Available online at www.sciencedirect.com
ScienceDirect
Systematic and Applied Microbiology 30 (2007) 319–330
SYSTEMATIC AND APPLIED MICROBIOLOGY
www.elsevier.de/syapm

Phylogenetic diversity and activity of aerobic heterotrophic bacteria from a hypersaline oil-polluted microbial mat

Raeid M.M. Abed[a,*], Burhanuddin Zein[a], Assad Al-Thukair[b], Dirk de Beer[a]

[a]*Max-Planck Institute for Marine Microbiology, Celsiusstraße 1, D-8359 Bremen, Germany*
[b]*King Fahd University of Petroleum and Minerals, P.O. Box 157, Dhahran 31261, Saudi Arabia*

Received 13 September 2006

Abstract

The diversity and function of aerobic heterotrophic bacteria (AHB) in cyanobacterial mats have been largely overlooked. We used culture-dependent and molecular techniques to explore the species diversity, degradative capacities and functional guilds of AHB in the photic layer (2 mm) of an oil-polluted microbial mat from Saudi Arabia. Enrichment isolation was carried out at different salinities (5% and 12%) and temperatures (28 and 45 °C) and on various substrates (acetate, glycolate, *Spirulina* extract and crude oils). Counts of most probable number showed a numerical abundance of AHB in the range of $1.15–8.13 \times 10^6$ cells g^{-1} and suggested the presence of halotolerant and thermotolerant populations. Most of the 16S rRNA sequences of the obtained clones and isolates were phylogenetically affiliated to the groups *Gammaproteobacteria*, *Bacteriodetes* and *Alphaproteobacteria*. Groups like *Deltaproteobacteria*, *Verrucomicrobia*, *Planctomycetes*, *Spirochaetes*, *Acidobacteria* and *Deinococcus-Thermus* were only detected by cloning. The strains isolated on acetate and glycolate belonged to the genera *Marinobacter*, *Halomonas*, *Roseobacter* and *Rhodobacter* whereas the strains enriched on crude oil belonged to *Marinobacter* and *Alcanivorax*. Members of the *Bacteriodetes* group were only enriched on *Spirulina* extract indicating their specialization in the degradation of cyanobacterial dead cells. The substrate spectra of representative strains showed the ability of all AHB to metabolize cyanobacterial photosynthetic and fermentation products. However, the unique in situ conditions of the mat apparently favored the enrichment of versatile strains that grew on both the cyanobacterial exudates and the hydrocarbons. We conclude that AHB in cyanobacterial mats represent a diverse community that plays an important role in carbon-cycling within microbial mats.

Keywords: Cyanobacterial mats; Aerobic heterotrophic bacteria; Arabian Gulf; Carbon cycle; Oil biodegradation; 16S rDNA cloning; Cultivation; Bacterial diversity

Introduction

The upper few millimeters in microbial mats, dominated by cyanobacteria and aerobic heterotrophic bacteria (AHB), represent biologically the most active layer with respect to carbon cycling. During daytime, this part is supersaturated with photosynthetically produced oxygen [8,48] whereas during the night, anoxic conditions prevail due to continued respiration activities of AHB (i.e. on cyanobacterial fermentative products) and sulfide production. Respiration in light is thought to be higher than in the dark because of the utilization of

*Corresponding author. Tel.: +49 421 2028832; fax: +49 421 2028690.
E-mail address: rabed@mpi-bremen.de (R.M.M. Abed).

0723-2020/$ - see front matter © 2006 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2006.09.001

soluble photosynthates by AHB [7,16,31]. AHB are presumed to be involved in the utilization of the complex, mostly polymeric carbon compounds of dead cyanobacterial cells [41]. As a result of these aerobic activities, most of the oxygen produced by photosynthesis is immediately respired, making aerobic respiration as important as photosynthesis for the carbon budget within the mat. Even though the importance of AHB for the carbon cycle of mats has been evident for many years, they have been treated in previous studies merely as a "bulk community". Most progress in research of microbial mats has been made with respect to the macroscopically and microscopically most striking organisms namely the cyanobacteria and bacteria of the sulfur cycle. In comparison, insights into the diversity and individual function of AHB are very scarce.

Degradation of oil derivatives by oil-polluted cyanobacterial mats has recently been demonstrated [2–4,9]. Evidence was obtained that degradation of hydrocarbons was chiefly performed by AHB [3]. However, these aerobic bacteria present a largely unknown community. Furthermore, the presence of oil-degrading AHB in mats raises the questions whether these populations are different than those involved in the degradation of photosynthates and if they play a role in carbon cycling within mats. Our hypothesis is that there are at least two functional guilds, (a) degraders of the autochthonous carbon compounds from cyanobacteria (soluble photosynthates, exopolymers and cell material) and (b) degraders of the external, allochthonous carbon compounds (mostly hydrocarbons) from oil. In order to test this hypothesis, an oil-polluted cyanobacterial mat from Saudi Arabia was chosen. We recently demonstrated the ability of this mat to degrade *n*-octadecane, pristane, phenanthrene and dibenzothiophene at different salinities and temperatures [2]. This mat was expected to harbor many types of halotolerant and thermotolerant AHB that are adapted to the seasonally extreme conditions [2].

In this study, we used culture-dependent and independent (16S rDNA cloning) approaches to gain insights into the species diversity and function of AHB, a largely unexplored group, in the photic layer (2 mm) of an oil-polluted cyanobacterial mat from Saudi Arabia. Our cultivation approach involved both direct plating and serial dilution isolation techniques and focused on AHB that grew on cyanobacterial exudates and hydrocarbons. Representative isolates were tested for their potential to grow on different organic compounds.

Materials and methods

Description of the studied mat

A cyanobacterial mat was collected in November 2002 from the low intertidal flat of Dawhat Al-Daffi, north of Jubail, at the Arabian Gulf coast of Saudi Arabia. The mat had a dry leathery texture with visible precipitated salts on its surface. Because of its tidal position, the mat experiences a daily fluctuation in salinity and temperature that may reach 15% and 40 °C during low tide and drop to 5% and 25 °C during high tide, respectively. Seasonal differences in salinity and temperature are also dramatic (5% and 15 °C in winter and 25% and 50 °C in summer). At the time of sampling, the air temperature was 30 °C and the salinity of the overlying water was 5%. An additional feature of the selected mat is its continuous exposure to oil pollution from nearby oil terminals. Frozen mat samples for molecular work as well as live mat samples for enrichment cultivation were collected.

