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ORIGINAL PAPER

Pyrene-degradation potentials of *Pseudomonas* species isolated from polluted tropical soils

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Abstract Three *Pseudomonas* species isolated from oil polluted soils in Lagos, Nigeria were studied for their pyrene degradation potentials. These isolates exhibited broad substrate specificities for hydrocarbon substrates including polycyclic aromatic hydrocarbons, petroleum fractions and chlorobenzoates. All three isolates tolerated salt concentrations of more than 3%. They resisted ampicillin, cenfuroxime, but susceptible to ofloxacin and ciprofloxacin. *Pseudomonas* sp. strain LP1 exhibited growth rates and pyrene degradation rates of 0.018 h^{-1} and 0.111 mg l^{-1} h^{-1} respectively, while *P. aeruginosa* strains LP5 and LP6 had corresponding values of 0.024, 0.082 and 0.017, 0.067 respectively. The overall respective percentage removal of pyrene obtained for strains LP1, LP5 and LP6 after a 30-day incubation period were 67.79, 66.61 and 47.09. Resting cell assay revealed that strain LP1 had the highest uptake rate. Strains LP1, LP5, and LP6 also used the *ortho*-cleavage pathway. Enzyme study confirmed activity of catechol 1,2-dioxygenase in all with values 0.6823, 0.9199, and 0.8344 μmol min^{-1} mg^{-1} respectively for LP1, LP3 and LP6. To the best of our knowledge, ours is the first report of pyrene-degraders from the sub-Saharan African environment.

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Introduction

Petroleum is a major source of energy in the world today and Nigeria is one of the major oil producing countries (Amund et al. 1987). Polycyclic aromatic hydrocarbons (PAHs) are present as natural constituents of fossil fuels and are formed anthropogenically as a result of incomplete combustion of organic materials. They are, therefore, present in relatively high concentrations in refined products of fossil fuel and in every environment where fossil fuels and other organic materials are combusted. This, in combination with global transport phenomenon, results in their worldwide distribution (Kanaly and Harayama 2000). Pyrene is a pericondensed, four-ring polycyclic aromatic hydrocarbon (PAH). It is a regulated contaminant at sites polluted with petroleum (Kazunga and Aitken 2000). Interest in the biodegradation of PAHs such as pyrene is spurred in part by its mutagenicity and shared structure with carcinogenic PAH such as benzo(a)pyrene (Cerniglia 1992; Kanaly and Harayama 2000; Cheung and Kinkle 2001). Bacteria capable of degrading a wide range of hydrocarbons abound in the soil environment. Whereas microorganisms capable of utilising two- and three-ring PAHs as sole carbon and energy sources have been well-documented in the literature, until the late 1980s there was no literature on microorganisms capable of using four and five ring PAHs as sole carbon and energy sources (Heitkamp et al. 1988). Pyrene is mainly degraded by actinomycetes such as *Mycobacterium* and *Rhodococcus* (Grosser et al. 1991; Walter et al. 1991; Kastner et al.

1994; Dean-Rose and Cerniglia 1996; Schneider et al. 1996; Kanaly and Harayama 2000; Derz et al. 2004). However, there have also been reports of few non-actinomycete degraders of pyrene, particularly the pseudomonads (Caldini et al. 1995; Bouchez et al. 1995). Sarma et al. (2004) isolated a strain of the enteric bacterium *Leclercia adecarboxylata* capable of degrading pyrene from oily sludge contaminated soil. Das and Mukherjee (2007) reported the pyrene-induced production of biosurfactant by a *Bacillus* and two *Pseudomonas* species.

