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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 2.2×10^4 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.

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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

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; HMN, heptamethylnonane; MPN, most-probable number.

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biogenic origin are produced in living organisms such as bacteria, phytoplankton, plants and metazoans (Chen *et al.*, 1998; Fischer-Romero *et al.*, 1996; Tissot & Welte, 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 operation 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 denitrifiers belonging to the class *Gammaproteobacteria* that have been isolated from river sediment (genus *Dechloromonas*) (Chakraborty *et al.*, 2005) and ditch sediment (strain HdN1) (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 validly 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 Térénez beach (=TB) in Plougasnou (France) and from the harbour of Le Dourduff en Mer (=LD) in Plouézoc'h (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 Wilhemshaven (Germany). The second sample originated from a sampling station of the Black Sea (=BS) located off the Romanian coast. Sediment cores were collected with polyacryl 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 HCO_3^-/CO_2^--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 denitrifiers: 100 mg $MnCl_2 . 4H_2O$ l^{-1} and 29 mg $CuCl_2 . 2H_2O$ l^{-1}. Nitrate was used at a final concentration of 5 mM, and resupplied after consumption. Anoxic conditions in enrichments were achieved solely by degassing and flushing with N_2/CO_2 (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 N_2/CO_2 (90 : 10, v/v). Subcultures contained 150 ml medium, 20 ml of the initial enrichment, 19 ml heptamethylnonane (HMN) 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), benzoate (4 mM), n-hexane (1 %, v/v, in HMN) and toluene (1 %, v/v, in HMN). 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 ($<C_{12}$) and aromatic hydrocarbons were diluted in HMN. 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 (Roti-Aqua-Phenol, pH 4.5–5.0; Roth) prewarmed to 60 °C and 10 % (w/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-isoamyl alcohol (Roti-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 I (1 U μl^{-1}; Promega), in a mixture containing DNase 10× buffer (Promega), DTT (0.1 mol l^{-1}; Roche) and RiboLock RNase inhibitor (40 U μl^{-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 RNeasy 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 rRNA gene and cloning. About 2 μg RNA was reverse transcribed using RevertAid H^- M-MuLV reverse transcriptase (Fermentas) and 20 pmol of the primer GM4r (Muyzer *et al.*, 1995), following the manufacturer's instructions. After completion of the reverse transcription reactions, PCR amplifications were performed with the universal 16S rRNA gene bacterial primers GM4r and GM3f (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 $Miniprep_{96}$ 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′-GCGCCAGCAGCCGCGGTAA-3′) and GM4r (Muyzer *et al.*, 1995).

Phylogenetic analyses. Insert-containing clones were partially sequenced and fragments were analysed 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 phylotype. At least one representative of each unique phylotype 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://evolution.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 DNADIST then 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 polycarbonate filters (GTTP filters; pores, 0.2 μm; Millipore) as previously described (Snaidr *et al.*, 1997; Fuchs *et al.*, 2000). The following oligonucleotide probes were used: EUB338 (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 EUB338; 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

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 l^{-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 as 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 1×10^7 cells ml^{-1} (for n-hexane) to 6×10^7–6×10^8 cells ml^{-1} (for toluene).

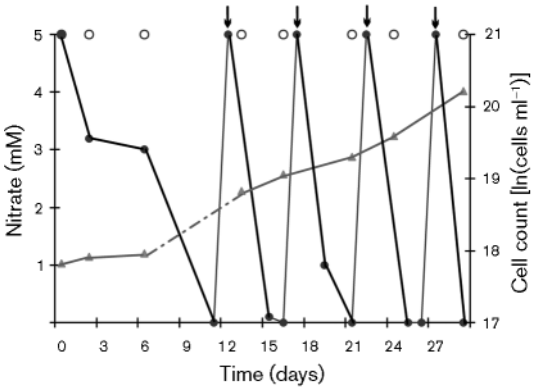

Fig. 1. Nitrate reduction and cell numbers in an enrichment culture from LD sediments on toluene (1 %, v/v, in carrier phase) (subculture of the enrichment). Samples for determination of cell numbers in the enrichment culture (▲) as well as nitrate consumption in the enrichment (●) and in a substrate-free control (○) were withdrawn using N_2-flushed syringes. Downward arrows indicate addition of nitrate.

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 (Rosselló-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 rRNA-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 Térénez 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.

