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ORIGINAL ARTICLE

Arsenic precipitation by an anaerobic arsenic-respiring bacterial strain isolated from the polluted sediments of Orbetello Lagoon, Italy

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Abstract

Aims: To isolate and characterize an anaerobic bacterial strain from the deeper polluted lagoon sediment able to use as electron acceptors [As(V)] and sulfate (SO_4^{2-}), using lactate as an electron donor.

Methods and Results: Methods for isolation from polluted lagoon sediments included anaerobic enrichment cultures in the presence of As(V) and SO_4^{2-}. Reduction of As(V) to As(III) was observed during the growth of the bacterial strain, and the final concentration of As(III) was lower than the initial As(V) one, suggesting the immobilization of As(III) in the yellow precipitate. The precipitate was identified by energy dispersive spectroscopy X-ray as arsenic sulfide. Scanning electron microscopy (SEM) revealed rod-shaped bacterial cells embedded in the precipitate, where net-like formations strictly related to the bacterial cells were visible. The surface of the precipitate showed the adhesion of bacterial cells, forming clusters. Transmission electron microscopy (TEM) also highlighted precipitates inside the bacterial cells and on their surface. Following 16S rRNA sequencing, the bacterial strain 063 was assigned to the genus *Desulfosporosinus*.

Conclusions: This study reports, for the first time, the isolation from the polluted lagoon sediments of a strain capable of respiring and using As(V) and SO_4^{2-} as electron acceptors with lactate as the sole carbon and energy source with the formation of an arsenic sulfide precipitate.

Significance and Impact of the Study: The identification of these properties provides novel insight into the possible use of the anaerobic strain in bioremediation processes and also adds to the knowledge on the biogeochemical cycling of arsenic.

Introduction

Arsenic is a toxic metalloid widely distributed in nature and commonly associated with the ores of metals like copper, lead and gold (Nriagu 2002). The main sources of arsenic are natural and it also originates as the result of human activities (Nordrstorm 2002). Moreover, arsenic is often associated with pyrite, one of the most ubiquitous minerals, and is commonly present in sulfide mineral deposits (Nriagu 2002).

The predominant form of inorganic arsenic in aqueous, aerobic environments is arsenate [As(V)], whereas arsenite [As(III)] is more prevalent in anoxic environments, and of these two, As(III) is more toxic (Smedley and Kinniburgh 2002). Micro-organisms can use arsenic compounds as electron donors or electron acceptors and possess arsenic detoxification mechanisms, with pumps at the membrane level able to remove As(III) from cells, eventually after As(V) reduction (Ahmann *et al.* 1994; Newman *et al.* 1997a).

Dissimilatory reduction for anaerobic respiration involves arsenate as the terminal acceptor of electrons. Arsenate-respiring bacteria are widespread and metabolically active in nature. The first report of an arsenic-respiring bacterial strain concerned the strain MIT-13, a microorganism with the shape of a *Vibrio* (Ahmann *et al.* 1994), subsequently named *Geospirillum arsenophilus* (Lovley and Coates 1997). Although a limited number of species have been identified, it is clear that the phenomenon is polyphyletic, i.e., the organisms in question are phylogenetically diverse (Oremland and Stolz 2003).

The concomitant reduction of sulfate and arsenate is not frequent. The importance of this combined reduction lies in the fact that stimulation of sulfate reduction and the ensuing generation of sulfide is an effective mechanism for the precipitation of metals, including arsenite (Newman *et al.* 1997b). This is useful in reducing mobility and risk.

The arsenic contamination in Orbetello Lagoon, Italy, is mostly because of ash, dust and debris originating from a fertilizer production plant located on a bank of the lagoon. Closed down in the late 1980s, the fertilizer production plant used pyrite (FeS_2) containing high concentrations of arsenic and other impurities such as zinc and lead, as raw materials for the production of sulfuric acid. The pyrite used in the production cycle was extracted from the Colline Metallifere, a chain of hills located in an internal area of Tuscany not far from Orbetello Lagoon, which shows anomalous arsenic concentrations (Donati *et al.* 2005).

