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FEMS Microbiology Ecology 42 (2002) 37–49

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Anaerobic biodegradation of squalene: Using DGGE to monitor the isolation of denitrifying Bacteria taken from enrichment cultures

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Received 13 December 2001; received in revised form 12 May 2002; accepted 27 May 2002

First published online 24 July 2002

Abstract

The process of enrichment and subsequently isolation of squalene degrading denitrifying bacteria has been developed. The enrichment method used in this study targeted denitrifying bacteria, therefore an initial enrichment incubation using nitrate amendments under anaerobic conditions was performed before squalene amendment. Denaturant gradient gel electrophoresis (DGGE) analysis of polymerase chain reaction (PCR)-amplified DNA fragments prepared from extracted DNA was used to compare the composition of bacterial communities at various steps of enrichment cultures and the diversity of the 80 isolated strains obtained by classical culture methods. After 8 months of anaerobic incubation, the squalene biodegradation rate reached 80%. The community composition changed substantially during the incubation time. The enrichment cultures were dominated by 12 phylotypes, of which eight corresponded to cultivatable strains. Their identities were established by sequencing V3–V5 16SrRNA PCR fragments directly or after excision of DGGE bands and comparing the sequences with those available in GenBank. Most of the isolates were Proteobacteria of the gamma subgroup; among them, seven novel denitrifying bacteria which were capable of using squalene as the sole carbon source, were isolated and characterized.

Keywords: Denitrification; Squalene; Isoprenoid; Biodegradation; Denaturant gradient gel electrophoresis

1. Introduction

Lipids are generally considered to be less labile than carbohydrates and proteins and to have the potential to be preserved in marine sediments. The composition and lability of individual classes of lipid is an important issue both for predicting the potential for preservation of material in the sedimentary record and for assessing sedimentary inputs from biomarker distribution [1]. Among lipids a range of lability is apparent and depends upon the environmental conditions. Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6*E*,10*E*,14*E*, 18*E*,22-hexaene), an acyclic triterpene compound (C_{30}), which is an important synthetic precursor of steroids, is generally considered to be one of the most abundant natural biogenic isoprenoid compounds [2–4]. Isoprenoids are present in a large fraction of the organic matter on earth [5] and their chemical structure is generally considered as recalcitrant towards degradation. The biodegradation of these compounds by aerobic bacteria has been extensively studied [6–8] and it is generally accepted that the metabolism of these compounds requires molecular oxygen for mono- or di-oxygenase catalyzing oxygen insertion into the molecule. Under oxygen-depleted conditions, anaerobic bacteria must apply a different biochemical strategy [5]. Available data concerning the anaerobic bacterial degradation of isoprenoids in general are still very limited [9–11] and more studies of the reactions which modify isoprenoid distribution in microbially active sediments are essential [12].

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0168-6496/02/$22.00
PII: S0168-6496(02)00304-5

Pure culture isolation is an inescapable step in assessing the metabolic pathways of biodegradation. However, previous studies investigating isolation of hydrocarbon utilizing bacteria have shown discrepancies between the metabolic potential of the bacterial communities within the sediment and that of pure isolates. This reflected the difficulty of isolating organisms capable of using such substrates as sole carbon and energy sources from hydrocarbon degrading mixed cultures. This may be due to (i) the inability of some organisms to grow on the solidified medium although it may have the same composition as the liquid selective medium or (ii) an insufficient number of colonies screened to detect variation among the phenotypically similar but genotypically different isolates. Testing the capability of numerous denitrifying bacteria to degrade recalcitrant compounds is fastidious and time consuming and consequently the isolation step is often limited to only a few colonies exhibiting different morphologies. This increases the risk of missing relevant strains in low number that show similar morphology to irrelevant strains. Whenever the metabolic potential of a community has been demonstrated, molecular techniques such as denaturant gradient gel electrophoresis (DGGE) make it possible to know its structure without isolation. After DNA extraction of the community, polymerase chain reaction (PCR) products of 16S rRNA V3–V5 gene fragments can be separated according to their electrophoretic mobility along a denaturating gradient. DGGE can sort DNA fragments of identical length but with different nucleotide sequences. DGGE can detect up to 95% of all possible single base substitutions among sequences of up to 1000 bp in length [13]. After electrophoresis, major DGGE bands corresponding to uncultured strains can be excised from the gel for subsequent phylogenic analysis. In this study, after investigating the metabolic potential of a marine community of the sediment for squalene degradation under anaerobic conditions, we have simultaneously used culture-based and molecular methods to monitor the dynamics of the microbial community through each step of bacterial isolation. More than 80 bacterial strains have been isolated at random and sorted according to the electrophoretic mobility on DGGE. Comparison of the fingerprint of the isolate obtained from the selective medium and DGGE pattern of the enrichment cultures has allowed us to adjust the isolation strategy and to identify the metabolically important members of this community able to degrade squalene in anaerobiosis.

2. Materials and methods

2.1. Sampling

The isolation procedure was carried out on sediment collected at Carteau cove (Gulf of Fos, French Mediterranean coast). The sampling site is located 12 miles west of Marseille near the mouth of the river Rhône on the French Mediterranean coast. Undisturbed sediment cores were taken by hand in plexiglass tubes at 5 m water depth [14]. Sediment cores were stored at 4°C during transport to the laboratory and analyzed upon arrival.

2.2. Procedure for enrichment cultures and isolation of bacteria

Squalene degrading denitrifying pure cultures were obtained from enrichment cultures of denitrifying bacteria subsequently selectively cultured in the presence of squalene. 1-ml portions of sediment were used to inoculate the enrichment medium which consisted of 5 ml filtered (0.2 μm, cellulose acetate filters) natural sea water supplemented with 10 mM KNO_3, as the electron acceptor. Thirteen denitrifying enrichment cultures were incubated in 22-ml vials sealed with rubber stoppers (sample T-init) at 20°C. No carbon source was added for the first 2 months in order for the bacterial communities to consume any existing organic matter present in the sediment (Fig. 1). The nitrate concentration (10 mM) was maintained throughout the incubation period by adding sterile nitrate. When the rate of nitrate consumption decreased, indicating a depletion in the organic matter of the sediment, 8 mg of squalene was added to each vial as the sole carbon and energy source (sample R-T0). Anaerobic conditions were obtained by flushing nitrogen through the vials for 1 h, cultures were then incubated under these conditions. Squalene degradation was monitored over an 8-month period (sample R-T1, after 8 months of incubation). A 0.2-ml aliquot was then taken from one of these vials and transferred to a flask containing 50 ml of fresh artificial sea water (ASW, [15]) supplemented with nitrate (10 mM) and squalene (0.7 mM). This subculture was incubated for 4 months under the same conditions as above (sample R2). Killed controls were prepared using autoclaved sediments, nitrate and squalene.