Because of the ubiquitous nature and recalcitrance of pyrene, and the significance of this for bioremediation, there is a growing interest in the ability of pyrene-degrading strains to also degrade other related PAHs and other components present in petroleum (Churchill et al. 1999). The role of pyrene-degrading organisms in the fate of mixture of PAHs in the environment has also been the subject of investigation by various workers (Trzesicka-Mlynarz and Ward 1995; Juhasz et al. 1997). Vila et al. (2001) identified a novel metabolite 6,6′-dihydroxy-2,2′-biphenyl dicarboxylic acid resulting from the *ortho* cleavage of both central rings of pyrene. While Krivobok et al. (2003) identified Pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1, thus suggesting the presence of two ring-hydroxylating dioxygenases. It has been suggested that products derived from pyrene transformation, particularly by pseudomonads and *Bacillus* species have the potential to accumulate in PAH-contaminated sites and that such products may significantly interfere with the removal of other PAHs (Kazunga and Aitken 2000).

In spite of the existing body of knowledge on pyrene metabolism, there is virtually no report of study of pyrene degradation in the tropical African environment and in Nigeria especially where gas flaring and unabated release by automobiles are loading the environment with cocktails of PAHs. Improving on the available bank of microbial resources (isolates) and information is crucial to the proper management of petroleum-polluted sites. In this paper we report the isolation and characterisation of three strains of *Pseudomonas* able to grow on pyrene as sole source of carbon and energy.

Materials and methods

Sample sites

Soil samples were collected from three contaminated sites MVOA, DGED and LAS. MVOA samples were collected from a mechanic village at Egbeda, Lagos, with a long history of contamination with spent engine oil, diesel, gasoline and transformer fluid. DGED samples were collected from a diesel generator exhaust-contaminated soil, at Dopemu-Agege, Lagos. LAS is a diesel generator site soil, dark and thick with soot. The samples were collected at a depth of 10–12 cm with sterile trowel after clearing debris from the soil surface.

Enrichment and isolation of pyrene-degrading bacteria

Bacteria able to degrade pyrene were isolated on pyrene minimal salt medium (MSM) by continual enrichment method (Churchill et al. 1999). The mineral medium described by Kastner et al. (1994) was used. The medium contains per litre Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4 \cdot 7H_2O$, 0.20 g. The pH of the medium for bacteria was adjusted to 7.2 and fortified with nystatin at 50 μg/ml to suppress fungal growth. Trace elements solution (1 ml per litre) described by Bauchop and Elsden (1960) was sterilised separately and added aseptically to the medium. Contaminated soil sample (5.0 g) was added to 45 ml MSM containing 50 ppm of pyrene. Enrichment was carried out with shaking (175 rpm) for 4–5 weeks in the dark until there was turbidity. After 5 consecutive transfers, pyrene degraders were isolated by plating out dilutions from the final flask on Luria–Bertani (LB) agar. Several of the colonies that appeared were further purified by subculturing once onto LB agar. Ability to degrade pyrene was confirmed by inoculating washed LB broth grown culture (18 h) into fresh MSM flask supplemented with 50 ppm pyrene as a sole carbon source and observing (OD_{500} of 0.2 was considered significant). Pure isolates were maintained at −20°C in glycerol: LB broth medium (50:50).

Identification and characterisation of isolates

Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the scheme of Cowan and Steel (Barrow and Feltham 1995). This was complemented by using the ID32 E system for the identification of Enterobacteriaceae and other non-fastidious Gram-negative rods. The ID32 E kit was used according to the manufacturer's specifications. (Biomerieux Inc., Durham, NC, USA).

Antibiotic sensitivities of isolates were carried out using multidiscs. Salt tolerance was tested in LB broth containing NaCl ranging from 1 to 10% (w/v). Incubation was carried out at room temperature (27 ± 2.0°C) for 2 weeks with shaking and daily observation for growth as indicated by turbidity.

Substrate specificity

The ability of the isolates to grow on pure hydrocarbon substrates was evaluated in liquid media amended with 50 ppm of respective hydrocarbons as a sole carbon and energy source. Incubation was carried out under similar

conditions as described above. Degradation was monitored by cell increases and visual observation for turbidity. The hydrocarbons tested include naphthalene anthracene, phenanthrene, benzene, phenol, biphenyl and dibenzothiophene. Isolates were also tested for growth on crude oil and petroleum products. Liquid hydrocarbons were autoclaved and separately added to sterile MSM at 0.1% (v/v). Incubation was carried out for 21 days.

