Journal of Hazardous Materials 215–216 (2012) 243–251

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Metabolic versatility of Gram-positive microbial isolates from contaminated river sediments

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ARTICLE INFO

Article history:
Received 15 November 2011
Received in revised form 21 February 2012
Accepted 22 February 2012
Available online 2 March 2012

Keywords:
Gram-positive
Biodegradation
Aromatic compound
Heavy metal
Biopolymer

ABSTRACT

Gram-positive bacteria from river sediments affected by the proximity of a petrochemical industrial site were isolated and characterized with respect to their ability to degrade a wide range of aromatic compounds. In this study we identified metabolically diverse Gram-positive bacteria capable of growth on wide range aromatic compounds in the presence of heavy metals and with the ability to accumulate biopolymers. Thirty-four isolates that were able to use 9 or more common aromatic pollutants, such as benzene, biphenyl, naphthalene etc. as a sole source of carbon and energy included members of *Bacillus*, *Arthrobacter*, *Rhodococcus*, *Gordonia*, *Streptomyces*, and *Staphylococcus* genus. *Rhodococcus* sp. TN105, *Gordonia* sp. TN103 and *Arthrobacter* sp. TN221 were identified as novel strains. Nine isolates were able to grow in the presence of one or more metals (mercury, cadmium, nickel) at high concentration (100 mM). Seven isolates could degrade 15 different aromatic compounds and could grow in the presence of one or more heavy metals. Two of these isolates were resistant to multiple antibiotics including erythromycin and nalidixic acid. One third of isolates could accumulate at least one biopolymer. Twelve isolates (mainly *Bacillus* sp. and *Arthrobacter* sp.) accumulated polyphosphate, 3 *Bacillus* sp. accumulated polyhydroxybutyrate, while 4 isolates could accumulate exopolysaccharides.

1. Introduction

The demand for novel bacterial strains applicable in bioremediation efforts continues to grow as human activities continuously release hazardous materials into environment. At the same time, contaminated sites are rich source of novel and versatile microorganisms capable of bioremediation [1–3]. Aromatic compounds are among the most prevalent and persistent in the environment, including both polyaromatic hydrocarbons (PAHs) and monoaromatic hydrocarbon groups that have been reported as toxic, carcinogenic and mutagenic [4,5]. Metal contamination (i.e. cadmium, chromium, mercury) also poses serious environmental problem in many ecosystems decreasing metabolic activity and diversity [6]. Contaminated sites usually contain complex mixture of aromatic pollutants and toxic heavy metals [6,7]. These hazardous and persistent compounds are introduced into aquatic environment from a range of different sources, with the sediments acting as the major repository of deposition [8]. The release of hydrocarbons into aquatic environments which contain low concentrations of inorganic nutrients often produce high carbon to nitrogen or carbon to phosphorus ratios, this coupled with lower oxygen levels associated with sediments results in generally harsh environmental conditions unfavorable for microbial growth [9,10].

Aerobic Gram-positive bacteria are primarily chemoorganotrophic and produce spores in response to environmental stress such as harsh chemical or physical conditions [11,12]. The best studied representatives of this group include human-pathogens such as *Bacillus anthracis* and antibiotic producing *Streptomycetes* [13,14]. More recently, representatives of the *Bacillus* and *Streptomyces* genus have also been reported for the ability to synthesize other valuable biopolymers such as polyhydroxyalkanoates (PHA) [15–17]. Gram-positive bacteria are well known for their varied morphologies, physiologies, and metabolic properties that allow them to prosper in a wide range of environments. With the exception of genus *Rhodococcus* [18], they are relatively little studied in respect to their ability to degrade environmental pollutants such as aromatic hydrocarbons. Hydrocarbon-degrading bacteria were firstly isolated almost a century ago, and most of the known aromatic degraders today are Gram-negative bacteria such as *Pseudomonas* related *Burkholderia*, *Acinetobacter* and *Sphingomonas*

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doi:10.1016/j.jhazmat.2012.02.059

species [3]. Although in soil contaminated by PAHs besides *Pseudomonas* strains, Gram-positive *Mycobacterium*, *Arthrobacter* and *Nocardia* species have often been found to be selectively stimulated [19,20], less effort has been devoted to further study their bioremediation potential.

The main aim of this study was to isolate robust Gram-positive microorganisms that could potentially thrive in the variety of contaminated sites including mixed heavy metal and aromatic pollutions, which do not carry multiple antibiotic resistance markers and are capable to cope with a range of stress environmental conditions. Using a selective cultivation approach, we have set out to isolate and characterize Gram-positive bacteria from river sediments affected by proximity of a petrochemical industrial site with the respect to their ability to degrade a wide range of aromatic hydrocarbon compounds. To further expand our understanding of the isolated aerobic aromatic hydrocarbon degraders, the assessment of their metabolic versatility and application potential was extended by analyzing their ability to grow in the presence of heavy metals, often found at hydrocarbon contaminated sites [7,21], resistance to antibiotics (associated with the presence of plasmids encoding heavy metal degradation genes [22]), to accumulate inorganic polyphosphate (polyPi), polyhydroxybutyrate (PHB) and to produce exopolysaccharides (EPS).

2. Materials and methods

2.1. Reagents

Benzene, toluene, phenol, biphenyl, styrene, naphthalene and other aromatic compounds used as a sole carbon source were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). Salts such as $CdSO_4$, $NiCl_2$, $HgCl_2$, $CuSO_4$ and $FeCl_3$ as well as antibiotics (ampicillin, nalidixic acid, erythromycin, kanamycin, rifampicin, and tetracycline), Congo Red dye and cyclohexamide were also of the highest available grade from Sigma–Aldrich (St. Louis, MO, USA). Tryptone soy broth, soluble starch, casein, bacteriological agar and other media components were purchased from Becton Dickinson (Sparks, MD, USA). KAPA Robust 2G Hotstart Ready Mix was purchased from KAPA Biosystems (Woburn, Massachusetts, USA). Oligonucleotide primers were obtained from Invitrogen (Darmstadt, Germany). QIAEX II gel purification and QIAquick PCR purification kits were purchased from QIAGEN (Hilden, Germany).