For each enrichment culture (R-T0, R-T1, R2), the sediment or culture slurries were serially diluted and appropriate dilutions were spread onto solid TBA medium containing 10 mM KNO_3, 1 g of lactate, 1 g of Na-acetate, 1 g of glucose, 2 g of yeast extract [Merieux], 1 g of Biotrypcase [Merieux] and 15 g of agar [Merieux] per liter of ASW. The anaerobic plates were incubated for 2 weeks at 20°C in a desiccator where oxygen was removed using the Gas Pak system [Becton Dickinson]. For each of the samples, R-T0, R-T1 and R2, about 20 colonies, exhibiting different morphologies, were picked out from the different dilutions and re-plated to check for purity. From sample R2, bacteria were also isolated under aerobic conditions, in which a set of agar plates were incubated at the same temperature but in the presence of oxygen. Twelve strains were isolated under these conditions.

Anaerobic isolates originating from samples R-T0, R-T1, and R2 were called 0sq, 1sq and 2sq respectively,

whereas aerobic isolates originating from R2 samples were called 2Asq. All isolates were stored in liquid nitrogen in the presence of glycerol (20%).

2.3. Bacterial activities and number quantification

Denitrifying activities were performed according to Bonin et al. [14]. Enumeration of denitrifying bacteria by the N_2O-MNP technique was performed according to Bonin [16] with the help of Cochran tables [17].

2.4. Nutritional pattern

Biolog GN microplates (Biolog TNC, Hayward, CA, USA) were used to investigate the carbon patterns of the strains. Cells suspension was adjusted to the turbidity standard GN-NENT provided by the manufacturer (Biolog TNC, Hayward, CA, USA) and inoculated in the 96 wells of the microplates. Utilization of carbon sources was detected as an increase in the respiration of cells in the well of a microplate, leading to irreversible reduction of the tetrazolium dye. A positive utilization reaction was indicated when a purple color formed in a well. After 2 days incubation at 20°C the breathprint pattern that resulted was read with an automated microplate reader.

2.5. Growth conditions

The basic medium consisted of autoclaved ASW supplemented with iron sulfate (0.1 mM), potassium phosphate (0.33 mM) and squalene (0.7 mM) as the carbon source. Aerobic growth experiments (50 ml of culture) were performed in 250-ml Erlenmeyer flasks shaken on a reciprocal shaker (96 rev. min^{-1}, 5 cm amplitude). Anaerobic growth experiments were performed in 100-ml serum flasks containing 50 ml of the same medium supplemented with KNO_3 (30 mM). Anaerobic conditions were obtained as described above, and cultures were magnetically stirred at 300 rpm. For each experiment, two flasks were inoculated: the first to monitor growth and nitrate reduction, the second to measure substrate degradation. Control experiments without cells but in the presence of squalene and nitrate were carried out in parallel. Growth was monitored by measuring the optical density at 610 nm with a Shimatzu UV 240 spectrophotometer during 25 days and 100 days for aerobic and anaerobic incubations respectively.

2.6. Chemicals and analytical procedures

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6*E*,10*E*,14*E*,18*E*,22-hexaene) (Aldrich) was purified using column

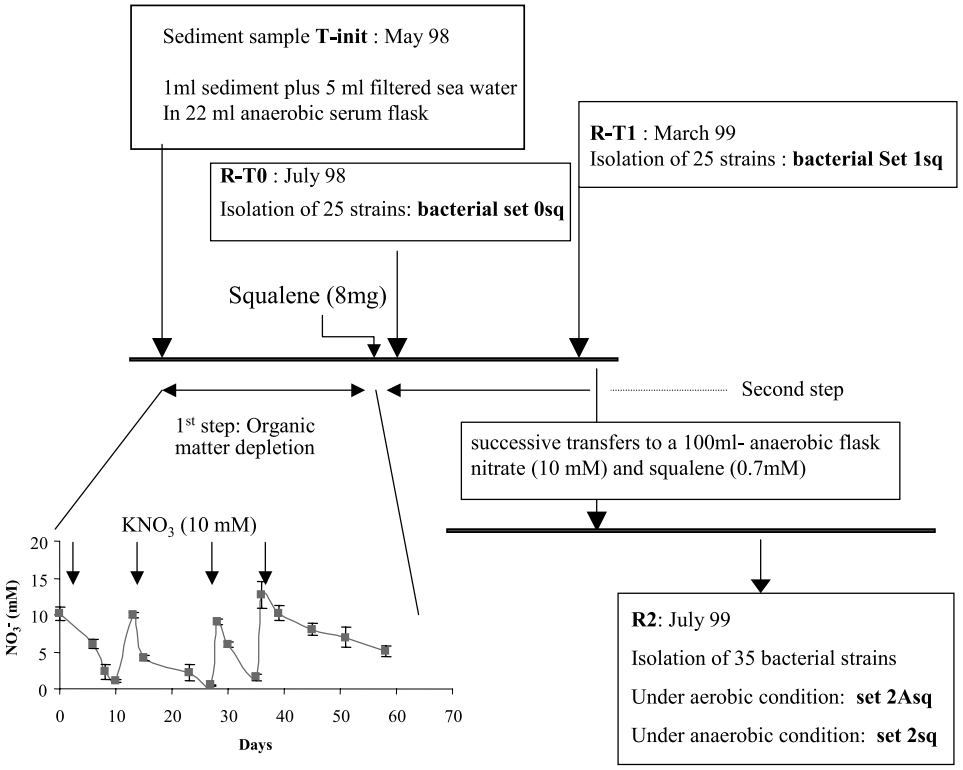

Fig. 1. Procedure developed for the enrichment culture and the isolation of squalene degrading bacteria. Enrichment cultures were inoculated with 1 ml of sediment (sample T-init). For the first 2 months (first step), no carbon source was added and nitrate concentration (10 mM) was maintained throughout the incubation period by adding sterile nitrate. When the rate of nitrate consumption decreased, indicating a depletion in the organic matter of the sediment, 8 mg of squalene was added to each vial as the sole carbon and energy source (sample R-T0). After 8 months of incubation (sample R-T1), two successive transfers were performed in fresh medium. The second subculture was incubated for 4 months under the same conditions (sample R2). R-T0, R-T1, and R2 samples were used to isolate bacterial strains. Anaerobic isolates originating from R-T0, R-T1, and R2 samples were called 0sq, 1sq and 2sq, respectively, whereas aerobic isolates originating from R2 samples were called 2Asq.

chromatography on silica gel (Kieselgel 60+0.5% H_2O) with hexane as the eluent.

For cultures containing sediment, the aqueous and solid phases were separated at the end of the incubation period. The aqueous phase was continually extracted with chloroform overnight, whilst the wet solid phase was extracted ultrasonically with isopropanol/hexane (4:1, v/v) [18]. The chloroform and hexane extracts were combined, dried over anhydrous Na_2SO_4, filtered and concentrated by rotary evaporation to yield the residual substrate. In the case of the isolates, the contents of the flasks were extracted by continual extraction with chloroform for 24 h; this results in a neutral extract containing the residual substrate. Following the evaporation of the solvents, the different extracts were taken up in 400 μl of a mixture of pyridine and bis(trimethyl)trifluoroacetamide (3/1, v/v) and allowed to silylate at 50°C for 1 h. Following evaporation, the dry residue was taken up in ethyl acetate (1 mg ml^{-1}) and analyzed. The residual substrate was quantified (calibration using an external standard) by gas chromatography/electron impact mass spectrometry (GC/EIMS). GC/EIMS analyses were carried out with an HP 5890 series II plus gas chromatograph connected to an HP 5972 mass spectrometer (Hewlett-Packard). Operating conditions were as follows: 30 m×0.25 mm (i.d.) capillary column coated with HP 5 (Hewlett Packard); the oven temperature was programmed from 60 to 130°C at 30°C min^{-1} and then from 130 to 300°C at 4°C min^{-1}; helium carrier gas pressure was maintained at 1.05 bar until the end of the temperature program and then programmed from 1.05 to 1.5 bar at 0.04 bar min^{-1}; injector (on column) temperature, 50°C; electron energy, 70 eV; source temperature, 170°C; cycle time, 1.5 s.

