Synergistic Utilization of Dichloroethylene as Sole Carbon Source by Bacterial Consortia Isolated from Contaminated Sites in Africa

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Abstract The widespread use and distribution of chloroethylene organic compounds is of serious concern owing to their carcinogenicity and toxicity to humans and wildlife. In an effort to develop active bacterial consortia that could be useful for bioremediation of chloroethylene-contaminated sites in Africa, 16 combinations of 5 dichloroethylene (DCE)-utilizing bacteria, isolated from South Africa and Nigeria, were assessed for their ability to degrade *cis*- and *trans*-DCEs as the sole carbon source. Three combinations of these isolates were able to remove up to 72% of the compounds within 7 days. Specific growth rate constants of the bacterial consortia ranged between 0.465 and 0.716 d^{-1}, while the degradation rate constants ranged between 0.184 and 0.205 d^{-1}, with 86.36~93.53 and 87.47~97.12% of the stoichiometric-expected chloride released during growth of the bacterial consortia in *cis*- and *trans*-DCE, respectively. Succession studies of the individual isolates present in the consortium revealed that the biodegradation process was initially dominated by *Achromobacter xylosoxidans* and subsequently by *Acinetobacter* sp. and *Bacillus* sp., respectively. The results of this study suggest that consortia of bacteria are more efficient than monocultures in the aerobic biodegradation of DCEs, degrading the compounds to levels that are up to 60% below the maximum allowable limits in drinking water.

Keywords: aerobic, biodegradation, consortium, dichloroethylene, synergism

INTRODUCTION

The ubiquity of chlorinated hydrocarbons in the environment continues to be the basis for public and regulatory concern because of their recalcitrance and potential for bioaccumulation [1]. A large number of these compounds are recognized to be potentially mutagenic, carcinogenic and/or toxic to the liver, kidney or neural system [2-4]. Currently, more than 15,000 different chlorinated organic compounds are produced annually for a variety of industrial and commercial uses, including solvents, lubricants, and intermediates in the chemical industry, plasticizers, crop protection, pharmaceuticals, and medical equipment [5]. Apart from the industrial production, more than 2,000 halogenated chemicals are known to be released into the biosphere by various marine organisms, higher plants and ferns, insects, bacteria, fungi, and mammals [6,7].

In particular, higher chlorinated aliphatic hydrocarbons such as tetrachloroethylene (PCE) and trichloroethylene (TCE) are widely used as degreasing and dry-cleaning solvents [8]. The widespread use of these volatile organic compounds is of serious concern to human health, owing to their carcinogenicity and toxicity [9]. Through natural attenuation, these industrially-important solvents can be degraded anaerobically, by pure and mixed cultures of anaerobes, via reductive dehalogenation to less-chlorinated ethylenes; dichloroethylenes (DCEs) and vinyl chloride (VC) [10-12]. Under certain conditions these daughter products can be degraded further by sequential dechlorination through various intermediates to non-toxic products such as ethene [13-15], and ethane [16,17].

However, reductive dechlorination of PCE and TCE is often incomplete, resulting in the accumulation of DCEs and VC in subsurface ecosystems [12]. The innate toxicity of DCEs and their tendency to be reduced to VC, a known human carcinogen, has led to their classification as priority groundwater pollutants by the U.S. Environmental Protection Agency. Furthermore, the biodegradation of DCEs to ethene is often a limiting factor in the use of reductive dechlorination as an approach for the remediation of chloroethylene contaminated sites. There-

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fore, an understanding of factors affecting the transformation of DCEs is critical for evaluating natural attenuation and for establishing engineering procedures for chlorinated-solvent remediation [14]. Despite the observed aerobic oxidation of DCEs to CO_2 in microcosms and enrichment culture studies [18,19], aerobic utilization of these compounds as the sole carbon source has only been reported in very few bacteria [20,21]. Also, because chloroethene organics still continue to be among the most common contaminants frequently observed in groundwater systems [22-24], the quest for an effective way of remediating sites contaminated with this group of compounds is on the increase. Previously, we isolated five organochloride-degrading bacteria and characterized their dehalogenase activities [25,26]. In this paper, the characterization of some consortia of these isolates for aerobic utilization and degradation of *cis*- and *trans*-DCEs as sole carbon and energy source was described. To the best of our knowledge, this is the first report of aerobic degradation of DCEs by consortia of bacteria indigenous to contaminated sites in Africa.

