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Carbazole angular dioxygenation and mineralization by bacteria isolated from hydrocarbon-contaminated tropical African soil

- Authors
- Authors and affiliations
- L. B. Salam
- M. O. Ilori
- O. O. Amund
- M. Numata
- T. Horisaki
- H. Nojiri

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Abstract

Four bacterial strains isolated from hydrocarbon-contaminated soils in Lagos, Nigeria, displayed extensive degradation abilities on carbazole, an *N*-heterocyclic aromatic hydrocarbon. Physicochemical analyses of the sampling sites (ACPP, MWO, NESU) indicate gross pollution of the soils with a high hydrocarbon content (157,067.9 mg/kg) and presence of heavy metals. Phylogenetic analysis of the four strains indicated that they were identified as *Achromobacter* sp. strain SL1, *Pseudomonas* sp. strain SL4, *Microbacterium esteraromaticum*strain SL6, and *Stenotrophomonas maltophilia* strain B_A. The rates of degradation of carbazole by the four isolates during 30 days of incubation were 0.057, 0.062, 0.036, and 0.050 mg L^{-1} h^{-1} for strains SL1, SL4, SL6, and B_A. Gas chromatographic (GC) analyses of residual carbazole after 30 days of incubation revealed that 81.3, 85, 64.4, and 76 % of 50 mg l^{-1} carbazole were degraded by strains SL1, SL4, SL6, and B_A, respectively. GC-mass spectrometry and high-performance liquid chromatographic analyses of the extracts from the growing and resting cells of strains SL1, SL4, and SL6 cultured on carbazole showed detection of anthranilic acid and catechol while these metabolites were not detected in strain B_A under the same conditions. This study has established for the first time carbazole angular dioxygenation and mineralization by isolates from African environment.

Keywords

Biodegradation Carbazole *Achromobacter Stenotrophomonas Microbacterium Pseudomonas*

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Introduction

Carbazole ($C_{12}H_9N$, dibenzopyrrole diphenylenimine, CAS no. 86-74-8) is a nonbasic tricyclic aromatic *N*-heteroatomic compound. It has a molecular weight of 167.21 g/mol, boiling and melting point of 355 and 246 °C, water solubility of 1.2 mg/l, vapor pressure of 1×10^{-4} Pa (Peddinghaus et al. 2012), and octanol/water partition coefficient (log K_{ow}) of 3.72 (Blum et al. 2011). It is one of the π-excessive heterocycles (electron-rich rings) and is more recalcitrant than dibenzofuran but less than dibenzothiophene (Balaban et al. 2004). It is one of the major *N*-heterocyclic aromatic hydrocarbons in fossil fuels (coal, crude oil, oil derived from oil shales pyrolysis) and is also found in cigarette smoke and from coal and wood combustion (Odabasi et al. 2006). However, its release into the environment from diverse anthropogenic sources is of serious health and environmental concern, as carbazole is both mutagenic and toxic and classified as “benign tumorigen” (Smith and Hansch 2000; Nojiri and Omori 2007).

Interest in the study of bacterial degradation of carbazole is spurred partly because of the ubiquitous nature of the pollutant and its mutagenic and toxic activities. In addition, carbazole is a structural analog of dioxins, and carbazole-degrading enzymes can partly function as dioxin-degrading enzymes (Nojiri and Omori 2007). Three major degradation pathways have been reported for carbazole: lateral dioxygenation at carbon positions 3 and 4; monohydroxylation at carbon positions 1, 2, and 3; and angular dioxygenation at carbon positions 1 and 9a (Grifoll et al. 1995; Lobastova et al. 2004).

Some microbes reported in literature degrade carbazole via angular dioxygenation, a novel type of oxidative attack that occurred at the ring-fused position and mediated by a multicomponent enzyme, carbazole 1,9a-dioxygenase (CARDO) with addictive preference for angular positions (Nojiri et al. 2001; Nojiri 2012). In angular dioxygenation, carbazole is dioxygenated at angular (C9a) and adjacent (C1) carbon atoms to produce an unstable hemiaminal (1-hydro-1,9a-dihydroxycarbazole) which is spontaneously cleaved to form 2′-aminobiphenyl-2,3-diol. This metabolic intermediate is converted to anthranilic acid via *meta*-cleavage and subsequent hydrolysis. Anthranilic acid is converted to catechol by dioxygenation at the C1 and C2 positions followed by

spontaneous deamination and decarboxylation reactions. Formed catechol is converted to a tricarboxylic acid (TCA) cycle intermediate via *ortho*-cleavage or *meta*-cleavage pathways (Nojiri and Omori 2002; Nojiri 2012).

Elevated concentrations of heavy metals are introduced into soil compartments through various anthropogenic activities such as petroleum exploration, waste disposals, and corrosion of metals in use among others. Toxicity of heavy metals to autochthonous microbial community in soil has been reported to also inhibit biodegradation of pollutants in co-contaminated sites (Sandrin et al. 2000). To tolerate heavy metals stress, microorganisms have evolved various resistance mechanisms such as efflux pumps, enzymatic detoxification, permeability barriers, intra- and extracellular sequestration, and reduction (Nies 1999).

In spite of existing body of knowledge on carbazole metabolism, there is virtually no report of study of carbazole angular dioxygenation and mineralization in the African environment in general and Nigeria especially where gas flaring and indiscriminate release of various hydrocarbon mixtures is preponderant. In addition, improving on the available bank of autochthonous microbial resources is crucial to the proper management of polluted sites as cleanup strategies are environment specific. In this paper, we report for the first time, the isolation and characterization of a novel *Microbacterium* sp. and two other bacterial strains from African environment capable of angular dioxygenation and mineralization of carbazole.

