Rhizosphere colonization and arsenic translocation in sunflower (Helianthus annuus L.) by arsenate reducing Alcaligenes sp. strain Dhal-L

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Rhizosphere colonization and arsenic translocation in sunflower (Helianthus annuus L.) by arsenate reducing Alcaligenes sp. strain Dhal-L

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Received: 18 February 2013 / Accepted: 21 April 2013 © Springer Science+Business Media Dordrecht 2013

Abstract In the present study, six arsenic-resistant strains previously isolated were tested for their plant growth promoting characteristics and heavy metal resistance, in order to choose one model strain as an inoculum for sunflower plants in pot experiments. The aim was to investigate the effect of arsenic-resistant strain on sunflower growth and on arsenic uptake from arsenic contaminated soil. Based on plant growth promoting characteristics and heavy metal resistance, Alcaligenes sp. strain Dhal-L was chosen as an inoculum. Beside the ability to reduce arsenate to arsenite via an Ars operon, the strain exhibited 1-amino-cyclopropane-1-carboxylic acid deaminase activity and it was also able to produce siderophore and indole acetic acid. Pot experiments were conducted with an agricultural soil contaminated with arsenic (214 mg kg\(^{-1}\)). A real time PCR method was set up based on the quantification of ACR3(2) type of arsenite efflux pump carried by Alcaligenes sp. strain Dhal-L, in order to monitor presence and colonisation of the strain in the bulk and rhizospheric soil. As a result of strain inoculation, arsenic uptake by plants was increased by 53 %, whereas ACR3(2) gene copy number in rhizospheric soil was 100 times higher in inoculated than in control pots, indicating the colonisation of strain. The results indicated that the presence of arsenate reducing strains in the rhizosphere of sunflower influences arsenic mobilization and promotes arsenic uptake by plant.

Keywords Arsenic · ACR3 · q-PCR · PGP · Sunflower · Arsenic uptake

Abbreviations

As(III) Arsenite
As(V) Arsenate
ACC 1-Amino-cyclopropane-1-carboxylic acid
PGP Plant growth-promoting
q-PCR Real time quantitative PCR
MIC Minimum inhibitory concentration
DF Dworkin and Foster
LB Luria–Bertani
TSB Tryptic soy broth
TMMG Tris mineral medium supplemented with gluconate
ACCD ACC deaminase
THB Total heterotrophic bacteria
ARB Arsenic-resistant bacteria
ICP-MS Inductively coupled plasma-mass spectrometry
TSA Tryptic soy agar

Introduction

Arsenic mainly occurs in the environment in two inorganic forms: arsenite \([\text{As(III)}]\) and arsenate \([\text{As(V)}]\). In soil, the metalloid can be present as soluble compound or associated with iron and manganese oxyhydroxides and its toxicity risk depends on rate of transfer from soil compartments to soil solution, plants, groundwater, and more generally, to the food chain. Microbial detoxification is based on the expression of the \(\text{Ars}\) operon (Mukhopadhyay et al. 2002):
once As(V) is reduced by arsenate reductase (ArsC), As(III) is pumped out of the cell via transmembrane efflux pump (ArsB or ACR3 types). Two families of transmembrane efflux pumps are known: the ArsB and the ACR3 family. The ACR3 type is more widespread in nature being found in prokaryotes, animals and plants (Fu et al. 2009), remarkably in arsenic hyper accumulating plant Pteris vittata. ACR3(2) in particular, has been found in Alphaproteobacteria (Achour et al. 2007) and in Betaproteobacteria (Pohlmann et al. 2006; Bachate et al. 2009). In plants, As(V) acts as an analogue of phosphate and it is transported across the plasma membrane via phosphate co-transport systems, whereas As(III) enters non-specifically through aquaglyceroporins. Some plants respond to biological—and environmental—stresses by synthesizing “stress” ethylene from the precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) (Morgan and Drew 1997). Plant growth-promoting (PGP) bacteria that produce the enzyme ACC deaminase cleave ACC and lower the level of stress-induced ethylene, facilitating the formation of longer roots in plants growing in the presence of heavy metals (Grichko et al. 2000), thus causing an enhanced uptake of inorganic contaminants through modification of root architecture and of root uptake system of plant (Arshad et al. 2007). Recently it was demonstrated that the presence of arsenic-resistant bacteria (ARB) in rhizosphere influence arsenic content in plants (Xiong et al. 2010). Yang et al. (2012) found that the inoculation of ARB increased the accumulation efficiency of P. vittata (L.) by 13–110 %. These findings indicate that arsenic accumulating plants in a synergistic use with ARB could represent a promising approach for remediation of arsenic contaminated soils. Although sunflower (Helianthus annuus L.) has been proposed for phyto-extraction purposes because it uptakes relatively high concentrations of metals and can have high biomass yield (Marchiol et al. 2007), literature is scarce about its ability to translocate arsenic. Arsenic uptake by food crops should not exceed the limit of 1 mg kg\(^{-1}\) (Bhattacharya et al. 2010) and health hazards for food poisoning might be present when crops grow in soils with arsenic content higher than 200 mg kg\(^{-1}\) (Warren et al. 2003).

