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ESTUDIO DE LOS GENES IMPLICADOS EN EL METABOLISMO DEL ARSÉNICO EN CULTIVOS Y EN SISTEMAS NATURALES

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Estudio de los genes implicados en el metabolismo del arsénico en cultivos y en sistemas naturales

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Sabiduría ante todo; adquiere sabiduría;

Y sobre todas tus posesiones adquiere inteligencia.

Engrandécela y ella te engrandecerá.

Proverbios 4:7

A mi madre

Has d'arribar-hi, és el teu destí,
però no forcis gens la travessia.
És preferible que duri molts anys,
que siguis vell quan fondegis l'illa,
ric de tot el que hauràs guanyat
fent el camí, sense esperar
que et doni més riqueses.
Itaca t'ha donat el bell viatge,
sense ella no hauries sortit.
I si la trobes pobra, no és que Itaca
t'hagi enganyat. Savi, com bé t'has fet,
sabràs el que volen dir les Itaques.

(Itaca, Lluís Llach)

Resumen

La presencia de arsénico en aguas potables y de riego es un problema económico, social y ambiental de extrema importancia, especialmente en varios países de América Latina (principalmente en el Norte de Chile y de Argentina). Los microorganismos respiradores que reducen As(V) a arsenito As(III) son diversos y pueden estar implicados en la movilización del arsénico del sedimento a fuentes de agua potable. Para entender cómo contribuye este metabolismo a la biogeoquímica del arsénico la presente tesis explora, por un lado, tanto la diversidad de comunidades microbianas en ambientes naturales salinos con presencia (p.ej. el Salar de Ascotán) o ausencia (Laguna Tebenquiche) de arsénico, como la distribución y diversidad de los genes involucrados en este metabolismo (arsenato reductasa *arrA* y *arsC*) y, por el otro, el aislamiento y caracterización de cultivos puros y su implicación en la formación de minerales sulfurosos de arsénico en estos ambientes salinos.

Con la construcción de bibliotecas genéticas se pudo caracterizar la diversidad microbiana del gen ribosómico 16S rRNA, encontrando diferencias marcadas entre las bacterias presentes en ambientes salinos con y sin arsénico. La diversidad de genes involucrados en la reducción del As(V), se estudió con cebadores específicos descritos en estudios previos pero optimizando su aplicación a muestras naturales con contenidos elevados de arsénico. Se estudió el gen *arrA* presente en microorganismos que respiran arsenato y lo reducen a arsenito y el gen *arsC* que está ligado a la detoxificación del arsénico en el citoplasma y su expulsión mediante bombas de excreción ligadas a la membrana. La mayoría de las secuencias recuperadas formaron grupos nuevos pero relacionados con los clones obtenidos en ambientes similares pero de menor concentración de arsénico reportados en la literatura, y emparentados con el grupo Firmicutes. Observamos que, tanto por el estudio de los genes 16S rRNA como de los genes funcionales de As, los grupos más abundantes siempre fueron Firmicutes y Gammaproteobacteria en muestras de agua y de sedimento del Salar de Ascotán.

En un conjunto de muestras naturales con diferentes concentraciones de arsénico encontramos una relación entre la concentración de arsénico y la presencia, mediante la técnica del número más probable, de bacterias reductoras de arsénico muy abundantes a mayores concentraciones y ausentes a bajas concentraciones. La presencia del gen *arrA* se detectó a lo largo de todo el gradiente. En cambio, el gen *arsC* sólo fue encontrado en muestras con bajas concentraciones de arsénico. Estos datos indican la presencia en estos sistemas de bacterias relacionadas con la reducción del arsénico sugiriendo un papel importante en su movilización a fuentes de aguas naturales.

Finalmente, los estudios de cultivos de enriquecimientos y aislamiento de cepas procedentes del Salar de Ascotán, nos permitió disponer de una nueva cepa del genéro *Shewanella* capaz de reducir As y sulfato en forma anaeróbica, una peculiaridad que la diferencia de otras *Shewanella* spp. aisladas previamente de ambientes contaminados con arsénico. Además, estos estudios nos ayudaron a comprender mejor el ciclo biogeoquímico del arsénico a nivel geológico, ya que se demostró que la presencia y actividad de bacterias reductores de arsenato está ligada a la formación de minerales sulfurados de arsénico a gran escala, confirmando el origen biológico de estos minerales.

Resum

La presència d'arsènic a l'aigua potable i de rec és un problema econòmic, social i ambiental d'extrema importància, especialment en diversos països d'Amèrica Llatina (principalment al nord de Xile i a l'Argentina). Els microorganismes respiradors que redueixen arsenat As(V) a arsenit As(III) són diversos i poden estar implicats com a possibles catalitzadors en la mobilització de l'arsènic del sediment a fonts d'aigua potable. Per entendre com aquest metabolisme contribueix a la biogeoquímica de l'arsènic aquesta tesi explora, per una banda, la diversitat de comunitats microbianes en ambients naturals salins amb presència (p.ex. el Salar d'Ascotán) o absència (Laguna Tebenquiche) d'arsènic, així com la distribució i diversitat dels gens involucrats en aquest metabolisme (arsenat reductasa *arrA* i *arsC*) i, d'altra banda, l'aïllament i caracterització de cultius purs i la seva implicació en la formació de minerals sulfurosos d'arsènic en aquests ambients salins.

Amb la construcció de biblioteques genètiques es va poder caracteritzar la diversitat microbiana del gen ribosòmic 16S rRNA, trobant diferències marcades entre els bacteris presents en ambients salins amb i sense arsènic. La diversitat de gens involucrats en la reducció de l'As(V) es va estudiar amb encebadors específics descrits en estudis previs però optimitzant la seva aplicació en mostres naturals amb continguts elevats d'arsènic. Es va estudiar el gen *arrA* present a microorganismes que respiren arsenat i el redueixen a arsenit i el gen *arsC* que està lligat a la detoxificació de l'arsènic al citoplasma i la sev a expulsió mitjançant bombes d'excreció lligades a la membrana. La majoria de les seqüències recuperades van formar grups nous relacionats amb els clons obtinguts en ambients similars descrits a la literatura però de menor concentració d'arsènic, i emparentats amb el grup Firmicutes. Observem que, tant per l'estudi dels gens 16S rRNA com dels gens funcionals d'As, els grups més abundants sempre van ser Firmicutes i Gammaproteobacteria en mostres d'aigua i de sediment d'Ascotán.

En un conjunt de mostres naturals amb diferents concentracions d'arsènic vam trobar una relació entre la concentració d'arsènic i la presència, mitjançant la tècnica del número més probable, de bacteris reductors d'arsènic molt abundants a altes concentracions i absents a baixes concentracions. La presència del gen *arrA* es va detectar al llarg de tot el gradient. En canvi, el gen *arsC* només va ser trobat a mostres amb baixes concentracions d'arsènic. Aquestes dades indiquen la presència en aquests sistemes de bacteris relacionats amb la reducció de l'arsènic suggerint un paper important en la seva mobilització a fonts d'aigua natural.

Finalment, els estudis de cultius d'enriquiment i l'aïllament de soques del Salar d'Ascotán, ens va permetre disposar d'una nova soca del gènere *Shewanella* capaç de reduir As i sulfat de manera anaeròbica, una peculiaritat que la diferencïa d'altres *Shewanella* spp. aïllades previament d'ambients contaminats amb arsènic. A més a més, aquests estudis ens van ajudar a comprendre millor el cicle biogeoquímic de l'arsènic a nivell geològic, ja que es va demostrar que la presència i activitat de bacteris reductors d'arsenat està lligada a la formació de minerals sulfurats d'arsènic a gran escala, confirmant l'origen biològic d'aquests minerals.

Summary

Presence of arsenic in drinking and irrigation waters is a serious concern of great economic, social, and environmental importance in numerous locations across South America (mainly in the Northern Chile and Argentina). As respiring microorganisms that reduce As(V) to As(III) are diverse and can be involved in the As mobilization from the sediment to drinking water sources. The present PhD thesis explores the links between the microbial metabolism and the biogeochemical cycling of As in saline systems of Northern Chile. On the one hand, the microbial diversity and the distribution and diversity of arsenic functional genes (i.e., arsenate reductases *arrA* and *arsC*) were studied in saline environments with high (e.g., Salar de Ascotán) and low (e.g., Laguna Tebenquiche) As concentrations. On the other hand, characterization of microbial enrichments and bacterial pure cultures were carried out to link bacterial activity and the origin of arsenic minerals in the environment.

The 16S rRNA gene clone libraries showed consistent differences in bacterial community composition in saline environments with high and low concentrations of As. As functional genes were amplified with specific primers previously described in the literature and methodological improvements were conducted in this work to optimize the amplification in hypersaline, As-rich natural samples. The gen *arrA* is present in anaerobic microorganims that respire As(V) to As(III). The gen *arsC*, in turn, is used for detoxification trough membrane pumps. Most of the sequences found formed new clusters distantly related to previously known sequences obtained from environments with presence of As, within the Firmicutes. Both 16S rDNA and arsenic functional genes showed Firmicutes and Gammaproteobacteria as the predominant bacterial groups in water and sediment samples of Salar de Ascotán.

Along a set of natural samples with increasing arsenic concentrations we found a positive relationship between arsenic concentration and abundance of arsenic reducing bacteria determined by MPN. The gen *arrA* was detected throughout the gradient. Conversely, the gen *arsC* was only found in the samples with the lowest arsenic concentrations. These data showed a widespread occurrence of bacteria related to the arsenic cycling, suggesting a key role of arsenic reducing bacteria in the arsenic mobilization to the aqueous phase.

Finally, through classical microbiology studies we obtained a new strain of *Shewanella* from sediments of Ascotán that reduced both As and sulfate under anaerobic conditions, a specific trait not previously reported in other *Shewanella* spp isolated from environments contaminated with arsenic. In addition, these studies gave further clues for a better comprehension of the arsenic cycle at the geological level. We showed that the presence and activity of arsenic reducing bacteria was linked to the formation of sulfurous arsenic minerals at a large scale, giving further evidence for the biological origin of the wide arsenic mineral layers found in Salar de Ascotán.

ÍNDICE

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INTRODUCCIÓN GENERAL

El trabajo del pensamiento se parece a la perforación de un pozo:
el agua es turbia al principio, más luego se clarifica.
(Proverbio Chino)

INTRODUCCIÓN GENERAL

Todos los seres vivos viven entre ciertos límites de tolerancia a distintos factores ambientales (temperatura, humedad, salinidad, etc.). Por debajo y por encima de estos límites no pueden vivir. Sin embargo, se encuentran seres vivos, especialmente bacterias, en los lugares más insospechados: en las salinas donde la concentración de sal es muy alta, en los hielos antárticos con temperaturas bajísimas, en manantiales cargados de ácidos, en fuentes hidrotermales con temperaturas muy altas, etc. A los organismos que viven en estos ambientes extremos se les llama extremófilos.

Algunos de estos ambientes extremos se encuentran en las planicies de altura de los Andes Centrales, que se extienden en parte de Perú, Bolivia, Chile y Argentina, entre los 3500 y 4500 metros sobre el nivel del mar. En ellas, numerosas cuencas endorreicas forman lagos y salares de diversos tamaños, los cuales constituyen islas de hábitats acuáticos dentro de una matriz desértica. Estos humedales presentan una gran variabilidad y heterogeneidad, tanto espacial como temporal y se caracterizan por una alta fragilidad ecológica. Su composición biogeoquímica es, en algunos casos, única en el mundo. Como caso extremo, en estos ambientes han sido descritos ecosistemas acuáticos con concentraciones naturales de arsénico de hasta 80 mg por litro. Los mecanismos que han desarrollado los organismos del plancton a lo largo de la evolución para sobrevivir bajo estas condiciones son muy poco conocidos.

En contraposición a tradicionales y extensos trabajos de geología y geoquímica, la diversidad y mecanismos de adaptación de los microorganismos que habitan estos ambientes han sido muy poco estudiados. Sin embargo, el potencial de estos ambientes como fuente de nuevos microorganismos con aplicaciones útiles es enorme. Un caso relevante es el de los microorganismos capaces de procesar el arsénico.

La presencia de arsénico en aguas potables y de riego es un problema económico, social y ambiental de extrema importancia. En varios países de América Latina (especialmente en el Norte de Chile y de Argentina) por lo menos cuatro millones de personas beben en forma permanente agua con niveles de arsénico que ponen en riesgo su salud, presentando índices muy elevados de enfermedades ligadas a exposición prolongada a este elemento.

El objetivo de la presente memoria es caracterizar comunidades microbianas en un ambiente extremo por métodos microbiológicos y moleculares e intentar discernir las variables que controlan la estructura de las comunidades planctónicas en ambientes altamente contaminados por arsénico.

Esta investigación se enmarca en el proyecto BIOARSÉNICO financiado por la Fundación BBVA, que está orientado a la búsqueda de microorganismos de importancia geomicrobiológica y biotecnológica y al conocimiento del ciclo biogeoquímico del arsénico en esta zona y su posible gestión y biorremediación.

1. ARSÉNICO

El arsénico (As) es un elemento que se encuentra en los sistemas naturales en una gran variedad de formas químicas, incluyendo formas inorgánicas, As(III) y As(V), y formas orgánicas metiladas. El potencial redox (Eh) y el pH son los factores más importantes que controlan la especiación química del As. Pertenece al grupo de los metaloides, ya que muestra propiedades intermedias entre los metales y los no metales. Entre los metales formadores de oxianiones (p.ej.; As, Se, Sb, Mo, V, Cr, U, Re) es posible que el arsénico sea el único elemento sensible a la movilización a valores de pH cercanos al neutro (pH 6.5-8.5), y tanto bajo condiciones de oxidación como de reducción. El arsénico puede estar en el ambiente en varios estados de oxidación (0, +3 y +5) pero en aguas naturales se encuentra sobre todo como arsenito As(III) o arsenato As(V). En aguas superficiales se puede encontrar en forma orgánica debido a la actividad biológica y por efecto de la contaminación industrial.

La mayoría de los metales tóxicos están en solución en forma de cationes (p.ej. $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}), que generalmente son más insolubles cuanto más alto sea el pH. A valores de pH cercanos al neutro la solubilidad de la mayoría de estos cationes se encuentra limitada por la precipitación o co-precipitación formando óxidos, hidróxidos, carbonatos, fosfatos o, más probablemente, por la adsorción fuerte a los óxidos metálicos acuosos, la arcilla o la materia orgánica. En cambio, la mayoría de los oxianiones, incluido el arsenato, tienden a adsorberse menos fuertemente al aumentar el pH (Dzombak y Morel, 1990). Bajo ciertas condiciones, estos aniones pueden persistir en solución en concentraciones relativamente altAs(decenas de mg L^{-1}) incluso a los valores de pH cercanos al neutro. Por lo tanto, los oxianiones que conforman los elementos tales

como Cr, As, U y Se son algunos de los contaminantes más comunes en las aguas subterráneas y superficiales.

En relación a otros elementos formadores de oxianiones, el As es el más problemático debido a su movilidad relativa en una amplia gama de condiciones redox. El selenio es móvil como seleniato (SeO_4^{2-}) bajo condiciones oxidantes pero se inmoviliza bajo condiciones de reducción, debido a la fuerte adsorción de su forma reducida selenita (SeO_3^{2-}), o debido a la reducción del metal.

El cromo se puede movilizar como Cr(VI), especie estable del oxianión bajo condiciones oxidantes, pero la especie catiónica Cr(III) en ambientes reductores se comporta como otros cationes (es decir es relativamente inmóvil en los valores de pH cercanos al neutro). Otros oxianiones tales como el molibdato, el vanadato, el uranilo y el renio parecen ser menos móviles en condiciones reductoras. En ambientes reductores ricos en azufre, muchos de los metales traza forman sulfuros insolubles. El arsénico se distingue por ser relativamente móvil bajo condiciones reductoras. Éste puede encontrarse en un orden de concentraciones de mg L^{-1} cuando el resto de los elementos formadores de oxianiones está presente en concentraciones del orden de μg L^{1} (Smedley & Kinniburgh, 2002).

Bajo condiciones oxidantes, la forma H_2AsO^{4-} es dominante a pH bajo (menor pH 6.9), mientras que a un pH más alto, la forma $HAsO_4^{2-}$ empieza a ser dominante ($H_3AsO_4^{0}$ y $AsO4^{3-}$ pueden estar presentes en condiciones extremadamente ácidas y alcalinas respectivamente, Fig. 1). Bajo condiciones reductoras, a pH menor a 9.2, la especie predominante es $H_3AsO_3^{0}$. Las distribuciones de estas especies en función del pH se muestran en la Fig. 2 A y B. En presencia de concentraciones altas de compuestos de azufre reducidos son significativas las especies disueltas de As-sulfurado. En condiciones reductoras ácidas se favorece la precipitación del oropimente (As_2S_3), del rejalgar (AsS) o de otros minerales sulfurados que contienen As(Cullen & Reimer, 1989). Como consecuencia, en aguas con alto contenido de As no se encuentran concentraciones altas de sulfuros libres (Moore *et al.,* 1988).

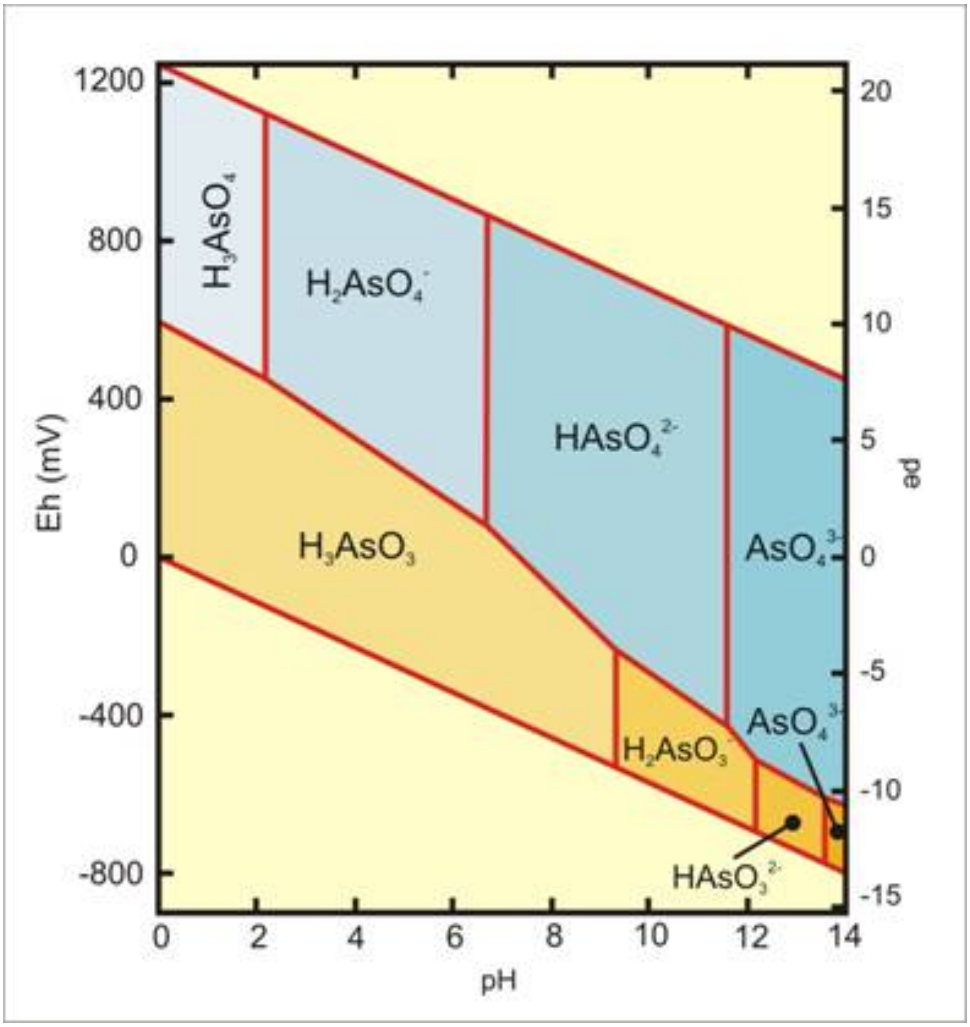

Figura. 1. Diagrama Eh-pH de las especies acuosas del arsénico en un sistema de As–O_2–H_2O a 25º C y 1 bar de presión total (Smedley & Kinniburgh, 2002).

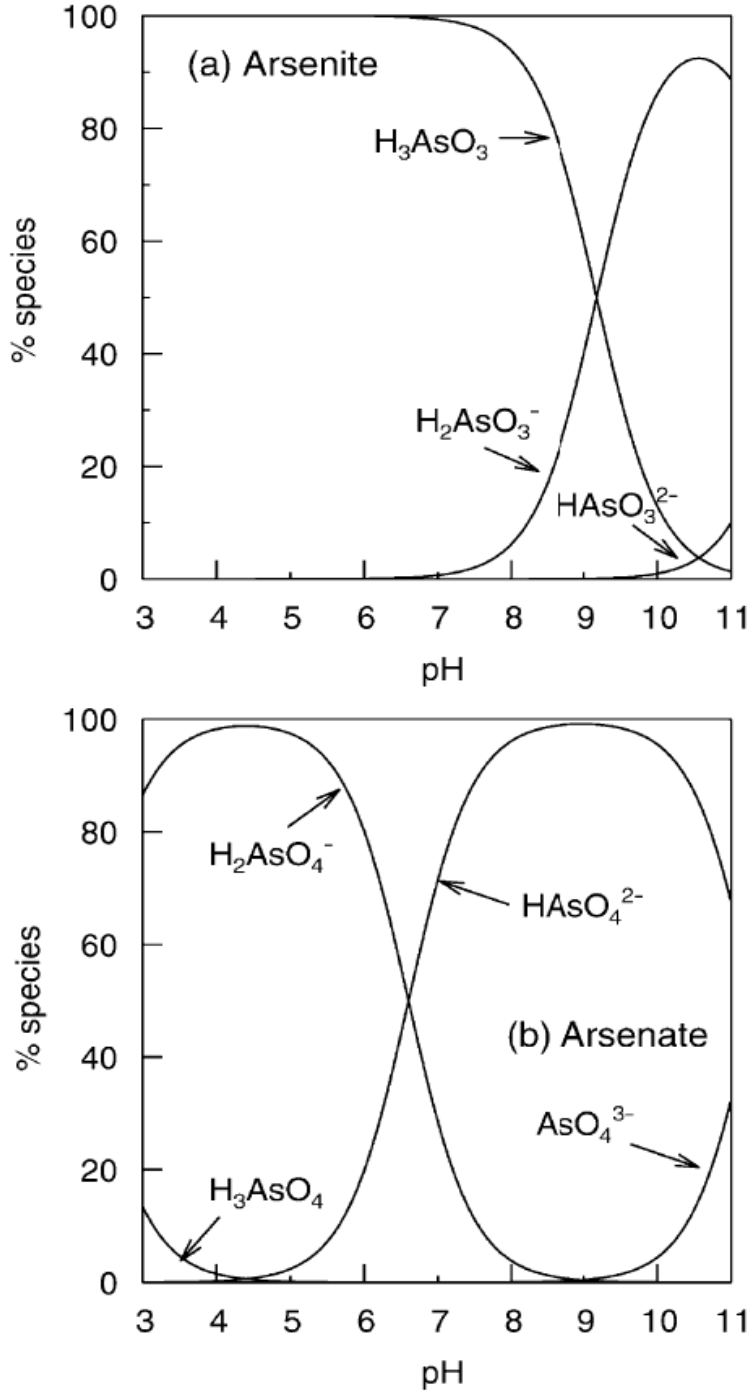

Figura.2. Especies de arsenito y arsenato en función de pH (fuerza iónica de 0.01M). Las condiciones redox se han elegido tales que el estado de oxidación indicado domina el especiación en ambos casos (Smedley & Kinniburgh, 2002).

1.1 Mineralogía del As

El arsénico se encuentra en suelos y minerales y, a través del polvo, puede entrar en el aire y en el agua. El arsénico se encuentra por lo general en la superficie de las rocas, combinado con azufre o metales como Mn, Fe, Co, Ni, Ag o Sn. El principal mineral de arsénico es el FeAsS (arsenopirita, pilo); otros arseniuros metálicos son los minerales $FeAs_2$ (löllingita), NiAs(nicolita), CoAsS (cobalto brillante), NiAsS (gersdorfita) y $CoAs_2$ (esmaltita). Los arsenatos y tioarsenatos naturales son comunes y la mayor parte de los minerales de sulfuro contienen arsénico. El As_4S_4 (rejalgar) y el As_2S_3 (oropimente) son los minerales más importantes que contienen azufre. El óxido arsenolita, As_4O_6, se encuentra como producto de la alteración de otros minerales de arsénico debido a los agentes atmosféricos, y también se recupera de los polvos colectados durante la extracción de Ni, Cu y Sn. También se obtiene este mineral al calcinar los arseniuros de Fe, Co o Ni en presencia de aire u oxígeno.

1.2 *Abundancia y distribución*

Las concentraciones de arsénico en aguas naturales varían más de cuatro órdenes de magnitud dependiendo de la fuente de As, la cantidad disponible y el ambiente geoquímico. En condiciones naturales, los rangos más grandes y concentraciones más altas de As se encuentran en aguas subterráneas, como resultado de la fuerte interacción agua-roca y las favorables condiciones físicas y geoquímicas de los acuíferos para la movilización y la acumulación de As. Las concentraciones de As que se encuentran en la Tabla 1 proporcionan una visión general de los rangos de concentración y su variación en diversos lugares de la hidrosfera y la litosfera (Smedley & Kinniburgh, 2002).

Los rangos de concentraciones de As encontrados en aguas naturales son amplios, extendiéndose de menos de 0.5 $\mu g\ L^{-1}$ a más de 5000 $\mu g\ L^{-1}$. Las concentraciones en aguas potables deben ser menores de 10 $\mu g\ L^{-1}$ y preferiblemente menores de 1 $\mu g\ L^{-1}$. Existen zonas en el mundo con alto contenido de As en aguas subterráneas, hasta el punto de que, en algunas áreas concretas, más del 10% de los pozos para obtener agua potable están afectados (excediendo los 50 $\mu g\ L^{-1}$) y en casos más extremos la afectación excede el 90% (Croal *et al*., 2004).

Tabla 1. Concentraciones de arsénico en rocas, sedimentos, suelos, minerales y otros depósitos. (Reproducido de Smedley & Kinniburgh, 2002).

Localización de ambientes acuáticos	Concentración promedio de As(µg l^{-1})	Referencias
Rain water		
Baseline		
Maritime	0.02	Andreae (1980)
Terrestrial (w USA)	0.013–0.032	Andreae (1980)
Coastal (Mid-Atlantic, USA)	0.1(<0.005–1.1)	Scudlark and Church (1988)
Snow (Arizona)	0.14 (0.02–0.42)	Barbaris and Betterton (1996)
Non-baseline:		
Terrestrial rain	0.46	Andreae (1980)
Seattle rain, impacted by copper smelter	16	Crecelius (1975)
River water		
Baseline		
Various	0.83 (0.13–2.1)	Andreae et al. (1983) Seyler and Martin (1991)
Norway	0.25 (<0.02–1.1)	Lenvik et al. (1978)
South-east USA	0.15–0.45	Waslenchuk (1979)
USA	2.1	Sonderegger and Ohguchi(1988)
Dordogne, France	0.7	Seyler and Martin (1990)
Po River, Italy	1.3	Pettine et al. (1992)
Polluted European rivers	4.,5–45	Seyler and Martin (1990)
River Danube, Bavaria	3 (1–8)	Quentin and Winkler (1974)
Schelde catchment, Belgium	0.75–3.8 (up to 30)	Andreae and Andreae (1989)
High-As groundwater influenced:		
Northern Chile	190–21,800	Cáceres et al. (1992)
Northern Chile	400–450	Sancha (1999)
Córdoba, Argentina	7–114	Lerda and Prosperi (1996)
Geothermal influenced		
Sierra Nevada, USA	0,20–264	Benson and Spencer (1983)
Waikato, New Zealand	32 (28–36)	McLaren and Kim (1995)
Madison and Missouri Rivers, USA	44 (19–67)	Robinson et al. (1995)
Mining influenced		
Ron Phibun, Thailand	218 (4,8–583)	Williams et al. (1996)
Ashanti, Ghana	284 (<2–7900)	Smedley et al. (1996)
British Columbia, Canada	17.5 (<0.2–556)	Azcue et al. (1994)
Lake water		
Baseline		
British Columbia	0.28 (<0.2–0.42)	Azcue et al. (1994, 1995)
Ontario	0,7	Azcue and Nriagu (1995)
France	0.73–9.2 (high Fe)	Seyler and Martin (1989)
Japan	0.38–1.9	Baur and Onishi (1969)
Sweden	0.06–1.2	Reuther (1992)

Geothermal influenced		
Western USA	0.38–1000	Benson and Spencer (1983)
Mining influenced		
Northwest Territories, Canada	270 (64–530)	Bright et al. (1996)
Ontario, Canada	35–100	Azcue and Nriagu (1995)
Estuarine water		
Baseline		
Oslofjord, Norway	0,7–2,0	Abdullah et al. (1995)
Saanich Inlet, British Columbia	1.2–2.5	Peterson and Carpenter (1983)
Rhone Estuary, France	2.2 (1.1–3.8)	Seyler and Martin (1990)
Krka Estuary, Yugoslavia	0.13–1.8	Seyler and Martin (1991)
Mining and industry influenced		
Loire Estuary, France	up to 16	Seyler and Martin (1990)
Tamar Estuary, UK	2.7–8.8	Howard et al. (1988)
Schelde Estuary, Belgium	1.8–4.9	Andreae and Andreae (1989)
Seawater		
Deep Pacific and Atlantic	1.0–1.8	Cullen and Reimer (1989)
Coastal Malaysia	1.0 (0.7–1.8)	Yusof et al. (1994)
Coastal Spain	1.5 (0.5–3.7)	Navarro et al. (1993)
Coastal Australia	1.3 (1.1–1.6)	Maher (1985)
Groundwater		
Baseline UK	<0.5–10	Edmunds et al. (1989)
As-rich provinces (e.g. Bengal	10–5000	Das et al. (1995)
Basin, Argentina, Mexico, northern		Nicolli et al. (1989)
China, Taiwan, Hungary)		Del Razo et al. (1990)
		Hsu et al. (1997)
Mining-contaminated groundwaters	50–10,000	Wilson and Hawkins (1978)
		Williams et al. (1996)
Geothermal water	<10–50,000	Baur and Onishi (1969)
		Ellis and Mahon (1977)
Arsenical herbicide plant, Texas	408	Kuhlmeier (1997a,b)
Mine drainage		
Various, USA	<1–34,000	Plumlee et al. (1999)
Iron Mountain	up to 850,000	Nordstrom and Alpers (1999)
Ural Mountains	400	Gelova (1977)
Sediment porewater		
Baseline, Swedish Estuary	1.3–166	Widerlund and Ingri (1995)
Baseline, clays, Saskatchewan, Canada	3.2–99	Yan et al. (2000)
Baseline, Amazon shelf sediments	up to 300	Sullivan and Aller (1996)
Mining-contam'd, British Columbia	50–360	Azcue et al. (1994)
Tailings impoundment, Ontario, Canada	300–100,000	McCreadie et al. (2000)
Oilfield and related brine		
Ellis Pool, Alberta, Canada	230	White et al. (1963)
Searles Lake brine, California	up to 243,000	White et al. (1963)

1.3 *Distribución en el mundo*

Se han determinado concentraciones de As que están por encima de los 50 mg L^{-1} en un gran número de acuíferos en diversas partes del mundo. Éstos proceden tanto de fuentes naturales de enriquecimiento como de fuentes relacionadas con la minería. Las áreas más significativas están en Argentina, Bangladesh, Chile, China, Hungría, India (Bengala occidental), México, Rumanía, Taiwán, Vietnam y EE.UU. (Fig. 3). El arsénico también se encuentra asociado a aguas geotermales, que incluyen fuentes termales en varias áreas de Argentina, Japón, Nueva Zelanda, Chile, Kamchatka, Islandia, Francia y EE.UU. Estudios recientes han revelado que la calidad del agua subterránea en otras áreas tales como Nepal, Myanmar y Camboya presenta concentraciones de As que exceden 50 mg L^{-1} en algunas fuentes.

Hasta hace poco tiempo el As no estaba incluido entre los elementos analizados rutinariamente para las pruebas de calidad de agua, por lo que muchas de las fuentes de agua con alto contenido de As habían pasado desapercibidas. Las aguas subterráneas con problemas están comenzando a ser reportadas, aumentando el número conocido de países y personas afectadas por esta problemática. La revisión de la legislación sobre aguas potables ha provocado una reevaluación de la situación en muchos países.

En términos de exposición de la población a altas concentraciones de As, el mayor problema se encuentra en la cuenca de Bengala con más de 40 millones de personas que beben diariamente agua que contiene altas concentraciones de As (Croal *et al*., 2004). El descubrimiento reciente del enriquecimiento a gran escala de As en Bangladesh ha impuesto la necesidad de estudiar la situación en acuíferos aluviales para todo el mundo.

Algunas áreas donde se realizan actividades mineras, también muestran problemas de As, debido a la oxidación de los minerales de sulfuro. En áreas de influencia minera el problema del As puede ser grave y las aguas se cargan con concentraciones de As a veces superiores al rango de mg L^{-1}. Se han identificado áreas mineras relacionadas con problemas de As en agua en muchas partes del mundo, incluyendo Ghana, Grecia, Tailandia y los EE.UU. (Fig. 3). Sin embargo, las incidencias de As en estos tipos de acuíferos se limitan a áreas localizadas.

Bajo circunstancias especiales, existen aguas subterráneas con alto –As (con concentraciones de As por encima de los estándares del agua potable). Estas circunstancias

están relacionadas con el ambiente geoquímico y con la hidrogeología pasada y presente (Smedley & Kinniburgh, 2002). Paradójicamente, estos contenidos altos de As en aguas subterráneas no se relacionan necesariamente con áreas rocosas ricas en As, sino con su movilización.

Figura 3. Distribución en el mundo de acuíferos documentada con problemas de As importantes. Las áreas en azul son lagos. También se indican las zonas contaminadas por operaciones mineras y las de origen geotérmico. (Reproducido de Smedley & Kinniburgh, 2002).

1.4 *Efectos del As sobre la salud de las personas*

El arsénico es uno de los elementos más tóxicos para el ser humano. Los humanos pueden estar expuestos al arsénico a través de la comida, el agua y el aire. La exposición también puede producirse a través del contacto de la piel con el suelo o el agua que contengan arsénico. La exposición al As inorgánico puede causar varios efectos sobre la salud, como irritación del estómago e intestinos, disminución de la producción de glóbulos rojos y blancos, cambios en la piel e irritación de los pulmones. Se ha sugerido que la ingestión continuada de cantidades significativas de arsénico inorgánico puede intensificar las posibilidades de desarrollar cáncer, especialmente de piel, pulmón, hígado y linfa (Greenwood & Earnshaw, 1997).

1.5 *Efectos del As en el medio ambiente*

Como ya se ha mencionado, el arsénico puede encontrarse de forma natural en el suelo y minerales en pequeñas concentraciones y puede entrar en el aire, el agua y la tierra a través de las tormentas de polvo y las aguas de escorrentía. En consecuencia, la contaminación por arsénico está muy extendida debido a su fácil dispersión (Emsley, 2001). De hecho, debido a las actividades humanas, sobre todo la minería y las fundiciones, el arsénico se moviliza y puede encontrarse en muchos lugares donde no existiría de forma natural.

El ciclo del arsénico ha sufrido modificaciones notables como consecuencia de la intervención humana que resulta en grandes cantidades de arsénico en el medio ambiente y en organismos vivos. El arsénico es emitido mayoritariamente por industrias productoras de cobre, pero también durante la producción de plomo, zinc y en la agricultura. Éste no puede ser eliminado una vez introducido en el medio ambiente, por lo que se esparce y causa efectos negativos sobre la salud de los humanos y los animales en amplias regiones alejadas del foco original.

Las concentraciones de arsénico inorgánico que están presentes en las aguas superficiales experimentan un proceso de bioacumulación a lo largo de las cadenas tróficas, desde algas y plantas acuáticas hacia peces y aves, y aumentan las posibilidades de altera-

ción del material genético de los peces y, a dosis crecientes, pueden causar la muerte en las aves por envenenamiento (Wright, 2003).

2. GENÉTICA Y GEOQUÍMICA

Las bacterias son excepcionales en su diversidad metabólica, debido a su capacidad de obtener energía de innumerables reacciones de oxidación y de reducción. La genética bacteriana ayuda a entender cómo funcionan estos metabolismos. Una vez que se comprenda cómo funcionan los genes y los productos que catalizan reacciones geoquímicamente relevantes, así como las condiciones que regulan su expresión, podremos comenzar a predecir cuándo y en qué medida estos metabolismos influyen en los ciclos geoquímicos. Este conocimiento permitirá desarrollar una base para descifrar los estrechos vínculos entre la biología y las características químicas y físicas de nuestro planeta. En la Tabla 2 se presentan los genes que realizan reacciones de oxidación o reducción de As.

Tabla 2. Reacciones catalizadas por microorganismos que metabolizan As.

Metabolismo	Reacción	Genes
Reducción del arsenato mediante la oxidación del lactato	$CH_3CHOHCOO^- + 2HAsO_2^- 4 + 3H^+ \rightarrow CH3COO^- + 2HAsO_2 + 2H_2O + HCO^{-3}$	*arrA, arrB*
Reducción del arsenato mediante la oxidación del acetato	$CH_3COO^- + 4HAsO_4^{2-} + 7H^+ \rightarrow 2HCO_3^- + 4HAsO_2 + 4H_2O$	*arrA, arrB*
Oxidacion del arsenito, dependiente del oxígeno	$2HAsO_2 + 2H_2O + O_2 \rightarrow 2HAsO_4^{2-} + 4H^+$	*aroA, aroB*

2.1 *Transformaciones de As*

Los microorganismos que transforman As(V) y As(III) son diversos en su filogenia (Fig. 4) y fisiología. Hasta ahora, los estudios genéticos más avanzados sobre la reducción de As(V) se han realizado principalmente con el sistema de detoxificación del As de cepas de *Staphylococcus aureus* y *E. coli* (Broer *et al.,* 1993, Dey & Rosen 1995). Estos sistemas no generan energía.

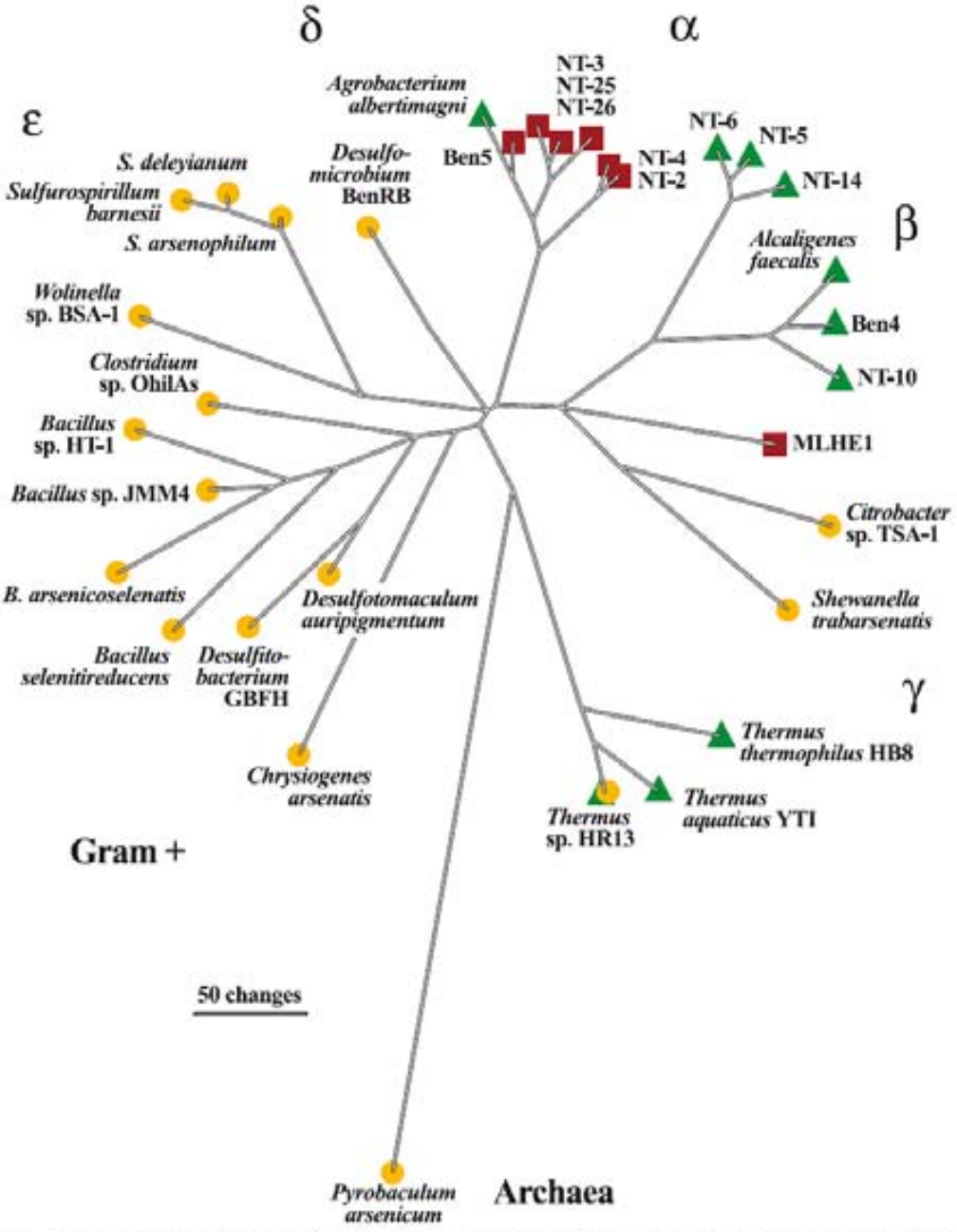

Figura 4. Diversidad filogénetica de procariotas metabolizadoras de arsénico. Procariotas respiradoras de As, desasimilatoriAs(DARPs, circulo amarillo), oxidadores heterotróficos de arsenito (HOAs, triángulo verde) y oxidadores quimiolitotróficos de arsenito (CAOs, cuadro rojo). *Thermus* sp. cepa HR13 es capaz de respirar As(V) y oxidar As(III) (Oremland *et al.,* 2003).

La Figura 5 muestra los 3 sistemas enzimáticos principales para la transformación del As. Existen dos clases de microorganismos que utilizan el As bien como fuente de energía o bien como aceptor de electrones en la respiración, según su estado redox: los oxidantes quimiolitotróficos de As(III) y los reductores heterotróficos de As(V), respectivamente.

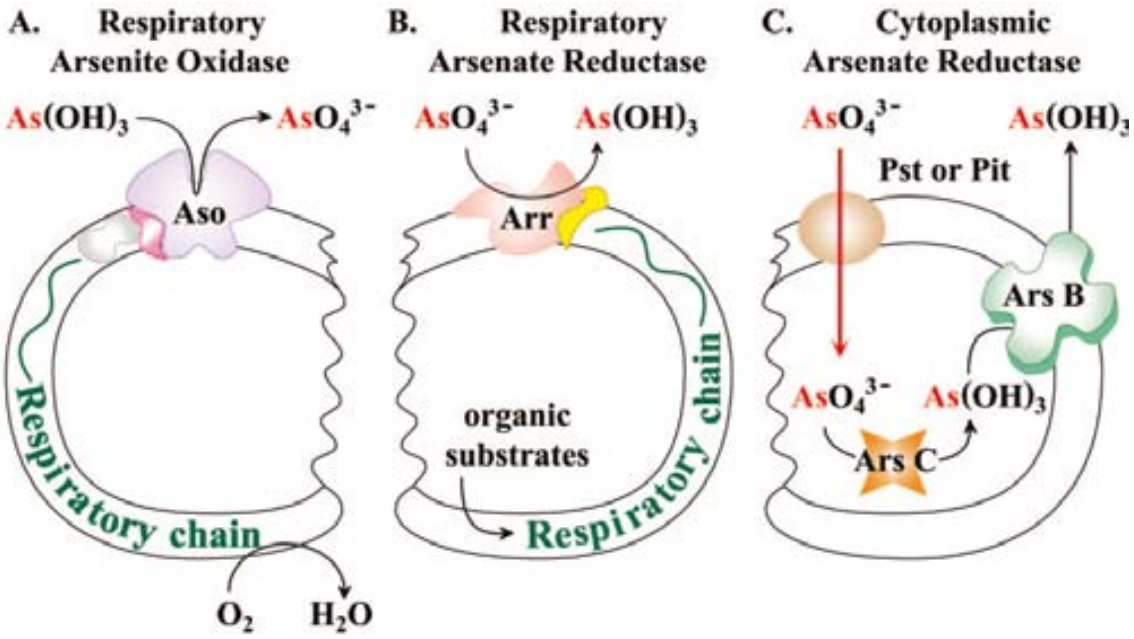

Figura 5. Localización y función celular de los tres sistemas enzimáticos que procesan As: arsenito oxidasa, arsenato reductasa respiratoria y arsenato reductasa citoplasmática (Silver & Phung, 2005). Los genes *aox y aro* también tiene la misma función de arsenito oxidasa, pero no se muestran en la figura.

2.1.1 *Mecanismos de oxidación de As(III)*

Los microorganismos quimiolitotróficos obtienen energía debido a la oxidación de As(III) y la reducción del oxígeno o del nitrato (Oremland *et al.,* 2002). Entre estos se encuentran Alpha- y Gamma-*Proteobacterias* (Fig. 4). Sin embargo, no todas las bacterias capaces de oxidar As(III) obtienen energía de este proceso. Algunas bacterias heterotróficas oxidantes de As(III) no parecen utilizar el As(III) como donador de electrones, sugiriendo que la oxidación del As(III) puede ser fortuita o una forma de detoxificación para dichas especies (Anderson, 1995, Croal et al., 2004). Éstos incluyen a miembros de los géneros *Alcaligenes, Pseudomonas* y *Thermus*. Los mecanismos de oxidación del arsenito involucran a los genes *aoxAB* y *aroAB.*

La oxidación bacteriana del arsenito a arsenato es ampliamente conocida, especialmente en aislados aeróbicos de ambientes con altas concentraciones de arsénico. No está claro si la oxidación de arsenito está limitada a unas pocas cepas de cada especie. Por ejemplo, aunque dos aislados de *Alcaligenes faecalis* tienen esta capacidad, esta actividad no ha sido encontrada en otros cultivos de colección de esta especie (Osborne & Erlich, 1976, Phillips & Taylor, 1976). Aunque la mayoría de los aislados ambientales no tienen esta capacidad, la actividad enzimática de arsenito oxidasa ha sido detectada en varios grupos tanto de bacterias como arqueas (Newman *et al.,* 1998).

Los genes *aoxA* y *aoxB* requeridos para la oxidación de As(III), se han identificado en un cultivo de la Betaproteobacteria ULPAs1 (Weeger *et al*., 1999), donde los estudios y análisis bioquímicos indican que estos genes codifican para enzimas que son miembros de la familia de las DMSO reductasas.

Otro par de genes que codifica oxido reductasas de As(III) son *aro*A y *aro*B, que se han identificado en la cepa NT-26, una bacteria quimiolitotrófica que oxida As(III) (Croal *et al.,* 2004)

Respecto a la secuencia de aminoácidos, estos genes tienen una similitud muy alta: *aroA* y *aoxB* tienen un 48% de similitud entre sí, mientras que *aroB* y *aoxA* tienen un 52% de similitud. Las secuencias de estos genes también están altamente relacionadas con las proteínas que han sido secuenciadas e identificadas en *Alcaligenes faecalis,* un

oxidador de As(III), las arqueas *Aeropyrum pernix* y *Sulfolobus tokodaii* y con un organismo fotosintético como *Chloroflexus aurantiacus* (Santini & van den Hoven, 2004).

2.1.2 *Mecanismos de reducción de As(V)*

Diversos grupos de bacterias heterotróficas pueden utilizar As(V) como aceptor terminal de electrones en la respiración anaeróbica. Estos organismos abarcan a miembros de las subdivisiones Gamma-, Delta-, y Epsilon- *Proteobacteria,* bacterias Gram-positivo, bacterias termófilas y Arqueas del reino *Crenarchaeota*. La mayoría de las especies respiradoras de As(V) utilizan la oxidación del lactato a acetato para la reducción de As(V) a As(III), aunque algunos aislados pueden oxidar el acetato a CO_2 y/o utilizar H_2 como donador de electrones (Huber *et al.*, 2000). Aunque la mayoría de procariotas respiradores de As(V) son anaerobios estrictos, algunos son aerobios facultativos.

El arsenato es reducido a arsenito por las bacterias como mecanismo de detoxificación o como un aceptor terminal de electrones durante la respiración anaerobica. Los mecanismos de respiración del arsenato y detoxificación del arsenato se atribuyen a los genes que codifican la arsenato reductasa *arrAB* y al operón *arsRDABC*, respectivamente.

Se han aislado muchas bacterias que tienen la capacidad de usar el arsenato como un aceptor terminal de electrones por la vía de respiración anaerobia. El sistema genético de la especie *Shewanella* sp. ANA-3 (Saltikov et al., 2003), ha puesto al descubierto que este organismo posee dos sistemas independientes de reducción de As(V) (Fig. 6). El primero pertenece al sistema de detoxificación Ars que no participa en la respiración anaerobica. El segundo sistema incluye dos genes, *arrA* y *arrB*, requeridos para la reducción respiratoria del arsenato.

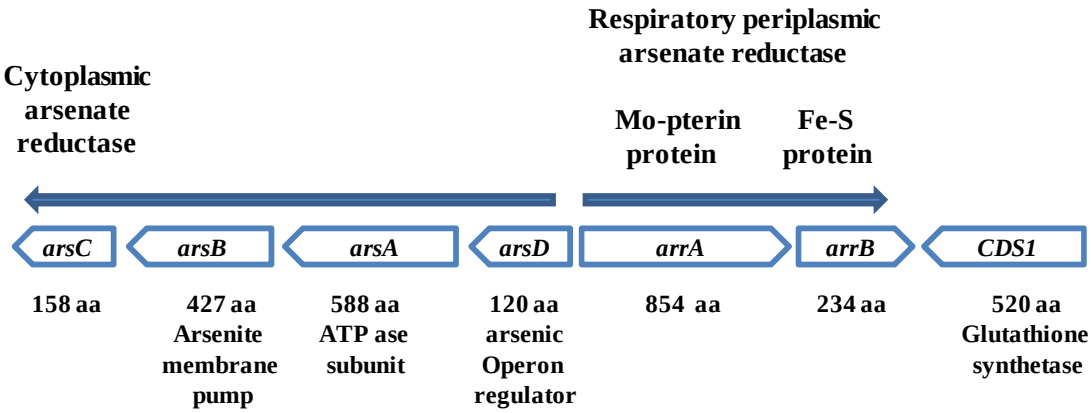

Figura 6. Organización molecular de los genes para la reducción de arsenato por respiración *arrAB* y por detoxificación *arsC* presentes en la cepa de *Shewanella* sp. ANA-3. aa, número aminoácido (Croal *et al.*, 2004).

Genes arrA y arrB

El operón que engloba estos genes está próximo al operón *ars* en el genóforo, pero se transcribe en sentido contrario. Estos genes parecen estar bajo el control de un promotor que detecta la anaerobiosis, y existe un activador adicional específico de As que ayuda a regular la expresión del gen *arr*. *arrA* codifica una proteína que comparte características con las oxidoreductasas con molibdopterina. Estos son sitios ricos en cisteína ligados a sulfuros de hierro y al dominio de molibdoproteína (Fig. 7). *arrB* se transcribe a continuación de *arrA* y su producto traducido también contiene sulfuros del hierro (Croal *et al.*, 2004).

