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ORIGINAL PAPER

Two naphthalene degrading bacteria belonging to the genera *Paenibacillus* and *Pseudomonas* isolated from a highly polluted lagoon perform different sensitivities to the organic and heavy metal contaminants

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Abstract Two bacterial strains were isolated in the presence of naphthalene as the sole carbon and energy source from sediments of the Orbetello Lagoon, Italy, which is highly contaminated with both organic compounds and metals. 16S rRNA gene sequence analysis of the two isolates assigned the strains to the genera *Paenibacillus* and *Pseudomonas*. The effect of different contaminants on the growth behaviors of the two strains was investigated. *Pseudomonas* sp. ORNaP2 showed a higher tolerance to benzene, toluene, and ethylbenzene than *Paenibacillus* sp. ORNaP1. In addition, the toxicity of heavy metals potentially present as co-pollutants in the investigated site was tested. Here, strain *Paenibacillus* sp. ORNaP1 showed a higher tolerance towards arsenic, cadmium, and lead, whereas it was far more sensitive towards mercury than strain *Pseudomonas* sp. ORNaP2. These differences between the Gram-negative *Pseudomonas* and the Gram-positive *Paenibacillus* strain can be explained by different general adaptive response systems present in the two bacteria.

Keywords Arsenic · Heavy metals · Naphthalene · EC50 · 16S rRNA gene · *Paenibacillus* · *Pseudomonas*

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Introduction

Simultaneous contamination by organic pollutants and toxic heavy metals are often present in the same polluted area, and contemporary biodegradation capability of organic contaminants and resistance to heavy metal leads to a valorization of native isolates (Springael et al. 1993). Therefore, investigations of the behavior of bacteria isolated from such extreme sites might provide information on microbial adaptability in sites with mixed pollution. Therefore, it gains importance in studies on the physiology of bacteria growing in the presence of organic pollutants and of toxic elements. However, next to reports on the toxicity of organic compounds and metals, observations of the effects of different contaminants on microbial cells are relatively rare (Dore et al. 2003).

The biodegradation of polycyclic aromatic hydrocarbons (PAHs) has been studied intensively and many bacterial strains have been isolated for their ability to use different PAHs as a source of carbon and energy. Complete mineralization of different PAHs has been described for bacterial strains belonging to different phylogenetic groups (Cerniglia and Heitkamp 1990). Naphthalene is commonly used as a model for studying PAH metabolism by bacteria because it is the simplest and most soluble PAH, as well as a frequent constituent of PAH-contaminated environments (Peters et al. 1999).

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Next to the presence of catabolic activities, the capability of those bacteria to cope with highly toxic monoaromatic compounds that are often co-pollutants of PAHs also affect the behavior of bacteria in the sites contaminated by mixed pollutants (Baldwin et al. 2000). In addition, the presence of toxic elements such as heavy metals and metalloids also affect degraders of organic pollutants (Pepi et al. 2008).

Sites with mixed pollution from organic contaminants, heavy metals and metalloids, allow growth of adapted bacterial strains facing with both types of contaminants. Studies frequently report that site contamination by both organic compounds and metals are causing difficulties for bioremediation applications. Bacterial strains that are able to interact with more than one contaminant are therefore of particular interest for bioremediation processes (Wetzel and Van Vleet 2003).

Lagoons represent a buffer zone between terrestrial and marine ecosystems and are frequently subject to anthropogenic pollution resulting from urbanization and industrialization. The Orbetello Lagoon, Tuscany, Italy, is an example of such ecosystem; it is located in an urbanized area, which was previously highly industrialized and was therefore subjected to the input of various pollutants. Organic contaminants and heavy metals and metalloids were detected in sediments of the lagoon (Focardi 2005).

The present work focuses on the characterization of two bacterial strains isolated from sediments of the Orbetello Lagoon polluted by organic contaminants and toxic elements. Their capability of growth in the presence of different organic contaminants and the effect of toxic elements were investigated. For the first time, two naphthalene-degrading isolates were compared for their capability to grow in the presence of different monoaromatic compounds and heavy metals and metalloids in order to obtain information on bacterial strains adaptation in environments polluted by multiple contaminants, as the Orbetello Lagoon.

