B subunit, as an intranasal vaccine to prevent experimental urinary tract infection by *Proteus mirabilis*. *Infection and Immunity* 72 (12) (Dec): 7306-10.

Li, Z., X. Wang, Z. Bian, S. Li, H. Zheng, B. Zhao, and J. Chen. 1992. *Proteus penneri* isolated from the pus of a patient with epidural abscess. *Kansenshogaku Zasshi.the Journal of the Japanese Association for Infectious Diseases* 66 (2) (Feb): 144-8.

Lindberg, B. 1998. Polysaccharides: Structural diversity and functional versatility. In: Dimitriu S. (ed) Marcel Dekker, New York.

Liu, C., X. Fu, L. Liu, X. Ren, C. K. Chau, S. Li, L. Xiang, et al. 2011. Sequential establishment of stripe patterns in an expanding cell population. *Science* 334 (6053) (Oct 14): 238-41.

Lominski, I., and A. C. Lendrum. 1947. The mechanism of swarming of *Proteus*. *The Journal of Pathology and Bacteriology* 59 (4) (Oct): 688-91.

Long, S. R. 1996. Rhizobium symbiosis: Nod factors in perspective. *The Plant Cell* 8 (10) (Oct): 1885-98.

Loomes, L. M., B. W. Senior, and M. A. Kerr. 1992. Proteinases of *Proteus* spp.: Purification, properties, and detection in urine of infected patients. *Infection and Immunity* 60 (6) (Jun): 2267-73.

Lüderitz, O., M. A. Freudenberg, C. Galanos, V. Lehmann, E. Th Rietschel, and D. H. Shaw. 1982. Lipopolysaccharides of gram negative bacteria. *Curr. Top. Membr. Transp.* 17 : 79-151.

Lugtenberg, B., H. Bronstein, N. van Selm, and R. Peters. 1977. Peptidoglycan-associated outer membrane proteins in gammegatine bacteria. *Biochimica Et Biophysica Acta* 465 (3) (Mar 17): 571-8.

Lukomski, S., L. Serwecinska, A. Rozalski, J. Dziadek, P. Staczek, and A. Jaworski. 1991. Cell-free and cell-bound hemolytic activities of *Proteus penneri* determined by different hly determinants. *Canadian Journal of Microbiology* 37 (6) (Jun): 419-24.

MacIntyre, D. L., S. T. Miyata, M. Kitaoka, and S. Pukatzki. 2010. The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences of the United States of America* 107 (45) (Nov 9): 19520-4.

Macleod, S. M., and D. J. Stickler. 2007. Species interactions in mixed-community crystalline biofilms on urinary catheters. *Journal of Medical Microbiology* 56 (Pt 11) (Nov): 1549-57.

Magana-Plaza, I., and J. Ruiz-Herrera. 1967. Mechanisms of regulation of urease biosynthesis in *Proteus rettgeri*. *Journal of Bacteriology* 93 (4) (Apr): 1294-301.

Magana-Plaza, I., C. Montes, and J. Ruiz-Herrera. 1971. Purification and biochemical characteristics of urease from *Proteus rettgeri*. *Biochimica Et Biophysica Acta* 242 (1) (Jul 21): 230-7.

References

Majdalani, N., and S. Gottesman. 2005. The rcs phosphorelay: A complex signal transduction system. *Annual Review of Microbiology* 59 : 379-405.

Majdalani, N., M. Heck, V. Stout, and S. Gottesman. 2005. Role of RcsF in signaling to the rcs phosphorelay pathway in *Escherichia coli*. *Journal of Bacteriology* 187 (19) (Oct): 6770-8.

Manos, J., and R. Belas. 2006. The genera *Proteus, Providencia,* and *Morganella.* In *The prokaryotes: An evolving electronic resource for the microbiological community.* Springer-Verlag ed. Vol. 6, 245-269. New York: M. Dworkin et al.

Marolda, C. L., and M. A. Valvano. 1998. Promoter region of the *Escherichia coli* O7-specific lipopolysaccharide gene cluster: Structural and functional characterization of an upstream untranslated mRNA sequence. *Journal of Bacteriology* 180 (12) (Jun): 3070-9.

Marolda, C. L., M. F. Feldman, and M. A. Valvano. 1999. Genetic organization of the O7-specific lipopolysaccharide biosynthesis cluster of *Escherichia coli* VW187 (O7:K1). *Microbiology (Reading, England)* 145 (Pt 9) (Pt 9) (Sep): 2485-95.

Massad, G., C. V. Lockatell, D. E. Johnson, and H. L. Mobley. 1994. *Proteus mirabilis* fimbriae: Construction of an isogenic *pmfA* mutant and analysis of virulence in a CBA mouse model of ascending urinary tract infection. *Infection and Immunity* 62 (2) (Feb): 536-42.

Massad, G., H. Zhao, and H. L. Mobley. 1995. *Proteus mirabilis* amino acid deaminase: Cloning, nucleotide sequence, and characterization of *aad*. *Journal of Bacteriology* 177 (20) (Oct): 5878-83.

Mathoera, R. B., D. J. Kok, C. M. Verduin, and R. J. Nijman. 2002. Pathological and therapeutic significance of cellular invasion by *Proteus mirabilis* in an enterocystoplasty infection stone model. *Infection and Immunity* 70 (12) (Dec): 7022-32.

Mathur, S., N. A. Sabbuba, M. T. Suller, D. J. Stickler, and R. C. Feneley. 2005. Genotyping of urinary and fecal *Proteus mirabilis* isolates from individuals with long-term urinary catheters. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology* 24 (9) (Sep): 643-4.

McCarter, L., and M. Silverman. 1989. Iron regulation of swarmer cell differentiation of *Vibrio parahaemolyticus*. *Journal of Bacteriology* 171 (2) (Feb): 731-6.

McClerren, A. L., P. Zhou, Z. Guan, C. R. Raetz, and J. Rudolph. 2005. Kinetic analysis of the zinc-dependent deacetylase in the lipid A biosynthetic pathway. *Biochemistry* 44 (4) (Feb 1): 1106-13.

McCoy, A. J., H. Liu, T. J. Falla, and J. S. Gunn. 2001. Identification of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. *Antimicrobial Agents and Chemotherapy* 45 (7) (Jul): 2030-7.

McGrath, B. C., and M. J. Osborn. 1991. Localization of the terminal steps of O-antigen synthesis in *Salmonella typhimurium*. *Journal of Bacteriology* 173 (2) (Jan): 649-54.

McLean, R. J., J. C. Nickel, K. J. Cheng, and J. W. Costerton. 1988. The ecology and pathogenicity of urease-producing bacteria in the urinary tract. *Critical Reviews in Microbiology* 16 (1): 37-79.

McManus A. T., Moody E. E., and Mason A. D. 1980. Bacterial motility: A component in experimental *Pseudomonas aeruginosa* burn wound sepsis. *Burns* 6 : 235-9.

Meier-Dieter, U., K. Barr, R. Starman, L. Hatch, and P. D. Rick. 1992. Nucleotide sequence of the *Escherichia coli rfe* gene involved in the synthesis of enterobacterial common antigen. molecular cloning of the *rfe-rff* gene cluster. *The Journal of Biological Chemistry* 267 (2) (Jan 15): 746-53.

