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Full Length Research Paper

Biodegradation of pyrene by a newly isolated *Proteus vulgaris*

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This work was aimed at identifying a new strain which can degrade pyrene effectively and quickly. Strain 4Bi was identified as *Proteus vulgaris* based on several morphological, biochemical characters and 16S rRNA phylogeny by using conventional methods, API and 16S rRNA gene sequence analysis. 16S rRNA gene sequence of the bacterium has been deposited in GenBank database under the accession number FJ799903. Carbon free mineral medium (CFMM plus pyrene) used for *P. vulgaris* 4Bi was excessively sufficient for positive improvement of bacterial growth and pyrene removal. The growth rates and pyrene degradation rates were increased depending on the time in CFMM. At a biodegradation rate with an initial pyrene concentration of 0.5 mg /ml, 71.5% of pyrene was degraded in 7 days. Pyrene-degradation metabolites were detected by HPLC and TLC analyses. Pyrene is a highly stable compound containing four fused aromatic rings and is one of the representative higher molecular weight-polycyclic aromatic hydrocarbons that are recalcitrant to biodegradation. Whereas, our bacterium could degrad pyrene effectively and quickly. With our study, the strain of *P. vulgaris* was firstly reported among pyrene-degrading bacteria. The ability of this strain is very useful for the remediation of pyrene one of PAH pollutants. Also, degradation metabolites detected in the study are not toxic or nonpersistent compounds in nature. These results indicated that this novel pyrene-degrading strain has a strong potential for further bioremediation processes without undesirable metabolites.

Key words: Polycyclic aromatic hydrocarbons, pyrene, *Proteus vulgaris,* identification, biodegradation, metabolites.

INTRODUCTION

Pyrene is one of the polycyclic aromatic hydrocarbons (PAHs) which have been considered to be priority pollutants by the United States Environmental Protection Agency (US EPA) (Yan et al., 2004). Pyrene ($C_{16}H_{10}$) has a low biodegradability and high persistence in the environment (Yan et al., 2004). Pyrene possessing four benzene rings is a by-product of gasification processes and other incomplete combustion processes. Due to its chemical structure is highly recalcitrant and resistant to microbial degradation (Seo et al., 2009). The structure of pyrene is found in the molecule of carcinogenic PAHs. Pyrene could become a neurotoxicant and skin or sense organ toxicant, very toxic to terrestrial and aquatic lives with long lasting effects. Biodegradation is an important method of PAH removal from contaminated sites (Peng et al., 2008). Therefore, pyrene as indicator for PAHs-contamination monitoring in environmental samples and as a model compound of the PAH group to study the photocatalytic degradation and biological degradations of PAHs was investigated by various studies (Klankeo et al., 2009; Dong et al., 2010). The microbial PAHs-degradation is an effective strategy to remove pollutants from the environment by bioremediation. Diverse microorganisms including algae, heterotrophic bacteria, cyanobacteria and fungi have been reported to play a role in PAH degradation (Kim et al., 2005; Seo et al., 2007; Klankeo et al., 2009; Seo et al., 2009). A large number of bacteria isolated from the environment have the ability to degrade pyrene as summarised in Table 1.

Pyrene degradation by both pure and mixed cultures

Table 1. Pyrene-degrading bacteria isolated from the environment.

Bacteria	References
Mycobacterium sp.	Cheung and Kinkle (2001), Vila et al. (2001), Sho et al. (2004) and Kim et al. (2005)
Rhodococcus sp.	Walter et al. (1991)
Sphingomonas sp.	Liu et al. (2004) and Baboshin et al. (2008)
Sphingomonas paucimobilis	Kastner et al. (1998)
Sphingomonas yanoikuyae JAR02	Rentz et al. (2008)
Bacillus cereus	Kazunga and Aitken (2000)
Burkholderia cepacia	Juhasz et al. (1997)
Cycloclasticus sp. P1	Wang et al. (2008)
Pseudomonas fluorescens	Caldini et al. (1995)
Pseudomonas stutzeri	Kazunga and Aitken (2000)
Stenotrophomonas maltophilia	Boonchan et al. (1998) and Juhasz et al. (2000)
Novosphingobium pentaromativorans	Sohn et al., (2004)
Leclercia adecarboxylata PS4040	Sarma et al. (2004)
Paracoccus sp.	Zhang et al. (2004)
Diaphorobacter sp. and *Pseudoxanthomonas sp.*	Klankeo et al. (2009)
Saccharothrix xinjiangensis PYX-6T	Hu et al. (2004)
Yeosuana aromativorans GW-1T	Kwon et al. (2006)

has been reported before (Kazunga and Aitken, 2000; Ho et al., 2000). However, to our knowledge, no previous report shows *P. vulgaris* growth on pyrene. This knowledge about *Proteus* sp. is scarce, although the biomass and diversity of this genus members were higher in contaminated sites. Many researches have reported the potential biodegradation/biotransformation of xenobiotics by the members of *Proteus* genus. *Proteus* species from various environments are able to utilize and degrade many variety of toxic materials. Among these compunds are different heavy metals, pesticides, insecticides, herbisides, other toxic xenobiotics, reactive textile dyes, crude oil, used motor oil, phenolic compounds, formaldehyde, formic acid, trichloroethane and methyl tert-butyl ether. *Proteus* sp. have an important place in environmental pollution bioremediation (Okerentugba and Ezeronye, 2003; Patil et al., 2008; Abdulsalamand Omale, 2009; Zhang et al., 2009; Saratale et al., 2010). We hypothesized that *P. vulgaris* would have potential capacities to degrade pyrene.

The enzymes and genes involved in PAHs degradation have not yet been fully elucidated. This has been made difficult due to the low aqueous solubility of PAHs especially HMW-PAHs. Microbial diversity and their application in biodegradation of PAH have been reviewed (Cheung and Kinkle, 2001; Jain et al., 2005). However, recent works on new microbial diversity and ecosystem responses in the environment have pointed out that several microorganisms in the environment remain to be studied. Thus, it was also thought that a number of microorganisms including PAH-degrading micro-organisms are still undiscovered. In order to accumulate more information to improve the success of bioremediation, it is beneficial to search for novel microorganisms that have the ability to degrade PAHs especially HMW-PAHs like pyrene (Cheung and Kinkle, 2001; Peng et al., 2008). Therefore, the aims of this study was to assess the ability of novel strain isolated from biofilm-waters of petrochemical industry plant as a different origin to metabolize pyrene.

