CHARACTERISATION OF HEXANE-DEGRADING MICROORGANISMS FROM WASTE GAS BIOFILTERS

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In loving memory of Inge Naismith

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I. Introduction

BIOFILTRATION: TREATMENT OF WASTE GAS

The increased ecological awareness in the 80s and early 90s of the last century led to stricter environmental guidelines. Waste air was not merely regarded as a source of odour nuisance but also in terms of its health and environmental impacts. Thus, biological waste gas treatment was no longer used almost solely for odour control as in the 1960s and the application of biological waste gas treatment for the elimination of solvents in industrial waste gas followed. Waste gas emissions are a common problem in various branches of industry such as the chemical and pharmaceutical industry, the automotive, the furniture, the textile, the agricultural, and the food industry. Biological waste gas treatment is applicable when processes emit high volume loads containing degradable pollutants in relatively low concentrations. Only if pollutants are more concentrated than 5 g/m^3, chemical or physical treatments are more suitable (Engesser et al., 1997). Because investment and operation costs of biological air remediation have proven to be considerably lower than those of chemical and physical techniques biological waste gas treatment has become very dominant (Ottengraf, 1986; Ottengraf and Diks, 1992; Menig et al., 1997). Apart from being cost-effective biological air remediation ideally leads to a complete degradation of the pollutants to carbon dioxide, water, salt and biomass (Engesser, 1992; Converse et al., 2003). Three forms of biological waste gas treatment have established on the market: biofilters, bioscrubbers and trickling filters, the biofilter being the most commonly applied version (Sabo et al., 1996). The simplest construction of a biofilter is the open biofilter. Generally, the pre-treated waste gas is pumped through the filterbed in an upward direction. The packing material usually consists of organic material such as heather-peat mixtures, wood chops, preferably crushed tree roots or tree bark compost. Apart from the advantage of being able to dispose off the organic packing material after usage as compost, the organic filter material also supports the microbiological population of the biofilter with additional nutrients and provides enough surface area for the microorganisms. Most organisms are thought to live in the water layer surrounding the packing material (Ottengraf, 1986). When air flows around the packing material a continuous mass transfer between the gas and water phase occurs. Biological degradation or consumption of the compounds in the water layer drives the continuous mass transfer by retaining the concentration gradient (Ottengraf and Diks, 1992). Thus, biofiltration is not a classical filtration process because the compounds are actually degraded and converted. Most studies on the degradation of hydrophobic substances such as hexane have focused on the technical biofiltration performance (Morgenroth et al., 1996; Budwill and Coleman, 1997; Kastner et al., 1999; Zhu et al.,

2004). This study rather focuses on the actual degrading microorganisms of the biofilter population. The ability to convert alkanes has been described for a variety of bacteria. The most commonly mentioned genera are *Pseudomonas, Acinetobacter, Arthrobacter, Mycobacterium*, and *Nocardia* (Britton, 1984; Witholt et al., 1990). More recently *Alcanivorax, Rhodococcus, Nocardioides,* and *Gordonia* have been shown to utilise alkanes including short-chained gaseous alkanes such as hexane (Kummer et al., 1999; Saadoun et al., 1999; Hamamura et al., 2001; Kasai et al., 2002; Saadoun, 2003).

CULTIVATION-DEPENDENT AND -INDEPENDENT APPROACHES

A number of combined culture-dependent and -independent studies have been conducted. Wagner and co-workers (1993) found the community structure obtained by the application of cultivation-independent rRNA-targeting probes to deviate substantially from that obtained by culturing. During an investigation of grassland soils the cultivation-independent cloning of environmental 16S rDNA led to a microbial community structure that did not correlate with the cultivation-dependent investigation (Felske et al., 1999). Usually, the enrichment seamed to have introduced the bias. Fatty acid analyses of cell pellets during enrichment phases showed strong shifts and pronounced deviations from the fatty acid profile of the original samples (Piehl, 2002). Studies have been conducted to isolate some bacteria whose rDNA is common in cultivation-independent analysis of ecosystems but even despite the wide spectrum of growth media employed the isolation of many of these strains failed (e.g. Rheims et al., 1999). Since current culture techniques do not always satisfy the need of providing a balanced picture of the microbial community, future developments in the study of bacterial diversity should include improvements in cultivation methods to approach as closely as possible the conditions of natural habitats (Palleroni, 1997). Thus, the isolation approach may not be designed to isolate only the fast-growing, easy-to-handle strains. In contrast, the scientist must also try and cultivate the fastidious strains as these may be of importance within the ecosystem to be analysed. Many properties that make these organisms important members of the living World are amenable to observation only through the study of living cultures. Additionally, analysis of phospholipid fatty acids (PLFAs) is not entirely isolation independent because interpretations are based on known fatty acid profiles (Spring et al., 2000). The same holds true for interpretations of data obtained by fluorescence *in situ* hybridisation (FISH) which obviously also depend on knowledge of known taxa. Concerning FISH the dependence on known taxa can be partly overcome by applying the full cycle rRNA approach. Culture-independent approaches may describe the *in situ* situation more realistically whereas cultured organisms offer deeper insights into their physiology and genetics that are not easily accessible *in situ*. Therefore, the combination of isolating organisms and cultivation-independent methods is favourable.

Application of two cultivation-independent methods

Lipids, especially fatty acids, have long been used in phylogenetic and taxonomic classification (Lechevalier, 1977; Lechevalier and Lechevalier, 1988). The lipid analysis has been applied to assess microbial communities in mud, soil, rhizosphere, sediments, and biotechnological applications (White et al., 1996; Lipski et al., 2001; Knief et al., 2003; Timke et al., 2005). Using certain fatty acids as signature lipid biomarkers provides a quantitative means of measuring viable microorganisms (Findlay et al., 1989), microbial community composition (Fredrickson et al., 1995), and community nutritional/physiological status (Guckert et al., 1986; Sikkema et al., 1995). Unlike most other biomarkers, phospholipids are quickly degraded; degradation ranging from minutes to a few hours after cell death (White and Ringelberg, 1997). This rapid degradation of phospholipids to diglycerides makes phospholipid fatty acids (PLFAs) excellent biomarkers for viable cells (White et al., 1997). Because different groups of microorganisms synthesise a variety of PLFAs through various biochemical pathways (White et al., 1997), they are effective taxonomic markers, useful to define community composition. However, there is an overlap in PLFA composition between different species, and as such, it is not possible to define each species with a unique pattern of PLFA. Moreover, because only about 0.1 –1% of the bacterial taxa have been isolated so far (Amann, 1995; Pimm et al., 1995; Hugenholtz et al., 1998), the number of available PLFA or FAME profiles is still limited.

Since the introduction of rRNA-targeting oligonucleotide probes (Stahl et al., 1988), the limitations of cultivation-dependent methods have partly been circumvented because the *in situ* detection, identification, and quantification of microorganisms were strongly facilitated. Over the past decade, the composition of complex microbial communities has in many studies been analysed by rRNA-targeting nucleic acid probes. A commonly applied method is the fixation of whole cells, which are then directly enumerated after *in situ* hybridisation. Usually fluorescently labelled probes are used giving the method the name fluorescence *in situ* hybridisation (FISH). The reasons for targeting probes to 16S/18S rRNA of the small subunit of the ribosome or to the 23S/28S rRNA of the large subunit are numerous and have been discussed in depth (Woese, 1987). In sum, the large amount of rRNA in most cells is advantageous as well as the apparent lack of lateral gene transfer, a decent length of 16S and 23S, of about 1500 and 3000 nucleotides, respectively, and the range of variable to highly conserved sites. Additionally, an essential prerequisite for the construction of specific probes are large sequence databases, which have expanded rapidly over the last decade. Not even 15 years ago data sets only contained several hundred rRNA sequences, now they contain some ten thousands. Furthermore, rRNA-targeting probes may also be designed based on sequences obtained from uncultured species by using 16S rDNA clone libraries. Additionally, FISH gives not only qualitative but also quantitative information.

The application of these two cultivation-independent methods in combination with the isolation of bacteria from the biofilters enables a comprehensive analysis of the microbial ecology of these systems.

II. Materials and Methods

Unless stated otherwise, all of the chemicals were purchased from Merck, Darmstadt, Germany, Sigma-Aldrich, Taufkirchen, Germany, or from Riedel-de-Häen, Seelze, Germany.

ANALYSES OF BIOFILTER PACKING MATERIAL

Sample collection and physicochemical analyses

The sample Alberta originates from a laboratory-scale, peat-based biofilter used for the elimination of hexane. The sample was donated by Karen Budwill (Alberta Research Council, Vegreville, Canada) in December 1997. Samples Hamm A and B were collected from two sites of a biofilter used for hexane-waste gas treatment in an oil mill (Hamm, Germany) in December 1997. The size of the filterbed was 300 m^2 and the filling height was about 1.5 m. The waste gas was pumped through the filterbed in an upward direction. The biofilter packing material consisted of crushed tree roots and samples were dug up from a depth of about 0.7 m. Site A was closer to the inlet of the waste gas into the biofilter (~ 3 m distance) than site B (~ 12 m distance). Additional samples were taken from the biofilter in Hamm in May 2001. The samples were taken from the same site, near site A but from two different depths, at 0.2 m for sample O and at 0.7 m for sample U. It has to be noted that the packing material of the biofilter in Hamm was replaced in June 1999. For pH-measurements, 10 g of fresh filter material were stirred in 25 ml of 1 M KCl for 30 min before being analysed with a pH electrode. Between 40 and 50 g of fresh filter material were dried at 80°C for 48 h to assess the water content.

Direct enumeration of biofilter samples

For direct counts, 10 g of fresh filter material were stirred in 100 ml of Ringer solution (0.9% NaCl, 0.042% KCl, 0.024% $NaHCO_3$) for 30 min. Subsequently, 5 ml of the solution were fixed with 15 ml of 4% paraformaldehyde (PFA) and stored at 4°C. To ease direct enumeration, fixed samples were diluted about 1:100 with sterile 0.9% NaCl solution to yield about 100 cells per microscopic field. Between 1 and 5 ml of the subsamples were stained with DAPI (4'–6–diamidino–2–phenylindole; final concentration, 2 mg l^{-1}) (Porter and Feig, 1980) in a filtration tower for 5 min, and then filtered on black polycarbonate membrane filters (pore size 0.2 µm, Nuclepore) by application of gentle vacuum pressure (≈ 2 kPa). If less than 4 ml of the subsamples were needed, the subsamples were added to sterile-filtered water in the filtration tower so a total volume of 5 ml was obtained to

encourage an even distribution of the cells on the filter. Filters were mounted in melting-point bath oil (M-6884; Sigma). At least 400 DAPI-stained cells were counted by examining at least 20 randomly selected microscopic fields.

MICROBIOLOGICAL METHODS

Origin and cultivation of strains

Origin of strains and isolation procedure

Enrichment cultures were started with the samples of the laboratory scale biofilter from Alberta and with the samples of the sites A and B of the industrial biofilter in Hamm (see above). Approximately 1 g of biofilter packing material was placed in 50 ml of minimal medium (see below) together with 2 µl of hexane for one of the laboratory scale biofilter subsamples and 10 µl of hexane for all other enrichments in airtight 100 ml screw-cap flasks capped with butyl rubber septa. After 6 to 7 passages in minimal medium containing hexane as sole carbon source the enrichment cultures were diluted and 0.1 ml of the dilutions 1×10^{-4} to 1×10^{-7} were used to inoculate agar plates of minimal medium which were then kept in excicators with hexane added to the atmosphere. Usually, 1 ml of hexane was used per 5 l excicator volume. This isolation procedure was carried out at 30°C at a pH of 7 and for the Hamm samples also at a pH of 5 because the Hamm biofilter exhibited a low pH. Strains were then separated to obtain pure strains.

Cultivation

The minimal medium used to grow the organisms was prepared of 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.5 g $MgSO_4 \times 7\ H_2O$, 0.01 g $FeSO_4 \times 7\ H_2O$, and 1 g $(NH_4)_2SO_4$. One millilitre of a trace element solution containing per litre 3 g Na_2-EDTA, 0.05 g $MnCl_2 \times 2\ H_2O$, 0.19 g $CoCl_2 \times 6\ H_2O$, 0.041 g $ZnCl_2$, 0.006 g H_3BO_3, 0.024 g $NiCl_2 \times 6\ H_2O$, 0.002 g $CuCl_2$, and 0.018 g $NaMoO_4 \times 2\ H_2O$ with the pH adjusted to 6 was added in addition to 5 ml of a vitamin solution (0.01 g thiamine, 0.02 g nicotinic acid, 0.02 g pyridoxine-hydrochloride, 0.01 g *p*-aminobenzoic acid, 0.02 g riboflavin, 0.02 g panthotein acid, 0.001 g biotin and 0.001 g cyanocobalamin in 1 l of distilled water adjusted to a pH of 7). The medium solution was then filled up to 1 litre with double distilled water. Hexane was the sole carbon source and was added to the gas phase. The majority of the strains could be transferred onto nutrient broth medium containing 5 g l^{-1} of peptone derived from pancreatically digested meat and 3 g l^{-1} of meat extract. For solid media, 15 to 16 g l^{-1} of agar were added.

Polyphasic classification of strains

Morphology

Colour and morphology of three-day-old colonies grown, unless stated otherwise, on trypticase soy broth (TSB, Becton Dickinson, Heidelberg, Germany) plates were described and cells were examined microscopically under phase contrast using a Zeiss Axioskop microscope equipped with a 100 × oil immersion lens. For size measurements of cells, images were recorded using a charge-coupled device camera (Spot RT slider, Visitron systems, Puchheim, Germany) and analysed with the image analysis software package KS300 (Zeiss).

Fatty acid analysis

Unless indicated otherwise all cells were grown on trypticase soy broth plates (TSB, Becton Dickinson, Heidelberg, Germany) containing 1.5% (w/v) of agar at 25°C for 3 days. Saponification, methylation and extraction of fatty acids were performed as described previously (Sasser, 1990). Fatty acids were identified by gas chromatography-mass spectrometry with a Hewlett-Packard model 5890 series II gas chromatograph equipped with a 5% phenyl methyl silicone capillary column (0.25 mm by 30 m) and a type 5972 mass selective detector. The carrier gas was helium. The injection volume of the samples was 2 µl. The injector temperature was 250°C, the column temperature was increased from 120 to 240°C at a rate of 5°C/min and the gas chromatography-mass spectrometry transfer line temperature was 280°C. Fatty acid methyl esters (FAMEs) were identified on the basis of their retention times and their mass spectra. For verification of the position of double bonds and cyclopropyl groups, dimethyl disulfide adducts and dimethyloxazoline derivates of the FAMEs were analysed (Nichols et al., 1986; Zhang et al., 1987). The positions of double bonds and of cyclopropene, hydroxy, and methyl groups were determined from the carboxyl group of the fatty acid molecule according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN, 1977). The dendrogram of the fatty acid profiles is based on Euclidian distances calculated using the Ward linkage method (Systat Version 10).

Analyses of isoprenoid quinones, polar lipids, mycolic acids and acyl type of peptidoglycan

Polar lipids and isoprenoid quinones were extracted according to Minnikin et al. (1984). Polar lipids were analysed applying two-dimensional silica gel thin-layer chromatography (Minnikin et al., 1984). The isoprenoid quinones were separated using the method described previously (Kroppenstedt, 1982). Ubiquinones were identified further using RP-18 F_{254}-TLC-plates with Q10 as a standard. The menaquinone extracts were analysed with a

Hewlett-Packard 1050 series model HPLC as described previously (Kroppenstedt, 1985). Mycolic acids were extracted and analysed by the method of Minnikin et al. (1975). The acyl type of the peptidoglycan was analysed by a photometric test (Uchida and Aida, 1977).

Physiological tests

Cells harvested for physiological tests were usually grown on nutrient broth or minimal medium at 25°C for 3 days.

Catalase and oxidase tests were carried out with 3.5% hydrogen peroxide and tetramethyl-1.4-phenyldiamine-dihydrochloride, respectively (McFaddin, 1980). The KOH-test (Buck, 1982), the aminopeptidase-test (Cerny, 1978), and Gram-staining were performed according to standard protocols.

The physiological profile of strains belonging to the *Proteobacteria* and *Flavobacteria* was determined using API 20NE strips (bioMérieux sa, Marcy-l'Etoile, France) and microplates that allow testing of 95 substrates simultaneously (Biolog GN MicroPlate, Hayward). In addition, API 50 carbohydrate substrate strips and API 50 CHB/E medium (both bioMérieux sa, Marcy-l'Etoile, France) were used to determine the assimilation and the oxidation patterns of some strains, respectively. Additionally, growth tests were carried out in 20 ml glass tubes closed with teflon-coated air tight lids, to which 5 ml of minimal medium and following substrates were added as sole carbon source: D-alanine, D-threonine, L-aspartic acid, L-lysine, L-arabinose, D-fructose, D-glucose, succinate, citrate and β-hydroxybutyric acid. Generally, the carbon sources were prepared as 20% (w/v) aqueous solutions that were autoclaved or filter sterilised. The carbon sources were then added to the media to obtain identical final concentrations of carbon atoms.

The ability to grow on different alkanes (pentane, hexane, heptane, octane, nonane, decane) was tested. Ten microlitres of the different alkanes were added and 50 µl of 20% β-hydroxybutyric acid served as a positive control.

Growth at temperatures ranging from 4 to 45°C was tested, as well as the ability to grow at different pH-levels. Temperature tests were carried out in minimal medium or nutrient broth as described above. Growth at different pH values was carried out in minimal medium, which was treated with citrate-phosphate buffer below pH 6, with phosphate buffer from 6 to 8 and with Tris-HCl buffer at a pH value above 8. In order to avoid misinterpretations due to different buffering systems the unmodified minimal medium (see above) was merely adjusted to different pH values in the controls and not buffered additionally. With the aim of determining the possible oligotrophic character of a strain, nutrient broth (see above) was diluted (1:5; 1:10; 1:25; 1:50; 1:100) and buffered with K_2HPO_4 and KH_2PO_4 to pH 7. Except for the temperature growth tests, these experiments were carried out at 30°C in tube rollers. The former were carried out in diagonally placed glass tubes to increase the surface area of the media. The tubes were shaken twice daily to avoid the settling of cells and the

limitation of oxygen or hexane. Growth was monitored by measuring the optical density (OD) at 578 nm.
Nutrient broth was used as a pre-inoculation medium for the majority of strains and minimal medium plus hexane was used for those strains that could not grow on NB. For the inoculation of the tubes, cells grown for 3 to 5 days on agar plates were transferred into 3 ml of 0.9% NaCl until a turbidity of Mc Farland standard 1 was reached (McFaddin, 1980). To ensure comparability all test tubes were then inoculated with 0.1 ml of these cell suspensions. Sterility of vitamins and alkanes was monitored by adding them onto solid nutrient broth medium. In certain occasions grown cells of test tubes were brought onto agar plates to verify their identity.

MOLECULAR BIOLOGICAL METHODS

Fluorescence In Situ Hybridisation (FISH)

Cultivation and fixation of strains

Pure cultures of strains were cultivated in 5 ml of nutrient broth or minimal medium (with hexane or β-hydroxybutyric acid added as carbon sources) and harvested in exponential growth phase ($OD_{578\,nm}$ between 0.5 and 0.8). Strains belonging to the *Firmicutes* and *Actinobacteria* were fixed with ethanol (100%) at a final concentration of 50% and stored at –18°C. All other strains (5 ml) were fixed with 15 ml of 4% paraformaldehyde (PFA) solution (Amann et al., 1990) overnight at 4°C or for 4 h at room temperature and centrifuged at 11200 × *g* for 20 min. Pellets were washed twice in 20 ml phosphate-buffered saline (PBS; 10 mM $Na_2HPO_4 \times 2H_2O$ adjusted to pH 7.4 with 10 mM $NaH_2PO_4 \times H_2O$; final concentration of NaCl, 130 mM) and centrifuged. Cells were then resuspended in 1:1 phosphate-buffered saline-ethanol and stored at –18°C.

FISH analyses of biofilter samples

Aliquots of biofilter material treated with Ringer solution (see above) were fixed as described for the reference strains with both, PFA solution and with ethanol. For *in situ* hybridisation, 3 µl of each fixed sample were spotted onto precleaned (washed in 1% HCl and rinsed with 70% ethanol) and gelatin-coated [0.075% gelatin–0.01% $CrK(SO_4)_2$] slides. The slides were then dried at 42°C for 10 min. The slides were placed in 50, 80, and 100% ethanol for 3 min each. After dehydration the samples were covered with 8 µl hybridisation buffer Manz et al. (1992) and 1 µl probe (50 ng/µl). Oligonucleotides were synthesised and fluorescently labelled with Cy3 (Amersham), at the 5' end by Thermo Electron (Ulm,

Germany). The rRNA-targeted oligonucleotides used are summarised in Table 1. Samples were hybridised at 46°C for 90 min in isotonically equilibrated humid chambers. Afterwards, samples were treated with a posthybridisation wash as described by Manz et al. (1992) at 48°C for 15 min. Sodium chloride concentrations in the washing buffer were selected according to the formulae of Lathe (1985). Slides were briefly rinsed with distilled water, air dried and counterstained with 10 µl of DAPI (1 mg $litre^{-1}$) at 4°C for 10 minutes in the dark and rinsed with distilled water. Slides were subsequently mounted in Vectashield (Vector Laboratories Inc., Burlingame, Calif., USA). DAPI- and probe-conferred fluorescence signals were examined by use of a Zeiss Axioskop epifluorescence microscope equipped with the filter sets no. 01 (Zeiss) and an HQ-Cy3 filter set (AF Analysentechnik, Tübingen, Germany). Microscopic photographs were recorded by use of a Spot RT slider CCD camera (Visitron systems, Puchheim, Germany).

Table 1. Data on probes used in the present study.

Probe	Target (position)[a]	Sequence (5'→3')	Applied stringency (formamide concentration [%])	Reference
XAN818	*Xanthomonas* branch 16S rRNA, (818–835)	CAACATCCAGTTCGCATC	10	(Friedrich et al., 1999)
EUB338	Bacterial 16S rRNA, (338–355)	GCTGCCTCCCGTAGGAGT	10	(Amann et al., 1990)
NEV817[b]	*Nevskia ramosa* and relatives 16S rRNA, (817–832)	CGTCAAGTTCTCATCG	10	(Friedrich et al., 2003)
ALF968	α- proteobacterial 16S rRNA, (968–986)	GGTAAGGTTCTGCGCGTT	35	(Neef, 1997)
BET42a	β- proteobacterial 23S rRNA, (1027–1043)	GCCTTCCCACTTCGTTT	35	(Manz et al., 1992)
CF319a	*Cytophaga-Flavobacterium* cluster of the CFB phylum 16S rRNA, (319–336)	TGGTCCGTGTCTCAGTAC	35	(Manz et al., 1996)
GAM42a	γ- proteobacterial 23S rRNA, (1027–1043)	GCCTTCCCACATCGTTT	35	(Manz et al., 1992)
HGC69a	*Actinobacteria* 23S rRNA, (1901–1918)	TATAGTTACCACCGCCGT	20	(Roller et al., 1994)
LGC354a[c]	*Firmicutes* 16S rRNA, (354–371)	TGGAAGATTCCCTACTGC	35	(Meier et al., 1999)
LGC354b[c]	*Firmicutes* 16S rRNA, (354–371)	CGGAAGATTCCCTACTGC	35	(Meier et al., 1999)
LGC354c[c]	*Firmicutes* 16S rRNA, (354–371)	CCGAAGATTCCCTACTGC	35	(Meier et al., 1999)
PLA46	Planctomycetal 16S rRNA, (46–63)	GACTTGCATGCCTAATCC	35	(Neef et al., 1998)
RHO66	*Rhodococcus* 16S rRNA, (66–84)	CCCCGAAAGGCCTTACCGC	35	This study
SPH120	*Sphingomonas* 16S rRNA, (120-137)	GGGCAGATTCCCACGCGT	35	(Neef et al., 1999)
NON338	Negative control	ACTCCTACGGGAGGCAGC	10	(Wallner et al., 1993)

[a] *Escherichia coli* numbering (Brosius et al., 1981).
[b] Applied with helper oligonucleotides: H571 and H796 (Friedrich et al., 2003).
[c] Applied in combination

PCR amplification of 16S rDNA

Bacterial DNA was extracted from pure cultures with either the FastDNA® SPIN Kit for Soil (BIO 101, Vista, USA) or with the DNeasy™ Tissue Kit (Qiagen, Hilden, Germany). Nearly complete 16S rDNA PCR products were obtained using the two universal bacterial PCR primers GM3F and GM4R (Muyzer, 1995 #475). PCR was carried out with 10 to 20 ng of template DNA, 2.5 units of Taq DNA polymerase (Life Technologies, Gaithersburg, MD, USA), 0.5 µM of each primer, 200 µM of each deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$, and 1 × PCR buffer, adjusted to a final volume of 100 µl with water. In the case of some bacteria belonging to the *Firmicutes* or *Actinobacteria* the usage of Q-Solution (Qiagen) led to a higher PCR-yield. PCR was carried out using a model Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany) programmed with a primary denaturation at 95°C for 4 min, followed by 10 cycles consisting of 95°C for 1 min, 49°C for 1 min and 72°C for 2 min, and 25 cycles at 95°C for 1 min, 44°C for 1 min, and 72°C for 2 min and a final extension of 72°C for 10 min. Two microlitres of each PCR product were analysed on 0.8% agarose gels in 1 × TAE buffer (DNA Typing Grade®, Life Technologies, Gaithersburg, MD, USA). A Low DNA mass ladder (Life Technologies, Gaithersburg, MD, USA) served as standard. PCR products were purified using the QIAquick™ PCR purification kit (Qiagen). Their concentration was then measured using the Pico Green® dsDNA quantification kit (Molecular Probes, Eugene, Or., USA) and a fluostar fluorescence reader (SLT Instruments, Crailsheim, Germany). The PCR products were sequenced in full length or partially by GATC GmbH (Konstanz, Germany) or by GAG BioScience (Bremen, Germany). Sequences were assembled using the DNAMAN programme (Lynnon BioSoft, Quebec, Canada) and especially the overlapping regions of two partial sequences were analysed using the Chromas programme (Technelysium Pty. Ltd, Helensvale, Australia).

The edited 16S rDNA sequences were added to the sequence database (“ssujun02.arb”) available for the software package ARB (Ludwig et al., 2004). The sequences were aligned using the ARB_EDIT tool and the alignment was corrected manually. Phylogenetic trees were calculated by applying neighbour joining, maximum parsimony, and maximum likelihood. Bootstrapping was applied in combination with maximum parsimony by a 100 × resampling of the sequence data used. The 16S rDNA sequences obtained in this study were submitted to the EMBL database and are available under the following accession numbers: AJ313017 to AJ313028, AJ555474 to AJ555478, and AJ864344 to AJ864347.

