Isolation and Initial Characterization of a Bacterial Consortium Able To Mineralize Fluorobenzene

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Fluorinated compounds are known to be more resistant to microbial degradation than other halogenated chemicals. A microbial consortium capable of aerobic biodegradation of fluorobenzene (FB) as the sole source of carbon and energy was isolated by selective enrichment from sediments collected in a drain near an industrial site. A combination of three microbial strains recovered from the enriched consortium was shown to be necessary for complete FB mineralization. Two of the strains (F1 and F3) were classified by 16S rRNA analysis as belonging to the *Sphingobacterium*/*Flavobacterium* group, while the third (F4) falls in the β-*Proteobacteria* group, clustering with *Alcaligenes* species. Strain F4 was consistently found in the liquid cultures in a much greater proportion than strains F1 and F3 (86:8:6 for F4, F1, and F3, respectively). Stoichiometric release of fluoride ions was measured in batch and fed-batch cultures. In batch cultures, the consortium was able to use FB up to concentrations of 400 mg liter^{-1} and was able to utilize a range of other organic compounds, including 4-fluorophenol and 4-fluorobenzoate. To our knowledge this is the first time biodegradation of FB as a sole carbon source has been reported.

The advances in organic synthesis have led to the introduction of numerous new organic compounds into the environment, whose susceptibilities to biotreatment processes are unknown. Fluoroaromatics are being increasingly used in a wide range of agrochemical and pharmaceutical products, due to the need to find environmentally acceptable alternatives to chlorinated compounds (17). The diversity of structures and the chemical inertness of many halogenated organics pose particular problems and challenges for microbial degradation (10). Some authors propose that the recalcitrance of a halogenated organic compound usually becomes greater with the increase of the electronegativity of the substituents; thus, the recalcitrance of F-C is greater than that of Cl-C, Br-C, and I-C (9).

The biodegradation of a vast range of halogenated aromatic compounds, especially chlorinated compounds, has been described (13, 22), but scant information is available on the metabolic and cometabolic fate of fluorinated aromatic compounds in bacteria. Examples of biodegradation of fluorinated compounds most commonly found in the literature involve fluorobenzoic acids (7, 15, 20, 21, 24) and fluorophenols (1, 2, 23). Although degradation under aerobic conditions is usually reported, anaerobic degradation of fluorobenzoates under denitrifying conditions has also been reported (26). The existence of various metabolic pathways, some of which may lead to the formation of inhibitor metabolites, has been reported (15, 24, 25). In some cases, as in the degradation of fluoroacetate, a specific enzyme is responsible for the cleavage of the C-F bond (12). Studies on the metabolism of 2-flurobenzoate have shown that cleavage of the C-F bond occurs incidentally during oxygenase attack on the aromatic ring (20). It has been reported that biodegradation of fluorophenol occurs through a phenol hydroxylase (2). Some authors suggested that 3-fluorobenzoate, 4-fluorobenzoate, and difluorobenzoate are metabolized via 4-fluorocatechol (4, 5, 24). The oxidation of fluorobenzene to produce fluorocatechol by a *Pseudomonas putida* strain growing on fructose-containing medium has been described (19).

To our knowledge, growth of bacteria on fluorobenzene (FB) as the sole source of carbon has not yet been reported. This paper describes the enrichment, isolation, and characterization of a microbial consortium capable of using fluorobenzene as the sole source of carbon and energy.

MATERIALS AND METHODS

Enrichment of FB degraders. A variety of soil and rhizosphere samples collected from a contaminated drain in northern Portugal, which has received the discharge of chemical industry effluents for more than 50 years (fine chemistry, agrochemicals), were combined as the initial inoculum for the FB enrichments. Rhizosphere soil (approximately 5 g) was used to inoculate 250-ml flasks containing 50 ml of sterile minimal salts medium (3) and FB, supplied in the liquid culture as the sole carbon and energy source, at a concentration of 50 mg liter^{-1}. Cultures were incubated on a rotary shaker (100 rpm) at 25°C. Half of the suspension was removed and replaced with fresh medium at 6- to 7-day intervals. Growth was monitored by measuring the optical density at 600 nm, and liberation of fluoride was monitored using an ion-selective electrode. When growth on FB was established, samples of the culture were periodically spread onto minimal salts agar plates and onto nutrient agar (NA) plates and were incubated in a sealed jar containing FB in the vapor phase.

