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

Karine Alain[1,2,3], Jens Harder[4], Friedrich Widdel[4] and Karsten Zengler[5, *]

[1] NRS, IUEM – UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Place Nicolas Copernic, F-29280 Plouzané, France
2 Université de Bretagne Occidentale (UBO, UEB), Institut Universitaire Européen de la Mer (IUEM) – UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Place Nicolas Copernic, F-29280 Plouzané, France
[3] Ifremer, UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Technopôle Pointe du diable, F-29280 Plouzané, France
[4] Department of Microbiology, Max Planck Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany
[5] University of California, San Diego, Department of Bioengineering, 9500 Gilman Drive, La Jolla, CA 92093-0412, USA

*: Corresponding author : Karsten Zengler, email address : kzengler@ucsd.edu

Abstract:

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.

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 & Welte, 1984). Furthermore, in addition to hydrocarbons of biogeochemical or biogenic origin, anthropogenic activities, such as off-shore 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 mono- and polyaromatic hydrocarbons. Since hydrocarbons can be highly toxic to a wide variety of life, 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 microorganisms 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

54 several anaerobic phototrophic, nitrate-, iron-, sulphate-reducing, and fermenting
55 bacteria have been isolated or enriched over the last decades (Heider *et al.*,
56 1999; Widdel *et al.*, 2010). The activity of sulphate-reducing bacteria in oil
57 reservoirs and in on- and offshore oil operation has been of great interest from an
58 industrial perspective, since detrimental souring (production of sulphide) has
59 been associated with this group of bacteria. One of the strategies to control
60 souring has been the addition of nitrate to oil reservoirs and surface facilities,
61 which can have a direct impact on the sulphate-reducing population (Gieg *et al.*,
62 2011). The anaerobic degradation of aromatic hydrocarbons and alkanes with
63 nitrate as terminal electron acceptor has been previously demonstrated and
64 extensively studied in freshwater environments. Almost all the nitrate-reducing
65 strains isolated so far from terrestrial and freshwater environments belong to the
66 *Betaproteobacteria*, and more especially to the genera *Thauera, Azoarcus* and
67 *Georgfuchsia* (Dolfing *et al.*, 1990; Evans *et al.*, 1991; Fries *et al.*, 1994; Hess *et*
68 *al.*, 1997; Rabus & Widdel, 1995b; Ehrenreich *et al*., 2000; Weelink *et* al., 2009).
69 Two of the few exceptions so far are hydrocarbon-degrading denitrifiers
70 belonging to the *Gammaproteobacteria* that have been isolated from river
71 sediment (genus *Dechloromonas)* (Chakraborty *et al.*, 2005) and ditch sediment
72 (strain HdN1) (Ehrenreich *et al*., 2000; Zedelius *et al*., 2011). *Betaproteobacteria*
73 that dominate the oxidation of hydrocarbons in freshwater environments,
74 however, are commonly not dominant in marine sediments. Furthermore, nitrate-
75 reducing microorganisms of marine origin capable of hydrocarbon degradation
76 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 microorganisms involved in the process The alkyl-substituted monoaromatic hydrocarbon toluene was chosen as model substrate since 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 on our understanding of the role of these organisms in hydrocarbon degradation in marine settings and on practices by the oil industry to reduce souring by addition of nitrate.

METHODS

Sources of organisms, media and cultivation procedures. Enrichment cultures and enumeration of viable nitrate-reducers were 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 harbor 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 one was collected in the North Sea (=NS), in a small harbor (Horumersiel) located near Wilhemshaven (Germany). The second one originated from a sampling station of the Black Sea (=BS) located off the Romanian coast. Sediments cores were collected with polyacryl tubes and stored under nitrogen. The upper four cm of the sediment cores were used for this work.

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 (Widdel *et al.*, 2004) with minor modifications to accommodate the needs of denitrifiers: 100 mg/l $MnCl_2.4H_2O$ and 29 mg/l $CuCl_2.2H_2O$. 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 of sodium sulfide or 4 mM of freshly prepared sodium ascorbate were 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 of homogenized sediments, 150 ml of 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 performed in duplicates in addition to one control without substrate.

The most probable-number (MPN) method was used in five replicates series with 10-fold dilutions in liquid medium, and calculations were done using standard tables. MPN 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 a period of 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 used chemicals were of analytical grade.

Growth indicators, analytical procedures and chemical analyses. In the initial enrichment cultures, growth was monitored by quantifying the 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 high-performance liquid chromatography (HPLC), as detailed below.

The initial enrichment cultures were further transferred (inoculum size: 25%) in 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 the metabolism) level. Cells were observed under a light microscope (Zeiss; x100 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, Germany), connected to an HT300 autosampler (WICOM; GAT GmbH Bremerhaven, Germany). The eluent was 20 mM NaCl in aqueous ethanol (45% v/v). The flow rate was 1 ml/min and the temperature of the column was constant at 50°C. Nitrate (retention time: 3.3 min) and nitrite (retention timer: 2.3 min) were detected at 220 nm with an UV detector. Data acquisition and processing were performed with the Clarity software (DataApex, Czech Republic). 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 gas chromatography as described before (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 previously (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 GmbH, Karlsruhe, Germany) prewarmed at 60 °C and SDS (sodium dodecyl sulphate) 10% (w/v). 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 GmbH, Karlsruhe, Germany), and finally with one volume of 100% chloroform. Nucleic acids in the aqueous phases were subsequently precipitated by addition of cold isopropanol, washed with 70% ethanol, dried and resuspended in RNAse-free deionized water. An aliquot of the suspended nucleic acids was digested with RNase-free DNaseI (1 U/µl, Promega, Mannheim, Germany), in a mixture containing DNase 10×buffer (Promega, Mannheim, Germany), dithiothreitol (DTT 0.1 mol/l, Roche) and RiboLock™ ribonuclease inhibitor (40 U/µl, Fermentas GmbH, St. Leon Rot, Germany), according to the manufacturer instructions. The reaction was stopped by the addition of stop-solution (ethylene glycol tetraacetic acid (EGTA), pH 8.0, 20 mM; Promega, Mannheim, Germany). The removal of DNA was confirmed by PCR with universal primers. RNA aliquots were further purified with RNeasy Mini purification columns (Qiagen, Hilden, Germany). 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 the RNA extraction and storage were RNase-free.

