

Isolation and Characterization of Diverse Halobenzoate-Degrading Denitrifying Bacteria from Soils and Sediments

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Denitrifying bacteria capable of degrading halobenzoates were isolated from various geographical and ecological sites. The strains were isolated after initial enrichment on one of the monofluoro-, monochloro-, or monobromo-benzoate isomers with nitrate as an electron acceptor, yielding a total of 33 strains isolated from the different halobenzoate-utilizing enrichment cultures. Each isolate could grow on the selected halobenzoate with nitrate as the terminal electron acceptor. The isolates obtained on 2-fluorobenzoate could use 2-fluorobenzoate under both aerobic and denitrifying conditions, but did not degrade other halobenzoates. In contrast, the 4-fluorobenzoate isolates degraded 4-fluorobenzoate under denitrifying conditions only, but utilized 2-fluorobenzoate under both aerobic and denitrifying conditions. The strains isolated on either 3-chlorobenzoate or 3-bromobenzoate could use 3-chlorobenzoate, 3-bromobenzoate, and 2- and 4-fluorobenzoates under denitrifying conditions. The isolates were identified and classified on the basis of 16S rRNA gene sequence analysis and their cellular fatty acid profiles. They were placed in nine genera belonging to either the α -, β -, or γ -branch of the *Proteobacteria*, namely, *Acidovorax*, *Azoarcus*, *Bradyrhizobium*, *Ochrobactrum*, *Paracoccus*, *Pseudomonas*, *Mesorhizobium*, *Ensifer*, and *Thauera*. These results indicate that the ability to utilize different halobenzoates under denitrifying conditions is ubiquitously distributed in the *Proteobacteria* and that these bacteria are widely distributed in soils and sediments.

Halogenated aromatic compounds are widely distributed in the environment as a result of their widespread use as herbicides, insecticides, fungicides, solvents, fire retardants, pharmaceuticals, and lubricants. Chlorinated compounds are used more frequently than fluorinated or brominated compounds (23). Several of these chemicals cause considerable environmental pollution and human health problems due to their persistence and toxicity. In addition, a large number of halogenated organic compounds are naturally produced by plants, marine organisms, insects, bacteria, fungi, and mammals and released into the environment (11).

Biodegradation of numerous halogenated aromatic compounds has been reported under both aerobic and anaerobic conditions (for reviews, see references 6, 13, 14, 22, and 26). Halobenzoates have been studied as model chemicals for biodegradation of halogenated aromatic compounds, and their aerobic degradation pathways in different bacteria are well characterized (for reviews, see references 6, 7, 13, 21, 26, and 35). The capability to degrade halobenzoates under aerobic conditions has been found in diverse bacteria isolated from various geographical and ecological sources.

The observed functional and taxonomic diversity of halobenzoate-degrading bacteria under aerobic conditions raises questions about the diversity of these catabolic properties under anaerobic conditions. Investigations with enrichment cultures under different anaerobic electron acceptor conditions have demonstrated halobenzoate degradation coupled to denitrification, sulfidogenesis, iron reduction, or methanogenesis (10,

15, 19, 34). Under methanogenic conditions, halobenzoate degradation is initiated by reductive dehalogenation (30), and this process has been extensively studied (6, 14). Under denitrifying conditions, different halobenzoate isomers can be degraded by enrichment cultures established with soils and sediments from various geographical locations (15, 16, 34). Anaerobic utilization of halobenzoate was dependent on and coupled to denitrification, and denitrifying consortia could be maintained with halobenzoate as the sole carbon source. Only one denitrifying chlorobenzoate- and bromobenzoate-degrading bacterium has previously been isolated (17), and the strain was identified as a genomovar of the species *Thauera aromatica* (28). In addition, a number of fluorobenzoate-degrading denitrifying bacteria have been isolated (25, 31, 34). Our previous work with enrichment cultures suggested that denitrifying bacteria capable of degrading halobenzoates are widespread in different environments; however, there is little information on their taxonomic or biochemical diversity. It is thus of interest to examine this functional group of denitrifying bacteria in detail. In this paper, isolation of pure cultures from the enrichment cultures established with sediments and soils from several different geographical and ecological sites was pursued. The objective was to systematically examine growth on halobenzoate as a sole carbon source, nitrate dependency of halobenzoate degradation, substrate specificity, and taxonomic diversity of halobenzoate-degrading denitrifying bacteria.

MATERIALS AND METHODS

Enrichment cultures. Sediment and soil samples were collected from various environmental and geographical locations, including estuarine sediments (Arthur Kill, N.J.; Lubeck, Maine; Hopewell Rock, Canada; Alma, Canada; and Shihwa Lake, Korea), freshwater sediments (Passion Puddle, New Brunswick, N.J.; Blue Mountain Lake, N.Y.; Lake Vesijärvi, Finland; Kyungan River, Korea; and Anyang River, Korea), and soils (Rutgers Golf Course, Piscataway, N.J., field soil, Wyoming; El Yunque Forest, Puerto Rico; Guanica Forest, Puerto Rico; Viikki, Finland; and Salpausselkä, Finland). Samples were transferred in sealed glass jars and stored at 4°C until used. Strict anaerobic techniques for medium preparation, culture handling, and sampling were followed throughout

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the enrichment process. Initial enrichment cultures were established by using 10% (vol/vol) sediment or soil slurry in a minimal salts medium with 30 mM nitrate (32) under argon. One-hundred-milliliter aliquots of the slurry were dispensed in 160-ml serum bottles, sealed with rubber stoppers, and crimped with aluminum seals under a headspace of argon. Halobenzoates (2-fluorobenzoate, 3-fluorobenzoate, 4-fluorobenzoate, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2-bromobenzoate, 3-bromobenzoate, or 4-bromobenzoate; >97% purity; Aldrich Chemical Co., Milwaukee, Wis.) were fed from deoxygenated stock solutions to a final concentration of 100 μ M. All enrichment cultures, including autoclaved sterile controls, were incubated in the dark at 30°C. The utilization of halobenzoates was monitored over time, and halobenzoates were refed when depleted. A total of halobenzoates of 1 mmol/liter⁻¹ was fed before cultures were subcultured.