In the present study, pot experiments with sunflower plants inoculated with an arsenic-resistant strain were set up in order to test the mobility of arsenic in relation to the presence of As(V) reducing bacteria. Sunflower was chosen because it is a well established crop in temperate zones and it can give rise to non-food products such as oil for industrial use.

In order to select a microorganism to use as inoculum for sunflower seeds, six isolates from an agricultural soil contaminated by arsenic (Bachate et al. 2009) were tested for PGP characteristics and arsenic and other heavy metal resistance. Alcaligenes sp. strain Dhal-L resulted to be the best candidate since it had several PGP characteristics and it was able to reduce As(V) to As(III) in heterothrophic conditions via an ArsC and to transport As(III) out of the cell via an ACR3(2) type As(III) efflux pump (Bachate et al. 2009).

The effect of Alcaligenes sp. strain Dhal-L on plant growth and arsenic uptake from soil was evaluated with the objective to evidence the role of As(V) reducing bacteria in the process of metalloid mobilization in rhizospheric environment. ACR3(2) was used as genetic marker to monitor root colonisation in real time quantitative PCR experiments (q-PCR) specifically developed.

### Materials and methods

#### Bacterial strains and growth conditions

Six arsenic-resistant strains previously isolated from an arsenic contaminated soil were tested for PGP characteristics and for the minimum inhibitory concentration (MIC) of different heavy metals. The strains, identified on the basis of their 16S rRNA sequence, belonged to Arthrobacter sp. strain Dhal-A, Alcaligenes sp. strain Dhal-L, Alcaligenes sp. strain Dhal-N, Oceanomonas doudoroffii Dhal-Rw, Variovorax paradoxus Dhal-F and Bacillus megaterium Rice-A. Dworkin and Foster (DF) mineral medium (Dworkin and Foster 1958), Luria–Bertani (LB) broth or tryptic soy broth 1:10 diluted (TSB 0.1×, Difco) were used as liquid growth media for bacterial strains as and when mentioned. When required, 1.5 % (w/v) NaCl was added to the liquid medium to prepare the solid medium. MIC was determined in tris mineral medium at low phosphate content (0.12 g L\(^{-1}\) of Na\(_2\)HPO\(_4\)) to avoid heavy metal precipitation (Sadouk and Mergeay 1993), supplemented with 0.6 % (w/v) gluconate (TMMG) and containing different concentrations of heavy metals: NiCl\(_2\), ZnSO\(_4\), CuSO\(_4\), CdSO\(_4\) and HgCl\(_2\). For inoculation, the isolated strains were grown in TSB 0.1× medium at 30 °C. Cells in the exponential phase were collected by centrifugation at 6,000 rpm for 10 min, washed with sterile distilled water and centrifuged again. Bacterial inoculum was prepared by suspending cells in sterile distilled water to get an inoculum density of ca 10\(^7\) cells mL\(^{-1}\). One mL of inoculum was seeded into duplicate 100-mL vials containing 19 mL of TMMG supplemented with the appropriate amounts of different heavy metals. After inoculation, vials were incubated at 30 °C for 5 days and the growth was measured spectrophotometrically at 600 nm. The strains were maintained in glycerol stocks at −70 °C.
Determination of PGP characteristics

