Anaerobic utilization of toluene by marine alpha- and gammaproteobacteria reducing nitrate

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Aromatic hydrocarbons are among the main constituents of crude oil and represent a major fraction of biogenic hydrocarbons. Anthropogenic influences as well as biological production lead to exposure and accumulation of these toxic chemicals in the water column and sediment of marine environments. The ability to degrade these compounds in situ has been demonstrated for oxygen- and sulphate-respiring marine micro-organisms. However, if and to what extent nitrate-reducing bacteria contribute to the degradation of hydrocarbons in the marine environment and if these organisms are similar to their well-studied freshwater counterparts has not been investigated thoroughly. Here we determine the potential of marine prokaryotes from different sediments of the Atlantic Ocean and Mediterranean Sea to couple nitrate reduction to the oxidation of aromatic hydrocarbons. Nitrate-dependent oxidation of toluene as an electron donor in anoxic enrichment cultures was elucidated by analyses of nitrate, nitrite and dinitrogen gas, accompanied by cell proliferation. The metabolically active members of the enriched communities were identified by RT-PCR of their 16S rRNA genes and subsequently quantified by fluorescence in situ hybridization. In all cases, toluene-grown communities were dominated by members of the Gammaproteobacteria, followed in some enrichments by metabolically active alphaproteobacteria as well as members of the Bacteroidetes. From these enrichments, two novel denitrifying toluene-degrading strains belonging to the Gammaproteobacteria were isolated. Two additional toluene-degrading denitrifying strains were isolated from sediments from the Black Sea and the North Sea. These isolates belonged to the Alphaproteobacteria and Gammaproteobacteria. Serial dilutions series with marine sediments indicated that up to \(2.2 \times 10^4\) cells cm\(^{-3}\) were able to degrade hydrocarbons with nitrate as the electron acceptor. These results demonstrated the hitherto unrecognized capacity of alpha- and gammaproteobacteria in marine sediments to oxidize toluene using nitrate.

INTRODUCTION

Hydrocarbons are naturally widespread in marine sediments and can originate from several natural and anthropogenic sources. Petroleum hydrocarbons produced during diagenesis of organic-rich sediments and oil emitted by near-surface hydrocarbon seepages constitute a natural source of hydrocarbons in sediments. Some other hydrocarbons of...
biogenic origin are produced in living organisms such as bacteria, phytoplankton, plants and metazoans (Chen et al., 1998; Fischer-Romero et al., 1996; Tissot & Welse, 1984). Furthermore, in addition to hydrocarbons of biogeochemical or biogenic origin, anthropogenic activities, such as offshore production, transportation or tanker accidents, municipal or industrial wastes and runoff, are responsible for additional inputs of petroleum hydrocarbons into the marine environment.

The main constituents of petroleum hydrocarbons are branched and unbranched alkanes, cycloalkanes, as well as monoaromatic and polyaromatic hydrocarbons. As hydrocarbons can be highly toxic to a wide variety of living organisms, the degradation of these contaminants and of petroleum compounds in general is of great importance. The aerobic degradation of aromatic hydrocarbons and alkanes has been studied since the beginning of the 20th century, and numerous aerobic hydrocarbon-degrading micro-organisms have been isolated (e.g. Austin et al., 1977; Gibson & Subramanian, 1984; Teramoto et al., 2009). Even though hydrocarbons are among the least chemically reactive molecules, microbial-mediated degradation has also been demonstrated under anoxic conditions and several anaerobic phototrophic, nitrate-, iron-, sulphate-reducing and fermenting bacteria have been isolated or enriched in recent decades (Heider et al., 1998; Widdel et al., 2010). The activity of sulphate-reducing bacteria in oil reservoirs and in onshore and offshore oil operations has been of great interest from an industrial perspective, as detrimental souring (production of sulphide) has been associated with this group of bacteria. One of the strategies used to control souring has been the addition of nitrate to oil reservoirs and surface facilities, which can have a direct impact on the sulphate-reducing population (Gieg et al., 2011). The anaerobic degradation of aromatic hydrocarbons and alkanes with nitrate as terminal electron acceptor has been previously demonstrated and extensively studied in freshwater environments. Almost all the nitrate-reducing strains isolated so far from terrestrial and freshwater environments belong to the class Betaproteobacteria, and more especially to the genera Thauera, Azoarcus and Georgfuchsia (Dolfing et al., 1990; Evans et al., 1991; Fries et al., 1994; Hess et al., 1997; Rabus & Widdel, 1995b; Ehrenreich et al., 2000; Weelink et al., 2009). Two of the few exceptions so far are hydrocarbon-degrading denitifiers belonging to the class Gammaproteobacteria (Chakrabarty et al., 2005) and ditch sediment (strain Hdsn1) (Ehrenreich et al., 2000; Zedelius et al., 2011). Betaproteobacteria that dominate the oxidation of hydrocarbons in freshwater environments, however, are commonly not dominant in marine sediments. Furthermore, nitrate-reducing micro-organisms of marine origin capable of hydrocarbon degradation have so far not been validated described. To date, fully characterized anaerobic hydrocarbon-degrading strains from marine sediments are all iron- or sulphate-reducing bacteria.

