Study of a hexane-degrading consortium in a biofilter and in liquid culture: biodiversity, kinetics and characterization of degrading strains

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Abstract

A gasoline-degrading consortium, originating from a Mexican soil, was used to study its hexane-degradation kinetics in liquid culture and in a biofilter with mineral support. The biodiversity of the consortium depending on the culture conditions and electron and energy source (gasoline, hexane in liquid or hexane in the biofilter) was analyzed using a 16S rRNA-based approach. Significant differences between the populations were observed, indicating a probable adaptation to the substrate. Two strains, named SP2B and SP72-3, isolated from the consortium, belonged to *Actinomycetes* and demonstrated a high metabolic potential in hexane degradation. Even though the SP2B strain was related to *Rhodococcus ruber* DSM 43338^T by phylogenetic studies, it displayed enlarged metabolic properties in hexane and other short-alkane degradation compared with the collection strain.

Introduction

Alkanes are largely produced by geochemical processes and represent metabolical intermediates in numerous organisms. They are also used worldwide in gasoline. They are therefore continuously released on a large scale in the atmosphere and constitute a main source of environmental organic pollution. Many studies have focussed on the use of n-alkane-using microorganisms in the bioremediation of oil-spill environments and contaminated sites (Atlas, 1981). The physical properties of n-alkanes strongly influence their rates of oxidation. Linear alkanes ranging from C10 to C18 and above are readily degraded and support abundant growth of many different microorganisms; short-chain alkanes (C5 to C10) are more soluble and consequently more cytotoxic for microorganisms (Leahy & Colwell, 1990). Amongst them, n-hexane (C_6H_{14}) is largely used in gasoline, as an extraction solvent in industries and as a constituent of domestic products. The molecule is chemically inert, hydrophobic and volatile, with a high Henry's law coefficient, and displays a reduced mass transfer into microorganisms from the gaseous phase to the aqueous phase. Cytotoxicity is displayed by partitioning into and disrupting the lipid bilayer. Hexane belongs to the volatile organic compounds (VOCs), which represent the major atmospheric pollutants responsible for smog formation and other harmful health effects such as cancer. Biofiltration systems have been used for many years to remove VOCs from contaminated air. This technology, well adapted for the treatment of large volumes of air containing low pollutant concentrations, provides environmentally friendly, low-cost alternatives to more traditional, energy-consuming treatment methods. Previous studies for efficiency improvement have therefore mainly focussed on the technical part of the process rather than on the microbiological aspects. In addition, most microbiological studies involved isolation techniques, which allowed the identification of new species (Reichert *et al.*, 1998), but not the characterization of entire populations, because most organisms are not cultivable in

the standard media used for isolation (Amann *et al.*, 1995). Although molecular techniques have already been used to describe the population of a toluene-degrading biofilter (Moller *et al.*, 1996) and of a phosphate-removing biofilm (Gieseke *et al.*, 2001), little is known about population dynamics in biofilters in relation to changing operating conditions.

In this article, we describe and compare a parallel approach of hexane degradation in a bench-scale biofilter supplied with hexane vapors and in a gasoline-adapted consortium cultivated by using a liquid-microcosm approach. Subsequently, the effect of the carbon source and support material (liquid and solid) on the gasoline-adapted-consortium population has been analyzed with molecular techniques, to understand better the changes observed in degradation capabilities. We have then focussed on a hexane-degrading strain, SP2B, an *Actinomycete* identified as *Rhodococcus ruber* and isolated from this consortium. This strain will be used as a reference organism for further studies to improve hexane-biofiltration processes.

Materials and methods

Microbial material and cultivation

The experiments were carried out with a microbial consortium, obtained from a 260 L biofilter previously operated for one year in the Department of Chemical Engineering (UAM-Iztapalapa, Mexico) with gasoline vapors. This consortium was then readapted in our laboratory, in 1 L liquid culture using 750 μL gasoline/Methyl *t*-butyl ether (MTBE) (95/1 volume in volume, v/v) mix as the sole carbon and energy sources with mineral medium number 1 (RT, 120 r.p.m.). This medium contained ($g\,L^{-1}$): NH_4Cl, 0.5; $(NH_4)_2SO_4$, 0.5; $NaNO_3$, 0.5; K_2HPO_4, 1.95; $MgSO_4 \cdot 7H_2O$. 0.57; $CaSO_4 \cdot 2H_2O$, 0.375; $FeSO_4 \cdot 7H_2O$, 0.0225 and 10 mL of trace-element solution (Balch *et al.*, 1979). After 1 month, 3 mL aliquots were used to study hexane degradation in liquid conditions. A 50 mL aliquot from the same gasoline-MTBE culture, concentrated down to 1.5 mL, was used to inoculate 44 g of mineral support (vermiculite) for biofiltration experiments. A 3 mL aliquot from the same culture was conserved for biodiversity analysis.

