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Aerobic degradation of di- and trichlorobenzenes by two bacteria isolated from polluted tropical soils

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Abstract

Two polychlorinated biphenyl (PCBs)-degrading bacteria were isolated by traditional enrichment technique from electrical transformer fluid (Askarel)-contaminated soils in Lagos, Nigeria. They were classified and identified as *Enterobacter* sp. SA-2 and *Pseudomonas* sp. SA-6 on the basis of 16S rRNA gene analysis, in addition to standard cultural and biochemical techniques. The strains were able to grow extensively on dichloro- and trichlorobenzenes. Although they failed to grow on tetrachlorobenzenes, monochloro- and dichlorobenzoic acids, they were able to utilize all monochlorobiphenyls, and some dichlorobiphenyls as sole sources of carbon and energy. The effect of incubation with axenic cultures on the degradation of 0.9 mM 1,4-dichlorobenzene, 0.44 mM 1,2,3- and 0.43 mM 1,3,5-trichlorobenzene in mineral salts medium was studied. Approximately, 80–90% of these xenobiotics were degraded in 200 h, concomitant with cell increase of up to three orders of magnitude, while generation times ranged significantly ($P < 0.05$) from 17–32 h. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were detected in crude cell-free extracts of cultures pre-grown with benzoate, with the latter enzyme exhibiting a slightly higher activity (0.15–0.17 μmol min^{-1} mg of $protein^{-1}$) with catechol, suggesting that the *meta*-cleavage pathway is the most readily available catabolic route in the SA strains. The wider substrate specificity of these tropical isolates may help in assessing natural detoxification processes and in designing bioremediation and bioaugmentation methods.
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1. Introduction

Chlorinated benzenes (CBz), with 12 possible isomers, are common and widespread environmental pollutants with reports of contamination in coastal marine sediments, freshwater lake sediments, sewage sludge, wastewater, groundwater, rivers and estuaries, and soils (Oltmanns et al., 1988; Boyd et al., 1997; Wu et al., 2002). They have been widely used in industrial, agricultural and domestic products such as pesticides, soil fumigants, disinfectants, toilet deodorants, solvents, and precursors for the production of dyes and silicone coatings (Golden et al., 1993; Fetzner, 1998). As a result of their persistence, toxicity, and bioaccumulation potential, the production, use and discharge of CBz are subject to regulation in most developed countries. Unfortunately, partly due to limited economic resources, these compounds are not subject of any regulation in most African countries. Consequently, unrestricted use, accidental release and illegal discharge into the environment often continue unabatedly.

Due to the hazards presented by CBz in the environment, information on their rates and extent of biodegradation is of great interest. If bioremediation or bioaugmentation of

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contaminated matrices are to be effectively used, isolation and characterization of organisms capable of degrading CBz are necessary. Increased knowledge about physiological properties and substrate range will help determine the process conditions that should be used and the range of transformations that can be obtained in practical treatment systems.

In recent years, several organisms have been isolated that were able to degrade CBz (Mars et al., 1997; Sommer and Gorisch, 1997; van der Meer et al., 1998; Janssen et al., 2001; Pollmann et al., 2001). Organisms able to use CBz as sole carbon and energy sources produced a chlorocatechol as an intermediate by the action of a chlorobenzene 1,2-dioxygenase and a dehydrogenase. The chlorocatechols are usually further degraded via a modified *ortho*-cleavage pathway involving an intradiol dioxygenase (Reineke and Knachmuss, 1988; van der Meer et al., 1991, 1992; Sommer and Gorisch, 1997; Mars et al., 1999), or alternatively through a *meta*-cleavage pathway (Bartels et al., 1984; Arensdorf and Focht, 1994; Mars et al., 1999). Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle (TCA).

Although there have been numerous reports of aerobic biodegradation of monochlorobenzenes and dichlorobenzenes (diCBz) (Spain and Nishino, 1987; Oltmanns et al., 1988; Sommer and Gorisch, 1997), reports of isolation of bacteria capable of trichlorobenzene (triCBz) degradation are more limited (van der Meer et al., 1991; Sander et al., 1991). In addition, to the best of our knowledge, there has been no previous report of the biodegradation of chlorinated aromatic compounds by organisms indigenous to the tropical African environment, even though CBz pollution of most environmental matrices is present in this part of the world. Indeed, most studies that have established biodegradation pathways and kinetics have used isolates obtained in temperate regions of the developed world. It is unlikely, however, that identical xenobiotic-degrading bacteria are uniformly distributed around the globe due to differing ambient environmental conditions, soil composition, organic carbon soil inputs, and many other factors. We herein report the degradation of diCBz and triCBz by two bacterial strains both of which were previously demonstrated to possess competent PCB degradative capabilities (Adebusoye et al., 2004).

2. Materials and methods

2.1. Chemicals

Analytical grades of chlorobenzene congeners including, 1,2-diCBz (98.6% purity), 1,3-diCBz (99.6% purity), 1,4-diCBz (98.6% purity), 1,2,3-triCBz (>99% purity), 1,2,4-triCBz (>99% purity), 1,3,5-triCBz (>99% purity), 1,2,4,5-tetraCBz (99.6% purity), 1,2,3,5-tetraCBz (99.2% purity) and 1,2,3,4-tetraCBz (99.3% purity) were purchased from AccuStandard, Inc., New Haven, CT. All standards were solutions of chlorobenzene congeners with 95% purity purchased from Ultra Scientific (North Kingstown, RI). Askarel fluid was generously provided by the National Electric Power Authority (NEPA) Transformer Workshop, Ijora, Lagos, Nigeria. All other chemicals used were reagent grade or better.

