

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/279967867>

Characterization of the arsenite oxidizer Aliihoeflea sp. strain 2WW and its potential application in the removal of arsenic from groundwater in combination with Pf-ferritin

Article in *Antonie van Leeuwenhoek* · July 2015

DOI: 10.1007/s10482-015-0523-2 · Source: PubMed

CITATIONS

7

READS

125

4 authors:



Anna Corsini

University of Milan

29 PUBLICATIONS 372 CITATIONS

[SEE PROFILE](#)



Milena Colombo

University of Milan

22 PUBLICATIONS 710 CITATIONS

[SEE PROFILE](#)



Gerard Muyzer

University of Amsterdam

622 PUBLICATIONS 38,289 CITATIONS

[SEE PROFILE](#)



Lucia Cavalca

University of Milan

141 PUBLICATIONS 1,859 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Vestfold Hills [View project](#)



Microbial transformations of arsenic: Perspectives for biological removal of arsenic from water [View project](#)

Characterization of the arsenite oxidizer *Aliihoeflea* sp. strain 2WW and its potential application in the removal of arsenic from groundwater in combination with *Pf*-ferritin

Anna Corsini · Milena Colombo ·
Gerard Muyzer · Lucia Cavalca

Received: 15 May 2015 / Accepted: 29 June 2015
© Springer International Publishing Switzerland 2015

Abstract A heterotrophic arsenite-oxidizing bacterium, strain 2WW, was isolated from a biofilter treating arsenic-rich groundwater. Comparative analysis of 16S rRNA gene sequences showed that it was closely related (98.7 %) to the alphaproteobacterium *Aliihoeflea aestuari* strain N8^T. However, it was physiologically different by its ability to grow at relatively low substrate concentrations, low temperatures and by its ability to oxidize arsenite. Here we describe the physiological features of strain 2WW and compare these to its most closely related relative, *A. aestuari* strain N8^T. In addition, we tested its efficiency to remove arsenic from groundwater in combination with *Pf*-ferritin. Strain 2WW oxidized arsenite to arsenate between pH 5.0 and 8.0, and from 4 to 30 °C. When the strain was used in combination with a *Pf*-ferritin-based material for arsenic removal

from natural groundwater, the removal efficiency was significantly higher (73 %) than for *Pf*-ferritin alone (64 %). These results showed that arsenite oxidation by strain 2WW combined with *Pf*-ferritin-based material has a potential in arsenic removal from contaminated groundwater.

Keywords Arsenite oxidation · Arsenic · *Aliihoeflea* · Ferritin · Groundwater

Introduction

Arsenic (As) is present in various environments, originating from either natural or anthropogenic sources. In aquatic systems, arsenic is primarily present as arsenite, As(III), under anaerobic conditions, or as arsenate, As(V) under aerobic conditions. Bacteria play an important role in arsenic transformations, thus influencing arsenic mobility, bioavailability and toxicity (Smedley and Kinniburgh 2002). The oxidation state of arsenic is crucial, because it affects its mobility and the efficiency with which it can be removed in remediation processes. As(V) tends to associate with oxyhydroxides and clay minerals, and is therefore less mobile than As(III).

Both forms of arsenic are toxic to biological systems and they induce distinct types of cellular damage. Because of its structural analogy to phosphate, As(V) can enter the cells via phosphate membrane transport systems and disrupt metabolic

Electronic supplementary material The online version of this article (doi:10.1007/s10482-015-0523-2) contains supplementary material, which is available to authorized users.

A. Corsini · M. Colombo · G. Muyzer · L. Cavalca (✉)
DeFENS - Dipartimento di Scienze per gli Alimenti, la
Nutrizione e l'Ambiente, Università degli Studi di Milano,
via Celoria 2, 20133 Milan, Italy
e-mail: lucia.cavalca@unimi.it

G. Muyzer
Microbial Systems Ecology, Department of Aquatic
Microbiology, Institute for Biodiversity and Ecosystem
Dynamics, University of Amsterdam,
1090 GE Amsterdam, The Netherlands

reactions that require phosphorylation; As(III) is transported into the cell by aquaglyceroporins and exerts its toxicity by binding thiol groups in proteins, impairing their function (Oremland and Stolz 2003). Nevertheless, certain bacteria have evolved mechanisms to resist arsenic, allowing them to grow in environments containing levels of arsenic that would be toxic to most other organisms. Microbial resistance to concentrations greater than 10 mM As(III) and 100 mM As(V) is regarded as very high, while resistance to 300–500 mM As(V) or 30 mM As(III) is a hyper tolerance property (Jackson et al. 2003). It has also been reported that arsenic resistance property is not limited to the arsenic contaminated sites, as several highly resistant isolates have been isolated from environmental samples having very low or negligible arsenic content (Gosh and Sar 2013).

Arsenic resistance strategies include active extrusion of As(III) from the cell as well as As(III) oxidation. The extrusion is based on the presence of an arsenite efflux system encoded by the two unrelated families of arsenite transporters (arsB and ACRp) (Achour et al. 2007). As(III) oxidation is a detoxification mechanism, but also an energy-generating reaction that bacteria perform in chemolitho-autotrophic or -heterotrophic growth (Stolz et al. 2002; Lugtu et al. 2009). As(III) oxidation is a widespread mechanism in phylogenetically different bacteria comprising members of the *Aquificales* and *Thermus*, as well as *Alpha*-, *Beta*- and *Gammaproteobacteria*, such as *Alcaligenes*, *Thiomonas*, *Hermiimonas*, *Agrobacterium*, *Polaromonas* and *Pseudomonas* (Cavalca et al. 2013a), isolated from arsenic-contaminated soils (Chitpirom et al. 2009; Bachate et al. 2012) and groundwaters (Liao et al. 2011).

Human exposure to arsenic typically occurs through drinking water and the World Health Organization (WHO) has revised the limit for total arsenic in drinking water to 10 $\mu\text{g L}^{-1}$ (WHO, 2001). Arsenic adsorption onto iron-based sorbents (i.e., iron oxides, and zero and bivalent-valent iron nanoparticles) has been developed to counteract arsenic contamination of water (Mondal et al. 2006; Corsini et al. 2014b). The adsorption phase often requires a pre-oxidation of As(III) to As(V), as As(III) is less easily removed by the positively charged surfaces of sorbents. Utilization of As(III)-oxidizing bacteria is considered a cost-effective and eco-friendly alternative to the use of chemical oxidants (Ito et al. 2012).

Here, we compare the physiological characteristics, arsenic metabolism and genes of *Aliihoeflea* sp. strain 2WW and its most closely-related relative *Aliihoeflea aestuarii* strain N8^T (Roh et al. 2008). Furthermore, the removal of arsenic from natural ground water was assessed in a combined system consisting of cells of strain 2WW and *Pf*-ferritin-based material (hereafter referred as *Pf*-ferritin), which is a nano-iron storage protein obtained from *Pyrococcus furiosus* (Jacobs et al. 2010).