This report describes an anaerobic arsenic- and sulfur-respiring bacterial strain isolated from the sediments of Orbetello Lagoon and illustrates its involvement in the biogeochemical cycle of arsenic and in its immobilization reducing risks.

Materials and methods

Study area and sediment sampling

Orbetello Lagoon is located on the Tyrrhenian coast of central Italy (42°26′34″N, 11°13′29″E), covering an area of about 2300 ha with a water depth of about one metre. Total As concentration reached 155·13 ± 0·08 mg kg^{-1} (d.w.) at level 30–50 cm (Focardi 2005). Deep sediment samples were extracted from cores sampled by a scuba diver using a soft sediment plastic-barrel coring device (inside diameter 7 cm). The cores were carefully capped to prevent any moisture loss and shipped to the laboratory. Samples for microbiological analyses were aseptically collected in sterile sacs (Wirlpak, Nasco), by using a spatula sterilized at 121°C for 15 min, from the cores at the 30–50 cm level and maintained at 4°C until treatment in the laboratory.

Enrichment cultures and isolation of bacterial strain

An aliquot of 1·0 g of the sediment sample was added to vials containing 50 ml of minimal medium, composed of 100 mmol l^{-1} of Tris–HCl (pH 7·2), 5 mmol l^{-1} of NH_4Cl, 0·1 mmol l^{-1} KH_2PO_4, 0·5 mmol l^{-1} of K_2SO_4, 0·1 mmol l^{-1} of $CaCl_2$, 10 mmol l^{-1} of $MgCl_2$ and 100 mmol l^{-1} of NaCl per litre of double-distilled water. The enrichment cultures were amended with 14 mmol l^{-1} of sodium sulfate (Na_2SO_4), 10 mmol l^{-1} of dibasic sodium arsenate, 20 mmol l^{-1} of sodium lactate and 1 mmol l^{-1} of cysteine under an N_2 atmosphere. The medium contained a vitamin mix and a mineral mix (Newman *et al.* 1997b). The medium culture was prepared according to the Hungate technique for anaerobic cultures, by boiling for 5 min to degas O_2. The medium was cooled to room temperature under N_2 flux. Sealed vials were steam heat-sterilized (Miller and Wolin 1974). Determinations were carried out in duplicate. Inoculated vials were mixed and incubated at 28°C in the dark for 15 days. Colonies growing in these conditions and responsible for precipitate formation were isolated by successive colony transfers in agar shake tubes (Pfenning *et al.* 1981) and were maintained in 20 mmol l^{-1} of sodium lactate, 10 mmol l^{-1} of disodium arsenate, added as disodium hydrogen arsenate ($Na_2HAsO_4{\cdot}7H_2O$), and 14 mmol l^{-1} of sulfate. Single yellow colonies were picked from each shake tube for further growth and precipitation. The isolated bacterial strain was maintained in vials in the presence of 5% sterile DMSO at −80°C.

Growth of the isolated bacterial strain in anaerobic conditions

The isolated bacterial strain was tested for growth in mineral medium with low phosphate content, in the presence of 20 mmol l^{-1} of sodium lactate as the electron donor, 5 mmol l^{-1} of As(V) added as disodium hydrogen arsenate ($Na_2HAsO_4{\cdot}7H_2O$) and 14 mmol l^{-1} of sulfate. Samples were taken directly from the cultures and used for analyses. A control without bacteria added was treated in the same way. Growth in the presence of the same medium culture and under the same conditions was also prepared in aerobic conditions. Medium culture with sodium azide-killed cells, in the presence of arsenate and sulfate, was also used as control.

Determination of As(V)-reduction to As(III)

Aliquots of 1 ml were harvested at different times (0, 1, 2, 3, 4, 6, 8, 12, 14 and 21 days) using syringes to measure cell growth at OD 600 nm and to determine As(V) and As(III) concentration by spectrophotometric analysis according to method described by Cummings *et al.* (1999).

