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"Ecophysiological and molecular characterization of estuarine microbial mats"

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Departament de Microbiologia

UNIVERSITAT DE BARCELONA

VII. CHARACTERIZATION OF HETEROTROPHIC BACTERIA ISOLATED FROM THE PHOTIC ZONE

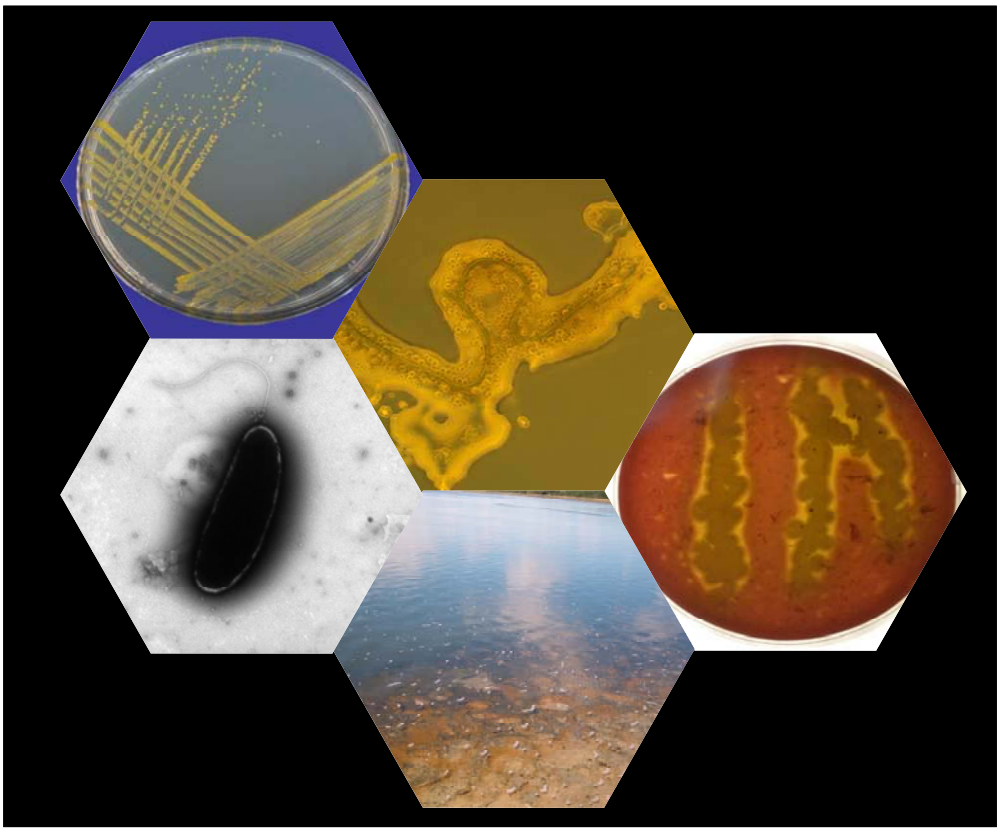

Figure VII. "To be astonished at anything is the first movement of the mind toward discovery" Louis Pasteur (1822–1895).

From top left to right: Pure culture of *Sphingomonas* sp. Camargue / Micromanipulated cyanobacteria *Lyngbya* sp. surrounded by heterotrophic growth / Haemolytic activity of *Pseudoalteromonas* sp. EBD onto a SWYP-blood agar plate / Transmission electron micrograph of a Negative-staining cell of *Pseudoalteromonas* sp. EBD / Ebro delta microbial mat.

- Introduction and objectives of the study

Microbial mats are areas of intense primary production. The availability of both dissolved and particulate organic carbon as a result of excretion from and lysis of cyanobacteria results in the active growth of heterotrophic bacteria in mats. As a consequence, one can expect that the cyanobacteria and the heterotrophic bacteria will undergo interactions ranging from beneficial to harmful.

Some studies have reported the harmful interactions between cyanobacteria and heterotrophic bacteria. For example, lysis of cyanobacteria by gliding bacteria with high G+C contents has been reported by many workers (Shilo, 1970; Marshall, 1989; Rashidan and Bird, 2001). It has also been reported the benefit to heterotrophic bacteria of dissolved organic carbon excreted by cyanobacteria and its role in the establishment of food chains (Worm and Søndergaard, 1998). Other benefits to cyanobacteria from associated heterotrophic bacteria include the exchange of vitamins and other growth factors leading to enhanced cyanobacterial growth, as well as sources of nitrogen (Steppe *et al*., 1996).

Respiring bacteria counteract the chemical changes in O_2, CO_2 and pH induced by photosynthesis and a mutualistic relationship between cyanobacteria and associated heterotrophic bacteria has frequently been suggested (Whitton, 1973). As an example, Worm and Søndergaard (1998) studied the ecology of heterotrophic bacteria attached to the mucilaginous colonies of the cyanobacteria *Microcystis* sp., and demonstrated that *Microcystis* offers attachment sites for a very dynamic community of heterotrophic bacteria which contributes significantly to the carbon metabolism in the photic zone.

During photosynthesis, organic compounds are produced and partially released into the environment. A number of studies have shown that these compounds can readily be assimilated and recycled by associated heterotrophic bacteria (Bauld and Brock, 1974; Bateson and Ward, 1988; Epping *et al*., 1999). Bateson and Ward (1988) have shown that this extracellular release mainly consists of glycolate, the main product of photorespiration.

Aerobic heterotrophic and sulfate-reducing bacteria are expected to play a major role in inorganic carbon mineralization in the photic zone, due to changing diurnal oxic and anoxic conditions. Although sulfate-reducing bacteria are well studied in hypersaline microbial mats (Caumette *et al*., 1991; Teske *et al*., 1998), few studies have focused on the diversity and the role of aerobic heterotrophic bacteria (Van Trappen *et al*., 2002; Jonkers and Abed, 2003). Jonkers and Abed (2003) reported the phylogenetic identification of apparent dominant populations of aerobic heterotrophs in a hypersaline mat. Their studies with those heterotrophs and their potential to use a range of photosynthetic compounds suggested that the isolated strains were specialized in the degradation of photosynthates excreted by primary producers in the photic zone.

Several studies have shown the importance of aerobic heterotrophic bacteria of the microbial mat photic zone in the degradation of pollutants (Cohen, 1992; McGowan *et al.*, 2004). The microhabitat surrounding cyanobacteria assure the availability of O_2, nutrients and nitrogen source that are prerequisites for a successful aerobic degradation, and the close proximity between primary producers and consumers facilitate their interaction and reinforce their interdependence. For this reason, the heterotrophic population is well adapted to alternative aerobic and anaerobic conditions within the mat which is a clear advantage for a potential capacity of degrading refractory organic pollutants. A diverse range of genera of microorganisms have been able to degrade organic pollutants in soils, such as *Pseudomonas*, *Alcaligenes*, *Rhodococcus*, *Corynebacterium*, *Marinobacter*, *Sphingomonas* etc. (Bartha and Atlas, 1977; Van Hamme and Ward, 2001; McGowan *et al.*, 2004).

The aim of this study was (i) the isolation and characterization of heterotrophic bacteria associated with cyanobacteria in the photic zone of microbial mats, giving a special emphasis in their metabolic capacities and their interactions with the primary producers, and (ii) the isolation and characterization of members of the *Sphingomonas* sp. genus in estuarine microbial mats, and the detection of the sphingolipids of this strain and in mat samples as an indicator of a 'potential' capability of the community in degrading pollutants. This last objective is in relationship with chapter III 'Validation of the SLB approach' because it pretended the combination of the characterization of new species and the signature lipid biomarker approach.

- Material and methods

Isolation and characterization of aerobic heterotrophic bacteria associated with cyanobacteria

➢ Micromanipulation of cyanobacteria and isolation of surrounding heterotrophs

Microbial mats from the Ebro delta were sampled in October 2003 and maintained as a microcosm in an incubator chamber under light/dark conditions. Cyanobacterial layer was removed and transferred to 1.5 ml-tubes with 1 ml of sea water and mixed by vortex. Mineral medium for the growth of cyanobacteria (MN) agar plates without cycloheximide were inoculated with several drops of the inoculum and then were allowed to dry. Filamentous cyanobacteria *Microcoleus* sp. and *Lyngbya* sp., were selected and micromanipulated onto MN agar plates. Once the filament was isolated in an empty part of the MN agar, the agar piece was cut and placed onto a new plate to follow the heterotrophic growth surrounding the filamentous cyanobacteria. The MN agar plates with the isolated cyanobacteria were incubated at 20–25ºC under light/dark conditions. Once the bacterial growth was checked by contrast phase microscopy, the agar block with the filamentous cyanobacteria and the surrounding bacterial growth, was stamped onto 'Sea water yeast peptone' (SWYP) agar and onto MN agar, to evaluate the growth of the associated heterotrophic bacteria. The isolated heterotrophic bacterium was growth on SWYP agar plates incubated at 25ºC.