Materials and methods

Bacterial strains

Strain 2WW was isolated from an arsenic contaminated groundwater present in an aerobic biofilter, after an enrichment procedure on As(III) (Corsini et al. 2014a). The genome of *Aliihoeflea* sp. strain 2WW was sequenced (Cavalca et al. 2013b). Strain 2WW was deposited in The Netherlands Culture Collection of Bacteria (NCCB) under the accession number NCCB 100463. *A. aestuarii* strain N8^T (DSM 19536) was obtained from the German Culture Collection of Microorganisms and Cell Cultures (DSMZ).

Growth conditions

Strain 2WW was tested for chemolithotrophic growth supported by As(III) as electron donor, in heterotrophic and autotrophic conditions. Chemolitho-heterotrophic growth was performed in basal mineral medium with low phosphate content (BBWM) supplemented with different amounts (0.04, 0.2 and 0.4 % w/v) of yeast extract (YE) (BBWM-Y). Chemolitho-autotrophic growth was performed in BBWM supplemented with NaHCO_3 (9.5 mM) and devoid of vitamins (BBWM-C). Blanks were run in the absence of As(III). See the Electronic Supplementary Material for additional details on BBWM composition and preparation. All experiments were performed in triplicate in 20 mL fresh medium with 5 % (v/v) two-day old inoculum grown in BBWM-Y or in BBWM-C. The experiments were conducted at 30 °C with shaking at 150 rpm. The specific growth rate (μ) was calculated using the formula (Pirt 1975):

$$\mu = 1/\text{OD}_0 \times (\text{OD}_t - \text{OD}_0)/(\text{T}_t - \text{T}_0)$$

where μ (h^{-1}) is the specific growth rate of the initial bacterial concentration within a given time; OD_t and OD_0 represent the $\text{OD}_{600\text{nm}}$ of the culture at time t and time 0, respectively; and T_t and T_0 represent corresponding times (h). The specific growth rate was determined at different times for each type of media, and the mean specific growth rate (μ) was calculated.

Physiological characterization

Strains 2WW and N8^T were maintained in glycerol stocks at -70°C . Precultures were grown at 28°C in BBWM-Y (0.2 % w/v YE) at 30°C . The motility was checked by phase-contrast microscopy. Gram staining was conducted according to standard procedures on overnight grown cells. Catalase and oxidase activities were determined by observing bubble production in a 3 % (v/v) hydrogen peroxide solution and by using tetra-methyl-para-phenylenediamine, respectively. Phenotypic data of strains 2WW and N8^T were determined by the API 20 NE identification system (BioMerieux, France). Growth was scored after 24 and 48 h incubation at 30°C . The tests were repeated on two separate occasions.

The use of alternative electron donors was tested under aerobic conditions in BBWM-C separately supplemented with: thiosulfate (2 mM), elemental sulphur (6 g L^{-1}), acetate (6 g L^{-1}), gluconate (6 g L^{-1}), glucose (6 g L^{-1}), glutamate (6 g L^{-1}), benzoate (6 g L^{-1}) and toluene (6 g L^{-1}).

As(III) (0.075–0.750 g L^{-1}) and As(V) (0.150–18.75 g L^{-1}) resistance of the strains was evaluated by determining the Minimum Inhibitory Concentration (MIC) in BBWM-Y (0.2 % w/v YE).

The effect of temperature (5 – 30°C), pH (5.0 – 8.0) and osmotic pressure (0.1 – 10 % w/v NaCl) on the growth of the strains was examined in BBWM-Y (0.2 % w/v YE).

As(III) oxidation assay

The effect of temperature (5 , 15 and 30°C) and pH (5.0 , 6.0 , 7.0 and 8.0) on the oxidation of As(III) by growing cells of strain 2WW was evaluated. A two-day old culture grown in BBWM-Y (YE 0.2 % w/v) at 30°C was used as inoculum (5 % v/v). The As(III) oxidation experiment was carried out in triplicate flasks each containing 20 mL of BBWM-Y

supplemented with 75 mg L^{-1} As(III). Three flasks without As(III) were also inoculated to compare the growth of the microorganisms in the absence of As(V) or As(III). Three control flasks without inoculum were prepared in order to check for abiotic transformations of As(III). All flasks were incubated under aerobic conditions on a rotary shaker at 150 rpm. At successive incubation times, 2 mL of cell suspension were collected to determine growth by absorbance spectroscopy at 600 nm and to analyze total As, As(III) and As(V) content by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies, Santa Clara, CA, USA) as described below. The specific growth rate of strain 2WW was determined as above described.

DNA extraction

Genomic DNA was isolated from the reference strain N8^T and strain 2WW using the Microbial DNA Extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The yield and quality of DNA were analysed by agarose gel electrophoresis.

PCR amplification of 16S ribosomal rRNA and arsenic-related genes

PCR reactions of 16S rRNA were performed using primers 27F and 1495R (Weisburg et al. 1991). Amplification of arsenite oxidase gene *aioA* was performed with primers aoxBM1-2F and aoxBM3-2R according to the protocol of Quéménéur et al. (2008). Amplification of the arsenate reductase gene *arsC* was conducted with primers P52F and P323R according to Bachate et al. (2009). Amplifications of *arsB*, *ACR3(1)* and *ACR3(2)* genes coding for different types of As(III) efflux pump, were conducted with primer pairs darsB1F/darsB1R, dacr1F/dacr4R and dacr5F/dacr4R, respectively, according to Achour et al. (2007). Additional details on PCR conditions are provided in the Electronic Supplementary Material.

Comparative sequence analysis of arsenic-related genes

Arsenic gene sequences *aioA*, *ACR3(1)* and *ACR3(2)* were compared to sequences available in GenBank using BlastX (Altschul et al. 1990). Phylogenetic

analysis of the deduced amino acid sequences was performed using MEGA software version 4 (Tamura et al. 2007). Phylogenetic trees were constructed using the neighbor-joining distance method based on p-distance. A total of 1000 bootstrap replications were calculated. The nucleotide sequence data were deposited in the EMBL database under the accession numbers: HF565048, HF570940 and HF570939 for the 16S rRNA, *ACR3(2)* and *AioA* of strain 2WW, respectively; and HG000666 for *ACR3(1)* of strain N8^T.

Arsenic removal kinetics of *Pf*-ferritin

Pf-ferritin was provided by BiAqua B.V. (The Netherlands). *Pf*-ferritin is a thermostable protein from the hyperthermophilic archaeon *Pyrococcus furiosus*, and it is loaded with nanoscale ferric iron particles (Jacobs et al. 2010), and stabilized onto sand, used as a carrier (2.74 mg ferritin g⁻¹ dry sand). The *Pf*-ferritin has been developed for phosphate removal from water (Jacobs et al. 2010).