Evaluation of pyrene biodegradation

Pyrene degradation was carried out by inoculating replicate 250-ml flasks containing 50 ml of MSM already supplemented with pyrene as a sole carbon and energy source at concentration of 100 ppm. Flasks were inoculated with 0.5 ml of MSM-washed 18 h LB agar-grown cells and subsequently incubated with shaking at 150 rpm for 30 days at room temperature.

At each time point, entire replicate flasks were sacrificed. Metabolic reactions were stopped with the addition of hexane. Previous experiments in our laboratory have shown that hexane extraction lyses cells and that identical substrate recoveries are obtained using killed-cell controls (Adebusoye et al. 2007a, b).The total viable counts (TVCs) in each flask were determined after plating out aliquots of appropriate dilutions onto nutrient agar.

Analytical methods

Residual pyrene was determined by a method modified from Das and Mukherjee (2007). Residual pyrene was extracted from culture (20 ml) with equal volume of hexane. After vortexing hexane-culture mixture for 2 min, it was centrifuged at 10,000*g* for 10 min to remove cell debris and separate aqueous and organic phases. Hexane extract was concentrated and re-constituted in 20 ml of hexane. Pyrene concentration was determined on a 6305 UV/Vis Spectrophotometer at 335 nm and the amount of pyrene read on a standard curve. Control tubes inoculated with heat-killed cells were similarly treated.

Alternatively, undegraded pyrene was quantified using gas chromatographic (GC) analysis in order to validate the results obtained from spectrophotometric method described above. Residual pyrene was extracted according to the method described by Sarma et al. (2004). Briefly, culture (20 ml) was extracted once with an equal volume of toluene and then twice with an equal volume of chloroform. After the solvents were vented off, the residual pyrene was dissolved in acetone, and concentrated to 2 ml. Pyrene concentrations in the acetone were determined using a Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector (FID) and 30 m long HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 μm). The injector and detector temperatures were maintained at 300 and 320°C respectively. The column temperature was programmed to rise from 60 to 500°C for 27 min. The GC was programmed at an initial temperature of 60°C; this was held for 2 min, then ramped at 12°C/min to 205°C and held for 16 min.

Resting cell assay

Pyrene uptake by resting cells was measured spectrophotometrically as described by Stringfellow and Aitkens (1995). Pyrene-grown cells were harvested by centrifugation and washed twice in 50 mM KH_2PO_4 buffer (pH 7.2). Cells were resuspended in buffer (20 mM phosphate buffer containing 150 M NaCl, pH 7.0) to a final volume of 3.0 ml and $O.D_{600}$ of 1.0 in a 3.5 ml quartz curvette in a UV/Vis spectrophotometer (Jenway 6305). About 60 ng of pyrene (in 10 μl of acetone) was injected into the curvette. Decrease in A_{273} was measured from 0.5 to 30 min post-addition of pyrene. Acetone without pyrene served as control. A second control was set up for heat-killed cells to check decreases in pyrene concentration resulting from adsorption. Decrease in pyrene content was calculated from a standard curve of pyrene and result expressed as nanogram of pyrene uptake by 2.0×10^7 bacterial cells.

Detection of ring-fission enzymes

The methods of Stanier et al. (1966) and Rothera (1908) were used thus: bacterial strains were grown on pyrene (0.1 w/v, MSM) for 15 days. Cells were harvested from 30 ml broth culture by centrifugation (10,000*g* for 10 min, 4°C). Cells were resuspended in 4 ml Tris–HCl buffer (0.02 M pH 8.0) in a test tube. Catechol solution (4 ml, pH 8.0, 0.01 M) was then added to the mixture and shaken for 5 min. The appearance of a yellow colour within a few minutes as a result of formation of 2-hydroxymuconic semi-aldehyde indicated *meta*-fission. When yellow colour failed to appear the mixture was shaken for additional 2 h at 30°C and tested for *β*-keto-adipic acid by the method of Rothera (1908) thus: a small quantity of Rothera's reagent was added to the bottom of a test tube. A few drops of the test liquid were added to the powder with a Pasteur's pipette just to moisten it. The mixture was left for 1 min for the colour to develop. Appearance of a purple colour within 1 min indicated *ortho*-cleavage. The test tube was held against a sheet of white paper so that any colour change could be quickly noticed.