Most probable number counts

Most probable number (MPN) counts for the studied mat at different salinities and temperatures were compared to a control mat in order to obtain estimates of the abundance of AHB and to check for the presence of halotolerant and thermotolerant populations. The control mat was experimentally established in a glass aquarium using inoculums from a polluted (Etang de Berre, France) and two pristine (near l'Ampolla, Spain, and Horumersiel, Wilhelmshaven, Germany) mats [27]. This mat was grown under controlled salinity of 4% and temperature of 30 °C in a green house. The 2 mm photic zones (measured using oxygen microsensors) of the mat samples (ca. 1 g each) were cut in small pieces using a sterile scalpel and homogenized gently in 10 ml autoclaved seawater medium (see below). Sodium pyrophosphate was added to these suspensions as a dislodgment agent in a final concentration of 0.001 M [12] followed by vigorous vortex for 15 min at room temperature. These suspensions were used for further inoculations. MPN counts were performed in microtiter plates. Each well received 180 µl of autoclaved seawater medium (see below) amended with a mixture of 10 mM acetate and 5 mM succinate. Twenty microliter of the bacterial suspension was added to each well in the first row and mixed thoroughly with the medium. Twenty microliter of the suspension from the first row was transferred to the adjacent row. This procedure was repeated until row 11 (dilution 10^{-12}) and row 12 was left as a blank (medium without bacteria). MPN counts were performed at the following conditions of salinity (S) and temperature (T): (a) $S = 12\%$, $T = 45\,^{\circ}C$ (termed hereafter as HSHT), (b) $S = 5\%$, $T = 45\,^{\circ}C$ (termed as LSHT), (c) $S = 12\%$, $T = 28\,^{\circ}C$ (termed as HSLT) and (d) $S = 5\%$, $T = 28\,^{\circ}C$ (termed as LSLT). MPN counts were calculated using the MPN computer program developed by Clarke and Owens [11].

Construction of a 16S rDNA clone library

A clone library was constructed from the photic layer (2 mm) of the mat obtained from Saudi Arabia in order to study the bacterial diversity within this layer. Nucleic acids were extracted from the mat's photic layer as previously described [1]. Polymerase chain reaction (PCR) was performed on the DNA extract using the GM3 and GM4 primers [29]. The PCR products were purified using the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany) and were cloned using the *TOPO TA Cloning Kit* (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The clones obtained were screened for the presence of inserts and the positive clones were sequenced with an ABI PRISM 3100 genetic analyzer (applied Biosystems, Foster City, Calif.).

Enrichments and isolation of AHB

All enrichments were performed on a defined artificial seawater medium supplemented with a single carbon source. The medium contained $MgCl_2 \cdot 6\ H_2O$ (5.6 g l^{-1}), $MgSO_4 \cdot 7\ H_2O$ (6.8 g l^{-1}), $CaCl_2 \cdot 2\ H_2O$ (1.47 g l^{-1}), KCl (0.66 g l^{-1}) and KBr (0.09 g l^{-1}). Hypersaline media of 5, 8 and 12% (w/v) final total salinity were obtained by adding appropriate amounts of NaCl. After autoclaving, KH_2PO_4 and NH_4Cl solutions were added to the medium in final concentrations of 0.15 and 0.2 g l^{-1}, respectively. Autoclaved solutions of trace elements mixtures [47], selenite and tungstate and vitamins [17] were then added (1 ml each l^{-1}). Solid media were prepared with 1% (w/v) agar.

Direct plating and serial dilution were used as isolation techniques. Different populations of AHB were isolated at different conditions of temperature and salinity and on different carbon sources. The following enrichments were performed: (a) at various combinations of two salinities and two temperatures (as described above for the MPN) using a mixture of 10 mM acetate and 5 mM succinate as a carbon source, (b) serial dilutions on glycolate (photoexcretion product), acetate (fermentation product) and S*pirulina* extract (complex polymeric substances of dead cyanobacteria) and (c) serial dilutions on two types of crude oil; Casablanca oil and Maya oil [14]. Glycolate and acetate were used at a final concentration of 10 mM while *Spirulina* extract (Sigma, Steinheim, Germany) was used at a concentration of 0.05% (w/v). The two oils were used at a concentration of 1% (w/v). The medium had a salinity of 8% and all enrichments were incubated at 28 °C.

All enrichments were done in a 20 ml screw-cap culture tubes filled with 10 ml culture medium under aerobic conditions. Growth was monitored by comparison to abiotic (medium with substrate and without bacteria) and biotic (medium with bacteria and without substrate) controls. Axenic strains were obtained from high dilutions by plating on agar medium containing the same carbon source.

Denaturing gradient gel electrophoresis and phylogenetic analysis

Denaturing gradient gel electrophoresis (DGGE) was performed on the serial dilutions and the obtained isolates in order to check whether the obtained strains were axenic and whether they were present in the high dilution levels. Nucleic acids were released from the strains as well as the dilutions by subjecting the obtained pellets after centrifugation (washed several times and resuspended in 30 µl fresh medium) to 3–5 cycles of freeze (in liquid N_2) and thaw (at 65 °C). PCR for the amplification of 16S rRNA genes was carried out using the primers GM5F with GC-clamp in combination with the universal 907R [29]. DGGE was carried out using a Bio-Rad D-Code system and was run at 60 °C and at a constant voltage of 200 V for 3.5 h. The DGGE bands were excised manually, the DNA was left to diffuse out in buffer overnight, and PCR re-amplified. The amplification products were sequenced in both directions.

The 16S rRNA gene sequences of the obtained strains (ca. 1400 bp) and the DGGE bands (ca. 450 bp) were analyzed using the ARB software [24]. Phylogenetic trees were constructed based on almost complete 16S rRNA sequences (>1300 bp) by applying different methods integrated in the ARB software such as maximum likelihood, maximum parsimony and neighbor joining. Partial sequences were not included in the calculation of trees. Trees calculated using different methods were essentially equivalent. The final trees were minimized for simplicity in presentation.

For determining the number of operational taxonomic units (OTUs), similarity matrices among the sequences of the clones and the isolates were calculated with the ARB program. One OTU was defined for sequences which have more than 97% similarity. Rarefaction curves were calculated using the freeware program aRarefactWin (available at http://www.uga.edu/~strata/software/Software.html).

Substrate utilization

Ten representative strains were tested for growth on selected organic compounds, including cyanobacterial exudates (Table 2) and several alkanes (Table 3), in order to study the role of AHB in carbon cycling within cyanobacterial mats. Individual substrate was added in a final concentration of 10 mM. The used alkanes were filter sterilized using solvent-resistant cellulose filters (0.2 µm pore size) prior to use and were added in a final

concentration of 0.5–2% depending on the toxicity and the solubility of the compound. All incubations were done in test tubes using 10 ml of artificial seawater medium. Each tube was inoculated with 100 µl of cultures pre-grown on acetate. The cultures were incubated at 28 °C in the dark with continuous shaking. Growth on individual substrate was measured by following changes in optical density at 660 nm against biotic (without a substrate) and sterile (without bacteria) controls.

Results

MPN counts

MPN estimates at different combinations of salinities and temperatures yielded counts between 1.15–8.13 × 10^6 cells g^{-1} of AHB in the mat from Saudi Arabia (Table 1). At 5% salinity and 28 °C (termed as LSLT), the control mat yielded higher MPN counts than the studied mat. When the salinity was kept at 5% and the temperature increased to 45 °C (= LSHT), the MPN counts for both mats were comparable and the difference between the two counts was statistically insignificant. The MPN counts at 45 °C were lower than at 28 °C at both incubation salinities (Table 1). The most dramatic difference between the studied mat and the control mat were observed in case of the 12% salinity, regardless of the incubation temperature. The MPN value for the mat from Saudi Arabia was 11 folds higher than the control mat at 28 °C whereas it reached 12.4 fold higher at 45 °C.

