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Degradation of crude oil by an arctic microbial consortium

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Abstract The ability of a psychrotolerant microbial consortium to degrade crude oil at low temperatures was investigated. The enriched arctic microbial community was also tested for its ability to utilize various hydrocarbons, such as long-chain alkanes (*n*-C_{24} to *n*-C_{34}), pristane, (methyl-)naphthalenes, and xylenes, as sole carbon and energy sources. Except for *o*-xylene and methylnaphthalenes, all tested compounds were metabolized under conditions that are typical for contaminated marine liquid sites, namely at pH 6–9 and at 4–27°C. By applying molecular biological techniques (16S rDNA sequencing, DGGE) nine strains could be identified in the consortium. Five of these strains could be isolated in pure cultures. The involved strains were closely related to the following genera: *Pseudoalteromonas* (two species), *Pseudomonas* (two species), *Shewanella* (two species), *Marinobacter* (one species), *Psychrobacter* (one species), and *Agreia* (one species). Interestingly, the five isolated strains in different combinations were unable to degrade crude oil or its components significantly, indicating the importance of the four unculturable microorganisms in the degradation of single or of complex mixtures of hydrocarbons. The obtained mixed culture showed obvious advantages including stability of the consortium, wide range adaptability for crude oil degradation, and strong degradation ability of crude oil.

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Introduction

Hydrocarbons are the major pollutants in marine environments. They are derived from terrestrial and freshwater run-off, offshore oil production, refuse from coastal oil refineries, shipping activities, and accidental spillage of fuels and other petroleum products. Although the majority of hydrocarbon-derived contaminations occur in cold marine environments, most of the investigations have been performed at higher temperatures, namely between 20 and 35°C (Raghukumar et al. 2001; Ko et al. 1999; Geerdink 1997; Margesin and Schinner 1997a, b; Rosenberg et al. 1992). For a long time, decreased solubility was considered to be responsible for the recalcitrance of hydrophobic compounds observed in temperate and cold environments. Several recent reports, however, have indicated that some bacteria may have adapted to the low solubility of hydrophobic environmental chemicals and that generalizations about the bioavailability of hydrocarbons might be inappropriate (Wick et al. 2002a; Bastiaens et al. 2000; Friedrich et al. 2000; Grosser et al. 2000; Guerin and Boyd 1992). Indeed, hydrocarbons are degraded at rates which exceed their rates of dissolution in the aqueous phase, demanding special uptake mechanisms to be employed by hydrocarbon-degrading microorganisms (Leahy and Colwell 1990; Thomas et al. 1986).

In cold climates, psychrophilic and psychrotolerant microorganisms play an important role in the biodegradation of organic matter. These bacteria are distinguished by their minimum, optimum, and maximum growth temperatures, which are <0, <15, and <20°C, for psychrophilic and 0–5, >15, and >20°C for psychrotolerant bacteria (Morita 1975). Former studies concerning degradation of hydrocarbons by psychrotolerant bacteria were performed with single bacterial strains (Whyte et al. 1998; Geerdink et al. 1996) and in the presence of complex compounds such as yeast extract (Hamme van et al. 2000). The obtained cell yields under these conditions in most cases do not correspond

to the degradation of hydrocarbons. Furthermore, during subcultivation of the strains plasmids, which encode enzymes with essential degradation capabilities, can be lost. Former investigations have shown that linear and in some cases branched alkanes from crude oil or diesel fuel are degradable while aromatic compounds were hardly attacked (Raghukumar et al. 2001; Whyte et al. 1998; Atlas and Bartha 1992). These studies indicate that pure cultures can metabolize only a limited range of hydrocarbons. Consequently, mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil or diesel fuel (Leahy and Colwell 1990). Such mixed cultures display metabolic versatility and superiority to pure cultures (Hamme et al. 2000). In a consortium, single strains can complement each other, e.g. by co-metabolic turnover reactions or by interactions with substrates (e.g. via biofilm formation) and carry out finally a more effective degradation process. Pelz et al. (1999) have proved for example that in a consortium metabolic and physiological weaknesses of primary degraders of hydrocarbons can effectively be compensated by recruitment of other organisms from the mixed culture with appropriate complementary physiology.

Based on the fact that most hydrocarbon-derived pollutions occur at low temperatures, attempts were made to enrich a microbial consortium effective in crude oil degradation in cold habitats. For these studies, physiological experiments and molecular biological techniques were employed to analyze the microbial community in the consortium.

Materials and methods

Source of bacteria

During an expedition to Spitzbergen in 1998, arctic sea ice and seawater samples were collected at Svalbard (Norway) and transported to the laboratory at a temperature of 2–10°C. Samples from different habitats were mixed and used as inoculum for the enrichment culture.

Crude oils

Two different crude oils (nos. 1 and 2) were used for degradation experiments. Both consisted of linear and branched alkanes (pristane and phytane) and diverse aromatic components (e.g. toluene, xylenes, ethylbenzene, (methylated) naphthalenes, dibenzothiophenes, phenanthrenes, and acenaphthenes) in varying concentrations. The oils were derived from different offshore North Sea oil fields. Unless otherwise stated in the text, oil no. 1 was used.

