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Degradation of chlorobenzenes by a strain of *Acidovorax avenae* isolated from a polluted aquifer

Magdalena V. Monferrán, José R. Echenique, Daniel A. Wunderlin *

Universidad Nacional de Córdoba-CONICET, Facultad de Ciencias Químicas, Dep. Bioquímica Clínica-CIBICI, Haya de La Torre esq, Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina

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Abstract

A subsurface microbial community was isolated from a polluted site of Suquía River (Córdoba—Argentina), acclimated during 15 days in aerobic conditions using 1,2-dichlorobenzene (1,2-DCB) as the sole carbon source. From this acclimated community, we isolated and identified by 16S rDNA analysis a strain of *Acidovorax avenae*, which was able to perform the complete biodegradation of 1,2-DCB in two days affording stoichiometric amounts of chloride. This pure strain was also tested for biodegradation of chlorobenzene (CB); 1,3-DCB and 1,4-DCB, giving similar results to the experiments using 1,2-DCB.

The aromatic-ring-hydroxylating dioxygenase (ARHDO) α-subunit gene core, encoding the catalytic site of the large subunit of chlorobenzene dioxygenase, was detected by PCR amplification and confirmed by DNA sequencing. These results suggest that the isolated strain of *A. avenae* could use a catabolic pathway, via ARHDO system, leading to the formation of chlorocatecols during the first steps of biodegradation, with further chloride release and subsequent paths that showed complete substrate consumption.
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1. Introduction

Chemical contamination of aquifers has been documented and poses a potential health hazard (Aelion et al., 1987). The ability of polluted aquifers to recover from chemical contamination depends primarily on biological reactions that, in the subsurface, may be faster than chemical and physical reactions. Because the subsurface biological community is composed primarily of bacteria, it is essential to investigate the interaction of its microbial community with different xenobiotic compounds, including biodegradation and adaptation processes (Aelion et al., 1987).

The potential health hazard of a given xenobiotic compound is a function of its persistence in the environment as well as the toxicity of the corresponding chemical class (Aelion et al., 1987). Persistence is a function of the biotransformation rate, which influences the compound's form, residence time, and mobility in the subsurface environment. The ability of the subsurface microbial community to adapt to xenobiotics and the time necessary for the adaptation process to occur will

* Corresponding author. Tel.: +54 351 433 4164; fax: +54 351 433 3048.
E-mail address: dwunder@mail.fcq.unc.edu.ar (D.A. Wunderlin).

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be principal factors determining persistence for less readily degraded compounds (Aelion et al., 1987).

The widespread use of chlorobenzenes (CBs) during the last decades has led to their common occurrence in the environment. CBs are of great concern because of their toxicity, persistence and accumulation in the food chain (Aelion et al., 1987). Chlorobenzene (MCB) and 1,2-dichlorobenzene (1,2-DCB) have been identified as priority pollutants by the US Environmental Protection Agency (EPA) (www.epa.gov/fedrgstr/epa-meetings/2004/january/Day-30/m1973.htm). While the amount of CBs can be high in polluted soils and sediments, their concentration in the aqueous phase is rather low, due to their poor water solubility (Yalkowsky and Valvani, 1980; Rapp, 2001). However, adsorption of CBs to either colloid particles in surface water or to the organic matrix of sediments (van der Meer, 1997) is quite common. Furthermore, CBs are distributed worldwide adsorbed to sediments (Akiko et al., 1993; Meharg et al., 2000; Nikolaou et al., 2002).

In a study of water quality of Suquía River (Córdoba—Argentina), DCB isomers were found in river water samples (data not published) at different points along the river.

Chlorinated aromatic compounds are considered among the most problematic categories of environmental pollutants, which are non-degradable or slowly degradable by microorganism (van der Meer, 1997). Nevertheless, bacteria that are able to use these compounds as sole source of carbon and energy have been isolated from polluted environments (Schraa et al., 1986; Spain and Nishino, 1987; van der Meer et al., 1987; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995). Field studies at contaminated sites have shown that river sediments, exposed to CBs, degraded them faster than sediments from unpolluted sites (Aelion et al., 1987). Furthermore, chlorinated benzenes are chemically stable in nature, their photochemical degradation does not play an important role in soil and aquatic environment (www.epa.gov/safewater/dwh/t-voc/o-dichlo.html). So, biological degradation could be considered as a feasible process to eliminate these compounds (Schraa et al., 1986).

Bacterial metabolism of CBs is particularly interesting, because it requires a specific combination of several catabolic pathways. Bacteria able to biodegrade these compounds, attack CBs by a dioxygenase and, after rearomatization, the resulting chlorocatechols undergo ortho cleavage to yield CO_2, HCl and H_2O (van der Meer, 1997). Dechlorination is a significant step towards its degradation, bacterial contacts with chlorinated benzenes liberates chloride which is assumed as evidence of mineralization.