Preparation of cell extracts and enzyme assay

Cell extract was prepared according to the method of Phillips et al. (2001). About 2 ml of bacterial culture was

centrifuged and the pellet was resuspended in 1 ml of basal medium. Cells were lysed by the addition of 10 µl toluene. Cell debris and unbroken cells were removed by centrifugation (16*g*, 30 s) and the supernatant was immediately used for the experiment or kept on ice for not more than 10 min. Deactivated cell extracts were made by boiling the extract for 15 to 30 min.

Catechol 1,2-dioxygenase and catechol 2,3 dioxygenase activities in the crude cell extracts were evaluated by the methods of Ka-Leung et al. (1990) and Kataeva and Golovleva (1990) respectively. The protein contents of cell extracts were determined with the method of Bradford (1976). The concentration of protein was read off a standard curve prepared with bovine serum albumin (0–10 µg).

Results

Isolation and characterisation of pyrene degraders

Pyrene-degrading strains namely, LP1 and LP5 were obtained from MVOA and DGED sites respectively while, the third strain, LP6 originated from the soil obtained from LAS. The colony strain LP1 measured 2–3 mm with irregular edges on LB agar and it spread on the line of streaking on blood, chocolate and MacConkey agar. It was Gram-negative motile rods, oxidase- and catalase-positive. It failed to ferment almost all sugars tested and was indole, urease and citrate negative, attributes suggestive of *Pseudomonas* sp. It produced no pigment, thus clearly showing that it is not *Pseudomonas aeruginosa*. Cultural morphologies of LP5 showed circular, raised and smooth edge translucent colony (3–4 mm in diameter) while colonies of LP6 measured about 10 mm and were cream with a round, and smooth edge. Both strains were Gram-negative, motile rods that shared a lot of biochemical characteristics with LP1, however, they grew at 42°C on nutrient agar. Additionally, strain LP5 was urease- and malonate-positive, and was also positive for the pigment pyoverdin. On the other hand, LP6 utilized citrate and was positive for the pigment pyorubrin. These characteristics indicate that both isolates were strains of *Pseudomonas aeruginosa.* All three isolates tolerated salt concentrations of more than 3%. They all resisted ampicillin, cenfuroxime, but were susceptible to ofloxacin and ciprofloxacin. Only LP6 was resistant to both gentamycin and terramycin (Table 1).

Table 1 Antibiotic resistance patterns of pyrene-degrading isolates

Antibiotic	Isolate		
	LP1	LP5	LP6
Norfloaxacin (10 µg)	S	R	R
AX (20 µg)	R	R	R
Ofloxacin (5 µg)	S	S	R
Chloramphenicol (10 µg)	S	ND	R
Cenfuroxime (30 µg)	R	R	R
Ampicillin (25 µg)	R	R	R
Gentamycin (10 µg)	S	S	R
Nitrofurantion (100 µg)	R	ND	R
Ciprofloxacin (50 µg)	S	S	S
Terramycin (50 µg)	S	S	R

R: resistant; S: susceptible; ND: not determined

Table 2 Substrate specificity of pyrene degrading isolates

Substrate	Isolate		
	LP1	LP5	LP6
Naphthalene	+	–	++
Phenanthrene	++	–	++
Anthracene	++	–	++
Pyrene	+++	+++	++
Dibenzothiophene	++	++	++
Biphenyl	+++	–	+
Benzene	+	+	++
Catechol	+++	+++	++
Phenol	–	–	–
Toluene	+	+	+
Hexane	–	–	–
Crude oil	+++	+++	++
Diesel	++	+++	++
Engine oil	+++	+++	+
Kerosene	+	+	+
Benzoate	+++	++	++
2-Chlorobenzoate	++	+++	+
3-Chlorobenzoate	+++	–	++
4-Chlorobenzoate	+++	–	–
1,4-Dichlorobenzene	+++	+	+
2,4-Dichlorobenzoate	+++	++	+
2,5-Dichlorobenzoate	+	+++	+
2,6-Dichlorobenzoate	++	+++	+