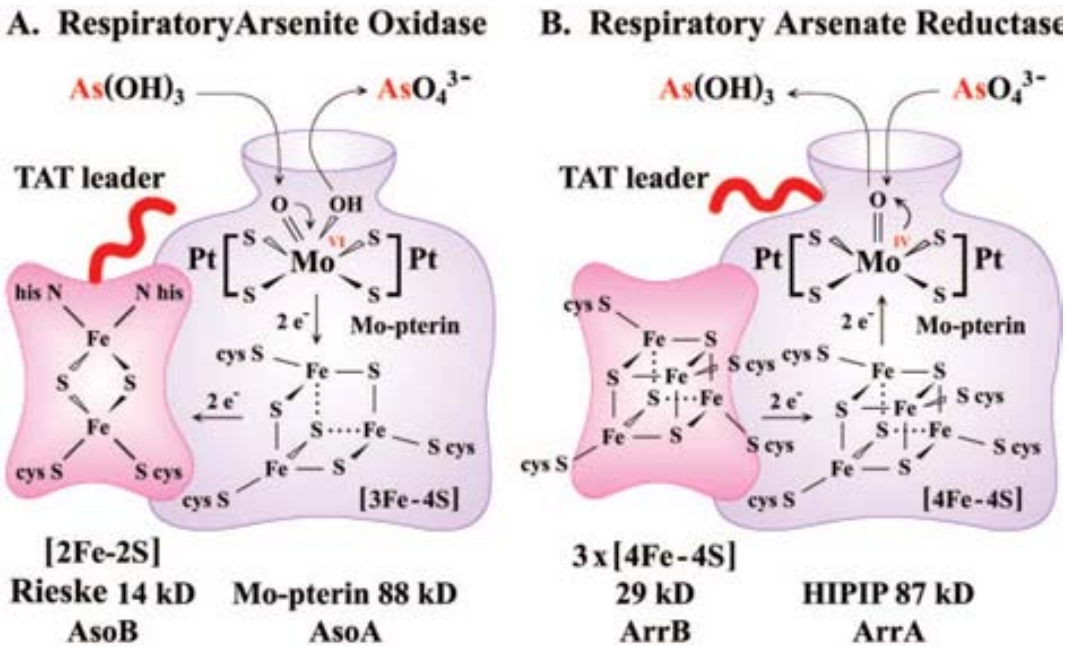

Figura 7. Modelos de los heterodímeros arsenito oxidasa y arsenato reductasa. Los centros activos se muestran en la parte superior en forma de embudos (Silver & Phung, 2005).

Los genes *arr* parecen estar altamente conservados entre diversos grupos filogenéticos de bacterias reductoras de As(V). Así, por ejemplo, las secuencias de aminoácidos de estas proteínas entre la bacteria Gram-positiva *Desulfitobacterium hafniense* tiene un 68% de similitud con la proteína ArrA de *Shewanella* sp. ANA-3, y un 61% de similitud con ArrB.

Genes de entrada en las celúlas

El arsenato es un análogo del fosfato y tiene la capacidad de entrar en el citoplasma principalmente a través del sistema transportador de fosfato Pit, un sistema de alta afinidad/alta velocidad. Se ha descrito que en *E. coli* y otras cepas que dependen del sistema Pit, no hay crecimiento en presencia de arsenato (10 mM). Sin embargo, los mutantes *pit* de *E. coli* dependen del sistema transportador de fosfato Pst y puede crecer en presencia de arsenato. Una vez el As(V) ha entrado en la célula, inhibe la fosforilación oxidativa y la síntesis de ATP. El As(III) en cambio entra en la célula a través de los

transportadores de acuagliceroporinas. El As(III) es mucho más tóxico que el As(V) debido a su gran afinidad por los grupos sulfidrilos (Croal *et al*., 2004).

Genes ars

El estudio del sistema de detoxificación mejor conocido es el codificado por el operón *ars* del plasmido R773 de *E. coli* (Hedges & Baumberg, 1973). Este operón incluye cinco genes, *arsRDABC*. El gen *arsC* codifica una arsenato reductasa citoplasmática que se encuentra ampliamente distribuida en muchas bacterias y arqueas. ArsC es una pequeña proteína monomérica de cerca de 135 aminoácidos, que interviene en la reducción de As(V) a As(III) en el citoplasma. *arsB* que es una bomba de arsenito que está junto al gen *arsC*. Aunque el As(III) es más tóxico, éste puede ser excretado activamente por la vía de ArsB, que es un transportador específico de As(III) (Silver & Phung, 2005).

3. DESCRIPCIÓN DEL ENTORNO DE TRABAJO

Los ambientes extremos, como el Desierto de Atacama, permiten suponer la presencia de hábitats excepcionales que favorezcan el desarrollo de microorganismos de importancia biotecnológica. En estos ambientes existen procesos biogeoquímicos que incluyen diversas reacciones y efectos (oxidantes, reductores, catalizadores, complejantes, entre otros) de elementos como Fe, S, N, C, As. Estos procesos han intervenido e intervienen en la formación de yacimientos de minerales y en la diagénesis de los sedimentos. Por este motivo, la zona constituye un laboratorio natural para el estudio de los procesos microbiológicos que pueden ser explotados en desarrollos biotecnológicos.

En el sector andino, entre los 14º y 27º de latitud sur, se encuentran numerosos y variados depósitos salinos entre los que se destacan las cuencas endorreicas de lagos y salares (Fig. 8). Los salares son cuerpos evaporíticos que incluyen niveles de agua subterránea y lagunas superficiales. Estos ambientes suelen ser hipersalinos, y poseen fauna, flora y microbiota autóctonas. En el caso del sector chileno de la franja mencionada, los salares no son tan numerosos o extensos como en Bolivia o Argentina, pero son de una gran variedad y se extienden sobre todo el territorio desde la zona litoral hasta la Al-

ta Cordillera. Incluyen desde cuerpos salinos fosilizados en la parte occidental, hasta otros en plena formación, como sucede en la Alta Cordillera, con todo un amplio repertorio de cuerpos salinos correspondientes a los estados intermedios.

Estos ambientes han sido estudiados desde los puntos de vista geológico (Chong 1984, Risacher & Fritz 1991 a y b), limnológico y de las poblaciones de invertebrados (Hurlbert *et al*., 1984, Alpers & Whittemore 1990, Igarzábal 1991, Servant-Vildary & Mello 1993, Grosjean 1994, López *et al.*, 1999 y Zúñiga *et al.*, 1999) y de ecología microbiana (Demergasso *et al.*, 2004). Los únicos estudios de microbiología clásica han consistido en obtener cultivos puros de halófilos en la laguna de Tebenquiche en el Salar de Atacama (Zúñiga *et al.*, 1991, Prado *et al.*, 1993, Lizama *et al.*, 2001, 2002).

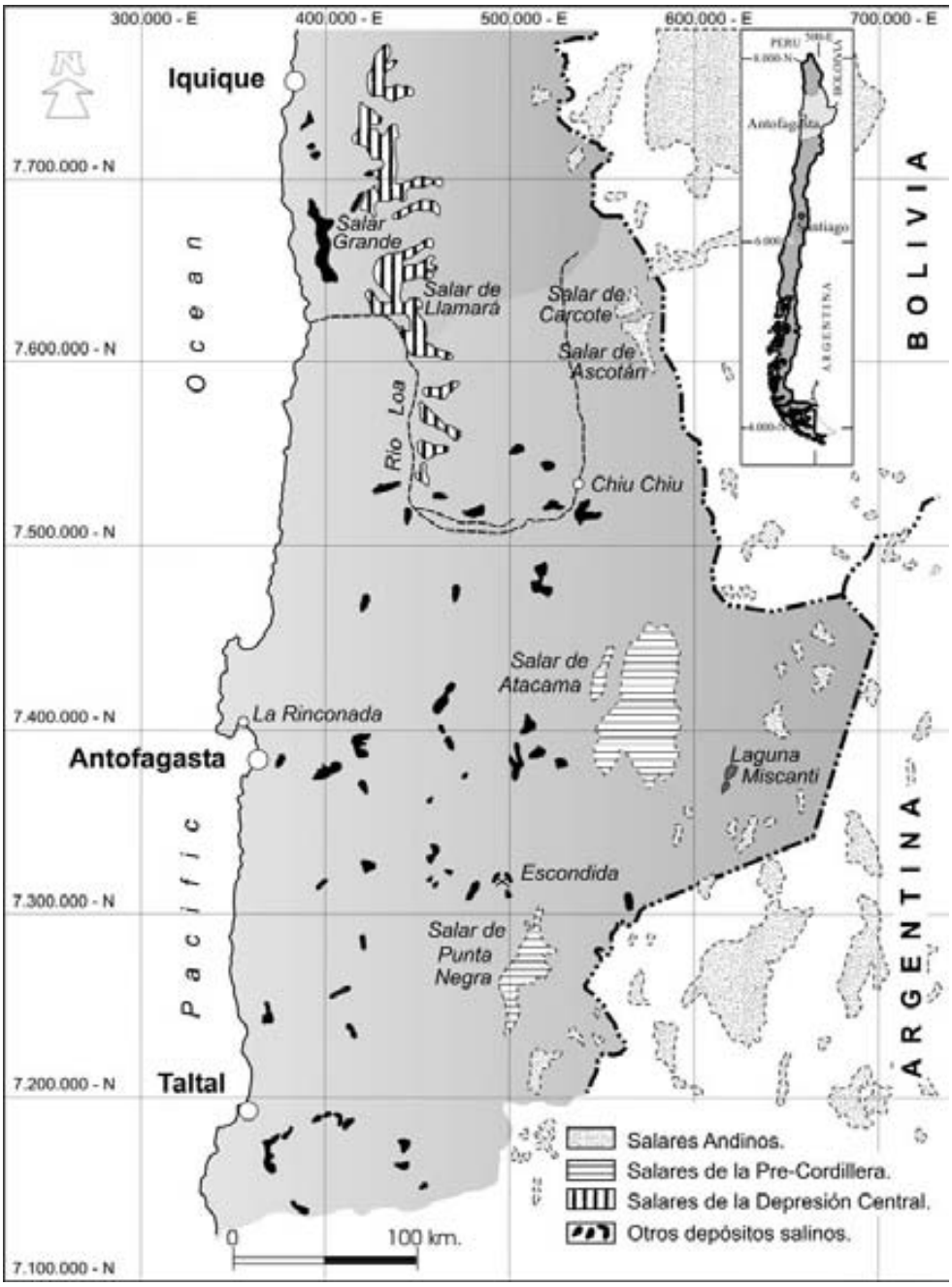

Figura 8. Mapa del Desierto de Atacama que muestra la localización de algunos salares y las características de los diferentes depósitos salinos (Reproducido de Demergasso *et al.*, 2004).

Los salares se clasifican de acuerdo a su ubicación geográfica (Chong, 1984), dado que ésto implica también importantes diferencias entre ellos (Fig.9). Esta división incluye, de oeste a este, a los salares de la Cordillera de la Costa, de la Depresión Central, de la Depresión Preandina y a los salares y lagos andinos. En la Cordillera de la Costa sólo hay un salar (El Salar Grande) que se caracteriza por ser una cuenca rellena exclusivamente de cloruro de sodio, seca, sin salmueras y que no recibe sobrecarga. Se le puede considerar un salar "fósil". En la Depresión Central hay gran variedad de cuencas que reciben una recarga irregular desde el oriente, principalmente en forma de aguas subterráneas y en grandes avenidas estacionales. Los salares de la Depresión Preandina son los más antiguos y de mayor tamaño (Salar de Atacama) y se encuentran en una etapa de "fosilización" ya que sólo reciben una recarga significativa en su parte norte, oriental y sur. Finalmente los salares andinos están en plena evolución (Salar de Ascotán). A continuación se detallarán las características de los dos salares estudiados en el presente trabajo de tesis el Salar de Atacama y el Salar de Ascotán.

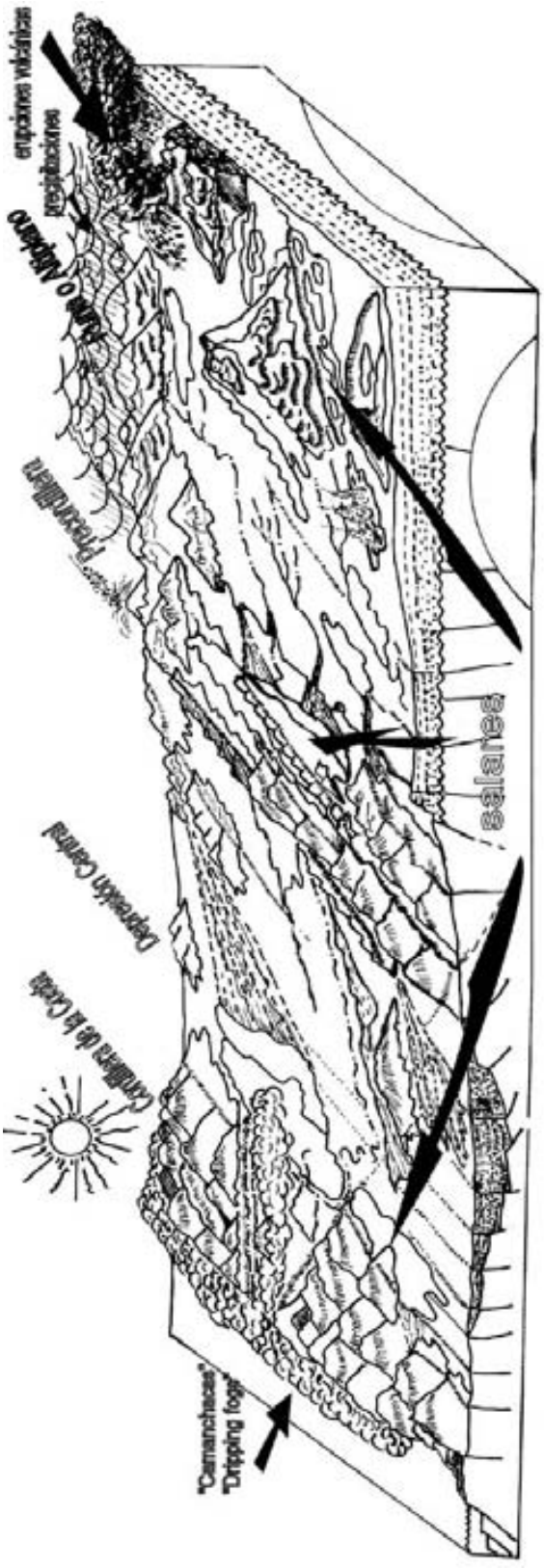

Figura 9. Perfil esquemático de la geomorfología y distribución de cuencas endorreicas en el Norte de Chile (Imagen reproducida de la Tesis doctoral presentada por Cecilia Demergasso).

3.1 *Salar de Atacama*

La cuenca del Salar de Atacama (Figs. 8 y 10) ocupa una depresión geológica de origen estructural, elongada en dirección N-S, dispuesta entre la Cordillera de Domeyko por el oeste y la Cordillera de los Andes por el este, entre aproximadamente 22º30' y 24º15' de latitud Sur. Su superficie es de 13.300 km^2. Posee dos depresiones separadas por la Cordillera de la Sal: la depresión del Salar de Atacama que ocupa 2200 km^2 y está en la parte más baja a 2300 m sobre el nivel del mar y la depresión del Llano de la Paciencia.

Los parámetros climáticos que inciden significativamente sobre el proceso de recarga hídrica presentan una distribución notablemente diferente entre la mitad occidental y la mitad oriental de la cuenca. Esta situación se traduce en que la mitad oeste de la cuenca parece aportar una proporción menor al 5% de la recarga hídrica que beneficia los acuíferos que se ubican en la depresión.

El promedio anual de agua caída sobre toda la cuenca del salar se calcula en 69 mm (Bevacqua, 1992). Las precipitaciones se producen principalmente entre los meses de diciembre y marzo y, en menor cantidad, entre junio y agosto. La media anual de precipitaciones registrada en las estaciones Toconao, San Pedro y Peine oscilan entre 20.4 y 41.7 mm (Ministerio de Obras Públicas, 1987). Los cauces superficiales que escurren al interior de la cuenca del Salar de Atacama convergen todos en la depresión.

La evapotranspiración y la evaporación representan los únicos procesos de descarga natural que tienen lugar en la cuenca del salar. La evaporación total anual determinada a partir de estanques de evaporación en las estaciones Peine, Toconao y San Pedro es de 2993, 3186 y 3189 mm, respectivamente (Ministerio de Obras Públicas, 1987). Sin embargo, la presencia de un gran número de variables que inciden sobre la tasa de evaporación (salinidad del agua, profundidad del nivel freático, fuerte variación térmica en el ciclo diario, textura de los sedimentos que cubren la superficie, etc.) dificulta el cálculo de la descarga global.

En el núcleo salino del salar, el nivel freático se sitúa entre 40 y 50 cm bajo la superficie. Al norte del núcleo el nivel freático se encuentra a una profundidad de entre 5 y 12 m. Existen niveles de arcilla que provocan el confinamiento del acuífero más superficial, impidiendo la evapotranspiración de las aguas subterráneas en dicho sector, excepto

en el área de las lagunas y vertientes (manantiales) del borde este del salar. Las aguas del acuífero emergen en diferentes puntos del salar. En el margen nor-oriental se encuentra la laguna de Burros Muertos y un río que une al Llano del Tambillo y que genera una línea de lagunas como son Chaxa, Barros Negros y otras. El otro punto destacable se encuentra en la parte sur-oriental donde están la Laguna Salada, la Laguna Brava y dos más pequeñas sin nombre. El afloramiento de las aguas del acuífero se hace a través de pozos artesianos como Tilopozo, Tambillo y el Pozo 3, o formando dolinas por disolución de la superficie salina con formas redondeadas de colapso como son el conjunto Tebenquiche, Baltinache, Cejas y dos dolinas cercanas a éstas. La laguna Tebenquiche es una de las lagunas más grandes que se encuentran en la parte norte del Salar de Atacama. La caracterización y limnología de la laguna fue llevada a cabo por Zuñiga *et al.,* (1991).

Figura 10. Imágenes del Salar de Atacama. De arriba a bajo y de izquierda a derecha: Laguna Tebenquiche, dolina (Ojos del Salar), Salar de Atacama, Laguna Chaxas y Laguna Cejar.

3.2 *Salar de Ascotán*

El Salar de Ascotán se ubica en la II región de Chile, emplazado en una hoya hidrográfica que se extiende a territorio boliviano. Forma parte de un sistema de cuencas endorreicas que albergan salares que, además, incluye Carcote (también conocido como San Martín) y Ollagüe. Ascotán es el cuerpo evaporítico de mayor tamaño con 230 km^2, ubicado en el Altiplano a unos 200 km al noroeste de Calama (Figs. 8 y 11).

Ambos salares (Ascotán y Carcote) se implantan en la parte más baja de una depresión flanqueada al Este y al Oeste por cadenas volcánicas terciarias y cuaternarias de orientación general Norte-Sur. Colinda al Oeste con la cuenca del río Loa. Al Este, la frontera con Bolivia no sigue la línea divisoria de aguas pues pasa dentro de la cuenca de drenaje de Ascotán. Los sistemas volcánicos son complejos y los más representativos de la parte Occidental son Aucalquincha (6476 msnm), Miño (5611 msnm), Chela (5644 msnm), Palpana (6028 msnm) y Polapi (5949 msnm). A su vez, en la parte oriental, destacan Araral (5698 msnm), Ascotán (5498 msnm) y Ollagüe (5863 msnm), además de otros que se ubican en territorio boliviano.

Es un salar de tipo "playa" con limos salinos y costras de sales (yeso, halita). Se observa un complejo sistema de lagunas superficiales elongadas Este-Oeste o Suroeste-Noreste alimentadas por vertientes que surgen de la orilla oriental del salar. Cerca de la orilla oeste del salar se encuentran lagunas más pequeñas y menos numerosas. Las principales características morfométricas y climatológicas del salar son; altura: 3716 m, superficie de la cuenca: 1757 km^2, superficie del salar: 243 km^2, superficie de las lagunas 18 km^2, precipitaciones: 100 - 150 mm/año, evaporación potencial: 1630 mm/año y temperatura media: 5.8°C.

El Salar de Ascotán es una unidad geológica dinámica en la cual interactúan principalmente factores como evaporación, precipitación, y escorrentías superficiales y subterráneAs(Herrera *et al.*, 1997). El mecanismo recurrente en estos cuerpos evaporíticos es una recarga, superficial y subterránea, de aguas de diferente salinidad que ingresan a la cuenca. Al igual que en los restantes salares andinos hay una proveniencia predominante de iones que tienen su origen tanto de la lixiviación de rocas volcánicas como de la acción misma del volcanismo circundante. Posteriormente, la elevada tasa de evaporación produce la precipitación de minerales evaporíticos formando costras salinas. Las salmueras se van renovando en forma constante por movimientos ascendentes de aguas subterráneas

menos salinas. Este fenómeno determina distintos tipos de costras formadas principalmente por cloruros y sulfatos y minoritariamente por otros 15 minerales por lo menos.

La cuenca del Salar recibe aportes de agua de las cadenas volcánicas que lo limitan, aunque mayoritariamente provienen de la cadena Oriental. En este último caso se produce una descarga de 12 vertientes principales con aguas que surgen de la base de las coladas de lava circundantes y cuyas aguas discurren hacia el oeste. Debido a la importancia de los volúmenes de agua se forman esteros (arroyos) que penetran kilómetros en el cuerpo del Salar formando, además, algunos rosarios de lagunas. Los aportes de la parte oeste son, en comparación, secundarios y la mayoría se infiltra como aguas subterráneas.

La extracción de agua del sistema se produce antrópicamente mediante aprovechamiento desde pozos y, de manera natural, a través de evaporación directa desde superficies de agua libre y de aquella cercana a la superficie que asciende por capilaridad, por descarga directa de la cuenca en forma de aguas subterráneas hacia el Salar de Carcote y por evapotranspiración a través de la vegetación (Guerra *et al.,* 1997).

Ascotán, además, tiene una recarga que proviene desde el sur, sector de las quebradas Perdiz y El Inca en la parte sur oriental de la hoya hidrográfica, en territorio boliviano. En el caso del agua de las cadenas volcánicas, éstas acceden principalmente a través de fracturas en las rocas con una contaminación menor. En cambio la que viene de la parte sur-oriental lo hace a través de sedimentos que incorporan una mayor cantidad de sólidos disueltos. En la parte sur del salar, por efectos de gradiente geotérmico, asociado a volcanismo activo, las aguas se encuentran a temperaturas de hasta 30ºC.

Los aportes salobres del Este del salar presentan sus puntos representativos bien agrupados. Son aguas de tipo Na-(Ca) /Cl. Las aguas del Sur del salar también son de tipo Na-(Ca) / Cl, pero se diferencian ligeramente de las aguas Orientales por un leve aumento de HCO_3 y Na. Las aguas de aporte del sector Occidental de la cuenca tienen una composición mucho más variada. Tienen un contenido porcentual más elevado en sulfato que las aguas del Este y del Sur. Casi todas las aguas de aporte de Ascotán tienen influencia termal aunque no se puede correlacionar el termalismo con ningún parámetro químico de las aguas.

En Ascotán, es común encontrar en el mineral de borato nódulos lenticulares de color amarillo y rojo de algunos centímetros de espesor que se extienden por unos pocos metros y que han sido caracterizados como compuestos con contenido de arsénico. Estós nódulos son de minerales de arsénico rejalgar y oropimente.

Figura 11. Imágenes del Salar de Ascotán. De arriba abajo y de izquirda a derecha: Laguna Turquesa, sedimento del Salar, Volcán de Ascotán, Vertiente 4 y zona de explotación de boratos.

4. ARSÉNICO EN LAS AGUAS DE LA II REGIÓN CHILENA

El problema de la contaminación con As en la Región de Antofagasta presenta aspectos muy diversos que deben ser abordados por diferentes disciplinas. En líneas generales se admite que esta contaminación se debe a causas tanto naturales como antrópicas, aunque el origen geológico de la contaminación con As de las aguas de la Segunda Región de Chile es claro (Fritsch *et al.,* 1999). Las fuentes de agua donde se abastece la empresa Aguas de Antofagasta para proveer de agua potable a la población de la Región de Antofagasta, tienen un contenido de As promedio de 0.33 mg/L. El mayor impacto de la actividad industrial (emisiones de polvo y material partículado de empresas mineras) se representa en la contaminación del aire con As y la acumulación del elemento en suelos y sedimentos. La concentración y especiación de As en los ambientes acuáticos está gobernada por numerosos procesos tanto abióticos como generados por actividad biológica (Fig. 12). Los informes elaborados en el evento de contaminación del Río Loa del año 1997 registraron niveles extremos de más de 6 mg/L de As, y estimaron que la resuspensión de los lodos y sedimentos acumulados por años en el río, junto con una modificación de parámetros como el oxígeno disuelto, el pH, el potencial redox y la salinidad, generaron un cambio en las formas de los compuestos del As y con esto una removilización del As antes fijado en el sedimento. El elevado contenido de arsénico en los minerales, unido al proceso de producción de cobre, la principal actividad económica del norte de Chile, son factores que hace peligrar la sostenibilidad productiva y ambiental de la minería chilena.

La Región de Antofagasta en concreto está afectada por la contaminación con arsénico en agua, aire y sedimentos y son frecuentes los episodios periódicos de muerte de peces en la cuenca del Río Loa. Existen evidencias de la presencia en esta región de microorganismos involucrados en el ciclo biogeoquímico del As(respiradores, reductores), así como de ambientes propicios para el desarrollo de otras capacidades metabólicas relacionadAs(oxidadores de arsénico). Por lo tanto se hace necesario conocer la microbiota y estudiar los factores ambientales que pueden afectar la viabilidad-actividad de estos microorganismos y, en consecuencia, a su contribución al ciclo biogeoquímico del arsénico en la zona.

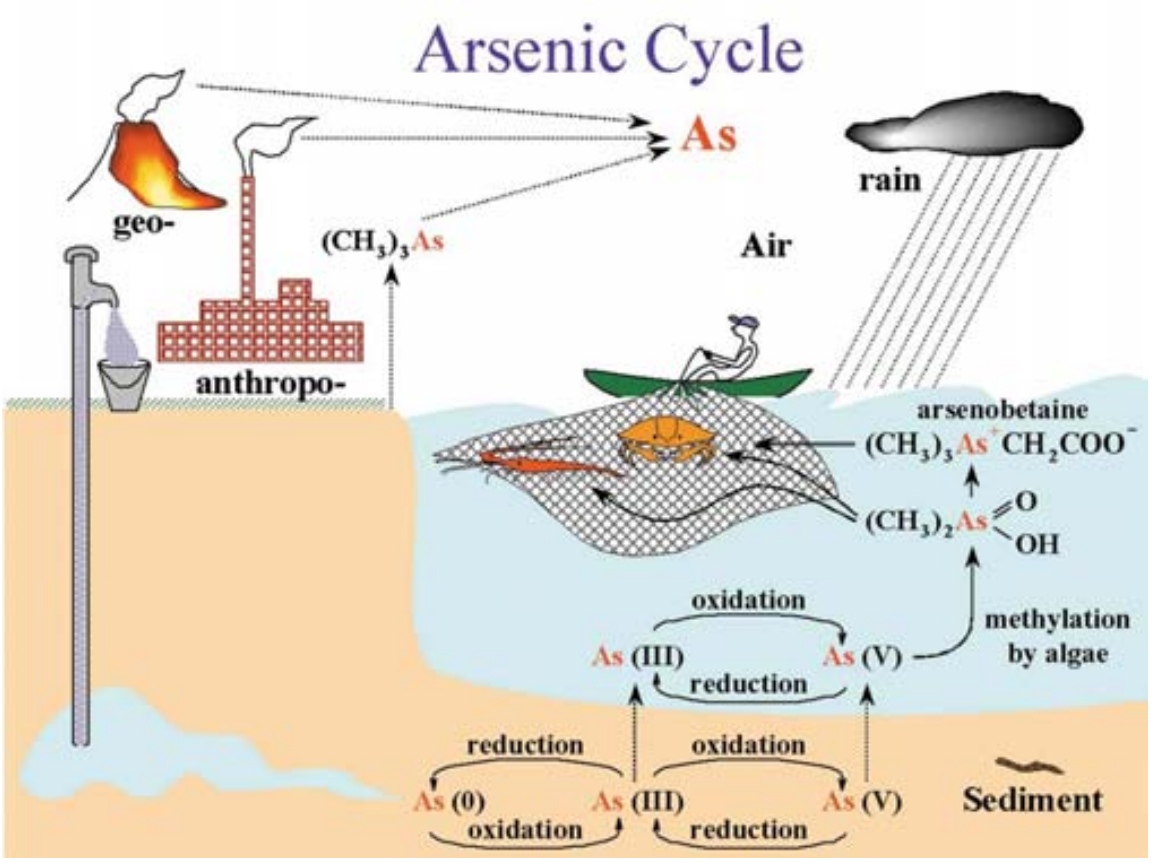

Figura 12. Ciclo biogeoquímico del As (Mukhopadhyay *et al*., 2002).

5. OBJETIVOS Y ESTRUCTURA DE LA PRESENTE TESIS DOCTORAL

Partiendo de estos antecedentes, el objetivo principal del presente trabajo de tesis doctoral es caracterizar las comunidades microbianas que se encuentran en estas cuencAs(Salar de Atacama y Salar de Ascotán) por métodos microbiológicos y de ecología microbiana molecular e intentar identificar las variables principales que controlan la estructura de las comunidades microbianas en ambientes altamente contaminados por arsénico. Esta investigación está orientada también a la búsqueda de microorganismos de importancia geomicrobiológica y biotecnológica. Por otra parte, el conocimiento de la biodiversidad microbiana y sus aplicaciones útiles en estos frágiles sistemas, sumará argumentos para su protección, dada la intensa explotación de los recursos hídricos que padece el desierto de Atacama.

A continuación se detalla de forma escueta el contenido de cada capítulo:

En los capítulos 1 y 2 se obtuvo información acerca de la diversidad genética de las comunidades microbianas en la Laguna de Tebenquiche del Salar de Atacama y en el Salar de Ascotán. Este estudio se llevo a cabo mediantes técnicas moleculares, a partir de una exploración con una técnica de huellas dactilares y la construcción de bibliotecas genéticas del gen ribosómico 16S rRNA. En este estudio se determinó la heterogeneidad de los salares estudiados y se identificaron los gradientes de salinidad y de concentración de arsénico como las causas más importantes de dicha variabilidad. Estos capítulos proporcionan un marco de referencias para los estudios sobre genes y microorganismos relacionados con el As del resto de la memoria.

En el capítulo 3 se estudió la presencia de los genes de arsénico involucrados en los sistemas de respiración y detoxificación (genes *arrA* y *arsC,* respectivamente), en un amplio repertorio de muestras ambientales con distintas concentraciones de arsénico. Este análisis se llevó a cabo mediante la amplificación de estos genes por PCR y el recuento de bacterias reductoras de arsénico por número más probable. Se obtuvo una distribución diferencial de estos genes en las muestras analizadas. El gen *arrA* se encontró en todas las muestras. En cambio, el gen *arsC* solo se detectó en muestras con concentraciones de As menores a 4 mg/L.

En el capítulo 4 se caracterizó la diversidad genética de genes de arsénico implicados en la respiración (gen *arrA*) y detoxificación (gen *arsC*) en un sistema modelo. Este estudio se realizó mediante la construcción de bibliotecas genéticas y secuenciación en dos muestras con alta y baja concentración de As procedentes del Salar de Ascotán. Se observó que existe una baja diversidad de taxones bacterianos que poseen los genes que respiran y detoxifican arsénico, pero con una elevada microdiversidad dentro de ellos.

En el capítulo 5 se relacionó la actividad microbiana con la precipitación de sulfuros de arsénico. Este estudio se realizó a través de cultivos de enriquecimiento, análisis microscópicos de SEM y TEM y el estudio de la composición isotópica y atómica de los precipitados As/S. Se demostró que la precipitación microbiana de sulfuros de arsénico, contribuye a la formación de minerales de arsénico de origen biológico en el medio natural, dando a este peculiar metabolismo una relevancia geológica.

Finalmente, en el capítulo 6 se describe el aislamiento de cultivos puros de bacterias capaces de precipitar minerales de arsénico que explicarían la formación de estos depósitos en la naturaleza. Este aislamiento se realizó mediante técnicas de cultivos para microorganismos anaerobios. Las cepas bacterianas se identificaron filogenéticamente como pertenecientes al grupo de las Gammaproteobacteria (*Shewanella* sp. y *Pseudomonas* sp.).

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Capítulo I

Novelty and spatio-temporal heterogeneity in the bacterial diversity of hypersaline Lake Tebenquiche (Salar de Atacama)

Demergasso, C., L. Escudero, E. O. Casamayor, G. Chong, V. Balagué & C. Pedrós-Alió, (2008) Extremophiles 12: 491-504.

La candidata Lorena Escudero ha participado en todas las campañas de muestreo y en los experimentos descritos en esta publicación, responsabilizándose de los trabajos de biología molecular (extracción de ADN, amplificación por PCR, electroforesis desnaturalizante, DGGE) y análisis filogenético de secuencias y en la construcción de árboles filogenéticos con el programa ARB, así como en la discusión de los planteamientos experimentales y los resultados obtenidos.

Aquel que pregunta es un tonto por cinco minutos,
pero el que no pregunta permanece tonto por siempre.
(Proverbio Chino)

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ORIGINAL PAPER

Novelty and spatio–temporal heterogeneity in the bacterial diversity of hypersaline Lake Tebenquiche (Salar de Atacama)

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Abstract Lake Tebenquiche is one of the largest saline water bodies in the Salar de Atacama at 2,500 m above sea level in northeastern Chile. Bacteria inhabiting there have to deal with extreme changes in salinity, temperature and UV dose (i.e., high environmental dissimilarity in the physical landscape). We analyzed the bacterioplankton structure of this lake by 16S rRNA gene analyses along a spatio–temporal survey. The bacterial assemblage within the lake was quite heterogeneous both in space and time. Salinity changed both in space and time ranging between 1 and 30% (w/v), and total abundances of planktonic prokaryotes in the different sampling points within the lake ranged between two and nine times 10^6 cells mL^{-1}. Community composition changed accordingly to the particular salinity of each point as depicted by genetic fingerprinting analyses (denaturing gradient gel electrophoresis), showing a high level of variation in species composition from place to place (beta-diversity). Three selected sites were analyzed in more detail by clone libraries. We observed a predominance of Bacteroidetes (about one third of the clones) and Gammaproteobacteria (another third) with respect to all the other bacterial groups. The diversity of Bacteroidetes sequences was large and showed a remarkable degree of novelty. Bacteroidetes formed at least four clusters with no cultured relatives in databases and rather distantly related to any known 16S rRNA sequence. Within this phylum, a rich and diverse presence of *Salinibacter* relatives was found in the saltiest part of the lake. Lake Tebenquiche included several novel microorganisms of environmental importance and appeared as a large unexplored reservoir of unknown bacteria.

Keywords 16S rRNA gene · Bacteroidetes · Biodiversity · Clone libraries · DGGE · Ecology · Extremophiles · Fingerprinting · Gammaproteobacteria · Hypersaline · Salar · Salt lake · Tebenquiche

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Introduction

Diversity of microbial communities is believed to be very large and poorly characterized (see overview in Pedrós-Alió 2006). Yet, its knowledge is of interest for several reasons, both practical and theoretical. Bacterial diversity is a reservoir of potentially interesting genes for biotechnology and medicine, and the large seed-bank of bacterial taxa hidden in natural communities should be of interest to better delineate both the taxonomy and the evolutionary

relationships among microorganisms (Baldauf 2003; Pedrós-Alió 2007). In this respect, the ocean and freshwater lakes have received a lot of attention (Glöckner et al. 1999; Giovannoni and Rappé 2000). However, bacterial diversity in saline lakes have been studied sparsely (Bowman et al. 2000; Humayoun et al. 2003; Jiang et al. 2006; Wu et al. 2006), despite the fact that they are numerous and widespread (Williams 1996). Microorganisms from such environments have potentially interesting enzymes (Oren 2002), and some authors have claimed that the physiology and ecology of microorganisms in hypersaline environments may be relevant for a better understanding of both the early stages of life on Earth (Kunte et al. 2002) and potential life in Mars evaporitic environments (Mancinelli et al. 2004).

Lake Tebenquiche is one of the largest hyperhaline high-altitude water bodies in the Salar de Atacama (Chile). A preliminary limnological characterization of this lake was carried out by Zúñiga et al. (Zúñiga et al. 1991). Next, a large collection of bacteria and archaea were isolated in pure culture. Results have been reported for moderately halophilic Gram-negative rods (Prado et al. 1991) and Gram-positive cocci (Valderrama et al. 1991), heterotrophic halophilic microorganisms (Prado et al. 1993) and extreme halophilic *Archaea* (Lizama et al. 2001, 2002). Most of the bacteria isolated belonged to the Gammaproteobacteria, especially members of the genera *Vibrio*, *Halomonas* (including *Deleya* and *Volcaniella*), *Acinetobacter*, *Alteromonas*, *Psychrobacter* and *Marinococcus*. The only other groups that were recovered with some frequency were the High and Low GC Gram positives. No Bacteroidetes were recovered. The real extent of bacterial diversity within the system remains still unexplored, because it is well known that isolation in pure culture selects some of the microorganisms present in the sample and that those able to grow in culture are in many occasions, not the most abundant ones in nature (Staley and Konopka 1985; Amann et al. 1995; Pedrós-Alió 2006).

Here, we present a detailed study of Lake Tebenquiche covering spatial heterogeneity and changes in time of the bacterioplankton composition by genetic fingerprinting on the environmental 16S rRNA gene pool. We also constructed clone libraries from selected sampling sites to obtain a more precise description of the bacterial diversity. In a previous paper (Demergasso et al. 2004), we carried out a general fingerprinting survey of the bacterial and archaeal diversity in other undersampled and remote athalassohaline environments from the Atacama Desert. Thus, it was of additional interest to compare the sequences retrieved by molecular methods and check whether any of the isolates could be found among them. Our goal was to use Lake Tebenquiche as a model to determine the degree of spatial heterogeneity in this kind of shallow lakes and to explore whether bacterial taxa specific to systems with intermediate salinities existed.

Materials and methods

Description of Lake Tebenquiche

The Salar de Atacama is a huge system (about 2,900 km^2) with several different water bodies in its interior. Lake Tebenquiche is one of the largest and it is located in the northern part of the Salar (Fig. 1). A summary of the geographical coordinates and other parameters of the locations sampled can be found in Table 1 and in Demergasso et al. (2004). Information on the geochemistry of this system can be found in Risacher et al. (1999) and in Zúñiga et al. (1991).

The hydrochemistry of salt lakes and marshes within the Salar de Atacama basin shows significant differences (Carmona et al. 2000). Water inputs have a wide range of

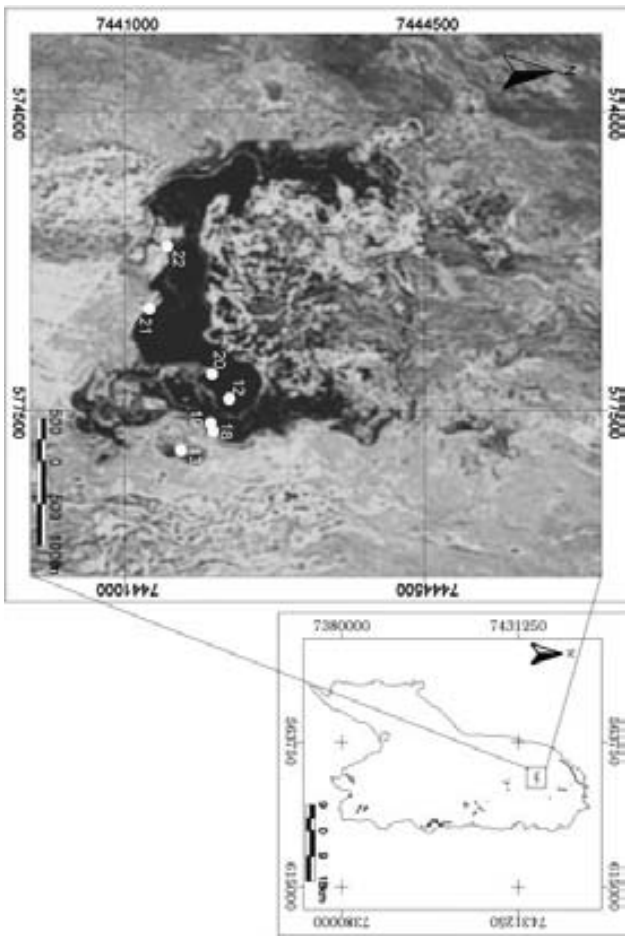

Fig. 1 Map of Lake Tebenquiche, showing its location within the Salar de Atacama and the sampling sites

Table 1 Geographical location, physicochemical and biological parameters for the samples analyzed

Samples	UTM coordinates		Date	Code[b]	Salinity (%)	pH	Temperature (°C)	Chlorophyll *a* ($\mu g\ L^{-1}$)	Prokaryotes (cells mL^{-1})
	North	East							
Tebenquiche 18[a]	7441459	578149	5 August 1999	At18Aug	7.98	8.40	14.0	ND	3.60E+06[c]
Tebenquiche 12	7441646	577767	15 March 2000	At12Mar	5.74	8.00	17.0	0.590	2.35E+06[c]
Tebenquiche 18[a]	7441459	578149	13 October 2000	At18Oct	14.80	7.68	17.7	0.333	5.69E+06[d]
Tebenquiche 19	7441428	578054	13 October 2000	At19Oct	14.80	7.77	20.4	0.025	4.40E+06[d]
Tebenquiche 20	7441446	577475	13 October 2000	At20Oct	5.45	7.73	19.1	0.035	3.40E+06[d]
Tebenquiche 12[a]	7441646	577767	13 October 2000	At12Oct	29.60	7.10	22.5	0.075	4.36E+06[d]
Tebenquiche 21	7440717	576714	13 October 2000	At21Oct	1.07	7.16	17.3	0.048	6.20E+06[d]
Tebenquiche 22	7440919	575978	13 October 2000	At22Oct	1.08	7.19	27.6	0.094	8.95E+06[d]
West Doline 13	7441082	578370	5 August 1999	At13Aug	0.90	8.94	10.6	ND	9.40E+05[c]
East Doline 13	7441082	578370	15 March 2000	At13Mar	1.25	8.50	18.0	1.67	1.24E+05[c]
Burro Muerto 14	7424309	584195	5 August 1999	At14Aug	4.75	8.01	21.5	9.92	8.88E+05[c]
Burro Muerto 14	7424309	584195	16 March 2000	At14Mar	7.10	7.60	22.0	4.20	2.41E+05[c]
Burro Muerto 15	7424506	584490	13 October 2000	At15Oct	0.49	7.89	28.6	2.18	1.03E+07[d]
Cejas 11	7449820	580386	15 March 2000	At11Mar	19.00	8.00	17.0	0.06	9.04E+04[c]

All the sampled systems are shallow (less than 50 cm depth) lakes with the exception of the dolines (small sinkholes) that are 10 m deep

ND not determined

[a] Samples selected for clone libraries

[b] Internal code for all the sampling series carried out in the Atacama region (see also Demergasso et al. 2004)

[c] Values obtained by flow cytometry (SD < 1%)

[d] Values obtained by epifluorescence microscopy (SD < 4%)

compositions and flows, draining very different geological formations surrounding the Salar. In addition, the evaporation along the flow path and within the Salar itself, contributes to the heterogeneity. A spatial distribution of pore brines within the Salar nucleus in two zones has been proposed (Risacher and Alonso 1996): calcium-rich brines (of Na–Ca–(Mg)–Cl type) are present in its south-western part, while sulfate-rich brines (of Na–(Mg)–SO_4–Cl type) are found towards the eastern part. This distribution agrees with the 87Sr/86Sr data, suggesting different water sources feeding each area (Carmona et al. 2000).

Sampling and measurements

Lake Tebenquiche was visited in August 1999 (winter) and March 2000 (summer). In October 2000 (spring), an intensive sampling expedition was carried out, and six different points in the lake were sampled to investigate the spatial heterogeneity of the microbial assemblage (Fig. 1). For comparison, additional samples were taken at two nearby dolines (small sinkholes) and at Burro Muerto, a shallow lake south of Lake Tebenquiche but within the Salar de Atacama. The environments sampled showed a variety of salinities and other physicochemical conditions (Table 1). An Orion model 290 pH meter was used to measure temperature and pH. Salinity was measured using an Orion model 115 conductivity meter.

Water samples were transferred to plastic bottles and kept in an icebox with ice until further processing. Samples for chlorophyll analysis were filtered through 25-mm-diameter Whatman GF/F glass fiber filters. The filters were placed in aluminum foil and kept frozen. Chlorophyll *a* concentration was determined by fluorescence of acetone extracts (Yentsch and Menzel 1963) with a Turner Designs Fluorometer.

Total bacterial number was determined by flow cytometry in 1.8 ml samples fixed with 200 µl of paraformaldehyde:glutaraldehyde (1 and 0.05% final concentration, respectively) in criovials. Vials were frozen until processing in the laboratory. The protocol followed was that of Gasol and del Giorgio (2000) and Gasol et al. (2004). Briefly, 100-µl aliquots were stained with Syto13 (Molecular Probes, Eugene, OR, USA), a suspension of fluorescently labeled beads was added at a known concentration and the samples were counted in a FACScalibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). In some cases, bacteria were counted by epifluorescence microscopy using the DNA-specific dye 4', 6-diamidino-2-phenylindole (DAPI) with a Leica DMLS epifluorescence microscope.

Nucleic acid analyses: DGGE, clone libraries and 16S rRNA sequences analyses

Between 20 and 650 ml of water was filtered through 0.2-μm polycarbonate membranes (Nuclepore Millipore, Bedford, MA, USA) and stored at −70°C. Filters were incubated with lysozyme, proteinase K and sodium dodecyl sulfate (SDS) in lysis buffer as described previously (Schauer et al. 2000). DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated with ethanol. The extracted genomic DNA was used as target in the PCR to amplify 16S rRNA genes. Bacterial fragments suitable for subsequent denaturing gradient gel electrophoresis (DGGE) analysis were amplified with the primer combinations 358fGC-907r as described previously (Dumestre et al. 2002). A 6% polyacrylamide gel was obtained with a gradient of DNA-denaturant agent 40–80% (100% denaturant agent is defined as 7 M urea and 40% deionized formamide). Around 800 ng of PCR product was loaded for each sample and the gels were run at 100 V, 60°C for 16 h in a CBS DGGE-2000 system (CBS Scientific Company, Del Mar, CA, USA). The gels were stained with the nucleic acid dye SybrGold for 45 min, and visualized with UV in a Fluor-S MultiImager (Bio-Rad, Hercules, CA, USA) with the Multi-Analyst software (Bio-Rad, Hercules, CA, USA). High-resolution images (1,312 × 1,034 pixels, 12-bits dynamic range) were saved as computer files. Then the picture was analyzed using the gel plotting macro tool of the NIH-Image software package version 1.62 (National Institute of Health, USA). After background subtracting, the intensity of each band was measured integrating the area under the peak and was expressed as percent of the total intensity in the lane. The error measured among replicates was less than 4%. Bands were excised from the gels, reamplified and purified for sequencing as reported (Casamayor et al. 2001). Bands that provided sequence between 450 and 540 bp length were submitted to GenBank with accession numbers AJ487523 to AJ487534 and AJ568004 to AJ568014.

Cloning and RFLP analysis were performed as previously described (Ferrera et al. 2004). 16S rRNA genes were amplified by PCR with the universal primers 27f and 1492r. PCR amplifications were digested with the restriction enzyme HaeIII (Invitrogen Corporation, Madison, WI, USA), and the RFLP patterns of the clones were compared. Chimeric sequences were identified by using the CHECK_CHIMERA (Maidak et al. 2000) and by visual inspection of the BLAST search outputs.

Sequences were sent to BLAST search (www.ncbi.nlm.nih.gov) to determine the closest relative in the database. A similarity matrix was built with the ARB software package (Technical University of Munich, Munich, Germany; www.arb-home.de). Partial sequences were inserted into the optimized and validated tree available in ARB (derived from complete sequence data), by using the maximum-parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology. The respective ARB tools were used to perform maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) analyses for full sequences. The calculation methods were combined with different filters, and the resulting phylogenetic trees were compared manually to obtain a final consensus tree. Results from the three types of analyses were essentially identical and only the maximum parsimony trees are shown. The sequence data have been submitted to the EMBL database under accession numbers AY862726 to AY862797.

Results

Table 1 provides the geographical location, physicochemical and biological parameters for all the samples used in the present study. The different sampling sites in Lake Tebenquiche have also been indicated in Fig. 1. Despite the different seasons at which samples were collected, the temperature range was moderate, between 14°C in winter and 27.6°C in one spring sample. On the other hand, salinity of the samples ranged between 1 and 30% (w/v) even within the same sampling date. Likewise, salinity also changed considerably between sampling dates at the same sampling site (see different visits to sites 12 and 18 for example). Chlorophyll *a* was generally low and ranged by one order of magnitude between 0.03 and 0.6 μg l^{-1}. In contrast, chlorophyll *a* was much higher in the very shallow Lake Burro Muerto, where resuspension from the sediments is an important factor. Bacterial numbers changed by a factor of two (3–6 × 10^6 cells ml^{-1}) with only two exceptions. There was no correlation between temperature, salinity, chlorophyll *a* and bacterial numbers.

Heterogeneity in space and time

Six different sampling sites on the lake were chosen as representative of the different water-inundated areas, and depth of water was less than 50 cm at all sampling sites. We analyze the possible heterogeneity within the system in time and space by DGGE (Fig. 2), and identification of the excised and sequenced DGGE bands is shown in Table 2. We observed major differences in the composition of the bacterial assemblage among the different sampling locations within Lake Tebenquiche (see lanes 3–8, all taken on the same date, i.e., 13 October 2000). The general grouping of the DGGE lanes was in agreement with the local salinity at each place (see Table 1). There were at least three types of assemblages. Lanes 3 and 4 were identical. This made sense, since the samples had been collected very closely in

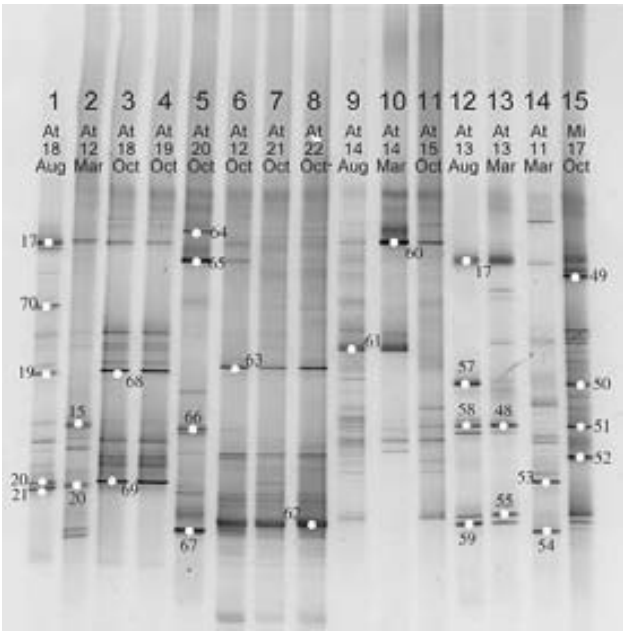

Fig. 2 Negative image of a denaturing gradient gel electrophoresis (DGGE). *Bands* that were cut off from the gel are labeled with the *same number* as in Table 2, and in Figs. 4 and 5. When *bands* across several *lanes* could be identified as being the same, they all have the *same number*. Sample from the freshwater Lake Miscanti is added for comparison (see also Demergasso et al. 2004)

space and their salinity was the same. Lane 5 was from a close sampling location, but the salinity was three times lower, and finally lanes 6–8 formed a third cluster. Therefore, we observed change in the bacterial assemblages among places because of the environmental variability and topographic complexity, within the lake.

We also analyzed some samples from other water bodies in the Salar de Atacama to see whether the same assemblage would be found all over the complete system. Sample in lane 14 corresponds to Lake Cejas, north of Lake Tebenquiche; samples in lanes 12 and 13 correspond to two dolines (West and East dolines located in the sampling point 13 of our general survey in this area), a few hundred meters east from Lake Tebenquiche (Fig. 1); and samples in lanes 9 and 10 correspond to Burro Muerto (sampling points 14 and 15), south of Tebenquiche. As could be expected, the band patterns from these water bodies were completely different from those in Lake Tebenquiche.

Finally, we examined samples taken on the same site (Lake Tebenquiche) but at different seasons to evaluate changes with time. Samples in lanes 2 and 6 were taken at site 12 in fall and spring, respectively. Most bands were different on the two sampling dates. Samples in lanes 1 and 3, in turn, were taken in winter and spring at site 18, east of the previous one. In this case, although a few bands did not appear in both sampling dates, the patterns were relatively similar and the most intense bands were the same (bands 17, 19 and 20). This is coherent with the salinities of the samples. The two samples from site 12 differed in salinity by almost six times, while the two samples from site 18 only differed by a factor of two. Comparing lanes corresponding to the dolines (12 and 13) and lanes corresponding to Burro Muerto (9–11) taken at different times of the year, it appears that the assemblages in these two areas were less variable with time. Thus, changes with time were quite important in Lake Tebenquiche, but the differences were associated to the changes in salinity caused by the variable hydrographic regime and did not conform to a seasonal succession.