Table 1. Percentages of hybridized cells with group-specific probes relative to total DAPI cell counts

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.

Enrichment culture	Percentage of cells hybridized with probe				
	EUB338	ALF968	BET42a	GAM42a	CF319a
Toluene (TB)	88	ND	ND	80.7	ND
Toluene (LD)	98	ND	35.7	45.9	1.4
n-Hexane (LD)	91	5.0	ND	41.8	19.8
Toluene (ML)	82	ND	ND	12.9	18.3
n-Hexane (ML)	95	ND	ND	52.6	6.0
Toluene (NS)	93.3	1.5	>1.0	79.8	ND
Toluene (BS)	91.3	73.7	5.3	3.3	ND

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 rRNA 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 Dourduff 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

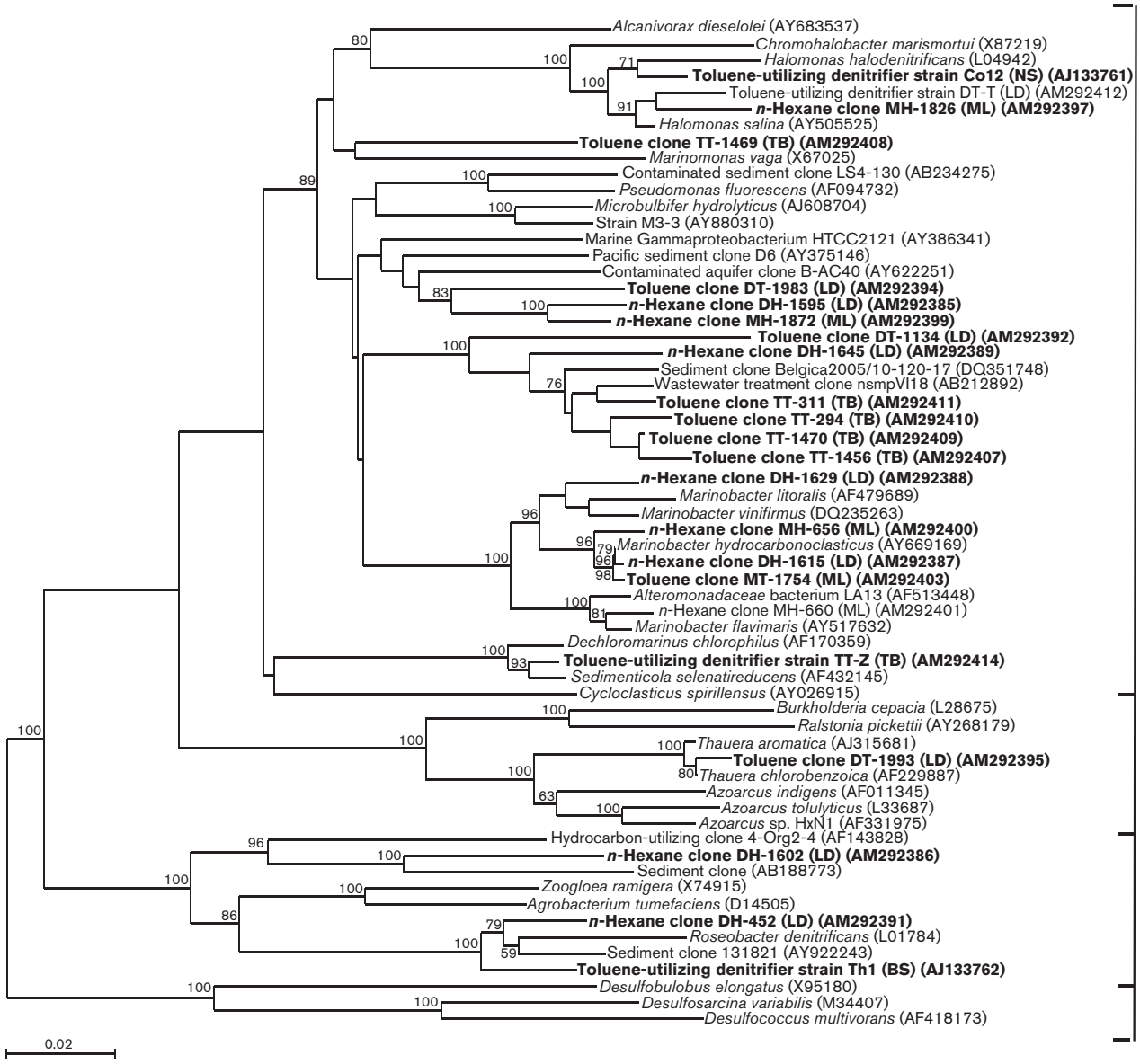

Fig. 2. Phylogenetic reconstruction showing the affiliations of the 16S rRNA gene sequences of the new isolates and clone phylotypes from the *n*-hexane and toluene enrichment cultures performed with TB, ML and LD sediments, and of the toluene-degrading denitrifiers isolated from NS and BS sediments, 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.