Bacterial diversity in the photic layer

The 16S rDNA clone library was constructed from the photic zone of the studied cyanobacterial mat in order to obtain a first insight into the bacterial diversity within this layer (Figs. 1 and 2). The calculated rarefaction curve (Fig. 1) showed that the clone library was far from saturation and the obtained 77 sequences were not enough to describe the entire bacterial diversity in this layer. The clone library had a homologous coverage of 33.8% and the obtained sequences were distributed among 51 OTUs. Most sequences belonged to the *Gammaproteobacteria* (23) and the *Bacteriodetes* (21) groups. The remaining sequences were distributed among the phyla *Alpha-* (2) and *Deltaproteobacteria* (6), *Spirochaetes* (3) *Planctomycetes* (3), *Verrucomicrobia* (5), *Acidobacteria* (3) and *Deinococcus-Thermus* (4). The gammaproteobacterial clones were related to the genus *Marinobacter* and to different sulfur-oxidizing and purple sulfur bacteria. Within the *Bacteriodetes* group, the sequences fell into four clusters and all sequences had less than 94% sequence similarity to their closest relatives. The remaining clones were mostly affiliated to other environmental clones obtained from a wide range of habitats. Our clone library included sequences related to halotolerant and thermotolerant species (e.g. *Marinobacter* spp. and *Halothiobacillus hydrothermalis*), UV and solar radiation resistant species (e.g. *Deinococcus*) as well as to species isolated from oil contaminated sites (e.g. *Marinobacter aquaeoli*, *Geothrix fermentas* and *Holophaga foetida*).

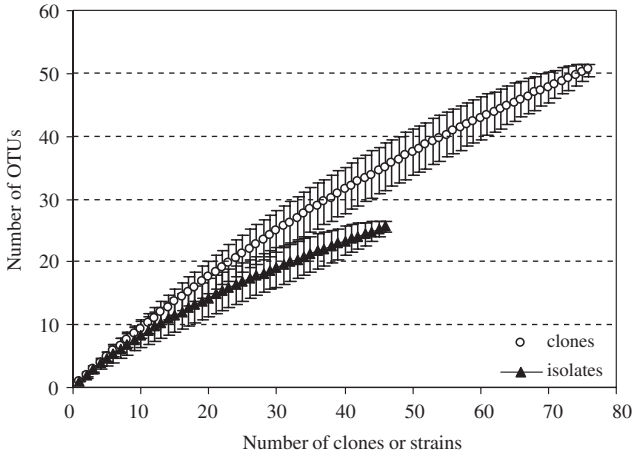


Fig. 1. Calculated rarefaction curves of observed OTUs richness among the clones and the isolates obtained from the photic zone of the studied mat.

Table 1. Most probable number (MPN) counts (cell number g^{-1}) of (AHB) in an oil-polluted microbial mat from Saudi Arabia

Incubation	Salinity (%)	Temperature (°C)	MPN estimates		95% confidence limits	
			Control mat	Saudi mat	Control mat	Saudi mat
LSLT	5	28	1.10×10^7	8.13×10^6	0.50–2.30	3.72–10.78
HSLT	12	28	4.60×10^5	5.07×10^6	1.94–10.95	2.17–10.18
LSHT	5	45	9.27×10^5	1.15×10^6	4.26–20.02	0.64–3.19
HSHT	12	45	1.15×10^5	1.43×10^6	0.52–2.55	0.52–2.51

The serial dilutions were incubated at different combinations of salinities (LS: 5%; HS: 12%) and temperatures (LT: 28 °C; HT: 45 °C). Counts were performed on the top 2 mm photic layer of the mat.

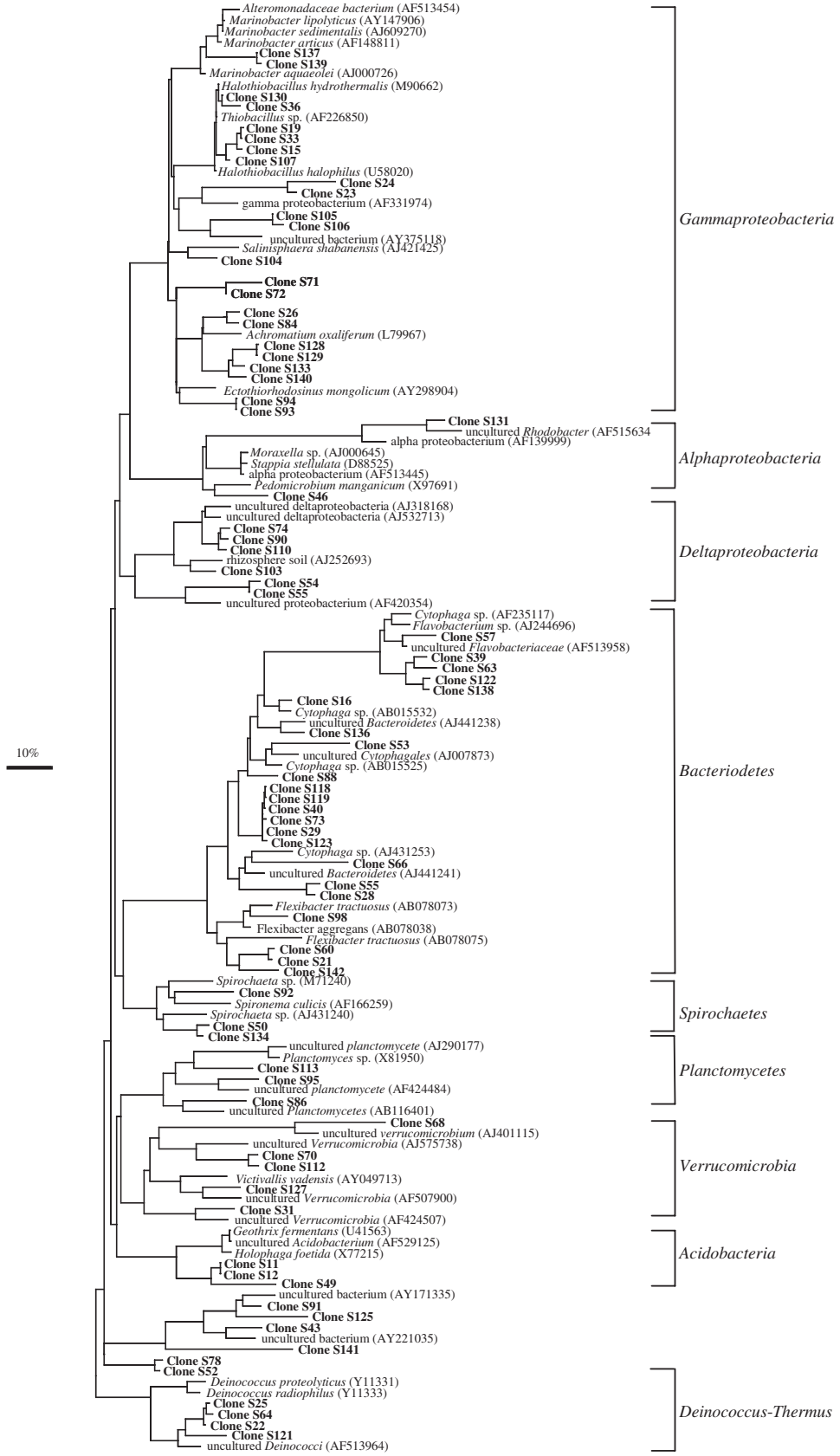


Fig. 2. Unrooted phylogenetic tree showing the affiliations based on 16S rRNA genes of the clones obtained from the photic layer (2 mm) of the mat from Saudi Arabia and selected sequences from various bacterial groups. Most clones were affiliated to the groups *Gammaproteobacteria* and *Bacteriodetes*. The tree was simplified for clarity by omitting all sequences between clusters. The bar indicates 10% sequence divergence.