Materials and methods

Study area and sediment sampling

Orbetello Lagoon is located on the Tyrrhenian coast of Central Italy (42°26′34″N, 11°13′29″E), covering an area of about 2,300 ha with a water depth of about 1 m. Total PAHs concentrations were at levels of 0.70 ± 0.18 mg kg^{-1} in 0–20 cm sediments, and 6.96 ± 0.26 mg kg^{-1} in 30–50 cm ones (Focardi 2005). Concerning heavy metals and metalloids concentrations in sediments, maxima values were the followings: total Hg 7.34 ± 0.11 mg kg^{-1} in 0–20 cm sediments; total Pb $3{,}276.93 \pm 0.23$ mg kg^{-1} at level 30–50 cm; total Cd 14.69 ± 0.12 mg kg^{-1} at level 0–20 cm; total Cu $2{,}624.66 \pm 0.231$ mg kg^{-1} at level 30–50 cm; total Zn $3{,}876.51 \pm 0.25$ mg kg^{-1} at level 0–20 cm; and total As 155.13 ± 0.08 mg kg^{-1} at level 30–50 cm (Focardi 2005). Sediment samples were collected in March 2005, both at superficial (0–20 cm) and at deeper (30–50 cm) levels. Superficial sediment sampling was carried out using a grab sampler. A sterile portion for microbiological analyses was immediately deposited in sterile sacks (Whirl-Pak, Nasco) and maintained at 4°C in the dark until arrival at the laboratory. Deeper samples were extracted from cores sampled by a scuba diver using a soft-sediment plastic-barrel coring device (inside diameter 7 cm). Samples for microbiological analyses were aseptically collected from the cores at the 30–50 cm level and were maintained at 4°C until their treatment in the laboratory.

Enrichment cultures and isolation of bacterial strains

Sediment samples (1.0 g) were added to flasks containing 50 ml of liquid mineral salts basal medium (MSBM), with the following composition: 1.5 g of KH_2PO_4, 7.9 g of $Na_2HPO_4{\cdot}2H_2O$, 0.8 g of NH_4Cl, 0.1 g of $MgSO_4{\cdot}7H_2O$, and 10 ml of Pfenning's trace element solution, per litre of double distilled water. The composition of the trace element solution per liter of double distilled water was the following: 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.03 g of $MnCl_2{\cdot}4H_2O$, 0.3 g of H_3BO_3, 0.2 g of $CoCl_2{\cdot}6H_2O$, 0.01 g of $CuCl_2{\cdot}2H_2O$, 0.02 g of $NiCl_2{\cdot}6H_2O$, 0.03 g of $Na_2MoO_4{\cdot}2H_2O$, and 0.05 g of $FeCl_3$. Sediment-MSBM suspension was supplemented with 0.2% (w/v) naphthalene as the enrichment substrate and incubated without shaking at 30°C in the dark. After 30 days without further transfers, enrichment cultures showing turbidity were selected for isolation experiments. A sample (100 µl) from each culture was spread on solid MSBM plates supplemented with 100 mg of naphthalene added on the plate lid, thus allowing vapors to reach the cells of microorganisms as the sole carbon and energy source. After incubation for 7 days, colonies showing different morphologies were selected as candidate naphthalene-degrading strains. The purity of colonies was also detected on a complex culture medium Plate Count Agar (PCA), containing 5.0 g of tryptone (Difco), 2.5 g of D-glucose (BDH), and 2.5 g of yeast extract (Oxoid) per liter of double distilled water. Isolated strains were stored as liquid culture containing 30% sterile glycerol (v/v) in liquid nitrogen.

Bacterial strains characterization and 16S rDNA analysis

After growth on the solid medium of the bacterial isolate, the colony morphology was observed under a stereomicroscope (Optika, mod. 500). Morphology of bacterial cells was

observed by microscopic analyses (Diaplan, Leitz). Gram determination was carried out (Gram stain kit, Carlo Erba) and catalase and oxidase activities were determined according to Smibert and Krieg (1981). For 16S rDNA sequencing of the 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 16S 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 IU "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. Finally, the mixtures were incubated at 72°C for 10 min and at 60°C for 10 min; 2 µl of each amplification mixture was analyzed by agarose gel (1.2% w/v) electrophoresis in TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA) containing 0.5 µg ml^{-1} (w/v) ethidium bromide. Sequencings were carried out at the Bact 16S biomolecular research service (CRIBI Biotechnology Centre, University of Padua, Italy). A partial 1,367 bp, 16S rDNA sequence was determined for naphthalene degrading isolate ORNaP1, and a 1,506 bp 16S rDNA sequence for the isolated strain ORNaP2.

Sequence accession numbers

16S rDNA nucleotide sequences obtained in this study were submitted to the GenBank database and have been assigned the following accession numbers: DQ401157 for the *Paenibacillus* sp. ORNaP1 strain and EF025737 for *Pseudomonas* sp. ORNaP2.

Analysis of sequence data

Consensus sequence of isolates ORNaP1 and ORNaP2 were compared with those deposited in GenBank using the BLAST program (Altschul et al. 1997). For phylogenetic analysis, 16S rDNA sequences of ORNaP1, ORNaP2 and related sequences retrieved from databases (GenBank and RDPII) were aligned with ClustalW, included in the MEGA 3.1 software package (Kumar et al. 2004). The resulting alignments were checked manually. Phylogenetic analyses were conducted using MEGA version 3.1. The phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei 1987). Sequence divergences among strains were quantified using the Kimura 2-parameter distance model (Kimura 1980). For treatment of gaps, the "Complete Deletion" option was chosen. A total of 1,000 bootstrap replications were calculated. The trees were unrooted.

