Isolation and properties of a pure bacterial strain capable of fluorobenzene degradation as sole carbon and energy source

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Summary

A pure bacterial strain capable of aerobic biodegradation of fluorobenzene (FB) as the sole carbon and energy source was isolated by selective enrichment from sediments collected from a polluted site. 16S rRNA and fatty acid analyses support that strain F11 belongs to a novel genus within the α-2 subgroup of the Proteobacteria, possibly within a new clade related to the order *Rhizobiales*. In batch cultures, growth of strain F11 on FB led to stoichiometric release of fluoride ion. Maximum experimental growth rate of 0.04 h^{-1} was obtained at FB concentration of 0.4 mM. Growth kinetics were described by the Luong model. An inhibitory effect with increasing FB concentrations was observed, with no growth occurring at concentrations higher than 3.9 mM. Strain F11 was shown to be able to use a range of other organic compounds, including other fluorinated compounds such as 2-fluorobenzoate, 4-fluorobenzoate and 4-fluorophenol. To our knowledge, this is the first time biodegradation of FB, as the sole carbon and energy source, by a pure bacterium has been reported.

Introduction

Haloaromatic compounds have been produced industrially on a large scale for several decades, becoming common environmental pollutants of soil, water and air. Fluorinated compounds are among these, because of their useful properties, which make them suitable for a wide range of applications, including aerosol propellants, surfactants, refrigerants, plastics, anaesthetics, pesticides, plant growth regulators, medicines, adhesives and fire retardants (Key *et al*., 1997). The recalcitrance of these compounds is usually related to the number and location of the fluorine substituents in the molecule. In addition, the high electronegativity of fluorine confers a strong polarity to the carbon-fluorine bond. This bond has also one of the highest bond energies in nature (Key *et al*., 1997), strongly contributing to the high stability of the fluorinated molecules. There is still scant information on the biodegradation of fluoroaromatics, when compared with chloroaromatics. The most common examples found in the literature include fluorobenzoic acids (Engesser and Schult, 1989; Oltmanns *et al*., 1989; Schlömann *et al*., 1990; Song *et al*., 2000; Vargas *et al*., 2000) and fluorophenols (Reinscheid *et al*., 1998; Wunderwald *et al*., 1998; Vargas *et al*., 2000). In general, fluoraromatic compounds are biodegraded under aerobic conditions, although anaerobic degradation has also been reported (Song *et al*., 2000; Vargas *et al*., 2000). Among fluorinated compounds, fluorobenzene (FB) has been an object of less attention and few studies are available on its biodegradation. Fluorobenzene is mainly used as a solvent in the pharmaceutical industry, as an insecticide and as a reagent for plastic and resin polymers production. Its physical-chemical properties and environmental fate characteristics indicate that FB may be persistent, essentially, in air (http://www.epa.gov/chemrtk/flurbenz/c14602tp.pdf). The degradation of FB by a microbial consortium has been only reported recently (Carvalho *et al*., 2002), despite the numerous studies on the biodegradation of its chlorinated analogue (Reineke and Knackmuss, 1984; Mars *et al*., 1997; Rapp and Timmis, 1999). The conversion of FB to fluorocatechol by a *Pseudomonas putida* strain growing in fructose-containing medium has also been described (Lynch *et al*., 1997). However, to our

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knowledge, utilization of FB as the single source of carbon and energy by a single strain bacterium has not been yet reported. This work describes the isolation and characterisation of a pure bacterial culture that can utilize FB as a single source of carbon for its growth.

Results and discussion

Enrichment and isolation of a FB-degrading pure culture

A sediment sample collected at a polluted site in northern Portugal, was inoculated into a FB-containing mineral medium. After a few months of selective enrichment, a consortium was obtained which was able to grow in batch culture with FB as the sole carbon and energy source, as indicated by fluoride release and disappearance of the parent compound. Pure strains were obtained by streaking the culture onto Nutrient Agar (LAB M) plates. Isolates were re-inoculated into minimal salts liquid medium (MM) (Caldeira *et al*., 1999) containing FB 0.5 mM as the carbon source, and cultures were monitored for growth and fluoride liberation. When a positive result was obtained, samples of the culture were spread onto Nutrient Agar medium to verify the purity of the culture. From this process a single strain, referred in this study as strain F11, capable of FB degradation was obtained.