2.2. Physico-chemical properties of the soil sample

Soil samples for microbial isolations and physico-chemical parameters were collected (up to the depth of 10 cm) randomly from a PCB-polluted soil at the Transformer Workshop of the Power Holding Company of Nigeria (PHCN; formerly, NEPA), Ijora, Lagos. This site has been heavily polluted with electrical transformer fluids (Askarel; a blend of PCBs and CBz) for decades. The samples were placed into sterile bottles and transported immediately to the laboratory for further work. The physico-chemical composition of the soil was determined using standard protocol of Black (1965) as well as Bray and Kurtz (1945).

2.3. Isolation and maintenance of bacterial strains

Chlorobenzene-degraders indigenous to the soils were enriched and isolated using a mineral salts (MS) medium previously described by Kim and Picardal (2000). Soil (1 g) was added to 99 ml MS medium in a flask and supplemented with 0.1% (v/v) Askarel fluid as the sole carbon and energy source. Enrichment flasks were incubated at 25 °C on a gyratory shaker table for 30 days. Utilization of the compound in enrichment cultures was evidenced by a visual decrease in the amount of oil, a color change in the medium, and a visual increase in turbidity. When growth had occurred, the enrichment culture was transferred to fresh MS medium using a 1% inoculum and incubation continued. This procedure was repeated for five successive transfers. Pure cultures were isolated from enrichments by plating out appropriate dilutions onto nutrient agar plates. Single colonies were transferred to MS medium containing Askarel or CBz to confirm their ability to grow on these substrates. Isolates able to utilize selected CBz (as described below) were selected for further characterization and study. Bacterial isolates were preserved in nutrient broth supplemented with 0.5% yeast extract and 20% glycerin at −80 °C.

2.4. Identification of the bacterial strains

Substrate utilization patterns were obtained using the API 20 E system (bioMerieux Vitek, Inc., Hazelwood, MO). Identification of the organisms was achieved on the basis of 16S rRNA gene analysis. Genomic DNAs were obtained from cultures of isolates grown overnight in MS medium supplemented with 2.5 mM benzoate using UltraClean DNA Prep Kit (MoBio Laboratories, Inc., Solana Beach, CA). Two eubacterial PCR primers: forward primer 63f and reverse primer 1387r, were used to amplify approximately 1300 bp of the 16S rRNA gene (Marchesi et al.,

1998). The resulting PCR-generated fragments were gel-purified, and cloned into the PCR 2.1-TOPO plasmid vector (Invitrogen Life Technologies, Carlsbard, CA). Recombinant plasmids were EcoR1-digested to confirm the presence of rDNA inserts. Recombinant plasmids with correct rDNA inserts were subsequently sequenced on an ABI 3700 sequencer (AP Biotech), and were probed against the GenBank database with the BLAST algorithm.

2.5. Growth and degradation studies

Cells used in growth and degradation studies were grown aerobically at room temperature (≈25 °C) in MS medium supplemented with 2.5 mM benzoate. Cultures were harvested by centrifugation and washed twice in MS medium lacking benzoate, and resuspended in the same medium to an optical density of 0.5–0.6 at 600 nm.

Growth and degradation studies were performed in anaerobic culture (Balch) tubes containing 6 ml of MS medium and selected CBz with enough air headspace (21 ml) to maintain aerobic conditions over the course of the study. The carbon sources used for the study included 1,4-diCBz, 1,2,3-triCBz, and 1,3,5-triCBz. Tubes were inoculated with 10^5–10^6 cells/ml and crimp-sealed with Teflon-coated, butyl rubber stoppers to prevent losses due to volatilization or sorption. With the exception of 1,4-diCBz that was supplied at a target concentration of 132 ppm (0.9 mM), all other congeners were added in 5-μl aliquots to a final concentrations of 78–100 ppm (0.53–0.68 mM for diCBz and 0.43–0.55 mM for triCBz) from stock solutions created by dissolving the substrates in 2,2,4,4,6,8,8-heptamethylnonane (HMN), a multi-branched, non-biodegradable carrier to improve mass transfer. Sealed tubes were incubated horizontally on an orbital shaker table at room temperature. At each time point, entire replicate tubes were sacrificed in time-course experiments. The reaction was stopped by adding 5 ml of hexane, vortexed for 1–2 min and thereafter, mixed continuously on a tube rotator for 12 h. The hexane extracts and aqueous phases were separately collected and stored at −4 °C for analysis. Cell numbers were determined using an acridine orange direct counting method as previously described (Kepner et al., 1994). Mean generation times (T_g) and specific growth rates (μ) were calculated using non-linear regression of growth curves from the first 100–150 h during which growth rates were maximal. Regression, correlation, and variance analyses were performed using Prism version 2.01 (GraphPad Software, San Diego, CA, USA).