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, gamma-proteobacteria 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

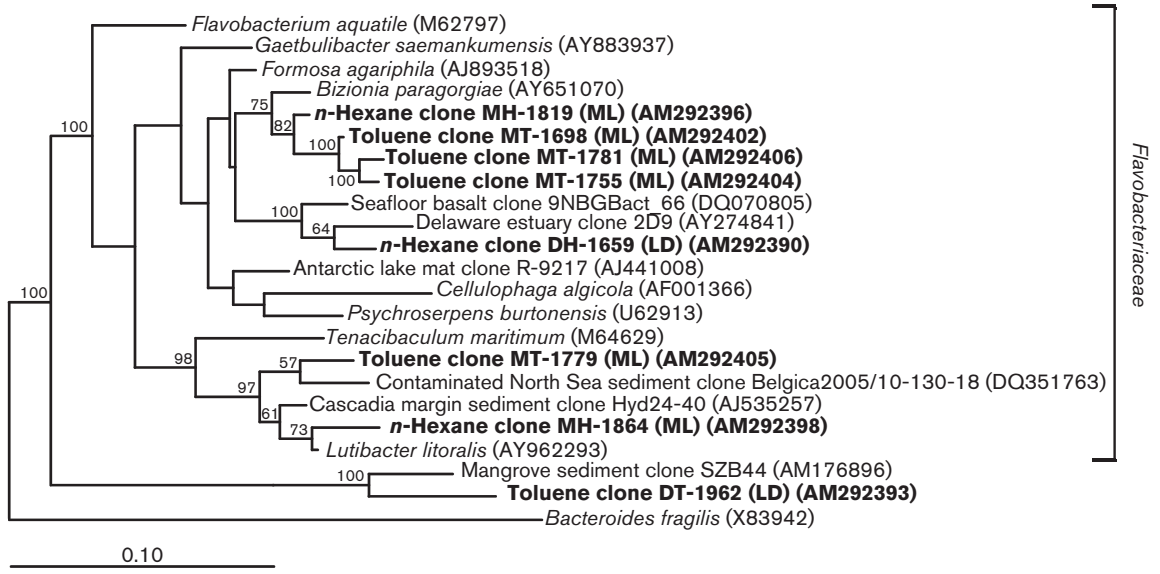

Fig. 3. Phylogenetic reconstruction showing the affiliations of the 16S rRNA gene sequences of the clones from the *n*-hexane and toluene enrichment cultures performed with ML and LD sediments with selected reference sequences of the *Bacteroidetes*. Sequences from this study are given in bold. The tree topology shown was obtained with the maximum-likelihood algorithm, with 100 bootstrap replicates. Bar, 10 % 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 NS 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-oxidizers 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 Col2, isolated from NS sediment, consisted of oval-shaped to spherical cells (Fig. 4c)

