Characterization of bacterial consortia capable of degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions

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Received 14 February 2002; received in revised form 5 June 2002; accepted 5 June 2002

First published online 22 June 2002

Abstract

4-Chlorobenzoate and 4-bromobenzoate were readily degraded in denitrifying enrichment cultures established with river sediment, estuarine sediment or agricultural soil as inoculum. Stable denitrifying consortia were obtained and maintained by serial dilution and repeated feeding of substrates. Microbial community analyses were performed to characterize the 4-chlorobenzoate and 4-bromobenzoate degrading consortia with terminal restriction fragment length polymorphism (T-RFLP) and cloning of 16S rRNA genes from the cultures. Interestingly, two major terminal restriction fragments (T-RFs) in the 4-chlorobenzoate degrading consortia and one T-RF in the 4-bromobenzoate utilizing consortium were observed from T-RFLP analysis regardless of their geographical and ecological origins. The two T-RFs (clones 4CB1 and 4CB2) in 4-chlorobenzoate degrading consortia were identified as members of the β-subunit of the Proteobacteria on the basis of 16S rRNA sequencing analysis. Phylogenetic analysis of 16S rRNA genes showed that clone 4CB1 was closely related to Thauera aromatica while clone 4CB2 was distantly related to the genera Limnobacter and Ralstonia. The 4-chlorobenzoate utilizing consortium mainly consisted of one T-RF, which was identical to clone 4CB2 in spite of different enrichment substrate. This suggests that degradation of 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions was mediated by bacteria belonging to the β-subunit of the Proteobacteria. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Halobenzoate degradation; Denitrification; Terminal restriction fragment length polymorphism

1. Introduction

Enrichment culture studies have previously demonstrated the degradation of 3-chlorobenzoate, 4-chlorobenzoate, 2-bromobenzoate, 3-bromobenzoate and 4-bromobenzoate under denitrifying conditions [1–4]. Benzoates with chlorine or bromine in the meta or para position were readily degraded within 30 days after an initial spike of substrate [1]. Isolation of pure cultures capable of degrading 3-chlorobenzoate and 3-bromobenzoate, belonging to the genera Ochrobactrum, Thauera and Pseudomonas, was successful from several of the sites tested [1,5]. In contrast, repeated attempts to isolate pure cultures degrading 4-chlorobenzoate or 4-bromobenzoate failed although anaerobic transformation of 4-chlorobenzoate by pure cultures was reported previously [6,7].

Since isolation of a pure culture was not successful, community analyses using molecular techniques were considered to characterize the 4-chlorobenzoate and 4-bromobenzoate degrading denitrifying enrichment cultures. Terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA gene was used to fingerprint the microbial communities in the enrichment cultures [8]. The T-RFLP patterns can be considered as a representation of the operational taxonomic units (OTUs) present in a microbial community. The number of distinct terminal restriction fragments (T-RFs) represents the number of different OTUs in a community. The T-RFLP technique...
has been used to profile Archaeal and Eubacterial communities in various environments such as activated sludge, sediments and agricultural soils [9–12] as well as microbial populations related to anaerobic aromatic compound degradation [13–16]. Furthermore, T-RFLP analysis has been applied to investigate genetic diversity of functional genes encoding for ammonia oxidation, methane oxidation, nitrite reduction, nitrous oxide reduction and methane production from various environmental samples [17–21]. However, this technique does not provide information for the identification of each T-RF. A combination of 16S rRNA gene cloning and screening of clones by TRFLP has been successful in identifying various peaks within a fingerprint [11,13–15,19]. In this study, T-RFLP analysis and 16S rRNA gene cloning were applied to characterize bacterial consortia capable of degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions.