Diversity in Lake Tebenquiche

Three samples were chosen from the previous ones for a more in depth analysis of the community by clone libraries. On the one hand, those for At12Oct and At18Oct were representative of two of the DGGE patterns found in Lake Tebenquiche (Fig. 2). On the other hand, At18Aug was intended to provide a comparison with At18Oct at a different time of the year. Based on results from both partial and complete sequences, a similarity level of ≥97% was chosen to define operational taxonomic units (OTUs). The resultant OTUs are shown in Table 3 with the number of clones belonging to each OTU found in each library, the clones sequenced completely and their closest relative as identified with BLAST. The assemblage was clearly dominated by Bacteroidetes and Gammaproteobacteria, whereas the remaining phylogenetic groups were represented only by a few clones. A general tree constructed with full sequences is shown in Figs. 3 and 4, and more details are provided in Table 3.

Bacteroidetes grouped consistently into four clusters (Fig. 4). The best-represented cluster was Atacama-I, and it was very distantly related to the genus *Psychroflexus* (at the 87% level). Atacama-I also included three DGGE bands from Lake Tebenquiche and six more bands from other aquatic saline systems in Northern Chile (Fig. 5), showing that this cluster could be abundant and widely distributed in the area. Two clones from a survey of a hypersaline endoevaporitic microbial mat in Eilat (Israel) (Sorensen et al. 2005) showed similarities between 95 and 97% to sequences in cluster Atacama-I as well. Even though we sampled environments with salinities ranging between freshwater and 36.4%, sequences from this cluster were only retrieved from samples within the range of 3–15%, suggesting that it may contain bacteria adapted to intermediate salinities. This cluster had sequences from both libraries from site 18 and none from site 12. Since the salinity of the latter at the time of sampling was 29.6% (Table 1), this again suggests that members of the

Table 2 Codes (DGGE band-sampling site), accession numbers and closest relatives for selected DGGE bands shown in Fig. 2

Code	Length (bp)	Accession number	Phylogenetic group	Closest relative	Accession number	Similarity (%)
15-AT13	508	AJ568007	Alfaproteobacteria	Alfaproteobacterium clone SOGA1	AJ244780	93.1
17-AT18	505	AJ487527	Bacteroidetes	*Psychroflexus torquis* ACAM 627	AF001365	90.7
19-AT18	490	AJ487529	Bacteroidetes	Bacterial clone DG890	AY258122	93.4
20-AT18	Ss	Ss	Proteobacteria	Proteobacterium clone Sva0071		81.0
21-AT18	515	AJ487530	Gammaproteobacteria	Gammaproteobacterium clone ML602J-47	AF507818	96.9
48-AT13	Ss	Ss	Proteobacteria	Proteobacterium strain TB66		82.0
49-MI17	490	AJ568011	Bacteroidetes	Marine clone ATAM173a_2	AF359539	94.8
50-MI17	499	AJ568012	Bacteroidetes	Bacterial clone 13	AF361196	97.8
51-MI17	502	AJ568013	Alfaproteobacteria	*Sagitula stellata*	U58356	94.2
52-MI17	514	AJ568014	Betaproteobacteria	Alcaligenaceae clone LA1-B29N	AF513937	98.2
53-AT11	472	AY862809	Gammaproteobacteria	Gammaproteobacterium clone ML602J-47	AF507818	96.4
54-AT11	508	AY862810	High GC Grampositive	Actinobacterium clone ML602M-15	AJ575527	98.4
55-AT13	507	AY862808	High GC Grampositive	Actinobacterium clone SV1-7	UA 575517	97.8
56-AT13	513	AY862806	Bacteroidetes	*Cellulophaga marinoflava*	D12668	91.0
57-AT13	516	AY862807	Bacteroidetes	Bacterial clone 13	AF361196	98.1
58-AT13	Ss	Ss	Alfaproteobacteria	*Roseobacter* clone 253		82.0
59-AT13	506	AJ568009	High GC Grampositive	Actinobacterium clone SV1-7	UA 575517	98.8
60-AT14	521	AJ568006	Bacteroidetes	*Psychroflexus torquis* ACAM 627	AF001365	95.0
61-AT14	540	AJ568005	Gammaproteobacteria	*Thiomicrospira crunogena*	AF064545	94.4
62-AT22	402	AY862805	Bacteroidetes	*Salinibacter ruber* POLA 18	AF323503	87.2
63-AT12	518	AY862804	Bacteroidetes	Eubacterium clone KEppib22	AF188173	83.4
64-AT20	500	AY862801	Bacteroidetes	*Psychroflexus torquis* ACAM 627	AF001365	95.9
65-AT20	521	AY862802	Bacteroidetes	*Psychroflexus torquis* ACAM 627	AF001365	95.7
66-AT20	500	AY862803	Alfaproteobacteria	Alfaproteobacterium clone SOGA1	AJ244780	97.2
67-AT20	Ss	Ss	High GC Grampositive	Bacterial clone FukuN101		86.0
68-AT18	514	AY862799	Bacteroidetes	Bacterial clone DG890	AY258122	86.6
69-AT18	526	AY862800	Gammaproteobacteria	Gammaproteobacterium clone ML602J-47	AF507818	97.0
70-AT18	495	AY862798	Algae	Diatom PENDANT-26 chloroplast	AF142938	97.9

Ss sequences too short to be submitted to GenBank

Atacama-I cluster could not survive at high salinities. We split this cluster into three OTUs at a similarity ≥97% (Fig. 5). OTU-1, only found in Northern Chile up to the present, was the most numerous in the sites of intermediate salinity and also included sequences from Ascotán (Nch31 and 34-AS6), a different salt flat system located at 4,000 m a.s.l. in Northern Chile (Demergasso et al. 2004). The Bacteroidetes cluster Atacama-I, therefore, showed a rich diversity at different levels of similarity and appeared to be a novel and phylogenetically complex group of bacteria inhabiting intermediate salinity environments. Cluster Atacama-II and Cluster Atacama-III again were not related to any cultivated bacterium and distantly related to any sequence in the databases (90% similarity) with the only closer clone relatives (99–95% similarity) obtained from the mat in Eilat. Finally, the last Bacteroidetes cluster (Atacama-IV) was retrieved from the At12Oct library exclusively and from the most intense DGGE band at sampling sites 12, 21 and 22 (Fig. 2, Table 2), and comprised some sequences related to *Salinibacter* spp. (95–96% similarity) (Fig. 4), and other group of sequences distantly related to *Salinibacter* (85% similarity) or to clones from the microbial mat in Eilat (91–95% similarity). These sequences were only found in the highest salinity sample, indicating a rich and diverse presence of *Salinibacter* relatives in Lake Tebenquiche. Altogether, the diversity of Bacteroidetes sequences in Lake Tebenquiche was large and showed a remarkable degree of novelty.

Proteobacteria were the second most abundant group of sequences. The beta subdivision was represented by only one DGGE sequence (Table 2), but no clones were retrieved. Conversely, the gamma subdivision was the best-represented group, followed by the alpha subdivision. The

Table 3 Clones and DGGE bands obtained from Lake Tebenquiche organized by OTUs and, when applicable, by clusters according the labeling shown in Table 1 and in Figs. 3, 4, 5, and 6

Cluster	OTU	Clones[a]			Total	DGGE bands	Complete sequences	Closest relative	%
		AT12Oct	AT18Aug	AT18Oct					
Bacteroidetes									
Atacama-I	1		2 (B3, D8)	17 (A5, A7, B4, B5, B11, C1, C4, C5, C6, C7, C10, C12, D4, 1B2, 1B4, 1B5)	19	2 (31-AS6, 34-AS6)	4 (18OctA5, C1, C5, D4)	Hypersaline mat clone E2aA05	95–96
	2		5 (A4, B8, C11)		5	1 (17-AT18)	1 (18AugB8)	Hypersaline mat clone E2aA05	95
	3			2 (C9, D9)	2		1 (18OctC9)	Hypersaline mat clone E4aA01	97
	NA					6 (60-AT14, 2-LL3, 1-AS8, 5-AS8, 64-AT20, 65-AT20)		*Psychroflexus* sp.Lakes Xiaochaidan and Gahail-2 clones	
Atacama-II	4			1 (C3)	1				
	5			2 (1B3)	2		1 (18Oct1B3)	Hypersaline mat clone E4aF11	99
	6			4 (A8, A10, D1)	4	1 (19-AT18)	1 (18Oct1A10)	Hypersaline mat clone E4aF11	95
								Lake Xiaochaidan clone	96
	7		1 (A9)		1	1 (68-AT18)			
Atacama-III	8	2 (A6, B3)			2	1 (63-AT12)	2 (12OctA6, B3)	Hypersaline mat clone E2aB05	95–96
Atacama-IV	9	1 (A2)			1		1 (12OctA2)	*Salinibacter* sp.	96
	10	2 (A9, B5)			2	1 (62-AT22)	1 (12OctA9)	*Salinibacter* EHB-2	94–95
	11	1 (A8)			1		1 (12OctA8)	Hypersaline mat clone E4aG09	95
	12	1 (A10)			1		1 (12OctA10)	Hypersaline mat clone E4aG09	91
Gammaproteobacteria									
Atacama-V	31		3 (A6, A10, B10)	9 (B2, B3, D7, D8)	12	4 (69-AT18, 21-AT18, 53-AT11, 32-AT11)	4 (18OctB2, B3, D7, 18AugB10)	Mono Lake clone ML110J-38	96–97
								Lake Xiaochaidan	90–99
	30		5 (A2, B1, C12, D7)	1 (D5)	6			Mono Lake clone ML602J-47	95–97
Atacama-VI	29	1 (A7)			1		1 (12OctA7)	*Alkalispirillum mobile*	94
	28	1 (B1)			1			*Arhodomonas aquaeolei*	90
Atacama-VII	27			2 (B6, C2)	2	2 (22-AT14, 61-AT14)	18OctB6	*Thiomicrospira* sp.	94

Table 3 continued

Cluster	OTU	Clones[a]			Total	DGGE bands	Complete sequences	Closest relative	%
		AT12Oct	AT18Aug	AT18Oct					
Other gammaproteobacteria	26	1 (B2)			1			Marine clone CHAB-III-7	90
	25		1 (A1)		1			*Francisiella* sp.	89
Other proteobacteria									
Atacama-VIII	13		1	1 (B9)	2			Alfaproteobacterium	
	14		1 (A11)		1	2 (15-AT13, 38-AT13, 66-AT20)	1 (18AugA11)	*Roseobacter gallaeciensis* AY881240	93
Other clones	15			1 (E4)	1		1 (18OctE4)	Alfaproteobacterium GL3 microbial mat AF344287	97
	16			1 (B12)	1			Epsilonproteobacteria clone NCh-18OctD10	99
	17			1 (D10)	1		1 (18OctD10)	Epsilonproteobacteria Clone ATRB90-23 AM159463	88
Other bacteria									
High G+C Gram+	18	1 (B10)			1	2 (40-AT13, 55-AT13)	1 (12OctB10)	Actinobacterium clone R7 UAC575503	95
Low G+C Gram+	19	1 (B4)			1		1 (12OctB4)	Mono Lake clone ML615J-28 AF454301	95
	20			3 (D2, D11, E5)	3		1 (18OctE5)	Guaymas Basin clone GZKB110 AJ853603	88
								Clone AT18OctD2 AY862749	99
	21	1 (A1)		1 (E3)	2		2 (18OctE3)	Clone GZKB80 AJ853574	88
	22			3 (D2, D11, E5)	3			Clone CS_B020	89
	23	1 (B7)			1		1 (12OctB7)	KB1 group clone UKB347769	96
Chloroplasts	24	3 (A11)			3		1 (12OctA11)	Cyanobacterial environmental clone OCS162 AF001659	83
	25		8 (A3, A12, D6)		8	2 (18-AT18, 70-AT18)	1 (12OctA12)	*Dunaliella salina* chloroplast AF547096	96

[a] The total number of clones sharing the same RFLP pattern is shown. The clones sequenced are identified within parentheses

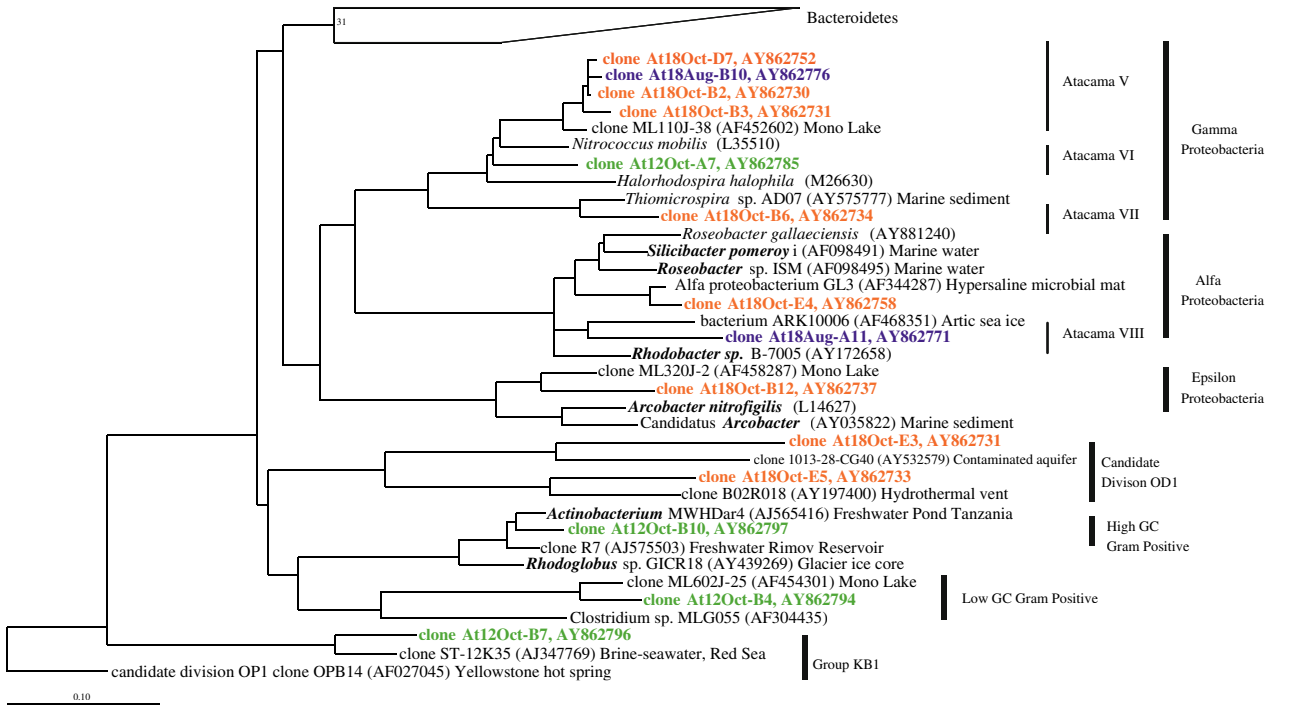

Fig. 3 Phylogenetic tree constructed with almost complete sequences retrieved form 16S rDNA clone libraries from Lake Tebenquiche. The Bacteroidetes subtree has been collapsed for clarity. *Scale bar* 0.10 mutations per nucleotide position

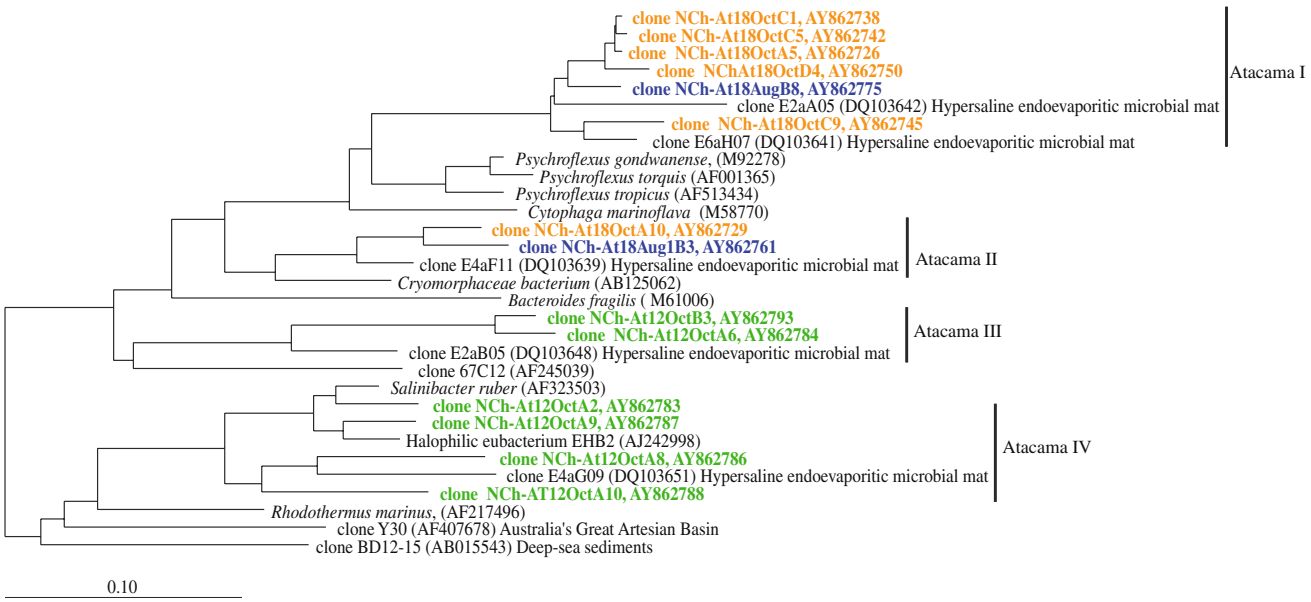

Fig. 4 Phylogenetic tree for Bacteroidetes constructed with almost complete sequences retrieved from 16S rDNA clone libraries from Lake Tebenquiche. *Scale bar* 0.10 mutations per nucleotide position

latter were all within the *Roseobacter* group, but with low similarity to all cultivated strains reported so far. Finally, the epsilon subdivision was represented by only two clones. A tree based on full 16S rRNA gene sequences is shown in Fig. 3, and Fig. 6 includes all the partial sequences obtained from DGGE. Most sequences were included within three clusters in the gamma-Proteobacteria, distantly related (92–93%) to *Nitrococcus mobilis*, *Halorhodospira halophila* and *Thiomicrospira* sp. (Fig. 6, Table 3). Cluster Atacama-V contained sequences from Lake Tebenquiche from sampling site 18, with salinity ranging between 8 and 15%, from Lake Cejas (another

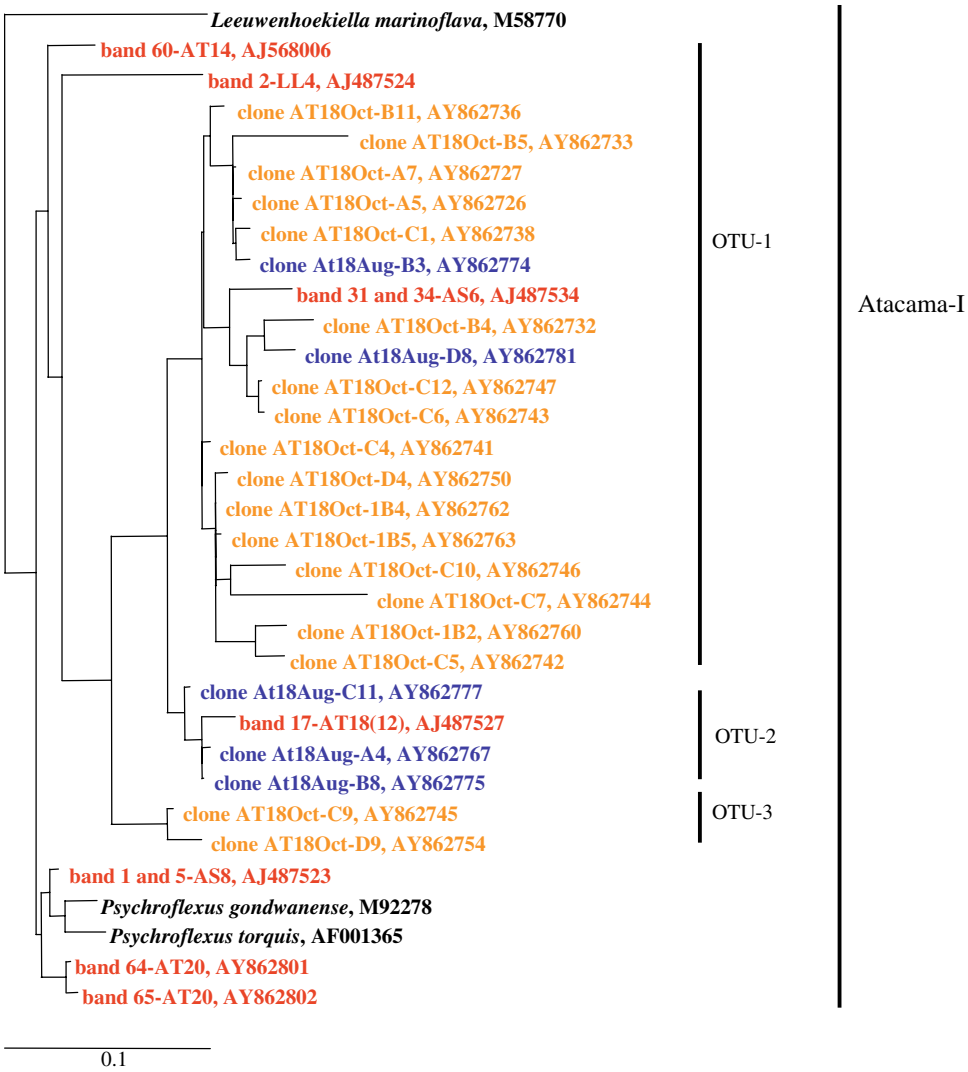

Fig. 5 Phylogenetic tree including partial sequences from DGGE bands and clones for the cluster Atacama-I. *Scale bar* 0.10 mutations per nucleotide position. The code includes the band number in Fig. 2 and Table 2, and the code for the natural environment from which the 16S rRNA gene sequence was originally obtained. Bands with site codes *-AS* and *-LL* were obtained from previous work (Demergasso et al. 2004)

shallow lake on the Salar de Atacama, relatively close to Lake Tebenquiche, Table 1), and from Mono Lake, another highly saline lake in California, USA (Figs. 3, 6), but the clones were not closely related to any other bacterium or environmental sequence. Judging from the number of clones and the intensity of the DGGE bands, this cluster appeared to be important in the lake. The Alphaproteobacteria sequences found were related with environmental clones retrieved from relatively high salinity environments (Fig. 3) and, again, had no close relatives isolated in pure cultures.

Finally, the remaining divisions of bacteria were represented by very few sequences. One clone and two bands belonged to the High G+C Gram-positive bacteria, one clone (related to some Mono Lake clones) was affiliated to the Low G+C Gram-positive bacteria and a series of clones was associated with yet poorly defined lineages: two clones could be affiliated with the candidate division OD1; three more clones associated with uncultivated clones CS_B020 and BD1-5 from marine sediments; and one clone was related to sequences of the KB1 group, from sediments in hypersaline brines (Fig. 3).

The percentage contribution to the total assemblage of the different groups is shown in Fig. 7, together with the percentage contribution to total band intensity in DGGE gels for the same samples. The recovered groups were the same in both the techniques: *Psychroflexus*-like (Atacama-I), *Salinibacter*-like (Atacama-IV), and DG890 cluster (Atacama-II) among the Bacteroidetes, and Mono Lake cluster (Atacama-V) and *Thiomicrospira*-like (Atacama-VII) cluster for the Gammaproteobacteria (Fig. 7). Since primers used for cloning and DGGE are very different, the good coincidence between techniques gives support to the idea that the targeted bacterial assemblage was considerably well sampled. The least abundant groups were usually absent from the DGGE results (Fig. 7) due to the fact that

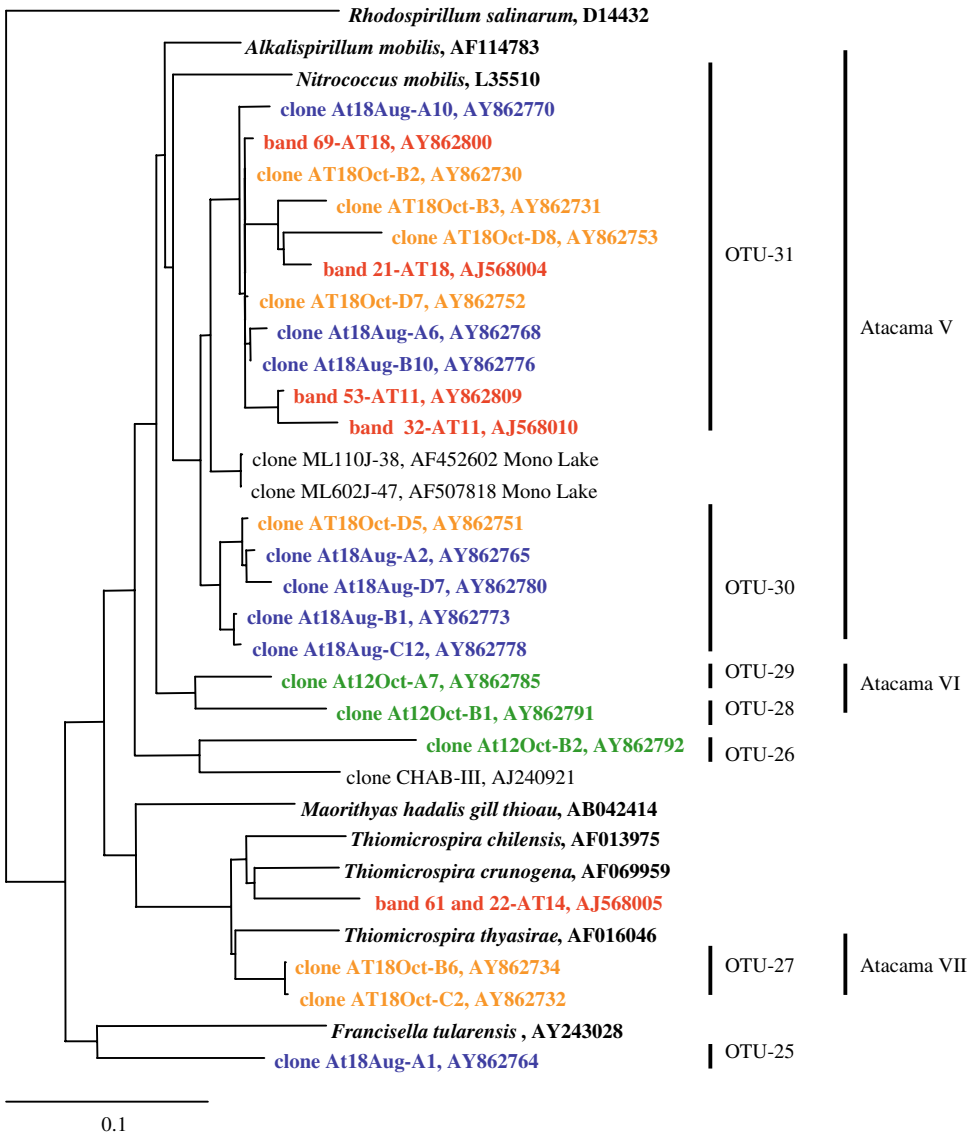

Fig. 6 Phylogenetic tree including partial sequences from DGGE bands and clones for the Gammaproteobacterial clusters. *Scale bar* 0.10 mutations per nucleotide position. The code includes the band number in Fig. 2 and Table 2, and the code for the natural environment from which the 16S rRNA gene sequence was originally obtained. See also previous work (Demergasso et al. 2004)

faint bands in DGGE gels are very difficult to sequence (Sánchez et al 2007).

Discussion

At a global level, the biodiversity of the Earth's aquatic systems can be approached by sampling different ecosystems, each with a different diversity. In this respect, both low and high salinity systems have received considerable attention. At the seawater end, many studies have been performed in the last 15 years (Giovannoni et al. 1990; Giovannoni and Rappé 2000; Pommier et al. 2007). At the other end, crystallizer ponds from solar salterns have also been studied extensively (Benlloch et al. 1996, 2001, 2002; Rodríguez-Valera et al. 1999; Casamayor et al. 2000, 2002; Oren 2002; Estrada et al. 2004; Pedrós-Alió 2005; Maturrano et al. 2006). Aquatic systems with intermediate salinities, however, have not received much attention. In the case of solar salterns, ponds with intermediate salinities show relatively high levels of heterotrophic activities (Gasol et al. 2004). The case of Lake Tebenquiche shows that such systems hold the potential to reveal a considerable degree of phylogenetic novelty at different levels. Thus, we found sequences that are candidates for new branches in the bacterial phylogenetic tree, novel clusters within the well-characterized bacterial groups and a large microdiversity within these novel clusters.

Consistently, all three libraries were dominated by the same two large phylogenetic groups: Bacteroidetes and Gammaproteobacteria. All the other groups represented minor components. The identity of the particular OTUs

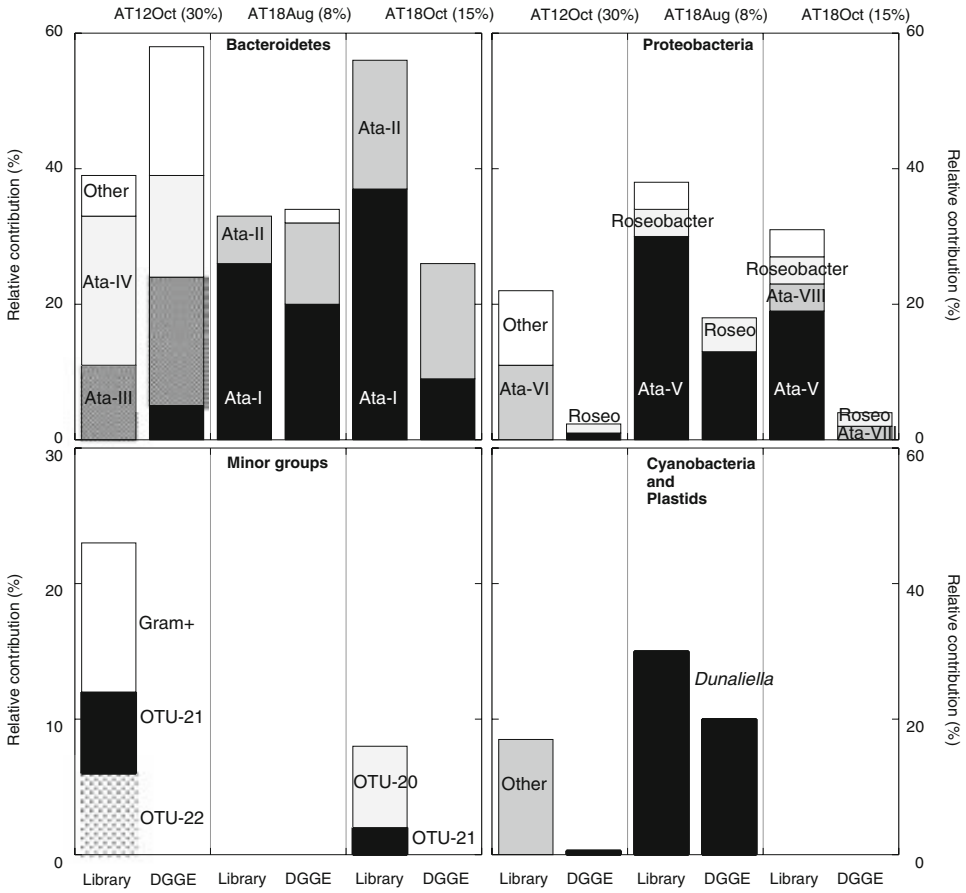

Fig. 7 Relative contribution to total community assemblage at three sites in Lake Tebenquiche as determined from DGGE band intensity or percent of clones in libraries. Salinity is indicated in *parentheses* for each site (w/v)

within these large groups, however, was very different between library At12Oct (the highest salinity point) and the other two, and substantially different between At18Aug and AT18Oct (the same site with intermediate salinities in winter and spring, respectively). Bacteroidetes accounted for 39% of the clones in the At12Oct library, mostly distantly associated (similarities ranged between 91 and 95%) to the halophilic bacterium *Salinibacter ruber* isolated from crystallizer ponds (salinities up to 35%) in coastal solar salterns (Antón et al. 2002). Since this library came from the most saline sample in Lake Tebenquiche (29.6%), it makes sense that *Salinibacter*-like sequences were retrieved in abundance. Certainly, the potential for new extremely halophilic members of the Bacteria exists within this cluster. In clone libraries At18Aug and At18Oct, Bacteroidetes were the most abundant groups followed by Gammaproteobacteria. Salinity was about twice in October (14.8%) than in August (7.9%), and most Bacteroidetes clones were distantly related to *Psychroflexus* spp. (similarities ranged between 87 and 92%), mainly in October. The most abundant cluster of Gammaproteobacteria was associated with uncultured bacteria from Mono Lake (Humayoun et al. 2003), a saline alkaline lake in the USA, as well as the few clones belonging to the Low and High G+C groups of bacteria were also associated to sequences from Mono Lake. As indicated above, aquatic systems with intermediate salinities have not received much attention, and our data will help for future comparisons.

A noteworthy finding is the consistent predominance of Bacteroidetes. A similar predominance was found in Salar de Huasco (C. Dorador, K.P. Witzel, C. Vargas, I. Vila, J.F. Imhoff, unpublished data) where both DGGE and clones libraries were done. Salar de Huasco is north of the areas studied here and it is found at 3,800 m a.s.l. Bacteroidetes were also the most abundant groups of bacteria in clone libraries from the water column of Lake Chaka (Jiang et al. 2006) with a salinity of 32.5%. In contrast, a study of several lakes in the Tibetan plateau (Wu et al. 2006) found Bacteroidetes to account for only 5–10% of the total cell count by fluorescent in situ hybridization, in the most saline lakes. When DGGE was carried out, however, Bacteroidetes were the most represented group accounting for almost half of the sequences retrieved. Preliminary FISH counts in Lake Tebenquiche indicated that Bacteroidetes accounted for 3–17% of the total count (average 8%). This discrepancy between PCR-related methods and FISH deserves further research, because in marine systems FISH usually gives higher representation of Bacteroidetes than cloning or DGGE (Cottrell and Kirchman 2000). Obviously, the high salinity Bacteroidetes are very

different from the marine representatives of this group and better FISH probes have to be designed for them. Alternatively, most of the Bacteroidetes would have low ribosomal content in the case where they were inactive under in situ conditions being difficult to detect by FISH but still recovered by DNA amplification methods.

A large number of bacteria have been isolated in pure culture from Lake Tebenquiche in the past (Prado et al. 1991, 1993, 2006; Valderrama et al. 1991), but none of them could be retrieved in our molecular study of the same lake. The difference between microorganisms retrieved from natural systems by molecular and pure culture methods is a well-known phenomenon (Amann et al. 1995; Pedrós-Alió 2007). Usually, it is attributed to inability of microbiologists to find suitable conditions to tame those microbes that are abundant in nature. Microorganisms isolated from a given system but absent from clone libraries strongly indicate that they were at low abundances but can potentially become dominant. Thus, the topographic complexity and environmental heterogeneity of Lake Tebenquiche make "visible" for the molecular methods different fractions of the whole collection of microorganisms present in the system, presumably the ones best adapted to each particular environment.

Conclusions

We found that the bacterial community composition in Lake Tebenquiche was very heterogeneous both in space and time, in close relationship to the salinity of the water. This system is highly variable depending on the groundwater inputs in different zones of the lake basin, and thus, water with very different salinities can coexist in the lake. In addition, hypersaline places within the lake can be diluted by dynamic freshwater inputs and bacteria have to adapt locally to the new conditions. These differences in salinity override any seasonal changes that would be mild anyway in this tropical region. Overall, this intermediate salinity lake showed a remarkable degree of novelty in its bacterial assemblage, both in terms of deep lineages and of microdiversity within known clusters. All these novel sequences belong to the diversity of the system and, therefore, to microorganisms that are relevant for the ecosystem functioning. The challenge now is to isolate them in pure culture to analyze in detail their specific adaptations to the dynamic perturbations found in Lake Tebenquiche.

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Capítulo II

Bacterial diversity in the sediment and water column of Salar de Ascotán (Northern Chile), a hypersaline high-altitude, arsenic-rich wetland

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(Manuscript in preparation)

Las cosas simples son las más extraordinarias
y sólo los sabios consiguen verlas.
(El Alquimista, Paulo Coelho)

Bacterial diversity in the sediment and water column of Salar de Ascotán (Northern Chile), a hypersaline high-altitude, arsenic-rich wetland

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Keywords: arsenic respiring microorganisms, bacterial diversity, hypersaline lake, salar, Firmicutes, Alphaproteobacteria.

ABSTRACT

The diversity of bacterial communities present in water and sediment samples of Salar de Ascotán, a hypersaline, arsenic-rich, wetland located at an average altitude of 3700 m, was examined using denaturing gradient gel electrophoresis (DGGE) and clone libraries of the bacterial 16S rRNA gene in the samples collected during several sampling expeditions covering different seasons. Different arsenic concentrations were measured in water samples (0.9 to 212 mg/L total arsenic) and in sediments (781 to 6,504mg/L total arsenic). These are some of the highest values of total arsenic reported so far from a natural environment. Overall, the different water samples were mostly dominated by Alphaproteobacteria whereas Firmicutes (i.e. *Natroanaerobium* and *Halanaerobium*) were mostly found in sediments as shown by DGGE fingerprinting and sequencing. We also observed that Betaproteobacteria and Firmicutes (*Fusibacter* and *Clostridium*-like) were more often found at arsenic concentrations below 4 mg/L, whereas Bacteroidetes and Gammaproteobacteria were found at higher As concentrations. Two samples were chosen for more detailed analysis by clone libraries, one from sediment (high arsenic concentration) and the other from water (low arsenic concentration). The water sample showed lower bacterial diversity than the sediment sample, and a marked phylogenetic segregation according to habitat type was found for Firmicutes and Proteobacteria.

Epsilonproteobacteria were also abundant in water whereas Deltaproteobacteria and Bacteroidetes were only found in the sediment. Most of the sequences recovered from Ascotán constituted defined new clusters within their respective groups.

INTRODUCTION

High altitude saline lakes are found in upland areas of the Andes, North and Central America, East Africa, Asia and Europe. These wetlands are exposed to particularly harsh environmental stresses, such as extreme aridity, high UV radiation, high concentration of toxic metals (e.g. arsenic), extreme variations in diurnal and seasonal temperatures, and a wide range of salinities. These are remote environments, difficult to reach and mostly inhabited by microorganism. However, only a handful of studies are available on the bacterial community composition and environmental factors prevailing in these environments (Demergasso *et al.*, 2004, Wu *et al.*, 2006, Dorador *et al.*, 2008). Understanding the factors that control the composition and diversity of microbial communities is important for understanding how bacterial populations are linked to biogeochemical processes.

Salar de Ascotán is an ideal site for such studies because it has strong geochemical gradients, a relatively simple microbial community, and one of the highest reported natural concentrations of total arsenic. Salar de Ascotán is part of an evaporitic basin system at an average altitude of 3,700 m in the High Andes of northern Chile (Chong, 1984). The Salar de Ascotán is an environment very rich in arsenic. This is a partially due to the As-rich red and yellow nodules and lenses, some centimeters thick and a few meters in diameter included inside the borate deposits, (mostly in the form of ulexite (Demergasso *et al.*, 2007). These nodules are rich in the arsenic minerals realgar and orpiment.

The saline crusts are mainly composed of chlorides (halite) and sulfates (gypsum) with important borate ore deposits composed mostly of ulexite with significant amounts of arsenic sulfide minerals (Demergasso *et al.,* 2007). This system exhibits high spatial and temporal variability with water salinities ranging from freshwater to salt-saturated brines (Risacher *et al.*, 1999). Hydrothermal processes associated with volcanism have led to the accumulation of arsenic compounds, which play an active role in the biogeochemical cycles operating at the Salar and these characteristics

can be expected to affect the composition and activity of microbial communities.

The present study aims to compare the phylogenetic compositions and diversity of bacterial assemblages present at different arsenic concentrations in water samples and in sediments by both 16S rRNA DGGE fingerprinting in a wide set of samples and gene cloning and sequencing in two selected samples. Overall, we found low diversity in the system as compared to previous studies in other salt lakes in the area with low As concentration. These analyses provided a framework for future studies on the interaction of hydrologic and microbial processes affecting arsenic speciation, solubility and mobility in extreme environments.

MATERIALS AND METHODS

Site description.

Salar de Ascotán is part of an evaporitic basin system in the High Andes of northern Chile (Chong, 1984). It is located between 22°25' and 22°45' South latitude and 68°30' and 68°10' West longitude at an average altitude of 3,700 m (Fig. 1). Salar de Ascotán is an athalassohaline environment located at the bottom of a tectonic basin surrounded by volcanic chains in east–west direction, including some active volcanoes over 5,000 m high, with the highest altitudes close to 6,000 m. The geological setting is dominated by volcanic structures and includes acidic (rhyolites) and intermediate (andesites) rocks of Tertiary and Quaternary age. The evaporitic basin contains palaeoshore lines indicating the existence of a former saline lake with deeper bathymetry. Climate is characterized by large daily thermal oscillations. High solar irradiation and strong and variable winds cause intense evaporation (about 1,640 mm/year) while precipitation is about 120 mm/year (Mardones-Pérez, 1997). Water inputs are surface drainage from the snow fields of volcanoes and underground geothermal waters with spring waters commonly reaching 23 to 25 °C. In the eastern border of the basin there are thermal springs (Ojos del Coñapa) with 2,700 to 3,000 mg L^{-1} of total dissolved solids. The saline crusts are mainly composed of chlorides (halite) and sulfates (gypsum) with important borate ore deposits composed mostly of ulexite with significant amounts of arsenic sulfide minerals (Demergasso *et al.,* 2007). This system exhibits large spatial and temporal heterogeneity with water salinities ranging from freshwater to salt-saturated brines (Risacher *et al.*, 1999).

Sample collection and processing

Four sampling expeditions to Salar de Ascotán were conducted in November 2004, August 2005, June 2006 and April 2007 (Table 1). Ten sampling sites were selected within the basin (P1-P2, P6-P11) and three thermal springs ("vertientes" V4, V6 and V10) for sampling both water and sediments (Table 1 and Fig. 1). Overall, 24 samples were processed for DNA analysis. Temperature and pH were measured with a pH meter Orion model 290. A conductivity meter Orion model 115 was used for salinity, conductivity and total dissolved solids. Oxygen was measured with a Thermo Orion model 9708. Water samples were kept in polyethylene 2-L bottles in an icebox until further processing. Microbial counts were carried out with water and sediment samples collected *in situ* in sterilized vials. Samples were fixed *in situ* with 4% formaldehyde (vol/vol, final concentration) overnight at 4ºC. Counts were done by epifluorescence microscopy with a staining with a DNA-specific dye, 4′, 6-dia-midino-2-phenylindole (DAPI) in a Leica DMLS epifluorescence microscope. Arsenic concentrations were measured using hydride generation atomic absorption spectroscopy (HG-AAS) after acid digestion of the for the sediment samples (Demergasso *et al.*, 2007).

Total DNA extraction

Between 800 and 1000 mL of water was filtered through 0.2 μm polycarbonate membranes (Nuclepore). The filters were stored at −20 °C in 1 mL lysis buffer (50 mM Tris–HCl pH=8.3, 40 mM EDTA and 0.75M sucrose) (Demergasso *et al.*, 2008). For sediment samples, nucleic acids were extracted from 25 to 50 g sediment (wet weight), and were actively vortexed in a salt solution (1x PBS buffer, Tween 20 at 10% v/v) at 12.56 rad s^{-1}. The supernatant was filtered and processed as previously done with water samples.

Filters were incubated with lysozyme and proteinase K (Demergasso *et al.*, 2008) and genomic DNA was extracted with a High Pure Template Preparation Kit (Qiagen, Duesseldorf, Germany).

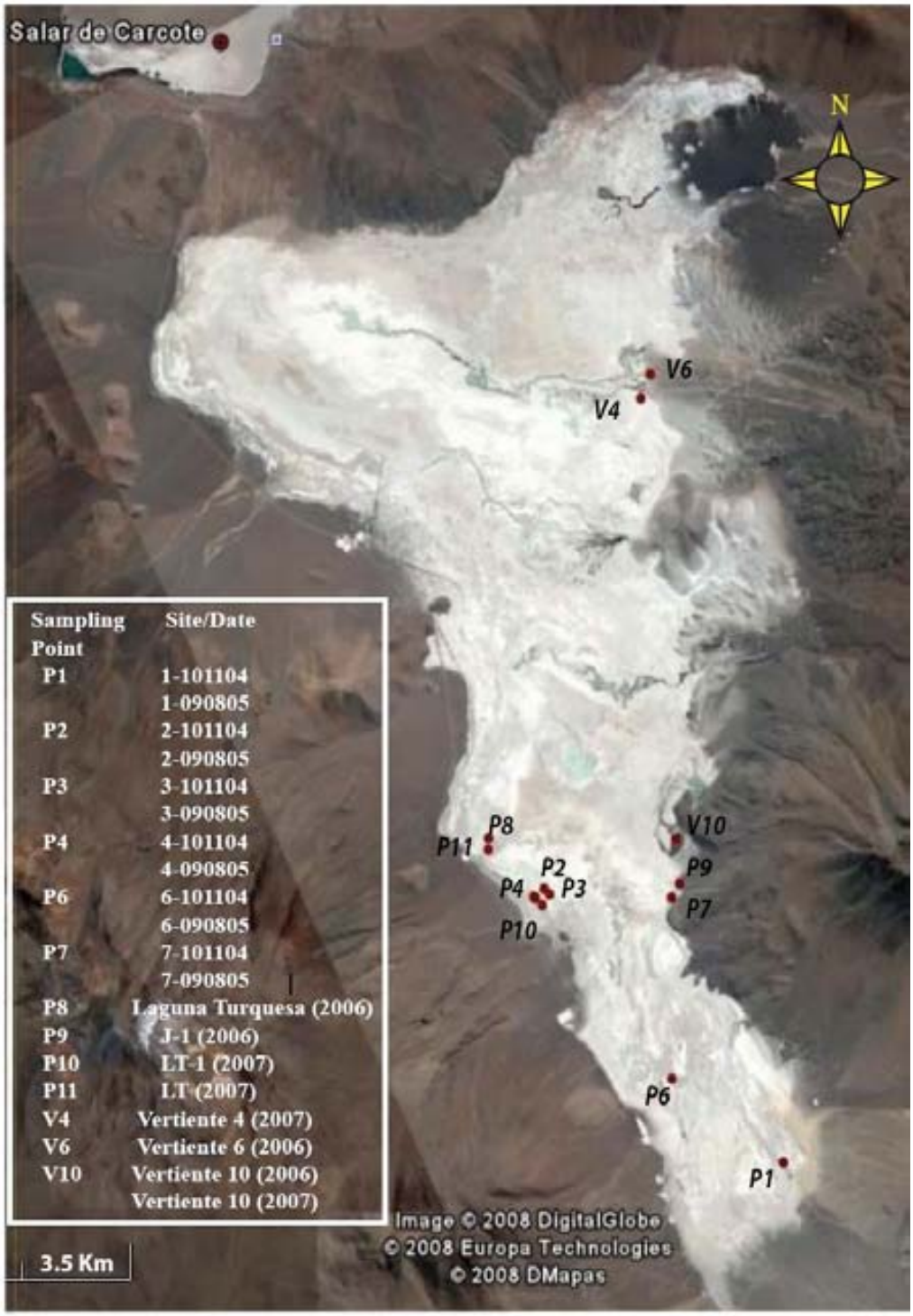

Fig. 1. Map of Salar de Ascotán showing the location of the sampling spots.

PCR amplification and DGGE of the 16S rRNA gene

Genomic DNA was used as target in the PCR to amplify the 16S rRNA gene using the bacterial universal primer set 358F with a GC clamp and 907R. The 16S rRNA gene fragments were run in a denaturing gradient gel electrophoresis (DGGE) as previously described (Muyzer *et al.*, 1998, Casamayor *et al.*, 2002). DGGE, excision of bands and reamplification were performed as previously reported (Casamayor *et al.*, 2000).

Clone library construction of the 16S rRNA gene

The purified genomic DNA was PCR amplified with primers 27F MOD (5'-AGR(AG) GTT TGA TCM(AC) TGG CTC AG-3') and 1492R MOD (5'-GGY(CT) TAC CTT GTT AY G ACT T-3') and cloned into the pCR2.1 vector with the TOPO TA cloning kit Catalog #4500-01 (Invitrogen Carlsbad, California) and transformed into TOP10 chemically competent cells. The transformed cells were grown on Luria-Bertani plates containing 50 mg of Kanamycin mL^{-1}, 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) m L^{-1}, as recommended by the manufacturer and incubated overnight at 37°C. Cloning and sequencing were carried out as previously described (Demergasso *et al.*, 2008). OTUs were determined by RFLP with enzyme HAE III. All sequences from the same OTU were found to be >97% identical.

Rarefaction curves, the total number of phylotypes in each clone library were estimated by calculating the nonparametric richness estimator Chao1 and clustering analysis were carried out with DOTUR program (Schloss & Handelsman, 2005).

Phylogenetic analysis

Sequences of the 16S rRNA gene were sent to BLAST search (http://www.ncbi.nlm.nih.gov) to determine the closest relative in the database and were further aligned using the alignment tool in Greengenes (http://www.greengenes.lbl.gov).
Aligned sequences were inserted into the optimized and validated maximum likelihood, tree available in ARB (http://www.arb-home.de), by the maximum-parsimony criterion. In this program the optimized initial tree topology is not affected by the added sequences. Sequences were deposited in GenBank under accession numbers FM879025 to FM879135.

Results

Geographical location, physiccochemical data and biological parameters for all sites sampled in Salar de Ascotán are shown in Fig. 1 and Table 1. Samples were obtained in different seasons (austral fall, spring and winter) and temperatures ranged between 3ºC and 25ºC. Salinity values were between 0.1 and 7.6 g L^{-1}, conductivity ranged between 0.3 and 79.1 mS and total dissolved solid (TDS) between 143 and 92,300 mg L^{-1}. Sample P6 collected in winter 2005 was a brine with the highest values of salinity, conductivity, and TDS. All water samples were well oxygenated. We found total arsenic concentration values between 0.7 and 212 mg L^{-1} in water samples, and between 780 and 6,504 mg L^{-1} in the sediments. These are some of the highest values of total arsenic reported so far for natural samples (Smedley & Kinniburgh, 2002). Sulfate concentrations were also high: between 160 and 3,650 mg L^{-1}, as well as chloride and sodium (1,100 to 5,100 mg L^{-1} and 431 to 8,900 mg L^{-1} res- pectively) (Table 2). Calcium, magnesium and potassium concentrations were also very high, ranging between 30 and 1,000 mg L^{-1} (Table 2).

Concentrations of DAPI-stained cells ranged between 1.5 x 10^5 and 2.0 x 10^7 cells mL^{-1} in the water samples and between 5.8 x 10^5 and 1.9 x10^8 cells g^{-1} in the sediments (Table 1).

The bacterial community composition was analyzed by 16S rDNA PCR-DGGE. Between 3 and 12 DGGE bands were found per sample (Table 1). The main DGGE bands (in terms of intensity and frequency of appearance) were excised and sequenced (39 bands from 70 different DGGE band positions observed). We could not always obtain high quality sequences from the excised bands, a limitation of DGGE that has been described previously (Casamayor *et al.,* 2000) . The sequences affiliated with Alpha-, Gamma-, Beta- and Delta-Proteobacteria, Firmicutes, Bacteroidetes, Cyanobacteria and algal plastids (Table 3). Overall, the different water samples were mostly dominated by Alphaproteobacteria (up to 37% of total sequences analyzed) whereas Firmicutes were mostly found in sediments (33%) (Table 4 and Fig. 2). We also observed differences in bacterial community composition in water samples above and below 4 mg/L total As. Betaproteobacteria and Firmicutes were more often found at low arseni c concentrations, whereas Bacteroidetes and Gammaproteobacteria were found at higher As concentrations.