+++: Luxuriant growth; ++: Growth; +: Poor growth; –: No growth

Substrate susceptibility of isolates

Results summarized in Table 2 show that all the isolates utilized pyrene but not phenol and hexane. Strain LP1 grew very well on naphthalene, phenanthrene, anthracene, dibenzothiophene, biphenyl and some congeners of chlorobenzoate (CBA) as sole carbon and energy sources while, these pollutants were weakly utilized by LP6. Interestingly, LP5 failed to grow on naphthalene, phenanthrene, anthracene and biphenyl. Additionally strain LP1 grew excellently well on crude oil, diesel and engine oil, but only fairly well on kerosene. LP5, on the other hand,

only grew poorly on crude oil and kerosene, fairly well on diesel, but failed to grow at all on engine oil. LP6 grew slightly on all the petroleum cuts tested (Table 2).

Biodegradation of pyrene

The growth kinetics of the LP strains on pyrene are illustrated in Fig. 1 and Table 3. The organisms exhibited slight lag phases, especially strains LP1 and LP5. Growth was gradual with concomitant decreases in pyrene concentration. LP1 grew from an initial density of 13.3 $\times$ 10^6 cfu/ml to peak at 2.0 $\times$ 10^8 cfu/ml resulting in over 15-fold increase. Similar growth dynamics was obtained with LP5 though with over 20-fold cell increase at a growth rate of 0.024 h^{-1} which was the highest observed. In the case of strain LP6, there was a rapid cell increase between days 5 and 6 compared to other time points. By day 12, sharp drop in cell density was obtained which peaked and remained relatively stationary for more than 6 days before assuming a decreasing trend.

The rates of pyrene utilization observed as quantified by spectrophotometric technique were 0.111, 0.082 and 0.067 mg l^{-1} h^{-1} respectively for LP1, LP5 and LP6. In the control tubes, no apparent decrease of the substrate was observed. This indicates that the depletion of the pyrene from the basal media was due to biodegradation rather than to non-specific losses such as compound volatility or adsorption to the glass tubes or mere accumulation of the PAH substrate in cells. Although strain LP5 grew with the least doubling time (28.4 h), the best pyrene degrader was LP1 with nearly 68% degradation. The overall percentage removal of pyrene observed for other strains were 67 (LP5) and 47 (LP6) after a 30-day incubation period. It is noteworthy that in spite of decline in growth observed from day 21, degradation of the pyrene substrate lasted the experimental period. It is noteworthy that these results were comparable with those obtained during GC analysis (GC fingerprints not shown).

Rate of pyrene uptake

Figure 2 shows the rate of uptake of pyrene by the isolates. The organisms exhibited divergent pyrene uptake rate. The uptake rate peaked for LP1 within 10 min at 14 ng/2.0 $\times$ 10^7 bacteria cells; LP5 peaked within 6 min at 7.5 ng/2.0 $\times$ 10^7 bacteria cells, while LP6 recorded a value of 9 ng/2.0 $\times$ 10^7 bacteria cells within 10 min.

Enzyme activities

Cell suspensions of the three isolates failed to show appearance of yellow colouration in the presence of catechol, indicating the absence of *meta*-cleavage of catechol by these organisms. The appearance of purple colour upon testing for β-keto-adipic acid showed the presence of the *ortho*-cleavage pathway in the three isolates. Furthermore, analysis of the cell-free extracts confirmed the presence of *ortho*-cleavage enzymes for the metabolism of catechol. The respective specific activities of catechol 1,2-dioxygenase obtained for LP1, LP5 and LP6 were 0.6823, 0.9199, and 0.8344 µmol min^{-1} mg of $protein^{-1}$ (Table 4).