Phylogenetic affiliation of the obtained strains

A total of 47 strains of AHB were isolated from the photic layer of the studied mat (Fig. 3). The sequences of the strains were distributed among 21 OTUs. The calculated rarefaction curves showed lower diversity of the isolates than the clones (Fig. 1). Enrichments on acetate and succinate at various combinations of salinities and temperatures resulted in the isolation of 28 strains that belonged to the *Gamma-* (22) and *Alphaproteobacteria* (6). The gammaproteobacterial strains constituted three clusters (cluster I, II and III, Fig. 2) closely affiliated to the halophilic *Halomonas* and *Marinobacter* strains. Out of 6 *Halomonas*-related isolates, five strains were obtained at 12% salinity and had more than 96% sequence similarity to each other and 95% to the closest relative *Halomonas salicampi* (cluster I). Within the *Marinobacter* group, one cluster consisted exclusively of strains that were isolated at the 10^{-6} dilution level and shared more than 96.5% sequence similarity to each other (cluster II) whereas the other cluster contained strains from both low and high dilution levels and had more than 94% sequence similarity to each other (cluster III). The six alphaproteobacterial strains formed two clusters (IV and V). The first cluster (IV) had two strains that were phylogenetically related to strains from the genera *Rhodobacter* and *Roseobacter* while the second cluster (V) consisted of four strains that were isolated at 5% salinity and 45 °C (LSHT strains) and were related to *Stappia stellulata* and *Moraxella* sp.

The serial dilutions on acetate and *Spirulina* extract showed growth up to 10^{-7} dilution level and glycolate up to 10^{-9}. The strains displayed a single band on DGGE gels indicating that they were axenic (Fig. 4). The bacterial strains that dominated the lower dilutions were different than those who dominated the higher dilution levels. The sequences of the DGGE bands showed that the strains were dominant in the dilutions from which they were isolated except for strains S71, A61 and A74 (Fig. 4). The phylogenetic affiliation of three strains isolated on acetate (A61, A62 and A74) revealed relatedness to species from the *Alphaproteobacteria* (Fig. 3) while one strain (i.e. A71) belonged to the *Deltaproteobacteria*. The strains isolated on glycolate (G91, G92 and G93) fell next to the halophilic *Halomonas* species within the *Gammaproteobacteria* (Fig. 3). From the enrichments on *Spirulina* extract, three isolates (S71, S72 and S73) were obtained. Strain S71 belonged to the *Alphaproteobacteria* and was closely related to sequences from *Rhodobacter* and *Roseobacter* species, whereas strain S72 and S73 fell within sequences of uncultured *Bacteriodetes*.

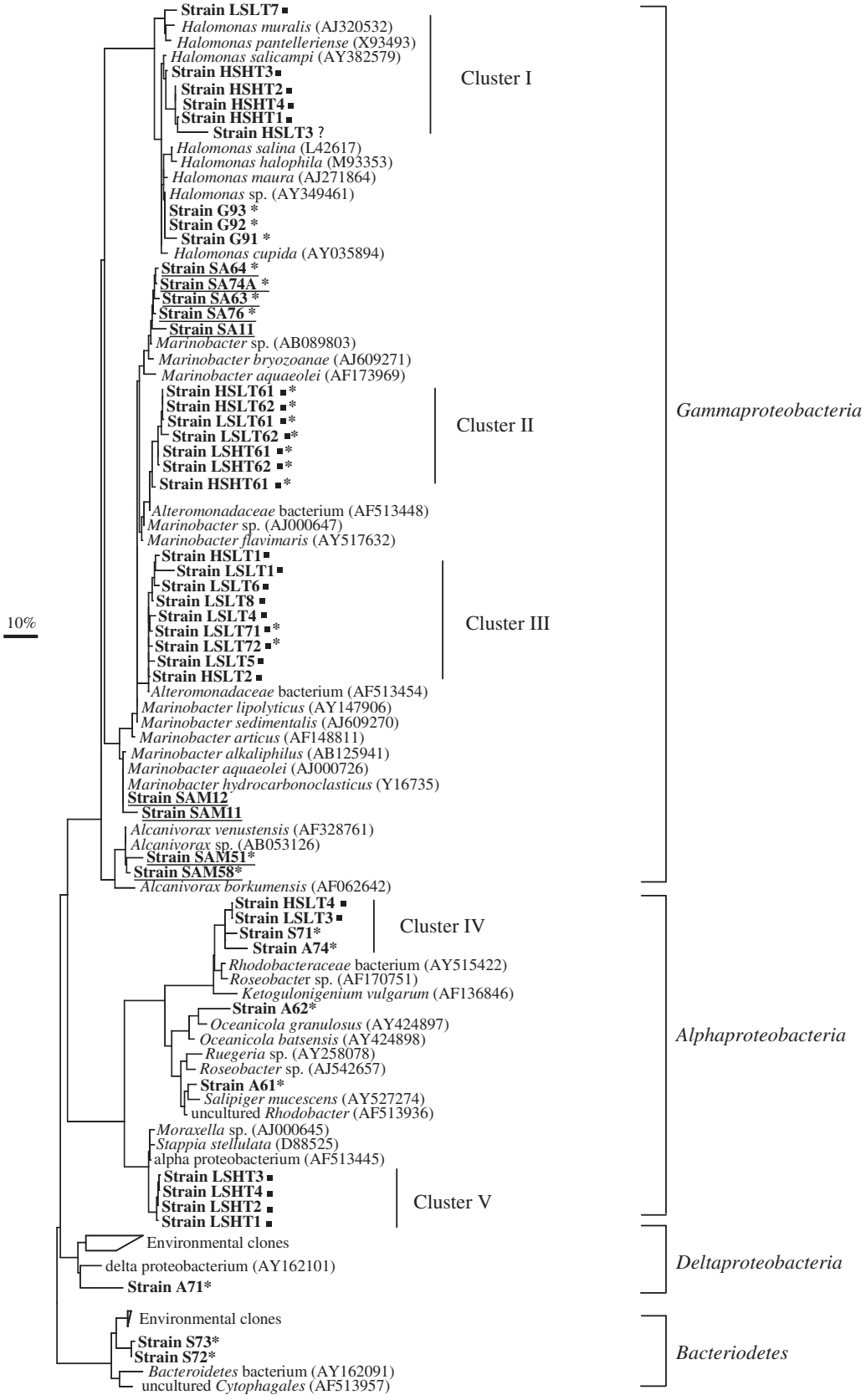
Phylogeny of the strains isolated on oil

The strains enriched on crude oil fell phylogenetically in three clusters within the *Gammaproteobacteria* (underlined strains in Fig. 3). The highest dilution levels, at which growth was detectable, were 10^{-5} and 10^{-7} for Maya oil and Casablanca oil, respectively. All isolated strains from low and high dilutions on Casablanca oil formed one cluster within species of the genus *Marinobacter*. These strains shared 94–98% sequence similarity to *Marinobacter bryozoanae*. The remaining two clusters included isolates from enrichments on Maya oil and were related to known oil-degrading aerobic bacteria within the genera *Marinobacter* and *Alcanivorax*. The strains SAM11 and SAM12 were obtained from the lowest dilutions (10^{-1} and 10^{-2}, respectively) and had 95.9% and 97.8% sequence similarity to *Marinobacter hydrocarbonclasticus*, respectively whereas SAM51 and SAM58 were obtained from the highest dilution (10^{-5}) and had 92% and 96% sequence similarity to *Alcanivorax* sp., respectively.