Due to analogy with phosphates, in the present study it was tested for the ability to adsorb inorganic arsenic forms. As(III) and As(V) adsorption capacity of *Pf*-ferritin was tested in artificial system by adding 12.8 g L⁻¹ *Pf*-ferritin and 50 mL of Milli-Q water, spiked with 200 µg L⁻¹ As(III) or As(V), chosen on the base of arsenic content of the groundwater used in the present study. Experiments were carried out in polypropylene tubes for 48 h in shaking condition at 150 rpm at 15 °C. 10 mL of suspension were collected every 24 h for 1 week, centrifuged, syringe-filtered through 0.22 µm filters (Millipore), and treated as described below for arsenic species quantification at ICP-MS.

Arsenic removal assay by combined system: *Pf*-ferritin and bacterial cells

The assays were conducted in a system composed of *Pf*-ferritin and cells of strain 2WW. The arsenic removal efficiency of the combined system and of its single components was evaluated in an artificial system (Milli-Q water supplemented with 200 µg L⁻¹ As(III)) and in natural system (arsenic-contaminated groundwater), sampled from a public-supply well in the province of Cremona (Italy). Physico-chemical characterization revealed that the

groundwater was anoxic (with a redox potential (Eh) value of -113 mV, and no dissolved oxygen) and had the following characteristics: temperature of 15.8 °C; pH value of 7.6; CaCO₃ 282 µg L⁻¹; organic C 2.11 µg L⁻¹; dissolved S-SO₄ 267 µg L⁻¹; dissolved P-PO₄ 312 µg L⁻¹; dissolved N-NO₃ 685 µg L⁻¹; dissolved N-NH₄ 2680 µg L⁻¹; dissolved Fe 760 µg L⁻¹ and dissolved Mn 97 µg L⁻¹. Arsenic concentration in the groundwater was 171 µg L⁻¹ with AsIII as the main As species. Preparation and inoculation of resting cells were performed as specified in the Electronic Supplementary Material. Experiments were carried out in triplicate for 48 h in shaking condition at 150 rpm at 15 °C, at pH 7.58, according to the in situ parameters of the sampled groundwater, and at pH 6.41 for Milli-Q water system. A final cell density of 10⁷ cell mL⁻¹ and 12.8 g L⁻¹ of *Pf*-ferritin were used. Abiotic controls with 12.8 g L⁻¹ of *Pf*-ferritin were prepared. Biotic controls without *Pf*-ferritin with cell density of 10⁷ cell mL⁻¹ were prepared to check for the As(III) oxidation capacity of the strain. At the end of the experiments, 10 mL of cell suspensions were collected, centrifuged, syringe-filtered through 0.22 µm filters (Millipore), and treated for arsenic species quantification at ICP-MS, as described below.

Analytical methods

Total As was determined in samples previously acidified with HNO₃. For the measurement of As speciation, As(V) and As(III) species were separated on the basis of their selective retention on a WATERS Sep-Pak Acell Plus QMA cartridge (Waters, MA, USA). As(V) is retained in the cartridge, while As(III) passes through and is collected. The procedure was performed according to Kim et al. (2007). Total As, As(III), As(V) contents were determined in the artificial system and in the groundwater by ICP-MS. Dissolved Mn, Fe and P content were determined in the groundwater by ICP-MS.

Standards of As for concentrations ranging from 0 to 1 mg L⁻¹ were prepared from sodium arsenite NaAsO₂ (Sigma) solution, while standards of Mn, Fe and P concentrations ranging from 0 to 1.5 mg L⁻¹ was prepared from a multi standard solution (Agilent Technologies). For all the measurements by ICP-MS an aliquot of a 2 mg L⁻¹ of an internal standard solution (⁴⁵Sc, ⁸⁹Y, ¹⁵⁹Tb, Agilent Technologies) was

added to samples and the calibration curve to give a final concentration of $20 \mu\text{g L}^{-1}$. The instrument was tuned daily with a multi-element tuning solution for optimised signal-to-noise ratio.

Statistical analysis

Data represent the mean values obtained from at least three replicates. The values were subjected to two-tailed *t* test ($P < 0.05$) and to one-way analysis of variance (ANOVA) with Tukey-b test using *SPSS Statistics for Windows*, ver. 20.0 (IBM Corp., Armonk, NY).

Results and discussion

Physiological traits of strains 2WW and N8^T

Physiological characterization of strains 2WW and N8^T was performed regarding either common or arsenic-related features (Table 1).

Colonies of strain 2WW on agar plates were circular and pale cream and cells were Gram-negative stained, motile and rod-shaped. Strain 2WW was oxidase, catalase and urease positive. Fermentation of glucose and hydrolysis of aesculin, gelatin and *p*-nitrophenyl- β -D-galactopyranoside (PNPG) did not occur. These characteristics were shared also by N8^T type strain, in accordance with the description by Roh et al. (2008). Differently from strain N8^T, strain 2WW was arginine hydrolase negative. The carbon sources utilized by strain 2WW for growth included lactate, glucose, gluconate, adipate, and malate. Arabinose, mannose, mannitol, and N-acetyl-glucosamine were weakly utilized for growth. Strain 2WW showed a lower temperature threshold for growth (5°C) than N8^T, which did not start growing until 17°C . Strain 2WW grew in the presence of up to 8 % (w/v) NaCl, whereas strain N8^T grew up to 10 % (w/v) NaCl. The two strains were able to grow at comparable pH values. Yeast extract as sole carbon and energy source supported the growth of both strains. Differently from strain N8^T, strain 2WW presented very weak growth on complex medium such as tryptic soy broth (TSB).

Although the two strains were isolated from sediment and groundwater samples, they were strictly aerobic strains. Their presence in sediment and water may be related to the occurrence of oxic niches in

these suboxic/anoxic environments (Reise 2002). Aquifers are considered dynamic environments in terms of oxic-anoxic states due to seasonal variation in the depth distribution of groundwater and to digging wells operation (Fan et al. 2008).

Both strains were resistant to As(V) with MIC values of 18.75 g L^{-1} , and less resistant to As(III) with MIC values of 0.375 and 0.225 g L^{-1} for 2WW and N8^T strains, respectively.

As(III), elemental sulfur or thiosulphate did not supported chemolithoautotrophic growth of both strains. Differently from strain 2WW, which was able to oxidize As(III) in heterotrophic conditions, strain N8^T was unable to perform As(III) oxidation. Both strains did not reduce As(V).