Naphthalene degradation assay and naphthalene determination in bacterial cultures

Isolated strains ORNaP1 and ORNaP2 grown in liquid MSBM (in the presence of naphthalene added in crystal form as the sole carbon and energy source, incubated at 300 rpm at 28°C) were inoculated in three different 250 ml Erlenmeyer flasks containing 50 ml of MSBM. At different times, samples in triplicate and a control with no bacteria added were collected and the biomass measured in terms of CFU ml^{-1}, by spreading 100 µl of the cultures, or of appropriate dilutions, on PCA medium. Plates were incubated at 28°C and colonies counted after 24 h. Each whole sample was then stored at −20°C for subsequent chemical analyses. For naphthalene quantification, the whole content (10 ml) of the ORNaP1 and ORNaP2 cultures and of the control with no bacteria added, in MSBM in the presence of 500 µg ml^{-1} of naphthalene, were placed in 50 ml screw-top Teflon vials with 5 ml headspace. A liquid–liquid sample extraction was carried out following the EPA Method 3510 C (USEPA 1996). Forty millilitres of a dichloromethane/hexane (DCM/Ex) solution (50/50 v/v) were added to each sample and sonicated for 60 min (C.E.I.A., mod. CP823). Phase separation was made possible by maintaining samples undisturbed for 5 min and then 10 ml of DCM/Ex solution were transferred to screw-top analysis vials with 22 ml headspace and analyzed by HPLC. Naphthalene concentrations in the extract from bacterial cultures were measured using a Waters 474 scanning fluorescence detector. Chromatographic separation was performed on a Supelcosil™ LC-PAH HPLC chromatographic column (250 × 4.6 mm i.d., particle size 5 µm, Supelco) with an acetonitrile/water gradient of 60:40 (hold 3 min, ramp to 100:0 within 30 min and hold 10 min) and a flow rate of 1.5 ml min^{-1}. Fluorescence detection was performed by following the wavelength program to selectively detect the compounds. Analyses were carried out by calibrating the HPLC system with naphthalene in acetonitrile using an external standard method. At least three standard solutions of known concentrations were used to arrange calibration curves and a good linearity was obtained, with R correlation coefficient values above 0.99. The detection limit for the HPLC system ranged from 0.01 to 0.5 µg ml^{-1}. Quality control was carried out with blank analysis, and with repeated analyses of the same standard. The recovery range for naphthalene was 69.4–100%.

Carbon source utilization

The following carbon sources were tested for growth of the two bacterial strains: acenaphthene, phenanthrene,

anthracene, fluoranthene, fluorene, pyrene, benzo(*a*)anthracene, sodium-salicylate, catechol, gentisate, phenol, benzene, toluene, ethylbenzene, *o*-xylene, 4-chlorophenol, chlorobenzene, and diesel fuel, added at a concentration of 500 μg ml^{-1}, as the sole carbon and energy sources, to 50 ml MSBM in 250 ml conical flasks. Naphthalene-induced bacterial strains were used as inocula and cultures were incubated at 28°C in the dark, at 300 rpm. Growth was checked by measuring the optical density, at a wavelength of 600 nm using an UV-visible spectrophotometer (Jenway, mod. AC30). A blank with the medium culture alone, with no bacteria added, was also analyzed. All chemicals were of analytical grade and obtained from Sigma or Fluka (Milan, Italy).

Toxicity tests in the presence of monoaromatic compounds

The two isolated bacterial strains were tested for growth in the presence of increasing concentrations of monoaromatic BTEX compounds such as benzene, toluene, ethylbenzene, *m*-xylene, added at different toxic concentrations. Samples of 1.0 ml of overnight cultures were incubated in 99.0 ml of YEPG medium containing (per litre of double distilled water): 5.0 g of tryptone, 2.5 g of D-glucose, and 2.5 g of yeast extract. Ten millilitres were distributed in 18 ml test tubes sealed with screw-top caps. The different monoaromatic concentrations were then added to exponentially growing cells and the tubes incubated at 28°C for 24 h. The optical density of the cultures, as a measure of microbial growth, was detected at a wavelength of 600 nm by an UV-visible spectrophotometer (Jenway, mod. AC30); a blank with the medium culture alone, with no added bacteria, was also analyzed.