The identity of the strain was also revealed by 16S rRNA phylogeny. In addition, we wanted to prove microbial degradation of pyrene by using this bacterium in pyrene-supplemented culture conditions and information on metabolites of degradation. The present work describes the characterization of novel pyrene-degrading strain including the first representative of *P. vulgaris* that is able to utilize pyrene as sole carbon source.

MATERIALS AND METHODS

Bacterial strain

A bacterium (designated strain 4Bi) was isolated from biofilm in waters of a petrochemical industry plant located at Izmir, Turkey. The strain was identified by the cultural, morphological, biochemical methods and the 16S rRNA gene sequencing. During all the assays, stock cultures of microorganism were grown and maintained at 30 and 4°C, respectively by periodic transfer on tryptic soy agar (TSA) slants (17 g tryptone, 3 g soytone, 10 g dextrose, 5 g NaCl, 3.5 g K_2HPO_4, 1.5% agar in 1,000 ml distilled water).

Isolation and enrichment of strain 4Bi

To isolate and enrich strain 4Bi, samples taking from cooling

solution and thin biofilm layers of cooling tower of a petrochemical industry were passed aseptically through tenfold dilutions of Ringers solution to yield final concentrations of 10^{-4} and 10^{-5}. Spread plates were prepared from each dilution (0.1 ml) on TSA supplemented with pyrene solution (0.5 mg pyrene/1.0 ml acetone) and incubated at 30°C for 3 days. The isolates were picked from each plate into Erlenmeyer flasks (250 ml) containing tryptic soy broth (TSB) (plus pyrene) (TSA without agar) (100 ml) and grown at 30°C with gyratory shaking (200 rev min^{-1}) for 3 days.

Phenotypic characterization of strain 4Bi

The strain was tested for some cultural, morphological and biochemical properties like utilization of different carbohydrates as carbon source, five proteins as nitrogen source and some enzyme activities by the conventional methods and the commercial API identification kit (bioMérieux, France). A single well isolated colony from TSA (young cultures, 24 h old) was added into an ampule of API NaCl 0.85% medium (5 ml) and then this suspension was used in each well of the kits and incubated in a humidity chamber at 37°C for 24 h. After incubation, the colour reactions are read (some with the aid of added reagents) and the reactions (plus the oxidase reaction done separately) are converted to a seven-digit code which is called the 'analytical profile index' from which name the initials "API" are derived. The code can be fed into the on-line manufacturer's database for the identification using a computer software containing an APILAB Plus Program.

DNA extraction and PCR amplification

Genomic DNA was extracted from 1 ml of isolate cultured (almost 10^9 bacterial cell) in the deionized water using the ZR fungal/bacterial DNA MiniPrep™ extraction kit (ZymoResearch). All steps of the kit protocol were applied carefully. The DNA extracts required five additional 70% ethanol washes to eliminate salts and to obtain an amplifiable DNA. DNA concentrations and purity (A260/A280 ratio) were determined with a spectrophotometer (Varian 300). An A260/A280 ratio of 1.8 to 2.1 was considered acceptable for PCR-based procedures (Gonzalez et al., 2006). The extracted DNA was used as template to amplify the 16S rRNA genes by PCR with the universal forward primer 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and reverse primer 1522R (5´-AAGGAGGTTATCCANCCRCA-3´) (Thermo Scientific) (Lee et al., 2003). DNA was amplified by FastStart Taq DNA Polymerase dNTPack kit (Roche). PCR reaction (50 µl) contained 5 µl of each primer, 200 to 500 ng of genomic DNA, PCR reaction-polymerase buffer (with 20 mM $MgCl_2$), 1 µl of PCR Grade Nucleotid Mix (dNTP Mix), 10 µl of GC-RICH solution, 5 U/µl of FastStart Taq DNA polymerase and brought to 50 µl of deionized water. The amplification was performed with a thermal cycler (GeneAmp® PCR system 9700). Amplification conditions included a denaturation step for 5 min at 95°C followed by 35 cycles consisting of 55 s at 95°C, 40 s at 50°C and 1.5 min at 72°C and a final extension step for 7 min at 72°C. These conditions were modified from Ausubel et al. (1997).

The amplification was done with a a thermal cycler (Gene Amps PCR System 9700; Applied Biosystems).

Sequencing analysis and sequence access number

Amplified PCR products were analyzed by agarose gel electrophoresis. Also, DNA markers (5000 and 10000 bp, Fermentas DNA ladder) as control sequence were separated on 1% agarose gel by agarose gel electrophoresis containing 15 µl of ethidium bromide (10 mg/ml) for visualization on an ultraviolet light box (254 nm) (Ausubel et al., 1997). PCR products were sequenced by RefGen (Gen Researches and Biotechnolgy Centre), Turkey, and compared with those available in the GenBank database by using basic alignment search tool, BLAST of the NCBI to identify whether they aligned with closely related organisms (Lee et al., 2003; Adiguzel et al., 2009). This was done using the NCBI website: (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The 16S rRNA gene sequences of the species most closely related to our strain were retrieved from the database. The related sequences were preliminarily aligned with the default settings of CLUSTAL X (Thompson et al., 1997), and complete sequence alignments were performed using BLAST and manual comparison to secondary structures. The phylogenetic analysis and phylogenetic tree was inferred using the MEGA 4.0 (Tamura et al., 2007; Kumar et al., 2008).

Sequence alignment and analysis of similarity of the 16S rRNA genes were performed with CLUSTALX and MEGA 4.0 programs. The sequence obtained in this paper have been deposited in the GenBank database. The accession number for this fragment (1382 bp) is FJ799903.

