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ORIGINAL ARTICLE

Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello Lagoon, Italy, and their characterization

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Abstract

Aims: The aim of this study was to isolate arsenic-resistant bacteria from contaminated sediment of the Orbetello Lagoon, Italy, to characterize isolates for As(III), As(V), heavy metals resistance, and from the phylogenetic point of view.

Methods and Results: Enrichment cultures were carried out in the presence of 6·75 mmol l^{-1} of As(III), allowing isolation of ten bacterial strains. Four isolates, ORAs1, ORAs2, ORAs5 and ORAs6, showed minimum inhibitory concentration values equal or superior to 16·68 mmol l^{-1} and 133·47 mmol l^{-1} in the presence of As(III) and As(V), respectively. Isolate ORAs2 showed values of 1·8 mmol l^{-1} in the presence of Cd(II) and 7·7 mmol l^{-1} of Zn(II), and isolate ORAs1 pointed out a value of 8·0 mmol l^{-1} in the presence of Cu(II). Analysis of 16S rRNA gene sequences revealed that they can be grouped in the three genera *Aeromonas, Bacillus* and *Pseudomonas*. Phylogenetic analysis of the four more arsenic-resistant strains was also performed.

Conclusion: Isolates are highly resistant to both As(III) and As(V) and they could represent good candidates for bioremediation processes of native polluted sediments.

Significance and Impact of the Study: This study provides original results on levels of resistance to arsenic and to assigning genera of bacterial strains isolated from arsenic-polluted sediments.

Introduction

Arsenic is a metalloid widely distributed in soils and natural waters, released both from natural and anthropogenic sources, from the weathering of rocks or by mining industries and agricultural practices. It is found in the oxidation states +5 (arsenate), +3 (arsenite), 0 (elemental arsenic) and −3 (arsine). It has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC 1987) and the US Environmental Protection Agency (EPA 1988). The most common oxidation states of arsenic in the environment are the pentavalent As(V) and trivalent As(III) forms (Cullen and Reimer 1989). Of these two, As(III) is more toxic and can inhibit various dehydrogenases (Ehrlich 1996); arsenite (AsO^{2-} or AsO_3^{2-}) is able to bind sulfhydryl groups of proteins and dithiols such as glutaredoxin. Arsenate (AsO_4^{3-}) acts as a structural analogue of phosphate and inhibits oxidative phosphorylation by producing unstable arsenylated derivatives (Da Costa 1972). Its biogeochemical cycle strongly depends on microbial transformations, which affects the mobility and the distribution of arsenic species in the environment (Mukhopadhyay *et al.* 2002). Although arsenic is generally toxic to life, it has been demonstrated that micro-organisms can use arsenic compounds as electron donors, electron acceptors or possess arsenic detoxification mechanisms (Ji and Silver 1992; Ahmann *et al.* 1994; Cervantes *et al.* 1994; Newmann *et al.* 1997; Stolz

et al. 2002). Arsenic resistance and metabolizing systems occur in three patterns, the widely found *ars* operon present in most bacterial genomes and many plasmids, the more recently recognized *arr* genes for the periplasmic arsenate reductase functioning in anaerobic respiration as a terminal electron acceptor, and the *aso* genes for the periplasmic arsenite oxidase functioning as an initial electron donor in aerobic resistance to arsenite (Silver and Phung 2005).

Several remediation processes have been described for arsenic removal (Jeckel 1994) based on chemical oxidation of arsenite to arsenate followed by alkaline precipitation (Gupta and Chen 1978; Hering *et al.* 1997; Bothe and Brown 1999; Gregor 2001). The major disadvantages of these processes are that they generate additional pollution and are expensive, thus the need to experiment with alternative methods of arsenic remediation based on its biological oxidation. Concerning arsenic bioremediation in sediments, natural attenuation is a promising approach, as it exploits the activity of indigenous microorganisms capable of iron oxidation, thereby enhancing the removal of arsenic by direct adsorption and/or coprecipitation (Fukushi *et al.* 2003; Mulligan and Yong 2004).

The source of arsenic contamination in the Orbetello Lagoon is mostly due to ash, dust and debris originating from a fertilizer production plant located on a bank of this lagoon. Closed down in the late 1980s, the fertilizer production plant used pyrite (FeS_2) containing high concentrations of arsenic and other impurities like Zn and Pb, as a raw material for sulphuric acid production. 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 the Orbetello Lagoon, which show anomalies in arsenic concentrations (Arisi Rota and Vighi 1971; Donati *et al.* 2005).

The aim of this study was to isolate arsenic-resistant bacteria from arsenic-contaminated sediments of the Orbetello Lagoon, to be used for possible bioremediation processes. Moreover, the research was aimed to evaluate the bio-available arsenic in contaminated sediments, using bacteria as a bio-indicator.