Rhodococcus ruber DSM 43338^T and *Gordonia amicalis* DSM 44461^T were cultivated as suggested by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, in medium 65 containing ($g\,L^{-1}$): glucose, 4; yeast extract, 4 and malt extract, 10; pH was adjusted at 7.2 with KOH (10 M).

Experimental biofilter and operating conditions

The biofilter consisted of a cylindrical glass column, 0.042 m in inner diameter and 0.21 m in height (bed volume: 0.29 L). It was filled with vermiculite mixed with mineral medium number 2 containing ($g\,L^{-1}$): $NaNO_3$, 18; K_2HPO_4, 1.95; $MgSO_4 \cdot 7H_2O$, 0.57; $CaSO_4 \cdot 2H_2O$, 0.375; $CaCl_2$, 0.0825; $FeSO_4 \cdot 7H_2O$, 0.0225 and 10 mL of a solution of trace elements. Trace elements were ($g\,L^{-1}$): $MnSO_4 \cdot H_2O$, 0.012; $ZnSO_4 \cdot 7H_2O$, 0.013; $CuSO_4 \cdot 7H_2O$, 0.0023; $CoCl_2 \cdot 6H_2O$, 0.0015 and H_3BO_3, 0.0015. This material had an initial water content of 70% and an initial pH of 7. It was inoculated with the gasoline-degrading consortium as indicated above. A mixture (%/%) of hexane- and water-saturated airs was passed up through the column, in order to obtain a hexane inlet concentration of about $5 \pm 0.2 g\,m^{-3}$, corresponding to a constant hexane loading rate of $93.8 \pm 4\ g_{hexane}\,m^{-3}\,h^{-1}$. Empty bed residence time (EBRT) was 3.2 min. The column temperature was $24 \pm 2\,^{\circ}C$. The mineral medium (50 mL) was added at the top of the column by spraying it through a nozzle on the 19th and the 28th days of the experiment.

The elimination capacity (EC) of the hexane, defined as the difference between inlet and outlet concentrations divided by the EBRT, was expressed as g hexane per m^3 per h. Removal efficiency (Ef), defined as the difference between inlet and outlet concentrations divided by inlet concentration, was expressed in percent.

Microcosms experiments

Hexane-degradation rates, by the consortium and by the isolated strains, in liquid culture, were studied in duplicate in 118 mL flasks, sealed with Mininert teflon valves (Alltech, Templemars, France). Hexane (2 μL), corresponding to about $6\,mg\,L^{-1}$ of hexane in liquid phase, was added to the flasks, containing 20 mL of inoculated mineral medium number 1, at the beginning of each cycle (a cycle starts with the renewal of the flask atmosphere by opening it under a laminar hood). An abiotic control was made in the same condition without inoculation. Flasks were incubated at 30 °C and agitated at 120 r.p.m. Hexane was measured periodically from the headspace by gas chromatography. Specific hexane-degradation rate was calculated from the slope of the consumption kinetics divided by the protein content.

The alkane-utilization pattern (n-C5, n-C6, n-C8, n-C10, n-C11, n-C12, n-C15, n-C16, n-C18, n-C20, pristine) of strains isolated from the consortium was tested in a microcosm by qualitative analyses of CO_2 production in the headspace.

Analytical methods

Hexane concentrations from the inlet and outlet streams of the biofilter and of the headspace of the microcosms were measured with a gas chromatograph equipped with an flame ionization detector (Hewlett Packard HP6890 Series GC,

Alltech, Deerfield, IL) and an HP-5 capillary column. The CO_2 concentration contained in the outlet of the biofilter and in the headspaces of the microcosms was measured with thermal conductivity detector gas chromatography (Shimadzu, Duisburg, Germany) using a concentric CTR1 column (Alltech). Operating conditions were as already reported (Teran Perez *et al.*, 2002). The production rate of CO_2 was expressed as g per $m^3_{gas\ phase}$ per h.