Determination of the GC-content of DNA

The GC-content of DNA was analysed by high-performance liquid chromatography (HPLC) according to Mesbah et al. (1989). Generally, 25 µl of genomic bacterial DNA-solution (extracted as described above and containing 5 to 25 µg of DNA) and of reference λ-DNA were heated in a boiling water bath for 2 min and rapidly cooled in an ice water bath. Then 50 µl of 30mM sodium acetate buffer (pH 5.3), 5 µl of 20 mM $ZnSO_4$, and 3 µl nuclease (1 mg/ml in sodium acetate buffer; 340 U/ml) were added. After two hours of incubation at 37°C 5 µl of 0.1 M glycine hydrochloride buffer (pH 10.4) and 5 µl of bovine intestinal mucosa alkaline phosphatase (200 U/ml in glycine buffer) were added. After six hours of incubation at 37°C the samples were centrifuged at 10.000 x g for 4 min and stored at -20°C. For the chromatography we used a C18 reversed phase column at 37°C. The mobile phase consisted of 12% methanol in 20 mM triethylaminephosphate (pH 5.1; mixture of 40 ml 0.5 M triethylaminephosphate of pH 5.1 with 750 ml HPLC-water and with 120 ml methanol, HPLC-grade, filled up to 1000 ml with HPLC-water). The high-performance liquid chromatography was performed with a 1050 HPLC of the Hewlett-Packard Series, which had a diode array multiple wavelength detector and an ODS-hypersil column. The column flow rate was 1 ml/minute. The maximum pressure was about 160 bar and the injection volume was most commonly 5 µl. Samples were injected twice and calculated separately. The correction factor y was based on the peak areas of the λ-DNA (49,858 mol% G+C) reference. With the help of formulae 1 and 2 the data could be corrected according to formulae 3. The GC-content was presented in mol%.

$$y = \frac{M_{measured}}{M_{real}} \tag{1}$$

$$M_{measured} = \frac{G+C}{G+C+A+T} = \frac{G}{G+T} \tag{2}$$

$$M_{real} = \frac{M_{measured}}{y} \tag{3}$$

LABELLING OF HEXANE-DEGRADING MICROORGANISMS

Labelling of filter material biomass and fatty acid analyses

Deuterated hexane was used as a tracer in combination with phospholipid fatty acid (PLFA) analysis for the characterisation of hexane-degrading microbial communities of the Hamm biofilter. In May 2001 samples were collected from the biofilter for stable-isotope-based labelling. Replicates of 20 g of the packing material were incubated in 500 ml screw-cap flasks capped with butyl rubber septa with 20 µl of either deuterated hexane (Deutero GmbH, Kastellaun, Germany) or normal hexane as a control for 9 to 24 days. Five-gram portions of these samples served PLFA analysis and the remaining material was used for total cell count analysis and for FISH (see above) to monitor possible shifts of the microbial community during the incubation. The lipids were extracted, fractionated on silica columns and methylated to FAMEs by mild alkaline methanolysis as described previously (Schinner et al., 1995; von Keitz et al., 1999). FAMEs were then analysed as described above.

Quantification of labelled FAMEs

The quantification of the deuterated FAMEs was based on the calculation of the percentage of labelled molecules in comparison to the total amount. All calculations were performed with averages of the mass spectra from the entire area of each FAME peak. In most cases, the molecular ions (M) were used for the calculations. However, for the molecular ions of cyclopropane and monounsaturated fatty acids, the intensities were lower than those of the saturated fatty acids. Therefore, M-32 fragments were analysed for these compounds. All fatty acids that accounted for more than 1% of the total fatty acids of a sample were monitored for deuterated ions and the degree of labelling was calculated based on the abundance of the unlabelled ion and the isotopically modified ions (isotopomeres). The sum of all isotopomeres was corrected for the naturally occurring isotopes (^{2}H,^{13}C, and ^{18}O). The mass spectra of the FAMEs of the unlabelled samples gave the natural distribution of ions and isotopomeres. The percentages were then used for correction. The abundances of all isotopomeres were added after subtraction of the proportion of isotopomeres, which resulted from their natural occurrence (formulae 1).

$$L = \sum_{i=1}^{n} A_{M+i} \quad - \quad A_M \cdot I_i \tag{1}$$

where L is the sum of the corrected abundances of isotopomeres, A_{M+i} stands for the abundances of the isotopomeres, M is the mass of the unlabelled molecular ion, i is the increase in this mass related to the number of incorporated isotopes, and n is the maximum

mass increase caused by the incorporation of isotopes. A_M is the abundance of the unlabelled molecular ion and I_i the correction factor that was determined for each isotopomere.
The corrected abundance (L) was transformed to percentages, in which P is the labelled portion of the fatty acid (formulae 2).

$$P = \frac{L}{L + A_M + \sum_{i=1}^{n} A_M \cdot I_i} \cdot 100 \qquad (2)$$

III. Results

CHARACTERISATION OF THE BIOFILTERS UNDER INVESTIGATION

Both biofilters under investigation were used for hexane removal with hexane being the main component of the inlet gas. In the Alberta biofilter bacterial growth was enhanced by the weekly addition of a buffered nutrient solution (pH 7 to 7.5) (Budwill and Coleman, 1997). The biofilter in Hamm did not receive such treatment and the pH of the packing material was as low as 2.6 in May 2001 (Table 2). Cell concentrations of the Hamm samples were about one order of a magnitude lower than of the Alberta samples. The water content of the Alberta packing material was the highest of all samples (Table 2). In contrast to the Alberta biofilter material, which consisted of coarse sphagnum peat the Hamm biofilter material was very coarse, consisting of crushed tree roots, which were generally up to several decimetres long (see Fig. 1). One other difference lay in the treatment of the packing material. In an attempt to enhance the mass transfer of *n*-hexane from the gas to the

Figure 1. Photograph showing the coarse packing material in the Hamm full-scale industrial biofilter.

liquid phase, the Alberta pet fibres had been treated with silicone oil at about 10% (v/v) before they had been loaded into the biofilter column.

Table 2. Physicochemical properties and cell concentrations of the samples.

Sample		pH	Water content [%]	Cell concentration[a]
Alberta	(1997)	7.0	76.5	7.8×10^{10} (1.1×10^{10})
Hamm A	(1997)	3.0	65.5	7.2×10^{9} (1.9×10^{9})
Hamm B	(1997)	4.2	71.0	5.5×10^{9} (4.6×10^{8})
Hamm O	(2001)	2.8	72.0	6.3×10^{9} (1.1×10^{8})
Hamm U	(2001)	2.6	71.4	5.3×10^{9} (5.6×10^{8})

[a] Reported as mean (standard deviation) for three replicates and given in counts per gram dry weight.

DIFFERENTIATION OF THE ISOLATED BACTERIA

In total, 277 strains were isolated from the biofilter packing material of both biofilters. Strains forming colonies of identical appearance were grouped together. If already all analysed members of one of these pre-groups showed the same fatty acid profile, only randomly selected representatives of the remaining members were analysed. Additionally, not all strains survived cultivation procedures. Thus, fatty acid analyses were carried out for 151 isolates. One strain was a eukaryotic organism. Based on the fatty acid profiles of the prokaryotic strains a dendrogram was calculated (Fig. 2). The strains were separated into 10 different clusters at Euclidian distances ranging from 2 to 31.

The strains were differentiated into two main branches at a Euclidian distance of 169 (Fig. 2). Clusters A-F lack anteiso-branched fatty acids and 10-methyl fatty acids (Tables 4-8). In contrast, clusters G-J contain strains with either 10-methyl fatty acids or mainly iso- or anteiso-branched fatty acids (Tables 9-12). At a Euclidian distance of 113 cluster G is separated from clusters H-J. Strains belonging to cluster G contain 18:0 10 methyl whereas the clusters H-J are dominated by iso- and /or anteiso-branched fatty acids. The assignment of strains to the large cluster G is discussed below. At a distance of 155 cluster A is separated from clusters B-F. Fatty acid profiles in Cluster A are dominated by 18:1 *cis*11, accounting for 66 to 89% (Table 4). Based on origin and morphological features, which are underlined by special features in the fatty acid profiles of strains within a cluster, some clusters are separated into groups. Origin and enrichment conditions of the prokaryotic

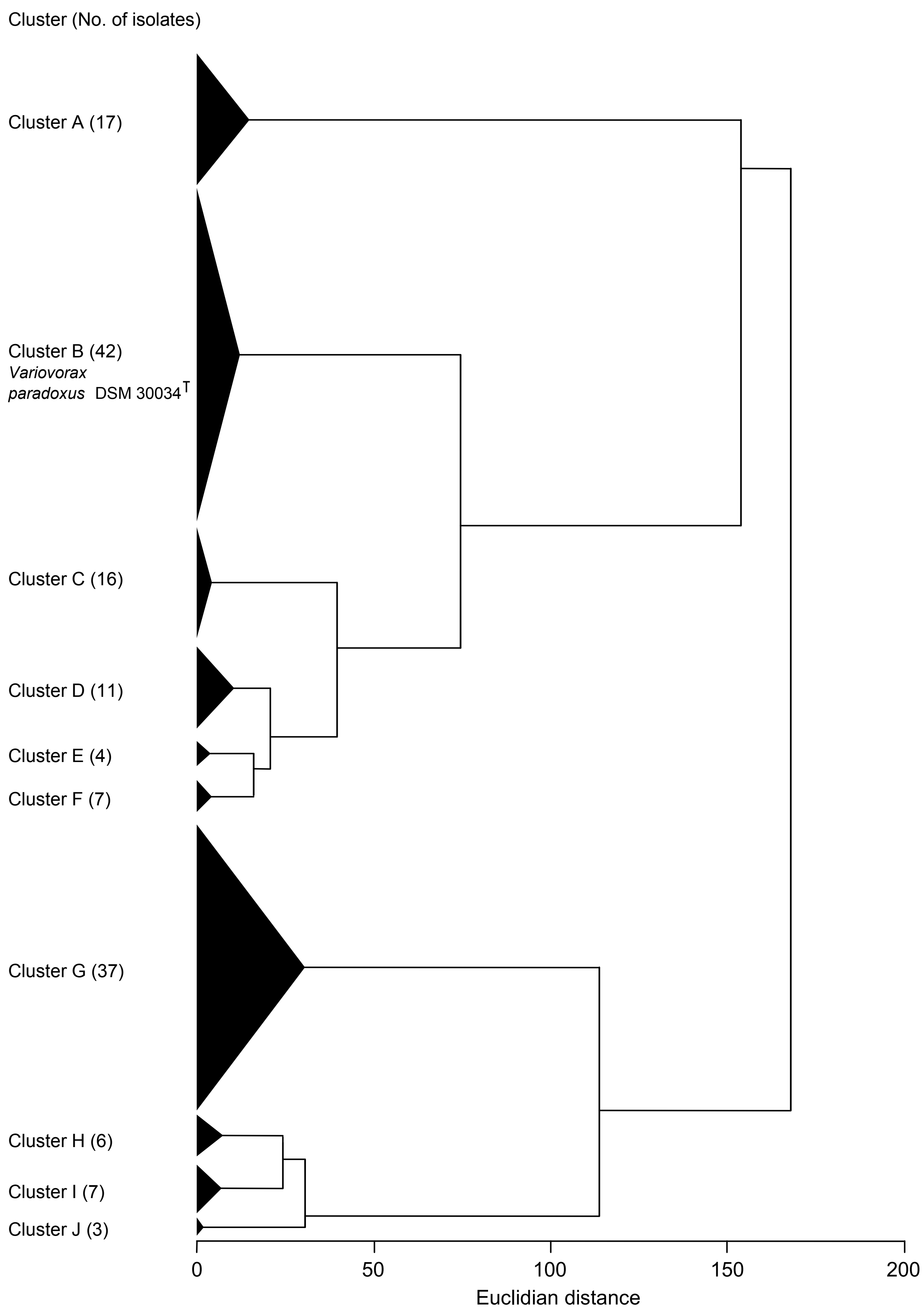

Figure 2. Dendrogram generated from fatty acid profiles.

groups and their assignment to clusters are shown (Table 3). Thirteen and 18 groups originated from the Alberta and Hamm biofilters, respectively. The eukaryotic organism originated from site A of the Hamm biofilter. From the Alberta biofilter three groups were isolated which dominated in numbers (n) and these were enriched both with lower and higher hexane content. The two most abundantly isolated groups from the Hamm samples originated from both sites of the biofilter. The less abundant groups were each isolated from one site only. None of the groups isolated from Hamm were isolated under both pH conditions (Table 3). Group identification numbers were assigned during the isolation and cultivation procedures and maintained thereafter.

Table 3. Origin of bacterial groups. The number of strains of each group (n), their affiliation to clusters based on fatty acids, and the applied enrichment conditions are shown. For the Alberta biofilter only one sample was obtained and enrichments were carried out with a low and a high hexane content (see Materials and Methods). In Hamm two sites were sampled and initial enrichment steps were carried out at pH 5 and 7.

Alberta					Hamm						
			hexane enrichment concentration					pH 5		pH 7	
Group number	cluster	n	low	high	Group number	cluster	n	site A	site B	site A	site B
1	B	39	✔	✔	8	I	2				✔
2	I	1		✔	9	I	1			✔	
3	A	14	✔	✔	10	F	7				✔
4	J	3		✔	12	C	14			✔	✔
5	G	16	✔	✔	16	G	17	✔	✔		
6	D	1		✔	17	E	4		✔		
7	I	1		✔	18	H	5		✔		
11	C	1	✔		19	D	3		✔		
13	A	1	✔		20	D	3	✔			
14	I	1		✔	21	D	3	✔			
15	A	1		✔	22	D	1			✔	
24	G	1		✔	23	A	1				✔
29	I	1	✔		25	H	1			✔	
					27	G	2		✔		
					28	G	1				✔
					30	B	3				✔
					31	C	1				✔

Within cluster A following groups were differentiated: group 3, 13, 15 and 23, with the later three groups each represented by one strain only (Tables 3 and 4). Group 15 was treated separately within this cluster due to its distinct colony morphology and the fairly high

percentage of 16:1 *cis*9 (4.6%). Group 23 was separated from group 3 on the basis of the fatty acid profile with its high percentage of 19:0 cyclo (7-9%) and the lack of fatty acids with less than 16 carbon atoms. Additionally, group 23 was isolated from the industrial biofilter in contrast to the other groups belonging to cluster A. Apart from the absence of 18:1 *cis*11 11 methyl group 13 fits into the range of the fatty acid profile of group 3 which generally has a wider variety of fatty acids than group 13 (Table 4). The fatty acid profile of group 13 consisted of 6 fatty acids in contrast to group 3 which exhibited at least 8 fatty acids and the majority of strains had 11 fatty acids.

Colonies of the representatives of group 3 appeared shiny and moist and were entire or undulate. The colour of the colonies was pale yellow-beige or a transparent-white. It was fairly common that a more intensely coloured centre of the colonies was surrounded by a paler and less raised rim. Some strains of this group appeared more adhesive than others. The cells were non-motile rods and generally 0.5 to 1.0 µm wide and 1.5 to 5 µm long, with an occasional length ranging up to 9 µm. Oxidase and catalase tests were positive. Gram-staining was negative and both the aminopeptidase and KOH-tests were positive. The quinone type and polar lipids of four strains of group 3 were analysed. The strains contained ubiquinone 10 (Q-10) and showed a large assortment of polar lipids. Apart from diphospatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and an unidentified aminophospholipid, two to three lipids were present containing neither amino- nor phosphate-groups. Additionally, some strains contained phosphatidylcholine (PC) and two unidentified phospholipids without an amino group.

The colonies of group 13 were beige-white with a slightly orange tone and they appeared slightly dry although being shiny. Group 13 was represented by one strain only and could not be maintained during the cultivation process. Thus, no further tests were made. Group 13 was dominated by the presence of an octadecenoic acid with few other detectable fatty acids.

Group 15 showed very small pink-pigmented colonies and characteristically, did not yield a high biomass on agar plates on any of the used media. In liquid culture growth was even poorer. Thus, the cell mass grown on three agar plates of the strain MN 8.1d.1a representing group 15 was harvested for the preparation of FAMEs.

Group 23 consists of two clones (MN45.1 and MN45.2a), which originated from the same colony-forming unit, thus they were treated as one strain. MN45.1 was chosen to be the representative clone. The fatty acid profile was not affected strongly by growth on hexane or on nutrient broth dishes (Table 4). The colonies grown on hexane were one day older and this might also have caused the shift from 18:1 *cis*11 to 19:0 cyclo11-12. The fatty acid profile of group 23 was characterised by the dominance of 18:1 *cis*11 and 19:0 cyclo11-12 with 18:1 *cis*11 contributing more than 84% of the total fatty acid profile (Table 4). Group 23 showed pale-yellow colonies, was catalase- and oxidase-positive, and stained Gram-negative.

Table 4. Fatty acid profiles of groups, 3, 13, 15, and 23, which are affiliated with cluster A. The fatty acid profile of group 23, represented by strain MN45.1, under different growth conditions is included in this table.

Fatty acid[a] [%]	Group 3 n = 14		Group 13 n = 1	Group 15 n = 1	Group 23 n = 1	
	Mean	Range			nutrient broth	hexane
14:0	0.2	0-0.9				
15:0	0.1	0-0.6				
14:0 2OH	1.8	0-5.2	3.4			
14:0 3OH				0.6		
16:1 *cis*9	0.1	0-0.7		4.6		
16:1 *cis*11	0.7	0-2.6	0.4			
16:0	17.4	9.6-28.7	12.8	3.4	1.3	2.5
17:1 *cis*11	3.5	0.8-10.5	1.9			
17:0	0.6	0-2.6	0.5		2.3	0.5
18:1 *cis*11	72.5	65.8-81.8	81.0	87.4	88.5	84.7
18:0	0.3	0-0.7		4.0	2.3	3.7
18:1 *cis*11 11methyl	2.1	1.3-3.7				
19:0 cyclo11-12	0.5	0-0.8			5.6	8.6

[a] Examples of abbreviations for fatty acids: 16:0, hexadecanoic acid; 18:1 *cis*9, *cis*-9-octadecenoic acid; 16:0 3OH, 3-hydroxyhexadecanoic acid.

Cluster B consists of two groups of isolated strains and *Variovorax paradoxus* DSM 30034^T, which was used as a reference strain. The two groups, 1 and 30, from this cluster originated from Alberta and Hamm, respectively (Table 3).

Chromatograms obtained from members of group 1 always showed four distinct peaks (with mean ECLs of 9.952, 10.018, 10.470, and 11.225) which were small but always present and typical for this group and were neither present in group 30 nor in *V. paradoxus* (Table 5). Because of their low abundance the fatty acids of the first three peaks could not be identified completely (Fig. 3). The fourth peak, which was not a fatty acid, always showed 75 and 117 as the main ions and was not identified.

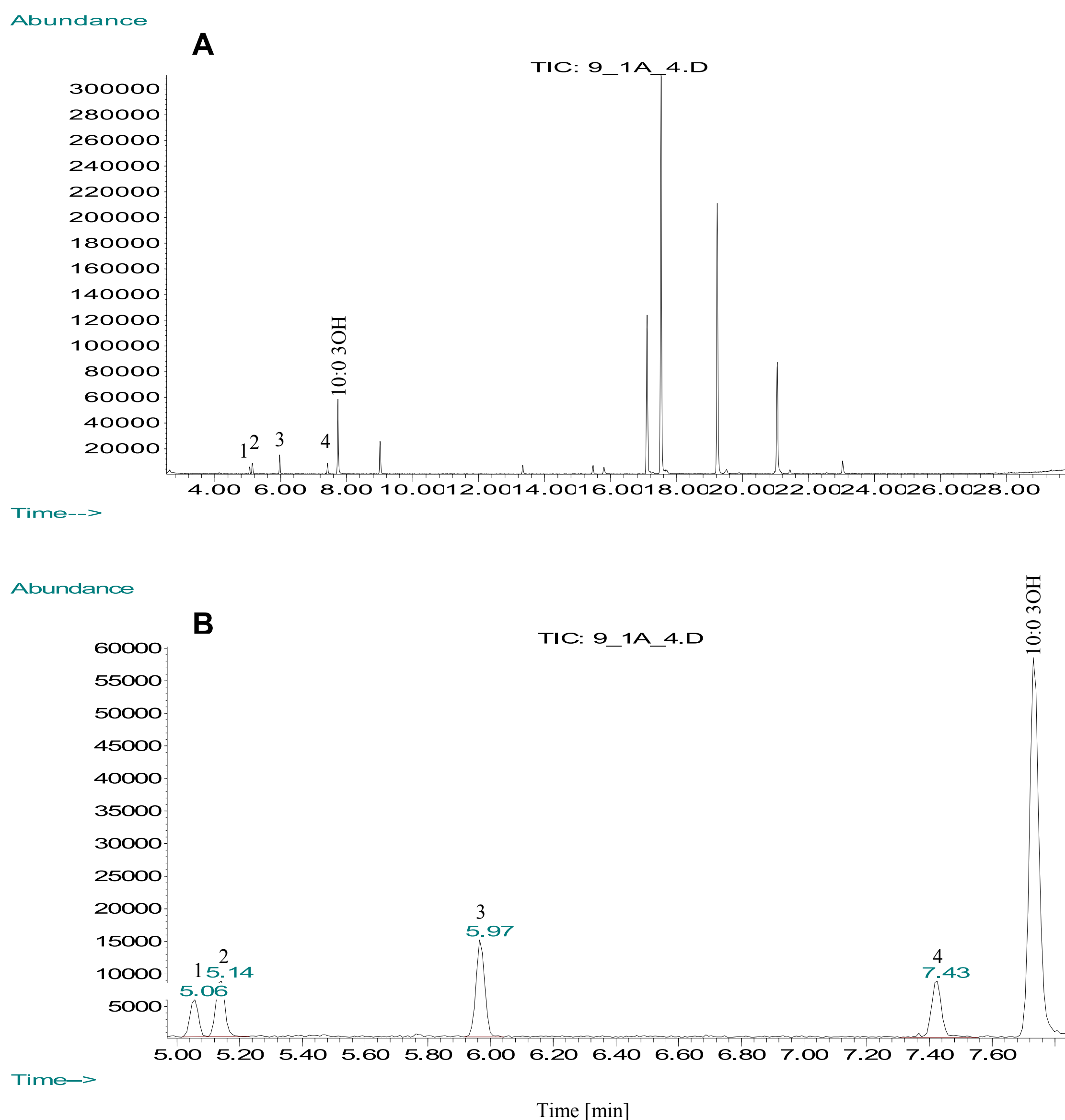

Figure 3. Gas chromatogram of FAMEs of group 1. A: The entire chromatogram of strain MN9.1a.4. B: In the partial gas chromatogram the four non-identified Peaks 1 – 4, which are typical for group 1, and the peak of the fatty acid 10:0 3OH are shown.

Another interesting characteristic of group 1 was the existence of two different colony morphotypes that were found for all member strains of the group, especially on TSB medium. The colonies were either shaped irregularly, transparently yellow, flat, and of larger diameter (5 to 6 mm) or raised in a convex way, whitish and non-transparent, and of about 2 to 4 mm in diameter (Fig. 4a). The yellow morphotype displayed undulate margins and swarming. After about six days the colour of the yellow morphotype intensified in the centre of the colony. The trait to produce both morphotypes was never lost completely. But both morphotypes could be maintained separately for a couple of generations. Profiles of fatty acid composition of both types did not show any difference. Analysis of the polar lipids

revealed one additional component for the yellow morphotype: Whereas diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), and a phospholipid with an amino group (PL_{N1}) were present in both morphotypes the yellow morphotype additionally showed phosphatidylglycerol (PG). As representatives of the benzoquinone type the isolates exhibited ubiquinone-8 (Q8). Cells of group 1 stained Gram-negative, KOH and aminopeptidase tests were positive, but a few strains showed a positive aminopeptidase reaction only after 24 hours. Catalase and oxidase tests were both positive. The cells were rod-shaped cells of 0.5-1 µm × 1-2 µm.

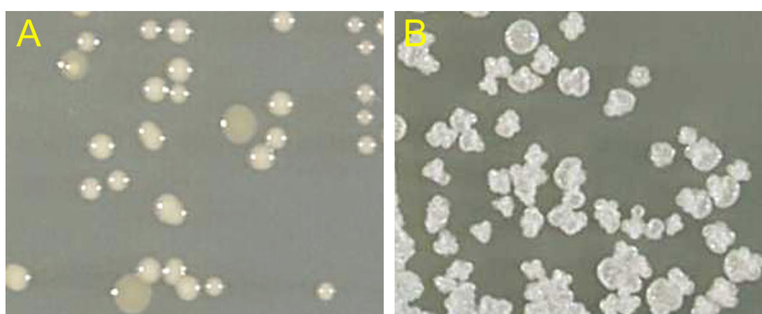

Figure 4. Colony morphologies of two isolates. A: Members of group 1 exhibited two morphotypes, B: Colonies of group 12 showed irregular margins and raised rims.

Group 30 formed larger, fairly flat beige-white, umbonate colonies with a large undulate margin. The rim was almost completely transparent. The colonies of two of the strains of group 30 appeared to be drier than the other strain of this group. In addition, their rims contained slightly raised structures and they were also somewhat more yellow than the colonies of the other strain. All strains reacted positive in terms of oxidase, catalase, KOH, and aminopeptidase. They stained Gram-negative. Individual cells were small rods of about 0.4 µm × 0.8 µm up to 0.5 µm × 1 µm. Some cells almost appeared coccoid, however, small rods dominated.

Strains of group 1 and 30 show close resemblance to *Variovorax paradoxus* with hexadecanoic acid and 17:0 cyclic acid as the dominant fatty acids. Colonies of *Variovorax paradoxus* are flat and beige and there is a higher morphological similarity to those of group 30 than to those of group 1. The resemblance of the fatty acid profile of group 30 and *Variovorax paradoxus* is also larger than that of group 1 towards the latter. This is also because group 30 and *Variovorax paradoxus* lack the four early peaks, which are typical for group 1 (Table 5).

Table 5. Fatty acid profiles of *Variovorax paradoxus* DSM 30034[T] and groups 1 and 30, which are affiliated with cluster B.

	Group 1 n = 39		*V. paradoxus* DSM 30034[T] n=2	Group 30 n = 3	
Fatty acid[a] [%]	Mean	Range	Range	Mean	Range
8:0 3OH			0-0.3		
10:1[b]	0.5	0.3-0.6			
10:1[c]	0.6	0.3-0.9			
10:0				0.0	0-0.1
10:1[d]	1.2	0.4-1.6			
ECL 11.225[e]	0.8	0.4-1.0			
10:0 3OH	4.5	2.4-6.5	1.3-1.8	1.5	0.9-1.9
12:0	2.9	2.5-3.6	2.5-3.6	2.7	2.2-3.0
13:0				0.0	0-0.1
14:0	0.9	0-1.3	0.6-0.9	1.0	0.8-1.1
15:1 *cis*9			0.5-0.8	0.3	0-0.9
15:0	0.8	0.4-1.5	5.7-6.0	1.8	0.1-5.0
14:0 2OH	0.7	0.3-1.1	0.7-0.9	0.6	0-1.0
16:1 *cis*9	16.9	12.9-24.1	11.0-13.6	15.4	10.5-19.8
16:1 *cis*11			1.8-1.8	0.2	0-0.8
16:0	37.5	33.6-40.9	31.9-32.2	39.4	38.2-40.5
17:0 cyclo9-10	22.2	15.8-27.6	28.7-29.3	27.3	22.7-33.8
17:0	0.6	0.1-1.1	1.6-3.0	0.6	0.2-1.1
16:0 2OH	0.0	0-0.3		0.0	0-0.1
18:1 *cis*11	11.7	5.9-14.2	7.8-10.4	8.1	6.0-10.3
18:0	0.5	0.4-0.9		0.2	0-0.4
19:0 cyclo11-12	1.0	0.2-1.7	0.5-1.0	0.8	0.2-1.5

[a] For examples of abbreviations, see Table 4. [b,c,d] most likely unsaturated straight chain decenoic acid, but the position of the double bond was not determined due to the low abundance of these fatty acids. [e] This peak was not a fatty acid but present in all members of group 1.

Cluster C was divided into group 12 and two single isolates with the group numbers 11 and 31. The two single isolates were not placed into group 12 because of the presence of 10:0 3OH, the absence of 14:0 3OH (Table 6), and distinct morphological features. The fatty acid profile of group 11 showed a closer resemblance with group 12 than with that of group 31 (Table 6). But group 11 originated from Alberta in contrast to groups 12 and 31. The high abundance of 18:0 was a distinct feature of group 31. The colonies of the only strain representing group 31 had a slightly more yellowish-beige colour than those of group 12. The strain was lost during the cultivation procedures and could not be analysed further.

Group 11 often formed punctiform colonies of about 0.6 to 1 mm in diameter. Colonies were beige-white. This group was oxidase and catalase positive but the catalase reaction was slow and weak. KOH and aminopeptidase tests were also positive. The aminopeptidase reaction was slower than that of, e.g., group 30 from cluster B. Cells stained Gram-negative and were usually rods of 0.5 µm × 1 µm. The longest rods reached a length of about 2 to 2.5 µm. The shortest rods almost appeared coccoid.