Materials and methods

Sampling and physicochemical analysis

Soil samples were obtained from three different sampling points within Lagos metropolis (Fig. 1). These are Abandoned Coal Power Plant (ACPP), Ijora-Olopa (latitude 6° 28′ 1″ N and longitude 3° 22′ 47″ E); Mechanic Workshop (MWO), Okokomaiko (latitude 6° 28′ 23″ N and longitude 3° 11′ 14″ E); and NEPA Substation (NESU), UNILAG (latitude 6° 31′ 18″ N and longitude 3° 23′ 47″ E). Soil samples were taken at a depth of 10–12 cm using sterile hand trowel after removing the debris from the soil surface. Samples for physicochemical analysis were collected in clean black polythene bags, while samples for microbiological analysis were collected in sterile screw-capped bottles. Immediate analysis of the samples were carried out within 5 h of collection or stored at 4 °C. The pH of the soil samples was determined with a pH meter (Jenway 3051). Moisture and total organic contents were determined gravimetrically, while total nitrogen content was determined by macro-Kjeldahl digestion method described by Black (1965). Heavy metals composition of the soils was determined using atomic absorption spectrophotometer (Alpha 4, AAS) following mixed acid digestion and extraction of the soil samples (Mwegoha and Kilampa 2010). The

total hydrocarbon content of the soil was extracted using *n*-hexane:dichloromethane solvent systems (1:1, *v*/*v*) according to the method described by Obayori et al. (2008a, b).

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Fig. 1

Map of Lagos State, Nigeria showing the sampling points used in this study. The location of ACPP (*black circle*), MWO (*light gray circle*), and NESU (*gray circle*) and their description were indicated on the map

Isolation of carbazole-degrading bacteria

The carbon-free mineral medium (CFMM) described by Habe et al. (2002) was used. The medium contained per liter of distilled water NH_4NO_3, 3.0 g; Na_2HPO_4, 2.2 g; KH_2PO_4, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.05 g; and $CaCl_2 \cdot 2H_2O$, 0.05 g. The medium was supplemented with yeast extract (0.005 g). The pH of the medium for bacteria was adjusted to 7.0 and nystatin included at 50 μg/ml. Nalidixic acid (20 μg/ml) was also added to the medium for selective isolation of actinomycetes. Contaminated soil sample (5 g; for actinomycetes the soil was air-dried) was added to 45 ml of CFMM medium containing 50 mg l^{-1} carbazole. Enrichment was carried out by shaking for 4 to 5 weeks in the dark at room temperature until there was turbidity. After five transfers, carbazole 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 on LB agar. Ability to degrade carbazole was confirmed by inoculating pure isolates into fresh CFMM medium flasks containing carbazole (50 ppm) as sole carbon and energy source and observing for turbidity.

Identification and molecular characterization of carbazole-degrading isolates

Pure cultures of carbazole-degrading isolates were identified based on their colonial morphology, cellular morphology, biochemical characteristics according to the identification scheme of Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Genomic DNA from the six carbazole-degrading isolates was extracted using standard protocols (Ausubel et al. 1990). Using the purified genomic DNA as template, the gene coding for 16S rRNA was amplified using the universal primers 27f (5′-AGAGTTTGATC{A/C}TGGCTCAG-3′) and 1378r (5′-CGGTGTGTACAAGGCCCGGGAACG- 3′) (Heuer et al. 1997). The amplification reaction mixture contained 20 pmol each of universal primers, 10 μl of Ex *Taq* buffer (Mg^{2+}plus), 2.5 mM of each dNTPs, 2.5 U (0.5 μl) of Ex *Taq* polymerase (Takara Bio, Shiga, Japan), and 1.0 μl of purified genomic DNA in a total volume of 100 μl. Amplification conditions consisted of an initial

denaturation step at 95 °C for 3 min, 30 amplification cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min with the final extension at 72 °C for 7 min before cooling to 4 °C. The PCR product was cloned into the plasmid vector pT7Blue® (Novagen, USA) after purification by centrifugation using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, Wis.). *Escherichia coli* strain DH5α (Toyobo, Tokyo, Japan) was transformed by the resultant plasmids. Nucleotide sequencing was carried out by the chain termination method using the ABI Prism 3730xl DNA Sequencer (Applied Biosystems, UK) according to the manufacturer's instructions. The 16S rRNA nucleotide sequence obtained from both strands was aligned (CLUSTAL W) and the homology search for 16S rRNA were performed in the DDBJ/EMBL/GenBank database using the Basic Local Alignment Search Tool (BLAST) program. Phylogenetic tree with retrieved sequences from NCBI database was constructed using neighbor joining algorithm within the program MEGA 5.1 (The Biodesign Institute) and bootstrapped with 500 repetitions.

Substrate specificity of carbazole-degrading isolates

Biodegradation ability of carbazole degraders on different hydrocarbon substrates was evaluated in CFMM containing the respective hydrocarbons as sole carbon and energy source at a concentration of 100 mg l^{-1}. Stock solution of the substrates was prepared by dissolving 1 g of respective hydrocarbon substrates in 10 ml dimethyl sulfoxide (DMSO) and filter-sterilized the solution using hydrophobic filter (Advantec, JP020AN). Sterile CFMM (5 ml) was prepared in test tubes and 5 µl of stock solution of the respective hydrocarbons was added. Carbazole degraders were added at 1 % (10^6 cfu/ml; *v*/*v*). Incubation was carried out at room temperature in the dark for 14 days. Test tubes prepared as above but without carbon source serve as control. Degradation was monitored by cell increases and visual observation for turbidity. The polyaromatic and heterocyclic aromatic hydrocarbons tested include naphthalene, fluorene, acenaphthene, pyrene, anthranilic acid, dibenzothiophene, dibenzothiophene-sulfone, dibenzofuran, 3,3′-dimethoxybenzidine, and *p*-hydroxybenzoic acid. Liquid hydrocarbons like crude oil and engine oil were autoclaved and added separately to the sterile CFMM at 0.1 % (*v*/*v*).