Melchers, F., V. Braun, and C. Galanos. 1975. The lipoprotein of the outer membrane of *Escherichia coli*: A B-lymphocyte mitogen. *The Journal of Experimental Medicine* 142 (2) (Aug 1): 473-82.

Mengin-Lecreulx, D., and J. van Heijenoort. 1994. Copurification of glucosamine-1-phosphate acetyltransferase and N-acetylglucosamine-1-phosphate uridyltransferase activities of *Escherichia coli*: Characterization of the *glmU* gene product as a bifunctional enzyme catalyzing two subsequent steps in the pathway for UDP-N-acetylglucosamine synthesis. *Journal of Bacteriology* 176 (18) (Sep): 5788-95.

Merino, S., A. Aguilar, X. Rubires, D. Simon-Pujol, F. Congregado, and J. M. Tomas. 1996a. The role of the capsular polysaccharide of *Aeromonas salmonicida* in the adherence and invasion of fish cell lines. *FEMS Microbiology Letters* 142 (2-3) (Sep 1): 185-9.

Merino, S., X. Rubires, A. Aguilar, and Tomas. 1996b. The O:34-antigen lipopolysaccharide as an adhesin in *Aeromonas hydrophila*. *FEMS Microbiology Letters* 139 (2-3) (Jun 1): 97-101.

Michael, F., E. Vinogradov, C. Q. Wenzel, B. McIntosh, J. Li, J. C. Hoe, J. C. Richards, and A. D. Cox. 2009. Phosphoethanolamine is located at the 6-position and not at the 7-position of the distal heptose residue in the lipopolysaccharide from *Neisseria meningitidis*. *Glycobiology* 19 (12) (Dec): 1436-45.

References

Miller, S. I., A. M. Kukral, and J. J. Mekalanos. 1989. A two-component regulatory system (phoP phoQ) controls *Salmonella typhimurium* virulence. *Proceedings of the National Academy of Sciences of the United States of America* 86 (13) (Jul): 5054-8.

Mobley, H. L., and J. W. Warren. 1987a. Urease-positive bacteriuria and obstruction of long-term urinary catheters. *Journal of Clinical Microbiology* 25 (11) (Nov): 2216-7.

Mobley, H. L., and R. Belas. 1995. Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. *Trends in Microbiology* 3 (7) (Jul): 280-4.

Mobley, H. L., and R. P. Hausinger. 1989. Microbial ureases: Significance, regulation, and molecular characterization. *Microbiological Reviews* 53 (1) (Mar): 85-108.

Mobley, H. L., B. D. Jones, and J. L. Penner. 1987b. Urease activity of *Proteus penneri*. *Journal of Clinical Microbiology* 25 (12) (Dec): 2302-5.

Mobley, H. L., M. D. Island, and R. P. Hausinger. 1995. Molecular biology of microbial ureases. *Microbiological Reviews* 59 (3) (Sep): 451-80.

Mobley, H. L., R. Belas, V. Lockatell, G. Chippendale, A. L. Trifillis, D. E. Johnson, and J. W. Warren. 1996. Construction of a flagellum-negative mutant of *Proteus mirabilis*: Effect on internalization by human renal epithelial cells and virulence in a mouse model of ascending urinary tract infection. *Infection and Immunity* 64 (12) (Dec): 5332-40.

Mollet, C., M. Drancourt, and D. Raoult. 1997. *rpoB* sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology* 26 (5) (Dec): 1005-11.

Moran, A. P., E. T. Rietschel, T. U. Kosunen, and U. Zahringer. 1991. Chemical characterization of *Campylobacter jejuni* lipopolysaccharides containing N-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. *Journal of Bacteriology* 173 (2) (Jan): 618-26.

Morgenstein, R. M., and P. N. Rather. 2012. Role of the Umo proteins and the rcs phosphorelay in the swarming motility of the wild type and an O-antigen (*waaL*) mutant of *Proteus mirabilis*. *Journal of Bacteriology* 194 (3) (Feb): 669-76.

Morgenstein, R. M., B. Szostek, and P. N. Rather. 2010. Regulation of gene expression during swarmer cell differentiation in *Proteus mirabilis*. *FEMS Microbiology Reviews* 34 (5) (Sep): 753-63.

Morona, R., L. Van Den Bosch, and C. Daniels. 2000. Evaluation of Wzz/MPA1/MPA2 proteins based on the presence of coiled-coil regions. *Microbiology (Reading, England)* 146 (Pt 1) (Pt 1) (Jan): 1-4.

Morona, R., M. Mavris, A. Fallarino, and P. A. Manning. 1994. Characterization of the *rfc* region of *Shigella flexneri*. *Journal of Bacteriology* 176 (3) (Feb): 733-47.

Morris, N. S., D. J. Stickler, and R. J. McLean. 1999. The development of bacterial biofilms on indwelling urethral catheters. *World Journal of Urology* 17 (6) (Dec): 345-50.

Morrison, D. C., and D. M. Jacobs. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* 13 (10) (Oct): 813-8.

Morrison, D. C., and J. L. Ryan. 1992. Bacterial endotoxic lipopolysaccharides: Immunopharmacology and pathophysiology. Vol. 1.

Moskowitz, S. M., R. K. Ernst, and S. I. Miller. 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *Journal of Bacteriology* 186 (2) (Jan): 575-9.

Mulks, M. H. 1985. Microbial IgA proteases. In *Bacterial enzymes and virulence.* CRC Press, Inc. ed., 81-104. Boca Raton, Fla.: I. A. Holder.

Nichols, W. A., B. W. Gibson, W. Melaugh, N. G. Lee, M. Sunshine, and M. A. Apicella. 1997. Identification of the ADP-L-glycero-D-manno-heptose-6-epimerase (*rfaD*) and heptosyltransferase II (*rfaF*) biosynthesis genes from nontypeable *Haemophilus influenzae* 2019. *Infection and Immunity* 65 (4) (Apr): 1377-86.

Nicolle, L. E. 2005. Catheter-related urinary tract infection. *Drugs & Aging* 22 (8): 627-39.

Nicolle, L. E. 2012. Urinary catheter-associated infections. *Infectious Disease Clinics of North America* 26 (1) (Mar): 13-27.

Nielubowicz, G. R., and H. L. Mobley. 2010. Host-pathogen interactions in urinary tract infection. *Nature Reviews. Urology* 7 (8) (Aug): 430-41.

Nikaido, ,Hiroshi. 1970. Structure of cell wall lipopolysaccharide from *Salmonella typhimurium*. *European Journal of Biochemistry* 15 (1): 57-62.

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews : MMBR* 67 (4) (Dec): 593-656.

Nikaido, H., and M. Vaara. 1987. Outer membrane. In *Escherichia coli and Salmonella typhimurium: Cellular and molecular biology.* American Society for Microbiology ed., 7-22. Washington, D. C.: .

References

Nixdorff, K., H. Fitzer, J. Gmeiner, and H. H. Martin. 1977. Reconstitution of model membranes from phospholipid and outer membrane proteins of *Proteus mirabilis*. role of proteins in the formation of hydrophilic pores and protection of membranes against detergents. *European Journal of Biochemistry / FEBS* 81 (1) (Nov 15): 63-9.