Biomass production was determined as the protein contents of microcosm flasks at the initial and final steps of the experiments. Aliquots of 1 mL were harvested from each microcosm flask and resuspended in 0.5 N NaOH for microbial cells lysis. The suspension was then boiled for 5 min and dilutions of this suspension were mixed with the Bradford reagent (Kit BIO-RAD Protein Assay, Bio-Rad, France) and stored for 10 min before colorimetric measurements at 595 nm. A calibration curve with bovine serum albumin was plotted for quantitative determinations.

Isolation of hexane-degrading strains

Appropriate dilutions (from 10^{-2} to 10^{-7}) of the consortium grown in liquid hexane were streaked onto noble-agar plates containing 23 g L^{-1} agar and mineral medium number 1 (pH 7). The plates were incubated at 30 °C in sealed containers with hexane, in the gas phase, used as the sole energy and carbon source. One milliliter of hexane was added to 10 g of activated carbon allowing a gradual release of hexane vapors in the headspace of the 2 L sealed container. Different colony types were purified by several successive transfers to the agar plates. Culture purity was assessed using a phase-contrast microscope (Nikon, Champigny sur Marne, France), before identification by sequencing of 16S rRNA gene and checking for the ability to degrade hexane.

DNA base composition and DNA–DNA hybridization studies

At least 2 g of cells were harvested from 3 L culture in medium 65, washed with sterile physiological solution and stored at −20 °C in isopropyl alcohol (50%, v/v). The G+C content of DNA and the level of biding of the strain DNA were determined at the DSMZ. The DNA was isolated and purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977) and the G+C content determined by HPLC of deoxyribonucleosides following the method of Mesbah *et al.* (1989). DNA–DNA hybridization was carried out at the DSMZ as described by De Ley *et al.* (1970), with the modification described by Huß *et al.* (1983) and Escara & Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories Inc, Oberlin, NH). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Molecular ecology of the samples

Extraction of the total genomic DNA was performed from three samples: a 3 mL aliquot taken from the original gasoline-MTBE culture after 1 month, a 20 mL liquid culture taken at the end of the hexane kinetic experiment in a microcosm, both concentrated down to 500 µL, and 5 g of the inoculated vermiculite in biofilter experiments after 27 days. Extractions were made using a FastDNA spin kit for soil (QBiogene, France). This method relies on mechanical cell lysis by bead beating (FastPrep DNA extractor, QBiogene) followed by a selective DNA adsorption to microporous silicate filters. The bound DNA was then washed with ethanol in the presence of chaotropic salts and finally eluted in a low-salt buffer. Moreover, the genomic DNA was extracted from 4 mL of the isolated strain with the Wizard Genomic DNA Purification Kit (Promega, Charbonnières les Bains, France). Cells were preliminary treated with lysozyme for 30 min (1.2 mg mL^{-1}). The 16S rRNA gene was amplified using universal eubacterial primers Fd1 (5′-AGAGTTTGATC CTGGCTCAG-3′; position 8 to 27, forward, *Escherichia coli* numbering) and R6 (5′-TACGGCTACCTTGTTACG-3′; position 1494 to 1475, reverse, *E. coli* numbering), allowing amplification of nearly the whole gene. Reaction was as follows: 95 °C for 2 min, 40 cycles of 30 s at 95 °C, 30 s at 50 °C, 2 min at 72 °C and a final extension of 7 min at 72 °C. PCR products from the consortia were cloned into Topo XL plasmid (Invitrogen, Cergy-Pontoise, France). PCR products from the isolated strains were cloned into a pGEM-T Easy vector (Promega). Both experiments were performed according to manufacturers' instructions. After transformation, recombinant clones containing an insert of the correct length were selected by direct PCR amplification using primers specific to the plasmid, as already described (Thomsen *et al.*, 2001; Miranda-Tello *et al.*, 2004). The 16S rRNA gene clone library was screened using Amplified Ribosomal DNA Restriction Analysis (ARDRA) with *Hae*III and *Rsa*I (Promega). Selected clones, representing each profile family, were sequenced using the plasmid primers (T7 and M13rev; Genome Express, Grenoble, France). Sequences were aligned using the Ribosomal Database Project (http://rdp.cme.msu.edu/html) and analyzed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Both chromatograms and alignments were manually verified and pairwise evolutionary distances were calculated using the method of Jukes & Cantor (1969). A 100-bootstrapped phylogenetic tree was constructed with 744 unambiguous bp using TREECON (http://iubio.bio.indiana.edu/soft/molbio/evolve/draw/treecon) with the Neighbor-Joining method (Saitou & Nei, 1987).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences reported in the paper have been deposited in GenBank and assigned accession numbers

AY887067, AY887068 and AY898614 for strains SP2B, SP72-3 and SPE2-6, respectively.