Bacterial growth on plate medium supplemented with pyrene

Carbon-free mineral medium (CFMM) of Yuting et al. (2003) and Habe et al. (2004) was prepared in plates with some modifications. The CFMM used as the basal medium contained (per liter of distilled water) $(NH_4)_2SO_4$ 2.0 g, NaH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2 \cdot 7H_2O$ 0.1 g; and 100 ml of the stock solution (0.5 mg pyrene/1.0 ml acetone). For preparation of agar plates, 15 g/l of agar was added. The final pH of the medium was adjusted to 7.0 with 1 N HCl and the medium was sterilized (121°C for 20 min) prior to the addition of pyrene. The prepared CFMM includes pyrene as only one carbon source. The fresh-broth culture of *P. vulgaris* 4Bi in TSB (24 h culture at 30°C) was spreaded aseptically on CFMM plates supplemented with pyrene as the source of carbon and energy. The stock solution (0.5 mg/ml) of pyrene was made in acetone and was sterilized by millipore micro syringe filter (0.45 µm pore size). Pyrene was purchased from Sigma (purity 95%). Acetone solution of pyrene was added to CFMM agar and equal volume of acetone was used for control in dublicate. The inoculated plates were sealed with parafilm to prevent evaporation. After 72 h of cultivation (at 30°C), colonies of *P. vulgaris* 4Bi were appeared on plate media.

The pyrene utilization on CFMM plates by colonies was observed as clear zones (haloes) around them (Figure 1). With observation of growth and clear zones on the pyrene agar-plates, we suspected that *P. vulgaris* 4Bi was a bacterium degrading pyrene.

Biodegradation experiments

P. vulgaris 4Bi cultivated in TSB at 30°C for 24 h was used as inoculum. The cells were collected by centrifugation (8000 rpm for 10 min) and were washed twice in 0.85% NaCl. The initial biomass of the bacterium was determined by cell growth and the initial density of bacterial suspension was obtained by measuring the OD_{560} of 0.1 level of inoculum. Cell growth was determined through the colony-forming units (CFU) on CFMM agar (CFMM plus 1.5% agar). The agar plates were incubated for 1 week at 30°C. The pH of the culture medium was maintained at pH 7.0 with automated pumping of 5 M NaOH. The experiments were modified according to the previously reported methods of Romero et al. (2002) and Kim et al. (2005). A cell suspension of 0.1 ml was added into a 100-ml flask with 20 ml of liquid CFMM plus 0.5 mg/ml pyrene. The flask was incubated at 30°C for 7 days in a rotary shaker at 150 rpm. CFMM containing pyrene only and CFMM with inoculum but without pyrene were the negative control and growth control, respectively.

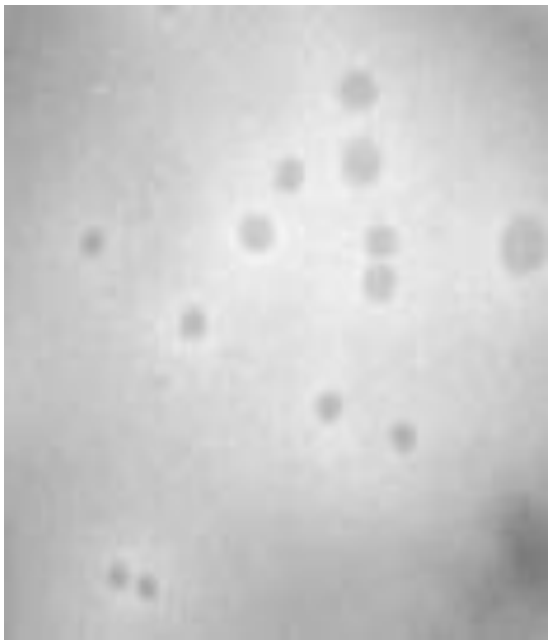

Figure 1. Photograph of bacterial colonies and clear zones of pyrene utilization on CFMM agar supplemented with pyrene (0.5 mg/ml) (incubation at 30°C, 72 h).

The optical density (OD) and the colony-forming units (CFU/ml) on CFMM agar plates were determined. The agar plates were incubated for 7 days at 30°C. To detect pyrene concentration from the cultured broth, the bacterial cells were harvested by centrifugation at room temperature (14,000 g, 15 min) and washed thoroughly with sterile 50 mM sodium phosphate buffer pre-chilled at 4°C. The buffer pH was adjusted to the same value as the previous culture medium with 5 M NaOH and 5 M phosphoric acid. For the determination of pyrene consumptions, while the flasks were incubated at 30°C and 150 rpm, the whole flasks were extracted after 1, 2, 3, 4, 5, 6 and 7 days. The samples were immediately mixed with 200 ml of cold ethyl acetate.

The extracts were dried over anhydrous NA_2SO_4, and evaporated to dryness at 40°C *in vacuo* and the residue was dissolved in acetonitrile (1.5 ml) to remove insoluble particles by filtration through a 0.2 µm membrane fillter and an aliquot of 20 µl was taken for the determination of residual pyrene concentrations by high performance liquid chromatography (HPLC) analysis. Triplicate samples were taken to examine the remaining pyrene concentration and total cell growth.

HPLC analysis

A series of pyrene solutions from 0.1 to 10 mg/ml was prepared and used to establish a standard curve for quantification. HPLC was performed with a liquid chromatograph (Shimadzu) equipped with an LC-10A pump, a SIL-9A automatic injector and an UV/Visible-Diode array detector at 254 nm. The separation column, Inerstil ODS column equipped with a guard column packed with the same stationary phase was 4.6 mm inside diameter x 150 mm. The mobile phase was acetonitrile:water (25:75, v:v). The flow rate was 1.0 ml/min. Each sample was injected at least three times and the mean calculated. Residual pyrene concentrations were expressed as mg/g of dry weight (Romero et al., 2002; Klankeo et al., 2009).

Pyrene was identified by its retention time under these conditions (21.7 min) in comparison with a commercially available standard and controlled with a calibration curve obtained with different pyrene concentrations.