Nummila, Kim, IIkka Kilpeläinen, Ulrich Zähringer, Martti Vaara, and IIkka M. Helander. 1995. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Molecular Microbiology* 16 (2): 271-8

O'Brien, I. G., G. B. Cox, and F. Gibson. 1970. Biologically active compounds containing 2,3-dihydroxybenzoic acid and serine formed by *Escherichia coli*. *Biochimica Et Biophysica Acta* 201 (3) (Mar 24): 453-60.

O'Connor, E. T., A. Piekarowicz, K. V. Swanson, J. M. Griffiss, and D. C. Stein. 2006. Biochemical analysis of Lpt3, a protein responsible for phosphoethanolamine addition to lipooligosaccharide of pathogenic *Neisseria*. *Journal of Bacteriology* 188 (3) (Feb):1039-48.

O'Hara, C. M., F. W. Brenner, A. G. Steigerwalt, B. C. Hill, B. Holmes, P. A. Grimont, P. M. Hawkey, J. L. Penner, J. M. Miller, and D. J. Brenner. 2000. Classification of *Proteus vulgaris* biogroup 3 with recognition of *Proteus hauseri* sp. nov., nom. rev. and unnamed *Proteus* genomospecies 4, 5 and 6. *International Journal of Systematic and Evolutionary Microbiology* 50 Pt 5 (Sep): 1869-75.

O'Hanley, P. 1996. *Urinary tract infections: Molecular pathogenesis and clinical management.* Mobley, H. L. T. & Warren, J. W.

Onishi, H. R., B. A. Pelak, L. S. Gerckens, L. L. Silver, F. M. Kahan, M. H. Chen, A. A. Patchett, et al. 1996. Antibacterial agents that inhibit lipid A biosynthesis. *Science* 274 (5289) (Nov 8): 980-2.

Orskov, F., and I. Orskov. 1992. *Escherichia coli* serotyping and disease in man and animals. *Canadian Journal of Microbiology* 38 (7) (Jul): 699-704.

Osborn, M. J., J. E. Gander, and E. Parisi. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. site of synthesis of lipopolysaccharide. *The Journal of Biological Chemistry* 247 (12) (Jun 25): 3973-86.

Paulsen, I. T., A. M. Beness, and M. H. Saier Jr. 1997. Computer-based analyses of the protein constituents of transport systems catalysing export of complex carbohydrates in bacteria. *Microbiology (Reading, England)* 143 (Pt 8) (Pt 8) (Aug): 2685-99.

Payne, S. M. 1988. Iron and virulence in the family *Enterobacteriaceae*. *Critical Reviews in Microbiology* 16 (2): 81-111.

Pearson, M. M., A. Yep, S. N. Smith, and H. L. Mobley. 2011. Transcriptome of *Proteus mirabilis* in the murine urinary tract: Virulence and nitrogen assimilation gene expression. *Infection and Immunity* 79 (7) (Jul): 2619-31.

Pearson, M. M., D. A. Rasko, S. N. Smith, and H. L. Mobley. 2010. Transcriptome of swarming *Proteus mirabilis*. *Infection and Immunity* 78 (6) (Jun): 2834-45.

Pearson, M. M., et al. 2008. Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. *Journal of Bacteriology* 190 (11) (Jun): 4027-37.

Peerbooms, P. G., A. M. Verweij, and D. M. MacLaren. 1984. Vero cell invasiveness of *Proteus mirabilis*. *Infection and Immunity* 43 (3) (Mar): 1068-71.

Penner, J. L., and J. N. Hennessy. 1980. Separate O-grouping schemes for serotyping clinical isolates of *Proteus vulgaris* and *Proteus mirabilis*. *Journal of Clinical Microbiology* 12 (3) (Sep): 304-9.

Penner, J. L., N. A. Hinton, G. R. Whiteley, and J. N. Hennessy. 1976. Variation in urease activity of endemic hospital strains of *Proteus rettgeri* and *Providencia stuartii*. *The Journal of Infectious Diseases* 134 (4) (Oct): 370-6.

Perch, B. 1948. On the serology of the *Proteus* group. *Acta Pathol. Microbiol. Scand.* 25 : 703-14.

Perry, M. B., and L. L. MacLean. 1994. The structure of the polysaccharide produced by *Proteus vulgaris* (ATCC 49990). *Carbohydrate Research* 253 (Feb 3): 257-63.

Plaut, A. G. 1983. The IgA1 proteases of pathogenic bacteria. *Annual Review of Microbiology* 37 : 603-22.

Pollack, J. R., and J. B. Neilands. 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. *Biochemical and Biophysical Research Communications* 38 (5) (Mar 12): 989-92.

References

Popoff, M. Y., and L. Le Minor. 1985. Expression of antigenic factor O:54 is associated with the presence of a plasmid in *Salmonella*. *Annales De l'Institut Pasteur.Microbiologie* 136B (2) (Sep-Oct): 169-79.

Radziejewska-Lebrecht, J., A. S. Shashkov, E. V. Vinogradov, H. Grosskurth, B. Bartodziejska, A. Rozalski, W. Kaca, L. O. Kononov, A. Y. Chernyak, and H. Mayer. 1995. Structure and epitope characterization of the O-specific polysaccharide of *Proteus mirabilis* O28 containing amides of D-galacturonic acid with L-serine and L-lysine. *European Journal of Biochemistry / FEBS* 230 (2) (Jun 1): 705-12.

Radziejewska-Lebrecht, J., D. Krajewska-Pietrasik, and H. Mayer. 1990. Terminal and chain-linked residues of D-galacturonic acid: Characteristic constituents of the R-core regions of *Proteeae* and *Serratia marcescens*. *Syst. Appl. Microbiol.* 13 : 214-9.

Raetz, C. R. 1990. Biochemistry of endotoxins. *Annual Review of Biochemistry* 59 : 129-70.

Raetz, C. R. 1996. Bacterial lipopolysaccharides: A remarkable family of bioactive molecules. In *Escherichia coli and Salmonella: Cellular and molecular biology.* American Society for Microbiology Press ed., 1035-1063. Washington: Neidhardt F C, Curtiss III R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, Schaechter M & Umbarger H E.

Raetz, C. R., and C. Whitfield. 2002. Lipopolysaccharide endotoxins. *Annual Review of Biochemistry* 71 : 635-700.

Raetz, C. R., C. M. Reynolds, M. S. Trent, and R. E. Bishop. 2007. Lipid A modification systems in gram-negative bacteria. *Annual Review of Biochemistry* 76 : 295-329.

Rahav, G., E. Pinco, F. Silbaq, and H. Bercovier. 1994. Molecular epidemiology of catheter-associated bacteriuria in nursing home patients. *Journal of Clinical Microbiology* 32 (4) (Apr): 1031-4.

Rather, P. N. 2005. Swarmer cell differentiation in *Proteus mirabilis*. *Environmental Microbiology* 7 (8) (Aug): 1065-73.

Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik, C. Whitfield, D. Coplin, N. Kido, et al. 1996. Bacterial polysaccharide synthesis and gene nomenclature. *Trends in Microbiology* 4 (12) (Dec): 495-503.