Results and discussion

Hexane-degradation kinetics in the consortium cultivated in microcosms

The consortium adapted on gasoline/MTBE was able to use hexane as a unique carbon and energy source in a microcosm. Successive cycles of hexane degradation demonstrated an increasing consumption rate in the consortium (data not shown). After 100 h of adaptation, the consortium totally degraded hexane within approximately 16 h, with a maximum specific consumption rate of 0.07 ± 0.001 $mg_{hexane}\,h^{-1}\,mg_{protein}^{-1}$. Compared with hexane-degradation rates previously described in the literature for pure cultures such as *Pseudomonas aeruginosa,* which was able to degrade $0.055\,mg_{pentane}\,h^{-1}\,mg_{protein}^{-1}$ (Garnier *et al.*, 1999), this new consortium displayed high capabilities of degradation. The consortium was thus used as inoculum to further studies on hexane degradation in biofilter experiments.

Biofilter experiments

After 1 week, corresponding to the acclimation period of the microbial population, a maximum EC of $50\,g\,m^{-3}\,h^{-1}$ and a CO_2 production rate of $30\,g\,m^{-3}\,h^{-1}$ were reached in the biofilter (Fig. 1). The subsequent decrease of EC during the following 10 days was attributed to the drying of the packing material and/or nutrient limitations, as previously described (Morales *et al.*, 1999), although CO_2 production remained at a constant level. The addition of a mineral medium to the biofilter on day 19 led to a rapid increase in EC and CO_2 production rate to $80\,g\,m^{-3}\,h^{-1}$. During the following days of the experiment, a constant removal efficiency (Ef) of $64 \pm 10\%$ was obtained in the biofilter, with an EC of $60 \pm 10\,g\,m^{-3}\,h^{-1}$ and a CO_2 production rate of approximately $80 \pm 10\,g\,m^{-3}\,h^{-1}$. The EC of hexane reached in our biofilter is much higher than those reported previously in laboratory-scale biofilters: EC of $21\,g\,m^{-3}\,h^{-1}$, Ef of 99% (Morgenroth *et al.*, 1996) and EC of $32\,g\,m^{-3}\,h^{-1}$, Ef of 39% (van Groesnetijn & Lake, 1999). The biofilter was unpacked at day 27 for a 5 g sampling for DNA extraction. The support was then packed again, the hexane load restored, allowing the biofilter to reach again the former EC and CO_2 production levels. During the 55 days of the experiment, 19.8 g of hexane were eliminated and 23.1 g of CO_2 were produced, indicating a conversion of approximately 40% of the consumed hexane carbon into CO_2.

Part of the hexane carbon being converted into biomass and possibly organic intermediates can explain this observation, as previously reported in the case of the biofiltration of toluene (Morales *et al.*, 1999). The biomass production resulting from the hexane consumption was not sufficient to clog the biofilter as indicated by the absence of a pressure drop (data not shown).

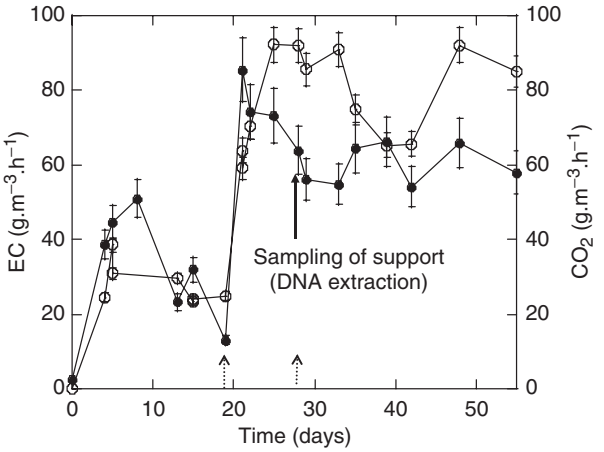

Fig. 1. Elimination capacity (EC) and CO_2 production rate with time in the hexane-biofilter inoculated with the gasoline-degrading consortium. Solid and open symbols represent EC and CO_2 production rate, respectively. Dotted arrows correspond to the addition of a fresh medium.