Group 12 formed a large group of isolates from both sites of the biofilter in Hamm. Typical for group 12 was the raised rim of most of the colonies, which were white with irregular

margins and between 1.9-2.5 mm (Fig. 4b). Cells were rod shaped and stained Gram-negative. The KOH test was positive but the aminopeptidase test was only weakly positive after 24 hours. The catalase reaction was also very weak for some strains, but the oxidase test was always positive. The polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), and one unidentified phospholipid with an amino group. The quinone type was ubiquinone-8 (Q8). The fatty acid profile was dominated by unbranched fatty acids with even numbers of carbon atoms. Main fatty acids were hexadecanoic acid, its unsaturated form 16:1 *cis*9, and 18:1 *cis*11.

Table 6. Fatty acid composition of groups 11, 12, and 31 affiliated with cluster C.

Fatty acid[a] [%]	Group 11 n = 1	Group 12 n = 14		Group 31 n = 1
		Mean	Range	
10:0 3OH	1.5			1.0
12:0	2.5	0.0	0-0.4	2.7
14:0	3.0	4.4	3.8-5.3	0.7
15:0	0.1	0.1	0-0.3	
14:0 2OH		0.6	0-1.1	0.5
14:0 3OH		3.1	0.8-4.6	
16:1 *cis*9	39.8	25.8	22.9-30.1	23.5
16:1 *trans*9		0.9	0-2.2	0.4
16:1 *cis*11		0.4	0-0.7	
16:0	31.8	29.1	26.9-32.4	19.1
17:0 cyclo 9-10	0.8	6.5	3.8-8.7	16.2
17:0		0.3	0-1.2	0.9
16:0 2OH		0.1	0-0.3	
18:1 *cis*11	20.2	27.1	22.3-29.2	20.7
18:1 *trans*11		0.8	0-2.5	
18:0	0.2	0.3	0-0.5	13.5
18:1 *cis*11 11methyl	0.1			
19:0 cyclo 11-12		0.3	0-0.9	0.7
18:1 2OH		0.0	0-0.2	

[a] For examples of abbreviations, see Table 4.

Five different groups contributed to cluster D. These groups were separated on the basis of morphological features and especially based on the fatty acid profiles (Table 7).
High amounts of 17:1 *cis*11 and 17:0 separated group 6 from the other groups within cluster D. No other group isolated from these biofilters exhibited such a high percentage (38%) of 17:1 *cis*11. The colonies of group 6 formed plain, yellow and moist colonies. This group was represented by one strain only and could not be maintained during the cultivation process.
Characteristic for group 19 within cluster D was the high amount of 16:1 *cis*9 and the presence of 16:0 2OH and 16:0 3OH. The colonies of group 19 were shiny and appeared

transparent-white to opaquely beige. They were fairly flat with a diameter of 2.5 to 2.9 mm. The colonies were circular with defined edges. Individual cells were rods of about 0.5-1 µm × 1-2 µm which stained Gram-negative. They tested catalase, oxidase, aminopeptidase and KOH positive. The main fatty acids of this group were hexadecanoic acid and 18:1 *cis*11, and the fatty acid profile was dominated by long chain fatty acids. The polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), an unidentified phospholipid with a primary amino group, and one unidentified lipid containing an amino but no phosphate group. The major respiratory quinone was ubiquinone-8 (Q8).
Among other features, the presence of 14:0 2OH separated group 20 from groups 6 and 19, and the absence of 12:0 separated it from groups 21 and 22 (Table 7). Group 20 was very fastidious concerning cultivation but three strains could be maintained. In 3-day-old cultures, dimensions of rods of strain MN122.2a, chosen to be the reference strain of group 20, were 0.5 ± 0.1 µm by 1.9 ± 0.7 µm (n= 400 sized cells). Cells usually occurred singularly (Fig. 5a). Strain MN122.2a exhibited positive KOH- and aminopeptidase tests and Gram-staining was negative. The catalase test was only weakly positive and the oxidase test was negative. Colonies were circular and normally yellow, only small and young colonies were less pigmented. Most colonies ranged from 1.0 to 1.5 mm but colonies from 0.5 to 3 mm in diameter were not unusual. Colonies were shiny, opaque, and raised. Their colour intensified with age, three months old viable colonies can turn brownish-yellow or greyish-yellow in the centre, which is surrounded by a paler whitish rim. MN122.2a and MN149.3, both members of group 20, had a GC content of 62.5% (± SD = 0.17) and 64.4% (± SD = 0.74), respectively. Group 20 showed no growth on TSB agar plates, thus the fatty acid profiles had to be derived from colonies grown under hexane on minimal medium and only one strain (MN122.2a) yielded enough cells for fatty acid analysis on nutrient broth with unaltered pH.

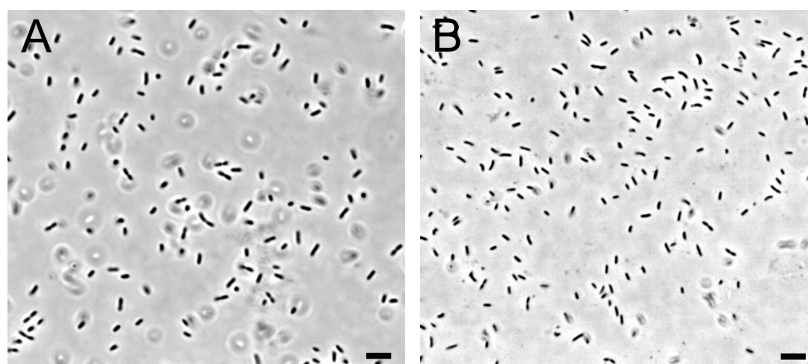

Figure 5. Microscopic photographs of cells. The scale bars indicate 5µm. A: MN122.2a of group 20, B: MN 154.3 of group 21.

Fatty acid profiles of group 21 and 22 differed mainly in terms of the absence of 16:0 iso and 16:1 *cis*11 and the high amounts of 19:0 cyclo 11-12 acid in group 21 (Table 7). Cells were small rods (Fig. 5b). In 4-day-old cultures, dimensions of cells of strain MN154.3 (group 21) were 0.6 ± 0.1 µm by 0.9 ± 0.3 µm (n=1200). The colonies were transparently yellow and circular. KOH and aminopeptidase tests were positive and Gram-staining was negative. Catalase and oxidase tests were both positive. MN154.3 and MN157.2, both belonging to group 21, had a similar GC content of 62.8% (± SD= 0.19) and 62.5% (± SD = 0.04), respectively.

The colonies of group 22, which is represented by one strain only, were variable in colour. Therefore, four variants were analysed. Generally, the colonies of group 22 varied between yellow, orange and brown. They had an average size of 1-3 mm in diameter, were umbonate, and surrounded by a flat, pale white rim. The colour was more intense in the centre of the colonies. The colour variants could be maintained separately for 1-2 generations on agar plates. For all four variants fatty acid patterns were obtained (Tab. 7). The fatty acid profile was dominated by fatty acids with an even number of carbon atoms. *Cis*-11-octadecenoic acid was the main fatty acid. Interestingly, a fairly high abundance of both 16:0 iso and 16:1 *cis*11 was found. The cells of group 22 were rods, which were longer than those of group 21. Additionally, cells seemed slightly curved at times. Gram-staining was negative, KOH and aminopeptidase test were positive. Catalase reaction was only weakly positive but oxidase reaction was clearly positive. The GC content was 64.9% (± SD = 0.07).

Table 7. Fatty acid compositions of groups 6, 19, 20, 21, and 22 affiliated with cluster D. The fatty acid profiles of group 22 were derived from four different colour variants.

Fatty acid[a] [%]	Group 6 n = 1	Group 19 n = 3		Group 20 n = 3		n = 1[b]	Group 21 n = 3		Group 22 4 variants of strain MN28	
				on hexane		on nutrient broth				
		Mean	Range	Mean	Range		Mean	Range	Mean	Range
8:0									0.2	0-0.7
9:0									0.4	0-1.5
ECL 10.852		0.7	0.6-0.7							
ECL 11.625									1.0	0-2.3
12:0							5.6	4.4-8.4	6.2	3.1-11.1
ECL 12.882		1.0	1.0-1.0							
12:0 2OH									0.3	0-0.7
14:0 iso									0.9	0-2.0
14:1									0.1	0-0.2
14:0		3.6	3.4-3.8	0.4	0.1-0.4		0.2	0-1.2	7.7	5.3-9.1
15:0 iso									0.1	0-0.5
15:0	1.6								0.4	0-1.0
14:0 2OH				4.0	2.3-6.1	3.9	0.7	0-2.0		
14:0 3OH		1.5	1.1-2.2				3.9	1.4-5.7	3.3	2.2-5.0
16:0 iso									8.7	4.1-13.6
16:1 c/t 5									2.2	1.8-2.8
16:1 *cis*9		12.7	11.9-14.3	0.1	0-0.3		5.5	2.1-7.9	4.7	3.2-6.2
16:1 *cis*11	0.4	0.3	0.2-0.3						6.3	4.7-10.7
16:0	5.5	27.2	25.7-29.3	27.2	26.1-28.8	20.2	28.0	20.7-31.1	15.9	12.7-19.5
17:0 cyclo9-10		9.3	5.4-11.5	0.1	0-0.4		5.1	0.8-7.7		
17:1 *cis*9	2.1									
17:1 *cis*11	38.3			0.0	0-0.1	2.1			0.1	0-0.3
17:0	10.3	0.3	0.3-0.3						0.0	0-0.2
ECL 17.441									0.4	0-0.9

Table 7. Continued

18:1 *cis*7									0.2	0-0.8
16:0 2OH		0.4	0.4-0.4							
16:0 3OH		1.3	0.7-2.1							
18:2 5,11									0.6	0-1.4
18:1 *cis*9							0.6	0-1.4		
18:1 *cis*11	39.2	34.9	32.9-38.5	43.7	38.7-48.1	62.8	31.5	17.2-45.0	39.3	31.5-50.9
18:0		0.9	0.7-1.0	0.1	0-0.2		2.4	1.5-3.3		
18:1 *cis*11 11methyl	1.7			15.6	14.4-17.9	3.6	1.1	0.3-1.7	0.1	0-0.4
19:0 cyclo 11-12	1.0	6.4	3.5-8.0	9.1	7.2-11.7	7.3	14.8	7.4-27.7	0.7	0-3.0
18:1 2OH		0.1	0-0.2				0.1	0-0.7		
17:0 11methoxy							0.1	0-0.5		
19:0 cyclo 2OH							0.7	0.4-1.2		

[a] For examples of abbreviations, see Table 4.

[b] Only strain MN122.2a of group 20 was grown on nutrient broth.

The clusters E and F were not further subdivided into groups (Table 8).
The colonies of group 17, representing cluster E, were flat, shiny, and appeared transparent but with a yellowish tone. The cells were relatively large rods (0.5-0.7 µm × 2-4 µm). This group was catalase and oxidase positive with a very strong catalase reaction for all strains. The fatty acid profile was characterised by large amounts of hexadecanoic acid and cyclic acids with 17 or 19 carbon atoms. Cis-11-octadecenoic acid was the fourth most abundant fatty acid of group 17 and is known as the precursor of 19:0 cyclo (Table 8).
The strains of group 10, forming cluster F, formed small (colony-diameter: 0.2-1.7 mm), flat, whitish, and shiny colonies on agar plates. They were oxidase and catalase positive. Growth on agar plates of the different media used in this study was always slower than that of most other groups in this study. Thus, the fatty acid profile was obtained from 4-5 day old cultures. Individual cells were larger than those of groups belonging to cluster A or group 20. Cells also appeared to have sharper ends compared to the normal rod-like shape and stained Gram-negative.

Table 8. Fatty acid profiles of clusters E and F consisting of the groups 17 and 10, respectively.

Fatty acid[a] [%]	Cluster E: Group 17 (n = 4)		Cluster F: Group 10 (n = 7)	
	Mean	Range	Mean	Range
12:0	1.1	1-1.4		
12:0 2OH	0.5	0.3-0.7		
14:0	0.5	0.3-0.6	0.3	0-0.8
15:0			0.7	0-1.8
14:0 3OH	1.9	1.1-2.8	0.4	0-0.9
16:1 *cis*9	5.3	2.9-7.9	1.5	0-3.2
16:0	29.4	24.7-35.4	17.7	11.5-24.6
17:0 iso			2.5	0-8.7
17:0 cyclo9-10	28.7	23.9-32.6	4.6	0.3-9.6
17:0			0.7	0-3.2
16:0 3OH	1.7	0.5-2.9	0.5	0-1.3
18:1 *cis*11	9.6	6.9-13.3	21.0	2.8-42.4
18:1 *cis*12			0.3	0-1.4
18:0	0.6	0.6-0.7	0.7	0-3.2
18:1 *cis*11 11methyl			5.3	0-16.7
19:0 cyclo11-12	20.6	15.5-24.9	42.5	28.1-58.1
19:0 cyclo 2OH			0.7	0-1.6
ECL 10.852	0.6	0-1.1	0.2	0-0.6
ECL 12.882	0.8	0-1.4	0.4	0-1.3

[a] For examples of abbreviations, see Table 4.

The fatty acid 18:0 10 methyl contributed at least 3% to the total fatty acid profile in all groups belonging to cluster G. The cluster was separated into 5 groups with the group numbers 5, 16, 24, 27, and 28 (Table 9). Groups 5 and 27 were treated as two separate groups because of slight differences in colony morphology and their different origin being Alberta and Hamm, respectively. The cluster was chosen this large because it consisted of two main branches. One included group 5 and group 27 strains. And the other branch consisted of one pure group 16 branch and one branch including strains of group 5, 16, 24, and 28. The analysis ignored the fact that group 16 always exhibited the fatty acid 10:0 and never the fatty acids 16:0 10methyl and 17:0 cyclo, whereas group 5 did not contain 10:0 but always showed the later two (Table 9). It was rather the quantitative differences in the fatty acid profile from some of the group members that led to the mixed branch. So even strains form originally the same colony forming units were separated by this cluster analysis. Thus, the cluster was treated as a larger one and the groups were assigned based more on qualitative differences in the fatty acids and other group traits.
Individual cells of group 5 were rods, coccoidal rods or cocci (1 µm × 1 – 2 µm) that occurred singly, or in small clusters (pairs and chains up to 9 µm long). The pairs and chains were straight or curved. Strains of group 5 formed circular, shiny, opaque, moist, and whitish pink colonies of 1.2 to 1.8 mm in diameter. All colonies had smooth edges and some strains were more viscous than others. Gram-staining was positive and aminopeptidase- and KOH-tests were negative. Catalase activity was present but oxidase tests were negative. The analysis of polar lipids of two strains of group 5 showed identical polar lipids. In addition to diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and a phosphatidylinositolmannoside (PIM), an additional phosphatidylinositolmannoside, a glycolipid (GL) and phosphatidylglycerol (PG) were present. The major fatty acids were hexadecanoic acid, 18:1 *cis*9, tuberculostearic acid, and 16:1 *cis*10. In this group these four fatty acids accounted for up to 90% of the total fatty acids with an average of 79%. Mycolic acids were corynemycolic acids and aldehydes were detectable in the fatty acid profiles.
The two strains of group 27 formed white colonies with a moist shine and fairly small individual cells, which were Gram-positive. The strains showed catalase activity, but no oxidase activity. There was a high similarity of the fatty acid profiles of groups 27 and 5 (Table 9).
Generally the fatty acid profile of group 16 was less diverse than that of group 5 (Table 9). In contrast to groups 5 and 27 the fatty acid 10:0 was always present in group 16. Additionally, morphological and biochemical features of group 16 differed from those of group 5. The colonies of group 16 were about 1 to 4 mm in diameter. The strains formed white, apricot, and bright orange coloured colonies. The colonies appeared either moist or, especially when grown on hexane, very dry. Even a single strain showed this variety of colony characteristics. Cells in the early growth phase were rods, which occasionally

occurred in chains of up to 10 cells. Some rods appeared fairly long and thin. Cells in the exponential and stationary growth phases tended to be slightly more coccoid. Gram-staining was positive and aminopeptidase- and KOH-tests were negative. Catalase activity was present and oxidase tests were negative. Analysis of polar lipids showed diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylinositolmannoside (PIM) to be present. The menaquinone was identified to be MK-9(H_2). Analysis of mycolic acid methyl esters revealed nocardomycolic acids with a number of carbon atoms similar to or slightly larger than those of *Gordonia terrae,* which was used as a reference organism. The peptidoglycan type was determined to be the glycolyl type.

Beside groups 5, 27, and 16 cluster G included two additional groups. Members of the groups 24 and 28 showed 14:0, 16:1 *cis*10, 16:0, and 18:1 *cis*9 as their major fatty acids and about 3% 18:0 10 methyl (Table 9). The colonies of group 28 were yellow or occasionally slightly orange and consisted of small rod-shaped cells. Group 24 also formed bright, yellow to orange colonies. Group 24 and group 28 originated from Alberta and Hamm, respectively (Table 3). Group 28 exhibited a wider variety of fatty acids than group 24 (Table 9). In addition, the reproducibility of the FAME method can be inferred from the high similarity of the fatty acid profiles from two analyses of MN44b, which were obtained one and a half years apart (Table 9).

Table 9. Fatty acid profiles of groups 5, 16, 24, 27, and 28 from cluster G. Groups 24 and 28 consist of only one strain each, but for group 28, two profiles are shown to demonstrate the reproducibility of the FAME method with the 1st and 2nd analysis being 1.5 years apart.

Fatty acid[a] [%]	Group 5 n = 16		Group 16 n = 17		Group 24 n = 1	Group 27 n = 2		Group 28 n = 1	
	Mean	Range	Mean	Range		Mean	Range	1st	2nd
8:0	0.1	0-0.2	1.7	0.9-2.8	0.8			1.7	2.0
9:0			0.1	0-0.4				0.4	0.4
10:0			1.9	1.0-3.3				4.5	4.8
11:0								0.3	
12:1 *cis*5								0.6	0.8
ECL 11.841								0.5	
12:0	0.1	0-0.3	0.2	0-0.3				1.5	1.8
13:0	0.1	0-0.3				0.2	0-0.4	0.2	
14:1 *cis*11	0.1	0-0.4						0.3	0.5
14:0	5.4	4.7-6.4	1.9	1.4-2.6	4.8	7.7	6.8-8.5	7.3	7.6
15:1 *cis*10	0.3	0-0.8				1.0	1.0-1.0		
15:0	4.3	0.4-11.0	0.4	0-0.8		8.6	8.2-9.1	2.1	0.6
16:1 *cis*7	0.2	0-0.7	0.1	0-0.1	1.4			0.4	0.5
16:1 *cis*9	1.8	1.1-3.3			0.9	1.9	1.9-1.9	3.0	3.4
16:1 *cis*10	13.2	8.1-16.6	7.2	5.1-10.5	8.1	14.9	14.0-15.8	10.4	11.0
16:0	33.8	28.9-38.2	37.4	33.5-41.1	34.3	29.5	29.2-29.8	28.0	30.0
16:0 10methyl	1.1	0.4-1.7				1.9	1.7-2.1	0.2	
17:1 *cis*9	2.8	0.5-7.1	0.5	0-1.8		4.0	3.4-4.5	2.4	0.8
17:0 cyclo	2.7	0.8-4.5				3.4	2.9-3.9		
17:0	1.4	0.3-2.9	1.1	0.7-2.3		1.8	1.5-2.0	1.0	0.3
17:0 10methyl	2.2	0.3-3.2				3.5	3.0-4.0	0.2	
18:1 *cis*9	18.1	9.4-34.7	30.2	26.1-40.7	43.7	9.2	8.2-10.1	28.8	30.0
18:0	0.9	0.2-1.5	6.0	2.6-8.2	1.3	0.2	0-0.4	0.8	0.8
18:0 10methyl	13.7	9.1-19.3	12.6	5.6-18.0	3.2	11.6	11.0-12.3	3.2	4.4
19:0 cyclo 11-12	0.1	0-1.3							
19:1 *cis*7	0.2	0-1.3				0.7	0-1.4		
20:0					1.5			0.4	0.1
22:0								1.8	0.2

[a] For examples of abbreviations, see Table 4.

Within cluster H a single strain was separated from the other cluster members (group 18) because the later showed a larger variety in fatty acids than the strain assigned to group 25 (Table 10). Colonies of group 18 were pale white and generally small with diameters of 0.3 - 0.9 mm. The fatty acids 15:0 anteiso and 17:0 anteiso predominated. Individual cells had a typical rod shape. Group 18 was Gram-positive and the catalase reaction was also positive. The oxidase-test was negative. The strain assigned to group 25 formed shiny creamy-white, circular, and raised colonies. Older colonies appeared thicker and opaque. Single cells were rods, which were larger than those of group 18, and stained Gram-positive. The KOH-test was negative and the aminopeptidase-test gave a weakly positive result. Catalase-test was positive and the oxidase-test gave only weakly positive results. The GC-content of MN30.1b.1 representing group 25 was 58.8% (± SD = 0.01).

Table 10. Fatty acid composition of groups 18 and 25 affiliated to cluster H.

Fatty acid[a] [%]	Group 18 n = 5		Group 25 n = 1
	Mean	Range	
14:0 iso	0.0	0-0.1	
14:0	0.4	0-0.7	
15:0 iso	5.8	3.2-8.6	2.8
15:0 anteiso	33.1	25.0-50.7	51.7
15:0	0.4	0.2-0.7	
16:0 iso	9.9	4.3-12.4	2.9
16:0	5.2	0.3-9.4	
17:0 iso	1.9	0.6-3.2	
17:0 anteiso	43.3	38.4-49.3	42.6

[a] For examples of abbreviations, see Table 4.

The dendrogram based on the fatty acid compositions (Fig. 2) revealed that the isolated strains belonging to cluster H could be separated from those belonging to cluster I at a Euclidian distance of 24. Cluster I was dominated by 15:0 anteiso accounting for at least 37% of the total fatty acids. In contrast to cluster H the contribution of 17:0 anteiso to the total fatty acid profile was less than 13% (Tables 10 and 11). Cluster I was separated into six groups, which consisted mainly of single strains (Table 11). Group 2 showed a high content of 13:0 anteiso (6.5%). The fatty acids 15:0 anteiso, 15:0 iso, and 13:0 anteiso contributed more than 95% to the total FAME profile (Table 11). Another interesting feature of group 2 was the absence of fatty acids longer than 15:0 anteiso. The colonies of group 2 were yellow, opaque, and matt. The tested strain was catalase-and oxidase-positive. Cells were Gram-

positive and mostly arranged in tetrads. Group 7 generally formed creamy-white, circular colonies of 0.2 to 0.7 mm in diameter. Larger colonies of group 7 could turn pale whitish yellow or yellow. Individual cells of this group were cocci, which often appeared in pairs and occasionally in tetrads. The member strain of group 7 was catalase- and oxidase-positive and stained Gram-positive.

Groups 8 and 9 were the only two groups within this cluster that originated from the Hamm biofilter, from sites B and A, respectively (Table 3). Group 8 consisted of two clones from the same isolated colony. The colonies were flat, yellowish and had an average diameter of about 1.8 mm reaching a maximum diameter of up to 5 mm. Older colonies turned brownish beige but kept a transparent shine. The margins of those colonies were irregular and whitish. This group was Gram-positive and catalase-positive. The oxidase reaction was only weakly positive. Particularly dominating in group 8 were 14:0 iso, 15:0 iso, and 15:0 anteiso (Table 11). The high percentage of 14:0 iso was a distinct feature of this group. Colonies of group 9 were mainly between 1.3 and 2.6 mm in diameter, circular, convex, smooth, matt, dry, and yellow. The member strain of group 9 was catalase-positive and oxidase-negative and stained Gram-positive.

Colonies of group 14 consisted of small rods which stained Gram-positive, were yellow, 0.8 - 1.3 mm in diameter, circular and had a tendency to grow into the agar. Catalase is produced. The contribution of 16:0 and 17:0 anteiso, each accounting for more than 12% of the total fatty acid profile, separated group 14 from the other groups in this cluster. In contrast to the other groups of this cluster, group 29 formed white colonies without any other colour tone. Almost 82% of the fatty acid profile of group 29 consisted of 15:0 iso, 15:0 anteiso, 17:0 iso, and 17:0 anteiso (Table 11). The fatty acid 17:0 iso contributed more than 6% to the total fatty acid profile. This fairly high percentage was a distinct feature of group 29 within this cluster. Strains of groups 8 and 29 were the only isolates in this study, which exhibited 16:1 iso 5 and 17:1 iso 5. Group 29 contained 2.6% of the uncommon fatty acid 17:1 iso 5.

Table 11. Fatty acid profiles of the groups affiliated with cluster I. Except for group 8 all other groups consisted of one strain only.

Fatty acid[a] [%]	Group 2 n = 1	Group 7 n = 1	Group 8 n = 2		Group 9 n = 1	Group 14 n = 1	Group 29 n = 1
			Mean	Range			
11:0 anteiso	0.6						
13:0 iso	1.2	0.6			0.6		
13:0 anteiso	6.5	1.0			1.1		
14:0 iso	2.2	3.6	19.9	15.0-24.8	1.0	0.4	1.1
14:0	0.8	2.1	0.3	0-0.6	0.9	4.3	0.5
15:0 iso	25.6	26.9	16.9	14.2-19.6	17.2	8.5	19.5
15:0 anteiso	63.1	61.5	41.1	37.8-44.5	62.0	54.8	49.1
15:0						2.6	0.8
16:1 iso 5			5.3	4.4-6.3			1.4
16:0 iso		1.7	12.1	9.6-14.6	5.6	4.4	4.6
16:1 *cis*9		0.6					
16:1 c/t 5[b]			0.4	0-0.9			3.2
16:0		2.1	0.3	0-0.6	1.3	12.1	4.0
17:1 iso 5			0.3	0-0.6			2.6
17:1 iso 10			0.5	0-1.0			
17:0 iso			0.3	0-0.6	1.1	0.4	6.4
17:0 anteiso			2.5	2.3-2.7	8.7	12.4	6.7
17:0						0.3	

[a] For examples of abbreviations, see Table 4.
[b] *Cis* or *trans* configuration.

Cluster J was defined and separated from clusters H and I at a Euclidian distance of 30. The presence of hydroxylated fatty acids was a typical feature of cluster J in comparison with clusters H and I. Cluster J only consisted of group 4.

All colonies of group 4 were circular and orange with a moist, transparent shine. The majority of the colonies were 3-6 mm in diameter. Colonies on agar plates had a strong, characteristic smell and formed viscous threads when pulled with the loop. Cells were rods of 0.5-1 µm width and 1-1.7 µm length. Strains of this group were catalase and oxidase positive. The Gram reaction was negative, and the KOH and aminopeptidase tests were positive. Generally, the dominating fatty acids were those with uneven numbers of carbon atoms such as 15:0 iso, 17:1 iso *cis*9, 17:0 iso 3OH, and 15:0 iso 2OH (Table 12). When grown on hexane the fatty acid profile was dominated stronger by 15:0 iso, which contributed 64%. Group 4 had a menaquinone and the dominating polar lipids were phosphatidylethanolamine (PE) in addition to one not further identified lipid that neither contained a phosphate nor an amino group. Moreover, two additional, unidentified lipids contained amino but no phosphate groups.

Table 12. Fatty acid composition of cluster J represented by group 4. The fatty acid profile when grown on hexane as the sole carbon source is also shown.

Fatty acid[a] [%]	Group 4 growth on		
	hexane n = 1	TSB n = 3	
		Mean	Range
13:0 iso			
15:0 iso	64.1	41.1	38-44.9
16:0 iso	2.8		
16:1 *cis*9		0	0-0.2
15:0 iso 2OH	10.8	12.6	9.2-15.3
16:0	5.6	1.0	0.8-1.2
15:0 iso 3OH		2.4	2.1-2.7
17:1 iso *cis*9	8.8	19.3	17.4-22.7
17:0 cyclo	2.9	1.2	1-1.3
17:0 iso	2.5	1.7	1.5-2.0
16:0 3OH		0.1	0-0.4
17:0 iso 3OH	2.4	18.1	16.8-18.9
ECL: 13.526		2.3	0-3.9
ECL: 14.381		0.2	0-0.5

[a] For examples of abbreviations, see Table 4.