MATERIALS AND METHODS

Chemicals and Media

Cis-dichloroethylene (*cis*-DCE; 97%) was obtained from Fluka and *trans*-dichloroethylene (*trans*-DCE; 98%) from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). The minimal salts medium (MSM) used for enrichment cultures was modified from that reported by Hartmans *et al.* [27] by reducing the concentration of phosphate to 20 mM, ammonium to 10 mM and chloride concentration to 0.02 mM.

Isolation and Identification of the Bacterial Isolates

The bacterial strains used in this study are listed in Table 1. They were isolated from soil samples collected from chlorinated hydrocarbon-contaminated sites using culture enrichment techniques, and tentatively identified as described elsewhere [21]. Pure cultures of the isolates were grown in nutrient broth (Difco), stored on nutrient agar (Difco) slants at 4°C as working stock cultures, and preserved from overnight broth cultures in 80% (v/v) glycerol at −20°C. The definitive identification of the isolates was done by cloning, sequencing, and analysis of their 16S rRNA gene sequences. Total genomic DNA was isolated from LB-grown cells using QIA amp DNA mini kit (Qiagen) and used directly as the template for PCR amplification. The 16S rRNA genes of the bacterial isolates were amplified with the oligonucleotide primers: 63f and 1387r as described by Marchesi *et al.* [28]. Amplification products were cloned into plasmid pGEM-T easy vector (Promega) as specified by the manufacturer. The ligation mixture was used to transform electrocompetent *E. coli* DH5α′ cells, and ampicillin-resistant white colonies were selected from X-Gal plates. Plasmid DNA was isolated from the positive clones and checked by restriction analysis as well as amplification of the insert with the same primer set used for amplifying the 16S rRNA genes. Cloned 16S rRNA genes were cycle sequenced using the Spectrumedix SCE2410 genetic analysis system with 24 capillaries. The Big Dye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems) was used for the sequencing reactions. Both DNA strands (SP6 and T7) were sequenced to ensure accuracy. The 16S rRNA gene sequences of the bacterial isolates were compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov) by using BLAST [29] to determine the most similar sequences. The DNA sequences of the bacterial isolates have been deposited into GenBank under the following Accession Numbers; DQ228950, DQ228951, DQ228954, DQ228955, and DQ228956.

Table 1. Bacterial isolates used in this study

Isolate code	Source	Identity
NG_1	Refuse dumpsite (Nigeria)	*Bacillus cereus*
NG_2	Soil sample (Nigeria)	*Bacillus* sp.
NG_3	Soil sample (Nigeria)	*Pseudomonas aeruginosa*
SA_1	Soil sample (South Africa)	*Acinetobacter haemolyticus*
SA_2	Soil sample (South Africa)	*Achromobacter xylosoxidans*

Growth profile and Chloride Release Assay

The bacterial consortia were prepared by mixing equal volumes of the standardized suspensions (OD_{600} = 1.0) of each isolate in the consortium to make up a total volume of 1 mL. This was used to inoculate 100 mL of MSM containing either *cis*- or *trans*-DCE at a final concentration of 1.0 and 1.5 mM, respectively, and incubated at 30°C and at 150 rpm over a period of 7 days and sampled daily. Total bacterial counts were carried out using the standard spread plate technique [30,31]. Each organism in the consortium was identified based on their cultural characteristics on the culture plates and their population estimated as a percentage of the total population of all the organisms present in the mixture to determine the dominant strain at the different sampling times. The specific growth rate constant was estimated as described elsewhere [32] while the degradation rate constant was estimated according to LaGrega *et al.* [33]. Chloride release was measured spectrophometrically [34] but modified by adding 200 μL of 0.25 M $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ in 9 M HNO_3, and 400 μL of $Hg(SCN)_2$ in 95% ethanol to 1 mL of the supernatant, obtained after the removal of cells by centrifugation at 16,000 × g for 2 min. After 10 min, the absorbance of the $Fe(SCN)^{2+}$ product was measured at 460 nm using a UV/Visible spectrophotometer, Ultrospec 2000 (Pharmacia Biotech). Three replicates were used for each set of experiments.