Media

The enrichment medium contained (g l^{-1}) NaCl 28.13; KCl 0.77; $CaCl_2 \times 2\ H_2O$ 0.02; $MgSO_4 \times 7\ H_2O$ 0.5; NH_4Cl 1.0; $FeCl_2$ 0.001; yeast extract 0.5. The pH was adjusted to 7.0. After autoclaving the medium, the following sterile solutions were added: 1 ml of tenfold trace element solution as described for medium 141 (DSMZ 1998); 100 ml containing KH_2PO_4 (2.3 g) and $Na_2HPO_4 \times 2\ H_2O$ (2.9 g) (pH 7.2); 100 ml containing Na-acetate (0.5 g), Na-succinate (0.5 g), DL-malate (0.5 g), Na-pyruvate (0.5 g), D-mannitol (0.5 g), and glucose (2.0 g) (pH 6.8), to give a total volume of 1000 ml. For preparation of a mineral medium, the carbon sources and yeast extract were omitted. Isolation of strains was performed on TSA agar plates (Sifin, Berlin, Germany).

Enrichment and cultivation

Enrichment was performed in a one-liter flask containing 500 ml of enrichment medium with 10 ml of crude oil. As inoculum, 10 ml of the sample from the arctic sea ice was applied. Incubation was performed at 4°C for 6 weeks without regular shaking, as described by Mohn et al. (1997). To avoid anaerobic conditions, the flask was mixed and aerated manually each third day. Microbial growth was monitored by direct cell counting using a Neubauer counting chamber. After significant cell growth had been achieved, the bacteria were subcultivated in 100-ml Erlenmeyer flasks (triplicates) containing 22.5 ml of minimal medium (final concentration: $4–5 \times 10^7$ cells ml^{-1}). Crude oil was added as a single source of carbon and energy (1 ml l^{-1}). The flasks were sealed tightly with screw caps (Whyte et al. 1998) containing PTFE covered with silicone septa to avoid abiotic loss of the substrate. After initial growth was obvious, the cultures were aerated once a day and mixed thoroughly. Uninoculated control flasks (duplicates) were incubated and aerated in parallel to monitor abiotic losses of the substrates. In addition, the following carbon sources were used in the growth experiments: 1 mM naphthalene, methylnaphthalenes, *n*-tetracosane (*n*-C_{24}), *n*-tetratriacontane (*n*-C_{34}), 0.5 mM pristane, and 0.1 mM xylenes.

Gas chromatography

For chemical analysis, samples with a volume of 23.5 ml were extracted with 5 ml hexane, dried over Na_2SO_4, and stored at 4°C in 2-ml glass vials sealed with screw caps. Gas chromatographical measurement of the hexane soluble fraction of the crude oil during the degradation experiments was accomplished using a Perkin Elmer Autosystem Gas Chromatograph (GC), (Überlingen, Germany) fitted with a flame ionization detector (FID). The used column was a 30 m × 0.32 mm (ID) glass capillary column Rtx-5 (Restek, Bad Soden, Germany) with a film thickness of 0.25 µm. Hydrogen was used as the carrier gas. Injector mode was performed

with a split volume of 90 ml, at 300°C; injection volume was 2 µl. The oven program started at 35°C, held for 2 min, and was raised at 4°C min^{-1} to 310°C, and held for 10 min. Detector temperature was adjusted to 300°C. Phenanthrene- d_{10} was used as internal standard (15 mg/l); 2 µl was added to 50 µl of the sample.

For calculation of total crude oil degradation, peak areas (from triplicate samples) were quantified by integration. These values were expressed as the percentage degraded relative to the amount of the corresponding components, which remained in the appropriate abiotic control samples.

Mass spectrometry

Analysis with gas chromatograph-mass spectrometer (GC-MS) was performed as described by Annweiler et al. (2000). The measurements were carried out with a Carlo Erba 4160 GC, using a DB-5 HT capillary column (J & W Scientific, Folsom, USA) with a length of 60 m; (ID 0.32 mm) and a film thickness of 0.25 µm. Carrier Gas and temperature program were applied as described for the GC measurements. The GC was coupled with a CH7A mass spectrometer (Finnigan, Germany). The conditions were as follows: ionization mode, EL+; ionization energy, 70 eV; emission current, 200 µA; temperature of source 250°C; mass range m/z 50–500.

16S rDNA amplificaton and cloning

Cells were harvested from 2-ml cultures by centrifugation and resuspended in 100 µl of minimal medium. An aliquot of 1 µl was used as a template for amplification of 16S rRNA genes with 27F and 1492R primers (Lane 1993). The PCR was performed in a total volume of 100 µl containing 50 pmol of each primer, 200 µM of each dNTP, 30 µg bovine serum albumin, 20 mM Tris–HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. Negative controls without DNA template were included in every reaction set. After applying a hot start (Muyzer et al. 1993) with 2.5 U *Taq* DNA polymerase (GibcoBRL Life technologies, Karlsruhe, Germany), 30 cycles of 94°C for 1.5 min, 46°C for 1.5 min, and 72°C for 1.5 min were run in a DNA thermal cycler Gene Amp PCR System 2400 (Perkin–Elmer, Überlingen, Germany).

The amplicons were separated on 1.0% agarose gel stained with ethidium bromide and purified with the QIAquick purification Kit (Qiagen, Hilden, Germany) or with the Qiaex Gel extraction Kit (Qiagen). Purified 16S rDNA fragments were cloned by the TA-TOPO cloning kit (Invitrogen, Karlsruhe, Germany) in pCR2.1-TOPO vector following the manufacturer's instructions. For DGGE and sequencing, plasmids were isolated and purified with a QIAprep Spin Miniprep Kit (Qiagen).

Denaturing gradient gel electrophoresis (DGGE)

Primers GM5clamp and 907R were used to amplify variable regions of the 16S rDNA in a touchdown PCR, as described by Sahm et al. (1999) and by Buchholz-Cleven et al. (1997) with the following modifications: as template served 0.5–1.0 µg of DNA extract, prepared from 2 ml bacterial culture with the QIAamp DNA Mini Kit (Qiagen). For reamplification of PCR bands, DNA was excised from the gel and eluted overnight in 100 µl H_2O MilliQ at 4°C. For the new amplification, 1 µl of eluted DNA served as the template.