Determination of degradation metabolites

For the purification of metabolites, 100-ml cultures were filtered through glasswool to remove residual pyrene crystals and further treated as described earlier (Rehmann et al., 1998). Extraction residues dissolved in a suitable volume of methanol were fractionated by thin layer chromatography (TLC). TLC was carried out by using precoated 500-,um-Silica Gel 60 DC plates (20 x 20 cm) (Merck). A two step solvent system was applied to separate metabolites using a mixture of methanol (55% v:v) and acidified water (45% v:v; water:acetic acid 24:1) for 30 min followed by 100% methanol for 15 min at 2 ml/min. This solvent system was used for further separation of bands enriched in metabolites. Several bands were isolated and extracted with methanol in an ultrasonic bath. These bands (Rf, 0.10 to 0.90) showed an HPLC elution profile with a major metabolite peak and these were further purified by multiple injections into an HPLC. The subsequent collection of these metabolite peaks resulted in the accumulation of small amounts (0.5 to 1.8 mg) of purified metabolites (Grifoll et al., 1992). UV-Vis spectra of the metabolites were scanned during HPLC analysis as described earlier in a Diode array detector at 254 nm (Varian 300).

Additionally, identification of all metabolites were performed by comparison of commerciallly available standards and by comparison of their retention times.

RESULTS AND DISCUSSION

Phenotypic identification of strain 4Bi

Strain 4Bi isolated from wastewater of a petrochemical industry was identified and used in all pyrene degradation assays. This strain was identified by the physiological methods and the 16S rRNA gene sequencing. The strain was straight rods under the microscope, gram-negative and motile. According to API-test results, the biochemical profiles seemed to ferment some carbohydrates by the strain. The APILAB PLUS program revealed this strain could also be related closely to *P. vulgaris* (98% identity to carbohydrate utilization pattern of *P. vulgaris*). Phenotypic characterization results of strain 4Bi was given in Table 2. API characterization provided the corresponding result with an ‘analytical profile index’ 98%, suggesting a good match to *P. vulgaris*. For 16S rRNA phylogeny, genomic DNA extraction (~13000 bp) (Figure 2a), PCR amplification and sequencing analysis were done. The expected size of the fragment sequenced from the 16S rRNA gene was 1382 bp (Figure 2b). The partial 16S rRNA gene fragment was over 99% similarity to that of type strain *P. vulgaris* DSM 30118 (AJ233425). The phylogenetic tree analysis suggested that strain 4Bi belongs to *P. vulgaris*. The position of strain 4Bi in the phylogenetic tree is presented in Figure 3. The genus *Proteus* was placed in the family Enterobacteriaceae. To demonstrate that pyrene utilization of our bacterium, the colonies surrounded by clear zones in CFMM agar with

Table 2. Phenotypic characteristics of the pyrene-degrading *Proteus vulgaris* 4Bi.

Characteristic	Results
Colony morphology and sizes[a]	0.66-1.5 mm in diameter, circular, smooth
Cell morphology and sizes	Straight rods, 0.4-0.8 µm by 1.0-2.5 µm
Motility[b]	+ (spreading growth)
Gram reaction	Gram (-)
Growth on MacConkey plate	+
Optimum growth temperature	CFU, 35°C; pyrene degradation, 30°C
Optimum growth pH	CFU, pH 7.0; pyrene degradation, pH 6.5
Production of catalase	+
Production of oxidase	-
Reduction of NO^{3-}	
NO^{2-} Production	+
N_2 Production	+
Utilization of C and N sources[c]	
Ortho-nitro-fenil-β-D-galactopyranoside	-
Arginine	-
Lisin	-
Ornitin	-
Sodium citrate (Use of citrate)	-
Sodium thiosulfate (Production of H_2S)	+
Urea	+
Tryptophane (Production of indole)	+
Creatine sodium pyruvate (Production of acetoin)	_
Gelatine	+
Glucose	+
Inositole	-
Sorbitole	-
Saccharose	+
Mellibiose	-
Amigdalin	+
Rhamnose	-
Arabinose	-
Lactose	-
Mannitole	-
Pyrene[d]	+

a: tested on nutrient agar at 35°C (24 h OLD culture); b: tested in semi-solid deep nutrient agar at 35°C (24 h old culture); c: tested using the API 20E ıdentification System; d: tested in carbon free mineral medium (CFMM) supplemented with 0.5 mg/ml pyrene at 30°C; +: positive; -: negative.

pyrene solution in acetone were obtained after a 3 day incubation (Figure 1). The growth profile of strain 4Bi determined by the optical density and CFU/ml (total viable count) are shown in Figure 4. The amounts of residual pyrene in the culture fluids were also given in this figure.

The novel pyrene-degrading bacterium isolated for this study was identified as *P. Vulgaris* using its morphology, biochemical characteristics and 16S rRNA gene sequence. So far, there have been no reports on the degradation of pyrene by bacteria in the genus Proteus. A study was performed by an indigenous hydrocarbon-degrading bacterial consortium composed of Proteus sp. and members of other genera (Lee et al., 1993). Nonetheless, bacteria belonging to genus Pseudomonas with nearest genus Proteus have been demonstrated to degrade pyrene. These include *Pseudomonas* sp. LP1 (Obayori et al., 2010), *Pseudomonas fluorescens* 29 L (Husain, 2008), *P. aeruginosa* strains LP5 and LP6 (Obayori et al., 2008) which can degrade. On the contrary, many researches reported that these PAH degrading pseudomonads and some other genera commonly not utilize HMW-PAHs such as pyrene (Daugulis and McCracken, 2003; Story et al., 2004).