Substrate utilization

Growth spectra on different organic compounds that occur internally (autochthonous) in microbial mats showed a unique substrate utilization pattern for each species (Table 2). The strains obtained on oil enrichments exhibited good growth on tested photosynthates and fermentation products similar to the other strains. The fatty acids that were readily utilized by most tested strains were acetate, lactate, succinate, butyrate, fumarate and pyruvate. In contrast, none of the strains grew on citrate and malate. Other fatty acids like glycolate, formate, propionate supported the growth of certain strains but not others. All strains grew well on glycerol but not on methanol. Strain S72 showed the best growth on alcohols among all strains. Strains A62 and S72 could not grow on any of the tested amino acids whereas strain SAM58 grew on all of them. Only the strains SAM58 and S71 were able to grow on the compatible

Fig. 3. 16S rRNA-based phylogenetic reconstruction of the AHB strains obtained from the mats of Saudi Arabia. The strains were isolated from incubations at different combinations of salinities (LS: 5%; HS: 12%) and temperatures (LT: 28 °C; HT: 45 °C) using a mixture of acetate and succinate as a substrate (marked with black square). Other strains were isolated on different substrates including Casablanca oil (SA strains, underlined), Maya oil (SAM strains, underlined), glycolate (G strains), acetate (A strains) and *Spirulina* extract (S strains). The strains labeled with an asterisk were obtained from higher dilution levels. All strains were affiliated to the groups *Gamma-*, *Alpha-*, *Deltaproteobacteria* and *Bacteriodetes*. The tree was simplified for clarity by omitting all sequences between clusters. The bar indicates 10% sequence divergence.


Strain LSLT7
Halomonas muralis (AJ320532)
Halomonas pantelleriense (X93493)
Halomonas salicampi (AY382579)
Strain HSHT3
Strain HSHT2
Strain HSHT4
Strain HSHT1
Strain HSLT3 ?
Cluster I
Halomonas salina (L42617)
Halomonas halophila (M93353)
Halomonas maura (AJ271864)
Halomonas sp. (AY349461)
Strain G93 *
Strain G92 *
Strain G91 *
Halomonas cupida (AY035894)
Strain SA64 *
Strain SA74A *
Strain SA63 *
Strain SA76 *
Strain SA11
Marinobacter sp. (AB089803)
Marinobacter bryozoanae (AJ609271)
Marinobacter aquaeolei (AF173969)
Strain HSLT61 *
Strain HSLT62 *
Strain LSLT61 *
Strain LSLT62 *
Strain LSHT61 *
Strain LSHT62 *
Strain HSHT61 *
Cluster II
Alteromonadaceae bacterium (AF513448)
Marinobacter sp. (AJ000647)
Marinobacter flavimaris (AY517632)
Strain HSLT1
Strain LSLT1
Strain LSLT6
Strain LSLT8
Strain LSLT4
Strain LSLT71 *
Strain LSLT72 *
Strain LSLT5
Strain HSLT2
Cluster III
Alteromonadaceae bacterium (AF513454)
Marinobacter lipolyticus (AY147906)
Marinobacter sedimentalis (AJ609270)
Marinobacter articus (AF148811)
Marinobacter alkaliphilus (AB125941)
Marinobacter aquaeolei (AJ000726)
Marinobacter hydrocarbonoclasticus (Y16735)
Strain SAM12
Strain SAM11
Alcanivorax venustensis (AF328761)
Alcanivorax sp. (AB053126)
Strain SAM51*
Strain SAM58*
Alcanivorax borkumensis (AF062642)
Gammaproteobacteria
10%
Strain HSLT4
Strain LSLT3
Strain S71*
Strain A74*
Cluster IV
Rhodobacteraceae bacterium (AY515422)
Roseobacter sp. (AF170751)
Ketogulonigenium vulgarum (AF136846)
Strain A62*
Oceanicola granulosus (AY424897)
Oceanicola batsensis (AY424898)
Ruegeria sp. (AY258078)
Roseobacter sp. (AJ542657)
Strain A61*
Salipiger mucescens (AY527274)
uncultured Rhodobacter (AF513936)
Moraxella sp. (AJ000645)
Stappia stellulata (D88525)
alpha proteobacterium (AF513445)
Strain LSHT3
Strain LSHT4
Strain LSHT2
Strain LSHT1
Cluster V
Alphaproteobacteria
Environmental clones
delta proteobacterium (AY162101)
Strain A71*
Deltaproteobacteria
Environmental clones
Strain S73*
Strain S72*
Bacteroidetes bacterium (AY162091)
uncultured Cytophagales (AF513957)
Bacteriodetes

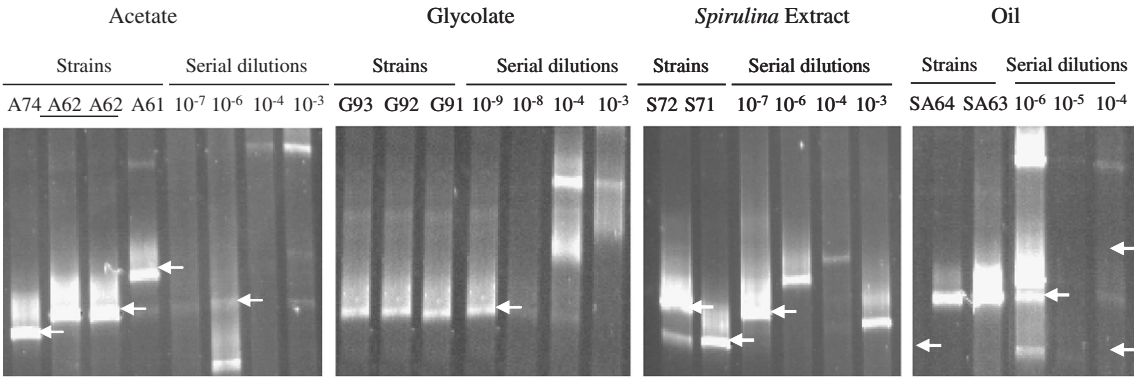


Fig. 4. DGGE bandings of PCR-amplified 16S rRNA fragments obtained from isolated strains and from serially diluted mat suspensions after enrichment on acetate, gylcolate, *Spirulina* extract and Casablanca oil. Arrows show the bands that were sequenced. Note that the isolated strains displayed a single band indicating that they were axenic. Some of the strains were present in the high dilutions whereas others not.

solute betain. All strains grew well on most tested carbohydrates.

Growth on alkanes showed differences among strains (Table 3). The strains SAM58, SAM11, SA64 and SA64 showed growth on most used alkanes. Growth was more pronounced on longer chain alkanes. Strain A62, A74, S71 and G93 did not grow on any of the alkanes even after extended period of incubation. Interestingly, strains S72 and G91 that were originally isolated on *Spirulina* extract and glycolate, respectively, showed a good capability to grow on longer chain alkanes. Growth of these strains on pentane, hexane and octane was not detected.