The hot start was followed by 25 cycles at 94°C for 1.0 min, 62°C for 1.0 min, and 72°C for 3.0 min. The annealing temperature was decreased by 0.4°C in every cycle until a touchdown at 52°C was achieved. From cycle 11 to 25, the elongation time was increased (0.4 min). Additionally, five cycles were carried out at 94°C for 1.5 min, 52°C for 1.5 min, and 72°C for 7.4 min. The DGGE was performed with a DGGE Biorad DCodeTM System (Biorad Laboratories Inc., München, Germany) as described previously (Muyzer et al. 1996).

The gels contained denaturing gradients ranging from 20 to 80%. A 150 ml of 80% denaturing acrylamide solution contained 22.5 ml acrylamide (40%, 37.5:1, Biorad Laboratories Inc, München, Germany), 3 ml 50 × TAE, 48 ml formamide (deionized, Roth, Germany), and 50.4 g urea. This solution was stored in a dark bottle. A 0% denaturing acrylamide solution contained the same ingredients except for urea and formamide. Electrophoresis was run in 1 × TAE at 55°C for 5 or 20 h at 200 or 100 V, respectively.

Sequencing and phylogenetical analysis

The Taq DyeDesoxy Terminator Cycle Sequencing kit (Applied Biosystems) was used to directly sequence the purified PCR products. Sequencing reactions were analyzed on the 373S DNA sequencer (Applied Biosystems). Both strands of the amplification product were sequenced using primers 27F, 787F, 787R, 1175R, 1099F, and 1492R (Lane 1993). Sequences were compared to the available 16S rDNA primary structures present in the EMBL database by using the Fasta3 program. To detect chimera, the chimera_check program at RDPII was used.

Results

Enrichment of a crude oil degrading consortium

A crude oil degrading bacterial consortium was enriched at 4°C from a mixture of arctic sea ice and seawater samples derived from Spitzbergen. For enrichment and cultivation, the cultures were not permanently shaken because continuous shaking at 120 rpm inhibited

microbial growth significantly (data not shown). Microscopic examination revealed the oil droplets to be covered by a biofilm before effective degradation was observed (Fig. 1a). After approximately 19 days of cultivation at 4°C, the size of the oil droplets decreased significantly (Fig. 1b) and finally the droplets disappeared. The center of the droplets was not occupied by microorganisms. Cells in the vicinity of the oil droplets were found to be very motile and did not form any aggregates. By applying gas chromatography, the degradation of saturated hydrocarbons in crude oil was analyzed. During the degradation process, *n*-alkanes