The aromatic ring hydroxylating dioxygenase (ARHDO) is an important enzyme complex in the aerobic degradation of many aromatic compounds, constituted by three to four protein subunits (Kahl and Hofer, 2003). The subunit characteristics and the composition of such dioxygenase system vary considerably. However, all of them consist of a hydroxylase, which binds and oxidizes the organic substrate, and a short transport chain that provides electrons to the system. Based on the number of subunits, and on some characteristics features, ARHDOs have been grouped into five classes: IA, IB, IIA, IIB, and III (Kahl and Hofer, 2003).

ARHDOs play important roles in the cycling of organic carbon in many environments. Thus, genes coding for this enzymes are useful markers for bacteria that are able to use aromatic hydrocarbons as growth substrates (Taylor et al., 2002).

Previous studies on ARHDO system have demonstrated that the key for its catalytic properties reside on the core of the large or α-subunit (Erickson and Mondello, 1993; Kimura et al., 1997; Mondello et al., 1997).

In this work we examined the ability of a native subsurface microbial community, isolated from a polluted site at Suquía River, to degrade 1,2-DCB after acclimatizing. From this acclimatized community, we report the isolation and identification of a strain of *Acidovorax avenae*, which was able to perform aerobic biodegradation of not only 1,2-DCB, but also CB, 1,3-DCB and 1,4-DCB. Additionally, we identified and characterized in this strain the α-subunit gene core, encoding the potential catalytic site of the large subunit of chlorobenzene dioxygenase. Thus, suggesting a putative CB-degradation pathway, initially mediated by the ARHDO enzymatic system.

To the extend of our knowledge this is the first report on biodegradation of CB and DCB isomers by *A. avenae* as well as the first report on the presence of α-subunit gene core, encoding for ARHDO in this strain.

2. Materials and methods

2.1. Chemicals

Chlorobenzene (CB); 1,2- 1,3- and 1,4-dichlorobenzene (DCBs), and 3,5-dichlorocatechol (3,5-DCC) were obtained from Sigma-Aldrich Co. Solvents and other chemicals used were all reagent-grade.

2.2. Analytical methods

CB and DCBs were extracted from culture medium by solid phase extraction (SPE) using SPE-C18 cartridges (Merck, LiChrolut® RP-18) and methanol as eluting solvent. The eluate was analyzed by HPLC using a Hewlett Packard model HP 1100 chromatograph, using UV detection at 205 nm. A Phenomenex C18 column (4.6 mm I.D., 25 cm length, luna type 5 μm C18-2), and

acetonitrile:water (65:35) as mobile phase. Chromatographic separations were performed at a flow rate of 1.5 ml min^{-1}; temperature 20 °C. Under these conditions, 1,2-DCB showed a retention time of 7.76 min and a detection limit of 0.25 mg l^{-1} (1.7 μM), CB, 1,3-DCB and 1,4-DCB showed retention times of 5.54, 8.89 and 7.00 min, respectively, and detection limits of 0.30, 0.10, and 0.30 mg l^{-1} (2.67, 0.68 and 2.04 μM) respectively. Extraction and HPLC procedures were performed to verified extraction efficiency using culture medium supplemented with 0.136 mM 1,2-DCB. The average recovery for 1,2-DCB was (78 ± 5%). A similar procedure was applied to CB, 1,3-DCB and 1,4-DCB, affording 82 ± 3%, 75 ± 2% and 80 ± 5% recovery, respectively.

Free inorganic chloride was determined using a modification of the procedure of the mercury (II) thiocyanate method (Pesce and Wunderlin, 2004).

2.3. Acclimation and pure strain isolation

River sediment was sampled in sterile flask from Suquía River at a polluted site (Bajo Grande) (Pesce and Wunderlin, 2000). Sediment (20 g) was suspended in 200 ml of mineral salt medium (MSM) containing per liter: 112 mg $MgSO_4 \cdot H_2O$, 5 mg $ZnSO_4 \cdot H_2O$, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 340 mg KH_2PO_4, 670 mg $Na_2HPO_4 \cdot 7H_2O$, 17 mg $CaSO_4$, 0.22 mg $FeSO_4 \cdot 7H_2O$, and 0.613 g $(NH_4)_2SO_4$. The pH of MSM was adjusted to 7.0 (Spain and Nishino, 1987) and sterilized before use. This suspension was used as inoculum for enrichment cultures, which were shaken at 100 rpm and incubated at 25 °C. Considering that CBs are highly volatile, acclimation experiments were performed in a 500 ml Erlenmeyer flask, connected by a sealed union to a Dewar flask equipped with a purge valve. Aerobic conditions were kept by introducing pure oxygen (99.9%) through a needle valve. During oxygen addition, the Dewar flask was filled with liquid air to prevent loss of substrate by volatilization. Thereafter, the system was closed and magnetically stirred until sampling or further oxygen addition (every 24 h). Sampling was performed in a biological safety cabinet (Class II, Jouan-France,) to prevent contamination with environmental microorganisms. Bacterial growth was determined at 600 nm on a Shimadzu, MultiSpect 1501 spectrophotometer.