2.6. Analytical procedure

Chlorobenzene concentrations in hexane extracts were determined using a Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard Co., Palo Alto) equipped with an electron capture detector and a 30-m DB-5 megabore capillary column (J&W Scientific, Folson, CA). Chlorobenzenes were quantified using four-point calibration curves constructed using standards bracketing the concentration expected in samples showing no degradation. GC efficiency and accuracy was constantly ascertained by injections made from standards prior to sample analysis.

2.7. Enzyme assays

Cells for use in enzyme assays were grown in MS medium containing 2.5 mM benzoate for 18–24 h. Cells were harvested by centrifugation, washed twice with potassium phosphate buffer (50 mM, pH 7.3), and resuspended in the same buffer. Washed cell suspensions were disrupted by repeated (twice) passage through a Manual-Fill Mini-Cell (FA-003) French press (20 000 lb/in^2; Aminco, SLM Instruments Inc., Urbana, IL). Unbroken cells and debris were removed by centrifugation for 30 min at 12 000*g* at 4 °C. The clear supernatant was kept on ice and used immediately after preparation.

Enzyme assays of cell extracts were performed at room temperature. Catechol 1,2-dioxygenase activity was measured spectrophotometrically by the absorbency of the *ortho*-cleavage product (*cis*,*cis*-muconate) at $\lambda = 260$ nm as described by Nakasawa and Nakasawa (1957) and modified by Ka-Leung et al. (1990). Catechol 2,3-dioxygenase was measured according to the procedure of Kataeva and Golovleva (1990) as modified by Kaschabek et al. (1998). The protein contents of cell-free extracts were determined by the modified Lowry method (Galli and McCarty, 1989), using bovine serum albumin as the standard.

3. Results

3.1. Physico-chemical compositions of the Ijora soil

The physico-chemical characteristics of the Ijora soil is shown in Table 1. The results show a general lack of mineral nutrients which are required in relatively large amounts for efficient and normal functioning of microbial cells. Total phosphorus, nitrogen, and organic matter content obtained were 0.074%, 0.012%, and 0.26%, respectively.

3.2. Isolation and identification of chlorobenzene-growing bacterial strains

Our enrichment and isolation procedures produced three isolates able to utilize Askarel, biphenyl, all three

Table 1
Physico-chemical composition of the Ijora soil

Parameter	Mean determination
Physical appearance	Dark, sticky and very oily
pH	6.1
Organic matter (%)	0.26
Moisture (%)	8.15
Ash (%)	74.9
Total phosphorus as P_2O_5 (%)	0.074
Total nitrogen (%)	0.012

monochlorobiphenyls and selected chlorobenzenes in batch culture. Two isolates, SA-2 and SA-6, were selected for further studies due to their ability to grow on several di- and trichlorobenzenes. On nutrient agar, SA-2 produced big, circular, smooth, convex and cream-white colonies while colonies of SA-6 were flat and had light green color pigmentation that diffused relatively quickly. Both strains are motile, Gram-negative, and catalase-positive. The organisms were classified on the basis of 16S rRNA gene analysis by comparison of the obtained sequence data with known sequences in the GenBank (Maidak et al., 1997; Stoesser et al., 1997). DNA analysis of the approximately 1300 nucleotides revealed that SA-2 (accession number DQ 854842) is highly homologous (98%) to the type strain of *Enterobacter aerogenes* 16S rRNA gene sequence with accession number AF 395913. SA-6 (1438 nucleotides, accession number DQ 854841) had 99% homology with *Pseudomonas* sp. Bu34 with accession number AF 031152. Analyses of substrate specificity by the API 20 E test system supported the identification of the organisms, and were subsequently identified as *Enterobacter* sp. strain SA-2 and *Pseudomonas* sp. strain SA-6.

Table 2
Substrate range of bacterial strains

Substrate	SA-2	SA-6
Biphenyl	+	+[b]
Benzoate	+[a]	+[b]
Naphthalene	+	+
2-Monochlorobiphenyl	+	+
3-Monochlorobiphenyl	+	+
4-Monochlorobiphenyl	+	+
2,3-Dichlorobiphenyl	+	+
2,2′-Dichlorobiphenyl	+	+
2,4′-Dichlorobiphenyl	+	+
1,2-Dichlorobenzene	+	+
1,3-Dichlorobenzene	+	+
1,4-Dichlorobenzene	+	+
1,2,3-Trichlorobenzene	+	+
1,2,4-Trichlorobenzene	+	+
1,3,5-Trichlorobenzene	+	+
1,2,3,4-Tetrachlorobenzene	−	−
1,2,3,5-Tetrachlorobenzene	−	−
1,2,4,5-Tetrachlorobenzene	−	−

+, growth; −, no growth. Culture supernatant fluid turned a yellow color that was permanent ([a]) and disappeared ([b]). All compounds were supplied as sole carbon sources in MS medium at 100 ppm except benzoate presented at a concentration of 2.5 mM (305 ppm). All compounds with the exception of benzoate were supplied in a HMN carrier to improve mass transfer.

3.3. Substrate spectrum of SA strains and growth characteristics on 1,4-diCBz

In this study, growth is defined as an increase in cell numbers of at least one order-of-magnitude and concomitant disappearance of the parent compound relative to uninoculated controls. The SA-2 and SA-6 isolates were able to utilize 1,2-, 1,3- and 1,4-diCBz; 1,2,3-, 1,2,4- and 1,3,5-triCBz; as well as all the three monochlorobiphenyls and various dichlorobiphenyls chlorinated on both rings as sole carbon and energy sources (Table 2). They, however, failed to grow with 1,2,3,4-, 1,2,3,5- and 1,2,4,5-tetracholorobenzene. The degradative potential for chlorobenzenes was not lost during repeated growth on non-selective (non-inducing) substrates.