Biodiversity of the consortium under three experimental conditions

Total genomic DNA was extracted from the three following samples: (1) the original consortium readapted for 1 month using gasoline/MTBE as the unique carbon and energy source; (2) the same consortium after the hexane-degradation kinetics in a liquid microcosm; and (3) the same consortium adapted to hexane degradation in a biofilter on vermiculite after 27 days of the experiment.

The 16S RNA genes, theoretically corresponding to most of the eubacteria present in the three samples, were amplified and cloned. Sequencing was limited to the clones containing a significantly different insert. The two restriction enzymes tested in this experiment (*Hae*III and *Rsa*I) allowed the differentiation of 16 different profiles out of 36 clones for the sample from the column, 10 profiles out of 23 clones for the sample on hexane in liquid culture and five profiles out of 22 clones for the initial consortium on gasoline.

After sequencing one clone for each profile, a phylogenetic tree was constructed with representatives of the different phylogenetic groups (Fig. 2). All the clones identified in the original consortium, adapted to gasoline, belonged to the class of *Proteobacteria*, whether it was the *Alphaproteobacteria*, *Betaproteobacteria* or *Gammaproteobacteria*. Several studies (MacNaughton *et al.*, 1999) reported that Gram-negative bacteria usually dominate the system in hydrocarbon contaminated environments. The majority of the clones belonged to *Gammaproteobacteria*,