Other chemical analyses and cell count

The sulfate content was measured using the barium sulfate technique (Tabatabai 1974), and the sulfide content was measured using the methylene blue method (Franson 1981). Lactate and acetate concentrations were determined by HPLC on a polysulfonate ion-exclusion column (Hamilton PRP X 300; Hamilton, Bonaduz, Switzerland) with UV detection at 210 nm. Cells were stained with 4′,6-diamidino-2-phenylindole and counted.

Scanning electron microscopy

For scanning electron microscopy (SEM), samples were fixed with 2·5% paraformaldehyde + 2·5% glutaraldehyde in 0·1 mol l^{-1} cacodylate buffer pH 7·2 overnight at 4°C. After washing three times for 30 min each at 4°C in the same buffer, they were postfixed with 1% osmium tetroxide + 0·15% ruthenium red in cacodylate buffer for 1 h at 4°C. Specimens were washed in three changes of distilled water for 15 min each at 4°C, then block stained with 1% uranyl acetate in distilled water. They were then washed in distilled water and dehydrated in a graded acetone series. Samples were adsorbed onto poly-L-lysinate coverslips and then dried by the critical point method using CO_2 in a Balzers Union CPD 020 (Balzers, Wiesbaden, Germany). They were then attached to aluminium stubs using carbon tape and sputter-coated with gold in a Balzers MED 010 unit. Observations were performed using a JEOL JSM 5200 scanning electron microscope (JEOL, Tokyo, Japan). Elemental analysis was performed by energy dispersive spectroscopy (EDS) (ISIS, Oxford University, Oxford, UK) coupled with the SEM.

Transmission electron microscopy

For transmission electron microscopy (TEM), the samples were fixed and dehydrated as described above. Infiltration and embedding were performed in Epon-based resin. Thin sections were cut with a Reichert Ultracut ultramicrotome using a diamond knife, collected on copper grids, stained with uranyl acetate and observed with a JEOL 1200 EX II electron microscope.

Isolate characterization and identification by 16S rDNA gene sequencing

Following the growth of the bacterial isolate in shake tubes, Gram determination was carried out (Gram stain kit; Carlo Erba, Milan, Italy). For 16S rDNA sequencing of isolated bacterial strains, a single colony was suspended in 50 μl of double-distilled water and treated for 5 min at 100°C. Amplification of 16S rRNA gene was performed as previously reported (Pepi *et al.* 2007). Sequencing was carried out at the Bact 16S biomolecular research service (CRIBI Biotechnology Centre, University of Padua, Italy). A partial (1492 bp) 16S rDNA sequence was determined for the anaerobic arsenate-respiring bacterial isolate 063. The sequence was deposited in the GenBank database with the accession number GQ214051. The consensus sequence of isolate 063 was compared with those deposited in GenBank using the BLAST program (Altschul *et al.* 1997).

Analysis of sequence data

The 16S partial sequence of strain 063 was compared at the prokaryotic small subunit rDNA on the Ribosomal

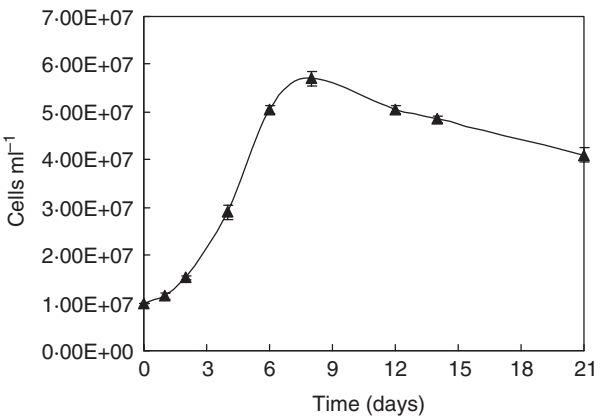

Figure 1 Growth of the isolated bacterial strain 063 in minimal medium in the presence of lactate 14 mmol l^{-1} as the electron donor and As(V) 5 mmol l^{-1}. Triangles show single data points that are representative of duplicate cultures.