The aim of this study was to elucidate nitrate-dependent degradation of hydrocarbons in various marine sediments and to determine the identity of potential micro-organisms involved in the process. The alkyl-substituted monoaromatic hydrocarbon toluene was chosen as model substrate as it is a widespread hydrocarbon that has been intensely studied. Additional experiments were also performed with the short-chain aliphatic alkane n-hexane. The findings have implications for our understanding of the role of these organisms in hydrocarbon degradation in marine settings and for practices by the oil industry to reduce souring by addition of nitrate.

METHODS

Sources of organisms, media and cultivation procedures.

Enrichment cultures were made and enumeration of viable nitrate-reducers was performed from marine sediments collected from five different sites. Two samples were coastal sediments from La Manche (France), an epicontinental sea of the Atlantic, and were collected respectively from a subtidal station from Ténez beach (=TB) in Plougasnou (France) and from the harbour of Le Douruff en Mer (=LD) in Plouézec (France). A third sample was collected from a polyhaline (17% salinity) Mediterranean lagoon (=ML) located near the Etang de Berre (France). This sediment was collected in a station where deposits of petroleum residues were covered by saltwater. In addition, two samples were used to perform enrichment cultures and isolations with toluene, as well as counting series. The first was collected in the North Sea (=NS), in a small harbour (Horumersiel) located near Wilhelmshaven (Germany). The second sample originated from a sampling station of the Black Sea (=BS) located off the Romanian coast. Sediment cores were collected with pycnacryl tubes and stored under nitrogen. The upper 4 cm of the sediment cores was used.

Procedures for preparation of media and for cultivation under anoxic conditions were as described elsewhere (Widdel & Bak, 1992). Cultures were incubated at 20 °C in HCO₃⁻/CO₂-buffered full marine mineral medium, supplemented with vitamins and trace elements as described by Widdel et al. (2004) with minor modifications to accommodate the needs of denitifiers: 100 mg MnCl₂, 4H₂O L⁻¹ and 29 mg CuCl₂. 2H₂O L⁻¹. Nitrate was used at a final concentration of 5 mM, and resupplied after consumption. Anaerobic conditions in enrichments were achieved solely by degassing and flushing with N₂/CO₂ (90:10, v/v). In pure cultures, 0.5 mM sodium sulphide or 4 mM freshly prepared sodium ascorbate was used in addition to establish reducing conditions (Widdel et al., 2004). Ascorbate did not serve as a growth substrate for the isolated strains. Toluene and n-hexane were prepared as described elsewhere (Ehrenreich et al., 2000; Widdel et al., 2004) and resupplied when consumed. Enrichment cultures were performed in butyl-rubber-stopper-sealed 250 ml flat glass bottles containing 8 ml homogenized sediments, 150 ml mineral medium and 16 ml of the substrate-containing carrier phase, under a headspace of N₂/CO₂ (90:10, v/v). Subcultures contained 150 ml medium, 20 ml of the initial enrichment, 19 ml heptamethylnonane (HMM) and 190 µl of the aromatic or aliphatic hydrocarbon. All the enrichment cultures were made in duplicate in addition to one control without substrate.

The most-probable number (MPN) method was used in five replicate series with 10-fold dilutions in liquid medium, and calculations were done using standard tables. MPNs were performed with the following substrates: acetate (20 mM), benzene (4 mM), n-hexane (1%, v/v, in HMM) and toluene (1%, v/v, in HMM). This experiment was incubated over 90 days at 20 °C in the dark. In MPN series and to test
the ability of the isolates to grow on different substrates, water-soluble substrates were added from concentrated, separately sterilized stock solutions in water to yield the indicated concentrations, and short-chain alkanes (\(<\mathrm{C}_{12}\)) and aromatic hydrocarbons were diluted in HMM. Growth experiments with aromatic hydrocarbons in the presence of oxygen were carried out as described elsewhere (Rabus & Widdel, 1995b). All chemicals used were of analytical grade.

### Growth indicators, analytical procedures and chemical analyses

In the initial enrichment cultures, growth was monitored by quantifying gas production in a gas-tight syringe, and determining the nitrogen content of the gas by trapping of the carbon dioxide, as described previously in detail (Rabus et al., 1999). In addition, more accurate measurements of nitrate and nitrite contents were performed by HPLC, as detailed below.

The initial enrichment cultures were further transferred (inoculum size: 25 %) to fresh media and incubated under the same conditions. In these subcultures, the time course of growth and activity were monitored with precision at the microbiological (cell counts) and chemical (reactants and products of metabolism) level. Cells were observed under a light microscope (Zeiss; 100 × magnification) and enumerated using a Neubauer chamber (depth 0.02 mm).