Characterization of FB-degrading bacterial consortium. Bacterial strains consistently recovered from the degrading culture were purified by repetitive streaking onto NA agar medium. Isolates and combinations of the isolates were reinoculated into minimal salts liquid medium containing FB. Growth and liberation of fluoride were monitored. When a positive response was obtained, samples of the culture were spread onto NA agar medium to verify the proportion of each of the strains, and the procedure was repeated for several transfers. Strains involved in FB degradation were analyzed further by 16S rRNA gene characterization. The 16S rRNA genes of the three strains were amplified by PCR using the primer set f27 and r1492 (18) under standard PCR conditions (30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C) with *Taq* DNA polymerase

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(Promega). The template DNA was obtained by boiling washed cell suspensions for 5 min and using 1 to 2 μl of the supernatant. The amplified fragments were cloned into the pGEM T-Easy vector (Promega) and sequenced by Alta Bioscience, University of Birmingham, Birmingham, United Kingdom (*Taq* DyeDeoxy Terminator Cycle Sequencing and Model 373A gel apparatus; Applied Biosystems) using 16S-specific primers f27 and r519 (18). The 16S rRNA gene sequences were aligned using the BioEdit program (version 4.8.8) (14) and analyzed using the programs SEQBOOT (100 iterations), DNADIST (Kimura 2-parameter), NEIGHBOR, DNAPARS, and CONSENSE of the PHYLIP package (8). 16S rRNA sequences were obtained from the National Center for Biotechnology Information taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy). An alignment of 34 sequences by 670 nucleotides was used.

Analytical methods. Biodegradation was detected through the measurement of the fluoride ions in the culture supernatant using an ion-selective electrode (model CH-8902; Mettler-Toledo GmbH, Urdorf, Switzerland), after centrifuging the bacterial culture for 5 min. A calibration curve was prepared by using freshly prepared standard solutions of sodium fluoride in minimal salts medium. When required, the chloride concentration was determined as described previously (3).

Chemicals. All chemicals were of the highest purity grade available (Sigma-Aldrich Chemie, Steinheim, Germany; Difco Laboratories, Detroit, Mich.; Merck, Darmstadt, Germany).

Nucleotide sequence accession numbers. The 16S rRNA sequences of strains F1, F3, and F4 have been deposited in GenBank under accession no. AF38016159, AF380160, and AF380161, respectively.

RESULTS AND DISCUSSION

Isolation of FB-degrading consortium. A microbial consortium capable of using FB was isolated after 4 months of selective enrichment by repeated subcultures. The consortium was able to grow on and degrade FB when the compound was supplied as the sole source of carbon and energy. Biodegradation was detected through the measurement of the fluoride ion liberation, and in control experiments, with no bacterial inoculum, no fluoride release was observed. When samples of the enrichment culture were plated onto nutrient agar plates and incubated in a sealed jar containing FB in the vapor phase, five distinct colony types were initially revealed, whereas colonies obtained in minimal medium agar plates under the same conditions were very small and undistinguishable. Subsequent plating of the repeated subcultures of the degrading consortium in liquid medium revealed that only three of the observed colonial types were consistently recovered. Inoculation of minimal salts medium containing FB with a combination of those three strains, after purification on agar plates, led to bacterial growth and FB degradation. However, inoculation with isolates singly or in two-strain combinations failed to produce a culture capable of growth. This may be due to biodegradation being performed in a cooperative fashion by the three bacteria. When bacterial strains were grown individually in minimal medium supplemented with glycerol and FB, growth was readily obtained, but fluoride release did not occur, even when, after growth on glycerol, medium was replaced with fresh minimal salts medium in the presence of FB as the sole source of carbon. However, we may speculate that fluoride-containing aromatic intermediates were formed as a first step in the biotransformation of FB by single strains.

Growth of the three-member degrading consortium in batch and fed-batch cultures. A typical growth curve of the consortium in batch suspension cultures supplied with FB at 50 mg liter^{-1} is shown in Fig. 1. Liberation of fluoride was observed from the beginning of the experiment, and based on that ca. 75% of the supplied FB was degraded. The frequent sampling of the shake-capped flasks during the experiment may have

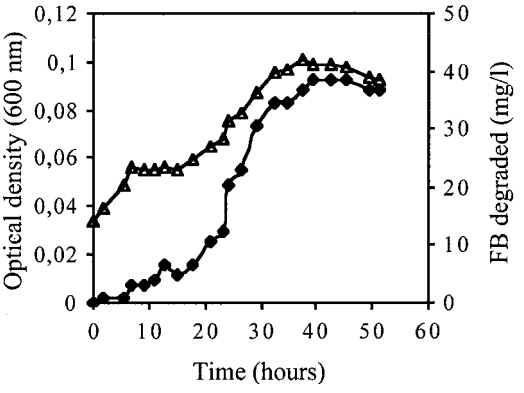

FIG. 1. Biodegradation of FB by the consortium during a batch culture at 50 mg liter^{-1}. Optical density (△) and FB degradation based on halide release (♦) are indicated.

