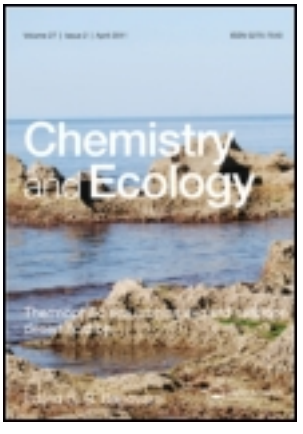

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As(V)-reduction to As(III) by arsenic-resistant *Bacillus* spp. bacterial strains isolated from low-contaminated sediments of the Oliveri-Tindari Lagoon, Italy

Marcella Ruta[a], Milva Pepi[a]*, Carlo Gaggi[a], Emanuele Bernardini[a], Silvia Focardi[b], Eleonora Magaldi[a], Simone Gasperini[c], Margherita Volterrani[a], Angiola Zanini[d] and Silvano E. Focardi[a]

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Five arsenic-resistant bacterial strains designated MT1, MT2, MT3, V1 and V2 were isolated from sediments of the Oliveri-Tindari Lagoon (Italy), which comprises six small lakes whose sediments contain low arsenic concentrations. Phylogenetic analysis of the 16*S* rRNA gene sequences assigned them to the genus *Bacillus*. *Bacillus* sp. strain MT3 showed higher tolerance to As(III) and As(V), as indicated by minimum inhibitory concentrations of 14 and 135 $mmol^{-1}$, respectively. *Bacillus* sp. strain V1 showed growth inhibition at 14 $mmol^{-1}$ in the presence of As(III) and at 68 $mmol^{-1}$ in the presence of As(V), whereas the arsenic resistance of *Bacillus* sp. strain MT1 was 10 and 27 $mmol^{-1}$ for As(III) and As(V), respectively. The strains *Bacillus* spp. MT2 and V2 showed low levels of As(III) and As(V) resistance, as it was unable to grow at concentrations >7 and 14 $mmol^{-1}$, respectively. The isolated arsenic-resistant *Bacillus* spp. strains were able to reduce As(V) to As(III), especially *Bacillus* spp. strain MT3. This study suggests that the isolated bacterial strains play a role in the arsenic biogeochemical cycle of arsenic-poor sediments in the Oliveri-Tindari Lagoon.

Keywords: arsenic; resistance to arsenic; sediments; *Bacillus*

1. Introduction

Arsenic is widely distributed but relatively rare, ranking as the twentieth most abundant element in the continental crust [1]. Over 99% of arsenic occurs in rocks [2]. Concentrations in sedimentary rocks are often much higher than in igneous or metamorphic rocks [3]; the former contain ∼2–400 mg As·kg^{-1}, the latter seldom more than ∼1.5–3 mg As·kg^{-1} [3,4]. This arsenic is most often associated with sulfur compounds, principally arsenopyrite [5]. The primary source of arsenic in soils and sediments is the parent material from which they are derived [6]. Human activities such as mining and herbicide use, as well as natural processes including the weathering of As-bearing minerals, contribute to the mobilisation of arsenic into ground and surface waters [3]. Geothermal

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fluids, which are capable of leaching rocks, are also a significant source of arsenic [7]. In aqueous environments arsenic primarily occurs as arsenite [As(III)] and arsenate [As(V)] [1].

Arsenic is well known for its toxic properties [8]. Despite its toxicity, a number of microorganisms can use either the oxidised or the reduced form of arsenic in their metabolism [9,10]. In many cases, these arsenic-resistant microorganisms have been obtained from arsenic-contaminated soils and sediments, such as mine/smelter waste, or from geothermal areas [2,11–14]. Microorganisms are commonly resistant to arsenic not only in contaminated areas, but also in arsenic-free environments [15,16]. The biological relevance of arsenic is twofold and is primarily related to its toxicity and/or its involvement in oxidation–reduction reactions that conserve energy in microbial metabolism [17]. The biogeochemical cycle of this element depends significantly on microbial transformations affecting the distribution and mobility of arsenic species in the environment [18]. Increasing evidence suggests that biotic transformations, in particular those involving arsenic and iron as either electron donors or acceptors, are key factors influencing As distribution and speciation [17].

The Oliveri-Tindari Lagoon, situated in a coastal area of north-eastern Sicily (Italy) comprises six small lakes named Verde, Mergolo della Tonnara, Marinello, Nuovo, Fondo Porto and Porto Vecchio. Over the last century, this lagoon system has been characterised by a rapid geomorphologic evolution that has modified the morphology of the lakes [19]. There is no impact from direct urban wastes, and low-level anthropogenic impact only occurs during summertime due to tourism in the prime line bay. The basins have different water sources; the external ones are mainly supplied with seawater through infiltration or through direct contributions during storms. The most significant input into the three internal lakes comes from superficial waters containing dissolved material and dust from the nearby ground, which is frequently used for grazing and agriculture [20]. The overall environmental conditions can be defined as good [21], and recent investigations have shown very low levels of contamination in this area [22,23].