Metal tolerance assay

Carbazole-degrading isolates were grown in LB broth for 18 h at room temperature. Cells were harvested by centrifugation (7,000 × *g*; 10 min), washed twice with sterile phosphate buffer, and resuspended in the same buffer solution. The cell concentration of bacterial suspensions was determined by measuring the optical density of the samples at 600 nm and relating the value to a calibration curve (10^{10} cfu L^{-1} = 1 OD unit).

Stock solutions (1 M) of metal salts namely, $HgCl_2$, $CrCl_3$, $NiSO_4$, and $Pb(NO_3)_2$ were prepared in distilled water, filter-sterilized using 0.22-µm membrane filters, and stored in sterile bottles in the dark at 4 °C. Dilutions to 1, 5, 10, and 15 mM of Hg^{2+}, Cr^{3+}, Ni^{2+}, and Pb^{2+} were made from the stock solutions into LB broth. The media were dispensed in 5-ml aliquots and inoculated with 50 µl (1 %, *v*/*v*) inoculum. Each of the experiment was conducted in triplicates. LB broth not amended with heavy metals and inocula serves as controls. Growth of the inocula was measured by absorbance at 600 nm and occasional viable count assay. Resistance was assayed by determining the maximum tolerance concentrations (MTCs) for the isolates after 10 days of incubation. MTC is defined as the highest concentration of metal, which do not affect the viable counts of organisms.

Biodegradation studies

Evaluation of carbazole biodegradation

Carbazole degradation was assessed by inoculating replicate 250-ml flasks containing 50 ml of CFMM medium already supplemented with carbazole as a sole carbon and energy source at concentration of 50 mg l^{-1}. Flasks were inoculated with 0.5 ml of CFMM-washed 18 h LB-grown cells and subsequently incubated with shaking for 30 days at room temperature. Culture flasks prepared as stated above but inoculated with heat-killed cells were used as controls. Biodegradation was monitored by assaying for residual carbazole using gas chromatography (GC) equipped with flame ionization detector (FID) concomitant with withdrawing a flask from the lot at intervals of 3 days and plating out aliquots of appropriate dilutions (in triplicates) onto nutrient agar for total viable counts (TVC).

Extraction of residual carbazole for gas chromatography

Residual carbazole was extracted twice by adding 10 ml of hexane to broth culture (50 ml) in flask and shaken vigorously for 30 min using a mechanical shaker. After removing the aqueous phase with separating funnel, the solvent was allowed to vent off in a preheated oven (170 °C) overnight to about 1 ml to concentrate the analyte (carbazole). The residual carbazole concentration was determined by injecting 1 µl of the resultant solution for gas chromatographic analysis.

Gas chromatographic analysis

Residual carbazole was determined by gas chromatography equipped with flame ionization detector (GC/FID). A standard carbazole (1 µl; 50 mg l^{-1}) was first injected into the GC/FID to obtain a standard chromatogram, which gave a peak area for standard carbazole. This was carried out to identify the run time and

retention time for carbazole prior to injection of the test sample. Afterwards, hexane extract (1 µl) was injected into GC/FID. Carbazole concentrations in the hexane were determined using a Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector. The column SE-30 is 60 m long. The carrier gas was nitrogen. The injector and detector temperature were maintained at 220 and 250 °C, respectively. The column was programmed at an initial oven temperature of 70 °C for 2 min, then ramped at 10 °C/min to 200 °C and held for 5 min.

Metabolites detection from growing and resting cells of carbazole degraders using gas chromatography-mass spectrometry (GC-MS)

Growing cultures (5 ml) of the isolates were centrifuged (13,000 rpm, 2 min) to remove the residual substrate and the cells. The supernatants were twice extracted with ethyl acetate (4,000 rpm, 10 min) after acidification to pH 2 with 1 N HCl. The ethyl acetate layer was dried with anhydrous sodium sulfate and concentrated by a rotary evaporator under reduced pressure at 20 °C. The concentrated ethyl acetate extracts were derivatized with methylation with PTAH, m-(trifluoromethyl)-phenyltrimethylammonium hydroxide (TMTFTH). For resting cells, bacterial cells were cultivated in 200 ml of CFMM/carbazole medium at 30 °C for 2–3 days. The cells were harvested by centrifugation (5,000 × *g*, 4 °C) for 15 min and washed twice with CFMM buffer. The resultant cells were distributed in 5-ml aliquot into test tubes and 50 µL of a 50-ppm stock solution of carbazole in DMSO were added to each tube of resting cells. The reaction mixture was incubated at 30 °C on a rotary shaker (300 rpm) for 1, 2, 3, 24, and 48 h. GC/MS analysis was performed on a JEOL JMS-K9 Ultra Quad GC/MS (JEOL Ltd., Tokyo, Japan) interfaced with an Agilent Technology 6890 N Network GC system equipped with a splitless injector. A capillary column InertCap® (5 % phenyl-95 % methylpolysilarylene; I.D. 0.25 mm, length 15 m, film thickness 0.25 µm) (GL Sciences Inc. Japan) was used as the analytical column. Each sample (1 µl) was injected into the column at 80 °C in the splitless mode. After 2 min at 80 °C, the column temperature was increased to 280 °C at 16 °C min^{-1}. The head pressure of the helium carrier gas was 65 kPa.

HPLC analysis of anthranilic acid metabolites

High-performance liquid chromatography (HPLC) was carried out to detect anthranilic acid metabolites from anthranilic acid-grown cultures of strains SL1, SL4, and SL6, respectively. Anthranilic acid (AN; 50 mg l^{-1}) was supplied as sole sources of carbon and energy and cultures were incubated in the dark at room temperature for 14 days. HPLC analysis was done using Shimadzu model 2010 HT (Kyoto, Japan) equipped with a variable wavelength photodiode array detector and fitted with uBondapak C18 column model WAT 025875 (250 mm length; 4.6 mm I.D.; 5 µm thickness; WATERS Scientific). Acetonitrile extracts

from growing cells culture (5 µl) were analyzed using acetonitrile and water (60:40 *v*/*v*) mobile phase, at a flow rate of 2 ml min^{-1}. Column temperature was set at 30 °C. Major products were monitored at an absorbance of 254 nm and identified with reference to retention times of standards used.