contributed to loss of FB through volatilization, thus explaining yields lower than 100%. Biodegradation experiments performed in sealed flasks showed stoichiometric release of fluoride for FB concentrations in the range of 50 to 250 mg liter^{-1}, corresponding to 0.5 to 2.7 mM FB, after incubation periods of 72 h (Fig. 2). There was also a linear increment of final biomass achieved with increasing amounts of FB (data not shown). Growth on FB occurred at higher concentrations, although growth was visibly slower, and after an extended incubation period of 192 h, at concentrations of 400, 600, and 800 mg liter^{-1}, the amount of fluoride released corresponded to 70, 45, and 7%, respectively, of the theoretically possible. A fed-batch culture was established in order to verify the cumulative utilization of FB by the microbial consortium. Successive additions of FB were made, in amounts required to supply the culture with 50 mg liter^{-1} at each feeding stage (ca. 30-h intervals). Based on fluoride release, the FB added to the fed-batch culture was used by the microbial consortium (Fig. 3). Formation of bacterial aggregates was sometimes noticed, which may explain the fact that fluoride release was not always concomitant with a rise of optical density at 600 nm (Fig. 3). The results suggest that there is no formation of dead-end products during FB metabolism by the three bacterial strains. Lynch et al. (19) have shown that the oxidation of FB during

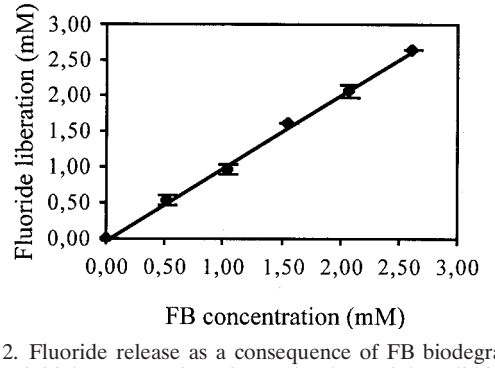

FIG. 2. Fluoride release as a consequence of FB biodegradation, versus the initial concentration of FB. The slope of the solid intercept line (slope ≅ 1.0 mM F^-/mM FB) evidences the stoichiometric release of fluoride.

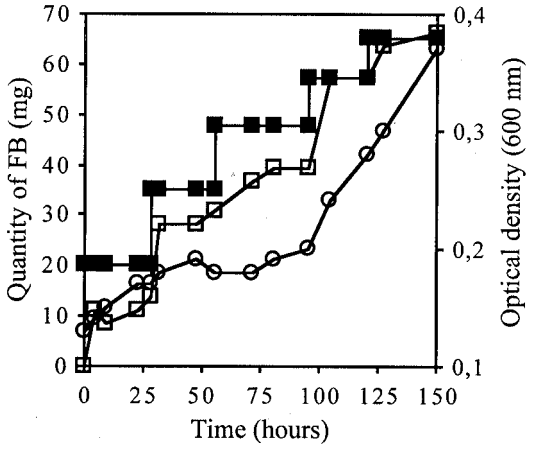

FIG. 3. Biodegradation of FB during a fed-batch culture. Cumulative supply of FB (■), cumulative FB degraded based on fluoride release (□), and optical density (○) are indicated.

growth of *Pseudomonas putida* on a rich medium led to the accumulation of fluorocatechol, which inhibited growth of that strain at concentrations higher than 1,000 mg liter^{-1}.

Catabolic activities of the degrading consortium and constituting strains. A range of organic compounds were tested as growth substrates for the degrading consortium. FB-grown cultures were inoculated into mineral salts medium containing aromatic and aliphatic compounds, and growth and halide release after 72 h of incubation were measured (Table 1). The consortium was able to grow on benzoate, benzene, phenol, indole, and catechol and was able to completely dehalogenate

TABLE 1. Growth of the degrading consortium on various substrates supplied at 50 mg liter^{-1} in liquid cultures and correspondent halide release

Substrate	Growth[a]	% Halide release
Benzene	+ (0.47)	
Benzoate	+ (0.45)	
Phenol	+ (1.47)	
Catechol	+ (1.23)	
Indole	+ (0.80)	
3-Chloro-1-propanol	+ (0.40)	74
1,3-Dichloro-2-propanol	−	0
Fluorobenzene	+ (1.29)	100
Chlorobenzene	−	0
Iodobenzene	−	ND[c]
Bromobenzene	−	ND
4-Fluorophenol	+ (0.67)	100
4-Chlorophenol	+ (0.70)	87
4-Nitrophenol	+ (0.46)[b]	
4-Fluorobenzoate	+ (0.32)	58
4-Chlorobenzoate	−	0
3-Chloro-4-fluoroaniline	+ (0.25)	57[d]
2-Fluorobenzoate	−	21

[a] The values in parenthesis indicate the ratio of biomass increase related to the initial amount of biomass, measured as the optical density at 600 nm.