Materials and methods

Study area and sediment sampling

The Orbetello Lagoon is located in Tuscany, on the west coast of Italy (42°26′34″N, 11°13′29″E) and covers an area of approx. 2300 ha with an average water depth of approx. 1 m. Sediment samples were collected in March 2005 in various sites located along a presumed pollution gradient from the former fertilizer plant (Fig. 1). During sampling, superficial (0–20 cm) and deeper (30–50 cm)

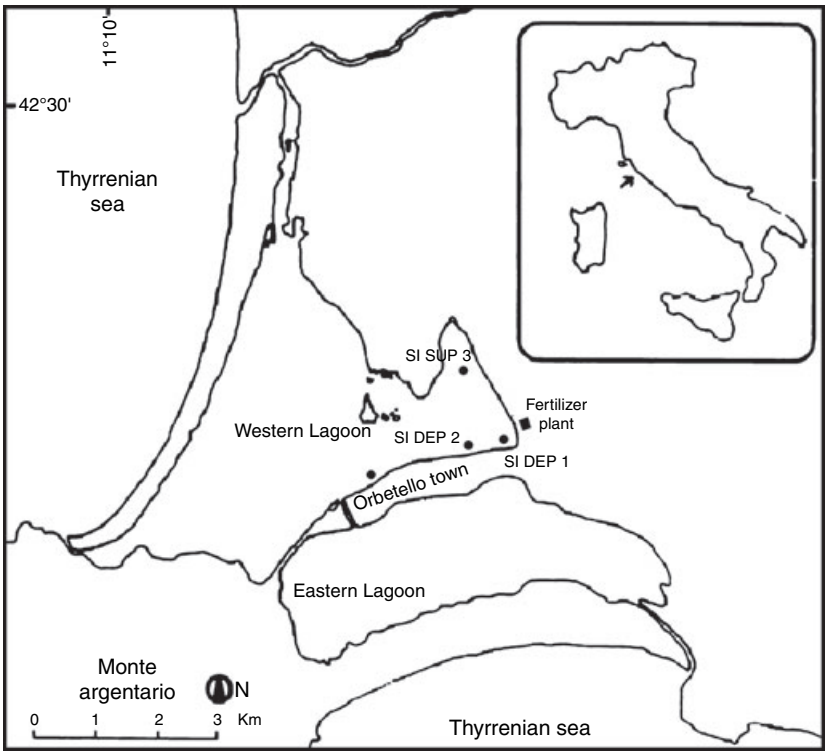

Figure 1 Site map of the study area, the Orbetello Lagoon, Tuscany, Italy. Sampling sites ORB-SUP, SI-SUP3, SI-DEP2 and SI-DEP1 were located 2·5, 1·5, 1·0 and 0·4 km, respectively, from the fertilizer plant.

sediments were collected. The former were collected by using a grab sampler with a square section. Sterile portions, for microbiological analyses, were immediately deposited into sterile sacks (Whirl-Pak, Nasco), and maintained at 4°C, in the dark, until their arrival in laboratory, where they were processed immediately. Deeper samples were extracted from a core, 50 cm long, sampled by a scuba diver using a soft-sediment plastic-barrel coring device (inside diameter 7 cm). Cores, carefully capped to prevent any loss of moisture, were shipped to the laboratory. Samples for microbiological analyses were aseptically collected from cores, at the 30–50 cm level, and maintained at 4°C. Sediments, both from superficial and deeper samples, were air dried and homogenized prior to chemical analyses. In total, 109 superficial and 23 deeper sediment samples were collected. Four sediment samples representative of different degrees of arsenic contamination were used for bacteria isolation.

Chemical analyses

For determination of metal content, dried sediment subsamples of up to 0·5 g were placed in Teflon vessels and extracted in a Milestone EthosD microwave oven (Bergamo, Italy), using concentrated nitric acid (HNO_3) according to EPA Method 3051. This method allows the extraction and subsequent determination of elements considered mobile in the environment and included in most aqueous transport mechanisms of pollution. After cooling, samples were filtered and the volume corrected by using ultra-pure water. Certified material and reagents without samples added were also analysed following the same methods. Metal determination was performed by a Perkin Elmer Elan 6100 ICP-Mass Spectrometer (ICP-MS, Monza, Italy). The efficiency of the extraction procedure and accuracy of analyses were ensured by digestion and analyses of Standard Reference Materials: SRM 1646a (Estuarine Sediments). Metal recovery varied between 94% and 98%, confidence limits for the SRM. All samples were analysed by ICP-MS in triplicate in order to check precision. Analytical precision of replicates ($n = 3$) was within 5%. Final results, obtained as mean values of ICP-MS determination conducted in triplicate, were expressed as mg kg^{-1} dry weight (d.w.).

Enrichment cultures and bacteria isolation

An aliquot of 1·0 g from each sediment sample was added to a 1000-ml flask containing 200 ml of a complex culture medium plate count agar (PCA), containing 5 g of tryptone (Difco), 2·5 g of D-glucose (BDH, Milan, Italy), and 2·5 g of yeast extract (Oxoid) per litre of double-distilled water. Trivalent arsenic As(III) was also added as sodium *m*-arsenite (*m*-$NaAsO_2$) at a concentration of 6·75 mmol l^{-1}. Inoculated flasks were mixed and incubated static at 30°C in the dark. Enrichment cultures showing turbidity after 2 weeks of incubation were subcultured by streaking onto Petri dishes containing the same culture medium of the enrichments solidified with 1·6% of agar (Bacto-Agar, Difco), in the presence of As(III) at a concentration of 1·35 mmol l^{-1}, assuming that selection for highly arsenic-resistant bacteria was operated in the enrichment cultures. Colonies different in shape, colour and margins appearing on inoculated plates were streak purified at least three times on the same complex solid medium, in the presence of the same concentration of As(III); isolates were stored at −80°C in 30% sterile glycerol.