Percentage of growth rates inhibition in the presence of toxic elements

The two bacterial strains were tested for growth in the presence of trivalent and pentavalent arsenic forms and of different heavy metals. Tested elements were the following As^{3+}, As^{5+}, Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, starting from salts as reported above. Each bacterial strain was grown in YEPG medium without toxic addition, and at the exponential growth, different concentrations of a form of arsenic or of heavy metals were added, and growth was detected at different times by measuring the optical density at 560 nm by an UV-visible spectrophotometer (Jenway, mod. AC30), and values of percentage of growth were reported by using growth in the absence of toxic as the 100%. For the measurement of the toxic effects, the investigated compounds showed above were added to exponentially growing cultures as described by Heipieper et al. (1995). Growth inhibition caused by the toxicants was measured by comparing the differences in growth rate μ (h^{-1}) between intoxicated cultures (μ, toxicant) with that of control cultures (μ, control). The growth inhibition of different concentrations metals and arsenic was defined as the percentage of the growth rate of intoxicated cultures compared to control cultures without toxicant addition.

$$\text{Inhibition growth } (\%) : \frac{\mu, \text{toxin}}{\mu, \text{control}} \times 100$$

Results

Isolation of bacterial strains in the presence of naphthalene and their characterization

Enrichment cultures were set up with polluted sediments of the Orbetello Lagoon in order to isolate bacterial strains capable of using naphthalene as the sole carbon and energy source. Naphthalene was chosen as a model for PAH degradation in general. This screening approach led to the isolation of two bacterial strains able to grow with naphthalene as sole C- and energy source, named ORNaP1 and ORNaP2. Their identification was carried out based on morphologic and phenotypic properties and by using 16S rRNA sequencing analyses. The heterotrophic and strictly aerobic strain ORNaP1 showed colonies with round form, entire margins, umbonate profile, Gram-positive and endospore-forming. Strain ORNaP2 was also heterotrophic and facultative anaerobic; colonies had oval form, entire margins, flat profile, and were Gram-negative. Microscopic analyses showed rod cells in the two isolates.

16S rDNA gene sequences analyses and phylogenetic tree

Bacterial isolates were further identified by 16S rDNA gene sequencing. An almost complete sequence of the gene encoding 16S rRNA of the ORNaP1 isolate was determined (1,367 bp), and BLAST analysis assigned it to the genus *Paenibacillus*. The closest relatives of the ORNaP1 strain were *P. validus* (AB073203), *Paenibacillus* sp. KBC101 (AB186915), *P. validus* strain PR-P9 (AF353697), *P. naphthalenovorans* strain PR-N1 (AF353681), and *P. validus* strain DSM3037 (D78320), the first three of which had a 99% 16S rRNA gene sequence identity, whereas the latter two strains had 97 and 96%, respectively. An almost complete sequence of the gene encoding 16S rRNA of the ORNaP2 strain was determined (1,506 bp). Comparison of the nucleotide sequence with those of the small 16S ribosomal subunits deposited in the ribosomal database of the project RDP II assigned the isolated bacterial strain to the genus *Pseudomonas*. The closest relatives of the ORNaP2

strain were *Pseudomonas* sp. SN1 (AY683884), *P. chloritidismutans* (AY017341), *P. putida* ASK-1 (AY277620), *P. stutzeri* 24a63 (AJ312173), *Pseudomonas* sp. SB1 (AY683883), all with 99% 16S rRNA gene sequence identity.

Phylogenetic analyses were performed on ClustalW aligned sequences of the 16S rRNA gene. Based on 16S rRNA similarity, ORNaP1 showed an apparent relationship with bacteria belonging to the *Paenibacillus* assemblage and formed a stable phylogenetic group within a heterogeneous cluster of *P. validus*, consisting mainly of PAH-degrading bacteria. The phylogenetic tree obtained based on 16S rDNA comparisons showed the position of ORNaP1 and type strains of the genus *Peanibacillus* and polycyclic aromatic hydrocarbon-degrading *Paenibacillus* isolates (Fig. 1).

Paenibacillus sp. ORNaP1 position in the phylogenetic tree is close to the naphthalene-degrading strain *P. validus* PR-P9 AF353697 (Daane et al. 2001). Daane et al. (2002) proposed a new species, *P. naphthalenovorans,* whose strains were able to utilize naphthalene and other aromatic contaminants as growth substrates. The ability of bacteria belonging to the genus *Paenibacillus* to degrade PAHs is well known. Pichinoty et al. (1986) described *Bacillus gordonae* sp. nov., later to be emended as *P. validus* (Heyndrickx et al. 1995), which was able to use naphthalene and aromatic contaminants.

A comparison of the 16S rRNA sequences of the isolated strain ORNaP2 with those of other strains belonging to the genus *Pseudomonas* showed that the tree resulting from phylogenetic analysis clustered ORNaP2 in a clade containing *Pseudomonas* sp. strain SN1, *Pseudomonas* sp. SB1, and *P. chloritidismutans*. The closest to the *Pseudomonas* sp. ORNaP2 strain in a different clade was a *P. stutzeri* strain (Fig. 2).