[b] Based on disappearance of the yellow color of the culture supernatant at 450 nm, all of the compound was utilized.

[c] ND, not determined.

[d] Based only on chloride, no fluoride release was found. Inoculum was supplied to an initial optical density of approximately 0.04, and the halide release was determined as the percentage of total halide content on each of the substrates. The data are the means of two independent measurements.

TABLE 2. Utilization of carbon sources by single strains on solid media[a]

Substrate	Growth of strains in solid medium			
	F1	F3	F4	Consortium
Catechol	+	+	+	++
Indole	−	−	+	++
Benzene	+	−	+	+
Benzoate	++	−	−	++
Phenol	−	−	+	++
3-Chloro-1-propanol	+	−	−	++
4-Fluorobenzoate	−	−	−	++
3-Chloro-4-fluoroaniline	−	−	−	−
4-Nitrophenol	−	−	−	++
4-Fluorophenol	−	−	−	++
4-Chlorophenol	−	−	−	++

[a] Symbols: ++, significant growth; +, sparse growth; −, no growth.

fluorobenzene and 4-fluorophenol and completely remove nitrophenol from the cultures. There was significant activity on 4-chlorophenol, 4-fluorobenzoate, and 3-chloro-1-propanol and a lower level of activity on 2-fluorobenzoate. The consortium did not grow on the FB analogues chlorobenzene, iodobenzene, or bromobenzene or on the chlorinated substrates 1,3-dichloro-2-propanol and 4-chlorobenzoate. Although growth was not significant with 3-chloro-4-fluoroaniline, a significant amount of chloride was liberated, but fluoride was not detected in culture supernatants. When growth was visible by an increase in optical density within 72 to 96 h, the cultures were fed again with the corresponding substrate in order to confirm its utilization. Subsequently, each of the three bacterial strains was tested for growth as single strain in minimal salts solid medium supplied with compounds previously shown to be utilized by the consortium (Table 2). Bacterial strain F3 did not show utilization of any of the tested substrates on their own, except for catechol, whereas strains F1 and F4 were able to utilize more of the substrates, although none of the fluorinated compounds.

Microbiological characterization. In previous studies, bacteria reported to degrade fluorobenzoic acids included mainly strains of *Alcaligenes* spp. and *Aureobacterium* spp. (21) and *Pseudomonas* sp. (6, 15, 25). Various *Rhodococcus* spp. have been shown to degrade fluorophenol (2). In this study, the consortium after growth on FB consistently presented F4 in much higher proportions than F1 and F3, typically 86:8:6% for F4, F1, and F3, respectively (from six independent experiments, with standard deviations of 8, 4, and 5%, respectively). The 16S ribosomal DNA sequence analysis suggested that the predominant one of the three strains (F4) falls in the β-*Proteobacteria* group, clustering with *Alcaligenes* species and showing 95% identity with *Denitrobacter permanens* (GenBank accession no. Y12639), a denitrifier isolated from an activated-sludge system (unpublished data). The other two strains (F1 and F3) belong to the *Flavobacteriaceae*, within the CFB (*Cytophaga-Flexibacter-Bacteroides*) group. Strain F1 clustered together with *Sphingobacterium multivorum* (accession no. AB020205; unpublished data), *Sphingobacterium talpophilum* (accession no. D14020 and X67851; unpublished data) and an unclassified "benzene-decomposing bacterium S21" (accession no. AJ279491; unpublished data), showing 97% identity to *S.*

multivorum. Strain F3 clustered within a rather solid group of *Chryseobacterium*/*Flavobacterium* species, showing highest sequence identity (93%) with *Chryseobacterium* sp. FR2 (accession no. AF217562; unpublished data), but did not closely associate with any of the organisms included in this study, suggesting that F3 is the representative of a new species within the *Flavobacteriaceae*.

The predominance of F4 in the degrading consortia suggests that this strain may have a prevalent role in the process. F4 groups with the genus *Alcaligenes*: as a matter of fact, strains belonging to this genus or related ones are frequently isolated as chloroaromatic degraders (5, 11, 16), and degradation of fluorobenzoic acids by *Alcaligenes* sp. has been also reported (21). All three strains were able to grow on catechol, strain F4 grew as a pure culture on benzene and phenol, and strain F1 was able to utilize benzoate and benzene. The data suggest that the three strains can be involved in the degradation of FB and its breakdown products. The exact degradation pathway and the role of each of the strains are still unknown and are being investigated. Nevertheless, the relevance of this consortium as the first known example capable of degrading FB is evident.

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