Catechol dioxygenase assay

Two milliliters of strains SL1, SL4, and SL6 cultures were harvested by centrifugation at the late logarithmic phase from CFMM medium containing carbazole and were suspended in 1-ml CFMM. Cells were lysed by the addition of 20 µl toluene and after vigorous mixing, unbroken cells and cell debris were removed by centrifugation at 16,000 × *g* for 30 s. The clear supernatants were immediately used for the assay or placed on ice for not more than 10 min. Activity assays were performed using GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, USA). The reaction was initiated by the addition of 100 µl catechol solution (100 µM) to a reaction mixture in a 1-cm light path quartz cuvette containing 800 µl phosphate buffer and 100 µl of crude lysate. The blank cuvette contained the same amount of enzyme in the same buffer with the exception of catechol. Activities of catechol 1,2-dioxygenase and 2,3-dioxygenase were monitored at 260 and 375 nm, respectively (Wang et al. 2008).

Results

Physicochemical properties of soil samples

The physicochemical properties of the soil samples was conducted to provide information about limiting nutrients, pollutants, and other physical factors that could determine the types of microorganisms recovered from the soils. The pH of the three sampling sites was weakly acidic (5.4–6.1); conductivity and total hydrocarbon content were highest at MWO site (318 µs/cm; 157,067.9 mg/kg), while the nitrogen content of the three study sites was generally less than 1 %. The presence of various heavy metals such as lead, cadmium, and nickel in reasonable concentrations further corroborated the extent of pollution of the sampling sites (Table 1).

Table 1
Physicochemical properties of the study sites

Parameter	NESU	MWO	ACPP
pH	5.80	6.10	5.40
Moisture (%)	7.89	6.85	11.1

Parameter	NESU	MWO	ACPP
Conductivity (µs/cm)	159.4	318	67.4
Total organic carbon (%)	1.01	1.93	3.10
Total hydrocarbon content (mg/kg)	216	157,067.9	13,382
Potassium (mg/kg)	0.28	2.10	18.4
Nitrogen (%)	0.05	0.10	0.18
Phosphorus (mg/kg)	0.19	1.34	363.4
Iron (mg/kg)	28.83	2.27	ND
Lead (mg/kg)	0.06	0.11	4.70
Zinc (mg/kg)	0.47	3.31	ND
Copper (mg/kg)	5.10	ND	ND
Manganese (mg/kg)	3.24	1.83	ND
Cadmium (mg/kg)	1.12	ND	ND
Nickel (mg/kg)	3.42	4.34	ND

ND Not detected; *MWO* Mechanic workshop, Okokomaiko; *ACPP* Abandoned coal power plant soil, Ijora-Olopa; *NESU* NEPA substation, UNILAG

Identification and characterization of carbazole-degrading isolates

Phenotypic characterization of carbazole-degrading strains SL1, SL2, and SL3 isolated from ACPP site showed that the strains were aerobic, motile, Gram-negative rods that are oxidase and catalase positive and urease negative. They failed to ferment most of the sugars tested with exception of glucose, xylose, galactose, and mannose. They are positive for nitrate reduction and show negative reaction to H_2S and indole production. Colonies on LB agar appeared circular, round, and smooth with no pigmentation. Based on these characters, they were putatively identified as *Achromobacter* species.

Strain SL4 isolated from MWO site was Gram-negative, aerobic, nonspore-forming, motile rod. Colonies on LB agar are smooth, circular, and muddy white in appearance. The strain was positive for oxidase, catalase, and nitrate reductase but negative for indole, methyl red, Voges-Proskauer, gelatinase, urease, and amylase. Colonies utilized glucose, fructose, arabinose, and

galactose but failed to ferment xylose, lactose, and raffinose. It was putatively identified as *Pseudomonas* species.

Strain SL6 also isolated from MWO site was obligately aerobic, Gram-positive, nonspore-forming, irregular rods occurring singly or in clusters. On LB agar, SL6 was circular, smooth, translucent, yellow-pigmented, opaque, low-convex, moist colonies with entire margins. Strain SL6 colonies are catalase positive but negative for oxidase, methyl red, Voges-Proskauer, indole, gelatinase, and H_2S production. It was positive for starch hydrolysis and is unable to utilize all the sugars tested with exception of mannitol, and salicin. Based on these characters, they were putatively identified as *Corynebacterium* species

Strain B_A isolated from ACPP site was a Gram-negative, aerobic, motile, nonspore-forming, rod-shaped bacterium. Colonies of strain B_A on LB agar were circular, smooth, glossy, and convex with a pale yellow appearance. Biochemical tests indicated that strain B_A was positive for oxidase, catalase, and gelatinase and urease negative. It exhibited negative activities for nitrate reduction as well as H_2S and indole production. Most of the sugars tested such as glucose, arabinose, mannitol, mannose, inositol, rhamnose, sucrose, sorbitol, melobiose, maltose, and inositol supported the growth of strain B_A. It was thus putatively identified as a *Xanthomonas* sp.

However, molecular characterization of the six strains based on sequencing of 16S rRNA partial fragments showed that strains SL1, SL2, and SL3 (1,383 bp) shares 99 % identity with nucleotide sequences of *Achromobacter* spp. deposited at the NCBI database. Strain SL4 (1389 bp) also shares 99 % identity with other *Pseudomonas* spp. in the database, while strain SL6 (1374 bp) shares 99 % identity with *Microbacterium esteraromaticum* strains. Strain B_A(1397 bp) was found to cluster with *Stenotrophomonas maltophilia* strains sharing 99 % identity. The nucleotide sequences of the six strains SL1, SL2, SL3, SL4, SL6, and B_A were deposited at the DDBJ, EMBL, and GenBank nucleotide sequence databases and were assigned the accession numbers **AB646575.2**, **AB646576.2**, **AB646577.2**, **AB646578.2**, **AB646579.2**, and **AB646574**, respectively. Four (SL1, SL4, SL6, B_A) of the six isolates that displayed better degradative ability on carbazole were selected for further study. A phylogenetic tree constructed using neighbor joining algorithm of the six carbazole-degrading bacteria and related strains retrieved from NCBI database is shown in Fig. 2.