Nitrate and nitrite were measured by HPLC on an IBJ A3 High Speed NOx anion exchange column (4 × 60 mm) (Sykam), connected to an HT300 autosampler (WICOM; GAT). The eluent was 20 mM NaCl in aqueous ethanol (45 %, v/v). The flow rate was 1 ml min\(^{-1}\) and the temperature of the column was constant at 50 °C. Nitrate (retention time 3.3 min) and nitrite (retention time 2.3 min) were detected at 220 nm with a UV detector. Data acquisition and processing were performed with the Clarity software (DataApex). Ammonium was measured using the indophenol formation reaction (Marr et al., 1988).

Concentrations of toluene and n-hexane in samples from the carrier phase were determined by GC as described previously (Rabus & Widdel, 1995a; Zengler et al., 1999).

### Total RNA extraction

Total RNA was extracted from the 50 ml enrichment cultures (after one transfer) by using a modification of a protocol described by Oelmüller et al. (1990). After centrifugation, pelleted cells were resuspended in STE buffer (10 mM Tris/HCl pH 8.3, 1 mM EDTA pH 8.0, 100 mM NaCl pH 8.0) and ribonucleic acids were extracted by successive additions of hot acidic phenol (Roth-Aqua-Phenol, pH 4.5–5.0; Roth) prewarmed to 60 °C and 10 % (v/v) SDS. After addition of 3 M sodium acetate solution, aqueous phases were extracted with one volume of hot phenol. Then, aqueous phases were collected and extracted with equal volumes of buffered (pH 4.5–5.0) phenol/chloroform-isomyl alcohol (Roth-Aqua-PCI 25:24:1; Roth), and finally with one volume of 100 % chloroform. Nucleic acids in the aqueous phases were subsequently precipitated by addition of cold 2-propanol, washed with 70 % ethanol, dried and resuspended in RNase-free deionized water. An aliquot of the suspended nucleic acids was digested with RNase-free Dnase 1 (1 U \(\mu\)l\(^{-1}\); Promega), in a mixture containing Dnase 10 x buffer (Promega), DTT (0.1 mol \(\mu\)l\(^{-1}\); Roche) and RiboLock RNase inhibitor (40 U \(\mu\)l\(^{-1}\); Fermentas), according to the manufacturers' instructions. The reaction was stopped by the addition of stop-solution (EGTA, pH 8.0, 20 mM; Promega). Removal of DNA was confirmed by PCR with universal primers. RNA aliquots were further purified with RNasey Mini purification columns (Qiagen). Deionized water used to prepare buffers and solutions for RNA extraction was treated (0.1 %) with diethylpyrocarbonate (DEPC), then autoclaved for 20 min at 121 °C. Plastic wares used for RNA extraction and storage were RNase-free.

### RT-PCR amplification of the 16S RNA gene and cloning

About 2 \(\mu\)g RNA was reverse transcribed using RevertAid H\(^{+}\) M-MuLV reverse transcriptase (Fermentas) and 20 pmol of the primer GM4r and GM4m (Muyzer et al., 1995), following the manufacturer’s instructions. After completion of the reverse transcription reactions, PCR amplifications were performed with the universal 16S RNA gene bacterial primers GM4r and GM4m (Muyzer et al., 1995). 16S rRNA gene libraries were constructed by pooling products of two parallel RT-PCR amplifications from the duplicate enrichments. The combined PCR products were then cloned directly using the TOPO TA Cloning kit (pCR4-TOPO suicide vector) and Escherichia coli TOP10F competent cells, according to the manufacturer’s specifications (LifeTechnology). To reduce cloning biases, clones of two parallel cloning experiments were combined to construct each library. Plasmid DNA from each clone was extracted using the Montage Plasmid Miniprep kit (Millipore), according to the manufacturer’s recommendations. Plasmids were checked for the presence of inserts on agarose gels, and then plasmids containing correct-size inserts were used as template for sequencing.

Inserts were sequenced by Taq cycle on an ABI 3130XL sequencer (Applied Biosystems), using the following primers: GM3f (Muyzer et al., 1995), 520f (5’-GC GCCAGCACCCCGGTATA-3’) and GM4r (Muyzer et al., 1995).

### Phylogenetic analyses

Insert-containing clones were partially sequenced and segments were analyzed using the DNASTAR Lasergene 6 package. These partial sequences were aligned in MEGALIGN using the CLUSTAL W program, and adjusted to the same size. Sequences displaying more than 97 % similarity were considered to be related and grouped in the same phytype. At least one representative of each unique phytype was completely sequenced. Sequences were assembled with the SeqMan program (DNASTAR Lasergene 6 software). Sequences were checked for chimera formation by comparing phylogenetic tree topologies constructed from partial sequences. To identify putative close phylogenetic relatives, sequences were compared with those in available databases by use of BLAST (Altschul et al., 1990). Sequences were then aligned to their nearest neighbours using the SeaView4 program with the Muscle Multiple Alignment option (Gouy et al., 2010). Alignments were refined manually and trees were constructed by using the PHYLIP version 3.69 software (http://volution.genetics.washington.edu/phylip) on the basis of evolutionary distance (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981). The robustness of inferred topologies was tested by using 100–1000 bootstrap replicates (Felsenstein, 1985).