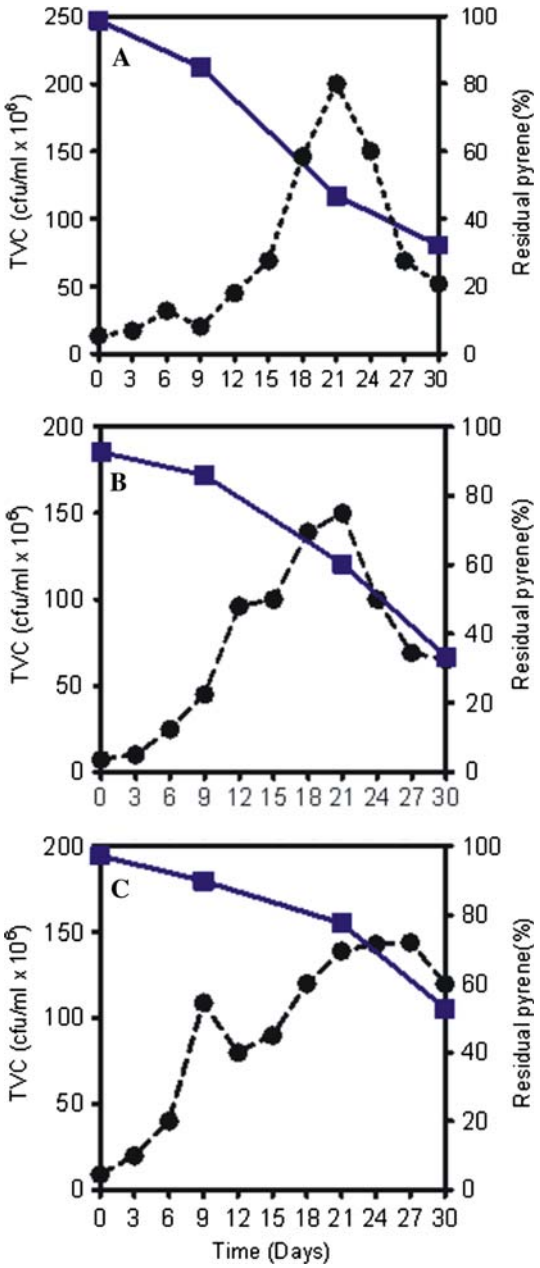

Fig. 1 Growth dynamics of LP1 (**a**), LP5 (**b**) and LP6 (**c**) strains in minimal medium supplemented with 100 mg l^{-1} pyrene showing total viable count, TVC (●) and residual pyrene (■). Data points represent the means of three replicate tubes and were determined with reference to pyrene recovered from heat-killed controls

Table 3 Growth kinetics of pyrene degrading isolate

	Isolate		
	LP1	LP5	LP6
Growth rate (h^{-1})	0.018	0.024	0.017
Doubling time (h)	39.4	28.4	41.7
Rate of degradation (mg l^{-1} h^{-1})	0.111	0.082	0.067

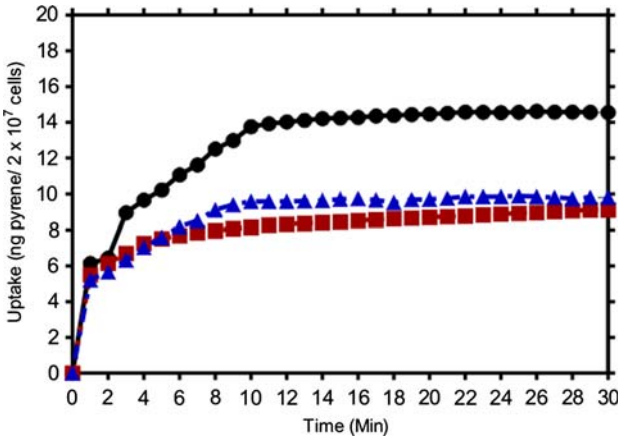

Fig. 2 Pyrene uptake by LP1 (●), LP5 (■) and LP6 (▲) strains. No significant pyrene uptake was observed in heat-killed control tubes as well as uninoculated tubes