RT-PCR amplification of 16S rRNA and cloning. About 2 µg of RNA were reverse transcribed using the RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon Rot, Germany) and 20 pmol of the primer GM4r (Muyzer *et al.*, 1995), following the manufacturer's instructions. After completion of the RT reactions, PCR amplifications were performed with the universal 16S rDNA 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. Then the combined PCR products were cloned directly using the TOPO TA Cloning® kit (pCR®4-TOPO® suicide vector) and *E. coli* TOP10F competent cells, according to the manufacturer's specifications (Lifetechnology, Carlsbad, CA, USA). 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, Schwalbach, Germany), 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, Foster City, CA, USA),

213 using the following primers: GM3f (Muyzer *et al*., 1995), 520f (5'-GCG CCA GCA
214 GCC GCG GTA A-3') and GM4r (Muyzer *et al*., 1995).
215
216 **Phylogenetic analyses.** Insert-containing clones were partially sequenced and
217 fragments were analysed using the DNASTAR Lasergene 6 package (Madison,
218 WI, USA). These partial sequences were aligned in Megalign using the Clustal W
219 program, and adjusted to the same size. Sequences displaying more than 97%
220 similarity were considered to be related and grouped in the same phylotype. At
221 least one representative of each unique phylotype was completely sequenced.
222 Sequences were assembled with the SeqMan program (DNASTAR Lasergene 6
223 software, Madison, WI, USA). Sequences were checked for chimera formation by
224 comparing phylogenetic tree topologies constructed from partial sequences. To
225 identify putative close phylogenetic relatives, sequences were compared to those
226 in available databases by use of BLAST (Altschul *et al.*, 1990). Then, sequences
227 were aligned to their nearest neighbours using the SeaView4 program with the
228 Muscle Multiple Alignment option (Gouy *et al.*, 2010). Alignments were refined
229 manually and trees were constructed by the PHYLIP (PHYlogeny Inference
230 Package) version 3.69 software (http://evolution.genetics.
231 washington.edu/phylip/getme.html) on the basis of evolutionary distance (Saitou
232 & Nei, 1987) and maximum likelihood (Felsenstein, 1981). The robustness of
233 inferred topologies was tested by using 100 to 1000 bootstrap resampling
234 (Felsenstein, 1985). Phylogenetic trees were generated using the SEQBOOT,
235 DNAPARS, DNAML and DNADIST then Neighbour-Joining. Rarefaction curves

were calculated with the freeware program aRarefactWin (http://www.uga.edu/strata/software/Software), with confidence intervals of 95%.

Nucleotide sequence accession numbers. The clone sequence data reported in this article appear in the EMBL, GenBank and DDBJ sequence databases under the accession numbers AM292385 to AM292411. The nucleotide accession numbers of the isolates are AM292412, AM292414, AJ133761 and AJ133762.

Cell fixation and fluorescent *in situ* hybridization (FISH). Culture subsamples (from the initial enrichment cultures and subcultures) were fixed at room temperature for 2 to 4 h with formaldehyde (3% final concentration), washed twice with phosphate-buffered saline solution (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*); ARCH915 (specific for *Archaea*) (Amann *et al.*, 1990; Manz *et al.*, 1992; Manz *et al.*, 1996; Neef, 1997). The labeled GAM42a and BET42a probes were used, respectively,

with the unlabeled 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 (Ulm, Germany).

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/l yeast extract 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, 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 sole organic substrate (each 1% v/v in carrier phase). Upon depletion of nitrate and nitrite during the first 12 to 18 days of incubation, nitrate was resupplied in increments of 5 mM. After 2½ 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 six weeks, 15.5 to 22.7 mM nitrate was consumed in the cultures on toluene and 16.8 to 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 into 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 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% of the toluene and ~15-18% of the *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 were 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 such 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 closed 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-size bacilli, as well as coccoid cells. Numerous cells were in division. Cell numbers

328 increased four to eight folds during that incubation and reached 1×10^7 cells/ml
329 (for *n*-hexane) to 6×10^7–6×10^8 cells/ml (for toluene).
330
331 **Phylogenetic affiliations of active bacteria from enrichment cultures, and**
332 **respective abundances**
333 Active prokaryotes within the enrichment cultures were identified by extracting
334 total RNA followed by analysis of the 16S rRNA genes obtained through RT-PCR
335 amplification. No PCR products were obtained from controls in which reverse
336 transcriptase was omitted, confirming the absence of contaminating DNA during
337 RNA preparation. In all cases, nearly full length 16S rRNA genes could be
338 amplified from crDNA with universal bacterial primers. A total of 48 to 53 insert-
339 containing crDNA clones were randomly selected from clone libraries and a
340 partial sequence of ~500 bp was obtained for each clone. Sequences differing
341 less than 3% were considered as a single relatedness group based (Rosselló-
342 Mora & Amann, 2001) and grouped as a single phylotype. One representative for
343 each phylotype was sequenced in full. Rarefaction curves were calculated from
344 the clone library phylotypes. All calculated rarefaction curves reached the
345 saturation limit, assuring that the vast majority of bacterial diversity in the
346 enrichment cultures was detected. The relative proportion of each taxonomic
347 group was determined by fluorescent *in situ* hybridization, carried out with group-
348 specific rRNA-targeted oligonucleotide probes (Table 1). Phylogenetic analyses
349 of the rRNA gene sequences revealed that the bacterial community in marine
350 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 rDNA similarity) to known bacterial genera with cultivated representatives, indicating that so far unkown 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 the *Gammaproteobacteria* and *Bacteroidetes* (Fig. 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 reached already 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 clone library. Only 13 % of