➢ Morphological and biochemical characterization of the isolate

The morphological characteristics, motility and presence of flagella were observed by phase contrast and TEM microscopy of negative-stained cells. The isolated strain was characterized by means of the tests explained in 'Table II.11. Biochemical characteristics' in chapter 'II. General Material and Methods'. The preparation of samples for testing the accumulation of PHA and the determination by HPLC was also performed as described in the mentioned section.

- 16S rDNA sequencing and phylogeny reconstruction

16S rDNA fragment was amplified and sequenced as it was described in chapter 'II. General Material and Methods'. The relationship of the sequenced fragment was visualized in a phylogenetic tree constructed from a ClustalW alignment, using the Neighbor-Joining algorithm and the Jukes and Cantor model (Jukes and Cantor, 1966). The Genbank accession number for the 16S rDNA sequence of the isolated *Pseudoalteromonas* sp. EBD is DQ218321.

- Detection of inhibitory compounds produced by the isolate and interaction activity assay with filamentous cyanobacteria

The isolated strain affiliated with members of the *Pseudoalteromonas* genus, was tested for its capacity of producing antibacterial and auto-inhibitory compounds. The method used for the detection of inhibitory compounds is explained in 'Table II.11. Biochemical characteristics' in chapter 'II. General Material and Methods'. The target bacterial strains were *Halomonas* sp. EBD and *Stappia* sp. EBD (del Campo, 2005), other aerobic heterotrophic bacteria isolated from Ebro delta and Camargue microbial mats in our group and gram-positive and gram-negative bacteria listed in Table II.10 of the 'General Material and Methods' chapter.

In order to clarify the relationship between cyanobacteria and the isolated bacterial strain, an experiment based on the inhibition of the photosynthetic processes of cultured cyanobacteria to avoid the extracellular emission of photosynthate was designed. Several filamentous cyanobacteria previously obtained in our group (Urmeneta *et al*., 2003) were cultured in MN agar plates supplemented with DCMU ([3-(3',4'-dichlorphenyl)-1,1-dimethylurea], an inhibitory compound of the photosystem II) and the cyanobacterium EBD11 (member of the LPP-group) was selected as being unable to growth in 2 μM DCMU. Filamentous cells of EBD11 were transferred to liquid MN+DCMU and maintained in an incubator chamber under light/dark conditions for 48 h. After the incubation, the cells were pelleted by centrifugation (1500 rpm, 10 min) and washed 3 times with MN+DCMU. The washed pellet was suspended in certain volume of MN+DCMU and then it was spread onto MN or MN+DCMU agar

plates that could be previously inoculated with 100 µl suspension of *Pseudoalteromona*s sp. EBD (Table VII.1). The suspension of *Pseudoalteromona*s sp. EBD was obtained by growing the strain in SWYP broth for 24 h at 30ºC. The cells were pelleted by centrifugation and the pellet was washed four times with Ringer ¼ to avoid the presence of broth. The inoculated agar plates were incubated in an incubator under light/dark conditions for 10 days (the growth was followed by phase contrast microscopy every 24 h).

Table VII.1. Experimental design of the interaction activity assay with filamentous cyanobacteria and the isolated heterotroph.

Experimental design	
MN+ *Pseudoalteromonas* sp. EBD	Negative growth control of the heterotroph on MN
MN+DCMU+ *Pseudoalteromonas* sp. EBD	Control of the innocuous effect of DCMU on the heterotroph
MN+cyanobacterium	Control of the recovery of the cyanobacterium without DCMU
MN+DCMU+cyanobacterium	Control of the effect of DCMU on the cyanobacterium
MN+ *Pseudoalteromonas* sp. EBD+cyanobacterium	Assay of interaction
MN+DCMU+ *Pseudoalteromonas* sp. EBD+ cyanobacterium	Assay of interaction

- FISH of cyanobacterial samples

The top cyanobacterial layer of fresh samples of Ebro delta microbial mats, was scrapped with a sterile razor blade, and the samples fixed as detailed in 'Fluorescence *in situ* hydridization' section of the chapter 'II. General Material and Methods'. Samples of cyanobacterial cultures maintained in MN agar plates were also fixed. Fixed samples were filtered through a 0.2 µm-filter and then hybridized with PSA184 (30% formamide) and EUB388 (20% formamide) probes as described in 'II. General Material and Methods'.

Isolation and characterization of members of the* Sphingomonas *genus in estuarine mats and detection of sphingolipids

- Isolation and characterization of the *Sphingomonas* strains

The isolation of the *Sphingomonas* strains was done as it was described in the section 'Isolation of microorganisms for microbial mat samples' of 'II. General Material and Methods'. The isolated strain was characterized by means of the tests explained in 'Table II.11. Biochemical characteristics' (except from the tests 6, 7, 9, 10, and 12). The accumulation of PHA was tested by growing the strain in M55-fructose for 48 h that was transferred to a M55+fructose nitrogen-limited media, and analyzed by HPLC.

- 16S rDNA sequencing and phylogeny reconstruction

16S rDNA fragment was amplified and sequenced as described above. The phylogenetic tree was constructed using the Neighbor-Joining algorithm and the Jukes and Cantor model (Jukes and Cantor, 1966). The Genbank accession number for the 16S rDNA sequence of the isolated *Sphingomonas* sp. Camargue is DQ218322.

- Analysis of sphingoid bases from sphingolipids in *Sphingomonas* strains and microbial mat samples

Total lipid extracts were extracted from cell cultures of the isolated strain (*Sphingomonas* sp. Camargue), from pure cultures of *Sphingomonas paucimobilis* CECT 599, *Novosphingobium capsulatum* CECT 4388, and microbial mat samples from Ebro delta and Camargue mats sampled on November 2004. The total lipid samples were treated and analyzed for sphingoid bases by the method described by Leung and collaborators (Leung *et al.*, 1999) described in the section 'Polar lipid fraction' of 'II. General Material and Methods'.

- Results

Isolation and characterization of aerobic heterotrophic bacteria associated with cyanobacteria

➢ Micromanipulation of cyanobacteria and isolation of surrounding heterotrophs

After 48 h, the micromanipulated cyanobacteria were surrounded by bacterial growth (Fig. VII.1A, B) and negative controls revealed that bacterial growth did not come from the micromanipulator hook or from the process itself. The 'contaminating' bacteria proliferated only surrounding the cyanobacterial sheaths and the phototroph did not seem to be affected by the heterotrophic growth. Moreover, filamentous cyanobacteria did not divide or degrade after the micromanipulation although, inside *Lyngbya* sheaths, the presence of active bacteria was observed by phase contrast microscopy.

The surrounded heterotrophic growth was spread onto SWYP agar plates in order to isolate the growing morphologies but only one heterotrophic strain was isolated. The isolate was unable to grow alone onto MN agar plates.

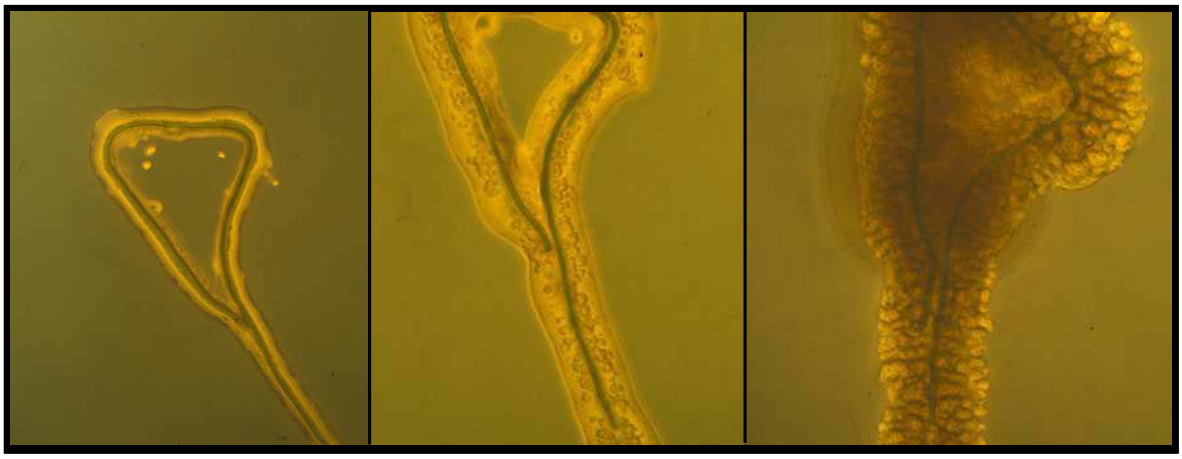

Figure VII.1A. Micromanipulated filamentous cyanobacteria *Lyngbya* sp.

Cyanobacteria micromanipulated onto MN agar plates and the surrounding heterotrophic growth after 2, 4, and 12 days of incubation respectively (200×).

Figure VII.1B. Micromanipulated filamentous cyanobacteria *Lyngbya* sp.

Filamentous cyanobacteria micromanipulated onto MN agar plates and their surrounding heterotrophic growth after 2 (left) and 4 (right) days of incubation respectively (200×).