Table 1: Physical and chemical parameters of sample collected from Salar de Ascotán

Sampling day	*Sampling location*	*Sample type*	*UTM coordinates Grid 19K*		*pH*	*Salinity*	*Conductivity*	*TDS*	*Temp.*	*Dissolved oxigen*	*Total As*	*DAPI count*	*DGGE bands*
(dd/mm/yy)			*East*	*North*		(‰)	*(mS)*	(m g/L)	° C	*(mg/L)*	*(mg/L)*	*(cell/mL)*	
											(mg/Kg)	(*cell/ g)*	
10-11-2004	P1	Water	581436	7601844	6.9	0.1	0.30	143	15.5	n.d	3.5	7.70E+05	10
spring	P2	Water	573971	7609154	8.5	0.7	1.35	659	16.0	n.d	10.6	7.00E+05	9
	P6	Water	577712	7604308	7.5	7.1	12.43	6940	17.5	n.d	212	2.60E+05	9
	P3	Sediment	573971	7609154	-	-	-	-	-	-	-	1.30E+06	9
	P4	Sediment	573679	7609029	-	-	-	-	-	-	-	1.80E+06	10
	P7	Sediment	577712	7604308	-	-	-	-	-	-	-	5.80E+05	8
09-08-2005	P1	Water	581420	7601876	7.8	33	41.40	31500	10.0	n.d	4.4	6.70E+05	n.d
winter	P2	Water	573967	7609154	8.0	76	79.10	92300	14.0	n.d	6.5	5.00E+05	n.d
	P6	Water	577706	7604253	7.2	309	193.40	628000	17.0	n.d	183	1.60E+05	6
	P3	Sediment	574012	7609154	-	-	-	-	-	-	781	2.30E+06	7
	P4[a]	Sediment	573669	7609035	-	-	-	-	-	-	1210	3.00E+06	12
	P7	Sediment	577770	7609035	-	-	-	-	-	-	6504	6.80E+05	2
22-06-2006	P8	Water	572171	7610653	6.5	10.5	18.50	10200	3.0	7.5	28.0	4.25E+07	n.d
winter	V-6	Water	577180	7622870	6.0	0.5	1.04	493	16.0	10.8	0.9	1.20E+06	n.d
	V-10	Water	577782	7610573	7.5	1.5	2.85	1390	16.0	8.8	1.6	1.50E+06	n.d
	P9[a]	Water	577921	7609328	5.0	1.8	3.90	1750	0.0	8.9	3.4	3.93E+06	4
	P8	Sediment	572171	7610653	-	-	-	-	-	-	-	5.12E+07	6
	V-6	Sediment	577180	7622870	-	-	-	-	-	-	-	5.47E+07	n.d
	V-10	Sediment	577782	7610573	-	-	-	-	-	-	-	1.92E+08	5
	P9	Sediment	577921	7609328	-	-	-	-	-	-	-	6.03E+07	n.d
20-04-2007	P10	Water	573881	7608410	8.4	6.2	11.50	6003	4.9	7.1	10.0	8.14E+06	7
fall	P11	Water	572247	7610325	8.2	3.5	6.56	3410	17.4	6.4	4.0	2.61E+06	7
	V-4	Water	576823	7622927	8.6	2.1	4.10	2120	20.4	7.8	0.7	4.88E+06	8
	V-10	Water	577586	7610212	8.1	3.0	5.57	2950	24.5	8.0	1.0	9.27E+05	3

TDS total dissolved solids; MPN most probable number; n.d: not determined; n.g: no growth; P: sampling site; V: "vertiente", thermal spring.

[a] Samples selected for clone libraries

Table 2. Chemical analyses in water sample collected in 2006 and 2007 from Salar de Ascotán

Sampling day	*Sampling location*	*Cl^-*	*SO_4^{2-}*	*Ca*	*Mg*	*Na*	*K*	*Total As*
(dd/mm/yy)		*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*
22-06-2006	P8	3009	3650	550	758	8922	1220	28
	V-6	1139	181	152	40	600	67	0.9
	V-10	1849	366	249	88	954	96	1.6
	P9	4561	683	594	216	2216	217	3.4
20-04-2007	P10	5100	1370	280	253	2650	297	10.0
	P11	2300	423	110	93	1190	117	4.0
	V-4	1210	160	145	27	431	30	0.7
	V-10	1990	259	262	68	735	47	1.0

*Chemical analyses of samples from 2004 and 2005 were not carried out.

Deltaproteobacteria were found in sediments only, together with some Gammaproteobacteria (Table 4 and Fig. 2).

After the DGGE fingerprinting survey, two samples were chosen for more detailed analysis of bacterial community composition by constructing clone libraries. Samples P9 (water) and P4 (sediment) were selected because they had some of the highest (16.1 mM) and lowest (2.4 mM) total As concentrations respectively, and they showed very different DGGE fingerprints (data not shown).

The clones that were successfully sequenced are shown in Table 5. Twelve different phyla were retrieved from the sediment sample: Firmicutes, Delta- and Gammaproteobacteria were the most abundant groups, followed by Bacteroidetes, Alphaproteobacteria and Verrucomicrobia (Table 5). Minor groups (c. 1-2% of total clones) were Epsilonproteobacteria, candidate divisons OP11, WS3, JS1, and *Deinococcus Thermus*. In turn, the water sample showed only three phyla: Firmicutes (50% of total clones), Epsilonproteobacteria (34%), and Gammaproteobacteria (16%) (Tables 4 and 5, Fig. 2).

Table 3. Blast output for sequenced DGGE bands from water and sediment samples in Salar de Ascotán

Band number	*Length (bp)*	*Phylogenetic group*	*Closest relative*	*Accession number*	*% Similarity*
Water					
5-P1-2004	539	Betaproteobacteria	*Polynucleobacter* sp. SUWAF016	AB447552	99
7-P1-2004	538	Betaproteobacteria	*Polynucleobacter* sp. SUWAF016	AB447552	99
3-P1-2004	539	Betaproteobacteria	*Polynucleobacter* sp. SUWAF016	AB447552	98
6-P1-2004	483	Betaproteobacteria	Uncultured beta proteobacterium clone PRD18D11	AY948031	98
1-P9-2006	419	Firmicutes	*Fusibacter* sp. enrichment culture clone 22-7A	EU517558	97
1-P1-2004	430	Cyanobacteria	Uncultured cyanobacterium clone SIMO-2257	AY711623	97
3-P9-2006	427	Firmicutes	*Fusibacter* sp. enrichment culture clone 22-7A	EU517558	96
8-P1-2004	492	Alphaproteobacteria	Uncultured *Rhodobacter* sp. clone KS-13	EU809813	96
12-P10-2007	533	Bacteroidetes	*Flavobacteriaceae* bacterium 4	FJ152552	96
1-P6-2005	539	Gammaproteobacteria	*Halothiobacillus* sp. NP36	EU196319	96
2-P6-2004	461	Algae	Uncultured phototrophic eukaryote clone PM2-29	EF215817	96
15-P2-2004	552	Bacteroidetes	*Flavobacteriaceae* bacterium CNU041	EF217417	95
23-P11-2007	493	Alphaproteobacteria	*Loktanella rosea* isolate IMCC1504	EU687492	95
18-P11-2007	528	Firmicutes	*Anoxybacillus flavithermus* clone LK4	AJ810551	93*
3-P6-2005	529	Gammaproteobacteria	*Halothiobacillus* sp. NP36	EU196319	93*
24-V10-2007	358	Gammaproteobacteria	Gamma proteobacteria MN 154.3	AJ313020	93*
28-P2-2004	404	Alphaproteobacteria	*Loktanella vestfoldensis strain LMG22003*	DQ915611	92*
2-P6-2005	509	Betaproteobacteria	*Thiobacillus* sp. EBD bloom	DQ218323	91*
19-V4-2007	492	Alphaproteobacteria	*Brevundimonas* sp. WPCB153	EU880921	90*
16-P2-2004	467	Bacteroidetes	Uncultured bacterium clone Hot Creek 2	AY168735	90*
21-P1-2004	433	Alphaproteobacteria	*Loktanella* sp. *MOLA 317*	AM945550	89*
22-P2-2004	524	Alphaproteobacteria	Uncultured alpha proteobacterium clone JL-WNPG-T46	FJ203409	85*
25-P11-2007	423	Alphaproteobacteria	Uncultured bacterium clone LB_B4	EF429642	81*
9- P11-2007	520	Alphaproteobacteria	*Ruegeria* sp. 3X/A02/236	AY576770	80*
10-V4-2007	428	Alphaproteobacteria	Uncultured bacterium clone PB_B7	EF429656	79*
22-V4-2007	428	Alphaproteobacteria	Uncultured bacterium clone MQ_B15	EF429637	78*
2-P9-2006	410	Firmicutes; Clostridia	*Clostridiales* bacterium UXO5-23	DQ522110	75*
Sediment					
11-P4-2005	488	Deltaproteobacteria	*Desulfohalobium utahense* strain EtOH3	DQ067421	97
10-P4-2005	517	Firmicutes	Uncultured firmicute isolate ikaite un-c22	AJ431344	96
6-P4-2005	420	Firmicutes	*Halanaerobium* sp. M2	EF522947	96
20-P4-2005	538	Gammaproteobacteria	*Halothiobacillus* sp. NP36	EU196319	95
13-P7-2004	409	Alphaproteobacteria	Uncultured *Rhodobacteraceae,* isolate EG7	AM691097	94*
14-P7-2004	364	Firmicutes	Uncultured firmicute isolate ikaite un-c22	AJ431344	92*
4-P3-2004	387	Bacteroidetes	*Psychroflexus* sp. YIM C238	AF513434	89*
26-P7-2004	365	Firmicutes	Uncultured Gram-positive bacterium clone LR-638	DQ302448	89*
24-P3-2004	476	Alphaproteobacteria	Uncultured alpha proteobacterium clone T66ANG3	AJ633984	88*
5-P3-2005	418	Deltaproteobacteria	Uncultured *Desulfotignum* sp. clone A15-30-22	AJ966329	87*
4-P4-2005	484	Gammaproteobacteria	Uncultured gamma proteobacterium clone WN-FSB-209	DQ432134	77*
21-P3-2005	454	Cyanobacteria	*Leptolyngbya* sp. 0BB19S12	AJ639895	74*

*Sequences containig Ns and gaps that produced an artificially low similarity value.

Table 4. Percentage of recovered sequences for different taxa after sequencing of DGGE bands in water and sediments (left side). Relative abundances after cloning two selected samples with different total arsenic concentrations (right side).

	DGGE bands (% of total number of bands analyzed)				Clone libraries (relative abundances)	
	Water (%)			Sediment (%)	Water (%)	Sediment (%)
Taxa	All samples	<4 mg/L As	≥4 mg/L As	All samples	P9 (3.4 mg/L As)	P4 (1210 mg/Kg As)
Alphaproteobacteria	37	44	38	17	nd	5
Betaproteobacteria	18	22	8	nd	nd	nd
Gammaproteobacteria	11	6	15	17	16	12
Deltaproteobacteria	nd	nd	nd	17	nd	14
Epsilonproteobacteria	nd	nd	nd	nd	34	1
Firmicutes	15	22	8	33	50	50
Bacteroidetes	11	nd	23	8	nd	7
Cyanobacteria	4	6	nd	8	nd	nd
Plastid	4	nd	8	nd	nd	nd
Others	nd	nd	nd	nd	nd	11
Samples analyzed	9	5	4	3	1	1
Bands or clones analyzed	27	20	7	12	64	83

nd, not detected

Range total As in water (mg/L) 0.7-212

Range total As in sediments (mg/L) 781-6,504

Sample P9 yielded 100 % Firmicutes in DGGE

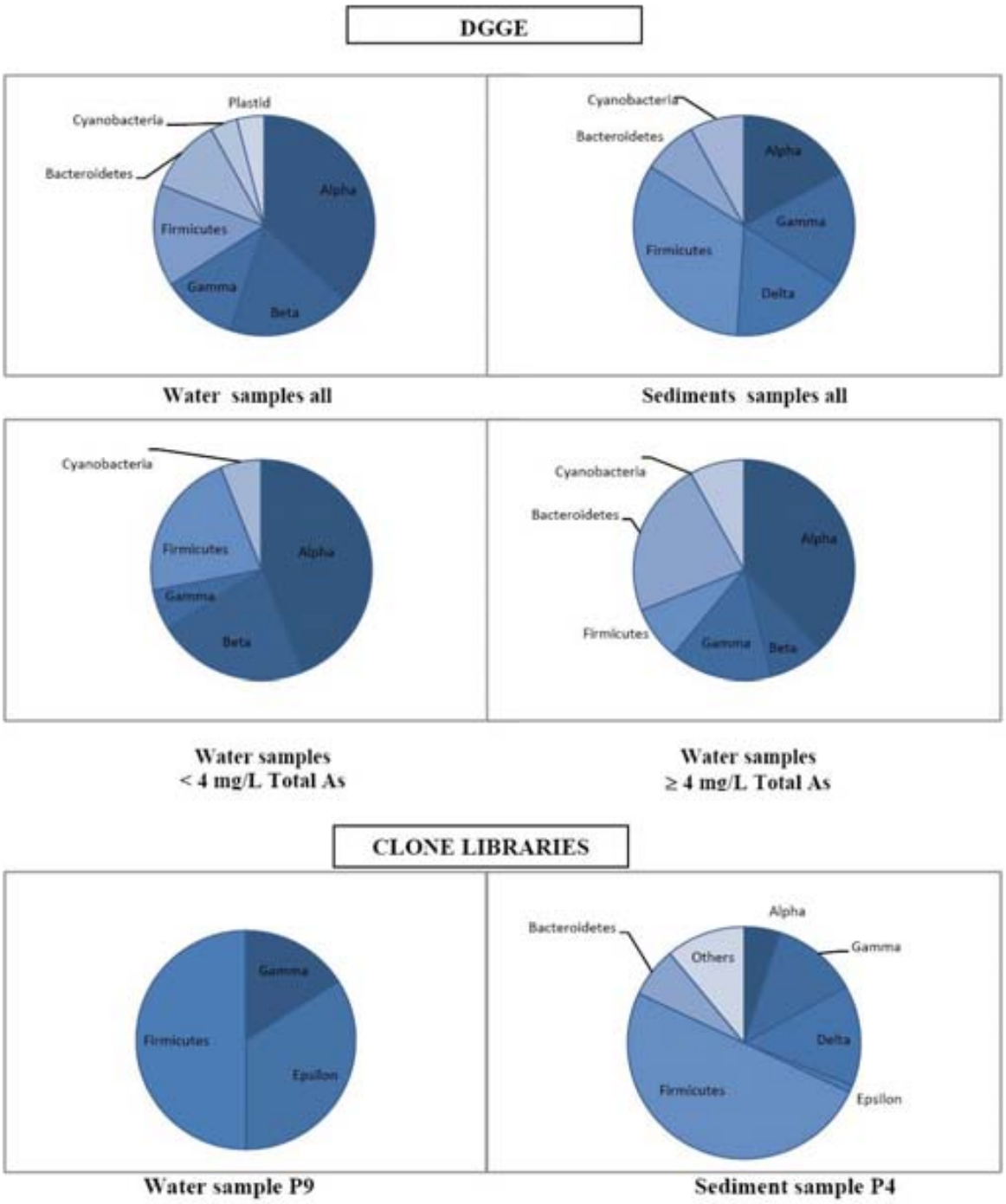

Fig. 2. Percentage of recovered sequences for different taxa after sequencing DGGE bands in water and sediment samples. Relative abundances after cloning two selected samples with different total arsenic concentrations.

Table 5. Clones obtained from sediment P4 and water P9 samples

Clones	*Phylogenetic group*	*Closest relative in Genbank*	*Accession number*	*% Similarity*
Water				
Wat-1	Gammaproteobacteria	*Pseudomonas* sp. KAR75	EF451704	98
Wat-2 to Wat-5	Gammaproteobacteria	*Pseudomonas* sp. p50	EU864269	99
Wat-6	Gammaproteobacteria	*Pseudomonas* sp. BSs20166	EU365518	99
Wat-7 to Wat-10	Gammaproteobacteria	*Pseudomonas* sp. A40	EU369174	98
Wat-11, Wat-12	Epsilonproteobacteria	*Sulfurospirillum* sp. NO3A	AY135396	88
Wat-13 to Wat-31, Wat-63, Wat-64	Epsilonproteobacteria	*Sulfurospirillum* sp. NO2B	AY135395	97 to 98
Wat-32 to Wat-53	Firmicutes	*Fusibacter* sp. enrichment culture clone 22-7A	EU517558	98
Wat-54	Firmicutes	Uncultured bacterium clone PL-5B5	AY570558	97
Wat-55 to Wat-60	Firmicutes	Uncultured bacterium clone SC172	EU735637	99
Wat-61	Firmicutes	*Clostridiales* bacterium UXO5-23	DQ522110	98
Wat-62	Firmicutes	Uncultured bacterium clone 91-133	EF157160	86
Sediment				
Sed-1, Sed-2	Alphaproteobacteria	Uncultured bacterium clone E4aB11	DQ103616	94
Sed-3, Sed-4	Alphaproteobacteria	Uncultured *Rhodobacteraceae* bacterium	AM691100	97
Sed-5	Gammaproteobacteria	Uncultured gamma proteobacterium clone WN-HWB-157	DQ432376	93
Sed-6	Gammaproteobacteria	Uncultured gamma proteobacterium clone WN-HWB-157	DQ432376	93
Sed-7	Gammaproteobacteria	Uncultured bacterium clone Asc-s-2	EF632658	90
Sed-8, Sed-9	Gammaproteobacteria	Uncultured bacterium clone Asc-s-14	EF632659	94
Sed-10	Gammaproteobacteria	Uncultured bacterium clone Asc-s-85	EF632660	94
Sed-11, Sed-12	Gammaproteobacteria	Uncultured bacterium clone LCKS000B50	EF201723	92
Sed-13	Gammaproteobacteria	*Gamma proteobacterium* A59	AB302343	78
Sed-14	Gammaproteobacteria	Unclassified gamma proteobacterium, isolate EG19	AM691086	91
Sed-15	Deltaproteobacteria	Uncultured bacterium clone SMI1-GC205-Bac3j	DQ521796	84
Sed-16	Deltaproteobacteria	Benzene mineralizing consortium clone SB-9	AF029042	94
Sed-17	Deltaproteobacteria	Uncultured bacterium clone Hua-s-72	EF632806	85
Sed-18, Sed-19	Deltaproteobacteria	Uncultured bacterium clone Asc-s-4	EF632652	94
Sed-20	Deltaproteobacteria	Uncultured bacterium clone Asc-w-7	EF632705	96
Sed-21, Sed-22	Deltaproteobacteria	Uncultured bacterium clone Asc-w-16	EF632707	95
Sed-23	Deltaproteobacteria	*Desulfosalina propionicus* strain PropA	DQ067422	91
Sed-24,Sed-25	Deltaproteobacteria	Uncultured *Desulfobacteraceae* bacterium clone New3Dsbb	DQ386174	93
Sed-26	Deltaproteobacteria	Uncultured delta proteobacterium clone ML-A-19	DQ206407	96
Sed-27	Epsilonproteobacteria	*Thiomicrospira* sp. CVO	U46506	91
Sed-28 to Sed-33	Firmicutes	Uncultured Firmicute isolate ikaite un-c22	AJ431344	93
Sed-34 to Sed-36	Firmicutes	*Halanaerobium acetoethylicum*	HAU86448	96
Sed-37	Firmicutes	Uncultured *Clostridiales* bacterium clone D1Dbac	DQ386212	87
Sed-38, Sed-84	Firmicutes	*Halanaerobium* sp. M2	EF522947	98
Sed-39- Sed-44	Firmicutes	Uncultured firmicute	UFI431344	97
Sed-45	Firmicutes	*Halanaerobium* sp. AN-BI5B	AM157647	98
Sed-46	Firmicutes	Uncultured firmicute isolate ikaite un-c22	AJ431344	97
Sed-47	Firmicutes	Uncultured *Halanaerobiaceae* bacterium clone 2P90	EF106367	96
Sed-48 to Sed-54	Firmicutes	Uncultured organism clone MAT-CR-H2-A11	EU245101	93-97
Sed-55	Firmicutes	Uncultured organism clone MAT-CR-H1-G03	EU245086	79
Sed-56 to Sed-62	Firmicutes	Uncultured bacterium clone Hua-s-44	EF632801	98
Sed-63	Firmicutes	*Halanaerobium acetoethylicum*	X89071	91
Sed-64	Firmicutes	*Bacillus arseniciselenatis* DSM 15340T	AJ865469	92
Sed-76 to Sed-79	Firmucutes	Uncultured low G+C Gram-positive bacterium	DQ432454	95-97
Sed-65, Sed-66	Bacteroidetes	Uncultured CFB group bacterium clone ML1228J-16	AF449766	94
Sed-67	Bacteroidetes	Uncultured organism clone MAT-CR-P1-A12	EU245984	95
Sed-68	Bacteroidetes	*Flexibacteraceae bacterium* DG1392	DQ486489	94
Sed-69	Bacteroidetes	*Flexibacteraceae bacterium* CL-GR63	EF211828	83
Sed-70	Bacteroidetes	Uncultured CFB group bacterium clone ML1228J-16	AF449766	94
Sed-71	Candidate division JS1	Uncultured organism clone MAT-CR-P1-C02	EU245991	93
Sed-72	Candidate division op11	Uncultured bacterium clone B02R017	AY197399	89
Sed-73	Candidate division op11	Uncultured bacterium clone B02R017	AY197399	92
Sed-74	Candidate division TM6	Uncultured bacterium clone SLB630	DQ787724	86
Sed-75	*Deinococcus thermus*	Uncultured *Deinococci* bacterium clone LA7-B27N	AF513964	91
Sed-80	Candidate division Hyd24-32	Uncultured bacterium clone Zplanct34	EF602495	92
Sed-81	Candidate division Hyd24-32	Uncultured bacterium clone Zplanct34	EF602495	95
Sed-82	Verrucomicrobia	Uncultured bacterium clone C5lks8	AM086108	90
Sed-83	Verrucomicrobia	Uncultured *Verrucomicrobia* bacterium clone LD1-PB12	AY114330	88

Phylogenetic trees were constructed for the three main groups of sequences retrieved: Firmicutes, Proteobacteria and Bacteroidetes (Figs. 3-5). The Firmicutes sequences recovered from the water sample belonged to the *Clostridiales* whereas in sediments they grouped with *Halanaerobium* and *Natroanaerobium* (Fig. 3). Thus, a marked segregation according to habitat type was apparent in this phylum. The *Clostridiales* sequences formed two clusters, one related to *Fusibacter* and the other to *Clostridium sporogenes*. The *Halanaerobium* sequences were distantly related to several anaerobic, moderately halophilic, *Halanaerobium* species (Oren, 2000). Some additional sequences were very distantly related to *Halocella cellulosilytica*, clustering with a novel group of uncultured Firmicutes. Clones related to a group of *Natroanaerobium* were associated with uncultured bacteria from Mono Lake (Humayoun *et al.*, 2003), a saline alkaline lake in the U.S.A. Only one of the sequences retrieved from the sediments belonged to the Clostridiales. This sequence was distantly related to *Desulforoporosinus auripigmenti*, a well know As(V) reducing bacterium. The Proteobacteria were also very different in water and in sediments (Fig. 4). Epsilonproteobacteria were very abundant in water but only one sequence was retrieved from the sediments (Fig. 2). The opposite was true for the Deltaproteobacteria. The Gammaproteobacteria were retrieved from both, but the water sequences formed two clusters related to *Pseudomonas*, while the sediment sequences formed three clusters related to environmental clones from hypersaline environments (Fig. 4). The Epsilonproteobacteria from the water samples formed two clusters related to *Sulfurospirillum*. Both clusters consisted of many sequences that were closely related but not identical. The Deltaproteobacteria from the sediments were distributed among several genera: *Geobacter, Desulfolignum, Desulfosalina* and *Desulfobulbus.* Within the Gammaproteobacteria we observed sequences from water samples to be closely clustered with the Pseudomonaceae whereas sequences form sediments where more distantly related (Fig. 4) but were associated to clones from Artic sea ice, Antarctic lake water, hypersaline microbial mats in Guerrero Negro, hypersaline alkaline Mono Lake and very distantly related to *Halorhodospira halophila* strain SL1, an isolate from Wadi Natrum, Egypt (Imhoff & Suling, 1996),

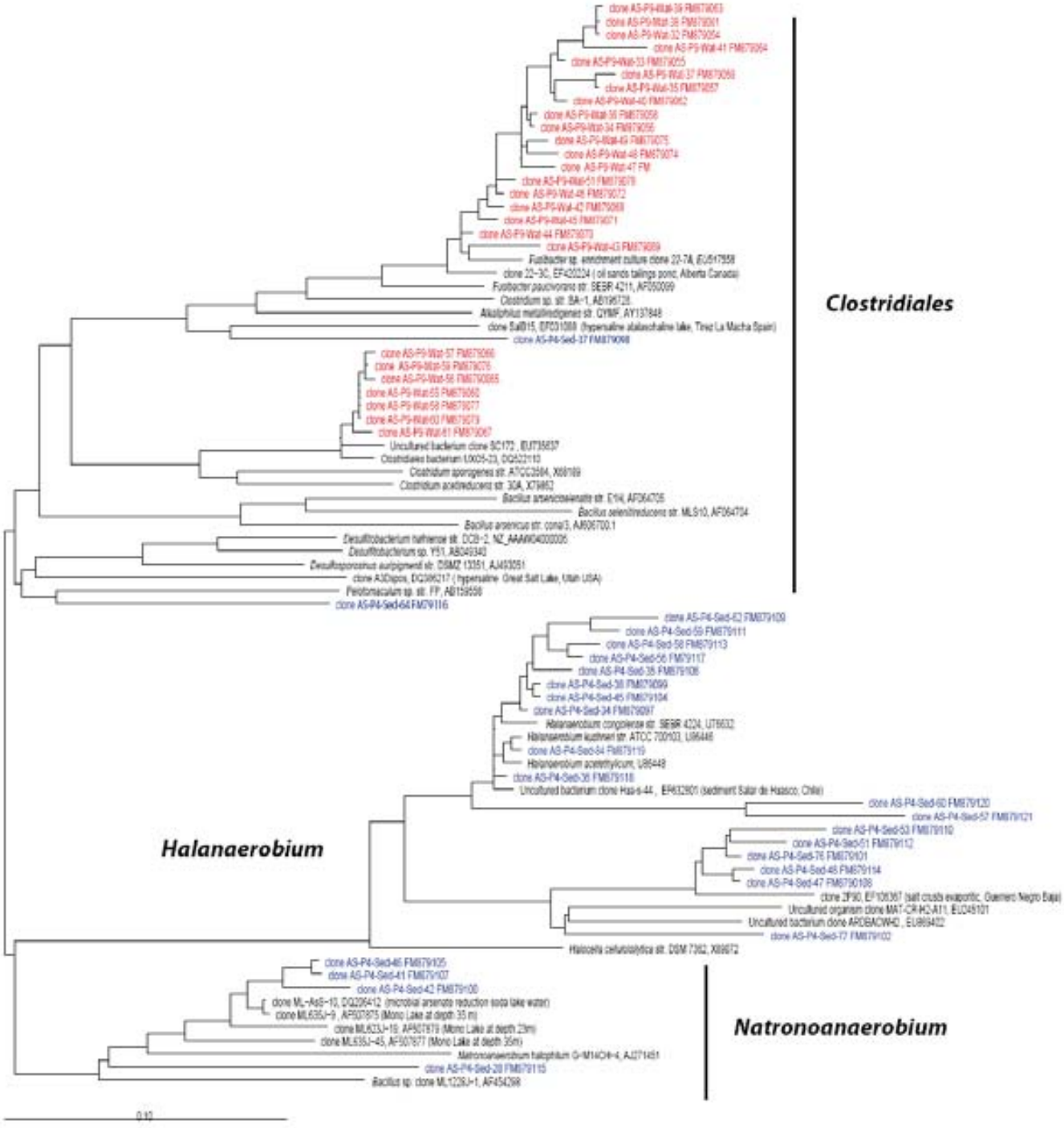

Figure 3. Phylogenetic tree for the Firmicutes sequences retrieved from Salar de Ascotán in water samples (red) and sediment samples (blue). The tree was originally constructed by Maximun Likelihood using complete or nearly complete 16S rRNA sequences.. Sequences were added by parsimony using ARB software facilities.

Alkalilimnicola ehrlichei, an arsenite oxidizing bacterium, (Oremland *et al.*, 2002) and *Alkalilimnicola halodurans,* isolated from the soda-depositing Lake Natron (Yakimov *et al.*, 2001). One clone was closely related to *Shewanella* sp. TS29, a well known respirer of As and sulfate isolated from an arsenic-contaminated environment in Hubei Province, China (Cai *et al.*, 2009).

In a separate study we isolated in pure culture a bacterium that is very close to both *Shewanella* sp. TS29 and the Ascotán clone (see Chapter 6).

Bacteroidetes sequences were retrieved from sediments only and most of them formed a cluster related to clones from hypersaline environments (Fig. 5). The remaining clones belonged to Verrucomicrobia (2 OTUs), and candidate divisions HyD24-32 (2 OTUs), JS1 (1 OTUs), and OP11 (2 OTUs).

Figure 6 shows rarefaction curves for both libraries considering different levels of similarity in 16S rRNA to define OTUs (100, 99 and 97%). The sediment library showed very high slopes at all levels of similarity. Accordingly, the coverage calculated was very low (around 10%, Table 6). The water library showed lower slopes, particularly at 99% and 97% similarity levels, and coverage was higher (Table 6). Figure 7 show clustering analyses for both libraries, showing a very clear difference in the distribution of density any the two libraries sample.

Discussion

High altitude wetlands are exposed to particularly harsh environmental stresses including aridity, high UV radiation, negative water balance, extreme differences in temperature between day and night and a wide range of salinity conditions (Demergasso *et al.,* 2004, Dorador *et al*., 2008).

Microbial diversity in high altitude wetlands of the Altiplano has been studied recently (Demergasso *et al.*, 2008, Dorador *et al.*, 2008) and revealed the predominance of Bacteroidetes and Proteobacteria through a wide range of salinities and sites (see Chapter I).

In the case of Salar de Ascotán, however, one or an additional stress is posed by the high arsenic concentrations present in both water and sediments.

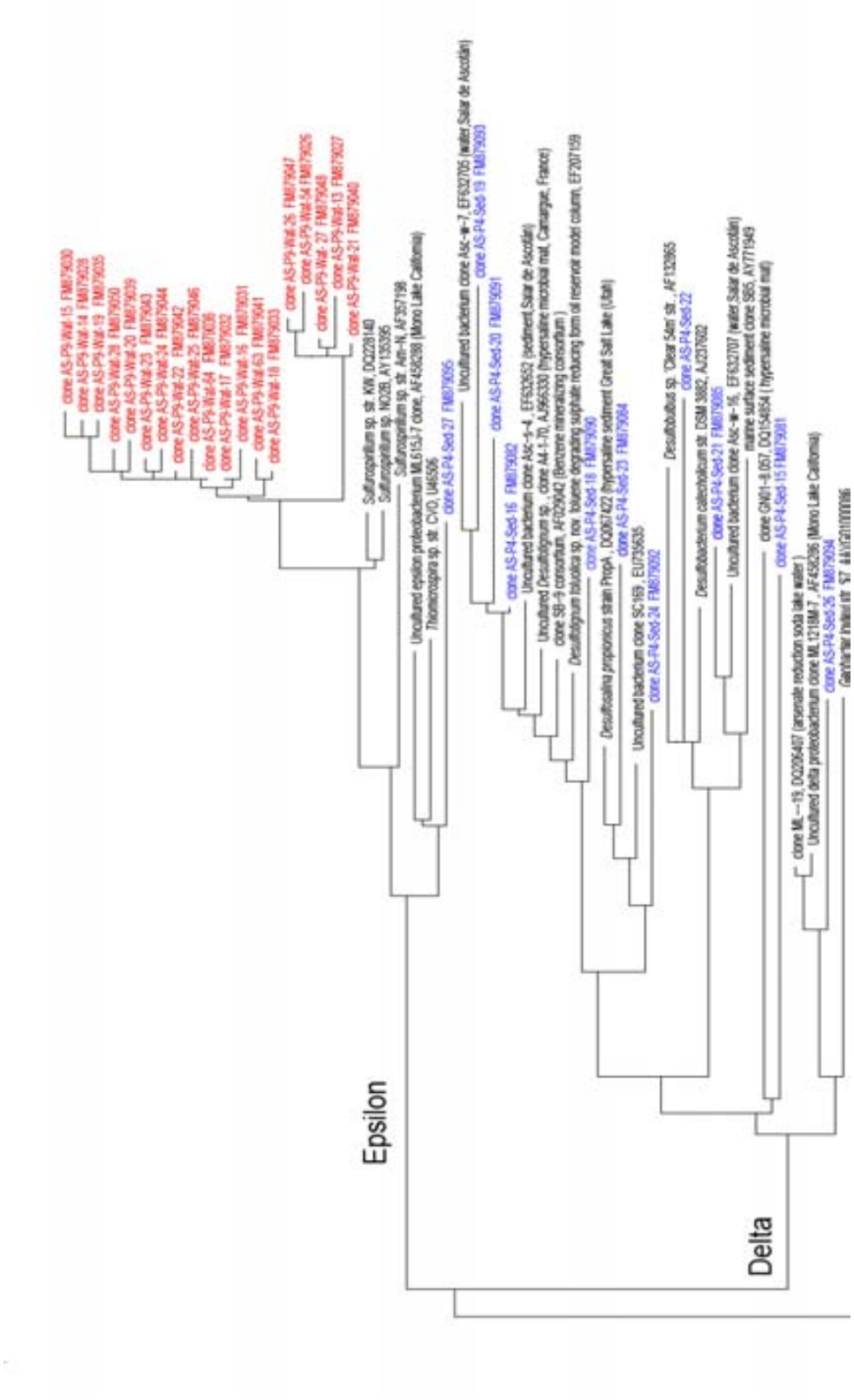

Figure 4. Phylogenetic tree for the Proteobacteria sequences retrieved from Salar de Ascotán in water samples (red) andr sediment samples (blue). The tree was originally constructed by Maximun Likelihood using complete or nearly complete 16S rRNA sequences. Sequences were added by parsimony using ARB software facilities.

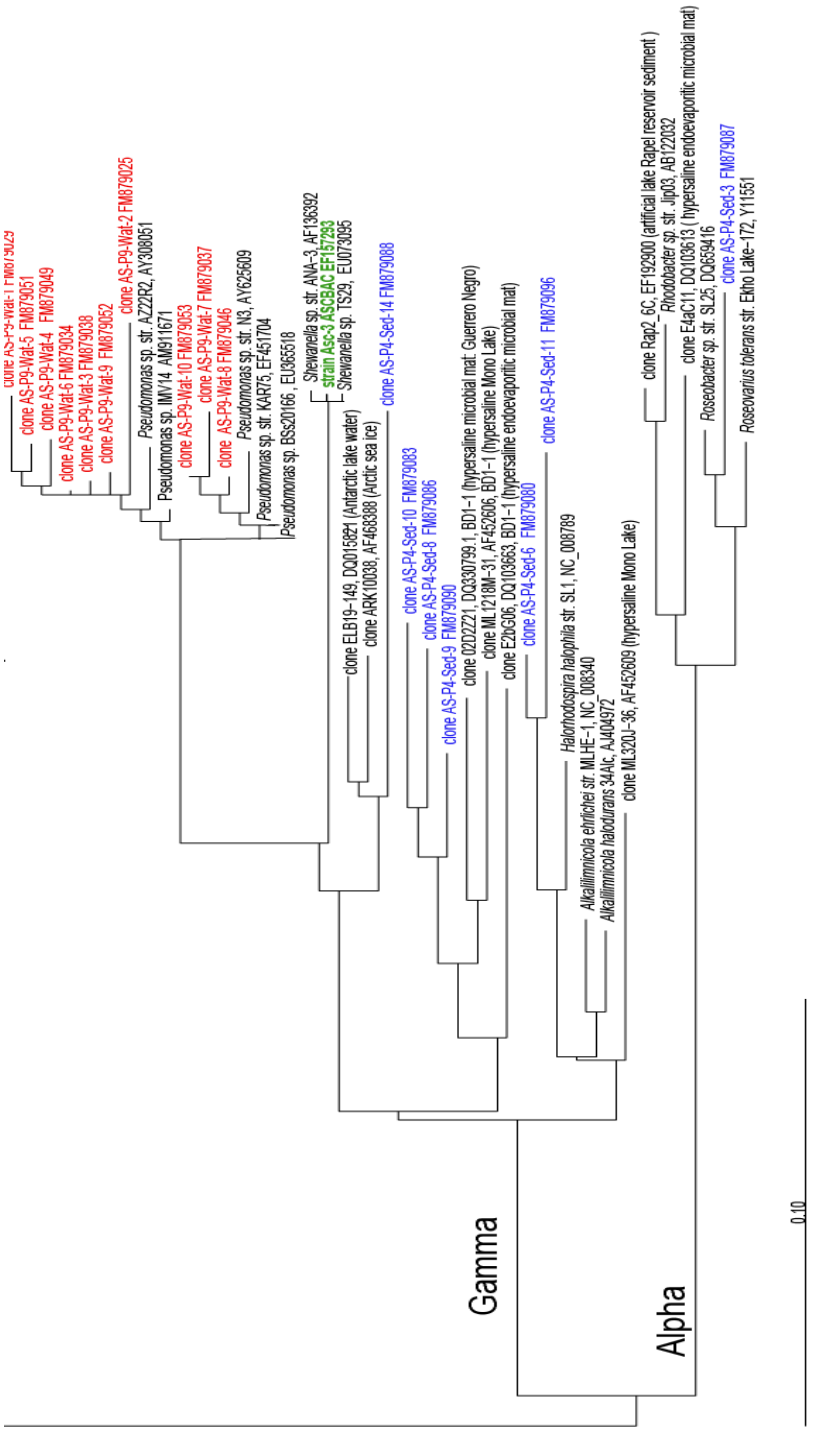

Figure 4. Continued

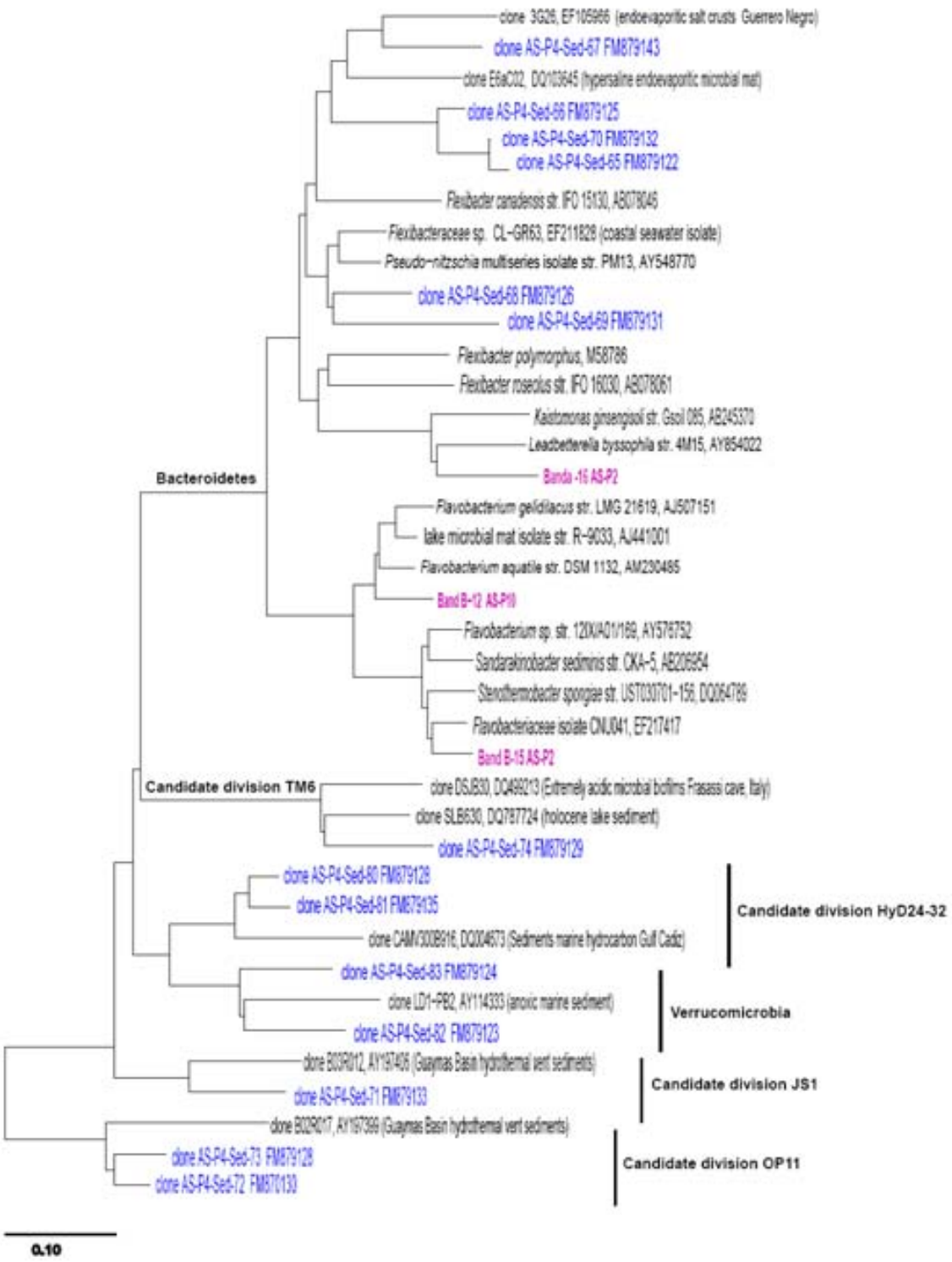

Figure. 5. Phylogenetic tree for the remaining sequences retrieved from Salar de Ascotán in water samples (red) and sediment samples (blue). The tree was originally constructed by Maximun Likelihood using complete or nearly completed 16S rRNA sequences. Sequences were added by parsimony using ARB software facilities.

Table 6. Summary data for clone libraries from Salar de Ascotán and predicted values of OTUs, S_{Chao1} and coverage at different % of similarity

Sample	Number of clones analyzed	Number of phylotypes			Chao1			Coverage (%)	
		Unique	99%	97%	Unique	99%	97%	99%	97%
P9	59	55	25	10	497	42.5	25	54.5	60.0
P4	77	77	70	62	3003	590	447	9.1	11.4

The total arsenic concentrations determined here (up to 86 mM) are among the highest ever recorded in aquatic environments (see Table 1 in the Introduction, Smedley and Kinniburgh, 2002). We expected that this would be reflected in the diversity and composition of the microbial community.

Accordingly, we decided to study the bacterial diversity and compared it to that of a similar, but As-poor system: Laguna Tebenquiche, studied in the previous Chapter.

There have been several microbiological studies of As-rich environment. These have involved isolation of As-metabolizing microbes from aquifers in Shanyin County, China (Fan *et al.*, 2008), or from soils in Shangyan Province, China (Cai *et al*., 2009), microcosm experiments with sediments from a Cambodian aquifer (Lear *et al.*, 2007), and detection of arsenic oxidation and reduction genes in geothermal springs in Yellowstone National Park, U.S.A. (Hamamura *et al.*, 2009). The most complete studies of As metabolism, microbiology, and environmental genetics have been carried out in Lakes Mono and Searles in California, EE.UU. (Oremland *et al*., 2002, Saltikov *et al.*, 2003, Oremland *et al.*, 2005, Hollibaugh *et al.*, 2006, Kulp *et al.*, 2006).

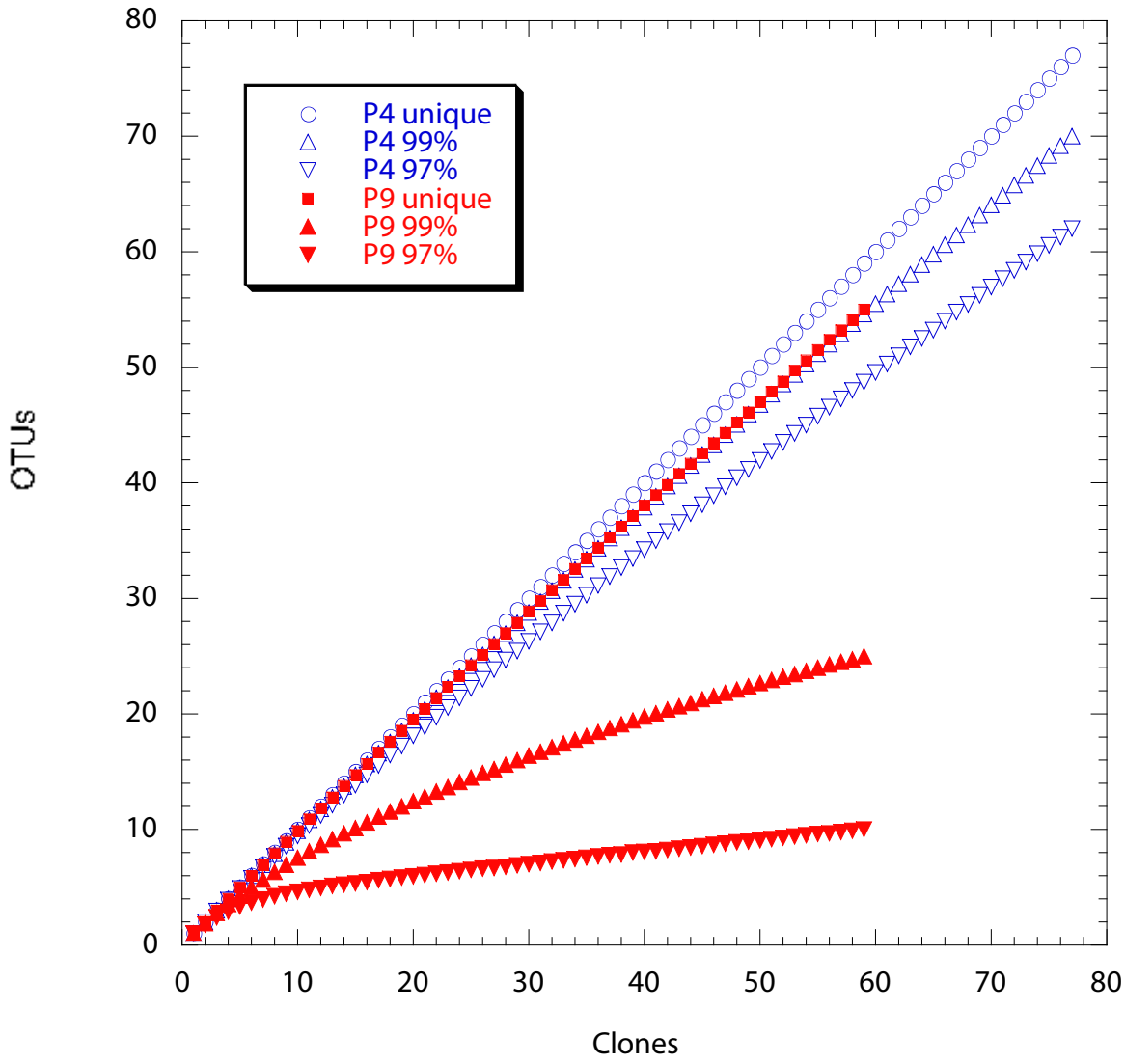

Figure 6. Rarefaction curves at different percentage of similarity for water and sediment samples.

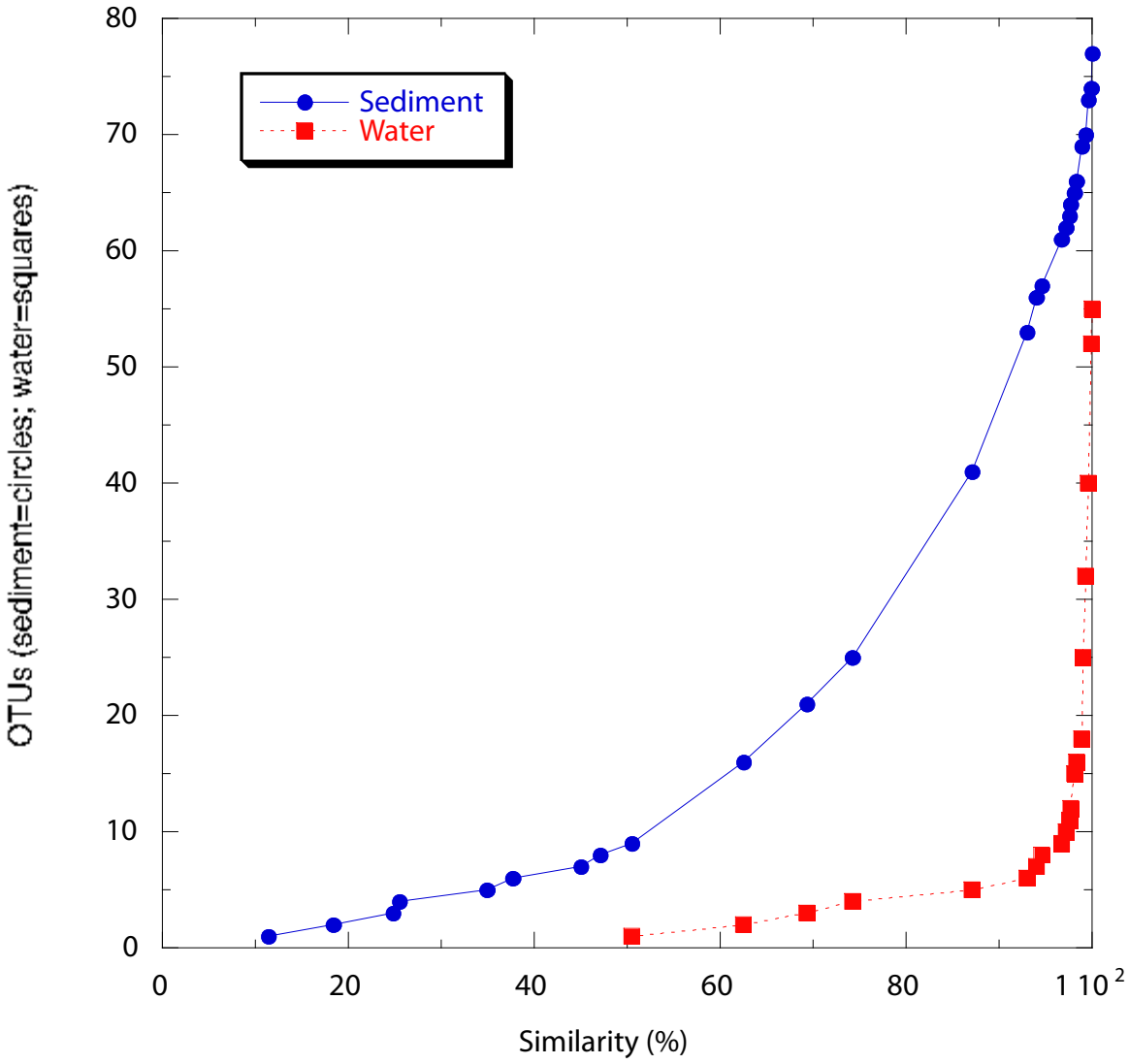

Figure 7. Clustering analyses of the water and sediment samples of the Salar de Ascotán.

However, the *in situ* composition of the bacterial assemblage has only been analyzed in the study of geothermal springs (Hamamura *et al*., 2009) and in Mono Lake (Oremland *et al*., 2002). Thus, it was of interest to compare the bacterial assemblage in Salar de Ascotán to a) that of alkaline hypersaline, As-rich, Mono Lake and b) to that of hypersaline, As-poor, Laguna Tebenquiche.

As mentioned before, Salar de Ascotán is a very heterogeneous system. In effect, the DGGE band patterns were different for different samples. If our purpose had been to determine the bacterial diversity in Salar de Ascotán exclusively, we would have had to examine many more samples and construct several clone libraries.

Our purpose, however, was to analyze genes relevant for arsenic processing (Chapter 4). Therefore, one sediment sample and one water sample were chosen on the basis of the As concentrations and the positive amplification of the different arsenic genes analyzed (see Chapters 3 and 4).

Previously, Demergasso *et al*., (2004) had collected and analyzed by DGGE several samples from Salar de Ascotán in August 1999 and March 2000. These samples included two springs and a few water samples from both trickling water over the salt crust and lagoons. Proteobacteria and Bacteroidetes were the two main groups of bacteria in all samples. There was a tendency for more Proteobacteria in the spring waters and more Bacteroidetes in the more saline lagoon waters. However, a large fraction of the bands did not provide quality sequences and between 15 and 75% of the total band intensity in each lane of DGGE gel remained unidentified. The most commonly retrieved bands at the time were distantly related to *Psychroflexus torquis* (90-95% similarity) and other uncultured Bacteroidetes sequences. A few were related to *Leifsonia* and some of them were Gammaproteobacteria.

In the present study we sampled the same types of environments, but not exactly at the same spots. Again, it was difficult to obtain good sequences from the DGGE gels. We could only retrieve a total of 27 sequences from water samples. Most of these (18) belonged to Proteobacteria, while 4 were Firmicutes, 3 Bacteroidetes and 2 cyanobacteria-chloroplasts. The differences with the groups found in the study of 2004 are substantial. Part of the differences may be due to the different salinities of the samples, that were generally higher in

2004, and part to the As concentrations, that were generally higher in the present study. This might explain the relatively lower detection of Bacteroidetes sequences in the present study than in the previous one. Moreover, the sequences found here might have escaped our 2004 study because they formed faint bands that were not properly detected by DGGE (Sánchez *et al.*, 2007). They would, thus, form part of the large percentage of unidentified sequences (Demergasso *et al.*, 2004).