Only one eukaryotic organism (MN169.2a, group 26) was isolated from the Hamm biofilter. This organism contained 36% of 18:2 *cis, cis*9,12, 55% 18:1 *cis*9 and 9% 16:0. This isolate

was catalase and oxidase positive. Aminopeptidase test was weakly positive and KOH and Gram tests were negative. The quinone type was ubiquinone with 11 isoprene units. Polar lipids consisted of phosphatidylethanolamine, phosphatidylinositol, one non-phosphorylated lipid with positive ninhydrin-reaction, one unidentified aminophospholipid, and two additional phospholipids.

Physiological tests

Physiological tests applying BIOLOG or API test systems were carried out with the majority of the groups, which stained Gram negative (Table 13). In nine cases the BIOLOG microplates and the API 20NE test strips tested for the same substrate. Both results are displayed because different results were possibly based on diverging media in both systems (Table 13). Across all groups, only in four cases more strains grew better on the carbon sources of the API strips than on the corresponding carbon sources of the BIOLOG microplates.

Table 13. Physiological reaction profile of some representatives of eleven groups. The percentage of positive reactions is given when more strains were tested. The number (n) of strains tested is indicated in parentheses. When equal percentages reacted positive for both treatments of group 3 those positive reactions were caused by the same strains. This was not the case for group 1, e.g. in the API 20NE strip system other strains grew on mannose than in the microplates. Group 4 did not show growth on the carbon sources in the API strips but in many cases all strains grew on the same carbon source in the BIOLOG microplates.

Substrates[1]:	Gr.1 (n = 8)	Gr. 3 (n = 5)	Gr. 4 (n = 3)	Gr. 11: MN33.2	Gr. 12 (n = 2)	Gr. 17 (n = 3)	Gr. 19 (n = 3)	Gr. 20: MN122.2a	Gr. 21: MN154.3	Gr. 22: MN28	Gr. 30 (n = 2)
α-cyclodextrin	0	20	100	–	0	0	0	–	–	–	0
dextrin	0	60	100	–	0	67	67	–	–	–	0
glycogen	0	60	100	–	0	100	67	–	–	–	0
tween 40	100	100	100	–	100	100	100	–	–	+	100
tween 80	100	100	100	–	100	33	100	–	–	–	50
N-acetyl-D-galactosamine	0	20	0	–	0	0	33	–	–	–	100
N-acetyl-D-glucosamine	0	60	0	–	0	0	100	–	–	–	100
N-acetyl-D-glucosamine[2]	0	60	0	–	0	0	100	–	–	–	100
adonitol	100	0	0	–	0	0	0	–	–	–	50
L-arabinose	38	20	100	+	0	0	100	–	–	–	50
L-arabinose[2]	38	20	0	+	0	0	100	–	–	–	50
D-arabitol	88	20	0	+	0	0	67	–	–	–	50
cellobiose	0	20	0	–	0	0	0	–	–	–	0
i-erythritol	0	0	0	–	0	0	0	–	–	–	0
D-fructose	100	60	100	+	100	67	100	–	–	–	100
L-fucose	0	0	100	–	0	67	100	–	–	–	0
D-galactose	13	40	100	+	0	67	100	–	–	–	50
gentiobiose	13	60	100	–	0	67	67	–	–	–	0

Table 13. Continued

Substrates[1]:	Gr.1	Gr. 3	Gr. 4	Gr. 11	Gr. 12	Gr. 17	Gr. 19	Gr. 20	Gr. 21	Gr. 22	Gr. 30
α-D-glucose	100	60	100	–	0	100	100	–	–	+	50
glucose[2]	75	40	0	+	0	0	100	–	–	–	50
m-inositol	0	20	100	–	0	67	100	–	–	–	0
α-D-lactose	0	20	100	–	0	67	67	–	–	–	0
lactulose	0	0	100	–	0	67	67	–	–	–	0
maltose	0	60	100	–	0	67	100	–	–	–	50
maltose[2]	0	60	0	–	0	0	100	–	–	–	0
D-mannitol	100	0	67	+	0	0	100	–	–	–	100
D-mannitol[2]	100	0	0	+	0	0	100	–	–	–	100
D-mannose	38	0	100	+	0	0	100	–	–	–	50
D-mannose[2]	13	60	0	+	0	0	100	–	–	–	50
D-melibiose	0	60	33	–	0	33	0	–	–	–	0
β-methyl-D-glucoside	0	20	100	–	0	33	33	–	–	–	0
D-psicose	88	0	100	+	0	67	67	–	–	–	0
D-raffinose	0	20	100	–	0	67	67	–	–	–	0
L-rhamnose	0	40	100	–	0	33	67	–	–	–	0
D-sorbitol	100	0	100	+	0	67	100	–	–	–	50
sucrose	0	0	100	–	0	67	67	–	–	–	0
D-trehalose	0	100	100	–	0	67	100	–	–	–	0
turanose	0	0	100	–	0	67	100	–	–	–	50
xylitol	100	0	100	–	0	67	67	–	–	–	50
methyl pyruvate	100	80	100	+	100	100	100	+	–	+	100
mono-methyl succinate	100	40	67	+	100	100	100	–	–	+	100
acetic acid	88	60	100	–	100	100	100	–	–	–	100
cis-aconitic acid	75	20	100	–	100	100	100	–	–	+	100
citric acid	88	0	100	–	100	100	100	–	–	–	50
citric acid[2]	0	0	0	–	100	0	33	–	–	–	100
formic acid	75	20	100	–	100	100	100	–	–	–	100
D-galactonic acid lactone	0	20	100	–	0	67	100	–	–	–	0
D-galacturonic acid	0	20	100	–	0	100	100	–	–	–	0
D-gluconic acid	100	0	100	+	100	100	100	–	–	–	100
gluconic acid[2]	100	0	0	+	100	100	100	–	–	–	50
D-glucosaminic acid	0	0	100	–	0	100	100	–	–	–	0
D-glucuronic acid	0	20	100	–	0	100	100	–	–	–	50
α-hydroxybutyric acid	100	0	67	–	0	100	100	–	–	–	100
β-hydroxybutyric acid	100	100	67	+	100	100	100	+	–	+	100
γ-hydroxybutyric acid	0	0	0	+	100	100	33	–	–	–	0
p-hydroxy phenylacetic acid	100	0	0	+	0	100	100	–	–	–	100
phenylacetic acid[2]	0	0	0	–	100	100	100	–	–	–	50
itaconic acid	0	0	33	–	0	33	33	–	–	–	50
α-keto butyric acid	88	40	100	+	0	100	100	–	–	+	100
α-keto glutaric acid	100	20	100	–	100	100	67	–	–	–	100
α-keto valeric acid	50	20	100	–	0	0	33	–	–	–	0
D,L-lactic acid	100	20	100	+	100	100	100	–	–	–	100
malonic acid	0	0	100	–	0	67	100	–	–	–	0
propionic acid	88	60	100	–	100	100	100	–	–	–	100
quinic acid	100	0	100	–	100	67	100	–	–	–	100
D-saccharic acid	0	0	100	–	0	67	100	–	–	–	50
sebacic acid	100	40	33	–	100	67	100	–	–	–	100
caprate[2]	38	0	0	–	100	33	100	–	–	–	50
succinic acid	100	0	0	+	100	100	100	–	–	+	100

Table 13. Continued

Substrates[1]:	Gr.1	Gr. 3	Gr. 4	Gr. 11	Gr. 12	Gr. 17	Gr. 19	Gr. 20	Gr. 21	Gr. 22	Gr. 30
Bromo succinic acid	100	0	0	+	100	100	100	–	–	+	100
adipate[2]	13	0	0	–	100	0	100	–	–	–	100
malate[2]	100	40	0	+	100	67	33	–	–	–	100
succinamic acid	100	0	67	+	100	100	100	–	–	+	100
glucuronamide	0	0	67	–	0	100	67	–	–	–	0
alaninamide	75	60	100	–	100	100	67	–	–	+	100
D-alanine	100	60	33	–	0	100	100	–	–	–	50
L-alanine	75	60	100	+	0	100	100	–	–	–	50
L-alanyl-glycine	75	80	100	+	0	100	100	–	–	+	50
L-asparagine	100	40	100	+	100	100	100	–	–	–	100
L-aspartic acid	100	80	100	+	100	100	100	–	–	–	100
L-glumatic acid	100	100	100	+	100	100	100	–	–	+	100
glycyl-L-aspartic acid	0	100	100	–	0	100	67	–	–	–	50
glycyl-L-glutamic acid	38	100	100	–	0	100	67	–	–	+	50
L-histidine	100	40	0	–	0	100	100	–	–	–	100
hydroxy L-proline	25	40	100	–	0	0	100	–	–	–	0
L-leucine	100	100	100	–	100	100	67	–	–	–	100
L-ornithine	0	40	100	–	0	33	67	–	–	–	0
L-phenylalanine	100	40	100	–	50	100	100	–	–	–	100
L-proline	100	100	100	+	100	100	100	–	–	–	100
L-pyroglutamic acid	100	0	100	+	100	100	100	–	–	–	100
D-serine	0	0	0	–	0	100	33	–	–	–	0
L-serine	75	20	100	+	0	100	100	–	–	–	50
L-threonine	100	20	100	–	0	100	100	–	–	+	50
D,L-carnitine	0	0	33	–	0	67	100	–	–	–	0
γ-amino butyric acid	75	0	0	–	0	0	100	–	–	–	100
urocanic acid	0	20	0	–	100	33	100	+	–	+	50
inosine	100	0	67	–	100	100	100	–	–	–	100
uridine	63	0	100	–	0	0	0	–	–	–	50
thymidine	0	0	33	–	0	0	0	–	–	–	0
phenyl ethylamine	0	0	0	–	0	0	100	–	–	–	0
putrescine	0	0	0	–	0	0	67	–	–	–	0
2-amino ethanol	0	0	0	–	0	0	100	–	–	–	0
2,3-butanediol	0	0	33	–	50	0	67	–	–	–	0
glycerol	88	20	100	+	0	67	100	–	–	–	50
D,L-α-glycerol phosphate	0	20	100	–	0	67	100	–	–	–	50
glucose-1-phosphate	38	0	100	–	0	33	100	–	–	–	0
glucose-6-phosphate	38	20	0	–	0	0	100	–	–	–	50
Reduction of nitrates[2]	0	60	0	+	0	100	100	–	–	–	50
indole production[2]	0	0	33	+	0	0	0	–	–	–	50
arginine dihydrolase[2]	0	0	0	–	0	0	0	–	–	–	0
urease[2]	63	0	100	–	0	0	0	–	–	–	50
hydrolysis (ß-glucosidase)[2]	0	100	100	–	0	0	0	–	–	–	0
hydrolysis (protease)[2]	0	0	100	–	0	0	0	–	–	+	0
β-galactosidase[2]	0	20	100	–	0	0	100	+	–	–	0
acidification[2]	0	0	0	–	0	0	0	–	–	–	0

[1] Utilisation of substrate unless the reaction or enzyme is specified. Physiological tests were performed either with the help of BIOLOG microplates or [2] API 20NE test strips

The lack of growth of group 21 and only four positive results of group 20 in both, BIOLOG microplates and API 20NE test strips, led to the selection of other tests and the design of new tests. MN154.3, belonging to group 21 and chosen as the type strain of this group, and MN122.2a being type strain of group 20 were thus tested in the API 50 carbohydrate substrate strips to obtain assimilation patterns and API 50 CHB/E media were used to obtain oxidation patterns. Due to occasionally long lag phases (see Fig. 8) these tests were monitored for 10 days. MN154.3 did not show positive results for neither the oxidation nor the assimilation tests. Apart from a positive esculin reaction, MN122.2a reacted negative in all assimilation tests. In the oxidation test kit the esculin test was also positive after 24 hours. Additionally, MN122.2a caused positive reactions in the oxidation test of galactose, 5-keto-gluconate and weakly positive reactions for L-arabinose and D-fucose. All other reactions were also negative implying that neither BIOLOG nor several API test kits yield adequate reaction profiles of these strains. Hence, tests for oligotrophy of the strains belonging to groups 20 and 21 were performed to study the substrate assimilation at lower nutrient concentrations. Growth of all member strains of group 20 was not enhanced at lower nutrient concentrations and thus this group showed no sign of oligotrophy (Fig. 6). Group 3, which grew readily and served as a control group in the following experiments also showed no sign of oligotrophy. In the four least diluted reaction tubes, the yield of groups 3 and 20 measured as optical density was higher when the medium was less diluted (Fig. 6). The highest reached optical densities of strains MN122.2a and MN149.3 corresponded exactly with the dilution factors of the medium for all diluted treatments. Only the undiluted treatments showed optical densities lower than expected based on the diluted yields. This was a result of the measuring procedures though. None of the member strains of group 21 grew in nutrient broth and dilution of the media had no effect. Therefore, oligotrophy was probably not a cause for the lack of growth when using the test kits. Following tests showed that group 21 is capable of growing up to measurable cell densities (Fig. 7 and 8), hence the problem of certain bacteria not growing to cell densities > 10^6 cells per ml (Rappé et al., 2002) can also be excluded for this group.

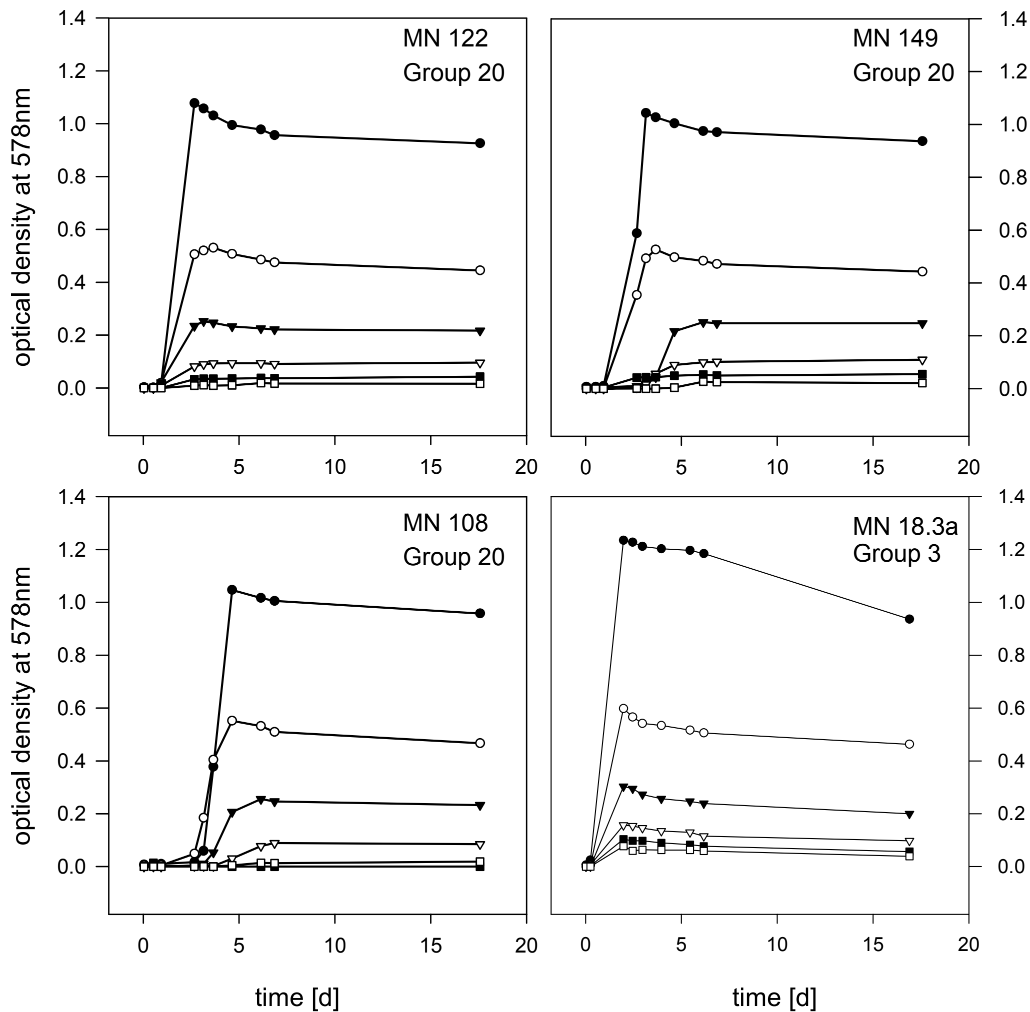

Figure 6. Growth curves of strains in nutrient broth of different dilution rates. MN122.2a, MN108, and MN149.3 belong to group 20 and MN18.3a belonging to group 3. ● Undiluted nutrient broth, ○ dilution 1:5, ▼ dilution 1:10, ▽ dilution 1:25, ■ dilution 1:50, and □ dilution 1:100.

Testing differing pH-values of growth media

The two groups 3 and 20 differed in terms of their pH-optima for growth (Fig. 7). Group 20 reached highest optical densities at pH 7 (Fig. 7b). This group also grew at pH 6 and to a limited extent at pH 5. In contrast, MN18.3a belonging to group 3, reached its highest optical density at pH 9, also exhibiting growth at pH 7 and 8. Cells of MN18.3a belonging to group 3 aggregated during growth in liquid media. This impaired measurements of optical density. Formation of aggregates was not observed during the exponential growth phase. The lag phase of growth at pH 9 was >10 days. The growth results of the tubes with unmodified buffering systems, which served as controls for the possible enhancement or inhibition effects of different buffering systems, showed no other growth results than the

tubes with buffering systems adjusted for the different pH-values and are thus not presented. MN154.3 as the representative strain of group 21 being very fastidious in terms of cultivation did not exhibit any growth in this experiment. However, another strain of group 21, MN157.2, grew at pH values of 4, 6, and 7 (Fig. 7). Growth at pH 4 was noticeable after one day; however, a higher OD was attained at pH 7, which was characterised by a long lag phase of about two weeks. Growth at pH 6 commenced even later. MN176 (group 21) did not show any growth at a pH value of ≥7. Thus, a more likely reason for the inability of group 21 to grow in standard test kits than its pH-optimum differing from the pH-values found in the test media may lay in the design of test kits being based on strains with short lag phases.

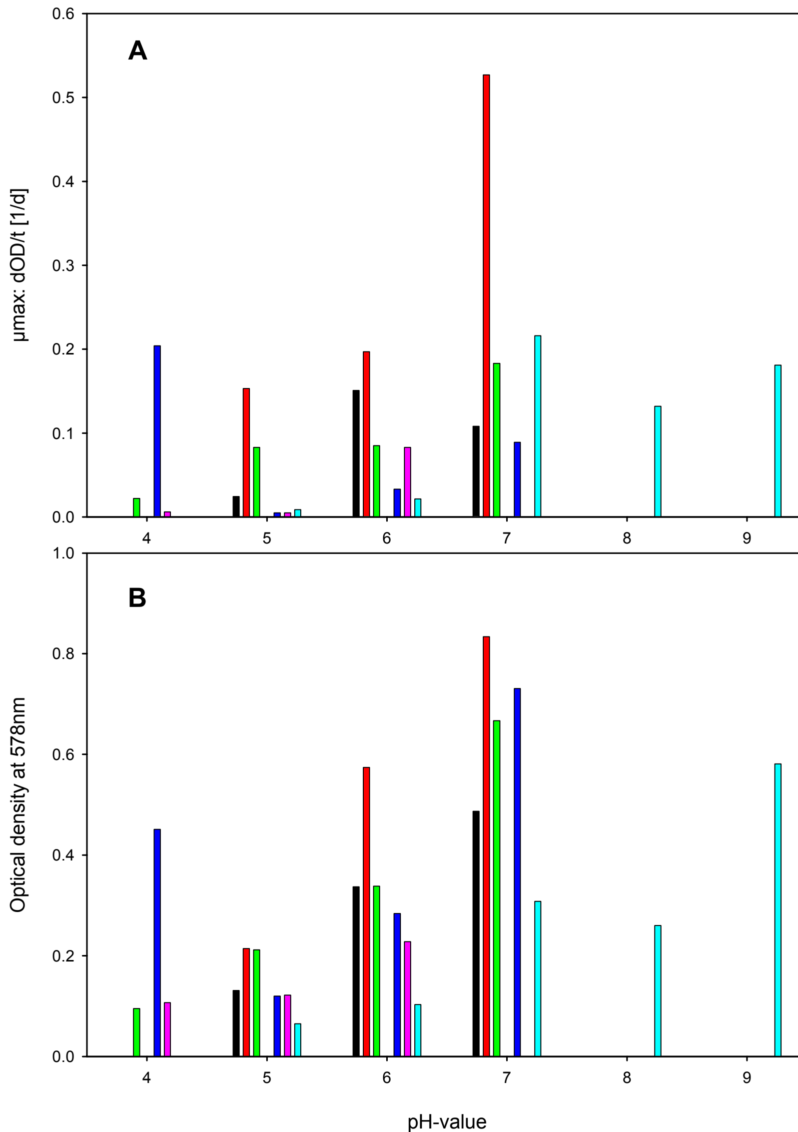

Figure 7. Maximum growth rates (A) and maximum obtained optical densities (B) of strains at different pH-values in the growth media. ▬ MN122.2a, ▬ MN108, and ▬ MN149.3 belong to group 20, ▬ MN157.2 and ▬ MN176 to group 21, and ▬ MN18.3a to group 3.

Growth using different carbon sources

Particularly long lag phases occurred at growth using different carbon sources. Two strains of group 20 exhibited lag phases of about 55 days on two different substrates. MN18.3a, representing group 3, showed a lag phase of about 30 days when growing on succinate as the sole carbon source (Fig. 8 and 9). Succinate also resulted in long lag phases for all three strains of group 20 (20 days for two of the strains and 55 days for MN122.2a).

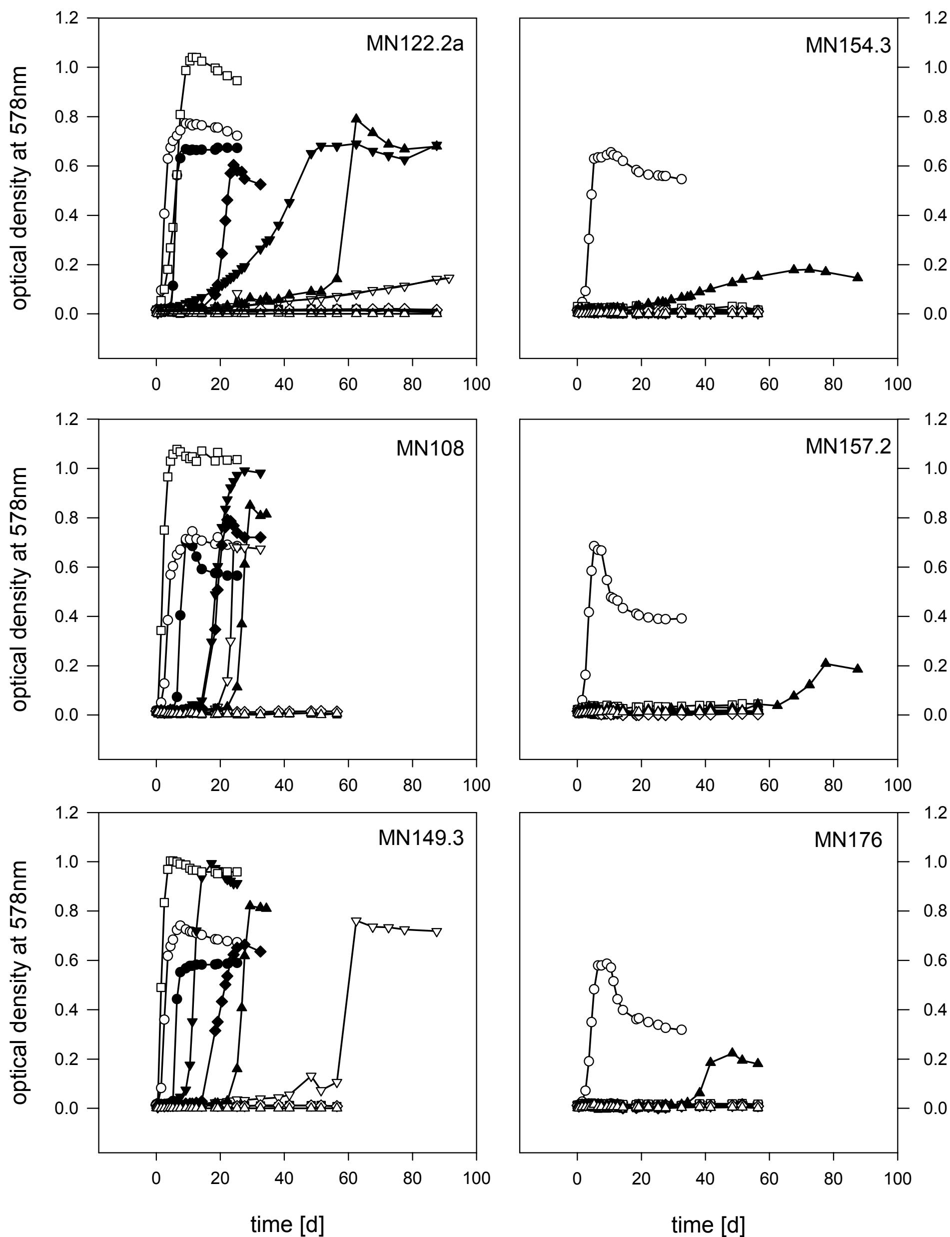

Figure 8. Growth curves of strains on different carbon sources. MN122.2a, MN108, and MN149.3 belong to group 20 (left column of diagrams) and MN154.3, MN157.2, and MN176 to group 21 (right column of diagrams). Growth on ● L-arabinose, ○ β-hydroxybutyric acid, ▼ D-fructose, ▽ D-alanine, ■ D-threonine, □ D-glucose, ◆ L-aspartic acid, ◇ L-lysine, ▲ succinate, and △ citrate.

Group 21 showed only weak growth on succinate but also only after a prolonged lag phase (Fig. 8). Group 21 grew well only on hydroxybutyric acid, which had been chosen as the positive control. Apart from succinate members of group 21 did not grow on any of the other carbon sources in this experiment. Group 20 did not grow on threonine, lysine and citrate. In one case it grew only poorly on alanine, however, the two other strains exhibited good growth on alanine after a prolonged lag phase. Strain MN18.3a representing group 3 grew

on all substrates except for fructose and citrate (Fig. 9). Especially the occurrence of long lag phases in these tests on a variety of carbon sources led to a thorough examination of the grown microorganisms. The analyses clearly demonstrated the authenticity of the isolates.

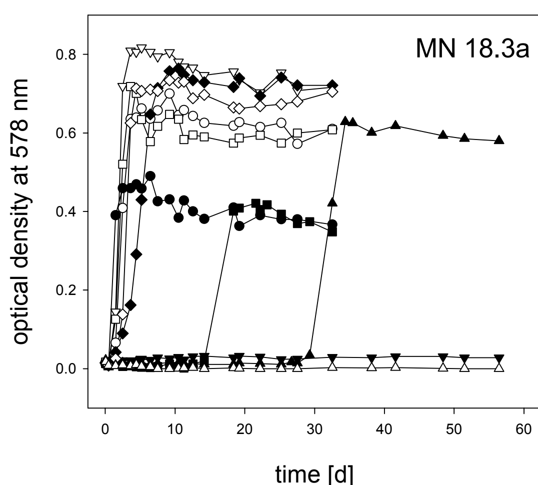

Figure 9. Growth curves of MN18.3a belonging to group 3 on different carbon sources. Growth on ● L-arabinose, ○ β-hydroxybutyric acid, ▼ D-fructose, ▽ D-alanine, ■ D-threonine, □ D-glucose, ◆ L-aspartic acid, ◇ L-lysine, ▲ succinate, and △ citrate.