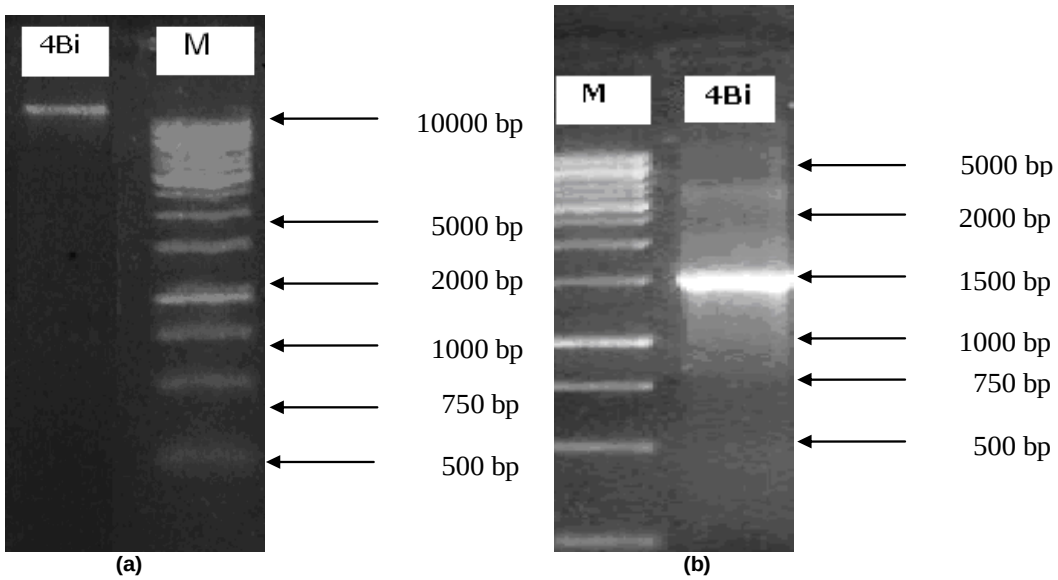

Figure 2. Results of genomic DNA (a), and PCR product (b) of strain 4Bi on 1% agarose gel electrophoresis. Lane 4Bi: genomic DNA of strain 4Bi (a); lane 4Bi: PCR product of strain 4Bi (b); lane M: DNA markers fermentas,10000 bp (a) and 5000 bp (b).

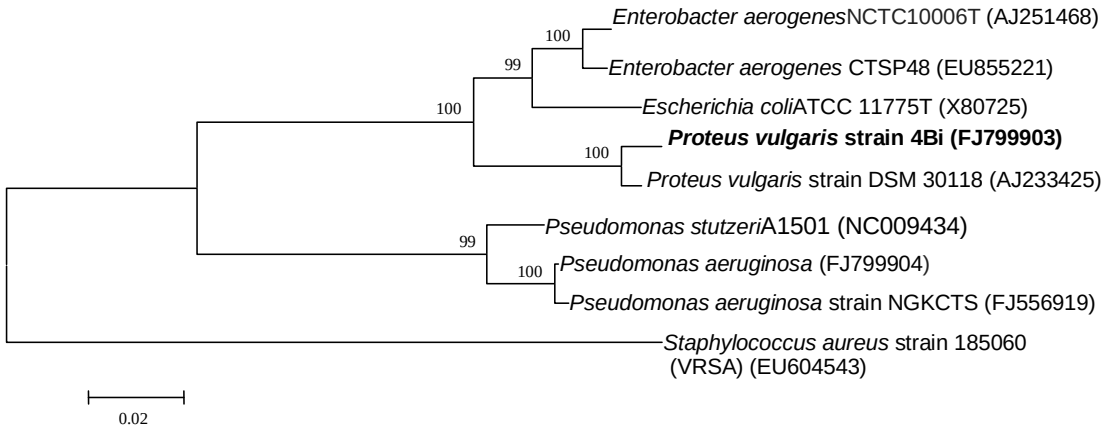

Figure 3. Phylogenetic tree of *P. vulgaris* strain 4Bi (FJ799903) based on 16S rDNA sequence analysis. The tree was constructed with the similarity and neighbor by MEGA 4.0. Accession numbers are given in parenthesis after the taxonomic assignment. Bar represents 0.02 (2%) sequence divergence.

Sphingomonas paucimobilis EPA 505 has also received attention as a possible HMW-PAHs (pyrene, etc.) degrading organism (Daugulis and McCracken, 2003).

Growth properties of *P. vulgaris* strain 4Bi during pyrene-biodegradation and detection of biodegradation

Although, the amount degraded by strain 4Bi was relatively small in the first two days (in lag phase and initial exponential phase), there was however sharpe increase in the consumption of pyrene at day 3 (Figures 4 and 5). It was also observed that the increment of bacterial cell was corresponded to the biodegradatin of pyrene. On day 7, bacterial growth was detected as 4.9 ± 1.80 CFU × 10^7/ml and 71.5% of the pyrene was degraded the maximum (Figures 4 and 5). The pyrene consumption in CFMM by *P. vulgaris* 4Bi as sole C-source is shown in Figure 5. The plot of percent pyrene degradation against incubation time (Figure 5) demonstrated that *P. vulgaris* 4Bi possess ability to

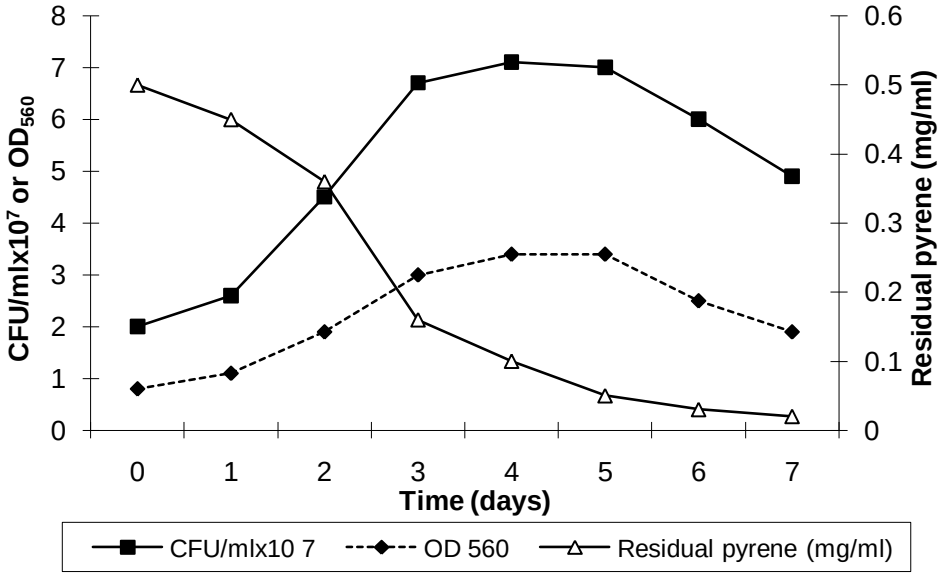

Figure 4. Growth profile of *P. vulgaris* strain 4Bi as CFUx10^7/ml, OD_{560} and residual pyrene. Culture conditions: 100 ml-erlenmeyer flasks containing 0.5 mg/ml pyrene/ml CFMM, pH 7, 30°C, rotary shaker 150 rpm. Each point represents the average value obtained with triplicate flasks. Error bars indicate one standart deviation.