During the acclimation period, quantitative assays of 1,2-DCB were performed daily by HPLC. 1,2-DCB dissolved in methanol was added directly to the slurry to obtain an initial concentration of 0.068 mM. Sterile controls were prepared with steam sterilized river sediments. Once substrate was exhausted, fresh substrate was added to restore the initial concentration. Acclimation was considered to be complete when we observed substrate disappearance after 2 days incubation. Further microorganism acclimation was obtained by 10 serial subcultures of the starting acclimated slurry.

Biodegradation assays were evaluated in MSM using CB and DCBs isomers as sole carbon source. CBs degradation was analyzed by substrate concentration (HPLC), bacterial growth (turbidity at 600 nm), chloride formation, and pH. Assays were carried out using the same equipment and methods described for the acclimation procedure. Strain isolation was performed from the acclimated inoculum on MSM plates, with the addition of CBs. Growth was achieved at both 30 and 370 °C under aerobic conditions. Results reported here correspond to strain growth at 37 °C. We did not find significant differences between strains growth at different temperatures.

Based on the hypothesis that different CBs could be degraded by diverse microorganisms, present in this population acclimated to 1,2-DCBs, bacterial strains were isolated from cultures using different CBs as sole carbon source. Isolated strains were tested for production of acid from the corresponding substrate as described in the literature (Spain and Nishino, 1987; Haigler et al., 1988). A pure strain was isolated for each substrate, based upon the colony characteristics (size, color and macroscopic morphology). When pure strains were growth on MSM agar, supplemented with bromothymol blue, we observed changes in the color of the culture medium, pointing out a drop in pH associated with the biodegradation of each substrate. This drop was assumed as indicative of the production of acids during CBs biodegradation. Sterile controls, with or without addition of CBs, did not change color after 7 days incubation.

2.4. Extraction of metabolites

Metabolite identification was attempted by extracting the culture medium of 1,3-DCB experiment with pure strains, using SPE-C18 cartridge and methanol as eluting solvent. Vials were stored at −20 °C until analyzed by HPLC. During chromatographic analysis we identified the presence of chlorocatechols by comparison to a 3,5-DCC standard. Additional identification attempts were performed by extracting the headspace of culture medium using solid phase micro extraction (SPME) fiber (Supelco 57300-U, 100 μm polydimethylsiloxane coating). Headspace SPME was carried out using 100 ml glass vials, half-filled with 50 ml culture medium, supplemented with 1 g NaCl to increase ionic strength, equilibred at 50 °C, absorption for 30 min at 50 °C with magnetic stirring, followed by thermal desorption onto the injector of a gas chromatograph couplet to a Shimadzu QP5050 mass spectrometry (GC-MS), equiped with capillary column HP5: 25 m, 20 mm i.d., 30 μm coating. GC conditions were: injector 250 °C, desorption time 3 min, column oven temperature: 30 °C for 3 min, increased until 250 °C at 7 °C min^{-1}, 250 °C for 3 min, carrier gas: helium at 1 ml min^{-1}.

2.5. Molecular techniques

DNA samples from each pure strain culture were obtained by washing three times with distilled water, suspended in 10 mM Tris pH 8.0, and boiled 10 min.

Bacterial gene encoding 16S rRNA was amplified by PCR using universal primers as previously described (Watt et al., 2001). To amplify ARHDO gene segments, consensus primers were used with conditions previously described (Kahl and Hofer, 2003).

Nucleotide sequence data were obtained by DNA sequencing (Macrogen, Korea) employing the same primers used for PCR amplification. The *adhA1* nucleotide sequence reported in this paper is available at GenBank^{TM}/EMBL Data Bank with accession number AY554272.