Growth at different temperatures

None of the tested strains showed psychro- or thermotolerant growth at 4 and 45°C, respectively (Fig. 10 and 11). MN18.3a belonging to group 3 grew fastest at 37 and 30°C and almost equally well at 23°C. Even though the optical density attained was lower, growth also occurred at 13°C (Fig. 10). MN 18.3a exhibited ascending growth rates with ascending temperatures (Fig. 12). Generally, group 20 grew well at 23, 30, and at 37°C (Fig. 11). The growth rate of strain MN149.3 decreased with lowering the temperature from 37 to

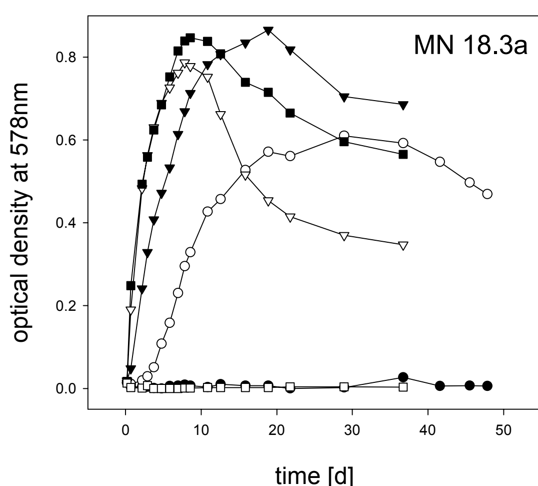

Figure 10. Growth curves of MN18.3a belonging to group 3 at different temperatures: ● 4°C, ○ 13°C, ▼ 23°C, ▽ 30°C, ■ 37°C, and □ 45°C.

13°C (Fig. 12). All three strains of group 21 grew at 23°C with some also growing at 30 and slightly at 37°C. In contrast to groups 20 and 3 hardly any growth was observed at 13°C for the strains of group 21 (Fig. 10 and 11). Group 3 reached higher optical densities than groups 20 and 21, but this could also be caused by group 3 being tested on nutrient broth medium instead of minimal medium with added carbon source (Fig. 10 - 12).

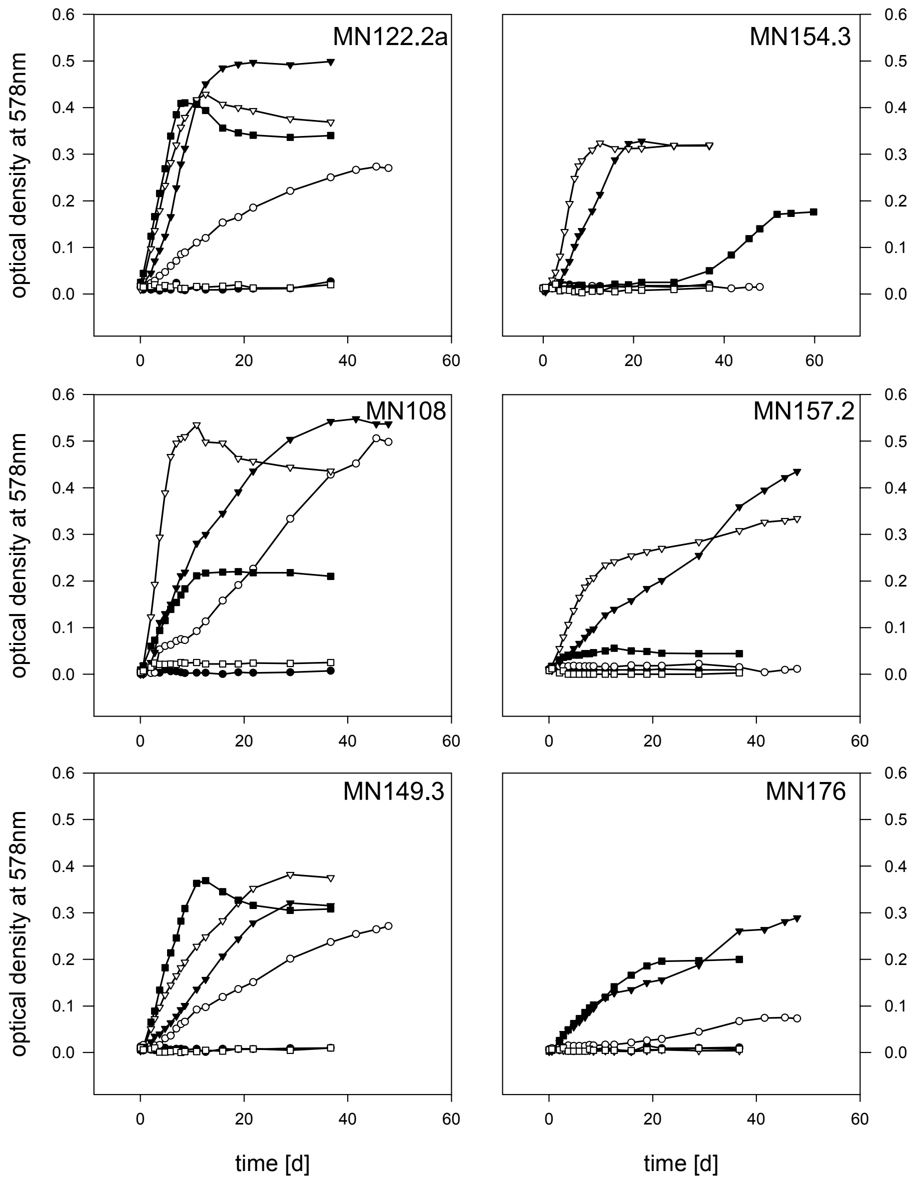

Figure 11. Growth curves of groups 20 and 21 at different temperatures. MN122.2a, MN108, and MN149.3 belong to group 20 (left column of diagrams) and MN154.3, MN157.2, and MN176 to group 21 (right column of diagrams). Growth at: ● 4°C, ○ 13°C, ▼ 23°C, ▽ 30°C, ■ 37°C, and □ 45°C.

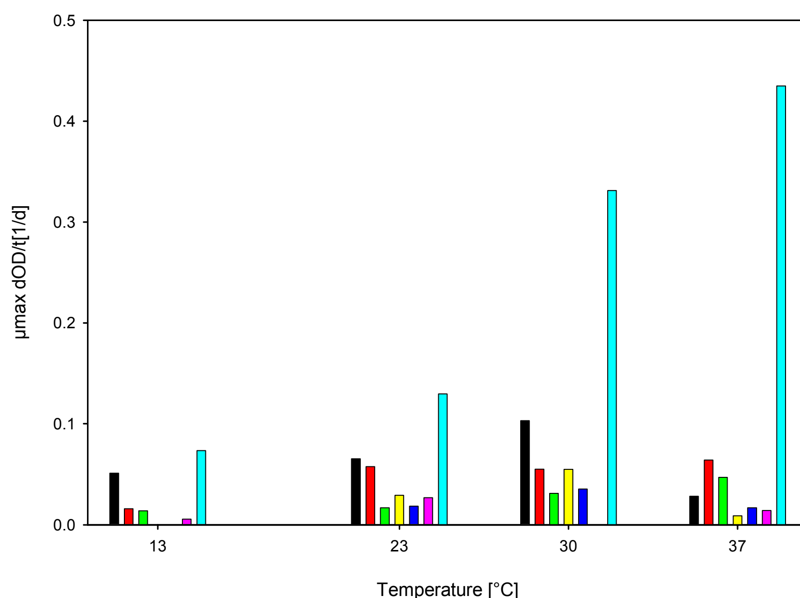

Figure 12. Maximum growth rates of strains at different incubation temperatures. ▬ MN122.2a, ▬ MN108, and ▬ MN149.3 belong to group 20, ▬ MN154.3, ▬ MN157.2 and ▬ MN176 to group 21, and ▬ MN18.3a to group 3.

Growth on short-chained alkanes

Apart from characterising the fastidious groups 20 and 21 the ability of several other groups to grow on a variety of short-chained alkanes was of interest to detect potential biocatalysts. Thus, growth experiments on different alkanes ranging from pentane to decane were carried out with 38 strains representing 16 groups. These tests were carried out with all three strains belonging to group 20. MN122.2a grew well on all given alkanes except for heptane and octane (Table 14). Growth on nonane was slow until a high growth rate was achieved after 18 days, resulting in a high OD of almost 1.2. MN149.3 could grow on all alkanes provided apart from heptane. Growth began later on decane and octane, with the growth rate being lower on decane. The optical densities reached were also lower with the former two carbon sources. Nevertheless, this group grew on a broad range of short-chained alkanes.

Representatives of groups 3, 5, 16, and 23 were able to grow on several alkanes (Table 14). In contrast, MN157.2, a member of group 21, which was normally cultivated on hexane as the sole carbon source showed hardly any growth on alkanes. MN176 belonging to the same group showed a larger variety of possible growth substrates in this experiment. MN154.3 grew best on hexane and on the positive control (Table 14).

Because long lag phases or long periods of slower growth were not unusual in several groups (Table 14), lack of growth must not be overrated. But even with a lengthy duration of the experiments it occurred twice that MN154.3 of group 21, which was held on hexane as the standard carbon source, did not grow at all in any of the test tubes of an experiment. In

one experiment MN122.2a of group 20 did not grow in any of the test tubes of one entire experiment that lasted 35 days. These negative growth results are not presented but they need to be considered when comparing strains of a same group. Therefore, it can not be concluded that a negative result implies a general lack of ability of the strain to grow on the tested substrate. This is especially true for the two fastidious groups 20 and 21.

Group 3 generally only grew slightly on hexane if at all (Table 14). Most strains belonging to group 3 tested for the results in Table 14 did not grow on octane. However, the strain MN34.1 grew slowly on octane, reaching an optical density of almost 1 after about 34 days. Thus, testing one strain of a group cannot represent the entire group. Therefore, individual strains are named in the following table.

Generally groups 5 and 16 grew very well on hexane (Table 14), but the lag phase lasted up to two weeks for strain MN5.1a from group 5. The addition of nitrate showed no consistent effects on the growth on hexane of groups 5 and 16.

Several groups grew only on β-hydroxybutyric acid, which was used as the positive control in these experiments (Table 14). Members of group 4, 22, and 23 reached high biomass on agar plates with minimal medium and hexane as the sole carbon source in a gas tight reaction chamber. However, there was no growth in liquid minimal medium with hexane as carbon source (Table 14). Group 22 did not even grow in any of the minimal media test tubes. Additionally, in all experiments strains of group 1 grew on a variety of alkanes with fairly low final optical densities. Generally, members of the groups 5, 16, 20, and 21 reached higher optical densities when grown on hexane than the other strains.

Table 14. Growth of several groups on different alkanes. Lag times are presented as the number of days (d) of the lag time duration.

Group	strain	Growth on															
		pentane		hexane		heptane		octane		nonane		decane		control[1]		hexane + KNO_3	
1	[2]MN3.2b	–		–		w	10	w	27	–		(+)	27	+++	0	–	
1	[3]MN34.2a.2	–		–		–		(+)	6	(+)	7	(+)	8	+++	0	w	27
1	[4]MN36.2	(+)	3	–		–		(+)	11	–		–		+++	0	–	
1	[5]MN39.1a	w	4	(+)	3,5	(+)	2	w	0	(+)	0	w	0	+++	0	(+)	3,5
1	[5]MN39.1b	w	4	w	4	–		w	3	w	3	(+)	0	+++	0	w	4
3	[6]MN1	+	3	(+)	3	–		–		+++	8	+++	3,5	++	19	n.d.	
3	[6]MN4	(+)	6	–		–		–		++	4	++	3	+	8	n.d.	
3	[6]MN11.3	+	4	(+)	4	–		–		–		++	2,5	++	+	n.d.	
3	[6]MN18.3a	+	3,5	w	4	–		+	4,5	++	4,5	++	2,5	++	5,5	n.d.	
3	[6]MN21.1	w	4	–		–		–		++	4,5	++	3	+	5	n.d.	
3	[7]MN34.1	+	2	–		w	10	+++	8	+++	0	+	1,5	+++	4,5	(+)	2
4	[5]MN13.3d	–		–		–		+	0	+	0	+	0	–		–	
5	[2]MN5.1a	–		+++	15	+++	5	+++	3	+++	2	+++	2	+++	0	+++	12
5	[6]MN12	+	8	+	3	–		w	3	+	2	+	2	+++	0	n.d.	
5	[4]MN31.1	–		+++	7	+++	4	–		n.d.		+++	2	+++	0	+	4
5	[6]MN38	++	2	++	0	–		w	2,5	+	2	++	0	+++	0	n.d.	
11	[8]MN33.2	–		–		–		–		–		–		+++	0	–	
12	[5]MN43	–		–		–		w	2,5	(+)	0	+	0	+++	0	–	
12	[5]MN48.1a	–		–		–		w	3	(+)	0	+	0	+++	0	–	
12	[8]MN49	–		–		–		–		–		–		+++	0	n.d.	
14	[8]MN60.3	–		–		–		–		–		–		+++	7	n.d.	
16	[5]MN110a	+	0	+++	0	–		(+)	0	+	0	++	0	+++	0	+++	0
16	[5]MN118.1a	w	4	+	2	w	3	++	2	(+)	0	+++	0	+++	0	+	2
16	[2]MN126.2	–		–		–		–		–		++	0	+++	0	++	0
16	[4]MN129	+	2	+++	2	w	9	–		+++	0	+++	0	+++	0	++	4
16	[5]MN152	(+)	3	(+)	0	+	0	+	0	++	0	+++	0	+++	0	++	0
19	[5]MN87	–		–		–		(+)	0	–		+	0	+++	0	–	
20	[8]MN108	+	0	–		–		–		–		–		++	0	–	
20	[8]MN122.2a	+++	0	+++	0	–		–		++	18	++	6	++	0	+++	0
20	[8]MN149.3	++	0	+	0	–		(+)	6	++	2	(+)	3	++	0	n.d.	
21	[4]MN154.3	+	7	++	3	–		–		w	10	+	7	++	4	–	
21	[8]MN157.2	(+)	3	–		–		–		–		–		++	9	n.d.	
21	[4]MN176	+	5	++	3	–		–		(+)	13	++	7	++	4	n.d.	
22	[4]MN28	–		–		–		–		–		–		–		–	
23	[8]MN45.1	(+)	0	–		–		++	8	+++	0	+++	0	+++	0	n.d.	
25	[4]MN30.1b.1	–		–		–		–		–		–		+++	0	–	
26[9]	[5]MN169.2a	–		–		–		(+)	0	–		(+)	0	–		–	
30	[8]MN45.2b.1	–		–		–		–		–		–		+++	0	–	

[1] Growth on β-hydroxybutyric acid; Duration of experiments [2] 35, [3] 49, [4] 19, [5] 10, [6] 30, [7] 50, and [8] 39 days; [9] Eucaryotic strain; Growth efficiency based on O.D.$_{max}$ and maximum growth rate: – no growth, w = very weak growth, (+) = slight growth, + = growth, ++ = strong growth, +++ = very strong growth; n.d. = not determined.

Phylogenetic analysis

For representative strains of 17 groups, nearly complete 16S rDNA sequences were determined. The phylogenetic analysis of the sequences of these 17 groups revealed affiliations to various bacterial groups (Fig. 13 and 14, Table 15). Some of the 16S rDNA sequences were highly similar to the ones of previously described bacterial species: The sequence of strain MN45.1 of group 23 was identical (100%) to that of *Xanthobacter flavus* strain JW/KR-1 (Rainey and Wiegel, 1996) (Fig. 13), the ones of MN36.2 of group 1 and MN45.2b.1 of group 30 to *Variovorax paradoxus* IAM12373 (each 99.4%), MN 101.b of group 19 to *Burkholderia multivorans* LMG13010^T (99.3%), and MN182.2 of group 17 to *Pandoraea pnomenusa* LMG18087^T (99.9%) (Fig. 14). Additionally, strain MN33.2 (single strain of group 11) shared 99.1% 16S rDNA sequence similarity with the type strain of *Acidovorax delafieldii*. In addition the 16S rDNA sequence of MN38 of group 5 was highly similar to the one of *Rhodococcus erythropolis* ATCC53968 (99.8%), the one of MN110a of group 16 to the sequence of *Gordonia polyisoprenivorans* DSM44302^T (99.9%), the 16S rDNA sequence of MN60.3 being the only representative of group 14 was highly similar to *Cellulosimicrobium cellulans* IFO16148 (99.7%), MN47.2a of group 8 shared high similarity with *Brevibacillus choshinensis* IFO15518^T (99.6%), and MN8.1d.1c, the only representative of group 7 with *Micrococcus luteus* LMG381 (99.3%) (Table 15). For eight of the strains isolated in the present study partial sequences were obtained. The sequence lengths were about 700 bp (approximately positions 100 to 800, *Escherichia coli* numbering Brosius et al., 1981). Three of these partial sequences were sequences of member strains of groups for which already nearly complete 16S rDNA sequences were used for taxonomic affiliation. The other four partial sequences were affiliated with the *Actinobacteria* (Table 15). Following, the strains with sequenced 16S rDNA are presented within their phylogenetic groups, which are *Alpha-, Beta-* and *Gammaproteobacteria* as well as *Actinobacteria* and *Firmicutes*.

Alphaproteobacteria

Although being affiliated with *Sphingomonas* MN122.2a has no close relative among previously described species of the genus (16S rDNA sequence similarities $\leq$ 97.0%). The strain actually represents a new species of *Sphingomonas* that will be described in more detail in the discussion. MN149.3 (EMBL accession number: AJ863567) and MN122.2a, both belonging to group 20, showed a 99% homology with just one mismatch in 639 bp. Additionally, MN18.3a (group 3) is also suggested to be a new *Sphingomonas* species. Group 10 (7 members), represented by strain MN51a, is affiliated with the family "*Bradyrhizobiaceae*" with *Bosea thiooxidans* DSM9653^T (99.0% sequence similarity) being its closest described bacterial relative (Fig. 13).

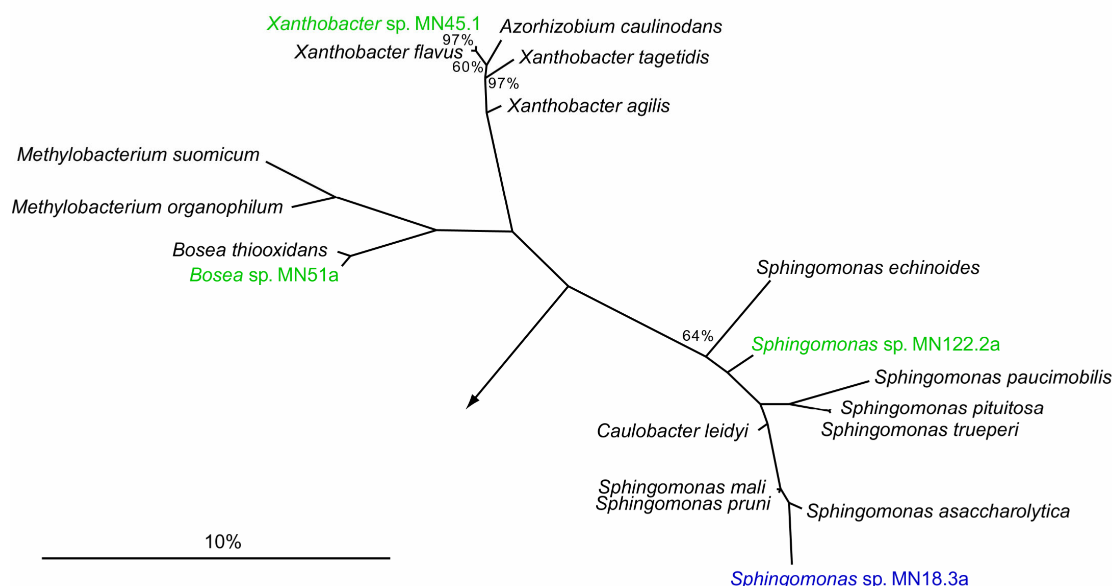

Fig. 13. Phylogenetic tree showing the affiliation of 16S rDNA sequences to representatives of the *Alphaproteobacteria.* The tree was calculated by maximum likelihood analysis. A conservation filter of 50% for the *Alphaproteobacteria* was used. Bootstrap values higher than 60% are given. The scale bar indicates 10% estimated sequence divergence. The EMBL accession numbers of the sequences are as follows (clock-wise from *Bosea* sp. MN51a to *Caulobacter leidyi*): AJ313022, AJ250796, D32226, AY009404, X94204, AJ313028, X94200, X99469, X94198, AB021370, AJ313019, U37337, AJ243751, X97776, Y09639, AJ555475, Y09637, Y09638, AJ227812.
EMBL accession numbers of outgroup organisms: D84026, M22509, L15475, U00096, X06684, X95917, AB021389, and AJ005447. Colour coding: blue and green indicate strains from the experimental biofilter and the full-scale biofilter, respectively.

Beta- and *Gammaproteobacteria*

MN154.3 representing group 21 shows the largest 16S rDNA sequence similarity to *Nevskia ramosa.* The 16S rDNA similarity of MN154.3 and the type-strain of *Nevskia ramosa* was < 94.1%. Both obtained sequences of group 21 (MN154.3 and MN157.2) were identical in the 730 bp long sequence identified for strain MN157.2 (EMBL accession number: AJ863569). The group accounts for a new genus within the deep-branching *Gammaproteobacteria* (Fig. 14) and will be described in more detail lateron. In addition, the strain MN28 (group 22) also forms a new genus within the deep-branching *Gammaproteobacteria* with the largest 16S rDNA sequence similarity to *Nevskia ramosa* of the described species with 89.6% and group 21 with 90.2%. The highest 16S rDNA similarity of group 22 is merely 94% to uncultured *Bacteria*. A more detailed description follows in the discussion section. Apart from the priory mentioned members of the genera *Acidovorax, Variovorax, Burkholderia,* and *Pandoraea* group 12 is also placed within the *Betaproteobacteria*. Group 12, consisting of 14 strains and represented by strain MN43, is

affiliated with the genus *Cupriavidus,* showing a sequence similarity of 98.6% with *Cupriavidus basilensis* DSM11853^T (Fig. 14).

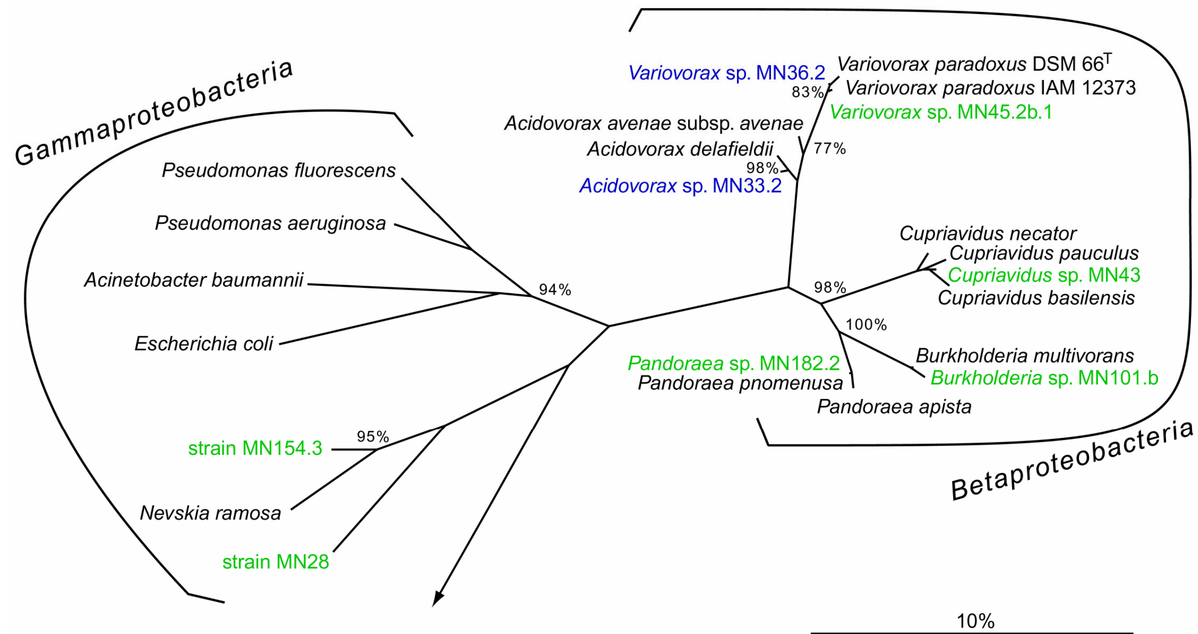

Fig. 14. Phylogenetic consensus tree showing the affiliation of 16S rDNA strain sequences to representatives of the *Beta-* and *Gammaproteobacteria.* The tree was calculated by maximum likelihood analysis. A conservation filter of 50% for the *Beta-* and *Gammaproteobacteria* was used. Bootstrap values higher than 60% are given. The scale bar indicates 10% estimated sequence divergence. The accession numbers of the sequences are as follows (clock-wise from strain MN28 to *Pandoraea* sp. MN182.2): AJ555478, AJ001343, AJ313020, AE000129, X81660, X06684, Z76662, AJ555476, AF078764, AB021421, AJ313017, AJ420329, D30793, AJ555474, M32021, AF085226, AJ313021, AF312022, Y18703, AJ555477, AF139172, AF139174, AJ313026.
EMBL accession numbers of outgroup organisms: D01260, D11345, D30768, M11223, X53854, X69159, X74915, and Z21936. Colour coding: blue: strains from the experimental biofilter; green: strains from the full-scale biofilter

Actinobacteria and *Firmicutes*

The remaining five nearly complete 16S rDNA sequences could be assigned to bacterial species previously described belonging to the *Actinobacteria* and *Firmicutes* (Table 15). Four other groups are affiliated with the *Actinobacteria* based on their partial 16S rDNA sequences. Groups 2, 9, 18, and 25 are affiliated with *Micrococcus luteus* (99.6%), *Kocuria rhizophila* (99.8%), *Leifsonia shinshuensis* (99.3%), and *Brevibacterium sanguinis* (99.7%), respectively (Table 15). The identification of the strains of groups 2, 9, and 18 based on the partial 16S rDNA sequences is generally in agreement with the fatty acid profiles of these groups and their corresponding reference strains. Currently, no fatty acid profile of the

recently described species *B. sanguinis* exists, and thus the fatty acid profile could not be compared with that of group 25.

The strain MN47.2a affiliated with the genus *Brevibacillus* represents the only member of the *Firmicutes* among the 16S rDNA-sequenced strains in the present study (Table 15). In contrast, based on 16S rDNA sequences, 9 groups belonging to the *Actinobacteria* were isolated from the biofilters (Table 15). Strains MN38 and MN110a belong to the dominating groups of the strains isolated from the laboratory- and the full-scale biofilters, respectively. Interestingly, the fatty acid profile of group 27, isolated from the Hamm biofilter, shows a high similarity to that of group 5, which is affiliated with the species *Rhodococcus erythropolis*. However, 16S rDNA sequences are not available for the strains of group 27.

Table 15. Phylogenetic affiliation based on 16S rDNA sequences of strains belonging to the *Actinobacteria* and *Firmicutes*.

Strain (EMBL accession number)	Group	Sequence length [bp]	Phylogenetic position	Strain	EMBL accession number	Similarity [%]
MN9.1a.3 (AJ864345)	2	730	*Micrococcus luteus*	DSM20030^T	AJ536198	99.6
MN38 (AJ313018)	5	1430	*Rhodococcus erythropolis*	ATCC53968	AF001265	99.8
MN8.1d.1c (AJ313024)	7	1472	*Micrococcus luteus*	LMG381	M38242	99.3
MN47.2a (AJ313027)	8	1482	*Brevibacillus choshinensis*	IFO15518^T	D78459	99.6
MN59 (AJ864344)	9	730	*Kocuria rhizophila*	ATCC9341	AF542072	99.8
MN60.3 (AJ313025)	14	1472	*Cellulosimicrobium cellulans*	IFO16148	X83809	99.7
MN110a (AJ313023)	16	1475	*Gordonia polyisoprenivorans*	DSM44302^T	Y18310	99.9
MN177 (AJ864347)	18	740	*Leifsonia shinshuensis*	JCM10591^T	AB028940	99.3
MN30.1b.1 (AJ864346)	25	730	*Brevibacterium sanguinis*	DSM15677^T	AJ564859	99.7

Affiliation of non-sequenced strains

For the nine remaining bacterial groups no 16S rDNA sequence data was obtained and seven of the nine groups were characterised and identified based on phenotypic data. Groups 6 and 31 were lost during the cultivation process and could not be assigned to certain taxa.