Discussion

This study provides insights into the diversity of cultured and uncultured AHB in an oil-polluted mat and their role in metabolizing cyanobacterial exudates and petroleum compounds. The photic zone of the studied mat had total cell numbers of AHB in the range of $1.15–8.13 \times 10^6$ cells g^{-1}, which are comparable to previously reported counts for other microbial mats [19]. Halotolerant and thermotolerant populations of AHB were most likely present, as inferred from the MPN estimates. Our clone library and culture collection showed that most of the aerobic bacteria in the photic zone belonged to the groups *Gammaproteobacteria*, *Bacteriodetes* and *Alphaproteobacteria*. AHB isolates exhibited growth on cyanobacterial exudates as well as on short and long chain alkanes.

Molecular versus culture-based diversity of AHB

Sequence analysis of the obtained clones and isolates showed clear differences in their bacterial diversity. The calculated rarefaction curves and the number of OTUs suggested lower bacterial diversity in the strains than in the clones. Except for two *Marinobacter*-related clones, none of the remaining clones had representatives in the culture collection. Similar observations were reported in other studies [34,38,46]. This could be attributed to the insufficient number of sequenced clones or to the preferential PCR amplification of other bacterial groups (e.g. cyanobacteria, sulfur-oxidizing and purple sulfur bacteria) that are also present in the photic zone. On the other hand, the isolated AHB could either represent populations that were present in low numbers and whose detection was not possible by cloning or that the major populations were cultivation-resistant. In all cases, the information obtained from culture-dependent and independent approaches complement each other in bacterial diversity studies.

Our clone library showed the dominance of *Gammaproteobacteria* and *Bacteriodetes* groups in the photic zone. These groups have also been detected form an entire core (oxic and anoxic layers) of the same mat by applying DGGE fingerprinting and band sequencing [2]. The *Gammaproteobacteria* was represented well in the clone library as well as in the culture collection. However, the sequences obtained by cloning were mainly related to bacteria that are involved in the sulfur cycle. The *Bacteriodetes* clones showed higher diversity than the *Bacteriodetes* isolates. Although it is not possible to predict the physiological capabilities of individual phylotypes, certain groups are known to contain aerobic bacteria with heterotrophic mode of life. For example, *Bacteriodetes* group contains typically aerobic bacteria that are specialized in the degradation of complex macromolecules [35] such as exopolymeric substances (EPS) or dead cyanobacteria [41]. *Planctomycetes* species are typical facultative aerobic chemoorganotrophs, growing either by fermentation or respiration of sugars [25]. *Planctomycetes* species were shown to be abundant

Table 2. Substrate spectra of representative strains of AHB on compounds previously identified as excretion products (E; [44]), fermentation products (F; [41]) or compatible solutes (C; [13]) of cyanobacteria

Substrate	Strains									
	Isolated on cyanobacterial exudates						Isolated on crude oil			
	A62	A74	S71	S72	G91	G93	SAM58	SAM11	SA64	SA76
Fatty acids										
Acetic acid	++	++	++	++	++	++	++	++	++	++
Glycolic acid[E]	−	−	−	+	++	++	−	++	−	+
Lactic acid[F]	+	+	+	++	++	−	+	++	++	−
Citric acid[E]	−	−	−	−	−	−	−	−	−	−
Malic acid	−	−	−	−	−	−	−	−	−	−
Succinic acid[E]	++	+	++	++	++	++	++	++	++	++
Butyric acid	+	−	+	++	++	+	++	++	++	−
Methyl-succinic acid[E]	−	−	+	−	−	−	−	+	+	−
Fumaric acid[E]	+	+	+	++	++	++	++	++	++	++
Pyruvic acid[E]	+	−	+	++	++	++	++	++	++	++
Hydroxybutyric acid[E]	−	−	−	+	++	++	++	++	++	++
Iso-valeric acid[E]	−	−	−	−	−	−	−	−	+	−
Propionic acid	++	−	−	−	++	+	++	++	++	++
Formic acid[E,F]	−	−	−	−	+	−	+	−	+	−
Alcohols										
Methanol	−	−	−	−	−	−	−	−	−	−
Ethanol[F]	−	++	+	+	−	+	−	+	−	−
Propoanol	+	−	−	+	−	−	−	−	−	−
Butanol	−	−	−	−	−	−	−	−	+	−
Glycerol[E]	++	++	++	++	++	++	++	++	++	++
Amino acids										
Glycine	−	+	−	−	++	++	+	−	−	−
Betain[C]	−	−	++	−	−	+	+	−	−	−
Alanine	−	+	++	−	++	++	++	++	++	++
Sugars										
Glucose	++	++	++	++	++	++	+	++	−	++
Fructose	++	++	++	++	++	++	+	++	++	−
Galactose	++	−	++	+	++	+	+	+	++	+
Sucrose	++	++	++	++	++	++	+	++	++	++
Ribose	+	++	++	++	++	++	++	++	++	++

(++): maximum growth reached within 3 days; (+): maximum growth reached with 10 days and (−): no or very slow growth. Growth was monitored by following changes in optical density at 660 nm against biotic (without a substrate) and sterile (without bacteria) controls.

in oxic marine sediments [23,28,36]. Other bacterial groups such as *Alpha-* and *Deltaproteobacteria*, *Verrucomicrobia*, *Deinococcus-Thermus*, and *Acidobacteria* have been reported in other oxic cyanobacteria-dominated ecosystems [4,22,37,38,50], thus can potentially grow heterotrophically on cyanobacterial exudates.

Diversity of cultivated AHB species

Cultivation of AHB was necessary in order to distinguish them from other functional groups and to study the physiological capabilities of individual strains. Culture-based techniques are still widely used in diversity studies and in testing and expanding hypotheses in microbial ecology although, enrichment isolation is known to underestimate bacterial diversity [5]. Our isolation attempts involved substrates that were previously identified as important exudates of cyanobacteria within mats and were believed to serve as carbon source for AHB [6,7]. Glycolate was identified as the main compound among photosynthates during hyperoxic and alkaline conditions [7] and acetate is a known fermentation product of cyanobacteria [6,20,30,41,42]. The use of *Spirulina* extract was successfully tested here in order to isolate the AHB that are specialized in the degradation of complex polymeric substrates and dead cyanobacterial cells.

Table 3. Substrate spectra of representative strains of AHB on various alkanes

Substrate	Chemical formulae	Strains									
		Isolated on cyanobacterial exudates						Isolated on crude oil			
		A62	A74	S71	S72	G91	G93	SAM58	SAM11	SA64	SA76
Pentane	C_5H_{12}	−	−	−	−	−	−	+	−	+	−
Hexane	C_6H_{14}	−	−	−	−	−	−	+	−	+	+
Octane	C_8H_{18}	−	−	−	−	−	−	−	−	+	+
Decane	$C_{10}H_{22}$	−	−	−	+	+	−	+	+	+	+
Dodecane	$C_{12}H_{26}$	−	−	−	+	++	−	++	+	++	+
Tetradecane	$C_{14}H_{30}$	−	−	−	++	+	−	++	++	++	++
Hexadecane	$C_{16}H_{34}$	−	−	−	++	+	−	+	++	++	++
Octadecane	$C_{18}H_{38}$	−	−	−	++	+	−	++	++	++	++

(++): fast growth; (+): growth and (−): no or very slow growth. Growth was monitored by following changes in optical density at 660 nm against biotic (without a substrate) and sterile (without bacteria) controls.