Minimum inhibitory concentrations

As(III) and As(V) resistance in isolated bacterial strains was evaluated by minimum inhibitory concentration (MIC) tests. Aliquots of 1·0 ml of overnight cultures were incubated in 99·0 ml of Mueller Hinton Broth (Oxoid) and 10 ml were distributed in 18 ml test tubes with radial cups. The MIC tests were carried out at different concentrations of As(III) added as sodium *m*-arsenite (*m*-$NaAsO_2$), (from 0 to 16·68 mmol l^{-1}); As(V) added as disodium hydrogen arsenate ($Na_2HAsO_4{\cdot}7H_2O$), (from 0 to 133·47 mmol l^{-1}); Cu(II) added as copper dichloride ($CuCl_2$), (from 0 to 8 mmol l^{-1}); Cd(II) added as cadmium dichloride ($CdCl_2$), (from 0 to 2 mmol l^{-1}); Zn(II) added as zinc dichloride ($ZnCl_2$), (from 0 to 7·7 mmol l^{-1}). Tubes were incubated in a rotary drum at 30°C for 24 h. The optical density of the cultures, as a measure of microbial growth, was detected at a wavelength of 600 nm by an UV-visible spectrophotometer (Jenway, mod. AC30, Milan, Italy); a blank with the sole medium culture, without bacteria, was also analysed. Experiments were carried out in duplicate.

Isolates characterization and 16S rDNA analyses

After growth of the bacterial isolates on Petri dishes, the colony morphology was observed under a stereomicroscope. Gram determination was carried out (Gram stain kit; Carlo Erba, Milan, Italy). Catalase and oxidase activity were determined according to Smibert and Krieg (1981). For 16S rDNA sequencing of bacterial isolated strains, a single colony was suspended in 50 μl double-distilled water and treated for 5 min at 100°C. Amplification of 16S rRNA gene was performed using 10 ng of genomic DNA in 20 μl of 1X 'Amplitaq' buffer (10 mmol l^{-1} Tris-HCl; 50 mmol l^{-1} KCl; 1·5 mmol l^{-1} $MgCl_2$; 0·001%

gelatin) with 150 ng each of the primers 27f (5′-GAGAG-TTTGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCT-ACCTTGTTACGA-3′), 250 μmol l^{-1} each dNTPs and IU of 'Amplitaq' (Perkin-Elmer). The reaction mixtures were incubated at 95°C for 90 s and then cycled 35 times through the following temperature profile: 95°C for 30 s, annealing temperature (T_a) for 30 s and 72°C for 4 min. T_a was 60°C for the first five cycles, 55°C for the next 5 cycles and 50°C for the last 25 cycles. Lastly, the mixtures were incubated at 72°C for 10 min and at 60°C for 10 min; 2 μl of each amplification mixture was analysed by agarose gel (1·2% w/v) electrophoresis in TAE buffer (0·04 M Tris-acetate, 0·001 M EDTA) containing 0·5 μg ml^{-1} (w/v) ethidium bromide. Sequencing was carried out at the Bact 16S biomolecular research service (CRIBI Biotechnology Centre, University of Padua, Italy). A partial (first 500 bp) 16S rDNA sequence was determined for six arsenic-resistant bacterial isolates (i.e. ORAs3, ORAs4, ORAs7, ORAs8, ORAs9 and ORAs10); more complete sequences were determined for the four bacterial strains ORAs1, ORAs2, ORAs5 and ORAs6.

Sequence accession numbers

The sequences obtained in this study were deposited in the GenBank database. The accession numbers for the 16S rDNA nucleotide sequences are: DQ308548 (strain ORAs1), DQ308549 (strain ORAs2), DQ401145 (strain ORAs3), DQ401147 (strain ORAs4), DQ308550 (strain ORAs5), DQ308551 (strain ORAs6), DQ401148 (strain ORAs7), DQ401146 (strain ORAs8), DQ401149 (strain ORAs9) and DQ401150 (strain ORAs10).

Analysis of sequence data

Consensus sequence of the arsenic-resistant isolated strains ORAs1, ORAs2, ORAs3, ORAs4, ORAs5, ORAs6, ORAs7, ORAs8, ORAs9 and ORAs10 were compared with those deposited in GenBank by using the BLAST programme (Altschul *et al.* 1997). For phylogenetic analysis, 16S rDNA sequences of the ORAs1, ORAs2, ORAs5 and ORAs6 isolates, and related sequences retrieved from databases (GenBank and RDPII), were aligned with ClustalW included in the MEGA 3·1 software package (Kumar *et al.* 2004). The resulting alignments were checked manually. Phylogenetic analyses were conducted using MEGA version 3·1. The phylogenetic tree was inferred using the neighbour-joining method (Saitou and Nei 1987). Sequence divergences among strains were quantified by using the Kimura-2-Parameter distance model (Kimura 1980). For treatment of gaps the 'Complete Deletion' option was chosen. A total of 1000 bootstrap replications were calculated. The tree was unrooted.