Classification of strain F11

Strain F11 was classified by 16S rRNA gene sequence analysis and by its guanosine + cytosine and fatty acids contents.

From BLAST searches and subsequent phylogenetic analysis (Fig. 1), it became evident that the rRNA sequence of F11 clusters with that (Acc. N° AF008564) of an unpublished bacterium (strain FH6) isolated from a crude-oil degrading enrichment by Bost F.D. and Morris P.J., Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, USA. In our phylogenetic analysis, all algorithms used resulted in strain F11 and FH6, forming an unequivocal cluster supported by the highest bootstrap value (100%). An association between this cluster and *Rhodobium orientis* was often found, but with low bootstrap values (29–44%).

The guanosine + cytosine content (mol% G + C) of strain F11 genomic DNA was determined by the high-performance liquid chromatography technique described by Mesbah and colleagues (1989) at the BCCM/LMG Culture Collection Laboratories, University of Gent, Belgium and revealed to be 62.9%.

Fatty acids analysis was performed at DSMZ, Braunschweig, Germany (http://www.dsmz.de) by gas-chromatography. The main fatty acids extracted from strain F11 were 16:0, 18:1 ω7c and 19:0 cyclo ω8c. This profile does not match closely with that of any described bacterium present in the databases, but the combination of the principal fatty acids together with the presence of 3-hydroxy fatty acids (14:0 3-OH, 16:0 3-OH and 18:0 3-OH) is diagnostic for the *Rhizobiales*, which fits with the 16S rRNA sequence analysis. These results support that strains F11 and FH6, sharing 95% overall identity at the 16S rRNA level, could be members of a novel genus within the α-2 subgroup of the Proteobacteria. Unfortunately, no further information is available on strain FH6 to allow any deeper comparison with strain F11.

Monitoring FB degradation in liquid phase

Degradation of FB by strain F11 was tested in batch suspension cultures. A typical growth curve is shown in Fig. 2. The initial concentration of FB, analysed in the liquid phase, was 0.6 mM and not the 1 mM actually fed to the culture. This is related with Henry's partition coefficient of FB, which determines the distribution of this volatile compound between the gas and the liquid phases. The total amount of FB initially fed to the culture (1 mM) was completely defluorinated and a stoichiometric liberation of fluoride was observed from the beginning of the experiment. A concomitant increment of biomass was also observed. Similar results were obtained for the FB-degrading consortium from which strain F11 was isolated (data not shown).

Growth kinetics on FB

F11 growth kinetics was characterized using a batch method (Fig. 3). Control cultures were set up for each FB concentration in order to quantify for physical losses, which consisted of sterile flasks containing only FB and MM. In these cultures liberation of fluoride ion and degradation of FB were not observed. The highest growth rate observed for strain F11 (0.04 h^{-1}) was obtained when the FB concentration was 0.4 mM. An inhibitory effect with increasing FB concentrations was observed, and no growth was found for concentrations higher than 3.9 mM. This fact may be attributed to the possible negative effect that FB exerts on the microbial cell membrane because of its highly lipophilic character. Generally, the toxicity of an organic solvent correlates with its hydrophobicity, expressed by the logarithm of the partition coefficient between octanol and water (log $P_{O/W}$-value). Solvents with a log $P_{O/W}$-value between 1 and 5, like FB, are highly toxic to whole-cells (Sikkema *et al*., 1994). Several studies have demonstrated this toxic effect for cells grown in toluene (Weber *et al*., 1994; Isken *et al*., 1999). Degradation studies conducted with chlorobenzene (CB) have also demonstrated microbial inhibitory effects. Ferreira Jorge and Livingston (1999) showed a strong inhibitory effect of CB,