Effect of pH and temperature on As(III) oxidation by growing cells of strain 2WW

Strain 2WW oxidized 75 mg L^{-1} As(III) at different temperatures (Fig. S1) and pH values (Fig. S2). In the presence of As(III) the strain did not grow more than in its absence, evidencing that the As(III) oxidation reaction was not used for gaining energy, but for detoxification purposes. As(III) was oxidized to As(V) in the early exponential growth phase. After the complete oxidation to As(V), the OD₆₀₀ value increased between 24 and 32 h of incubation time, evidencing that the depletion of As(III) decreased the toxicity level of the medium, thus relieving the strain, in accordance with the MIC values for both arsenic species (Table 1). The oxidation was complete within 24 h at 30°C and within 96 h at 15°C . Partial oxidation (75 %) of As(III) occurred in 15 days at 5°C , indicating that strain 2WW is a psychrotolerant As(III)-oxidizer. To date, partial As(III) oxidation has been demonstrated in *Sinorhizobium* strain M14 at 10°C (40 % of 375 mg L^{-1} As(III)) (Drewniak and Sklodowska 2007), in *Bordetella* sp. strain SPB-24 and in *Achromobacter* sp. strain SPB-31 at 8°C (20 % of 75 mg L^{-1} As(III)) (Bachate et al. 2012), and complete oxidation of 300 mg L^{-1} As(III) in *Polaromonas* strain GM1 at 4°C (Osborne et al. 2010). Temperature influenced the specific growth rate of strain 2WW regardless of the presence of As(III). Specific growth rate (h^{-1}) of cells growing with As(III) were 0.006, 0.13 and 0.23 at 5, 15 and 30°C , respectively; whereas it was 0.006, 0.16 and 0.24 for cells growing in absence of As(III) at 5, 15 and 30°C , respectively.

Table 1 Physiological characterization of *Aliihoeflea* sp. strain 2WW (NCCB 100463) and the *Aliihoeflea aestuarii* type strain N8^T (DSM 19536)

Characteristic	2WW	N8 ^T
Morphology	Rod-shaped	Rod-shaped
Gram reaction	Negative	Negative
Motility	+	+
Temperature range for growth (°C)	5–30	17–37
NaCl % (w/v) range for growth	0.1–8.0	0–10.0
pH range of growth	5.0–8.0	5.0–8.0
Nitrate reduction	–	–
Indole production	–	–
Arginine hydrolase	–	+
Urease	+	+
Oxidase	+	+
Catalase	+	+
Fermentation of glucose	–	–
Aesculin hydrolysis	–	–
PNPG hydrolysis	–	–
Gelatine hydrolysis	–	–
Assimilation of		
Glucose	+	+
Arabinose	w	+
Mannose	w	–
Mannitol	w	w
Citrate	–	–
Phenylacetic acid	–	–
Capric acid	–	–
N-acetyl-glucosamine	w	w
Gluconate	+	+
Adipic acid	+	+
Malate	+	+
Lactate	+	+
Yeast extract	+	+
MIC As(V)	18.75 g L ^{–1}	18.75 g L ^{–1}
MIC As(III)	0.375 g L ^{–1}	0.225 g L ^{–1}
Growth on*		
As(III)	–	–
S ⁰	–	–
(S ₂ O ₃) ^{2–}	–	–
Oxidation of As(III) [§]	+	–
Reduction of As(V) [§]	–	–

+ positive; – negative;

w weak reaction;

* chemolithoautotrophic condition; § heterotrophic condition

As(III) oxidation occurred between pH 5.0 and 8.0 at 48 h of incubation: it was complete at pH 6.0, 7.0 and 8.0, while it was partial (46 %) at pH 5.0, when bacterial growth was affected, similarly to *Bordetella* sp. and in *Achromobacter* sp. strains previously reported (Bachate et al. 2012).

As(III) was never oxidized to As(V) in abiotic controls, thus evidencing the role of the strain in arsenic conversion in Milli-Q water and in natural groundwater (data not shown).

These arsenic-related features of strain 2WW reported so far, make it a potential candidate to be

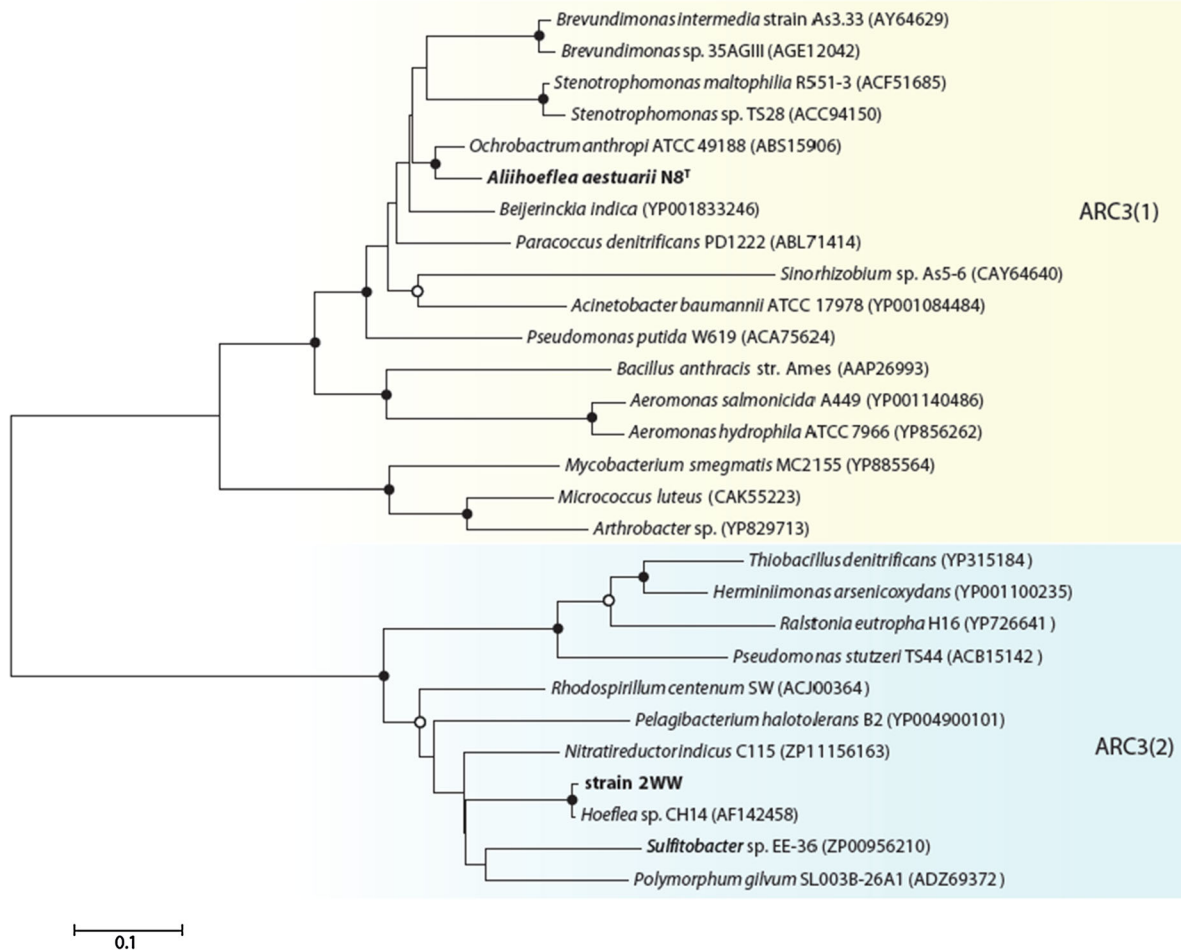


Fig. 1 Phylogenetic relationships of deduced amino acid sequences of arsenite efflux pump genes *ACR3(1)* and *ACR3(2)* retrieved from *Aliihoeflea* sp. strain 2WW and *Aliihoeflea aestuarii* strain N8^T (in bold). The evolutionary

history was inferred using the Neighbor-Joining method. Solid circles are bootstrap values between 75 and 100; the open circles are bootstrap values between 50 and 74 (out of 1000 iterations). The bar indicates 1 % sequence difference

used in bioremediation systems that couple biological oxidation and removal of arsenic by sorbent materials.