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Fig. 2

Phylogenetic tree resulting from neighbor joining analysis of 16S rRNA showing the phylogenetic positions of carbazole-degrading strains SL1, SL2, SL3, SL4, SL6, and $B_A$and related species of the

genus *Achromobacter*, *Pseudomonas*, *Microbacterium*, and *Stenotrophomonas* retrieved from NCBI GenBank. Accession number of each microorganism used in the analysis is shown before the species name

Substrate specificity of carbazole-degrading isolates

Substrate spectrum analysis of carbazole degraders isolated in this study on various aromatic and heteroaromatic hydrocarbon substrates was conducted to ascertain the substrate utilization pattern of each isolate. The analysis revealed different utilization patterns. All the isolates failed to grow on naphthalene and dibenzofuran while none of the isolates grows luxuriantly on all the polycyclic aromatic hydrocarbons tested. In addition, shared specificity for dibenzothiophene-sulfone (DBT-S), carbazole, *N*-ethyl carbazole, anthranilic acid, crude oil, and engine oil by all the isolates were also observed (Table 2).

Table 2

Substrate specificity test

Substrate	Isolates			
	SL1	SL4	SL6	B[A]
Naphthalene	–	–	–	–
Fluorene	+	–	++	+
Acenaphthene	++	++	+	++
Pyrene	+	+	+	+
Carbazole	+++	+++	+++	+++
Dibenzofuran	–	–	–	–
Dibenzothiophene	+	–	+	–
Dibenzothiophene-sulfone	+++	+++	++	++
3,3′-dimethoxybenzidine	++	+++	–	+
N-ethyl carbazole	++	+	++	+
Anthranilic acid	+++	+++	++	+
p-hydroxybenzoic acid	++	+	+	–
Crude oil	+++	+++	+++	++
Engine oil	++	++	+	+

+++ Luxuriant growth (>10^6 cfu/ml after 5 days of incubation); ++ Weak growth (>10^6 cfu/ml after 1 week of incubation); + Poor growth (>10^6 cfu/ml after 2 weeks of incubation); – No growth (<10^6 cfu/ml after 2 weeks of incubation)

Metal tolerance of carbazole-degrading isolates

Metal tolerance assay of the carbazole degraders on various heavy metals was conducted to determine the tolerance limit of the isolate to various concentrations of heavy metals. The assay revealed different resistance patterns. All the isolates (SL1, SL4, and SL6) showed resistance to 1 mM mercury and also resisted 1–5 mM of nickel and lead, respectively. Chromium concentrations of 1–5 mM were tolerated by strains SL4 and SL6 while strain SL1 tolerated up to 10 mM.

Biodegradation studies

The utilization of carbazole as a sole source of carbon and energy by the four strains was confirmed by an increase in cell population with a concomitant loss of carbazole. Population dynamics of the four isolates grown on 0.3 mM carbazole and incubated at room temperature in the dark for 30 days are shown in Fig. 3. Growth of strains SL1, SL4, and B_A on carbazole reaches its peak on day 12 with a population of 7.4×10^9, 8.1×10^9, and 7.4×10^9 cfu/ml, respectively, while it took strain SL6 15 days to reach its highest population of 7.8×10^8 cfu/ml. The rate of degradation of carbazole by the four isolates after 30 days of incubation are 0.057, 0.062, 0.036, and 0.050 mg l^{-1} h^{-1} for strains SL1, SL4, SL6, and B_A, respectively (Table 3). Gas chromatographic analyses of residual carbazole after 30 days of incubation showed that 81.3, 85, 64.4, and 76 % of the initial concentration of carbazole were degraded by strains SL1, SL4, SL6, and B_A, respectively (Table 3).

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Fig. 3

Population dynamics of strains SL1 (*inverted triangle*), SL4 (*upright triangle*), SL6 (*square*) and B_A (*diamond*) on carbazole after 30 days of incubation in the dark at room temperature. Carbazole was supplied at a concentration of 0.3 mM. Data points represent the mean of three replicate flasks. *Error bars* were eliminated for clarity

Table 3

Growth kinetics of the isolates on carbazole

Isolates	Specific growth rate, μ (h^{-1})	Mean generation time, ΔT_d(h)	% Degradation	Degradation rate (%/h)	Rate of degrada (mg L^{-1}
*Achromobacter*sp. strain SL1	0.0229	30.0	81.3	0.113	0.057
Pseudomonas sp. strain SL4	0.0238	29.0	85.0	0.118	0.062
*M. esteraromaticum*strain SL6	0.0125	55.4	64.4	0.089	0.036
*S. maltophilia*strain B_A	0.0233	29.5	76.4	0.106	0.050

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

Detection of metabolites of carbazole biodegradation

In order to identify the intermediates produced during the degradation of carbazole by strains SL1, SL4, SL6, and B_A, ethyl acetate extracts of growing and resting cells cultures were analyzed by GC-MS. TMTFTH-derivatized acidified extracts contained metabolites with the same GC retention times and mass spectra as the TMTFTH-derivatives of authentic AN. TMTFTH-derivatized AN had two GC peaks believed to be derived from compounds that were methylated at a COOH group and at both COOH and NH_2 groups. Mass-fragmentation patterns (mass spectra) of TMTFTH-derivatized AN obtained from growing and resting cells ethyl acetate extracts of strains SL1, SL4, and SL6 as well as TMTFTH-derivatives of authentic anthranilic acid were displayed in Fig. 4. It is noteworthy that anthranilic acid was neither detected in the growing cells nor resting cells ethyl acetate extracts of strain B_A. When AN was used as carbon source in culture, catechol (CAT) was obtained as the major metabolite (in strains SL1, SL4, SL6) by HPLC. Furthermore, a small amount of *cis*,*cis*-muconate was also detected in strain SL1.