Owing to their limited aqueous solubility, the chlorobenzene substrates were dissolved in HMN and added at an initial concentration of 100 ppm in the medium. The concentration given represents the total mass in both the aqueous and HMN phases, divided by the aqueous volume. However, in order to establish the non-biodegradability of HMN carrier, growth studies were performed in MS medium supplemented only with HMN. A slight to moderate increase in cell numbers was observed. Over the course of the experiment, this increase was relatively small for SA-6 (Fig. 1b). In the case of SA-2 (Fig. 1a), however, an almost 10-fold increase in cell numbers was observed after approximately 120 h, likely due to continued cell division by the robust inoculum or utilization of endogenous substrates. It is also possible that cells were not uniformly distributed on filters used for cell counts and that the high numbers observed at this single time point are an artifact. This increase in cell number was not observed at the next sampling period and the results indicated no major cell

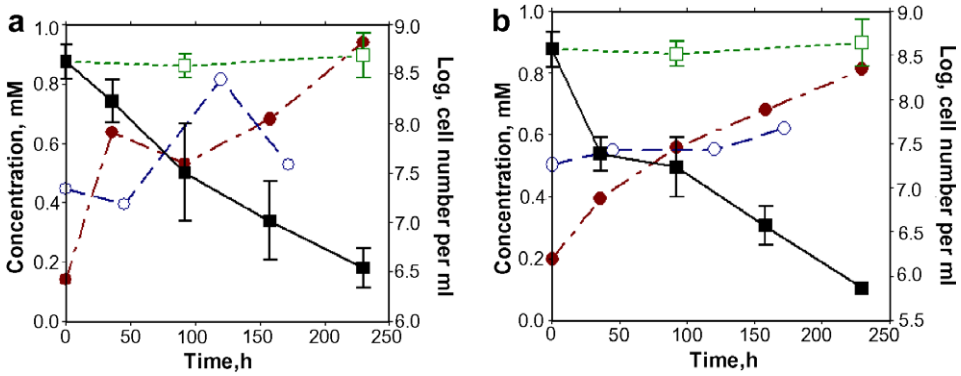

Fig. 1. Degradation of 1,4-diCBz by benzoate-grown cells of SA-2 (a) and SA-6 (b). (■) CBz concentration in inoculated tubes; (□) CBz concentration in uninoculated controls; (●) cell numbers in experimental tubes; (○) cell numbers in control tubes containing HMN only. Symbols represent the mean of three replicate tubes. Large error bars (stnd dev) were due to differential response of cells in replicate tubes.

Table 3
Aerobic utilization of chlorobenzene congeners by *Enterobacter* sp. strain SA-2 and *Pseudomonas* sp. strain SA-6

Substrate[a,b]	SA-2				SA-6			
	T_g (h)	μ (h^{-1})	Final substrate concentration (mM)	Percent degradation	T_g (h)	μ (h^{-1})	Final substrate concentration (mM)	Percent degradation
1,4-DiCBz	29	0.02	0.18 ± 0.12	80 ± 13	32	0.02	0.10 ± 0.02	89 ± 2.5
1,2,3-TriCBz	19	0.04	0.07 ± 0.00	84 ± 0.78	17	0.04	0.04 ± 0.01	91 ± 2.6
1,3,5-TriCBz	20	0.03	0.05 ± 0.01	88 ± 3.3	19	0.04	0.04 ± 0.02	91 ± 5.6

T_g, mean generation time; μ, specific growth rate. Concentration data and percent degradation values are means ± standard deviations for triplicate cultures. Percent degradation values have been calculated with reference to the amount recovered from uninoculated control tubes. 1,4-diCBz, 1,2,3- and 1,3,5-triCBz substrates were supplied at respective concentrations of 0.90 mM (132 ppm), 0.44 mM (81 ppm), and 0.43 mM (79 ppm). The diCBz was incubated for 230 h while the triCBz isomers were incubated for 202 h.

increase on the HMN carrier alone. The population increases recorded during CBz degradation experiments were only sustained when a CBz was supplied, thus ruling out possibility of co-metabolism during growth on HMN.

The degradation pattern of 1,4-diCBz by strains SA-2 and SA-6 is shown in Fig. 1. Washed cell suspension of both isolates, previously grown with benzoate as the source of carbon, transformed 1,4-diCBz at approximately equal rates. Utilization of the substrate was visually indicated by increased turbidity and slight darkening of the growth medium towards the end of the experiment. The main feature of the growth patterns on 1,4-diCBz, as illustrated in Fig. 1, is a steady increase in population densities without an observable lag time. Strain SA-2 for instance, grew with a generation time of 29 h while that of SA-6 was 32 h (Table 2). Degradation was slightly greater when SA-6 was used as inoculum, nevertheless, both strains converted more than 80% of the initial 0.90 mM concentration of 1,4-diCBz in 230 h (Table 2).