Arsenic genotyping of strains 2WW and N8^T

The presence of different operons encoding genes for arsenic transformation was assessed in the two strains. Strains 2WW and N8^T possessed different types of arsenite efflux pumps *ACR3(2)* and *ACR3(1)*, respectively, whereas the *arsB* gene was not detected. According to Castillo and Saier (2010), *ACR3*-type As(III) efflux pumps are present as footprints of *ars* operons in those strains lacking *arsB*. The deduced amino acid sequence of the *ACR3(2)* gene of strain

2WW was highly homologous (97 % positive) to arsenite transporter of *Hoeflea* sp. strain CH14 (acc. num. AFI42458) (Fig. 1). The deduced amino acid sequence of the *ACR3(1)* gene of strain N8^T had 93 % homology to arsenite transporter of *Ochrobactrum anthropi* ATCC 491 (acc. num. ABS15906) (Fig. 1). The phylogenetic analysis clearly separated the *ACR3* sequences into the two types: *ACR3(1)* of strain N8^T grouped with those of *Brevundimonas* and *Stenotrophomonas* species, whereas *ACR3(2)* of strain 2WW clustered together with those of other *Alphaproteobacteria* retrieved in deep seawaters and hot springs mud (Huo et al. 2012; Lai et al. 2012). Bacteria presenting arsenic operons or arsenic-related genetic footprints

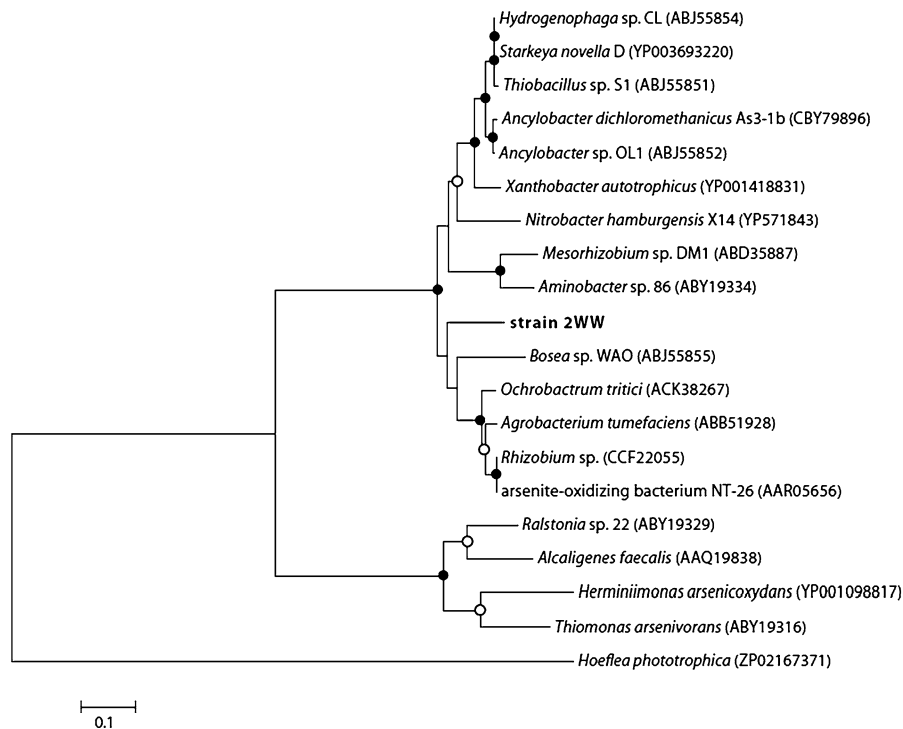


Fig. 2 Phylogenetic relationships deduced amino acid sequence of arsenite oxidase gene *aioA* of *Aliihoeflea* sp. strain 2WW (in **bold**). The evolutionary history was inferred using the Neighbor-Joining method. *Solid circles* are bootstrap values

between 75 and 100; the *open circles* are bootstrap values between 50 and 74 (out of 1000 iterations). The bar indicates 1 % sequence difference. The sequence of *Hoeftia phototrophica* (ZP02167371) was used as an outgroup

are normally retrieved either from contaminated and pristine environments (Cai et al. 2009), indicating the ancient origin of arsenic metabolisms from primordial cells.

The arsenate reductase gene *arsC* was not amplified in strain 2WW nor in N8^T, indicating that the ability of the strains to resist high As(V) concentrations (MIC of As(V) 18.75 g L⁻¹) was not related to the ability to reduce As(V) to As(III). For these microorganisms, alternative As(V) resistance mechanisms might rely on the absence of an efficient As(V) transporter (Bertin et al. 2011), or on arsenic sequestration in exopolysaccharides matrix as in *Herminiimonas arsenicoxydans* (Muller et al. 2007). On the contrary, the As(III) resistance level comparable for both strains (MIC of As(III) 0.375 and 0.225 g L⁻¹ for 2WW and N8^T strains respectively), could rely on extrusion of As(III) by *ACR3(2)* and *ACR3(1)*. Bacteria might have evolved As(III) resistance mechanisms before As(V) during the early stages of life, as As(III) is the major form of arsenic in reductive environments (Kruger et al. 2013), such as those prevalent during

early Earth. Our findings suggest that As(III) efflux pumps in *Aliihoeflea* spp. strains 2WW and N8^T are not related to As(V), but to As(III) resistance.

A gene fragment corresponding to *aioA* was found in strain 2WW in accordance with its ability to oxidize As(III) heterotrophically, but not in strain N8^T (Fig. 2). The deduced amino acid sequence was 89 % homologous to a large subunit of the arsenite oxidase of an As(III)-oxidizing bacterium (strain NT26, acc. num. AAR05656) and 88 % similar to *Ochrobactrum tritici* (acc. num. ACK38267) and 87 % similarity to that of *Agrobacterium tumefaciens* 52 (acc. num. ABB51928). Phylogenetic analysis of deduced amino acid sequences indicated that the sequence of strain 2WW clustered together with *Alphaproteobacteria* members of *Brucellaceae* and *Rhizobiaceae* families (Fig. 2). The close affiliation of *aioA* to arsenite oxidase of members of *Brucellaceae* and *Rhizobiaceae* families rather than to that of known *Phyllobacteriaceae* (i.e., *Mesorhizobium* and *Aminobacter*) could suggest the occurrence of horizontal gene transfer (HGT) among these bacteria. Lieutaud