The position of the second new sequence of *Pseudomonas* sp. ORNaP2, indicated by phylogenetic analysis, was close to the *Pseudomonas* sp. strains SN1 and SB1 isolated from PAH-contaminated sediments of Venice Lagoon, Italy, which were capable of using naphthalene and biphenyl as sole carbon and energy sources, respectively (Marcon et al. 2007) and to the species *P. chloritidismutans* (Wolterink et al. 2002) in the same clade. The fact that *Pseudomonas* sp. strain ORNaP2 showed higher similarity with *Pseudomonas* sp. SN1 and SB1, suggests that similar environments, in this case the lagoons of Orbetello and Venice, with contamination by petroleum hydrocarbons, metals and metalloids, select similar bacterial strains with common characteristics (Marcon et al. 2007). Bacterial strains belonging to the genus *Pseudomonas* sp. showing the ability to degrade naphthalene and other PAHs were previously described (Ben Said et al. 2007; Siunova et al. 2007).

Naphthalene degradation by the two isolated bacterial strains

Higher efficiency was observed for naphthalene degradation in strain *Pseudomonas* sp. ORNaP2 compared to *Paenibacillus* sp. ORNaP1. When grown in aerobic conditions in the presence of naphthalene as the sole carbon and energy source, strain *Paenibacillus* sp. ORNaP1 showed an increase in biomass from 10^4 to 10^6 CFU ml^{-1} after 20 h of incubation, with a corresponding decrease in naphthalene concentration from an initial concentration of 500 mg l^{-1} to a residual concentration of about 60 mg l^{-1}

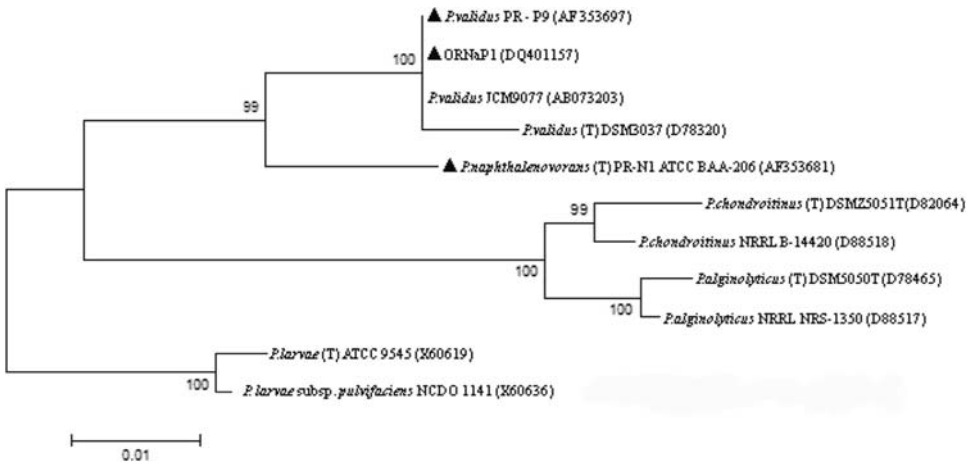

Fig. 1 Unrooted phylogenetic tree based on 16S rDNA sequence comparisons, showing the position of ORNaP1 and representative species of the genus *Paenibacillus*. *Black triangles* show polycyclic aromatic hydrocarbon-degrading *Paenibacillus* isolates. The branching pattern was generated using neighbor-joining methods and the bootstrap values, shown at the nodes, were calculated from 1,000 replicates. Bootstrap values lower than 70% are not shown. The *scale bar* indicates substitutions per nucleotide. The GenBank accession numbers for the 16S RNA sequences are given in *parentheses* after the strain