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Fig. 4

Mass-fragmentation patterns (mass spectra) data of TMTFTH-derivatized anthranilic acid obtained from growing and resting cells ethyl acetate extracts of strains SL1, SL4, and SL6. Panels *A*, *C*, and *E* shows mass spectra data for methylated anthranilic acid from ethyl acetate extracts of resting cells culture of strains SL1, SL4, and SL6. Panels *B*, *D*, and *F* shows mass spectra data for anthranilic acid (*N*-methyl, methyl ester), anthranilic acid (*N*, *N*-dimethyl,

methyl ester), and anthranilic acid (*N*-methyl, methyl ester) from ethyl acetate extracts of growing cells culture of strains SL1, SL4, and SL6. Panels *A1*, *C1*, and *E1* shows mass spectra data for standard anthranilic acid (methylated) obtained from the database for the resting cell extracts. Panels *B1*, *D1*, and *F1* shows mass spectra data for standard anthranilic acid (*N*-methyl, methyl ester; *N*, *N*-dimethyl, methyl ester; *N*-methyl, methyl ester) obtained from the database for the growing cell extracts

The three carbazole-degrading strains also degrade catechol via the *ortho* pathway as reflected in increase activity (increase in absorbance spectra values) at 260 nm when monitored using UV-Vis spectrophotometer thus indicating the formation of *cis*,*cis*-muconate via catechol 1,2-dioxygenase activity for the three strains (Fig. 5). At absorbance value of 375 nm, there is consistent decrease in absorbance spectra values, which indicates that the three carbazole-degrading strains lack ability to degrade catechol using the *meta* pathway. A simplified metabolic pathway of carbazole degradation by the three isolates (SL1, SL4, SL6) showing the metabolites identified using GC-MS, HPLC, and UV-Vis is presented in Fig. 6.

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Fig. 5

Enzymatic transformation of catechol to *cis*,*cis*-muconic acid by lysate of carbazole-grown cells. The reaction was started in a sample cuvette containing 100 ml of cell lysate in 800 ml of phosphate buffer, pH 7.5, by the addition of 100 mM catechol. Optical absorption spectra were recorded at periodic intervals of 0, 2, 4, 6, 8, and 10 min. Increase in absorption spectra at 260 nm indicate conversion of catechol to *cis*,*cis*-muconic acid by the three isolates (**a**). Consistent decreases in absorption spectra were observed at 375 nm (**b**)

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Fig. 6

Simplified degradation pathway of carbazole in *Achromobacter* sp. strain SL1, *Pseudomonas* sp. strain SL4, and *M. esteraromaticum* strain SL6. Only the metabolites identified are shown. Compounds: *I*, carbazole; *II*, anthranilic acid; *III*, catechol; *IV*, *cis*,*cis*-muconic acid; *TCA*, tricarboxylic acid

Discussion

The use of autochthonous microorganisms inhabiting hydrocarbon-polluted niches for biodegradation and bioremediation has been widely accepted as a formidable approach due to avalanche of successes recorded by researchers (Jain et al. 2005; Andreoni and Gianfreda 2007).

Carbazole degraders are isolated from polluted environments through classical continuous enrichment method. Majority of carbazole degraders reported in literature are aerobic, Gram-negative bacteria with the exception of very few carbazole degraders such as *Nocardioides aromaticivorans* IC177 (Inoue et al. 2005) and *Gordonia* sp. F.5.25.8 (Santos et al. 2006) that are aerobic, Gram-positive bacteria. Carbazole degraders isolated in this study are members of the genera *Achromobacter*, *Pseudomonas*, *Microbacterium*, and *Stenotrophomonas*, respectively. Aside from *Pseudomonas* species, reports on carbazole degradation by other isolated bacteria genera in this study are very few or nonexistent (Inoue et al. 2005; Farajzadeh and Karbalaei-Heidari 2012).

The genus *Achromobacter* is widely distributed in nature. They are nutritionally versatile with propensities for degradation of anthropogenic compounds such as hydrocarbons, polycyclic aromatic hydrocarbons, heterocyclic aromatic hydrocarbons, and polychlorinated biphenyls (Hong et al. 2008; Ilori et al. 2008; Eixarch and Constanti 2010; Kaczorek et al. 2013). In this study, *Achromobacter* sp. strain SL1 isolated from ACPP site, which exhibited specific growth rate and doubling time of 0.0229 h^{-1} and 30.0 h degrades 81.3 % of 50 ppm (0.3 mM) carbazole within 30 days with a rate of degradation of 0.057 mg l^{-1} h^{-1}. Reports on carbazole degradation by *Achromobacter* spp. are scanty and only two reports existed globally, which detailed carbazole biodegradation by *Achromobacter* species. Inoue et al. (2005) isolated *Achromobacter* sp. strain IC074 that degrade carbazole and harbor carbazole degradative genes *carR*, *carAa*, and *carAc* highly homologous to *Novosphingobium* sp. strain KA1. Similarly, Farajzadeh and Karbalaei-Heidari (2012) reported the isolation of an *Achromobacter* sp. strain CAR1389, which degraded 90 % of 6 mM carbazole within 7 days.