Phylogenetic trees were generated using the SEQBOOT, DNAPARS, DNAML and DNASIS programs and neighbour-joining. Rarefaction curves were calculated with the freeware program aRarefactWin (http://strata. uga.edu/Software/Software.html), with confidence intervals of 95 %.

### Cell fixation and fluorescence in situ hybridization (FISH)

Culture subsamples (from the initial enrichment cultures and subcultures) were fixed at room temperature for 2–4 h with formaldehyde (3 % final concentration), washed twice with PBS (10 mM sodium phosphate pH 7.2, 130 mM NaCl), and then stored in PBS/ethanol (1:1) until analysis. FISH was performed on C. Plastic wares used previously described (Snaidr et al., 1997; Fuchs et al., 2000). The following oligonucleotide probes were used: EUV338 (specific for most groups of the domain Bacteria); ALF968 (specific for the Alphaproteobacteria, with the exception of Rickettsiales); BET42a (specific for the Betaproteobacteria); GAM42a (specific for most Gammaproteobacteria); CF319a (specific for some groups of the Cytophaga–Flavobacterium group of the Bacteroidetes); and ARCH915 (specific for Archaea) (Amann et al., 1990; Manz et al., 1992, 1996; Neef, 1997). The labelled GAM42a and BET42a probes were used, respectively, with the unlabelled competitors BET42a and GAM42a. Hybridization with probe NON338 (control probe complementary to EUV338; Wallner et al., 1993) was performed as a negative control. For each probe and sample, 200–700 cells counterstained with DAPI (4,6-diamidino-2-phenylindole) were counted using an

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epifluorescence Zeiss microscope. All probes were labelled with Cy3 (indocarbocyanine) dye at the 5’ end and purchased from ThermoHybaid.

**Isolation, purity control and maintenance of strains.** Toluene-degrading denitrifiers were isolated from enrichment cultures via repeated agar dilution series (Widdel & Bak, 1992) overlaid with the hydrocarbon diluted in HMN, then followed by dilutions to extinction in liquid medium. Purity of the isolates was confirmed by microscopic observations (notably after addition of 0.5 g yeast extract 1−1 or 5 mM glucose) and sequencing. For maintenance, strains were grown on the same hydrocarbon as used for the enrichment, stored at 4 °C and transferred every 3 weeks.

**DNA G+C content.** The G+C content was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb, Braunschweig, Germany) (Mesbah et al., 1989).

**RESULTS**

**Enrichment of toluene- or n-hexane-utilizing denitrifying bacteria**

Anaerobic nitrate-dependent degradation of hydrocarbons in marine sediments was investigated by enrichment cultures performed with three marine sediments (TB, LD and ML, see Methods). The alkyl-substituted monoaromatic hydrocarbon toluene and the short-chain aliphatic alkane n-hexane were chosen as model substrates since they have been most intensely studied among their class. Enrichment for anaerobic prokaryotes oxidizing hydrocarbons with nitrate (5 mM) as electron acceptor was performed at 20 °C in artificial seawater, with toluene or n-hexane as the sole organic substrate (each 1 %, v/v, in carrier phase). Upon depletion of nitrate and nitrite during the first 12−18 days of incubation, nitrate was resupplied in increments of 5 mM. After 2.5 weeks and consumption of 2.5 mM (for TB and LD sediments) and 12 mM (for ML sediment) nitrate, gas production ceased in control cultures, indicating that the endogenous organic compounds from the sediments usable by the indigenous denitrifiers were depleted. From here on, gas production in the enrichment cultures containing hydrocarbons increased gradually, indicating enrichment of n-hexane- or toluene-utilizing microbes, reducing nitrate. After incubating the cultures for 6 weeks, 15.5−22.7 mM nitrate was consumed in the cultures on toluene and 16.8−17.3 mM in the cultures on n-hexane, representing, respectively, a theoretical consumption of 19−28 and 24−25 % of the added hydrocarbons. Subsequently, these cultures were transferred to new media. These positive subcultures were incubated and surveyed over a period of 29 days. Growth in these enrichment cultures was monitored by cell counts and determination of nitrate reduction by HPLC. Additionally, production of gas in these cultures was measured (Fig. 1). All enrichment cultures showed intermediate nitrite accumulation. Formation of ammonium was not detected, indicating that ammonification did not play a significant role in these enrichments. After 29 days of incubation, between 25 and 30 mM nitrate was consumed in the cultures on toluene and between 10 and 12 mM in the cultures on n-hexane. This corresponded to a theoretical oxidation of ~33−40 % the toluene and ~15−18 % n-hexane via denitrification, based on an assumption of complete oxidation of the hydrocarbons. In fact, GC measurements revealed nearly complete disappearance of toluene at this point. Besides a small physical loss (potential absorption in the stopper), the hydrocarbons were utilized for denitrification and biomass formation. It had been shown previously for the pure culture of strain HdN1 that less than 60 % of electrons derived from complete oxidation of the alkane was consumed by nitrate reduction (Ehrenreich et al., 2000). Incomplete oxidation of the hydrocarbon and formation of intermediates could theoretically also contribute to the discrepancy, although this has not yet been observed in denitrifying pure cultures. For the cultures on n-hexane, data are not as comprehensive as data on toluene, since n-hexane concentration was not monitored. Nevertheless, as nitrate depletion was observed in these cultures and nitrate consumption was close to zero in the controls without n-hexane, n-hexane is likely to sustain microbial growth. At the end of the incubation period, similar cell types were observed in duplicate enrichment cultures on toluene or on n-hexane. In all cases, cultures were dominated by short rod-shaped morphotypes, normal-sized bacilli as well as coccoid cells. Numerous cells were in division. Cell numbers increased four- to eightfold during that incubation and reached 1 × 10⁷ cells ml⁻¹ (for n-hexane) to 6 × 10⁶−6 × 10⁸ cells ml⁻¹ (for toluene).