Measurement of DCE Degradation

Two milliliters of the standardized (OD_{600} = 2) potassium phosphate buffer cell suspension of the bacterial

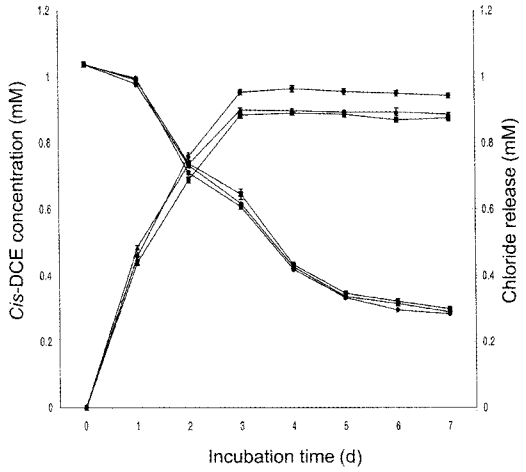

Fig. 1. Biodegradation profile of *cis*-dichloroethylenes (*cis*-DCE) and chloride release by the different bacterial consortia: (■) consortium 1 (Con. 1), a mixed culture of SA_1, SA_2, and NG_3; (▲) consortium 2 (Con. 2), a mixed culture of NG_2, NG_3, SA_1, and SA_2; (●) consortium 3 (Con. 3), a mixed culture of SA_1, SA_2, NG_1, NG_2, and NG_3. Data points are the mean of triplicate cultures and the error bars indicate one standard deviation above and below the mean.

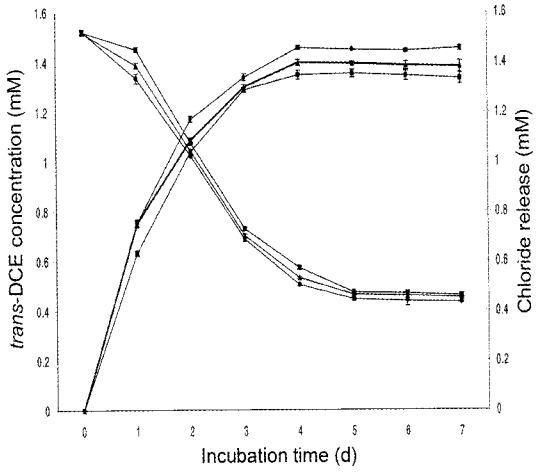

Fig. 2. Biodegradation profile of *trans*-dichloroethylenes (*trans*-DCE) and chloride release by the different bacterial consortia: (■) consortium 1 (Con. 1), a mixed culture of SA_1, SA_2, and NG_3; (▲) consortium 2 (Con. 2), a mixed culture of NG_2, NG_3, SA_1, and SA_2; (●) consortium 3 (Con. 3), a mixed culture of SA_1, SA_2, NG_1, NG_2, and NG_3. Data points are the mean of triplicate cultures and the error bars indicate one standard deviation above and below the mean.

consortia were used to inoculate 150 mL MSM in 250 mL serum bottle (headspace; 98 mL of air). The serum bottles were crimp-sealed with Teflon-faced butyl rubber stoppers (Wheaton). *Cis*- or *trans*-DCEs were added as the sole carbon source at a final concentration of 1.0 and 1.5 mM, respectively, and monitored daily by injecting 100 μL headspace sample with a 100 μL Hamilton gas-tight syringe into a gas chromatograph equipped with a flame ionization detector and injector at 200°C and a packed column at 100°C. Uninoculated MSM containing *cis*- or *trans*-DCE was used as negative controls and the concentration of the compounds indicated above that of losses of the negative controls.

RESULTS AND DISCUSSION

Although hydrocarbon-oxidizing bacteria are known to have evolved enzymes active on DCEs [35-38], and thermodynamic calculations suggest that DCEs contain sufficient energy to support aerobic growth of bacteria [39], results of this study revealed that very few organisms are capable of aerobic utilization of DCEs. Several enrichment cultures, with *cis*- or *trans*-DCE as the sole carbon source, yielded only five bacterial isolates capable of utilizing DCE as the sole carbon source. The identity of the bacterial isolates based on the biochemical tests was further confirmed by the analysis of their 16S rRNA gene sequences as shown in Table 1. Contrary to the initial characterization of isolate SA_2 as *Pseudomonas* sp., the analysis of their 16S rRNA genes identified the isolate as *Achromobacter xylosoxidans*. This further confirmed the broad and vague phenotypic definition which allowed the genus *Pseudomonas* to become a dumping ground for incompletely characterized polarly flagellated, Gram-negative, rod-shaped, aerobic bacteria, thus allowing a large number of species to be accommodated in the genus *Pseudomonas* [40]. However, the identity of the other four isolates remained the same. Previously, the dechlorination potential and the dehalogenase activities of the axenic cultures of these isolates were reported [21]. Therefore, in an effort to develop active bacterial consortia that may have greater application for the bioremediation of sites contaminated with chloroethylenes, various combinations of these bacterial isolates were assessed for their potential to biodegrade DCEs.

