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Fluorene biodegradation potentials of *Bacillus* strains isolated from tropical hydrocarbon-contaminated soils

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Two fluorene-degrading Gram-positive *Bacillus* strains, putatively identified as *Bacillus subtilis* BM1 and *Bacillus amyloliquefaciens* BR1 were isolated from hydrocarbon- and asphalt-contaminated soils in Lagos, Nigeria. The polluted soils have a relatively high total hydrocarbon content (16888.9 and 9923.1 mg/kg, respectively), very low concentrations of macronutrients and the total organic carbon was less than 4%. The two strains tolerated NaCl concentration of up to 7% while strain BR1 exhibited moderate growth at 10%. Shared resistance to ceftriazone and cotrimozaxole were exhibited by both strains while only strain BM1 was resistant to both amoxycilin and streptomycin. The rate of degradation of fluorene (50 mg/L) by the two isolates, after 30 days of incubation were 0.09 and 0.08 mg/L/h for strains BM1 and BR1, respectively. Gas chromatographic analyses of residual fluorene, revealed that 56.9 and 46.8% of 50 mg/L fluorene was degraded in 12 days by strains BM1 and BR1. However, after 21 days on incubation, 86 and 82% of 50 mg/L fluorene were degraded by strains BM1 and BR1, respectively. To the best of our knowledge, this is the first report highlighting flourene degradation potential of *Bacillus* strains isolated from tropical African environment.

Key words: Biodegradation, fluorene, hydrocarbon-contaminated soils, *Bacillus* spp.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds composed of two or more fused benzene rings. They are found in fossil fuel and result from incomplete combustion of organic compounds and other forms of pyrolysis and pyrosynthesis (Kanaly and Harayama, 2010). They have low aqueous solubility, are highly lipophilic and often persist in soil and sediments. PAHs are of concern because of their genotoxic effect for humans and environmental persistence.

Fluorene is a non-alternant PAH composed of two benzene rings between which is tucked a five-membered ring. It is sparingly soluble in water (1.992 mg/L) and has been found to persist in ground water and sediments at coal and oil gasification sites. It is found as constituent of refined coal derivatives such as creosotes as well as in vehicle exhausts. It is classified by the United States Environmental Protection Agency (USEPA) as a priority pollutant (Keith and Telliard, 1979).

The mutagenicity of its derivatives, which are used in pharmaceutical industry and as dyestuff, has been established (Shibutani et al., 1998). The interest in the biodegradation of fluorene is due in part to its persistence

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and genotoxicity as well as its potential for use as model for study of other rather carcinogenic non-alternant PAHs.

Unlike many of the other lower molecular weight PAHs such as naphthalene, phenanthrene and anthracene, fluorene degraders are not as readily isolated from the environment. However, a wide range of bacteria spanning both Gram-positive and Gram-negative genera including *Pseudomonas, Sphinghomonas, Burkholderia, Brevibacterium, Micrococcus*, *Arthrobacter* and *Terrabacter* have been reported (Grifoll et al., 1992; Grifoll et al., 1994; Grifoll et al., 1995; Monna et al., 1993; Trenz et al., 1994; Wattiau et al., 2001; Mukesh et al., 2012). Equally, there have been reports on degradation of fluorene in mixed cultures or by consortia (Gomes et al., 2006; Arulazhagan et al., 2010).

The non-alternant structure of fluorene offers a variety of routes for the initiation of its aerobic degradation by bacteria. Essentially, the pathways for its degradation to the intermediates of tricarboxylic acid (TCA) cycle have been described (Kasuga et al., 1997; Habe et al., 2004; Habe et al., 2005). It usually proceeds by initial di-oxygenation at the 1, 2 or 3, 4 positions with consequent dehydrogenation of the resulting cis-dihydrodiol and subsequent meta cleavage. Alternatively, there is mono-oxygenation at the 9-carbon position to 9-hydroxyfluorene and dehydrogenation to 9-fluorenone (Schuler et al., 2008).

Although there is a considerable information in the literature on the metabolism and genetics of fluorene degradation, there is virtually no report of study of fluorene degradation in the tropical African environment and in Nigeria especially where gas flaring, unabated release by automobiles and at times deliberate sabotage of oil pipelines are loading the environment with plethora 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 degradation of fluorene by pure isolates of two strains of *Bacillus* species from hydrocarbon-polluted sites in Lagos, Nigeria.