- Morphological and biochemical characterization of the isolate

Cells of the isolated strain were Gram-negative rods, facultative anaerobes, weakly oxidase and catalase-positive, 0.5–0.8 μm wide and 1.7–4 μm long (Fig. VII.2). When the organism was grown in SWYP broth; the cells occur single or in pairs. Colonies on SWYP agar medium had no pigmentation, and were uniformly round, 2 mm in diameter, beige, circular, regular, smooth and mucous. The strain was motile by means of one polar flagellum (Fig. VII.3). The isolated bacteria did not form endospores, and did not accumulate poly-β-hydroxybutyrate (tested by HPLC and using Nile red). The salinity range of growth was 1.5–10% NaCl, the temperature range of growth was 4–35ºC (being the optimum at 25–30ºC), and the pH range of growth was 5–10 (being the optimum around 7). Tables VII.2–4 summarize the biochemical characteristics, the susceptibility to certain antibiotics, the use of different carbohydrates and the enzymatic activities (Fig. VII.4) as described in 'II. Material and Methods'. The 'Oil displacement activity (ODA)' was positive suggesting a biosurfactant activity of the strain.

Total lipids were extracted, fractionated and analyzed for fatty acids (FAMEs, fatty acid methyl esters) and quinones, as described in 'II. General Material and Methods'. LC/MS/MS analysis indicated that ubiquinone-8 accounted for 96% of total

quinone content of the strain. The other quinones detected were ubiquinone-7 and 6. Menaquinones were not detected. Fatty acid composition was analyzed and the main fatty acids detected were 14:0, 16:0, *a*17:0, and *br*16:1.

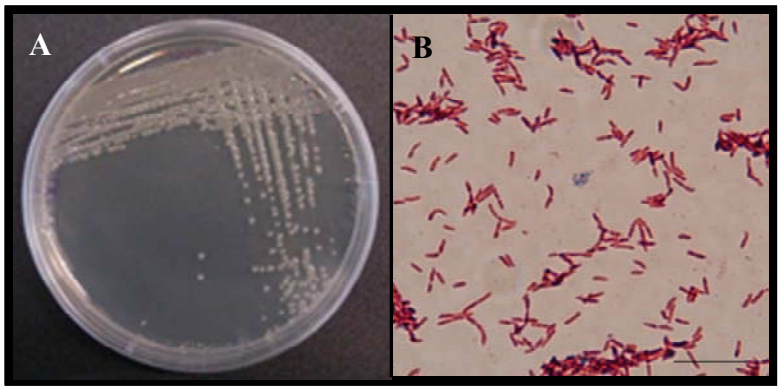

Figure VII.2. Morphology (A) and Gram-staining micrograph (B) of the isolated strain. (Bar = 10 μm).

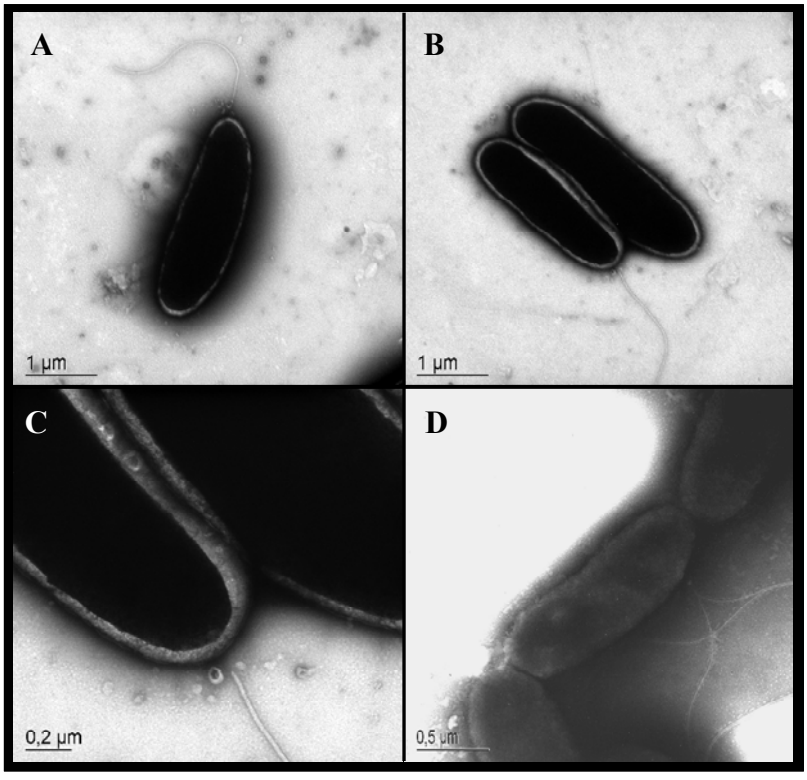

Figure VII.3. Transmission electron micrographs of negative-staining cells of the isolate.

From A–D, details of the morphology and the single polar flagellum.

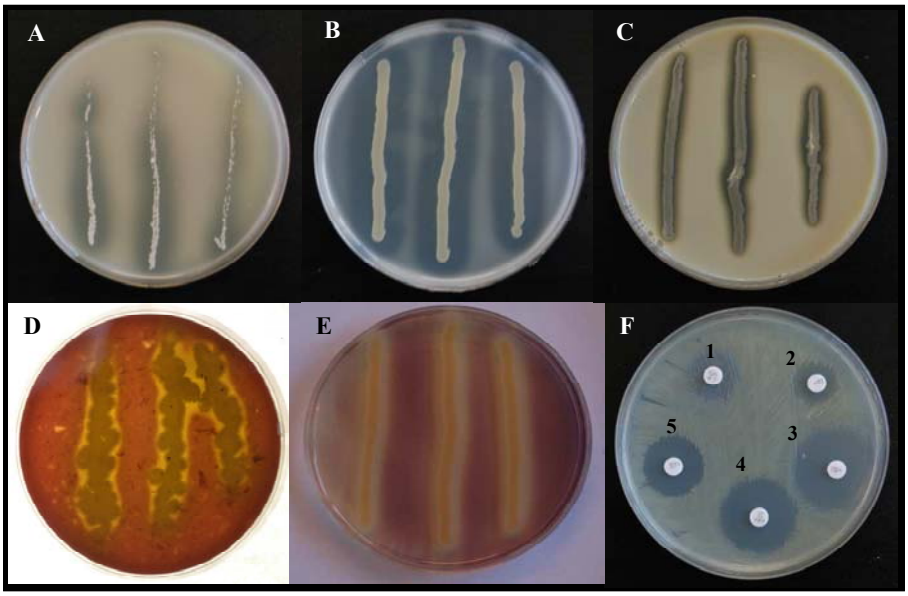

Figure VII.4. Enzymatic activities and antibiotic sensitivity onto agar plates.

(A) DNAse activity; (B) Gelatinase activity; (C) Caseinase activity onto SWYP+skimmed milk plate; (D) Haemolytic activity; (E) Amylase activity; (F) Antibiogram: 1. Trimetroprim 1.25 μg, 2. Streptomycin 10 μg, 3. Cloramphenicol 30 μg, 4. Ampicillin 10 μg, 5. Gentamycin 10 μg.

Table VII.2. Morphobiochemical characterization.

Characteristic	Result[1]	Characteristic	Result
Gram	–	**Growth on McConkey**	–
Motility	+	**Accumulation of PHB**	–
Flagellar arragement	Polar	**Susceptibility to[2]:**	
Oxygen sensitivity	Facultative	**Ampicillin (10 μg)**	Sensitive
Oxidase	weakly +	**Trimetroprim (1.25 μg)**	Sensitive
Catalase	+	**Streptomycin (10 μg)**	Sensitive
Growth with NaCl (%)	1.5–10%	**Gentamycin (10 μg)**	Sensitive
Tº range of growth	4–35ºC	**Cloramphenicol (30 μg)**	Sensitive
pH range of growth	5–10	**Isoprenoid quinone**	Q-8
Pigmentation	–	**Oil displacement activity**	+

[1]Result: + positive, – negative. [2]Tested by antibiogram. PHB: polyhydroxybutyrate.

Table VII.3. Biochemical characteristics based on the API20NE, API ZYM and plate tests.

Characteristic	Result[1]	*CHARACTERISTIC*	Result[1]
Reduction of nitrates to nitrites	–	**Assimilation of glucose**	+
Reduction of nitrates to N_2	–	**Assimilation of arabinose**	–
Indole formation	–	**Assimilation of mannose**	–
Fermentation of D-Glucose	–	**Assimilation of mannitol**	–
Arginine dihydrolase	–	**Assimilation of *N*-acetyl-glucosamine**	+
β-Galactosidase	–	**Assimilation of D-Maltose**	+
α-Galactosidase	–	**Assimilation of potassium gluconate**	–
β-Glucuronidase	–	**Assimilation of capric acid**	–
α-Glucosidase	–	**Assimilation of adipic acid**	–
α-Mannosidase	–	**Assimilation of malic acid**	–
α-Fucosidase	–	**Assimilation of citrate**	–
Alkaline phosphatase	+	**Assimilation phenylacetate**	–
Esterase (C4)	+	**DNase activity**	+
Esterase lipase (C8)	+	**Caseinase activity**	+
Lipase (C14)	d[2]	**Amylase activity**	+
Leucine arylamidase	+	**Cellulase activity**	–
Valine arylamidase	+	**PHB accumulation**	–
Cystine arylamidase	–	**PHB degradation**	–
Trypsin	–	**TBT degradation (plate)**	+
α-Chemotrypsin	–	**Oil degradation (plate)**	+
Acid phosphatase	+	**Alginase activity**	–
Naphtol-AS-BI-phosphohydrolase	+	**Haeomytic activity**	+
Urease	–	**Hydrolysis of Esculine**	+
		Hydrolysis of gelatine (protease)	+

[1]Result: + positive, – negative. [2]doubtful result. TBT: Tributyrin, PHB: polyhydroxybutyrate.