374 the cells hybridized with probe GAM42a. Most of the sequences of *Bacteroidetes*
375 from the toluene-grown enrichment cultures clustered in three neighboring
376 phylotypes affiliated with the family *Flavobacteriaceae*. Sequences of
377 *Gammaproteobacteria* were all related to the genus *Marinobacter.*
378
379 *n-hexane-grown cultures from a Mediterranean lagoon*
380 Similar to the toluene enrichment, the bacterial community enriched on *n*-hexane
381 from the ML sediments was also composed of *Gammaproteobacteria* and
382 *Bacteroidetes* (Fig. 2 and 3). In that case again, *Gammaproteobacteria* were
383 quantitatively dominant in the enrichment cultures, as demonstrated by
384 hybridization with probe GAM42a (Table 1). The clone library comprised
385 sequences for *Marinobacter* spp., distantly related to cultivated members, and
386 sequences affiliated with the genus *Halomonas*. *Halomonas* species can grow
387 anaerobically using either nitrate or nitrite, on a wide range of organic substrates
388 (Martinez-Canovas *et al.*, 2004).
389
390 *Toluene-grown cultures from Le Dourduff en Mer*
391 Hybridization of toluene cultures from LD sediment also indicated dominance of
392 *Gammaproteobacteria* (Table1). Two phylotypes affiliated with this subclass did
393 not have any close cultivated representative. However, several sequences from
394 the library of this site were related to the genus *Thauera* (97-98% 16S rRNA
395 similarity with sequences of *Thauera* species) of the *Betaproteobacteria*. Whole-
396 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 in marine habitats and their presence at this site is likely due to the location of the collection site near a river mouth. It might therefore be assumed that these *Betaproteobacteria* 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 of *Bacteroidetes*, *Gamma*- and *Alphaproteobacteria* (Fig. 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 to 99% 16S rDNA similarity with sequences 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 terminal electron acceptor (Gauthier *et al.*, 1992; Huu *et al.*, 1999). Although it has previously been demonstrated that *Marinobacter* species are able to utilize alkanes, their capability 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 *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 members' alkylbenzene utilization has not been demonstrated prompted isolation of denitrifying toluene-oxidizers from the enrichment cultures with toluene by repeated agar dilutions 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

443 anaerobic conditions on toluene, *m*-xylene, and diverse organic acids, using
444 nitrate as a terminal electron acceptor (Table 2). Phylogenetic analyses of the
445 16S rRNA gene revealed that this strain belonged to the genus *Halomonas* within
446 the *Gammaproteobacteria* (Fig. 2). Members of the genus *Halomonas* are
447 composed of mostly marine and moderately halophilic prokaryotes with
448 phenotypically very diverse capabilities (Sanchez-Porro *et al.*, 2010; Ventosa *et*
449 *al.*, 1998). Most *Halomonas* species are aerobes, but can also grow
450 anaerobically using either nitrate or nitrite as electron acceptor. Some
451 *Halomonas* species have been described to degrade benzoate or phenol under
452 aerobic conditions (Alva & Peyton, 2003). However, the ability of this validly
453 described species to grow anaerobically on aromatic compounds has not been
454 described.
455
456 Cells from strain TT−Z, isolated from TB sediments, were rod-shaped and motile
457 (Fig. 4b). Strain TT−Z grew organotrophically on toluene, *m*-xylene, and on
458 variety of organic acids, using nitrate as a terminal electron acceptor (Table 2).
459 Analysis of the 16S rRNA gene revealed that strain TT−Z was affiliated with the
460 genus *Sedimenticola* among the *Gammaproteobacteria*. It was closely related to
461 the species *Sedimenticola selenatireducens* (96% 16S rDNA similarity), a strain
462 able to grow anaerobically on 4-hydroxybenzoate coupled to selenate reduction
463 (Narasingarao & Haggblom, 2006).
464

Two additional toluene-utilizing denitrifiers were isolated from enrichment cultures and repeated agar dilutions series using sediments from the North Sea (NS) and the Black Sea (BS) as inoculum source. Strain Col2, isolated from North Sea 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 Black Sea 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 new species within the *Alphaproteobacteria*.

Abundance of hydrocarbon degrading nitrate-reducers in marine sediments

Albeit 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 1,400 mg N m^{-2} day^{-1} (Herbert, 1999).