**ACC deaminase activity**

The ability to use ACC as sole nitrogen (N) source is a consequence of enzymatic activity of ACC deaminase (ACCD). The bacteria were first cultured in TSB 0.1× and then transferred into tubes with DF medium containing 3.0 mmol L⁻¹ ACC instead of (NH₄)₂SO₄ as N source. Solution of ACC (0.5 M) (Sigma-Aldrich) was filter sterilized (0.2 μm) and the aliquots of filtrate were frozen at −20 °C. Prior to inoculation, the ACC solution was thawed and appropriately added to sterile DF medium. Following inoculation with the appropriate strain, the cultures were incubated at 30 °C on a rotary shaker at 200 rpm for 48 h. Growth was positive when the cultures developed turbidity. The ability of a strain to utilize ACC was verified by inoculating the strain in control tubes containing DF medium without any N source, and incubating the tubes in the mentioned conditions for 10 days. The absence of growth confirmed the utilization of ACC as N source.

In order to evaluate the effect of As(V) on ACCD activity, bacterial strains were grown in DF medium supplemented with 3 mmol L⁻¹ ACC in the presence and absence of 2 mmol L⁻¹ of As(V) added as Na₂HAsO₄·7H₂O solution. Growth was determined by measuring the OD at 600 nm after incubation at 30 °C for 72 h. The ACCD activity of cell-free extracts was determined by monitoring the amount of α-ketobutyrate generated by the hydrolysis of ACC (Saleh and Glick 2001), by comparing the absorbance (540 nm) of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 mmol L⁻¹ according to Penrose and Glick (2003) and Dell’Amico et al. (2008). The mixtures containing no cell suspension or no ACC were used as controls. The effect of arsenic on ACCD activity was evaluated both, by comparing the ACCD activity of the cell-free extract obtained from cells grown in the presence or absence of As(V) and by adding As(V) to the ACCD reaction buffer in the assay carried out on the cell-free extract obtained from cells grown in the presence of As(V). Total protein content of cell suspensions and of toluene-disrupted cells was determined by the method of Bradford (1976).

**Indole acetic acid production**

The bacteria were cultured for 4 days in flasks containing 20 mL of DF medium supplemented with 0.5 mg mL⁻¹ of tryptophane. After incubation, cell suspension (1 mL) was transferred into a tube and mixed vigorously with 2 mL of Salkowski’s reagent (150 mL of concentrated H₂SO₄, 250 mL of distilled H₂O, 7.5 mL of 0.5 M FeCl₃·6H₂O) (Gordon and Weber 1951) and allowed to stand at room temperature for 20 min. Development of pink colour in the cell suspensions indicated positive reaction for indole acetic acid production.

**Synthesis of siderophores**

Siderophore secretion by strains was qualitatively detected by the conventional method of Schwyn and Neilands (1987) using blue agar plates containing the ternary complex chrome azurol S/iron(III)/hexadecyltrimethylammonium bromide as an indicator. Change in the dye colour from blue to orange indicated production of siderophore.

**Seed inoculation and pot experiments**

The soil used in pot experiments was an agricultural soil from Scarlino (Toscany, Italy). According to Cozzolino et al. (2010), it was moderately alkaline (pH 8.1), with a medium content of organic matter (11.5 g kg⁻¹) and a low content of available P (7.4 mg kg⁻¹). The site is heavily polluted by arsenic; the cause of such pollution is mainly the use of such soil as a sink of residues from mining extraction of pyrite associated with arsenopyrite, carried out in the last century. Total arsenic content of soil was 214 mg kg⁻¹; 181 mg kg⁻¹ was associated with well crystallised Fe hydroxides, 25 mg kg⁻¹ was specifically sorbed and 0.25 mg kg⁻¹ was present as labile fraction. The count of total heterotrophic bacteria (THB) and ARB present in the soil at the sampling time was 8.07 × 10⁹ (±1.26) and 3.37 × 10⁶ (±0.46) CFU g⁻¹ soil d.w., respectively (Cavalca et al. 2010).

The bacterium used as inoculum was *Alcaligenes* sp. strain Dhal-L, isolated from a lentil grown field. It is able to reduce 2 mM As(V) to As(III) completely in 72 h, which is transported out of the cell via an ACR3(2) type efflux pump (Bachate et al. 2009).