Isolation of halobenzoate-degrading denitrifying bacteria. The primary enrichment cultures on 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 3-bromobenzoate, and 4-bromobenzoate were serially diluted up to 10⁻³ after 1 mM halobenzoate had been degraded. After substrate loss was observed, the 10⁻³ dilution samples were streaked onto minimal salts (32) plates solidified with Noble agar (Difco, Detroit, Mich.) containing 10 mM nitrate as the electron acceptor and 1 mM benzoate in combination with either 1 mM 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 3-bromobenzoate, or 4-bromobenzoate as the carbon sources. The plates were incubated in an anaerobic jar under argon at 30°C. After 2 weeks of incubation, single colonies were picked with a sterile capillary tube and restreaked onto fresh minimal salts plates. This isolation procedure was repeated until the colony morphology appeared homogeneous on a dissecting microscope. The purity of isolates was checked on minimal salts plates containing 1 mM succinate and 10 mM nitrate.

To confirm the utilization of halobenzoates, isolates were pregrown on minimal salts plates containing 10 mM nitrate, 1 mM benzoate, and 1 mM succinate under an atmosphere of argon. Cells were then transferred to 10 ml of liquid minimal salts medium (32) and tested for the degradation of halobenzoate (100 μ M) under denitrifying conditions.

Growth of isolates on halobenzoate. To test for growth on halobenzoates under denitrifying conditions, cells were pregrown in liquid medium with 30 mM nitrate as electron acceptor, 5 mM succinate, and 200 μ M halobenzoate as carbon and energy sources. The cells were then collected by centrifugation, washed twice with fresh medium, and inoculated in Hungate test tubes (Bellco, Vineland, N.J.) containing 10 ml of fresh liquid medium with 10 mM nitrate and different concentrations of halobenzoates (0, 0.2, 0.5, and 1 mM). Duplicate tubes were prepared for each concentration. Cultures without substrates were used as controls. Growth was monitored by measuring the optical density at 620 nm directly from the Hungate test tubes with a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y.).

To determine the nitrate dependency of halobenzoate degradation, cells were inoculated in Hungate test tubes with or without 0.5 mM halobenzoate or 10 mM nitrate. Each condition was tested in duplicate tubes and growth of cells was monitored as described above.

Substrate utilization. The utilization of benzoate, hydroxybenzoates, and halobenzoates (>97% purity; Aldrich Chemical Co.) was tested with a dense cell suspension of each isolate after growth in liquid medium with 5 mM succinate and 200 μ M halobenzoate under denitrifying conditions. The cultures were then fed 200 μ M test substrate and incubated under either aerobic or denitrifying conditions. After 14 days of incubation, the loss of substrates was determined by high-performance liquid chromatography (HPLC).

Determination of cellular fatty acids. All of the isolates were grown with 10 mM succinate and 30 mM nitrate under anaerobic conditions for 24 h as described previously (27). Cells were harvested by centrifugation, and the cellular fatty acids were saponified, methylated, and extracted by the procedure of the SHERLOCK Microbial Identification System (MIDI, Inc., Newark, Del.). The cellular fatty acid composition was then determined by gas chromatography with an HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, Calif.) and the SHERLOCK Microbial Identification System.

16S rRNA gene amplification, sequencing, and phylogenetic analysis. The genomic DNAs of isolates were extracted and purified as described previously (29). PCR amplification of the 16S rRNA gene of each isolate was performed with a GeneAmp PCR system 2400 as described previously (29). The partial sequence (600 nucleotides) of the 16S rRNA gene was obtained by using internal 16S rRNA oligonucleotide sequencing primers 27F and 685R (18) and an ABI 377A automated sequencer (Perkin-Elmer).

The reference 16S rRNA gene sequences of the species *Acidovorax avenae*^T (AF078759), *Paracoccus denitrificans*^T (Y16927), *Ensifer adhaerens*^T (AF191739), *Ochrobactrum anthropi*^T (D12794), *Bradyrhizobium elkanii*^T (U35000), *Pseudomonas stutzeri*^T (U25432), *Thauera aromatica*^T (X77118), and *Azoarcus toluolicus*^T (L33694) were obtained from the GenBank database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, Md.) and were aligned by using the PILEUP program of the Genetics Computer Group (GCG) software package (2).

The phylogenetic analysis was carried out according to Kimura's two-parameter method (20) and neighbor-joining topology (24). The SEQBOOT program was used to obtain the confidence level for neighbor-joining analysis with a 100 bootstrap data set (5).

Analytical methods. All enrichment cultures were monitored periodically for the loss of halobenzoate and nitrate. Approximately 1 ml of well-mixed slurry was sampled with sterile syringes preflushed with argon, centrifuged in a microcentrifuge, and filtered through a 0.45- μ m-pore-size filter (Millipore). Samples from pure culture studies were centrifuged if necessary, filtered, and frozen (-20°C) until analyzed. Halobenzoates and other aromatic compounds were analyzed by high-performance liquid chromatography (model LC-10AS; Shimadzu Corp., Kyoto, Japan) with a reverse-phase C₁₈ column (Sperisorb 4.6 by 250 nm, 5- μ m particle size; Phenomenex, Torrance, Calif.) and UV detection at 280 nm as previously described (34). The concentrations of nitrate and nitrite were monitored with an ion chromatography system (Dionex DX-100, Sunnyvale, Calif.) with conductivity detection as previously described (34).

Nucleotide sequence accession number. The 16S rRNA sequences for the halobenzoate-degrading isolates have been deposited in GenBank under accession numbers AF229856 to AF229888.