This study focuses on arsenic-resistant bacterial strains isolated from sediments of the Oliver-Tindari Lagoon, where a low arsenic content was detected. The aim of the study was to investigate microorganisms involved in the biogeochemical arsenic cycle and the role of arsenic-resistant bacterial strains in mobilising arsenic in sediments with low arsenic contents.

2. Material and methods

2.1. *Study area and sediment sampling*

The study area of the Oliveri-Tindari Lagoon is a nature reserve in north-eastern Sicily, Italy (38°08′134″ N; 15°03′366″ E), comprising six small lakes and covering an area of 420.435 m^2 (Figure 1). Three of the lakes (Verde, Mergolo della Tonnara and Marinello) formed a long time ago, whereas the others developed more recently (Nuovo, Fondo Porto and Porto Vecchio). As reported in a Military Geographic Institute map of 1856, this lagoon system originated ∼140 years ago, when the first three of the six current lakes were already present. The Oliveri-Tindari Lagoon undergoes changes due to marine, terrestrial and atmospheric processes [19]. Sediment samples (0–10 cm) were collected manually using Plexiglas tubes (i.d. 10 cm) in 2005. Collected samples were maintained at 4 °C and transported to the laboratory. Sterile sediment subsamples were collected and processed within twelve hours for microbiological analyses.

2.2. *Chemical analyses*

Dried sediment samples of up to 0.5 g were placed in Teflon vessels and extracted in a Milestone EthosD microwave oven using concentrated nitric acid (HNO_3) following EPA Method 3051. After

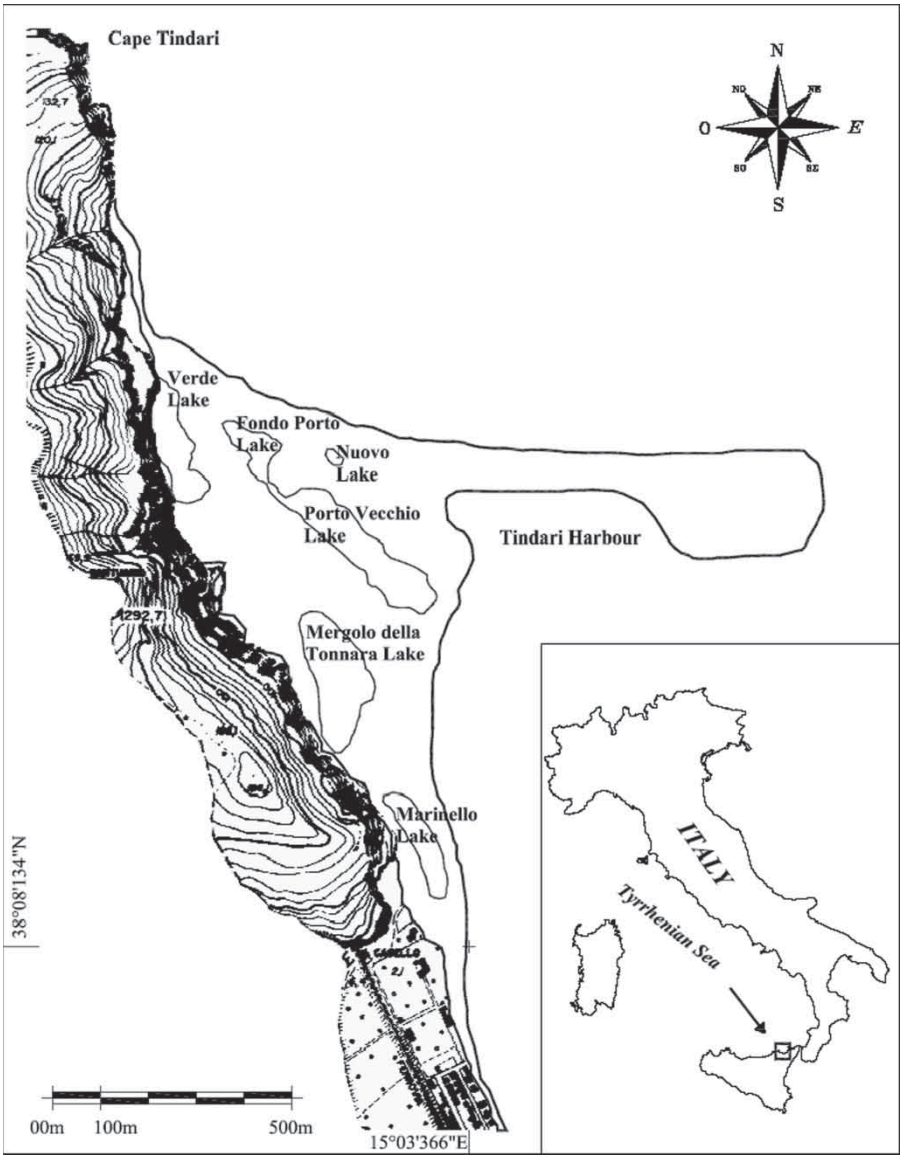

Figure 1. Study area: the Oliveri-Tindari Lagoon system, north-eastern Sicily, Italy.

cooling, samples were filtered and the volume corrected using ultra-pure water. Certified materials and reagents with no added samples were also analysed as controls. Arsenic (As) was determined by atomic absorption using the hydride generation technique. The method of standard additions was used for the analysis of all extracts. The efficiency of the extraction procedure and accuracy of analyses were ensured by digestion and analysis of Standard Reference Materials: SRM 1646a (Estuarine Sediments) from the National Institute of Standards and Technology (NIST) of the U.S. Department of Commerce (Gaithersburg, MD, USA). All samples were analysed in triplicate to check precision. The analytical precision of the replicates ($n = 3$), indicated by the percent coefficient of variation (% CV), was within 5%.