Seeds were treated according to Dell’Amico et al. (2008) prior to inoculation with *Alcaligenes* sp. strain Dh-al-L cell suspension. Seeds of *H. annuus* were washed in 70 % (v/v) ethanol for 1 min, rinsed with sterile water and put into a solution of sodium hypochlorite (1 % v/v) for 3 min. Finally, they were washed in copious amount of sterile distilled water. Seed sterility was verified by incubating 10 seeds onto LB agar plates at 30 °C for 10 days and observing bacterial growth. Bacterial cell suspension was prepared by growing *Alcaligenes* sp. strain Dh-al-L in 200 mL DF medium containing 3 mmol L⁻¹ ACC, as the ACCD is an inducible enzyme (Grichko et al. 2000). The cells were harvested by centrifugation (11,000×g for 30 min) and suspended in CaSO₄ until final OD (600 nm) of 0.35 was achieved (i.e., about 10⁹ CFU mL⁻¹). For inoculation, sterile seeds were soaked in 40 mL bacterial suspension and gently stirred in the dark at room temperature for 16 h before sowing.
temperature for 2 h, after which they were removed from the suspension by using sterile pliers and sown. Pot experiments were set up in triplicate using 250 g of soil (sieved using 2 mm mesh size). Five pots were used for each treatment: inoculated and control seeds, and three seeds were sown in each pot. Pots were placed in growth chamber with a 16/18 h photoperiod, 25/18 °C temperature, UR 80/60 %.

Plants (12 for each treatment) were carefully removed from pots at 0, 27 and 50 days. Two soil fractions were collected from root surrounding: the bulk fraction loosely adherent to roots was obtained by washing the roots extensively with water, and the rhizosphere fraction strongly adherent to roots was obtained by manually shaking the roots. The collected soil was dried at 80 °C in an oven and sieved using 2 mm mesh size. Five pots were used for each treatment: inoculated and control seeds, and three sets of pots were prepared from sodium arsenite (NaAsO$_2$) (Sigma-Aldrich). Cells were grown in TMMG medium for 24 h at 30 °C (Chicago). The suspension by using sterile pliers and sown. Pot experiments were set up in triplicate using 250 g of soil (sieved using 2 mm mesh size). Five pots were used for each treatment: inoculated and control seeds, and three sets of pots were prepared from sodium arsenite (NaAsO$_2$) (Sigma-Aldrich). Cells were grown in TMMG medium for 24 h at 30 °C (Chicago).

Arsenic content in plant biomass

To determine the total arsenic content in shoot biomass, samples (0.2 g) were digested in a mixture of concentrated HNO$_3$ and HClO$_4$ (4:1, v/v) (Chen et al. 2004). After digestion, the volume of each sample was adjusted to 20 mL using double deionized water. The arsenic content was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Agilent Technologies). Standards of arsenic for concentrations ranging from 0 to 1 mg L$^{-1}$ were prepared from sodium arsenite (NaAsO$_2$) (Sigma-Aldrich) solution. Analytical data were compared by applying t-Student test using SPSS version 17.0 (SPSS Inc., Chicago).

Microbiological analysis

The number of THB and ARB in soil was determined by conventional plating techniques on 1:10 diluted tryptic soy agar (TSA 0.1×) in the absence or in the presence of 15 mmol L$^{-1}$ of As(V).

DNA extraction

DNA extraction from Alcaligenes sp. Dhal-L was performed by UltraClean Microbial DNA Isolation kit (MO BIO, USA). Cells were grown in TMMG medium for 24 h at 30 °C. The DNA was extracted from approximately 3.0 × 10$^8$ CFU mL$^{-1}$. At the different incubation times, DNA was extracted from bulk and rhizospheric soil (0.5 g) by using PowerSoil DNA Isolation Kit (MO BIO, USA). After the quantification of DNA concentration by determining OD$_{260nm}$ in a SmartSpec 3000 spectrophotometer (Bio-Rad), each soil DNA was used for quantitative real-time PCR (q-PCR) experiments to quantify 16S rRNA and ACR3(2) genes, in order to monitor the presence and the colonisation of Alcaligenes sp. Dhal-L.