Assays for NO_3^- and NO_2^- concentrations were carried out using the procedure of Treguer and Lecorre [19]. The nitrous oxide concentration in the tubes was determined by GC (HP 5890) using an electron capture detector [20]. Chromatography operating conditions were: 8-ft-long 'Porapak Q' column (mesh 50:80); oven temperature: 80°C; injector temperature: 180°C; detector temperature: 250°C; carrier gas N_2 flow rate: 20 ml min^{-1}.

Ammonium was recovered from the sediment as follows. Pore waters were removed by centrifugation of sediments (5000×*g*, 5 min). Supernatant was stored and KCl (2 M) was added to the sediment pellet at KCl:sediment proportion of 1/1 (v/v) and the mixture was homogenized and then shaken for 1 h at 0°C [21]. The KCl extractable pools obtained after centrifugation at 5000×*g* for 5 min were added to the supernatant. Ammonium concentration was determined by the phenol/hypochlorite method [22].

2.7. Nucleic acid extraction

DNA extraction was performed on the entire vial for enrichment samples R-T0 and R-T1 and on 1/5 of the enrichment sample (R2). Cells lysis and DNA extraction were performed as described by Zhou et al. [23]. Briefly, the suspensions were repeatedly frozen and thawed, and subsequently incubated in the presence of SDS (1% w/v) and proteinase K (60 μg ml^{-1}) for 30 min at 60°C. DNA was extracted by applying CTAB (1%, w/v) (hexadecylmethylammonium bromide), phenol, chloroform and isoamylalcohol and precipitated from the aqueous phase by the addition of 0.6 volume of isopropyl alcohol. DNA extracted from R-T0 and R-T1 were subsequently purified by gel filtration chromatography in order to remove the humic acid (G25 Pharmacia).

2.8. PCR amplification of 16S rRNA gene fragments

A conserved region of approximately 568 bp from the 16S rDNA was amplified using the primer GM5F (5′-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and a mixture of two reverse primers, 907R (5′-CCGTCAATTCCTTT(A/G)AGTTT-3′) and 907RA (5′-CCGTCAATTCATTTGAGTTT-3′) [24]. A 40-nucleotide GC-rich sequence was attached to the 5′ end of the primer GM5F to improve the detection of sequence variation in the amplified DNA fragments by subsequent DGGE [25]. These primers are specific for almost all eubacterial 16S rDNA gene fragments. As the template for amplification, 100 ng of DNA extract was added to the PCR mixture. The PCR reaction volume was adjusted to 100 μl for analysis of the enrichment culture samples or to 25 μl for the analysis of pure strains patterns. PCR amplifications were carried out in 20 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, containing 0.2 mM of each deoxyribonucleotide triphosphate, 50 pM of each oligonucleotide primer, and 0.5 U of Taq polymerase (Boehringer Mannheim). PCR conditions, including the hot start and a touchdown for primer annealing, were similar to those used by Muyzer et al. [26]. The thermal cycling program was as follows: initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, and a touchdown primer annealing from 65 to 55°C for 1 min (annealing temperature decreased 1°C for each second cycle to a final temperature of 55°C), annealing at 55°C for 1 min for the next 14 cycles and then a primer extension at 72°C for 1 min. A final extension at 72°C for 5 min was then performed.

2.9. DGGE analysis

DGGE was carried out using a Dcode system (Bio-Rad Laboratories Inc, Hercules, CA, USA). Various amounts, depending on the DNA amplification, were loaded beside migration standard (16S rRNA V3–V5 gene fragments of *Clostridium perfringens*, 560 bp, G+C = 52.8%, *Marinobacter hydrocarbonoclasticus*, 580 bp, G+C = 51% and *Micrococcus luteus*, 571 bp, G+C = 57.6%) onto 1-mm-thick, 6% (w/v) polyacrylamide gels with a denaturation gradient from 30 to 50%, and electrophoresis was run for 5.5 h at

150 V using 1×TAE (40 mM Tris–HCl [pH 8.3], 20 mM acetic acid, 1 mM EDTA) as the electrophoresis buffer. Subsequently, the gels were incubated for 30 min in 1×TAE containing ethidium bromide (0.5 μg ml^{-1}) and photographed under UV transillumination. The bands located at the top and the bottom of the gel corresponded to 572- and 571-bp PCR fragments of 16S rRNA gene with G+C% of 49 and 57.6% respectively.

2.10. Sequencing of 16S rDNA gene fragment

PCR products of partial 16S rRNA gene from the isolates were sequenced using both strands by MWG Biotech (Germany). After PCR of the DNA from the enrichment cultures and DGGE analyses, major DGGE bands that do not correspond to DGGE profile of strains were excised from the gel. Each single band was removed from the gel by diffusion in distilled water and re-amplified by PCR under the same conditions. After DGGE analysis of the re-amplified band to verify the purity, the products of PCR were sequenced.

2.11. Comparative analysis of 16S rRNA sequences

The 16S rRNA partial sequences were aligned with the same region of the closest relative strains available in the GenBank database by using the BLASTN facility (http://www2.ncbi.nlm.nih.gov/BLAST/). Sequence alignment was achieved using Clustalw [27]. The phylogenetic tree was constructed using the neighbor-joining method [28]. A bootstrap analysis with 100 replicates was carried out to check the robustness of the tree. Finally, the tree was plotted using the Tree View program [29].

Nucleotide sequence accession number. The sequences obtained in this study are available from EMBL under the accession numbers AJ429499, AJ439500 and from AJ458192 to AJ458202.

3. Results

3.1. sampling site

The sediment was silty and contained 17 g of organic carbon kg^{-1} dried sediment. Nitrate and ammonium concentrations in interstitial water were 3.7 and 230 μM respectively. At this site, denitrification rate ranged from 7 to 10 μmol l^{-1} day^{-1} and the most probable numeration gives values of about 5×10^5 denitrifying bacteria ml^{-1}. These data were in accordance with those found at the same site [14].

3.2. Degradation of squalene in the different enrichment cultures

The isolation strategy was defined in two successive steps (Fig. 1). The first step corresponds to the simultaneous enrichment of denitrifiers and the depletion of endogenous organic matter (from May to July 1998). The second step following squalene addition permitted the selection of denitrifying-squalene degrading bacteria. In sediment slurries the concentration of squalene was monitored over 8 months (from July 1998, sample R-T0, to March 1999, sample R-T1). After 3 and 6 months of incubation 15 and 50% of the squalene had been respectively depleted when compared to the sterile control. An additional 2 months did not show an increase in the percentage of degradation. In the controls, none of the compounds disappeared over the three first months; and after 8 months of incubation, 95% still remained.