3. Results

3.1. Acclimation, enrichment and isolation of pure strains using chlorobenzenes as the sole carbon source

Starting with river sediment sampled from Suquía River, at a polluted site located 3.5 Km downstream from the wastewater plant of Córdobacity (Argentina) (Pesce and Wunderlin, 2000), the microbial population was acclimated to minimal medium (MSM), using 1,2-DCB as sole carbon source. After this acclimation period to 1,2-DCB, cultures were enriched for CBs-degrading microorganisms. Cultures were incubated with different CBs, starting with 1,2-DCB. We found that the biodegradation of 0.068 mM 1,2-DCB began after 15 days of acclimation. Successive addition of 1,2-DCB into the culture afforded complete degradation after 2 days (data not shown).

To evaluate the inhibitory concentration of 1,2-DCB, the acclimated inoculum was incubated with increasing amounts of this substrate (0.068, 0.136 and 0.170 mM), resulting in a linear increase of optical density (OD) at 600 nm, a drop in pH due to the production of acid metabolites, and the release of stoichiometric amounts of chloride as well (Schraa et al., 1986; Haigler et al., 1988). However, biodegradation of 1,2-DCB by the acclimated inoculum was inhibited at 0.272 mM. Sterile controls with sterile MSM plus steam sterilized inoculum did not show either chloride formation or increase in OD. To test if changes observed in pH and chloride formation were due to chemical photochemical degradation, we prepared additional controls with sterile MSM plus CBs, without addition of steam sterilized inoculum. No changes in these parameters were observed during the time analyzed.

Biodegradation of different chlorobenzenes (CB, 1,3-DCB and 1,4-DCB) (0.136 mM) produced a similar drop in pH as well as stoichiometric release of chloride.

Cultures acclimated to 1,2-DCB showed the presence of three different strains, which were isolated on the basis of colony morphology. Each isolated strain was tested for 1,2-DCB biodegradation. Results showed that only one strain was able to perform the complete biodegradation of this substrate. It should be noted that the isolation procedure used can lost microorganisms that cannot grow on agar plates. This limitation could be overcome by studying changes in the microbial community by PCR-DGGE-DNA techniques (Lyautey et al., 2005), which is out of the scope of the present paper.

Later on, cultures acclimated to 1,2-DCB were also used to evaluated the capability to biodegrade CB, 1,3-DCB and 1,4-DCB under the same conditions previously used for 1,2-DCB. Likewise to 1,2-DCB, each culture containing CB, 1,3-DCB and 1,4-DCB showed the presence of three different strains, with only one strain able to perform the complete biodegradation of the corresponding substrate. Further studies were carried out by using these pure strains having biodegradation ability.

3.2. Biodegradation using pure strains

Isolated strains were inoculated into 200 ml MSM with the corresponding substrate as sole carbon source to confirm the specific CB/DCBs degradation. Thus, each inoculum was supplemented with the corresponding substrate at 0.068 mM. The ability of pure strains to degrade the substrate was tested using the same protocol described for inoculum acclimation. Fig. 1 shows results obtained for different substrates, which were biodegraded by pure strains, as demonstrated by substrate consumption, increased turbidity, changes in pH, and released chloride.

Considering that dichlorocatechol has been proposed as a common metabolite produced during biodegradation of CB and DCBs (Schraa et al., 1986; Spain and Nishino, 1987; Haigler et al., 1988; Sander et al., 1991), we evaluated the ability of the pure strain that degraded 1,3-DCB, to degrade the corresponding metabolite 3,5-dichlorocatechol (3,5-DCC). Thus, increasing amounts (0.006, 0.011, 0.022 and 0.045 mM) of 3,5-DCC were added to MSM inoculated with pure strains. 3,5-DCC biodegradation was evaluated as previously described for CB and DCBs. We found a linear relationship between 3,5-DCC added and the increase in turbidity, demonstrating bacterial growth. Interestingly, we also observed stoichiometric production of chloride during biodegradation of 3,5-DCC (Fig. 2). However, both HPLC and GC-MS analysis did not detect transient metabolites produced during the biodegradation of CBs. We suspect that enzymes located downstream in the catabolic pathway of CBs are probably converting chlorocatechols at a rate that does not allow significant accumulation of these intermediates.