RESULTS

Halobenzoate degradation by enrichment cultures. The utilization of halobenzoates by denitrifying enrichment cultures established with sediment and soil from different geographical and ecological sites was observed (data not shown). In general, similar activities were observed with inocula from diverse sources, including estuarine and freshwater sediments and agricultural and forest soils. Complete loss of 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 3-bromobenzoate, and 4-bromobenzoate was observed within 30 to 70 days in most cultures. Stable cultures were maintained by repeated feeding of these halobenzoates. 3-Fluorobenzoate was not degraded in any of the enrichment cultures. Slow utilization of 2-chlorobenzoate was only observed in the enrichment cultures from Passion Puddle, N.J., and Kyungan River, Korea, but the activity was not maintained during subsequent refeeding. In contrast to 2-chlorobenzoate, utilization of 2-bromobenzoate was observed within 60 to 90 days in about half of the enrichment cultures, and activity was maintained after refeeding.

Isolation of halobenzoate-degrading denitrifying bacteria. The halobenzoate-degrading enrichments were subcultured, and isolation of denitrifying bacteria was pursued. Using a combination of benzoate and halobenzoate as the carbon sources, a large number of isolates were obtained from the active enrichment cultures of each halobenzoate. They were all capable of degrading benzoate under denitrifying conditions, and 33 isolates out of a total of 122 isolates were able to degrade halobenzoates under denitrifying conditions. These isolates were obtained mainly from enrichment cultures of 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate, or 3-bromobenzoate. The strain designations and sources of isolates are listed in Table 1. No 2-bromobenzoate-degrading bacteria could be isolated from the 2-bromobenzoate-utilizing enrichment cultures, although a number of benzoate-degrading denitrifying bacteria were isolated (data not shown). Neither 4-chlorobenzoate- nor 4-bromobenzoate-degrading isolates were obtained from any of the enrichment cultures after repeated attempts. Further studies were focused on the 33 isolates capable of degrading 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate, and 3-bromobenzoate under denitrifying conditions.

Growth and requirement of nitrate for halobenzoate degradation. Growth on the respective halobenzoates under denitrifying conditions was verified for each strain. For example, the growth of strains 2FB3, 4FB10, and 3CB2 on different concentrations of halobenzoates is shown in Fig. 1. The doubling time of each strain was calculated from the growth curve on the respective halobenzoate (Table 1). The isolates from 2-fluorobenzoate enrichment cultures had doubling times from 11 to 26 h. These growth rates were higher than those of the other isolates growing on 4-fluorobenzoate, 3-chlorobenzoate,

TABLE 1. Geographical and ecological origins and doubling time of isolates and closely matched species by 16S rRNA sequence

Substrate	Strain	Ecological and geographical source	Doubling time (h) ^a	Closely matched species	% Similarity ^b
2-Fluorobenzoate	2FB1	Agricultural soil, Wyo.	20	<i>Pseudomonas oleovorans</i>	99.7
	2FB2	Estuarine sediment, Arthur Kill, N.J.	21	<i>Azoarcus toluolyticus</i>	99.8
	2FB3	Pond sediment, Passion Puddle, N.J.	26	<i>Bradyrhizobium elkanii</i>	98.2
	2FB4	River sediment, Kyungan, Korea	ND ^c	<i>Acidovorax avenae</i>	96.7
	2FB5	River sediment, Kyungan, Korea	11	<i>Acidovorax avenae</i>	95.0
	2FB6	Agricultural soil, Wyo.	15	<i>Azoarcus toluolyticus</i>	99.6
	2FB7	River sediment, Kyungan, Korea	21	<i>Acidovorax avenae</i>	95.4
	2FB8	River sediment, Kyungan, Korea	ND	<i>Ensifer adhaerens</i>	97.4
	2FB9	Agricultural soil, Viikki, Finland	ND	<i>Pseudomonas stutzeri</i>	97.0
	2FB10	Agricultural soil, Viikki, Finland	ND	<i>Ochrobactrum anthropi</i>	99.2
	2FB11	Estuarine sediment, Shihwa, Korea	ND	<i>Pseudomonas stutzeri</i>	97.9
4-Fluorobenzoate	4FB1	Estuarine sediment, Arthur Kill, N.J.	58	<i>Thauera aromatica</i>	99.3
	4FB2	Estuarine sediment, Arthur Kill, N.J.	35	<i>Thauera aromatica</i>	99.3
	4FB3	Estuarine sediment, Arthur Kill, N.J.	151	<i>Pseudomonas stutzeri</i>	99.0
	4FB4	Agricultural soil, Wyo.	95	<i>Pseudomonas stutzeri</i>	99.7
	4FB5	Agricultural soil, Wyo.	18	<i>Pseudomonas stutzeri</i>	99.7
	4FB6	Agricultural soil, Wyo.	58	<i>Ensifer adhaerens</i>	99.3
	4FB7	Estuarine sediment, Arthur Kill, N.J.	51	<i>Pseudomonas stutzeri</i>	98.4
	4FB8	Pond sediment, Passion Puddle, N.J.	ND	<i>Paracoccus denitrificans</i>	100.0
	4FB9	Pond sediment, Passion Puddle, N.J.	ND	<i>Ochrobactrum anthropi</i>	99.2
	4FB10	Estuarine sediment, Shihwa, Korea	36	<i>Azoarcus toluolyticus</i>	99.9
	4FB11	Marine sediment, Hopewell Rock, Canada	114	<i>Mesorhizobium loti</i>	96.9
	4FB12	River sediment, Anyang, Korea	ND	<i>Ochrobactrum anthropi</i>	98.9
	4FB13	Agricultural soil, Viikki, Finland	ND	<i>Ochrobactrum anthropi</i>	96.8
	4FB14	River sediment, Kyungan, Korea	ND	<i>Ochrobactrum anthropi</i>	98.9
3-Chlorobenzoate	3CB2	Estuarine sediment, Arthur Kill, N.J.	34	<i>Thauera aromatica</i>	99.3
	3CB3	Agricultural soil, Wyo.	37	<i>Thauera aromatica</i>	98.9
	3CB4	Estuarine sediment, Arthur Kill, N.J.	133	<i>Ochrobactrum anthropi</i>	99.2
	3CB5	Agricultural soil, Wyo.	131	<i>Ochrobactrum anthropi</i>	99.2
	3CB6	Estuarine sediment, Shihwa, Korea	117	<i>Pseudomonas stutzeri</i>	97.5
	3CB7	River sediment, Kyungan, Korea	147	<i>Pseudomonas stutzeri</i>	97.9
	3-Bromobenzoate	3BB1	Agricultural soil, Wyo.	20	<i>Thauera aromatica</i>
3BB2		Estuarine sediment, Arthur Kill, N.J.	38	<i>Pseudomonas stutzeri</i>	98.9

^a Doubling time was measured for growth on halobenzoates under denitrifying conditions.