3.4. Growth characteristics on trichlorobenzenes

Growth of the bacterial strains on 1,2,3- and 1,3,5-triCBz resulted in a population increase of three orders-of-magnitude while generation times ranged from 17 to 20 h (t = 36–202 h) (Table 3). In both cases, growth of cells, disappearance of the parent compound proceeded without a noticeable lag period (Figs. 2 and 3). Growth rates for both organisms were quite similar regardless of whether the cells were grown on 1,2,3-triCBz or 1,3,5-triCBz. This may suggest that Cl-substitution patterns have little effect on the ability of these organisms to utilize these two trichlorobenzene isomers.

3.5. Catabolic enzyme activities in cell-free extracts

To investigate which degradation pathway was used by the SA strains for growth on aromatic compounds, the presence of enzymes involved in the *meta*- and *ortho*-cleavage pathways for dissimilation of catechol was determined using intact cells of washed benzoate grown cultures. Using the method of Stanier et al. (1966), pelleted cells of strain SA-6 turned brilliant yellow within few seconds of addition of catechol solution, apparently due to production of *meta*-cleavage product, while SA-2 turned brownish yellow. Further metabolism of the intermediate of *meta* pathway was suggested when the yellow color of the assay faded during a 24-h incubation.

Assays of the cell-free extracts confirmed the presence of both *ortho*- and *meta*-cleavage enzymes for the metabolism of catechol. The specific activities of catechol 1,2-dioxygenase were, respectively, 0.13 and 0.12 μmol min^{-1} mg of

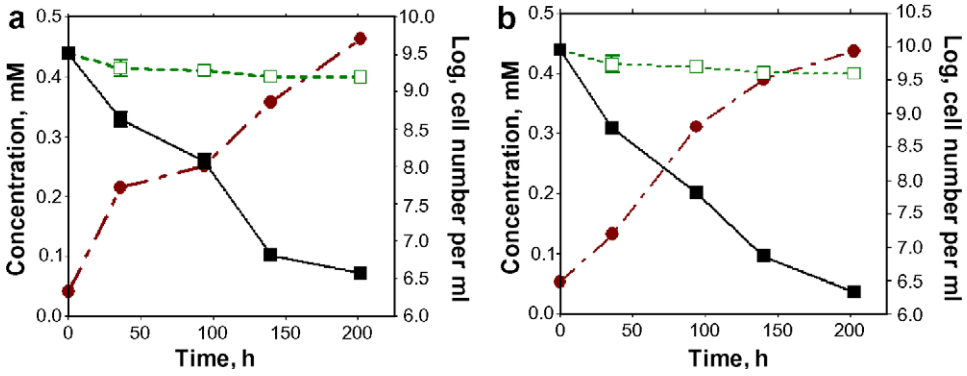

Fig. 2. Degradation of 1,2,3-triCBz by benzoate-grown cells of SA-2 (a) and SA-6 (b). (■) CBz concentration in inoculated tubes; (□) CBz concentration in uninoculated controls; (●) cell numbers. In the controls without cells, 1,2,3-triCBz was not utilized and minimal abiotic loss occurred. Data represent the mean of three replicate tubes. Error bars are not observable since they are smaller than the symbol size.

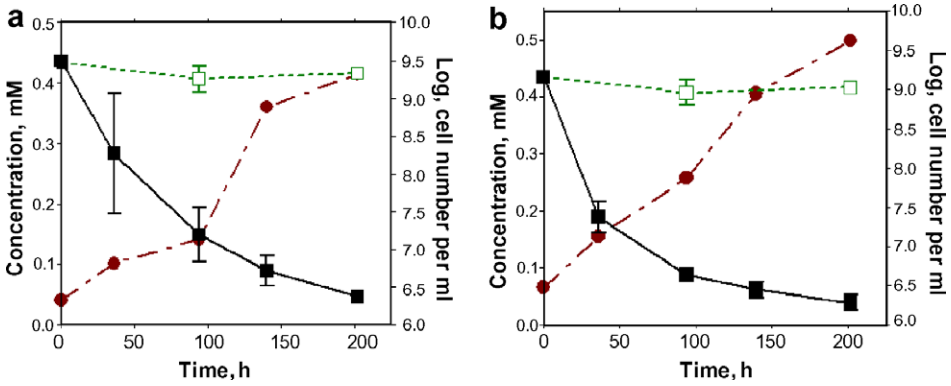

Fig. 3. Degradation of 1,3,5-triCBz by benzoate-grown cells of SA-2 (a) and SA-6 (b). (■) CBz concentration in inoculated tubes; (□) CBz concentration in uninoculated controls; (●) cell numbers. In the controls without cells, 1,3,5-triCBz was not utilized and minimal abiotic loss occurred. Data represent the mean of three replicate tubes. Large error bars (stnd dev) were due to differential response of cells in replicate tubes.

protein^{-1} for SA-2 and SA-6. Specific activities of catechol 2,3-dioxygenase were slightly higher, 0.17 and 0.15 μmol min^{-1} mg of protein^{-1}, respectively, for SA-2 and SA-6.