Results

Chemical analysis of sediments

Arsenic contamination in superficial sediments of the Orbetello Lagoon showed higher values in the area close to the former fertilizers plant (SI). In this area, the mean arsenic concentration in sediments ($n = 66$) was of 27·25 mg kg^{-1} (d.w.), with a minimum of 5·38 and a maximum of 92·28 mg kg^{-1} (d.w.). In the area far from the plant and close to the urban area (ORB), mean arsenic concentration in sediments ($n = 43$) was of 7·58 mg kg^{-1} (d.w.), with a range from 1·47 to 22·76 mg kg^{-1} (d.w.). In Table 1, As, Cd, Cu and Zn concentrations in superficial sediments collected from the cited areas are reported. Among the 109 superficial sediment samples, four representatives of both areas (SI and ORB) were used for arsenic-resistant micro-organism isolation. The same four sediment samples were also analysed at deeper levels. Relative metal concentrations are shown in Table 2. A higher level of arsenic was detected in sample SI-DEP2 with a concentration of 155·13 mg kg^{-1} (d.w.), in the same sample high levels of

Table 1 Heavy metals content (mg kg^{-1}) (d.w.) in surface sediments (0–20 cm) of the Orbetello Lagoon, Italy

Area	As	Cd	Cu	Zn
ORB ($n = 43$)				
Mean	7·58	0·65	40·67	166·20
Minimum	1·47	0·04	3·12	6·38
Maximum	22·76	2·77	233·18	840·56
SD	5·11	0·61	42·85	187·32
SI ($n = 66$)				
Mean	27·25	2·38	222·09	643·07
Minimum	5·38	0·14	13·16	46·84
Maximum	92·28	14·69	2209·64	3876·51
SD	17·72	2·15	281·68	569·27

ORB = close to the urban area of the Orbetello Lagoon; SI = close to the area of the fertilizer plant; SD = standard deviation.

Table 2 Heavy metals content (mg kg^{-1}) (d.w.) in surface (SUP) (0–20 cm) and deeper (DEP) (30–50) sediments representative of contaminated areas (SI) closer to the former fertilizer plant and of the less contaminated area (ORB)

Area	As	Cd	Cu	Zn
ORB-SUP	14·82	1·37	233·18	840·56
SI-SUP1	27·19	3·44	344·49	857·82
SI-DEP1	75·25	7·80	735·05	1619·42
SI-SUP2	87·69	14·69	2209·64	3876·51
SI-DEP2	155·13	12·85	2624·66	3861·02
SI-SUP3	92·28	5·68	349·70	1081·71

contamination by Cd, Cu and Zn were also detected (Table 2).

Enrichment and isolation of arsenic-resistant bacteria

A total of ten arsenic-resistant bacterial strains were isolated from contaminated sediment samples of the Orbetello Lagoon, and were named ORAs1, ORAs2, ORAs3, ORAs4, ORAs5, ORAs6, ORAs7, ORAs8, ORAs9 and ORAs10. All the enrichment cultures prepared with arsenic rich sediments (arsenic concentration of >14·82 mg kg^{-1} d.w.) allowed isolation of bacterial strains after 2 weeks of incubation at 30°C. The isolation of bacterial strains was possible on the basis of their ability to grow in the presence of As(III) added at a concentration of 6·75 mmol l^{-1}, selecting for highly arsenic-resistant bacterial strains, and according to the criteria of different colony morphology. Strains ORAs1, ORAs2, ORAs9 and ORAs10 were Gram-positive, all the others were Gram-negative. All strains isolated were negative for oxidase. Strains ORAs1, ORAs2, ORAs5, ORAs9 and ORAs10 were positive for catalase while the others were negative.

Minimum inhibitory concentrations

MICs in the presence of As(III) showed the same values in isolates ORAs1, ORAs2, ORAs3, ORAs5, ORAs7 and ORAs8, with inhibition of growth at 16·68 mmol l^{-1}. Growth of strains ORAs4 and ORAs10 was inhibited by 13·34 mmol l^{-1} of As(III), while strain ORAs6 proved to be the most resistant to As(III), still growing in the presence of 16·68 mmol l^{-1} of the trivalent form of the metalloid (Fig. 2a). Additions of different concentrations of As(V) showed the two strains ORAs4 and ORAs8 to be the most sensitive, with MICs at 80·0 mmol l^{-1}. Growth of bacterial strains ORAs3, ORAs6, ORAs7 and ORAs9 was inhibited in the presence of 133·47 mmol l^{-1} of As(V), whereas the remaining three strains ORAs1, ORAs2 and ORAs10 were still able to grow at the same level of concentration (Fig. 2b).