^b The sequence similarities were measured with 600 bp of 16S rRNA gene sequences and compared with the type strains of each species by using the olddistance program in the GCG package.

^c ND, not determined.

or 3-bromobenzoate. The isolates from 4-fluorobenzoate enrichment cultures had doubling times ranging from 18 to 58 h, except for strains 4FB3, 4FB4, and 4FB11, which were over 95 h. Strains 3CB2 and 3CB3 grew faster on 3-chlorobenzoate under denitrifying conditions than the other 3-chlorobenzoate-degrading strains. Strains 3BB1 and 3BB2 growing on 3-bromobenzoate under denitrifying conditions had doubling times of 20 and 38 h, respectively.

The anaerobic degradation and growth on halobenzoate were dependent on the presence of nitrate, as shown for strains 2FB6, 4FB10, and 3CB2 (Fig. 2). Without nitrate, the isolates did not grow on halobenzoate, but they did grow after the medium was spiked with nitrate. Similar results were obtained for the other halobenzoate-degrading isolates (data not shown). In addition, tests of alternative electron acceptors (10 mM NO_3^- , NO_2^- , SO_4^{2-} , SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, SeO_4^{2-} , or SeO_3^{2-} , or in the presence of oxygen) with succinate as a carbon source showed that the isolates could grow with O_2 , NO_3^- , or NO_2^- as an electron acceptor (data not shown).

Substrate specificity. The test of substrate specificity with benzoate, hydroxybenzoates, and halobenzoates showed that all of the isolates degraded benzoate, 3-hydroxybenzoate, and 4-hydroxybenzoate under both denitrifying and aerobic condi-

tions (Table 2). Most of the strains could use 2-hydroxybenzoate under aerobic conditions, but none degraded it under denitrifying conditions. The isolates from the 2-fluorobenzoate enrichment cultures appeared to be limited in their substrate ranges, and with the exception of two strains, did not utilize other halobenzoates under either denitrifying or aerobic conditions. In contrast, most strains from the 4-fluorobenzoate enrichment cultures could use 2-fluorobenzoate and 4-fluorobenzoate under both denitrifying and aerobic conditions. Interestingly, 3-chlorobenzoate and 3-bromobenzoate degradation under denitrifying conditions was observed with two strains isolated on fluorobenzoate. The isolates from the 3-chlorobenzoate and 3-bromobenzoate enrichment cultures used 2-fluorobenzoate under both denitrifying and aerobic conditions and utilized 4-fluorobenzoate, 3-chlorobenzoate, and 3-bromobenzoate under denitrifying conditions.

Cellular fatty acid analysis. The halobenzoate-degrading strains were grown on succinate under denitrifying conditions, and cellular fatty acids were extracted and analyzed by gas chromatography. Cluster analysis grouped the 33 strains into three clusters on the basis of their fatty acid profiles (Fig. 3). Cluster I was separated from clusters II and III at a Euclidian distance of over 30, while clusters II and III diverged at a