2.3. *Growth conditions of microorganisms with different metabolisms*

Growth experiments involving microorganisms with different metabolisms in the presence of arsenic were carried out using sediment samples collected from the lakes of the Oliveri-Tindari Lagoon as inocula. Heterotrophic aerobic microorganisms were tested for growth in a complex medium containing 5.0 g tryptone, 2.5 g yeast extract and 1.0 g D-glucose per litre of distilled water (TYEG) in the presence of As(III) as 1.35 mM sodium *m*-arsenite (*m*-$NaAsO_2$) and As(V) as 1.35 mM disodium hydrogen arsenate ($Na_2HAsO_4 \cdot 7H_2O$). The complex TYEG medium, treated for anaerobic conditions using the Hungate technique [24], was used to grow fermentation microorganisms in the presence of As(III) in the form of *m*-$NaAsO_2$ added at a concentration of 1.35 mM. Microbial growth in anaerobic conditions, in the presence of only As(V) added as 5 mM disodium hydrogen arsenate ($Na_2HAsO_4 \times 7H_2O$) an appropriate concentration for the final electron acceptor, or As(V) 5 mM plus 14 mM SO_4^{2-} as electron acceptors, was tested in a minimal medium (MM) containing 20 mM lactate, 0.14 g KH_2PO_4, 0.25 g NH_4Cl, 0.50 g KCl, 0.15 g $CaCl_2 \times 2H_2O$, 1.0 g NaCl and 0.62 g $MgCl_2 \times 6H_2O$ per litre of double-distilled water. Ten millilitres of a vitamin solution containing 0.005 mg *p*-aminobenzoic acid, 0.02 mg biotin, 0.05 mg nicotinic acid, 0.05 mg calcium pantothenate, 0.05 mg thiamine HCl, 0.1 mg pyridoxine HCl (B6) and 0.001 mg cyanocobalamin (B12) were also added, along with 10 mL of a trace element solution containing 0.001 concentrated HCl, 0.1 mg $MnCl_2 \times 4H_2O$, 0.12 mg $CoCl_2 \times 6H_2O$, 0.07 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.025 mg $NiCl_2 \times 6H_2O$, 0.015 mg $CuCl_2 \times 2H_2O$, 0.025 mg $Na_2MoO_4 \times 2H_2O$, and 1.5 mg $FeCl_2 \times 4H_2O$ per litre of double-distilled water. All preparations and manipulations took place under strict anoxic conditions using the Hungate technique [24]. Aerobic chemolithoautotrophic bacteria were investigated in the presence of 5 mM of As(III) as the energy source, in a mineral salts (MS) medium containing 0.25 g NH_4Cl, 1.0 g K_2HPO_4, 1.5 g KH_2PO_4, 0.125 g $MgSO_4 \times 7H_2O$ and 10 mL trace element solution. Cultures were incubated in the dark at 28 °C. As for the Most Probable Number (MPN) technique, five series of five tubes or anaerobic vials were inoculated with 1 g (f.w.) of the original sample and related serial dilutions and then incubated at the appropriate temperatures. Positive tubes or vials showing turbidity (indicative of microbial growth) were registered and related MPN·g^{-1} (f.w.) were assigned according to the McCrady tables.

2.4. *Enrichment cultures and isolation of bacterial strains*

Enrichment cultures were grown in a complex TYEG medium. Individual 250 mL flasks were filled with 50 mL of complex medium and inoculated with 0.5 g of sediment samples in the presence of 1.35 mM As(III) added as *m*-$NaAsO_2$. As(III) was chosen as the most toxic form of arsenic. The inoculated flasks were incubated at 28 °C for four weeks; microscopic analysis was used to detect growth throughout the incubation period. When microbial growth was detected, a 100 μL aliquot of each culture was first transferred and spread onto the complex medium in the presence of the same initial concentration of As(III) and with the addition of 1.6% of agar and then incubated at 28 °C for 48 h. After this period, colonies showing different morphologies were selected. Colonies were subcultured at least three times, and isolated strains were stored as liquid cultures containing 30% sterile glycerol (v/v) in liquid nitrogen.

2.5. *Bacterial strain characterisation and 16S rRNA gene analysis*

After growth of the bacterial isolates on a solid medium, colonies were observed under a stereomicroscope. Gram reactions were determined following the standardised method of bacterial cells staining (Gram stain kit, Carlo Erba). For the 16*S* rDNA sequencing of isolated bacterial

strains, a single colony was suspended in 50 μL double-distilled water and treated for 5 min at 100 °C. Amplification of the 16*S* rRNA gene was performed using 10 ng of genomic DNA in 20 μL of 1× 'Amplitaq' buffer (10 mM Tris–HCl; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin) with 150 ng each of the primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTACGA-3′), 250 μM each of dNTPs and 1 U of 'Amplitaq' (Perkin–Elmer). The reaction mixtures were incubated at 95 °C for 1 min and 30 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 five 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 a TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) containing 0.5 $\mu g \cdot mL^{-1}$ (w/v) ethidium bromide. Sequencing was carried out at the Bact 16*S* Biomolecular Research Service (BMR) (CRIBI Biotechnology Centre, University of Padua, Italy).