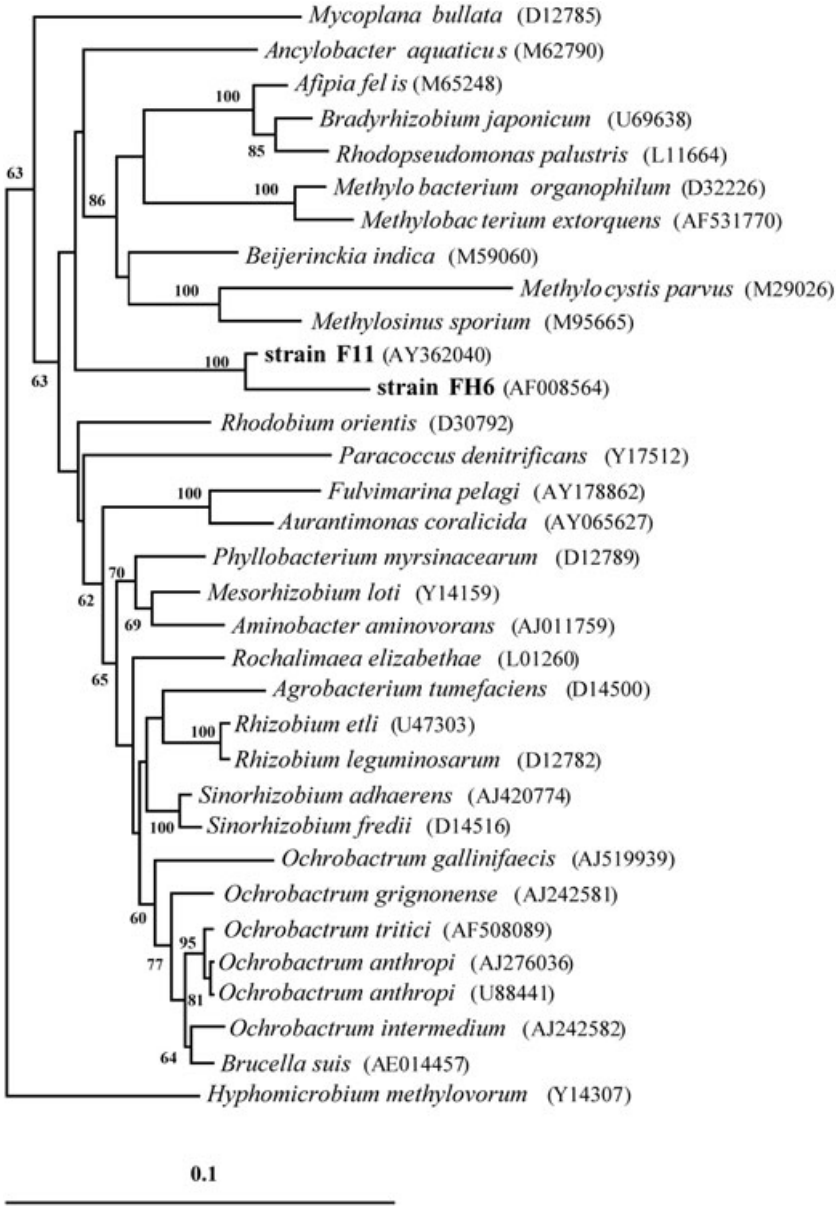

Fig. 1. Phylogenetic tree obtained by neighbour-joining analysis of 16S rRNA sequences. The 16S rRNA gene of strain F11 was amplified by polymerase chain reaction using the primer set f27-r1492 (Lane, 1991) (30 cycles of 60 s at 55°C, 90 s at 72°C and 60 s at 94°C) with Taq DNA polymerase (MBI Fermentas, Lithuania). The amplified fragments were cloned into the pGEM T-Easy vector (Promega) and sequenced by STAB Genomica, Lisbon, Portugal (Taq DyeDeoxy Terminator Cycle Sequencing and Model 373 A gel apparatus, Applied Biosystems) using vector primers and 16S-specific primer f357 (Lane, 1991). The 16S rRNA gene sequences were aligned using the BioEdit programme (version 4.8.8) (Hall, 1999) and analysed using the programmes DNAML, SEQBOOT (100 iterations), DNADIST (Kimura 2-parameter), NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1995). 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 1124 nucleotides was used. *Escherichia coli* 16S rRNA sequence was used to root the tree. Only bootstrap values higher than 60% are reported at the nodes. The bar represents 0.1 substitutions per site. The GenBank accession numbers are indicated between brackets.

supplied via gas phase, at concentrations above 1.4 mM. Fritz and colleagues (1992) have also observed cessation of microbial growth at relatively low CB concentrations (≥3.5 mM).