To estimate the abundance of cultivable toluene or *n*-hexane-degrading denitifiers, most-probable numbers (MPN) were calculated by 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 La Manche were similar, with slightly higher numbers obtained from the oil-polluted harbor 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 as for acetate (10^4 compared to 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 capability 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 *Alpha*- 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 hydrocarbons 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 chemoorganoheterotrophs involved in the decomposition of organic matter in natural habitats (Bernardet *et al.*, 2002). In brief, we cannot unambiguously conclude from this 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 capability are probably widely distributed in these sediments. Although the composition of the

534 enriched community differed from one habitat to the other, one can conclude that
535 hydrocarbons in marine sediments favour growth of phylogenetically more
536 diverse communities of denitrifiers, than what has been found in freshwater
537 sediments where numerous studies have repeatedly confirmed the dominance of
538 *Betaproteobacteria*. Surprisingly, even coastal sediments and sediments
539 obtained from petroleum-contaminated harbors, were not dominated by
540 *Betaproteobacteria*. Furthermore, none of the new microbial isolates was
541 affiliated to the *Betaproteobacteria*. Why the marine environment favours
542 hydrocarbon-degrading denitrifying microorganisms affiliated to different
543 phylogenetic lineages than those prevailing in freshwater environments can only
544 be speculated about at this time. The hypothesis that *Betaproteobacteria* able to
545 oxidize hydrocarbons might adapt to the marine environment was not supported
546 by our study. The isolation of new types of toluene-degrading denitrifiers from
547 marine habitats now permits a comparison of pathways involved in anaerobic
548 hydrocarbon degradation among the different groups of denitrifying *Alpha*-, *Beta,*
549 and *Gammaproteobacteria*, and to gain insights into the evolution of these
550 environmentally relevant capacities.
551
552 Furthermore, the closely related sequences detected in enrichment cultures
553 grown from sediments of different origins, implies that some hydrocarbon-
554 degraders could be widespread within the marine environment. To what extent
555 these denitrifying microorganisms participate in the degradation of hydrocarbons
556 in different marine environments is still unknown. However, nitrate, although less

abundant in the ocean than sulphate, is an energetically favorable 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 favorable 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 microorganisms 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 on 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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REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Alva, V. A. & Peyton, B. M. (2003). Phenol and catechol biodegradation by the haloalkaliphile *Halomonas campisalis*: influence of pH and salinity. *Environ Sci Technol* **37**, 4397-4402.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919-1925.

Austin, B., Calomiris, J. J., Walker, J. D. & Colwell, R. R. (1977). Numerical taxonomy and ecology of petroleum-degrading bacteria. *Appl Environ Microbiol* **34**, 60-68.

Bernardet, J. F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049-1070.

Chakraborty, R., O'Connor, S. M., Chan, E. & Coates, J. D. (2005). Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. *Appl Environ Microbiol* **71**, 8649-8655.

Chen, J., Henderson, G., Grimm, C. C., Lloyd, S. W. & Laine, R. A. (1998). Termites fumigate their nests with naphthalene. *Nature* **392**, 558-559.

Dolfing, J., Zeyer, J., Binder-Eicher, P. & Schwarzenbach, R. P. (1990). Isolation and characterization of a bacterium that mineralizes toluene in the abscene of molecular oxygen. *Archives of Microbiology* **154**, 336-341.

Ehrenreich, P., Behrends, A., Harder, J. & Widdel, F. (2000). Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. *Arch Microbiol* **173**, 58-64.

Evans, P. J., Mang, D. T., Kim, K. S. & Young, L. Y. (1991). Anaerobic degradation of toluene by a denitrifying bacterium. *Applied and Environmental Microbiology* **57**, 1139-1145.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17,** 368-376.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evol* **30,** 783-791.

Fischer-Romero, C., Tindall, B. J. & Jüttner, F. (1996). *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freswater lake. *Int J Sys Bacteriol* **46**, 183-188.

Fries, M. R., Zhou, J., Chee-Sanford, J. & Tiedje, J. M. (1994). Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. *Applied and Environmental Microbiology* **60**, 2802-2810.

Fuchs, B. M., Zubkov, M. V., Sahm, K., Burkill, P. H. & Amann, R. (2000). Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. *Environ Microbiol* **2**, 191-201.

Fukui, M., Suwa, Y. & Urushigawa, Y. (1996). High survival efficiency and ribosomal RNA decaying pattern of *Desulfobacter latus*, a highly specific acetate-utilizing organism, during starvation. *FEMS Microbiol Ecol* **19**, 17-25.

Gauthier, M. J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. & Bertrand, J.-C. (1992). *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremly halotolerant, hydrocarbon-degrading marine bacterium. *Int J Sys Bacteriol* **42**, 568-576.

Gibson, D. T. & Subramanian, V. (1984). Microbial degradation of aromatic hydrocarbons. In *Microbial degradation of aromatic compounds*, pp. 181-252. Edited by D. T. Gibson. New York: Marcel Dekker, Inc.

Gieg, L. M., Jack, T. R. & Foght, J. M. (2011). Biological souring and mitigation in oil reservoirs. *Appl Microbiol Biotechnol* **92**, 263-282.

Gouy, M., Guindon, S. & Gascuel, O. (2010). SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* **27**, 221-224.

Heider, J. & Fuchs, G. (1997). Anaerobic metabolism of aromatic compounds. *Eur J Biochem* **243**, 577-596.

Heider, J., Spormann, A. M., Beller, H. R. & Widdel, F. (1999). Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev* **22**, 459-473.

Herbert, R. A. (1999). Nitrogen cycling in coastal marine ecosystems. *FEMS Microbiol Rev* **23**, 563-590.

Hess, A., Zarda, B., Hahn, D., Häner, A., Stax, D., Höhener, P. & Zeyer, J. (1997). In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. *Applied and Environmental Microbiology* **63**, 2136-2141.

Hubert, C., Nemati, M., Jenneman, G. & Voordouw, G. (2005). Corrosion risk associated with microbial souring control using nitrate or nitrite. *Appl Microbiol Biotechnol* **68**, 272-282.