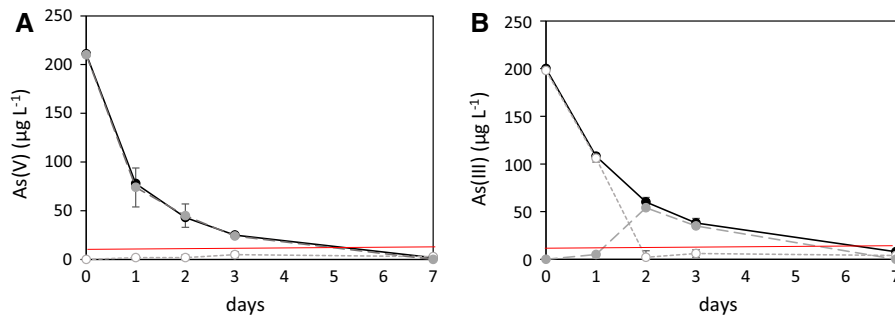


Fig. 3 Adsorption kinetics of *Pf*-ferritin in Milli-Q water supplemented with $200 \mu\text{g L}^{-1}$ As(III) (a) and in As-contaminated groundwater (b) at 15°C (mean $\pm$ std. dev.). The red

line indicates the maximum threshold of As allowed by the World Health Organization for drinking water ($10 \mu\text{g L}^{-1}$); filled circle total As, open circle As(III); grey circle As(V)

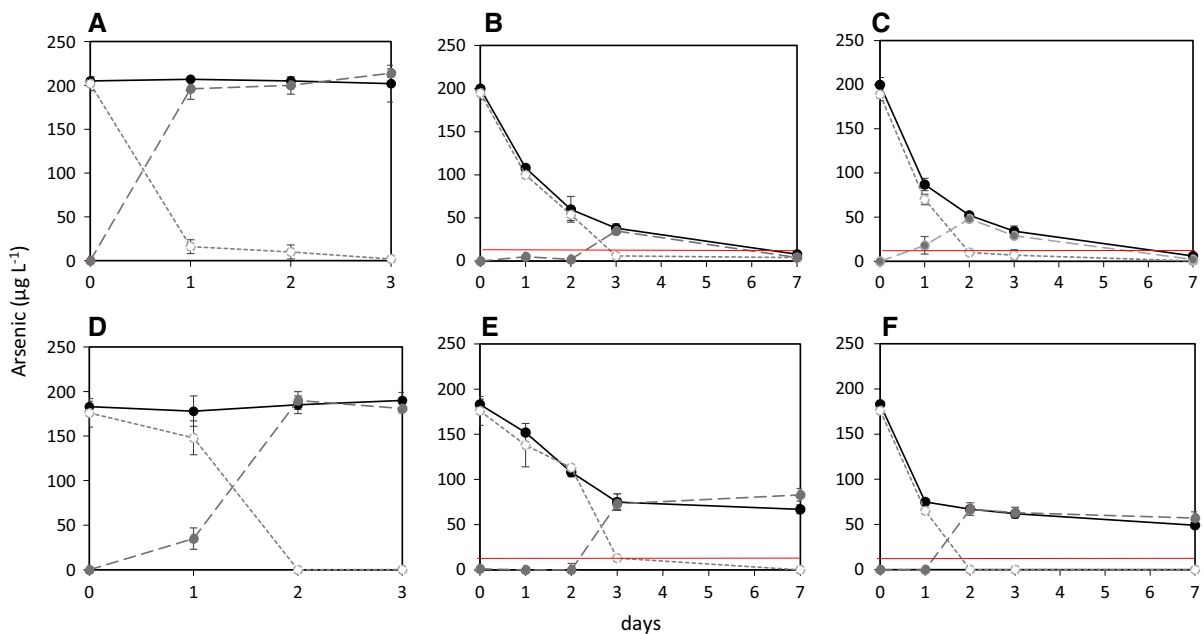


Fig. 4 Effect of *Pf*-ferritin on As(III) oxidation of resting cells of *Aliihoeflea* sp. 2WW strain in Milli-Q water supplemented with $200 \mu\text{g L}^{-1}$ As(III) (a, b and c) and in As-contaminated groundwater (d, e and f) at 15°C (mean $\pm$ std. dev.). The red

line indicates the maximum threshold of As allowed by the World Health Organization for drinking water ($10 \mu\text{g L}^{-1}$); filled circle total As, open circle As(III); grey circle As(V)

et al. (2010) proposed HGT in prokaryotic communities to explain the strong similarities of AioA proteins in *Ralstonia* spp. and in *Achromobacter* sp. strain SY8, suggesting that *Ralstonia* acquired the corresponding gene cluster from another betaproteobacterium. Subsequently, Heinrich-Salmeron et al. (2011) found that HGT of the *aioA* gene occurred between bacteria belonging to different classes or phyla, such as in *Agromyces* and *Bosea*, two unrelated bacteria belonging to *Actinobacteria* and *Alphaproteobacteria*,

respectively. The phylogenetic analysis of *aioA* gene of strain 2WW agrees with this hypothesis.

Kinetics of arsenic removal from water by *Pf*-ferritin

The arsenic adsorption of *Pf*-ferritin was tested in an artificial system, Milli-Q water, to which $200 \mu\text{g L}^{-1}$ As(III) or As(V) was added. *Pf*-ferritin was able to remove 96 % As(III) and 99 % As(V) from water after

7 days of incubation, showing higher affinity for As(V) than As(III) (Fig. 3). When As(III) was the initial As species in solution, As(V) was detected after 2 days of incubation, suggesting an abiotic oxidation of As(III). As(V) was adsorbed completely after 7 days. Our observations indicated that nano-iron loaded onto *Pf*-ferritin has As(III) oxidizing activity, in accordance with results previously reported by Katsoyiannis et al. (2008) related to zero-valent nano-iron.

Arsenic removal from water by a combined system of *Pf*-ferritin and bacterial cells

In the absence of *Pf*-ferritin, resting cells of strain 2WW oxidized almost 200 $\mu\text{g L}^{-1}$ As(III) in one day in Milli-Q water (Fig. 4a), whereas complete oxidation occurred in 2 days in groundwater (Fig. 4d). This indicated that the strain possessed the ability to convert As(III) to As(V), but not that the metalloid was adsorbing on cell surfaces or to accumulate it within the cell. Although adsorption mechanisms by arsenic resistant bacteria have been recently claimed as practical application for metalloid removal (Aryal and Liakopoulou-Kyriakides 2014), the application of strain 2WW can be envisaged only in conjunction with sorbent materials for arsenic removal from water.

Pf-ferritin removed up to 96 % of As in 7 days (8 $\mu\text{g L}^{-1}$ As left in solution) from Milli-Q water (Fig. 4b). The removal efficiency of *Pf*-ferritin was strongly reduced (64 % removal) when tested in natural groundwater, leaving 68 $\mu\text{g L}^{-1}$ arsenic in solution (Fig. 4e). As evidenced in the kinetics study in Milli-Q water, *Pf*-ferritin was able to completely oxidize As(III) to As(V) in groundwater within 3 days as indicated by the decrease of As(III) and increase of As(V).