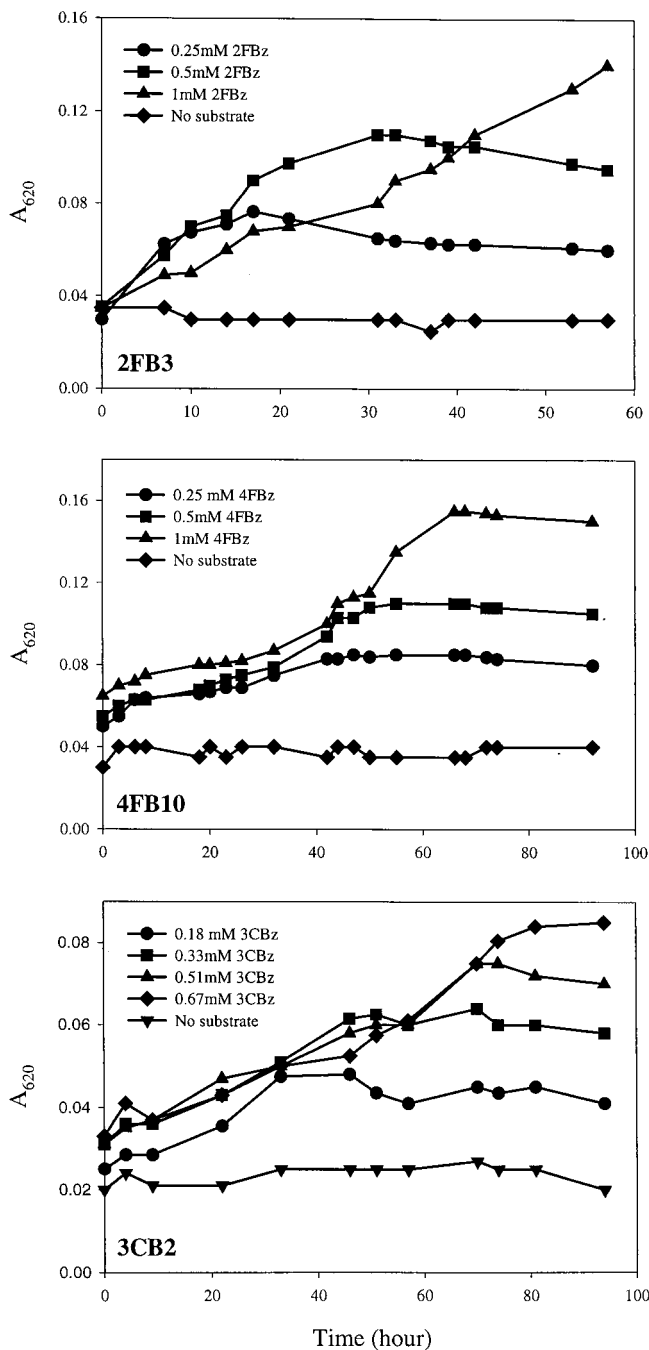


FIG. 1. Growth of strains 2FB3, 4FB10, and 3CB2 on 2-fluorobenzoate (2FBz), 4-fluorobenzoate (4FBz), and 3-chlorobenzoate (3CBz), respectively, under denitrifying conditions. Different concentrations of halobenzoate were spiked in denitrifying medium containing 10 mM nitrate.

Euclidian distance of 14. *Ochrobactrum anthropi* NCTC 12168^T and *Pseudomonas stutzeri* ATCC 17588^T, used as reference strains, were included in clusters I and II, respectively, while strains from the genera *Azoarcus* and *Thauera* were located in cluster III. There was no specific grouping of the strains based on either isolation substrate or source of inoculum. Each of these main clusters contained 2-fluorobenzoate-, 4-fluorobenzoate-, and 3-chlorobenzoate-degrading isolates.

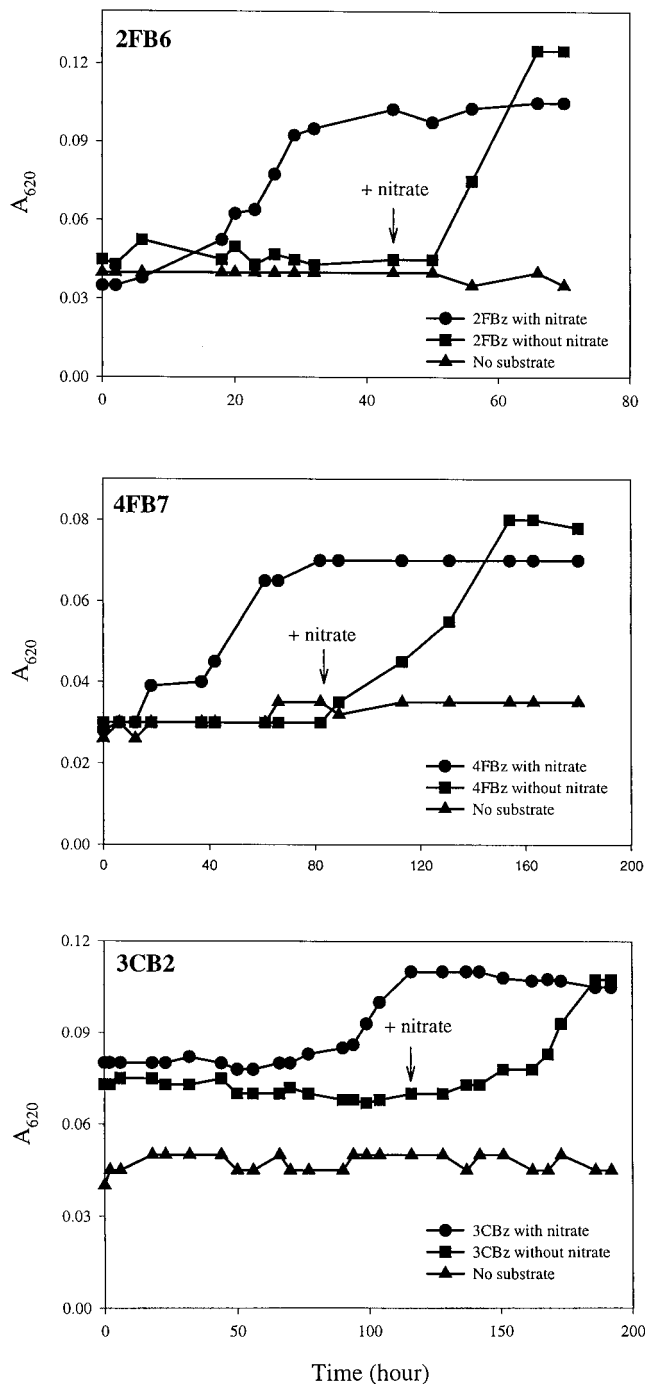


FIG. 2. Nitrate-dependent growth of strains 2FB6, 4FB7, and 3CB2 on 2-fluorobenzoate (2FBz), 4-fluorobenzoate (4FBz), and 3-chlorobenzoate (3CBz) (0.5 mM), respectively. Nitrate-free cultures were spiked with 10 mM nitrate as indicated by the arrow.

Phylogenetic analysis. Phylogenetic analysis was done on the basis of the partial 16S rRNA sequences (600 nucleotides) of all isolates. The 33 strains were grouped into three clusters (Fig. 4 and Table 1) in agreement with fatty acid analysis. The closely matched species of the isolates were searched in the GenBank database, and the percent similarities with type strains of each species were calculated by using the olddistance

TABLE 2. Substrate utilization by halobenzoate-degrading isolates under aerobic and denitrifying conditions