**Phylogenetic affiliations of active bacteria from enrichment cultures, and respective abundances**

Active prokaryotes within the enrichment cultures were identified by extracting total RNA followed by analysis of
the 16S rRNA genes obtained through RT-PCR amplification. No PCR products were obtained from controls in which reverse transcriptase was omitted, confirming the absence of contaminating DNA during RNA preparation. In all cases, nearly full-length 16S rRNA genes could be amplified from complimentary rDNA (crDNA) with universal bacterial primers. A total of 48–53 insert-containing crDNA clones were randomly selected from clone libraries and a partial sequence of ~500 bp was obtained for each clone. Sequences differing less than 3% were considered as a single relatedness group (Rossello-Mora & Amann, 2001) and grouped as a single phylotype. One representative for each phylotype was sequenced in full. Rarefaction curves were calculated from the clone library phylotypes. All calculated rarefaction curves reached the saturation limit, ensuring that the vast majority of bacterial diversity in the enrichment cultures was detected. The relative proportion of each taxonomic group was determined by FISH, carried out with group-specific RNA-targeted oligonucleotide probes (Table 1). Phylogenetic analyses of the rRNA gene sequences revealed that the bacterial community in marine sediments enriched on toluene or n-hexane consisted of several phylotypes affiliated to the gammaproteobacteria (Fig. 2). Although the percentage of gammaproteobacteria in these different enrichments varied (Table 1), based on whole-cell hybridization they represented (for the most part) the main phylotypes.

**Toluene-grown cultures from Térénéz beach.** Whole-cell hybridization applied to toluene-grown cultures from TB sediment revealed that more than 80% of the cells detectable by DAPI staining yielded a hybridization signal with probe GAM42a, specific for most groups of gammaproteobacteria (Table 1). All the detected phylotypes were only distantly related (<93% 16S rRNA gene sequence similarity) to known bacterial genera with cultivated representatives, indicating that so far unknown species were involved in nitrate-dependent degradation of toluene at this site.

**Toluene-grown cultures from a Mediterranean lagoon.** The toluene-grown enrichment cultures from ML sediment resulted in sequences belonging to members of the classes *Gammaproteobacteria* and *Bacteroidetes* (Figs 2 and 3). In these cultures, only 82% of the cells hybridized with probe EUB338 specific for the bacterial domain. This quite low hybridization signal might be explained by the fact that some cells had already reached the stationary growth phase due to substrate depletion and therefore exhibited a decreased cellular RNA content (Fukui et al., 1996). Only 18% of the DAPI-stained cells yielded a hybridization signal with probe CF319a. This probe was specific for only two phylotypes of bacteroidetes among the four phylotypes detected in the clone library. Only 13% of the cells hybridized with probe GAM42a. Most of the sequences of bacteroidetes from the toluene-grown enrichment cultures clustered in three neighbouring phylotypes affiliated with the family *Flavobacteriaceae*. Sequences of gammaproteobacteria were all related to the genus *Marinobacter*.

**n-Hexane-grown cultures from a Mediterranean lagoon.** Similar to the toluene enrichment, the bacterial community enriched on n-hexane from the ML sediments was composed of gammaproteobacteria and bacteroidetes (Figs 2 and 3). In that case again, gammaproteobacteria were quantitatively dominant in the enrichment cultures, as demonstrated by hybridization with probe GAM42a (Table 1). The clone library comprised sequences for *Marinobacter* spp., distantly related to cultivated members, and sequences affiliated to the genus *Halomonas*. *Halomonas* species can grow anaerobically using either nitrate or nitrite, on a wide range of organic substrates (Martínez-Cánovas et al., 2004).