Primer design for As(III) efflux pump ACR3(2) gene

In order to target specifically ACR3(2) gene coding for As(III) efflux pump of Alcaligenes sp. Dhal-L, PCR primers for SYBR green q-PCR were designed using Primer3 software (Rozen and Skaletsky 2002). Primers were designated as: acr475F (5’-AGCTCGGTCTTTATTCCA TC-3’) and acr611R (5’-GTTCAGGAACACTGGATC A-3’). Primers were designed by using the nucleotide sequence of ACR3(2) gene of Alcaligenes sp. Dhal-L (accession number AM983535), previously detected in the strain (Bachate et al. 2009). Specificity of the primer pair was tested by q-PCR (conditions indicated below) using DNA extracted from arsenic-resistant Gram-positive and Gram-negative strains previously isolated from Bangladesh soil (Bachate et al. 2009) and from Alcaligenes sp. Dhal-L as a positive control. PCR amplification gave an attended 136 bp amplicon in Alcaligenes sp. Dhal-L, only. Sequence analysis of the fragment was performed using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) with the respective primers. The forward and the reverse samples were run on a 310A sequence analyzer (Applied Biosystems). Sequences were compared with the entire GenBank/EMBL nucleotide and amino acid databases using the BlastN and BlastX query programs (http://www.ebi.ac.uk/Tools/blastall/index.html). Sequences were identical to ACR3(2) of Alcaligenes sp. strain Dhal-L, thus confirming that the primers specifically amplified the target gene of the strain.

Quantitative real-time PCR of ACR3(2) and 16S rRNA gene

q-PCR experiment was conducted in MJ Mini cycler (Bio-Rad). The reactions were set up in 20 μL with 1 × SsoFast EvaGreen Supermix (Bio-Rad). Two q-PCR reactions were separately set up: the first with primer pair acr475F/611R, the second with primer pair BACT1369F/PROK1492R (0.2 μmol L$^{-1}$ each). Primer pair BACT1369F (5’-CG GTGAATACGTTCYCGG-3’) and PROK1492R (5’-GGTACCTTGTTCGCACCTT-3’) targeting a 123 bp region of 16S rRNA gene were developed by Suzuki et al. (2000).

Real-time PCR conditions were as follows: 95 °C for 3 min, 40 cycles of 95 °C for 15 s (denaturation), 55 °C for 15 s (annealing), and 72 °C for 15 s (elongation). Sterile milliQ water was used as no template control (NTC).
instead of template DNA. At the end of the q-PCR a dissociation curve with melting temperature (Tm) of the amplified products was generated. The conditions were as follows: 95 °C for 1 min, 65 °C for 1 min and 60 cycles of 65 °C for 1 s with increase to 95 °C by increments of 0.5 °C s⁻¹. The curves obtained were used to ensure PCR product specificity by observation of a single melting peak. The single PCR product of target DNA was analyzed by using 2 % agarose gel electrophoresis. The gel was visualized under a UV transilluminator (Bio-Rad) after staining with 0.5 μg mL⁻¹ ethidium bromide.

Calibration curve

The concentration of standard DNA was measured by using a spectrophotometer and agarose gels. The gene copy number was calculated as follows:

\[
\text{Copy number (mmol/μL) = DNA concentration (g/μL) / average genome size (bp)} \\
\times 660^a \times A^b,
\]

where 660^a = average molecular weight of one base pair and A^b = Avogadro’s number = 6.02 × 10^23 molecules/mol.

Serial dilutions of total DNA of \textit{Alcaligenes} sp. Dhal-L were prepared to obtain final concentrations of 1.2, 2.4 × 10⁻¹, 4.8 × 10⁻², 9.6 × 10⁻³ and 1.9 × 10⁻³ ng corresponding to 1.6 × 10⁵, 3.2 × 10⁴, 6.4 × 10³, 1.3 × 10², 2.5 × 10¹ gene copy number, respectively. Each dilution was run in triplicate to generate the calibration curve of both 16S rRNA and ACR3(2) gene. Sterile MilliQ water was used as NTC. q-PCR reactions were performed as described above using P1369F/1492R or acr475F/611R primers. C_T (cycle threshold) value, which is defined as the cycle number crossing the threshold point, was calculated automatically by the iCycler software (Version 3, Bio-Rad). The calibration curve was obtained by plotting the C_T value versus the logarithm of starting concentration of template DNA. The amplification efficiency (E) was calculated from the slope of the standard curve using the equation E = [10^(-1/slope) - 1].