The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria due to their remarkable degree of physiological and genetic adaptability. *Pseudomonas* is reputed to possess broad substrate affinity for different classes of hydrocarbons such as alicyclics, heterocyclics, and aromatics (Vankateswaran et al. 1995; Nojiri et al. 1999; Obayori et al. 2008b). *Pseudomonas* strain SL4 isolated in this study, which exhibited specific growth rate and doubling time of 0.0238 h^{-1} and 29 h degraded 85 % of 0.3 mM carbazole within 30 days with a rate of degradation of 0.062 mg l^{-1} h^{-1}. This value is lower than 98 % carbazole degradation in 56 h reported for *Pseudomonas* sp. XLDN4-9 (Li et al. 2006) but higher than 12 % carbazole degradation in 10 days reported for *Pseudomonas rhodesiae* KK1 (Yoon et al. 2002), respectively.

The genus *Stenotrophomonas* are known to exhibit diverse degradative abilities on hydrocarbons, aromatic hydrocarbons, polycyclic aromatic hydrocarbons,

heterocyclics, and xenobiotics such as pesticides and herbicides (Juhasz et al. 2002; Nayak et al. 2009; Guo et al. 2013). In particular, *S. maltophilia* strains have shown extensive catabolic versatility on high and low molecular weight PAHs such as phenanthrene, benzo(*a*)pyrene, dibenzo(*a*,*h*)anthracene, and coronene (Juhasz et al. 2000; Guo et al. 2013). Carbazole degradation by *Stenotrophomonas* species has been reported by Inoue et al. (2005). Also, Shintani et al. (2008) reported conjugal transfer of pCAR1, an (Inc) P-7 conjugative plasmid involved in the degradation of carbazole from *Pseudomonas* host to *Stenotrophomonas*recipients, though the plasmid is unstable in the recipient strains. However, in this study, *S. maltophilia* strain B_A isolated from ACPP site degraded 76.4 % of 0.3 mM carbazole within 30 days with rate of degradation of 0.05 mg l^{-1} h^{-1}. On carbazole, this strain also exhibited specific growth rate and doubling time of 0.0233 h^{-1} and 29.5 h, respectively. Although involvement of a *S. maltophilia* strain in carbazole degradation through conjugal transfer have been reported, nevertheless, globally, this is the first report highlighting natural carbazole degradative potential of a *S. maltophilia* strain.

The phylum *Actinobacteria* encompasses bacteria genera such as *Mycobacterium*, *Rhodococcus*, and *Gordonia* with unrivaled capability to degrade recalcitrant pollutants due to their metabolic versatility, genetic plasticity, and ability to survive in harsh environments (Larkin et al. 2005; Mutnuri et al. 2005; Kanaly and Harayama 2010). In this study, strain SL6 phylogenetically identified as *M. esteraromaticum* and isolated from MWO site degrades 64.4 % of 0.3 mM carbazole within 30 days with a rate of degradation of 0.036 mg l^{-1} h^{-1}. The growth kinetics of the isolate on carbazole indicates a specific growth rate and doubling time of 0.0125 h^{-1} and 55.4 h, respectively. Although the degradation rate is lower than 80 % in 30 days and 40 % in 10 days reported for *N. aromaticivorans* strain IC177 and *Gordonia* sp. strain F.5.25.8 (Inoue et al. 2005; Santos et al. 2006), it is however higher than 57 % reported for *Arthrobacter* sp. P1-1 (Seo et al. 2006). Nevertheless, it is noteworthy that globally this is the first report detailing carbazole degradation potential of a *Microbacterium* sp.

Angular dioxygenation, the initial dioxygenation of carbazole, is a distinct reaction mediated by CARDO, which exhibit high regioselectivity and addictive preference for the angular position as hydroxylation occurs at the ring-fused position (Nojiri and Omori 2007; Nojiri 2012). Angular dioxygenation result in complete mineralization of carbazole with the resulting intermediate, anthranilic acid converted to catechol, which is degraded via *ortho* or *meta* pathways to TCA cycle intermediate (Nojiri and Omori 2002).

In this study, three of the four carbazole-degrading bacterial strains, SL1, SL4, and SL6, cleaved carbazole angularly and methylated derivative of the COOH group and both the COOH and NH_2 groups of anthranilic acid were detected in the resting and growing cells cultures similar to those found from other carbazole-degrading strains that cleaved carbazole angularly. These results indicated that the degradation pathway for carbazole to anthranilic acid in these strains was similar to that of the most extensively studied carbazole degrader *Pseudomonas resinovorans* strain CA10 (Ouchiyama et al. 1993). Furthermore, anthranilic acid has been detected from the culture extracts of several carbazole degraders and is regarded as the main metabolite of carbazole angular dioxygenation (Ouchiyama et al. 1993; Gieg et al. 1996; Ouchiyama et al. 1998; Kirimura et al. 1999; Schneider et al. 2000; Kilbane II et al. 2002; Inoue et al. 2005). Degradation of carbazole to anthranilic acid is important because anthranilic acid is an easily degradable and harmless substrate, and various organisms for the tryptophan biosynthesis pathway (Gibson and Pittard 1968; Maeda et al. 2009) assimilate it.

To discern the fate of anthranilic acid produced by the isolates during growth on carbazole, anthranilic acid was used as the only source of carbon and energy for the isolates, and production of catechol was monitored using HPLC at absorbance of 254 nm. Catechol was detected in the three isolates with small amount of *cis*,*cis*-muconic acid in strain SL1. In addition, with exception of strains SL1, residual anthranilic acid was detected by HPLC in the culture extracts of strains SL4 and SL6. These results indicate the possibility that anthranilic acid was converted to catechol by anthranilate 1,2-dioxygenase and the catechol formed mineralized by the β-ketoadipate pathway via *ortho* cleavage by strains SL1, SL4, and SL6 as confirmed by catechol dioxygenase assay using UV-Vis spectrophotometry. Detection of catechol from anthranilic acid has been reported in previous studies on carbazole degraders with angular dioxygenation ability. Ouchiyama et al. (1993) detected anthranilic acid and catechol (when anthranilic acid was used as carbon source) from culture extracts of strain *P. resinovorans* strain CA10 using HPLC and GC-MS and regarded anthranilic acid and catechol as the main metabolites of carbazole biodegradation. Furthermore, aside from catechol, small traces of *cis*,*cis*-muconic acid was detected by HPLC when anthranilic acid was used as carbon source for strain CA10 (Ouchiyama et al. 1993).