2.6. *Sequence accession numbers*

The 16*S* rDNA nucleotide sequences obtained in this study were deposited in the GenBank database with GenBank accession nos. EF428913, EF428914, EF428915, EF428916 and EF428917 for isolated strains MT1, MT2, MT3, V1 and V2, respectively.

2.7. *Analysis of sequence data*

The consensus sequences of the five isolates were compared with those deposited in GenBank. The 16*S* partial sequence of strain 063 was compared at the prokaryotic small subunit rDNA on the Ribosomal Database Project II website [25] and the NCBI website using the BLAST program [26]. The 16*S* rDNA sequences retrieved from the databases were aligned using ClustalW included in MEGA software, Version 4.1 [27]. The phylogenetic tree was inferred by MEGA 4.1 (neighbour-joining method) [28]. Sequence divergences between strains were quantified using the Kimura-2-parameter distance model [29]. 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.

2.8. *Minimum inhibitory concentrations (MICs)*

The As(III)- and As(V)-resistance of isolated bacterial strains was assessed through MIC tests. One-millilitre aliquots of overnight cultures were incubated in 99.0 mL of Mueller Hinton broth (Oxoid, Milan, Italy), and 10 mL were distributed in 18 mL test tubes sealed with radial caps. MIC tests were carried out at different concentrations of As(III), added as *m*-$NaAsO_2$ (0–17 mM), and As(V), added as disodium hydrogen arsenate ($Na_2HAsO_4 \times 7H_2O$; 0–135 mM). Tubes were incubated in a rotary drum at 30 °C for 24 h. The optical density of the cultures, used as a measure of microbial growth, was detected at a wavelength of 600 nm by an UV–vis spectrophotometer (Jenway, model AC30); a blank with the culture medium alone (without bacteria) was also analysed. Experiments were carried out in duplicate.

2.9. *As(V) reduction to As(III) in the isolated arsenic-resistant bacterial strains*

Cultures of the five isolated arsenic-resistant bacterial strains were prepared in a Tris minimal medium plus glucose (TMMG) containing 100 mM Tris–HCl at pH 7.2, 11 mM glucose, 5 mM NH_4Cl, 0.1 mMKH_2PO_4, 5 mM K_2SO_4, 0.1 mM $CaCl_2$, and 10 mM $MgCl_2$ per litre of

deionised water in the presence of 1 mM As(III) and 2 mM As(V). Cultures were incubated at 28 °C. One-millilitre aliquots were harvested at different times to measure cell growth at an OD of 600 nm and to determine As(V) and As(III) concentrations by spectrophotometric analysis according to the method described by Cummings et al. [30]. The As(V) concentration was determined by acidifying a 100 μL sample in 100 μL of HCl (24 mM). One hundred microlitres of the acidified sample was then added to 900 μL of a reaction mixture containing the following: ammonium molybdate ($6\,g{\cdot}L^{-1}$), ascorbic acid ($10.8\,g{\cdot}L^{-1}$), potassium antimonyl tartrate ($0.136\,g{\cdot}L^{-1}$) and concentrated H_2SO_4 ($67.3\,mL{\cdot}L^{-1}$). Each component was stored as a separate solution. Samples were heated in a water bath at 78 °C for 10 min and placed on ice for 5 min. The absorbance at 865 nm was compared with acidified As(V) standards. The As(III) concentration was determined by oxidising a second sample in 100 μL of KIO_3 (5 mM) and HCl (48 mM) for 10 min and then reading the OD at 865 nm. Blanks of deionised water were used to calibrate the spectrophotometer. New plastic wares were used each time to avoid contamination from phosphate in detergents. Standard curves were prepared for 0–100 mM concentrations of both As(V) and As(III). The difference between oxidised and unoxidised samples represented the As(III) concentration. Experiments were carried out in triplicate.