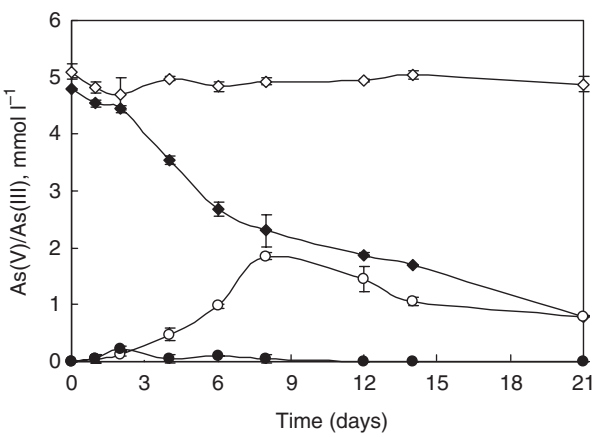

Figure 2 Detection of As(V) in the bacterial cells of the isolated strain 063 (♦) and related As(III) production (○) in cultures of the bacterial strain 063. The content of As(V) (◇) and of As(III) (•) in control cultures without bacteria added was constant, at 5 and 0 mmol l^{-1}, respectively. Symbols show single data points that are representative of duplicate cultures.

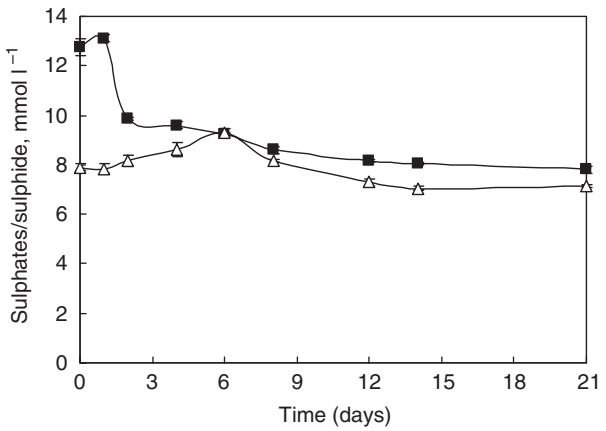

Figure 3 Detection of sulfates in the bacterial cells of the isolated strain 063 (■) and related sulfide production (Δ) in cultures of the bacterial strain 063. The sulfate and sulfide content in control cultures without bacteria added was constant throughout the experiment. Symbols show single data points that are representative of duplicate cultures.

Database Project II website (Cole *et al.* 2009). The 16S rDNA sequences retrieved from the databases were aligned with ClustalW included in the software MEGA version 4.0 (Kumar *et al.* 2008). The phylogenetic tree was inferred by MEGA 4.0 (neighbour-joining method) (Saitou and Nei 1987). Sequence divergences between strains were quantified using the Kimura-2-parameter distance model (Kimura 1980). The 'Complete Deletion' option was chosen to deal with gaps. Bootstrap analysis (1000 replicates) was used to test the topology of the neighbour-joining method data. The tree was unrooted.

Results

From enrichment cultures in the presence of As(V) and SO_4^{2-} as electron acceptors, and lactate as the sole carbon and energy source, an anaerobic bacterial strain was isolated and named 063. After 9 days of incubation, a yellow precipitate was apparent and colonies of yellow colour were also detected in solid medium. The precipitate did