Discussion

Degradation of pyrene and other PAHs in the environment occurs predominantly by microbial processes (Das and Mukherjee 2007). The recovery of only one species from each of the soils MVOA and DGEG is in tandem with earlier reports and may be attributed to the fact that enrichment medium imposes stress on the microbial community in order to select the required phenotype leading to reduction of species diversity and dominance of a single species (Stach and Burns 2002). Most of the high molecular weight-PAH degrading bacteria so far described are actinomycetes and the knowledge about biodegradation of pyrene by non-actinomycetes is scanty. However, a variety of non-actinobacteria, particularly pseudomonads, have also been reported to mineralize pyrene (Thibault et al. 1996; Juhasz et al. 1997; Sarma et al. 2004; Das and Mukherjee 2007). The three bacteria isolated in this study are members of the genus *Pseudomonas*. The fact that the three organisms are species of *Pseudomonas* is not surprising because they are a group of organisms with broad substrate specificity for dioxygenases, a factor which accounts for their metabolic diversity. The metabolic versatility of the pseudomonads isolated in the present study makes them unique and quite different from previously characterized strains.

Table 4 Specific enzyme activity of pyrene degrading isolate

Isolate	Enzyme activity (μmol min^{-1} mg^{-1})		
	Catechol 1,2-dioxygenase	Catechol 2,3-dioxygenase	Pathway
LP1	0.6823	0.0	*ortho*
LP5	0.9199	0.0	*ortho*
LP6	0.8344	0.0	*ortho*

The ability of these organisms to grow at salt concentrations higher than 3% is noteworthy. This may be important in their consideration for bioremediation purpose upon further study as salinity has been found by Kastner et al. (1998) to be a very important factor determining the survival of bacterial inoculum in soil during bioaugmentation. Antibiotics are produced by many soil microorganisms for competitive edge. Their persistence, after production, depends on many factors including climate, type of soil and antibiotic type. Many antibiotics are biodegradable in soil and many also have a long half life. Since antibiotics have both qualitative and quantitative effects on terrestrial microbial communities (Chander et al. 2005). For survival in soils, it is important and required to know the sensitivity and resistance patterns of microorganisms with potentials for use as seeds for bioremediation. Shared resistance to ampicillin and cenfuroxime by all the isolates could be attributed to the fact that these antibiotics are common in environmental compartments prompting evolution of resistance by autochthonous strains and hence, a survival strategy that allowed them to proliferate ahead of other members of the community. In any case, the metabolic versatility is an indication of the occurrence of multifunctional dioxygenases in these strains particularly LP1.

The broad spectrum of LP1 on PAHs may be attributed to the more diverse nature of petroleum products at the site from which it was recovered. This may also account for its better performance on crude and most of the petroleum products tested. LP1 also performed better on chlorobenzoates tested than LP5 and LP6. One reason for this may be the fact that in the mechanic village where this organism was isolated, apart from oil products, other pollutants such as coils, capacitor, and transformer oil containing polychlorinated biphenyls (PCBs) abound and the selective pressure exerted by these pollutants led to acquisition of such special ability (Wackett and Hershberger 2001; Adebusoye et al. 2007a).