MATERIALS AND METHODS

Sampling

Soil samples were collected from contaminated sites in Lagos, Nigeria, namely MWL (a mechanic workshop at Mebamu, Badagry) and APS (an asphalt-polluted soil along Lagos-Ibadan Highway). Soil samples were collected at a depth of 10-12 cm with sterile trowel after clearing debris from the soil surface. Samples for physicochemical analyses were collected in polyethylene bags, while those for microbiological analyses were collected in sterile screw-capped bottles. Samples were analyzed immediately upon arrival in the laboratory. Leftover samples were refrigerated at 4°C.

Physicochemical analysis of soil samples

The pH of the soil samples was determined with a pH meter (Jenway, 3051) in 1:1 soil solution in distilled water. The moisture content, organic carbon content, total nitrogen content, potassium content and available phosphorous were determined as described previously (Bray and Kurtz, 1945; Black, 1965; Chopra and Kanwar, 1998). Conductivity and total hydrocarbon content of the soil were determined as described by Salam et al. (2014). The heavy metal content of the soils was determined using atomic absorption spectrophotometer (Alpha 4, AAS) following mixed acid digestion and extraction of the soil samples.

Enrichment and isolation of fluorene-degrading bacteria

Bacteria able to degrade fluorene were isolated on fluorene mineral salts medium (MSM) by continual enrichment method. The mineral salts medium described by Kästner et al. (1994) was used. The medium contained in liter of distilled water: Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4.7H_2O$, 0.20 g. It was supplemented with yeast extract (0.005 g/L) as source of growth factors. After adjusting the pH to 7.2, the medium was fortified with 50 µg/mL of nystatin to suppress fungal growth. Sterile trace elements solution (1.0 mL/L) described by Bauchop and Elsden (1960) was aseptically added to the medium after sterilization. Contaminated soil (5 g) was added to 45 ml of MSM containing 50 mg/L of fluorene. Enrichment was carried out by incubation with shaking (180 rpm) at room temperature (28 ± 2°C) in the dark for four to five weeks until there was turbidity. After five consecutive transfers, fluorene degraders were isolated by plating out dilutions from the final flasks on Luria-Bertani (LB) agar. The colonies that appeared were further purified by sub culturing once onto LB agar. Ability to degrade fluorene was confirmed by inoculating washed LB broth grown culture in fresh MSM flask supplemented with 50 mg/L fluorene as sole carbon source.

Maintenance and identification of isolates

Pure bacterial isolates were maintained in glycerol/LB broth medium (1:1, v/v). Pure colonies of fluorene degraders sub cultured on LB agar supplemented with low percentage of fluorene (0.005%) were harvested with sterile inoculating loop, pooled and transferred to the medium. The mixture was shaken to homogenize and kept at -20°C.

Pure identification of fluorene-degrading isolates was carried out based on their colonial morphology, cellular morphology and biochemical characteristics according to the identification scheme of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Antibiotic sensitivities of the isolates were determined using standard multidisc.

Salt tolerance test of the pure isolates was conducted in LB broth supplemented with varying concentrations (1%-10%, w/v) of NaCl. Incubation was carried out with intermittent shaking at room temperature (28 ± 2°C) for two weeks.

Evaluation of fluorene biodegradation

Replicate 250 ml flasks containing 50 ml of MSM with 50 mg/L of fluorene as sole source of carbon were prepared. Flasks were inoculated with 0.5 ml of MSM-washed 18 - 24 h LB agar-grown cells to achieve an initial cell concentration of about 3.2×10^6 cfu/ml and subsequently incubated at 180 rpm in the dark for 21 days at room temperature (28 ± 2°C). Flasks containing fluorene as described above but inoculated with heat-killed cells were used as controls. Samples were withdrawn from each flask at 3 days interval and aliquots of appropriate dilutions were plated (in triplicates) onto nutrient agar for total viable counts (TVC).

Table 1. Physicochemical properties of hydrocarbon-contaminated sampling sites.