In a typical contaminated soil, inorganic nutrients especially the macronutrients is always limiting or lacking resulting in slow pollutant degradation even in the presence of carbon and energy required for growth (Giordani et al. 1998; Vidali 2001). For instance, the concentration of nitrogen and phosphorus at NESU and MWO site is very low, which may be due to their high demand by microorganisms for sugar phosphorylation, synthesis of amino acids, nucleic

acids, nucleotides, and other cellular processes (Andrew and Jackson 1996). In essence, amendment of these polluted soils with nitrogen and phosphorus is necessary to enhance biodegradation of organic pollutants.

Activity of soil microorganisms are optimized when between 38 and 81 % of soil pore space is saturated with water. Availability of water and oxygen are maximized in this range of water content (Maier 2009). Thus, the amount of available water for microbial growth and metabolism may limit hydrocarbon biodegradation in soil (Leahy and Colwell 1990). The observed low moisture content of the soils used in this study (6.85–11.1 %) could be ascribed to a hydrocarbon-mediated reduction in the water holding capacity of the soils (Dibble and Bartha 1979). The weakly acidic pH (5.40–6.10) observed at the hydrocarbon-contaminated sites used in this study could be attributed to hydrocarbon inputs. Earlier report indicates that environments, which receive hydrocarbon inputs, tend to be more acidic and very poor in nutrient content (Chikere and Okpokwasili 2002).

Heavy metals are ubiquitous and persistent environmental pollutants that are introduced into the environment through anthropogenic activities and other sources of industrial wastes. The presence of heavy metals such as iron, lead, cadmium, and nickel at the sampling sites used in this study indicate gross pollution as heavy oils and spent oils rich in heavy metals are indiscriminately disposed at these sites. Heavy metals in low concentrations are micronutrients, which play indispensable roles in cell growth and metabolic functions. However, at high concentrations, heavy metals induce oxidative stress and interfere with protein folding and function (Nies 1999). Bacteria to counteract heavy metals stress have devised various resistance mechanisms. These include formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Nucifora et al. 1989; Nies and Silver 1995; Outten et al. 2000). Isolation of carbazole degraders from MWO and ACPP sampling sites in spite of the high heavy metals presence especially at MWO may be due to the possibility of the degraders harboring genes for heavy metals resistance and the high hydrocarbon and organic carbon contents of the two sites. This assertion is buttressed by the fact that the carbazole degraders resisted elevated concentrations of heavy metals such as mercury, chromium, nickel, and lead. However, the relatively low hydrocarbon and organic carbon contents coupled with the presence of heavy metals at the NESU sampling site, UNILAG may be responsible for the inability to isolate carbazole degraders from this site.

The substrate spectrum analysis of the carbazole degraders isolated in this study on various hydrocarbon substrates revealed different utilization patterns. This may be attributed to the varied composition of the substrates and the diverse

nature of hydrocarbon products present at the site from which the isolates were recovered (Leahy and Colwell 1990). All the isolates failed to grow on naphthalene and dibenzofuran while none of the isolates grows luxuriantly on all the polycyclic aromatic hydrocarbons tested. In addition, shared specificity for DBT-S, carbazole, *N*-ethyl carbazole, anthranilic acid, crude oil, and engine oil by all the isolates were also observed. These findings corroborate earlier reports that carbazole degraders have limited substrate specificity for growth (Grosser et al. 1991; Kimura and Omori 1995). For example, two carbazole-degrading *Sphingomonas* strains, CB3 and CDH7, were reported to lack mineralizing ability for fluorene, naphthalene, dibenzothiophene, dibenzofuran, biphenyl, and phenanthrene (Shotbolt-Brown et al. 1996; Kirimura et al. 1999). It is noteworthy that the best studied carbazole degrader, *P. resinovorans* strain CA10, lack specificity for dibenzothiophene-sulfone (Takagi et al. 2002). However, carbazole-degrading strains isolated in this study grow luxuriantly on DBT-S indicating possibly the acquisition of novel degradative genes passively because of long exposure to various hydrocarbon products by the isolates. Capability to mineralize DBT-S has also been reported for carbazole-degrading actinomycetes, *N. aromaticivorans* strain IC177 (Inoue et al. 2005). Furthermore, the degradation of 3,3-dimethoxybenzidine (3,3 DMB) by some of the isolates is good news as 3,3 DMB is a congener of the known human bladder carcinogen, benzidine, and is classified by the International Agency for Research on Cancer as Group 2B carcinogens.

The luxuriant growth of the isolates observed on crude oil as compared to sparse growth on engine oil may be attributed to two factors. First, crude oil, a complex mixture of different chemical composition may favorably support growth of microorganisms better than refined petroleum product such as engine oil due to diverse nutrient options available in crude oil as source of carbon and energy. Second, at the ACPP and MWO sites where these isolates were recovered, different types of oil products may have been used for lubrication and fuelling of the coal plant coupled with indiscriminate disposal of spent engine oil and diesel at MWO site. These pollutants inevitably found their way into the soil along with product of coal combustion thereby resulting in adaptation of autochthonous organisms to the pollutants due to selective pressure and acquisition of degradative abilities.

We have described for the first time bacterial isolates from African environment exhibiting abilities for angular dioxygenation and mineralization of carbazole. We have also reported for the first time globally the involvement of a *Microbacterium* sp. in angular dioxygenation and mineralization of carbazole. Our results have shown that these isolates may, as well act on varieties of environmental hydrocarbon mixtures including polycyclic aromatics, heterocyclic aromatics, crude oil, and engine oil. This qualifies our isolates as

good candidates for bioremediation of sites not only polluted with carbazole and related dioxin compounds but also other hydrocarbons.

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