2.2. Sediment collection and bacterial isolation

Surface sediment samples (100 g; from 1 to 10 cm depth) were collected from the banks of the river Danube, within 5 km range of a petrochemical industrial site. An aliquot (5 g) was evaporated to dryness (100 °C, 60 min) and used for standard elemental analysis (Vario EL III; C, H, N, S/O Elemental Analyzer, Elementar Analysensysteme GmbH, Hanau-Germany). The rest of the sediment sample was dried for seven days in the presence of $CaCO_3$ (10%, w/w) at 40 °C, and then sieved under aseptic conditions to a particle size of approximately 3 mm. To sieved sample of dried sediment (1 g), sterile potassium phosphate buffer (9 ml; 50 mM, pH 7.4) was added and the suspension was vigorously mixed by vortexing for 5 min. Serial dilutions of this suspension (300 μl) were spread plated on starch casein agar (SCA) containing per liter: soluble starch (10 g), casein (1 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.5 g) and NaCl (3 g) and supplemented with antifungal cyclohexamide (75 mg l^{-1}). Plates were incubated at 30 °C for 7 days. Various isolates (225 in total) were selected by visual differentiation of contrasting colony morphology.

2.3. Bacterial aromatic compound degradation ability and growth conditions

To test the ability of the isolates to use various aromatic compounds as a sole source of carbon and energy, strains were transferred onto mineral salts medium (MSM) plates containing per liter of deionized water: $Na_2HPO_4{\cdot}12H_2O$ (9 g), KH_2PO_4 (1.5 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $CaCl_2$ (0.002 g), trace element solution (1 ml, [23]) and bacteriological agar (1.5 g). The carbon source was supplemented via vapor phase by adding it in the sterile Eppendorf plastic tip (50 μl) and placing the tip in the lid of the Petri dish. In the case of naphthalene, *o*-nitrophenol, biphenyl, a couple of crystals (5 mg) were similarly placed on the lid of a Petri dish. Plates were incubated at 30 °C for 5 days. Growth was confirmed by comparison with control plates without substrate and 20 mM glucose as carbon source.

To assess temperature growth characteristics of isolates they were grown on rich Luria Bertani (LB) plates [24] at 5 °C and 42 °C.

2.4. Identification of bacteria

Colony PCR was performed using KAPA2G Robust HotStart Ready Mix according to the manufacturers' manual. For amplification of 16S rDNA bacteria-specific primers: 27f and 1492r were used [25]. Obtained PCR products were sequenced using Applied Biosystems 3130 Genetic Analyser (Foster City, USA). Sequences were analyzed and assembled with SeqMan Pro software (DNASTAR Inc., USA. Homologues were identified in GeneBank using BLASTN algorithm [26], and in Ribosomal Database Project (RDP Release 10; http://rdp.cme.msu.edu; [27]) using Seqmatch tool. Alignment of obtained 16S rDNA sequences and type strain sequences taken from RDP, which were all trimmed to the same length, was performed with the CLUSTALW algorithm [28]. Phylogenetic tree was constructed by the maximum-likelihood algorithm with Jukes-Cantor distance correction and bootstrap resampling method with 1000 replicates, all included in the PHYLIP program package (http://bioweb2.pasteur.fr/; [29]). The tree was rooted using 16S rRNA gene sequence of *Pseudomonas putida* DSM 291^T (D84020) as an outgroup.

The sequences for 16S rRNA genes were deposited in GenBank under accession numbers: JN800323–JN800358, JN118576 and JN118577.

2.5. Growth of bacteria in presence of heavy metals and antibiotics

To examine the potential of isolates to grow in the presence of heavy metals, metal toxicity medium (MTM) was used containing per liter: sodium lactate (5.1 g), Na_2SO_4 (2.13 g), $CaCl_2$ anhydrous (0.06 g), NH_4Cl (1 g), $MgSO_4$ (1 g), yeast extract (0.05 g), tryptone (0.5 g), and PIPES (10.93 g) [30]. Metals used were Cd^{2+}, Ni^{2+}, Hg^{2+}, Cu^{2+}, Fe^{3+} and Zn^{2+}. Concentrations were calculated according to mining and spatial planning regulation of the Serbian Ministry of the Environment [31], and were 20 times higher than allowed by the regulation ($CdSO_4$, 111.3 mg l^{-1}; $NiCl_2$, 2.3 g l^{-1}; $HgCl_2$, 73 mg l^{-1}; $CuSO_4$, 5 mg l^{-1}; $FeCl_3$, 114 mg l^{-1} and $ZnCl_2$, 12.6 g l^{-1}). Growth of isolates was also tested in the presence of higher amounts of salts (CdSO4, 20.9 g l^{-1}; $NiCl_2$, 12.9 g l^{-1}; $HgCl_2$, 27 g l^{-1}; $CuSO_4$, 15.9 g l^{-1} and $FeCl_3$, 16.2 g l^{-1}) which constitute 100 mM concentrations of the corresponding metal ions.

To assess the ability of isolates to grow in the presence of heavy metals and to degrade aromatic compounds, lactate from the MTM was substituted with toluene (representative of monoaromatic compounds) and naphthalene (representative of PAHs).

Bacterial growth in the presence of antibiotics was determined by streaking the isolates on LB plates containing antibiotics (ampicillin, 100 μg ml^{-1}; nalidixic acid, 40 μg ml^{-1}; erythromycin, 30 μg ml^{-1}; kanamycin, 100 μg ml^{-1}; rifampicin, 20 μg ml^{-1}; tetracycline, 30 μg ml^{-1}) and growing the cultures for 48 h at 30 °C.