Huu, N. B., Denner, E. B., Ha, D. T., Wanner, G. & Stan-Lotter, H. (1999). *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. *Int J Syst Bacteriol* **49 Pt 2**, 367-375.

Jørgensen, B. B. (1983). Processes at the sediment-water interface. In *The Major Biogeochemical Cycles and Their Interactions*, pp. 477-509. Edited by B. Bolin & R. B. Cook. New York: J. Wiley & Sons.

Kemp, P. F., Lee, S. & Laroche, J. (1993). Estimating the growth rate of slowly growing marine bacteria from RNA content. *Appl Environ Microbiol* **59**, 2594-2601.

Manz, W., Amann, R., Ludwig, W., Wagner, M. & Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems ans solutions. *Syst Appl Microbiol* **15**, 593-600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. & Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142 (Pt 5)**, 1097-1106.

Marr, I. L., Cresser, M. S. & Ottendorfer, L. J. (1988). *Umweltanalytik. Eine allgemein Einführung*. Stuttgart: Thieme Verlag.

Martinez-Canovas, M. J., Quesada, E., Llamas, I. & Bejar, V. (2004). *Halomonas ventosae* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* **54**, 733-737.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of G+C content of deoxyribonuleic acid by high-performance liquid chromatography. *Int J Sys Bacteriol* **39**, 159-167.

Muyzer, G., Teske, A., Wirsen, C. O. & Jannasch, H. W. (1995). Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**, 165-172.

Narasingarao, P. & Haggblom, M. M. (2006). *Sedimenticola selenatireducens*, gen. nov., sp. nov., an anaerobic selenate-respiring bacterium isolated from estuarine sediment. *Syst Appl Microbiol* **29**, 382-388.

Neef, A. (1997). Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in Komplexen mikrobiellen Biozönosen. München: Technische Universität, München.

Oelmüller, U., Krüger, N., Steinbüchel, A. & Friedrich, G. C. (1990). Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. *J Microbiol Methods* **11**, 73-81.

Rabus, R. & Widdel, F. (1995a). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol* **163**, 96-103.

Rabus, R. & Widdel, F. (1995b). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Archives of Microbiology* **163**, 96-103.

Rabus, R., Wilkes, H., Schramm, A., Harms, G., Behrends, A., Amann, R. & Widdel, F. (1999). Anaerobic utilisation of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliated with *Azoarcus*/*Thauera* cluster. *Environ Microbiol* **1**, 145-157.

Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39-67.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Sanchez-Porro, C., Kaur, B., Mann, H. & Ventosa, A. (2010). *Halomonas titanicae* sp. nov., a halophilic bacterium isolated from the RMS Titanic. *Int J Syst Evol Microbiol* **60**, 2768-2774.

Snaidr, J., Amann, R., Huber, I, Ludwig, W. & Schleifer, K. –H. (1997). Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Appl Environ Microbiol* **63,** 2884-2896.

Spormann, A. M. & Widdel, F. (2000). Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* **11**, 85-105.

Teramoto, M., Suzuki, M., Okazaki, F., Hatmanti, A. & Harayama, S. (2009). *Oceanobacter*-related bacteria are important for the degradation of petroleum aliphatic hydrocarbons in the tropical marine environment. *Microbiology* **155**, 3362-3370.

Tissot, B. P. & Welte, D. H. (1984). Petroleum alteration. In *Petroleum formation and occurence*, pp. 459-469. New York: Springer-Verlag.

Ventosa, A., Nieto, J. J. & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62**, 504-544.

Wallner, G., Amann, R. & Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**, 136-143.

Weelink, S.A., van Doesburg, W., Saia, F.T., Rijpstra, W.I., Röling, W.F., Smidt, H. & Stams, A.J. (2009). A strictly anaerobic betaproteobacterium *Georgfuchsia toluolica* gen. nov., sp. nov. degrades aromatic compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor. *FEMS Microbiol Ecol* **70**, 575-85.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. In *The Prokaryotes*, pp. 3352-3392. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. Berlin, Heidelberg, New York: Springer-Verlag.

Widdel, F., Boetius, A. & Rabus, R. (2004). Anaerobic degradation of hydrocarbons including methane. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, pp. http://link.springer-ny.com/link/service/books/10125/. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrand. New York: Springer.

Widdel, F., Knittel, K. & Galushko, A. (2010). Anaerobic hydrocarbon-degrading microorganisms: an overview. In *Handbook of hydrocarbon and lipid microbiology*, pp. 1997-2021. Edited by K. N. Timmis. Berlin: Springer.

Zedelius, J., Rabus, R., Grundmann, O., Werner, I., Brodkorb, D., Schreiber, F., Ehrenreich, P., Behrends, A., Wilkes, H., Kube, M., Reinhardt, R. & Widdel, F. (2011). Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environ Microbiol Rep* **3**, 125-135.

Zengler, K., Richnow, H.H., Rosselló-Mora, R., Michaelis, W. & Widdel, F. (1999). Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **401**, 266.269.

Figure Legends and Tables

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 substrate-free control (○) were withdrawn using N_2-flushed syringes. Symbol ↓: additional nitrate.

Fig. 2. Phylogenetic reconstruction showing the affiliations of the 16S rRNA gene sequences of the 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 brackets. The tree topology shown was obtained by the Neighbour-Joining algorithm, with 1000 bootstrap replicates. The scale bar indicates 2% estimated sequence divergence.

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 by the maximum likelihood algorithm, with 100 bootstrap replicates. The scale bar indicates 10% estimated sequence divergence.