4. Discussion

Over the last several decades, there has been continued interest in the use of microorganisms as a means for *in situ* cleanup of xenobiotic compounds in contaminated soils and sediments. Two approaches can be envisioned for the application of this method: either the contaminated sites can be inoculated with specific, capable organisms (bioaugmentation), or the activity of indigenous organisms can be enhanced *in situ* by addition of appropriate nutrients and inducers (Morgan and Watkinson, 1989; Sylvestre and Sondossi, 1994). In the case of chloroaromatics, an acclimated, indigenous microbial population capable of degrading the pollutant may be lacking at some sites. In such cases, biodegradation may be limited and bioaugmentation may be the only practical option. The applicability of this approach, however, is often limited by the lack of suitable microorganisms. In this study, two bacterial strains capable of extensive growth on chlorinated aromatic compounds were successfully isolated from Askarel fluid-contaminated soil. However, for a successful bioremediation technique on a polluted soil, proper understanding of the properties of the contaminated site is required. Analysis of the soil used as the inoculum, revealed relatively low concentrations of N and P (Table 1), elements required for efficient and normal functioning of microbial cells (Stevens and Udall, 1981; Andrew and Jackson, 1996). In the absence of sufficient N and P, pollutant degradation reactions may be slow even though carbon and energy sources required for growth are available (Giordani et al., 1998; Lehtola et al., 1998; Vidali, 2001). This may partially explain the persistence of CBz in the soil in spite of the presence of a capable microbial population. In soils such as those from which our isolates were obtained, it may be necessary to adjust N and P concentrations to enhance biodegradation of the organic pollutant.

The isolation of *Enterobacter* sp. SA-2 is unusual in that it is an enteric organism – a group of bacteria that is rarely implicated in degradation of xenobiotic chemicals. Although dechlorination of DDT in pure systems of *E. aerogenes*, *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella pneumoniae* has been reported (Aislabie et al., 1997; Juhasz and Naidu, 2000), the abilities of an enteric organism to grow on PCBs as well as chlorinated benzenes, to the best of knowledge, is new and has never been documented elsewhere. Both SA-2 and SA-6 grew on a diverse range of environmental pollutants (Table 2). It is therefore possible that they possess unusual and unique multi-functional dioxygenases with relaxed substrate specificities, a property not previously known to occur in one organism. This is a desirable feature for bioremediation as contaminated sites usually contain a variety of organic contaminants (Cerniglia, 1992; Juhasz and Naidu, 2000). Additional work is required, however, to more fully characterize the physiology and molecular biology of these isolates.

The data regarding the utilization of CBz congeners by SA strains suggest that the compounds were utilized relatively quickly, and that positional location of the chlorine substituents on the aromatic nucleus, in the case of those CBz able to serve as growth substrates, presented no observable effect on degradation. This is further supported by the fact that degradation rates of 1,2,3- and 1,3,5-triCB did not differ significantly at the 5% level of probability. During degradation of 0.9 mM 1,4-diCB, more than 80% was converted in 220 h, and generation times were 28–32 h (Table 3). Clearly, however, this generalization is true only for diCBz and triCBz since tetraCBz was not degraded by either isolate (Table 2).

The initial oxidative attack by the chlorobenzene dioxygenase is assumed to proceed via the dihydrodiol of either 1,4-diCBz, 1,2,3- or 1,3,5-triCBz which should yield chlorocatechol upon enzymatic dehydrogenation and rearomatization as observed by darkening of culture tubes (Oltmanns et al., 1988; van der Meer et al., 1991; Sommer and Gorisch, 1997; Potrawfke et al., 1998), a color change that we noted in some of our incubations. The accumula-

tion of chlorocatechols during growth on chlorobenzenes has been described previously by other workers (Schraa et al., 1986; Spiess et al., 1995). The bacterium *Pseudomonas* sp. P51 was proposed to degrade 1,2- diCBz and 1,2,4-triCBz by dioxygenolytic reaction proceeding directly to 3,4-dichloro- and 3,4,6-trichlorocatechol, respectively (van der Meer et al., 1991). Subsequent degradation of the chlorocatechol intermediates proceeded through the modified *ortho* pathway in that strain as well as in *Pseudomonas* sp. RH0l, *Alcaligenes* sp. R3 and *Xanthobacter flavus* 14pl (Oltmanns et al., 1988; Sommer and Gorisch, 1997) with stoichiometric release of chloride. The amount of chloride released during metabolism of chlorobenzenes by strains SA-2 and SA-6 was not determined but the extent of growth suggested either partial or total mineralization of the transformed CBz. Consistent with earlier publications, the carbon for growth may have been derived from cleavage of the catechol intermediate with spontaneous release of inorganic chloride.

Previously-described bacteria which can use chlorobenzenes as their sole source of carbon and energy degrade CBz via the modified *ortho*-cleavage pathway, and it is generally accepted that the *meta*-cleavage pathway is not suitable for the degradation of haloaromatics (Bartels et al., 1984; Mars et al., 1997). However, results from enzyme analysis of crude cell-free extracts of SA-2 and SA-6 revealed the presence of both enzymes of the *ortho*- and *meta*-cleavage pathways with the latter exhibiting a slightly higher activity. In these isolates, it is possible that aromatic metabolism occurs via the *meta* catabolic route. This inference is further corroborated by the fact that cultures of these organisms frequently produce an intense, greenish-yellow metabolite (λ_{max} 375 nm) when incubated with benzoate. When compared with published spectra (Focht and Alexander, 1974; Furukawa and Chakrabarty, 1982), this was tentatively identified as 2-hydroxy-muconic semialdehyde. Since this colored metabolite was produced in some, but not all incubations with benzoate, there may be rapid turnover of the yellow *meta*-cleavage product or the alternate *ortho* pathway may predominate. In any event, additional work will be necessary to clarify degradation pathways in these isolates.