In a combined system of *Pf*-ferritin and bacterial cells, the removal efficiency of *Pf*-ferritin in Milli-Q water was 97 versus 96 % for *Pf*-ferritin alone (Fig. 4c). However, in groundwater the presence of the As(III)-oxidizing strain 2WW significantly enhanced the removal efficiency (73 versus 64 %) (Fig. 4f). Nevertheless, the synergistic effect of the two components did not lower the As level below the WHO level (10 $\mu\text{g L}^{-1}$), leaving 50 $\mu\text{g L}^{-1}$ in solution. With the combined system, the rate of As(III) oxidation was increased in both Milli-Q water and natural groundwater. This suggested that the activity of As(III) oxidation carried out by the strain

Table 2 Total As and main coexisting ions ($\mu\text{g L}^{-1}$) in groundwater samples at successive incubation time in adsorption experiments with *Pf*-ferritin in the presence and in the absence of bacterial cells

	As			Fe			Mn			P							
	Day			Day			Day			Day							
	0	1	2	7	0	1	2	7	0	1	2	7					
<i>Pf</i> -ferritin	183 ^d	152 ^c	108 ^b	75 ^a	67 ^a	1229 ^c	1032 ^c	607 ^b	654 ^b	287 ^a	153	140	125	130	118	340 ^b	<10 ^a
<i>Pf</i> -ferritin + strain 2WW	183 ^c	75 ^{a,b}	67 ^{a,b}	62 ^{a,b}	49 ^{a,ab}	1229 ^d	752 ^{a,c}	389 ^{a,b}	369 ^{a,b}	221 ^{a,ab}	153 ^c	122 ^b	111 ^b	115 ^b	92 ^{a,ab}	340 ^b	<10 ^a

Asterisks indicate statistical significance (*t* Student, $P < 0.05$) from the experiments with sole *Pf*-ferritin

Lower case letters refer to comparison among sampling times for each experiment; values followed by the same letters denote those not significantly different in each row ($P < 0.05$)

moderately counterbalanced the competition between arsenic and other ions present in groundwater, mainly phosphates.

The efficiency of As removal can be affected by several factors. Among the operational conditions, dosage and size of sorbent and agitation rate must be carefully taken into account (Kao et al. 2013). Moreover, the removal efficiency can decrease due to the presence in solution of competing components such as oxy-anions or bacterial cells. The potential competition of oxy-anions present in groundwaters is documented in the literature (Lievremont et al. 2003, Corsini et al. 2014b), whereas the possibility that As(III) oxidizing bacteria have a negative effect on arsenic retention by sorbents is still debated (Kim et al. 2010; Huang et al. 2011). The sharp decrease of phosphates present in groundwater after 24 h incubation with *Pf*-ferritin (Table 2) suggested that the decrease of the arsenic removal from groundwater is possibly related to competitive interactions between other coexisting ions rather than to the presence of 2WW cells in solution.

Conclusions

This study demonstrates the ability of strain 2WW to oxidize As(III) in a wide range of temperature and pH, in Milli-Q water as well as natural groundwater. The combined system of bacterial cells and *Pf*-ferritin was successful in the removal of arsenic from groundwater. Unfortunately, the removal was not efficient enough to meet the threshold concentration of 10 µg L⁻¹ drinking water enforced by the WHO, probably due to competition of phosphates for adsorption sites. Different operational conditions are under investigation and will help to improve the effectiveness of the system in the arsenic removal.

Acknowledgments This research was supported by CARIPLO Foundation (Project 2010-2221) and by PRIN (Project 2010JBNLJ7_004). The authors thank Dr. D.Y. Sorokin and Dr. Emily D. Melton for valuable comments.

References

Achour AR, Bauda P, Billard P (2007) Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. Res Microbiol 158:128–137

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
- Aryal M, Liakopoulou-Kyriakides M (2014) Bioremoval of heavy metals by bacterial biomass. Environ Monit Assess 187:4173
- Bachate SP, Cavalca L, Andreoni V (2009) Arsenic-resistant bacteria isolated from agricultural soils of Bangladesh and characterization of arsenate-reducing strains. J Appl Microbiol 107:145–156
- Bachate SP, Khapare RM, Kodam KM (2012) Oxidation of arsenite by two β -proteobacteria isolated from soil. Appl Microbiol Biotechnol 93:2135–2145
- Bertin PN, Heinrich-Salmeron A, Pelletier E, Goulhen-Chollet F, Arsene-Plöetze F, Gallien S, Lauga B, Casiot C, Calteau A, Vallenet D, Bonnefoy V, Bruneel O, Chane-Woon-Ming B, Cleiss-Arnold J, Duran R, Elbaz-Poulhichet F, Fonknechten N, Giloteaux L, Halter D, Koechler S, Marchal M, Mornico D, Schaeffer C, Smith AAT, Van Dorselaer A, Weissenbach J, Medigue C, Le Paslier D (2011) Metabolic diversity among main microorganisms inside an arsenic-rich ecosystem revealed by meta- and proteo-genomics. ISME J 5:1735–1747
- Cai L, Liu G, Rensing C, Wang G (2009) Genes involved in arsenic transformation and resistance associated with different levels of arsenic contaminated soils. BMC Microbiol 9:4
- Castillo R, Saier MH Jr (2010) Functional promiscuity of homologues of the bacterial ArsA ATPases. Int J Microbiol. doi:10.1155/2010/187373
- Cavalca L, Corsini A, Zaccheo P, Andreoni V, Muyzer G (2013a) Microbial transformations of arsenic: perspectives for biological removal of arsenic from water. Future Microbiol 8:753–768
- Cavalca L, Corsini A, Andreoni V, Muyzer G (2013b) Draft genome sequence of the arsenite-oxidizing strain *Aliihoe-flea* sp 2WW isolated from arsenic-contaminated groundwater. GenomeA 1(6):e01072-13
- Chitpirom K, Akaracharanya A, Tanasupawat S, Leepipatpi-boon N, Kim K-W (2009) Isolation and characterization of arsenic resistant bacteria from tannery wastes and agricultural soils in Thailand. Ann Microbiol 59:649–656
- Corsini A, Zaccheo P, Muyzer G, Andreoni V, Cavalca L (2014a) Arsenic transforming abilities of groundwater bacteria and the combined use of *Aliihoe-flea* sp. strain 2WW and goethite in metalloid removal. J Hazard Mater 269:89–97
- Corsini A, Cavalca L, Muyzer G, Zaccheo P (2014b) Effectiveness of various sorbents and biological oxidation in the removal of arsenic species from groundwater. Environ Chem 11:558–565
- Drewniak L, Skłodowska A (2007) Isolation and characterization of a psychrotolerant arsenite-oxidizing bacterium from a gold mine in Złoty Stok. Poland. Adv Mater Res 20–21:575
- Fan H, Su C, Wang Y, Yao J, Zhao K, Wang Y, Wang G (2008) Sedimentary arsenite-oxidizing and arsenate-reducing bacteria associated with high arsenic groundwater from Shanyin, Northwestern China. J Appl Microbiol 105:529–539
- Gosh S, Sar P (2013) Identification and characterization of metabolic properties of bacterial populations recovered