Regue, M., L. Izquierdo, S. Fresno, N. Jimenez, N. Pique, M. M. Corsaro, M. Parrilli, T. Naldi, S. Merino, and J. M. Tomas. 2005b. The incorporation of glucosamine into enterobacterial core

lipopolysaccharide: Two enzymatic steps are required. *The Journal of Biological Chemistry* 280 (44) (Nov 4): 36648-56.

Regue, M., L. Izquierdo, S. Fresno, N. Pique, M. M. Corsaro, T. Naldi, C. De Castro, D. Waidelich, S. Merino, and J. M. Tomas. 2005a. A second outer-core region in *Klebsiella pneumoniae* lipopolysaccharide. *Journal of Bacteriology* 187 (12) (Jun): 4198-206.

Regue, M., N. Climent, N. Abitiu, N. Coderch, S. Merino, L. Izquierdo, M. Altarriba, and J. M. Tomas. 2001. Genetic characterization of the *Klebsiella pneumoniae waa* gene cluster, involved in core lipopolysaccharide biosynthesis. *Journal of Bacteriology* 183 (12) (Jun): 3564-73.

Reid, G., and J. D. Sobel. 1987. Bacterial adherence in the pathogenesis of urinary tract infection: A review. *Reviews of Infectious Diseases* 9 (3) (May-Jun): 470-87.

Reissbrodt, R., W. Rabsch, A. Chapeaurouge, G. Jung, and G. Winkelmann. 1990. Isolation and identification of ferroxamine G and E in *Hafnia alvei*. *Bio-Metals* 3 : 54-60.

Reizer, J., A. Reizer, and M. H. Saier Jr. 1992. A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. *Protein Science : A Publication of the Protein Society* 1 (10) (Oct): 1326-32.

Ren, J., S. Sainsbury, S. E. Combs, R. G. Capper, P. W. Jordan, N. S. Berrow, D. K. Stammers, N. J. Saunders, and R. J. Owens. 2007. The structure and transcriptional analysis of a global regulator from *Neisseria meningitidis*. *The Journal of Biological Chemistry* 282 (19) (May 11): 14655-64.

Reynolds, C. M., S. R. Kalb, R. J. Cotter, and C. R. Raetz. 2005. A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of *Escherichia coli* lipopolysaccharide. identification of the *eptB* gene and Ca^{2+} hypersensitivity of an *eptB* deletion mutant. *The Journal of Biological Chemistry* 280 (22) (Jun 3): 21202-11.

Rick, P. D., and R. P. Silver. 1996. Enterobacterial common antigen and capsular polysaccharides. in: *Escherichia coli* and *salmonella*: Cellular and molecular biology. eds: Neidhardt F C, Curtiss R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, schaechter M & Umbarger H E, American Society for Microbiology press, Washington, D. C. : 104-22

Rick, P. D., G. L. Hubbard, and K. Barr. 1994. Role of the *rfe* gene in the synthesis of the O8 antigen in E*scherichia coli* K-12. *Journal of Bacteriology* 176 (10) (May): 2877-84.

References

Rietschel, E. T., and H. Brade.1992.Bacterial endotoxins.*Scientific American* 267 (2)(Aug):54-61.

Rietschel, E. T., H. Brade, O. Holst, L. Brade, S. Muller-Loennies, U. Mamat, U. Zahringer, et al. 1996. Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. *Current Topics in Microbiology and Immunology* 216 : 39-81.

Rietschel, E. T., L. Brade, B. Lindner, and U. Zähringer. 1992. Biochemistry of lipopolysaccharides. *In D. C. Morrison and J. L. Ryan (Ed.), Bacterial Endotoxic Lipopolysaccharides. CRC Press, Inc., Boca Raton, Fla.*: 3-41.

Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, and F. Di Padova. 1994. Bacterial endotoxin: Molecular relationships of structure to activity and function. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 8 (2) (Feb): 217-25.

Robbins, P. W., D. Bray, B. M. Dankert, and A. Wright. 1967. Direction of chain growth in polysaccharide synthesis. *Science (New York, N.Y.)* 158 (3808) (Dec 22): 1536-42.

Roberson, E. B., and M. K. Firestone. 1992. Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Applied and Environmental Microbiology* 58 (4) (Apr): 1284-91.

Roberts, I. S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology* 50 : 285-315.

Roberts, I. S., F. K. Saunders, and G. J. Boulnois. 1989. Bacterial capsules and interactions with complement and phagocytes. *Biochemical Society Transactions* 17 (3) (Jun): 462-4.

Roberts, I., R. Mountford, N. High, D. Bitter-Suermann, K. Jann, K. Timmis, and G. Boulnois. 1986. Molecular cloning and analysis of genes for production of K5, K7, K12, and K92 capsular polysaccharides in *Escherichia coli*. *Journal of Bacteriology* 168 (3) (Dec): 1228-33.

Rocchetta, H. L., L. L. Burrows, and J. S. Lam. 1999. Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiology and Molecular Biology Reviews : MMBR* 63 (3) (Sep): 523-53.

Rocha, S. P., J. S. Pelayo, and W. P. Elias. 2007. Fimbriae of uropathogenic *Proteus mirabilis*. *FEMS Immunology and Medical Microbiology* 51 (1) (Oct): 1-7.

Roland, K. L., L. E. Martin, C. R. Esther, and J. K. Spitznagel. 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *Journal of Bacteriology* 175 (13) (Jul): 4154-64.

Ronald, A. 2003. The etiology of urinary tract infection: Traditional and emerging pathogens. *Disease-a-Month : DM* 49 (2) (Feb): 71-82.

Rosenstein, I. J., J. M. Hamilton-Miller, and W. Brumfitt. 1981. Role of urease in the formation of infection stones: Comparison of ureases from different sources. *Infection and Immunity* 32 (1) (Apr): 32-7.

Rozalski, A., Z. Sidorczyk, and K. Kotelko. 1997. Potential virulence factors of *Proteus* bacilli. *Microbiology and Molecular Biology Reviews : MMBR* 61 (1) (Mar): 65-89.

Rund, S., B. Lindner, H. Brade, and O. Holst. 1999. Structural analysis of the lipopolysaccharide from *Chlamydia trachomatis* serotype L2. *The Journal of Biological Chemistry* 274 (24) (Jun 11): 16819-24.

Sabbuba, N. A., D. J. Stickler, E. Mahenthiralingam, D. J. Painter, J. Parkin, and R. C. Feneley. 2004. Genotyping demonstrates that the strains of *Proteus mirabilis* from bladder stones and catheter encrustations of patients undergoing long-term bladder catheterization are identical. *The Journal of Urology* 171 (5) (May): 1925-8.

Sabbuba, N. A., E. Mahenthiralingam, and D. J. Stickler. 2003. Molecular epidemiology of *Proteus mirabilis* infections of the catheterized urinary tract. *Journal of Clinical Microbiology* 41 (11) (Nov): 4961-5.

Sabbuba, N., G. Hughes, and D. J. Stickler. 2002. The migration of *Proteus mirabilis* and other urinary tract pathogens over foley catheters. *BJU International* 89 (1) (Jan): 55-60.

Samuel, G., and P. Reeves. 2003. Biosynthesis of O-antigens: Genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. *Carbohydrate Research* 338 (23) (Nov 14): 2503-19

Sanderson, K. E., and M. Demerec. 1965. The linkage map of *Salmonella typhimurium*. *Genetics* 51 (Jun): 897-913.