3. Results

3.1. *Enumeration of bacterial strains*

The highest mean As concentration of $19.80\,\mu g{\cdot}g^{-1}$ (d.w.) was detected in sediment samples from Lake Verde, and the lowest mean value of $3.60\,\mu g{\cdot}g^{-1}$ (d.w.) in sediment samples from Lake Mergolo della Tonnara (Table 1). Depending on the metabolic pathway, different culture media were used for Most Probable Number (MPN) analyses (Table 2). Heterotrophic microorganisms were cultured aerobically in a complex medium in the presence of 1.35 mM of As(III):MPN values ranged from 10^2 to $10^3\,g^{-1}$ (f.w.) in sediments collected from the six different lakes. Lakes Verde, Marinello and Nuovo showed the highest levels of heterotrophic arsenic-resistant microorganisms. The same three lakes showed higher levels of fermentation microorganisms able to thrive in the presence of 1.35 mM of As(III), again with values of $10^3\,MPN{\cdot}g^{-1}$ (f.w.). Values for anaerobic fermentation microorganisms ranged from 10 to $10^3\,MPN{\cdot}g^{-1}$ (f.w.). The biogeochemical arsenic cycle was also investigated in anaerobic culture media in the presence of 5 mM As(V) or, alternatively, 5 mM As(V) and 14 mM sulfates to test for dissimilatory respiration. Low levels of growth were detected under these two conditions; in some cases no growth was detected. The highest values were detected for Lake Verde sediments, in which 7.5 and $2.5\,MPN{\cdot}g^{-1}$ (f.w.) of microorganisms grew in the presence of As(V) and As(V) plus sulfates respectively, and for Lake

Table 1. Mean As concentrations in the lakes of the Oliveri-Tindari Lagoon, Sicily, Italy.

Sample	No. of samples	Mean	SD*
Lake Verde	8	19.88	6.03
Lake M. della Tonnara	8	3.60	1.33
Lake Marinello	8	10.24	4.40
Lake Nuovo	3	10.07	3.48
Lake Fondo Porto	6	18.86	1.20
Lake Porto Vecchio	4	14.83	7.03

Notes: Values are reported as $\mu g\ g^{-1}$ dry weight (d.w.). *The standard deviation indicates the variability within different samples collected from each lake. Geomorphological variability is present in the lake sediments of the Oliveri-Tindari Lagoon.

Table 2. MPN·g^{-1} (f.w.) of bacteria in sediments collected from the different lakes of the Oliveri-Tindari Lagoon, in the presence of arsenic and using different culture media.

	Culture media				
Sample	TYEG	TYEG an.	MM an.	MMS an.	MS
Lake Verde	1.5×10^3	1.1×10^3	7.5	2.5	1.5
Lake M. della Tonnara	1.4×10^2	4×10	1.5	0	2.5
Lake Marinello	1.1×10^3	1.5×10^3	9.5	4.5	1.1
Lake Nuovo	1.4×10^3	4.5×10^3	0.3	0	0.9
Lake Fondo Porto	1.4×10^2	1.5×10^2	0	0	2.5
Lake Porto Vecchio	4.5×10^2	20×10^2	0	2.5	4.5

Note: MPN·g^{-1} (f.w.), Most Probable Number per gram of fresh weight; TYEG, Tryptone, Yeast Extract, Glucose; MM, Minimal Medium, anaerobic; MMS, Minimal Medium Sulfates, anaerobic; MS, Mineral Salts.

Marinello sediments, in which 9.5 MPN·g^{-1} (f.w.) were detected in dissimilatory As(V) respiration and 4.5 MPN·g^{-1} (f.w.) when sulfates were added to As(V) as terminal electron acceptors. Chemolithoautotrophic bacteria, another metabolic group involved in the biogeochemical arsenic cycle, were investigated in a mineral medium in the presence of 5 mM As(III) as an electron and energy donor, with carbon dioxide as the sole carbon source. Growth was detected in all the tested sediment samples, albeit at low levels, with Lake Porto Vecchio showing the highest value of 4.5 MPN·g^{-1} (f.w.) (Table 2).

3.2. *Isolation and characterisation of arsenic-resistant bacterial strains*

Enrichment cultures showing turbidity allowed the isolation of five different arsenic-resistant bacterial strains. These five bacterial strains were isolated from Lake Mergolo della Tonnara (MT1, MT2 and MT3) and from Lake Verde (V1 and V2) (Table 3). Enrichment cultures were not grown for sediment samples collected from the other lakes in the lagoon system.

3.3. *Sequence data analysis*

16*S* rDNA gene sequence analysis was used to assign the isolated strains to bacterial strains. Strain MT1 showed 100% sequence similarity to *Bacillus thuringiensis* strain IECB T24 (AM747224), strain MT2 100% similarity to *Bacillus megaterium* strain OSS4 (EU124555), strain MT3 100% similarity to a strain of *Bacillus thuringiensis* (EF028102), strain V1 99% similarity to a strain of

Table 3. Bacterial strains isolated from sediments of the Oliveri-Tindari Lagoon in the presence of 100 μg·mL^{-1} of trivalent arsenic, As(III), added as m-sodium arsenite (*m*-$NaAsO_2$).

Sampling site	Isolated strain	Colony features	16*S* rRNA gene	Percentage identity
Lake M. della Tonnara	MT1	Ø 1.5 mm, cream, oval, matt, entire margins	*Bacillus*	100
Lake M. della Tonnara	MT2	Ø 2.0 mm, white, round, flat, matt, entire margins	*Bacillus*	100
Lake M. della Tonnara	MT3	Ø 1.0 mm, cream, oval, matt, entire margins	*Bacillus*	100
Lake Verde	V1	Ø 1.5 mm, cream, oval, matt, entire margins	*Bacillus*	99
Lake Verde	V2	Ø 1.0 mm, white, oval, flat, matt, entire margins	*Bacillus*	98

Note: Ø, diameter of the colony.