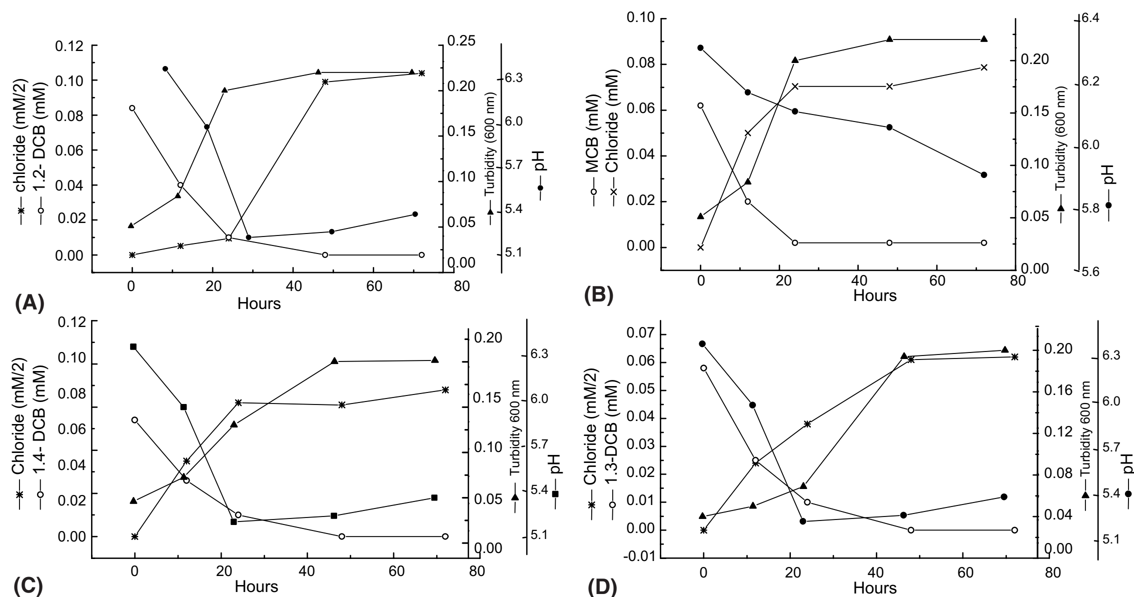

Fig. 1. Changes in culture turbidity (–▲–), chloride released (–✳–), substrate concentration (–○–), and pH (-●-) during growth of *A. avenae* CBA1 on 1,2-DCB (0.136 mM—1A), MCB (0.178 mM—1B), 1,3-DCB (0.136 mM—1C), and 1,4-DCB (0.136 mM—1D).

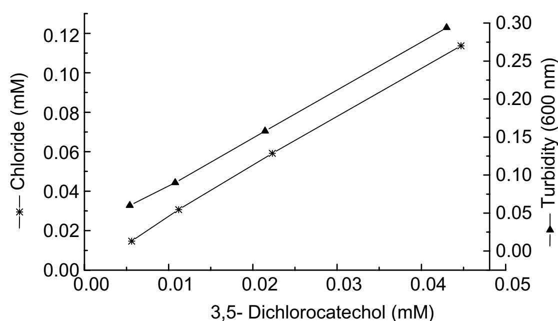

Fig. 2. End point determination of culture turbidity (–▲–) and chloride released (–✳–) after growth of *A. avenae* CBA1 on 3,5-dichlorocatechol. Data were determined from parallel batches of increasing amount of substrate 5 days after inoculation.

3.3. Identification of pure strains

Each one of the four strains, isolated from independent cultures in the presence of different CBs, showed the same biochemical pattern: gram negative non-fermentative bacilli, glucose oxidation-fermentation (alkaline), oxidase (+), motility (+), nitrate reduction (+), nitrite reduction (+), acetamide (+), citrate (+), urease activity (–), manitol (–), xylose (–), phenylalanine (–), esculin hydrolysis (–), pigment (–). This characterization was confirmed using API-NE (Bio Merieux) test for each isolated strain. In addition, 16S DNA analysis of four strains was performed as described in Materials and Methods. DNA fragment of 820 bp were obtained from each PCR reaction, and the samples were purified and sequenced in both directions by Macrogen Inc., Korea. The nucleotide sequences were compared in NCBI-BLAST Database (http://www.ncbi.nlm.nih.gov). We found, in the four strains, 100% identity with genes encoding 16S rRNA from *A. avenae*, confirming the presence of this strain.

3.4. Genetic characterization of the core α-subunit of ARHDO from *A. avenae*

Recently, Kahl and Hofer (2003) described a method to identify the core region of the large subunit of a class II ARHDO by PCR amplification using consensus primers. Following the protocol described by these authors, and utilizing as targets the DNA samples extracted from *A. avenae* strains, we obtained 730-bp PCR products. Further analysis by DNA sequencing revealed that nucleotide sequences from the *A. avenae* strains were identical. This strain was named *A. avenae* CBA1. The nucleotide sequence analyzed was typical for the core of α-subunit genes, and this putative gene was named *ahdA1*. The deduced protein sequence (224 amino acids), revealed that AhdA1 from *A. avenae* CBA1 is closely related to the α-subunit core of isopropylbenzene dioxygenase (IpbA1) from *Pseudomonas putida* RE204, and to biphenyl dioxygenase (BphA1) from *Ralstonia oxalatica* (81% and 77% identical amino acids, respectively). An alignment of these sequences is shown in Fig. 3. Homology to other dioxygenases, like benzene dioxygenase (BnzA) from *P. putida* BE81 was lower (50% identical amino acids).