Sareneva, T., H. Holthofer, and T. K. Korhonen. 1990. Tissue-binding affinity of *Proteus mirabilis* fimbriae in the human urinary tract. *Infection and Immunity* 58 (10) (Oct): 3330-6.

Savoia, D., P. Martinetto, A. Achino, and A. Pugliese. 1983. Adhesion of *Proteus* species to various cell types. *European Journal of Clinical Microbiology* 2 (6) (Dec): 571-6.

References

Scavone, P., V. Sosa, R. Pellegrino, U. Galvalisi, and P. Zunino. 2004. Mucosal vaccination of mice with recombinant *Proteus mirabilis* structural fimbrial proteins. *Microbes and Infection* 6 (9) (Jul): 853-60.

Schindler, M., and M. J. Osborn. 1979. Interaction of divalent cations and polymyxin B with lipopolysaccharide. *Biochemistry* 18 (20) (Oct 2): 4425-30.

Schnaitman, C. A., and J. D. Klena. 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiological Reviews* 57 (3) (Sep): 655-82.

Schoolnik, G. K., D. Lark, and P. O'Hanley. 1980. Bacterial adherence and anticolonization vaccines. *In J. S. Remington, and M. N. Swartz (Ed.), Current Clinical Topics in Infectious Diseases. McGraw-Hill Book Co., New York, N.Y.*: 85-102.

Senior, B. W. 1977. Typing of *Proteus* strains by proticine production and sensitivity. *Journal of Medical Microbiology* 10 (1) (Feb): 7-17.

Senior, B. W., L. M. Loomes, and M. A. Kerr. 1991. The production and activity *in vivo* of *Proteus mirabilis* IgA protease in infections of the urinary tract. *Journal of Medical Microbiology* 35 (4) (Oct): 203-7.

Senior, B. W., N. C. Bradford, and D. S. Simpson. 1980. The ureases of *Proteus* strains in relation to virulence for the urinary tract. *Journal of Medical Microbiology* 13 (4) (Nov): 507-12.

Seydel, U., H. Labischinski, M. Kastowsky, and K. Brandenburg. 1993. Phase behavior, supramolecular structure, and molecular conformation of lipopolysaccharide. *Immunobiology* 187 (3-5) (Apr): 191-211.

Seydel, U., M. Oikawa, K. Fukase, S. Kusumoto, and K. Brandenburg. 2000. Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. *European Journal of Biochemistry / FEBS* 267 (10) (May): 3032-9.

Shashkov, A. S., F. V. Toukach, N. A. Paramonov, A. Ziolkowski, S. N. Senchenkova, W. Kaca, and Y. A. Knirel. 1996. Structures of new acidic O-specific polysaccharides of the bacterium *Proteus mirabilis* serogroups O26 and O30. *FEBS Letters* 386 (2-3) (May 20): 247-51.

Shashkov, A. S., S. N. Senchenkova, E. V. Vinogradov, G. V. Zatonsky, Y. A. Knirel, E. Literacka, and W. Kaca. 2000. Full structure of the O-specific polysaccharide of *Proteus mirabilis* O24 containing 3,4-O-[(S)-1-carboxyethylidene]-D-galactose. *Carbohydrate Research* 329 (2) (Nov 3): 453-7.

Shimizu, N., T. Koyama, and K. Ogura. 1998. Molecular cloning, expression, and purification of undecaprenyl diphosphate synthase. No sequence similarity between E- and Z-prenyl diphosphate synthases. *The Journal of Biological Chemistry* 273 (31) (Jul 31): 19476-81.

Shrivastava, T., and R. Ramachandran. 2007. Mechanistic insights from the crystal structures of a feast/famine regulatory protein from *Mycobacterium tuberculosis* H37Rv. *Nucleic Acids Research* 35 (21): 7324-35.

Sidorczyk, Z., A. Swierzko, Y. A. Knirel, E. V. Vinogradov, A. Y. Chernyak, L. O. Kononov, M. Cedzynski, A. Rozalski, W. Kaca, and A. S. Shashkov. 1995. Structure and epitope specificity of the O-specific polysaccharide of *Proteus penneri* strain 12 (ATCC 33519) containing the amide of D-galacturonic acid with L-threonine. *European Journal of Biochemistry / FEBS* 230 (2) (Jun 1): 713-21.

Sidorczyk, Z., U. Zahringer, and E. T. Rietschel. 1983. Chemical structure of the lipid A component of the lipopolysaccharide from a *Proteus mirabilis* re-mutant. *European Journal of Biochemistry / FEBS* 137 (1-2) (Dec 1): 15-22.

Sidorczyk, Z., W. Kaca, and K. Kotelko. 1975. Studies on lipopolysaccharides of *Proteus vulgaris* serogroups. chemotypes of genus *Proteus* lipopolysaccharides. *Bulletin De l'Academie Polonaise Des Sciences.Serie Des Sciences Biologiques* 23 (9): 603-9.

Sidorczyk, Z., W. Kaca, H. Brade, E. T. Rietschel, V. Sinnwell, and U. Zahringer. 1987. Isolation and structural characterization of an 8-O-(4-amino-4-deoxy-beta-L-arabinopyranosyl)-3-deoxy-D-manno- octulosonic acid disaccharide in the lipopolysaccharide of a *Proteus mirabilis* deep rough mutant. *European Journal of Biochemistry / FEBS* 168 (2) (Oct 15): 269-73.

Siegman-Igra, Y., T. Kulka, D. Schwartz, and N. Konforti. 1994. Polymicrobial and monomicrobial bacteraemic urinary tract infection. *The Journal of Hospital Infection* 28 (1) (Sep): 49-56.

Silver, R. P., C. W. Finn, W. F. Vann, W. Aaronson, R. Schneerson, P. J. Kretschmer, and C. F. Garon. 1981. Molecular cloning of the K1 capsular polysaccharide genes of *E. coli*. *Nature* 289 (5799) (Feb 19): 696-8.

Silverblatt, F. J. 1974. Host-parasite interaction in the rat renal pelvis: A possible role for pili in the pathogenesis of pyelonephritis. *The Journal of Experimental Medicine* 140 (6) (Dec 1): 1696-711

References

Silverblatt, F. J., and I. Ofek. 1978. Effects of pili on susceptibility of *Proteus mirabilis* to phagocytosis and on adherence to bladder cells. In *Infections of the urinary tract.* University of Chicago Press ed., 49-59. Chicago: E. H. Kass and W. Brumfitt.

Skurnik, M., and P. Toivanen. 1993. *Yersinia enterocolitica* lipopolysaccharide: Genetics and virulence. *Trends in Microbiology* 1 (4) (Jul): 148-52.

Somerville, J. E.,Jr, L. Cassiano, B. Bainbridge, M. D. Cunningham, and R. P. Darveau. 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *The Journal of Clinical Investigation* 97 (2) (Jan 15): 359-65.

Soncini, F. C., E. Garcia Vescovi, F. Solomon, and E. A. Groisman. 1996. Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: Identification of PhoP-regulated genes. *Journal of Bacteriology* 178 (17) (Sep): 5092-9.

Stahl, S. J., K. R. Stewart, and F. D. Williams. 1983. Extracellular slime associated with *Proteus mirabilis* during swarming. *Journal of Bacteriology* 154 (2) (May): 930-7.