Table VII.4. Oxidative acid production from carbohydrates tested by the API50CH test.

Active component	*RESULT*[1]	*ACTIVE COMPONENT*	Result[1]
Glycerol	–	**Esculine / Ferric citrate**	+
Erythritol	–	**Salicin**	–
D-Arabinose	–	**D-Cellobiose**	–
L-Arabinose	–	**D-Maltose**	+
D-Ribose	–	**D-Lactose**	–
D-Xilose	–	**D-Melibiose**	–
L-Xilose	–	**D-Saccharose**	+
D-Adonitol	–	**D-Trehalose**	–
Methyl-β-D-xylopyranoside	–	**Inulin**	–
D-Galactose	–	**D-Melezitose**	–
D-Glucose	+	**D-Raffinose**	–
D-Fructose	+	**Starch**	+
D-Mannose	–	**Glycogen**	+
L-Sorbose	–	**Xylitol**	–
L-Rhamnose	–	**Gentiobiose**	+
Dulcitol	–	**D-Turanose**	–
Inositol	–	**D-Lyxose**	–
D-Mannitol	–	**D-Tagatose**	–
D-Sorbitol	–	**D-Fucose**	–
Methyl-α-D-mannopyranoside	–	**L-Fucose**	–
Methyl-α-D-glucopyranoside	–	**D-Arabitol**	–
N-Acetylglucosamine	+	**L-Arabitol**	–
Amygdalin	–	**Potassium gluconate**	–
Arbutine	–	**Potassium 2-ketogluconate**	–
		Potassium 5-ketogluconate	–

[1]Result: + positive, – negative.

- 16S rDNA sequencing and phylogeny reconstruction

On the basis of the phenotypic characteristics previously determined, the isolated strain was similar to *Pseudoalteromonas* or *Alteromonas* species (Gauthier *et al.*, 1995). Members of the genus *Pseudoalteromonas* are typical marine bacteria, which were previously classified under the genus *Alteromonas*. In order to clarify the taxonomy and phylogenetic position of the isolated strain, a phylogenetic analysis based on 16S rDNA sequences was performed. Analysis of the 16S rDNA sequence revealed that the isolated bacteria are members of the γ-subclass of the *Proteobacteria*. The isolated strain showed highest sequence similarity (99%) with *Pseudoalteromonas elyakovii*

(AB000389), although the first entry obtained after performing a Blast search in the Genbank database was *Pseudoalteromonas* sp. SM9913 (Chen *et al*., 2003).

Several phenotypic characteristics such as the capacity of growth at 4ºC, the absence of alginase activity, use of certain carbohydrates, and its facultative anaerobic growth can be used to differentiate the isolated strain from *Pseudoalteromonas elyakovii* (Sawabe *et al.*, 2000). According to the genotypic and phylogenetic studied described here, we conclude that the isolated strain should be classified as a member of the *Pseudoalteromonas* genus, and we propose the name *Pseudoalteromonas* sp. EBD until the DNA–DNA hybridization experiments could confirm the assignation of a new species.

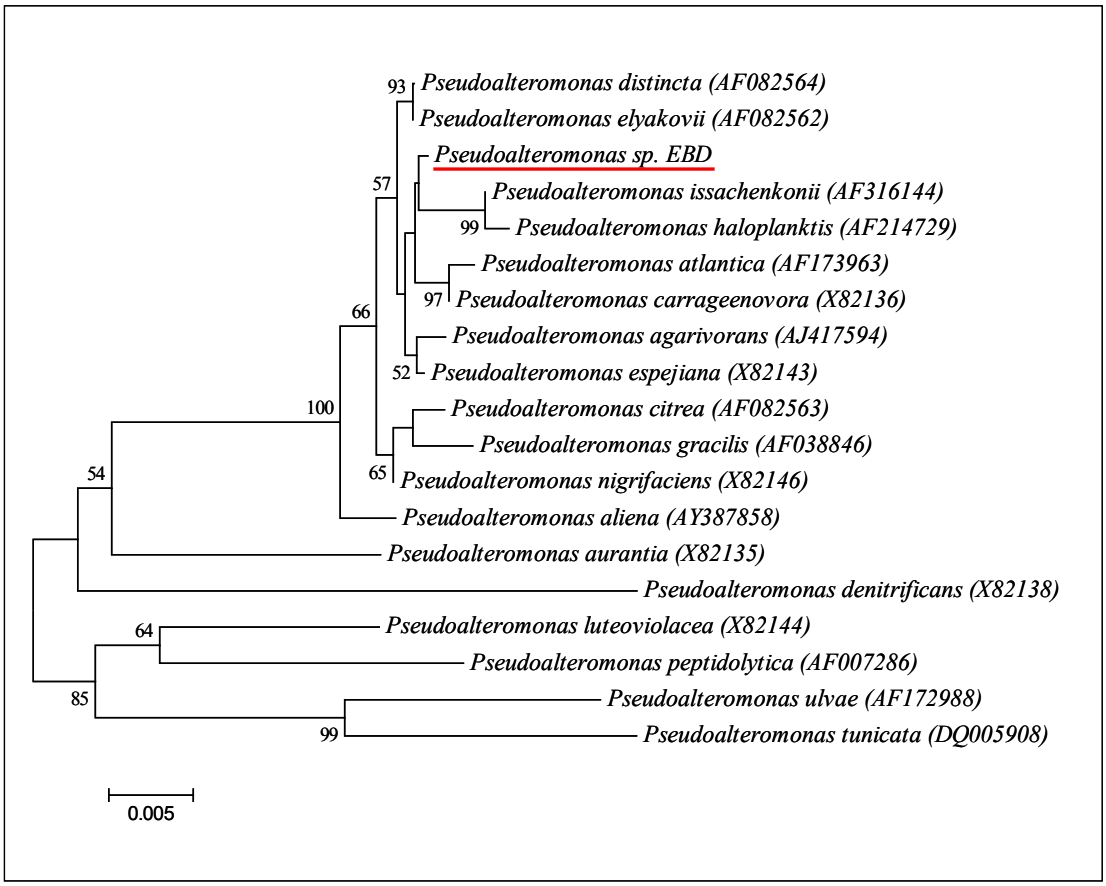

Figure VII.5. Phylogenetic tree showing the relationship of *Pseudoalteromonas* sp. EBD with strains of related *Pseudoalteromonas* species.

The tree was constructed using the Neighbor-joining algorithm (Saitou and Nei, 1987) and the Jukes and Cantor model (Jukes and Cantor, 1966). Numbers on the tree refer to bootstrap values on 1000 replicates. Nucleotide accession numbers are given in parentheses. The bar indicates a 0.5% estimated difference in nucleotide sequences.

- Detection of inhibitory compounds produced by the isolate and interaction activity assay with filamentous cyanobacteria

No antibacterial neither auto-inhibitory activity was detected using the supernatant obtained from the isolated strain using gram-negative and gram-positive microorganisms as targets. However, the possibility of an inhibitory activity by the isolated strain can not be excluded because the optimal conditions for the generation of inhibitory compounds would have not been found.

The interaction assay with filamentous cyanobacteria consisted on MN agar plates and MN+DCMU plates inoculated only with *Pseudoalteromonas* sp. EBD or with the selected cyanobacterium EBD11 (Table VII.1). After 10 days of incubation, the MN and MN+DCMU plates inoculated with *Pseudoalteromonas* sp. EBD showed extremely small colonies although the bacterial inoculum was washed four times in Ringer ¼ to avoid the presence of organic compounds. The other set of control plates, only inoculated with EBD11, reported that the cyanobacterium could return to its original state (growth of the cyanobacterium and recovery of its green color) in MN agar plates after being inhibited by DCMU. Therefore, this fact proved that the incubation of the cyanobacterium with DCMU was not lethal for the microorganism. In addition, those plates showed small colonies of heterotrophic bacteria surrounding the cyanobacterium.

Moreover, the other control plate of MN+DCMU inoculated only with the cyanobacterium revealed the same autochthonous heterotrophic population seen before (probably attached to the cyanobacterium even after the washes) that proliferated when the flux of photosynthates was blocked by the DCMU. However, the growth of that heterotrophic population was low and only observed under the microscope.