Two member groups of cluster A for which no 16S rDNA sequence were obtained are compared with reference strains with similar total fatty acid compositions (Table 16). Based on fatty acid profiles group 13 clustered closely with group 3 within cluster A and its fatty

acids fit well into the range of fatty acids of group 3 (Table 4). Group 13 showed a smaller number of fatty acids than group 3. Nevertheless, group 13 is compared to *Sphingomonas* species and information obtained on this group showed no contradiction to the genus description of *Sphingomonas*. On the basis of fatty acid profile and morphological features group 15 is best compared with the genus *Methylobacterium*. Growth of the strain representing group 15 in liquid media was characterised by a surface ring which appears indicative of an aerobic nature and is in agreement with growth descriptions of members of the genus *Methylobacterium* (Green, 1992). Additionally, the poor growth on nutrient agar is typical for this genus, which either grows slowly or not at all on nutrient agar (Green, 1992). The pigmentation of species within the genus *Methylobacterium* varies from pale pink to bright orange-red and the pigment is nondiffusible, which is also in accordance with the appearance of group 15.

Table 16. Fatty acid profiles of group 13 and 15 and of reference strains from the literature

Fatty acid[a] [%]	Group 13	*Sphingomonas mali*[b] IFO 15500^T	*S. sanguinis*[b] IFO 13937^T	Group 15	*Methylobacterium extorquens*[c] DSM 1337^T	*M. radiotolerans*[c] DSM 1819^T
14:0			1.0			
15:0			1.1			
14:0 2OH	3.4	8.4	5.0			
14:0 3OH				0.6	2.0	
16:1 *cis*9			6.7[d]	4.6	7.0	1.0
16:1 *cis*11	0.4		1.0			
16:0	12.8	11.8	13.6	3.4	4.0	4.0
17:1 *cis*11	1.9	9.3	2.4			
17:0	0.5		0.5		< 0.9	
18:1 *cis*11	81.0	70.5	64.6	87.4	79.0	89.0
18:1 *cis*13			3.5			
18:0			0.6	4.0	6.0	6.0

[a] For examples of abbreviations, see Table 4.
Data from [b] Kämpfer et al. (1997) and [c] Ahrens et al. (1997).
[d] summed feature: 16:1 *cis*9 and or 15:0 iso 2OH

Following, the groups without 16S rDNA sequence information belonging to cluster G are presented. The fatty acid profile of group 24 showed a high resemblance to that of the genus *Corynebacterium*.

The fatty acid profile of group 27 exhibited more than 10% of 18:0 10 methyl and showed a high similarity with group 5 (Table 9). Based on the fatty acid profile, 16S rDNA and additional phenetic properties group 5 appeared to represent strains of *Rhodococcus erythropolis* (see above and discussion and in particular tables 9 and 15). The fatty acid 17:0 cyclo, which was present in all strains of the groups 5 and 27, was found by other authors only in the reference strain *Rhodococcus coprophilus* (Yoon et al., 1997; Yoon et al., 2000). However, since the ECL value of 17:1 *cis*12 is close to that of 17:0 cyclo, it is possible that the fatty acid 17:1 *cis*12 presented in the literature on *Rhodococcus erythropolis* is actually 17:0 cyclo. Based on fatty acid analysis group 27 belongs to the actinomycetes and exhibits large similarities with the genus *Rhodococcus.*

A literature comparison of the fatty acid profiles of groups 24 and 28 with that of reference strains assigned these two groups to the *Corynebacterineae* (Bousfield et al., 1983; Bernard et al., 1991; Vuorio et al., 1999).

Cluster I contained mainly single strains and included members of the *Actinobacteria* and *Firmicutes* based on 16S rDNA analysis of groups 7 and 14 and group 8, respectively. The 16S rDNA sequence similarity of groups 2 and 7 was 98.9% (722 of 730 partial 16S rDNA sequence). Their fatty acid profiles clustered closely together in cluster I. Therefore, their fatty acid profiles were compared with the species *Micrococcus luteus* (as found previously Stackebrandt et al., 1995; Wieser et al., 2002) to which groups 2 and 7 were assigned based on partial and near full length 16S rDNA analysis, respectively. Interestingly, in contrast to group 7 and *M. luteus*, group 2 lacked fatty acids with more than 15 carbons. The fatty acid profiles of group 7 and *M. luteus* have more in common than either of the two with that of group 2. Nevertheless, the fatty acid profile of the species *M. luteus,* which has been separated into three biovars by Wieser et al. (2002), is heterogeneous enough to actually place groups 2 and 7 into one group.

Group 29 clustered closely with group 9, which was assigned to *Kocuria rhizophila* based on partial 16S rDNA sequences. However, in contrast to group 29, group 9 lacked monounsaturated fatty acids. Considering that the authors Kovács et al. (1999) did not show values less than 1% and the shift from 15:0 anteiso to 17:0 anteiso possibly being due to ageing of the cells, the fatty acid profile of group 9 showed a high similarity to that of *K. rhizophila*. In contrast, based on FAME group 29 could not be assigned to a certain family.

Cluster J is a single group cluster with no 16S rDNA sequence information. Generally, the fatty acid profile of group 4 (cluster J) is characterised by fatty acids typical for both, *Sphingobacteriaceae* and *Flavobacteriaceae*. But with the help of DMDS derivatisation 16:1 *cis*9 could be differentiated from the fatty acid 15:0 iso 2OH, both of which have the same

retention time. In contrast to the *Sphingobacteriaceae* only traces of 16:1 *cis*9 were found (Steyn et al., 1998). Thus, the fatty acid composition of group 4 most closely resembles those of *Chryseobacterium indologenes* and *C. gleum* (as found previously Yabuuchi et al., 1983; Segers et al., 1993; Hugo et al., 1999). However, group 4 could not be allocated to one of the two species, based on FAME. This is in accordance with a previous study stating that no clear differentiation of the *Chryseobacterium* species (except *C. meningosepticum*) could be achieved on the basis of FAME analysis (Hugo et al., 1999). Group 4 was allocated to the family *Flavobacteriacea* within the class *Flavobacteria.* Group 4 is the only group isolated from the biofilters, which belongs to the phylum *Bacteroidetes*.

FLUORESCENCE *IN SITU* HYBRIDISATION (FISH) OF BIOFILTER SAMPLES AND INDIVIDUAL STRAINS

The composition of the microbial community of the laboratory- and the full-scale biofilter was studied applying fluorescence *in situ* hybridisation using rRNA-targeting oligonucleotide probes. Overall, the two sampling sites of the Hamm biofilter showed similar bacterial community patterns in 1997. For both sampling sites the *Alphaproteobacteria* (ALF; 7.0-9.5%) formed the most dominant group, followed by the *Betaproteobacteria* (BET; 4.9-7.3%) and *Actinobacteria* (HGC; 4.1-5.0%) (Fig. 15). Members of the *Gammaproteobacteria* (GAM), *Cytophaga-Flavobacterium* cluster (CF), *Xanthomonas* branch (XAN), *Planctomycetes* (PLA), and *Firmicutes* with low G+C DNA-content (LGC) varied between 0 and 2.3% (Fig. 15). Members of the *Xanthomonas* branch accounted for portions that were in the same range as those for the *Gammaproteobacteria*. At both sites of the Hamm biofilter proportions of unidentified members of the *Bacteria* ranged between 33.9 and 45.5%. In the Alberta biofilter sample only 27.6% of the bacteria could not be affiliated using the probes applied. Moreover, for all three biofilter samples taken in 1997 12.3-47.9% of all DAPI-stained cells could not be detected using probe EUB338. The bacterial detection rates obtained with probe EUB338 are similar to the rates reported for other systems (Glöckner et al., 1996; Neef et al., 1996; Kalmbach et al., 1997; Stoffels et al., 1998). Usually, probe EUB338 is applied in combination with PFA-fixed samples. However, ethanol is more suitable for the fixation of Gram-positive bacteria (Roller et al., 1994). Thus, in samples with high proportions of members of the *Firmicutes* and *Actinobacteria*, bacterial detection rates may be underestimated when PFA is used as a fixative. Therefore, PFA- and ethanol-fixed samples were hybridised and enumerated when applying probes LGC354a, LGC354b, LGC354c, and HGC69a and percentages of EUB338-positive bacteria were corrected for samples with high proportions of bacteria belonging to the *Firmicutes* and *Actinobacteria*. Ethanol proved to be more effective than PFA for members of the *Actinobacteria*. The Alberta sample showed a large difference between

ethanol- and PFA-fixed samples. Detection rates (relative to those obtained with DAPI) obtained with probe HGC69a were merely 4.3% for the PFA-fixed sample but 37.5% for the ethanol-fixed sample. No consistent differences between the two fixatives for members of the *Firmicutes* with a low G+C DNA-content were found. For the Alberta sample the positive difference between detection rates for ethanol- and PFA-fixed samples were added to the proportions obtained with probe EUB338. This led to a higher detection rate with 87.7% of all DAPI-stained cells, being identified as bacteria with probe EUB338 (Fig. 15). In the Alberta filter the *Actinobacteria* (HGC) was the most dominant group followed by *Alpha-, Betaproteobacteria*, and *Cytophaga-Flavobacterium* cluster. XAN and GAM reached similar percentages: 2.0 and 1.8%, respectively (Fig. 15).

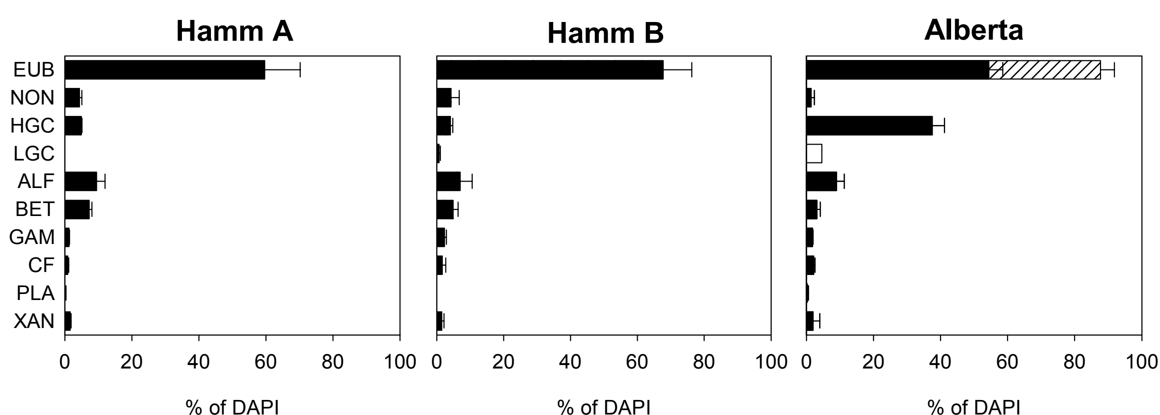

Fig. 15. Probe-specific counts relative to direct enumeration with DAPI (percentages) ■. Percentages of EUB338-positive bacteria corrected for samples with high proportions of members of the *Firmicutes* ▨. Percentages of LGC when PFA fixation was more efficient than ethanol-fixation □. Percentages given are corrected for negative control NON338. Sampling took place in 1997. Error bars indicate standard deviation of at least two replicates. Probe names are abbreviated forms of those given in Table 1.

Neither in the 1997 nor in the 2001 samples of the biofilters members of *Planctomycetes* hybridised with probe PLA46 contributed significantly, never reaching 1% (Fig. 15 and 16). Additionally, these counts were not subtracted from the counts obtained with probe EUB338, as these organisms generally do not hybridise with probe EUB338 (Neef et al., 1998).

It shall be noted that the packing material of the Hamm biofilter was replaced in the summer of 1999. Thus, the data on the bacterial community derived in 1997 (Fig. 15) and 2001 (Fig. 16) cannot be interpreted as time-series measurements. The proportion of unidentified bacteria was lower in the samples taken in 2001 with values as low as 4.2% in the upper and 12.8% in the lower biofilter layer. For the two samples incubated with deuterated hexane these values were in a similar range between 9.7 and 17.9%. The proportion of DAPI-stained

cells that could not be detected with probe EUB338 was in a similar range as in 1997 between 40.2 and 52.0%. The contribution of members of the *Actinobacteria* to the biofilter community was higher for the samples taken in 2001 than for those of the year 1997 attaining proportions of up to 25.8% (Fig. 16). The *Alphaproteobacteria* formed the second largest group accounting for 11.3 to 13.8% (Fig. 16). Members of the *Betaproteobacteria*, which contributed a significant portion to the microbial community in 1997, attained only less than 4.8% in all samples taken in 2001. Members of the *Gammaproteobacteria* contributed about 3% to the communities of both biofilter layers and XAN818-hybridised cells accounted for up to 1.7% in the lower biofilter layer. Member of the *Cytophaga-Flavobacterium* cluster attained 4.1% and 1.3% in the lower and upper biofilter layers, respectively. A comparison of the data retrieved before and after the labelling experiments with $[^2H_{14}]$ hexane showed no clear shift in terms of the composition of the microbial community (Fig. 16). Additionally, the increase of total microbial cells was not significant during the incubation experiment. Three replicates of the two lower layer samples, which were incubated with $[^2H_{14}]$ hexane for 24 days were counted. The means (and standard deviation) of the cell concentrations (given in counts per gram dry weight) of the incubated samples were 5.9×10^9 (4.8×10^8) and 5.6×10^9 (3.8×10^8). These cell concentrations lay within the range of the other cell concentrations obtained from the Hamm biofilter (see Table 2).

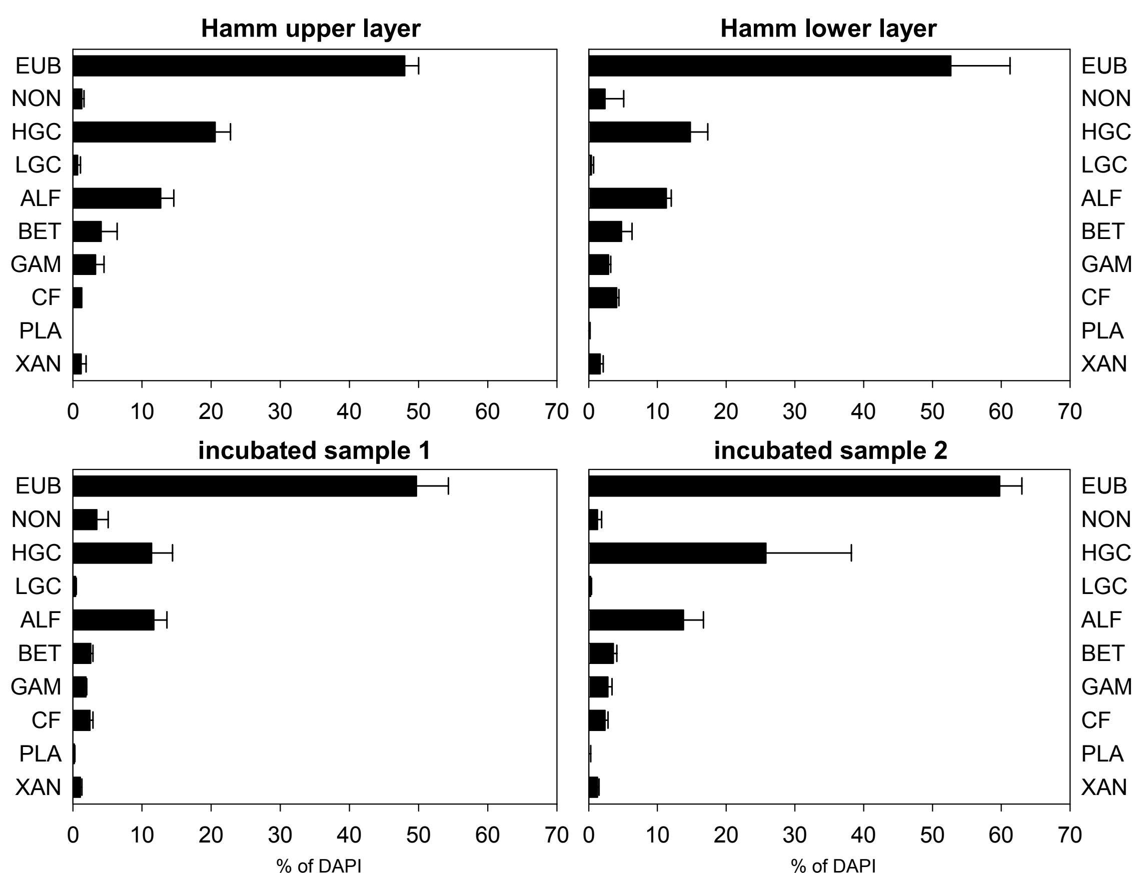

Fig. 16. Probe-specific counts relative to direct enumeration with DAPI (percentages) in 2001. Percentages given are corrected for the negative control NON338. Error bars indicate standard deviation of at least two replicates. Probe names are abbreviations of those given in Table 1. The incubated samples 1 and 2 were samples of the lower layer that were incubated with deuterated hexane for 24 days.

Hybridisation properties of the isolated strains were tested by hybridising them with rRNA-targeting probes. Of the tested groups all strains exhibiting hybridisation difficulties with probe EUB338 belong to the *Actinobacteria* (Table 17). Apart from group 14, *Cellulosimicrobium* sp., all groups conferring weak fluorescence or low detection rates after hybridisation with probe EUB338 also showed weak hybridisation signals and low detection rates with probe HGC69a. *Actinobacteria* have been shown to be poorly permeable for oligonucleotide probes without prior treatment (Macnaughton et al., 1994; Roller et al., 1994; Hahn et al., 1997; Schuppler et al., 1998; Davenport et al., 2000). However, applying several permeabilisation protocols to *Actinobacteria* isolated from biofilters no noticeable improvement of hybridisation efficacy was accomplished (Friedrich et al. 2003). Thus, pre-treatments were not applied in the present study. Taking the limited permeability for oligonucleotides into account, counts by FISH as described in the present study may underestimate the actual abundance of these bacteria (Fig. 15 and 16). It shall be noted that not all groups belonging to the *Actinobacteria* exhibited limited hybridisation efficacies. For

example groups 7 (*Micrococcus* sp.) and 18 (belonging to the *Microbacteriaceae*) conferred intense signals with probes EUB338 and HGC69a.
Figure 17 shows the limited portion of cells conferring probe fluorescence of the groups 5 belonging to the genus *Rhodococcus*. The probe RHO66 was designed for the *Rhodococcus* branch in the present study. However, because of the permeabilisation difficulties of this group the probe was not further optimised and thus not enumerated in the biofilter samples. In contrast, probe SPH120 hybridised with the majority of the cells of MN18.3a affiliated to *Sphingomonas* and was also detectable in biofilter samples (Fig. 17). The probe NEV817 only worked in combination with helper oligonucleotides.

Table 17. Hybridisation properties of representative group strains. The rRNA-targeting probes applied are described in Table 1.

Group	n[a]	EUB338	NON338	ALF968	BET42a	GAM42a	CF319a	HGC69a	LGC354	Other
1	2	+	–	–	+	–	–	–	–	
3	2	+	–	+	–	–	–	–	–	+[b]
4	1	+	–	–	–	–	+	–	–	
5	4	(+)	–	–	–	–	–	(+)	–	(+)[c]
7	1	++	–	–	–	–	–	++	–	
8	1	+	–	–	–	–	–	–	–	
9	1	+	–	–	–	–	–	(+)	–	
10	2	++	–	++	–	–	–	–	–	
11	1	++	–	–	++	–	–	–	–	
12	1	+	–	–	+	–	–	–	–	
14	1	(+)	–	–	–	–	–	+	–	
16	4	(+)	–	–	–	–	–	(+)	–	
17	3	++	–	–	+	–	–	–	–	
18	2	++	–	–	–	–	–	++	–	
19	1	+	–	–	+	–	–	–	–	
20	3	++[e]	–	++[e]	–	–	–	–	–	
21	2	++	–	–	–	–	–	–	–	+[d]
22	1	+	–	–	–	[+][f]	–	–	–	
23	1	++	–	++	–	–	–	–	–	
25	1	(+)	–	–	–	–	–	(+)	–	
27	2	(+)	–	–	–	–	–	(+)	–	
28	1	(+)	–	–	–	–	–	(+)	–	
30	1	++	–	–	+	–	–	–	–	

[a] Number of strains tested.
Probes used were [b] SPH120, [c] RHO66, [d] NEV817 applied with two helper oligonucleotides, as described previously (Friedrich et al., 2003).
[e] One strain only +
[f] Conferred fluorescence was weak
– no signal, (+) only a few cells positive, + majority of cells fluorescent, ++ all cells fluorescent brightly

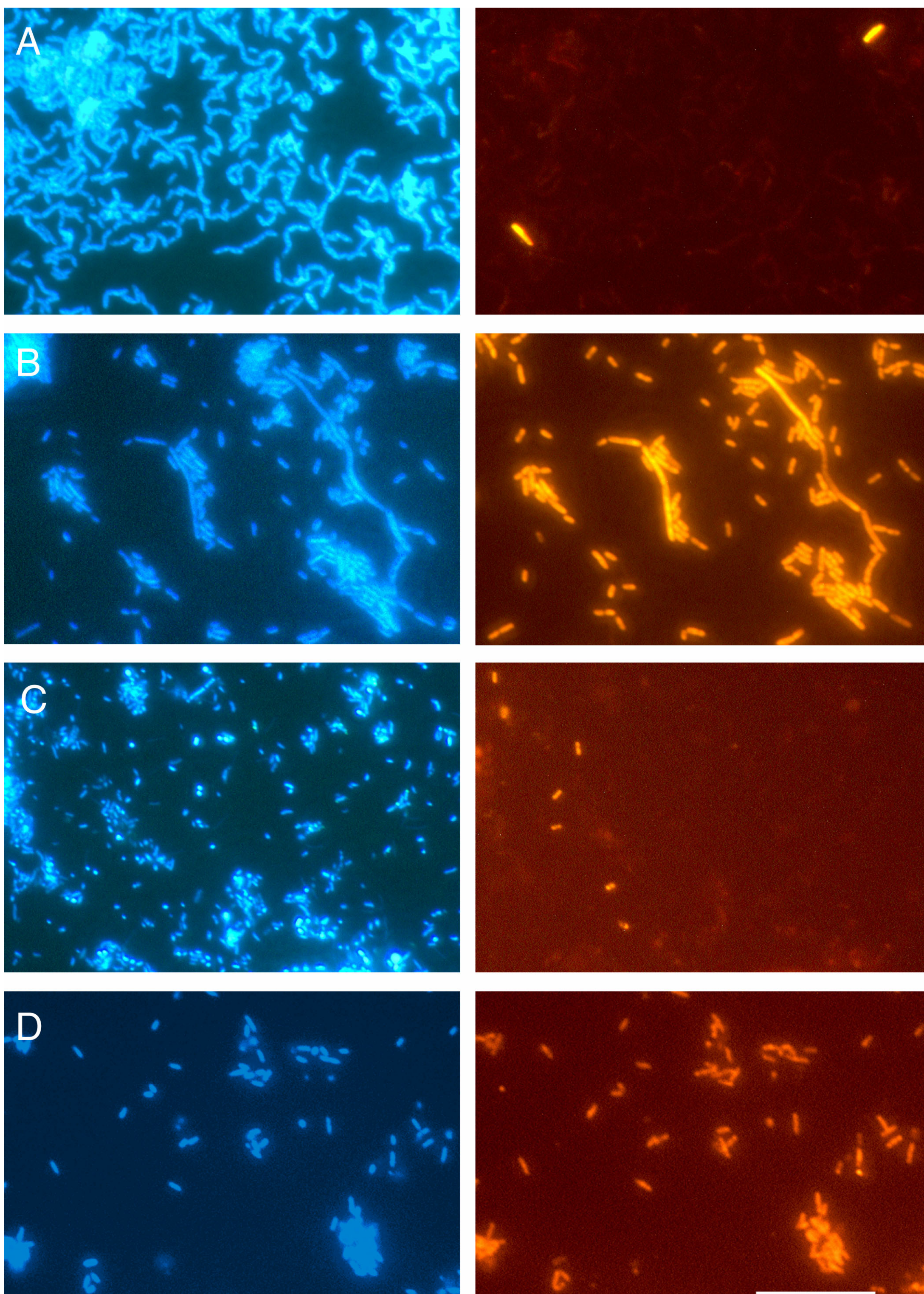

Figure 17. Microscopic photographs of fixed pure cultures (A, B, and D) and of biofilter packing material (C). The left panel shows images obtained after DAPI-staining of samples and the right panels shows the same field of views with CY3-confered fluorescence by probe-labelled cells. Applied probes: (A) RHO66, (B and C) SPH120, and (D) NEV817. NEV817 was applied with helper probes. A: *Rhodococcus* sp. MN38 (group 5); B: *Sphingomonas* sp. MN18.3a (group 3); C: SPH120 positive cells in the Alberta biofilter sample; D: deep-branching *Gammaproteobacterium* MN154.3 (group 21) . Bar represents 10 µm for all images.

For the three dominating taxonomic groups (*Actinobacteria, Alpha-,* and *Betaproteobacteria*), indicated by FISH counts, several groups were isolated from the samples of the Hamm biofilter. No strains isolated from the Hamm biofilter were assigned to the *Cytophaga-Flavobacterium* cluster and to the *Planctomycetes*. However, the later group was also by FISH only of minor importance (< 0.4% of DAPI). Also from the Alberta samples representative strains of the three dominating taxonomic groups (*Actinobacteria, Alphaproteobacteria*, and *Firmicutes*) were isolated (Table 18). In both of the two biofilters representatives of several bacterial taxa were isolated. Hence, the comprehensive isolation procedure of the present study revealed a high bacterial diversity.

Table 18. Taxonomic affiliation of strains and FISH analysis of pre-enrichment isolation samples

Detected taxon	Hamm biofilter		Alberta biofilter	
	Percentage by FISH[a]	Affiliated groups[b]	Percentage by FISH	Affiliated groups
Actinobacteria	4.5	9, 16, 18, 25, 27, 28	37.5	2, 5, 7, 14, 24
Firmicutes	0.4	8	4.6	29
Alphaproteobacteria	8.3	10, 20, 23	9.0	3, 6, 13, 15
Betaproteobacteria	6.1	12, 17, 19, 30, 31	3.1	1, 11
Gammaproteobacteria	1.8		1.8	
Deep branching Gamma-proteobacteria		21, 22		
Xanthomonas branch	1.5		2.0	
Cytophaga-Flavobacterium cluster	1.3		2.1	4
Planctomycetes	0.1		0.2	

[a] Mean percentages of the two sampling sites of the Hamm biofilter.
[b] Groups of strains affiliated with the respective taxon.

ISOTOPE-BASED LABELLING OF HEXANE DEGRADERS

Deuterated hexane ([$^2H_{14}$] hexane) was used as a tracer in combination with phospholipid fatty acid (PLFA) analysis for the characterisation of the hexane-degrading microbial community of the Hamm biofilter. PLFA profiles were determined for the samples of the upper and lower layers of the biofilter after 9 and 15 and after 9 and 24 days of incubation with deuterated hexane, respectively. To monitor a potential bias caused by deuterated hexane, one sub-sample of the upper and two sub-samples of the lower biofilter packing material were incubated with unmodified hexane for 15 and 24 days, respectively.