Stanislavsky, E. S., and J. S. Lam. 1997. *Pseudomonas aeruginosa* antigens as potential vaccines. *FEMS Microbiology Reviews* 21 (3) (Nov): 243-77.

Stevenson, G., A. Kessler, and P. R. Reeves. 1995. A plasmid-borne O-antigen chain length determinant and its relationship to other chain length determinants. *FEMS Microbiology Letters* 125 (1) (Jan 1): 23-30.

Stevenson, L. G., and P. N. Rather. 2006. A novel gene involved in regulating the flagellar gene cascade in *Proteus mirabilis*. *Journal of Bacteriology* 188 (22) (Nov): 7830-9.

Stickler, D. J. 2008. Bacterial biofilms in patients with indwelling urinary catheters. *Nature Clinical Practice.Urology* 5 (11) (Nov): 598-608.

Stickler, D. J., and R. C. Feneley. 2010. The encrustation and blockage of long-term indwelling bladder catheters: A way forward in prevention and control. *Spinal Cord* 48 (11) (Nov): 784-90.

Stickler, D., and G. Hughes. 1999. Ability of *Proteus mirabilis* to swarm over urethral catheters. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology* 18 (3) (Mar): 206-8.

Stone, K. J., and J. L. Strominger. 1971. Mechanism of action of bacitracin: Complexation with metal ion and C55-isoprenyl pyrophosphate. *Proceedings of the National Academy of Sciences of the United States of America* 68 (12) (Dec): 3223-7.

Storm, D. R., and J. L. Strominger. 1973. Complex formation between bacitracin peptides and isoprenyl pyrophosphates. the specificity of lipid-peptide interactions. *The Journal of Biological Chemistry* 248 (11) (Jun 10): 3940-5.

Susskind, M., B. Lindner, T. Weimar, H. Brade, and O. Holst. 1998. The structure of the lipopolysaccharide from *Klebsiella oxytoca* rough mutant R29 (O1-/K29-). *Carbohydrate Research* 312 (1-2) (Nov): 91-5.

Tamayo, R., B. Choudhury, A. Septer, M. Merighi, R. Carlson, and J. S. Gunn. 2005. Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar typhimurium lipopolysaccharide core. *Journal of Bacteriology* 187 (10) (May): 3391-9.

Tomasiewicz, H. G. 1990. *The macromolecular synthesis II operon of Escherichia coli*. University of Colorado ed. Colorado: .

Touze, T., A. X. Tran, J. V. Hankins, D. Mengin-Lecreulx, and M. S. Trent. 2008. Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. *Molecular Microbiology* 67 (2) (Jan): 264-77.

Trent, M. S., A. A. Ribeiro, S. Lin, R. J. Cotter, and C. R. Raetz. 2001a. An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: Induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. *The Journal of Biological Chemistry* 276 (46) (Nov 16): 43122-31.

Trent, M. S., A. A. Ribeiro, W. T. Doerrler, S. Lin, R. J. Cotter, and C. R. Raetz. 2001. Accumulation of a polyisoprene-linked amino sugar in polymyxin-resistant *Salmonella typhimurium* and *Escherichia coli*: Structural characterization and transfer to lipid A in the periplasm. *The Journal of Biological Chemistry* 276 (46) (Nov 16): 43132-44.

Trent, M. S., and C. R. Raetz. 2002. Cloning of EptA, the lipid A phosphoethanolamine transferase associated with polymyxin resistance. *J. Endotoxin Res.* 8 : 158.

Trent, M. S., C. M. Stead, A. X. Tran, and J. V. Hankins. 2006. Diversity of endotoxin and its impact on pathogenesis. *Journal of Endotoxin Research* 12 (4): 205-23.

Tzeng, Y. L., K. D. Ambrose, S. Zughaier, X. Zhou, Y. K. Miller, W. M. Shafer, and D. S. Stephens. 2005. Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. *Journal of Bacteriology* 187 (15) (Aug): 5387-96.

References

Uphoff, T. S., and R. A. Welch. 1990. Nucleotide sequencing of the *Proteus mirabilis* calcium-independent hemolysin genes (*hpmA* and *hpmB*) reveals sequence similarity with the *Serratia marcescens* hemolysin genes (*shlA* and *shlB*). *Journal of Bacteriology* 172 (3) (Mar): 1206-16.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiological Reviews* 56 (3) (Sep): 395-411.

Vaara, M., and P. Viljanen. 1985. Binding of polymyxin B nonapeptide to gram-negative bacteria. *Antimicrobial Agents and Chemotherapy* 27 (4) (Apr): 548-54.

Viau, C., V. Le Sage, D. K. Ting, J. Gross, and H. Le Moual. 2011. Absence of PmrAB-mediated phosphoethanolamine modifications of *Citrobacter rodentium* lipopolysaccharide affects outer membrane integrity. *Journal of Bacteriology* 193 (9) (May): 2168-76.

Vinogradov, E. 2002. Structure of the lipopolysaccharide core region of the bacteria of the genus *Proteus*. *Australian Journal of Chemistry* 55 (1-2): 61-7.

Vinogradov, E. 2011. Structure of the core part of the lipopolysaccharide from *Proteus mirabilis* genomic strain HI4320. *Biochemistry.Biokhimiia* 76 (7) (Jul): 803-7.

Vinogradov, E. V., D. Krajewska-Pietrasik, W. Kaca, A. S. Shashkov, Y. A. Knirel, and N. K. Kochetkov. 1989b. Structure of *Proteus mirabilis* O27 O-specific polysaccharide containing amino acids and phosphoethanolamine. *European Journal of Biochemistry / FEBS* 185 (3) (Nov 20): 645-50.

Vinogradov, E. V., D. Petrasik, A. S. Shashkov, IuA Knirel', and N. K. Kochetkov. 1988. The structure of O-specific polysaccharide of *Proteus mirabilis* 027 containing amino acids and phosphoethanolamine. *Bioorganicheskaia Khimiia* 14 (9) (Sep): 1282-6.

Vinogradov, E. V., J. E. Thomas-Oates, H. Brade, and O. Holst. 1994. Structural investigation of the lipopolysaccharide from *Proteus mirabilis* R45 (re-chemotype). *J. Endotoxin Res.* 1:199.

Vinogradov, E. V., K. Bock, B. O. Petersen, O. Holst, and H. Brade. 1997. The structure of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter* strain ATCC 17905. *European Journal of Biochemistry / FEBS* 243 (1-2) (Jan 15): 122-7.

Vinogradov, E. V., KatsaV, A. S. Shashkov, D. Petrashik, A. Rozhalski, IuA Knirel', and N. F. Kochetkov. 1989a. Structure of an O-specific polysaccharide of *Proteus mirabilis* 03, containing N-(2-hydroxyethyl)-D-alanine. *Bioorganicheskaia Khimiia* 15 (10) (Oct):1431-4.

Vinogradov, E. V., W. Kaca, A. S. Shashkov, D. Krajewska-Pietrasik, A. Rozalski, Y. A. Knirel, and N. K. Kochetkov. 1990. The structure of *Proteus mirabilis* O3 O-specific polysaccharide containing N-(2-hydroxyethyl)-D-alanine. *European Journal of Biochemistry / FEBS* 188 (3) (Mar 30): 645-51.