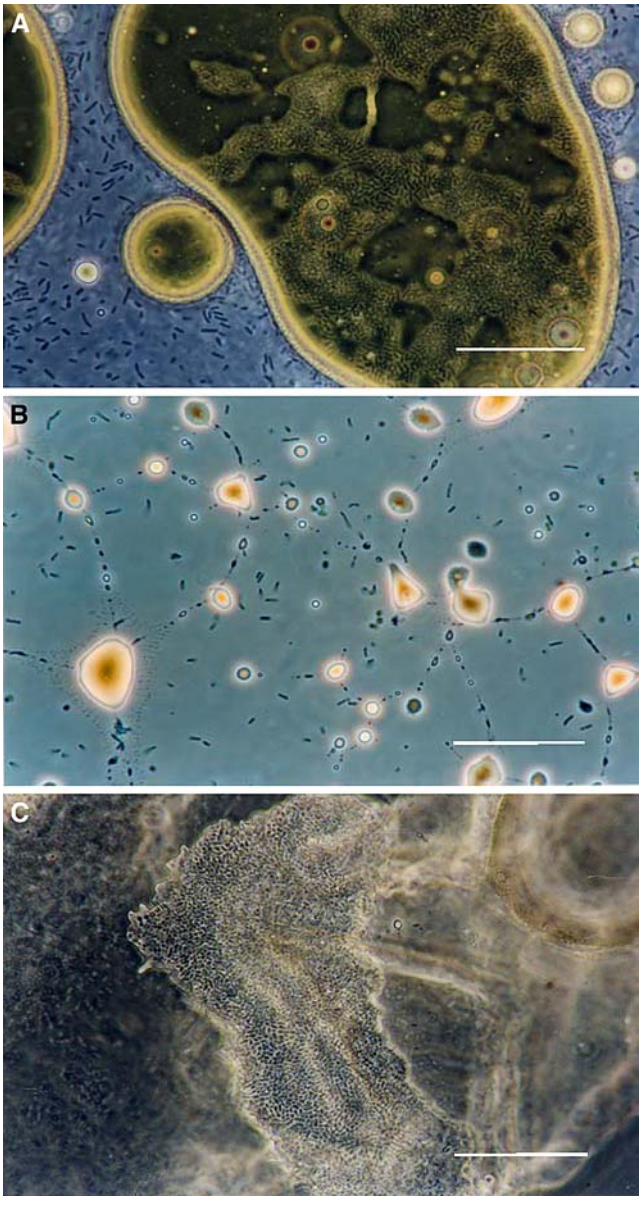


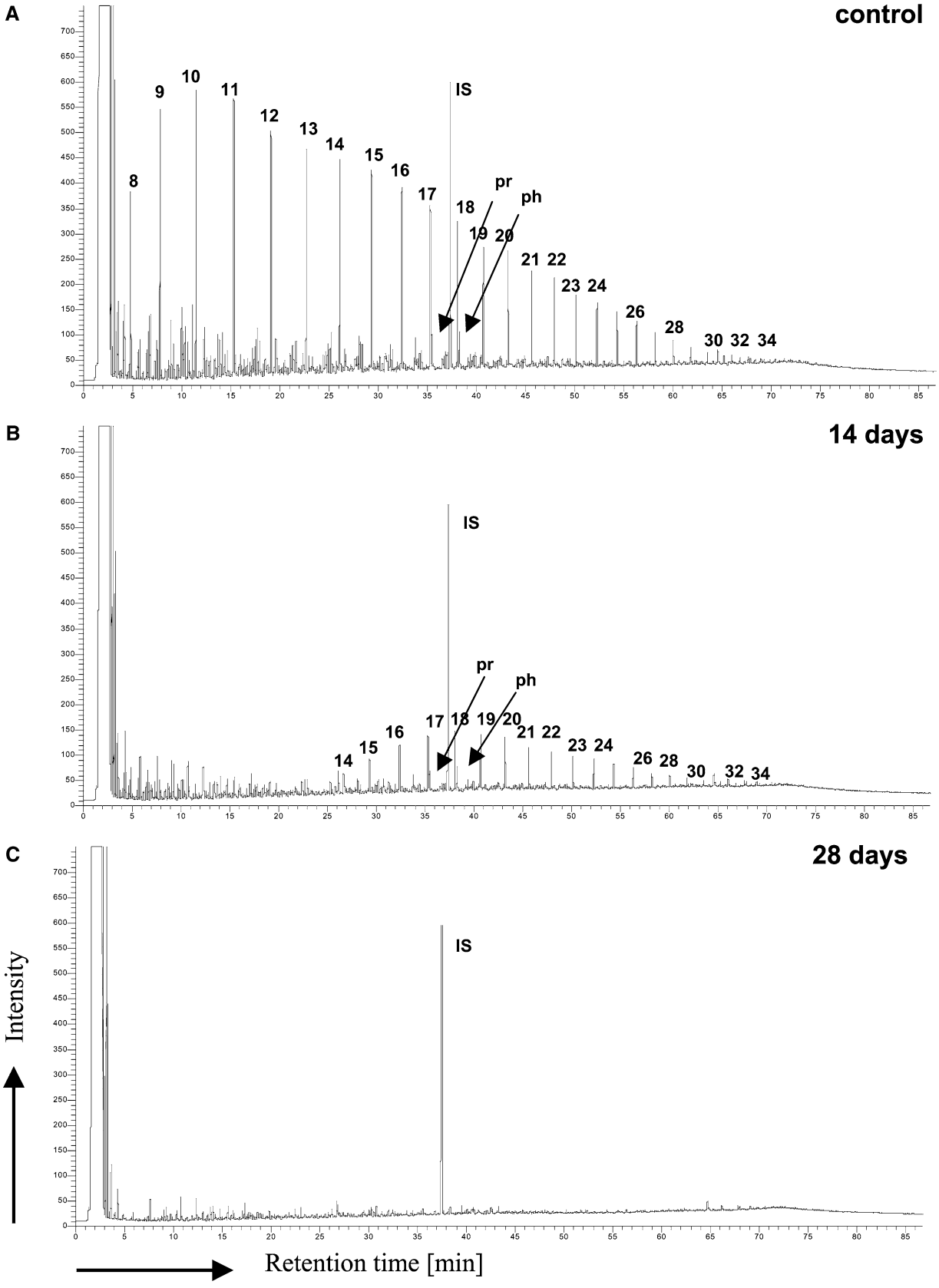
Fig. 1 Growth of the psychrotolerant consortium on crude oil (0.1%) at 4°C for 6 days (**a**) and 19 days (**b**). Biofilm formation after cultivation of the consortium at 4°C on *n*-C_{34} crystals (1 mM) for 4 weeks (**c**). Experiments were performed in a mineral medium at pH 7.0, *scale bar* 10 μm

Fig. 2 GC profiles of crude oil extracted from the aqueous phase after cultivation of the psychrotolerant consortium in a mineral medium with crude oil at 4°C. **a** abiotic control (28 days); **b** inoculated sample after 14 days of growth; **c** inoculated sample after 28 days. *IS* internal standard (phenanthrene d_{10}), 8–34, *n*-alkanes (*numbers* designate the number of C atoms), *pr* pristine, *ph* phytane. Alkanes, pristine, and phytane were identified by comparison of the retention time and mass spectra with authentic standards

(C_8–C_{34}) and the isoprenoids pristane and phytane were found to be completely eliminated after 28 days of incubation at 4°C (Fig. 2). Short-chain *n*-alkanes (C_8–C_{14}) were preferentially metabolized by the consortium. These components were used during the first 2 weeks. Afterwards, *n*-alkanes with a chain length of 15–34 carbon atoms were attacked in parallel with the isoprenoids pristane and phytane.