The isolation of AHB on glycolate and acetate resulted in the isolation of species related to the genera *Marinobacter* and *Halomonas*, *Roseobacter* and *Rhodobacter*. Species related to these genera were identified in other hypersaline cyanobacteria mats [19,39,40]. The growth of two *Bacteriodetes*-related species (out of 3) on the complex *Spirulina* extract confirms the role of *Bacteriodetes*-related AHB in the degradation of EPS and dead cyanobacteria [41]. The smaller molecules that result from this degradation step are believed to be taken up by other AHB. Using molecular tools, *Bacteriodetes*-related bacteria were shown to colonize the polysaccharide sheaths of filamentous cyanobacteria [3,37]. *Bacteriodetes*-related bacteria were also shown to dominate the microbial community following an experimental viral lysis of the filamentous cyanobacteria [45].

The AHB enriched on oil belonged to the genera *Marinobacter* and *Alcanivorax*. Casablanca oil resulted in the enrichment of only *Marinobacter* species whereas, on Maya oil, species from both genera were enriched. This could be attributed to the different chemical composition of the two oils [14]. Previous studies showed the genera *Marinobacter* and *Alcanivorax* to contain hydrocarbon-degrading species in different marine environments [10,15,18,43,49]. *Marinobacter hydrocarbonoclasticus*, *Marinobacter aquaeolei* and *Alcanivorax borkumensis* were shown to utilize various hydrocarbons as the sole source of carbon and energy [15,18,49]. The dominance of *Marinobacter*-related strains in our culture collection (23 of 47 strains) and the detection of sequences related to this genus in our clone library (two clones) hint to the abundance of this group in the studied mat.

Substrates spectra

The substrate spectra of the representative strains suggested the presence of two functional groups of AHB within the photic zone of the studied mat. The first group included strains that grew well on cyanobacterial exudates but not on alkanes while the second group included strains that grew well on both. Surprisingly, the two strains (SA72 and G91) exhibited good growth on tested alkanes although they were initially enriched on *Spirulina* extract and glycolate, respectively. This indicates that the unique in situ conditions of the studied mat apparently favored the enrichment of a number of versatile bacteria. The substrate spectra of the strains suggest an essential role of the AHB in the carbon cycling in the photic zone of oil-polluted cyanobacterial mats by metabolizing cyanobacterial exudates and/or petroleum compounds.

AHB and cyanobacteria were shown to constitute an ideal model consortium for hydrocarbon biodegradation in which cyanobacteria provide oxygen, fixed nitrogen and organics to the aerobic degraders [3,4,26,37]. Addition of simple cyanobacterial exudates like glucose and lipids were tested in soils and were shown to have stimulatory effect on hydrocarbons biodegradation rates [32,33]. Oil-degrading aerobic bacteria grew initially on these organics until depletion and then they degraded hydrocarbons. In a recent study on pulp and paper waste treatment systems, cyanobacterial exudates have been demonstrated to support the bacterial growth and stimulate the biodegradation of aliphatic and aromatic contaminants [21]. In spite of these reports, the exact role of cyanobacterial exudates on hydrocarbon degradation rates represents an interesting aspect that deserves further in-depth investigations.

In conclusion, AHB in oil-polluted mats represent a diverse community that contain populations capable of growing on autochthonous (photosynthates and fermentation products) and allochthonous (alkanes and oil constituents) organic compounds. AHB are thus essential in carbon cycling in cyanobacterial mats. Of special

importance are *Marinobacter* and *Bacteriodetes*-related AHB, thus further investigations on the distribution and the exact role of these groups in other pristine and polluted mat systems are required. The direct utilization of petroleum compounds by *Bacteriodetes*-related bacteria is interesting and deserves further studies. Further investigations to quantify the different AHB groups by fluorescence in situ hybridization (FISH) and to reveal their in situ role in the carbon cycle by stable isotope probing (SIP) are underway.

Acknowledgment

We would like to thank Lev Neretin, Fumio Inagaki and Miriam Weber for reviewing the manuscript and for their suggestions. We also thank Jacob Jacob for his practical assistance. This research was financially supported by the Deutsche Forschungsgemeinschaft (Grant BE 2167/4) and by the Max Planck Society.

References

[1] R.M.M. Abed, F. Garcia-Pichel, Long-term compositional changes after transplant in a microbial mat cyanobacterial community revealed using a polyphasic approach, Environ. Microbiol. 3 (2001) 53–62.

[2] R.M.M. Abed, A. Al-Thukair, D. de Beer, Bacterial diversity of a cyanobacterial mat degrading petroleum compounds at elevated salinities and temperatures, FEMS Microbiol. Ecol. 57 (2006) 290–301.

[3] R.M.M. Abed, J. Köster, The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds, Int. Biodeterior. Biodegradation 55 (2005) 29–37.

[4] R.M.M. Abed, N.M.D. Safi, J. Köster, D. de Beer, Y. El-Nahhal, J. Rullkötter, F. Garcia-Pichel, Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds, Appl. Environ. Microbiol. 68 (2002) 1674–1683.

[5] R.I. Amann, W. Ludwig, K.-H. Schleifer, Phylogenetic identification and in situ detection of individual microbial cells without cultivation, Microbiol. Rev. 59 (1995) 143–169.

[6] K.L. Anderson, T.A. Tayne, D.M. Ward, Formation and fate of fermentation products in hot-spring cyanobacterial mats, Appl. Environ. Microbiol. 53 (1987) 2343–2352.

[7] M.M. Bateson, D.M. Ward, Photoexcretion and fate of glycolate in a hot spring cyanobacterial mat, Appl. Environ. Microbiol. 54 (1988) 1738–1743.

[8] D.E. Canfield, D.J. Des Marais, Cycling of carbon, sulfur, oxygen and nutrients in a microbial mat, Science 251 (1994) 1471–1473.

[9] P. Caumette, Y. Cohen, J. Grimalt, R. Herbert, M. Kühl, Special Issue: role of microbial mats in the bioremediation of oil polluted coastal zones—Preface, Ophelia 58 (2004) 133–134.

[10] Y.-J. Chang, J.R. Stephen, A.P. Richter, A.D. Venosa, J. Brüggemann, S.J. Macnaughton, G.A. Kowalchuk, J.R. Haines, E. Kline, D.C. White, Phylogenetic analysis of aerobic freshwater and marine enrichment cultures efficient in hydrocarbon degradation: effect of profiling method, J. Microbiol. Methods 40 (2000) 19–31.

[11] T. Clarke, N. Owens, A simple and versatile microcomputer program for the determination of "most probable number", J. Microbiol Methods 1 (1983) 133–137.

[12] S.S. Epstein, J. Rossel, Enumeration of sandy sediment bacteria- search for optimal protocol, Mar. Ecol. Prog. Ser. 117 (1995) 289–298.

[13] E.A. Galinski, Osmoadaptation in bacteria, Adv. Microb. Physiol. 37 (1995) 273–328.

[14] T. Garcia de Oteyza, J.O. Grimalt, Molecular composition of the gas chromatography amenable fractions of Maya crude oil. A reference oil for microbial degradation experiments, Ophelia 58 (2004) 233–242.

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

[16] R.N. Glud, N.B. Ramsing, N.P. Revsbech, Photosynthesis and photosynthesis-coupled respiration in natural biofilms quantified with oxygen microsensors, J. Phycol. 28 (1992) 51–60.

[17] J. Heijthuijsen, T.A. Hansen, Interspecies hydrogen transfer in co-cultures of methanol-utilizing acidogens and sulfate-reducing or methanogenic bacteria, FEMS Microbiol. Ecol. 38 (1986) 57–64.