The four arsenic-resistant strains showing the highest level of resistance both to As(III) and As(V) (i.e. ORAs1, ORAs2, ORAs5 and ORAs6), were tested for resistance in the presence of ions of different metals, Cd(II), Cu(II) and Zn(II), measured as representative pollutants in the same sediment samples contaminated by arsenic. MICs in the presence of Cd(II) had the highest resistance level, at 1·8 mmol l^{-1}, for strain ORAs2, whereas growth of strains ORAs5 and ORAs6 was inhibited at 0·8 mmol l^{-1}, and that of strain ORAs1 at 0·4 mmol l^{-1} (Fig. 3a). MICs values in the presence of Cu(II) were 8·0 mmol l^{-1} for strain ORAs1, and 4·0 mmol l^{-1} for the others (Fig. 3b). Strain ORAs2 again showed the highest level of resistance in the presence of Zn(II), with a MIC value of 7·7 mmol l^{-1}, against inhibition of growth at 3·8 mmol l^{-1} for the remaining strains ORAs1, ORAs5 and ORAs6 (Fig. 3c).

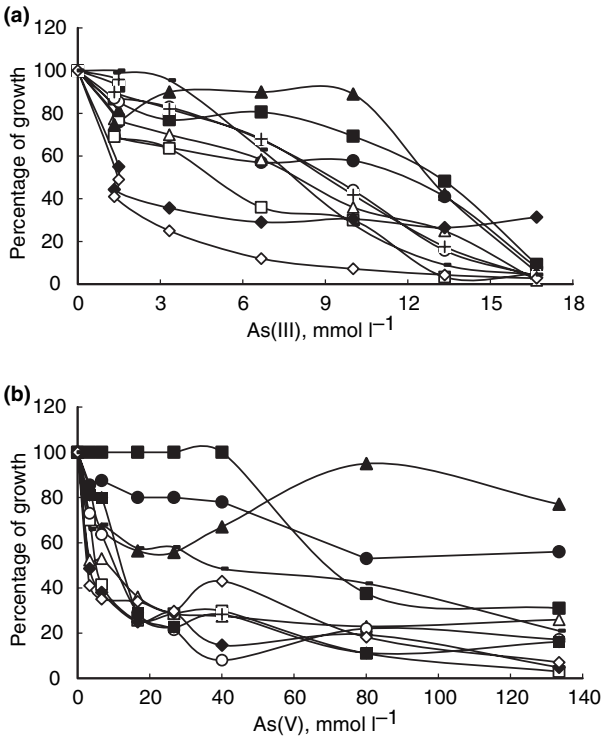

Figure 2 (a) MICs of the isolated strains (●) ORAs1; (■) ORAs2; (△) ORAs3; (□) ORAs4; (▲) ORAs5; (◆) ORAs6; (○) ORAs7; (+) ORAs8; (◇) ORAs9 and (-) ORAs10, in the presence of increasing concentrations (mmol l^{-1}) of As(III) added as *m*-$NaAsO_3$, and incubated 24 h at 30°C. (b) MICs of the same resistant strains as in (a), in the presence of increasing concentrations (mmol l^{-1}) of As(V) added as $Na_2HAsO_4x7H_2O$, and incubated 24 h at 30°C. Growth in the absence of metalloid, detected as O.D. at 600 nm, and stated as 100% for the different bacterial strains, were the followings: 0·9 in (●) ORAs1; 0·87 in (■) ORAs2; 0·72 in (△), ORAs3; 1·27 in (□) ORAs4; 0·97 in (▲) ORAs5; 1·17 in (◆) ORAs6; 1·02 in (○) ORAs7; 1·3 in (+) ORAs8; 1·11 in (◇) ORAs9 and 0·99 in (-) ORAs10. Results are means of duplicate determinations.

Sequence data analysis

Bacterial isolates were further identified by sequencing of their 16S rRNA genes. Almost complete sequence of the 16S rRNA gene was determined in isolates ORAs1 (1491 bp), ORAs2 (1480 bp), ORAs5 (1396 bp) and ORAs6 (1485 bp). Short sequences of the gene encoding 16S rRNA were determined in the isolated arsenic-resistant strains ORAs3 (397 bp), ORAs4 (382 bp), ORAs7 (410 bp), ORAs8 (441 bp), ORAs9 (397 bp) and ORAs10 (406 bp). Comparison of the obtained nucleo-