Parameter	MWL	APS
pH	5.01	6.12
Moisture (%)	7.49	9.64
Conductivity (µs/cm)	62.6	64.9
Total organic carbon (%)	1.94	3.14
Total hydrocarbon content (mg/kg)	16888.9	9923.1
Potassium (mg/kg)	9.40	8.10
Nitrogen (%)	0.10	0.15
Phosphorus (mg/kg)	1.52	0.08
Lead (mg/kg)	0.001	0.003

MWL, A mechanic workshop at Mebamu, Badagry; **APS**, An asphalt-polluted soil along Lagos-Ibadan Highway in Nigeria.

Extraction of residual fluorene

Residual fluorene was extracted by liquid-liquid extraction. Briefly, broth culture (50 ml) was extracted twice with an equal volume of hexane. After removing the aqueous phase with separating funnel, the organic fraction was concentrated to 1 ml and the residual fluorene concentration was determined by gas chromatography. Control flasks were also extracted similarly.

Analytical method

Hexane extracts (1.0 µl) of residual fluorene were analyzed with 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 carrier gas was nitrogen. The injector and detector temperatures were maintained at 300 and 320°C, respectively. The column temperature was programmed from 60 to 500°C for 27 min. The gas chromatograph column was programmed at an initial temperature of 60°C; this was held for 2 min, and then ramped at 12°C/min to 205°C and held for 16 min. Nitrogen column pressure was 37 psi, the hydrogen pressure was 9 psi and compressed air pressure was 13 psi. The software was Chem Station. Rev. A. 05. 01.

Statistical analysis

Mean generation times (T_d) and growth rate (K) of the isolate on fluorene was calculated using Prism version 5.0 (Graphpad software, San Diego, CA).

RESULTS AND DISCUSSION

Table 1 shows the physicochemical properties of the soils used in this study. The pH of the soils was acidic with a moisture content of 7.49 and 9.64%, respectively. The total hydrocarbon content of MWL (a mechanic workshop at Mebamu, Badagry) site is relatively higher (16888.89 mg/kg) compared to APS (an asphalt-polluted soil along Lagos-Ibadan Highway) site (9923.08 mg/kg). Concentrations of macronutrients such as nitrogen, phosphorus and potassium at the two sites were very low while the total organic carbon of the two sites was less than 4%. These results are not surprising as hydrocarbon contamination in soil have been reported to induce reduction in water holding capacity as well as a shift to acidic pH (Dibble and Bartha, 1979; Chikere and Okpokwasili, 2002).

Indigenous bacteria inhabiting hydrocarbon-contaminated niches have been widely employed with outstanding successes in the degradation of PAHs. This is because previous exposure to the compounds often results in evolution of adapted microflora that have acquired the necessary degradative genes and capable of transforming and mineralizing the compounds after a long period (Wackett and Hershberger, 2001). In this study, continual enrichment resulted in the isolation of several fluorene degraders. The best fluorene degraders from each of the two-soil sample were used. The two isolates were Gram-positive, endospore-forming, motile rods that are oxidase and catalase positive. They showed negative reaction to indole, urease and methyl red and failed to ferment xylose, galactose and raffinose. Colonial morphology of strain BM1 obtained from MWL indicated that it was circular in shape, dull cream in color, opaque, flat in elevation with round smooth edges and an entire margin. It was positive for citrate utilization, Voges-Proskauer, casein, gelatin and starch hydrolysis, failed to ferment lactose and arabinose, and reduces nitrate to nitrite. It was thus putatively identified as *Bacillus subtilis*. The colonies and biochemical characteristics of strain BR1 obtained from APS were quite similar to strain BM1. However, it had irregular rough edges, cream in color, ferment lactose and arabinose, reduced nitrate to nitrite and failed to hydrolyze casein. Based on these characteristics, it was putatively identified as *Bacillus amyloliquefaciens*. It is noteworthy that though, previous reports have established the propensity of PAHs degraders from the Nigerian environment (Ilori and Amund, 2000; Igwo-Ezikpe et al., 2006; Obayori et al., 2008; Salam et al., 2014), to the best of our knowledge, this is the first report of fluorene degraders from the Nigerian environment.