In this study we also analyzed sediment samples. In this case, the sequences retrieved belonged to Proteobacteria (2 alpha, 2 gamma and 2 delta), Firmicutes (4 bands), one Bacteroidetes and one Cyanobacterium.

Rarefaction analyses of the two clone libraries revealed saturation in the water sample (P9), but not in the case of the sediment sample (P4). In addition, coverage indicated that more than 54% of total diversity was detected in the water sample, but in the sediment only 11% of total diversity was detected. Sediments are environments where bacterial diversity is usually higher than in the water column, because of a larger number of microniches, and accumulation of sinking cells. The diversity found in the sediment sample was consistent with previously reported findings of other soda lakes where sediments had higher bacterial richness than the water column (Hollibaugh *et al.*, 2006). The very high arsenic concentrations found in Ascotán sediments did not seem, therefore, to reduce this general trend. Surprisingly, we observed the highest proportion of microdiversity to be located in the water column and this contradicts somehow our presumption of higher number of microniches in the sediment than in the water column. Marine (Acinas *et al.*, 2004) and freshwater plankton (Casamayor *et al*., 2002), as well as solar salterns (Benlloch *et al.*, 2002) and copper heaps (Demergasso *et al.*, 2005) show a high level of microdiversity as well. In turn, in soils and sediments this feature is more restricted to specific groups, for example the sulfate reducing bacteria (SRB) from salt marshes (Klepac-Ceraj *et al.*, 2004).

Despite the potential arsenic toxicity, phylogenetic bacterial diversity was higher in the anoxic sediment (16.1 mM total As) than in oxic water (2.4 mM total As) samples in Salar de Ascotán. Bacterial communities in the sediment and in water were dominated by Firmicutes in both cases. However, *Halanaerobium* and *Natroanaerobium* dominated in the sediment, while *Clo-*

stridiales and *Fusibacter* were dominant in the water. These results are different from those obtained along a salinity gradient in a Mediterranean solar saltern (Benlloch *et al*., 2002) where Firmicutes (low G+C Gram positive) were not detected. In Tebenquiche very few Firmicutes was detected as well (see Table 3, Chapter I). Firmicutes, however, were also found in laboratory isolates from Searles Lake. This lake is a salt saturated, alkaline, and As-rich water body (Kulp *et al.*, 2007). Clones related to a group of *Natroanaerobium* were also associated with uncultured bacteria from Mono Lake (Humayoun *et al*., 2003). And Firmicutes are involved in the As cycle, such as *Chrysiogenes arsenatis* and *Desulfo-porosinus auripigmenti*. Altogether, the presence of Firmicutes in Salar de Ascotán seems to be more related to the effects of arsenic than to those of salinity.

Another interesting group was the Betaproteobacteria (*Polynucleobacter* group at the lowest salinities). The *Polynucleobacter* group (Betaproteobacteria, *Burkholderiaceae*) is of great environmental relevance in freshwater habitats (Hahn *et al.*, 2005). These free-living heterotrophic bacteria contribute up to 60% of total bacterial cell numbers in the pelagic zone of freshwater habitats and have also been detected in groundwater. *Polynucleobacter* species are also important components of the bacterioplankton in many Arctic wetlands formed by thawing of Arctic permafrost and peats (Hahn *et al.*, 2005). The presence of *Polynucleobacter*-like bacteria in high-altitude water bodies such as Ascotán widens the distribution of this group to remote freshwater en-vironments of the Altiplano.

Epsilonproteobacteria, in turn, are a poorly studied group in inland waters and increasingly recognized as an ecologically significant player in sulfur-rich habitats, particularly in deep-sea hydrothermal environments, but also in terrestrial habitats including naturally sulfur-rich environments such as oil-field brines, hydrocarbon contaminated groundwater, uncontaminated groundwater, sulfur-rich springs, and limestone caves (Campbell *et al.*, 2006). Again, the presence of Epsilonproteobacteria in inland salt waters of a high-altitude environment widens the distribution of this group to a larger number of habitats than previously known.

Finally, it is interesting to note that very few groups of bacteria were found in abundance in either libraries: five in the sediment and just three in the

water sample (Fig. 2). This indicates low diversity and high dominance in the bacterial community composition. However, there was a large degree of microdiversity in all of them (Figs. 3 and 4). This was particularly striking when comparing the rarefaction curves constructed with different levels of similarity to define OTUs (Fig. 6). In the case of the sediment sample, the curves did not show a convergence towards and asymptote, indicating a large degree of both diversity and microdiversity. And in the water sample the differences among curves indicated a large degree of microdiversity and a more moderate degree of diversity (Fig. 7). Altogether, this suggests that the high As concentrations only allow the presence of a few large groups of bacteria (Bacteroidetes, Firmicutes, Proteobacteria). In fact, only some clusters within these groups were present and they were different in sediments and water. But the environment supported a very rich microdiversity. It has been suggested that this microdiversity (at the 99% similarity level) is explained by the simultaneous presence of different ecotypes (Acinas *et al*., 2004). Therefore, the environment is heterogeneous enough to provide a large array of niches, but the stress caused by As only allows a few bacterial groups to exploit these niches. This situation is certainly very different from that found in similar (except for As concentration) Lake Tebenquiche (Chapter I).

Acknowledgements

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Capítulo III

Environmental distribution of arsenate reductase genes in Andean wetlands with different arsenic concentration

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La verdadera locura quizá no sea otra cosa que la sabiduría misma que, cansada de descubrir las vergüenzas del mundo, ha tomado la inteligente resolución de volverse loca.
(Enrique Heine)

Environmental distribution of arsenate reductase genes in Andean wetlands with different arsenic concentration

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ABSTRACT

The presence of As processing genes was determined in a series of water samples and sediments from a wide array of As-rich environments in the Pre-Andean Depression and Altiplano of Northern Chile. A PCR approach was used to detect *arrA* and *arsC* genes. The performance of different primer sets, previously used with enrichments samples and laboratory strains, were tested in a wide range of complex environmental samples at a regional scale. In addition, we carried out estimations of viable As precipitating bacteria in most probable number incubations. A large proportion of total bacteria in sediments were retrieved as As precipitating bacteria, i.e., approximately half of total counts in four out of six samples from Salar de Ascotán. The arsenate reductase *arsC* gene was only found in natural samples with As concentrations lower than 4 mg/L. In turn, arsenate respiratory *arrA* was detected in all samples analyzed covering a range between 0.4 and 6,504 mg/L total As.

INTRODUCTION

Arsenic (As) is a toxic element found in natural environments such as geothermal springs, human-contaminated sites, and groundwater in volcanic areas. As-containing drinking waters are a serious concern for human health in numerous locations across South and North America (the American Midwest and the

Canadian Maritime Province), Asia (mainly in West Bengal and Bangladesh), but also in central Europe (Smedley & Kinniburgh, 2002). This issue has promoted considerable research on the biogeochemical processes that control the distribution and mobilization of As in aquatic environments (Croal *et al.*, 2004).

Understanding the microbiology of As mobilization is essential for possible bioremediation strategies. Malasarn *et al.* (2004) proposed that PCR amplification of the *arrA* gene could be used as a single molecular assay to detect the presence of As(V) reducing microorganisms in the environment. However, this possibility has not been tested in a significant number of natural habitats with different concentration of As. We carried out such a study in a large region and complemented it with MPN estimations of arsenic precipitating bacteria and presence of *arsC* genes.

From a geochemical point of view, As is present in the environment in different forms, but the major species existing in natural waters are arsenate [$HAsO_4^{2-}$; As(V)] and arsenite [H_3AsO_3; As(III)]. These two oxyanions readily interconvert, and their different properties determine whether they are sequestered in solid form or mobilized into the aqueous phase. The microbial metabolism undoubtedly exacerbates the problem of environmental As toxicity, by mobilizing As into drinking waters in shallow wells. Understanding the mechanisms behind the microbial transformations may therefore help to minimize its environmental impact.

Microorganisms that transform As(V) to As(III) are diverse in their phylogeny and overall physiology. Most of the work carried out on As(V) reduction comes from studies of the As related systems present in *Staphylococcus aureus* and *E. coli* (Hedges & Baumberg, 1973, Mobley *et al.*, 1983 Broer *et al.*, 1993). This reduction step does not generate energy but acts as a detoxification system.

Microorganisms that utilize As in their energy metabolism belong to two physiological types: the chemolithoautotrophic As(III) oxidizers and the heterotrophic As(V) reducers. Chemolithotrophs gain energy from coupling the oxidation of As(III) to the reduction of oxygen or nitrate. They include members of the bacterial genera *Alcaligenes, Pseudomonas*, and *Thermus*. Several As(III) oxidizing heterotrophs do not appear to utilize As(III) as an electron donor for respiration, suggesting that

As(III) oxidation may be incidental or a way for detoxification in these strains. A diverse group of heterotrophic bacteria can utilize As(V) as a terminal electron acceptor for respiration. These organisms include members of the Gamma-, Delta-, and Epsilonproteobacteria, Gram-positive bacteria, thermophilic Eubacteria and *Crenarchaeota* (Oremland & Stolz, 2003). Most As(V) respiring strains couple the oxidation of lactate to acetate to support As(V) reduction to As(III) and some strains can use acetate and/or H_2 as an electron donor. The better known As(V) respiring bacteria are obligate anaerobes, whereas only a few of them are facultative aerobes. One of these, *Shewanella* sp. strain ANA-3 has been particularly useful in genetic studies of As transformations (Saltikov & Newman, 2003, Croal *et al*., 2004).

Although As(III) is more toxic than As(V), it can be excreted via an As(III)-specific tranporter, ArsB (Oremland & Stolz, 2003). ArsC, cytoplasmic arsenate reductase is found widely distributed in microbes, and the *arsC* gene occurs in *ars* operons in most bacteria with genomes measuring 2 Mb or larger as well as in some archaeal genomes (Silver & Phung, 2005). The best studied mechanism of detoxification and resistance is the bacterial Ars system (Saltikov & Newman, 2003).

This *ars* operon encodes five genes, *arsRDABC*, that are co-transcribed from one promoter region (Fig. 1A). Briefly, *arsR* and *arsD* encode two regulatory elements: *arsR* encodes a helix-turn-helix repressor that binds the operator region of the *ars* operon as a dimer until As(III) or Sb(III) binds to it and induces its release. *arsD* is believed to be a *trans*-acting, inducer-independent, secondary regulator. It is expressed in the presence of As(III), and has no effect on the expression of the operon when ArsR is repressing the system. *arsA* encodes a membrane associated ATPase subunit that interacts with the *arsB* gene product. *arsB* is the efflux pump responsible for the extrusion of As(III) and Sb(III). In association with *arsA*, it uses the energy from ATP hydrolysis for this function; however, when ArsA is not present, ArsB is still functional. *arsC* encodes a small cytoplasmic arsenate reductase of 13 to 16 kD (Croal *et al.*, 2004).

The Arr system, in turn, comprises two genes *arrA* and *arrB*, required for respiratory arsenate reduction. The operon encoding these genes is close to the *ars* operon in the chromosome, but it is

divergently transcribed (Fig. 1 A and B). These genes appear to be under the control of a promoter that senses anaerobiosis, and preliminary data suggest that an additional As-specific activator exists to up-regulate *arr* gene expression. The protein codes for heterodimer periplasmic or membrane associated protein consisting of a larger molybdopterin subunit (ArrA) which contains an iron-sulfur center, perhaps a high potential [4Fe-4S] cluster, and a smaller [Fe-S] center protein (ArrB) (Silver & Phung, 2005).

Northern Chile, as part of the Andean Range, is an excellent place to study microbial implications in the As geochemical cycle. First, superficial and ground waters are relatively rich in As due to the volcanic-hydrothermal provenance of this element. As a consequence, As is also present in river sediments, where some of the microbial reactions using As oxianions for energy generation may mobilize it from the solid state to the aqueous phase, finally ending in drinking water sources. Contamination episodes have taken place in Loa River in several occasions with increased As levels. Microbial As mobilization activity has also been found in the sediments of Loa River (Demergasso *et al.*, 2003).

And second, geothermal processes and strong evaporation in the Andes region generate running waters rich in As and in other compounds. Salt flats in the Andes contain significant borate deposits (mostly in the form of ulexite) that include As-rich red and yellow nodules and lenses (Chong *et al.*, 2000). These nodules are rich in the As minerals rejalgar and orpiment.

The aim of the present study was to investigate the presence or absence of arsenate respiratory reductase (*arrA*) and arsenate reductase (*arsC*) genes in different As-rich wetlands surveyed on the Pre-Andean Depression and in the Altiplano on Northern Chile. We used a collection of degenerated primer sets based on the *arrA* and *arsC* genes for detecting these genes in environmental samples. This study provides a first overview of the distribution of As redox genes present in arsenate reducing bacteria along natural gradients of As.

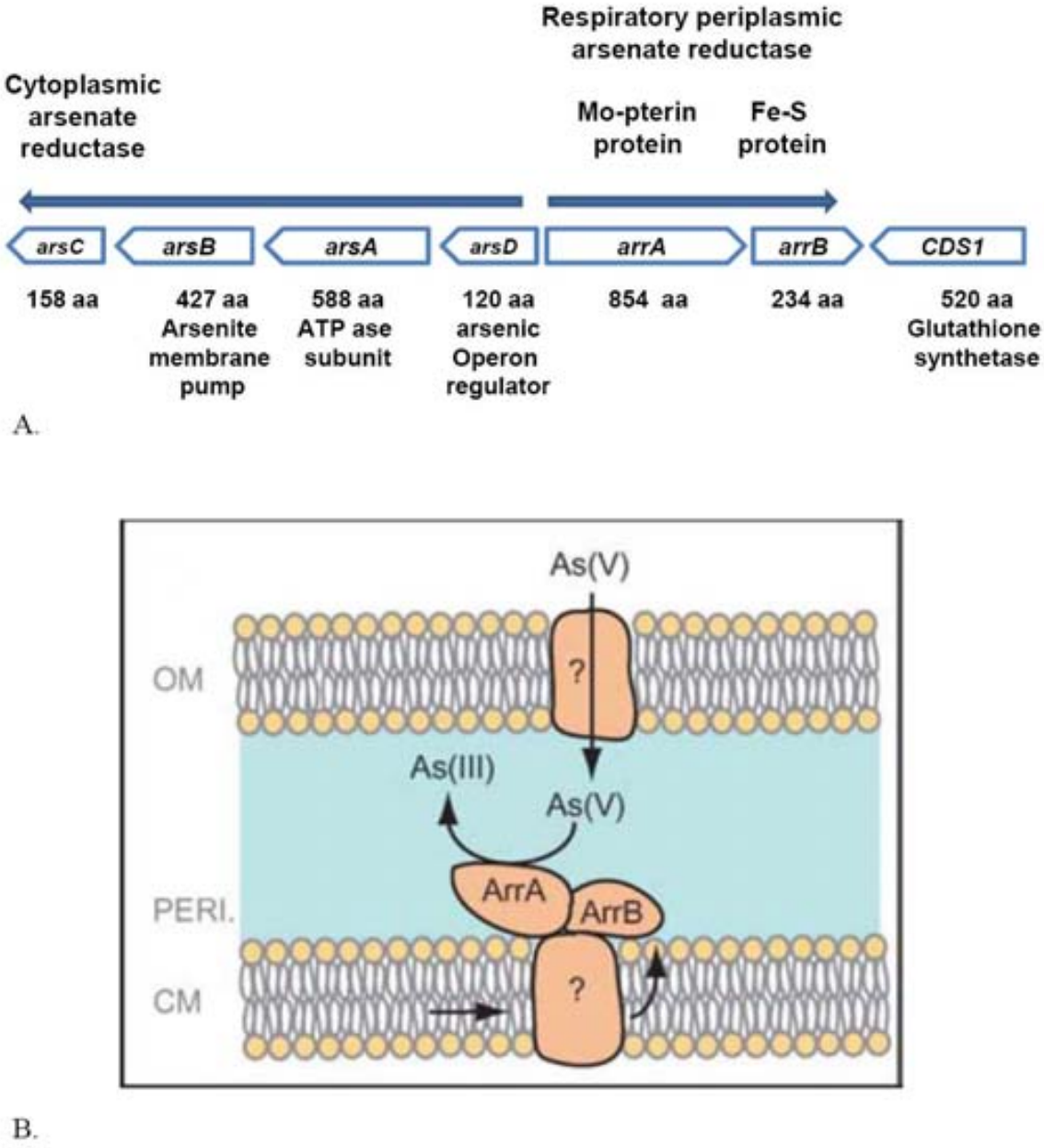

Figure 1. A. Genes for respiratory (*arrAB*) and cytoplasmatic (*arsC*) arsenate reductases of *Shewanella* sp. strain ANA-3. The two operons are divergently transcribed (horizontal arrows); aa, amino acid. **B.** Model pathway showing the major components involved in electron transfer for As(V) reduction by *Shewanella* sp. strain ANA-3. The arrows between the components point in the direction of electron flow. Question marks indicate unidentified components and/or proteins for which the role is unclear. Modified from Croal *et al*., 2004.

MATERIALS AND METHODS

General overview of the aquatic systems studied

A summary of the geographical location, altitude and other parameters of the systems studied is shown in Table 1. This survey was carried out to analyze factors that control the concentration, distribution in species, and arsenic mobility of (As) in surface waters of the II Region of Antofagasta. We chose different As-rich environments in Northern Chile (Fig. 2). They are located between 21º 78' and 23º 85' South latitude and 69º 65' and 67º 75' West longitude, and most of them are above 2500 m of altitude. These included different water supply sources (i.e. "tranques") and river inputs for the drinking water treatment plant for the cities of Antofagasta and Calama (i.e. Tranque Sloman, Tranque Santa Fe, Tranque Santa Teresa, Lequena, Quinchamale, Lasana, Toconce, Puritama, Vilama, Puentre Negro, Río Salado and Río San Pedro), several samples from the main Andean Salts deposits (Salar de Ascotán), samples from one selected geothermal environmental (Geiseres del Tatio) and incoming rivers (i.e. "quebradas") to Salar de Atacama (Quebrada de Jere and Quebrada Aguas Blancas). All these systems shared the presence of As in large or small concentrations (see Table 1).

Salar de Ascotán was studied in more detail because As concentrations were the highest found in the area. Salar de Ascotán is an athalassohaline environment located at the bottom of a tectonic basin surrounded by volcanic chains in east–west direction, including some active volcanoes over 5,000 m high, with the highest altitudes close to 6,000 m. Climate is characterized by large daily thermal oscillations. High solar irradiation and strong and variable winds cause intense evaporation (about 1,640 mm/year) while precipitation is about 120 mm/year (Mardones-Pérez, 1997). Water inputs are through surface drainage from the snow fields of volcanoes, from ground waters with a strong geothermal component and spring waters commonly reaching 23 to 25 °C. The saline crusts are mainly composed of chlorides (halite) and sulfates (gypsum) with important borate ore deposits composed mostly of ulexite with significant amounts of As sulfide minerals. This system exhibits high spatial and temporal variability with water salinities ranging from freshwater to salt-saturated brines (Risacher *et al*. 1999).

Sample collection and processing

Several sampling expeditions were carried out in this large area. Two sampling expeditions were conducted in May

2001 and July 2001 mainly focusing on sources of drinking water and also including El Tatio and Quebradas de Jere and Aguas Blancas. Four sampling expeditions specifically addressing the Salar de Ascotán were conducted in November 2004, August 2005, June 2006 and April 2007, including sediment sampling. In the 2007 expedition, additional samples were collected from Quebradas de Jere and Aguas Blancas, and Géiseres del Tatio (Tables 1 and 2). Overall, up to 48 samples were sampled from both water and sediment (Fig. 2). In water samples, temperature and pH were measured with a pH meter Orion model 290, whereas for salinity, conductivity and total dissolved solids a conductivity meter Orion model 115 was used. Oxygen was measured with a Thermo Orion sensor model 9708. Water samples were transferred to polyethylene 2-L bottles and kept in an icebox until further processing. Water and sediment samples were for total counts taken in sterilized vials to carry out microbial counts and MPN (Most Probable Numbers) estimations. Cells were fixed *in situ* with 4% formaldehyde (vol/vol, final concentration).

Total cells counts were done by epifluorescence microscopy with a DNA-specific dye, 4', 6-diamidino-2 phenylindole (DAPI) with a Leica DMLS epifluorescence microscope. As concentrations were measured by hydride generation atomic absorption spectroscopy (HG-AAS) previously described (Demergasso *et al.*, 2007).

Most probable numbers

Viable As-precipitating cells were detected by most-probable-number (MPN) incubations using fresh minimal medium (Newman *et al.*, 1997) modified by the addition of 0.008% yeast extract, and amended, after autoclaving, with sterile 20 mM sodium lactate, 10 mM sodium sulfate (Na_2SO_4), and 1 mM dibasic sodium arsenate ($Na_2HAsO_4.7H_2O$) under a N_2:CO_2:H_2 atmosphere (80:15:5, v/v). The highest decimal dilution was 10^{-6} and 5 tubes were analyzed for each dilution series. Enrichments were incubated in the dark, at 28ºC along two weeks. The presence of a yellow precipitate (i.e., As sulfide) was considered as a positive result.

Total DNA extraction

Between 800 and 1000 mL of water were filtered through 0.2 µm polycarbonate membranes (Nuclepore) and stored at −20 °C in 1 mL of lysis buffer (50 mM Tris–HCl pH=8.3, 40 mM EDTA and 0.75M sucrose) (Demergasso *et al.*, 2008).

Table 1. Field data for the environmental samples surveyed in Ascotán, Tatio and Quebradas in Atacama that were selected for DNA extraction and PCR amplification. Samples are sorted according incresing As concentration

Date	*Sampling site*	*Sample type*	*Altitude (m)*	*UTM coordinates Grid 19K*		*pH*	*Salinity*	*Conductivity*	*STD*	*Temperature*	*Dissolved oxigen*	*Total As*	*As^{+3}*
dd/mm/yy				North	East		g/L	mS	mg/L	ºC	mg/L	mg/L	mg/L
21-04-2007	Quebrada de Jere	water	2485	7435185	603405	7.20	0.1	0.30	145	17,0	7.80	0.04	-
29-05-2001	Quinchamale	water	3053	7577334	541584	7.76	0.8	1.59	782	12.9	11.90	0.15	BLD
29-05-2001	Lequena	water	3133	7604932	534945	7.79	0.3	0.64	303	2.7	11.40	0.16	0.013
29-05-2001	Vado Santa Barbara	water	-	7569791	540263	7.08	1.1	2.24	1110	13.2	8.57	0.16	0.001
29-05-2001	Lasana	water	2590	7537601	538252	8.52	0.8	1.64	802	13.6	8.83	0.17	0.005
01-08-2001	Vado Río salado	water	3150	7529707	547976	8.24	2.3	4.42	2290	12.6	3.70	0.51	0.001
30-05-2001	Cruce Río Salado	water	3150	7535060	567699	7.08	1.7	3.32	1690	12.1	5.81	0.56	0.008
30-05-2001	Toconce	water	4259	7536536	587831	7.63	0.2	0.47	224	6.7	6.03	0.67	0.002
29-05-2001	Puente Negro	water	2310	7516100	509925	8.4	2.2	4.12	2110	9.4	8.83	0.84	0.014
01-08-2001	Río San Pedro	water	4000	7468232	581017	8.66	1.5	3.16	1450	1.9	9.31	0.90	0.002
01-08-2001	Puritama	water	3475	7487326	598644	7.31	1.4	2.72	1360	33.0	4.05	1.20	0.02
31-07-2001	Quebrada Jere	water	2485	7435215	603015	8.13	0.2	0.40	187	10.0	7.10	1.30	0.003
01-08-2001	Vilama	water	4200	7470528	584125	7.57	1.4	2.77	1370	8.3	8.02	1.50	0.003
01-08-2001	Tatio bajo 1(40m downstream)	water	4278	7530227	601767	6.28	15.0	24.5	13800	22.9	4.20	1.70	0.02
28-05-2001	Tranque Sta. Teresa	water	2750	7570383	440665	8.5	4.3	7.88	4230	17.1	8.70	2.01	0.007
28-05-2001	Tranque Sta. Fe	water	2739	7578997	444521	8.35	4.3	7.80	4170	18.1	6.70	2.02	0.01
28-05-2001	Tranque Sloman	water	3100	7583404	446854	8.34	17.4	7.68	4120	17.4	3.54	2.13	0.007
21-04-2007	Quebrada Aguas Blancas	water	2485	7427145	603446	8.14	1.0	1.89	963	16.0	4.90	4.00	-
21-04-2007	Tatio-SL-2	water	4280	7530555	602449	2.50	6.9	12.15	6720	56.0	2.30	6.00	-
01-08-2001	Tatio 1	water	4278	7530227	601767	4.52	5.2	9.45	5010	66.9	2.31	11.90	8.00
21-04-2007	Tatio-SL-1	water	4278	7530388	602108	3.30	5.1	9.10	4830	52.0	2.80	14.00	-
30-07-2001	Quebrada Aguas Blancas	water	2485	7426588	602876	8.69	1.0	2.00	978	10.5	7.74	15.00	4.38
01-08-2001	Tatio 2	water	4280	7530414	602140	5.50	3.6	6.51	3480	34.9	2.28	29.70	1.88
21-04-2007	Tatio-SL-3	water	4284	7530214	601730	6.70	13.2	22.4	11800	78.3	1.50	36.00	-
2004-2007	Ascotan*	water	3748			5.0-8.6	0.1-670.4	0.3-193.4	143-628000	4.9-24.5	7.1-10.8	0.7-212	-
2004-2007	Ascotan*	sediment	3741			-	-	-	-	-	-	781-6504	-

*Data corresponds to several samples along this four year period (see Table 1, Chapter 2), BLD: below detection limits

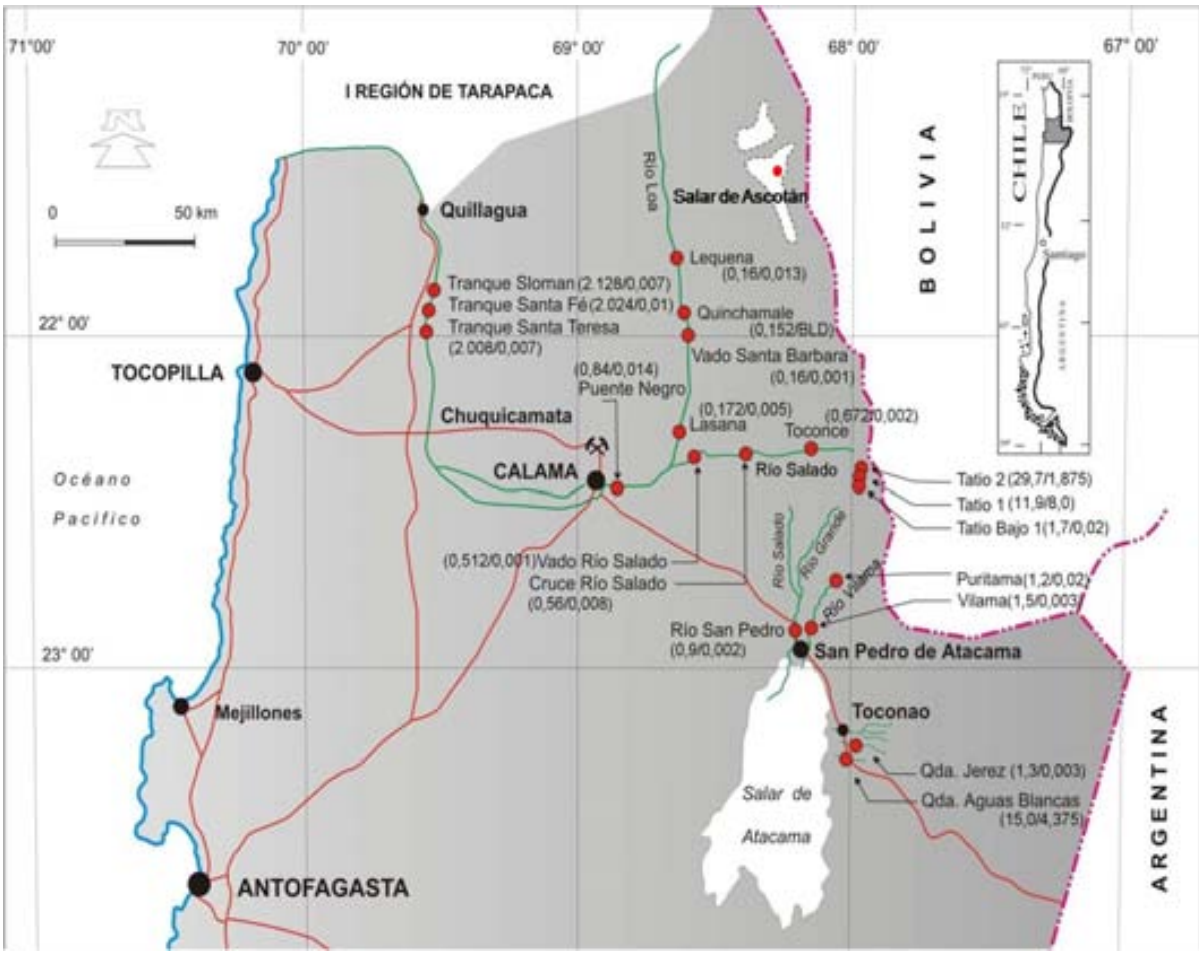

Figure 2. Map of Northern of Chile showing the location of the sampling spots. Numbers in parenthesis indicate total As concentration and As(III) concentration in mg/L.

Table 2. Chemical analyses of water samples from Salar de Ascotán

Sampling day	*Sampling location*	Cl^-	SO_4^{2-}	Ca^{2+}	Mg^{2+}	Na^+	K^+	*Total As*
(dd/mm/yy)		*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*	*(mg/L)*
22-06-2006	P8	3009	3650	550	758	8922	1220	28
	V-6	1139	181	152	40	600	67	0.9
	V-10	1849	366	249	88	954	96	1.6
	P9	4561	683	594	216	2216	217	3.4
20-04-2007	P10	5100	1370	280	253	2650	297	10.0
	P11	2300	423	110	93	1190	117	4.0
	V-4	1210	160	145	27	431	30	0.7
	V-10	1990	259	262	68	735	47	1.0
21-04-2007	Tatio-SL-1	3.30	160	109	183	1560	172	14
	Tatio-SL-2	4.47	226	158	0.31	2180	222	6.0
	Tatio-SL-3	8.14	37	245	38	3075	538	36
	Quebrada Aguas Blancas	0.40	333	100	73	378	56	4.0
	Quebrada Jere	0.08	40	28	21	78	26	0.04

For sediments nucleic acids were extracted from 25 to 50 g (wet weight) of sediment sample, resuspended and strongly vortexed in a salt solution (PBS buffer 1x, Tween 20 at 10% v/v) and the supernatant was filtered as previously described for water samples. Filters were incubated with lysozyme and proteinase K (Demergasso *et al*., 2008), and genomic DNA was extracted with a High Pure Template Preparation Kit (Qiagen, Duesseldorf, Germany). In order to purify and concentrate DNA solutions an additional step of ethanol precipitation was carried out.

PCR amplification of arrA and arsC functional genes

This study is based on genes for respiratory (*arrA*) and cytoplasmatic (*arsC*) arsenate reductases (Fig. 1, according to data provided for *Shewanella* sp. strain ANA-3 in Newman *et al*., 2004). Several PCR-amplifications with different primer combinations were undertaken in order to detect the presence of arsenate reducing bacteria along the different samples targeting the two key genes in the As metabolic pathways (Table 3).

We used three primers sets for the amplification of the As respiratory gene *arrA*. The first primer set *arrA*1, contained the *arrA*f and *arrA*r (Malasarn *et al.*, 2004) to amplify a ~160-200 bp fragment of the As respiratory gene. The optimized PCR conditions included incubation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 50°C for 40 seconds, and 72°C for one minute (Malasarn *et al*., 2004).

The concentration of each primer in a single reaction was 0.5 μM. The second primer set was *arrA*2 used in a nested PCR approach with primers AS1f, AS1r and AS2f (Lear *et al.*, 2007). This primer set was designed after comparison of conserved regions in the *arrA* genes from *Bacillus selenitireducens*, *Chrysiogenes arsenatis*, *Shewanella* sp. strain ANA-3, *Desulfitobacterium hafniense* DCB2, and *Wolinella succinogenes* (Lear *et al*., 2007). The nested-PCR with the primer combination AS2f and AS1r yielded a 625 bp product. The first PCR step was carried out with primers AS1f and AS1r by using a 5-min denaturation step at 94°C, followed by 35 cycles of a 30 s denaturation at 94°C, primer annealing of 30 s at 50°C, and a 1-min extension at 72°C. The second PCR amplification (nested) was done with primers AS2f and AS1r using the first PCR product as template. Nested PCR began with a 2 min denaturation step at 94°C, followed by 30 cycles of a 30 s denaturation at 94°C, primer annealing of 30 s at 55°C, and a 1 min extension at 72°C. Finally, the third primer set for gene *arrA* was HAArrA-D1f and HAArraA-G2r producing a 500 bp PCR product (Kulp *et al.*, 2006). PCR conditions were initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 94ºC for 30s, primer annealing at 53.5 ºC for 30 s, and extension at 72ºC for 30 s, with an additional step at 85º C for 10 s, each primer at the concentration of 0.33 μM.

The *arsC* gene was targeted using two primers sets (Table 3). The first primer set (*arsC1*) contained a mixture of primers; amlt-42-f, amlt-376-r, smrc-42-f and smrc-376-r (Sun *et al.*, 2004). PCR amplification conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 15 s (denaturation), 60°C for 15 s (annealing) and 72°C for 15 s (elongation), in a primer mixture of 0.25 μM each. These primers amplify a fragment of 353 bp of the *arsC* gene. The second set (arsC2) consisted of primers QarsC-f1 and QarsC-r1 (Saltikov *et al.*, 2005). PCR amplification conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s (denaturation), 60°C for 1 min (annealing) and 72°C for 30 s (elongation), at 0.5 μM each primer.

Presence of PCR amplification products was verified by electrophoresis in 1% agarose gels, stained with ethidium bromide. DNA sample reference 595 obtained from a coastal marine environment in Blanes Bay Microbial Observatory was used as negative control (Fig. 3).

Table 3. Different primer sets used in this study for PCR amplification of several genes involved in the arsenic cycle.

Targeted gene	Primer Set	Primer name	Primer sequence (5' – 3')	Amplicons lenght (bp)	Reference (system studied)
Arsenate respiratory reductase	*arrA 1*	arrAf	AAG GTG TAT GGA ATA AAG CGT TTG TBG GHG AYT	160-200	Malasarn *et al.* , 2004
		arrAr	CCT GTG ATT TCA GGT GCC CAY TY V GGN GT		(Haiwee Reservoir and *Shewanella* sp. strain ANA-3)
	arrA 2	AS1f	CGA AGT TCG TCC CGA THA CNT GG	625	Lear et al., 2007
		AS1r	GGG GTG CGG TCY TTN ARY TC		(Cambodian Sediments)
		AS2f (nested)	GTC CCN ATB ASN TGG GAN RAR GCN MT		
	arrA 3	HAArrA-D1f	CCG CTA CTA CAC CGA GGG CWW YTG GGR NTA	500	Kulp *et al.,* 2006 (Mono Lake and Searles Lake, California)
		HAArrA-G2r	CGT GCG GTC CTT GAG CTC NWD RTT CCA CC		
Arsenate reductase	*arsC 1 mix*	amlt-42-f	TCG CGT AAT ACG CTG GAG AT	334	Sun *et al.,* 2004
		amlt-376-r	ACT TTC TCG CCG TCT TCC TT		(Bacterial strain and plasmid)
		smrc-42-f	TCA CGC AAT ACC CTT GAA ATG ATC		
		smrc-376-r	ACC TTT TCA CCG TCC TCT TTC GT	-	
	arsC 2	Q-arsC-f1	GAT TTA CCA TAA TCC GGC CTG T		Saltikov *et al.,* 2005
		Q-arsC-r1	GGC GTC TCA AGG TAG AGG ATA A		(*Shewanella* sp. strain ANA-3)

Results

Table 1 and Fig. 2 show geographical location, physicochemical parameters and As concentrations for all sites sampled in the Pre-Andean Depression and the Altiplano of Northern Chile. All data for Salar de Ascotán (geographical location, physicochemical data and As concentrations) are available in Chapter II.

Samples were obtained in different seasons (austral spring and winter) and temperatures ranged between 1.9ºC in Río San Pedro and 78.3ºC in El Tatio. Salinity ranged between 0.1 and 7.6 g L^{-1}, conductivity ranged between 0.3 and 79.1 mS, and total dissolved solids (TDS) between 143 and 92,300 mg L^{-1}.

All water samples were well oxygenated. We found total As concentrations ranging between 0.7 and 212 mg L^{-1} in water samples, and between 780 and 6504 mg Kg^{-1} in the sediments. Ions such as sulfate, sodium and chloride showed higher concentrations in Salar de Ascotán that in the other samples. In Salar de Ascotán sulfate, chloride and sodium concentrations were between 37 and 3,650 mg L^{-1}, 1,100 to 5,100 mg L^{-1}, 431 to 8,900 mg L^{-1}, respectively (Table 2). Calcium, magnesium and potassium concentrations were also very high, ranging between 30 and 1000 mg L^{-1} (Table 2).

Bacterial abundance in Salar de Ascotán

DAPI-stained prokaryotic concentrations are shown in Table 4. Values ranged between 1.5 x 10^5 and 2.0 x 10^7 cells m L^{-1} in the brines and between 5.8 x 10^5 and 1.9 x 10^8 cells g^{-1} in the sediments. Most probable numbers (MPN) of As precipitating bacteria were determined for many of the samples. MPN ranged 5 orders of magnitude between 3.9 x 10^1 viable cells mL^{-1} and 1.6 x 10^6 cells g^{-1}. Both total bacteria and MPN varied considerably among the different samples.

arrA and *arsC* gene analysis

Fig. 4 and Table 4 show the score of PCR amplifications for different As genes according to the primer sets in the different samples. Amplification for arsenate respiratory reductase gene (*arrA*) was observed in most samples with the three primer sets. PCR products were detected in water and sediment samples with As concentrations between 0.04 and 6,504 mg/L total As. Each primer set produced a PCR fragment of the correct size (Fig. 3). Samples V-10 (2006), P8 (water), P8 (sediment) and P9 (sediment) did not produce a product with the primer sets *arrA*1 and *arrA*2 used for nested PCR. Primer set *arrA*3 did not produce a product with samples V6 (2006), P1-04, P2-04, P7-05, P8

(sediment), P9 (sediment) and P4-04 (sediment). Altogether, only two samples failed to produce an amplicon with the three primer sets. The *arrA* gene was also detected in most of the sediment of Salar de Ascotán where As minerals (i.e. realgar and orpiment) were found. On the other hand this gene was not found in the Blanes Bay sample Fig. 3. Amplification of the *arsC* gene fragment, with both primer sets, was observed only in the samples with the lowest As concentrations (Fig. 4).

PCR products with primer set *arsC1* were obtained in samples with As concentrations lower than 4 mg/L total As and with the primer set *arsC2* only in one sample. Some PCR products for primer set *arsC2* were nonspecific with two or three bands of different sizes in the agarose gel. Only six samples amplified simultaneously for the two genes: Quebrada Jere, V-4 (2007), V-10 (2007), water V-6 (2006), V-10 (2006) and P9.

Discussion

Although As is present only at around 1.8 ppm in the Earth's crust (Klein, 2002), its associated toxicity is a major problem in many parts of the world where drinking waters are heavily contaminated. The microbial anaerobic respiratory arsenate reductase releases previously immobilized underground As(V) and this has been reported as a major problem in newly drilled wells (Silver & Phung, 2005).

The World Health Organization has recommended As guideline values of 10 µg/L as the healthy limit in drinking waters. But As concentrations in water supplies in Northern Chile largely exceed this limit because of the abundance of natural sources, climatic conditions and microbiological activity. Detailed studies on the microbial species present in the area with arsenic redox biotransformation abilities are still needed to understand the role of microorganisms in the mobilization of arsenic from the sediment to groundwater and for bioremediation applications.

Abundant presence of arsenic in waters and sediments of the Pre-Andean Depression and Altiplano in Northern Chile is related to its location at the bottom of a tectonic basin surrounded by volcanoes. In the case of the hypersaline Salar de Ascotán the very high arsenic concentrations found (between 2.4 mM to 86 mM total arsenic) acts as an additional stressing factor.

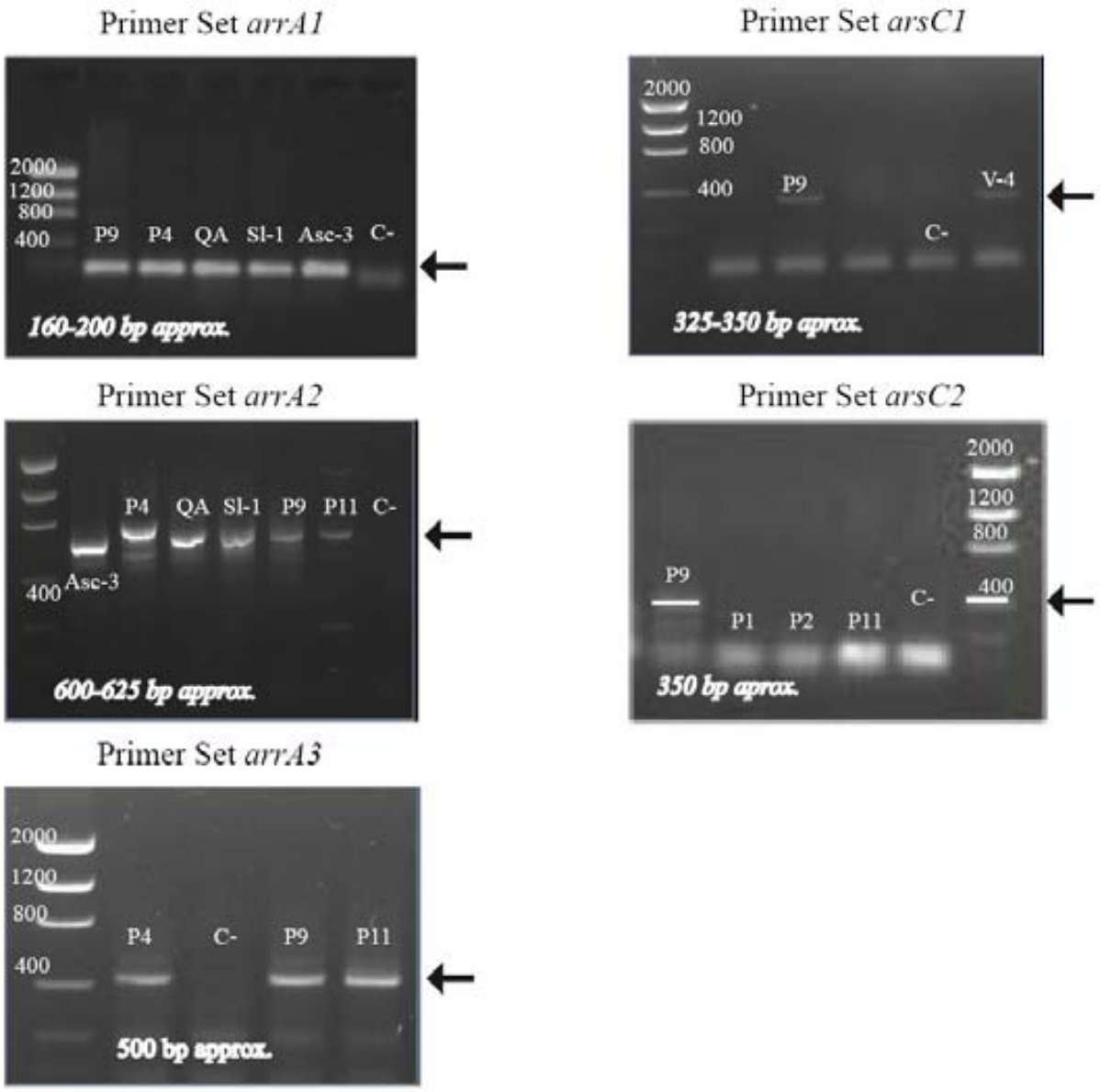

Figure 3. PCR products from some samples analyzed in this study. c- is the negative control. The arrows show the expected size of the amplicons.

Table 4. PCR results for environmental samples with different As concentrations using different primer sets. As concentrations are shown for water and sediment samples. MPN of arsenic reducing bacteria and total cells by DAPI counts are also shown. Primer sets nomenclature according Table 3.

SAMPLE	ORIGIN	Total As (mg/L) (mg/Kg)	MPN (cell/mL) (cell/g)	DAPI (cell/mL) (cell/g)	*arrA1*	*arrA2*	*arrA3*	*arsC1*	*arsC2*
Water									
Quebrada Jere	water Atacama 2007	0.04	ng	2.7E+06	+	+	+	+	+
V-4	water vertiente 4 Ascotán 2007	0.7	ng	4.9E+06	+	+	+	+	-
V-6	water vertiente 6 Ascotán 2006	0.9	ng	1.2E+06	+	+	-	+	-
V-10	water vertiente 10 Ascotán 2007	1.0	ng	9.3E+05	+	+	+	+	+/-
V-10	water vertiente 10 Ascotán 2006	1.6	ng	1.5E+06	-	-	+	+	-
P9	water J1 Ascotán	3.4	ng	3.9E+06	+	+	+	+	-
P1-04	water Ascotán 2004	3.5	nd	7.7E+05	+	+	-	-	-
P11	water Lag. Turquesa Ascotán 2007	4.0	3.3E+02	2.6E+06	+	+	+	-	+/-
Quebrada. Aguas Blancas	water Atacama 2007	4.0	ng	5.0E+05	+	+	+	-	-
P1-05	water Ascotán 2005	4.4	2.8E+05	6.7E+05	+	+	+	-	-
Tatio-SL-2	water Geiser Tatio 2007	6.0	ng	9.0E+05	+	+	+	-	-
P2-05	water Ascotán 2006	6.5	3.9E+01	5.0E+05	+	+	+	-	-
P10-07	water Lag. Turquesa-1 Ascotán 2007	10.0	ng	8.1E+06	+	+	+	-	+ *
P2-04	water Ascotán 2004	10.6	nd	7.0E+05	+	+	-	-	-
Tatio-SL-1	water Geiser Tatio 2007	14.0	2.3E+03	4.2E+06	+	+	+	-	-
P8	water Lag. Turquesa Ascotán 2006	28.0	ng	4.3E+07	-	-	+	-	+/-
Tatio-SL-3	water Geiser Tatio 2007	36.0	ng	1.8E+06	+	+	+	-	-
P6-05	water Ascotán 2005	183	1.4E+02	1.6E+05	+	+	+	-	-
P6-04	water Ascotán 2004	212	nd	2.6E+05	+	+	+	-	-
Sediment									
P3-05	sediment Ascotán 2005	781	9.2E+05	2.3E+06	+	+	+	-	-
P4-05	sediment Ascotán 2005	1210	1.6E+06	3.0E+06	+	+	+	-	-
P7-05	sediment Ascotán 2005	6504	2.4E+05	6.8E+05	+	+	-	-	-
P8	sediment Lag.Turquesa Ascotán 2006	nd	7.3E+02	5.1E+07	+	-	-	-	-
P9	sediment J1 Ascotán 2006	nd	7.9E+02	6.0E+07	+	-	-	-	+/-
V-10	sediment vertiente 10 Ascotán 2006	nd	3.2E+02	1.9E+08	+	-	+	-	-
V-6	sediment vertiente 6 Ascotán 2006	nd	2.3E+02	5.5E+07	+	+	+	-	-
P3-04	sediment Ascotán 2004	nd	nd	1.3E+06	+	+	+	-	-
P4-04	sediment Ascotán 2004	nd	nd	1.8E+06	+	+	-	-	+
P7-04	sediment Ascotán 2004	nd	nd	5.8E+05	+	+	+	-	-

nd: not determined; ng:no growth

+*: unspecific PCR products, +/- : weak band

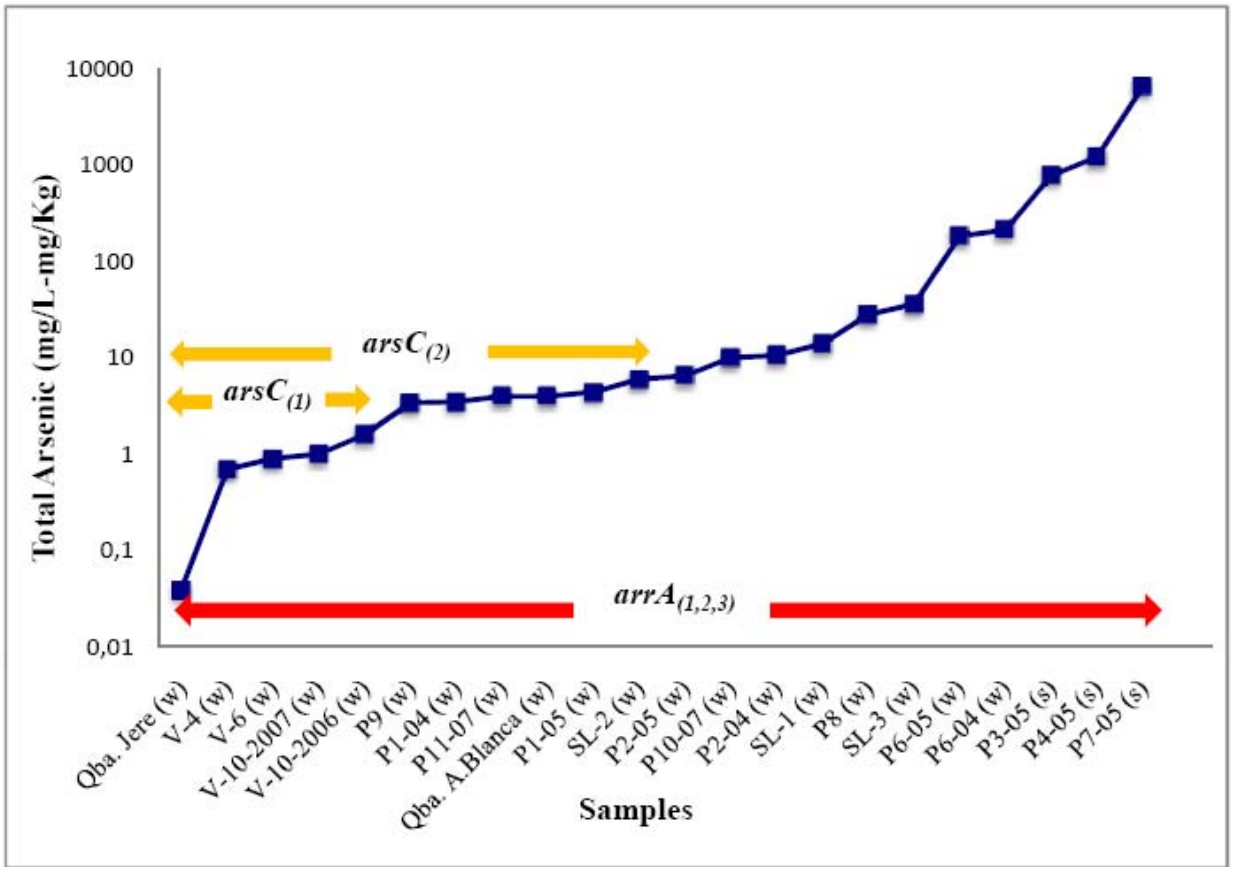

Figure 4. Presence or absence of As genes (*arrA* and *arsC*) in samples with increasing As concentration P4, P9 and P11 from Ascotán, SL-1 samples from of Tatio and QA from Quebrada Aguas Blancas in Atacama.

Total arsenic concentrations are towards the highest reported so far in aquatic environments. Thus, in Mono Lake arsenic concentrations are reported to be up to 0.2 mM (Kulp et al., 2006). In Cambodian aquifers, Shanyin County and Searles Lake arsenic concentrations are close to 0.17 mM, 0.01 mM, and 3.9 mM, respectively (Fan *et al.*, 2008, Oremland *et al.*, 2005).