2.6. Analysis of polyphosphate production

Total intracellular polyphosphates were assayed by a modified method described by McGrath and Quinn [32]. Isolates were grown in MSM medium (50 ml) with glucose (20 mM) as a carbon source for 5 days. Cells were harvested at 5000 rpm for 15 min at 4 °C and washed twice in 4 ml wash buffer (1.5 M NaCl; 0.01 M EDTA; 1 mM KF). Cell pellet was resuspended in 4 ml of wash buffer and sonicated on ice in two pulses of 10 s at 20 kHz, with 2 min interval. Obtained homogenate was centrifuged for 1 min at 14,000 rpm at 4 °C and polyphosphate was precipitated from the supernatant for 1 h at room temperature by adding equal volume of precipitation solution (0.5 M Tris–HCl pH 8; 0.25 M EDTA pH 8; 2.5% CTAB; 025 M NaCl, and 50% $BaCl_2$). Precipitated polyphosphate was collected by centrifugation at 14,000 rpm for 10 min at 4 °C. Pellet was washed twice with 4 ml of polyphosphate wash buffer (50 mM CH_3COONa and 1 mM KF in C_2H_5OH). Residue was resuspended in 2 ml of 0.154 M NaCl and incubated for 24 h at 28 °C. To determine the concentration of polyphosphate 200 μl of concentrated HCl was added to 1 ml of polyphosphate extract and incubated for 45 min at 100 °C. Phosphate concentration was determined according to Carter and Karl [33].

2.7. PHA polymer isolation and analysis

For the purpose of testing the strains for the ability to accumulate polyhydroxyalkanoate, MSM medium with decreased N concentration (NH_4Cl, 0.25 g l^{-1}) was used. Carbon source was glucose (20 mM) or toluene (150 μl supplied in the central column). Cultures (50 ml) were grown shaking at 200 rpm in an incubator at 30 °C. Cells were collected by centrifugation (5000 × *g* for 10 min at 4 °C) and washed twice with an equal volume of potassium phosphate buffer (50 mM, pH 7.4) and freeze-dried. The polymer content was determined by subjecting 5–10 mg lyophilized whole cells to acidic methanolysis according to published protocols [34,35]. The 3-hydroxyalkanoic acid methyl esters were assayed by GC using a Hewlett Packard HP6890 chromatograph equipped with a BP-20 capillary column (30 m × 0.25 mm, 0.25 mm film thickness; J & W Scientific) and a flame-ionization detector (FID). A temperature program of 60 °C for 3 min, temperature ramp of 5 °C/min, 200 °C for 1 min was used. Total PHA content was determined as a percent of cell dry weight (CDW).

2.8. Detection and quantification of exopolysaccharide producing isolates

For the assessment of EPS production based on colony color and morphology, strains were grown on tryptone agar plates (tryptone, 10 g l^{-1}; agar, 10 g l^{-1}) supplemented with Congo Red (40 mg ml^{-1}), and Coomassie Brilliant Blue (20 mg ml^{-1}) as described by Arciola et al. [36]. The plates were incubated at 30 °C for 4–7 days.

Exopolysaccharide isolation and quantification was carried out as described previously [37]. Strains were cultured in MSM media with glucose as a carbon source (25 ml) and nutrient broth media (NB; meat extract 2 g l^{-1}, yeast extract 2 g l^{-1}, bacto peptone 5 g l^{-1} and NaCl 4 g l^{-1}). Bacterial cells were removed by centrifugation (9000 × *g* for 30 min at 4 °C, Sorvall RC-5B Super Speed Centrifuge; Du Pont Instruments). The clear supernatant was collected and a solution of KCl (25%, w/v) was added to a final concentration of 1% (w/v). EPS were precipitated by adding three volumes of methanol (75 ml) and incubating at 4 °C for 16 h. Precipitated EPS were collected by centrifugation (10,000 × g for 30 min at 4 °C, Sorvall RC-5B Super Speed Centrifuge; Du Pont Instruments), finally dissolved in distilled water (2 ml), freeze-dried and weighed.

3. Results

3.1. Sediment sample properties and isolation and identification of aromatic hydrocarbon degrading bacteria

The water portion of the sediment sample (river water) was pH 6.5. Dried sediment sample contained 190.1 g kg^{-1} of total C, while there was 9.4 g kg^{-1} of total N, 23 g kg^{-1} of total H and 5.9 g kg^{-1} of S. It also contained 302.8 mg kg^{-1} of Zn, 90.1 mg kg^{-1} of Hg, 40.7 mg kg^{-1} of Ni, 3.1 mg kg^{-1} of Pb, and 1.7 mg kg^{-1} of Cd. The total carbon to nitrogen ratio was (C/N) 20:1.

Using selective isolation strategy (heat pretreatment and a medium stimulating growth and sporulation of *Actinobacteria*, [38]) 225 isolates based on differing colony morphology and growth characteristics were selected. They were screened for the ability to use 15 aromatic compounds (all common pollutants found in industrial effluents) as a sole source of carbon and energy (Fig. 1). From initial 225 isolates, 88 were not able to utilize any of substrates tested as a sole source of carbon and energy. 61% of the isolated strains were able to use at least one of the aromatic compounds. From these, 56 isolates could use up to three different compounds, 43 could use 3–8 compounds, 20 isolates could use 9–14 compounds, and 18 could use all 15 compounds tested. These last two groups (38 isolates) were selected for further study as wide-range aromatic degraders (Fig. 1). Toluene and Br-benzene could be utilized as a sole source of carbon and energy by all 38 isolates. On the other hand, *o*-nitrophenol and *p*-xylene could be utilized by 25 and 22 isolates, respectively (Fig. 1).

According to 16S rDNA identification out of these 38 isolates, 34 were Gram-positive with 16 strains belonging to *Bacillus* genus, 7 to *Rhodococcus*, 6 to *Arthrobacter*, two to *Streptomyces* genus (TN10 and TN121), two to *Gordonia* genus (TN103 and TN108) and one to *Staphylococcus* genus (TN104). We also isolated 4 Gram-negative bacteria, two *Sinorhizobium* sp. (TN21 and TN321), one *Acinetobacter* sp. (TN302) and one *Pseudomonas* sp. (TN301).