To establish enrichment cultures without sediment, successive transfers to a defined medium with squalene were made from sample R-T1. Consecutive subcultures confirmed the biodegradation previously observed in R-T1. Following two subcultures, a mixed culture was obtained called R2. About 15% substrate degradation had occurred after 45 days of R2-growth under denitrifying conditions.

3.3. DGGE analysis of bacterial communities and the relationship of DGGE profiles from enrichment cultures and isolates

From each enrichment step (R-T0, R-T1 and R2) about 20 strains were isolated at random and from different dilutions of the enrichment cultures. The DNA of dominant 16S rDNA-defined communities in the enrichment culture were monitored by DGGE analysis. The profiles were then compared to those of the isolates (Fig. 2). These DGGE analyses were performed prior to the addition of squalene (R-T0), after 8 months of incubation (R-T1), in the last subculture following 45 days incubation (R2) and with the four sets of isolated strains (0sq, 1sq, 2sq, 2sqA), i.e. 80 strains. The electrophoresis of fragments on a denaturing gel gradient results in drastic mobility shifts of DNA molecule at some position due to sequence-dependent melting of molecule domains [13]. All DGGE patterns were reproducible. Most of the strains gave a narrow band after DGGE. Two of them (#4 and #7) gave a broad band resulting probably from the existence of several cistrons encoding 16S RNAs with different sequences within a single genome [30]. Bands were referenced according to their electrophoretic mobility (#1–#11) (Fig. 2). Bands presenting the same mobility were assigned to the same number. The profile generated by the PCR product of aliquot R-T0 (lane 3) showed three bands. When this pattern (lane 3) was compared with electrophoretic mobility of the DNA fragments of the 0sq isolates (lanes 4–7), two bands of R-T0 matched with DGGE patterns obtained from 0sq bacterial set (patterns #1 and #4). With regard to the DGGE bacterial community profiles, addition of squalene causes a dramatic change in the community profile (Fig. 2, lane 8). Band patterns of R-T0 and R-T1 aliquots were notably

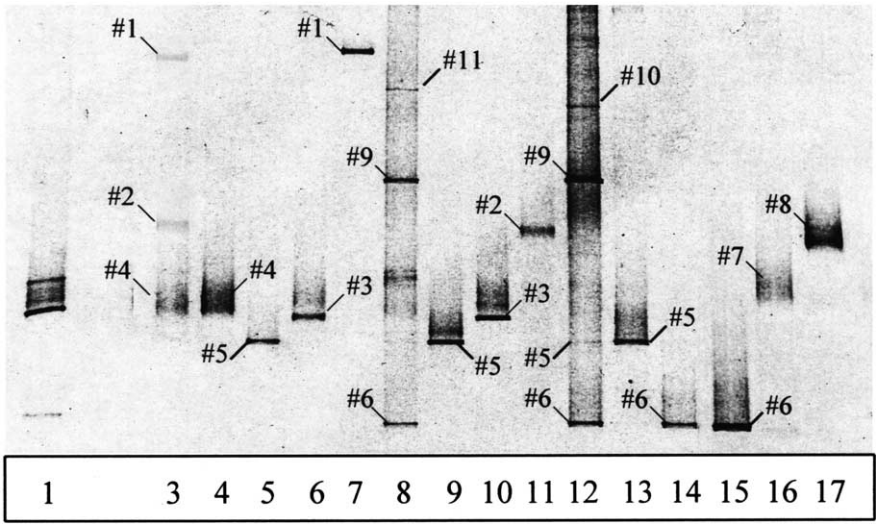

Fig. 2. Negative images of DGGE separation patterns of the 16S rRNA V3–V5 gene fragments obtained with primers specific for the domain Bacteria and template DNA extracted from R-T0, R-T1 or R2 enrichment cultures (lanes 3, 8, 12 respectively) and from strains isolated from the same enrichment cultures from 0sq set (lanes 4, 5, 6, 7), 1sq set (lanes 9, 10, 11), 2sq set in anaerobiosis (lanes 13 and 14) and 2Asq set in aerobiosis (lanes 15–17). Lane 1: migration standard from top to bottom (*Clostridium perfringens*, 560 bp, G+C = 52.8%; *Marinobacter hydrocarbonoclasticus*, 580 bp, G+C = 51%; and *Micrococcus luteus*, 571 bp, G+C = 57.6%).

different. The R-T1 aliquot showed many more intensive bands. We note the appearance of additional major bands referenced as # 6, #9 and #11. None of these bands matched with the DGGE pattern of 1sq isolates (lanes 9–11). In subculture R2 (lane 12), where most of the sediment was eliminated, two bands already observed in R-T1 pattern were still present and of greater intensity (bands #6 and #9). One fine band (band #10) and another matching with pattern #5 appeared. Band #11 which was present in aliquot R-T1 became undetectable. When R2 pattern was compared with electrophoretic mobility of the DNA fragment of the 2sq and 2Asq isolates, two bands of R2 matched with patterns of 2sq isolates (#6, lanes 14,15 and #5, lane 13). Among the strains isolated under aerobic conditions, two new patterns (#7, lane 16, and #8, lane 17) appeared within the 2Asq bacterial set.

The bacterial community from R2 was used to inoculate fresh squalene medium and incubated under aerobiosis (Com+O_2; Fig. 3). The DGGE profile of Com+O_2 presented 2 main bands (#6 and #7) that presented the same electrophoretic mobility as the 2Asq 71 (#6, lane 15, Fig. 2) and 2Asq64 (#7, lane 16, Fig. 2) isolates. The bacterial community from R2 was also used to inoculate fresh squalene medium and incubated under anaerobiosis (Com−O_2) (Fig. 3). The DGGE profile of the Com−O_2 subculture presented five bands including two bands corresponding to the 2sq31 (#5) and 2sq55 (#6) isolates.

For the four sets of isolates (0sq, 1sq, 2sq and 2Asq), information obtained from the DGGE patterns and from nitrate metabolism studies (strains were characterized as denitrifying, nitrate reducing and nitrate non-reducing) are summarized in Fig. 4. Among the isolates, numerous strains presented the same DGGE pattern. Among the 21 0sq isolates (Fig. 3A), 15 strains showed the DGGE pattern referenced as #4. Each of the other patterns referenced #1, #3 and #5 were represented by only two strains. Strains with the same DGGE pattern might present differences in nitrate respiration potential. For instance, among the 15 strains showing DGGE pattern #4, nine were denitrifiers, four nitrate reducers and two were not able to use nitrate as electron acceptor. Twenty strains were isolated from the enrichment culture R-T1 (Fig. 3B). They were distributed among three DGGE patterns (#2, #3 and #5). All the strains showing the same DGGE pattern presented the same nitrate metabolism. From subculture R2, pattern #6 was the major DGGE pattern observed among the 27 strains isolated under anaerobic conditions (2sq set) (Fig. 3C). Only two strains presented pattern #5. Greater biodiversity was observed among the 12 strains isolated from the same subculture R2 but under aerobic conditions (2Asq) (Fig. 3D). About half of the strains presented pattern #6, already observed with the strains isolated under anaerobic conditions. Only one DGGE pattern (#5) was found in all steps of anaerobic enrichment. In contrast, some DGGE patterns were found in only one set of isolates: #1 and #4 in 0sq bacterial set or #2 in 1sq bacterial set and patterns #7 and #8 in 2Asq. Among the 12 DGGE bands detected from the enrichment culture (R-T0, R-T1, R2, Com−O_2 and Com+O_2), only eight matched with the DGGE pattern of cultivable strains isolated from these cultures. Bands #9, #10 and #12, which did not correspond to the pattern observed with the isolates, were excised from the gel, re-amplified and re-run on a denaturing gel. Following electrophoresis, only one band was obtained for each sample (#9, #10 and #12; data not shown). Moreover the PCR product (#9, #10 and #12 re-amplified) can be sequenced. Thus, we believe that bands #9, #10 and #12 did not correspond