Viable arsenic respiring bacteria

The reduction of As(V) has been observed in sediment and water samples (Oremland & Stolz, 2003). Total bacterial numbers in the water and brine samples analyzed in this work (DAPI counts) were within the normal ranges found in planktonic environments. Bacterial abundance in the sediments containing the highest As content, however, was two to three orders of magnitude lower than the normal values usually found in sediments (Whitman *et al.*, 1998). In addition, a large proportion of these total bacteria were retrieved as arsenic precipitating bacteria in MPN incubations. For instance, we observed that arsenic precipitating bacteria accounted for approximately half of total counts in four out of six samples. MPN data from Mono Lake (another environment rich an arsenic, but with lower As concentrations than those reported in Salar de Ascotan) showed that arsenic respiring bacteria (AsRB) were much lower than total bacterial counts, reaching only 0.001% (Oremland *et al*. 2000). In fact, MPN of specific physiological groups higher than 1% of total counts is an unusual fact (Simu *et al.*, 2005). Overall, data suggests that arsenic-based metabolism in the rich arsenic sediments of Salar de Ascotán is a very significant process (Chapter V), and that above certain levels of As concentration the bacterial diversity in the system is strongly reduced (Chapter II).

PCR amplification of functional genes

Malasarn *et al*. (2004) proposed that PCR amplicons of the *arrA* gene could be used as a simple molecular assay to detect the presence of AsRB and, as a consequence, of arsenic contamination. However, this had not been tested in a suitable range of environmental samples. In fact, all the PCR primers published, both those reported in Malsarn *et al*. (2004) and in the other references shown in Table 2, were developed and tested with pure or enrichment cultures and not in environmental samples. Adapting the protocols to such samples

with substantial amounts of heavy metals and other potentially PCR inhibiting agents, required additional purification and washing steps (see Materials and Methods). In the present work we developed a protocol that successfully retrieved the target genes from a large range of environmental samples. Thus, we were in a position to test the proposal of Malasarn *et al*. (2004) for the first time.

Our initial hypothesis was that we would find the *arsC* gene in most environments, since it is widely distributed in many different bacteria that are not necessarily adapted to high As concentrations, but the gene would allow these non-specialists to tolerate its presence. On the other hand, we expected to find the *arrA* gene only in environments with higher concentrations of As, since this gene is only present in specialized As respiring bacteria. Our results showed just the opposite: the *arrA* gene was found in all the environments tested in Atacama while the *arsC* gene was only found in the environments with lower As concentrations and this requires a different explanation. First of all, neither one of the genes was found in the Mediterranean sample, showing that the genes are not present everywhere, at least in abundance enough to be retrieved by the primers used. So their presence in Atacama must be related to the general abundance of As in the area. One possible explanation is that bacteria can disperse from one environment to another in the Atacama area with relative ease. Tolerant bacteria with the *arsC* gene would be able to grow only at low As concentrations and, thus, even if they arrived at environments with higher concentrations of As they would not be able to compete there. The bacteria possessing the arrA gene, on the contrary, could grow in both types of environments because most of them can use other electron acceptors besides As for respiration. Finally, the absence of the *arsC* gene from the high As environments indicates that the As respiring bacteria cannot be of the *Shewanella* ANA3 type that have both the detoxification and the As respiring operons. In agreement with this, both isolates from Ascotán described in Chapter VI had the *arrA* gene and lacked the *arsC* gene. Obviously clarifying the issue will require further studies.

In any case, the proposal of Malasarn *et al*. (2004) would have to be modified as follows: the presence of both *arsC* and *arrA* genes simultaneously would be an indicator of low concentrations of As, while the presence of *arrA* plus the absence of *arsC* would be an

indicator of high As concentrations. Finally, absence of both genes would be an indicator of no As in the samples.

Both MPN and positive gene amplifications point to Salar de Ascotán as the most promising system to study the ecology and biogeochemistry of As respiring bacteria and as a source of pure cultures with potential biotechnological or bioremediation applications. Thus, we decided to pursue more intense studies in this system presented in Chapters IV to VI.

Acknowledgements

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Capítulo IV

Diversity of arsenic reducing genes in Salar de Ascotán, a high altitude salt flat (3700 m)

Lorena V. Escudero, Emilio O. Casamayor, Cecilia Demergasso and Carlos Pedrós-Alió

Hay problemas que el saber no soluciona. Algún día llegaremos a entender que la ciencia no es sino una especie de variedad de la fantasía, una especialidad de la misma, con todas las ventajas y peligros que la especialidad comporta.
(El libro del Ello, George Groddeck)

Diversity of arsenic reducing genes in Salar de Ascotán, a high altitude salt flat (3700 m)

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Keywords: arsenic respiring microorganisms, arsenic genes, arsenate reductase, *arrA*, *arsC*

ABSTRACT

The diversity of two types of arsenate reducing genes (*arrA* and *arsC*) was studied in water and sediment from Salar de Ascotán by PCR amplification, cloning, and sequencing. Two representative samples were selected from a survey previously carried out along a natural As concentration gradient where the presence of arsenate reducing genes was studied. Sample P4-05 was collected from a sediment with one of the highest As concentrations reported so far, while P9-07 was a water sample with a relatively low concentration of As. Genes *arrA* (linked to a respiratory process) and *arsC* (linked to a detoxification process) were amplified with different primer sets and the performance of the primers was compared. Most clones from sample P9 produced unspecific sequences with *arrA* primers. The situation was more favorable in the case of sample P9 with *arsC* primers and sample P4 with *arrA* primers. Lower diversity of *arrA* genes was observed in the water than in the sediment sample. This could be related to the lower bacterial diversity found in previous studies with the 16S rRNA gene. In water *arsC* sequences were closely related to Gamma-proteobacteria. In the sediment most of the *arrA* sequences formed new branches distantly related to Firmicutes sequences. The high abundance of *arrA* genes found in the sediment was consistent with the prevailing anaerobic conditions *in situ*. In addition, we found a good match among several bacteria with known potential to respire arsenic (through 16S rDNA analysis presented in Chapter II) and the presence of As(V) reductase genes. Altogether, genet-

ic data indicated that arsenic respiring bacteria play a significant role in the arsenic biogeochemistry in Salar de Ascotán.

INTRODUCTION

Arsenic pollution of groundwater used for drinking and irrigation needs has become a severe worldwide problem in recent years, that affects the health of millions of people, especially in Bangladesh, India and China (Chowdhury, 2004, Sun *et al.*, 2004). In some areas this is a new problem linked to human activities that modify the natural arsenic cycle. In some others, in turn, such as in Northern Chile, it is a natural process that has occurred for very long times. It has been suggested that the indigenous Atacameño people might be protected from the poisonous effects of arsenic in drinking water because of the many centuries of exposure to this element. The best known and largest of these exposed areas is the village of San Pedro de Atacama, where part of the inhabitants drink water containing around 600 µg/L arsenic (Smith *et al.*, 2000).

It is reasonable to believe that the natural contamination of much of the water in the region has been present throughout this time and in fact, much longer before. Therefore, bacteria have actively participated in the arsenic cycle of this area contributing to the actual distribution and mobilization of As. However, studies focused on the microbes that potentially release arsenic from sediments to groundwater have not been carried out. In fact, only a few studies have analyzed As reducing genes in natural environments in the whole world (Hollibaugh *et al.*, 2006, Kulp *et al.*, 2006, Lear *et al.*, 2007, Song *et al.*, 2009).

These studies targeted the gene *arrA* that encodes the As(V) reductase involved in respiration. Degenerate PCR primers were designed to amplify a diagnostic region of the gene in multiple As(V)-respiring isolates (Malasarn *et al.*, 2004). These authors showed that *arrA* was required for As(V) reduction and that the gene was expressed in contaminated sediments from Haiwee Reservoir in California, U.S.A. In this study, the sequences retrieved showed between 62 and 97% identity to *arrA* sequences from *Bacillus selenitireducens*, *Chrysiogenes arsenatis*, and *Shewanella* strain ANA-3.

Arsenate reduction genes were also studied in two As-rich soda lakes in California (Kulp *et al.*, 2006). Mono Lake and Searles Lake are hyperalkaline (pH 9.8), hypersaline (90 g/L salts in Mono Lake and 360 g/L in Searles

Lake), and As-rich (As concentration are 0.20 mM in Mono Lake and 3.9 mM in Searles Lake). The amplified *arrA* signal was strongest in surface sediments and decreased to undetectable levels deeper in the sediments of both lakes. But the signal was detected at greather depth in Mono Lake, despite the higher arsenate reduction activity observed in Searles Lake. The diversity of *arrA* genes in these lakes indicated that there were unique ArrA phylotypes found only in one of the two lakes. The sequences retrieved in this study were also different from those retrieved from the water column of Mono Lake (Hollibaugh *et al*., 2006). In both studies it was not possible to determine which species were responsible for the *arrA* genes found.

Another study was carried out with enrichment cultures from anaerobic sediments collected from a Cambodian aquifer (Lear *et al*., 2007). The sequences retrieved showed amino acid sequence similarities to *arrA* genes from *Chrysiogenes arsenatis* and *Geobacter uraniumreducens* (Song *et al.,* 2009).

Our aim was to identify both bacteria and As-related genes with the potential to metabolize or transform As in sediment and water from Salar de Ascotán. As has been reported in previous chapters, chemical conditions (As concentration, pH and salinity) in this system are extreme and very different from the environments studied in the literature. This model system is a valuable source of microorganisms and genes that may be useful in the bioremediation of contaminated waters. In the present chapter we summarize the results of PCR studies of different As-reducing genes, while results from enrichment cultures and isolation of pure cultures are shown in Chapters V and VI.

MATERIALS AND METHODS

General overview on the aquatic systems studied

A summary of the geographical location, altitude and other parameters of the samples analyzed can be found in Chapters II and III. We chose two different samples from Salar de Ascotán for the present study: sediment sample P4 and water sample P9 (Table 1, Fig 1).

Sample collection and processing

Temperature and pH in water samples were measured with a pH meter Orion model 290, whereas for salinity, conductivity and total dissolved solids a conductivity meter Orion model 115 was used. Oxygen was measured with a

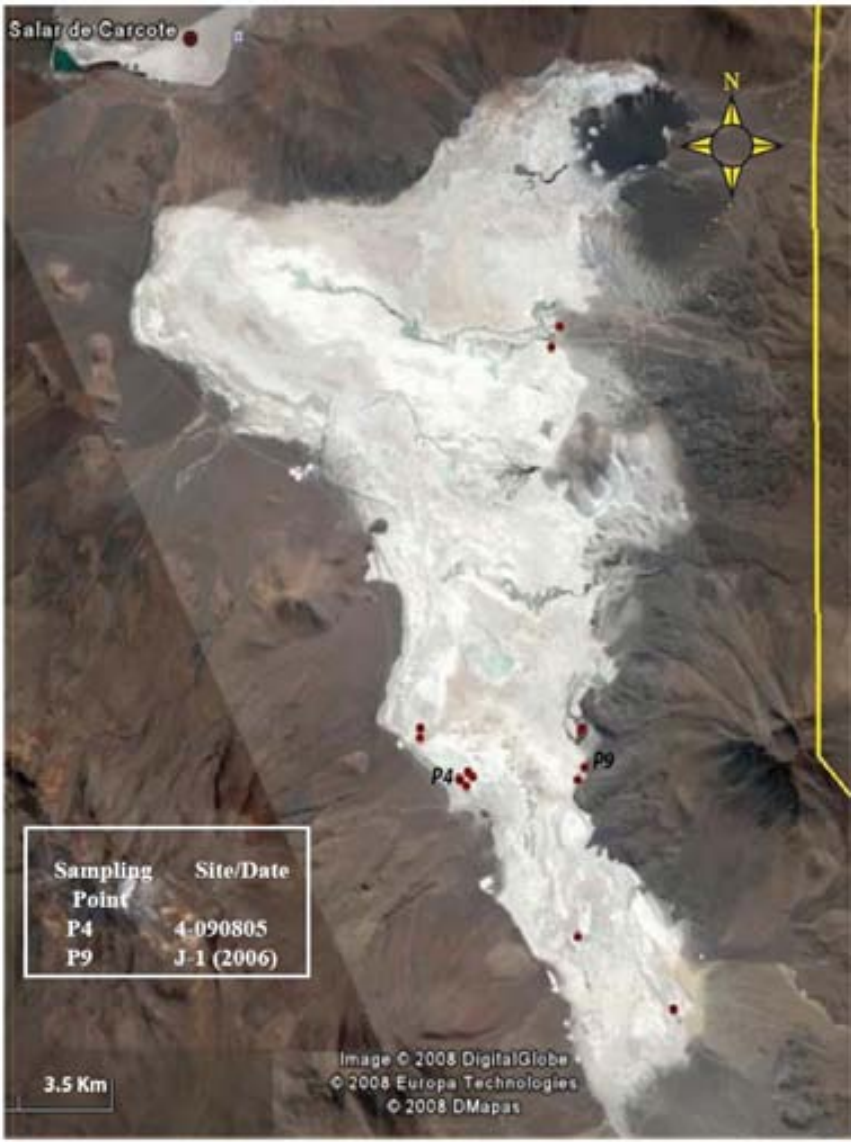

Figure 1. Map of Salar de Ascotán in Northern of Chile indicating sampling points P4 sediment and P9 water.

Table 1. Summary of field data for all samples from Salar de Ascotán. Samples selected for clone libraries were water sample P9 and sediment sample P4.

Date	*Sampling site*	*Sample type*	*Altitude (m)*	*UTM coordinates Grid 19K*		*pH*	*Salinity*	*Conductivity*	*STD*	*Temperature*	*Dissolved oxigen*	*Total As*
dd/mm/yy				North	East		g/L	mS	mg/L	ºC	mg/L	mg/L-mg/Kg
2004-2007	Ascotán*	water	3750	-		5.0-8.6	0.1-670.4	0.3-193.4	143-628,000	4.9-24.5	7.1-10.8	0.7-212
2004-2007	Ascotán*	sediment	3750	-		-	-	-	-	-	-	781-6504
09-08-2005	P4[a] Ascotán	sediment	3750	573669	7609035	-	-	-	-	-	-	1210
22-06-2006	P9[a] Ascotán	water	3750	577921	7609328	5.0	1.8	3.90	1750	0.0	8.9	3.4

[a] Selected for cloning of As functional genes

* Summary of values presented in Chapter II and III

Thermo Orion sensor model 9708. Water samples were transferred to 2 L polyethylene bottles and kept in an icebox until further processing.

Total DNA extraction

Between 800 and 1000 mL of water were filtered through 0.2 μm polycarbonate membranes (Nuclepore) and stored at −20 °C in 1 mL of lysis buffer (50 mM Tris–HCl pH=8.3, 40 mM EDTA and 0.75M sucrose). For sediments nucleic acids were extracted from 25 to 50 g (wet weight) of sediment, resuspended and vigorously shaked in a salt solution (1x PBS buffer, Tween 20 at 10% v/v) and the supernatant was filtered as previously described for water samples. Filters were incubated with lysozyme and proteinase K (Demergasso *et al.*, 2008), and genomic DNA was extracted with a High Pure Template Preparation Kit (Quiagen Duesseldorf, Germany). In order to purify and concentrate DNA solutions was carried out an additional step of ethanol precipitation.

PCR amplification of arrA and arsC genes

Several PCR-amplification runs with different primer combinations (see Table 2) were carried out in order to detect the presence of arsenate reducing genes in the different samples targeting two key genes in arsenate reduction (see Table 2, and a complete description of PCR amplification steps in Chapter III). For amplification of *arrA* we used three primer sets. The first primer set *arrA*1 was formed by *arrA*forward and *arrA*reverse (Malasarn *et al.*, 2004) to amplify a ~160-200 bp fragment of the arsenic respiratory gene. The second primer set named *arrA*2, used a nested PCR approach with primers AS1f, AS1r and AS2f (Lear *et al.*, 2007). Finally, the third primer set for gene *arrA* was HAArrA-D1f and HAArraA-G2r producing a 500 bp PCR product (Kulp *et al.*, 2006).

The *arsC* gene codes for a cytoplasmatic reductase that converts arsenate to arsenite. We targeted this gene using two primers sets (Table 2). The first primer set (*arsC1*) contained a mixture of primers amlt-42-f, amlt-376-r, smrc-42-f and smrc-376-r (Sun *et al.*, 2004). These primers amplified a fragment of 353 bp of the *arsC* gene. The second primer set (*arsC2*) contained primers QarsC-f1 and QarsC-r1 (Saltikov *et al.*, 2005). Presence of PCR amplification products was verified by electrophoresis in 1% agarose gels, stained with ethidium bromide. DNA obtained from a oligotrophic coastal environment free of As in the

Blanes Bay Microbial Observatory was used as negative control.

Cloning and sequencing arrA and arsC genes

PCR products were purified using a QUIAGEN PCR cleanup kit according to manufacturer instructions. Clone libraries were constructed using a TOPO TA cloning kit Catalog #4500-01 (Invitrogen Carlsbad, California). Between 80 and 140 clones were screened for the correct plasmid insert size and sent for sequencing to Macrogen (www.macrogen.com).

Phylogenetic analysis

Sequences were sent to BLASTX and BLASTN searches (http://www.ncbi.nlm.nih.gov) to determine the closest relative in the database. Multiple sequence alignment was performed using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). Nucleotide sequences were used for phylogenetic trees using neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. The trees were obtained using the MEGA 4 (http://www.megasoftaware.net) software with 1000 bootstrap replicates.

Results

Results of the survey of As-rich environments were presented in Chapter III. Two samples from Salar de Ascotán were chosen for clone library construction. These were chosen to maximize the potential diversity of genes retrieved. Thus, sample P4-05 was a sediment with one of the highest As concentrations (16.1 mM), while P9-07 was a water sample with a relatively low concentration of As(2.4 mM) (Table 1).Sample P9 produced amplicons for both genes, while sample P4 did not produce amplification for the *arsC* gene.

Clone libraries were built with these amplicons and results are summarized in Table 3. We experienced again the difficulties of working with natural samples from this region, that are highly saline and rich in heavy metals and other potential PCR inhibitors. Thus, only a fraction of the amplicons showed the expected insert size (see specific matches in Table 3). These were all sequenced. To check the specificity of the primers, amplicons sequences were translated to protein and blasted against the protein data base. The primers used had been designed with only a handful of available sequences from bacteria in pure cultures.

Table 2. Different primer sets used in this study for PCR amplification of several genes involved in the arsenic cycle.

Targeted gene	Primer set	Primer name	Primer sequence (5' – 3')	Amplicon lenght (bp)	Reference
Arsenate respiratory reductase	*arrA 1*	arrAf	AAG GTG TAT GGA ATA AAG CGT TTG TBG GHG AYT T	160-200	Malasarn *et al.* , 2004
		arrAr	CCT GTG ATT TCA GGT GCC CAY TY V GGN GT		
	arrA 2	AS1f	CGA AGT TCG TCC CGA THA CNT GG	625	Lear *et al* ., 2007
		AS1r	GGG GTG CGG TCY TTN ARY TC		
		AS2f (nested)	GTC CCN ATB ASN TGG GAN RAR GCN MT		
	arrA 3	HAArrA-D1f	CCG CTA CTA CAC CGA GGG CWW YTG GGR NTA	500	Kulp *et al* ., 2006
		HAArrA-G2r	CGT GCG GTC CTT GAG CTC NWD RTT CCA CC		
Arsenate reductase	*arsC 1 mix*	amlt-42-f	TCG CGT AAT ACG CTG GAG AT	334	Sun *et al.,* 2004
		amlt-376-r	ACT TTC TCG CCG TCT TCC TT		
		smrc-42-f	TCA CGC AAT ACC CTT GAA ATG ATC		
		smrc-376-r	ACC TTT TCA CCG TCC TCT TTC GT	~300	
	arsC 2	Q-arsC-f1	GAT TTA CCA TAA TCC GGC CTG T		Saltikov *et al.,* 2005
		Q-arsC-r1	GGC GTC TCA AGG TAG AGG ATA A		

It was not surprising, therefore, that several clones showed environmental sequences that were not related to the genes of interest. As detailed in Table 3 about 50% of the clones from sample P9 with *arrA* primers were unspecific. In contrast, most clones from sample P4 were specific. In the case of the primer sets for the *arsC* gene, no amplification was obtained from the sediment sample. The water sample produced a few clones, but all contained the target sequences (Table 3).

arrA gene diversity

Figure 2 B shows the proteins involved in the anaerobic respiratory arsenate reductase operon. *arrA* is a heterodimer periplasmic or membrane associated protein consisting of a larger molybdopterin subunit (ArrA) which contains an iron-sulfur center, probably a high potential [4Fe-4S] cluster, and a smaller [Fe-S] center protein (ArrB). In the present work, we scored as positive and specific PCR product when BlastX matched any of the protein domains associated with the anaerobic respiratory arsenate reductase operon.

Results from sediment sample P4 will be analyzed first. After PCR analysis, 102 clones (*arrA1*) and 43 clones (*arrA2*) were identified and sequenced. Up to 15% of the sequences obtained with *arrA1* primers were unspecific and were related to a TrK domain-containing protein or to a deoxyribodipyrimidine photolyase. No unspecific sequences were retrieved with the primer set *arrA2*. Most clones showed 65 to 71% aa identity clones from Mono Lake and Haiwee Reservoir (Table 4). The closest genes from isolated bacteria belong to arsenate respiratory reductase of *Chrysiogenes arsenatis* isolated from Ballarat Goldfields in Australia (Macy *et al.*, 1996), *Halanaerobiaceae bacterium* SLAS-1, an isolate from Searles Lake (California), and *Desulfosporosinus sp. Y5* an isolate of Onondaga Lake, New York.

Table 3. Total clones analyzed using four different primers set combinations in water and sediment samples from Salar de Ascotán. Specific and unspecific matches in the protein database (BlASTX) are indicated (number of clones and percentage of total sequenced clones) for each primer pair. Amplicon length in base pairs and translated aminoacids are shown. Targeted protein regions are expressed according to *Shewanella* ANA-3 numbering

Target gene	Primer Set	Amplicon length bp (aa lenght)	Position *Shewanella* ANA-3 (*arrA* protein 854 aa) (*arsC* protein 161 aa)	Water sample P9				Sediment sample P4			
Arsenate respiratory reductase				Total clones analyzed	Total clones sequenced	Specific matches* (%)	Unspecific match (%)	Total clones analyzed	Total clones sequenced	Specific matches* (%)	Unspecific match (%)
arrA 1	arrAf/arrAr	160-200 (40 -60)	300-350	98	21	11	10	140	102	87	15
						52%	48%			85%	15%
arrA 2	AS1f/AS1r/AS2f	625 (190-200)	160-350	63	20	9	11	47	43	43	0
						45%	55%			100%	0%
arrA 3	HAArrA-D1F/HAArrA-G2R	500 (110-150)	220-350	60	9	7	2	not attempted			
						78%	22%				
Arsenate reductase											
arsC 1	mixed primers set	334 (100-120)	31-148	60	9	9	0	no PCR product			
						100%	0%				

* arsenate respiratory reductase, molybdopterin oxidoreductase or iron-sulfur cluster binding protein were considered specific matches. *arsc2* did not porduce amplicons.

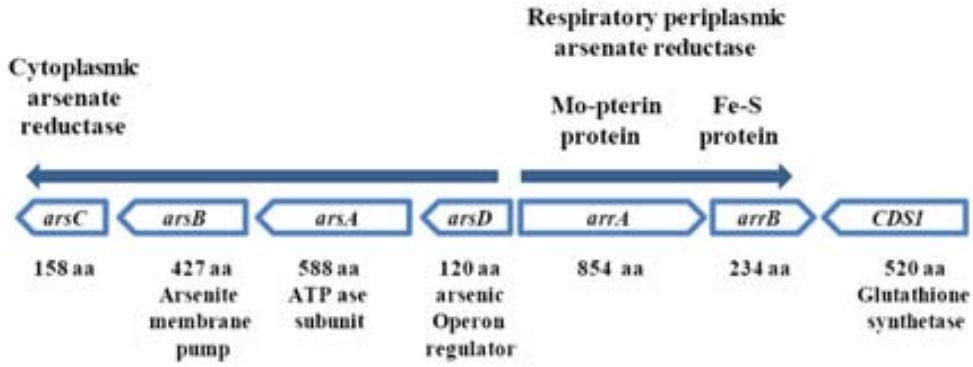

Figure 2B

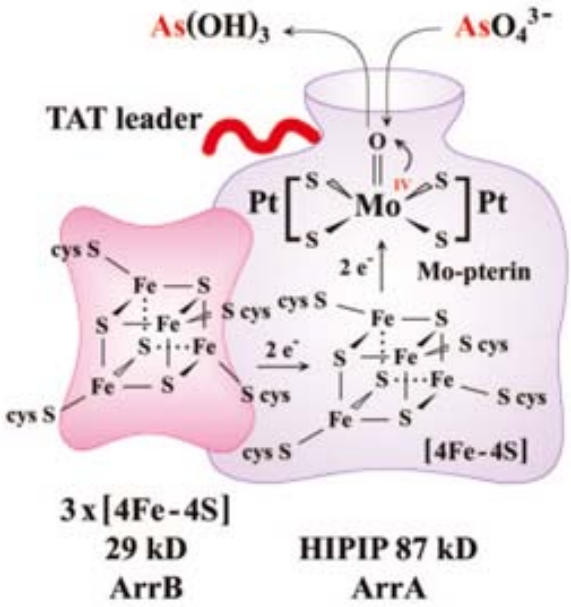

Figure 2. A. Genes for respiratory (*arrAB*) and cytoplasmatic (*arsDABC*) arsenate reductases of *Shewanella* sp. strain ANA-3. The two operons are divergently transcribed. Reproduced from Croal et al., (2004). **B**. Model for heterodimer arsenate reductase formed by proteins ArrA and ArrB. The funnel-shaped active site is show at the top. Also shown are embedded FeS cofactors, and amino acids (Cys or His) linking the [Fe-S] cofactors to the polypepetides. HIPIP, high potential iron protein (from Silver & Phung, 2005).

Using for the primer set *arrA2*, 15 clones showed 75% to 80% aa identity with arsenate respiratory reductase a clone from Mono Lake, and 28 clones showed 80% aa identity with arsenate reductase of *Halorhodospira halophila* SL1, a bacterium isolated from a salt lake mud (Imhoff & Suling, 1996). This sample did not produce amplicons with primer set *arrA3*, but the amount of DNA available for the analysis was probably too low and we did not have additional samples for further tries.

The *arrA* PCR products from sample P9 with the three primer sets were also cloned to construct three *arrA* clone libraries. After PCR analysis, 21 (*arrA1*), 20 (*arrA2)* and 9 (*arrA3*) clones were obtained with the right size of insert (Table 3). 48% of the sequences from *arrA1* in P9 were unspecific and related to acetate kinase, DNA topoisomerase I, histidine ammonia-lyase, tyrosyl-RNA synthetase and phosphopantothenate synthase (Table 5). Other clones showed 56% to 70% aa identity with arsenate respiratory reductase of *Sulfurospirillum barnessi* isolated from a selenate contaminated freshwater marsh in western Nevada (Stolz *et al.*, 1999), 50% aa identity with *Bacillus* sp. Rice-C isolated from soils of Bangladesh (S.P. Bachate, unpublished), and with the same microorganisms previously found in sample P4: *Halanaerobiaceae bacterium* SLAS-1, *Chrysiogenes arsenatis* and an environmental sequence from Haiwee Reservoir. Four sequences showed 59% identity with molybdopterin oxidoreductase of *Wolinella succinogenes* DSM1740 isolated from bovine rumen fluid (Herbel, 2002), and one sequence with *Alkalilimnicola ehrlichei* MLHE-1 (Oremland *et al*., 2002). With primer set *arrA2*, 55% of the sequences were unspecific and related to phospolipase C, histidyl-tRNA synthetase, translation initiation factor IF-2, recombination factor protein RarA, inner membrane translocator and hypothetical proteins (Table 5). The remaining sequences showed 67% identity with molybdopterin oxidoreductase of *Alkalilimnicola ehrlichei* MLHE-1 (Oremland *et al*., 2002) or 55% to 65% identity with iron sulfur cluster binding proteins from *Pseudomonas putida* GB-1, *Pseudomonas mendocina* and *Pseudomonas fluorescens* Pf01 isolated from soil and water samples. Primer set *arrA3* retrieved only nine clones; seven showed 65-75% aa identity with arsenate respiratory reductase of *Chrysiogenes arsenatis* or with an environmental sequence from Mono Lake, and the other two showed unspecific match with hypothetical proteins of Bacteroidetes.

Table 4. Closest match in protein and nucleotide database obtained after a BLASTX and BLASTN with DNA sequences obtained after cloning sample P4 from Ascotán sediment with primers set arrA1 and arrA2. Arsenate respiratory reductase domain. Cluster number are the same shown in the phylogenetic tree in Fig. 3.

Cluster	Closest relative GenBank	% Similarity	Total clones	Accesion number	Closest cultured relative	E value
				arrA1		
I	clone ML_C10 (Mono Lake)	85-87	1	DQ858368	*Halanaerobiaceae bacterium* SLAS-1	5E-04
	clone ML_A10(Mono Lake)		1	DQ858358	*Chrysiogenes arsenatis*	5E-04
	clone ML_G11(Mono Lake)		1	DQ858386		
IIA	clone HRR19 (Haiwee Reservoir)	87-90	3	AY707767	*Desulfosporosinus sp.* Y5	2e-05 to 2E-09
	clone HRR22 (Haiwee Reservoir)		1	AY707768	*Chrysiogenes arsenatis*	5E-09
	clone HRR23 (Haiwee Reservoir)		1	AY707770	*Desulfitobacterium hafniense* Y51	2E-06
IIB	clone HRR1 (Haiwee Reservoir)	75-84	2	AY707757	*Chrysiogenes arsenatis*	5E-07
	clone HRR4 (Haiwee Reservoir)		1	AY707760	*Sulfurospirillum barnesii*	5E-04
	clone HRR5 (Haiwee Reservoir)		3	AY707761	*Halanaerobiaceae bacterium* SLAS-1	3E-07 to 8E-07
	clone HRR6 (Haiwee Reservoir)		1	AY707762		
	clone HRR17 (Haiwee Reservoir)		2	AY707766		
	clone HRR19 (Haiwee Reservoir)		4	AY707767		
	clone HRR23 (Haiwee Reservoir)		1	AY707770		
	clone HRD9 (Haiwee Reservoir)		1	AY707754		
	clone ML44 (Mono Lake)		13	DQ155340		
IIC	clone ML44 (Mono Lake)	75-84	13	DQ155340	*Chrysiogenes arsenatis*	3E-07 to 8E-07
					Halanaerobiaceae bacterium SLAS-1	2E-06
III	clone ML32 (Mono Lake)	68-72	1	DQ155361	*Chrysiogenes arsenatis*	
	clone ML_F12 (Mono Lake)		3	DQ858383	*Desulfitobacterium hafniense* Y51	3E-07 to 8E-07
	clone HRR23 (Haiwee Reservoir)		1	AY707770		
	clone SL_S2B9 (Searles Lake)		3	DQ858426		
	clone ML_E11 (Mono Lake)		1	DQ858378		
IV	clone HRR19 (Haiwee Reservoir)	87-90	2	AY707767	*Chrysiogenes arsenatis*	5E-04
	clone HRR1 (Haiwee Reservoir)		1	AY707757		
V	clone HRR20 (Haiwee Reservoir)	87-90	1	AY707768	*Chrysiogenes arsenatis*	5E-04
	clone HRR22 (Haiwee Reservoir)		1	AY707769	*Desulfitobacterium hafniense* Y51	5E-04
	clone HRR19 (Haiwee Reservoir)		1	AY707767	*Alkaliphilus oremlandii* OhILAs	5E-08
	Halanaerobiaceae bacterium SLAS-1		1	EU723191	*Halanaerobiaceae bacterium* SLAS-1	5E-08
	Chrysiogenes arsenatis		1	AY660883		
VI	clone HRR19 (Haiwee Reservoir)	90	1	AY707767	*Chrysiogenes arsenatis*	5E-09
					Desulfosporosinus sp. Y5	5E-09
VII	clone HRR1 (Haiwee Reservoir)	81	2	AY707757	*Chrysiogenes arsenatis*	5E-08
					Bacillus selenitireducens MLS10	1E-09
VIII	clone HRR4 (Haiwee Reservoir)	78-93	3	AY707760	*Chrysiogenes arsenatis*	2E-05
	clone HRR5 (Haiwee Reservoir)		1	AY707761	*Bacillus arseniciselenatis*	1E-05
					Bacillus selenitireducens MLS10	1E-05
				arrA2		
	clone ML-C10 (Mono Lake)	75-80	15	DQ858368	*Halanaerobiaceae bacterium* SLAS-1	6E-61 to 1E-68
	Halorhodospira halophila SL1	80	28	CP000544	*Halorhodospira halophila SL1*	1E-74 to 8E-85

Figure 3 shows the phylogenetic relationships for most of the sequences of *arrA* obtained in this study in P4 (n=66) and P9 (n=7) samples.

The sequences were approximately 110 bp long. Selected *arrA* sequences from Mono Lake, Haiwee Reservoir and several other *arrA* sequences from the strains *Desulfosporosinus* sp. Y5, *Shewanella* sp. HAR-4, *Bacillus arseniciselenatis*, *Chrysiogenes arsenatis*, *Alkalilimnicola halodurans* and *Alkalispirillum mobile* are also shown in the tree. The *arrA* sequences we retrieved from sample P4 and P9 clustered in at least eight different groups. The first cluster included two sequences from water and one from sediments and was related to environmental sequences from Mono Lake. It was rather distantly related to *Alkalilimnicola halodurans* and *Alkalispirillum mobile.* Cluster II included the most sequences and all were from the sediments, but most sequences were closer to environmental clone from Mono Lake ML44. Three subclusters could be distinguished, all with a substantial number of clones, indicating that this was a very important group in Ascotán obviously containing substantial novel diversity.

Cluster III included sequencesfrom both water and sediment and, again was only distantly related to an environmental clone from Mono Lake. Cluster IV included sequences only from the sediment sample.

Cluster V included had sequences from both samples. It was distantly related to the Firmicutes *Desulfosporosinus* sp.Y5 and *Chrysiogenes arsenatis*. Clusters VI and VII included only sediment sequences and were relatively close to a cluster of sequences obtained from Haiwee Reservoir and Mono Lake. The last group (Cluster VIII) included only sediment sequences and was relatively close to a cluster of sequences obtained from Haiwee Reservoir and Mono Lake plus *Bacillus arseniciselenatis*.

arsC gene diversity

All the *arsC* gene products from sample P9 with the primer set arsC1 showed 90% to 98% aa identity with arsenate reductases of *Klebsiella pneumoniae*, *Acidiphilum multivorans* and *Serratia marcescens* (Table 5). The primer set *arsC2* did not produce amplicons. As mentioned before, no amplicons were obtained for the *arsC* gene in sample P4. Figure 4 shows the phylogenetic relationships of the *arsC* sequences obtained in this study, together with selected *arsC*

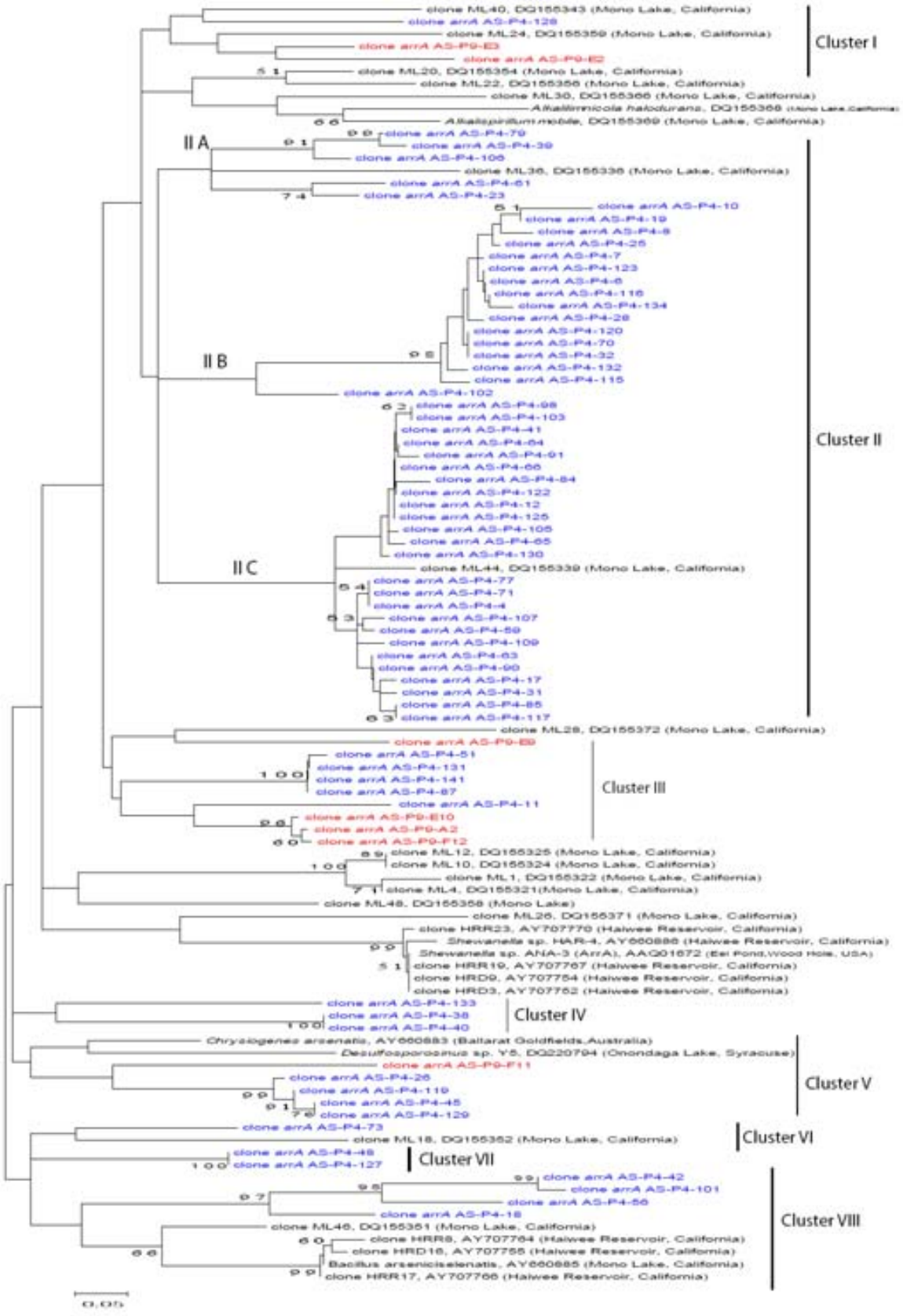

Figure 3. Phylogenetic relationships based on partial *arrA* sequences retrieved from water (red) and sediment (blue) samples from Salar de Ascotán. The tree was constructed by neighbor-joining. Bootstrap values for 1000 replicates are indicated at the nodes.

sequences obtained from *Klebsiella pneumoniae*, *Acidiphilum multivorans*, *Serratia marcescens* and other environmental sequences from As polluted soils and marine environments. One sequence was related to the alphaproteobacterium *Acidiphilum multivorum.* All the remaining sequences were essentially identical forming a cluster related to the gammaproteobacteria *Serratia marcescens* and *Klebsiella pneumoniae*.

Discussion

In Chapter II we analyzed the bacterial diversity (by construction of 16S rDNA clone libraries) of the same samples analyzed here. Thus, it would be of interest to try to match the arsenic reducing genes found here with the taxa retrieved in Chapter II. One difficulty is that the number of sequences of arsenic reducing genes from cultured bacteria is very limited. Another difficulty is that the phylogeny of functional genes does not exactly match the phylogeny of highly conserved ribosomal genes. For instance, in previous studies in Mono Lake and Searles Lake it was not clear with what bacterial species the *arrA* genes found affiliated (Hollibaugh *et al*. 2006). Finally, the As-reducing protein has several domains in common with other enzymes, for example the molibdopterin domain is found in many different enzymes. Therefore, this taxonomic assignment has to be attempted with caution.

The most abundant groups by 16S rDNA were the Firmicutes (about 50% of the clones) and the Gammaproteobacteria (15 to 20%), both in the water and in the sediment samples. *arrA* genes were very closely related to environmental clones from Mono Lake and Haiwee Reservoir. The similarity in amino acid sequence was very high (68 to 93%) in all cases. The similarity to isolates that could be taxonomically assigned was lower but still highly significant (see E values in Table 4). Most clusters retrieved with primer set *arrA1* (clusters I to VI) showed significant similarities to *Halanaerobium* (Firmicutes) and to *Chrysiogenes*. The latter organism forms its own phylum and was isolated from a reed-bed next to a gold field in Australia. The organism can grow respiring arsenate and has the arrAB operon. Thus, in principle, it could be a good candidate. However, no sequences close to *Chrysiogenes* were retrieved in the 16S rDNA analysis. On the other hand, Firmicutes of the Halanaerobiales order were the most abundant group of clones retrieved.

Table 5. Closest match in proteins database obtained after a BlastX search with the DNA sequences obtained after cloning sample P9 from Ascotán water column amplified with primers sets *arrA1* , *arrA2* , *arrA3* , and *arsC1* .

Protein	*Total Clones*	*Closest match*	*Phylogenetic group*	*E value*	*Accession Number*
		arrA1			
arsenate respiratory reductase	1	*Sulfurospirillum barnesii*	Epsilonproteobacteria	2E-09	AAU11840
	1	*Halanaerobiaceae* bacterium SLAS-1	Bacteria	1E-19	ACF74513
	1	*Chrysiogenes arsenatis*	Chrysiogenales	5E-15	AAU11839
	1	clone HRD4 (Haiwee Reservoir)	Bacteria	4E-14	AAU81544
	2	*Bacillus* sp. Rice-C	Firmicutes	7E-06 to 1E-07	CAQ48291
molybdopterin oxidoreductase	4	*Wolinella succinogenes* DSM 1740	Epsilonproteobacteria	5E-10 to 9E-19	
	1	*Alkalilimnicola ehrlichei* MLHE-1	Gammaproteobacteria	7E-09	NP_906980
					YP_741061
unspecific					
acetate kinase	3	*Flavobacterium* sp. MED217	Bacteroidetes	5E-12 to 6E-13	ZP_01061172
	2	*Polaribacter irgensii* 23-P	Bacteroidetes	2E-13 to 7E-13	ZP_01119205
	1	*Geobacter uraniireducens*	Deltaproteobacteria	2E-12	YP_001231404
DNA topoisomerase I	1	*Pseudomonas fluorescens* Pf-5	Gammaproteobacteria	8E-54	YP_261755
histidine ammonia-lyase	1	*Pseudomonas fluorescens* Pf0-1	Gammaproteobacteria	1E-78	YP_346098
tyrosyl-tRNA synthetase	1	*Campylobacter curvus* 595.92	Epsilonproteobacteria	4E-46	YP_001407884
phosphopantothenate synthase	1	*Pseudomonas fluorescens* Pf0-1	Gammaproteobacteria	2E-10	YP_351268
		arrA2			
molybdopterin oxidoreductase	1	*Alkalilimnicola ehrlichei* MLHE-1	Gammaproteobacteria	9E-69	YP_741061
iron-sulfur cluster binding protein	2	*Pseudomonas putida* GB-1	Gammaproteobacteria	1E-11 to 8E-14	YP_001671172
	1	*Pseudomonas mendocina* ymp	Gammaproteobacteria	5E-18	YP_001186129
	5	*Pseudomonas fluorescens* Pf0-1	Gammaproteobacteria	4E-03 to 2E-64	YP_346250
unspecific					
phospholipase C	3	*Pseudomonas fluorescens* strain MFN1032	Gammaproteobacteria	6E-04 to 4E-49	ABE73153
	1	*Pseudomonas fluorescens*	Gammaproteobacteria	6E-33	CAC18568
histidyl-tRNA synthetase	1	*Pseudomonas fluorescens*	Gammaproteobacteria	5E-05	YP_262033
translation initiation factor IF-2	1	*Alkaliphilus oremlandii*	Firmicutes	1.0E-5	YP_001513067
recombination factor protein RarA	1	*Pseudomonas mendocina*	Gammaproteobacteria	8E-01	YP_001187874
inner-membrane translocator	1	*Pseudomonas syringae* pv. B728a	Gammaproteobacteria	5.0E-48	YP_237496
hypothetical protein PflO1_0266	2	*Pseudomonas fluorescens* Pf0-1	Gammaproteobacteria	5.0E-09 to 2.0E-10	YP_345999
hypothetical protein PFL_0282	1	*Pseudomonas fluorescens* Pf-5	Gammaproteobacteria	1.0E-22	YP_257428
		arrA3			
arsenate respiratory reductase	4	*Chrysiogenes arsenatis*	Chrysiogenales	2.0E-29 to 4.0E-50	AAU11839
	1	clone ML-A3 (Mono Lake)		3.0E-41	ABJ53055
	1	clone ML-H12 (Mono Lake)		3.0E-45	ABJ53085
	1	clone ML-H7 (Mono Lake)		1.0E-48	ABJ53086
unspecific					
hypothetical protein BACCAP_03832	1	*Bacteroides capillosus* ATCC29799	Bacteroidetes	1.0E-13	ZP_02038208
hypothetical protein RUMGNA_02974	1	*Ruminococcus gnavus* ATCC 29149	Firmicutes	2.6E-01	ZP_02042184
		arsC1			
arsenate reductase	7	*Klebsiella pneumoniae*	Gammaproteobacteria	9.0E-59	YP_001965822
	1	*Acidiphilium multivorum*	Alphaproteobacteria	5.0E-59	BAA24824
	1	*Serratia marcescens*	Gammaproteobacteria	1.0E-50	NP_941245

It seems safe to conclude that Firmicutes of the *Halanaerobium* type were a very abundant group carrying out arsenic oxidation in the sediments of Salar de Ascotán. Clusters VII and especially cluster VIII were more closely related to *Bacillus arseniciselenatis* and *Bacillus selenitireducens*. Since no 16S rDNA clones related to these *Bacillus* species were found, the assignment of these two clusters must remain hypothetical. With primer set *arrA2* we found two groups of clones. One was again related to *Halanaerobium*, giving further support to the importance of this group in the sediments. The other cluster of *arrA* genes (28 clones) was closest to a *Halorhodospira halophila*, an alkaliphilic Gammaproteobacterium from hypersaline environments. The Gammaproteobacteria were, in effect, the second most abundant group in the 16S rDNA library from the sediments. The clones found were closest to environmental clones from hypersaline environments. The closest cultured representative, however, was precisely *Halorhodospira halophila* (see Figure 4 in Chapter II).

Thus, the two most abundant groups both in the 16S rDNA and the *arrA* libraries were *Halanaerobium* and *Halorhodospira*-like. This coincidence is compatible with the idea that the most abundant members of the community of Ascotán sediments were able to respire arsenic. This would also be consistent with the extremely high proportion of the total DAPI counts recovered as MPN of AsRB shown in Chapters III and V.

Two other groups within the Proteobacteria were abundant in the 16S clone libraries: Epsilonproteobacteria were very abundant in the water sample and Deltaproteobacteria in the sediment sample. But no *arrA* sequence could be ascribed to either of these two groups. Thus, it seems fair to conclude that Epsilon- and Deltaproteobacteria carry out other important functions in the ecosystem, but are not involved in As processing. The Epsilonproteobacteria sequences retrieved were closest to *Desulfospirillum*, a sulfur reducing anaerobic bacterium, while those of Deltaproteobacteria were closest to several sulfate reducing genera. Thus, these two groups may be responsible for a large part of sulfur cycling in Salar de Ascotán.

A second point of interest is the comparison of the sequences from Salar de Ascotán with those from other As-rich systems. There are only two natural environments where a similar study has been carried out: Lakes Mono and Searles and Haiwee Reservoir in the U.S.A. Mono and Searles Lakes are

hypersaline and alkaline, while Salar de Ascotán has a slightly acidic pH. Nevertheless, the sequences from Ascotán were always closest to environmental sequences from these two lakes, especially Mono Lake, suggesting that high As concentration may be a more important factor than pH or salinity in determining the composition of the bacterial assemblage.

In several cases, the Ascotán sequences formed their own clusters separated from those from Mono Lake (eg.: cluster II in Figure 3), indicating that the microbiotas of the two systems are similar but not identical. Moreover, these clusters showed a large degree of microdiversity. This structure was also observed in the 16S rDNA libraries presented in Chapter II. Diversity at a large scale (i.e.: phylum or family) was relatively low, but many different clusters with considerable microdiversity were found within the few phyla represented.

A final comparison is that between the water and the sediment sample. It is worth noting, that in all cases the clusters present in the water were different from those in the sediments (see for example, cluster III in Figure 3). This is the same pattern found with the 16S rDNA libraries (Chapter II). The diversity of both 16S and *arrA* genes was much lower in the water than in the sediments, adding some degree of robustness to the overall picture derived from this study. And the *arsC* gene could only be amplified from the water sample. This is in agreement with the results in Chapter III, where we found *arsC* to be retrievable only from systems with As concentrations lower than approximately 4 mg/L (like our water sample), but not from systems with higher concentrations (such as the sediment sample). Thus, retrieval of a large number of *arrA* clones but no *arsC* clones from the sediment is consistent with the extremely high concentration of As found in this sample.

In summary, we have shown that As concentration is a major factor in determining the diversity of bacteria in these hypersaline systems. A unique microbiota seems to be well adapted to these conditions and is composed of only a few major groups but with a rich microdiversity structure.

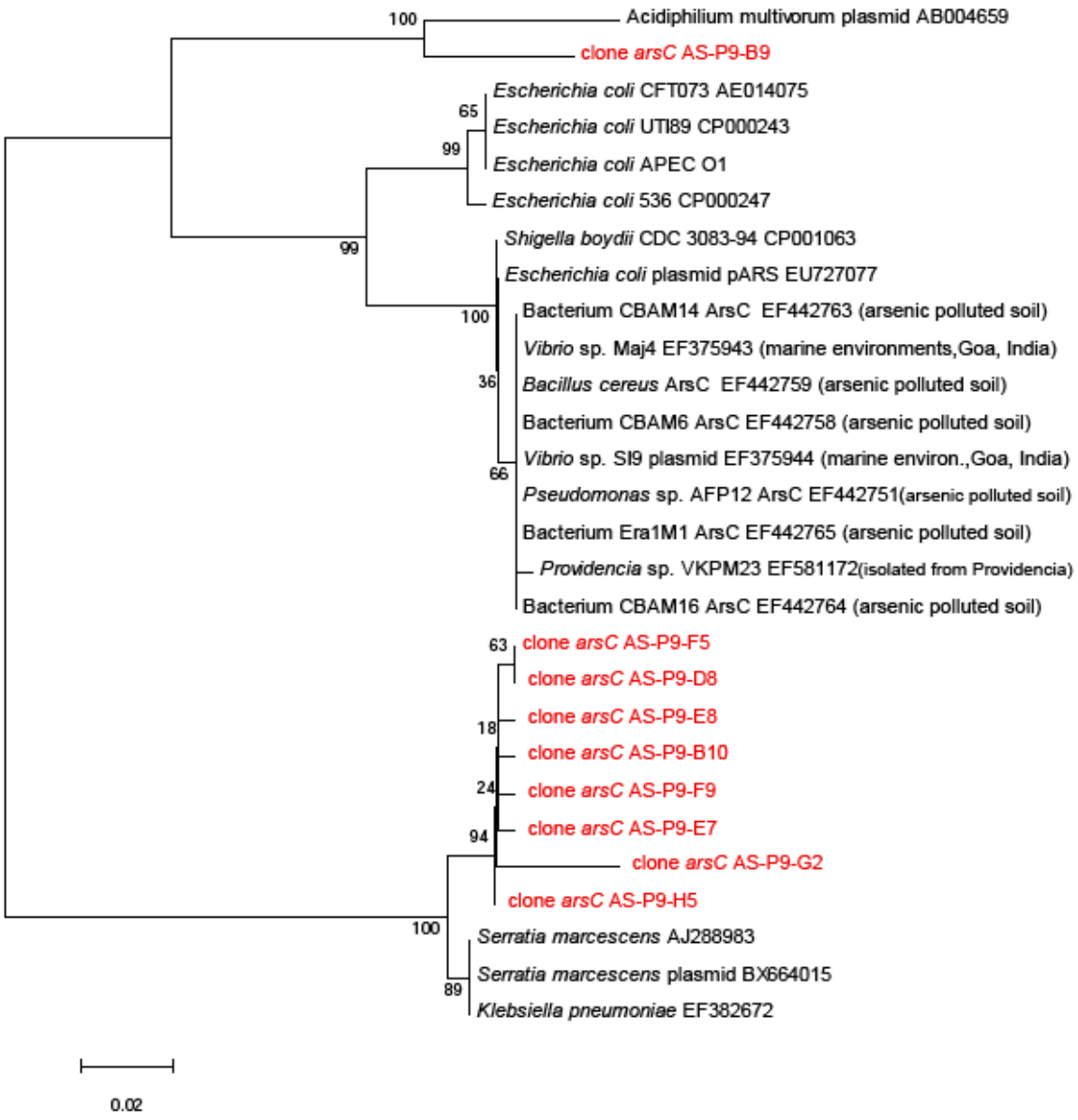

Figure 4. Phylogenetic relationships based on partial *arsC* sequences retrieved from water sample P4 from Salar de Ascotán. The tree was constructed by neighbor-joining. Bootstrap values for 1000 replicates are indicated at the nodes.