Phylogenetic tree of 34 Gram-positive TN isolates consists of two independent clades: *Actinomycetales* (*Gordonia, Arthrobacter, Rhodococcus* and *Streptomyces*), and *Bacillales* (*Bacillus* and *Staphylococcus*) (Fig. 2). All isolates shared 93–100% sequence homology with their closest matching sequence in GenBank. Isolates TN105, TN221 and TN103 were annotated as *Rhodococcus* sp., *Arthrobacter* sp., and *Gordonia* sp., respectively, sharing 93%, 94% and 95% sequence similarity to closest species. Upon phylogenetic tree construction, these strains appeared on longer branches than their closest relating species, showing greater genetic distance. Lower percent of similarity (93–95%) and their position in phylogenetic tree indicates that *Rhodococcus* sp. TN105, *Arthrobacter* sp. TN221 and *Gordonia* sp. TN103 are new strains (Fig. 2).

Out of 34 Gram-positive isolates, 16 were able to degrade all 15 compounds. This group consisted of 6 *Rhodococcus* sp., 5 *Bacillus* sp., 2 *Gordonia* sp., 2 *Arthrobacter* sp. (TN110 and TN201), and *Staphylococcus* sp. TN104. *Bacillus* sp. TN42, *Rhodococcus* sp. TN401, could grow on the rich medium on a wide range of temperatures (5–42 °C) while *Bacillus* sp. TN30 and *Rhodococcus* sp. TN113 were able to grow at 5 °C (TN30, TN113) and 12 other isolates were able to grow at 42 °C (TN10, TN22, TN41, TN105, TN106, TN107, TN109, TN122, TN125, TN128, TN130, TN222). From these, *Rhodococcus* sp. TN401 was distinct in its ability to grow on wide temperature range and also degrade all 15 aromatic compounds.

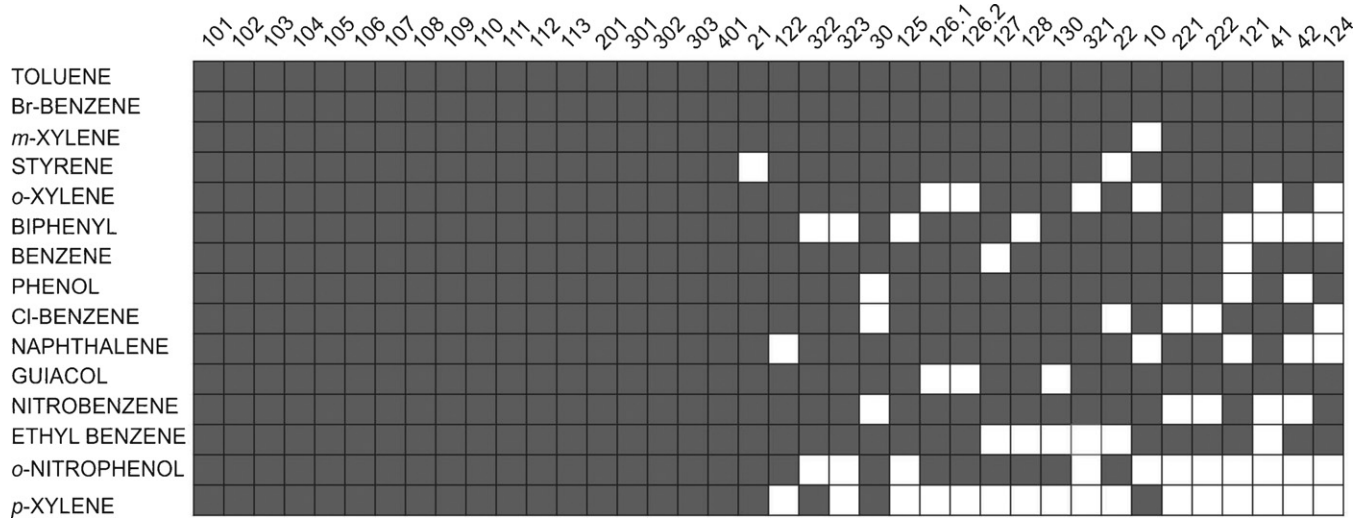

Fig. 1. Growth of bacteria isolated from contaminated surface river sediments on aromatic hydrocarbons. Strains were grown on solid MSM medium with aromatic hydrocarbon as a sole source of carbon and energy (growth (■) and absence of growth (□) on an aromatic compound).

3.2. Growth of isolates in the presence of heavy metals and antibiotics

We tested the ability of Gram-positive aromatic degrading isolates to grow in the presence of high concentrations of heavy metal ions (Cd^{2+}, Ni^{2+}, Hg^{2+}, Cu^{2+}, Fe^{3+} and Zn^{2+}) and a range of 6 different antibiotics and determined that 21 isolates could grow in the presence of at least one metal or antibiotic (Table 1). When tested on concentrations that were 20-fold higher than maximum concentrations recommended by the regulation on hazardous materials by the Serbian Ministry of the Environment, 31 isolates were able to grow in the presence of 17 μM Ni^{2+}, 16 in the presence of 535 μM Cd^{2+}, 5 in the presence of 270 μM Hg^{2+}, 3 in the presence of 892 μM Fe^{3+}, and 6 in the presence of 31.5 mM Cu^{2+}. None of the strains was able to grow in the presence of 92.5 mM Zn^{2+} (data not shown). However, when much higher salt concentrations that correspond to 100 mM concentrations of metal ions were used, these numbers were reduced to two isolates being able to grow in the presence of Ni^{2+}, one in the presence of Cd^{2+}, 4 isolates in the presence of Hg^{2+}, two in the presence of Fe^{3+} and 5 isolates in the presence of Cu^{2+} (Table 1). Among these, *Bacillus* sp. TN303 could grow in the presence of three different heavy metal ions (Cu^{2+}, Hg^{2+}, and Fe^{3+}).

Table 1
Wide-range aromatic degrading isolates able to grow in the presence of heavy metals (100 mM) and antibiotics.