Vinogradov, E., and J. Radziejewska-Lebrecht. 2000. The structure of the carbohydrate backbone of the core-lipid A region of the lipopolysaccharide from *Proteus mirabilis* serotype O28. *Carbohydrate Research* 329 (2) (Nov 3): 351-7.

Vinogradov, E., and K. Bock. 1999. The structure of the core part of *Proteus mirabilis* O27 lipopolysaccharide with a new type of glycosidic linkage. *Carbohydrate Research* 319 (1-4) (Jun 30): 92-101.

Vinogradov, E., and M. B. Perry. 2000. Structural analysis of the core region of lipopolysaccharides from *Proteus mirabilis* serotypes O6, O48 and O57. *European Journal of Biochemistry / FEBS* 267 (8) (Apr): 2439-46.

Vinogradov, E., J. Radziejewska-Lebrecht, and W. Kaca. 2000c. The structure of the carbohydrate backbone of core-lipid A region of the lipopolysaccharides from *Proteus mirabilis* wild-type strain S1959 (serotype O3) and its ra mutant R110/1959. *European Journal of Biochemistry / FEBS* 267 (1) (Jan): 262-9.

Vogel S.N., and Hogan M.M. 1990. Role of cytokines in endotoxic-mediated host response. In *Immunophisiology, role of cells and citokines in immunity and inflammation.* Oxford University press. ed., 238-258. Oxford: J. J. Hoppenheim and E.N. Shevack.

Vorachek-Warren, M. K., S. M. Carty, S. Lin, R. J. Cotter, and C. R. Raetz. 2002. An *Escherichia coli* mutant lacking the cold shock-induced palmitoleoyltransferase of lipid A biosynthesis: Absence of unsaturated acyl chains and antibiotic hypersensitivity at 12 degrees C. *The Journal of Biological Chemistry* 277 (16) (Apr 19): 14186-93.

Wang, L., and P. R. Reeves. 1998. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infection and Immunity* 66 (8) (Aug): 3545-51.

Wang, L., D. Liu, and P. R. Reeves. 1996. C-terminal half of *Salmonella enterica* WbaP (RfbP) is the galactosyl-1-phosphate transferase domain catalyzing the first step of O-antigen synthesis. *Journal of Bacteriology* 178 (9) (May): 2598-604.

References

Wang, Q., A. Torzewska, X. Ruan, X. Wang, A. Rozalski, Z. Shao, X. Guo, H. Zhou, L. Feng, and L. Wang. 2010. Molecular and genetic analyses of the putative proteus O antigen gene locus. *Applied and Environmental Microbiology* 76 (16) (Aug): 5471-8.

Wang, W. B., I. C. Chen, S. S. Jiang, H. R. Chen, C. Y. Hsu, P. R. Hsueh, W. B. Hsu, and S. J. Liaw. 2008. Role of RppA in the regulation of polymyxin b susceptibility, swarming, and virulence factor expression in *Proteus mirabilis*. *Infection and Immunity* 76 (5) (May): 2051-62.

Warren, J. W. 2001. Catheter-associated urinary tract infections. *International Journal of Antimicrobial Agents* 17 (4) (Apr): 299-303.

Wassif, C., D. Cheek, and R. Belas. 1995. Molecular analysis of a metalloprotease from *Proteus mirabilis*. *Journal of Bacteriology* 177 (20) (Oct): 5790-8.

Wazait, H. D., H. R. Patel, V. Veer, M. Kelsey, J. H. Van Der Meulen, R. A. Miller, and M. Emberton. 2003. Catheter-associated urinary tract infections: Prevalence of uropathogens and pattern of antimicrobial resistance in a UK hospital (1996-2001). *BJU International* 91 (9) (Jun): 806-9.

Weisgerber, C., and K. Jann. 1982. Glucosyldiphosphoundecaprenol, the mannose acceptor in the synthesis of the O9 antigen of *Escherichia coli.* biosynthesis and characterization. *European Journal of Biochemistry / FEBS* 127 (1) (Sep): 165-8.

Welch, R. A. 1987. Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infection and Immunity* 55 (9) (Sep): 2183-90.

Wenner, J. J., and L. F. Rettger. 1919. A systematic study of the *Proteus* group of bacteria. *Journal of Bacteriology* 4 (4) (Jul): 331-53.

White, K. A., I. A. Kaltashov, R. J. Cotter, and C. R. Raetz. 1997. A mono-functional 3-deoxy-D-manno-octulosonic acid (kdo) transferase and a kdo kinase in extracts of *Haemophilus influenzae. The Journal of Biological Chemistry* 272 (26) (Jun 27): 16555-63.

White, R. H. 1996. Biosynthesis of isoprenoids in bacteria. in: *Escherichia coli* and S*almonella*: Cellular and molecular biology. eds: Neidhardt F C, curtiss III R, ingraham J L, lin E C C, low K B, magasanik B, reznikoff W S, riley M, schaechter M & umbarger H E, american society for microbiology press, washington, D. C. : 637-41.

Whitfield, C. 1995. Biosynthesis of lipopolysaccharide O antigens. *Trends in Microbiology* 3 (5) (May): 178-85.

Whitfield, C., and M. A. Valvano. 1993. Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. *Advances in Microbial Physiology* 35 : 135-246.

Whitfield, C., P. A. Amor, and R. Koplin. 1997. Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer: Lipid A-core ligase and by determinants of polymer chain length. *Molecular Microbiology* 23 (4) (Feb): 629-38.

Williams, F. D., and R. H. Schwarzhoff. 1978. Nature of the swarming phenomenon in *Proteus. Annual Review of Microbiology* 32 : 101-22.

Winfield, M. D., T. Latifi, and E. A. Groisman. 2005. Transcriptional regulation of the 4-amino-4-deoxy-L-arabinose biosynthetic genes in Y*ersinia pestis*. *The Journal of Biological Chemistry* 280 (15) (Apr 15): 14765-72.

Woebking, B., G. Reuter, R. A. Shilling, S. Velamakanni, S. Shahi, H. Venter, L. Balakrishnan, and H. W. van Veen. 2005. Drug-lipid A interactions on the *Escherichia coli* ABC transporter MsbA. *Journal of Bacteriology* 187 (18) (Sep): 6363-9.

Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: Proposal for the domains archaea, bacteria, and eucarya. *Proceedings of the National Academy of Sciences of the United States of America* 87 (12) (Jun): 4576-9.

Wosten, M. M., L. F. Kox, S. Chamnongpol, F. C. Soncini, and E. A. Groisman. 2000. A signal transduction system that responds to extracellular iron. *Cell* 103 (1) (Sep 29): 113-25.

Wright, J. C., D. W. Hood, G. A. Randle, K. Makepeace, A. D. Cox, J. Li, R. Chalmers, J. C. Richards, and E. R. Moxon. 2004. *lpt6*, a gene required for addition of phosphoethanolamine to inner-core lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*. *Journal of Bacteriology* 186 (20) (Oct): 6970-82.

Wyckoff, T. J., C. R. Raetz, and J. E. Jackman. 1998. Antibacterial and anti-inflammatory agents that target endotoxin. *Trends in Microbiology* 6 (4) (Apr): 154-9.