In the set of MN and MN+DCMU plates inoculated with both the bacterial suspension of *Pseudoalteromonas* sp. EBD and EBD11, we could observe that the agar zones that had not been in contact with the cyanobacterial inoculum, presented the typical small colonies of *Pseudoalteromonas* sp. EBD observed in the control plates only inoculated with the heterotroph. On the contrary, those areas in which the cyanobacterial suspension was in contact with the agar previously inoculated with

Pseudoalteromonas sp. EBD, developed bigger colonies of an heterotrophic bacterial strain. Those bigger colonies growing onto the inoculum area could be attributed to a higher *Pseudoalteromonas* sp. EBD growth or to other heterotrophs that were accompanied the cyanobacterium inoculum. Comparing the extent of the heterotrophic colony growth with the control plates of MN+cyanobacterium (observation of small colonies), a higher growth of *Pseudoalteromonas* sp. EBD at the expense of other assimilable organic compounds apart from the photosynthates (blocked by the DCMU) could be possible (Fig. VII.6). In order to support that fact, some bigger colonies growing on the inoculum area were isolated on SWYP agar. The identity of the recovered heterotrophic microorganisms was tested by Gram-staining and 16S rDNA sequencing and the data obtained suggested the affiliation of the strains to the *Pseudoalteromonas* genus.

- FISH of cyanobacterial samples

To reveal the presence and localization of *Pseudoalteromonas* sp. EBD in cyanobacterial assemblages of the photic zone of Ebro delta mats, we used fluorescence *in situ* hybridization (FISH) with universal EUB388 and PSA184 probe for *Pseudoalteromonas* genus (Eilers *et al*., 2000). The whole-cell hybridization was performed with fresh samples and cyanobacterial cultures maintained in the laboratory in order to monitor differences in the number of *Pseudoalteromonas* sp. surrounded the cyanobacterial assemblage in natural samples and controlled cultures (Fig. VII.7).

In both kinds of samples, FISH clearly showed that members of the genus *Pseudoalteromonas* existed in the cyanobacterial assemblage and in cyanobacterial cultures. The cyanobacterial mixed assemblages fixed from fresh samples, revealed more fluorescence signals attributed to *Pseudoalteromonas* members, which is consistent with a clearly dominance of this genus under natural conditions. Although the FISH of target bacteria living on highly pigmented surfaces can be difficult due to high background fluorescence from cyanobacterial pigments (Bouvier and del Giorgio, 2003) positive signal have been detected around sheathed cyanobacteria.

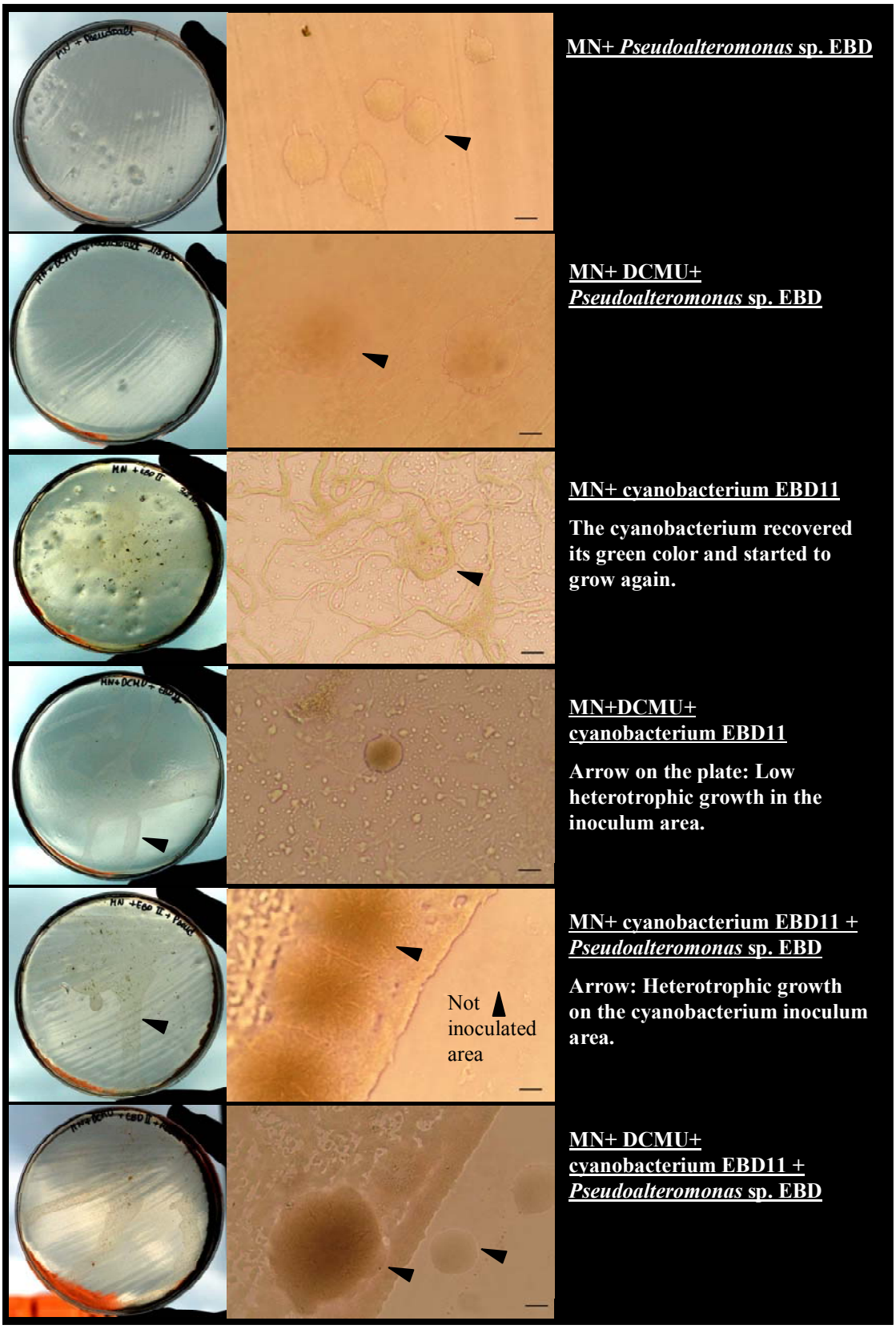

Figure VII.6. Interaction assay between cyanobacteria and *Pseudoalteromonas* sp. EBD. (Bar = 50 µm).

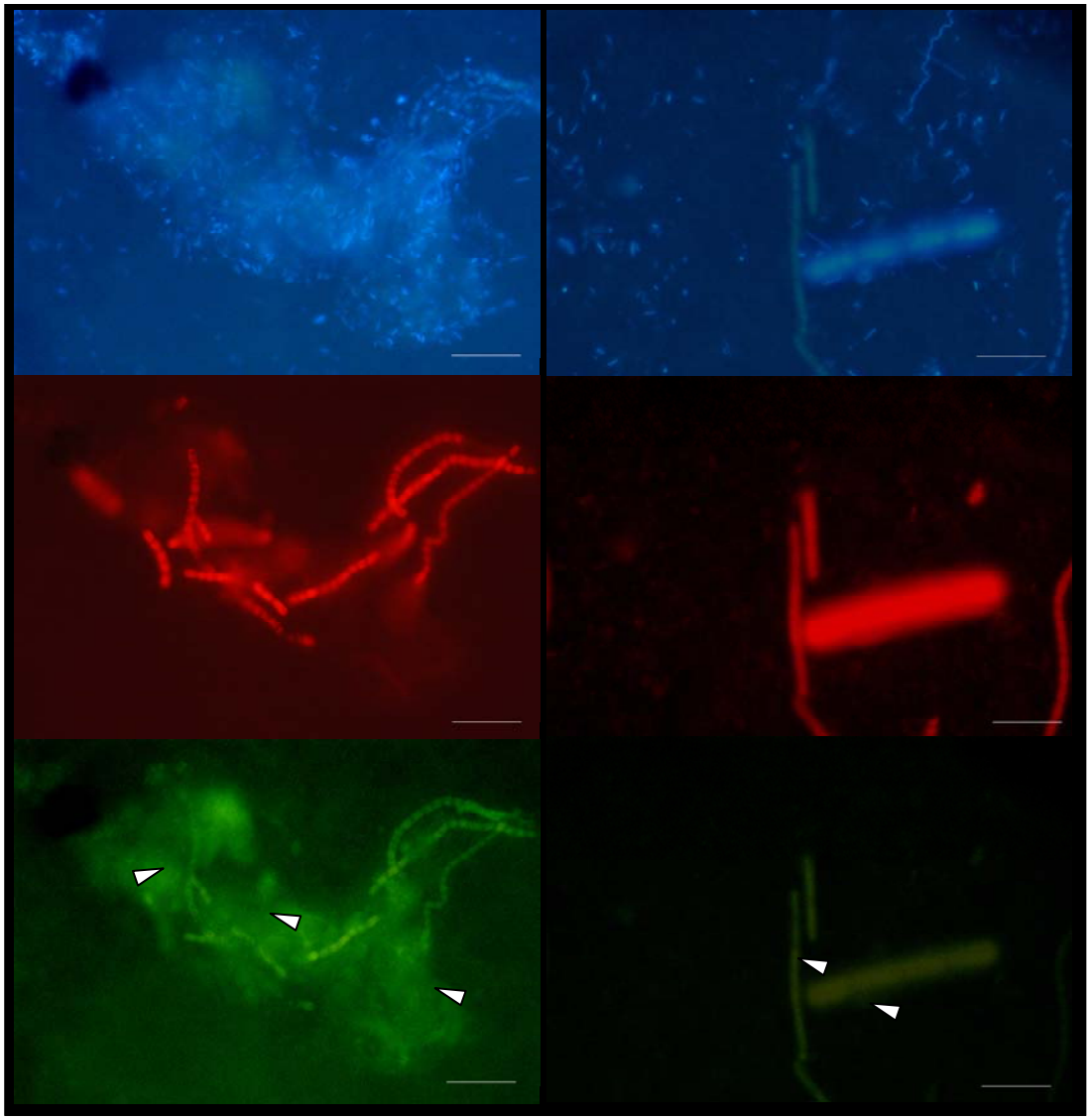

Figure VII.7. FISH of cyanobacterial assemblages with the PSA184 probe labeled with fluorescein (FITC).