Isolate	Metal[a] (100 mM)	Antibiotics					
		Am[b]	NA	Ery	Km	Rf	Tet
TN303	Cu^{2+}, Hg^{2+}, Fe^{3+}	+	+	+	+		
TN112	Cd^{2+}, Fe^{3+}				+		
TN221	Cu^{2+}, Ni^{2+}						
TN113	Cu^{2+}	+	+	+			
TN101	Hg^{2+}				+		
TN102	Cu^{2+}	+					
TN127	Ni^{2+}	+					
TN401	Cu^{2+}						
TN110	Hg^{2+}						
TN107		+		+			+
TN125		+	+				
TN104		+		+			
TN105		+					
TN106		+					
TN201			+				
TN30			+				
TN41						+	
TN42						+	
TN10		+					
TN111					+		
TN122				+			

[a] Isolates were grown on solid MT medium using lactate as a carbon source supplemented with metal salts ($NiCl_2$, $CdSO_4$, $HgCl_2$, $FeCl_3$ and $CuSO_4$) at appropriate concentrations to match 100 mM of metal ions.

[b] Am – ampicillin, 100 μg ml^{-1}; NA – nalidixic acid, 40 μg ml^{-1}; Er – erythromycin, 30 μg ml^{-1}; Km – kanamycin, 100 μg ml^{-1}; Rf – rifampicin, 20 μg ml^{-1}; Te – tetracycline 30 μg ml^{-1}.

Isolated strains had the ability to grow on toluene and naphthalene as sole source of carbon and energy in the presence of appropriate metals (data not shown), thus confirming that the ability to degrade aromatic compounds is not affected by the presence of heavy metal ions.

None of the isolated strains was resistant to all of 6 antibiotics tested, while 22 were resistant to at least one antibiotic (Table 1). Ampicillin resistance was exhibited by 10 isolates, while only one strain was resistant to tetracycline. Isolate *Bacillus* sp. TN303, which could degrade all 15 compounds, and able to grow in the presence of 3 different heavy metals at 100 mM concentration, was also resistant to 4 different antibiotics (ampicillin, nalidixic acid, erythromycin, kanamycin and tetracycline) (Table 1).

3.3. Polyphosphate accumulation

Inorganic polyphosphate was detected in *Bacillus*, *Arthrobacter*, *Rhodococcus*, and *Streptomyces* species (Fig. 3). Five *Bacillus* strains (TN107, TN109, TN130, TN126.2, and TN322) could accumulate polyPi under conditions tested, while TN322 showed the highest level of accumulation with 68 nmol Pi per mg of total protein that was 2.4 to 5.6-fold higher in comparison to other *Bacillus* sp. (Fig. 3). All 4 *Arthrobacter* strains (TN110, TN201, TN221, and TN222) accumulated polyPi to levels between 35 and 43 nmol Pi per mg of protein.

3.4. Polyhydroxyalkanoate accumulation

Three *Bacillus* strains (TN22, TN122, and TN128) were able to accumulate PHA from glucose and toluene as a carbon source. Polymer accumulated was short chain length PHA consisting of