Phospholipid fatty acid profiles of the upper and lower layers of the biofilter

The PLFA profiles of both layers of the biofilter, which is operated with an upward waste gas flow, were dominated by 16:0 iso, 16:0, 18:1 *cis*9, 18:0 10 methyl and 19:0 cyclo11-12 (Fig. 18 and 19). The fatty acids 16:0 iso and 18:1 *cis*9 were more abundant in the lower biofilter samples than in the upper ones, each contributing about 15% to the total fatty acid profile in the lower and only about 10% in the upper biofilter layers. The four most abundant fatty acids of the lower layer were 16:0 iso, 16:0, 18:1 *cis*9, and 18:0 10 methyl. In the upper biofilter layer 19:0 cyclo11-12 contributed about 10% and was more abundant than 16:0 iso. 17:0 iso contributed more to the PLFA profile in the upper than in the lower biofilter samples. 17:0 10 methyl and 18:2 *cis, cis*9,11 were more abundant in the lower biofilter samples (Fig. 18 and 19). Generally, the fatty acid profiles of the upper and lower samples were similar.

In situ [$^2H_{14}$] hexane labelling of hexane degrading microbiota

In both biofilter layers 7 of the more than 30 fatty acids of the PLFA profiles were labelled (Fig. 18 and 19). The marked fatty acids covered a wide variety of fatty acids such as saturated fatty acids (16:0, 18:0), monounsaturated fatty acids (16:1 *cis*10, 18:1 *cis*9), iso-branched fatty acids (16:0 iso), 10-methyl fatty acids (18:0 10 methyl), and cyclic fatty acids (19:0 cyclo11-12).

All fatty acids accounting for 5% or more of the total PLFA profile in the samples of the lower layer of the biofilter were labelled after incubation with deuterated hexane (Fig. 19). Interestingly, the fatty acids labelled in the samples of the lower biofilter layer were the same as those of the upper layer (Fig. 18 and 19). In the upper layer of the biofilter all but one (17:0 iso) of the fatty acids, which represented at least 5% of the total fatty acid profile were labelled (Fig. 18). In both layers none of the odd-numbered carbon chain iso/anteiso fatty acids were labelled. The unlabelled fatty acid 17:0 iso was less abundant in the lower

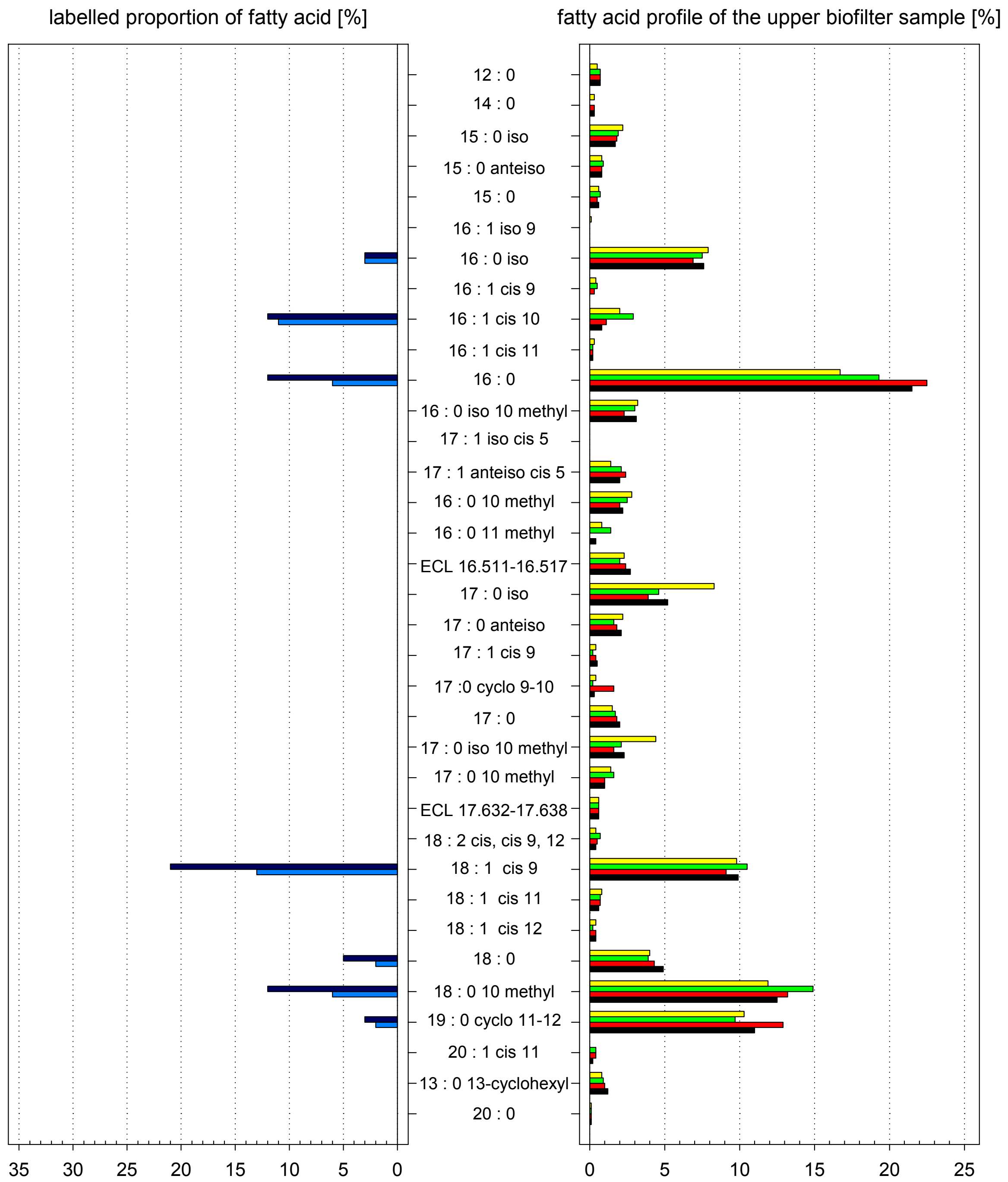

Figure 18. PLFA profile of the upper biofilter sample, and after 9 and 15 days of incubation with $[^2H_{14}]$ hexane and after 15 days with hexane. The labelled proportion of the fatty acid after 9 and after 15 days of incubation.

biofilter layer samples than in the upper ones. For 16:0 iso it was the opposite way and the degree of labelling increased from 3% after 9 days to about 10% after 24 days in contrast to

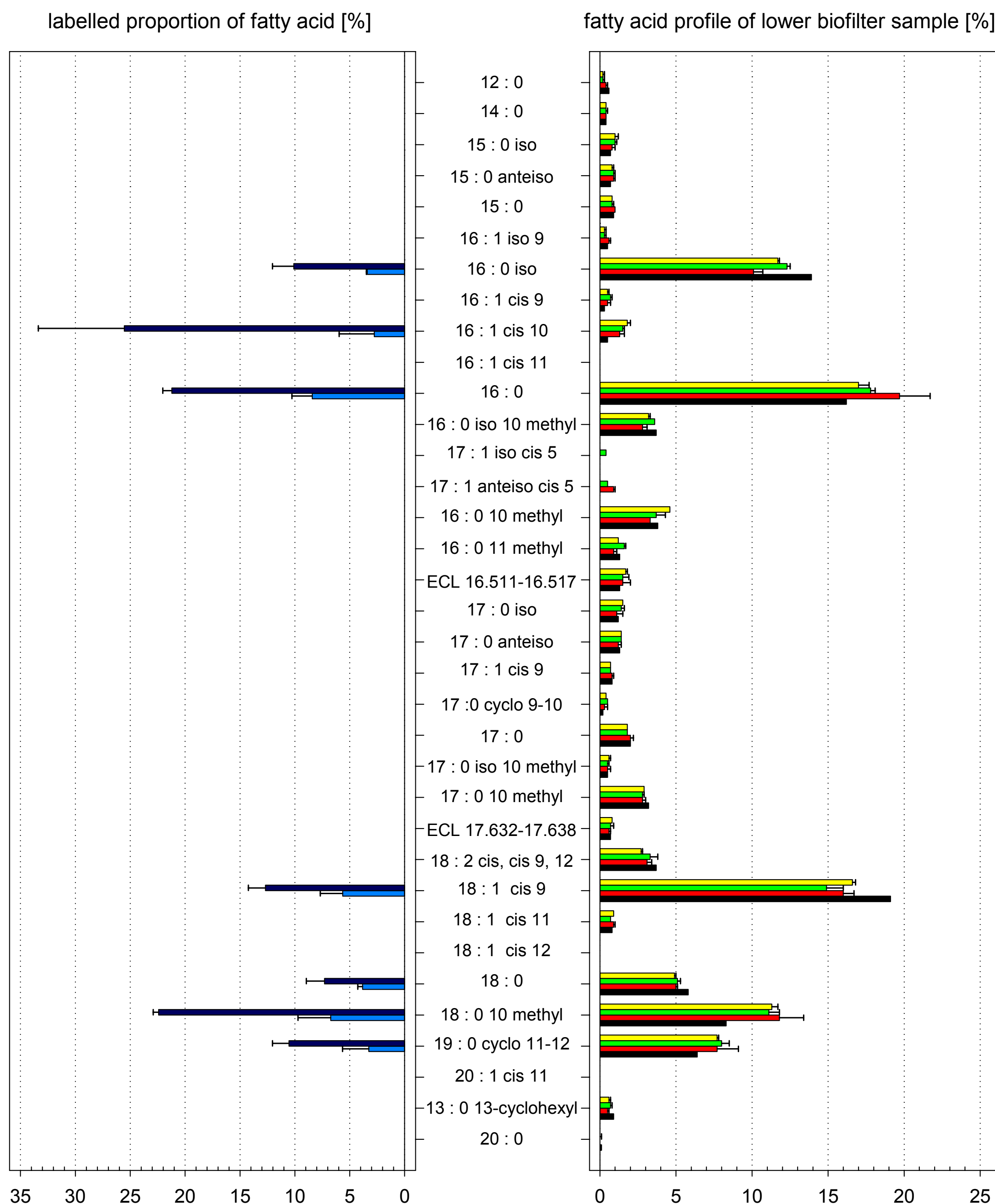

Figure 19. PLFA profile of the sample derived from the lower biofilter layer and the mean PLFA profile of two sub-samples each after 9 and 24 days of incubation with [$^2H_{14}$] hexane and after 24 days with hexane. The mean labelled proportion of the fatty acid of both sub-samples after 9 and after 24 days of incubation. Error bars indicate the standard deviation of two sub-samples.

the upper biofilter sample where it remained at 3% from day 9 to 15 (Fig. 18 and 19). The later is the only example of the degree of labelling of a deuterated fatty acid not increasing

with prolonged incubation time. The labelled fraction of all deuterated fatty acids varied from about 1 to 31% (Fig. 18 and 19). Already after 9 days, labelled portions of about 13% were reached for 18:1 *cis*9 in the upper biofilter sample (Fig. 18). In the upper layer material of the biofilter 18:1 *cis*9 formed the most strongly labelled fraction, attaining 21% after 15 days of incubation. After the same incubation time the three fatty acids 16:0, 16:1 *cis*10, and 18:0 10 methyl attained labelling ratios of 12%. Minor amounts of deuterated fatty acids were found for 18:0 (5%), 16:0 iso (3%), and 19:0 cyclo 11-12 (2%) after 15 days (Fig. 18). Three of the most strongly labelled fatty acids also belonged to the five most abundant fatty acids.

In the lower biofilter samples, which were incubated longer, 16:0 and 18:0 10 methyl also belonged to the most strongly labelled fatty acids with labelling ratios of more than 21%. The fatty acid 16:1 *cis*10 was also strongly labelled contributing relatively little to the total PLFA profile (Fig. 19). The fatty acids 16:0 iso, 18:1 *cis*9, and 19:0 cyclo 11-12 all reached at least 9%-labelling after 24 days of incubation. The fatty acid 18:0 showed the weakest labelling among the deuterated fatty acids of the lower biofilter samples with an average of 7% after 24 days.

Relationships between in situ [$^2H_{14}$] hexane labelling and biofilter isolates

The fatty acid profiles of the labelling experiment using biofilter samples were compared with those of the isolated hexane degrading microorganisms from Hamm (Table 19). The fatty acid profile of group 16 representing a numerously isolated group of both Hamm biofilter isolation sites includes all fatty acids labelled *in situ* from the biofilter except for 16:0 iso and 19:0 cyclo 11-12. The groups 27 and 28 contained the same five of the seven labelled fatty acids. These three groups represented the only groups that contained 16:1 *cis*10 and the diagnostic compound tuberculostearic acid (18:0 10 methyl) in their fatty acid profiles (Table 19). Additionally, these three groups were the only groups apart from group 21 (which averaged less than 1% of 18:1 *cis*9) which contained 18:1 *cis*9 in their FAME profile. In contrast, 19:0 cyclo is a fatty acid more common among the *Proteobacteria* and it contributed to all fatty acid profiles of the 10 groups from Hamm belonging to the *Alpha-, Beta-* and deep-branching *Gammaproteobacteria* (Table 19). This fatty acid occurred in large amounts within the groups 10 (up to 47%), 17 (up to 25%), and 21 (up to 28%) (Tables 7 and 8). Interestingly, the fatty acid 18:1 cis11, which usually precedes 19:0 cyclo 11-12, was of minor importance in the biofilter, not reaching more than 1% in the biofilter or any of the incubation samples. In all isolated proteobacterial groups 18:1 *cis*11 contributed substantially to the fatty acid profiles with an average of 32% among all groups (group means ranging from 8% to 89%). The fatty acid 18:0 occurred in proteo- and actinobacterial isolates and was present in 12 of the isolated groups, however, only in

two groups (16 and 31) it accounted for more than 5% (Table 19). However, it was shown in our lab that e.g. the experimental biofilter group 5, which usually contained less than 1.5% of 18:0, reached a proportion of up to 5% when grown on hexane. 16:0 was present in the eukaryotic isolate and all but one of the 17 bacterial isolates from Hamm (Table 19) and thus cannot be used to identify the dominant groups in the biofilter. 16:0 iso was present only in 5 groups and abundant only in groups 22 and 8 reaching up to 14 and 15%, respectively, and averaging 10% in group 18 (Tables 7, 11, and 19). Of the three fatty acids of the eukaryotic strain (group 26) two were labelled, but the signature fatty acid, 18:2 *cis, cis*9,12 for this group was not labelled and scarce, especially in the upper layer of the biofilter (Table 19 and Fig. 18).

Thus, each labelled fatty acid was present in at least three of the isolated groups from the industrial biofilter. Among the unlabelled fatty acids were 7 identified fatty acids, which could not be assigned to any of the groups isolated from this biofilter. Of two bacterial groups all fatty acids were present in the biofilter profile, but the major fatty acids of these groups (groups 23 and 25, see tables 4, 10, and 19) were not dominating in the fatty acid profile of the biofilter. When considering fatty acids that averaged more than 1% within the individual groups and when accounting for the fatty acids which are excluded in the PLFAs the groups 12, 17, 18, 19, 21, 30, and 31 had all their fatty acids represented in the biofilter profile. The groups 8, 9, 10, 16, 20, 22, 27, and 28 exhibited 1 or 2 fatty acids in the lab which were not among the fatty acids in the biofilter. But generally these fatty acids did not average more than 5% within the groups. The only exception among the actinobacterial groups was group 8, which averaged 20% of 14:0 iso. Within the proteobacterial groups only group 20 exhibited a PLFA absent in the biofilter which accounted for more than 5%; 18:1 *cis*11 11methyl averaged 16%. But because on nutrient broth 18:1 *cis*11 11methyl only accounted for 3.6% of the FAME in group 20, the absence of 18:1 *cis*11 11methyl in the PLFA of the biofilter should not be overrated. Due to the major waste gas component being hexane in the Hamm biofilter (Nolte, 1994) the focus should lie on the labelling experiment with deuterated hexane. Thus, at least one actinobacterial group containing 18:0 10methyl and 16:1 *cis*10 and at least one proteobacterial group exhibiting 19:0 cyclo seem to play major roles in hexane degradation in this biofilter. Additionally, one group containing 16:0 iso was labelled. Of the Hamm isolates four actinobacterial groups lacking 18:0 10methyl and 16:1 *cis*10 and one deep-branching gammaproteobacterial group exhibited 16:0 iso. Thus, based on the isolated groups at least 3 taxonomical groups dominated the Hamm biofilter in 2001.

Table 19. Presence of fatty acids of the labelling experiment with [$^{2}H_{14}$] hexane among the isolated groups.

Fatty acid	Presence of labelled and unlabelled fatty acids of the biofilter in groups:																	
	8	**9**	**10**	**12**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**25**	**26**	**27**	**28**	**30**	**31**
12:0	–	–	–	tr	(+)	+	–	–	–	+	++	–	–	–	–	+	+	+
14:0	(+)	(+)	(+)	+	+	(+)	(+)	+	(+)	(+)	++	–	–	–	++	++	(+)	(+)
15:0 iso	++	++	–	–	–	–	++	–	–	–	–	–	+	–	–	–	–	–
15:0 anteiso	++	++	–	–	–	–	++	–	–	–	–	–	++	–	–	–	–	–
15:0	–	–	(+)	tr	(+)	–	(+)	–	–	–	(+)	–	–	–	++	(+)	+	–
16:1 iso 9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16:0 iso	++	++	–	–	–	–	++	–	–	–	++	–	+	–	–	–	–	–
16:1 *cis*9	–	–	+	++	–	+	–	++	–	+	+	–	–	–	+	+	++	++
16:1 *cis*10	–	–	–	–	++	–	–	–	–	–	–	–	–	–	++	++	–	–
16:1 *cis*11	–	–	–	(+)	–	–	–	(+)	–	–	++	–	–	–	–	–	tr	–
16:0	(+)	+	++	++	++	++	++	++	++	++	++	+	–	++	++	++	++	+
16:0 iso 10methyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17:1 iso *cis*5	(+)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17:1 anteiso *cis*5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16:0 10methyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	(+)	–	–
16:0 11methyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ECL 16.511–16.517	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17:0 iso	(+)	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
17:0 anteiso	+	++	–	–	–	–	++	–	–	–	–	–	++	–	–	–	–	–
17:1 *cis*9	–	–	–	–	(+)	–	–	–	–	–	–	–	–	–	+	+	–	–
17:0 cyclo 9–10	–	–	+	++	–	++	–	++	–	+	–	–	–	–	+	–	++	++
17:0	–	–	(+)	(+)	(+)	–	–	(+)	–	–	–	+	–	–	+	(+)	(+)	+
17:0 iso 10methyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17:0 10methyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	tr	–	–
ECL 17.632–17.638	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18:2 *cis,cis*9,12	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–
18:1 *cis*9	–	–	–	–	++	–	–	–	–	(+)	–	–	–	++	++	++	–	–
18:1 *cis*11	–	–	++	++	–	++	–	++	++	++	++	++	–	–	–	–	++	++
18:1 *cis*12	–	–	(+)	(+)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18:0	–	–	(+)	(+)	++	(+)	–	(+)	tr	+	–	+	–	–	(+)	(+)	(+)	++
18:0 10methyl	–	–	–	–	++	–	–	–	–	–	–	–	–	–	++	+	–	–
19:0 cyclo11–12	–	–	++	(+)	–	++	–	++	++	++	(+)	++	–	–	–	–	(+)	(+)
20:1 *cis*11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13:0 13–cyclohexyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20:0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(+)	–	–

– fatty acid not present in the majority of the group strains; majority of the strains had: tr less than 0.1%, (+) 0.1-0.9%, + 0.91-5.0%, ++ more than 5%

IV. Discussion

Biofiltration is a frequently used and efficient biotechnological method for waste gas abatement. Gaseous pollutants are degraded as they pass through a packed bed reactor containing microorganisms growing on the reactor bed material. The degradation of hexane vapours by biofiltration was shown to be variable and often poor (Johnson and Deshusses, 1996; Morgenroth et al., 1996). Enhancement of biofiltration can be achieved with the inoculation of specific microorganisms, but generally the microbial communities evolving from the indigenous microorganisms found in the packing material are sufficient (Ottengraf and Diks, 1992; Coleman and Dombroski, 1995). Little is known about the microbial ecology of biofilter systems used for hexane elimination. Thus an improved understanding of the microbial communities capable of degrading hexane will be important for straightforward optimisation of biofilter performance by adjustment of operating parameters. This study focuses on the characterisation of the microorganisms isolated from the biomass of a laboratory- and a full-scale biofilter used for hexane abatement. Additionally, the microbial communities of the biofilters were studied *in situ* using whole cell FISH and PLFA analysis to complement the cultivation-dependent approach. The degradation of a hydrophobic and generally toxic compound such as hexane is of general microbiological interest.

TAXONOMIC AFFILIATION AND IDENTIFICATION OF THE ISOLATES

During this study a comprehensive isolation approach resulted in the cultivation of 278 strains that were assigned to one eukaryotic and 30 bacterial groups. Strains were allocated to clusters based on fatty acid profiles. Some clusters were further subdivided into groups because of differences in chemotaxonomic properties and certain fatty acids. Members of the isolated groups were assigned to taxonomic groups on the basis of their 16S rDNA sequences, physiological reaction profiles, and chemotaxonomic properties.
The isolated prokaryotic strains are members of several taxa of the *Bacteria*, being members of the *Alpha-, Beta-,* and *Gammaproteobacteria,* as well as the *Flavobacteria*, *Actinobacteria*, and *Firmicutes*.

Alphaproteobacteria

Comparisons of 16S rDNA gene sequences demonstrated that MN18.3a (group 3) and MN122.2a (group 20) cluster phylogenetically with species of the genus *Sphingomonas sensu stricto*. The fatty acid profiles of both groups were dominated by 16:0 and 18:1 *cis*11, but the contribution of hydroxylated fatty acids was less in both groups than for other species

of the genus *Sphingomonas* (Kämpfer et al., 1997; Takeuchi et al., 2001). A comparative study of GC-MS and GG-FID showed a potential underestimation of hydroxy fatty acids when using GC-MS in our lab. Thus, the lower abundance of hydroxy fatty acids in our *Sphingomonas* groups could be due to methological differences. In contrast to most other *Sphingomonas* species was the occurrence of 19:0 cyclo and especially of 18:1 *cis*11 11methyl, which contributed significantly (sum: 22-30%) in group 20 and partly (sum: 7-15%) in *Caulobacter leidyi,* also clustering with *Sphingomonas sensu stricto*. In a cross-journal examination only one species, *Sphingomonas aurantiaca* (Busse et al., 2003), exhibited 18:1 *cis*11 11methyl. Only three species belonging to *Sphingomonas sensu stricto* contained 19:0 cyclo, *S. wittichii* (Yabuuchi et al., 2001), *S. faeni* and *S. aurantiaca* (Busse et al., 2003). When grown on trypticase soy broth agar like group 3 *S. wittichii* reached the highest recorded 19:0 cyclo content with 5% (Busse et al., 1999). 16S rDNA analysis indicated that strain MN122.2a is most closely related to *Caulobacter leidyi* (97.0%)*,* which has a polar stalk and is morphologically distinct from MN122.2a and the other members of group 20. The species of the genus *Caulobacter* have been shown previously to be polyphyletic (Sly et al., 1999). *Caulobacter leidyi,* which was isolated from the hindgut of a millipede, belongs to the alpha-4 subclass of the *Proteobacteria* and is not related to the other species of the genus *Caulobacter* (Sly et al., 1999). In group 3 18:1 *cis*11 11methyl and 19:0 cyclo contributed in sum 1-5%. Based on 16S rDNA MN18.3a was most closely related to *Sphingomonas asaccharolytica* (97.7%). *S. asaccharolytica* exhibited no 18:1 *cis*11 11methyl and 19:0 cyclo when grown on trypticase soy broth agar. Additionally, it exhibited no 17:1 *cis*11, which together with 18:1 *cis*11 11methyl, was always present in all strains belonging to group 3 (Table 4). Apart from the 16S rDNA dissimilarities, also these differences of the fatty acid profiles found for *Sphingomonas asaccharolytica* and group 3 does not justify an assignment of group 3 to *S. asaccharolytica*. Individual cell sizes were occasionally slightly larger (group 20) and frequently larger (group 3) than given in the emended description of the genus *Sphingomonas* (Takeuchi et al., 2001). However, *S. pituitosa* also exhibits cells of 3 µm in length, which is more than a third longer than in those genus descriptions (Denner et al., 2001; Takeuchi et al., 2001). In 2003 another slightly emended description of the genus *Sphingomonas sensu stricto* was proposed by Busse and others. Cell length is now described to reach 2.7µm (Busse et al., 2003), thus group 20 would now fit into the new genus description but group 3 cells are still larger. Generally, features of groups 3 and 20 were in accordance with the genus description of *Sphingomonas sensu stricto* (Takeuchi et al., 2001; Busse et al., 2003). On the basis of 16S rDNA dissimilarities and differences in the fatty acid profiles of these two groups to their closest relatives the description of two new species within the genus *Sphingomonas* are in preparation. The suggested species are *Sphingomonas alkanivorans* sp. nov. (alkanum saturated aliphatic hydrocarbon, L. v. vorare to eat; L. adj. alkanivorans alkane-devouring),

type strain being DSM 14803^T, LMG 22841^T, MN122.2a^T (group 20) and *Sphingomonas purgata* (L. v. purgare to clean, because it has the ability to degrade alkanes), type strain being DSM15717^T, LMG 22843^T, MN18.3a^T (group 3).

Sequence similarities between the 16S rDNA gene sequences are 99.4% and 99.8% between *S. trueperi* and *S. pituitosa* (Denner et al., 2001) and *S. mali* and *S. pruni*, respectively. In contrast, the 16S rDNA similarities were merely up to 97.7% for groups 3 and 20 with their most closely related species of the genus *Sphingomonas*. Hence, groups 3 and 20 are phylogenetically distinct members of the genus showing larger 16S rDNA dissimilarities than the previously described species.