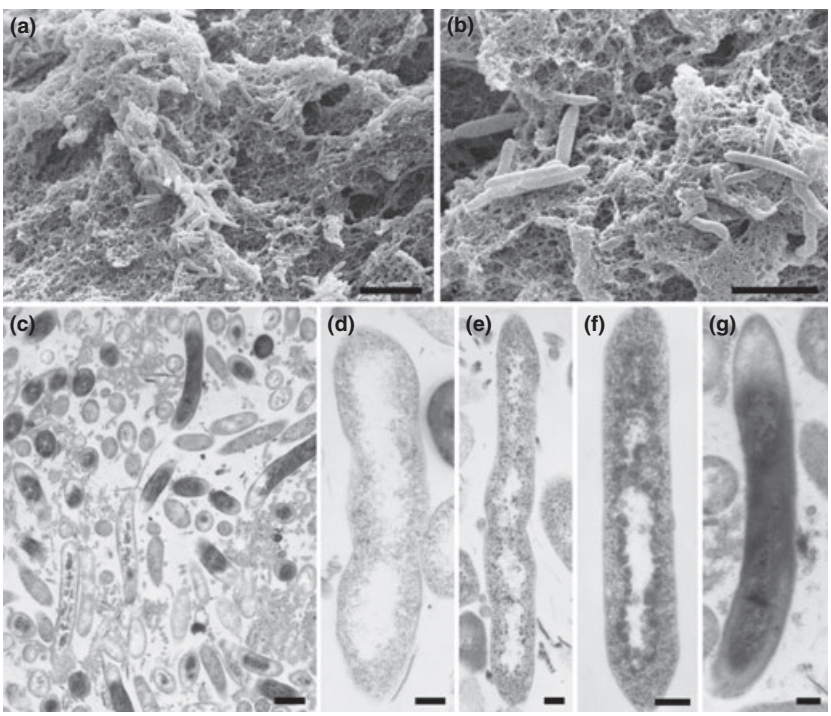

Figure 4 Image obtained by scanning electron microscopy (SEM) of the yellow precipitate in cultures of the isolated bacterial strain 063, showing a higher amount of precipitate with the clusters of adhered bacterial cells. Bar 10 μm (a). The same image as in a, observed at higher magnification, showing the rod-shaped cells of the isolated bacterial strain 063 included in the structure of the precipitate. Bar 5 μm (b). Image obtained by transmission electron microscopy (TEM) of a section of bacterial cells of the isolated strain 063, showing the rod-shaped cells with material of differing electron density included in the cytoplasm of the bacterial cells. Bar 1 μm (c). The subsequent image obtained by TEM shows a bacterial cell of the isolate 063 containing electron dense material distributed in the cytoplasm of the bacterial cell. Bar 200 nm (d). TEM image of a cell of the isolated bacterial strain 063, showing internal inclusions similar to the structures present in the immediately external part of the bacterium. Bar 200 nm (e). Image obtained by TEM of a bacterial cell of the isolate 063 with electron dense material included in the cytoplasm, in an area surrounding a vacuolar formation. Bar 200 nm (f). TEM image of a bacterial cell of the isolate 063 showing evident electron density and a probable entrance for the external material into the internal part of the cell. Bar 250 nm (g).

not form neither in the control culture without bacteria added nor in cultures with killed cells.

Maximum growth was detected after 5 days of incubation (Fig. 1), and the bacterial culture revealed the reduction of As(V) to As(III), showing a maximum after 6 days of incubation (Fig. 2). During growth of the isolated bacterial strain 063, As(V) reduction and As(III) production were observed, but not in a proportional manner (Fig. 2). A reduction in sulfates and the production of sulfides were also detected during growth of the bacterial cells of the isolated strain 063, although the concentration of sulfides decreased as the incubation time increased (Fig. 3). No growth of the isolated bacterial strain 063 was detected in aerobic conditions.

The SEM image of the yellow precipitate that originated in the cultures of the isolated strain 063 showed a significant deposition of material in the form of a net-like structure, with an appearance similar to a biofilm. Clusters of rod-shaped bacterial cells firmly adhered to the precipitate were evident, suggesting a close relationship between bacterial cells and the substrate (Fig. 4a). The same image was then observed at higher magnification, highlighting the net-like structure of the precipitate closely connected to the bacterial cells of the strain 063, which are also included in the structure of the precipitate (Fig. 4b).

The TEM image of the precipitate formed in cultures of the bacterial strain 063 showed a set of bacterial cells arranged in longitudinal and transverse sections, with evidence of low density external material between the different bacterial cells (Fig. 4c). In the same image, bacterial cells of the isolated bacterial strain 063 showed a differential uptake of material, probably in relation to the precipitate (Fig. 4c). A series of TEM images of different single cells of the isolated bacterial strain 063 showed uptake of electron-dense material. In the image shown in Figure 4d, the electron dense material was uniform inside the cytoplasm of the bacterial cell, except in the internal portion of the cell. The same image revealed a few points inside the cytoplasm with higher electron density (Fig. 4d). Here