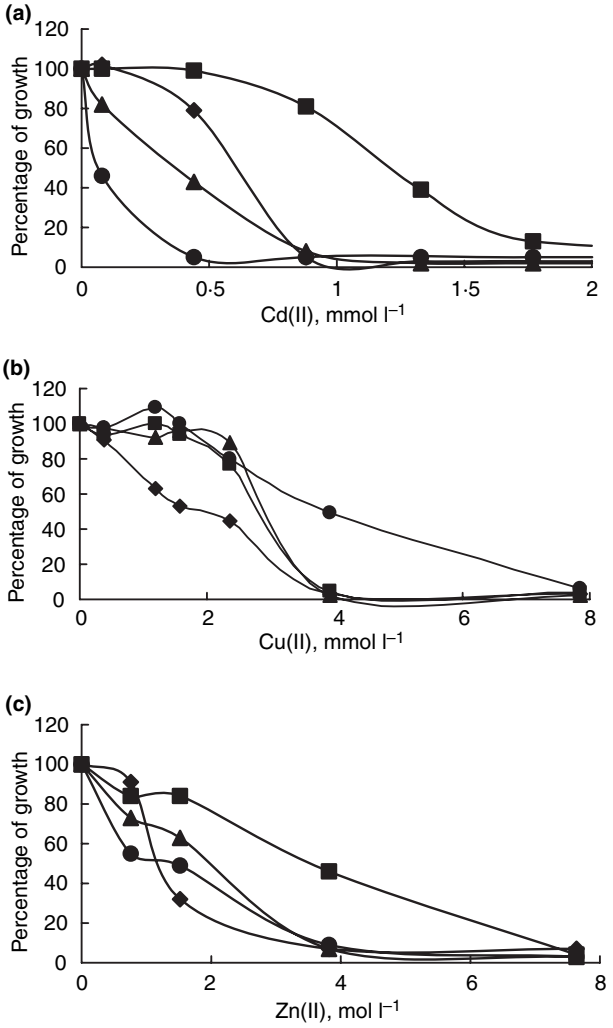

Figure 3 (a) MICs of the isolated strains (●) ORAs1; (■) ORAs2; (▲) ORAs5 and (◆) ORAs6, in the presence of increasing concentrations (mmol l^{-1}) of Cd(II) added as $CdCl_2$. (b) MICs of the same resistant strains as in (a), in the presence of increasing concentrations (mmol l^{-1}) of Cd(II) added as $CdCl_2$. (c) MICs of the same resistant strains as in (a), and in (b), in the presence of increasing concentrations (mmol l^{-1}) of Zn(II) added as $ZnCl_2$. All the cultures were incubated 24 h at 30°C. Growth in the absence of metals detected as O.D. at 600 nm, and stated as 100% for the different bacterial strains, were the following: 0·98 in (●) ORAs1; 0·72 in (■) ORAs2; 1·1 in (▲) ORAs5 and 1·05 in (◆) ORAs6. Results are means of duplicate determinations.

tide sequences with those of the small 16S ribosomal subunits deposited in the ribosomal database of the RDP II project assigned the isolated bacterial strains to the following genera: *Bacillus* including strains ORAs1, ORAs2, ORAs9 and ORAs10; *Aeromonas* including strains ORAs3, ORAs4, ORAs6, ORAs7 and ORAs8. Strain ORAs5 was, instead, a member of the genus *Pseudomonas* (Table 3).

Phylogenetic analysis

Phylogenetic trees were inferred for the four bacterial isolates showing the highest levels of arsenic-resistance. Analysis of 16S rDNA sequence indicated that strain ORAs1 belongs to the genus *Bacillus* with nearest type strain *B. sphaericus* (T) IAM13420 (D16280), and nontype strain *B. borotolerans* T-13B (AB199594), with a similarity score of 0·904 and 0·994, respectively. Again 16S rDNA sequence analysis assigned strain ORAs2 to the genus *Bacillus* with nearest type strain *B. thuringiensis* (T) ATCC10792 (AF290545), and nontype strain *Bacillus* sp. JS23b (DQ104990), with similarity score of 0·999 and 1·000, respectively. These two strains belong to phylum Firmicutes and to the genus *Bacillus* (Fig. 4a). Concerning strain ORAs5, 16S rRNA gene sequence showed that it belongs to the genus *Pseudomonas* with nearest type strain *P. plecoglossicida* (T) FPC951 (AB009457), and nontype strain *Pseudomonas* sp. MFY57 (AY331346), with a similarity score of 0·969 and 0·984, respectively. Analysis of 16S rDNA sequence identified strain ORAs6 as *Aeromonas* sp. with nearest type strain *A. bestiarum* (T) CIP7430 (X60406), and nontype strain *A. molluscorum* 93M (AY532688), with similarity score of 0·995 and 0·988, respectively. The latter two strains belong to gamma-Proteobacteria, one to the genus *Pseudomonas* and the other to the genus *Aeromonas* (Fig. 4b).

Discussion

We isolated ten bacterial strains that grow in the presence of elevated As(III) and As(V) concentrations. This feature is important to allow these bacteria to cope with the high arsenic concentrations in native polluted sediments of the Orbetello Lagoon. Comparison of this ecosystem with a similar one, the Venice Lagoon, reveals that arsenic concentrations detected in the two urban areas, both distant from the industrial district, resemble each other (Bernardello *et al.* 2006). The comparison of the industrially impacted areas such as Porto Marghera near the Venice Lagoon, and the area near the fertilizer plant analysed in this research, showed that levels of arsenic were high in both areas, although the Orbetello Lagoon impacted area reached greater peaks of contamination because of the use of pyrite with high arsenic content (Donati *et al.* 2005; Bernardello *et al.* 2006).

Isolates described in this work still need to be analysed in terms of their mechanisms of arsenic resistance, but at present we can select the most promising for bioremediation processes on the basis of their levels of arsenic resistance. We can compare MICs in the presence of As(III) and As(V) for the most resistant strains, 13·38 mmol l^{-1} and ≥133·47 mmol l^{-1}, respectively, with levels of arsenic

Table 3 Representative strains of heterotrophic arsenic-resistant bacteria isolated in the presence of As(III) from sediment samples collected at different sites in the Orbetello Lagoon, Italy