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IpbAa/Pputida         TYKGLIFANWDAEA PDLLTYLSDATPYMD VMLDRTEAGTQVITG MQKTVIPCNWKFAAE QFCSDMYHAGTVAHL
EbdAa/Pputida         TYKGLIFANWDAEA PDLLTYLSDATPYMD VMLDRTEAGTQVITG MQKTVIPCNWKFAAE QFCSDMYHAGTVAHL
BphA1/Roxalatica      TYKGLIFANWDAEA PDLKTYLSDAMPYMD VMLDRTEAGTTIVGG MQKWVIPCNWKFAAE QFCSDMYHAGTMSHL
BphA1/Ctestosteroni   TYKGLVFANWDPEA PDLKTYLSDAMPYMD VMLDRTEAGTEAIGG IQKWVIPCNWKFAAE QFCSDMYHAGTMSHD
BnzA/P08084           TYKGLIFANWDENA VDLDTYLGEAKFYMD HMLDRTEAGTEAIPG VQKWVIPCNWKFAAE QFCSDMYHAGTTSHL
TodC1/P13450          TYKGLIFANWDENA VDLDTYLGEAKFYMD HMLDRTEAGTEAIPG VQKWVIPCNWKFAAE QFCSDMYHAGTTSHL
BedC1/Q07944          TYKGLIFANWDENA IDLDTYLGEAKFYMD HMLDRTEAGTEVIPG IQKWVIPCNWKFAAE QFCSDMYHAGTTAHL
TcbAa/Pseudomonas sp  TYKGLIFANWDHDA PDLDTYLGEAKFYMD HMLDRTEAGTEAIPG VQKWVIPCNWKFAAE QFCSDAYHAGTTSHL
TecA1/U78099          TYKGLIFANWDHDA PDLDTYLGEAKFYMD HMLDRTEAGTEAIPG VQKWVIPCNWKLAAE QFCWDAYHAATTAHL
BphA1/X80041          TYKGLIFANWDSEA PDLDTYLGEAKFYMD HMLDRTEAGTEALPG IEKWVIQCNWKFAAE QFCSDMYHVGTTSHL
AhdA1/AY554272        DLQGPDFRNWDAEA PDLKTYLSDATPYMD VMLDRTEAGTEVIGG MQKWVIPCNWKFAAE QFCSDAYHAGTMAHL
BphA1/P37333          TYKGLVFANWDVQA PDLETYLGDARPYMD VMLDRTPAGTVAIGG MQKWVIPCNWKFAAE QFCSDMYHAGTTTHL

Invar                    G  F NWD  A  DL TYL  A  YMD  MLDRT AGT    G   K VI CNWK AAE QFC D YH  T   H


IpbAa/Pputida         AGVVSSLPPEMDLSQ VKLPSTGNQFRAKWG GHGTGWFNDDFSLLQ AIMGPKIVDYWTKGP NAERAQERLGNKLP
EbdAa/Pputida         AGVVSSLPPEMDLSQ VKLPSTGNQFRAKWG GHGTGWFNDDFSLLQ AIMGPKIVDYWTKGP NAERAQERLGNKLP
BphA1/Roxalatica      SGVLASLPPDMDLSQ VKLPTTGNQFRAQWG GHGTGWFKDDFGLLQ AITGPKIVEYWTKGT AAERAQKRLADVLP
BphA1/Ctestosteroni   SGVLAGLPPEMDLTQ IQLSKNGNQFRSAWG GHGAGWFINDSSILL SVVGPKITQYWTQGP AAEKAARRVPQ-LP
BnzA/P08084           SGILAGLPEDLEMAD LAPPTVGKQYRASWG GHGSGFYVGDPNLML AIMGPKVTSYWTEGP ASEKAAERLGSVER
TodC1/P13450          SGILAGLPEDLEMAD LAPPTVGKQYRASWG GHGSGFYVGDPNLML AIMGPKVTSYWTEGP ASEKAAERLGSVER
BedC1/Q07944          SGIIAGLPEDLELAD LAPPKFGKQYRASWG GHGSGFYIGDPNMML AMMGPKVTSYLTEGP AAEKAAERLGSIER
TcbAa/U15298          SGILAGLPDGVELAD LALPTVGKQYRAPWG GHGTGFFIGEPNLLL AIMGPKITSYWTEGP ASEKAAQRLGSVER
TecA1/U78099          SGILAGLPDGVELAD LAPPTVGKQYRAPWG GHGSGFFIGEPDLLL AIMGPKITSYWTEGP ASEKAAQRLGSVER
BphA1/X80041          SGLLAGLPDEIDIRE VQPPTTGIQYSAPWG GHGSGFYIGEMGTLA AVMGMKILEYYTSGP AAEKAAKRLGSAVR
AhdA1/AY554272        SGVLASLPPDMDLSQ VKLPTTGNQFRAQWG GHGTGWFKDDFGLLQ AITGPKIVEYWTKGT AAERAQKRLADVLP
BphA1/P37333          SGILAGLPPEMDLSQ AQIPTKGNQFRAAWG GHGSGWYVDEPGSLL AVMGPKVTQYWTEGP AAELAEQRLGHTGM