Results

Potential PGP and heavy metals resistance characteristics of bacterial strains

Arsenic-resistant isolates were tested for PGP characteristics and resistance to different heavy metals (Table 1). Strains \textit{Alcaligenes} sp. Dhal-L, \textit{O. doudoroffii} Dhal-Rw, \textit{V. paradoxus} Dhal-F and \textit{B. megaterium} Rice-A produced indole acetic acid. Strains Dhal-L, Dhal-N and Dhal-Rw were positive for siderophore production while \textit{Alcaligenes} sp. Dhal-L and Dhal-N were positive for ACC deaminase assay and their specific ACCD activities were 0.33 and 0.23 μmol hr⁻¹ μg⁻¹ of protein, respectively. Moreover, ACCD-specific activity of cell-free extract of these strains was not affected by the presence of 15 mM As(V). \textit{Alcaligenes} sp. Dhal-L and Dhal N were highly resistant to arsenic (MIC, 300 mmol L⁻¹ for As(V) and 70 mmol L⁻¹ for As(III)), nickel (MIC, 6 mmol L⁻¹) and zinc (MIC, 5 mmol L⁻¹). Isolate Dhal-Rw, although resisted to very high concentration of As(V), was sensitive to As(III) and to other heavy metals tested. \textit{Arthrobacter} sp. Dhal-L showed high resistance to all the heavy metals tested but did not show any PGP characteristics. Therefore, based on PGP and heavy metal resistance characteristics, \textit{Alcaligenes} sp. strain Dhal-L was chosen as inoculum for arsenic uptake experiment in association with sunflower plants.

Effect of \textit{Alcaligenes} sp. strain Dhal-L on arsenic uptake by plant

In sunflower plants inoculated with \textit{Alcaligenes} sp. strain Dhal-L the arsenic content was 1.18 and 1.96 mg kg⁻¹ d.w. biomass after 27 and 50 days of incubation, respectively, and it was significantly higher than in control plants (Table 2). There was increase in the plant biomass from 27 to 50 days and shoot biomass in inoculated plant was significantly higher after 50 days. Toxicity effects (i.e. appearance of chlorosis) were not recorded in plants during the experiment. Although the strain possessed an in vitro ACC-deaminase activity, it did not promote sunflower growth but possibly protected plants from suffering.

q-PCR primer design and specificity of amplification

To confirm the specificity of the designed primers acr475F and 611R for amplification of ACR3(2) of the inoculated strain, PCR reactions were performed on total DNA of \textit{Alcaligenes} sp. Dhal-L and of \textit{O. doudoroffii} Dhal-Rw, which also carries ACR3(2) gene (Bachate et al. 2009). An expected 136 bp fragment was obtained exclusively in strain Dhal-L. Sequence analysis of the PCR fragment showed 100 % homology with an internal region of the ACR3(2) gene of Dhal-L (accession number AM983535), thus confirming the specificity of the primers.

When tested by q-PCR using \textit{Alcaligenes} sp Dhal-L strain as template, dissociation curves produced a single melting peak with a T_m of 89 °C (Fig. 1), thus indicating the presence of a single amplicon. Additionally, dissociation curves produced by using soil DNA extracts contained only one peak corresponding to the amplified fragment in the standards, which confirmed the reaction specificity.
Furthermore, primers specificity was confirmed by the presence of a single band in agarose gel electrophoresis of q-PCR products (data not shown).

Standard curve performance in q-PCR of 16S rRNA gene and ACR3(2)

Standard curve was established from serial dilutions of Alcaligenes sp. Dhal-L DNA for both genes, based on the assumption that a single-copy of ACR3(2) is present in the closest sequenced genome of Cupriavidus metallidurans (Janssen et al. 2010). The standard curve of 16S rRNA gene was linear over four order of magnitude ($r^2 = 99\%$) from $10^2$ to $10^5$ gene copy numbers per reaction. The slope of the curve remained consistent between different runs and q-PCR efficiency of approximately 100% was obtained in each run (Fig. 2a). Similarly, the standard curve of ACR3(2) gene was linear over four order of magnitude ($r^2 = 95\%$) from $10^2$ to $10^5$ gene copy numbers per reaction and of the curve remained consistent between different runs with q-PCR efficiencies of approximately 80% (Fig. 2b).