824

825 **Fig. 4**. Phase contrast photomicrographs of novel marine denitrifying bacteria
826 isolated from enrichments cultures with toluene. (a) Strain DT-T originating from
827 muddy sediments from the harbor of Le Dourduff (LD), (La Manche, France), (b)
828 strain TT-Z originating from sandy sediments from Térénez (TB) (La Manche,
829 France), (c) strain Col2 originating from North Sea sediment (NS) and (d) strain
830 TH1 isolated from Black Sea sediment (BS). Bar, 5 µm.

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

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

n. d. not determined

*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*
- ARCH915 (35%): *Archaea*
- NON338 (10%): control probe

Hybridization with these probes did not exceed 0.1% of the DAPI stained cells in any enrichment culture.

Table 2. Physiological characteristics of the toluene-degrading denitrifying isolates.

Characteristics	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
Compounds 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)	−	−	n.d.	n.d.
n-hexadecane (1% in HMN)	−	−	n.d.	n.d.
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)	+	+	+	+
D/L-malate (2 mM)	+	+	+	+
Benzoate (2 mM)	+	+	+	+
Phenylacetate (1 mM)	+	+	+	−
Yeast extract (0.5%)	+	+	n.d.	n.d.
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	−	−	n.d.	n.d.
Acetate (5 mM) (agar plates)	+	+	n.d.	n.d.

[*]Each compound was tested twice at the concentration given in brackets, and positive cultures were transferred on the same substrate to confirm growth. Growth was monitored by optical density and confirmed by direct cell counts. Concentrations in percentages (vol/vol) refer to dilutions of hydrophobic compounds in heptamethylnonane (HMN) as an inert carrier phase. Symbols: +, growth; −, no growth; n.d. not determined.

[†]For the experiments carried out under oxic conditions, media were prepared without nitrate.

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

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	n. d.
Black Sea (BS)	2.2×10^5	1.8×10^3	6.0×10^1	n. d.

n. d. not determined

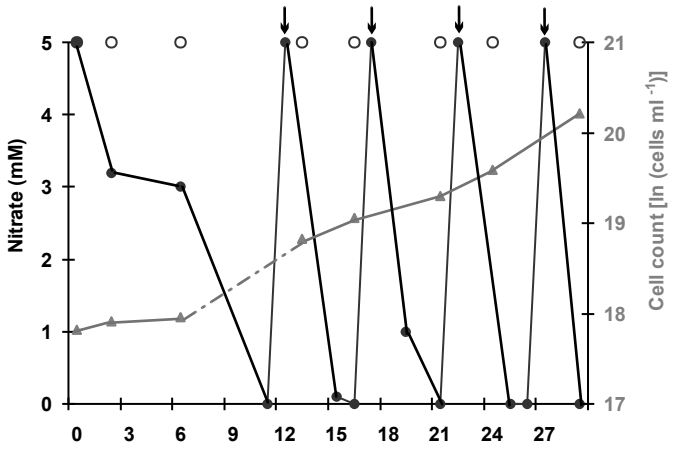

Nitrate (mM)
Cell count [ln (cells ml-1)]
0
1
2
3
4
5
17
18
19
20
21
0
3
6
9
12
15
18
21
24
27

Fig. 1.