The degradation behaviour of strain F11 was described by the Luong model (Luong, 1987), shown in Eq. 1 and chosen among the known kinetic models describing the substrate-inhibition phenomenon, as it better described the results obtained, particularly the potential toxic effect observed at high FB concentrations, where no microbial growth was observed. The Luong model was also proposed for describing the growth kinetics on other volatile organic compounds, such as 1,2-dichloroethene (Ferreira Jorge and Livingston, 1999). In this work, the application of the Luong model resulted in the following kinetic parameters: maximum growth rate (μ_{max}) = 0.078 h^{-1}, substrate saturation constant (K_S) = 0.16 mM, parameter indicative of the relation between the growth rate and the substrate concentration (n) = 2.3, and maximum substrate concentration above which growth is completely inhibited (S_m) = 3.9 mM (this last parameter determined experimentally). The high value obtained for the n parameter (higher than 1) suggests a strong inhibitory effect by FB, shown by the observed rapid drop in the growth rate (Luong, 1987).

$$\mu = \frac{\mu_{max} S}{K_S + S}\left[1 - \frac{S}{S_m}\right]^n \quad (1)$$

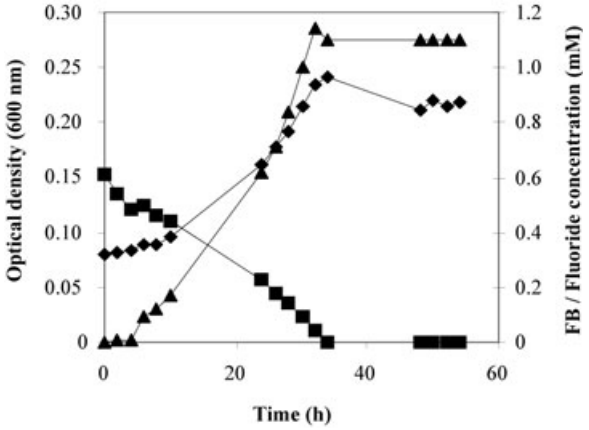

Fig. 2. Growth of strain F11 on 1 mM fluorobenzene (FB) in batch culture. The experiment was run, in duplicate, in 1 l sealed flasks, filled to one-fourth of their volume, containing MM supplied with 1 mM FB, at 25°C, and shaken on a rotatory shaker at 150 r.p.m. The flasks were closed with gas-tight rubber stoppers faced with a Teflon layer to prevent evaporation. Fluorobenzene was analysed by gas chromatography using a CP-Wasc 52 CB capillary column (Chrompack International B.V., Middelburg, the Netherlands), under a temperature regimen starting at 50°C for 2 min, increasing to 150°C at a rate of 25°C min^{-1} and reaching the final temperature of 250°C at a rate of 50°C min^{-1}. Injector and detector temperatures were 250°C. Culture samples (4.5 ml) were extracted with 2 ml of diethyl ether containing mesitylene as internal standard, by vortexing the extraction tube 1 min at maximum speed. The ether layer was analysed by split injection of 1 μl samples. The concentration of fluoride ions in the culture supernatant was measured as previously described (Carvalho *et al*., 2002). The represented FB concentrations are related to the concentrations determined in the liquid phase, which are in equilibrium with the gaseous phase, according to the Henry's coefficient. Optical density (◆), FB concentration in the culture medium (■) and FB degradation based on fluoride release (▲) are indicated.