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Capítulo V

Microbial precipitation of arsenic sulfides in Andean salt flats

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Geomicrobiology Journal, vol 24, pp 1-14

La candidata Lorena Escudero ha participado activamente en los experimentos descritos en dicha publicación responsabilizándose de los trabajos de Microbiología (aislamiento y cultivo de microorganismos anaerobios), Biología Molecular (extracción de ADN, amplificación por PCR, electroforesis desnaturalizante DGGE) y análisis filogenético de secuencias, Microscopía óptica y electrónica (SEM y TEM), así como en la discusión de los planteamientos experimentales y los resultados obtenidos.

Estamos unidos al inconsciente colectivo. A cualquier acción que cometamos, aunque sea anónima, el mundo le da una respuesta. Lo que hacemos a los otros, nos los hacemos a nosotros mismos.
(LaDanza de la realidad, Alejandro Jodorowsky)

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Microbial Precipitation of Arsenic Sulfides in Andean Salt Flats

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An abiotic origin has traditionally been assumed for the arsenic minerals realgar and orpiment associated with thermal springs. Microbial precipitation of arsenic, however, has been studied in pure cultures and the isotopic composition of arsenic sulfides associated with some borate deposits suggests a biotic origin for those minerals. The aim of the present study is to demonstrate the role of bacterial arsenic precipitation in the biogeochemical cycle of arsenic in such borate deposits. For this purpose both enrichment and pure cultures were obtained from the natural arsenic minerals and the composition and isotopic signatures of the arsenic sulfide minerals precipitated by the cultures and those associated with boron deposits from an Andean salt flat in northern Chile were compared. Based on the microbiological and chemical evidence gathered, it is concluded that bacteria contributed to the formation of the arsenic minerals. This interpretation is based on the consistent association of a variety of features that strongly indicate microbial involvement in the precipitation process. These include: (1) enrichment and isolation of cultures with arsenic precipitation capacity from arsenic mineral samples, (2) high numbers of arsenic-precipitating bacteria in the Andean minerals and brines, (3) chemical and mineralogical properties of precipitates experimentally formed under biotic and abiotic conditions, (4) similarities in stoichiometry between natural and laboratory obtained minerals, and (5) the consistent depletion in δ^{34}S values for natural versus laboratory obtained sulfides. Thus, microbial precipitation of arsenic sulfides is a geochemically relevant metabolism.

Keywords arsenic respiring microorganisms, sulfate reducing bacteria, borate deposits

INTRODUCTION

Determining how organisms that utilize or produce minerals may have influenced the chemical and physical features of the planet in the past, and how they continue to do so in the present, is a major challenge for biogeochemistry (Newman 2004). Many types of sedimentary minerals, formerly believed to have an inorganic origin, are now thought to have a complex origin with microbial participation. Progress has been made possible by the combination of two lines of research: Understanding microbial metabolisms involving minerals with pure cultures in the laboratory, and culture-independent analysis of genetic data that allow the description of microbial populations in situ (Macalady and Banfield 2003).

Recently, it has been reported that deep subsurface marine sediments contain a high number of living bacteria with turnover times comparable to those in surface environments (0.25–22 years) (Schippers et al. 2005). On longer time scales, isotopic signatures have evidenced microbial sulfate reduction activity at 3.47 Ga, indicating that this trait evolved early in Earth's history

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(Shen et al. 2001). Therefore, microbial activities relevant in mineralization processes are present in appropriate sedimentary environments and have been present almost since the origin of life. In addition, bacterial reduction of Mn and Fe, for example, has been established to account for a significant turnover of organic carbon in many environments (Heidelberg et al. 2002; Nealson et al. 2002). Among the elements recently shown to have a biological cycle are uranium (Lovley 2001), gold (Kashefi et al. 2001; Karthikeyan and Beveridge 2002) and arsenic (Oremland and Stolz 2003).

Arsenic is widely distributed in nature despite its low crustal abundance (0.0001%) and is commonly associated with the ores of metals such as copper, lead and gold, due to its chalcophilic character (Nriagu 2002). These natural arsenic sources are of utmost concern to human health on a global scale (Ball 2005). Anthropogenic point sources like smelter gas emissions and slag also contribute to arsenic found in the environment. Arsenic mobilization from aquatic sediments has been reported to involve a combination of chemical, physical, and microbial factors both in the laboratory and natural environments (Ahmann et al. 1997; Harrington et al. 1998; Ahmann 2002; Redman et al. 2002; Meng et al. 2003).

Bacteria have been identified that can oxidize and/or reduce arsenic compounds for energy conservation. These bacteria are present in many environments, including both aerobic and anaerobic water bodies and sediments (Robertson et al. 2000). Their activities are believed to mobilize and/or precipitate arsenic in the environment and, therefore, their metabolism may be important in detoxification and bioremediation as well as in contamination processes. Thus, interest in the arsenic cycle is clearly justified.

Northern Chile, as a part of the Andean Range, is an excellent place to study microbial implication in the arsenic geochemical cycle. First, ground waters are relatively rich in arsenic due to the volcanic-hydrothermal provenance of this element. As a consequence, arsenic is also present in river sediments, where some of the microbial reactions using arsenic oxianions for energy generation may mobilize arsenic from the solid to aqueous phases, resulting in contaminated drinking water sources. Contamination episodes have taken place in the River Loa with increased arsenic levels (Arroyo et al. 1999). Microbial arsenic mobilization activity has been also found in the arsenic contaminated sediments of the River Loa (Demergasso et al. 2003).

There are extensive salt flats in the Andes due to geomorphology and climate. These conditions include geothermal processes and evaporation of water rich in arsenic and other compounds. These salt flats contain significant borate deposits (mostly in the form of ulexite) that include As-rich red and yellow nodules and lenses, some centimeters thick and a few meters in diameter (Chong et al. 2000). These nodules are rich in the arsenic minerals realgar and orpiment. We have observed these features in Salar de Surire and Salar de Ascotán within the whole ulexite ore profile.

In the Salar de Atacama, ulexite appears in 50-cm-thick layers, related to calcium sulfates, in its eastern and northeastern edges. Such minerals are also common in equivalent environments on the Argentinian side of the Andes. (Alonso 1986). Moreover, major boron ore deposits present similar mineral assemblages and genesis around the world, such as in the U.S.A. (Tanner 2002) or in Turkey (Helvaci 1995; Helvaci and Orti 1998; Helvaci and Orti 2004). For this reason a similar biogeochemical origin would be expected for all of them. The Salar de Ascotán will be used as a model for these systems.

A number of bacteria are known to process arsenic through different metabolic pathways (Robertson et al. 2000). There are now over two dozen species of prokaryotes that are capable of conserving energy by linking the oxidation of an electron donor (either organic or inorganic) to the reduction of As(V) to As(III) (Oremland and Stolz 2003; Liu et al. 2004; Oremland et al. 2005). *Desulfosporosinus auripigmentum* (Newman et al. 1997; Labeda 2000; Stackebrandt et al. 2003), isolated from lake sediments in USA, and *Desulfovibrio* strain Ben-RB isolated from mud obtained from an arsenic-contaminated reed bed in Bendigo, Australia (Macy et al. 2000), are able to reduce As(V) to As(III) and S(VI) to S(-II).

Desulfosporosinus auripigmentum (Newman et al. 1997; Labeda 2000; Stackebrandt et al. 2003) precipitates arsenic trisulfide (As_2S_3) as a result of the reduction of both As(V) and S(VI). Cultures of *Desulfovibrio* strain Ben-RA (Macy et al. 2000) tended to precipitate As(V) as yellow arsenic sulfide (As_2S_3) even though the As reduction was not associated with energy conservation.

Other arsenic-reducing bacteria cannot precipitate As_2S_3 because they do not reduce S(VI), or because they do not reduce both As(V) and S(VI) to appropriate concentrations of As(III) and S(-II) (Newman et al. 1997; Macy et al. 2000). All these studies have been carried out with pure cultures in the laboratory. However, the isotopic composition of some arsenic sulfides associated to boron deposits in Turkey suggested the involvement of microbial sulfate reduction in the mineral formation (Palmer et al. 2004). This study attempts to demonstrate the involvement of bacteria in the formation of arsenic minerals in the Andean borate deposits.

MATERIALS AND METHODS

Field Locality (Geological Setting)

Salar de Ascotán is part of an evaporitic basin system in the High Andes of northern Chile and is described as an Andean salt flat (Stoertz and Ericksen 1974; Chong 1984). It is located between 22°25′ y 22°45′ South latitude and 68°30′ y 68°10′ West longitude at an average altitude of 3700 m. Salar de Ascotán is at the bottom of a tectonic basin surrounded by volcanic chains to the east and west, including some active volcanoes over 5000 m high, with the highest peaks of about 6000 m. The geological setting is dominated by volcanic structures and includes acidic (rhyolites) and intermediate (andesites) rocks of Tertiary and Quaternary age.

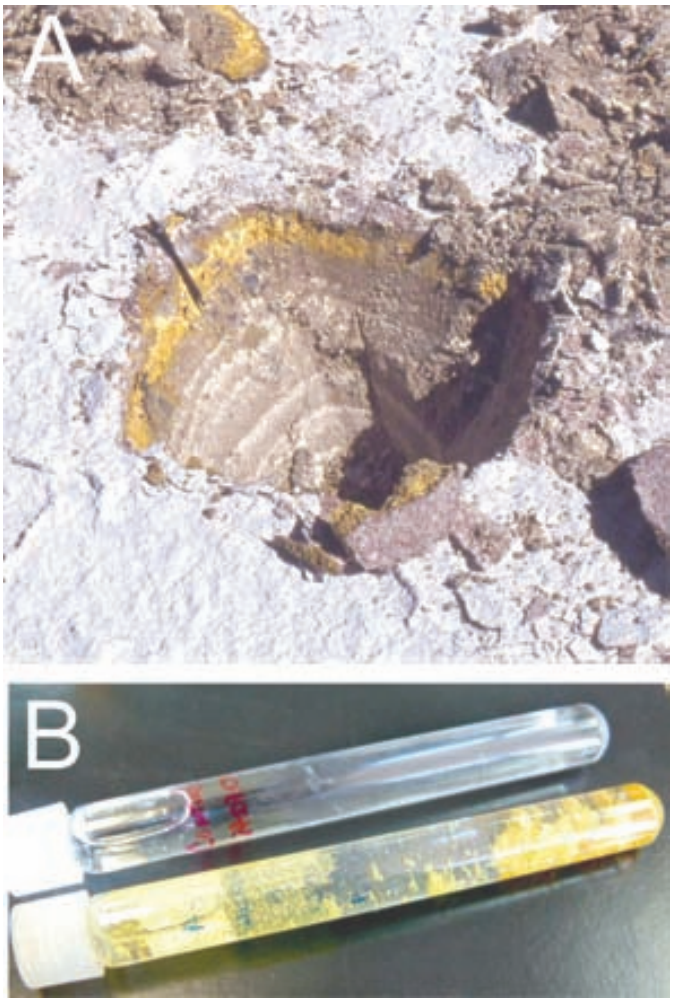

FIG. 1. (A) Position of the yellow material (3 to 5 cm thickness) in the stratigraphic column of Ascotán borate deposits. (B) Yellow precipitate in tubes inoculated with sediments from Ascotán compared to the uninoculated control.

The evaporitic basin contains palaeoshore lines indicating the existence of a former saline lake with deeper bathymetry. Climate is characterized by large daily thermal oscillations. High solar irradiation and strong and variable winds cause intense evaporation (about 4.5 mm/day) while precipitation is about 120 mm/year (Mardones-Pérez 1997). Water input is through surface drainage from the snow fields of volcanoes and underground waters and have a strong geothermal component with spring waters commonly reaching 23 to 25°C. In the eastern border of the basin there are thermal springs (Ojos del Coñapa) with 2700 to 3000 mg L^{-1} of total dissolved solids. The saline crusts are mainly composed of chlorides (halite) and sulfates (gypsum) with important borate ore deposits composed mostly of ulexite with significant amounts of arsenic sulfide minerals (Figure 1A).

Sampling Procedure and Measurements

Large chunks of ulexite with yellow or orange inclusions were extracted from the salt flat with a pull shovel. Samples were selected from these chunks with a sterile spatula and placed in sterile 250 mL polyethylene vials, which were then totally filled with water aseptically collected from the same deposit. Brine samples were also taken in sterilized vials to carry out microbial counts. The vials were stored in an icebox with ice, until further processing, in order to prevent oxidation and changes in microbial composition. Samples for X-ray diffraction (XRD), electron microscope observations, and energy dispersive X-ray microanalysis (SEM/EDS) were freeze-dried in the laboratory in order to preserve the sulfide mineralogy. An Orion model 290 pH meter was used to measure temperature and pH of the brines. Salinity was measured with an Orion model 115 conductivity meter. Two sampling expeditions were conducted, in August 2005 and June 2006.

Most Probable Numbers, Enrichment Cultures and Isolation

Culturable, arsenic-precipitating cells were detected by most-probable-number (MPN) incubations using fresh minimal medium (Newman et al. 1997) modified by the addition of 0.008% yeast extract, and amended, after autoclaving, with sterile 20 mM sodium lactate, 10 mM sodium sulfate (Na_2SO_4) and 1 mM dibasic sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) under an N_2:CO_2:H_2 atmosphere (80:15:5, v/v). The highest decimal dilution was 10^{-6} and 5 tubes were analyzed for each data point. Cultures were incubated in the dark, at 28°C. The presence of yellow precipitate was considered as a positive result.

Primary enrichment cultures were grown in the fresh, modified, minimal medium described above (Newman et al. 1997). Cultures were incubated in the dark, at 28°C. Primary enrichment cultures were started with sediment samples from Salar de Ascotán. These cultures took several weeks to precipitate arsenic sulfides. Subsequent cultures were maintained by periodic transfer to fresh medium. These cultures repeatedly grew in a few days.

Isolation was carried out by plating on the same medium used for enrichment, containing 2% purified Oxoid agar (Oxoid, Hants, England). Plates were incubated at 28°C, in an anaerobic jar, until the appearance of yellow colonies for further purification. The colonies could then be transferred to liquid medium.

Cell Microscopy

A Leica DMLS microscope was used for phase-contrast observations of morphology. Total cells counts were done by epifluorescence with a DNA-specific dye, 4′, 6-diamidino-2-phenylindole (DAPI) with a Leica DMLS epifluorescence microscope. Cells were fixed with 1.25% glutaraldehyde (final concentration) overnight at 4°C for SEM observation. The concentrated cell suspension from enrichment cultures was placed on polylysine coated glass coverslips (Marchant and Thomas 1983), fixed with osmium tetroxide, dehydrated, critical point dried and coated with gold. Samples were viewed using a Leica Stereoscan S 120 scanning microscope. Pure culture cells were fixed with 1.25% glutaraldehyde (final concentration) overnight

at 4°C, filtered and coated with gold. Samples were examined in a JEOL JSM-6360L scanning electron microscope.

Phylogenetic Analysis

An approximately 600 to 800 bp fragment of 16S rRNA gene was sequenced for strains CC-1, and Asc-3. A similarity matrix was built using those partial sequences with the ARB software package (Technical University of Munich, Munich, Germany; ⟨www.arbome.de⟩). Partial sequences were inserted into the optimized and validated tree available in ARB (derived from complete sequence data), by using the maximum parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology. Nucleotide sequence accession numbers at EMBL are: EF157293 and EF157294.

Growth Experiments

Strains CC-1 and Asc-3 were grown in the modified minimal medium described above amended with lactate (20 mM) and, as indicated (Fig. 5B), sulphate (10 mM), arsenate (1 mM) or sulphate (10 mM) plus arsenate (1 mM) as electron acceptors. Cysteine (1 mM) was also added. Cultures grown in the complete medium were inoculated (10%) into media with the different growth conditions. Two consecutive subcultures under the same conditions were carried out to avoid the presence of substrates from the initial complete medium. Cell numbers were determined by direct DAPI counts with an epifluorescent microscope, after 2 weeks of incubation. The appearance of an arsenic trisulfide precipitate was considered evidence of both sulfate and arsenate reduction. Sulfate reduction in the tubes without arsenic was observed by adding 1 mL of 5% $Fe(NH_4)_2(SO_4)_2$ after the incubation time. Tubes scoring positive for sulfate reduction turned black.

Arsenic Precipitation by Enrichment Cultures

A few milliliters from each enrichment culture were inoculated into several tubes with fresh medium. Additional tubes were inoculated with strain *D. auripigmentum*, ATCC Orex-4. An abiotic control was carried out in sterile medium without inoculum. Several tubes were sacrificed at each time point and opened in an anoxic glovebox (Coy Laboratory Products, Grass Lake, MI) (N_2:CO_2:H_2, 80:15:5). Medium was centrifuged at 2000 rpm for 5 minutes and filtered through a 0.2 μm cellulose filter; the filtrate was sealed and refrigerated at 4°C to preserve arsenic speciation until analysis. Total soluble arsenic was measured by Hydride Generation-Atomic Absorption Spectroscopy (HG-AAS). The precipitate was analyzed by X-ray diffraction and electron microscopy. Its chemical and isotope composition was also determined.

Analysis of Precipitates in Cultures and Natural Material

X-Ray Diffraction (XRD) . Samples taken from the enrichment and pure cultures 1 week after inoculation were opened in an anaerobic chamber. Precipitated material was recovered by filtration on 0.22 μm pore size Durapore filters and lyophilized. Natural material was directly placed on similar filters. Powdered microsamples of both materials (2 to 5 mg) were placed in 0.5 mm Ø (diameter) Lindemann glass tubes. X-ray diffraction analysis was performed using a Debye-Scherrer diffractometer fitted out with an Inel CPS-120 localization curved counter.

Electron Microscopy. Samples for SEM/EDS were mounted on stubs using a bi-adhesive carbon ribbon, and coated with carbon. Samples for TEM were mounted on a copper grid mesh stage with Mylar, and carbon coated. In addition to morphological observations, these samples were analyzed by SEM and TEM/EDS for determination of elemental composition, and by selected area electron diffraction (TEM/SAED) to establish whether the structures observed were crystalline or amorphous. Observations and SEM/EDS were carried out using a Leica Stereoscan S 120 scanning electron microscope. TEM was carried out with a Hitachi H 600 AB transmission microscope. Molar ratios were calculated from EDS analyses using realgar and orpiment standards.

Chemical Analyses. The relative arsenic and sulfur composition was determined from the average of 2 to 6 SEM-EDS spectra acquired from laboratory and natural materials, and compared to pure realgar and orpiment. Moreover, chemical analyses using Hydride generation atomic absorption spectroscopy (HG-AAS) were performed after acid digestion of the sediment sample.

Sulfur Isotopic Composition

$\delta^{34}S$ isotope analyses were carried out with natural and laboratory samples. An additional control experiment was carried out to check if there was some isotopic fractionation during chemical precipitation of As_2S_3, without changes in the oxidation state of either element, in the absence of microbial activity. Chemically precipitated As_2S_3 was obtained as previously described (Eary 1992) by adding an excess of $Na_2S \cdot xH_2O$ to a deoxygenated 0.15 *m* $NaAsO_2$ solution, buffered (pH 4 $\pm$ 0.2) with 0.1 *m* potassium hydrogen phtalate solution, at 25°C. The yellow precipitate was aged during 1 to 3 days and washed before analysis. Reagents used in the experiments were analytical grade solid Na_2SO_4 (Merck) and $Na_2S \cdot xH_2O$ (x = 7 a 9) (Merck) and were also isotopically analyzed to determine $\delta^{34}S$. The isotopic composition of sulfates was determined on $BaSO_4$ samples precipitated from previously dissolved sulfates. Sulfide samples were directly analyzed.

The sulfur isotopic composition was determined by continuous flow isotope ratio mass spectrometry (IRMS) and is expressed in the standard δ notation given by $\delta^{34}S = ([R_{sample}/R_{standard}]-1) \times 1000$, where $R = {}^{34}S/{}^{32}S$ is the isotopic abundance ratio. Values are expressed on a per mil (‰) basis as deviations from the international standard CDT (Jensen and Nakai 1962). Reproducibility of duplicate analysis of samples and internal standards were all better than 0.1 ‰ (1 σ) for $\delta^{34}S$ values.

Bacterial reduction of sulfate in cultures was allowed to proceed in sealed serum vials without loss of product. In such a closed system the isotopic difference between sulfate and sulfide develops following a Rayleigh distillation model (Canfield 2001). As sulfate depletion proceeds (Canfield 2001), the isotopic composition of the sulfide approaches that of the original sulfate, and after complete sulfate depletion, no fractionation information is preserved. Therefore, the isotopic enrichment factor ε can be calculated using the isotopic differences between dissolved sulfate and precipitated sulfide only when the fraction of sulfate remaining is greater than 95% (Mariotti et al. 1981; Canfield 2001). Then:

$$\varepsilon = [(\delta^{34}S_{\text{sulfide}} + 1000)/(\delta^{34}S_{\text{sulfate}} + 1000) - 1] \cdot 1000$$

where $\delta^{34}S_{\text{sulfide}}$ = isotopic composition of precipitated sulfide, $\delta^{34}S_{\text{sulfate}}$ = isotopic composition of dissolved sulfate.

In order to minimize the Rayleigh effect, the yellow precipitate was harvested as soon as it appeared in the cultures. The mass balance indicated that, at that point, the sulfate remaining fraction was greater than 70% and therefore, it is to be expected that the Rayleigh effect should be insignificant and the fractionation information similar to that observed in an open system. In any case, the Rayleigh effect would decrease the isotopic enrichment factor measured, and fortify the evidence for microbial sulfate reduction.

RESULTS

Bacterial Abundance in Salar de Ascotán

Three sediment samples and three brine samples taken from different ponds on the Salar de Ascotán on 9 August 2005 were analyzed for bacterial counts. Total bacterial numbers, as determined by epifluorescence, are shown in Figure 2A. Values ranged between 1.6 and 6.7 $\times$ 10^5 cells mL^{-1} in the brine samples and between 0.7 and 3.0 $\times$ 10^6 cells g^{-1} of sediment. Most probable numbers (MPN) of arsenic-precipitating bacteria were determined simultaneously (Figure 2A). MPN ranged 5 orders of magnitude between 3.9 $\times$ 10^1 cells mL^{-1} and 1.6 $\times$ 10^6 cells

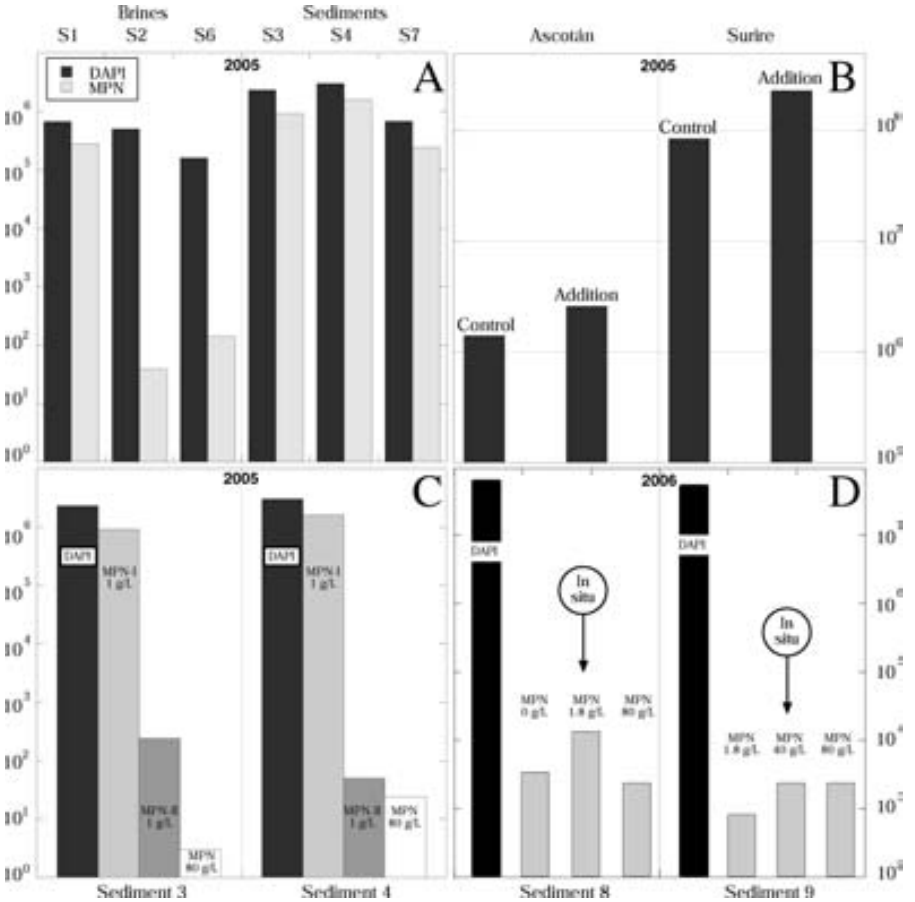

FIG. 2. (A) Total (DAPI) and MPN bacterial cell counts in sediments and brines from the May 2005 samples. (B) Test to control the efficiency of the DAPI method for counting cells in sediment samples from Ascotán and Surire salt flats. The two samples received, respectively, 1 $\times$ 10^6 and 2 $\times$ 10^7 cells g^{-1} from an arsenic-reducing culture obtained from Salar de Ascotán, (C) Effect of salinity on MPN in the May 2005 samples (MPN-I: with 1 g/L of salt, analysis carried out in August 2005; MPN-II: with 1 g/L and MPN 80 g/L salt, analysis carried out in May 2006). (D) Effect of salt concentration in the medium on MPN in two samples taken in 2006. The circles and arrows indicate the in situ salt concentration for each sample.

TABLE 1
Physicochemical parameters, arsenic-precipitating and total bacterial numbers in samples from Salar de Ascotán

Sample	Sample type	Coordinates	Date	pH	Salinity/per mil	As mg L^{-1}(A) mg Kg^{-1}(B)	DAPI count Cells mL^{-1}(A) Cells g^{-1}(B)
1	brine	7601876N581420E	03-08-2005	7.83	33	4.38 (A)	6.70E+05 (A)
2	brine	7609154N/573967E	03-08-2005	7.98	76	6.5 (A)	5.00E+05 (A)
6	brine	7604253N/577706E	03-08-2005	7.23	309	183 (A)	1.60E+05 (A)
3	sediment	7609154N/574012E	03-08-2005	ND	ND	781 (B)	2.30E+06 (B)
4	sediment	7609035N/573669E	03-08-2005	ND	ND	1210 (B)	3.00E+06 (B)
7	sediment	7609035N/577770E	03-08-2005	ND	ND	6504 (B)	6.80E+05 (B)
8	brine	7609328N/577921E	22-06-2006	5	1.8	3.4	3.9E+06 (A)
8	sediment	7609328N/577921E	22-06-2006	ND	ND	ND	6.03E+07 (B)
9	brine	7610653N/572171E	22-06-2006	6.50	10.5	28	4.25E+07 (A)
9	sediment	7610653N/572171E	22-06-2006	ND	41.4	ND	5.12E+07 (B)

[a]Note that units are different for brine (A) and sediment (B) samples.
ND = Not Determined.

g^{-1}. Both total bacteria and MPN varied considerably between samples.

This is probably due to the different salinities and arsenic concentrations of the samples (Table 1) indicating the heterogeneity of the ponds in the Salar de Ascotán. However, the very high MPN on four of the samples was surprising. Tests were carried out to check the reliability of the counts and additional samples gathered on 22 June 2006. The first test consisted of adding a known number of bacterial cells from a culture isolated from Salar de Ascotán (see later) to two natural samples. As shown in Figure 2B, the total cell counts increased as expected. It is therefore assumed that the total epifluorescence counts are reliable. Since salinity was so variable in this system two additional tests were carried out.

Two samples were selected where the MPN had been highest and this time it was determined at two different salinities without any salt added to the medium and with 8% NaCl added (Figure 2C). In all cases the MPN were several orders of magnitude lower than before. This is to be expected as the samples had been stored in the lab for several months and, thus, lower viability would be presumed. The differences between MPN at the two salinities were not consistent.

In sample Sediment 3, the MPN was one order of magnitude lower at the higher salinity, while in sample Sediment 4, the two values were essentially identical. On 22 June 2006 two additional fresh samples were obtained from the Salar de Ascotán and total counts and MPN were determined in media with different salinities (Figure 2D). At this sampling date, total counts were one order of magnitude higher than in the 2005 sampling, while MPN were about the same as the lower MPN from 2005. The salinity of the medium, again, did not have a marked influence on the results. Notwithstanding, arsenic-reducing bacteria could be detected in all samples, making up between 0.01 and 53% of the total bacterial assemblage. In general, MPN accounted for a larger proportion of the total count in sediments than in brines.

Enrichment Cultures and Isolation

Growth was shown by the appearance of a yellow precipitate (Figure 1B). Growth on Ascotán minerals was observed 12 weeks after inoculation. At this primary enrichment stage several cell morphologies were observed by phase contrast microscopy. Rod-shaped cells (2.5 $\times$ 1 μm) with terminal endospores and spirillum-like, motile microorganisms were the most apparent morphologies. Smaller straight and curved rods and cocci were also abundant. During successive enrichment stages, however, the most abundant and characteristic morphologies were lost, and other morphologies became predominant: straight and slightly curved rods (often paired), rods with pointed ends occurring singly or in pairs, and rod to pear-shaped cells with polar prostheca of varying lengths (Figures 3A–3D).

Some subcultures retained the rods with apparent terminal endospores, but most did not. These subcultures grew much faster and precipitates appeared after a few days of incubation. Soluble arsenic concentration decreased from an average of 77.8 to 33.1 mg/L after three days in the inoculated subcultures with Ascotán enrichment and in a *D. auripigmentum* (Labeda 2000) ATCC 700205™ pure culture, while remaining constant in the abiotic control (Figure 4).

Two strains, CC-1, and Asc-3 were isolated in pure culture. Both of them grew on lactate using sulfate or arsenate as electron acceptors, indicating that arsenate reduction was also associated with energy conservation (Figures 5A and 5B, conditions 1 to 4). Growth on lactate in the absence of an electron acceptor did not occur (conditions 5 and 6). When grown with both arsenate and sulphate, final yield was greater than with sulfate or arsenate alone (Figure 5A, conditions 1 and 2 vs 3 and 4). Cultures were

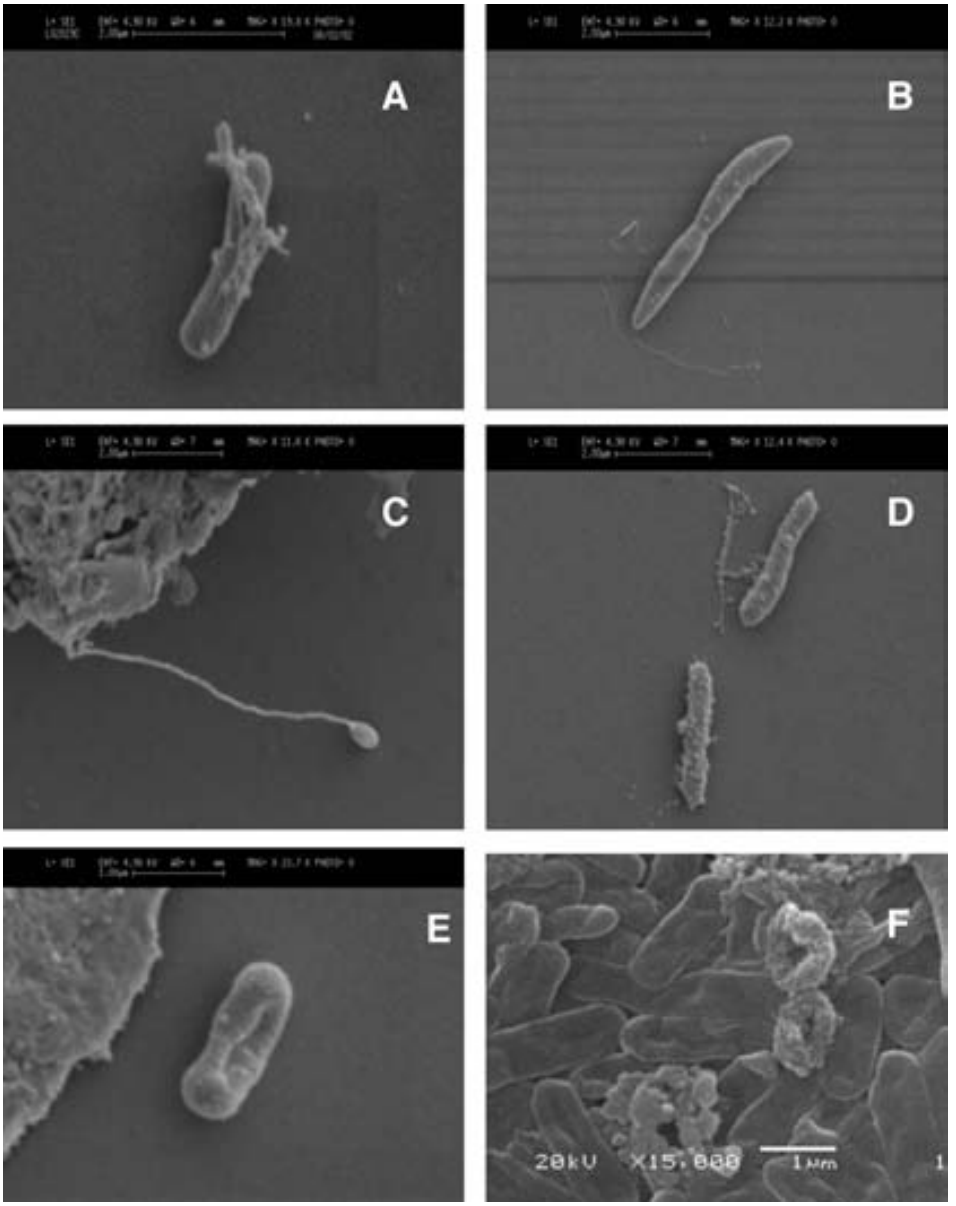

FIG. 3. SEM micrographs of cells observed in enrichment cultures (A–E) after successive steps of reinoculation in fresh medium, and of pure culture Asc-3 (F). Scale bar 2 μm for A to D, and 1 μm for E and F.

grown with and without cystein to check whether this made any difference (conditions 1 vs 2 and 5 vs 6). The two conditions resulted in the same final cell yield. Finally, a control without a sulfur source showed no growth (condition 6). Yellow arsenic sulfides precipitated when cells were grown in 10 mM sulfate and 1 mM arsenate (Figure 5C, conditions 1, 2). Yellow precipitate also appeared in the presence of arsenate and cystein (condition 4) but not in the absence of a source of sulfur (condition 6) nor in the absence of arsenate (condition 3). In order to show that in this culture sulfate had actually been reduced to sulfide, iron was added at the end of the incubation period and a black precipitate formed instantly (Figure 5C, condition 3). Only results for strain Asc-3 are shown in Figure 5, but results for strain CC-1 were identical.

Since salinity in Salar de Ascotán was very variable between sites and at differing times, the range of salinities allowing growth of these two strains was also tested. Both could growth in medium containing up to 3% NaCl after 2 weeks of incubation (0, 3, 5, 7, 10% were tested).

The nearest phylogenetic relative of Asc-3 strain was *Enterobacter* sp. BL-2 (Son et al. 2005) (97% sequence similarity). Strain CC-1, on the other hand, was related to *Pseudomonas* sp. PHLL (Gen Bank description) (99% similarity). Both microorganisms belong to the Gammaproteobacteria class. Both

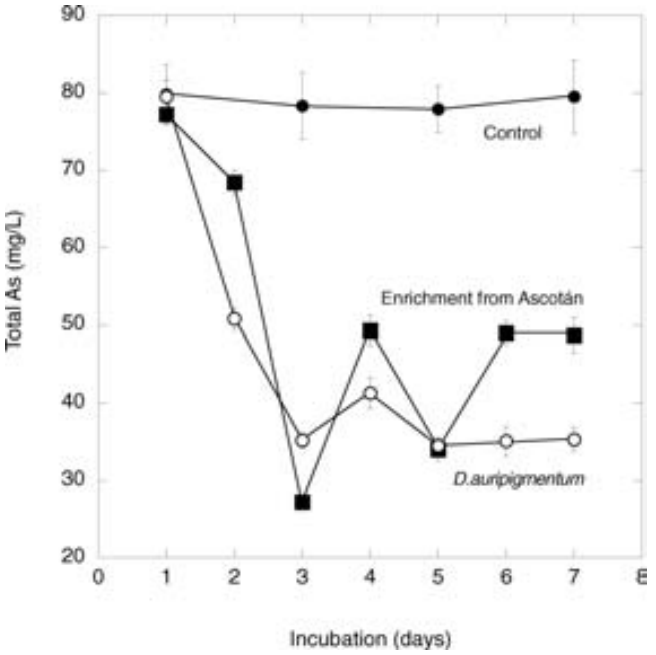

FIG. 4. Changes in the concentration of total soluble arsenic with time in an enrichment culture from Salar de Ascotán.

sequences showed a very low similarity (lower than 90%) to that of strains *D. auripigmentum* Orex-4 (Newman et al. 1997) and to *Desulfovibrio* strain Ben-RB and Ben-RA (Macy et al. 2000).

Characterization of Natural Material and Culture Precipitates

Arsenic-Bearing Natural Material. Bulk chemical analyses of Ascotán ulexite ore, using HG-AAS for As and gravimetry for S, showed concentrations of 0.47% As and 0.57% S (dry weight). The XRD analysis indicated a dominant mixture of calcite, ulexite, halite and quartz minerals. The arsenic-bearing minerals were under the detection limit of the technique. SEM observations showed an ulexite matrix with embedded electrodense grains, 1–3 μm in diameter (Figure 6A) and spicular crystals 0.3 to 0.5 μm wide. EDS analyses of the electrodense grains and crystals revealed sulfur and arsenic as the main components (Figures 6B, 7A). The relative arsenic/sulfur molar ratio in selected As-bearing crystals was 0.68 $\pm$ 0.07 on average (out of five determinations). This value was more closely related to the stoichiometry of orpiment than to that of realgar or pararealgar. The natural arsenic precipitate had an isotopic composition $\delta^{34}S_{CDT}$ = -0.55% as determined by IRMS (Table 2).

Laboratory Bacterially Produced Precipitate. Analysis of the precipitate produced by enrichment culture by XRD showed the presence of pararealgar As_4S_4 (Roberts et al. 1980) (JCPIDS 33-127 and 83-1013) with traces of uzonite (Popova and Polykov 1986) As_4S_5 (ASTM 39-331) (Table 2). SEM observations of the bacterially produced precipitate revealed a matrix of electrodense 0.24 to 0.6 μm thick fibres, 0.3 μm diameter spherical particles, and some framboids 2 μm in diameter (Figure 6C). EDS showed that fibers, spherical particles and framboids were composed of arsenic and sulfur. The As/S molar ratio determined on bacterial surfaces was similar to the stoichiometry of orpiment (Figure 7B). In the bulk, freshly produced precipitate, however, this ratio increased and was closer to that of pararealgar (Figure 7C), matching the XRD results (Table 2).

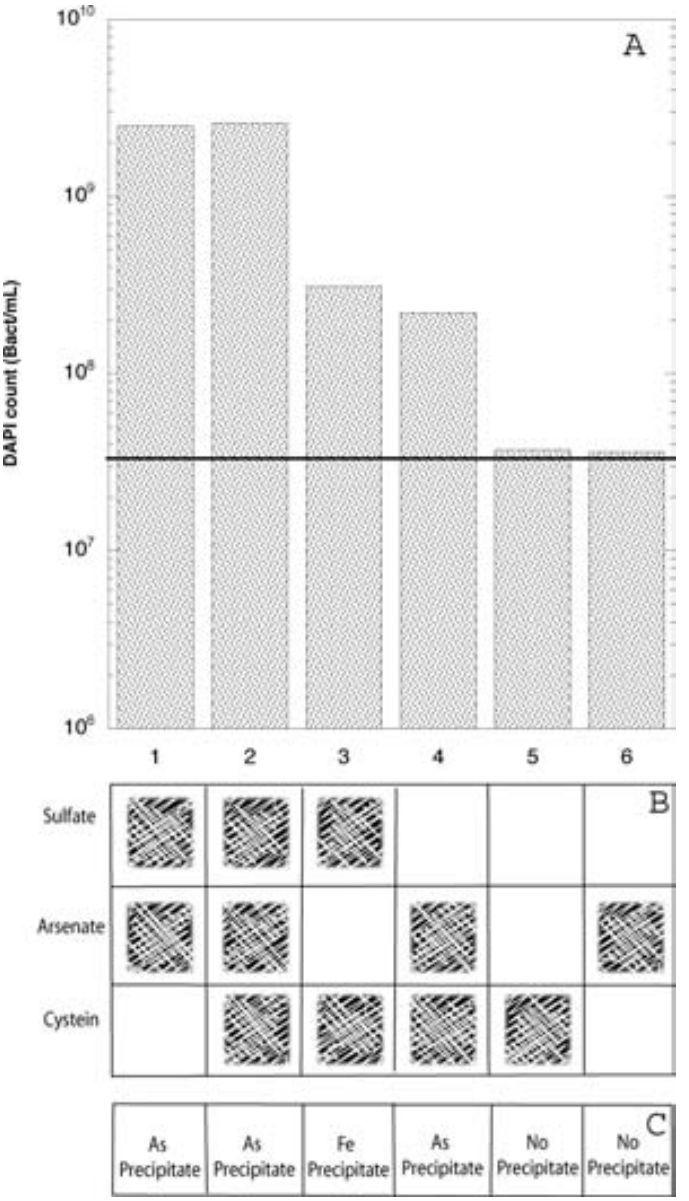

FIG. 5. (A) Growth of strain Asc-3 in modified minimal medium (Newman et al. 1997) with lactate (20 mM), and the addition of sulfate (10 mM), arsenate (1 mM), cysteine (1 mM) as indicated in the lower diagram (B). A culture of the strain grown in the same medium was inoculated (10%) into the experimental tubes at time 0. The black line shows the bacterial number at time 0 (3,5E+07). (B) Experimental conditions for each treatment. (C) Appearance of precipitates after incubation under the different experimental conditions.

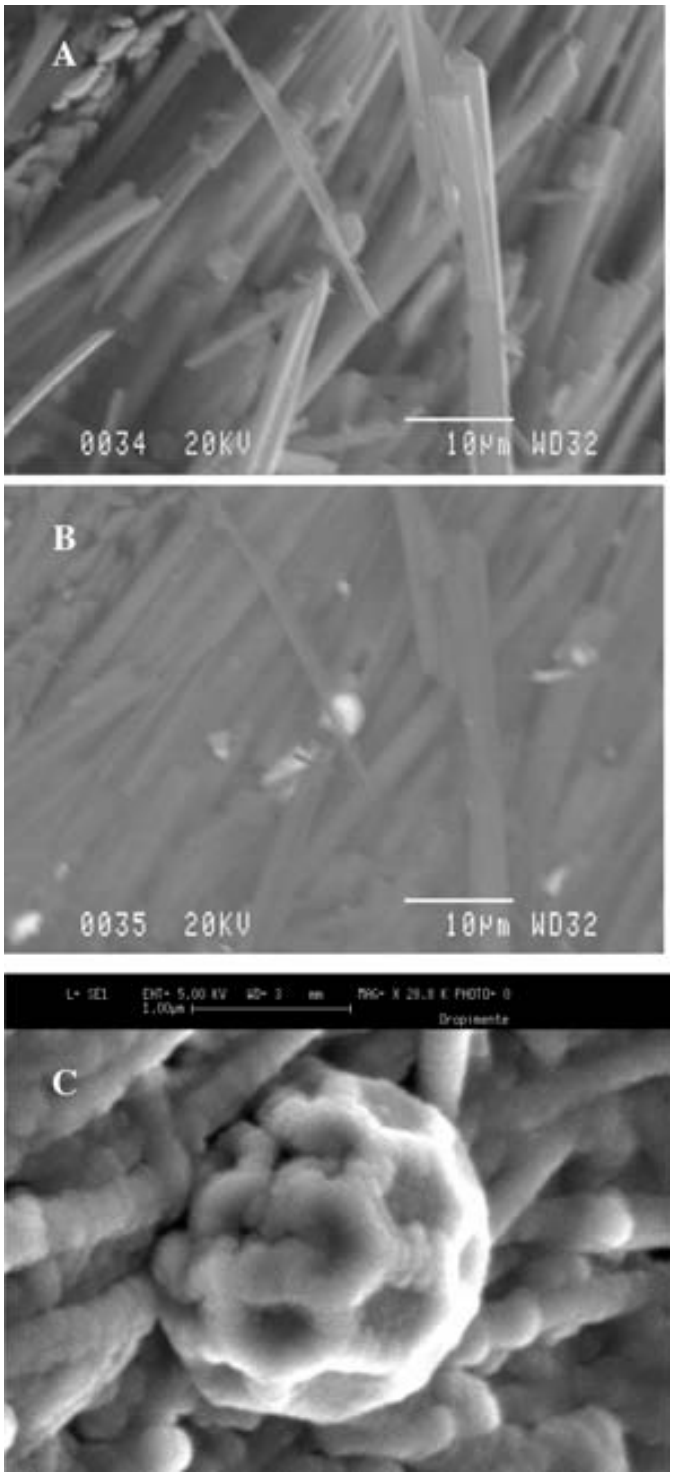

FIG. 6. (A) SEM micrograph of a natural sample from Salar de Ascotán. Note the fibrillar matrix of ulexite and the grain in the center. (B) SEM image of the same field taken with back-scattered scanning electrons. Note the lighter shade of the grain indicating the presence of heavy atoms. (C) SEM micrograph of laboratory-produced microbial precipitate. Note the framboid structure and the fibrillar structures surrounding it.

TEM observations revealed microfibers from 0.05 to 0.6μm thick and rectangular crystals from 0.05 to 0.3 μm in size that appeared to be fragments of the fibers. TEM-SAED confirmed the crystalline nature of these fragments. Likewise, TEM-EDS analyses confirmed that these crystals were formed by arsenic and sulfur. IRMS of the laboratory arsenic precipitate showed isotopic compositions of $\delta^{34}S_{CDT} = -0.55\%$ and –0.79 in two separate determinations (Table 2). The As/S molar ratio of the precipitate produced by pure cultures was closer to orpiment stoichiometry.

DISCUSSION

Bacterial Abundance in the Salar de Ascotán. Total bacterial numbers in the brine samples were within the normal range of values found in planktonic environments (Whitman et al. 1998). Abundance in the sediment samples with high As content (over 700 mg g^{-1}), however, was two to three orders of magnitude lower than the normal values usually found in sediments (Whitman et al. 1998). It is even more surprising that a very high percent of these were retrieved as arsenic-precipitating bacteria in MPN incubations. It is unusual to find MPN of a specific physiological group to make up more than 1% of the total count (Simu et al. 2005).

In the Ascotán samples collected in 2005, however, arsenic-precipitating bacteria accounted for approximately half of the total count in four out of six samples. This suggests that arsenic-based metabolisms must be very significant processes in this ecosystem with high arsenic content. The methodological tests carried out indicated that both the total and MPN counts were reliable despite the different salinities used in the MPN medium and the original in situ salinity. When counts were carried out again in June 2006, both total counts were higher and MPN lower than in 2005. It should be concluded that the rather variable conditions found in the Salar de Ascotán, both in space and time generate large differences in bacterial numbers.

Thus, neither the high nor the low numbers can be considered as representative. However, the high percent of MPN found in the 2005 sampling demonstrates that arsenic reducing bacteria may account, at times, for a very significant part of the bacterial assemblage. MPN results from Mono Lake showed that arsenate-respiring bacteria (AsRB) were much lower than total bacterial counts, accounting only 0.001% of the total population (Oremland et al. 2000). However the radioassay results in the same study measuring the reduction of ^{73}As (V) to ^{73}As (III) suggested that the population size of AsRB by MPN might have been underestimated by as much as two to three orders of magnitude (Oremland et al. 2000). If this were the case, Mono Lake would also show a significant percent of the total bacterial assemblage as bacteria able to reduce As.

Enrichment Cultures. Bacteria able to precipitate arsenic sulfide were successfully enriched from the natural arsenic-rich

TABLE 2
Isotopic signatures from natural and laboratory sulfur compounds and enrichments of different reduction processes

Substrate	Product	$\delta^{34}S‰$	Process	ε
Na_2SO_4		2.67 to 3.35		
	As_2S_3, As_4S_4,As_4S_5	−0.56 to −0.79	Precipitation in enrichment cultures	−3.5 to −3.9
	As_2S_3	ND	Precipitation in pure culture	ND
$Na_2S \cdot xH_2O$		1.73		
	As_2S_3	4.21 to 4.24	Chemical precipitation[c]	2.5
Sulfate in Ascotán brines		2.95 to 4.29 (3 samples)		
	Ascotán mineral As_2S_3	−0.55	Biogeochemical precipitation	−3.5 to −4.8
Gypsum		20.9		
	As_2S_3	−30.4 to −35.3	Biogeochemical precipitation[a]	−50.2 to −55.0
Sulfate				
	Metal sulfides in sediments		Biogeochemical precipitation[b]	<−4 to −46%
Sulfate				
	Sulfide		Reduction in pure cultures[b]	−4 to −46%

[a](Palmer et al. 2004).
[b](Canfield 2001).
[c](Eary 1992).

material. Soluble arsenic concentration decreased as a yellow precipitate appeared. Analysis of this precipitate showed it to be an arsenic sulfide with molar composition ranging between those of orpiment and realgar. The enriched bacteria showed a metabolic potential similar to that of these two isolated strains. However, they differed in the molar composition of the precipitate. This could be due to different culture conditions, which are known to alter the stoichiometry of the products. Alternatively,

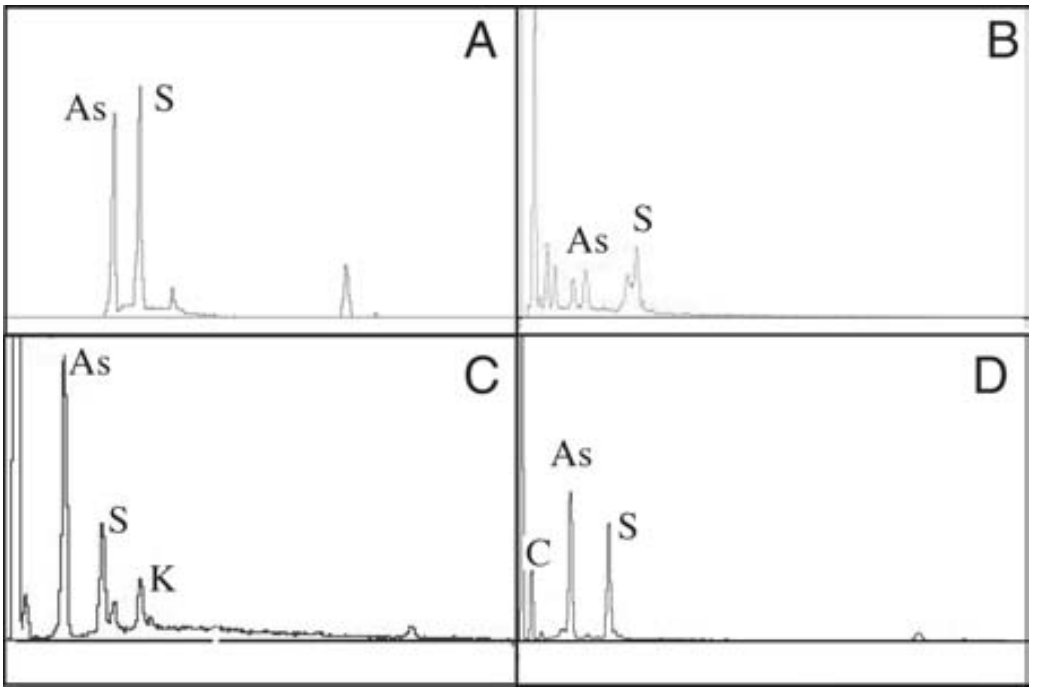

FIG. 7. (A) SEM-EDS spectrum of an electrodense grain in the ulexite matrix of an Ascotán sample (shown in Figures 6A and B). Calculated As/S molar ratio= 0.68 ± 0.07. (B) SEM-EDS spectrum of the laboratory-produced microbial precipitate, specifically from the surface of a cell from an enrichment culture. Calculated As/S molar ratio= 0.78 ± 0.03. (C) SEM-EDS spectrum of the laboratory-produced microbial precipitate, specifically from the bulk precipitate from an enrichment culture. Calculated As/S molar ratio= 1.13 ± 0.26. (D) SEM-EDS spectrum of the laboratory-produced microbial precipitate from an Asc-3 strain. Calculated As/S molar ratio= 0.57 ± 0.01.

this variation could be a characteristic of the different bacteria.