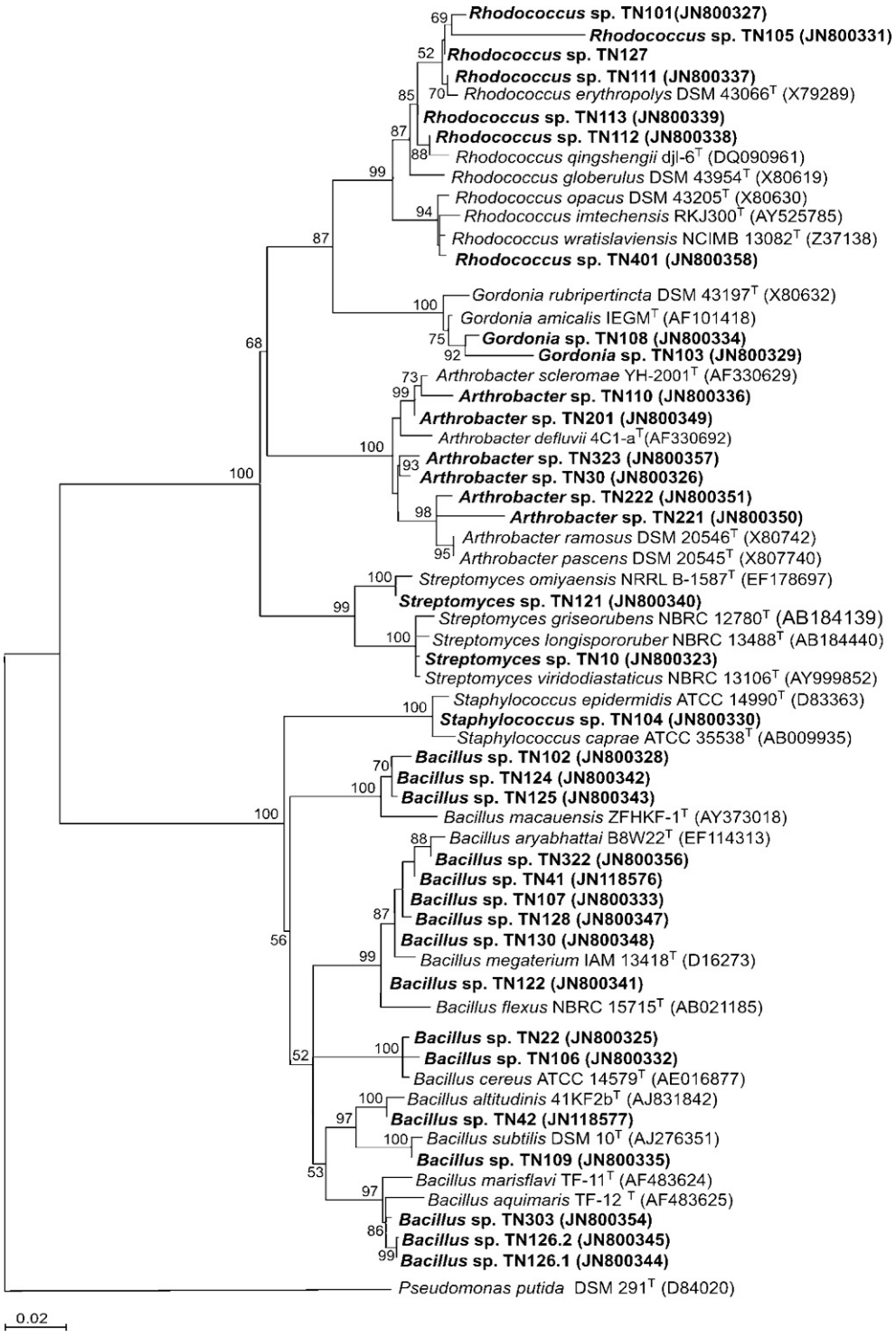

Fig. 2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship of Gram-positive TN isolates (designated in bold) and closely related strains. Bootstrap values at branch points are expressed as a percentage of 1000 replications. *Pseudomonas putida* DSM 291^T was used as an outgroup. GeneBank accession numbers are in brackets. The scale bar represents 0.02 substitutions per nucleotide position.

3-hydroxybutirate monomer units. Levels of polymer accumulation by each strain were similar when glucose and toluene was used as a carbon source (Fig. 4). However, strain TN22 could accumulate PHB to 12% and 10% of total dry cell weight from toluene and glucose, respectively, while the accumulation by strain TN122 was 2.5 and 3-fold lower in comparison to TN22 (Fig. 4). Strains TN22, TN128 and TN122 could degrade 11, 12 and 13 aromatic compounds, respectively (Fig. 1).

3.5. EPS production

EPS production was detected using Congo Red Stain, which is known to bind extracellular polysaccharides [36], in 4 strains when grown on tryptone agar, namely *Bacillus* sp. TN124, TN125, and TN128, and *Rhodococcus* sp. TN105 (Fig. 5A). Strains indicated as EPS producers stained much deeper red in comparison to the rest of isolates. In addition the stain was equally distributed throughout

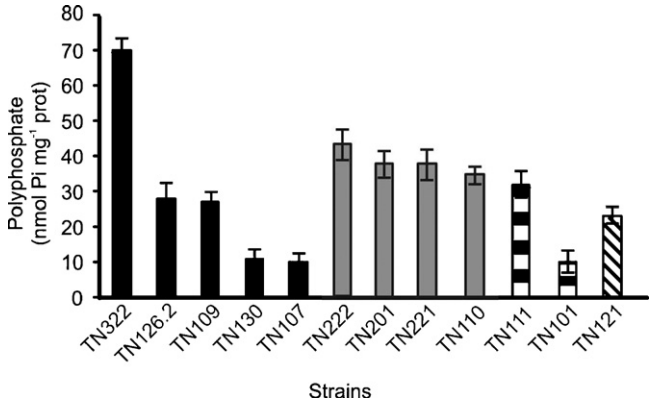

Fig. 3. Inorganic polyphosphate (polyPi) accumulation (nmol Pi mg^{-1} protein) by bacteria isolated in this study. Cultures were grown for 5 days in MSM medium with glucose (0.1%, w/v) as a carbon source. (■ *Bacillus*, ▩ *Arthrobacter*, ▬ *Rhodococcus*, and ▨ *Streptomyces* species). All data are the average of at least three independent experiments.

colonies, while it was restricted to the center of the colony in the strains that were not producing EPS.

Although strains grew equally well during the production of EPS in the liquid media (data not shown), *Rhodococcus* sp. TN105 accumulated 3.9-, 1.9- and 1.4-fold higher amounts of EPS in comparison to three *Bacillus* sp. TN 124, TN125 and TN128, respectively (Fig. 5B). Generally, the production of EPS was better in the minimal medium in comparison to nutrient broth. Isolate *Rhodococcus* sp. TN105 produced 1.4-fold more, while isolate *Bacillus* sp. TN128 produced 2.6-fold more EPS in the MSM in comparison to NB (Fig. 5B).

Strains that were able to produce EPS could not grow on MTM supplied with 100 mM concentration of any metal used, however *Bacillus* sp. TN125 and *Rhodococcus* sp. TN105 were able to grow in the presence of elevated concentrations of Ni^{2+} and Cd^{2+}, 17 μM and 535 μM, respectively (data not shown). Isolate TN125 could degrade 12 out of 15 aromatic compounds tested, while TN105 could degrade all 15 (Fig. 1). Neither of them was able to accumulate polyPi or PHA (Figs. 3 and 4), while both were resistant to ampicillin (Table 1).

4. Discussion

Many rivers suffer from intensive domestic and industrial wastewater pollution. As a consequence, river sediment contamination from various industrial effluents, spills and surface runoff

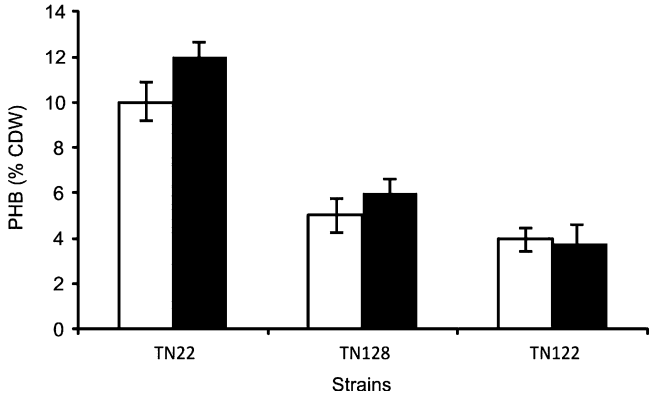

Fig. 4. PHB accumulation by aromatic degrading TN strains (expressed as a percentage of cell dry weight (CDW)). Pure cultures were grown under nitrogen limited conditions (0.07 g of N per liter of medium) with glucose (□; 20 mM; 1.44 g of C per liter of medium) and toluene (■; 150 μl supplied as vapor) as a carbon source.