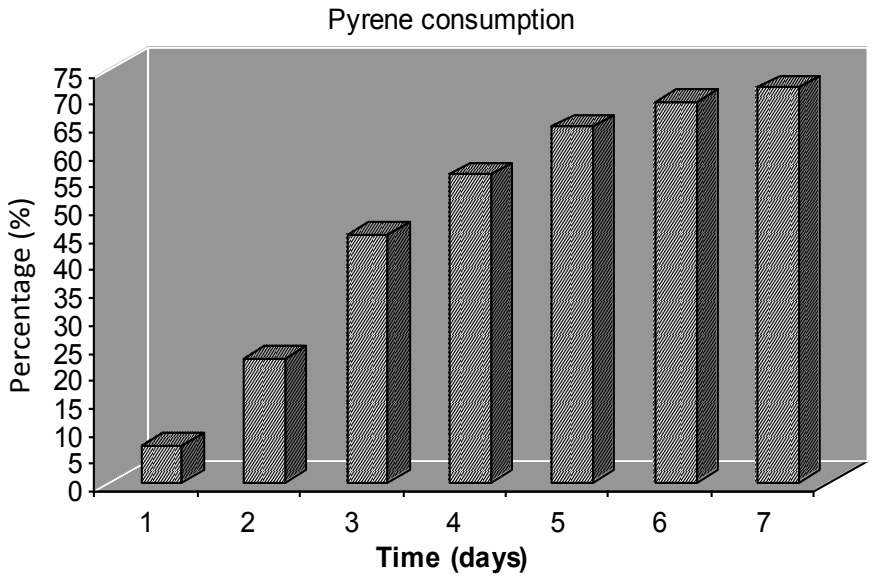

Figure 5. Percentage (%) consumption of pyrene by *P. vulgaris* strain 4Bi in CFMM plus pyrene (0.5 mg/ml) in 7 days. Bars represent the mean of triplicate cultures.

degrade pyrene as shown by the linear increase in pyrene degradation with time. This bacterium started degrading pyrene after 24 h and continued up to day 7. On 12 h, detectable amount of degradation was detected suggesting a lag phase of degradation. On day 1, strain 4Bi mediated pyrene degradation efficiency was about 6.8% that increased exponentially up to 4 days (about 55.8%) and there after with slow increase it became static on day 7 (71.5%). Further incubation of 10 and 14 days do not show any further increase in pyrene degradation. Whereas, on days 2, 3, 5 and 6, 4Bi was able to degrade about 22.5, 45.0, 64.5 and 69.0% pyrene respectively (Figure 5). Although, the number of viable cells (log10 CFU/ml) decreased considerably after 7 days

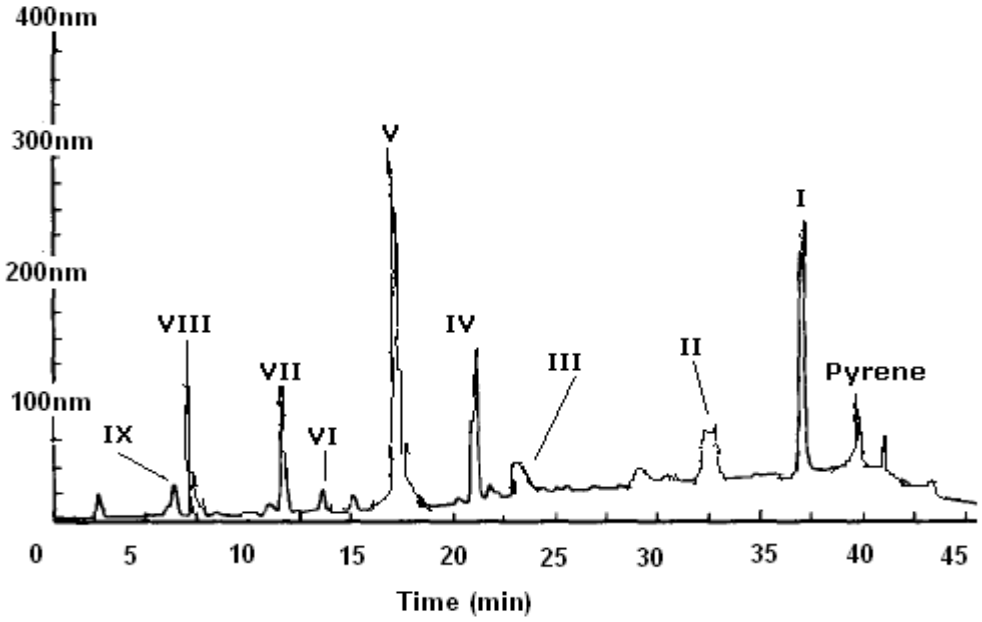

Figure 6. The HPLC profile for pyrene and its degradation metabolites after 7 days of incubation with *P. vulgaris* 4Bi in CFMM plus pyrene (0.5 mg/ml). UV absorbance for the chromatogram is shown with a vertical bar at time zero. The elution times of pyrene and its metabolites are shown under the representative peaks. These metabolites were identified as follows: I, cis-4,5-pyrene dihydrodiol; II, 2-carboxybenzaldehyde; III, phenanthrene 4-carboxylic acid; IV, phenanthrene 4,5-dicarboxylic acid; V, 4-phenanthroic acid; VI, pyrenol (1-hydroxypyrene); VII, phthalic acid; VIII, cinnamic acid and IX, trans-4,5-pyrene dihydrodiol.

of growth in CFMM, the strain 4Bi continued to degrade pyrene up to 7 days.