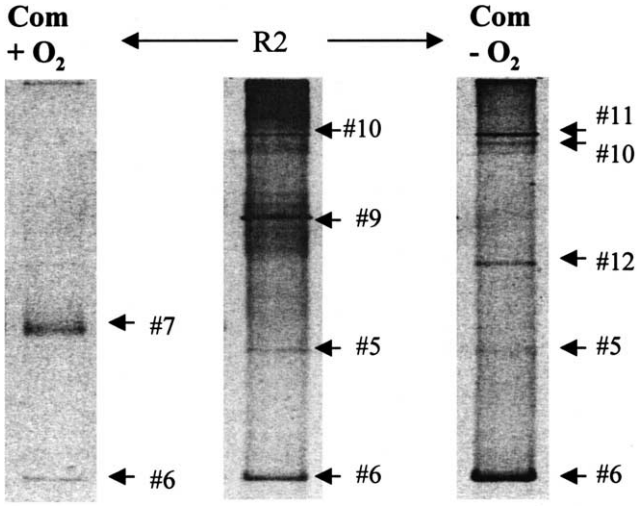

Fig. 3. DGGE separation pattern of PCR-amplified sequences of 16S rRNA genes derived from cultures inoculated with R2 sample, and incubated under aerobic conditions for 25 days (Com+O_2) or anaerobic conditions for 100 days (Com−O_2). This figure is a negative picture of an ethidium bromide-stained gel.

to the formation of heteroduplex, but were proof of the presence of unculturable strains in the bacterial community. However, the sequence of the PCR product of the #12 band contained too many ambiguous positions to be positioned in a phylogenetic tree with certainty. In contrast, band #11 was not amenable to sequencing due to high levels of background in the sequencing reaction which suggests that this band was composite, made up of two or more distinct sequences possessing similar electrophoretic mobility under the conditions used for DGGE.

3.4. Sequence and phylogenetic analysis

The 16S rRNA gene fragments of one or more strains representing each of the DGGE patterns and the PCR products of the unculturable strains (#9 and #10) were sequenced. From the 528 nucleotide sequences, a phylogenetic tree was constructed, in which the closest relative strains of each phylotype were indicated. For proper phylogenic reconstruction, longer sequences must be used but a 528 nucleotide stretch is sufficient to allow assignation of the 16S rDNA to a main line of descent and allows us to affiliate each isolate to a group or subgroup. Sequences obtained from various isolates showing the same DGGE pattern were identical. Thus, we refer now to all organisms represented by the same DGGE pattern and thus by the same sequence as a phylotype.

The range of identity to reference sequences contained in the databases was 99.8–80.3%. The sequences of the strains which we were unable to culture showed the lowest similarity with the sequences available in the databases. Phylogenetic analysis of the sequences (Fig. 5) revealed the restricted bacterial diversity of the enrichment cultures in the presence of squalene. Most of the isolates are Pro-

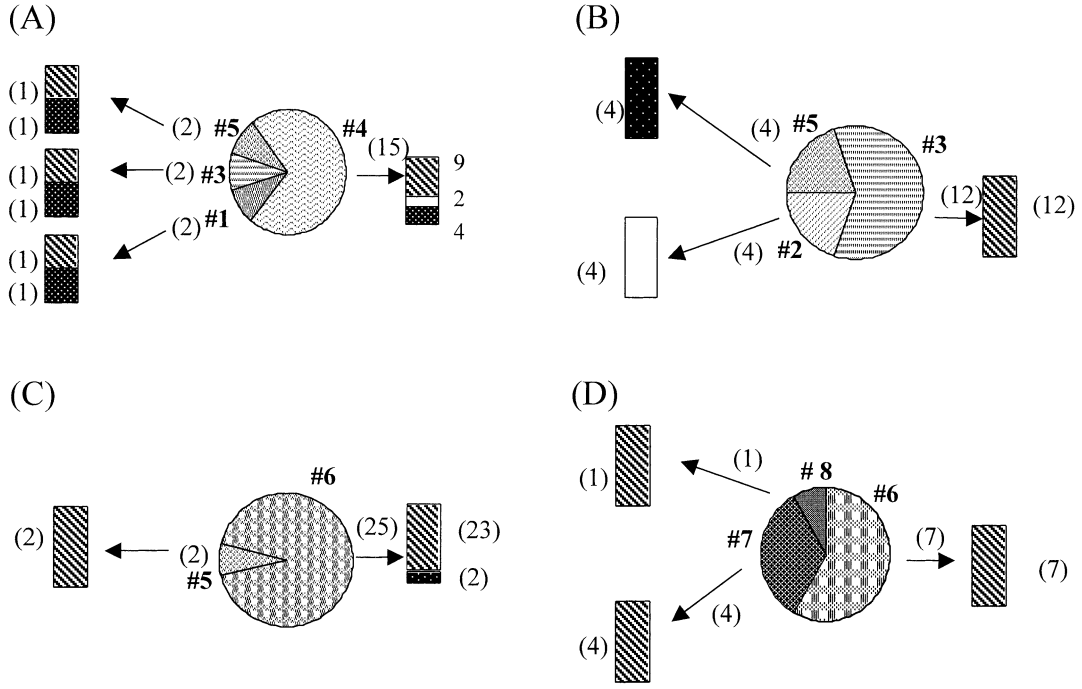

Fig. 4. Bacterial strains isolated from various steps of the isolation procedure (0sq bacterial set from R-T0: A; 1sq bacterial set from R-T1: B; 2sq bacterial set C; and 2Asq bacterial set D from R2) were characterized according their DGGE electrophoretic mobility patterns of the PCR-amplified V3–V5 fragments of 16S rRNA genes (pieces of circles). For each DGGE pattern all the strains were tested for nitrate utilization under anaerobic conditions. Arrows show the distribution of the strains belonging to the same DGGE pattern according their nitrate metabolism (vertical bar). □, Unable to use nitrate as electron acceptor; (dotted boxes) nitrate reducer only, or (striped boxes) denitrifier. Number in brackets represents the number of isolates for each characteristic.