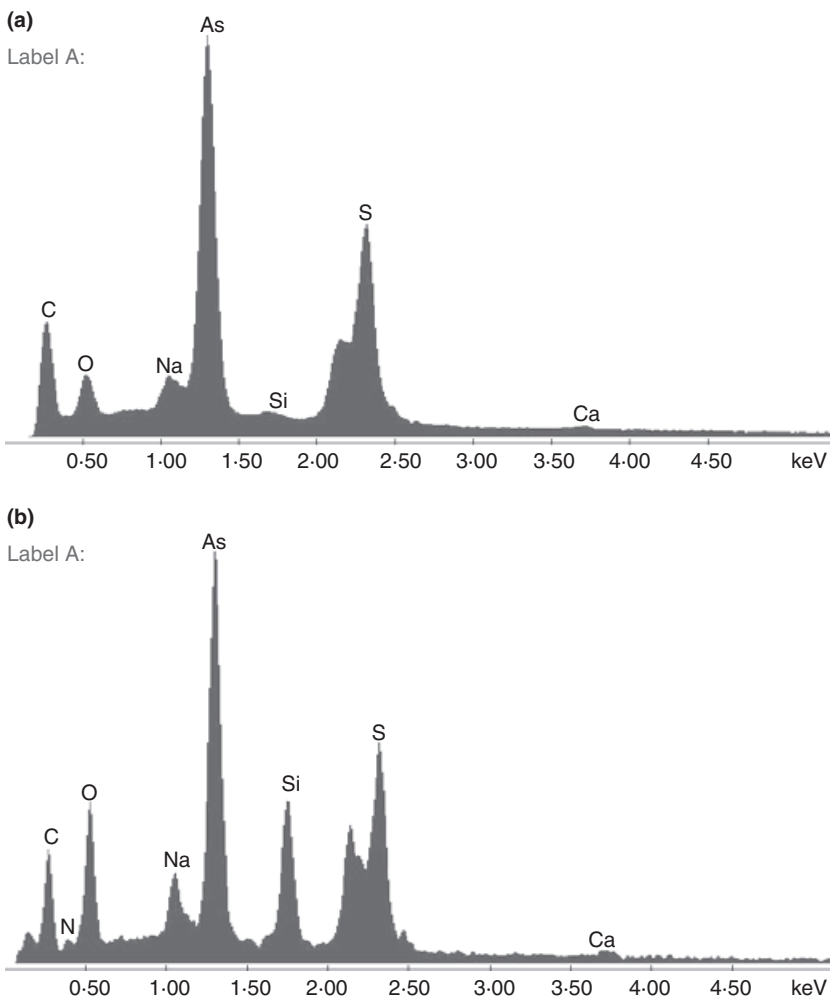

Figure 5 Energy dispersive X-ray spectroscopy (EDS) analysis of the yellow precipitate that originated in colonies of the isolated bacterial strain 063, showing peaks of As and S (a). EDS analysis of the bacterial cells of isolate 063, showing significant arsenic and sulfide distribution on the cell surface (b).

again it was possible to observe the uptake of electron dense material in the cytoplasm of the bacterial cell, except in the internal part, while black deposits were present in both the internal and external portions of the bacterial cell (Fig. 4e). In the subsequent image, significant electron density was highlighted in the cytoplasm of the bacterial cell; this material was localized and showed high density in the rim of the clear central area of the cell (Fig. 4f). Uniform electron density was observed in the cells of the strain 063, along with precipitated material near the cell wall of bacteria, highlighting a possible continuity between the external and internal parts of the bacterial cell (Fig. 4g).

Elemental analysis performed by EDS analysis of the precipitate obtained in the bacterial cell cultures showed important peaks for arsenic and sulfur, confirming that the precipitate is composed of arsenic and sulfur – an arsenic sulfide (Fig. 5a). Significant distribution of arsenic and sulfur on the cell surface was also seen in cells of the isolated strain 063 (Fig. 5b).