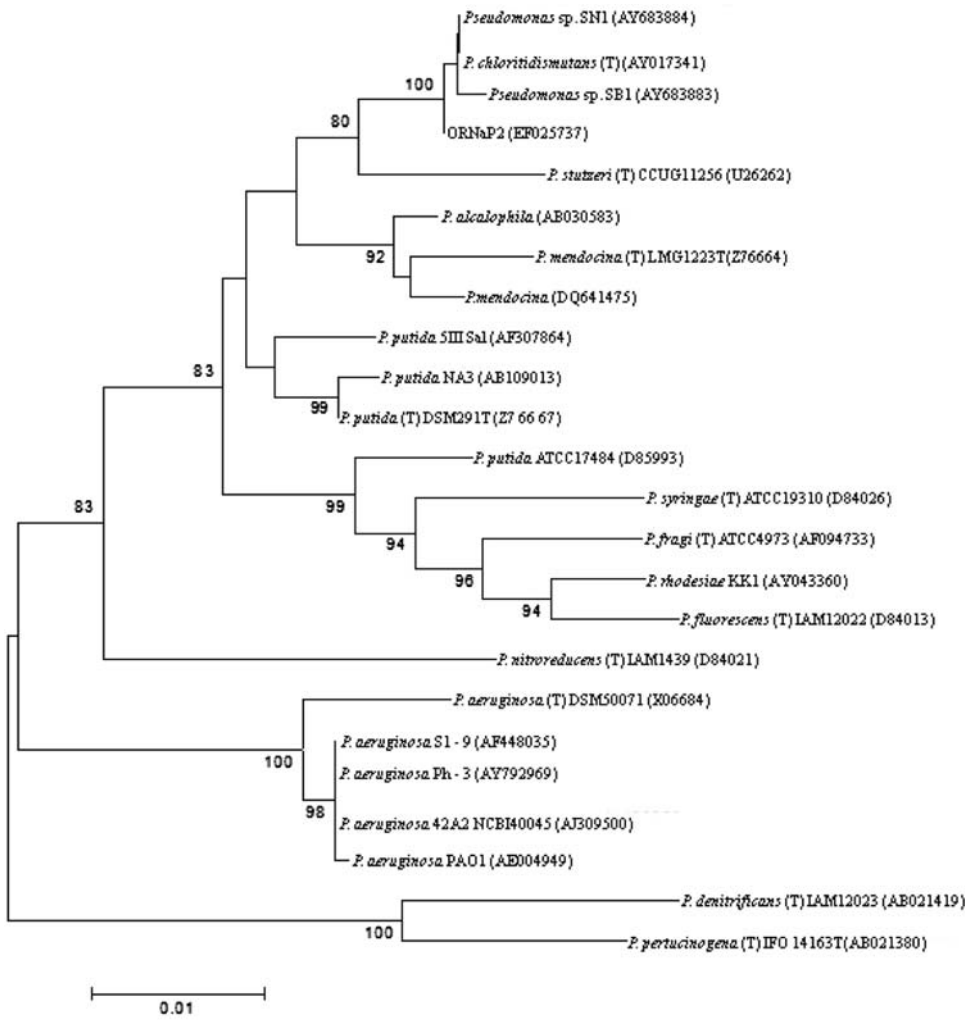

Fig. 2 Unrooted phylogenetic tree based on 16S rDNA sequence comparisons, showing the position of ORNaP2 and representative species of the genus *Pseudomonas*. The branching pattern was generated using neighbor-joining methods and the bootstrap values, shown at the nodes, were calculated from 1,000 replicates. Bootstrap values lower than 70% are not shown. The *scale bar* indicates substitutions per nucleotide. The GenBank accession numbers for the 16S RNA sequences are given in *parentheses* after the strain

(13%) at the end of incubation. In the control culture, with no bacteria added, the naphthalene concentration remained constant throughout the experiment (data not shown). A yellow color developed in the culture medium after 3 days of incubation at 28°C of the strain *Paenibacillus* sp. ORNaP1 when grown on naphthalene indicating the presence of the meta-cleavage pathway.

Growth of the *Pseudomonas* sp. ORNaP2 isolate in aerobic conditions and in the presence of naphthalene and of the corresponding substrate degradation showed an almost complete naphthalene degradation, showing a residue of 8%, after 24 h of incubation with 7.0×10^6 CFU ml^{-1}, and the highest biomass value of 1.0×10^7 CFU ml^{-1} and a 3% of naphthalene residue after 48 h of incubation. In the control culture, with no bacteria added, the naphthalene concentration remained constant throughout the experiment (data not shown).

Degradation rates of naphthalene for the two bacterial strains showed higher efficiency for strain *Pseudomonas* sp. ORNaP2, where a maximum value of milligrams of naphthalene per hour corresponding to 15.65 ± 0.02 was detected, compared to the *Paenibacillus* sp. ORNaP1 strain where the higher efficiency of degradation of 9.85 ± 0.014 mg naphthalene per hour. Best efficiencies of naphthalene degradation in both cases were reached after 24 h of incubation (data not shown).

Carbon source utilization by the isolated bacterial strains

In order to test the ability of both strains to degrade potential intermediates of naphthalene degradation, we carried out a series of growth experiments with the compounds listed as carbon sources. The two strains only showed growth with the compounds listed in Table 1. Next to naphthalene, *Pseudomonas* sp. ORNaP2 showed proper growth in the presence of sodium-salicylate and catechol. *Paenibacillus* sp. strain ORNaP1 was able to grow in the presence of gentisate, showing the absence of growth in the presence of sodium-salicylate (Table 1).