Strain	Substrate utilization (aerobic/denitrifying conditions) ^a												
	Bz	2OHBz	3OHBz	4OHBz	2FBz	3FBz	4FBz	2CBz	3CBz	4CBz	2BBz	3BBz	4BBz
2FB1	+/+	+/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB2	+/+	+/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB3	+/+	+/-	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB4	+/+	+/-	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB5	+/+	+/-	+/+	+/+	-/+	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB6	+/+	+/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB7	+/+	+/-	+/+	+/+	+/+	-/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-
2FB8	+/+	+/-	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
2FB9	+/+	+/-	+/+	+/+	-/+	+/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
2FB11	+/+	+/-	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
4FB1	+/+	+/-	+/+	+/+	-/+	+/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
4FB2	+/+	-/-	+/+	+/+	-/+	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
4FB3	+/+	+/-	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
4FB4	+/+	+/-	+/+	+/+	+/+	+/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
4FB5	+/+	+/-	+/+	+/+	+/-	+/-	+/+	-/-	-/+	-/-	-/-	-/+	-/-
4FB6	+/+	+/-	+/+	+/+	+/+	+/-	+/+	-/-	-/+	-/-	-/-	-/+	-/-
4FB7	+/+	+/-	+/+	+/+	-/-	+/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-
4FB10	+/+	-/-	+/+	+/+	-/+	-/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-
4FB11	+/+	+/-	+/+	+/+	+/+	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
3CB2	+/+	-/-	+/+	+/+	+/+	-/-	-/+	-/-	-/+	-/-	-/-	-/+	-/-
3CB3	+/+	+/-	+/+	+/+	+/+	+/-	-/+	-/-	-/+	-/-	-/-	-/+	-/-
3CB4	+/+	+/-	+/+	+/+	+/+	+/-	-/+	-/-	-/+	-/-	-/-	-/+	-/-
3CB5	+/+	+/-	+/+	+/+	+/+	-/-	-/+	-/-	-/+	-/-	-/-	-/+	-/-
3CB6	+/+	+/-	+/+	+/+	+/+	-/-	+/+	-/-	-/+	-/-	-/-	-/+	-/-
3CB7	+/+	+/-	+/+	+/+	+/+	-/-	+/+	-/-	-/+	-/-	-/-	-/+	-/-
3BB1	+/+	+/-	+/+	+/+	+/+	-/-	-/+	-/-	-/+	-/-	-/-	-/+	-/-
3BB2	+/+	+/-	+/+	+/+	+/+	-/-	-/+	-/-	-/+	-/-	-/-	-/+	-/-

^a +, complete loss of substrate; -, less than 10% loss of substrate. Substrate loss was determined by HPLC after incubation for 14 days. Bz, benzoate; OHBz, hydroxybenzoate; FBz, fluorobenzoate; CBz, chlorobenzoate; BBz, bromobenzoate.

program in the GCG package (Table 1). The strains in cluster I belonged to the genera *Paracoccus*, *Mesorhizobium*, *Ensifer*, *Ochrobactrum*, and *Bradyrhizobium* in the α -subunit of the *Proteobacteria*. The strains in cluster II were confirmed to belong to the genus *Pseudomonas* in the γ -subunit of *Proteobacteria*, which was also supported by fatty acid analysis. Strains in cluster III were identified as belonging to the genera *Azoarcus*, *Acidovorax*, and *Thauera* in the β -subunit of the *Proteobacteria*.

The data show that the halobenzoate-degrading strains are widely distributed in the *Proteobacteria*. 2-Fluorobenzoate-degrading isolates were widely assigned to the α -, β -, and γ -branches in the genera *Ochrobactrum*, *Bradyrhizobium*, *Acidovorax*, *Azoarcus*, and *Pseudomonas*, while the isolates from the 4-fluorobenzoate enrichment cultures were members of the genera *Paracoccus*, *Ensifer*, *Mesorhizobium*, *Ochrobactrum*, *Thauera*, *Azoarcus*, and *Pseudomonas*. The isolates obtained from 3-chlorobenzoate or 3-bromobenzoate enrichment cultures were placed in the genera *Ochrobactrum*, *Thauera*, and *Pseudomonas*.

DISCUSSION

A total of 33 isolates capable of degrading halobenzoates under denitrifying conditions were obtained from enrichment cultures utilizing 2-fluorobenzoate, 4-fluorobenzoate, 3-chlorobenzoate, and 3-bromobenzoate. The results of our experiments show that organisms able to degrade halobenzoates under denitrifying conditions can be isolated from enrichment cultures, and, regardless of the halogenated substrate used in each enrichment, they belong to different branches of the *Pro-*

teobacteria. With regard to the geographic and ecological characteristics of the sample sources, the results also indicate the widespread occurrence of these organisms. For example, the strains of the genera *Ochrobactrum* and *Pseudomonas* had diverse origins as well as halobenzoate-degrading capacities. In addition, strains of the genus *Thauera* were obtained from the estuarine sediment of Arthur Kill and field soil from Wyoming by using 4-fluorobenzoate, 3-chlorobenzoate, or 3-bromobenzoate, and strains of the genus *Azoarcus* capable of degradation of fluorobenzoates under denitrifying conditions were obtained from the estuarine sediments of Arthur Kill and Shihwa Lake (Table 1). Therefore, the diversity of species and various halobenzoate degradation patterns were independent of the geographical and ecological sources of the isolates.

Although 4-chlorobenzoate and 4-bromobenzoate were readily degraded in enrichment cultures with almost all inocula tested, repeated attempts did not result in isolation of pure cultures capable of degrading either 4-chlorobenzoate or 4-bromobenzoate. Previously, anaerobic transformation of 4-chlorobenzoate was demonstrated with pure cultures in the presence of nitrate. The coryneform bacterium strain NTB-1 was capable of converting 4-chlorobenzoate to 4-hydroxybenzoate under strict anaerobic conditions in the presence of nitrate, although hydroxybenzoate was not degraded further in the absence of oxygen (12, 36). *Thauera aromatica* strain T1, which is capable of degrading toluene and 4-hydroxybenzoate under denitrifying conditions, could use 4-chlorobenzoate as a carbon source under denitrifying conditions after insertion of a cosmid carrying the 4-chlorobenzoate dehalogenase genes from *Pseudomonas* sp. strain CBS3 (1). Although these pure