Yethon, J. A., and C. Whitfield. 2001. Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. *The Journal of Biological Chemistry* 276 (8) (Feb 23): 5498-504.

Yethon, J. A., D. E. Heinrichs, M. A. Monteiro, M. B. Perry, and C. Whitfield. 1998. Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. *The Journal of Biological Chemistry* 273 (41) (Oct 9): 26310-6.

References

Young, K., L. L. Silver, D. Bramhill, P. Cameron, S. S. Eveland, C. R. Raetz, S. A. Hyland, and M. S. Anderson. 1995. The *envA* permeability/cell division gene of *Escherichia coli* encodes the second enzyme of lipid A biosynthesis. UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase. *The Journal of Biological Chemistry* 270 (51) (Dec 22): 30384-91.

Zahringer, U., B. Lindner, and E. T. Rietschel. 1994. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry* 50 : 211-76.

Zamyatina, A., S. Gronow, C. Oertelt, M. Puchberger, H. Brade, and P. Kosma. 2000. Efficient chemical synthesis of the two anomers of ADP-L-glycero- and D-glycero-D-manno-heptopyranose allows the determination of the substrate specificities of bacterial heptosyltransferases. *Angewandte Chemie (International Ed.)* 39 (22) (Nov 17): 4150-3.

Zhao, H., X. Li, D. E. Johnson, and H. L. Mobley. 1999. Identification of protease and *rpoN*-associated genes of uropathogenic *Proteus mirabilis* by negative selection in a mouse model of ascending urinary tract infection. *Microbiology (Reading, England)* 145 (Pt 1) (Jan): 185-95.

Zhou, Z., A. A. Ribeiro, and C. R. Raetz. 2000. High-resolution NMR spectroscopy of lipid A molecules containing 4-amino-4-deoxy-L-arabinose and phosphoethanolamine substituents. different attachment sites on lipid A molecules from NH4VO3-treated *Escherichia coli* versus *kdsA* mutants of *Salmonella typhimurium*. *The Journal of Biological Chemistry* 275 (18) (May 5): 13542-51.

Zunino, P., V. Sosa, A. G. Allen, A. Preston, G. Schlapp, and D. J. Maskell. 2003. *Proteus mirabilis* fimbriae (PMF) are important for both bladder and kidney colonization in mice. *Microbiology (Reading, England)* 149 (Pt 11) (Nov): 3231-7

Zych, K., and Z. Sidorczyk. 1989. Lipopolysaccharides of *Proteus penneri* species novum. *Carbohydrate Research* 188 (Jun 1): 105-11.

9. List of Abbreviations

A-B-C-D-E

ABC = ATP-binding cassette

ACP = acyl carrier protein

ADP = adenosine diphosphate

AMP = antimicrobial peptides

AraN = 4-amino-4- deoxy-L-arabinosyl

ATF = ambient temperature fimbriae

ATP = adenosine-5'-triphosphate

C55-P = undecaprenyl-phosphate

C55-PP = undecaprenyl pyrophosphate

CAMP = cationic antimicrobial peptides

CAPs = cationic antimicrobial peptides

CaUTIs = catheter-associated urinary tract infections

CAZy = Carbohydrate-Active enZymes

CD14 = cluster of differentiation 14

CDS = protein coding sequences

CI = competitive index

CPS = capsular polysaccharide

D,D-Hep = D-*glycero*-D-*manno*-heptose

DNA = deoxyribonucleic acid

ECA = enterobacterial common antigen

EMBL = European Molecular Biology Laboratory

F-G-H-I

G + C = guanine plus cytosine

Gal = galactose

GalA = galacturonic acid

GalALys = amide of GalA with L-lysine

GalAN = amides of D-GalA

GalN = galactosamine

GalNAc = N-acetylgalactosamine

Gal*o*NAc = *N*-acetylgalactosamine residue in the open-chain form

Glc = glucose

Glc I = first Glc residue

Glc II = second Glc residue

GlcN = glucosamine

GlcN-1-P =   glucosamine-1-phosphate

GlcNGly = 2-glycylamino-2-deoxy-D-glucose

HDL = high density lipoprotein

Hep = heptose

Hep I = first Hep residue

Hep II = second Hep residue

Hex = hexose

HexN = hexosamine

HexNAc = N-acetyl-hexosamine

IFN-γ = Interferon-gamma

IgA = immunoglobulin A

IL-1 = interleukin-1

IL-10 = interleukin-10

IL-6 = interleukin-6

IL-8 = interleukin-8

J-K-L-M

JUMPStart = *J*ust *U*pstream of *M*any *P*olysaccharide- associated gene *Start*s

Kb = kilobase

Kdo = 3-Deoxy-D-manno-oct-2-ulosonic acid

Ko = D-glycero-D-talo-oct-ulosonic acid

L-Ara4N = L-arabinoso-4-amine

l-Ara*p*4N = 4-amino-4-deoxy-l-arabinopyranose

L,D-Hep = L-glicero-α-D-manno-heptose

LB = Luria-Bertani medium

LBP = LPS binding protein

lipid X = 2,3-diacylglucosamine 1-phosphate

LOSs = lipooligosaccharides

LPS = lipopolysaccharide

LTR4 = toll like receptor 4

ManNAc = N-acetylmannosamine

MIC = Minimal Inhibitory Concentration

MR/K = Mannose-resistant/*Klebsiella*-like fimbriae

MR/P = Mannose-resistant/*Proteus*-like fimbriae

MS = Mass Spectometry

N-O-P

N-terminal = amino-terminal

NAF = non-agglutinating fimbriae

NHS = non-immune human serum

NPN = 1-*N*-fenilnaftilamine

O-PS = O-specific polysaccharide

OM = outer membrane

OMP = outer membrane proteins

ops = operon polarity suppressor

ORF = Open Reading Frame

OS = oligosaccharide

OSMII = Macromolecular Synthesis Operon II

P = phosphate

PAGE = PolyAcrylamide Gel Electrophoresis

Pcho = phosphorylcholine

PEtN = phosphoethanolamine

PHPT = polyisoprenyl-phosphate hexose-1-phosphate transferases

PMF = *Proteus mirabilis* fimbriae

PMP = *Proteus mirabilis* P-like fimbriae

PmxB = polymyxin B

PNPT = polyisoprenyl-phosphate *N*-acetylhexosamine- 1-phosphate transferases

PPEtN = pyrophosphorylethanolamine

PPM = *Proteus-Morganella-Providencia*

Q-R-S

Qui4NAlaAla = 4-(L-alanyl-L-alanyl)amino-4,6-dideoxy-D-glucose

RNA = ribonucleic acid

S LPS = smooth LPS

sCD14 = soluble cluster of differentiation 14

SDS = Sodium Dodecyl Sulphate

SEM = scanning electron microscope

sIgA = secretory immunoglobulins A

STM = signature-tagged mutagenesis

T-U-V-Z

T6SS = type six secretion system

TNF = tumor necrosis factor

UCA = uroepithelial cell adhesin

UDP = uridine diphosphate

UMP = uridine monophosphate

Und-P = undecaprenolphosphate

UTIs = urinary tract infections

α-β

α-GalA = α-galacturonic acid

β-L-Ara4N = 4-amino-4-deoxy-β-L-arabinose