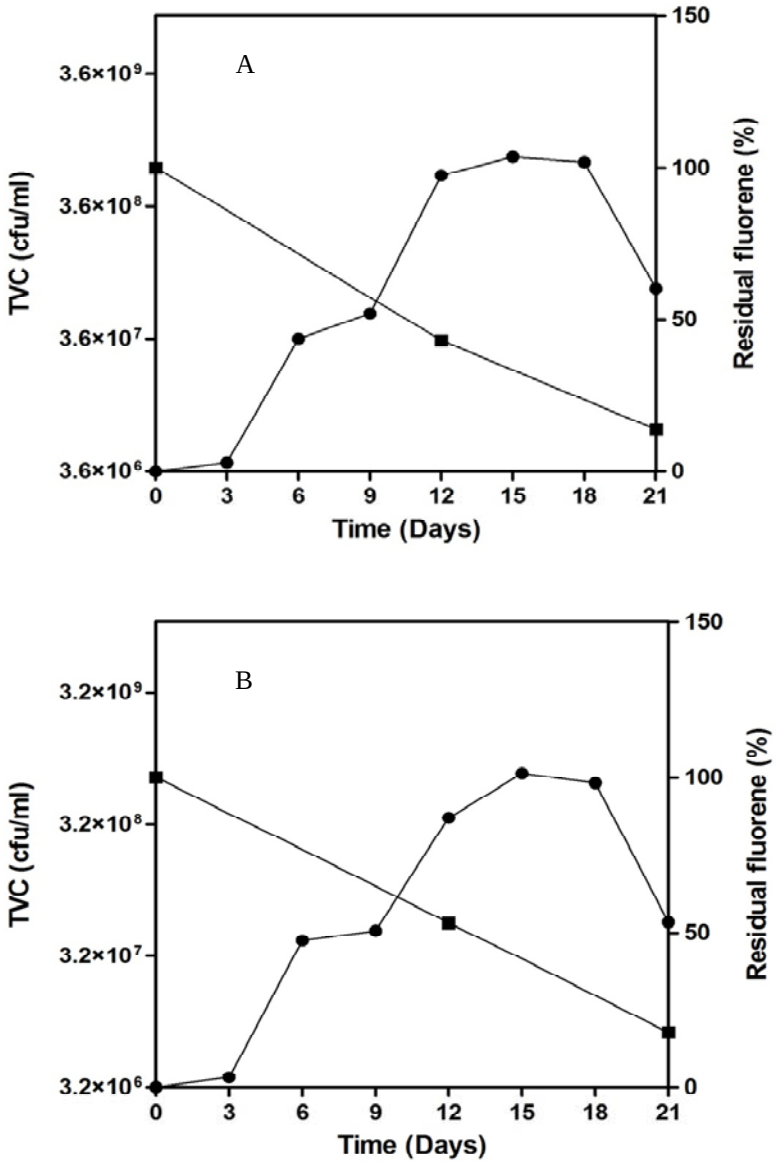

Figure 1. Growth dynamics of BM1 (A) and BR1 (B) strains in minimal medium amended with 50 mg L^{-1} fluorene showing total viable count, TVC (●) and residual fluorene (■). Data points represent the mean of three replicate flasks. In the case of population counts, error bars that represent standard deviation were removed for clarity. Residual fluorene were determined with reference to fluorene recovered from heat-killed controls.

The two isolates tolerated NaCl concentration of 7% with good and moderate growth exhibited by strain BR1 and BM1, respectively. However, at 10% NaCl concentration, only strain BR1 showed moderate growth. This physiological property favours the use of these strains as possible candidates for bioaugmentation purpose. Previous reports have shown that salinity could be a critical factor that determines the survival of allochthonous bacterial strains during bioremediation (Kästner et al., 1998; Obayori et al., 2008). In addition, the two strains resisted ceftriazone and cotrimozaxole but were susceptible to ofloxacin, gentamicin, and ciprofloxacin. Only strain BM1 was resistant to both amoxycilin and streptomycin. Shared resistance of strains BM1 and BR1 to ceftriazone and cotrimozaxole may be attributed to acquisition of resistant genes to these antibiotics through gene transfer, as soil environments are replete with these antibiotics, which could allow evolution of resistance by indigenous strains (Obayori et al., 2008).

The growth kinetics of the *Bacillus* strains on fluorene is illustrated in Figure 1 and Table 2. The two strains

Table 2. Growth kinetics of fluorene-degrading isolates.