Compared to abiotic controls, mass spectrometric analysis provided a clear indication of the degradation of diverse aromatic components in crude oil at 4°C (Fig. 3). Ethylbenzene, *m*-xylene, and *p*-xylene, e.g. were found to be utilized by the consortium (Fig. 3a). In contrast, *o*-xylene was not attacked. A temporary relative enrichment of *p*-xylene and ethylbenzene during crude oil degradation indicated a preferential use of *m*-xylene (Fig. 3a). Furthermore, several alkyl substituted benzenes with nine and ten carbon atoms and naphthalene were totally eliminated by the consortium (Fig. 3b–d), whereas other isomers remained unattacked. Even 2-methylnaphthalene was attacked during the degradation process, indicated by the relative enrichment of 1-methylnaphthalene. Total elimination of these two isomers, however, could not be observed (Fig. 3d). In contrast, other polyaromatic hydrocarbons (e.g. phenanthrene, dibenzothiophene, and acenaphthene) and saturated cyclic compounds (hopanes) were not attacked by the consortium even after 5 weeks of incubation at 4°C. In conclusion, the conversion of hydrophobic substrates like long-chain *n*-alkanes and naphthalene is astonishing, especially at 4°C. It can be estimated that the metabolism of these components occurs at rates which exceed their rates of dissolution in the medium.

Further physiological experiments were performed to find the optimal conditions for crude oil degradation. Bacterial degradation of crude oil was observed at neutral pH (7.0) in a temperature range between 4 and 27°C, with an optimum temperature of 20°C. The corresponding growth rates were found to be nearly identical (0.83–0.9 day^{-1}) in a temperature range from 15 to 27°C (Fig. 4). The degradation process was completed within 7 days at 20°C and within 28 days at 4°C. No degradation was observed at 30°C. Furthermore, crude oil degradation was observed in a pH range between 6 and 9 with an optimum at pH 8.5 (data not shown). At pH 8.5, up to 77% of the crude oil was degraded at 20°C within 7 days and up to 70% within 4 weeks at 4°C. The degradation pattern was reproducible when a



different crude oil was used as substrate (oil no. 2). In this case, 65% degradation was achieved at 4°C after 5 weeks and 71% at 20°C after 10 days (data not shown). From these results, it is obvious that the metabolic capability of the consortium is not restricted to one type of crude oil.