In situ monitoring of Alcaligenes sp. Dhal-L

At the initial time (0 day), copy number of 16S rRNA gene in soil was $3.4 \times 10^5$ (±0.77). In the bulk soil of the control pots, it increased to $2.2 \times 10^6$ (±0.25) after 27 days and decreased to $6.5 \times 10^5$ (±0.70) after 50 days. In the bulk soil of the inoculated pots 16S rRNA gene copy number was higher: $3.5 \times 10^6$ (±0.32) after 27 days and $2.0 \times 10^6$ (±0.18) after 50 days. Viable cell counts of THB of the bulk soil did not vary significantly after 27 days of incubation in control versus inoculated pots, being $5.25 \times 10^6$ (±0.77) and $5.60 \times 10^6$ (±0.94) CFU g$^{-1}$ soil d.w., respectively.

In rhizospheric soil, 16S rRNA gene copy number increased after 27 and 50 days, both in control and inoculated pots (Fig. 3). A similar trend was observed for the THB counts (Fig. 4a).

Signal of ACR3(2) gene was detected in the soil at concentration $1.5 \times 10^{-4}$ ng corresponding to $1.9 \times 10^4$ (±0.60) gene copy g$^{-1}$ of soil. This indicates the presence

### Table 1

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>PGP characteristic</th>
<th>MIC (mmol L$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IAA production</td>
<td>Siderophore</td>
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<tr>
<td></td>
<td></td>
<td>production</td>
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<tr>
<td>Arthrobacter sp. Dhal-A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alcaligenes sp. Dhal-L</td>
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<td>+</td>
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<tr>
<td>Alcaligenes sp. Dhal-N</td>
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<td>+</td>
</tr>
<tr>
<td>Oceanomonas doudoroffii Dhal-Rw</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Variovorax paradoxus Dhal-F</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Bacillus megaterium Rice-A</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td>As(V)</td>
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<td>Ni(II)</td>
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<td>&gt;650</td>
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<td>&gt;650</td>
<td>1</td>
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<tr>
<td></td>
<td>200</td>
<td>30</td>
</tr>
</tbody>
</table>

**PGP** plant growth promoting, **MIC** minimum inhibitory concentration, **IAA** indole acetic acid, **ACCD** 1-amino-cyclopropane-1-carboxylic acid deaminase, + positive, - negative, n.d. not determined, each value is average of two independent analyses

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total arsenic content (mg kg$^{-1}$ d.w.)</th>
<th>Shoot biomass (g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27 days</td>
<td>50 days</td>
</tr>
<tr>
<td>Inoculated</td>
<td>1.18*</td>
<td>1.96*</td>
</tr>
<tr>
<td>Control</td>
<td>0.77</td>
<td>1.76</td>
</tr>
</tbody>
</table>

* Statistically different from the control ($t$ Student, p = 0.1); d.w., dry weight

Furthermore, primers specificity was confirmed by the presence of a single band in agarose gel electrophoresis of q-PCR products (data not shown).
of indigenous bacteria carrying ACR3(2) gene, although their quantification might be different because values were not comprised in the range of the standard curve, being lower than the most diluted standard used in the calibration curve. At successive incubation times, ACR3(2) gene copy number was significantly higher in inoculated pots versus controls in the bulk and in the rhizospheric soil (Fig. 5). While in inoculated pots ACR3(2) gene significantly increased until the end of the experiment, in control bulk soil the increment was detected in the first 27 days of incubation only. These data suggested that the strain colonised the system and that it persisted until the end of the experiment. Inoculation effect was stronger than the rhizosphere effect. ARB count of rhizospheric soil increased and it was higher in inoculated than in control pots (Fig. 4b).

Fig. 2 Standard curves for 16S rRNA (a) and ACR3(2) (b) genes (n = 3). C(t) values are plotted against Log of initial quantity (ng) of total DNA.

Discussion

The study describes the arsenic solubilisation, root colonization and growth promoting potential of a bacterial
arsenic used as inoculum for sunflower plants in an arsenic-contaminated soil.