Bacillus weihenstephonensis (AM747231), and V2 98% similarity to *Bacillus sphaericus* strain AU3 (EF032669) (Table 3).

3.4. *Phylogenetic analysis*

The phylogenetic tree was inferred for the five bacterial isolates showing high levels of arsenic-resistance. Analysis of the 16*S* rDNA sequence indicated that strain MT1 was closely related to the strain *Bacillus* sp. OrAs10 (DQ401150) and *Bacillus* sp. OrAs9 (DQ401149) isolated from an impacted lagoon in Central Italy (Figure 2). Within the same clade, strain MT3 was similar to the arsenic-resistant *Bacillus* sp. JS 23b (DQ104990) (Figure 2). Strain V1 and the type strain of *Bacillus mycoides* ATCC6462 (AB021192) were grouped in a separate clade. Strain MT2 was closely related to the type strain of *Bacillus arsenicus* con a/3 (AJ606700), an arsenic-resistant bacterial strain isolated from a siderite concretion in West Bengal, India (Figure 2). Strain V2 resulted in a separate line, with the nearest strains included within a clade originated by *Bacillus borotolerans* T-13B (AB199594), *Bacillus fusiformis* Z1 (AY548950) isolated from Pakistan soil, and the arsenic-resistant bacterial strain *Bacillus* sp. ORAs1 (Figure 2).

3.5. *Minimum inhibitory concentrations*

Levels of resistance to trivalent arsenic for the five *Bacillus* spp. strains ranged between 7 and 14 mM, with *Bacillus* sp. strain MT3 showing the highest level of 13.35 mM (Figure 3A). As for

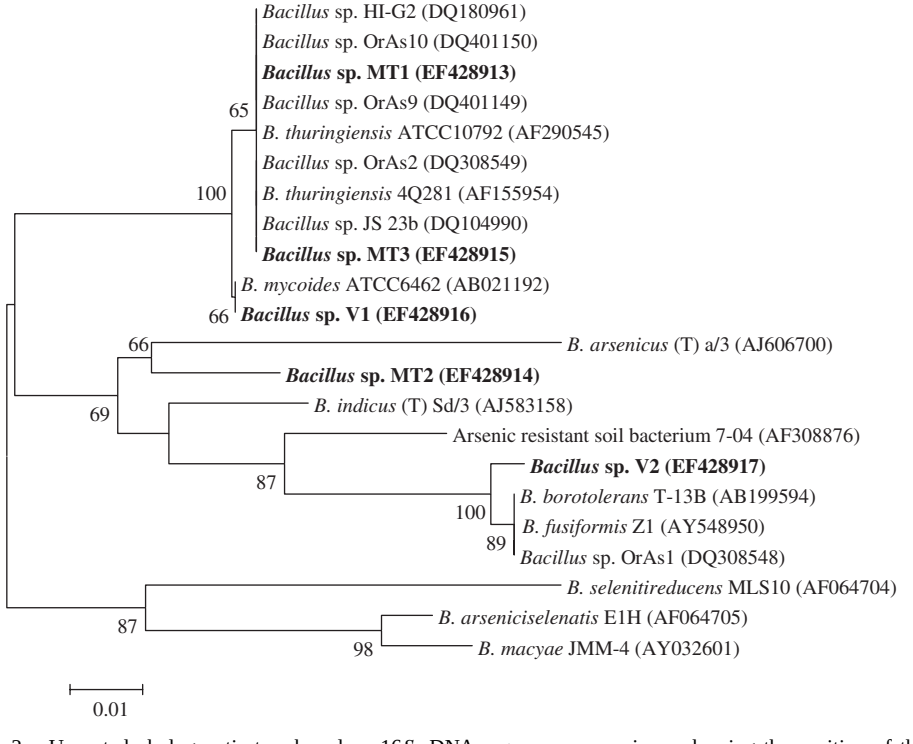

Figure 2. Unrooted phylogenetic tree based on 16*S* rDNA sequence comparisons showing the position of the five *Bacillus* spp. isolates and representative species of the genus *Bacillus*. The branching pattern was generated by neighbour-joining methods. Bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap values <60% are not shown. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16*S* rDNA sequences are given in parentheses after the strain.