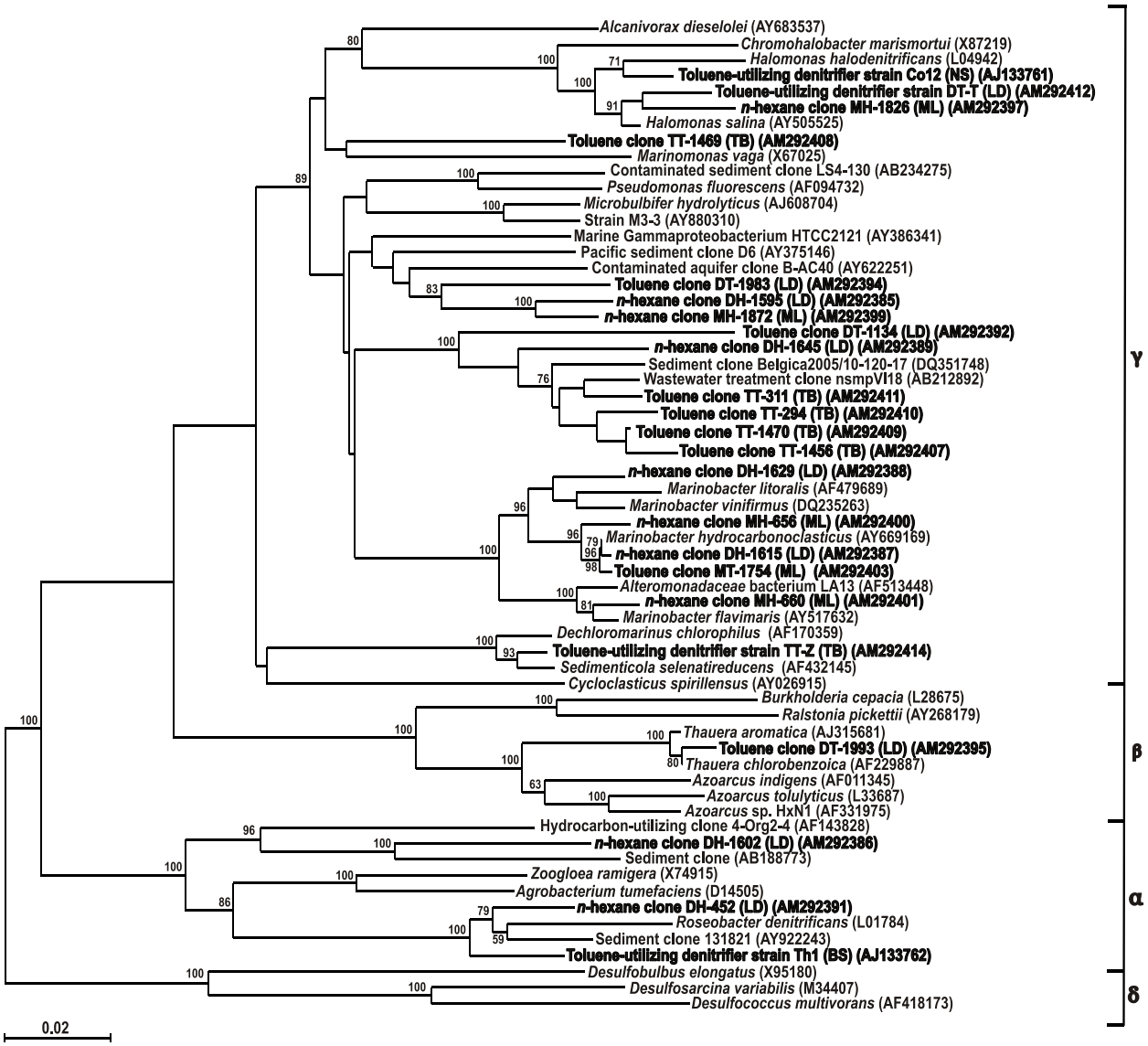
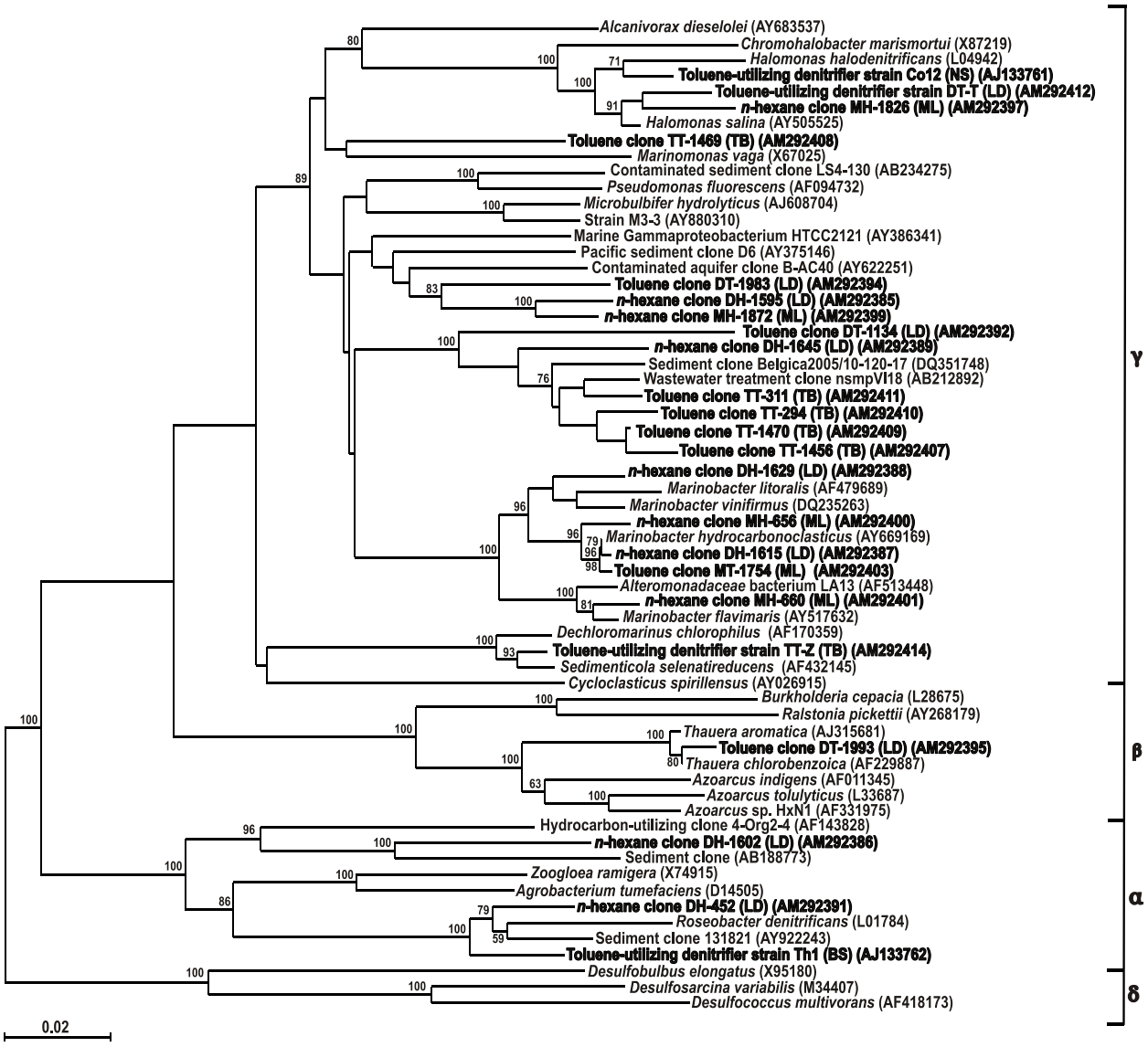