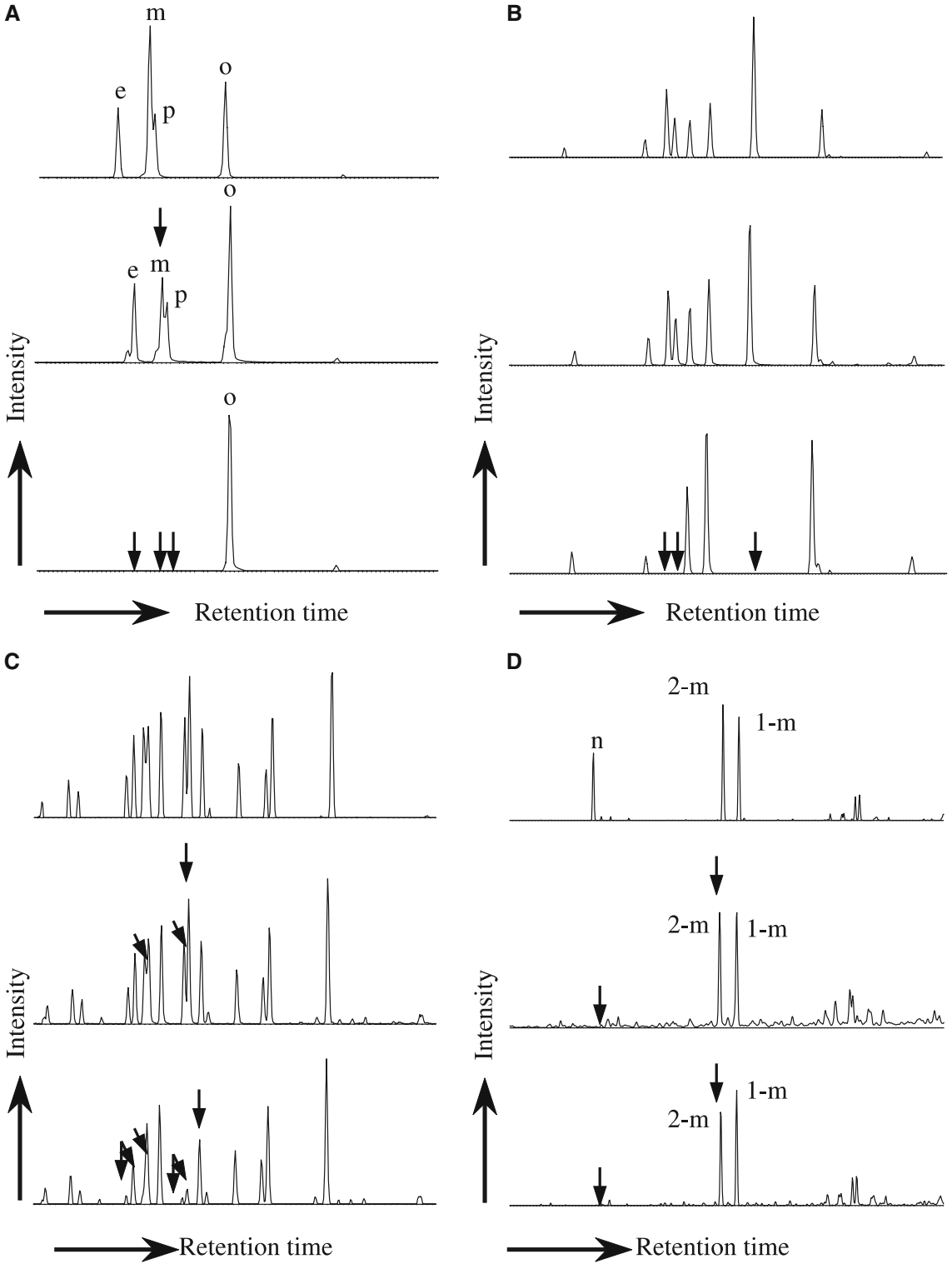


Fig. 3 Mass chromatograms of aromatic components of crude oil during microbial degradation at 4°C; *first line* from the top abiotic control after 28 days, *second line* inoculated sample after 7 days, *third line* inoculated sample after 28 days. **a** xylenes and ethylbenzene (ion chromatogram *m*/*z* 106), **b** alkylsubstituate benzenes with nine carbon atoms (ion chromatogram *m*/*z* 120), **c** alkylsubstituate benzenes with ten carbon atoms (ion chromatogram *m*/*z* 132), **d** naphthalene, and methylnaphthalenes (ion chromatograms *m*/*z* 128 + 144). *o* *o*-xylene, *m* *m*-xylene, *p* *p*-xylene, *e* ethylbenzene, *n* naphthalene, *1-m* 1-methylnaphthalene, *2-m* 2-methylnaphthalene, peaks in a and d were identified by coinjection of standards. *Arrows* indicate decreasing/disappearing peaks

Utilization of typical crude oil components

To analyze whether the decrease in oil components is due to mineralization or co-metabolic turnover reactions, various crude oil components (*n*-C_{24}, *n*-C_{34}, pristane, xylenes, and (methyl-)naphthalene) were tested as single sources of carbon and energy for growth. Incubation was performed at 4 and 20°C in the mineral medium at neutral pH (7.0). All substrates except *o*-xylene and methylnaphthalenes supported the growth of the consortium. Interestingly, hydrophobic *n*-alkane crystals (C_{24} and C_{34}) were covered by a biofilm, when applied as a single carbon source (Fig. 1c). The *n*-C_{24} crystals disappeared from the medium and the size of *n*-C_{34} crystals was significantly reduced. The growth rates for *n*-C_{24} at 4 and 20°C were 0.32 (day^{-1}) and 0.4 (day^{-1}), respectively. Corresponding values for *n*-C_{34} were found to be 0.19 (day^{-1}) at 4°C and 0.29 (day^{-1}) at 20°C. As already described for crude oil, pristane droplets were covered at 4°C by a biofilm, before they were utilized. The growth rates at 4 and 20°C were 0.31 (day^{-1}) and 0.72 (day^{-1}), respectively. From the three-xylene isomers, only *m*-xylene and *p*-xylene enabled microbial growth. Selective elimination of these two components from crude oil was also shown by mass spectrometry. During growth, biofilm formation could be observed on the surface of the medium as well. The corresponding growth rates were 0.15 (day^{-1}) at 4°C and 0.38 (day^{-1}) at 20°C for *m*-xylene and 0.21 (day^{-1}) at 4°C and 0.51 (day^{-1}) at 20°C for *p*-xylene. To a lower extent, biofilm formation was observed during the growth of the consortium on naphthalene. Thus, growth rates for this substrate were only 0.15 (day^{-1}) at 4°C and 0.22 (day^{-1}) at 20°C. In spite of the low growth rates, naphthalene crystals were eliminated from the medium. This was, however, accompanied by the formation of a yellow-brown-colored metabolite. Further growth experiments with 1-methylnaphthalene and 2-methylnaphthalene as sole carbon sources have shown that these substrates did not support growth even after 5 weeks of cultivation at various temperatures. These observations are in accordance with results obtained from GC–MS analysis, where only a slight decrease in 2-methylnaphthalene could be observed.

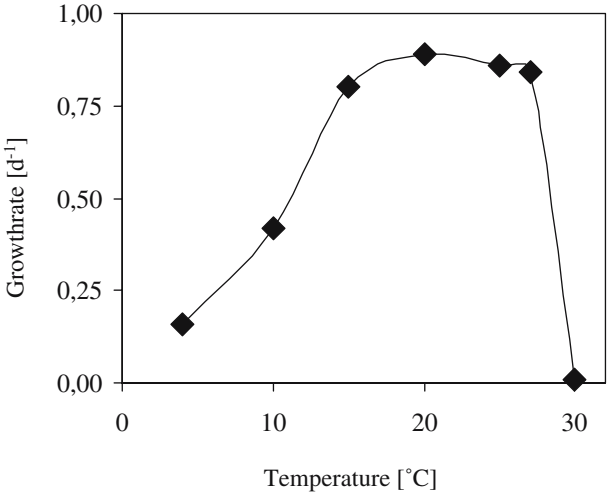


Fig. 4 Effect of temperature on growth of the consortium on crude oil. Growth rates of the psychrotolerant mixed culture were determined by cell counting in exponential growth phases at different temperatures with crude oil as sole carbon source in a mineral medium at pH 7.0. The increase in cell growth was correlated with the decrease in crude oil concentration (data not shown)

Phylogenetical analysis

To analyze the composition of the consortium, classical and molecular biological methods were used. At first, the culturable strains were separated from each other by streaking several times on TSA-agar plates. With this method, five bacterial isolates were obtained, which were described as P1, P3, P4, P5, and P6. Based on 16S rDNA sequences, the isolates were closely related to the following strains: P1, *Pseudoalteromonas elyakovii* (99.6%); P3, *Psychrobacter glacincola* (98.9%); P4, *Pseudomonas anguilliseptica* (97.8%); P5, *Pseudomonas synxantha* (95.3%); P6, *Agreia bicolorata* (96.6%).