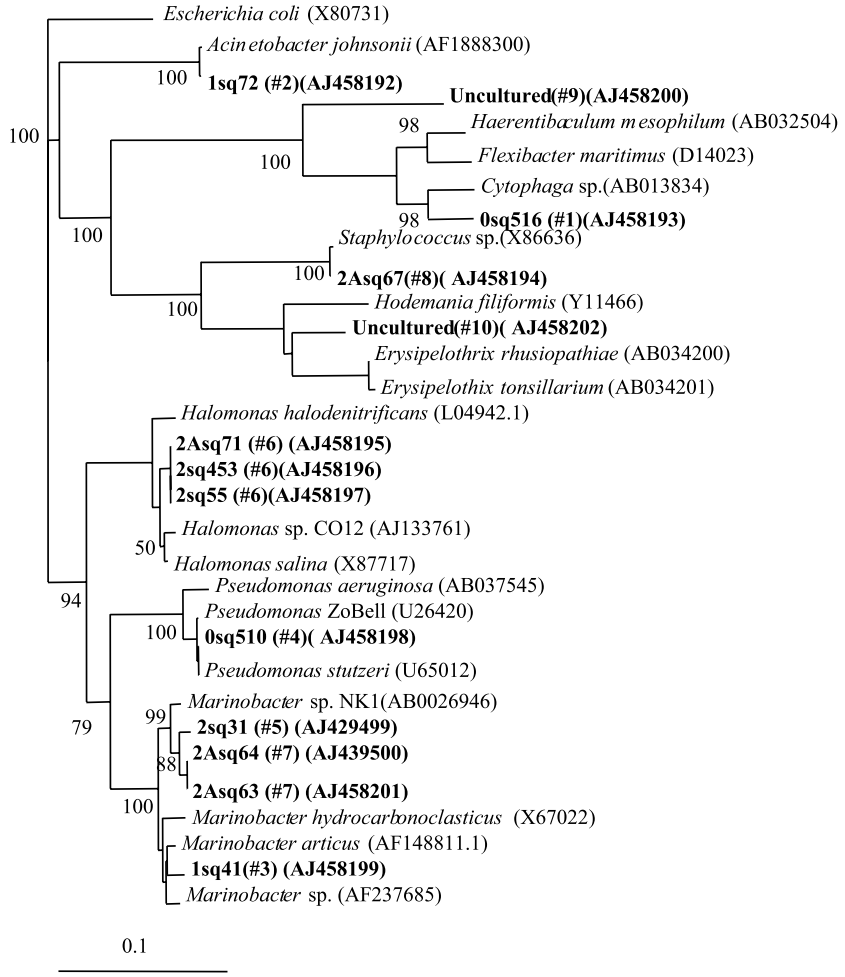

Fig. 5. Inferred phylogenetic relationship between environmental sequences from squalene enrichment cultures and closest reference sequences available in GenBank/EMBL. Neighbor-joining tree based on the partial sequence of 16S rDNA is rooted using *Escherichia coli*. Uncultured #9 and #10 are the sequences from the excised DGGE band. Comparison were made using 580 unambiguous positions. K_{nuc} value = 2. Bootstrap support value (100 replicates) above 50% are shown at nodes.

teobacteria (92.5%), (phylotypes #2–#7). Within the Proteobacteria, all isolates belonging to the gamma subdivision, were members of the genera *Marinobacter*, *Pseudomonas*, *Halomonas* and *Acinetobacter*. The sequences of the isolates 1sq41 (#3), 2sq31 (#5), 2Asq64 (#7) and 2Asq63 (#7) grouped with *Marinobacter* and showed about 98% similarity with one another. The group of sequences clustering with *Halomonas*, 2Asq71, 2sq55 and 2sq453 (#6), showed approximately 88% similarity with the *Marinobacter* group. Only about 7.8% of the isolates were affiliated to other groups. Green sulfur bacteria group were presented by phylotype #1 (2.9%) and low G+C Gram-positive bacteria of the *Bacillus/Clostridium* were presented by phylotype #8 (4.9%). Sequences of 2Asq67 and 0sq516 showed less than 65% similarity to the sequence clustering in the gamma Proteobacteria.

3.5. Analysis of the substrate utilization patterns of the isolates

The ability of the isolates to use different organic compounds as the sole carbon and energy source was studied. Results are reported in Table 1. Of the 31 carbohydrates tested only a few substrates can be used by the isolates. Most of the isolates were unable to metabolize the tested alcohols, only glycerol was used by 0sq516, 0sq510 and 1sq41. Apart from 2sq31, 1sq41, 2Asq63 and 2Asq67, which used less than four of the 28 organic acids tested, these kinds of substrates seem to be the ones which are used in preference by the other isolates.

The carbon utilization pattern for each strain, estimated by the total number of substrates oxidized in Biolog GN plates, was used for cluster analysis and the creation of a dendogram using Manhattan similarity indices (Fig. 6B). Examination of Fig. 6 suggests that there are quite a number of considerable differences between nutritional and phylogenetic analyses (Fig. 6A,B). According to the Manhattan indices, the tested strains were divided into three groups corresponding to: the *Bacillus/Clostridium* group (2Asq67), the CFB group (0sq516) and the proteobacterial group gamma subdivision for the other strains. Nutritional patterns did not permit the affiliation of strains belong-

Table 1
Nutritional screening of the different isolates that are able to degrade squalene

Substrate tested	Substrate used										
	0sq510 #4	0sq516 #1	1sq41 #3	1sq72 #2	2sq31 #5	2sq453 #6	2sq55 #6	2Asq71 #6	2Asq63 #7	2Asq64 #7	2Asq67 #8
Carbohydrates and derivatives tested: 31	8	12	3	3	0	5	3	1:	0	0	7
Alcohols tested: 3	0	2	1	0	0	0	0	0	0	0	0
Amino acids tested: 21	12	10	2	7	3	9	7	6	1	6	2
Organic acids and derivatives tested: 28	19	11	1	5	4	17	11	10	2	13	2
Other tested: 11	3	5	5	2	2	4	0	0	2	2	0

Carbohydrates and derivatives: α-D-glucose, α-D-lactose, adonitol, β-methyl-D-glucoside, cellibiose, D-arabitol, dextrin, D-fructose, D-galactose, D-mannitol, D-mannose, D-melibiose, D-psicose, D-raffinose, D-sorbitol, D-trehalose, gentiobiose, glucose-1-phosphate, glucose-6-phosphate, glycogen, *i*-erythritol, L-rhamnose, lactulose, L-arabinose, maltose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, sucrose, turanose, xylitol, *a*-cyclodextrine. Alcohols: 2-3-butanediol, 2-amino ethanol, glycerol. Amino acids: alaninamide, D-alanine, D-serine, glucuronamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy L-proline, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-carnitine. Organic acids and derivatives: acetic acid, α-glutaric acid, α-hydroxybutyric acid, α-keto valeric acid, α-ketobutyric acid, β-hydroxybutyric acid, bromosuccinic acid, *cis*-aconitic acid, citric acid, D-galactonic acid, lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D-L-lactic acid, D-saccharic acid, formic acid, γ-hydroxybutyric acid, itaconic acid, malonic acid, methylpyruvate, monomethyl succinate, *p*-hydroxyl phenylacetic acid, propionic acid, quinic acids, ebacic acid, succinamic acid, succinic acid, urocanic acid. Other: L-pyroglutamic acid, D-L-α-glycerol phosphate, γ-amino butyric acid, inosine, phenylethylamine, putrescine, thymidine, Tween 40, Tween 80, uridine *m*-inositol.

ing to the gamma subdivision of proteobacterial group to the genus level. Strains of the same phylotype revealed notable differences in nutritional patterns. For example, in the 16S rRNA gene sequence analysis all the phylotype #6 sequences were recovered as a monophyletic lineage, yet the corresponding lineage based on the substrate utilization patterns also contained members of the phylotypes #4 and #7.