Metabolic versatility of strain F11

The ability of strain F11 to use a number of aromatic compounds with a chemical structure similar to FB, was tested in batch cultures (Table 1). Strain F11 grew very well in benzoate, benzene, phenol, 4-fluorobenzoate, fluorobenzene and 4-fluorophenol, with the latest three compounds being completely defluorinated. Other halogenated (chloro-, bromo-, iodo-) benzenes did not serve as growth substrates for strain F11. Also, strain F11 did not use any of the four chlorinated compounds tested, during the 10-day period along which the experiment was carried out. The metabolic versatility of strain F11 was compared with the consortium from which it was isolated. The pattern of the carbon sources used by the two cultures was very similar, except for 3-chloro-4-fluoroaniline and 4-chlorophenol, which could only be used by the consortium and not by strain F11.

Acknowledgements

M.F.C. and R.F.J. acknowledge research grants from Fundação para a Ciência e Tecnologia (FCT), Portugal (BD/21839/99 and BPD/6977/2001 respectively). This work was supported in part by the European Community's Human Potential Programme under contract HPRTN-CT-2002-00213, [BIOSAP]. We wish to acknowledge Prof. Pedro Moradas-Ferreira (IBMC, Porto) for his support.

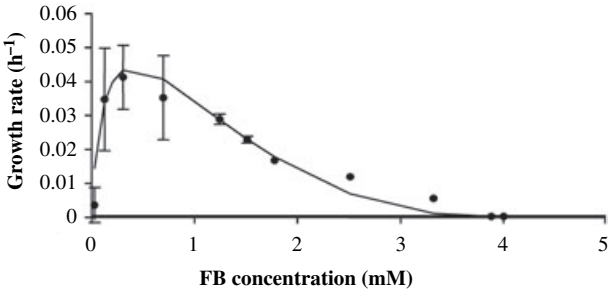

Fig. 3. Growth kinetics of strain F11 on fluorobenzene (FB). The experiments were carried out in MM with FB concentrations between 0 and 8 mM. Appropriate volumes of a FB pre-grown inoculum of strain F11 were added to 100 ml of MM to obtain initial culture optical densities (at 600 nm) of about 0.03. Flasks were sealed with rubber stoppers faced with a Teflon layer to minimize evaporation losses. The cultures were shaken on a rotatory shaker at 150 r.p.m. and 25°C. Samples were periodically taken for FB, fluoride and biomass concentration determinations. Experiments were repeated at least twice. For each FB concentration, the average of the growth rates was used. The obtained standard error of the mean was 10% and is represented at all tested concentrations with a 95% interval confidence. The represented FB concentrations are related to the concentrations determined in the liquid phase, which are in equilibrium with the gaseous phase, according to the Henry's coefficient. The kinetic parameters of the Luong model were estimated using the minimum sum of squares methodology. The solid line shows the Luong equation plotted with $\mu_{max} = 0.078\ h^{-1}$, $K_S = 0.16$ mM, $n = 2.3$ and $S_m = 3.9$ mM.

Table 1. Utilization of various aromatic compounds by strain F11 and by the consortium from which strain F11 was isolated. Growth was tested in 100-ml serum flasks filled to one-third of their volume. The flasks were closed with rubber stoppers and sealed with aluminium caps. Appropriate volumes of a FB pre-grown inoculum of strain F11 were used to obtain initial culture optical densities (at 600 nm) of about 0.03. Tests were run in duplicate in MM fed with each carbon source at a final concentration of 0.5 mM. The experiments were conducted at 25°C, and cultures were shaken on a rotatory shaker at 150 r.p.m. Samples were taken during a 10-day period and analysed for biomass and, when appropriate, for fluoride or chloride release.

Substrate	FB consortium	F11	% of substrate dehalogenation (strain F11)
4-Fluorobenzoate	+	++	100
2-Fluorobenzoate	NT	+	78
Benzoate	++	++	NA
Benzene	++	++	NA
Fluorobenzene	++	++	100
Bromobenzene	NT	–	ND
Iodobenzene	NT	–	ND
Chlorobenzene	–	–	0
3-Chloro-4-fluoroaniline	+	–	0
4-Chlorobenzoate	–	–	0
4-Chlorophenol	+	–	0
Phenol	++	++	NA
4-Fluorophenol	++	++	100

–, No growth; +, Growth; ++, Good growth; NA, Not applicable; NT, Not tested; ND, Not determined.

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