Invar                  G L  LP               G Q  A WG GHG G              G K   Y T G    E A  R


IpbAa/Pputida         N-RMVTQHMTIFPTC SFLPGINTIRTWHPR GPNEVEVWSFILVDA DAPEEIKDEYRRKNI FTFNQGGTYEQDDGE
EbdAa/Pputida         N-RMVTQHMTIFPTC SFLPGINTIRTWHPR GPDEVEVWPFILVDA DAPEEIKDEYRRKNI FTFNQGGTYEQDDGE
BphA1/Roxalatica      ANRMVLQHMTVFPTC SFLPGVNTVRSWHPR GPNEVEVWAFVVVDA DAPEEIKDEFRRQNI RTFNAGGVFEQDDGE
BphA1/Ctestosteroni   LD-MFGQHMTVFPTC SFLPGINTIRTWHPR GPNEVEVWAFVLVDA DAPEDIKEEFRLQNI RTFNAGGVFEQDDGE
BnzA/P08084           S-KLMVEHMTVFPTC SFLPGINTVRTLASA RAERGEVWAFTVVDA DAPDDIKEEFR-ARL RTFSPVACSSRTTG-
TodC1/P13450          S-KLMVEHMTVFPTC SFLPGINTVRTWHPR GPNEVEVWAFTVVDA DAPDDIKEEFRRQTL RTFSAGGVFEQDDGE
BedC1/Q07944          T-KIMLEHMTVFPTC SFLPGVNTIRTWHPR GPNEVEVWAFTVVDA DAPDDIKEEFRRQTL RTFSAGGVFEQDDGE
TcbAa/U15298          S-KLMVEHMTVFPTC SFLAGPNTVRTWHPR GPNEVEVWAFTVVDA DAPDDIKEEYRRQTL RTFSAGGVFEQDDGE
TecA1/U78099          S-KLTVEHMTVFPTC SFLLGANTVRTWHPR GPNEVEVWAFTVVDA DAPDDIKEEFRRQTV RTFSAGGVFEQDDGE
BphA1/X80041          S-QATGQHMTVFPTC SFLPGINTIRTWHPR GPHEIEVWSFTVVDA DAPAEIKEEYRRQTI RTFSAGGVFDQDDGE
AhdA1/AY554272        ANRMVLQHMTVFPTC SFLPGVNTVRSWHPR GPNEVEVWAFVVVDA DAPEEIKDEFRRQNI RTFNAGGVFEQDDGE
BphA1/P37333          VRRMVGQHMTIFPTC SFLPTFNNIRIWHPR GPNEIEVWAFTLVDA DAPAEIKEEYRRHNI RNFSAGGVFEQDDGE

invar.                       HMT FPTC SFL   N  R           EVWAF  VDA DAP  IK E RR     F          GE
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Fig. 3. Alignment of α-subunit core sequences deduced from amplified gene segments. Amino acid numbering is tentative for the B3B sequence. Invariant amino acids (invar) are indicated by their respective letters. All residues that, by analogy with the three-dimensional crystal structure of a class III naphthalene dioxygenase (Carredano et al., 2000), belong to the substrate-binding pocket are highlighted.

A close relationship between these dioxygenases is reflected in the phylogenetic tree (Fig. 4), which is based on the amino acid sequences from the α-subunit core genes corresponding to different dioxygenases.

4. Discussion

Acclimated bacteria, isolated from the sediment of Suquía River, were capable of degrading xenobiotic compounds such as CB and DCBs. Currently, it is believed that the adaptation process may involve one, or a combination of, the following steps: (i) induction or derepression of enzymes specific for degradation pathways of a particular compound; (ii) a random mutation that produces new metabolic capabilities, which allow degradation that was not previously possible; (iii) an increase in the number of organism presents in the degradation population (Spain et al., 1980). At sites where the community has been exposed to known pollutants for long periods, it is clear that adaptation can occur (Aelion et al., 1987).