BLAST analysis assigned the isolated strain 063 to the genus *Desulfosporosinus*, and the closest relatives was *Desulfosporosinus auripigmenti* strain OREX-4 (AJ493051) (formerly *Desulfotomaculum auripigmentum* type strain DSMZ 13351), with percentage identity values of 98. The phylogenetic tree showed the position of strain 063 in a separate clade, close to the strain *Desulfosporosinus hippei* DSM 8344 (Y11571) isolated from permafrost, and to the strains *Desulfosporosinus* spp. T2 (AF076526), T1 (AF076525) and S5 (AF076248) isolated from groundwater contaminated with motor fuel (Fig. 6).

Discussion

It has been reported that anaerobic bacteria can reduce As(V) to As(III) and sulfate reduction to elemental sulfur,

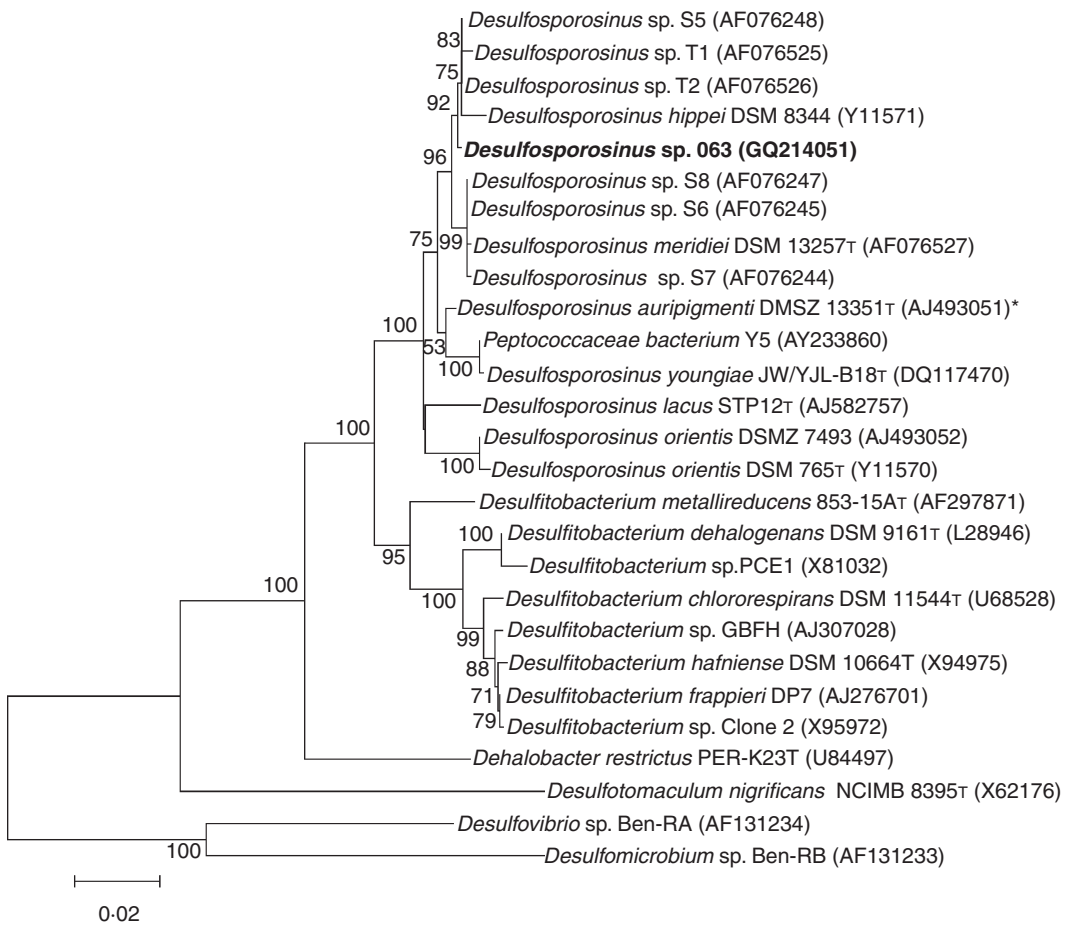

Figure 6 Unrooted phylogenetic tree based on partial 16S rRNA gene sequencing, showing the position of *Desulfosporosinus* sp. 063 strain (bold) among members of Peptococcaceae. The sequences of two δ-Proteobacteria arsenate/sulfate-reducing bacteria Ben-RA and Ben-RB were used as outgroups. Numbers indicate per cent bootstrap confidence. The scale bar indicates substitutions per nucleotide. The accession numbers for the 16S rRNA sequences are reported after the strain name in parenthesis. T: type strain. Sequence *AJ493051 is classified as *Desulfotomaculum auripigmentum*, which has recently been reclassified as *Desulfosporosinus auripigmenti* (Stackebrandt *et al.* 2003).

producing a yellow precipitate (Newman *et al.* 1997a,b; Lee *et al.* 2007). The decrease in sulfide concentration accompanied bacterial growth, indicating that reduction of As(V) to As(III) and formation of the As-S yellow precipitate was microbiologically mediated by strain 063. The dissimilatory metal-reducing bacterium *Shewanella* sp. HN-41 precipitates arsenic sulfide nanotubes upon reduction in thiosulfate and arsenate (Lee *et al.* 2007).

The isolated bacterial strain 063 has rod-shaped bacterial cells firmly connected to the precipitate and shows different behaviour to the bacterial arsenic-metabolizing strain YeAs, known to produce an arsenic sulfide mineral in particle form during bacterial growth (Ledbetter *et al.* 2007).

Arsenic and sulfur were detected in the precipitate that originated in the cultures of the isolated bacterial strain 063, as well as on the surface of the bacterial cells. Similar behaviour has previously been reported for other arsenic- and sulfur-precipitating bacterial cells (Newman *et al.* 1997a; Ledbetter *et al.* 2007; Lee *et al.* 2007).