2. Materials and methods

2.1. Source of consortia

4-Chlorobenzoate degrading denitrifying enrichment cultures were previously established with estuarine sediment from Arthur Kill, New Jersey, USA or agricultural soil from Wyoming, USA and 4-bromobenzoate degrading enrichment culture was established with river sediment from Kyungan River, Korea [1]. Enrichment cultures were initially fed 100 μM of 4-chlorobenzoate or 4-bromobenzoate and the loss of substrates was monitored using high performance liquid chromatography (HPLC) [1]. After initial loss, substrates were re-fed three times up to a total of 1 mM concentration. These enrichment cultures were subcultured (1:10) in denitrifying medium [22] and substrates were fed in the same way until 1 mM concentration had been utilized. The enrichment cultures were subcultured further and re-fed. The most dilute enrichment cultures (10−3 dilution) were used as inocula for a serial dilution from 10−1 to 10−8. Substrates were repeatedly fed until 1 mM concentration had been consumed as mentioned above. The most dilute cultures (10−11 dilution of original enrichment culture) were used as inocula for another serial dilution of 10−1 to 10−8 and the activities of halobenzoate degradation were maintained. Bacterial consortia from these cultures (10−19 dilution of original enrichment culture) were used to monitor the degradation of 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions.

2.2. DNA extraction

DNA was extracted from the consortia using a modified phenol:chloroform method as described previously [23]. The modification included four cycles of rapid freeze thaws and use of gene clean kit (Bio 101, Carlsbad, CA, USA) to purify DNAs.

2.3. 16S rRNA amplification and T-RFLP

16S rRNA genes from the extracted DNA samples were amplified with universal eubacterial primers 27F and 1522R [24]. The universal eubacterial primer 27F was labeled with 6-FAM (5-[6]-carboxy-fluorescein) on the 5′-end (Gibco-Life Technologies, Gaithersburg, MD, USA). PCR products were purified using gene clean kits and digested with MnlI or Hinfl (New England Biolabs, Beverly, MA, USA) for 6 h at 37°C. The digested samples were run on an ABI 310 automated sequencer. The sizes of fragments were compared with internal standards and determined by the GeneScan software (Applied Biosystems, Foster City, CA, USA).

2.4. Cloning and sequencing

16S rRNA genes from the samples were amplified with universal eubacterial primers 27F and 1522R and used for direct cloning with the TOPO-TA® cloning system (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instruction. The ligated plasmids were transformed in high transforming efficiency Escherichia coli TOP10F® (Invitrogen) following the manufacturer’s instructions. The transformed cells were plated on Luria–Bertrani agar plates containing 50 μg ml−1 kanamycin. White or blue color development of colonies after overnight incubation at 37°C was used to verify successful cloning of 16S rRNA genes on plasmids. The white colonies were picked for direct PCR amplification with M13R and T7 primers. Restriction fragment length polymorphism (RFLP) was performed to compare different patterns of restriction fragments (RFs) for each amplified PCR product using HaeIII restriction enzyme for digestion.

The partial sequences (850 nucleotides) of the 16S rRNA gene from the unique RFs were obtained by using internal 16S rRNA oligonucleotide sequencing primers 27F, 357F, 704F, 907R, 685R and 321R [24] and an ABI 310 automated DNA sequencer (Applied Biosystems).

2.5. Phylogenetic analysis

The phylogenetic analysis was carried out according to Kimura’s two-parameter method [25] and neighbor-joining topology [26]. The SEQBOOT program was used to obtain the confidence level for neighbor-joining analysis using a 100 bootstrapped data set [27].

The 16S rRNA gene sequences of the reference strains and species in the β- and γ-subunits of the Proteobacteria were obtained from the GenBank database (National Center for Biotechnology Information, National Library of Medicine). The nucleotide sequence accession numbers
are as follows: Thauera aromatica K172T (X77118), Thauera chlorobenzoica 3CB1 (AF123264), Thauera sele- 

natis AX7 (X68491), Burkholderia caryophylli ATCC 25418T (AB021423), Pandoraea promenusa LMG 18087T 

(AF139174), Pandoraea norimbergensis LMG 13019 (AF- 

139171), Limnobacter thiooxidans CS-K2T (AJ289885), 

Ralstonia thomaisi LMG 6866T (AJ270258), Ralstonia so- 

lanacearum ATCC 11696T (X67036), Pseudomonas sp. 