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

[19] H.M. Jonkers, R.M.M. Abed, Identification of aerobic heterotrophic bacteria from the photic zone of a hypersaline microbial mat, Aquat. Microb. Ecol. 30 (2003) 127–133.

[20] B.B. Jørgensen, D.C. Nelson, D.M. Ward, Chemotrophy and decomposition in modern microbial mats, in: J.W. Schopf, C. Klein (Eds.), The Proterozoic Biosphere: A Multidisciplinary Study, Cambridge University Press, Cambridge, 1992, pp. 287–293.

[21] A.E. Kirkwood, C. Nalewajko, R.R. Fulthorpe, The effects of cyanobacterial exudates on bacterial growth and biodegradation of organic contaminants, Microb. Ecol. 51 (2006) 4–12.

[22] E. Kolmonen, K. Sivonen, J. Rapala, K. Haukka, Diversity of cyanobacteria and heterotrophic bacteria in cyanobacterial blooms in lake Joutikas, Finland, Aquat. Microb. Ecol. 36 (2004) 201–211.

[23] E. LIobet-Brossa, R. Rossellò-Mora, R. Amann, Microbial community composition of Wadden Sea sediments as revealed by Fluorescence-*in-situ*-Hybridization, Appl. Environ. Microbiol. 64 (1998) 2691–2696.

[24] W. Ludwig, O. Strunk, R. Westram, L. Richter, H. Meier, A. Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A.W. Ginhart, O.

Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, K.-H. Schleifer, ARB: a software environment for sequence data, Nucl. Acids Res. 32 (2004) 1363–1371.

[25] M.T. Madigan, J.M. Martinko, J. Parker, Brock Biology of Microorganisms, Prentice-Hall, NJ, 2000.

[26] F. Musat, J. Harder, F. Widdel, Study of nitrogen fixation in microbial communities of oil-contaminated marine sediment microcosms, Environ. Microbiol. 8 (2006) 1834–1843.

[27] F. Musat, A. Wieland, F. Widdel, Marine sediment with surface contamination by oil in microcosms for microbiological studies, Ophelia 58 (2004) 217–222.

[28] N. Musat, U. Werner, K. Knittel, S. Kolb, T. Dodenhof, J.E.E. van Beusekom, D. de Beer, N. Dubilier, R. Amann, Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea, Syst. Appl. Microbiol. 29 (2006) 333–348.

[29] G. Muyzer, A. Teske, C.O. Wirsen, H.W. Jannasch, 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 (1995) 165–172.

[30] S.C. Nold, D.M. Ward, Photosynthate partitioning and fermentation in hot spring microbial mat communities, Appl. Environ. Microbiol. 62 (1996) 4598–4607.

[31] H.W. Paerl, B.M. Bebout, S.B. Joye, D.J. Des Marais, Microscale characterization of dissolved organic-matter production and uptake in marine microbial mat communities, Limnol. Oceanogr. 38 (1993) 1150–1161.

[32] S.S. Radwan, H.A. Al-Aawadi, M. Khanafer, Effects of lipids on *n*-alkane attenuation in media supporting oil-utilizing microorganisms from the oily Arabian Gulf coasts, FEMS Microbiol. Lett. 198 (2001) 99–103.

[33] S.S. Radwan, D. Al-Mailem, I. El-Nemr, S. Salamah, Enhanced remediation of hydrocarbon contaminated desert soil fertilized with organic carbons, Int. Biodeterior. Biodegradation 46 (2000) 129–132.

[34] A. Ranchou-Peyruse, R. Herbert, P. Caumette, R. Guyoneaud, Comparison of cultivation-dependent and molecular methods for studying the diversity of anoxygenic purple phototrophs in sediments of an eutrophic brackish lagoon, Environ. Microbiol. 8 (2006) 1590–1599.

[35] H. Reisenbach, The order *Cytophagales*, in: A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.-H. Shleifer (Eds.), The Prokaryotes, Springer, Berlin, Germany, 1991.

[36] A. Rush, M. Huettel, C.E. Reimers, G.L. Taghon, C.M. Fuller, Activity, distribution of bacterial populations in Middel Atlantic Bight shelf sands, FEMS Microbiol. Ecol. 44 (2003) 89–100.

[37] O. Sánchez, E. Diestra, I. Esteve, J. Mas, Molecular characterization of an oil-degrading cyanobacterial consortium, Microb. Ecol. 50 (2005) 580–588.

[38] C.M. Santegoeds, S.C. Nold, D. Ward, Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat, Appl. Environ. Microbiol. 62 (1996) 3922–3928.

[39] P. Sigalevich, M. Baev, A. Teske, Y. Cohen, Sulfate reduction and possible aerobic metabolism of the sulfate reducing bacterium *Desulfovibrio oxyclina*e in a chemostat co-culture with *Marinobacter* sp. strain MB under exposure to increasing oxygen concentrations, Appl. Environ. Microbiol. 66 (2000) 5013–5018.

[40] P. Sigalevich, Y. Cohen, Oxygen dependant growth of the sulfate reducing bacterium *Desulfovibrio oxyclinae* in co-culture with *Marinobacter* sp. strain MB in an aerated sulfate depleted chemostat, Appl. Environ. Microbiol. 66 (2000) 5019–5023.

[41] L.J. Stal, Physiological ecology of cyanobacteria in microbial mats and other communities, New Phytol. 131 (1995) 1–32.

[42] L.J. Stal, R. Moezelaar, Fermentation in cyanobacteria, FEMS Microbiol. Rev. 21 (1997) 179–211.

[43] K. Syutsubo, H. Kishira, S. Harayama, Development of specific oligonucleotide probes for the identification and in situ detection of hydrocarbon-degrading *Alcanivorax* strains, Environ. Microbiol. 3 (2001) 371–379.

[44] M.L.O. Teiser, Extracellular low molecular weight organic compounds produced by *Synechococcus* sp. and their roles in the food web of alkaline hot spring microbial mat communities, University of Oregon, Eugene, 1993.

[45] E.J. van Hannen, G. Zwart, M.P. van Agterveld, H.J. Gons, J. Ebert, H. Laanbroek, Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses, Appl. Environ. Microbiol. 65 (1999) 795–801.

[46] D.M. Ward, R. Weller, M.M. Bateson, 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community, Nature 345 (1990) 63–65.

[47] F. Widdel, F. Bak, Gram-negative mesophilic sulfate-reducing bacteria, In: A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer (Eds.), The Prokaryotes, second ed., Springer, New York, 1992, pp. 3353–3378.

[48] A. Wieland, M. Kühl, Short-term temperature effects on oxygen and sulfide cycling in a hypersaline cyanobacterial mats (Solar Lake, Egypt), Mar. Ecol. Prog. Ser. 196 (2000) 87–102.

[49] M.M. Yakimov, P.N. Golyshin, S. Lang, E.R.B. Moore, W.-R. Abraham, H. Lünsdorf, K. Timmis, *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium, Int. J. Syst. Bacteriol. 48 (1998) 339–348.

[50] G. Zwart, B.C. Crump, M.P.K. van Agterveld, F. Hagen, S. Han, Typical freshwater bacteria: analysis of available 16S rRNA gene sequences from plankton of lakes and rivers, Aquat. Microb Ecol. 28 (2002) 141–155.