From top to bottom image of the figure: DAPI staining; auto-fluorescence of cyanobacteria visualized through UN41007 Cy3 filter; fluorescein fluorescence signal. Arrows point to heterogeneous FISH staining (Bar = 10 μm).

Isolation and characterization of members of the **Sphingomonas** *genus in estuarine mats and detection of sphingolipids*

- Isolation and characterization of the *Sphingomonas* strains

The screening of *Sphingomonas* species by means of their resistance to streptomycin and their yellow pigmentation was performed with Ebro delta and Camargue mat samples. However, only one pigmented, streptomycin-strain was isolated from Camargue mats. Cells of the isolated strain were Gram-negative rods (0.6–0.8 μm wide and 1.5–2 μm long) (Fig. VII.8), strict aerobes, non-sporeforming, and non-motile. On nutrient agar 2, colonies of the strain were around 0.2 mm in diameter, circular, deep-yellow pigmented and smooth after 2 days of incubation at 30ºC. The isolated heterotroph was weakly catalase and oxidase positive, and accumulate poly-β-hydroxybutyrate (tested by HPLC and using Nile red). The salinity range of growth was 0–2.5% NaCl, the temperature range of growth was 20–37ºC (being the optimum at 25–30ºC), and the pH range of growth was 5–8 (being the optimum around 7). Tables VII.5–7 summarize the biochemical characteristics, the use of different carbohydrates and the enzymatic activities as it was previously described in 'II. General Material and Methods'. The fatty acids detected were 16:1ω7*c*, 16:0, 17:1, *cy*17:0, 17:0, 18:1ω7*c*, 18:1ω7*t*, 18:1, 18:0, and 19:1.

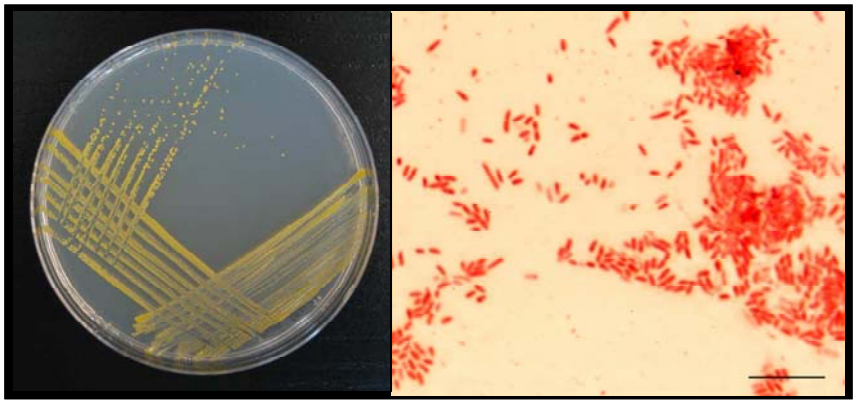

Figure VII.8. Morphology (A) and Gram-staining micrograph (B) of the isolated strain. (Bar = 10 μm).

Table VII.5. Morphobiochemical characterization.

Characteristic	Result[1]
Gram	–
Motility	–
Flagellar arragement	–
Oxygen sensitivity	Strict aerobes
Oxidase	+
Catalase	weakly +
Growth with NaCl (%)	0–2.5%
Tº range of growth	20–37ºC
pH range of growth	5–8
Pigmentation	Deep-yellow
Accumulation of PHB	+

[1]Result: + positive, – negative.

Table VII.6. Biochemical characteristics based on the API20NE, API ZYM and plate tests.

Characteristic	Result[1]	Characteristic	Result
Reduction of nitrates to nitrites	–	**Assimilation of glucose**	+
Reduction of nitrates to N_2	–	**Assimilation of arabinose**	+
Indole formation	–	**Assimilation of mannose**	+
Fermentation of D-Glucose	–	**Assimilation of mannitol**	–
Arginine dihydrolase	–	**Assimilation of N-acetyl-glucosamine**	+
β-Galactosidase	+	**Assimilation of D-Maltose**	+
α-Galactosidase	–	**Assimilation of potassium gluconate**	–
β-Glucuronidase	–	**Assimilation of capric acid**	–
α-Glucosidase	+	**Assimilation of adipic acid**	–
α-Mannosidase	–	**Assimilation of malic acid**	+
α-Fucosidase	–	**Assimilation of citrate**	–
Alkaline phosphatase	+	**Assimilation phenylacetate**	–
Esterase (C4)	+	**DNase activity**	–
Esterase lipase (C8)	+	**Caseinase activity**	–
Lipase (C14)	–	**Amylase activity**	–
Leucine arylamidase	+	**Cellulase activity**	–
Valine arylamidase	+	**PHB accumulation**	+
Cystine arylamidase	–	**TBT degradation (plate)**	+
Trypsin	–	**Oil degradation (plate)**	d[2]
α-Chemotrypsin	–	**Alginase activity**	–
Acid phosphatase	+	**Hydrolysis of Esculine**	+
Naphtol-AS-BI-phosphohydrolase	+	**Hydrolysis of gelatine (protease)**	–
Urease	–		

[1]Result: + positive, – negative. [2]doubtful result. TBT: Tributyrine, PHB: polyhydroxybutyrate.

Table VII.7. Oxidative acid production from carbohydrates tested by the API50CH test.

Active component	Result[1]	Active component	Result[1]
Glycerol	–	**Esculine / Ferric citrate**	+
Erythritol	–	**Salicin**	–
D-Arabinose	–	**D-Cellobiose**	+
L-Arabinose	+	**D-Maltose**	+
D-Ribose	–	**D-Lactose**	+
D-Xilose	+	**D-Melibiose**	–
L-Xilose	–	**D-Saccharose**	+
D-Adonitol	–	**D-Trehalose**	+
Methyl-β-D-xylopyranoside	–	**Inulin**	–
D-Galactose	+	**D-Melezitose**	–
D-Glucose	+	**D-Raffinose**	–
D-Fructose	+	**Starch**	–
D-Mannose	+	**Glycogen**	–
L-Sorbose	–	**Xylitol**	–
L-Rhamnose	+	**Gentiobiose**	–
Dulcitol	–	**D-Turanose**	–
Inositol	–	**D-Lyxose**	–
D-Mannitol	–	**D-Tagatose**	–
D-Sorbitol	–	**D-Fucose**	+
Methyl-α-D-mannopyranoside	–	**L-Fucose**	–
Methyl-α-D-glucopyranoside	–	**D-Arabitol**	–
N-Acetylglucosamine	+	**L-Arabitol**	–
Amygdalin	–	**Potassium gluconate**	–
Arbutine	–	**Potassium 2-ketogluconate**	–
		Potassium 5-ketogluconate	–

[1]Result: + positive, – negative.

- 16S rDNA sequencing and phylogeny reconstruction

The analysis of the 16S rDNA sequence revealed that the isolated bacteria was a member of the α-subclass of the *Proteobacteria*. The isolated strain showed highest sequence similarity (99%) with *Sphingomonas melonis* (AB055863; Buonaurio *et al*., 2002). Several phenotypic characteristics such as the inability growth at 3% NaCl and assimilate phenylacetate and the absence of oxidation activity onto glycerol differed from the described characters for *Sphingomonas melonis*; however, the high similarity of their 16S rDNA sequence and phenotypic characteristics suggested that the isolate may be a new strain of the same genus and species. Until DNA–DNA hybridization

experiments could confirm its assignation, we propose the name *Sphingomonas* sp. Camargue.