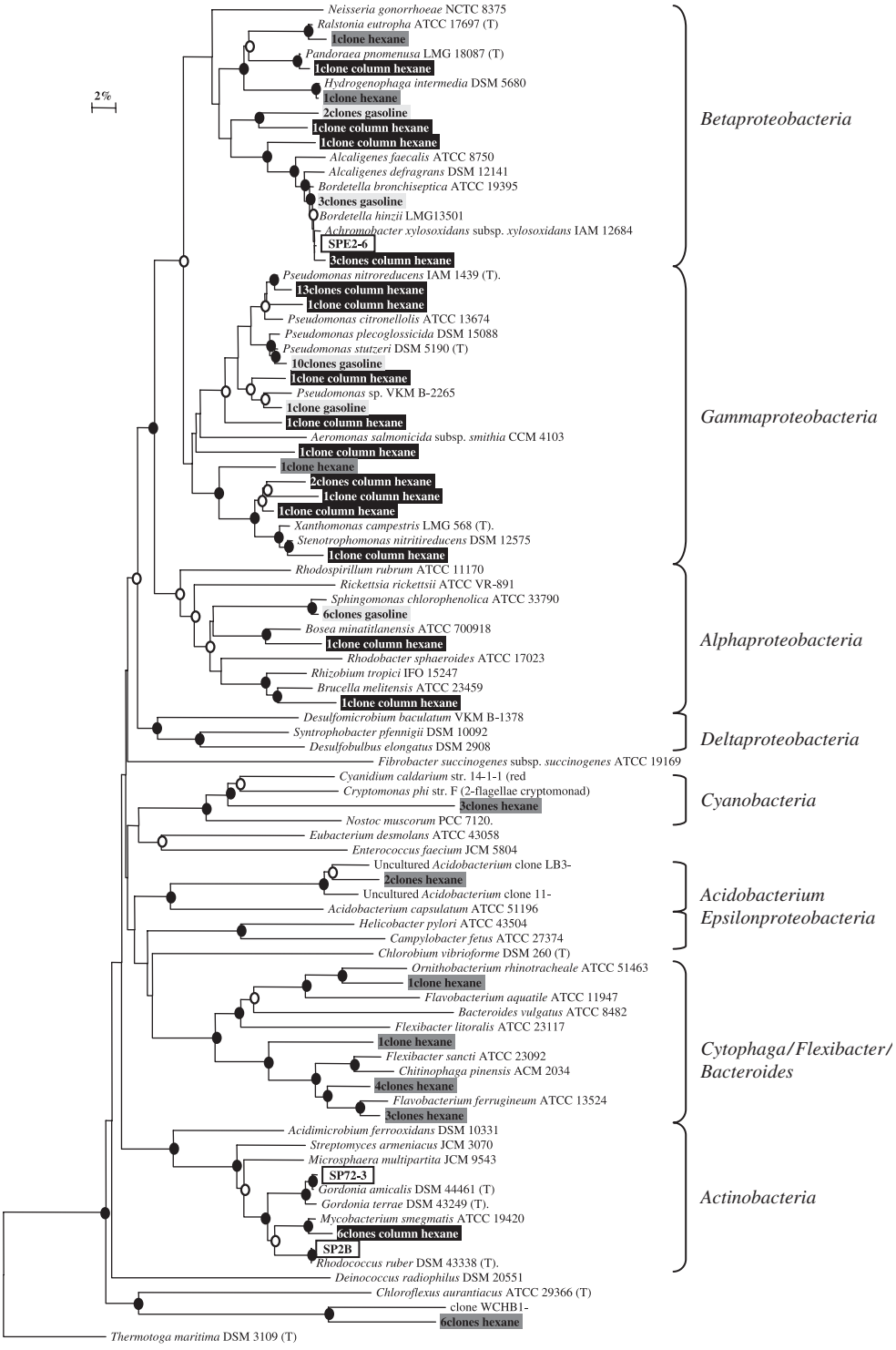

Fig. 2. Phylogenetic tree based on 744 unambiguous bp of the 16S rRNA gene sequences of the prokaryotes with the clones obtained from the three samples and the three strains isolated from the hexane consortium. Dark gray, hexane microcosms; black, biofilter; light gray, gasoline; bold and outlined, isolated strains. Solid circles, nodes with a bootstrap value higher than 80%; open circles, nodes with a bootstrap value between 50% and 80%.

the predominance of which has already been described in a recently spilled oil (Roling *et al.*, 2002). *Pseudomonas* was the most represented genus, a result in accordance with the literature as *Pseudomonas* spp. were frequently identified in soils contaminated with gasoline residues (Ridgway *et al.*, 1990).

The clones identified in the consortium after hexane-degradation kinetics in liquid were fairly different from those in the original consortium on gasoline. They are distributed over more diverse groups. This result seems quite surprising because oil is constituted of many different hydrocarbons and therefore the community of microorganisms able to grow on those substrates should be more diverse. However, many studies showed that the microbial-community diversity decreases remarkably during the degradation process of oil (MacNaughton *et al.*, 1999; Sonderkamp *et al.*, 2001; Roling *et al.*, 2002). The authors explain this decrease in biodiversity by oil being the combination of various complex chemicals that are individually strongly disturbing growth and are even more toxic when combined. This assertion could explain the reduction in population observed in the consortium on gasoline compared with the same consortium on hexane alone (for that reason a less-stressing substrate). Few clones belonged to *Proteobacteria*, *Acidobacteria* and *Cyanobacteria*. The most represented group corresponded to *Cytophaga-Flexibacter-Bacteroides*. This group has previously been reported to contain n-alkane-degrading microorganisms (Al-Hasan *et al.*, 1998; Friedrich *et al.*, 1999).

In the case of the sample taken from the biofilter, as in the case of the original consortium on gasoline, most of the clones belonged to the class of *Proteobacteria*. With hexane applied as the sole energy and carbon source in the column, few additional clones belonging to the *Actinomycete* group (*Actinobacteria*) were also detected. The small changes in the population might have resulted from the short time between inoculation and sampling. The conditions in the biofilter were more drastic than in the microcosm (less mobility, dryness of the filter, substrate accessibility, etc.), possibly explaining a slower growth of microorganisms. The short operating time of the biofilter during the experiment, correlated to the slower growth, could explain the absence of visible change in the community. A longer period of biofilter use, maybe a few months, would be thus necessary before studying the composition of the population. In biofilters used for hexane degradation, *Actinobacteria, Alphaproteobacteria*, *Gammaproteobacteria, Betaproteobacteria* and *Cytophaga-Flexibacter-Bacteroides* have been described (Friedrich *et al.*, 1999). The presence of these microorganisms is in perfect agreement with those found in the hexane consortium placed in liquid conditions and is also in favor of a longer period of biofilter use needed for a notable change in biodiversity.

During growth on hexane in liquid culture, hexane-degradation capacity of the consortium was improved with time (data not shown). One explanation for this phenomenon can be a selective enrichment of organisms able to transform the compound of interest (Leahy & Colwell, 1990). In the consortium, the apparent change in the population seems in good agreement with this result. The organisms found in microcosms on hexane could be involved in hexane degradation. It might be of interest to isolate strains from this consortium and to check their capacity to degrade hexane.