To identify the unculturable strains, 16S rDNA fragments derived from the consortium were cloned. Afterwards, DGGE fragments from the mixed culture and from the clones were amplified and analyzed by a corresponding gel (Fig. 5). Interestingly, variations in the band patterns of the mixed culture were observed depending on the growth phase. Only the samples from exponential and stationary phase together were able to display the full bacterial diversity of the investigated mixed culture (Fig. 5, lanes 1, 4, 7, 10, and 13). After the screening of 117 clones, all DGGE bands of the consortium could be assigned, either to DGGE fragments from the isolated strains or to corresponding DNA from selected clones (C51; C53; C56; C81), (Fig. 5).

Due to the fact that each step in PCR-mediated community analysis is potentially open to error or bias, the clones 51, 53, 56, and 81 were checked for the existence of chimeric sequences. Clone 51 was a hybrid, assembled under the conditions used in the PCR reaction. The clone displayed 94.5% similarity to *Marinobacter hydrocarbonoclasticus* over the first 1150 nucleotides and a similarity of 99.6% to *P. elyakovii* in the last 250 nucleotides. Obviously, the second part of the clone belongs to isolate P1. This strain displays the highest similarity to *P. elyakovii* (see above) over a sequence length of 1420 bp. Consequently, the DGGE band from the consortium, which corresponded to clone 51 was excised in duplicate and sequenced as well. Over a length of 550 bp, it displayed a similarity of 93.8% to *M. hydrocarbonoclasticus*, indicating that indeed a *Marinobacter* strain might be present in the community. On the level of 16S rDNA sequences, the clones C53, C56, and C81 displayed highest similarities to *Pseudoalteromonas atlantica* (99.2%), *Shewanella baltica* (97.4%), and *Shewanella frigidimarina* (99.9%). However, none of these clones contained chimeric sequences.

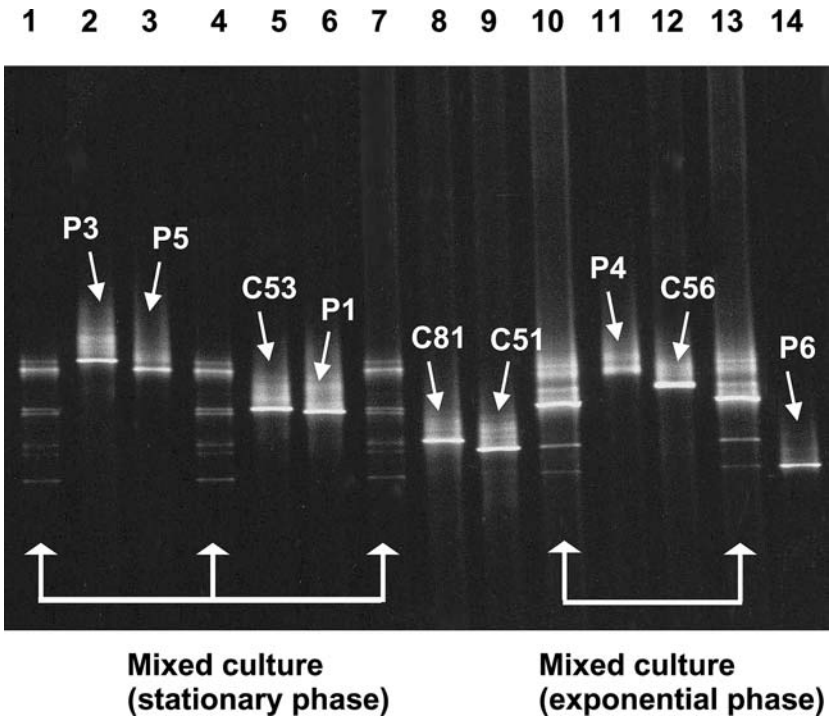


Fig. 5 Analysis of the microbial population by DGGE. *lanes 1, 4, and 7* show the band pattern of a sample taken in the stationary phase of growth after crude oil degradation at 4°C. The *lanes 10 and 13* show the corresponding band patterns of the consortium at the exponential growth phase. The remaining lanes belong to the following isolates or clones: *lane 2* isolate P3, *lane 3* isolate P5, *lane 5* clone 53, *lane 6* isolate P1, *lane 8* clone 81, *lane 9* clone 51, *lane 11* isolate P4, *lane 12* clone 56, *lane 14* isolate P6

Degradation capabilities of the pure strains and the defined mixed cultures

To analyze the potential role of culturable strains in the described degradation processes, all isolates (P1, P3, P4, P5, and P6) were tested separately for their ability to degrade crude oil components (*n*-alkanes (C_{24} and C_{34}), pristane, xylenes, naphthalene, methyl-naphthalenes). None of the five isolated strains showed significant growth with any of the mentioned substrates compared to the original consortium; biofilm formation could neither be observed.

Furthermore, the same strains were cultured separately and in defined mixed cultures with crude oil as the sole carbon source. The defined mixed cultures contained 2–5 isolates in various combinations. Only isolate P3 adhered to crude oil droplets and formed a biofilm when cultivated alone with the carbon source. No significant degradation of the substrate, however, was observed. These experiments clearly show that none of the culturable strains (separately or in combination) are effective in crude oil degradation (data not shown). From these experiments, it is obvious that the nonculturable strains are essential and play a key role in the degradation of crude oil.