Sample	Isolated strain	Colony features	Closest relative	Percent of identity
ORB-SUP	ORAs1	1·0 mm, white, oval, convex, matt, entire margins	*Bacillus fusiformis*	99
SI-SUP3	ORAs2	2·5 mm, white, round, flat, matt, entire margins	*Bacillus thuringiensis*	100
SI-DEP1	ORAs3	1·0 mm, cream, round, convex, moist, entire margins	*Aeromonas salmonicida*	98
SI-DEP1	ORAs4	1·0 mm, cream, round, convex, moist, entire margins	*Aeromonas salmonicida*	99
SI-DEP2	ORAs5	convex, moist, entire margins	*Pseudomonas* sp.	99
SI-DEP2	ORAs6	convex, moist, entire margins	*Aeromonas molluscorum*	99
SI-DEP2	ORAs7	convex, moist, entire margins	*Aeromonas salmonicida*	99
SI-SUP3	ORAs8	convex, moist, entire margins	*Aeromonas salmonicida*	99
SI-DEP2	ORAs9	1· mm, white, oval, flat, matt, entire margins	*Bacillus cereus*	100
ORB-SUP	ORAs10	2·0 mm, white, oval, flat, matt, entire margins	*Bacillus cereus*	100

resistance of bacterial strains used in industrial bioremediation processes, such as *Corynebacterium glutamicum,* which shows a natural resistance to arsenite up to 12 mmol l^{-1}, and superior to 400 mmol l^{-1} for arsenate, applied to remove arsenic from contaminated water (Mateos *et al.* 2006); and the 10·13 mmol l^{-1} level of resistance to arsenate detected in marine and nonmarine bacteria able to accumulate arsenic and used in bioremediation processes (Takeuchi *et al.* 2007). According to these examples, arsenic-resistant bacteria isolated from the Orbetello Lagoon showed levels of resistance high enough to be considered for possible bioremediation strategies of native sediments.

Studies of resistance to other heavy metals, among those present in the polluted sediments of the Orbetello Lagoon (Focardi 2005), pointed out a cadmium- and zinc-resistance in the *Bacillus* sp. strain ORAs2, similar to that found in some isolates from a galvanization plant in Berlin, Germany where the resistance levels to cadmium and zinc were of 1·5 mmol l^{-1} and 5·0 mmol l^{-1}, respectively (Dressler *et al.* 1991). A study conducted in *Bacillus subtilis* for genes induced by exposure to high levels of metal ions including Ag(I), Cd(II), Cu(II), Ni(II) and Zn(II) and the metalloid As(V), showed the involvement of different metal resistance determinants (e.g. *AseA, CzcD, CadA* and *ArsB*) (Moore *et al.* 2005). The fact that the arsenic-resistant bacteria characterized in this study are autochthonous enhances their uniqueness and utility in possible applications for bioremediation strategies in polluted sediments of the Orbetello Lagoon. In fact, they also showed the ability to grow in the presence of others heavy metals, co-pollutants in the same polluted sediments, conferring particular interest to these isolates.

The arsenic-resistant bacteria isolated in this study were grouped into three genera, *Aeromonas, Bacillus* and *Pseudomonas*, based on phylogenetic analysis of 16S rDNA sequence. Other reports showed the presence of these genera among arsenic-resistant bacteria. Water and sediment samples from two areas in New Zealand allowed isolation of bacterial strains oxidizing arsenite or using arsenate as an electron acceptor; among the seventeen isolated bacterial strains from this area, *Bacillus* and *Pseudomonas* were the most predominant genera, and one strain belonging to the genus *Aeromonas* was also detected (Anderson and Cook 2004). *Bacillus arsenicus* sp. nov., and *B. indicus* sp. nov., arsenic-resistant bacteria from West Bengal, India, were isolated, respectively, from a siderite concretion and an aquifer (Suresh *et al.* 2004; Shivaji *et al.* 2005). *Bacillus arseniciselenatis* sp. nov. was a dissimilatory bacterium growing in the presence of As(V) isolated from the anoxic mud of Mono Lake, California, an alkaline, hypersaline, arsenic rich body of water (Blum *et al.* 1998). The genus *Pseudomonas* is broadly represented among the arsenic-resistant strains isolated from environmental samples (Turner 1949; Ilyaletdonov and Abdrashitova 1981). *Pseudomonas aeruginosa* strains isolated from marine environments also showed arsenic-resistance (de Vincente *et al.* 1990). *Aeromonas* spp. are ubiquitous aquatic bacteria that cause serious infections. *Aeromonas* strains isolated from both urban and rural playa lakes and several rivers, showed antibiotic, metals and metalloids resistance, including arsenic, and resistance was carried on the plasmids (Huddleston *et al.* 2006). These isolates belong to genera frequently found to be arsenic-resistant and representative of the native environment, as *Aeromonas* strains are frequently found in association with fish, and *Bacillus* are recovered in sediment areas, because of the presence of spores in these bacteria enabling them to survive unfavourable conditions (Anderson and Cook 2004). Concerning the genus *Pseudomonas*, it is composed of ubiquitous bacteria endowed with a remarkable adaptability to diverse environments (de Vincente *et al.* 1990).