Table 1 Growth of the naphthalene-degrading bacterial strains *Paenibacillus* sp. ORNaP1 and *Pseudomonas* sp. ORNaP2 in mineral medium and in the presence of different organic pollutants as the sole carbon and energy source

Substrate	*Paenibacillus* sp. ORNaP1	*Pseudomonas* sp. ORNaP2
Naphthalene	+++	+++
Anthracene	++	−
Fluorene	−	±
Pyrene	±	−
Na-salicylate	−	+++
Catechol	−	+++
Gentisate	++	−
Diesel fuel	++	−

+++ very fast growth rates, ++ fast growth rates, ± slow growth rates, − no growth

Growth in the presence of monoaromatic compounds

Next to growth test, the two isolated bacterial strains were also subjected to toxicity tests in order to investigate their ability to tolerate the presence of several monoaromatic compounds which were added to exponentially growing cells in different concentrations (Fig. 3). Hereby, *Paenibacillus* sp. ORNaP1 showed a far higher sensitivity to these monoaromatic compounds, whereas higher levels of tolerance were detected for *Pseudomonas* sp. ORNaP2.

Effect of metals on growth of the two isolated bacteria

In order to quantify the toxicity of heavy metals known to be present in the Orbetello Lagoon as well, the toxic elements were added in different concentration to cultures of the two strains growing exponentially. The bacteria continued to grow exponentially but at reduced growth rates. By plotting the relative growth rates against the concentrations of the toxic elements, a direct correlation between the concentration of a compound and its growth-inhibiting effect was observed as previously described (Pepi et al. 2008). From these plots, the effective concentrations (EC50) of the metals leading to 50% growth inhibition were calculated and listed in Table 2. Like already observed for the sensitivity towards monoaromatic compounds, the two isolates showed a different sensitivity to the toxic elements. Contrary to the sensitivity towards BTE, *Paenibacillus* sp. ORNaP1 showed a higher tolerance to all tested heavy metals than the *Pseudomonas* strain. Only exception was of mercury, where the Gram-positive strain revealed a very high sensitivity with the EC50 being at the very low concentration of 0.005 mM (Table 2). In addition, in the presence of copper, *Paenibacillus* was slightly more sensitive than *Pseudomonas* sp. ORNaP2.

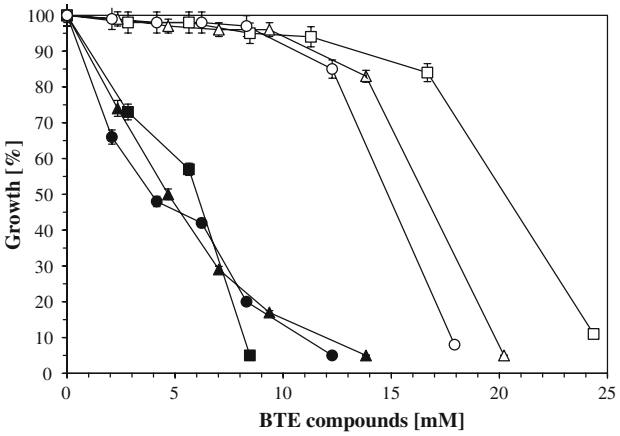

Fig. 3 Percentage of growth of strain *Paenibacillus* sp. ORNaP1 (*closed symbols*) and *Pseudomonas* sp. ORNaP2 (*open symbols*) in the presence of different concentrations of the monoaromatic BTEX compounds benzene (*filled square*, *open square*); toluene (*filled triangle*, *open triangle*); and ethylbenzene (*filled circle*, *open circle*). Growth was detected as optical density by measuring absorbance at a wavelength of 600 nm. Experiments were carried out in triplicate

Table 2 Toxicity, given as effective concentrations (EC50) that inhibit growth by 50%, of the isolated bacterial strains in the presence of metals

Toxic element	EC50 (mM)	
	Paenibacillus sp. ORNaP1	*Pseudomonas* sp. ORNaP2
As^{3+}	3.50 ± 0.18	0.45 ± 0.02
As^{5+}	6.00 ± 0.34	1.00 ± 0.05
Hg^{2+}	0.005 ± 0.0003	0.020 ± 0.001
Pb^{2+}	1.20 ± 0.06	0.05 ± 0.002
Cd^{2+}	0.35 ± 0.018	0.03 ± 0.001
Cu^{2+}	0.75 ± 0.038	0.90 ± 0.05
Zn^{2+}	0.65 ± 0.04	0.30 ± 0.015

The standard deviations of three independent experiments are shown

Discussion

In this contribution, we present for the first time a systematic approach to quantify the effects of toxic elements such as heavy metals, arsenic, and aromatic contaminants on bacteria isolated from a site known to be highly polluted with both classes of contaminants (Focardi 2005). These conditions presumably caused the selection of specialized extremophillic microbial population in the sediments of the Orbetello Lagoon from where the two bacteria were isolated for their ability to degrade naphthalene.