Our isolates grew on pyrene with growth rates of 0.017–0.024 h^{-1} lower than those previously reported for many actinomycetous pyrene-degraders, these values are better than previously reported for some pseudomonads. Thus, while Boldrin et al. (1993) reported 0.056 h^{-1} for

Mycobacterium BB1, Thibault et al. (1996) reported 0.014 and 0.013 h^{-1} for *Pseudomonas* sp. K-12 and B-24 respectively. Walter et al. (1991) also reported 0.023 h^{-1} for their *Rhodococcus* UW1. Compared to previous reports, the pyrene utilisation rates of our organisms are low. Whereas we report here values of 0.111, 0.082 and 0.067 mg l^{-1} h^{-1}), Dean-Ross and Cerniglia (1996) reported 0.56 mg l^{-1} h^{-1} for *Mycobacterium flavescens*. It is noteworthy however that growth rates on substrate and rate of utilisation are not intrinsic properties of isolates independently of culture conditions. For instance, Boldrin et al. (1993) had shown that pyrene crystal sizes greatly influence pyrene degradation rates by *Mycobacterium* species. The authors found that large crystals were utilised at the rate of 1.2 while smaller ones were utilised at the rate of 5.6 μg ml^{-1} h^{-1}. It has also been shown that variations in physico-chemical parameter such as pH may also significantly influence mineralization rates (Grosser et al. 1991; Kim et al. 2005).

The overall percentage utilisation of pyrene exhibited by strains LP1 and LP5 are well within the ranges previously reported for many pyrene-degraders, including actinomycetous: *Mycobacterium* sp. PYR1—52.4% (Heitkamp et al. 1988) *Rhodococcus* sp. UW1—72% (Walter et al. 1991) *Leclercia adecarboxylata*—61.5% (Sarma et al. 2004). The lower degradation recorded for LP6 (40.09%) is in tandem with its generally poor performance on most hydrocarbon substrates tested (Table 3). This may be due to possible effects of some of some of the metabolites which inhibit further degradation. This argument is buttressed by its early attainment of stationary phase, in spite of early similarities in utilisation of pyrene it shared with LP5.

Since bacteria initiate PAH degradation by the action of intracellular dioxygenase, the PAHs must be taken up by the cells before degradation can take place (Johnsen et al. 2005). Thus uptake rate is an important parameter amidst the gamut of factors worthy of consideration when assessing the biodegradation abilities of potential candidates for bioaugmentation or biostimulation. Recent report by Das and Mukherjee (2007) on the role of biosurfactant in the uptake of pyrene by *Bacillus* and *Pseudomonas* spp. has further underscored this. Our organisms demonstrated higher uptake rates than the non-biosurfactant enhanced resting cell of the two *Pseudomonas* strains reported by these authors. It is noteworthy that preliminary studies (data not shown) already showed that ours are poor biosurfactant producers with pyrene, suggesting therefore other mechanisms of uptake than biosurfactant production in the growth medium. The higher rate of uptake of pyrene recorded for LP1 (Fig. 2) is in consonance with its higher rate of utilisation. The same also applies to LP5, which had the lowest total uptake, but peaked only within 6 min. The rapidity of uptake may account for the relatively higher growth rate of this organism. The inconsistencies between growth rate and amount of pyrene utilised can be attributed to difference in the efficiency of conversion of the carbon source to biomass by the different strains.

Pathway of degradation of aromatic compounds usually involves the incorporation of two atoms of oxygen into the aromatic ring and subsequent cleavage of the dihydroxylated compound. Such cleavage could either be *ortho* (between the two hydroxylated carbons) or *meta* (between a hydroxylated and non-hydroxylated carbon; Cerniglia 1992). The three organisms in this study degraded catechol via the *ortho* pathway, but more interestingly, LP1 with the highest rate of degradation of pyrene showed the least enzyme activity. This is not unlikely however as the dioxygenase enzymes are highly specific and the ones involved in the cleavage of pyrene ring itself in this case are actually more crucial than those of the lower pathway. The rate of degradation is ultimately a function of interaction among a number of factors including rate of uptake and cellular physico-chemistry.

We have described for the first time organisms exhibiting pyrene catabolic phenotypes from Nigerian contaminated systems. Our results have shown that pyrene-degraders may, as well, act on a number of hydrocarbon environmental mixtures including aliphatic and aromatic compounds. Pyrene degraders are not so common in the environment; however, such organisms are needed for use in developing models and strategies for removing PAH pollutants. Obtaining them require careful and painstaking search.

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