Proteus sp. are common in the environment and have been reported to be able to utilize and/or degrade many variety of xenobiotics when given as a single source of carbon and energy as a result from bacterial catabolism. Despite the results on pyrene degradation presented by Heitkamp et al. (1988), there is little information on the microbial degradation of pyrene and other HMW-PAHs by pure bacterial cultures. By Proteus sp., the degradation of pyrene which is one of the 16 PAHs included in the list of 129 priority pollutants compiled by the U.S. Environmental Protection Agency (Heitkamp et al., 1988) has not been reviewed. This study reported for the first time, the utilization of pyrene as sole source of carbon and energy by *P. vulgaris*. The novel strain *P. vulgaris* 4Bi showed enhancement in biomass within 7 days and about 71.5% of pyrene degradation after 7 days of growth. At present, it is not clear whether the decrease in CFU/ml after 7 days is due to the exhaustion of pyrene as carbon and energy source. The maximum pyrene consumption (71.5% of the pyrene in CFMM) was detected on the 7th day. On the other hand, maximum daily pyrene consumption rate was observed on the 3rd day. At pH 7.0 and 30°C, *P. vulgaris* 4Bi showed a maximum degradation rate of 0.002 mg pyrene/ml per day while growing with a doubling time of 30 min. The lower biodegradation rates exhibited between 5th and 7th days probably reflect its lower bacterial cell counts and high metabolite contents. It was reported that proteins and pH-changers are known to be more common metabolites in culture samples in microbial degradation procedures (Juhasz et al., 1997; Okerentugba et al., 2003; Wu et al., 2009).

Determination of metabolites obtained from biodegradation

Strain 4Bi was able to use pyrene as sole source of carbon and energy; it degraded 71.5 of the pyrene within 7 days. During growth, a metabolites were detected in the culture fluid and characterized by HPLC and TLC. Most of the products were confirmed by comparison with chemical standards. The results of HPLC were shown in Figure 6 as chromatogram. The HPLC profile of 7 days incubation of strain 4Bi in CFMM medium was compared with that of negative control (without strain 4Bi). Only one peak was detected in negative control chromatogram. In Figure 6, the chromatogram showed nine additional peaks (I to IX) indicating different metabolites formed during the degradation of pyrene. The major metabolites, designated I and V presented retention times of 38.0 and 17.5 min, respectively. The HPLC peak detected at 40.2

min was undegraded pyrene. The data in Figure 4 shows that approximately 0.14 mg/ml is the amount of residual pyrene. No abiotic degradation of pyrene was detected in the control flask containing killed cells. Enriched metabolite fractions from preparative TLC were obtained as described earlier. Two of the bands resolved were injected into an HPLC. Separation was achieved with a methanol-water linear gradient of 0 to 100% (vol/vol) methanol in 15 min. The HPLC profile of one of these bands showed other major metabolite peaks, designated metabolite IV and VIII (Figure 6) which were isolated by repeated injections into an HPLC and subsequent collection.

The other bands resolved by TLC showed an HPLC elutionprofile with several peaks. These peaks were designated metabolites II, III, VI, VII and IX (Figure 6). *P. vulgaris* 4Bi grew on CFMM suplemented pyrene with an exponential growth pattern. On pyrene, this organism also grew with a short lag phase (Figure 4) and stationary phase was attained in 3 days (3th to 5th days) at population density of 7.1 ± 2.00 CFU x 10^7/ml. The highest growth rate of 9.06/h was recorded in the first 2 days of incubation. But the highest rate of residual pyrene in medium of 0.048/h was recorded at initial stationary phase (Figure 4). These results suggest that CFMM could be very useful in the bioremediation of environments contaminated with pyrene (Obayori et al., 2010). *P. vulgaris* 4Bi shares similar or identical pyrene-degradation metabolites (Figure 6) as strains of *Mycobacterium* sp. (Heitkamp et al., 1988; Rehmann et al., 1998). Thus, pyrene degradation by strain 4Bi produced several metabolites also produced by other pyrene degraders such as 2-carboxybenzaldehyde by *Mycobacterium* sp. strain KR2 (Rehmann et al., 1998), phthalic acid produced by *Mycobacterium* strain AP1 (Vila et al., 2001), cinnamic acid by *Mycobacterium* BB1 (Heitkamp et al., 1988), both of phthalic acid and cinnamic acid was produced by *P. fluorescens* 29 L (Husain, 2008). However, there were also metabolites such as phenanthrene derivatives that contributed to the uniqueness of pyrene-degradation pathways in strain 4Bi.

The occurrence of phenanthrene 4-carboxylic acid has been reported in pyrene metabolism by *Mycobacterium* sp. strain AP1 (Vila et al., 2001) and phenanthrene 4.5-dicarboxylic acid metabolism in *Mycobacterium* sp. strain KR2 (Rehmann et al., 1998) and *M. flavescens* also (Dean-Ross and Cerniglia, 1996). Cinnamic acid and phthalic acid were as highly polar metabolites of pyrene. However, these metabolites resulted from further metabolism of 4-phenanthroic acid, 4-hydroxyperinaphthenone or other chemical intermediates. The origin of these polar, single-ringed metabolites may be resolved in studies in which large quantities of each ring fission intermediate are synthesized and utilized as substrate for microbial metabolism (Heitkamp et al., 1988). The information on the chemical pathway for microbial catabolism of HMW-PAHs is scarce. Recently, several studies have described bacterial metabolites from different pyrene degradation pathways (Seo et al., 2009). We found the four products of ring oxidation, pyrene-*cis*-4,5-dihydrodiol, pyrene-*trans*-4,5-dihydrodiol, pyrenol and 2-carboxybenzaldehyde, and several products of ring fission, 4-phenanthroic acid, phenanthrene 4-carboxylic acid, phenanthrene 4,5 di-carboxylic acid, phthalic acid and cinnamic acid by HPLC analyses. None of these reports demonstrated detoxification or reduced carcinogenicity of the described metabolites. All these compouds are non-carcinogenic (Enke et al., 2007).