Isolated strains

Isolation and phylogenic identification

Three strains have been isolated from the consortium grown on hexane in liquid condition as described in the experimental procedures; strains were named SP2B, SPE2-6 and SP72-3, respectively. Genomic DNA of each strain was extracted and the 16S rRNA genes were amplified with primers Fd1 and R6, in order to clone the 1500 bp fragment into pGEM-T Easy vector. One clone, containing the right insert size, was selected for each cloning experiment and sequenced. The sequence analyses allowed the location of the isolated strains in the phylogenetic tree (Fig. 2). BLASTN analyses of each sequences showed that SP2B was related to *R. ruber* DSM 43338^T with 99% identity and SP72-3 was close to *G. amicalis* DSM 44461^T (99% identity), both belonging to the *Actinomycetes* order. SPE6-2 was identified as *Achromobacter xylosoxidans* ssp. *xylosoxidans* ATCC 9220 with 99% identity, a *Betaproteobacteria*. Amongst the three strains isolated from the consortium, two *Actinomycetes* (Gram positives) were identified. High-G+C, mycolic acid-containing *Actinomycetes* (*Rhodococcus*, *Gordonia*, *Nocardia*, etc.) are increasingly recognized as ideal candidates for the biodegradation of alkanes (Warhurst & Fewson, 1994; Hamamura & Arp, 2000; Koma *et al.*, 2003). These widely occurring organisms are of considerable environmental and biotechnological importance because of their broad metabolic diversity and array of unique enzymatic capabilities. They are well suited for bioremediation due to their capacity for long-term survival in the environment, their exceptional ability to degrade hydrophobic pollutants even in the presence of more readily assimilable carbon sources and their ability to produce biosurfactants (Bell *et al.*, 1998). These organisms have been found in the consortium in the biofiltration column. *Actinomycetes* therefore are highly interesting microorganisms, partly for their alkane-degradation capacities and partly for their apparent resistance to the restrictive biofiltration environment. No bacteria belonging to that group were found in the consortium in liquid conditions. Still, it is noteworthy that two different mineral

mediums were used in the two experiments, which might have influenced the diversity of the community. It is indeed reported that the specific effect of hydrocarbons on the microbial composition may rely on the study conditions (Leahy & Colwell, 1990). Moreover, the ARDRA approach with a few restriction enzymes might equally underestimate the diversity of microbial populations in the environmental sample.

Hexane-degradation kinetics of the isolated strains alone or in combination

Although SPE2-6 strain was isolated on hexane vapors, it did not significantly degrade hexane. On the contrary, SP72-3 metabolized 1.3 mg hexane into CO_2 in 15 h. Its specific maximum consumption rate was of 0.084 $mg_{hexane}\,h^{-1}\,mg_{protein}^{-1}$, which corresponds approximately to the same degradation capacity as the consortium. SP2B completely degraded 1.5 mg hexane within 33.5 h (Table 1), with a specific maximum consumption rate of 0.028 $mg_{hexane}\,h^{-1}\,mg_{protein}^{-1}$. This value is threefold lower than that of the consortium or SP72-3 alone. The complementary action of strains in breaking down hexane was further examined in liquid cultures. Reconstitutions with all possible combinations of two or three members of the consortium were made (Table 1). Mixing SPE2-6 and SP2B did not increase significantly SP2B degradation activity. In the same way, no improvement in SP72-3 hexane-degradation rate was observed whether it was grown with SPE2-6 and/or SP2B. These results confirm the absence of a role for SPE2-6 in hexane catabolism in the consortium. However, unexpectedly, when SP2B and SP72-3 were mixed, the two strains showed the same specific maximum consumption rate than SP72-3 alone. Two hypotheses can explain the lack of increase in hexane-degradation rate: (1) an inhibition of one strain degradation capacity by the other one; and (2) the delayed participation of SP2B because of its longer lag phase for hexane degradation.

Pseudomonas putida GPo1 (previously named *Pseudomonas oleovorans* GPo1) is well known as a hexane-degrading microorganism (Baptist *et al.*, 1963). To be able to compare its degradation rate with our isolated strains, it was tested in the same conditions. The *Pseudomonas putida* GPo1 hexane consumption rate was in the same range as that obtained for SP2B.