Alcanivorax dieselolei (AY683537)
Chromohalobacter marismortui (X87219)
Halomonas halodenitrificans (L04942)
Toluene-utilizing denitrifier strain Co12 (NS) (AJ133761)
Toluene-utilizing denitrifier strain DT-T (LD) (AM292412)
n-hexane clone MH-1826 (ML) (AM292397)
Halomonas salina (AY505525)
Toluene clone TT-1469 (TB) (AM292408)
Marinomonas vaga (X67025)
Contaminated sediment clone LS4-130 (AB234275)
Pseudomonas fluorescens (AF094732)
Microbulbifer hydrolyticus (AJ608704)
Strain M3-3 (AY880310)
Marine Gammaproteobacterium HTCC2121 (AY386341)
Pacific sediment clone D6 (AY375146)
Contaminated aquifer clone B-AC40 (AY622251)
Toluene clone DT-1983 (LD) (AM292394)
n-hexane clone DH-1595 (LD) (AM292385)
n-hexane clone MH-1872 (ML) (AM292399)
Toluene clone DT-1134 (LD) (AM292392)
n-hexane clone DH-1645 (LD) (AM292389)
Sediment clone Belgica2005/10-120-17 (DQ351748)
Wastewater treatment clone nsmpVI18 (AB212892)
Toluene clone TT-311 (TB) (AM292411)
Toluene clone TT-294 (TB) (AM292410)
Toluene clone TT-1470 (TB) (AM292409)
Toluene clone TT-1456 (TB) (AM292407)
n-hexane clone DH-1629 (LD) (AM292388)
Marinobacter litoralis (AF479689)
Marinobacter vinifirmus (DQ235263)
n-hexane clone MH-656 (ML) (AM292400)
Marinobacter hydrocarbonoclasticus (AY669169)
n-hexane clone DH-1615 (LD) (AM292387)
Toluene clone MT-1754 (ML) (AM292403)
Alteromonadaceae bacterium LA13 (AF513448)
n-hexane clone MH-660 (ML) (AM292401)
Marinobacter flavimaris (AY517632)
Dechloromarinus chlorophilus (AF170359)
Toluene-utilizing denitrifier strain TT-Z (TB) (AM292414)
Sedimenticola selenatireducens (AF432145)
Cycloclasticus spirillensus (AY026915)
Burkholderia cepacia (L28675)
Ralstonia pickettii (AY268179)
Thauera aromatica (AJ315681)
Toluene clone DT-1993 (LD) (AM292395)
Thauera chlorobenzoica (AF229887)
Azoarcus indigens (AF011345)
Azoarcus tolulyticus (L33687)
Azoarcus sp. HxN1 (AF331975)
Hydrocarbon-utilizing clone 4-Org2-4 (AF143828)
n-hexane clone DH-1602 (LD) (AM292386)
Sediment clone (AB188773)
Zoogloea ramigera (X74915)
Agrobacterium tumefaciens (D14505)
n-hexane clone DH-452 (LD) (AM292391)
Roseobacter denitrificans (L01784)
Sediment clone 131821 (AY922243)
Toluene-utilizing denitrifier strain Th1 (BS) (AJ133762)
Desulfobulbus elongatus (X95180)
Desulfosarcina variabilis (M34407)
Desulfococcus multivorans (AF418173)
0.02
γ
β
α
δ

Fig. 2.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Chromohalobacter marismortui (X87219)
Halomonas halodenitrificans (L04942)
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Toluene-utilizing denitrifier strain DT-T (LD) (AM292412)
n-hexane clone MH-1826 (ML) (AM292397)
Halomonas salina (AY505525)
Toluene clone TT-1469 (TB) (AM292408)
Marinomonas vaga (X67025)
Contaminated sediment clone LS4-130 (AB234275)
Pseudomonas fluorescens (AF094732)
Microbulbifer hydrolyticus (AJ608704)
Strain M3-3 (AY880310)
Marine Gammaproteobacterium HTCC2121 (AY386341)
Pacific sediment clone D6 (AY375146)
Contaminated aquifer clone B-AC40 (AY622251)
Toluene clone DT-1983 (LD) (AM292394)
n-hexane clone DH-1595 (LD) (AM292385)
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0.02
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Fig. 3.

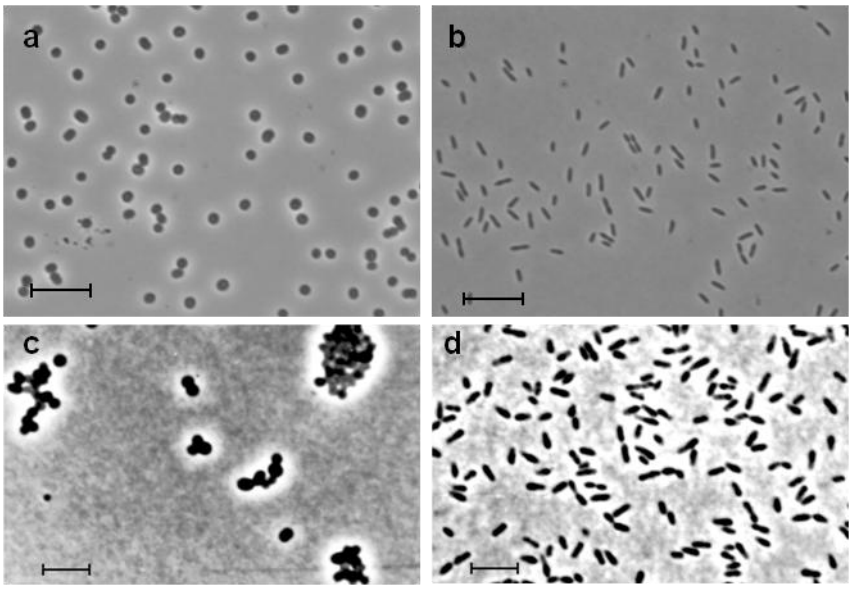

Fig. 4.