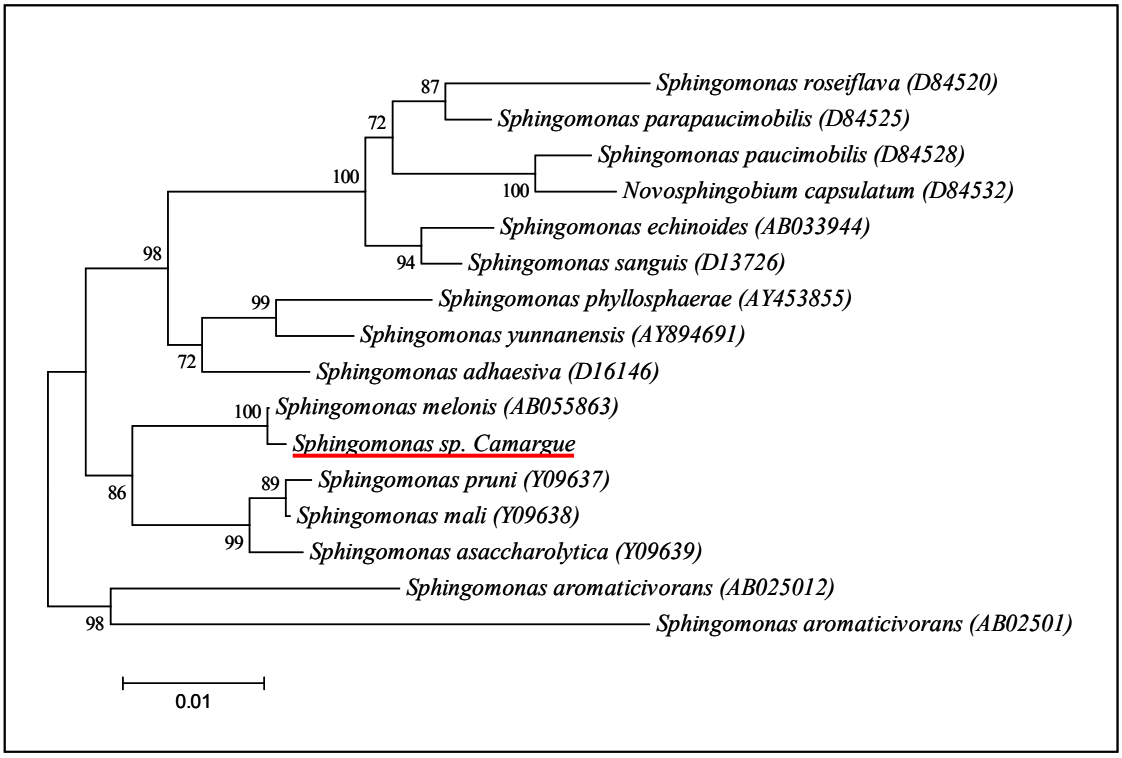

Figure VII.9. Phylogenetic tree showing the relationship of *Sphingomonas* sp. Camargue with strains of related *Sphingomonas* species.

The tree was constructed using the Neighbor-joining algorithm (Saitou and Nei, 1987) and the Jukes and Cantor model (Jukes and Cantor, 1966). Numbers on the tree refer to bootstrap values on 1000 replicates. Nucleotide accession numbers are given in parentheses. The bar indicates a 1% estimated difference in nucleotide sequences.

- Analysis of sphingoid bases from sphingolipids in *Sphingomonas* strains and microbial mat samples

Sphingomonas paucimobilis CECT 599, *Novosphingobium capsulatum* CECT 4388 were used as a positive control of the extraction and detection of long-chain sphingoid bases. The major sphingoid bases in *Sphingomonas paucimobilis* are C18:0 (dihydrosphingosine) and C21:1, as well as in *Novosphingobium capsulatum* (Yabuuchi *et al.*, 1990). The TMS-derivatized long-chain bases were fragmented to generate signature fragments (Leung *et al*., 1999).

The major sphingoid bases detected in *Sphingomonas* sp. Camargue were C14:0, C18:0 (dihydrosphingosine), and C21:1. In the Ebro delta sample only C18:0 (23 nmol g^{-1}) and C21:1 (47 nmol g^{-1}) sphingoid bases were detected. In the Camargue microbial mat sample the sphingoid bases C14:0, C15:0, C16:0, C18:0, C19:0 and C21:1 were detected (Table VII.8). The total sphingoid content in Ebro delta mat sample was 7×10^4 pmol g^{-1} dry weight and 4.3×10^5 pmol g^{-1} dry weight in Camargue sample.

Table VII.8. Quantification of the detected sphingoid bases in nanomoles per gram.

Sphingoid base	*Sphingomonas* sp. CMD	Ebro delta mat	Camargue mat
C14:0	103.31	0.00	69.25
C15:0	0.00	0.00	30.80
C16:0	0.00	0.00	136.30
C18:0	75.18	23.10	120.36
C19:0	0.00	0.00	20.83
C21:1	22.38	46.97	51.24

- Discussion and conclusions

In this study, only one heterotrophic strain was isolated from micromanipulated cyanobacteria. The isolation medium, SWYP is rich in organic compounds and this could be the reason for the low heterotrophic diversity recovered from cyanobacterial sheaths. Indeed, Jonkers and Abed (2003) showed the importance of the isolation media in the diversity of culturable heterotrophs in mats, and they suggested the use of individual substrates in enrichment media instead of complex rich media. They reported members of the genera *Rhodobacter*, *Roseobacter*, *Marinobacter*, *Halomonas* and populations affiliated to *Cytophaga/Flavobacterium* in a hypersaline microbial mat, and suggested the specialization of the isolated bacteria in the degradation of photosynthates excreted by the primary producers.

The isolated *Pseudoalteromonas* sp. EBD has proved to be a versatile bacteria with a wide range of pH, salinity and temperature conditions, and enzymatic capabilities (lipase, proteinase, hemolysin, DNase, amylase etc.) that can be active in different environmental conditions, such as the alkaline and acid phosphatase activity that can favor the use of organic phosphorus. These adaptations might allow the bacteria access to a more stable and lasting source of nutrients that is beneficial in oligotrophic marine environments (Holmström and Kjelleberg, 1999; Ivanova *et al*., 2003). Furthermore, the biosurfactant activity observed in the strain can explain the implication of *Pseudoalteromonas* sp. genus and other accompanying heterotrophic bacteria in the degradation of organic pollutants in cyanobacterial mats (Deppe *et al*., 2005; Chaillan *et al*., 2005).

Previous studies have observed a cooperative relationship between heterotrophic bacteria in the degradation of the brown algae *Fucus evanescens* (Ivanova *et al*., 2002a; Ivanova *et al*., 2002b). The heterotrophic community consisted of *Pseudoalteromona*s sp. and *Halomonas* sp. The *Pseudoalteromonas* partner must play an important role in the initial stages of algal degradation because of its enzymatic capacities, and *Halomonas* with a lower enzymatic capacity may use the low molecular weight compounds resulting from the degradation activity of its partner. In those experiments

Pseudoalteromonas was considered to be a saprophytic organism that degrades the algal thallus.

The quantitative importance of the *Halomonas* genus in Ebro delta and Camargue microbial mats have been also described (del Campo, 2005). For this reason, a similar association to that described in brown algae in Ivanova *et al*. (2002a), may explain the interaction between these two heterotrophic species in the photic zone. *Pseudoalteromonas* sp. cells are motile by means of a polar flagellum and can move towards nutrient sources and attach to the surface of cyanobacteria. Then, the production of bacterolytic enzymes and hemolysins may be responsible for the elimination of other bacteria inhabiting the cyanobacteria sheaths. The *Pseudoalteromonas* strain isolated in this study, presented hemolytic activity but apparently lacked a bacteriolytic response. Previous studies have observed that some members of the *Pseudoalteromonas* genus produce biologically active compounds as a result of their colonization strategies, symbiotic associations etc. (Sawabe *et al*., 1998; Lovejoy *et al*., 1998; Kalinovskaya *et al*., 2004; Rao *et al*., 2005). This lack of bacteriolytic activity can not excluded because maybe the optimal conditions for the generation of inhibitory compounds have not been found. In spite of the fact, no inhibitory activity was found using *Halomonas* sp. EBD as a target, which can explain the association between an *Halomonas* strain resistant to bacteriolytic activity of its partner. Moreover, this complex association of heterotrophic populations may also explain the polyhydroxyalkanoate (PHA) dynamic cycle in microbial mats. *Pseudoalteromonas* sp. EBD can not accumulate PHA but the studies performed by del Campo (2005) indicated that members of the *Halomonas* sp. genus isolated from microbial mats accumulated PHA. Indeed, *Halomonas* might take advantage of the wide range of easily assimilable organic compounds generated by *Pseudoalteromonas* to accumulate the excess of carbon source as reserve polymers.

The interaction assay between cyanobacteria and *Pseudoalteromonas* sp. EBD has been a preliminarily study of the role of the heterotrophic isolate in the recycling of organic compounds. The higher growth of *Pseudoalteromonas* sp. observed in cyanobacterial inoculum treated with DCMU is consistent with the growth of *Pseudoalteromonas* at the expense of other assimilable compounds apart from the

photosynthates (blocked by DCMU), for example the structural components of the cyanobacterial sheath.