Heitkamp et al. (1988) reported similar dihydrodiols and ring-cleavage products to ours from growing *Mycobacterium* sp. cultures. As different with our results, 4-hydroxyperinaphthenone was also produced, 2-carboxybenzaldehyde, phenanthrene 4-carboxylic acid and phenanthrene 4,5 di-carboxylic acid was not produced. The formation of pyrene-*cis*-4,5-dihydrodiol by dioxygenase and pyrene-*trans*-4,5-dihydrodiol by monooxygenase suggested multiple initial oxidative attacks on pyrene. However, Li et al. (1996) observed the oxidation of pyrene to dihydrodiol by *Mycobacterium* sp. strain Pyw2, but no ring cleavage products were observed. A novel metabolite, 6,6'-dihydroxy-2,2'-biphenyl-dicarboxylic acid was identified by Vila et al. (2001) from the degradation of pyrene by *Mycobacterium* sp. strain AP1. Fritsche (1994) detectedpyrene-cis-4,5-dihydrodiol, 4,5-dicarboxylic acid, protocatechuic acid from the degradation of pyrene by *Mycobacterium* sp. strain BBI. Liang et al. (2006) reported pyrene-4,5-dione formation and identified almost all the enzymes required during the initial steps of pyrene degradation in *Mycobacterium* sp. KMS. This study is different from other studies in the following two aspects. First, all the reported metabolites of pyrene degradation have been shown to investigate pyrene-degrading mycobacteria. This is an important shortage for biodegradation and bioremediation studies by other bacteria.

The metabolites information of degradation from our work can be used to understand the biodegradation mechanism of pyrene and to develop bioremediation technology. It is necessary that such studies are done by using other diverse bacteria for improving the biodegradation and bioremediation applications. Secondly, strain 4Bi degraded pyrene in high levels and rapidly by producing nontoxic and nonaccumulating metabolites. In early researches, *Ochrobactrum* sp. BAP5 (Wu et al., 2009), *P. fluorescens* 29 L (Husain, 2008), *Bacillus sp.* strain Py1, Py4 (Hou et al., 2007), *Rhodococcus sp.* UW1 (Walter et al., 1991) and the three *Burkholderia cepacia* strains (Juhasz et al., 1997) could utilize pyrene, respectively, 19% in 30 days, 82.38% in 6 days, 88% in 10 days, 84% in 14 h, 72% in 2 weeks, 95 to 100% in 7 to 10 days. The environmental/cultural conditions could strongly affect the physiological and biochemical properties of the microorganisms in the biodegradation and biotransformation of PAHs (Klankeo

et al., 2009). Moreover, a strain of *P. vulgaris* was firstly reported among pyrene-degrading microorganisms as the individual bacterium. Generally, LMW-PAHs tend to degrade at faster rates than those with HMW-PAHs (Juhasz et al., 1997; Kim et al., 2003). Also, PAH degrading pseudomonads commonly utilize naphthalene, anthracene and phenanthrene but not high molecular weight PAHs such as fluoranthene or pyrene and, less often (Story et al., 2004). For example, results of Daugulis and McCracken (2003) have shown that, individually, *Sphingomonas aromaticivorans* BO695 and *S. paucimobilis* EPA 505 can degrade only LMW-PAHs such as naphthalene, phenanthrene and fluoranthene.

The importance of *P. vulgaris* 4Bi in the rapid degradation of pyrene was clearly exhibit. A different study was made with *Pseudomonas* sp. LP1 and *P. aeruginosa* LP5 grown on pyrene plus CSL (corn step liquor) as the additional energy and carbon source (Obayori et al., 2010). Similarly, *Bacillus* sp. Py1 and Py4 together using peptone as the additional carbon sources was able to degrade 88% of pyrene in 8 h at 37°C (Hou et al., 2007). In a recent investigation of pyrene biodegradation, *Diaphorobacter* sp. and *Pseudoxanthomonas* sp. isolated from soil grown on CFMM supplemented with pyrene was used. These strains could degrade 99% of 100 mg/l pyrene after a 16-day incubation. The cell number was increased from 6.32 to 7.55 log CFU/ml and from 6.52 to 8.05 log CFU/ml, respectively, which were higher than those of growth control experiments (Klankeo et al., 2009). In our study, the cell number of *P. vulgaris* 4Bi increased from 2 to 4.9 CFU x 10^7/ml (Figure 4). Some of the other studies involved use of *Burkholderia cepacia* 2A-12 derived from oil-contamined soil which co-metabolically mineralizes pyrene when provided with a complex hydrocarbon co substrate such as crude oil separately in soil. This bacterium was able to also degrade pyrene by the addition of yeast extract as another carbon source (Kim et al., 2003).

Another work, *Mycobacterium vanbaalenii* PYR-1 (DSM 7251) degraded pyrene in TSB plus yeast extract (0.005%) plus a vitamin supplement (Kim et al. 2005). *Saccharothrix* sp. PYX-6 degraded pyrene in CFBM (carbon free basic medium) plus yeast extract (0.005%) (Hu et al., 2003). Yeast extract was thought to induce the degradability of pyrene (Kim et al., 2003). In this study, no carbon source other than pyrene was available which may have favored cell counts. In another study, the co-metabolicmineralization of pyrene was examined in soil, in sludge mixed homogenously into soil and in sludge-soil systems containing a lump of sludge (Klinge et al., 2001). Also, up to now, almost all pyrene degraders encountered in natural environments have been gram-positive bacteria (Walter et al., 1991). Recently, gram-negative strains have also been reported (Juhasz et al., 2000), but they only cometabolized pyrene upon addition of other organic chemicals (Ye et al., 1996) and did not degrade them as a sole carbon source except *Paracoccus* sp. (Zhang et al., 2004) as described here for strain 4Bi.

Conclusion

To date, only few organisms are known that are capable of utilizing PAHs with more than three fused benzene rings as sole carbon source. In this study, *P. vulgaris* 4Bi could effectively and quickly degrade pyrene. Moreover, the identification of *P. vulgaris* strain that is able to use pyrene as sole source of carbon and energy is documented for the first time. This is an important finding on the degradation of HMW-PAHs like pyrene. Phenanthrene-derivatives metabolites determined in this work are LMW-PAHs and half-life of them in nature take a short time than that of pyrene. Microbial degradation is a major factor affecting high persistence of pyrene in the environment. So, these metabolites from degradation of pyrene by *P. vulgaris* 4Bi is nonpersistent and have no risk for ecology and human health as not in pyrene. Our results emphasize that this strain extensively metabolized pyrene to nontoxic and nonaccumulating metabolites.

In conclusion, this bacterium may be useful for bioremediation applications and contribute to pyrene detoxification in polluted habitats.

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