Three of the sixteen different consortia, developed from the various combinations of the isolates, showed good potential for the aerobic utilization of DCEs. All three bacterial consortia degraded both *cis*- and *trans*-DCEs, removing about 72% of the initial compound within 7 days (Figs. 1 and 2). The specific growth rate constants of the bacterial consortia for *cis*- and *trans*- DCE ranged between 0.465~0.634 d^{-1}, and 0.662~0.716 d^{-1}, respectively, while the degradation rate constant ranged between 0.184~0.205 d^{-1} (Table 2). The pattern of degradation indicates that increasing the inoculum size and/or addition of certain nutrients may lead to the total removal of these compounds by the bacterial consortia. This is because some of the bacterial cells might have died as a result of the production of toxic metabolites while certain nutrients could be limiting to the growth of the organisms. The result of the chloride release assay in the growth medium revealed that up to 86.36~93.53 and 87.47~97.12% of the stoichiometric-expected chloride ion was released during growth of the bacterial consortia in *cis*- and *trans*-DCE, respectively (Figs. 1 and 2).

Various observations suggest that reductive dechlorina-

Table 2. Utilization of dichloroethylenes by the different bacterial consortium

Bacterial consortia	Specific growth rate constant (d^{-1})		Degradation rate constant (d^{-1})	
	cis-DCE	*trans*-DCE	*cis*-DCE	*trans*-DCE
Consortium 1[a]	0.465 (0.00781)	0.662 (0.00964)	0.184 (0.0113)	0.190 (0.003)
Consortium 2[b]	0.528 (0.0187)	0.702 (0.0187)	0.203 (0.001)	0.193 (0.0058)
Consortium 3[c]	0.634 (0.0219)	0.716 (0.0147)	0.205 (0.0058)	0.199 (0.0062)
NG_1	0.346 (0.017)	0.518 (0.047)	0.169 (0.000577)	0.171 (0.00231)
NG_2	0.358 (0.022)	0.461 (0.038)	0.173 (0.00208)	0.177 (0.00361)
NG_3	0.461 (0.039)	0.638 (0.013)	0.190 (0.00208)	0.167 (0.00252)
SA_1	0.391 (0.017)	0.610 (0.013)	0.178 (0.00208)	0.174 (0.00723)
SA_2	0.552 (0.0096)	0.667 (0.013)	0.198 (0.005)	0.172 (0.00503)

Values in parenthesis represent the standard deviation calculated from the triplicate assays obtained for each of the experiments.
[a]Mixed culture of SA_1, SA_2 & NG_3; [b]Mixed culture of NG_2, NG_3, SA_1 & SA_2; [c]Mixed culture of SA_1, SA_2, NG_1, NG_2 & NG_3.

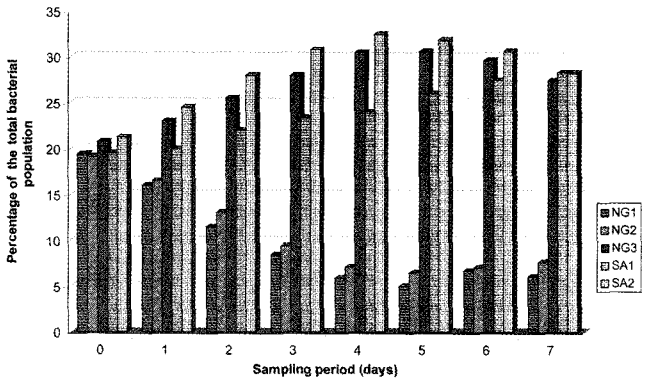

Fig. 3. Growth patterns of individual bacterial isolates within the mixed culture using *cis*-dichloroethylene as the sole carbon source. Total absolute bacterial population is 48.45, 105.61, 151.10, 251.47, 199.0, 142.5, 128.54, and 103.20 ($\times 10^5$ cfu/mL) at 0, 1, 2, 3, 4, 5, 6, and 7 days, respectively.