**Toluene-grown cultures from Le Douduff en Mer.** Hybridization of toluene cultures from LD sediment also indicated a dominance of gammaproteobacteria (Table 1). Two phylotypes affiliated with this subclass did not have any close cultivated representative. However, several sequences from the library of this site were related to the genus *Thauera* (97–98% 16S rRNA gene sequence similarity with those of *Thauera* species) of the class *Betaproteobacteria*. Whole-cell hybridization confirmed that a significant fraction (36%) of the enriched cells belonged to the *Betaproteobacteria*. Members of the genus *Thauera* are known as efficient alkane- or aromatic hydrocarbon-degrading denitrifiers and are widespread in freshwater environments. However, betaproteobacteria are rarely retrieved from marine habitats and their presence at this site is probably due to the location of the collection site near a river mouth. It might therefore be assumed that these betaproteobacteria

<table>
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<tr>
<th>Enrichment culture</th>
<th>Percentage of cells hybridized with probe</th>
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<tr>
<td></td>
<td>EUB338</td>
</tr>
<tr>
<td>Toluene (TB)</td>
<td>88</td>
</tr>
<tr>
<td>Toluene (LD)</td>
<td>98</td>
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<tr>
<td>n-Hexane (LD)</td>
<td>91</td>
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<tr>
<td>Toluene (ML)</td>
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<tr>
<td>Toluene (NS)</td>
<td>93.3</td>
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<tr>
<td>Toluene (BS)</td>
<td>91.3</td>
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*ND*, Not detected. Oligonucleotide probes (formamide concentration in hybridization buffer): EUB338 (35%), most groups of the domain Bacteria; ALF968 (20%), *Alphaproteobacteria* with the exception of *Rickettsiales*; BET42a + GAM42a-competitor (35%), *Betaproteobacteria*; GAM42a + BET42a-competitor (35%), most groups of *Gammaproteobacteria*; CF319a (35%), some groups of the *Cytophaga-Flavobacterium* group of the *Bacteroidetes*. Hybridization with these probes did not exceed 0.1%. NON338 (10%), control probe of the DAPI-stained cells in any enrichment culture.
Marine hydrocarbon degraders respiring nitrate

have a freshwater origin. The remaining sequences were related to the bacteroidetes and represented only a minor fraction of the enriched prokaryotes, as indicated by hybridization with probe CF319a.

**n-Hexane-grown cultures from Le Dourduff en Mer.** The denitrifying community grown on n-hexane from the same LD sediment comprised mainly bacteroidetes, gammaproteobacteria and alphaproteobacteria (Figs 2 and 3). The majority of cells grown with n-hexane also hybridized with probe GAM42a (Table 1). Sequences belonging to the gammaproteobacteria were diverse and clustered in four phylotypes. Most sequences were affiliated with phylotypes belonging to the genus *Marinobacter* (96–99% 16S rRNA gene sequence similarity with those of *Marinobacter* species). *Marinobacter* species are Gram-negative, halophilic bacteria able to grow heterotrophically on a wide range of substrates with oxygen or nitrate as a terminal electron acceptor (Gauthier et al., 1992; Huu et al., 1999). Although it has previously been demonstrated that *Marinobacter* species are

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**Fig. 2.** Phylogenetic reconstruction showing the affiliations of the 16S rRNA gene sequences of the new isolates and clone phylotypes from the n-hexane and toluene enrichment cultures performed with TB, ML and LD sediments, and of the toluene-degrading denitrifiers isolated from NS and BS sediments, with selected reference sequences of the proteobacteria. Sequences from this study are given in bold and the sediments used for these cultures are indicated in parentheses. The tree topology shown was obtained with the neighbour-joining algorithm, with 1000 bootstrap replicates. Bar, 2% estimated sequence divergence.
able to utilize alkanes, their ability to do so anaerobically with nitrate as a terminal electron acceptor has, to our knowledge, never been investigated. Other gammaproteobacteria sequences from this enrichment were related to environmental clone sequences from polluted habitats. Bacteroidetes represented a significant fraction of the DAPI-stained cells as demonstrated by FISH counts with probe CF319a (Table 1). Two phylotypes with no close cultivated relatives were found to belong to the class Alphaproteobacteria. A total of 5% of cells in the enrichment culture yielded a hybridization signal with probe ALF968 that covers the alphaproteobacteria.

In addition, FISH analysis demonstrated that the bacterial community enriched on toluene from BS sediment was strongly dominated by gammaproteobacteria, while the enrichment from BS sediment was dominated by alphaproteobacteria (Table 1).

**Isolation of marine toluene-degrading denitrifiers**

The presence of taxa for which alkylbenzene utilization has not been demonstrated prompted isolation of denitrifying toluene-utilizers from the enrichment cultures with toluene by repeated agar dilution series. New toluene-utilizing denitrifying strains were isolated and one representative strain of each taxon was described in more detail. Strain DT-T was isolated from the enrichment culture performed with LD sediment. Cells were motile and coccoid-shaped (Fig. 4a). The strain grew under anaerobic conditions on toluene, m-xylene and diverse organic acids, using nitrate as a terminal electron acceptor (Table 2).