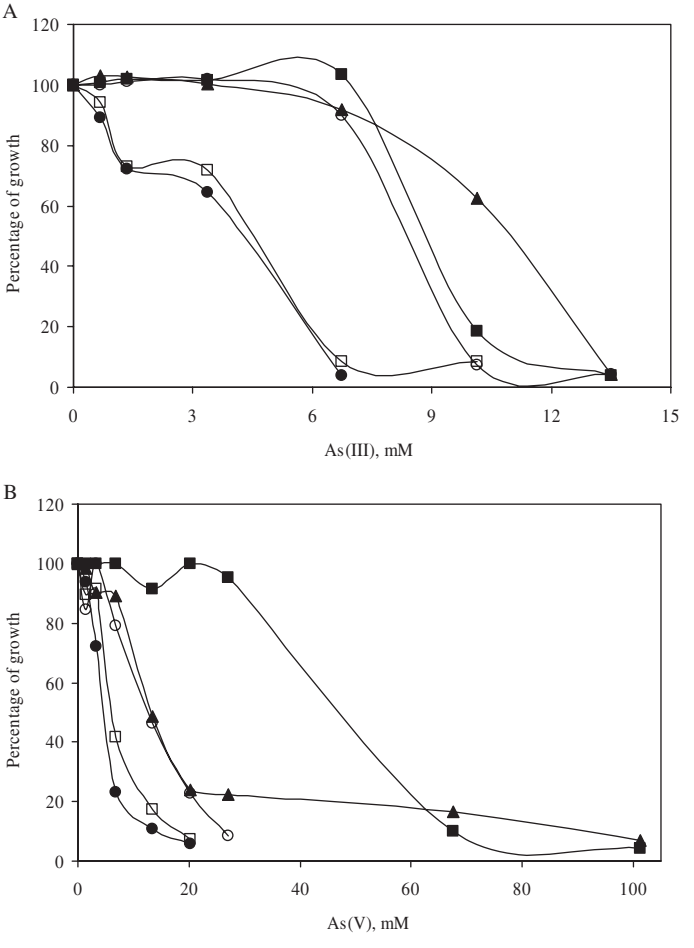

Figure 3. Minimum inhibitory concentrations for the five *Bacillus* spp. isolates MT1 (○), MT2 (●), MT3 (▲), V1 (■) and V2 (□) in the presence of (A) different concentrations of As(III), ranging from 0 to 17 mM, and (B) As(V), ranging from 0 to 135 mM.

the MICs of isolated bacterial strains in the presence of pentavalent arsenic, values ranged from 20 to 100 mM, with *Bacillus* sp. strain MT3 showing the highest MIC value (100 mM) for As(V) (Figure 3B). The five isolated bacterial strains were tested for growth in a mineral medium under both aerobic and anaerobic conditions; trivalent arsenic was added as selective pressure under aerobic conditions and pentavalent arsenic, alone or with sulfate, was added as a final electron acceptor. *Bacillus* sp. strain MT3 was again found to be the best adapted to arsenic and was able to grow in the presence of As(V) as an electron acceptor in dissimilatory respiration (data not shown).

3.6. *As(V) reduction to As(III) by the isolated bacterial strains*

To verify the presence of detoxification mechanisms in arsenic-resistant bacteria, the As(V) reduction and As(III) oxidation profiles of the isolates were studied. Four of the five isolated arsenic-resistant bacterial strains were tested in order to investigate the resistance mechanism to arsenic. Because this experiment was carried out in a minimal medium containing low concentrations of phosphate, strain MT1 was not able to grow under these conditions. Strain MT2 was able

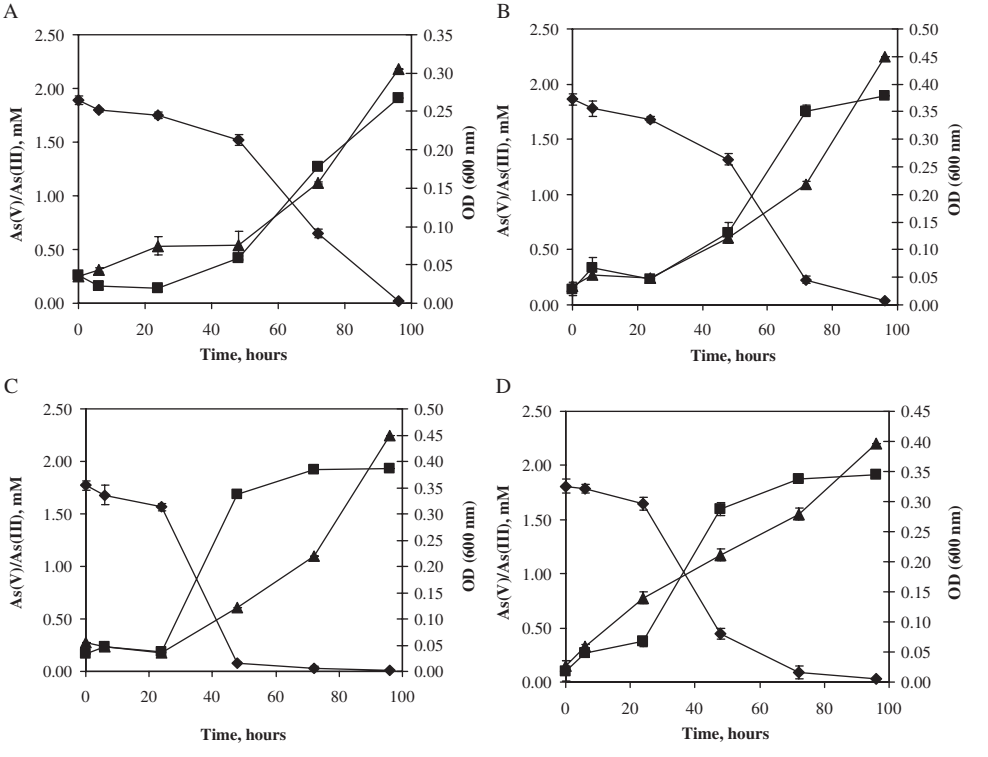

Figure 4. As(V) reduction to As(III) by the bacterial strains isolated from sediments of the Oliveri-Tindari Lagoon. Symbols represent the following parameters: (♦) As(V) concentrations; (■) As(III) concentrations, (▲) bacterial biomass (A) strain MT2; (B) strain V1; (C) strain MT3; (D) strain V2.

to completely reduce 2 mM of As(V) to As(III) in ~96 h, strains V1 and V2 in 72 h, and strain MT3 in 48 h (Figure 4). None of the isolates oxidised As(III) under aerobic conditions.