3CB6 (AF229885), Pseudomonas pictorum ATCC 23328T 

(ABO21392), Stenotrophomonas maltophilia LMG 10881 

(AJ131904), Stenotrophomonas nitritireducens L2T (AJ-

012229) and Ochrobactrum sp. 3CB4 (AF229883). The nu-

cleotide sequence accession numbers of T-RFs are 

AF229889 (clone 4CB1), AF229890 (clone 4CB2) and 

AF481735 (clone 4CB3).

3. Results and discussion

3.1. 4-Chlorobenzoate and 4-bromobenzoate degradation 

under denitrifying conditions

The degradation of 4-chlorobenzoate and 4-bromoben-

zoate from the most diluted consortia was monitored (Fig. 

1). The 4-chlorobenzoate degrading consortium from Ar-

thur Kill sediment completely used the initial 200 µM of 

4-chlorobenzoate within 5 days. The initial 100 µM of 

4-bromobenzoate was also completely degraded within 

5 days by the 4-bromobenzoate degrading consortium 

from Kyungan River sediment. The cultures were re-fed 

up to a total of 2.2 mM of 4-chlorobenzoate or 1.4 mM of 

4-bromobenzoate, respectively. A total of 2.2 mM of 4-

chlorobenzoate was utilized within 25 days and 16 mM of 

nitrate used as electron acceptor. In addition, 5 mM of 

nitrate was used while degrading 1.4 mM of 4-bromoben-

zoate. During the utilization of halobenzoates coupled to 

denitrification, an increase of turbidity of the cultures was 

observed (data not shown).

3.2. T-RFLP

T-RFLP analysis of the 4-chlorobenzoate degrading 

consortia yielded two major T-RFs of 171 and 175 bp 

length with MnlI restriction enzyme digestion (Fig. 2), 

however, three major T-RFs of 198, 326 and 335 bp length 

were observed with HindI restriction enzyme digestion 

(Fig. 2). The 4-chlorobenzoate degrading consortium 

showed only one T-RF with the two different restriction 

enzyme digestions (Fig. 2). Interestingly the three bacterial 

consortia enriched from three different sites using two dif-

ferent substrates had similar T-RFLP patterns. The T-RFs 

of the size of 171 bp length with MnlI digestion and 326 

bp length with HindI digestion were observed in all three 

consortia whereas the T-RFs of the size of 175 bp length 

with MnlI digestion, and 198 and 335 bp lengths with 

HindI digestion were observed in only the 4-chloroben-

zoate degrading consortia. These similar T-RFLP patterns 

must be due to an intensive enrichment of the original 

inocula. Thus, only one or two major bacterial populations 

were apparent based on T-RFLP analysis, which were 

well adapted for the use of 4-chlorobenzoate or 4-

bromobenzoate under denitrifying conditions in the labo-

ratory. These outcompeted other bacterial populations 

which were below the detection limit on T-RFLP analysis. 

The abundance of each bacterial population represented 

as an area of each T-RF varied in two 4-chlorobenzoate 

degrading consortia (Fig. 2). The consortium established 

using the Arthur Kill sediment had a larger bacterial popu-

lation of T-RFs with 171 bp length after MnlI digestion 

and 326 bp length after HindI digestion. In contrast, the 

consortium from Wyoming soil had more abundance of 

bacterial populations corresponding to T-RFs of 175 bp 

length after MnlI digestion and 198 and 335 bp lengths 

after HindI digestion. This discrepancy in the relative 

proportions of bacterial populations in the two cultures could
show a competitive relationship between two populations for 4-chlorobenzoate degradation.