Phylogenetic analyses of the 16S rRNA gene revealed that this strain belonged to the genus *Halomonas* within the Gammaproteobacteria (Fig. 2). Members of the genus *Halomonas* are composed mostly of marine and moderately halophilic prokaryotes with phenotypically very diverse capabilities (Sánchez-Porro et al., 2010; Ventosa et al., 1998). Most *Halomonas* species are aerobes, but can also grow anaerobically using either nitrate or nitrite as electron acceptor. Some *Halomonas* species have been described to degrade benzoate or phenol under aerobic conditions (Alva & Peyton, 2003). However, the ability of this species (*Halomonas campisalis*) to grow anaerobically on aromatic compounds has not been described.

Cells from strain TT-Z, isolated from TB sediments, were rod-shaped and motile (Fig. 4b). Strain TT-Z grew organotrophically on toluene, m-xylene and a variety of organic acids, using nitrate as a terminal electron acceptor (Table 2). Analysis of the 16S rRNA gene revealed that strain TT-Z was affiliated with the genus *Sedimenticola* among the Gammaproteobacteria. It was related closely to the type strain of *Sedimenticola selenatireducens* (ATCC BAA-1233; 96% 16S rRNA gene sequence similarity), which is able to grow anaerobically on 4-hydroxybenzoate coupled to selenate reduction (Narasingarao & Häggblom, 2006).

Two additional toluene-utilizing denitrifiers were isolated from enrichment cultures and repeated agar dilution series using sediments from the North Sea (NS) and the Black Sea (BS) as inoculum source. Strain CoI2, isolated from NS sediment, consisted of oval-shaped to spherical cells (Fig. 4c).
that were non-motile and tended to form loose aggregates in liquid culture. This isolate utilized toluene and a wide range of substrates via denitrification (Table 2). Similar to strain DT-T, this strain was affiliated to the Gammaproteobacteria and belonged to the genus Halomonas. This result underlines the great metabolic versatility of Halomonas species.

Strain TH1 originated from BS sediments and had rod-shaped (Fig. 4d), non-motile cells. This strain grew organotrophically on toluene and several organic acids (Table 2) and on the basis of its 16S rRNA gene sequence belongs to a novel species within the class Alphaproteobacteria.

**Abundance of hydrocarbon-degrading nitrate reducers in marine sediments**

Although nitrate in marine sediments is much less abundant than sulphate, it plays a key role in the anaerobic mineralization of organic matter, notably in coastal sediments (Jørgensen, 1983). As nitrate concentrations in coastal marine sediments are regulated by a complex range of physico-chemical and micro-biological factors, they can differ dramatically from one site to another, with denitrification rates reaching up to 1400 mg N m⁻² day⁻¹ (Herbert, 1999).

To estimate the abundance of cultivable toluene- or n-hexane-degrading denitifiers, MPNs were calculated based on five replicate anoxic serial dilutions carried out from the original sediments with 5 mM nitrate as electron acceptor. For comparison, MPN series were performed in parallel with benzoate and acetate. Benzoate was chosen as it is a common intermediate in the degradation of alkylbenzenes and polar aromatic compounds in freshwater denitrifying bacteria (Heider & Fuchs, 1997; Spormann & Widdel, 2000). Acetate is a key intermediate in the degradation and preservation of organic matter in marine sedimentary habitats. As it is the major fatty acid produced from breakdown of biomass by fermentation, it was expected to allow growth of numerous cultivable denitrifiers. Numbers of cultivable denitrifying prokaryotes utilizing different substrates in sediments from two sites of the sea at La Manche were similar, with slightly higher numbers obtained from the oil-polluted harbour samples (LD) (Table 3). MPN counts of hydrocarbon-degrading denitifiers in sediments from the petroleum-rich ML and NS sediment were substantially higher than for the BS, LD and TB samples (Table 3). The counts for toluene in these petroleum-rich sediments were only two orders of magnitude lower than for acetate ($10^4$ compared with $10^6$ cells cm⁻³), whereas the difference for the other sediments was three orders of magnitude and more. The results suggest that hydrocarbon-degrading denitrifiers are abundant, especially in coastal petroleum-rich sediments.

**DISCUSSION**

In the present study, we revealed the hitherto unrecognized ability of indigenous prokaryotes from marine sediments to degrade alkylbenzenes and alkanes anaerobically using nitrate as a terminal electron acceptor. Most of these toluene- or n-hexane-oxidizing denitifiers enriched from marine sediments represent new types of hydrocarbon-degraders. The majority of the metabolically active bacteria detected within the enrichment cultures belonged to the Alphaproteobacteria and Gammaproteobacteria, as well as the Bacteroidetes. Metabolic activity and growth in the enrichments was monitored by substrate consumption, nitrate reduction and cell counts. Although the main nitrate-reducing hydrocarbon-degraders were identified, not all sequences will belong to organisms directly involved in toluene or n-hexane degradation. A fraction of the bacterial community might have grown with metabolic intermediates derived from the assimilation of toluene or n-hexane by primary hydrocarbon-oxidizers. This may, for example, be the case for the enriched bacteroidetes species, as most bacteroidetes described so far are chemo-organoheterotrophs involved in the decomposition of...
### Table 2. Physiological characteristics of the toluene-degrading denitrifying isolates