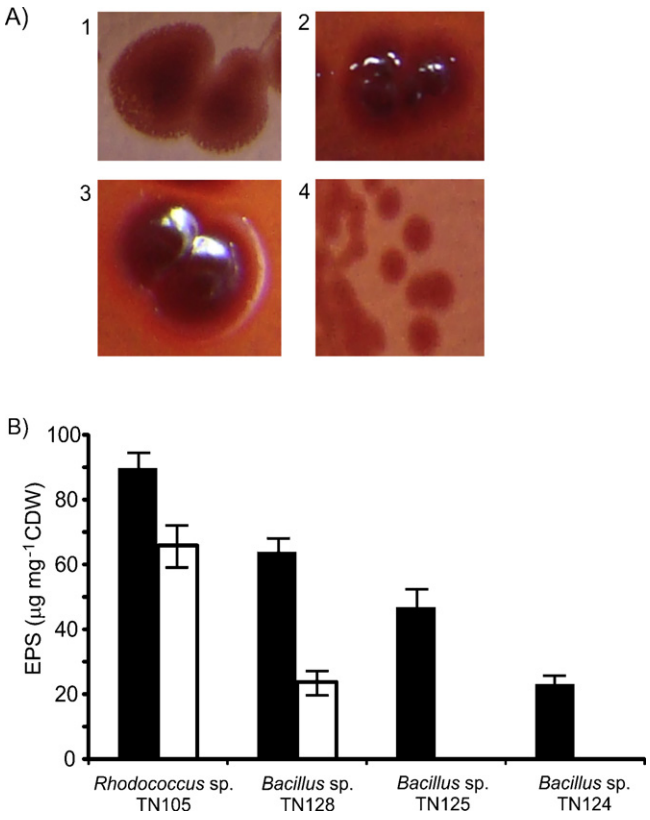

Fig. 5. Exopolysaccharide production by aromatic degrading TN strains: (A) Colonies of *Bacillus* sp. (TN124, TN125, and TN128; 1–3) and *Rhodococcus* sp. TN105 (4) upon staining with Congo Red dye; (B) EPS produced during growth in MSM (■) and NB (□) liquid media.

is of great concern worldwide. Aromatic compounds are among the most widespread hazardous pollutants in nature and pose an environmental threat due to their toxicity and recalcitrance. In particular, low volatile PAHs have great affinity for sediments [39]. Most organic carbon metabolism in running waters occurs on or in sediments [40]. Bacteria play a key role in organic carbon processing and influence many aspects of the chemistry and biology of river ecosystems [41]. Therefore, microbial transformation is regarded as one of the most important processes for PAH and other pollutants removal from the aquatic and sediment environments [1]. It is then anticipated, that although harsh, contaminated sediments can be rich source of bacterial diversity. Using direct cultivation approaches, many important microorganisms, mostly *Pseudomonas* and closely related species, have been isolated from the contaminated sites [42,43]. Indications that Gram-positive bacteria are present in those samples are there, and often come from the cultivation-independent approaches [44]. Thus a diverse group of Gram-positive, spore forming bacteria still remains an untapped source of bioremediation relevant microorganisms.

Indeed, river sediment exposed to petrochemical industry effluents was confirmed to be rich source of microorganisms. In this study, we managed to selectively (heat pretreatment and casein starch medium) isolate 34 metabolically versatile Gram-positive bacterial strains in respect to their potential remediation applications (Figs. 1 and 2). By 16S rDNA analysis coupled with biodegradation ability and other physiological characteristics, we confirmed that at least 3 isolates were novel strains of *Rhodococcus* (TN105), *Arthrobacter* (TN221) and *Gordonia* (TN103) genus. Out of all strains used in construction of the phylogenetic tree, strains *Rhodococcus imtechensis* RKJ300^T, *Rhodococcus quingshengii*

djl-6^T and *Arthrobacter defluvii* 4C1-a^T have the ability to degrade aromatic compounds [45–47]. Although strain TN401 shares 98% 16S rDNA sequence similarity with *R. imtechensis* and 96% with *R. quingshengii*, while strains TN201 and TN110 share 98% and 97% sequence similarity with *A. defluvii*, they degrade broader range of aromatic compounds and are resistant to presence of metal ions (Fig. 1, Table 1). Interestingly, isolates TN108 and TN103 clustered with *Gordonia amicalis* IEGM^T that was reported to desulphurize dibenzothiophene [48].

It is of great importance to the bioremediation potential of these isolates that so many single isolates could degrade such a wide range of aromatic compounds, including halogenated and nitro derivatives, as well as polyaromatic compounds e.g. naphthalene, as contaminated sites are never contaminated by a single pollutant rather a complex mixture. Usually, literature reports on strains as potentially good candidates for bioremediation studies are focusing on single substrate. However, we have recently reported *Bacillus* sp. PS11 that can degrade 16 different aromatic compounds [49]. Another comprehensive study was carried out by Stapleton et al. [10] in which they have tested 7 different hazardous compounds and found that 20% of isolated strains were able to utilize all of the substrates. Wide spectrum of diverse Gram-positive isolates capable of utilizing 15 different aromatic pollutants has not been reported before. This suggests that strains isolated in this study may be a rich source of potentially novel oxygenases that are involved in biodegradations with a broad substrate range which could be applied to other areas of biotechnology such as biocatalysis [50].