Image studies showed different electron densities in the internal portion of cells of the isolated bacterial strain 063, suggesting a different internalization of the precipitated material in different cells. Internalization of the precipitate of arsenic and sulfur has been observed in the internal part of the bacterial cells of *D. auripigmentum* (Newman *et al.* 1997b), later reclassified as *D. auripigmenti* (Stackebrandt *et al.* 2003). No uptake of the arsenic sulfide precipitate was detected in the internal portion of the *Schewanella* sp strain HN-41, also reported as capable of resulting in an arsenic sulfide precipitate during respiration (Lee *et al.* 2007).

This study suggests that the arsenic present as a pollutant in the sediments of Orbetello Lagoon (Focardi 2005) is subject to the activity of bacteria that determine its transformation from one chemical form to another and as a result the relative processing and mobility in the environment. This study has illustrated the adaptability of bacterial populations to the biogeochemical cycle of arsenic in the sediments of this polluted lagoon. Characterization of the adapted bacterial strains that are capable of precipitating arsenic could also provide an important basis for the biotechnological application of bioremediation to remove pollutants, in this case arsenic, i.e., to reduce mobility and risk in mine-contaminated environments.

Desulfosporosinus auripigmenti DSMZ 1335T (AJ493051), formerly *D. auripigmenum* (Stackebrandt *et al.* 2003), produces a yellow precipitate during growth, composed of arsenic trisulfide. This bacterial strain originated from surface sediments where contamination by arsenic and the presence of a biogeochemical cycle of this element was known (Aurilio *et al.* 1994). The selection carried out by the native environment seems to suggest an important role in the adaptation of the isolated bacterial strain 063.

Other strains that belong to the genus *Desulfosporosinus* and are closely related to the strain 063 originated from anaerobic groundwater polluted with gasoline and were able to reduce sulfates (Robertson *et al.* 2000). No arsenate respiration was reported in these bacterial strains.

The data here suggest that the isolated bacterial strain *Desulfosporosinus* sp. 063 probably plays a role in the biogeochemical cycle of arsenic in the native polluted sediments of Orbetello Lagoon, as the metabolism of the isolate was found to give rise to a precipitate of arsenic sulfide in laboratory conditions. Reduced risk and arsenic mobility by precipitation by the isolated bacterial strain *Desulforosporinus* sp. 063 in bioremediation processes is suggested.

Acknowledgements

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