Pure culture experiments using different substrates showed that both sulphate and arsenic reduction are associated with energy conservation. The 16S rRNA analysis revealed that those strains are different enough from *Bacteria* previously described able to precipitate arsenic sulphide in the specified conditions.

In summary, the enrichment and pure cultures showed that bacteria able to carry out the precipitation of arsenic sulfide were in fact present in high numbers in the environment and that such precipitation did not occur in the laboratory in the absence of the bacteria. Moreover, MPN data of arsenic-precipitating bacteria determined in samples from Salar de Surire, Cejar lagoon (Salar de Atacama) and River Loa sediments (data not shown), all in Northern Chile, suggests that these bacteria are not limited to the borate layers described here but, rather, that they are widespread through the different environments associated with the presence of arsenic.

Morphology and Chemistry of the Precipitates. One obvious difference between laboratory and environmental precipitates was the presence of a ulexite matrix in the latter. This was expected since precipitation of borate salts to form ulexite is a chemical process independent of the arsenic precipitation. Likewise, the environmental precipitate was mixed with a number of minerals such as calcite, quartz or halite that could not form in the cultures.

Analysis of the arsenic minerals showed both similarities and differences between the laboratory and environmental precipitates. First, the environmental precipitate had a molar ratio As/S of 0.68 ± 0.07, consistent with the mineral orpiment. On the other hand, the molar ratio of the laboratory precipitate using enrichment culture was 0.78 ± 0.03 (closer to orpiment) for the material precipitated on cell surfaces, but it was 1.13 ± 0.26 (closer to realgar) for the cell-free precipitate. Arsenic precipitate molar ratio produced by pure cultures was also consistent with orpiment stoichiometry (molar ratio As/S 0.57 ± 0.01). And second, the precipitates showed morphological differences: grains (1–3 μm in diameter) and spicules (0.3–0.5 μm thick) in sediments, fibers (0.2–0.6 μm), spheres (0.3 μm in diameter) and framboids (2 μm in diameter) in enrichment cultures. The pure culture precipitate shows an amorphous appearance composed mainly of grains (1–2 μm in diameter).

These differences can be attributed to the very different environments in which the precipitation occurred. In fact, the different molar ratios of the arsenic precipitated directly onto the cells and of those further away indicate that both minerals could be precipitated by the same bacterial consortium. It is worth noting that *D. auripigmentum* precipitates orpiment alone (Newman et al. 1997), which was the mineral present on cell surfaces in enrichment cultures. Perhaps different bacteria in the enrichments were responsible for the precipitation of the two minerals. Additionally, it could be suggested that the artificial culture conditions select only a fraction of the bacteria able to precipitate the As minerals present in Ascotán. For instance, all the experiments were carried out using lactate as electron donor, but inorganic electron donors may be important for As (V) reduction metabolism in some extreme systems (Oremland and Stolz 2005).

The framboid-like structures observed in the bacterial precipitate obtained in the laboratory (Figure 6C) are common for pyrite. These structures are typically formed via monosulfide precursors (Sawlowicz 1990), mainly in sedimentary rocks. As far as is known, no framboid-like structures composed of arsenic sulfide have been reported before. However, there are also other minerals which, under specific conditions, may reveal similar structures, e.g., copper (Sawlowicz 1990) and zinc (Sawlowicz 2000) sulfides. Different genetic processes have been postulated for these structures, from purely inorganic to the directly biogenic, and including indirect biogenic formation. Recently, a multistage process has been proposed for the formation of framboidal pyrite (Wilkin and Barnes 1997). According to Sawlowicz (2000) a colloidal stage is needed for framboid formation.

In conclusion, even though arsenic precipitates were found in both nature and cultures, the details of molar ratios, and morphology of minerals seemed dependent on the particular environment where the precipitation took place and the microorganisms involved. It cannot be expected that the cultures mimic all aspects of the natural environment. This is especially true if the completely different time scales involved in both processes are considered. Clearly, diagenesis might have occurred in the environmental sample, changing the structure and chemistry of the minerals.

Isotopic Fractionation of Precipitates. It is well known that enzymatic processes tend to enrich products in the lighter isotopes due to kinetic effects. This characteristic has been used extensively to implicate biological activity in geochemical processes (Boschker and Middelburg 2002). The biological arsenic sulfide precipitation is the result of microbial arsenic and sulfate reduction plus precipitation. Sulfate-reducing bacteria produce sulfide depleted in ^{34}S during their metabolism (Canfield 2001) and, thus, the isotopic composition of sedimentary sulfides provides an indication of the activity of sulfate-reducing bacteria in sulfide formation.

Natural sulfides produced from bacterial sulfate-reduction can reach ^{34}S depletion values of about $-50‰$ (Kaplan and Rittenberg 1964). Recently reported information by Palmer et al. (2004), confirmed the presence of $\delta^{34}S$ values as low as $-30‰$ in arsenic sulfides (orpiment and realgar) associated with the Emet (Turkey) borate deposits, suggesting microbially mediated sulfate reduction (Table 2). These $\delta^{34}S$ values were lighter than in previously reported data for dissolved sulfide in geothermal fluids, and also lighter than the range determined for sedimentary sulfides formed by sulfate-reducing bacteria (Canfield 2001).

Nevertheless, the isotopic composition of sedimentary sulfides depends on variables such as temperature, availability of dissolved sulfate and organic substrates (the last two factors

modify the specific rate of sulfate reduction), and substrate type (Harrison and Thode 1958; Kaplan and Rittenberg 1964; Kemp and Thode 1968; Chambers et al. 1975). Thus, the relevant variable is the enrichment ε, which shows the changes in the isotopic composition of the product of the biological process with respect to the substrate. Under optimal conditions (adequate temperature and abundant organic substrate) fractionation can be minimized due to the fact that the reaction controlling the rate of SO_4^{2-} reduction is its transformation to APS (adenosine-5′-phosphosulfate), that does not involve fractionation (Kemp and Thode 1968). This may happen in cultures, where fractionations are, commonly, smaller than under natural conditions (Canfield 2001). Under other conditions, fractionation may be extreme. Thus, sulfate-reducing bacteria in cultures have been shown to generate sulfide with ^{34}S enrichment values of $-4‰$ to $-40‰$ compared to sulfate (Kaplan and Rittenberg 1964; Chambers et al. 1975).

The laboratory arsenic sulfide precipitation experiments described in this work showed $\delta^{34}S$ –0.56 and –0.79, while the $\delta^{34}S$ of the initial sulfate added to the culture medium ranged between +2.67 and +3.35. Therefore, the ^{34}S enrichment values ranged between −3.5 and −3.9‰, which are in the lower range of reported values (Table 2). Sulfate $\delta^{34}S$ data obtained from the Salar de Ascotán brines ranged between 2.9 and 4.3‰ and sulfide precipitated in Ascotán showed $\delta^{34}S$ about −0.55‰. Thus the ^{34}S enrichment values ranged between −3.5 and −4.8‰ compared to sulfate (Table 2). Both laboratory and Ascotán precipitates, therefore, showed the same enrichment values. These were significantly different from chemically precipitated arsenic sulfide (without sulfate-reduction) that showed an enrichment value of +2.5 (Table 2). This strongly supports the involvement of microorganisms in the origin of the arsenic sulfides in Salar de Ascotán, and that natural conditions of sulfate-reduction in the salar were similar to the experimental ones, probably reflecting availability of organic substrates in both cases.

Considering the evidence presented here, the involvement of bacteria in the precipitation of arsenic minerals in Salar de Ascotán seems clear. It is very likely that the same is true for similar borate deposits in other parts of the world, such as the Argentinian side of the Andes, North America or Turkey. Thus, the microbial reduction of arsenic becomes a biogeochemically relevant process. Steps will be taken to characterize the in situ microbial community and to attempt to isolate in pure culture and identify the bacteria responsible for most of the in situ activity.

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Capítulo VI

Isolation of novel As(V) reducing halotolerant *Shewanella* spp. from an arsenic-rich sediment in hypersaline Salar de Ascotán (Northen Chile)

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No sé si el desierto puede ser amado,
pero es el desierto que esconde mi tesoro.
(Paulo Coelho)

Isolation of novel As(V)-reducing halotolerant *Shewanella* spp. from an As-rich sediment in hypersaline Salar de Ascotán (Northern Chile)

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Keywords: As respiring microorganisms, arsenate, arsenite, sulfate reducing bacteria, hypersaline lake, As genes.

ABSTRACT

A novel arsenate reducing Gram-negative halotoleratnt bacterium is reported in this work. In addition, preliminary information in provided on an isolate that has not been completely purified. Strains Asc-3 and CC-1 were isolated from sediments rich in As in Salar de Ascotán, Northern Chile. Both strains differed significantly from previously described arsenate reducing isolate strains *Shewanella* sp. ANA-3, MIT-13 and SES-3. Among others differences, both strains had the ability to grow on sulfate as unique electron acceptor. They were closer to the Firmicutes *Desulfosporosinus* Orex-4 and HN-41 in their capacity to grow on lactate with either arsenate or sulfate as electron acceptors. Asc-3 and CC-1 grew with NaCl up to 7% (v/v), had optimal temperature at 28ºC. Phylogenetically, the strain Asc-3 belonged to the Gammaproteobacteria group, within the genus *Shewanella*, close to other As(V) reducing *Shewanella*-like bacteria such as the strains ANA-3 and HN-41. Strain CC-1 was a Gammaproteobacterium of the *Pseudomonadaceae,* and this is the first report of an As(V) reducing *Pseudomonas*. The activity of arsenate-reducing bacteria mobilizes As from sediments and, therefore, the isolated strains are of great interest to carry out further studies on the mechanisms for As mobilization under reduced

anaerobic conditions. Salar the Ascotán is an excellent place for studying microbial implication in the As cycle and a natural source of bacterial strains able to carry out As transformations.

INTRODUCTION

Microbiological investigations of arsenic (As) redox biotransformation capabilities in laboratory strains are a necessary step for studying the impact of microbial processes on As mobilization. In nature As respiration takes place in the absence of oxygen and contributes to organic matter mineralization. In aquifers, these microbial reactions may mobilize As from the solid to the aqueous phase, ending in contaminated drinking water (Oremland & Stolz, 2003). In Northern Chile As mobilization is a serious problem for drinking water treatment plants. The objective of the present study was to isolate and characterize bacterial strains able to use arsenate as terminal electron acceptor from a natural As-rich environment.

Several As(V) reducing microorganisms have been isolated in pure culture, including members of both Bacteria and Archaea. These organisms have been isolated from a large set of environments, such as freshwater marshes, acid hot springs, groundwaters and alkaline hypersaline sediments (Blum, 1998, Laverman & Oremland, 1995, Macy *et al.*, 1996, Macy *et al.*, 2000). As(V) reducing bacteria can obtain energy by coupling it to an array of organic and inorganic electron donors. They are, therefore, metabolically versatile and phylogenetically diverse. Furthermore, several studies have revealed such organisms to be abundant and active *in situ* and, likely, they play key roles in mediating the reductive portion of the As cycle and in mobilizing As in aquatic environments.

The implication of microbes in the As cycling is quite significant (Mukhopadhyay *et al.*, 2002). Inorganic arsenate enters the microbial cytosol of may bacteria through the phosphate transport system, it is then reduced to arsenite, and further extruded out of the cell, either through channels or secondary transporters (Rosen, 2002). Arsenate-respiring microbes can release arsenite from arsenate-rich sediments, leading to As contamination of ground water (Oremland & Stolz, 2003). Arsenite-oxidizing microbes utilize the reducing power from As(III) oxidation to gain energy for cell growth (Stolz *et al.*, 2006). Microbes can also convert inorganic As into gaseous methylated arsenide (Bentley & Chas-

teen, 2002, Qin , 2006). Marine microorganisms can convert inorganic arsenicals to various water- or lipid-soluble organic As species. These include generation of di- and trimethylated As derivatives (DMA, TMA), arsenocholine, arsenobetaine, arsenosugars, and arsenolipids. Arsenobetaine can be degraded to inorganic As by microbial metabolism, completing the As cycle in marine ecosystems (Dembitsky & Levitsky, 2004). However, relatively little research has been conducted to investigate the effect of salinity on microorganisms that mediate biogeochemical As reactions.

Hypersaline lakes are common landscape components in many of the Earth's arid regions and can be very useful model systems to explore these effects. The peculiar conditions prevailing in the Andes of Northern Chile have enriched this area with significant As deposits (see Chapter II). Salar de Ascotán is an excellent place to study microbial implications in the As geochemical cycle (see Chapter III and IV) but also a natural source of new microbial strains involved in the As mobilization under reducing anaerobic conditions.

Saline lakes in the Andean region typically occupy closed-basin settings, where their size is controlled by the balance between evaporation and freshwater inputs from drainage basins. As a result, the salinity of a given lake may oscillate dramatically over both long and short time intervals in response to regional changes in climate, topography, or hydrologic conditions. To date, relatively little research has been conducted to investigate the effect of changing salinity on the resident populations of microorganisms that mediate various biogeochemical reactions in these environments. A better understanding of the response of microbial communities to changing salinity and the relative importance of particular electron acceptors at high salt concentrations may offer insight into the development of life in hypersaline conditions on this planet (Dundas, 1998, Knauth, 1998), as well as the potential for the existence on the As cycle of life in evaporated brines from extraterrestrial settings (van der Wielen, 2005).

In addition, research on this topic may help to better understand the broader ecological effects that may accompany salinity changes brought about by anthropogenic causes, including the diversion of tributary streams for irrigation or drinking water uses (Herbst, 1998).

MATERIALS AND METHODS

Field locality

Salar de Ascotán is part of an evaporitic basin system in the High Andes of northern Chile. Salar de Ascotán is at the bottom of a tectonic basin surrounded by volcanic chains to the east and west including some active volcanoes over 5,000 m high, with the highest peaks of about 6,000 m. The geological setting is dominated by volcanic structures and includes acidic (rhyolites) and intermediate (andesites) rocks of Tertiary and Quaternary age. Climate is characterized by large daily thermal oscillations. Saline crusts are mainly composed of chlorides (halite) and sulfates (gypsum) and this salt flat has important borate ore deposits composed mostly of ulexite with significant amounts of As sulfide minerals (Fig. 1A).

Sampling procedure and measurements

Large chunks of ulexite with yellow or orange inclusions were extracted from the salt flat with a pull shovel. Samples were selected from these chunks with a sterile spatula and placed in sterile 250 mL polyethylene vials, which were then totally filled with water aseptically collected from the same deposit. The vials were stored at 4ºC in an icebox until further processing a few hours later, in order to prevent oxidation and changes in microbial composition. Samples for electron microscope observations and energy dispersive X-ray microanalysis (SEM/EDS) were freeze-dried in the laboratory in order to preserve the sulfide mineralogy.

Enrichment cultures and isolation

As precipitating bacteria were isolated from sediments of Salar de Ascotán by adding 10 g sediment (in triplicate) into 20 mL of fresh modified minimal medium (Newman *et al.*, 1997a). The medium contained 0.1% yeast extract and was amended, after autoclaving, with sterile 20 mM sodium lactate, 10 mM sodium sulfate (Na_2SO_4) and 1 mM dibasic sodium arsenate ($Na_2HAsO_4.7H_2O$) under an N_2:CO_2:H_2 atmosphere (80:15:5 v/v). Enrichments were incubated in the dark, at 28ºC, for several weeks until yellow As sulfide precipitates were visible. The presence of yellow precipitate was considered as a positive result (Fig. 1B). Subsequent enrichments were maintained by periodic transfer to fresh medium. These cultures grew in a few days, reproducibility. The

As-precipitating bacteria Asc-3 and CC-1 were enriched, isolated and cultivated by serial dilution and plating onto the same medium used for enrichment supplemented with 2% purified Oxoid agar (Oxoid, Hants, England). Plates were incubated under anaerobic conditions at 28°C until the appearance of yellow colonies. Single colonies were re-streaked several times to obtain pure isolates, and transferred to liquid medium for confirmation of the ability to form yellow precipitates.

Growth experiments

The optimal temperature ranges and salinity tolerance of strains Asc-3 and CC-1 were determined by following direct cell counts in cultures incubated at pH 6.8 by growth kinetics on fresh medium supplemented with sodium lactate (20 mM) and sodium sulfate (10 mM) under anaerobic conditions. The range of temperatures studied was from 20°C to 37°C. The salinity range was between 0.5 and 10 % NaCl (studied at 28ºC). For each test we used two-steps dilution subcultures to rule out the presence of traces of substrates from the original medium. Alternative electron acceptors were also tested at concentrations shown in Table 1. Arsenate reduction was verified by presence of yellow precipitates. Sulfate reduction in tubes without As was observed by addition of 1 mL of 5 % stock solution $Fe(NH_4)_2(SO_4)_2$ which formed black precipitates (iron sulfide).

Optical and electron microscopy

Cell morphology was observed by phase-contrast microscopy in a Leica DMLS microscope. Total cells counts were done by epifluorescence with a DNA-specific dye, 4′, 6-diamidino-2-phenylindole (DAPI) with an OLYMPUS BX61 epifluorescence microscope (Porter & Feig, 1980). For SEM observations, cells were fixed overnight with glutaraldehyde (1.25% final concentration) at 4ºC. The concentrated suspension was placed on polylysine coated glass coverslips (Marchant HJ, 1983), fixed with osmium tetroxide, dehydrated, critical point dried and coated with gold. Samples were viewed using a Leica Stereoscan S120 scanning microscope.

A

B

Figure 1. A. Position of the yellow material (3 to 5 cm thickness) in the stratigraphic column of Ascotán borate deposits. **B.** Yellow precipitate in Asc-3 strain.

Analysis of precipitates in bacterial strains and natural samples

Electron microscopy: Samples for SEM/EDS were mounted on stubs using a bi-adhesive carbon ribbon, and coated with carbon. Samples for TEM were mounted on a copper grid mesh stage with Mylar, and carbon coated. In addition to morphological observations, these samples were analyzed by SEM and TEM/EDS for determination of elemental composition, and by selected area electron diffraction (TEM/SAED) to establish whether the structures observed were either crystalline or amorphous. Observations and SEM/EDS were carried out using a Leica Stereoscan S120 scanning electron microscope. TEM was carried out with a Hitachi H600 AB transmission microscope. Molar ratios were calculated from EDS analyses using realgar and orpiment standards.

C**hemical analyses**: The relative As and sulfur composition was determined from the average of 2 to 6 SEM-EDS spectra acquired from laboratory enrichments and natural samples, and compared to pure realgar and orpiment. Moreover, chemical analyses using Hydride generation atomic absorption spectroscopy (HG-AAS) were performed after acid digestion of the sediment sample.

Nucleic acid extraction and 16S rDNA analysis

Biomass from pure bacterial cultures was harvested by filtration on 0.2 μm polycarbonate membranes (Nuclepore®) and frozen until treatment. Total DNA from strain ASC-3 and CC-1 were extracted using the Ultra Clean Soil Kit as described by the manufacturer's instruction (Mo Bio Laboratories Solana Beach, CA, USA). The purified genomic DNA was used as target in a PCR reaction to amplify the bacterial 16S rRNA genes with primers 27F MOD (5'-AGR (AG) GTT TGA TCM(AC) TGG CTC AG-3') and 1492R MOD (5'-GGY(CT) TAC CTT GTT AYG ACT T-3'). PCR conditions were initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, primer annealing at 55 ºC for 1 min and extension at 72ºC for 2 min, with an additional step at 72º C for 10 min. PCR product was purified and sent for sequencing.

Amplification of *arrA* and *arsC* genes from the strains

For amplification of the As respiratory gene *arrA* and of the As reductase gene *arsC* we used three primers sets, as described in Table 2 and material and methods of Chapter III. Presence of PCR amplification products was verified by electrophoresis in 1% agarose gels, stained with ethidium bromide. DNA obtained from an As-free environment in Blanes Bay was used as negative control.

Phylogenetic analysis

Sequences of the 16S rRNA gene were sent to BLAST search (http://www.ncbi.nlm.nih.gov) to determine the closest relative in the database and were further aligned using the alignment tool in Greengenes (http://www.greengenes.lbl.gov).
Aligned sequences were inserted into the optimized and validated Maximum Likelihood tree available in ARB (http://www.arb-home.de), by the maximum-parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology. Sequences were deposited in Genbank under accession numbers EF15793 and EF15794.

Results

Enrichment and isolation

Previous studies by the most probable number approach (Chapters III and IV) had indicated the presence of As precipitating bacteria in As-rich samples (higher than 700 mg/Kg total As) of Salar de Ascotán and other system. We tried to isolate organisms that could respire and reduce both As(V) and sulfate. After several enrichment steps, followed by plating on Newman's agar medium, only yellow and white colonies were observed on the plates. Isolated colonies were separately inoculated into anaerobic As(V) medium and after one week of incubation yellow colonies were selected again. Then, colonies were transferred to liquid medium and yellow precipitates were observed again.

The white colonies in liquid medium did not produce precipitate. Selected strains were designated as Asc-3 (from sediment of Ascotán collected in 2004) and CC-1 (from sediment of Ascotán collected in 2005).

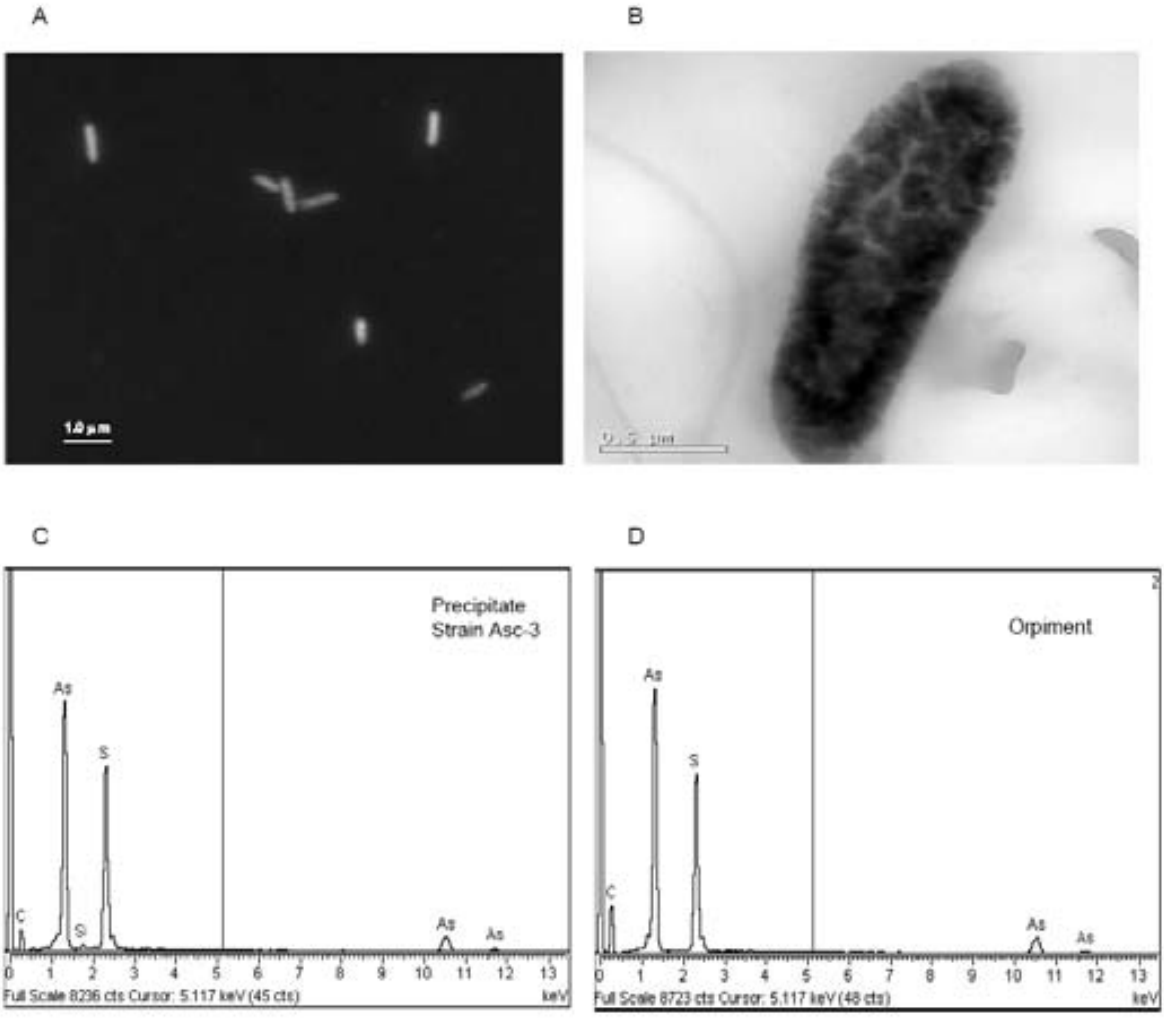

Figure. 2. A. Cells of the Asc-3 strain by epifluorescence microscopy. **B.** TEM micrograph of strain Asc-3 illustrating cell morphology. **C.** SEM-EDS spectrum of the laboratory produced microbial precipitate from an Asc-3 strain. **D.** SEM-EDS spectrum of orpiment mineral.

Cells and colony morphology

Strains Asc-3 and CC-1 were straight or slightly curved rods (often paired) motile and Gram-negative. Cells were approximately 1.0 to 2.0 µm in length and 0.5 to 0.8 µm in diameter (Fig. 2A). A transmission electron micrograph of strain Asc-3 is shown in Figure 2B. The colonies showed a bright yellow color due to precipitation of As_2S_3 when grown on 2% agar plates with arsenate and sulfate.

Growth conditions and substrates

Strains Asc-3 and CC-1 grew optimally at 28ºC (Table 1). Liquid cultures reached total bacterial numbers between 3.5 x 10^7 to 6.5 x 10^7cells/mL at the stationary phase with the medium used. Both strains showed growth respiring As(V) at temperatures ranging from 20 to 37ºC. The strains grew on lactate using sulfate or arsenate as electron acceptors, indicating that arsenate reduction was coupled to energy conservation (Table 1 conditions A to D). We did not detect growth on lactate in the absence of an electron acceptor (conditions E and F). When the strains grew with both arsenate and sulfate, the final yield was higher (up to one order of magnitude) than with sulfate or arsenate alone (Table 1.1, conditions A and B vs C and D). Cultures were grown with and without cysteine to check whether this made any difference (conditions A vs. B and E vs. F). The two conditions resulted in the same final cell yield. Finally, a control without a sulfur source showed no growth (condition F). Yellow As sulfide precipitates were observed when cells were grown in 10 mM sulfate and 1 mM arsenate (Table 1.2 conditions A, B). Yellow precipitate also appeared in the presence of arsenate and cystein (condition D) but not in the absence of a source of sulfur (condition F) or in the absence of arsenate (condition C). In order to show that in the last condition sulfate had actually been reduced to sulfide, iron was added at the end of the incubation period and a black precipitate instantly formed (Table 1.3, condition C). Since salinity in Salar de Ascotán was rather variable among sites and at differing times, the range of salinities allowing growth of these two strains was also tested.

Table 1. Summary of revelant physiological characteristics for strains ASC-3 and CC-1, as compared to strains ANA-3, OREX-4 and HN-41. Electron acceptors were tested using 20 mM lactate as the electron donor. Data for *Desulfosporosinus auripigmenti* OREX-4 are from Newman *et al.,* 1997. Data for *Shewanella* sp. strain ANA-3 are from Saltikov *et al.,* 2003. Data for *Shewanella* sp. HN-41 are from Lim *et al* ., 2008.

Physiological parameter	ASC-3	CC-1	ANA-3	OREX-4	HN-41
1.1 Growth					
A. Sulfate (10 mM)+ arsenate(1 mM)	+	+	+	+	+
B. Sulfate(10 mM) + arsenate(1mM) + cysteine(mM)	++	++	+	+	+
C. Sulfate(10 mM) + cysteine (1 mM)	+	+	-	+	+
D.Arsenate(1 mM) + cysteine (1mM)	+	+	+	+	+
E. Cysteine (1mM)	-	-			
F. Arsenate (1 mM)	-	-	+		
1.2 Precipitation of As sulfides					
A. Sulfate (10 mM)+ arsenate(1 mM)	+	+	-	+	+
B. Sulfate(10 mM) + arsenate(1mM) + cysteine(mM)	+	+	-	+	+
C. Sulfate(10 mM) + cysteine (1 mM)	-	-	-		
D.Arsenate(1 mM) + cysteine (1mM)	+	+	+	+	
E. Cysteine (1mM)	-	-	-		
F. Arsenate (1 mM)	-	-	-		
1.3 Precipitation of Fe sulfides					
A. Sulfate (10 mM)+ arsenate(1 mM)	-	-	-	-	-
B. Sulfate(10 mM) + arsenate(1mM) + cysteine(mM)	-	-	-	-	-
C. Sulfate(10 mM) + cysteine (1 mM)	+	+	-	+	+
D.Arsenate(1 mM) + cysteine (1mM)	-	-			
E. Cysteine (1mM)	-	-			
F. Arsenate (1 mM)	-	-			
Growth with					
NaCl %(w/v)					
0.5	+	+			
1.0	+	+			
2.0	+	+			
3.0	++	++			
3.5	+	+			
5.0	+	+			
7.0	+	+			
10.0	-	-			
Optimal Temperature ºC				25-30*	
20ºC	+	+			
28ºC	++*	++*			
37ºC	+	+			

Blank cells indicate that no data are avaible with regard to the specific variable. ++, growth optimal; +, growth was supported; *, optimal temperature; -, growth was not supported.

Strains Asc-3 and CC-1 showed optimal growth in synthetic medium containing up to 3.0% NaCl after two weeks of incubation (Table 1). Both strains were capable of growth and As(V) respiration at salinities ranging from 0.5 to 7.0 % (w/v) of NaCl, but not at 10%. Figs. 2 C and D show the element composition of the precipitates formed by culture Asc-3 and minerals *in situ*, have been presented in Chapter V.

16S rDNA phylogeny

Phylogenetic analyses of the complete 16S rDNA sequence of Asc-3 showed that the strain belonged to the genus *Shewanella* within the Gammaproteobacteria subdivision (Fig. 3, Tabla 2). In the phylogenetic tree in Figure 3 we included strains known to be As(V) reducing microorganisms such as the Epsilonproteobacteria *Sulfurospirillum barnesi* SES-3 (GenBank accession number U41564) and *Sulfurospirillum arsenophilum*. MIT-13 (GenBank accession number U85964) and the Firmicutes *Desulfosporosinus auripigmenti* OREX-4, *Desulfitobacterium hafniense* str. GBFH (GenBank accession number AJ307028), *Bacillus arseniciselenatis* str. E1H (GenBank accession number AF064705) and *Chrisiogenes arsenatis* str. BAL-1 (GenBank accession number X81319) were also included. These strains were very distantly related to the strains obtained from Salar de Ascotán. The later showed similarities of 80% with *Desulfosporosinus auripigmenti* OREX-4 and *Desulfitobacterium hafniense* str. GBFH and 78-80% similarity with *Sulfurospirillum barnesii* SES-3 and *Sulfurospirillum arsenophilum* MIT-13.

The genus *Shewanella* includes several strains that obtain energy for growth from the reduction of arsenate, such as *Shewanella* ANA-3 (GenBank accession number AF136392, isolated from an As treated wooden pier piling in the Eel Pond brackish estuary, Woods Hole), TS29 (EU073095, isolated from an As-contaminated environment, Huangshi City, China), HAR-4 (AY660887, isolated from Haiwee Reservoir sediments), and HN-41 (DQ0100165, a *Shewanella* sp. capable of both iron and arsenate reduction, that was isolated from intertidal flat sediments in Muan, Korea).

Table 2. BLAST results and ecophysiology of the Bacterial strains isolated from Salar deAscotán.

Strain	*Accession number*	*Phylogenetic group*	*Closest relative in GenBank*	*Source*	*Accession number*	*% Similarity*
Asc-3 ASCBAC	EF157293	Gammaproteobacteria	*Shewanella* sp. TS29	As-contaminated environment, Huangshi City, Hubei Province, China	EU073095	98
			Shewanella sp. ANA-3	As-treated wooden pier in a brackish estuary, Eel Pond Wood Hole, USA	AF136392	98
CC-1 ASCBAC	EF157294	Gammaproteobacteria	*Pseudomonas mosselii*	Soil, Nanjing, China	FJ455453	98
			Pseudomonas sp. DB-2	Soil near an oil refinery, Hangzhou, China	EU439402	98

Similarities between Asc-3 and other *Shewanella* species in the 16S rDNA nucleotide sequence were between 95 and 98%. Thus, the strain from Ascotán is clearly a novel bacterium probably at the species level. This point deserves further characterization beyond 16S rRNA to conclude it is a new species of *Shewanella*.

For CC-1 a partial 16S rDNA sequence was analyzed (612 bp). This strain belonged to the genus *Pseudomomas* within the Gammproteobacteria subdivision (Fig. 3 and Table 2). The partial sequence had similarities of 98% with *Pseudomomas plecoglossicida* (DQ095886) and *Pseudomomas putida* (AY952323) but was not related to any arsenate reducing bacterium. The lack of expected matches closely related to As bacteria, and the mixture of 16S rRNA sequences observed in the chromatogram provided by the sequencing service for CC-1 gene suggested that we were dealing with a mixed culture. Interestingly, *Pseudomonas* related 16S rRNA gene sequences were very abundant in Ascotán clone libraries (see Chapter II). Further work is therefore needed to purify and properly characterize this strain.

Amplification of arrA and arsC from the strains

Both strains Asc-3 and CC-1 showed positive amplifications for *arrA* gene with all primers sets tested (data not shown). Conversely they did not show amplification for any of the *arsC* gene primer combinations.

Discussion

In the present work we described two isolates, Asc-3 and CC-1, within the gamma Protebacteria group. These bacteria were capable of growth with arsenate as a terminal electron acceptor and with lactate as electron donor. They could also grow with sulfate as terminal electron acceptor. Asc-3 and CC-1 carried out the precipitation of As sulfide as a yellow precipitate. The compositional analysis showed this precipitate to be formed by an As sulfide with molar composition of orpiment, a mineral typically found in Salar de Ascotán. The first example of a sulfate-reducing bacterium capable of growth with arsenate as terminal electron acceptors was *Desulfosporosinus auripigmentum* OREX-4 (Newman *et al.*, 1997b). Both Orex-4 and our isolates could use either As or sulfate alone as electron acceptors.

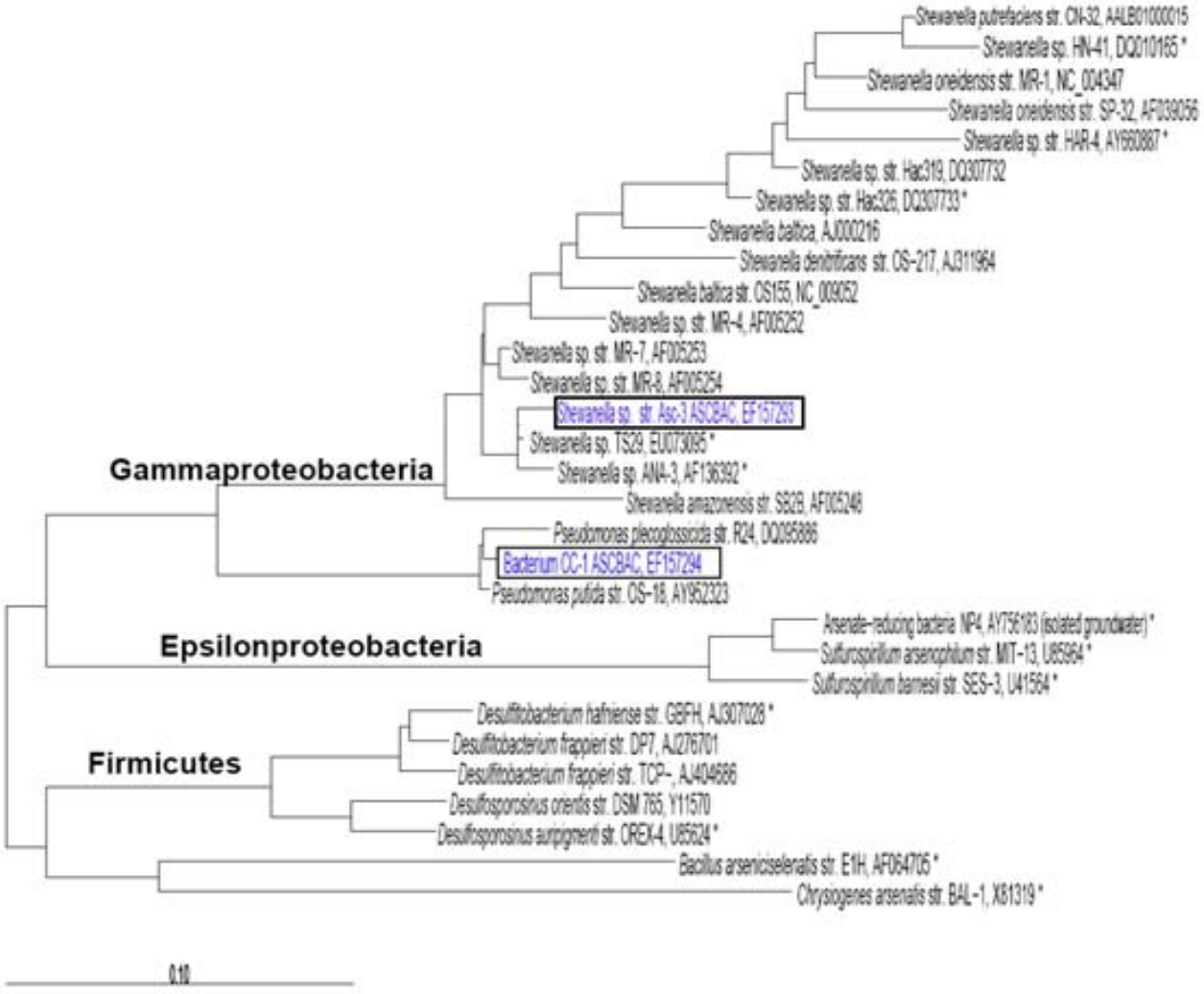

Figure 3. Phylogenetic tree based on complete 16S rRNA gene sequences showing the placement of strains Asc-3 and CC-1 (blue) in the gammaproteobacteria clade among members of the genus *Shewanella* and *Pseudomanas*. The position of currently recognized As(V)-reducing microorganisms (*) are also shown. The tree was constructed by parsimony using ARB.

Other dissimilatory arsenate reducing bacteria reported so far, e.g. strains SES-3 and MIT-13 were capable of reducing other sulfur compounds such as thiosufate and elemental sulfur, but not sulfate. Strain OREX-4 reduces arsenate before sulfate. This is not surprising given that arsenate is a better oxidant than sulfate and would thus yield a higher free energy when coupled to the oxidation of lactate to acetate at pH 7 according to the following reaction (Morel & Hering, 1993, Peters, 1974) :

$CH_3\text{-}CHOH\text{-}COO^- + 2\ HAsO_4^{2-} + 4\ H^+ \rightarrow CH_3\text{-}COO^- + 2\ HAsO_2 + CO_2\ (g) + 3\ H_2O$

$\Delta G0' = -172$ kJ/mol lactate

$CH_3\text{-}CHOH\text{-}COO^- + 0.5\ SO_4^{2-} + 0.5\ H^+ \rightarrow CH_3\text{-}COO^- + 0.5\ HS^- + CO_2\ (g) + H_2O$

$\Delta G0' = -89$ kJ/mol lactate

The stoichiometry of arsenate reduction in dual arsenate/sulfate cultures of Ascotán strains could not be explained by abiotic reduction of arsenate by sulfide. The rate of arsenate reduction in cultures Asc-3 and CC-1 amended with both arsenate and sulfate was always faster than those with sulfate alone, independent of either relative concentrations of arsenate and sulfate or the growing history of the inocula. It has been reported in some As reducing strains (e.g. *Desulfitobacterium* sp. strain GBFH) that there is a preference to use first the As and next the sulfate, to help bacteria to quickly decrease toxic As concentrations (from arsenate to arsenite that will be then excreted). This is a possibility to be further explored with the Ascotán strains.

Strain CC-1 was phylogenetically related to the genus *Pseudomonas* (98 % similarity with *Pseudomonas plecoglossicida* str. R24 and *Pseudomonas putida* str. OS-18). A link of the latter *Pseudomonas* spp. with arsenate reduction has not been described. *Pseudomonas* spp. are Gram-negative rods, historically classified as strict aerobes, although some species have been found to be facultative anaerobes (Iglewski, 1996). CC-1 grew anaerobically reducing arsenate and sulfate, and this had never been reported before in *Pseudomanadaceae.* The *Pseudomonas* abundantly found in the 16S rRNA gene clone libraries from water samples of Salar de Ascotán (see Chapter II), were only 95% similar to strain CC-1. However, we could not obtain a clear complete sequence of the 16S rDNA gene. Therefore, the strain needs to be further purified and studied in order to rule out contaminations or presence of a mixed

culture. Potentially, this is a new highly versatile *Pseudomonas* species of great interest. For this reason we report the preliminary data available here.

Asc-3 was related to the genus *Shewanella*. Only two *Shewanella* strains have been shown to use As as electron acceptor: ANA-3 (Saltikov *et al.*, 2003) and TS29 (Cai *et al.*, 2009). Arsenate therefore does not appear to be a common electron acceptor for *Shewanella* species. Recently, however, links with the As cycle have been formed for members of this genus with a detailed study of the reduction, precipitation, and transport of As species by *Shewanella* sp. str HN-41, a facultative and versatile iron reducing bacterium (Lim *et al.*, 2008). This strain can reduce As(V) and sulfate, and form As sulfide, but detailed characterization of HN-41 has not been published yet.

It is known that representatives of *Shewanella* are distributed widely in nature and members of this genus have been often isolated from very different environments such as Antarctic habitats, oil-rich sediments, deteriorated foods, and also as opportunistic pathogens of humans and aquatic animals. The genus *Shewanella* is at present a focus of detailed studies analyzing their physiological versatility and the diversity of ecological niches they can colonize (Tiedje, 2002). They are able to use a wide array of terminal electron acceptors in the absence of oxygen, such as ferric compounds, manganese oxides, molybdenum, sulfur and nitrate. This fact has a significant impact in mineral solubilization and mobilization in the environment. For instance, in anaerobic soils *Shewanella* uses Fe^{3+} as final electron acceptor, and the same could apply to As compounds in sediments (Tiedje, 2002). Myers and Myers (1992) related this versatility to the presence of numerous cytochromes in the external membrane, as this could facilitate electron transport and the dissimilatory reduction (Myers & Myers, 1992). These are key issues for efficient anaerobic respiration and important traits for mineral mobilization. The strain isolated from the sediment of Salar de Ascotán is of great interest as a model organism to study the mechanisms for As mobilization under reducing anaerobic conditions and high salt concentrations. The 16S rDNA analyses revealed that strain Asc-3 was closely related (similarity 98%) to *Shewanella* sp. str. ANA-3 and *Shewanella* sp. TS29 (a strain isolated from an As contaminated site, but data only available in GenBank). These three strains can grow anaerobically at temperatures between 28 to 37ºC and pH

6.8 to 7.0, using lactate as electron donor, and obtaining energy for growth from the reduction of arsenate. The difference existing between Asc-3 and ANA-3 (no data are available for TS29) is that Asc-3 was capable of sulfate reduction that precipitated as As_2S_3, whereas ANA-3 could not reduce sulfate. In the case of this isolate formation of As_2S_3 under anaerobic conditions is the result of metabolizing cysteine, when this aminoacid is included in the medium as a reducing agent (Saltikov *et al*., 2003). In addition, Asc-3 was strictly anaerobic, whereas ANA-3 could grow aerobically in LB medium. So far, sulfate reduction in As using bacteria had been found only in the Firmicutes *Desulfosporosinus auripigmentum* strain OREX-4 (Newman *et al*., 1997b). Thus, strain Asc-3 widens considerably the phylogenetic spectrum of bacteria able to carry out this peculiar combination of sulfate and arsenic precipitation.

In addition, in Chapter V and in the present Chapter, our results show that the relative As/S molar ratio of the precipitate was more closely related to the stoichiometry of orpiment than that of realgar or pararealgar. The As/S molar ratio was 0.60±0.06 for strain Asc-3 and 0.66±0.07 for orpiment (Fig. 2 C-D). Therefore, the data presented here provide strong evidence that the arsenic sulfide minerals in natural environments such as Salar de Ascotán are due to microbial activities.

Finally, both strains produced an amplicon when PCR was carried out with primers for the *arrA* gene, but not for the *arsC* gene. This is surprising because the latter gene is much more widespread among bacteria than the former. *arsC* is used to detoxify arsenic and is, thus, present in bacteria able to tolerate moderate arsenic concentrations. *arrA*, on the other hand, is required for anaerobic respiration of arsenate. This factor is apparently restricted to only a few groups. We showed in Chapter III that *arsC* could not be found in habitats with high As concentration but was present at lower As concentrations. *arrA* could be found in all habitats analysed in Atacama. Apparently, bacteria specialized in obtaining energy from arsenate respiration do not required detoxification genes such as *arsC*. The *arsC* gene of detoxification was not present in strain Asc-3 but, on the contrary, gene *arrA* was required for the strain to respire As(V).

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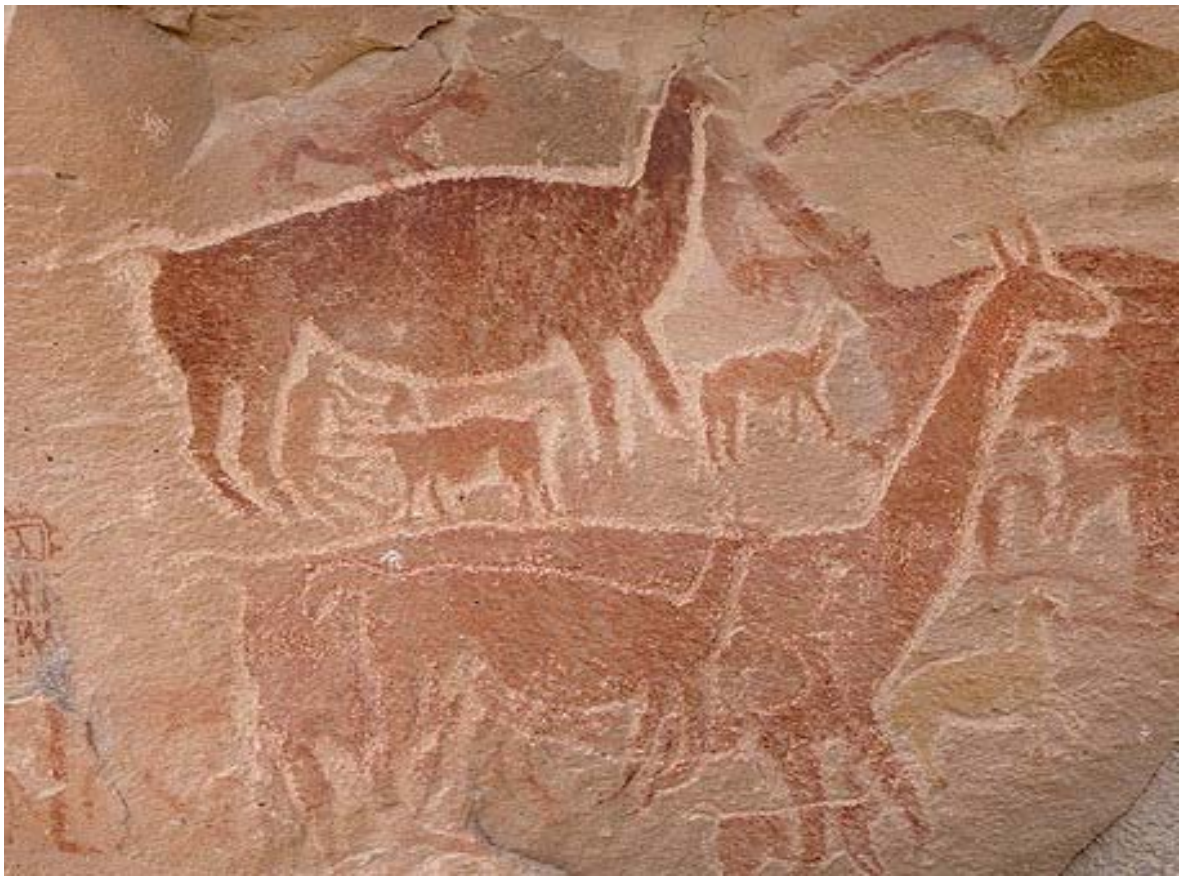

CONCLUSIONES GENERALES

Para las personas creyentes, Dios está al principio.
Para los científicos está el final de todas sus reflexiones.

(MaxPlanck)

CONCLUSIONES GENERALES

En la II Región de Chile, donde el agua escasea y el arsénico tanto de origen natural como antropogénico presiona históricamente a la población, el tratamiento de residuos industriales con arsénico es un tema de la más alta prioridad en los planes estratégicos de desarrollo. La presencia de microorganismos reductores de arsénico, incluso en sistemas de agua dulce, que favorecen la movilización del elemento desde sedimentos, sugiere la relevancia de la microbiología en el ciclo biogeoquímico del arsénico en la zona.

En el transcurso de la presente tesis se pudo observar la importancia de estos microorganismos, en ambientes con características específicas y únicas. En esta tesis el estudio de la biodiversidad asociada a parámetros naturales específicos como el arsénico, nos permitió entender la diferencia entre ambientes salinos ricos en arsénicos y otros pobre. Además se pudocomprender mejor la distribución de los genes involucrados en el ciclo biogeoquímico del arsénico en el Salar de Ascotán.

1. El estudio de la diversidad bacteriana mediante clonación y secuenciación del gen 16S rDNA en distintos ambientes salinos del Norte de Chile reveló una elevada diversidad filogenética, especialmente en muestras de sedimentos. En general, Firmicutes, Proteobacterias y Bacteroidetes fueron los grupos que se recuperaron con una mayor frecuencia.

2. La composición de la comunidad bacteriana en la Laguna Tebenquiche fue muy heterogénea en espacio y tiempo, condicionada por la salinidad del agua en cada punto de la laguna.

3. La Laguna Tebenquiche está dominada por los grupos de Gammaproteobacteria y Bacteroidetes (representado por miembros *Psychroflexus torquis* y *Salinibacter*). Dentro de estos grupos se observó un elevado grado de microdiversidad.

4. En el Salar de Ascotán se observó por PCR-DGGE del gen 16S rDNA que en las muestras de aguas el grupo más abundante fue de los Alfaproteobacterias y que en las muestras de sedimentos fueron los Firmicutes. Además se observó que en muestras con concentraciones de arsénico por debajo de 4

mg/L los grupos más frecuentes fueron Betaproteobacteria y Firmicutes. A concentraciones más altas de arsénico los grupos dominantes fueron Bacteroidetes y Gammaproteobacteria.

5. Se observó menor diversidad filogenética pero mayor microdiversidad en la muestra de agua (baja concentración de As) que en el sedimento (alta concentración de As).

6. Se encontró una marcada segregación filogenética dentro de Firmicutes y Proteobacterias condicionada al tipo de hábitat. Epsilonproteobacteria fueron más abundantes en la muestra de agua, mientras que Bacteroidetes y Deltaproteobacterias fueron exclusivos del sedimento.

7. El estudio de la presencia o ausencia de los genes de arsenato reductasa a través de PCR con distintos cebadores permitió detectar una distribución diferencial entre el gen *arrA y* el gen *arsC*. El gen *arrA* se encontró en todas las muestras analizadas desde un rango de concentración de As entre 0,4 a 6504 mg/L. El gen *arsC* sólo se encontró en las muestras con concentraciones inferiores a 4 mg/L de As.

8. Se observa que existe un nuevo grupo de clones relacionados con el gen *arrA,* en el Salar de Ascotán, muy alejados de todos los descritos previamente.

9. Los resultados recopilados en este trabajo –obtención de cultivos enriquecidos que precipitan arsénico y medición del fraccionamiento isotópico- permiten concluir que existe contribución de actividad microbiana en la precipitación de arsénico en forma de sulfuros en el mineral de ulexita de los salares asociados a procesos termales.

10. Se logró aislar dos cepas bacterianas reductoras de arsénico y sulfato desde el Salar de Ascotán, pertenecientes al grupo de Gammaproteobacteria: Asc-3 cercana a *Shewanella,* y CC-1 a *Pseudomonas.* La cepa CC-1 sería la primera Pseudomonaceae descrita como reductor de As en el caso de confirmarse.

Aprendí que no se puede echar marcha atrás,
que la esencia de la vida es ir hacia adelante.
La vida, en realidad, es una calle de sentido único.
(Agatha Christie)