- from arsenic contaminated ground water of North East India (Assam). *Water Res* 47:6992–7005
- Heinrich-Salmeron A, Cordi A, Brochier-Armanet C, Halter D, Pagnout C, Abbaszadeh-Fard E, Montaut D, Seby F, Bertin PN, Bauda P, Arsene-Ploetze F (2011) Unsuspected diversity of arsenite-oxidizing bacteria as revealed by widespread distribution of the *aoxB* gene in Prokaryotes. *Appl Environ Microbiol* 77:4685–4692
- Huang J-H, Elzinga EJ, Brechbuehl Y, Voegelin A, Kretzschmar R (2011) Impacts of *Shewanella putrefaciens* strain CN-32 cells and extracellular polymeric substances on the sorption of As(V) and As(III) on Fe(III)-(hydr)oxides. *Environ Sci Technol* 45:2804–2810
- Huo YY, Cheng H, Han XF, Jiang XW, Sun C, Zhang XQ, Zhu XF, Liu YF, Li PF, Ni PX, Wu M (2012) Complete genome sequence of *Pelagibacterium halotolerans* B2(T). *J Bacteriol* 194:197–198
- Ito A, Miura JJ, Ishikawa N, Teruyuki U (2012) Biological oxidation of arsenite in synthetic ground water using immobilised bacteria. *Water Res* 46:4825–4831
- Jackson CR, Jackson EF, Dugas SL, Gamble K, Williams SE (2003) Microbial transformations of As(III) and As(V) in natural environments. *Recent Res Dev Microbiol* 7:103–118
- Jacobs JF, Hasan MH, Paik KH, Hagen WR, van Loosdrecht MCM (2010) Development of a bionanotechnological phosphate removal system with thermostable ferritin. *Biotechnol Bioeng* 105:918
- Kao A-C, Chu Y-J, Hsu F-L, Liao VH-C (2013) Removal of arsenic from groundwater by using a native isolated arsenite-oxidizing bacterium. *J Contamin Hydrol* 155:1–8
- Katsoyiannis IA, Ruettimann T, Hug SJ (2008) pH dependence of Fenton reagent generation and As(III) oxidation and removal by corrosion of zero valent iron in aerated water. *Environ Sci Technol* 42:7424–7430
- Kim YT, Yoon H, Yoon C, Woo NC (2007) An assessment of sampling, preservation, and analytical procedures for arsenic speciation in potentially contaminated waters. *Environ Geochem Health* 29:337–346
- Kim HC, Lee CG, Park JA, Kim SB (2010) Arsenic removal from water using iron-impregnated granular activated carbon in the presence of bacteria. *J Environ Sci Health Part A Tox Hazard Subst Environ Eng* 45:177–182
- Kruger MC, Bertin PN, Heipieper HJ, Arsène-Ploetze F (2013) Bacterial metabolism of environmental arsenic-mechanisms and biotechnological applications. *Appl Microbiol Biotechnol* 97:3827–3841
- Lai Q, Li G, Yu Z, Shao Z (2012) Genome sequence of *Nitratireductor indicus* type strain C115. *J Bacteriol* 194:6990
- Liao VH, Chu YJ, Su YC, Hsiao SY, Wei CC, Liu CW, Liao CM, Shen WC, Chang FJ (2011) Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan. *J Contam Hydrol* 123:20–29
- Lieutaud A, van Lis R, Duval S, Capowiez L, Muller D, Lebrun R, Lignon S, Fardeau M-L, Lett M-C, Nitschke W, Schoepp-Cothenet B (2010) Arsenite oxidase from *Ralstonia* sp. 22. *J Biol Chem* 285:20433–20441
- Lièvremon D, N'negue MA, Behra PH, Lett M-C (2003) Biological oxidation of arsenite: batch reactor experiments in presence of kutnahorite and chabazite. *Chemosphere* 51:419
- Lugtu RT, Choi SC, Oh YS (2009) Arsenite oxidation by a facultative chemolithotrophic bacterium SDB1 isolated from mine tailing. *J Microbiol* 47:686–692
- Mondal P, Majumder CB, Mohanty B (2006) Laboratory based approaches for arsenic remediation from contaminated water: recent developments. *J Hazard Mater* 137:464–479
- Muller D, Médigue C, Koechler S, Barbe V, Barakat M, Talla E, Bonnefoy V, Krin E, Arsène-Ploetze F, Carapito C, Chandler M, Cournoyer B, Cruveiller S, Dossat C, Duval S, Heymann M, Leize E, Lieutaud A, Lièvremon D, Makita Y, Mangenot S, Nitschke W, Ortet P, Perdial N, Schoepp B, Siguier P, Simeonova DD, Rouy Z, Segurens B, Turlin E, Vaellenet D, Dorsselaer AV, Weiss S, Weissenbach J, Lett M-C, Danchin A, Bertin PN (2007) A tale of two oxidation states: bacterial colonization of arsenic-rich environments. *PLoS Genet* 3:0518–0530
- Oremland RS, Stolz JF (2003) The ecology of arsenic. *Science* 300:939–944
- Osborne TH, Jamieson HE, Hudson-Edwards KA, Nordstrom DK, Walker SR, Ward S, Santini JM (2010) Microbial oxidation of arsenite in a subarctic environment: diversity of arsenite oxidase genes and identification of a psychrotolerant arsenite oxidizer. *BMC Microbiol* 10:205–212
- Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell Scientific, Oxford
- Quéménéur M, Heinrich-Salmeron A, Muller D, Lièvremon D, Jauzein M, Bertin PN, Garrido F, Joulain C (2008) Diversity surveys and evolutionary relationships of *aoxB* genes in aerobic arsenite-oxidizing bacteria. *Appl Environ Microbiol* 74:4567–4573
- Reise K (2002) Sediment mediated species interactions in coastal waters. *J Sea Res* 48:127–141
- Roh SW, Kim KH, Nam YD, Chang HW, Kim MS, Shin KS, Yoon JH, Oh HM, Bae JW (2008) *Aliihoeflea aestuarii* gen. nov., sp. nov. a novel bacterium isolated from tidal flat sediment. *J Microbiol* 46:594–598
- Smedley PL, Kinniburgh DG (2002) A review of the source, behaviour and distribution of arsenic in natural waters. *Appl Geochem* 17:517–568
- Stolz J, Basu P, Oremland RS (2002) Microbial transformation of elements: the case of arsenic and selenium. *Int Microbiol* 5:201–207
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) Software Version 4.0. *Mol Biol Evol* 24:1596–1599
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA for phylogenetic study. *J Bacteriol* 173:697–703
- WHO (2001) Arsenic in drinking water. Fact sheet number 210. Available at <http://www.who.int/inf-fs/en/fact210.html> Accessed October 2013