The results demonstrated that the *Alcaligenes* sp. strain Dhal-L with PGP potential colonized sunflower rhizosphere and promoted arsenic uptake by plants. The application of As(V) reducing *Alcaligenes* sp. strain Dhal-L, carrying an ACR3(2) type efflux pump, significantly increased (53%) specific arsenic uptake in the shoots of sunflower over the control, thus supporting the evidence that ARB possessing an Ars operon influence arsenic mobilization in rhizosphere with consequent increase of arsenic uptake by plants (Meagher and Heaton 2005). Yang et al. (2012) found that inoculation with a mixed bacterial population increased arsenic uptake up to 110% higher than that of the control in arsenic hyper accumulating plant *P. vittata*. Such a high increase in arsenic uptake could be explained by the use of a mixed bacterial population versus a single strain. Jankong et al. (2007) found arsenic content in the roots and the fronds of rhizobacteria-inoculated *Pityrogramma calomelanos* was significantly higher than in the control mainly due to enhanced above-ground plant biomass. Arsenic uptake achieved by sunflower in the present study indicates that the plant is not a suitable candidate for phyto-extraction purposes. Although the quantity of arsenic present in sunflower shoot is not comparable to that obtained in other studies by using hyper accumulating plants (Wei et al. 2010), the results evidenced that the arsenic uptake by sunflower exceeded the limit reported for food crops (1 mg kg\(^{-1}\)) (Bhattacharya et al. 2010). This confirms the estimation made by Warren et al. (2003) which states that crops grown in soil with more than 200 mg kg\(^{-1}\) arsenic cause food poisoning. Moreover, consumption of rice and vegetables grown in arsenic-contaminated agricultural soils is the second most common route of arsenic poisoning in Bangladesh (Chowdhury 2004). Therefore, the uptake of arsenic by plants from contaminated soils presents a serious health hazard and use of arsenic contaminated and former industrial lands for agriculture is of great concern.

Different bacterial mechanisms produce effects on availability and transport of arsenic to the plant: As(V) reduction and production of more soluble As(III) (Yang et al. 2012), As(III) oxidation to As(V) that enters cells via PO\(_4^{3–}\) uptake systems (Tu and Ma 2003; Fayiga et al. 2008), less specific PGP characteristics that promote plant growth (Vogel-Mikuš et al. 2006; Jankong et al. 2007; Arriagada et al. 2007; Dell’Amico et al. 2008). Although *Alcaligenes* strain Dhal-L possessed an in vitro ACC-deaminase activity, it did not significantly promote sunflower growth. Sometimes, bacteria screened for their beneficial plant growth promoting attributes in vitro fail to perform in vivo due to poor colonization potential or out-competition by indigenous rhizosphere bacteria (Benizri et al. 2001).

Plate count method failed to detect significant differences in inoculated versus control pot experiments. q-PCR of 16S rRNA gene revealed an increase in the total bacterial population in inoculated pots, particularly in the rhizosphere fraction. Gene copy numbers revealed to be one order of magnitude higher than microbial counts, possibly due to low cultivability of soil bacteria. These data suggested that the rhizosphere exerted a positive effect on bacterial growth in general and that the strain colonized preferably rhizosphere soil, although it was detected also in the bulk. A new quantitative q-PCR method was set up to monitor the presence of inoculated strain by targeting the ACR(2) gene of the strain directly from the total DNA extracted from soil pots. New primer set acr475F/611R specific for q-PCR experiment targeting an internal region of the *ACR3(2)* gene of *Alcaligenes* sp. Dhal-L was designed for the first time in this study. The method proved to be sensitive with a detection limit of ca. 10 gene copies. Furthermore, melting curve analysis confirmed that the fluorescent signals obtained were originated from specific PCR products and not from primer dimers. The abundance of *ACR3(2)* gene in all samples was comprised in the range of linearity of the standard curve which was linear over four order of magnitude. The q-PCR analysis indicated that the inoculated strain *Alcaligenes* sp. Dhal-L, and possibly indigenous bacteria carrying *ACR3(2)* gene, colonized the rhizosphere of sunflower plants and that strain Dhal-L colonization continued to increase until the end of the experiment. There was a general positive effect of rhizosphere on growth of arsenic-resistant bacteria, their count being higher in rhizosphere than in bulk soil. In accordance to these findings, Xiong et al. (2010) found that arsenic...
resistance genes were different among P. vittata rhizosphere and bulk soil, which provides evidence for a strong linkage amongst the level of arsenic contamination, the rhizosphere, and the functional gene distribution.

The results reported in this study substantiate that the presence of strain Alcaligenes sp. Dhal-L enhanced arsenic uptake by sunflower plant. The q-PCR analysis for ACR3(2) gene here developed was suitable to monitor the inoculated strain and to detect presence and evolution of ARB in pot experiments, supporting the role of rhizosphere ARB in arsenic mobilization.

Acknowledgments This research was supported by PRIN-MIUR 2010: 2010JBNLJ7_004, from the Italian Ministry for University and Research, Rome, Italy.

References