The capability of one or more strains of set 1sq and 2sq representing each of the phylotypes to use squalene as sole source of carbon and energy were investigated. The growth cultures were incubated for 25 days under aerobic conditions or for 100 days under anaerobic conditions. The extent of biodegradation was compared with sterile controls. Results are reported in Table 2. Under aerobic conditions, four strains of 10 tested were able to degrade

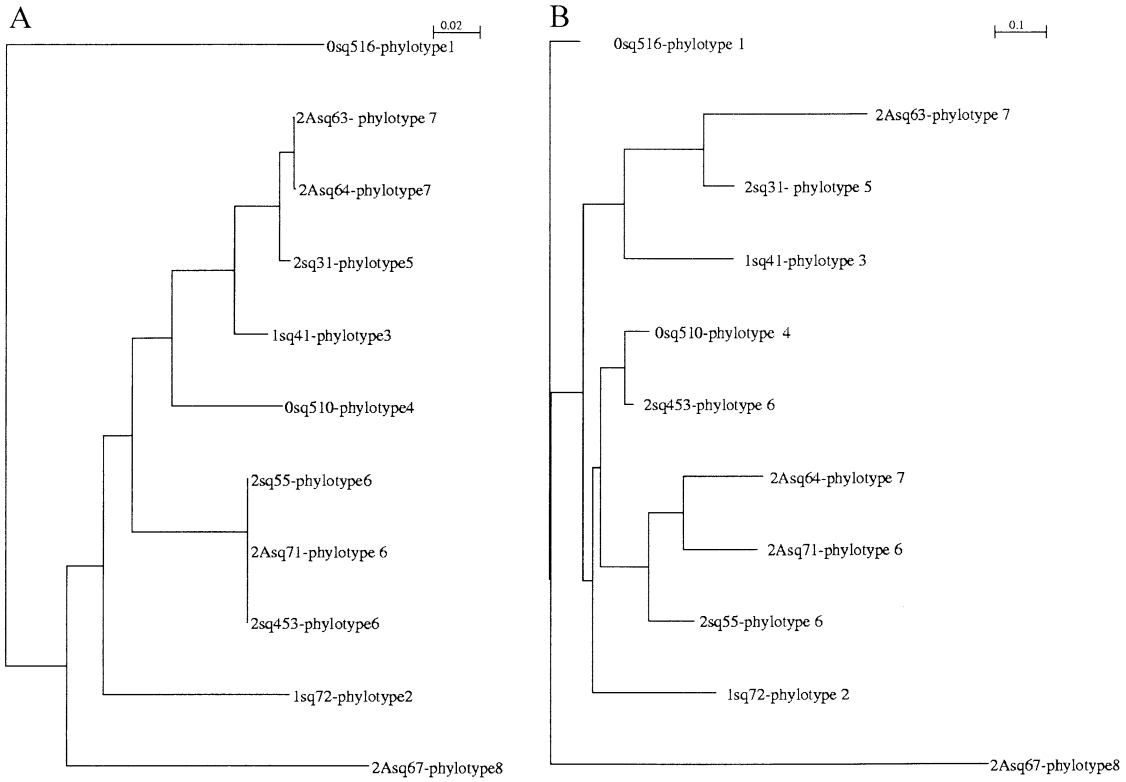

Fig. 6. Cluster analysis of DNA Sequences (A) and biolog (GN) substrate oxidation (B) of the isolated strain using Manhattan algorithm.

Table 2
Aerobic or anaerobic (in the presence of nitrate) degradation of squalene by different strains belonging to the different phylotypes isolated from R1 and R2 cultures

Strains	DGGE pattern	Aerobiosis		Anaerobiosis			
		OD (610 nm)	% biodegradation	OD (610 nm)	% biodegradation	Electrons from squalene[a] oxidation (mmol)	Electrons consumed by NO_3^-[b] reduction (mmol)
1sq31	5	0.35	0	n.d.	n.d.		
1sq41	3	0.23	4	n.d.	n.d.		
1sq72	2	0.27	6	n.d.	n.d.		
2sq31	5	6.96	99.5	9.48	77	4.58	6.04
2sq453	6	0.23	33	0.18	9	0.69	0.75
2sq55	6	0.22	17	0.16	16	0.95	1.01
2Asq71	6	0.23	5	n.d.	n.d.		
2Asq63	7	0.31	4	n.d.	n.d.		
2Asq64	7	6.99	99.8	0.57	27	1.60	1.67
2Asq67	8	0.45	5	n.d.	n.d.		

n.d.: not done. Growth experiments were carried out in anoxic flasks with a culture volume of 50 ml containing squalene (0.7 mM) and nitrate (10 mM); squalene consumption and nitrate reduction were quantified. At the end of the incubation time, no nitrite or negligible amounts of squalene biodegradation metabolites were observed. The amount of squalene assimilated was not significant.
[a]The complete oxidation of 1 mol squalene yields 170 mol electrons.
[b]The reduction of 1 mol nitrate to dinitrogen consumes 5 mol electrons.

large amounts of squalene (higher than 10%). These isolates were part of 2sq and 2Asq bacterial set, obtained by successive transfers in squalene medium. The anaerobic biodegradation of squalene was only tested on the four denitrifying strains that showed high percentage of squalene biodegradation in aerobiosis. After 100 days incubation, under denitrifying conditions, 2sq55 and 2sq453 strains exhibited lower percentage of degradation (16 and 9%, respectively) compared with those obtained from 2sq31 and 2Asq64 (77 and 27% respectively). Concomitantly with squalene degradation, nitrate consumption was observed for all anaerobic cultures. For all the denitrifying strains, the electron recovery was nearly complete, and the molar ratio between oxidized squalene and reduced nitrate were close to the theoretical ratio (see stoichiometry, Table 2).

4. Discussion–conclusion

Mixed populations are probably important in the degradation of complex substrates such as squalene especially under anaerobic conditions. However, the major difficulty is the identification of the metabolically relevant bacterial strains in recalcitrant substrate degradation. The classical approach involves the isolation of dominant morphologically different colonies, followed by screening of the isolates on the substrate. Previous studies, investigating the isolation of recalcitrant substrate utilizing bacteria, have shown a discrepancy between the metabolic potential of the enrichment cultures and that of the pure isolates. This may be due to (i) the apparent 'unculturability' of the metabolically important strains or (ii) an insufficient number of colonies screened to detect variation among isolates that present a similar phenotype or colony morphology but that are genotypically different. A probably large number of metabolically active species that remained 'unculturable' could indeed be cultured without undue difficulty but so far there has been insufficient effort to achieve this. Apart from the laborious and time consuming work involved in screening a large number of isolates the more serious limitation for strain isolation is the fact that liquid batch enrichment cultures typically select for faster growing organisms [31]. To circumvent this problem, molecular techniques are very helpful to achieve a more accurate picture of the molecular diversity of the enrichment cultures in order to focus the effort on the isolation of strains representing all the different relevant community members.