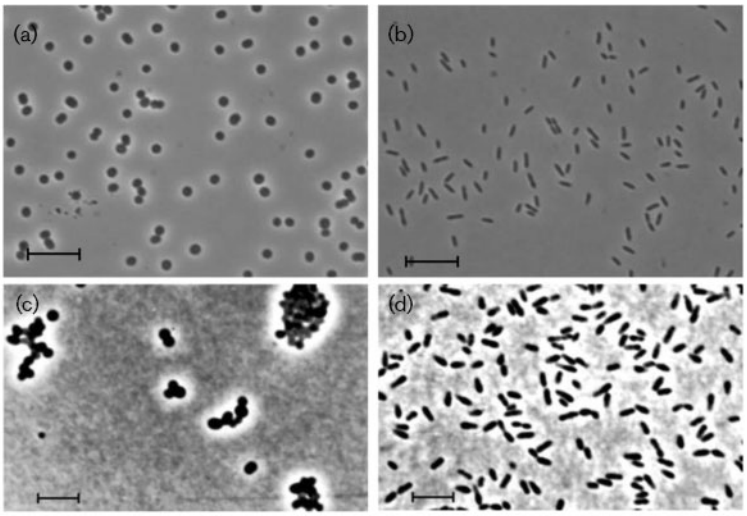

Fig. 4. Phase-contrast photomicrographs of novel marine denitrifying bacteria isolated from enrichment cultures with toluene. (a) Strain DT-T originating from muddy sediments from the harbour of Le Dourduff (LD; La Manche, France), (b) strain TT-Z originating from sandy sediments from Térénez (TB; La Manche, France), (c) strain Col2 originating from North Sea sediment (NS) and (d) strain TH1 isolated from Black Sea sediment (BS). Bars, 5 μm.

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 denitrifiers 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 denitrifiers 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.

Characteristic	Strain DT-T	Strain TT-Z	Strain Col2	Strain TH1
Phylogenetic affiliation	*Halomonas* sp.	*Sedimenticola* sp.	*Halomonas* sp.	*Oceanicola* sp.
Temperature range of growth (°C)	4–40	15–30	5–40	15–30
Temperature optimum (°C)	36	28	37	28
DNA G+C content (mol%)			68.4	64.9
Compound tested with NO_3^- as an electron acceptor				
Toluene (1 % in HMN)	+	+	+	+
Benzene (1 % in HMN)	−	−	−	−
o-Xylene (1 % in HMN)	−	−	−	−
m-Xylene (1 % in HMN)	+	+	−	−
p-Xylene (1 % in HMN)	−	−	−	−
Ethylbenzene (1 % in HMN)	−	−	−	−
n-Hexane (1 % in HMN)	−	−	ND	ND
n-Hexadecane (1 % in HMN)	−	−	ND	ND
Benzyl alcohol (1 mM)	+	−	+	−
Formate (5 mM)	−	+	−	+
Acetate (5 mM)	+	+	+	−
Propionate (5 mM)	+	+	+	−
n-Butyrate (5 mM)	+	+	+	−
Lactate (5 mM)	+	+	+	+
Succinate (2 mM)	+	+	+	+
Fumarate (2 mM)	+	+	+	+
DL-Malate (2 mM)	+	+	+	+
Benzoate (2 mM)	+	+	+	+
Phenylacetate (1 mM)	+	+	+	−
Yeast extract (0.5 %)	+	+	ND	ND
Pyruvate (2 mM)	+	+	+	+
Glucose (5 mM)	−	−	−	−
H_2/CO_2 (80 : 20, v/v) 2 bar	−	−	−	−
Compound tested with O_2 as an electron acceptor*				
Toluene (1 % in HMN)	−	−	ND	ND
Acetate (5 mM) (agar plates)	+	+	ND	ND

*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.

Sediment	MPN counts (cells cm^{-3}) of denitrifying bacteria with:			
	Acetate	Benzoate	Toluene	*n*-Hexane
Le Dourduff (LD)	9.2×10^5	5.4×10^4	5.4×10^3	3.5×10^2
Térénez (TB)	9.2×10^4	1.1×10^3	3.5×10^2	1.7×10^2
Mediterranean lagoon (ML)	1.1×10^6	2.8×10^5	2.2×10^4	1.1×10^4
North Sea (NS)	9.3×10^5	1.5×10^5	1.1×10^4	ND
Black Sea (BS)	2.2×10^5	1.8×10^3	6.0×10^1	ND

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-contaminated harbours were not dominated by betaproteobacteria. 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 gammaproteobacteria, 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 preferably 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.*, 2011; Hubert *et al.*, 2005) could provide conditions favourable for marine denitrifying bacteria. Although detrimental production of sulphite might be reduced by the addition of nitrate, the degradation of hydrocarbons accompanied by the production of large amounts of nitrogen gas would be the consequence.

Our results confirm that marine sediments are rich in nitrate-reducing micro-organisms able to degrade hydrocarbons and that these organisms are clearly different from their freshwater counterparts. The effect these denitrifying hydrocarbon degraders can have on the marine environment, especially in coastal regions where nitrate can be abundant, or on measures to prevent oil souring will be the focus of future studies.

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