Discussion

In contrast to earlier reports where psychrophilic or mesophilic single strains were used (Whyte 1998; Atlas and Bartha 1992; Raghukumar et al. 2001), the investigated consortium presented in this study is able to degrade not only linear alkanes but also isoprenoids (pristane and phytane) and several aromatic compounds (ethylbenzene, *m*-xylene/*p*-xylene, naphthalene). The consortium was able to co-metabolize isoprenoids with long-chain *n*-alkanes. In previous reports, isoprenoid metabolism was only observed after the complete elimination of the linear alkanes (Whyte et al. 1998; Atlas and Bartha 1992; Geerdink et al. 1996; Leahy and Colwell 1990; Westlake et al. 1974; Jobsen et al. 1972). In this study, C_8–C_{15} *n*-alkanes were preferably metabolized followed by C_{16}–C_{36} *n*-alkanes. This sequential degradation of *n*-alkanes was already reported by Peters and Moldowan (1993) and is also in agreement with previous investigations performed at low temperatures (Whyte et al. 1998), indicating that the solubility of individual components influences their bioavailability even at low temperatures.

The utilization of the diaromatic compound naphthalene by the consortium at 4°C, at least via co-metabolism, is remarkable. Leahy and Colwell (1990) reported on the degradation of asphaltenic compounds in mixed bacterial cultures to be dependent upon the presence of *n*-alkanes (12–18 carbon atoms in length). Also, the induction of enzymes for PAH degradation can depend on the presence of lower-molecular-weight aromatics such as naphthalene (Atlas and Bartha 1992). Saturated, cyclic high-molecular-weight compounds like hopanes were not degraded by the investigated consortium. Hopenes belong to the most persistent components of oil spillages in the environment. From the light aromatic crude oil fraction, toluene and *o*-xylene were not attacked by the consortium. The presence of these components obviously did not inhibit crude oil

degradation. Bacteria that degrade xylenes commonly fall into two classes: those that can degrade both *m*-xylene and *p*-xylene, and those that can degrade *o*-xylene only. It can be concluded that the metabolic pathway for utilization of *o*-xylene is missing in the consortium.

It is generally believed that bacteria could utilize only solubilized hydrocarbons (Britton 1984). Our results with the crystalline substrates (*n*-C24, *n*-C34, and naphthalene) indicate that the general view about substrate bioavailability might be inappropriate (Wick et al. 2002a; Bastiens et al. 2000; Friedrich et al. 2000; Grosser et al. 2000; Guerin and Boyd 1992). The bioavailability of hydrophobic compounds can be controlled by factors other than solubility because degradation of these substrates occurs at rates which exceed the rates of hydrocarbon dissolution (Jobson et al. 1972). It is obvious that microorganisms have developed various strategies, which enable them to utilize insoluble hydrocarbons efficiently. Biofilm formation, which was observed in most cases, could be applied as a strategy for the utilization of crystalline components (Bowman et al. 1997; Whyte et al. 1999; Wick et al. 2001, 2002a, b; Watkinson and Morgan 1990).

Although the microbial population was enriched at 4°C, the optimal temperature for growth and crude oil degradation was 20°C. Psychrotolerant bacteria usually possess an optimum temperature 10–20°C above the environmental temperatures from which they were isolated (Morita 1975). The culturable strains were closely related to *P. elyakovii* (99.6%), *Psychrobacter glacincola* (98.9%), *P. anguilliseptica* (97.8%), *P. synxantha* (95.3%), and *A. bicolorata* (96.6%). None of these strains, however, were previously reported to degrade crude oil. Interestingly, the DGGE band profile of the consortium was found to be dependent on the growth. Similar variations in bacterial profiles of mixed cultures were reported by Ferris et al. (1996) and Gillan et al. (1998). Furthermore, the detection limit for a bacterial member in a consortium was found to be $<1\%$ by applying DGGE (Polz and Cavanaugh 1998). Although the composition of microbial communities determined by the analysis of libraries of cloned PCR-amplified sequences is not quantitative (von Witzingerode et al. 1997), the predominance of sequence types suggests that the microorganisms represented by these sequences play an important role in the microbial activity within the psychrotolerant consortium.

The closest relative to the unculturable strain C51, *M. hydrocarbonoclasticus*, was isolated from seawater near a petroleum refinery (Gauthier et al. 1992). The strain displays a high tolerance against NaCl, up to 20% (Gauthier et al. 1992). Moreover, the reference strain *M. hydrocarbonoclasticus* utilizes linear saturated hydrocarbons (tetradecane, hexadecane, eicosane, and heneicosane) with high degradation rates. Growth to a lower extent was also observed with pristane, phenyldecane, and phenanthrene as carbon and energy sources (Gauthier et al. 1992). In general, *M. hydrocarbonoclasticus* belongs to the highly specialized hydrocarbonoclastic marine bacteria, which are present only in low numbers in "clean" marine environments but "bloom" (may account for up to 90% of microbial community) in response to oil spills or other hydrocarbon contamination events (Yakimov et al. 2002; Kasai et al. 2001; Syutsubo et al. 2001; Harayama et al. 1999). The close genetic relationship to *M. hydrocarbonoclasticus* suggests that the bacterial strain, corresponding to clone C51, may provide physiological capabilities to degrade crude oil components with respect to the overall degradation potential of the consortium.

Interestingly, a close relationship between C53 and *P. atlantica* was found. This strain exhibits the ability to switch the production of the adhesion EPS on and off. By this process, the bacterium moves from solid surfaces (such as seaweed or sand) to the Open Ocean and then back to a solid substrate to initiate biofilm formation (Corpe 1970). A 1.2-kb multicopy insertion sequence was found to be responsible for this phenomenon (Bartlett et al. 1988; Belas and Silverman 1989). The close relationship of C53 to *P. atlantica* may indicate that C53 could play an essential role in biofilm formation prior to the described degradation processes by the consortium. *S. baltica*, the closest related strain of C56 was originally isolated from an oil brine (Ziemke et al. 1998) but until now it has not been reported to degrade pollutants of environmental relevance.

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