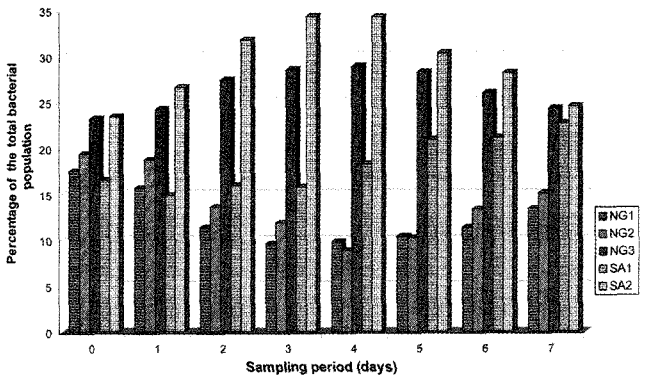

Fig. 4. Growth patterns of individual bacterial isolates within the mixed culture using *trans*-dichloroethylene as the sole carbon source. Total absolute bacterial population is 41.8, 64.03, 136.85, 179.55, 139.79, 113.4, 80.98, and 67.53 ($\times 10^5$ cfu/mL) at 0, 1, 2, 3, 4, 5, 6, and 7 days, respectively.

tion of chloroethene contaminants is often attributable to the activities of cooperative consortia of microorganisms rather than to a single species. A number of mixed cultures capable of growth and complete dechlorination of this group of compounds have therefore been identified [13-15,17,41]. This observation is not far-fetched in this study since the mixed cultures of the bacterial isolates utilized *cis*- and *trans*-DCE, better than the respective monocultures. This is evident by the increase in the amount of both compounds degraded by the different active consortia developed from the various combinations of the bacterial isolates (Figs. 1 and 2) and the corresponding increase in the stoichiometric-expected amount of chloride from both *cis*- and *trans*-DCE (Figs. 1 and 2). It has also been previously proposed that successful detoxification of recalcitrant organic chemicals may require the concerted effort of multispecies bacterial consortia. Several examples of the degradation of such structurally diverse organic compounds, including endosulfan [42], chloronitrobenzene [43], atrazine [44], and 1,3-dichloropropene [45] have been reported. This phenomenon also seems to be applicable to the current study since consortium 3, containing all the five bacterial isolates, demonstrated the greatest degradation potential when compared to the other two consortia.

The optimum pH and temperature for growth of these bacterial isolates in the presence of DCEs was found to be 7.0 and 30°C, respectively, which is similar to the optimum conditions reported for other organisms utilizing *cis*-DCE [20]. The bacterial isolates used in this study have also been previously reported to have high dehalogenase activity in the presence of other organochlorides at this pH and temperature [25,26]. Succession studies of bacterial consortium 3 revealed that the biodegradation process was initially dominated by *A. xylosoxidans* and later succeeded by the *Acinetobacter* sp. and *Bacillus* sp., respectively. A general increase in the population of *A. xylosoxidans* in the mixed culture was initially observed, while *Acinetobacter* sp. and *Bacillus* sp. decreased (Figs. 3 and 4). Thereafter, the population of *A. xylosoxidans* remained fairly constant, while those of *Acinetobacter* sp. and *Bacillus* sp. demonstrated a slight increase. This is not surprising though, since the versatility of *A. xylosoxidans* has been previously reported in many biodegradation processes [46]. Although, the intermediate products of the degradation of these compounds were not analyzed

in this study, it is evident that some kind of synergistic association exists among these bacteria with *A. xylosoxidans* initiating the breakdown of the compounds to less toxic ones that could be utilized by the other groups of organisms. This is the subject of an on-going study in our laboratory. However, this study clearly demonstrates that contaminated sites in Africa are a potential source of microorganisms with the inherent ability to degrade DCE. This finding is significant for the managed biostimulation and enhanced bioremediation of such sites polluted by chloroethylenes and other organochloride compounds. The results of this study also clearly demonstrate that consortia of bacteria are more relevant than monocultures in the bioremediation of environmental sites contaminated with this group of chlorinated hydrocarbon compounds. It is also worth noting that the concentration of *cis*- and *trans*-DCE detectable in the medium after degradation by these bacterial consortia is about 60% lower than the maximum allowable contaminant level for *cis*-DCE and *trans*-DCE (70 and 100 μg/L, respectively) in drinking water, mandated by the Environmental Protection Agency (http://www.epa.gov).

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