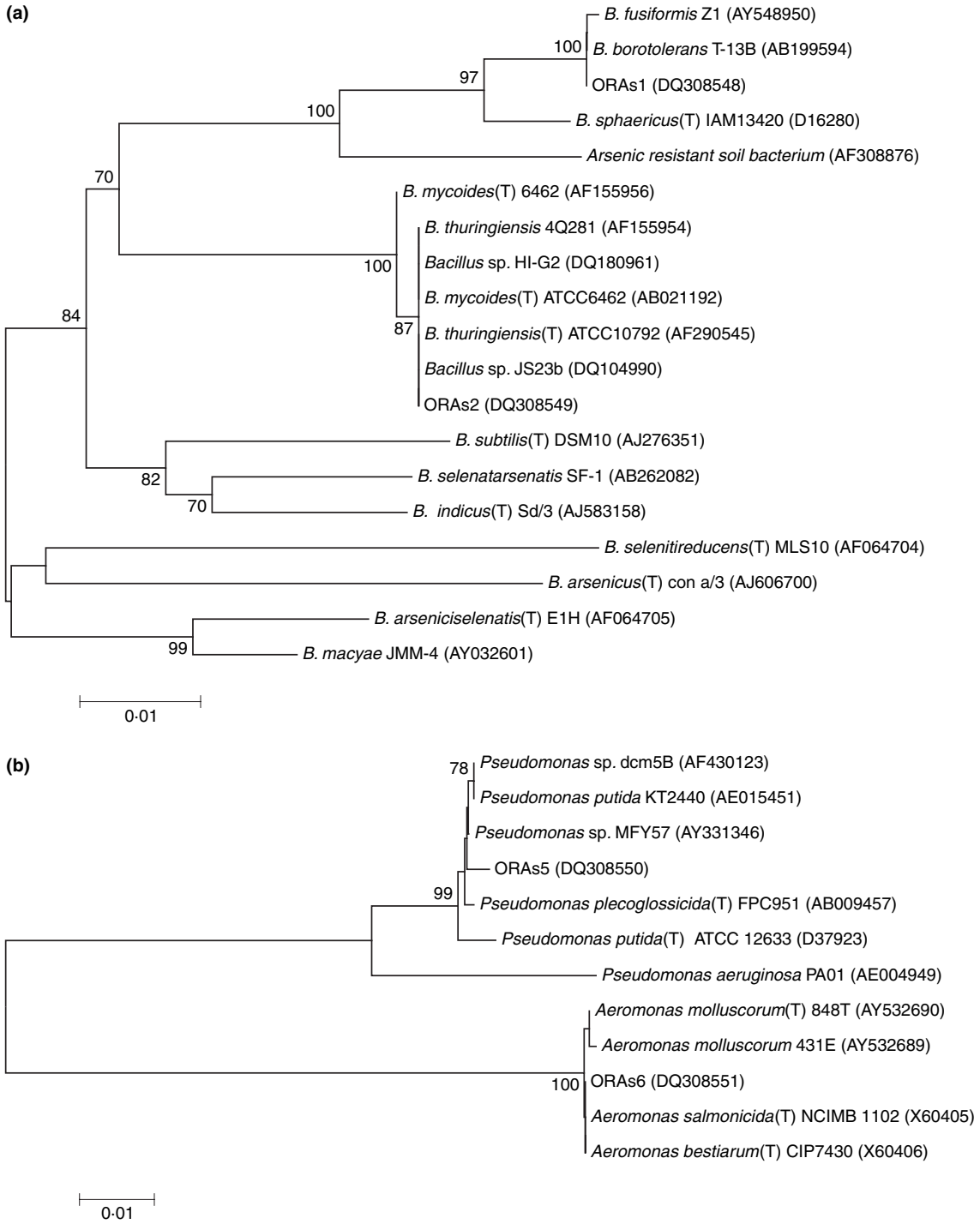

Figure 4 (a) Unrooted phylogenetic tree based on 16S rDNA sequence comparisons showing the position of ORAs1, ORAs2 and representative species of the genus *Bacillus*. The branching pattern was generated by neighbour-joining methods and the bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap values lower than 70% are not shown. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16S rDNA sequences are given after the strain in parenthesis. (b) Unrooted phylogenetic tree based on 16S rDNA sequence comparisons showing the position of ORAs5, ORAs6 and representative species of the subclass of Proteobacteria. The branching pattern was generated by neighbour-joining methods and the bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap values lower than 70% are not shown. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16S rDNA sequences are given after the strain in parenthesis.

Phylogenetic trees of the four most resistant bacterial strains showed two different localizations for the two *Bacillus* strains: ORAs1 is closest to *B. borotolerans* T-13B, isolated from soil, and the other named ORAs2 showed *Bacillus* sp. JS23b as the closest one, again isolated from soil. Concerning trees of ORAs5 and ORAs6, the closest strains were *Pseudomonas* sp. MFY57 and *Aeromonas salmonicida* (T) NCIMB1102, respectively. The first was of environmental origin (Bodilis *et al.* 2004); the second was isolated from clinical specimens and was determinant in the recognition that members of the genus *Aeromonas* formed a distinct line within the gamma subclass of the Proteobacteria (Martinez-Murcia *et al.* 1992).

Bacterial strains described showed high levels of arsenic resistance and are indigenous to polluted sediments of the Orbetello Lagoon, and could therefore represent good candidates for bioremediation processes. Physiological and molecular analyses will continue to be carried out.

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