In conclusion, the isolation of an enteric bacterium, *Enterobacter* sp. strain SA-2, with a unique ability to utilize several CBz as a sole source of carbon and energy is notable and suggests that the genes for catabolism of such compounds may be more widely distributed across microbial genera than previously believed. Furthermore, this is the first time that bacteria capable of aerobic dechlorination of chlorobenzenes have been isolated from tropical soils from the African continent. In addition, these isolates are able to utilize a variety of chlorinated and non-chlorinated aromatic compounds which are often co-contaminants at some polluted sites, including select CBz, PCBs, and PAHs (polynuclear aromatic hydrocarbons). The wide substrate range of these strains may help in assessing natural detoxification processes and in designing bioaugmentation methods, especially in Africa where the identity of co-contaminants is often poorly characterized. Although these isolates cannot degrade tetraCBzs, they may prove useful in degradation of the di- and tri-CBzs produced by reductive dechlorination of tetraCBzs, either *in situ* or in a reactor-based sequential anaerobic–aerobic treatment system.

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References

Adebusoye, S.A., Amund, O.O., Ilori, M.O., Picardal, F., Fuqua, C., Grindle, N., 2004. Rapid aerobic metabolism of polychlorinated biphenyls (PCBs) by novel microorganisms isolated from tropical soils contaminated with electrical transformer fluid. In: Presentation Abstr. 105th Meet. Am. Soc. Microbiol. abstr. Q118, New Orleans, USA.

Aislabie, J.M., Richards, N.K., Boul, H.L., 1997. Microbial degradation of DDT and its residues: a review. New Zealand J. Agric. Res. 40, 269–282.

Andrew, R.W.J., Jackson, J.M., 1996. Pollution and waste management. In: Environmental Science: The Natural Environment and Human Impact. Longman Publishers, Singapore, pp. 281–297.

Arensdorf, J.J., Focht, D.D., 1994. Formation of chlorocatechol meta cleavage products by a pseudomonad during metabolism of monochlorobiphenyls. Appl. Environ. Microbiol. 60, 2884–2889.

Bartels, I., Knackmuss, H.-J., Reineke, W., 1984. Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl. Environ. Microbiol. 47, 500–505.

Black, C.A., 1965. Methods of Soil Analysis. Agronomy No. 9 Part 2. Amer. Soc. Agron., USA.

Boyd, E.M., Killham, K., Wright, J., Rumford, S., Hetheridge, M., Cumming, R., Meharg, A.A., 1997. Toxicity assessment of xenobiotic contaminated groundwater using lux modified *Pseudomonas fluorescens*. Chemosphere 35, 1967–1985.

Bray, R.H., Kurtz, L.T., 1945. Determination of total organic and available forms of phosphorus in soils. Soil Sci. 59, 39–45.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351–368.

Fetzner, S., 1998. Bacterial dehalogenation. Appl. Microbiol. Biotechnol. 50, 633–657.

Focht, D.D., Alexander, M., 1974. Bacterial degradation of 1,1-diphenylethylene by mixed culture. Can. J. Microbiol. 20, 631–635.

Furukawa, K., Chakrabarty, A.M., 1982. Involvement of plasmids in total degradation chlorinated biphenyls. Appl. Environ. Microbiol. 44, 446–626.

Galli, R., McCarty, P.L., 1989. Biotransformation of 1,1,1-trichloroethane, trichloromethane, and tetrachloromethane by a *Clostridium* sp.. Appl. Environ. Microbiol. 55, 837–844.

Giordani, G., Donnelly, A., Azzoni, R., 1998. The uptake of inorganic phosphate by *Z. noltii* in the Basin d' Arcachon. In: Proceedings (Handbook) of Summer Conference. Society for Applied Microbiology, University of Lancaster, UK, pp. 22–23.

Golden, K.A., Wong, C.S., Jeremiasion, J.D., Eisenreich, S.J., Hallgren, J., Swackhamer, D.L., Engstrom, D.R., Long, D.T., 1993. Accumulation and preliminary inventory of organochlorines in Great Lakes sediments. Water Sci. Technol. 28, 19–31.

Janssen, D.B., Oppentocht, J.E., Poelarends, G.J., 2001. Microbial dehalogenation. Curr. Opin. Biotechnol. 12, 254–258.

Juhasz, A.L., Naidu, R., 2000. Enrichment and isolation of non-specific aromatic degraders from unique uncontaminated (plant and faecal

material) sources and contaminated soils. J. Appl. Microbiol. 89, 642–650.

Ka-Leung, N., Neidle, E.L., Ornston, N.L., 1990. Catechol and chlorocatechol 1,2-dioxygenases. Meth. Enzymol. 188, 122–130.

Kaschabek, S.R., Kasberg, T., Muller, D., Mars, A.E., Janssen, D.B., Reineke, W., 1998. Degradation of chloroaromatics: purification and characterization of a novel type of chlorocatechol 2,3-dioxygenase of *Pseudomonas putida* GJ31. J. Bacteriol. 180, 296–302.

Kataeva, I.A., Golovleva, L.A., 1990. Catechol 2,3-dioxygenase from *Pseudomonas aeruginosa* 2×. Meth. Enzymol. 188, 115–121.