SP72-3 was identified with phylogenetical studies as *G. amicalis* DSM 44461^T. The collection strain was tested for hexane-degradation capacity and was extremely efficient (0.3 $mg_{hexane}\,h^{-1}\,mg_{protein}^{-1}$). Other *Gordonia* strains have been described as using alkanes (Kummer *et al.*, 1999; Koma *et al.*, 2003). SP2B was identified as *R. ruber*. However, whereas SP2B showed an interesting capacity for hexane degradation, *R. ruber* DSM 43338^T was unable to degrade it.

Table 1. Specific hexane-degradation rates, in liquid microcosms, for isolated strains, single or in combination

	Isolated strains			Type strains			Combined cultures					
	SP2B*	SP72-3*	SPE2-6*	*Rhodococcus ruber** DSM 43338^T	*Gordonia amicalis*† DSM 44461^T	*Pseudomonas putida* GPo1†	SP2B/SP72-3†	SP2B/SPE2-6†	SP72-3/SPE2-6†	SP2B/SP72-3/SPE2-6†	Consortium*
Specific degradation‡	0.028 ± 0.001	0.084 ± 0.008	–§	– §	0.31	0.029	0.08	0.032	0.072	0.063	0.07 ± 0.001

*Experiments were carried out in duplicate.
†Experiments were carried out once.
‡Units: m $g_{hexane}\,h^{-1}\,mg_{protein}^{-1}$.
§No hexane degradation was detected.

Among the strains isolated from the consortium, two were effectively degrading hexane. Both strains were identified as already described microorganisms. *Gordonia amicalis* DSM 44461^T and SP72-3 are the most efficient for hexane degradation and should be interesting for bioremediation studies. SP2B and *R. ruber* DSM 43338^T present the same phenotypes regarding the carbon sources and various enzymatic activities tested by using commercial microplates of API ZYM and API 50CH systems (bioMerieux, Marcy I'Etoile, France; data not shown); however, their phenotypes are quite different concerning alkane degradation. Preliminary results showed that SP2B was able to degrade short-chain alkanes (C5 to C11), whereas the type strain *R. ruber* has no effect on those substrates. On the other hand, both strains are efficient for the degradation of alkanes ranging from C12 to C20. We confirm that both strains were identical regarding molecular characteristics like DNA–DNA relatedness and G+C composition. DNA–DNA relatedness studies provide a reliable way of distinguishing between representatives of species that share high 16S rRNA gene similarity (Goodfellow *et al.*, 1998). In the present study, strain SP2B showed a high level of DNA–DNA similarity (88.1%) with the type strain *R. ruber* DSM 43338^T, above the 70% cut-off point recommended for the assignment of organisms to the same genomic species (Wayne *et al.*, 1987). SP2B is therefore a strain of *R. ruber* when the recommendation of the *ad hoc* committee is considered. The G+C composition of strain SP2B was 69.1 M%, whereas 71 M% was determined for the type strain *R. ruber* DSM 43338^T (Bock *et al.*, 1996).

In our study, a few microorganisms have been identified from the hexane consortium, using culture-dependent methods. Despite the presence of some bias related to the protocol, molecular ecology allowed the identification of numerous microorganisms and the study of the population changes during the experiments performed in different physiological conditions. Because biodiversity may play a major role in enhancing bioreactor predictability and reliability (McGrady-Steed *et al.*, 1997; Naeem & Li, 1997), an understanding of the relative influence of the main microbial populations contained in biofilters is of great importance. Nevertheless, the respective influence on the hexane-degrading capacities of the microbial diversity and on the shifts in the community structure of the consortium are still unclear. Further studies on the optimum conditions for alkane degradation in biofilters are in progress. We have also focussed our work on the isolation of microorganisms, such as *Actinomycetes*, presenting high metabolic potential in hexane degradation. Besides the already described ability of the genus *Rhocococcus* to use different aliphatic and aromatic hydrocarbons (Warhurst & Fewson, 1994), this study demonstrated that alkane metabolism was strain-specific and not a general feature of the species. Investigating the genetic variability of both strains might therefore explain the differences highlighted in the alkane-degradation pattern. In particular, strain SP2B, closely related to *R. ruber* DSM 43338^T, will be used as a model for a genetic approach to identify better the genes involved in the hexane-degradation pathway and to compare them with those identified in *R. ruber* DSM 43338^T.

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