Isolate	Growth rate, K (h^{-1})	Mean generation time, ΔT_d (h)	Percentage (%) degradation[1] (day 12)	Percentage (%) degradation[1] (day 21)	Degradation rate (%/h)	Rate of degradation (mg $L^{-1}h^{-1}$)
BM1	0.016	44.9	56.9	86	0.17	0.09
BRI	0.015	45.2	46.8	82	0.16	0.08

[1]Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat-killed control flasks.

exhibited slight lag phases followed by gradual population increase with concomitant decrease in fluorene concentration. Strain BM1 grew from an initial population density of 3.6×10^6 cfu/ml to peak at 8.6×10^8 cfu/ml, resulting in over 200-fold increase in 15 days. It thereafter maintained a decreasing trend. During the exponential growth of the isolate on fluorene, it exhibited a growth rate and doubling time of 0.016 h^{-1} and 44.9 h, respectively. Similar growth pattern on fluorene was obtained with strain BR1, which increase from an initial cell density of 3.2×10^6 cfu/ml to 7.8×10^8 cfu/ml in 15 days. It also exhibited a growth rate and doubling time of 0.015 h^{-1} and 45.2 h during exponential growth on fluorene.

The rates of fluorene utilization as quantified by gas chromatographic (GC) analysis were 0.09 and 0.08 mg/L/h respectively for strains BM1 and BR1. These rates of degradation were lower than 0.8 ± 0.07 mg/L/h reported for fluorene-degrading *Pseudomonas putida* ATCC 17514 in 10 to 15 days (Rodrigues et al., 2005). Fluorene transformation by strains BM1 and BR1 were studied at 72 h intervals in MSM containing 50 mg/L fluorene. After 12 days of incubation, the residual fluorene content for strains BM1 and BR1 decreases to 43.06% (21.53 mg/L) and 53.22% (26.61 mg/L) corresponding to uptake of 56.94% (28.47 mg/L) and 46.78% (23.39 mg/L) fluorene, respectively. At the end of 21 days incubation, the residual fluorene content decreases further to 14% (7.0 mg/L) and 17.82% (8.90 mg/L) corresponding to uptake of 86% (43 mg/L) and 82.2% (41.1 mg/L) fluorene, respectively (Table 2). In the heat-killed control flasks, no apparent decrease of the substrate was observed, thus confirming that fluorene depletion from the MSM was due to biodegradation by the isolates rather than to non-specific abiotic losses such as substrate volatility or absorption to the glass tubes.

The percentages of fluorene degraded by these strains were lower than the 97% recorded for *Pseudomonas* sp. PSS6 (Mukesh et al., 2012). However, the difference is rather insignificant since the authors challenged the *Pseudomonas* strain with only 3 mg/L of fluorene compared to our 50 mg/L. Furthermore, fluorene degradation rates of the two strains is higher than 40.6% in 20 days reported for enteric bacterium *Leclercia adecarboxylata* isolated from oil sludge contaminated soil (Sarma et al., 2004). It is equally higher than the 81.87% in 50 days reported for *Rhodococcus ruber* ISO-2 isolated from automobile workshop sediments (Srujana and Khan, 2012).

The ability of the two strains to degrade fluorene may not be unconnected with the fact that they were isolated from sites where, non-volatile hydrocarbons, spent oils with its attendant PAHs, were indiscriminately dumped. This may have allowed autochthonous organisms to adapt and evolve necessary gene battery to degrade pollutants.

Previous reports have established the importance of prior exposure and adaptation to acquisition of degradative genes by autochthonous microorganisms. The mechanisms of adaptation include synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-impacted community through horizontal gene transfer (Top and Springael, 2003; Obayori and Salam, 2010).

Though, several researchers have highlighted degradative abilities of *Bacillus* strains on PAHs (Das and Mukherjee 2007; Lily et al. 2009; Yuliani et al. 2012), globally, only one report exists on fluorene utilization by *Bacillus* species. Hidayati et al. (2011) reported fluorene removal by a biosurfactant-producing *Bacillus megaterium*.

The presence of the amended crude biosurfactant increase fluorene removal by the organism. In this study, two species of *Bacillus* putatively identified as *Bacillus subtilis* BM1 and *Bacillus amyloliquefaciens* BR1 displayed extensive degradation on fluorene and to the best of our knowledge, this is the first report highlighting the fluorene degradative potentials of these two species of *Bacillus*.

Further works will focus on the environmental factors favorable for the application of these organisms, the metabolites produced and the degradative genes involved in the process.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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