Kepner, J., Raymond, L., Pratt, J.R., 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol. Rev. 58, 603–615.

Kim, S., Picardal, F., 2000. A novel bacterium that utilizes monochlorobiphenyls and 4-chlorobenzoate as growth substrates. FEMS Microbiol. Lett. 185, 225–229.

Lehtola, M., Miettinen, I., Vartianen, T., Martikainen, P.J., 1998. Purification of drinking water phosphorus and bacterial growth. In: Proceedings (Handbook) of Summer Conference. Society for Applied Microbiology, UK, pp. 16–17.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J., Woese, C.R., 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. 25, 109–110.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64, 795–799.

Mars, A.E., Kasberg, T., Kaschabek, S.R., van Agteren, M.H., Janssen, D.B., Reineke, W., 1997. Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. J. Bacteriol. 14, 4530–4537.

Mars, A.E., Kingma, J., Kaschabek, S.R., Reineke, W., Janssen, D.B., 1999. Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. J. Bacteriol. 181, 1309–1318.

Morgan, P., Watkinson, R.J., 1989. Microbiological methods for the cleanup of soil and ground water contaminated with halogenated organic compounds. FEMS Microbiol. Rev. 63, 277–300.

Nakasawa, T., Nakasawa, A., 1957. Pyrocatechase (*Pseudomonas*)[1]. Meth. Enzymol. A 17, 518–522.

Oltmanns, R.H., Rast, H.G., Reineke, W., 1988. Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl. Microbiol. Biotechnol. 28, 609–616.

Pollmann, K., Beil, S., Pieper, D.H., 2001. Transformation of chlorinated benzenes and toluenes by *Ralstonia* sp. strain PS12 *tecA* (tetrachlorobenzene dioxygenase) and *tecB* (chlorobenzene dihydrodiol dehydrogenase) gene products. Appl. Environ. Microbiol. 67, 4057–4063.

Potrawfke, T., Timmis, K.N., Wittich, R.-M., 1998. Degradation of 1,2,3,4-tetrachlorobenzene by *Pseudomonas chlororaphis* RW71. Appl. Environ. Microbiol. 64, 3798–3806.

Reineke, W., Knachmuss, H.-J., 1988. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. 42, 263–287.

Sander, P., Wittich, R.-M., Fortnagel, P., Wilkes, H., Francke, W., 1991. Degradation of 1,2,4-trichloro- and 1,2,4,5-tetrachlorobenzene by *Pseudomonas* strains. Appl. Environ. Microbiol. 57, 1430–1440.

Schraa, G., Boone, M.L., Jetten, M.S.M., van Neerven, A.R.W., Colberg, P.J., Zehnder, A.J.B., 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. Appl. Environ. Microbiol. 52, 1374–1381.

Sommer, C., Gorisch, H., 1997. Enzymology of the degradation of (di)chlorobenzenes by *Xanthobacter flavus* 14p1. Arch. Microbiol. 167, 384–391.

Spain, J.C., Nishino, S.F., 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp.. Appl. Environ. Microbiol. 53, 1010–1019.

Spiess, E., Sommer, C., Gorisch, H., 1995. Degradation of 1,4-dichlorobenzene by *Xanthobacter flavus* 14p1. Appl. Environ. Microbiol. 61, 3884–3888.

Stanier, R.Y., Palleroni, N.J., Doudoroff, M., 1966. The aerobic pseudomonas: a taxonomic study. J. Gen. Microbiol. 99, 159–271.

Stevens, T., Udall, M.K., 1981. The use of genetically engineered microorganisms in the environment. In: Impacts of Applied Genetics of Microorganisms Plants and AnimalsCongress of the United States of America. Office of Technology Assessment, Washington DC, pp. 117–132.

Stoesser, G., Sterk, P., Tuli, M.A., Stoehr, P.J., Cameron, G.N., 1997. The EMBL nucleotide sequence database. Nucleic Acids Res. 25, 7–13.

Sylvestre, M., Sondossi, M., 1994. Selection of enhanced polychlorinated biphenyl-degrading bacterial strains for bioremediation: consideration of branching pathways. In: Chaudhry, G.R. (Ed.), Biological Degradation and Bioremediation of Toxic Chemicals. Dioscorides Press, Oregon, pp. 47–73.

van der Meer, J.R., van Neerven, A.R.W., de Vries, E.J., de Vos, W.M., Zehnder, A.J.B., 1991. Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. J. Bacteriol. 173, 6–15.

van der Meer, J.R., de Vos, W.M., Harayama, S., Zehnder, A.J.B., 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiology 71, 159–178.

van der Meer, J.R., Werlen, C., Nishino, S.F., Spain, J.C., 1998. Evolution of a pathway for chlorobenzene metabolism leads to natural attenuation in contaminated groundwater. Appl. Environ. Microbiol. 64, 4185–4193.

Vidali, M., 2001. Bioremediation: an overview. Pure Appl. Chem. 73, 1163–1172.

Wu, Q., Milliken, C.E., Meier, G.P., Watts, J.E.M., Sowers, K.R., May, H.D., 2002. Dechlorination of chlorobenzenes by a culture containing bacterium DF-1, a PCB dechlorinating microorganism. Environ. Sci. Technol. 36, 3290–3294.