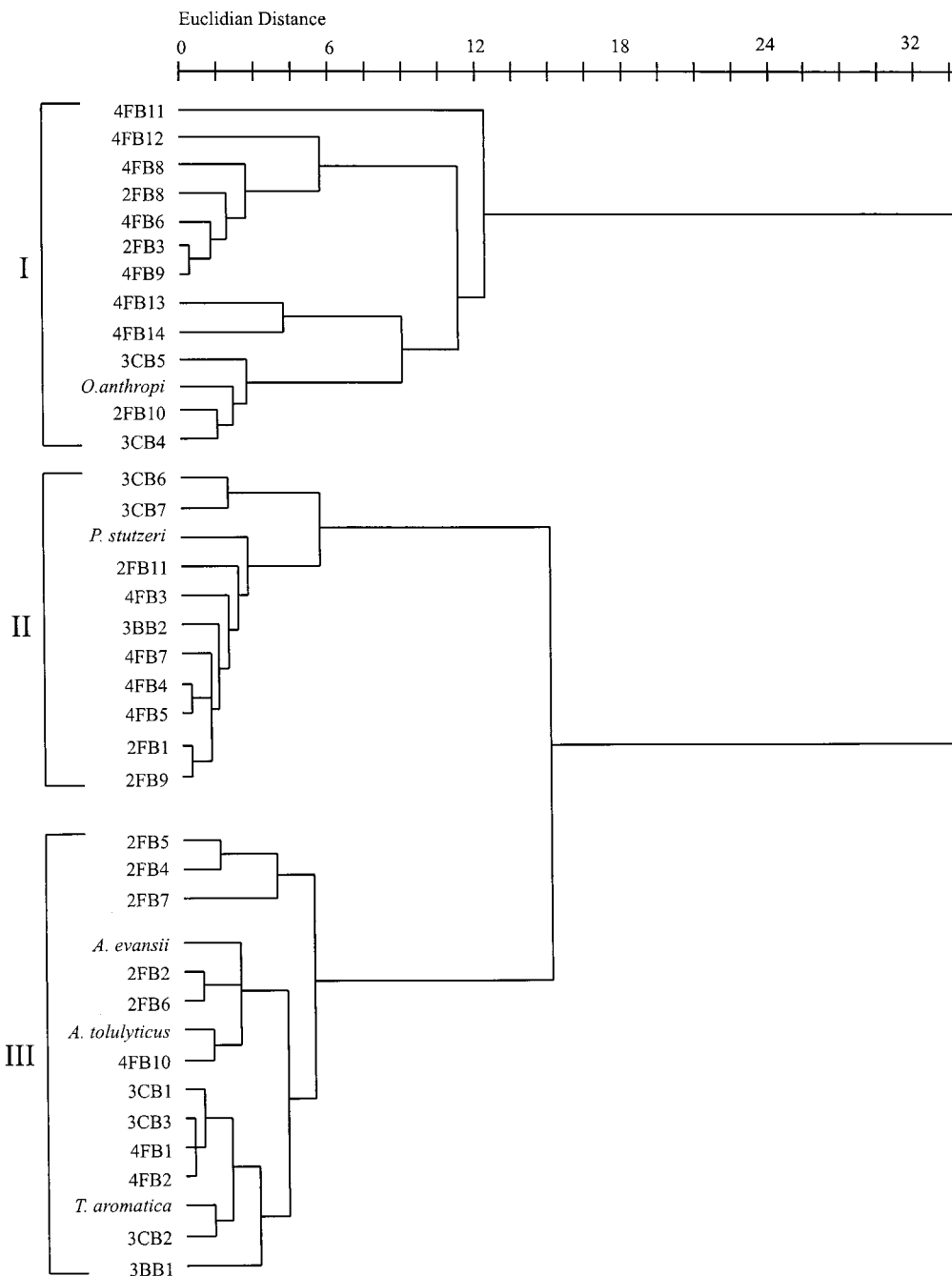


FIG. 3. Dendrogram of halobenzoate-degrading isolates on the basis of their cellular fatty acid profiles. Isolates were grown with 10 mM succinate and 30 mM nitrate for 24 h in the atmosphere of argon.

culture studies indicate the possibility of obtaining isolates from the 4-chlorobenzoate-utilizing enrichment cultures, the degradation of 4-chlorobenzoate may be carried out by the combined activity of the consortia, or the halobenzoate-degrading bacteria may require specific nutrients provided by other members of the consortium. However, it is not clear why the nutritional requirements of the 4-chlorobenzoate- and 4-bromobenzoate-degrading bacteria would be substantially different from those of the isolates obtained on the other halobenzoates.

Halobenzoate degradation under denitrifying conditions was

affected by both the halogen substituents and the substitution position. 2-Chlorobenzoate and 3-fluorobenzoate were not degraded by either enrichment cultures or pure cultures, although 3-chlorobenzoate, 3-bromobenzoate, 2-fluorobenzoate, and 4-fluorobenzoate were readily degraded. The negative results could be due to the regioselectivities of enzymes capable of degrading halobenzoates or to the toxicities of these compounds. In fact, inhibition of 2-fluorobenzoate degradation was demonstrated in the presence of 3-fluorobenzoate under denitrifying conditions, although benzoate utilization was not affected (34). The recalcitrance of 2-chlorobenzoate and 2-bro-