Our study has proven that under denitrifying conditions, a bacterial community from marine sediment was able to consume of substantial proportion of a recalcitrant substrate such as squalene (50% after 6 months incubation). In contrast to aerobic bacterial degradation which has been widely studied [6–8,32,33], the anaerobic degradation of alkenes is not well documented. To our knowledge this is the first paper giving direct evidence for the biodegradation of a multiply branched alkene under anaerobic conditions using nitrate as the electron acceptor. In the literature, there is only one report of an attempt to assess the degradation of squalene by measuring long-term methane production by methanogenic enriched cultures [34]. This author postulated that according to the stoichiometry, squalene is degraded with only 50% of the expected methane being produced; the only detected intermediate was acetate.

In view of our successful anaerobic degradation experiment with this marine bacterial community, we have attempted to isolate squalene degrading strains, the pure culture isolation step being imperative in assessing the metabolic pathways of biodegradation. A combination of culture-dependent and -independent methods were developed to investigate how selective enrichment cultures af-

fect species composition. DGGE separation of PCR-amplified 16S rRNA gene fragment was used to monitor the populations contained within enrichment cultures of squalene degrading denitrifying bacteria.

The molecular diversity of the bacterial community in each isolation step was estimated by the number of amplified 16S rRNA bands following the DGGE separation as performed by Muyzer [26]. The majority of the bands obtained were sequenced without interference, suggesting that each band represented one microorganism. With the exception of phylotype #11, each band was assumed to represent one phylotype. Because only one sequence out of the 12 analyzed might be a heteroduplex, we can conclude that the PCR conditions used for this study did not favor the formation of such bands and thus heteroduplex formation does not significantly interfere with PCR–DGGE analysis of the complex communities. However, for the analysis of R-T0, R-T1, R2, Com+O_2 and Com−O_2 we cannot ignore that one particular minor band contains more than one sequence or corresponds to a heteroduplex.

Comparison of the DGGE profiles of the enrichment samples with the DGGE pattern of the 80 isolates (Fig. 2) shows that several bands corresponding to isolates co-migrated with intensely stained DGGE bands in the enrichment culture DNA. For the R-T0 and R-T1 steps it is difficult to match the DGGE pattern of the isolates with the DGGE profile of the enrichment samples. At this stage none of the isolates were able to degrade squalene. The difference in the number and intensity of bands during the isolation procedure suggests that the microbial community underwent a decrease in species diversity as members of the bacterial population were subjected to selective enrichment. After several subcultures, required for the R2 step, two out of four intensely stained DGGE bands in the enrichment culture DNA matched with phylotypes #5 and #6 corresponding to strains isolated under anaerobic conditions. From the same enrichment culture (R2), the denitrifying strains isolated under aerobic conditions did not present the same DGGE pattern as those isolated under anaerobic conditions. This highlights again the bias of growth conditions on the strain isolation. Strains corresponding to phylotypes #7 and #8 were never isolated under anaerobic conditions. These latter bands (#7 and #8) were not visible in the DGGE profile of the enrichment samples (R2) but were detected in the subculture Com+O_2. Thus, molecular methods may inefficiently detect relatively rare sequences of low abundance populations or might bias against the sequences of some populations. The number of bands from the enrichment culture found on a DGGE gel may not accurately reflect the number of different sequences in the enrichment culture, but the most abundant species amplified should be represented in the band pattern [26]. However, from the cultivation and molecular evidence obtained in this study, we believe that 2sq31, 2sq55 and 2Asq64 are the main strains able to use squalene under denitrifying conditions from this bacterial community. Indeed, when cultures were inoculated with the community R2 (Com−O_2), the DGGE patterns at the end of the incubation period clearly showed the predominance of these strains in the community. However, unexpectedly, 2sq55 (phylotype #6) which reached the lowest degradation rate in the monoculture presented the most intensive band in the DGGE pattern. This may be due to (i) a drawback of DGGE approach (only qualitative information about the population composition can be obtained by this technique) [35] or (ii) synergetic growth of 2sq55 with 2sq31. It should be noted that bacterial numeration on plate seems to indicate that the concentration of 2sq55 was 100-fold higher than that of 2sq31 in R2 (data not shown). Without the help of DGGE, 2sq31 strain that seems to be in low number in R2 would probably never have been isolated. Strains corresponding to the #5 DGGE pattern represented only two strains among the 37 isolates. With regard to metabolic diversity of the isolates, it was interesting to note that strains representing the same phylotype (#5) isolated from R-T1 or R2 do not exhibit the same behavior towards squalene, only strains isolated from R2, after 8 months incubation were able to use this substrate as the sole carbon and energy source. Moreover, strains showing the same sequences (2sq55; 2sq453; 2Asq71 and 2Asq63; 2Asq64 for phylotypes #6 and #7, respectively) were notably different in their nutritional pattern.

Sequencing of the 16S rRNA gene fragments of the isolates and those of excised bands from the DGGE gels revealed a reduced spectrum in microorganisms probably due to very high selective pressure. However, our culture conditions did not allow us to isolate strains with DGGE pattern matching with bands #9, #10 and #12. Therefore, we can note a lower diversity with culture based analysis than with DGGE analysis. The closest strains from each isolate appeared to be bacteria isolated from the marine environment. Most of the sequences of the isolates (greater than 90%) were members of the gamma subdivision of the *Proteobacteria*. On the basis of information obtained from the DGGE profiles of the enrichment samples, we can note that all phylotypes grouping in this phylum were isolated. The main organisms able to degrade squalene are found in the genus *Marinobacter*. We have previously shown using culture methods that many bacteria belonging to this group can grow on isoprenoid compounds [9,10,36]. The bands corresponding to strains (phylotypes #9 and #10) appeared in R-T1 and are still present in R2. The appearance of these bands seems to indicate that the strains were positively selected under this growth condition and that they might use squalene or one of its metabolites as carbon source. These strains were assigned to CFB and *Bacillus*/*Clostridium* groups. We were unable to culture them because of the inadequacy of the growth conditions chosen for the isolation steps.

In conclusion, due to the complexity of the system that

we studied, DGGE has proven to be a valuable first step in obtaining information regarding the dynamics of the bacterial community and for tentatively identifying the relevant community members. Although, our study has shown that a phylotype included squalene degrading and non-degrading strains, this technique appeared to be useful in monitoring the fastidious screening procedures for the bacterial isolation. The combination of culture-dependent and -independent methods allows us for the first time to isolate two strains able to efficiently degrade squalene under denitrifying conditions. Sequencing of 16S rDNA fragments identified these organisms as belonging to *Marinobacter* genus which is known to be important in the biodegradation of hydrocarbons.

Acknowledgements

This work was supported by grants from the Centre National de la Recherche Scientifique and the Elf Aquitaine Society (Research Groupment HYCAR 1123) and from the REMER (Coopération Franco-Marocaine en sciences de la mer). Thanks are due to G. Muyzer and P. Lebarron for their helpful advice and Miss T. Bentley for her careful reading of the English.

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