The cyanobacterial sheath provides a microenvironment where essential nutrients are concentrated and used by the cyanobacteria, and where the photosynthates produced by the phototroph are available to the heterotrophs. The gelatinous sheath acts as a protective matrix that retains humidity, nutrients and is an attachment surface for the heterotrophs. For this reason, the sheath is not attacked by them when other assimilable organic matter is available (Lange, 1976), but in the absence of a more suitable substrates, the bacteria feed on the less desirable sheath. In this degradation stage, *Pseudoalteromonas* may degrade the cyanobacterial sheath mainly formed by carbohydrates especially glucose (Bertocchi *et al.*, 1990), and then the cell walls and membranes by producing proteinases and lipases. This degradation activity may provide assimilable monosaccharides and low molecular weight organic compounds for both members of the association.

The application of FISH onto cyanobacterial natural assemblages supported the dominant presence of *Pseudoalteromonas* surrounding the cyanobacterial sheath, but other studies need to be performed to clarify if these microorganisms are involved in the recycling of organic compounds in the photic zone, their preference for certain substrates, and the quantitative importance of this genus. The combination of techniques such as microautoradiography and FISH, and the recent development of new methods for assess their abundance, e.g. Real-time quantitative PCR (Skovhus *et al*., 2004), may help in future investigations.

Other aerobic heterotrophic bacteria with important metabolic capacities, such as *Sphingomonas* sp., have been detected in microbial mats (Wieland *et al*., 2003). Members of the *Sphingomonas* genus have been found to be opportunistic pathogens, to degrade copper pipes in distribution systems, and to produce root infections in plants (White *et al.*, 1996). However, some species can prevent phytopathogenic infections and others present capabilities to degrade a wide variety of refractory pollutants and biosynthesize valuable biopolymers. The biotechnological importance of this genus has

lead to an active research of new methods for their isolation and rapid detection in complex communities, such as microbial mats.

The application of the method proposed by Vanbroekhoven *et al*., (2004) has facilitated the isolation of a *Sphingomonas* strain in Camargue mat samples; however, no streptomycin-resistant, yellow-pigmented colonies were recovered from Ebro delta mats. This data is consistent with a different composition of heterotrophic strains in the photic zone of both microbial mat systems. In fact, DGGE-data obtained in chapter IV. 'Vertical microscale characterization of bacterial diversity and physiological status', suggested a dominance of members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, as well as γ- and α-Proteobacteria, in the photic zone of Camargue mats. Furthermore, previous studies performed in Orkney Islands microbial mats (Wieland *et al.*, 2003) reported evidences of the presence of the genus *Sphingomonas* in the upper layers of the mats by T-RFLP (terminal restriction fragment length polymorphism) of the *pufM* gene. In addition, recent studies on the diversity of the microbial endolithic community in gypsum crust (Hughes and Lawley, 2003) have evaluated the importance of this genus as heterotrophic component of phototrophic-based microbial systems.

The isolated strain had a high similarity with *Sphingomonas melonis* (Buonaurio *et al.*, 2002) with respect to their phenotypic characteristics and the 16S rDNA sequence, which suggest a classification of the isolated strain in the mentioned species. Since it has been reported that a large population of *Sphingomonas* members grows on the surface of many plants (Kim *et al*., 1998), it could be hypothesized that this kind of *Sphingomonas* species grows as an epiphyte on a phototrophic partner. This case appears favorable for a potential explanation of the relationship between cyanobacteria and *Sphingomonas* members in the photic zone. Indeed, other interactions between *Sphingomonas* genus and cyanobacteria have been previously described, for example the production of an anti-cyanobacterial compound by a *Sphingomonas* strain (Hibayashi and Imamura, 2003). In any case, more studies have to be performed in order to assess the active role of *Sphingomonas* species in the aerobic zone of microbial mats.

Although the isolated *Sphingomonas* do not require a salt-environment to growth, its potential capacity of living in a saline environment (range of growth 0–2.5% NaCl) reflects the importance of this genus in marine environments. *Sphingomonas* species can play an important role in the ecology of a wide range of saline communities (Cavicchioli *et al*., 1999). For this reason, this study was undertaken to gain insight on the presence and capabilities of *Sphingomonas* genus in mats; however, further studies need to be done to assess the capability of the isolated strain for degrading recalcitrant pollutant or for synthesizing biopolymers at a high rate (e.g. PHA) in future biotechnological applications.

The analysis of sphingoid bases following the protocol described by Leung *et al*. (1999) reported advantages in comparison with the sequential method discussed in chapter III. Firstly, the use of total lipid extracts instead of a sequential polar lipid phase reduces the amount of losses in each step of the sequential protocol. However, working with derivatized total lipid extracts could be problematic especially in environmental samples, for this reason the sequential protocol seems to be a better choice. Moreover, the acid hydrolysis proposed by Leung *et al.* (1999) was not enough to break the amide bond that links the hydroxy fatty acid to the sphingolipid structure, but this process was completely accomplished and the OH-FA was TMS-derivatized and quantified after the strong acid hydrolysis in the sequential protocol. Apart from that, the use of a DB-5 (or HP-5) GC column instead of an HP-1 has improved the separation of the sphingoid bases that tended to coelute in an HP-1 non-polar column. However, there is still a problem of separation of the sphingoid bases and another internal standard should be applied to avoid problems of resolution of structural similar compounds.

The use of chemotaxonomic characters for a suitable classification and detection of *Sphingomonas* members have been widely used (Busse *et al*., 1999; Leung *et al*., 1999). In this study, the predominance of sphingoid bases in Camargue mats *versus* Ebro delta mats (one order of magnitude more), and the isolation of *Sphingomonas* members only in Camargue samples reinforces the idea of a higher importance of this genus in the French mats. In spite of the fact, the predominance of sphingoid bases may also been attributed to other bacterial genus such as *Bacteroides* and *Sphingobacterium* (described in chapter 'I. Introduction', 'Sphingophospholipids'), which were also

detected by denaturing gradient gel electrophoresis (DGGE) in chapter IV. These observations suggest the combination of different methods to monitor the presence and importance of the *Sphingomonas* genus, e.g. sphingoid bases analysis and DGGE etc. (Leung *et al*., 1999; Leys *et al*., 2004).

Conclusions

- Filamentous cyanobacteria from microbial mats have been micromanipulated and the behaviour of their surrounding heterotrophic bacteria has been studied.

- A member of the *Pseudoalteromonas* genus, named *Pseudoalteromonas* sp. EBD has been isolated from the heterotrophic growth surrounding the micromanipulated cyanobacteria.

- The isolated *Pseudoalteromonas* sp. EBD has been physiological and taxonomically characterized. It showed haemolytic, lipase, protease, amylase, DNase, acid and alkaline phosphatase activities, oil displacement activity and a wide range of pH, salinity and temperature of growth.

- The 16S rDNA sequence of *Pseudoalteromonas* sp. EBD presented a 99% similarity with *Pseudoalteromonas elyakovii* but several phenotypic characteristics recommend its classification as a new species.

- No inhibitory or auto-inhibitory activity was detected in the supernatant of *Pseudoalteromonas* sp. EBD using other heterotrophic bacteria isolated from microbial mats (*Halomonas*, *Stappia*, etc) as a target.

- An interaction assay performed with cyanobacteria inhibited by DCMU and *Pseudoalteromonas* sp. EBD apparently indicates that *Pseudoalteromonas* sp. EBD can growth at the expense of other assimilable compounds apart from photosynthates generated by the cyanobacterium, for example the gelatinous cyanobacterial sheath.

- FISH experiments with cyanobacterial natural assemblages suggest the dominance of members of the *Pseudoalteromonas* genus surrounding the cyanobacterial sheaths.

- A *Sphingomonas* member, named *Sphingomonas* sp. Camargue, has been isolated from Camargue microbial mat samples on basis of their resistance to streptomycin and yellow pigmentation.

- *Sphingomonas* sp. Camargue has been physiological and taxonomically characterized. The isolated strain can accumulate polyhydroxybutyrate, and its biochemical and 16S rDNA sequence suggest its classification as a strain of the *Sphingomonas melonis* species.

- The sphingoid base composition of the isolated *Sphingomonas* sp. Camargue and from Ebro delta and Camargue mat samples has been evaluated. Dihydrosphingosine (C18:0) and C14:0, and C21:1 sphingoid bases have been detected in the pure culture and in the Camargue microbial mat sample. Camargue mat sample revealed one order of magnitude more of sphingoid bases than Ebro delta mats, and the isolation of *Sphingomonas* members only in Camargue samples reinforces the idea of a higher importance of this genus in these French mats.

• Publications and communications

- **Villanueva L., J. del Campo, A. Barberán, A. Navarrete, J. Urmeneta, and R. Guerrero.** Heterotrophic bacteria associated with cyanobacteria in the photic zone of estuarine microbial mats. In preparation.

- **Villanueva L., J. del Campo, A. Navarrete, A. Barberán, S. Demajo, R. Guerrero, and J. Urmeneta.** Characterization of heterotrophic bacteria involved in nutrient recycling in the photic zone. XX Congress of the Spanish Society for Microbiology. Cáceres (Spain), September 2005. Poster.