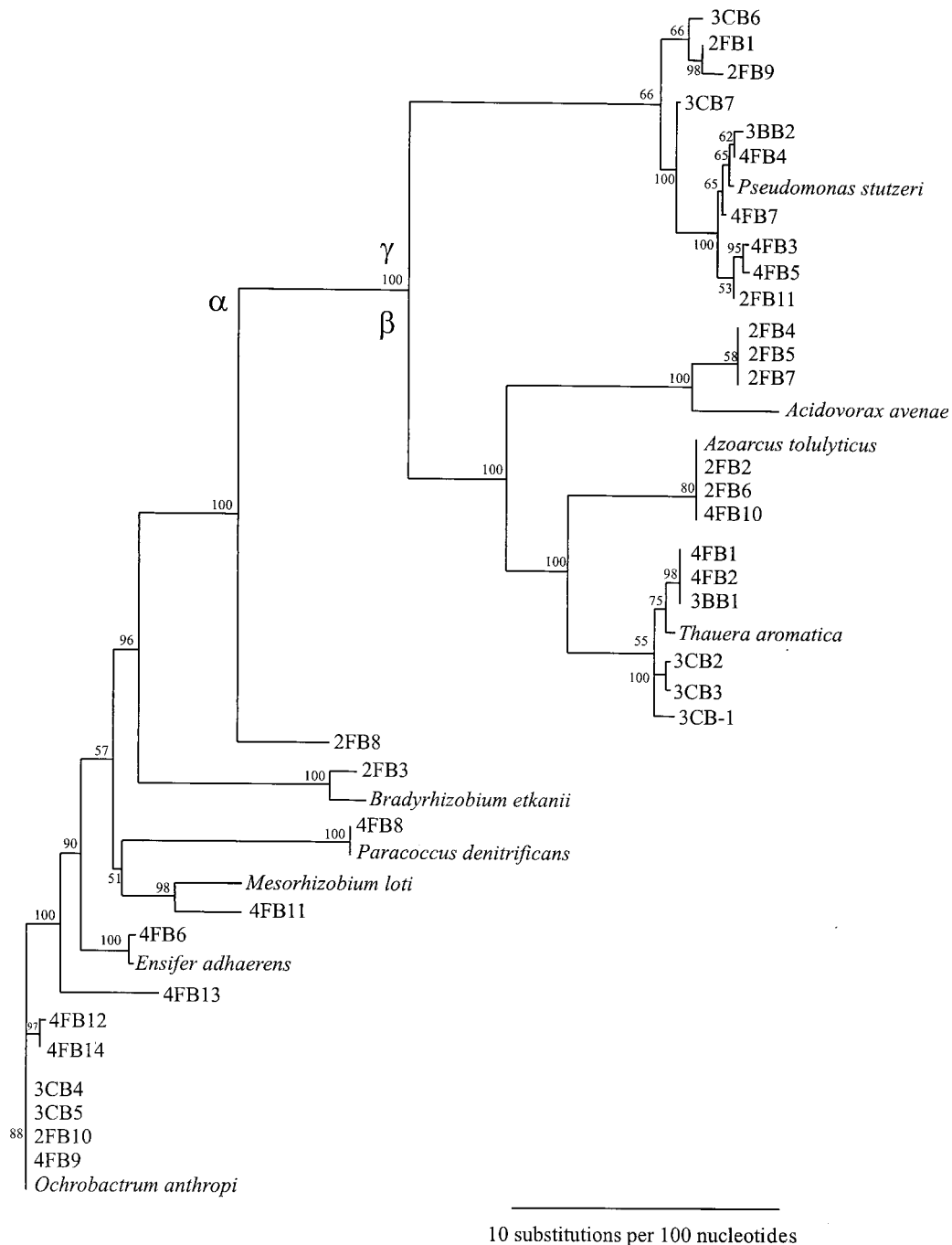


FIG. 4. Phylogenetic relationship of halobenzoate-degrading isolates. The tree was constructed with 600 bases of the 16S rRNA gene by using a neighbor-joining tree and a 100 bootstrap for the confidence level.

mobenzoate under denitrifying conditions is in agreement with previous enrichment culture studies (15, 16), although initial loss of 2-bromobenzoate was observed in some of the enrichment cultures. However, the activity of these 2-bromobenzoate-utilizing cultures was difficult to maintain during subculturing (unpublished results).

Selective enrichment followed by isolation yielded diverse halobenzoate-degrading denitrifying bacteria; nonetheless, enrichment may limit and bias the diversity of organisms obtained. Previous studies of the isolation of aerobic benzoate or

2,4-dichlorophenoxyacetate-degrading bacteria after selective enrichment showed that the numbers of isolates were limited and the isolates formed restricted groups (3, 4). Similar results were also seen with toluene-degrading denitrifiers isolated from various geographical locations (8, 33). The isolates were tightly grouped together, regardless of their geographical and ecological origins, and assigned to the genus *Azoarcus* (37). Later, these strains were differentiated into three closely related species: *Azoarcus toluityticus*, *Azoarcus toluvorans*, and *Azoarcus toluclasticus* (27). A study of phenol-degrading deni-

trifiers isolated from different geographical sources also showed a tight grouping of isolates in the genus *Azoarcus* (33). However, we isolated diverse groups of bacteria after enrichment in spite of the selective conditions represented by halobenzoate as a substrate and nitrate as an electron acceptor. For instance, four 4-fluorobenzoate-degrading isolates obtained from the same enrichment of Arthur Kill sediment (Table 1) were identified as either *Thauera aromatica* or *Pseudomonas stutzeri*, and these strains also had different growth rates. 3-Chlorobenzoate-degrading isolates obtained from a Wyoming soil sample were assigned to either *Thauera aromatica* or *Ochrobactrum anthropi* as well.

The isolation of bacteria from enrichment cultures could impose limits on the study of microbial diversity, because enrichment cultures create artificial conditions, which may not be favorable to some members of the microbiota. However, many strains of bacteria have been isolated after enrichments that increased the population and stimulated the slow-growing organisms. This is particularly true of microorganisms capable of degrading recalcitrant compounds, and enrichment cultivation is still important for the study of microbial populations with respect to taxonomic and functional diversity.

Halobenzoates are used as substrates by various bacteria under aerobic conditions (13). A considerable taxonomic diversity of aerobic bacteria capable of degrading halobenzoates was observed from the isolates from contaminated sites as well as from pristine environments (9). We have shown that under denitrifying conditions, halobenzoates were utilized by strains belonging to various branches of the *Proteobacteria*, and the microbial populations with this biochemical capability appear to be ubiquitously distributed in the environment. Thus, the taxonomic diversity of halobenzoate-degrading denitrifying bacteria has been addressed by traditional enrichment and isolation techniques in this study, although the limitation of enrichment is still present. Further studies of these isolates will unveil the functional diversity of halobenzoate degradation under denitrifying conditions.

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