Each compound was tested twice at the concentration given in parentheses, and positive cultures were transferred on the same substrate to confirm growth. Growth was monitored by measuring optical density and confirmed by direct cell counts. Concentrations in percentages (v/v) refer to dilutions of hydrophobic compounds in HMN as an inert carrier phase. +, Growth; −, no growth; ND, not determined.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain DT-T</th>
<th>Strain TT-Z</th>
<th>Strain Col2</th>
<th>Strain TH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylogenetic affiliation</td>
<td>Halomonas sp.</td>
<td>Sedimenticola sp.</td>
<td>Halomonas sp.</td>
<td>Oceanicola sp.</td>
</tr>
<tr>
<td>Temperature range of growth (°C)</td>
<td>4–40</td>
<td>15–30</td>
<td>5–40</td>
<td>15–30</td>
</tr>
<tr>
<td>Temperature optimum (°C)</td>
<td>36</td>
<td>28</td>
<td>37</td>
<td>28</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>68.4</td>
<td>64.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound tested with NO$_3$ as an electron acceptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene (1 % in HMN)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzene (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>o-Xylene (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>m-Xylene (1 % in HMN)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>p-Xylene (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ethylbenzene (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>n-Hexane (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>n-Hexadecane (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Benzy alcohol (1 mM)</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Formate (5 mM)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acetate (5 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Propionate (5 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>n-Butyrate (5 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Lactate (5 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Succinate (2 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fumarate (2 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DL-Malate (2 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzoate (2 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenylacetate (1 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Yeast extract (0.5 %)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pyruvate (2 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose (5 mM)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H$_2$/CO$_2$ (80:20, v/v) 2 bar</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Compound tested with O$_2$ as an electron acceptor*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene (1 % in HMN)</td>
<td>−</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acetate (5 mM) (agar plates)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*For the experiments carried out under oxic conditions, media were prepared without nitrate.

### Table 3. Most-probable numbers (MPNs) of cultivable bacteria degrading acetate, benzoate, toluene or $n$-hexane with nitrate as a terminal electron acceptor

ND, Not determined.

<table>
<thead>
<tr>
<th>Sediment</th>
<th>MPN counts (cells cm$^{-3}$) of denitrifying bacteria with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
</tr>
<tr>
<td>Le Dourduff (LD)</td>
<td>$9.2 \times 10^5$</td>
</tr>
<tr>
<td>Térenez (TB)</td>
<td>$9.2 \times 10^4$</td>
</tr>
<tr>
<td>Mediterranean lagoon (ML)</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>North Sea (NS)</td>
<td>$9.3 \times 10^5$</td>
</tr>
<tr>
<td>Black Sea (BS)</td>
<td>$2.2 \times 10^5$</td>
</tr>
</tbody>
</table>
organic matter in natural habitats (Bernardet et al., 2002). In
brief, we cannot unambiguously conclude from these data
alone that all active bacteria identified by molecular methods
are bona fide toluene- or n-hexane-utilizing denitrifiers.
However, successful isolation of toluene-oxidizing denitrifiers
belonging to the alpha- and gammaproteobacteria from
four different marine samples confirmed that marine
denitrifiers with this metabolic ability are probably widely
distributed in these sediments. Although the composition
of the enriched community differed from one habitat to
another, we conclude that hydrocarbons in marine sediments
favour growth of phylogenetically more diverse communities
of denitrifiers than has been found in freshwater sediments
where numerous studies have repeatedly confirmed the
dominance of betaproteobacteria. Surprisingly, even coastal
sediments and sediments obtained from petroleum-con-
taminated harbours were not dominated by betaproteobac-
teria. Furthermore, none of the new microbial isolates
was affiliated to the betaproteobacteria. Why the marine
environment favours hydrocarbon-degrading denitrifying
micro-organisms affiliated to phylogenetic lineages different
from those prevailing in freshwater environments can only
be speculated upon. The hypothesis that betaproteobacteria
able to oxidize hydrocarbons might adapt to the marine
environment was not supported by our study. The isolation
of new types of toluene-degrading denitrifiers from marine
habitats now permits a comparison of pathways involved
in anaerobic hydrocarbon degradation among the different
groups of denitrifying alpha-, beta- and gammaproteobac-
teria, and will allow us to gain insight into the evolution of
these environmentally relevant capacities.

Furthermore, the closely related sequences detected in
enrichment cultures grown from sediments of different
origins imply that some hydrocarbon-degraders could be
widespread within the marine environment. To what
extent these denitrifying micro-organisms participate in
the degradation of hydrocarbons in different marine
environments is still unknown. However, nitrate, although
less abundant in the ocean than sulphate, is an energetically
favourable electron acceptor and one would expect that it
is utilized preferentially over sulphate. The use of nitrate and
nitrite by the oil industry to prevent souring and control
corrosion in oil reservoirs and surface facilities (Gieg
et al., 2002). In

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