### 3.3. 16S rRNA gene cloning and phylogenetic analysis

More than 100 clones from 16S rRNA gene clonal libraries of 4-chlorobenzoate degrading consortia were randomly picked for PCR amplification and the amplified products were digested with *Hae*III restriction enzyme digestion to compare RFs. Three different types of RFs (4CB1, 4CB2 and 4CB3) in Arthur Kill sediment and two different types of RFs (4CB1 and 4CB2) in Wyoming soil samples were found (data not shown). Sequencing analysis of these RFs showed that clones 4CB1 and 4CB2 from two different samples were identical and belonged to the β-subdivision of the *Proteobacteria*, and clone 4CB3 was assigned to the γ-subdivision of the *Proteobacteria* (Fig. 3). Clone 4CB1 was closely related to *T. aromatica* with 99.3% sequence similarity and clone 4CB3 was closely related to *S. nitritireducens* with 97.5% sequence similarity. Clone 4CB2 was distantly related to *R. solanacearum* and *L. thiooxidans* with 92.7% and 93.8% sequence similarities. T-RFLP analysis of each RF showed that clone 4CB2 could be matched with the 171 bp length fragment after *Mnl*I digestion and the 326 bp length fragment after *Hin*fI digestion. Clone 4CB1 could be matched with the 175 bp length fragment after *Mnl*I digestion and the 326 bp length fragment after *Hin*fI digestion. In addition, clone 4CB1 generated a small T-RF with the size of 335 bp after *Hin*fI digestion due to incomplete digestion. Clone 4CB3 generated a 106 bp length fragment after *Mnl*I digestion and a 340 bp length fragment after *Hin*fI digestion, which were very small T-RFs in the T-RFLP of the 4-chlorobenzoate degrading consortium from Arthur Kill sediment.

Both 4-chlorobenzoate degrading consortia had similar

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**Fig. 2. T-RFLP analysis of 4-chlorobenzoate (A, B) and 4-bromobenzoate (C) degrading consortia with restriction endonuclease *Mnl*I or *Hin*fI. The numbers represent the length of T-RFs.**

![MnlI Digestion](image)

![Hinfl Digestion](image)
from mineralsalt plates containing 4-chlorobenzoate, tia. Three different denitrifying isolates were obtained. Conditions continued after characterization of the consortium consisting of clones 4CB1 and 4CB2. Thus, clone 4CB2 was considered as the major population degrading 4-bromobenzoate in this denitrifying consortium.

The presence of the Thauera sp. T-RF (4CB1) in 4-chlorobenzoate degrading denitrifying consortia is interesting because several T. aromatica and T. chlorobenzoica isolates obtained from the same sites were capable of degrading 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate or 3-bromobenzoate under denitrifying conditions [5]. In addition, clone 4CB2, distantly related to the genera Ralstonia and Limnobacter should be considered as a major population degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions.

Efforts to isolate pure cultures capable of degrading 4-chlorobenzoate or 4-bromobenzoate under denitrifying conditions continued after characterization of the consortia. Three different denitrifying isolates were obtained from mining salt plates containing 4-chlorobenzoate, benzoate and nitrate. None of these isolates utilized 4-chlorobenzoate but were able to use benzoate as a carbon and energy source under denitrifying conditions. 16S rRNA gene sequencing analysis of these isolates assigned them to the genera Acidovorax, Mesorhizobium and Stenotrophomonas (data not shown). These isolates were considered as minor populations of the halobenzoate utilizing consortia, which were not detected by T-RFLP analysis and 16S rRNA gene cloning although isolated under the plating conditions. However, the major populations (clones 4CB1 and 4CB2) were not isolated with this technique, although they were readily cultured in liquid media.

Different isolation techniques must therefore be considered to obtain pure cultures capable of degrading 4-chlorobenzoate or 4-bromobenzoate under denitrifying conditions.

Acknowledgements

We thank Dr. Bess B. Ward for her help to use instruments at Princeton University. This work was supported by grants from the United States Environmental Protection Agency (R822457) and the Office of Naval Research (N0014-99-1-0761).

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