Naphthalene degradation is well known in the genus *Pseudomonas* (Peng et al. 2008; Yen and Serdar 1988). *Pseudomonas* sp. ORNaP2 seems to contain the pathway of naphthalene degradation via 1,2-dihydroxynaphthalene,

salicylate and catechol described for *P. putida* G7 (Ghiorse et al. 1995). *Paenibacillus* sp. strain ORNaP1 grew in the presence of gentisate, an intermediate of naphthalene degradation, showing a different behavior with respect to the *P. naphthalenovorans* isolated from rhizosphere of salt march plants (Daane et al. 2002). Strain ORNaP1 also showed the ability to grow using diesel fuel, mainly composed of aliphatic compounds. Abilities to degrade aliphatic and aromatic hydrocarbons are not necessarily mutually exclusive in bacteria (Churchill et al. 1999).

Bacteria that metabolize aromatic hydrocarbons face the challenge of acquiring carbon and energy from compounds that are potentially toxic (Ramos et al. 2002; Sikkema et al. 1995). The inability of potential biodegrading populations to tolerate aromatic hydrocarbon toxicity may contribute to the persistence of pollutants in the environment. The mechanisms of toxicity are generally believed to be disruption of biological membranes (Heipieper et al. 1994; Sikkema et al. 1995) and the production of toxic metabolites (Park et al. 2004). The lipophilic character of aromatic hydrocarbons can alter membrane fluidity, permeabilize the membrane, and cause swelling of the lipid bilayer (Heipieper et al. 1994). Alteration of membrane structure can disrupt energy transduction and the activity of membrane-associated proteins (Heipieper et al. 1991; Sikkema et al. 1995).

In the present investigation, we have shown that *Pseudomonas* sp. ORNaP2 performed a higher level of tolerance to monoaromatic compounds compared to *Paenibacillus* sp. ORNaP1. A higher tolerance of *Pseudomonas* strains towards monoaromatic organic solvents has already been described and is based on the high adaptability of these bacteria mainly on the level of membrane composition as well as by active efflux pumps for organic solvents (Heipieper and de Bont 1994; Ramos et al. 2002).

On the contrary, *Paenibacillus* sp. strain ORNaP1 was much more resistant than strain *Pseudomonas* sp. ORNaP2 to most of the tested toxic heavy metals, with the exception of mercury and copper.

Like for organic solvents, also resistance to mercury and copper is at least partially related to active efflux systems transporting the compounds across the membranes of Gram-negative bacteria. Thus, like for the BTEX compounds, the higher tolerance to the two metals, mercury and copper, shows a similar trend related to the composition and the presence of active transport systems present in the cytoplasmic membrane of the *Pseudomonas* strain (Zhang and Rainey 2008).

In the presence of both As^{3+} and As^{5+} strain *Paenibacillus* sp. ORNaP1 showed higher EC50 values compared to *Pseudomonas* sp. strain ORNaP2. A similar behavior was previously pointed out for two arsenic-resistant bacterial strains, belonging to the genera *Bacillus* and *Pseudomonas*, isolated from sediments of the Orbetello Lagoon, where the Gram-positive bacterium showed higher tolerance to the presence of As^{3+} (Pepi et al. 2008).

The bacteria showed similar relative behaviors to cadmium and zinc with the *Paenibacillus* strain showing higher tolerance. It is known that the bacterial resistance mechanisms towards these two elements are related (Silver and Phung 2005) and most probably more effective in the Gram-positive than in the Gram-negative bacterium.

Concerning the far higher lead resistance of the *Paenibacillus* strain, it is known that such big differences of lead tolerance to Pb^{2+} occur in strains isolated from environmental samples. However, it is still unclear whether a single mechanism of Pb^{2+} resistance is responsible for this variability in bacterial resistance (Borremans et al. 2001).

So far, only a few studies described PAH-degrading bacteria having the capacities to cope with the presence of other pollutants. Most of these bacteria were isolated from extreme environments such as naphthalene-utilizing and mercury-resistant bacteria isolated from an acidic environment (Dore et al. 2003). The presence of heavy metals may suppress or reduce PAH degradation (Insam et al. 1996; Wild et al. 1991). Contamination by heavy metals combined with PAH contamination affects microbial and enzymatic activity in ecological systems (Amezcua-Allieri et al. 2005). Assessment of microbial natural attenuation in groundwater polluted with gasworks residues showed a rapid aerobic degradation of BTEX and PAHs (Shulze and Tiehm 2004).

In conclusion, the two isolated bacterial strains showed significant differences regarding their tolerance towards the potential contaminants present in the Orbetello Lagoon. *Pseudomonas* sp. ORNaP2 showed a higher tolerance to benzene, toluene, and ethylbenzene than *Paenibacillus* sp. ORNaP1. Contrary to that *Paenibacillus* sp. ORNaP1 could tolerate higher concentrations of arsenic, cadmium, and lead whereas it was more sensitive to mercury and copper. These differences between the Gram-negative *Pseudomonas* and the Gram-positive *Paenibacillus* strain can be explained by different general adaptive response systems present in the two bacteria.

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