There is often co-occurrence of aromatic hydrocarbons and heavy metals in polluted sites [7,21]. This usually poses difficulties for bioremediation applications [51]. Therefore, bacterial strains that are able to interact with more than one contaminant are of particular interest for bioremediation processes. As zinc, mercury and nickel could be detected in the sediment sample, we assessed the ability of the isolates to grow in the presence of high concentrations of these metals (100 mM). Indeed, we obtained 9 highly metal tolerant isolates with 50% of them being *Rhodococcus* sp. (Table 1). Metal tolerant strains are usually isolated from mine tailing sites [52], however the sediment sample in this study proved rich source of high metal tolerant bacteria. Only recently, Nithya et al. [53] isolated 24 strains from marine sediments exhibiting resistance to several heavy metals including arsenic, mercury, cobalt, cadmium, lead and selenium. Strains were considered resistant if they showed more than 50% growth in 25 mM concentrations of metals, which is 4-fold lower than concentrations reported here. However, they also isolated strains of *Bacillus* sp., *Staphylococcus* sp., and *Planococcus maritimus* that were resistant to cadmium, copper, mercury, and zinc, at 300 mM concentrations. This is the first comprehensive report of the Gram-positive microorganisms capable of a wide range aromatic degradation and being able to grow in the presence of high concentrations of heavy metals. Malik and Aleem [22] isolated metal resistant *Pseudomonas* sp. isolates from the river using 10-fold lower concentrations of metals in comparison to this study, however they suggested co-occurrence of metal and antibiotic resistance markers. Resistance to antibiotics is widespread in isolates from contaminated aquifers [10]. We also observed high incidence of resistance to ampicillin, nalidixic acid and erythromycin among a wide range aromatic degrading organisms that are resistant to heavy metals (Table 1). Nevertheless, we have not observed high incidence of multiple antibiotic resistances among wide-range aromatic degraders, as this is important when considering application of these strains in the field bioremediations. Only *Rhodococcus* sp. TN401 being resistant to copper was not sensitive to any of the antibiotics tested, making it the most suitable candidate for the application in mixed contamination studies.

Considering the high total carbon content of sediment sample and high C/N ratio, we decided to investigate the ability of isolates to accumulate range of biological polymers (polyPi, PHA, EPS). These biopolymers are usually produced in the response to environmental stress conditions, but also have industrial applications. In this way, the potential application ability of the isolated strains could be extended beyond bioremediations. Polyphosphates (polyPi), linear chain of phosphate residues linked by high-energy phosphoanhydride bonds are among the most widely distributed biopolymers [54]. Various roles of polyPi have been demonstrated, including energy preservation and sequestration of divalent cations while, it may also play an important role in the physiological adaptation of microbial cells during growth and development, and in their response to nutritional and environmental stresses [54]. For example, under osmotic or nutritional stress it has been demonstrated that *E. coli* can accumulate polyPi [55]. We have determined that among 34 Gram-positive wide range aromatic degraders, 11 could accumulate polyPi to various levels (Fig. 3). Fifty percent of isolated polyPi accumulators could also degrade all 15 aromatic compounds. PolyPi accumulation depends on the levels of Pi in the medium. *Bacillus* sp TN322 when grown in the presence of 75 mM Pi, was able to accumulate 68 nmol of Pi per mg of total protein, while previously reported *Bacillus cereus* accumulated 2 nmol Pi per mg protein when grown in rich medium supplemented with 2 mM Pi [56].

Polyhydroxyalkanoates are valuable bacterial biopolymers that usually accumulate under stress nutrient conditions, such as surplus of carbon coupled with limitation of nitrogen [15]. As sediment sample had C/N ratio of more than 20:1, we assumed that among 34 wide range aromatic degrading isolates we would be able to detect abundance of PHA producers. However, only 12% isolates had this ability (3 strains of *Bacillus* genus; Fig. 4). Reported levels of PHB accumulated by *Bacillus* sp. from glucose [57] are 2–8-fold higher in comparison to PHB accumulated by *Bacillus* species in this study. However, TN strains could accumulate PHB from toluene, which was not shown before. Accumulation of similar polymer poly(3-hydroxybuturate-co-3-hydroxyvalerate) to same level (10% CDW) was previously reported for *Rhodococcus aetherivorans* IAR1 [58]. Although co-accumulation of polyphosphates and polyhydroxyalkanoates was previously observed in *Pseudomonas* strains [59], we have not detected this ability in *Bacillus* isolates.

Exopolysaccharides are another important biopolymer secreted by microorganisms. Due to their many interesting physical and chemical properties, such as stabilizing, suspending, thickening, film-forming and water retention capability, EPS are used in various industrial sectors (detergents, textiles, adhesives, pharmaceutical) [60]. EPS are involved in biofilm formation and microbial protection against environmental stress and dehydration [61]. Considering the role of biofilms in adaptation to different environments and survival of microorganisms, it was suggested that biofilm-mediated bioremediation is a more effective than bioremediation with planktonic bacteria [61]. We have determined that among a wide range aromatic degrading isolates, there are 4 capable of EPS production including members of *Bacillus* and *Rhodococcus* genus (Fig. 5). It was previously shown that *Bacillus subtilis* and *P. putida* with the ability to produce EPS have increased Cd^{2+} adsorption capacity compared to strain with impaired EPS production [62]. Furthermore, EPS promoting effects in Cd^{2+} accumulation were more remarkable in Gram-positive *B. subtilis* cells than on Gram-negative *P. putida* cells [62]. In this study, strains producing EPS could not grow in the presence of 100 mM metal ions, however could tolerate elevated levels of metal ions. On the other hand, multiple resistance to antibiotics was shown to be more frequent among exopolysaccharide-forming strains [36]. In our study, two strains capable of EPS formation were resistant to ampicillin (Table 1).

5. Conclusion

As microbial transformation is of immense importance for PAH and other pollutants removal from various environments, continuous isolation of applicable bacteria is needed. Overall, we have isolated 34 metabolically versatile Gram-positive microorganisms from the river sediment with great application potential for mixed pollution bioremediation studies, as well as in other biotechnological applications. Of special interest are Gram-positive spore forming isolates (*Bacillus* and *Streptomyces*) that readily adapt to harsh environmental conditions and previously have not been regarded for remediation studies [51]. These diverse and robust isolates are a valuable addition to the current microbial and green biotechnology resources.

Acknowledgments

This work was supported by the grant 173048 from Ministry of Education and Science, Republic of Serbia. We also thank Dr Snezana Trifunovic from the Faculty of Chemistry, University of Belgrade for carrying out elemental analysis.

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