4. Discussion

Results demonstrate that bioavailable arsenic and bacteria that thrive in the presence of this metalloid are present in the sediments of the Oliveri-Tindari Lagoon, an area unaffected by human activity [31]. Geochemical studies carried out in north-eastern Sicily indicated a relatively high level of arsenic in the same geological formation [32]. Arsenic is present in higher concentrations in sedimentary rocks than in igneous or metamorphic rocks [3], and the rocks within the study area seem to have a sedimentary origin [33]. Arsenic concentrations in Canadian lake sediments showed a mean background content of 5.5 $\mu g \cdot g^{-1}$ (d.w.), with values ranging from 0.9 to 44 $\mu g \cdot g^{-1}$ (d.w.) [34,35].

This study confirms the presence of microorganisms with different metabolisms thriving in sites containing arsenic. Arsenic compounds have been abundant in the environment at near toxic levels since the origin of life. In response, microorganisms have developed mechanisms of arsenic resistance and enzymes that oxidise As(III) to As(V) or reduce As(V) to As(III), involving the formation and degradation of organoarsenicals such as methylarsenic compounds. Microbial metabolism and mobilisation (or immobilisation) are important processes in the global arsenic geocycle [8]. A full biogeochemical arsenic cycle driven in part by inorganic electron donors

occurs in Searles Lake, USA. Speciation changed arsenic from As(V) to As(III). Incubated anoxic sediment slurries displayed dissimilatory As(V)-reductase activity [10]. The microorganisms with different metabolisms highlighted in this study suggest the presence of bioavailable arsenic and of active microbial populations involved in its transformation [36]. In this study, we found different microorganisms that use arsenic in different ways, thereby suggesting a real interaction with autochthonous microorganisms at the level of native sediments. Some organisms have evolved biochemical mechanisms to exploit arsenic oxyanions, either as an electron acceptor (arsenate) for anaerobic respiration or as an electron donor (arsenite) to support the chemoautotrophic fixation of carbon dioxide into cell carbon [37,38]. Although overall levels of arsenic in the native lake sediment samples were low, aerobic and anaerobic bacteria thriving in the presence of arsenic were detected. This study of sediments from the Oliveri-Tindari Lagoon highlighted the presence of a biogeochemical arsenic cycle, although arsenic methylation was not addressed; studies by Jackson et al. [15] and Yamamura et al. [16] describe the use of this metalloid by microorganisms in non-polluted environments.

The five isolated bacterial strains that showed high levels of As(III)- and As(V)-resistance belonged to the genus *Bacillus*, whose presence in sediments is in part due to the resistance of their spores in these environments. Numerous bacteria belonging to the genus *Bacillus* and with levels of resistance higher than the ones found in this study have been recovered from different arsenic-contaminated sites [39,40].

Paenibacillus spp. belonging to the bacterial group Firmicutes and isolated from arsenic-free soils showed As(III) tolerance ranging from 2 to 5 mM, and As(V) from 35 to 150 mM [15]. *Bacillus* spp. isolated as part of this study ranged from 7 to 14 mM for As(III) and from 20 to 100 mM in the case of As(V), confirming similar tolerance pathways for bacteria isolated from similar non-impacted areas.

From a phylogenetic point of view, comparison of the five *Bacillus* spp. isolates with other arsenic-resistant bacterial strains belonging to the same genus and with type strains revealed a broad distribution of the five isolates and different associations among the different bacterial strains. Interesting correlations were highlighted with arsenic-resistant bacterial strains isolated from polluted sediments of a lagoon in Central Italy [14] and with an arsenic-resistant bacterial strain isolated from a siderite concretion in West Bengal, India [40].

The bacteria isolated from the arsenic-enrichment cultures were able to reduce As(V). The mechanism of As(V) reduction by isolates serves a detoxification purpose, as arsenate reductase is involved in As(V) reduction under aerobic conditions [8].

The isolated bacterial strains play a significant role in As(V) reduction to As(III), and the resulting change in arsenic species and mobility within lake sediments is ecologically important because it allows arsenic oxidising/reducing bacteria to control the biogeochemical cycle of sediments [41].

The field of geomicrobiology is rapidly expanding, as both geoscientists and biologists have come to recognise the importance of both disciplines in understanding the complexities of nature. Bacteria and other microorganisms have been extremely important for their direct and indirect effects on the geochemical cycling of metals and organic matter throughout the history of the Earth [42].

This study detected the presence of bioavailable arsenic in uncontaminated sediments of the Oliveri-Tindari Lagoon and of bacteria involved in the arsenic biogeochemical cycle and in the mobilisation of arsenic through reduction of arsenate to the more soluble arsenite.

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