In our case, adaptation of the native community to perform biodegradation of 1,2-DCB was evident after 15 days aerobic incubation. This time is shorter than those determined by Spain et al. (1980) during the

Fig. 4. Phylogenetic tree based on amino acid sequences of α-subunit core of initial dioxygenases from different bacteria. The names of bacteria strains and the GenBank accession number are indicated in parentheses. The distance scale indicates 10% substitutions and the numbers shown at the nodes indicate bootstrap percentages. The tree was constructed using α-subunit core sequences of group IV (Rapp and Gabriel-Jurgens, 2003).

biodegradation of 1,2-DCB. So, this result indicates that native microbial community can be adapted to perform biodegradation of these xenobiotics quite quickly.

From this adapted microbial community we isolated a strain of *A. avenae*. Our results indicate that the isolated strain of *A. avenae* was capable to biodegrade CB; 1,2-DCB, 1,3-, 1,4-DCB, and also 3,5-DCC. The capability of *A. avenae* to biodegrade all DCB isomers and CB is comparable with strains of *Burkholderia* sp. (Rapp, 2001; Rapp and Gabriel-Jurgens, 2003). However, this is not the usual situation with other bacteria, which are able to achieve biodegradation of some selected dichlorobenzenes but not all the isomers (Schraa et al., 1986; Haigler et al., 1988; van der Meer et al., 1991; Spiess et al., 1995).

Biodegradation of chlorinated benzenes have been previously described for *Alcaligenes*, *Pseudomonas* and *Xanthobacter* species, (Schraa et al., 1986; Spain and Nishino, 1987; van der Meer et al., 1987; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995) but there are no previous reports on biodegradation of CB and DCB isomers by *A. avenae*. Previous studies indicate that the degradation of CBs is initiated by ring-dioxygenation to yield chlorinated dihydrodiols. Further dehydrogenation of these dihydrodiols yields corresponding chlorocatechols, which serve as substrates for ring fission enzymes and subsequent reaction that result in elimination of chloride. Thus, the initial steps in the degradation of CBs are analogous to those described for bacterial degradation of other aromatic hydrocarbons, like benzene, toluene and ethylbenzene.

The substrate specificity of class IIB enzymes is usually determined by the α-subunit, which is critically responsible for the recognition and binding to the substrate, and thereby for substrate specificity (Erickson and Mondello, 1993; Mondello et al., 1997). Each α-subunit in these enzymes contains a Rieske [2Fe–2S] cluster and mononuclear iron (Jiang et al., 1996), which is believed to be the site of dioxygen activation and catalysis.

The *adhA1* gene of *A. avenae* CBA1, encoding the initial chlorobenzene dioxygenase sequence, was amplified by PCR using consensus primers. These primers were designed against the α-subunit core of enzymes, which predominantly dioxygenate benzene or its derivates (Kahl and Hofer, 2003). According to biochemical studies with different enzyme variants, these segments encode the major determinants of substrate and product spectra (Erickson and Mondello, 1993; Kimura et al., 1997; Mondello et al., 1997). They also encode all amino acid residues that, by analogy with the three-dimensional crystal structure of a related naphthalene dioxygenase (Carredano et al., 2000), form the substrate-binding pocket (Fig. 3).

Our results indicate that AdhA1 from *A. avenae* CBA1 has an amino acid sequence compatible with

ARHDO system. A comparison of the predicted amino acid sequences, obtained for the α subunit from *A. avenae* CBA1, reveals a great deal of amino acid conservation within each group, in the region that is proposed to contain the mononuclear-iron-binding site, where the common motif Glu-X_{3-4}-Asp-X_2-His-X_{4-5}-His is seen in all classes of terminal oxygenase components.

The AdhA1 from *A. avenae* CBA1 can be grouped most probably into class IIB, dioxygenases subfamilies (Rapp and Gabriel-Jurgens, 2003), This Rieske non-haem-iron oxygenases are now classified into groups based on their substrate specificity and the sequence of their α-subunits (Gibson and Parales, 2000), though this sequence exhibited higher amino acids sequence identity with IpbA1 and BphAa (from *P. putida* RE204 and *R. oxalatica*, respectively) than with chlorobenzenes dioxygenases from *Pseudomonas* sp. P51 (TcbAa) and *Burkholderia* sp. PS12 (TecA1), all of them grouped within group IIB (Fig. 4).

In conclusion, our results indicate that *A. avenae*, strain CBA1, is capable to perform complete biodegradation of CB and DCBs isomers. This ability is probably related to the presence of an ARHDO system in this strain, which show high homology with similar systems described for other aromatic compounds. We propose that AdhA1 could be responsible of CBs degradation assayed in this study. Further work is in progress to characterize this protein and to get evidences on its putative function in the biodegradation of CBs.

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