A detailed comparison was carried out using the biochemical results of groups 3 and 20 and all previously published biochemical data concerning the genus *Sphingomonas sensu stricto* (in particular Kämpfer et al., 1997; Denner et al., 2001; Buonaurio et al., 2002). Seventeen species assigned to the emended genus *Sphingomonas* have previously been tested using API 20 NE strips (Buonaurio et al., 2002; Yabuuchi et al., 2002; Li et al., 2004; Rivas et al., 2004). In agreement with all tested species of the genus *Sphingomonas* (including *S. asaccharolytica*), the five tested members of group 3 and strain MN122.2a, representing group 20, exhibited negative reactions for indole production, glucose fermentation, arginine dihydrolase, urease, and D-mannitol assimilation. All formerly tested *Sphingomonas* strains assimilated arabinose and glucose using the API 20 NE system. *Sphingomonas asaccharolytica* reacted positively concerning ß-glucosidase (esculin hydrolysis) and β-galactosidase activity. All strains of group 3 displayed esculin hydrolysis, but only one strain was β-galactosidase-positive when tested in API 20 NE system. Additionally, only two strains of group 3 assimilated glucose and only one strain assimilated arabinose. Thus the reaction profiles of the strains belonging to group 3 were not in agreement with that of *S. asaccharolytica*, the closest species affiliated with group 3 on the basis of 16S rDNA. In contrast to all tested *Sphingomonas* species strain MN122.2a did not exhibit β-glucosidase activity in the API 20 NE test system but it assimilated esculin in the API 50 carbohydrate substrate strips. The general problem concerning test results of members of group 20 has already been discussed (page 52). In accordance with 16 from 17 tested *Sphingomonas* type strains strain MN122.2a exhibited β-galactosidase activity, but in contrast to 16 *Sphingomonas* type strains it displayed no N-acetyl-D-glucosamine assimilation (Table 13). The biochemical characteristics of 11 *Sphingomonas* species (Denner et al., 2001) were compared to members of group 3 and group 20. The reaction profile of *S. adhaesiva* exhibited the same biochemical characteristic as the majority of strains of group 3 in 21 of 27 biochemical tests. In contrast *S. asaccharolytica* reacted in the same way as the majority of the strains of group 3 only in 16 out of 27 biochemical tests. The type strain of group 20, MN122.2a, showed best agreement of reaction profiles with *S. echinoides* and *S. paucimobilis*, with 22 and 19 identical reactions out of 27, respectively. However, this

reaction concurrence was based on shared negative results except for the presence of β-galactosidase activity.
Group 13 showed a large resemblance to group 3 and no contradiction to the genus description of *Sphingomonas* and thus seems to represent a member of the *Sphingomonadaceae* (Table 16).
MN51a (group 10) was phylogenetically related to *Bosea thiooxidans* (99.0% 16S rDNA similarity). Also the fatty acid profile of group 10 was in accordance to that of *Bosea thiooxidans* as described previously (Das et al., 1996; Ouattara et al., 2003) the only difference being the lack of 17:1 *cis*9, which contributes up to 3.6% to the fatty acid profile of *Bosea thiooxidans*. *Bosea thiooxidans* forms colonies on agar containing thiosulfate and succinate or yeast extract, which are smooth, mucoid, round and cream coloured, and about 1 to 1.5 mm in diameter. (Das et al., 1996). The colonies of group 10 are flat, round, whitish, and shiny and 0.2 – 1.7 mm in diameter. *Bosea thiooxidans* cells occur singly and are straight rods that are 0.85 µm wide by 1.4 to1.6 µm long. Cells of group 10 are larger and exhibit sharper ends than normal rods. Whereas the description of the colonies and single cells of the genus and species *Bosea thiooxidans* varied from the own observations, the deviations, such as mucus production, could be a consequence of different growth parameters. The only other member belonging to the genus *Bosea* that has been described only recently, *Bosea minatitlanensis*, has a similar fatty acid profile to *B. thiooxidans* (Das et al., 1996; Ouattara et al., 2003). FAME pattern similarities are stronger between *B. minatitlanensis* and *B. thiooxidans* than between either one and group 10. In spite of the low differences in their FAME profiles and the high 16S rDNA sequence similarity (98.9%) *B. minatitlanensis* forms a new species with a DNA-DNA hybridisation similarity of 44.2% with *B. thiooxidans* (Ouattara et al., 2003). Although the similarity of group 10 with *B. thiooxidans* is slightly higher than that between the two described *Bosea* species, a DNA-DNA hybridisation may be necessary to justify or deny the assignment of group 10 to *B. thiooxidans*. Nevertheless, as there are no contradictions group 10 is identified as *Bosea thiooxidans,* based on 16S rDNA.
On the basis of the 16S rDNA sequences group 23 was identified as *Xanthobacter flavus* and the appearance of the colonies and the fatty acid profile are also in agreement with the description of the genus *Xanthobacter* (Wiegel, 1992; Urakami et al., 1995; Padden et al., 1997). Apart from group 10 and group 23 also group 15 was placed within the family "*Methylobacteriaceae*". Based on morphological traits, for example the punctiform pink colonies with nondiffusible pigmentation, growth observations such as the surface ring when grown in liquid medium and poor growth on nutrient agar, and especially on fatty acid profiles e.g. the high (>87%) contribution of 18:1 *cis*11 and the presence of 14:0 3OH and 16:1 *cis*9 (Table 16), group 15 best matches with the genus *Methylobacterium*.

Betaproteobacteria

The strain MN36.2 of group 1 and strain MN45.2b.1 of group 30 shared high 16S rDNA sequence similarities with *Variovorax paradoxus* IAM12373 (each 99.4%). For many strains of *Variovorax paradoxus*, two stable colony types have been isolated: One type is round and convex with smooth margins and surfaces; the other type is round and umbonate with scalloped to smooth margins (Willems et al., 1991). Thus, the appearance of two morphotypes in group 1 conforms to former observations. The fatty acid profile of group 30 is also in agreement with the fatty acid profile of *Variovorax paradoxus* being the only described species of the genus. Group 1 also appears to be a member of the genus *Variovorax* with four distinct peaks in the fatty acid profile that separates this group from group 30 and the type strain of *Variovorax paradoxus* (Table 5). In agreement with the species description of *Variovorax paradoxus* both groups were catalase and oxidase positive, the rod-shaped cells stained Gram-negative, and ubiquinone-8 was exhibited by group 1. Group 1 and 30 differed concerning growth on N-acetyl-D-galactosamine and N-acetyl-D-glucosamine (both, in the BIOLOG and API systems): None of the tested strains of group 1 could grow on these carbon sources but both tested strains of group 30 did show growth (Table 13). Additionally, both strains of group 30 could grow on citrate in the API test system but none of the group 1 strains. The growth of *Variovorax* on citrate has been used to differentiate *Variovorax* from *Hydrogenophaga* (Willems et al., 1991), but for group 1 and 30 it is not a discriminatory trait because in the BIOLOG system only 50 to 88% of the tested strains of group 30 and 1, respectively, grew on citrate. Nevertheless, due to no additional traits differing from *Variovorax* group 1 and group 30 are assigned to the single-species genus *Variovorax* based on fatty acids, 16S rDNA and the mentioned other phenotypic traits.

Strain MN33.2 (single strain of group 11) shared 99.1% 16S rDNA sequence similarity with the type strain of *Acidovorax delafieldii*. The fatty acids of MN33.2 are also in the range of the fatty acid profiles reported for the genus *Acidovorax* (Table 6), and they correspond well with the fatty acid profile of *Acidovorax delafieldii* and *A. facilis*, which share nearly identical fatty acid profiles (Schloe et al., 2000). Also, the fatty acid profile of *A. delafieldii* described by Gardan et al. (2000) was nearly identical to that of MN33.2 with the two main differences being the slightly lower abundance of hydroxydecanoic acid in MN33.2 and the lack of 17:0 cyclo in *A. delafieldii*. In 2003 Gardan et al. did detect traces of 17:0 cyclo in *A. delafieldii*. Thus, the differences in fatty acid percentages of MN33.2 and *A. delafieldii* as well as *A. facilis,* obtained by Gardan et al. (2003), seem no larger than in-between-lab variations. Both species are very similar phenotypically, on the basis of cellular fatty acids and total protein profiles in spite of low DNA-DNA-homologies (Willems et al., 1990). One of four reactions to differentiate these two species is the growth on adipate (Willems et al.,

1990). MN33.2 did not grow on adipate (Table 13) and can thus be differentiated from *A. delafieldii*. Additionally, MN33.2 showed no growth on histidine and phyenylalanine which is in contrast to growth results of 14 strains of *A. delafieldii* (Schulze et al., 1999). Strain MN33.2 was gelatinase negative whereas the majority of strains of *A. delafieldii* and *A. facilis* are gelatinase positive. Thus, based on these phenotypic characteristics MN33.2 cannot be assigned to *Acidovorax delafieldii*, but in accordance with the 16S rDNA data and no contradicting phenotypic traits it is affiliated with the genus *Acidovorax*.
Strain MN182.2 of group 17 exhibited a highly (99.9%) similar 16S rDNA sequence to *Pandoraea pnomenusa* LMG18087^T (Fig. 16). Using fatty acid analysis, members of the genus *Pandoraea* can easily be distinguished from both *Burkholderia* and *Ralstonia* (now separated into *Ralstonia* and *Cupriavidus,* Vandamme and Coenye, 2004), species by the absence of 14:0 (Vandamme et al., 1997; Coenye et al., 2000). The fatty acid 14:0 was only found in traces (<0.7%) in the strains of group 17 and thus, supported the assignment of group 17 to the genus *Pandoraea*. Furthermore, the absence of the fatty acid 12:0 in group 19 and its presence in group 17 are in agreement with the differentiation characteristics for the two genera *Burkholderia* and *Pandoraea*. The fatty acid 12:0 has not been detected in *Burkholderia* species (Vandamme et al., 1997; Coenye et al., 2000). The 16S rDNA sequence of strain MN101.b of group 19 was highly similar to that of *Burkholderia multivorans* LMG13010^T (99.3%). Additionally, other phenotypic characteristics of group 19 were in agreement with those of the genus *Burkholderia* (Coenye et al., 2000; Coenye et al., 2001; Coenye et al., 2001). Thus, group 19 can be assigned to the genus *Burkholderia,* of which members have already been shown to degrade medium-chain length alkanes (Marín et al., 2001).
The genus description of *Pandoraea* (Coenye et al., 2000) is in accordance with the description of group 17 in respect to biochemical reaction properties, such as the absence of indole production and the lack of β-galactosidase activity, or morphological features like the relatively long length of cells (up to 4 µm). However, in group 17 the fatty acids 16:0 2OH and 18:1 2OH were not present in contrast to their reported presence in the genus description. The major fatty acids of *Pandoraea pnomenusa* have been described to be 16:0, 17:0 cyclo, and an unsaturated 18 chain-long fatty acid (Coenye et al., 2000). In contrast, in group 17 18:1 *cis*11 was only the forth most abundant fatty acid and 19:0 cyclo reached, on average, twice as high a percentage than 18:1 *cis*11. In contrast to *P. pnomenusa* some strains of group 17 were able to assimilate sucrose. Assimilation of D-glucose and maltose were also positive for some strains of group 17 in the BIOLOG system, but in the API system they showed no assimilation. This was in agreement with the profile of *Pandoraea pnomenusa,* which had also been obtained with the API system (Coenye et al., 2000). In contrast to *P. pnomenusa* group 17 assimilated citrate only in the BIOLOG and not in the API system. Additionally, in the species description of *Pandoraea pnomenusa,* urease

activity is present, but showed a negative reaction in all strains of group 17. The majority of *P. pnomenusa* strains have been isolated from patients with cystic fibrosis but there are also environmental strains included in this species. The two species *Pandoraea pulmonicola* and *P. pnomenusa,* which showed a 42% DNA-DNA hybridisation similarity with each other (Coenye et al., 2000), share 99.6% 16S rDNA sequence similarity. Therefore the high 16S rDNA sequence similarity of 99.9% is not sufficient evidence to identify group 17 as member of *P. pnomenusa*. Nevertheless, within this taxonomic group with high 16S rDNA sequence similarities between different species, group 17 can be assigned to the genus *Pandoraea*.

16S rDNA analysis indicated that strain MN43 (group 12) is most closely related (98.6%) to *Cupriavidus basilensis* (Fig. 14). The absence of assimilation of galactose, mannitol, mannose, and sorbitol has been used as a differential phenotypic marker to distinguish the genera *Ralstonia* and *Cupriavidus* from *Burkholderia* (Yabuuchi et al., 1995). Thus the reaction profile of group 12 was in agreement with the genera descriptions of *Ralstonia* and *Cupriavidus* (Table 13). In all 22 cases, biochemical reactions showed the same result for group 12 as for *Cupriavidus basilensis*, for which biochemical profiles have been described previously (Goris et al., 2001). In contrast to *C. campinensis* with which group 12 shares 98.5% 16S rDNA sequence similarity, group 12 exhibited no urease activity and could assimilate citrate both in the API and the BIOLOG system (Table 13). Assimilation of citrate and urease activity had been used previously to differentiate between *C. campinensis, C. basilensis, C. metallidurans* and *C. eutropha* (Goris et al., 2001). Therefore, group 12 is assigned to the genus *Cupriavidus* and DNA-DNA hybridisation of *Cupriavidus basilensis* and MN43 could clarify if MN43, representing group 12, belongs to a new species.

Even though the fatty acid profile of group 31 clustered with those of group 12, it cannot be assigned to the genus *Cupriavidus* because of the high (13.5%) abundance of 18:0, a fatty acid that is less abundant (generally <2%) in all described *Ralstonia* and *Cupriavidus* species (De Baere et al., 2001; Goris et al., 2001; Kageyama et al., 2005).

Gammaproteobacteria

Two groups (21 and 22) are members of the *Gammaproteobacteria* and both were placed within the order *Xanthomonadales*. They both affiliate with the family *Xanthomonadaceae* but could not be assigned to a certain genus because of too large differences in their 16S rDNA sequences. The closest genus within the phylogenetic tree based on 16S rDNA was *Nevskia* for both groups. Group 22 shared even less 16S rDNA similarities with *Nevskia ramosa*, the sole species of this genus, than group 21 (Fig. 14). Apart from the low sequence similarities (≤ 94.1%), groups 21 and 22 also differed strongly from *Nevskia ramosa* concerning morphological traits, for example groups 21 and 22 showed no slime formation, no presence of reserve-material globules and no stalks, even when they were incubated like

N. ramosa as described in Stürmeyer *et al*. (1998). Thus, two new genera are proposed to integrate groups 21 and 22 in the *Xanthomonadaceae*. The manuscript in preparation covers these two new genera of the deep-branching *Gammaproteobacteria*. It will include the description of *Alkanibacter difficilis* gen. nov., sp. nov. (suggested for group 21) and of *Imprimimonas variabilis* gen. nov., sp. nov. (suggested for group 22). Type strain of *Alkanibacter difficilis* gen. nov., sp. nov. (alkanum saturated aliphatic hydrocarbon, L. bacter n. rod; L. n. Alkanibacter gen. nov., because it is a rod that is capable of degrading alkanes; difficilis L. adj., because it is difficult to cultivate) is DSM 14804^T, LMG 22842^T, $MN154.3^T$. Type strain of *Imprimimonas variabilis* gen. nov., sp. nov. (L. adj. imprimis special, Gr. n. monas a unit; a special unit; variabilis, varying: because of the pigmentation variety) is $DSM15731^T$, LMG 22844^T, $MN28^T$. Group 22 exhibits a high 16:1 *cis*11 content. In the 2001 Hamm biofilter samples 16:1 *cis*11 did not reach more than 1%, but in other biofilters analysed in our lab this fatty acid contributed up to 13% (Alexandrino et al., 2001; Piehl, 2002) and was significantly labelled in isotope labelling experiments (Alexandrino et al., 2001; Knief et al., 2003). However in these studies this contribution could not be assigned to certain isolates or taxonomic groups. 16:1 *cis*11 is not commonly found in high abundances (>5% of total FAMEs) in presently known bacteria. The majority of mircoorganisms with a high content of 16:1 *cis*11 belong to the genera *Desulfobulbus, Desulforhopalus, Nitrospira, Pleurocapsa* or to the family *Methylococcaceae* (Bowman et al., 1993; Caudales et al., 2000; Lipski et al., 2001; Sass et al., 2002). The comprehensive isolation approach in the present study resulted in a new bacterial group containing 16:1 *cis*11 in significant amounts. Thus, group 22 may represent a group of organisms playing a role in several biofilters, but being difficult to cultivate and therefore overlooked in previous studies. The application of rRNA-targeting probes for members of this group to biofilters with high 16:1 *cis*11 contents may clarify its contribution.

Actinobacteria

More than one third of the bacterial groups isolated during this study belong to the class *Actinobacteria*. All groups affiliated with the order *Actinomycetales*. Three groups, each consisting of one strain only, belong to the family *Micrococcaceae*. Groups 2 and 7 are both assigned to *Micrococcus luteus* based on partial and near full length 16S rDNA analysis. The fatty acid profiles of group 7 and *Micrococcus luteus* showed a high similarity, but group 2 differed from the *M. luteus* type strain (DSM 20030^T) and group 7 by lacking fatty acids with more than 15 carbons. Generally, the *Micrococcus* and the closely related genus *Arthrobacter* have similar fatty acid profiles and cannot be identified on the basis of the fatty acid analysis (Koch et al., 1995; Stackebrandt et al., 1995). Additionally, none of these species showed fatty acid profiles excluding fatty acids with more than 15 carbons (Stackebrandt et al., 1995; Wieser et al., 2002). Cell and colony morphology of both groups

are in agreement with those of *Micrococcus luteus*. Thus, of both groups only group 7 is clearly identified as *Micrococcus luteus* due to the lack of longer chain fatty acids in group 2. Group 9 is identified as a member of the genus *Kocuria*. The partial sequence of the member strain of group 9 showed 99.8% 16S rDNA sequence similarity with strain ATCC9341 which has recently been reclassified from *M. luteus* to *Kocuria rhizophila* (Tang and Gillevet, 2003). Colonies of *K. rhizophila* have been described to be 1.5-2.5 mm in diameter, yellow and smooth (Kovács et al., 1999). This is in agreement with the appearance of the colonies of group 9, but in spite of Kovács et al. having used the same medium and growth conditions as in the present study *K. rhizophila* colonies have been described to have irregular edges. The colonies of group 9 in contrast were always circular and regular. However, *Kocuria rhizophila* ATCC9341 forms circular colonies on trypticase soy agar and two variants, one smooth and one rough have been observed if 5% sheep blood is added (personal communication: Dr. J. Tang, ATCC Collection). Thus, based on these data group 9 is assigned to *K. rhizophila*.

Group 18 belongs to the *Microbacteriaceae* and is placed in the genus *Leifsonia*. The fatty acid profiles of the member strains correspond well to those of the joint range from *Leifsonia aquatica and L. shinshuensis* (Bernard et al., 1991; Suzuki et al., 1999). The assignment to a described *Leifsonia* species based on FAME is not possible, however, the similarity of the partial 16S rDNA sequence was highest with *L. shinshuensis* (99.3%).

The sequence of the species *Brevibacterium sanguinis* (Wauters et al., 2004) shares 99.7% of the 730 sequenced bases of group 25. Thus, group 25 is placed within the genus *Brevibacterium*. Additionally, the fatty acid profile also lay in the range described for *Brevibacterium* (Funke and Carlotti, 1994) with 15:0 anteiso and 17:0 anteiso accounting for more than 75% of the total fatty acid profile, as generally observed in brevibacteria (Funke et al., 1997). The GC-content of 58.8% was slightly lower than the range of 60-70 mol% found in the literature for some *Brevibacterium* species. Moreover, it differed largely in terms of the GC-content of *Brevibacterium sanguinis* (69.9%). Gram-staining and catalase test were in agreement with the description of *B. sanguinis* but in contrast to the former the oxidase test was weakly positive instead of negative. Until the isolation of *B. sanguinis* group 25 shared the largest (97%) partial 16S rDNA similarity with *Brevibacterium iodinum*. Group 25 and *B. sanguinis* share only 92.3 – 97.7% 16S rDNA sequence similarity with other *Brevibacterium* species (Wauters et al., 2004). A comparison of group 25 with the newly described species *B. sanguinis* should be interesting due to the differing GC-contents and especially because most *B. sanguinis* strains were isolated from blood.

One other group was also placed in the suborder *Micrococcineae*. Group 14 is identified on the basis of 16S rDNA analysis as *Cellulosimicrobium cellulans*, which had been reclassified from *Cellulomonas* and now belongs to the family *Promicromonosporaceae* instead of to the *Cellulomonadaceae* (Schumann et al., 2001). The emended species description is in

agreement with the data of group 14. An additional predominant fatty acid of group 14 in comparison with the species description of Schumann et al. (2001) is 17:0 anteiso, which also belongs to the predominant fatty acids in former descriptions of strains classified as *Cellulosimicrobium* members (Bernard et al., 1991; Lednicka et al., 2000). The fatty acid profile of group 14 perfectly matches the fatty acid profiles of those *Cellulosimicrobium* strains. Thus, the fatty acid profile and other physiological traits additionally justify the identification of group 14.

The five remaining actinobacterial groups are assigned to the suborder *Corynebacterineae*. Based on fatty acid profiles group 24 is placed within the genus *Corynebacterium*. Yellow colonies of *Corynebacterium* are common; the orange tinted colour of group 24 has this far not been described in other *Corynebacterium* species. *Corynebacterium vitaeruminis* exhibited the most similar fatty acid profile to group 24 (Bousfield et al., 1983; Bernard et al., 1991). Just as for group 24 an identification based on fatty acids was not possible for group 28. Based on fatty acids group 28 is placed in the family *Mycobacteriaceae* within which the strongest resemblances were found to the genus *Mycobacterium*. The high number of fatty acids with 14 or less carbon atoms was a striking feature of group 28 and not found in the reference strains. In order to differentiate *Tsukamurella* from *Mycobacterium* it would be best to look at the menaquinones. Among the fast growing *Mycobacteria* bright yellow or orange colonies are fairly common; this is in agreement with the yellow to yellow-orange appearance of group 28.

Group 27 was assigned to the genus *Rhodococcus* and shared a high similarity with group 5, which was also identified to belong to the genus *Rhodococcus*. The assignment to the genus *Rhodococcus* was mainly based on the high 16S rDNA similarity of the group 5 strain with *Rhodococcus erythropolis* (99.8%) and was supported by the fatty acid profile and the presence of corynemycolic acids.

Finally, group 16 belongs to the genus *Gordonia*. This group exhibited the glycolyl type of the peptidoglycan and the polar lipids correspond to those of the genus *Gordonia*. The fatty acid profile was similar to that of other *Gordonia* species and additionally, the dominance of the menaquinone MK-9(H_2) separates this group from *Rhodococcus* and *Nocardia* and places it in the genus *Gordonia*. Additionally, 16S rDNA analysis assigns group 16 to *Gordonia polyisoprenivorans* with which it shares 99.9% similarity. Furthermore, the sequence of the two hyper-variable 16S rDNA regions previously suggested for the classification of *Gordonia* species (Arenskötter et al., 2001) were identical for *Gordonia polyisoprenivorans* DSM 44302^T and the sequenced member of group 16. Intragenetic relationships reveal similarity values of 95% up to 99.7% (Linos et al., 1999). Apart from the presence of phosphatidylglycerol no difference in chemotaxonomic characteristics between group 16 and *G. polyisoprenivorans* was found, thus there is no indication of group 16 representing a new species.

Firmicutes

Group 8 belongs to the genus *Brevibacillus* with a 16S rDNA sequence similarity of 99.6% with *Brevibacillus choshinensis*. In spite of the high sequence similarity of group 8 and *B. choshinensis,* the fatty acid profile differed quantitatively between both groups. The high content of 14:0 iso differed strongly from seven fatty acid profiles of *Brevibacillus* strains found in the literature (Kämpfer, 1994; Shida et al., 1995; Lipski et al., 1997). The content of 15:0 anteiso was less than that of *B. choshinensis* and 16:0 iso was more abundant in group 8 than in most other *Brevibacillus* species. The PCR primer BREV174F used by Shida et al. (1996) to detect the *Brevibacillus* cluster also matches group 8 *in silico*. Like *B. choshinensis* and four other *Brevibacillus* species the primer has one mismatch at base 5. Because *Brevibacillus agri* and *B. formosus* share a higher 16S rDNA similarity than group 8 and *B. choshinensis*, it is thus possible that group 8 forms a new *Brevibacillus* species. However, because the *Brevibacillus* species are difficult to distinguish on the basis of ARDRA, FAME, API and other phenotypic characters, it has been suggested that certain species might better be merged to give a more practically useful classification of the genus *Brevibacillus* (Logan et al., 2002). Additionally, a 16S rDNA bootstrap analysis has been shown to have a low discrimination potential for several *Brevibacillus* species including *B. choshinensis* (Logan et al., 2002). Group 8 is therefore identified as *Brevibacillus* sp.; based on the presented data it cannot be identified as *B. choshinensis*.

Group 29, consisting of one single strain, is also preliminary placed, based solely on FAMEs, in the class "*Bacilli*" and possibly in the family *Bacillaceae* or *Paenibacillaceae,* although an assignment to genera belonging to the *Actinobacteria* cannot be ruled out completely.

Flavobacteria

Only one group was placed in the class *Flavobacteria*. Group 4 was assigned to the genus *Chryseobacterium* but could not be identified at the species level. The API 20NE identification suggested *Chryseobacterium indologenes*. The Apilab identification was classed as very good (99.9%), but the reaction profiles of the strains had 3 or 4 contradicting reactions. The fatty acid profile of the strains belonging to group 4 fit well in the previously published range of those of other members of the genus *Chryseobacterium* (Segers et al., 1993). The assignment to the genus *Chryseobacterium* was underlined by the high abundance of 17:1 iso *cis*9. However, the production of indole, usually characteristic for this genus (Yabuuchi et al., 1983), was observed for only one of the three strains. Nevertheless, the polar lipids, the occurrence of menaquinone, the early orange pigmentation of the forming colonies, and the strong odour were in agreement with the genus *Chryseobacterium*.

Nevertheless, the 16S rDNA data discussed above in combination with other traits of the strains evidently indicated that a number of isolates can clearly be differentiated from known species. Hence, new bacterial species are proposed in the appendix.

PHYSIOLOGY OF THE ISOLATED STRAINS

The majority of the tested strains grew well in the BIOLOG microplates and the API 20NE strips. The meagre growth of group 20 and lack of growth of group 21 in these systems led to the design of further tests. An oligotrophic property of the tested groups 3 and 20 was absent, because groups 3 and 20 showed growth, which was proportional to the nutrient concentration. Additionally, oligotrophy was unlikely for group 21 because none of the member strains exhibited growth in any dilution of the medium. Apart from the problem of common test kits not being designed for oligotrophic bacteria, they are usually also not designed for bacteria with slower growth rates or longer lag phases. Additionally, the pH optimum of the tested bacteria may also differ from the pH value of the test medium, which was one of the reasons to test groups 20 and 21 at different pH values.
Testing growth parameters is inconvenient when working with strains as fastidious as the members of the groups 20 and 21. For example, both groups were isolated in fluid and solid media at a pH value of 5 and were maintained on agar plates at pH 5. However, during experiments testing growth at different pH values growth at pH 5 was found to be weak. In fact, for all three strains of group 20 highest growth yields were obtained at pH 7. Thus, the pH of the API and BIOLOG test kit media were probably not responsible for the poor growth of group 20. For groups 20 and 21 no growth was observed at pH $\geq$ 8 (Fig. 7). In contrast, the one strain tested belonging to group 3 obtained highest optical densities after a lag phase of 10 days at pH 9 and no noteworthy growth was observed below pH 7.
Lag phases of more than 20 days were not uncommon for these fastidious microorganisms. The lag phase was often substrate-dependent, for example all three groups tested exhibited a long lag phase on succinate. Because the physiological state of the inoculum affects lag time duration, it has to be emphasised that the growth curves from one strain were all started from the same inoculum tube within each figure. Especially groups 3 and 20, which grew on several carbon sources with different lag time durations would be interesting for lag phase studies and a proteomic approach. The long time spans should enable the detection of proteins, the synthesis of which depends on the carbon source. The length of lag phases may appear confusing if bacteria such as *Escherichia coli* are taken into account, but lag times of up to 8 days (Membre and Burlot, 1994), and even up to 30 days are not uncommon among bacteria (Baranyi, personal communication (Baranyi, 2002), Combase database for bacterial

growth curves http://www.combase.cc.) For mathematical modelling Mellefont (for example Mellefont et al., 2003) defines lag as “the time to increase above starting numbers”. Generally, the lag phase has been defined as the time during which the cells adjust to new environmental conditions. Lag time can be understood in terms of the amount of work to be done for this adjustment and the rate at which it is done (Robinson et al., 1998; Mellefont et al., 2003; Mellefont and Ross, 2003). However, bacterial responses to changes in environment are complex and challenging to characterise unambiguously.

During this study long lag phases were also observed when members of other groups (e.g. group 5) were grown on a variety of short- and medium-chain-length *n*-alkanes (Table 14). The genetics and enzymology of the metabolism of medium-chain-length *n*-alkanes have been well characterised. The enzymes involved oxidise the alkanes to the corresponding terminal acyl-coenzyme A derivates, which then enter the ß-oxidation cycle (van Beilen et al., 1994). In *Pseudomonas oleovorans* the expression of the genes required for the metabolism of alkanes is connected to the metabolic status of the cell through at least two checkpoints: The growth phase and the carbon source being used (through catabolic repression) (Yuste et al., 1998; Canosa et al., 1999). The genetic information for alkane oxidation of several pseudomonads is located in extrachromosomal DNA (OCT plasmid). The OCT plasmid (OCT for *n*-octane utilisation) contains the alk-genes that carry all genetic information for the proteins needed to transform the alkane to Acyl-CoA (van Beilen et al., 1994; Yuste et al., 1998). Thus, in these experiments lag time duration may also depend on plasmid frequency in the case of some carbon sources.

No psychro- or thermophilic growth at 4 or 45°C, respectively, was observed for the tested groups 3, 20 and 21. Generally the main growth lay between 23 and 37°C with slower growth at 13° C for groups 3 and 20. Group 21 showed no significant growth at 13°C and did not reach high optical densities at 37°C, which may indicate a narrower optimal temperature range for group 21 in comparison with groups 3 and 20.

In this experimental approach members of the groups 5, 16, 20, and 21 reached higher optical densities when grown on hexane than the other tested strains. But members of groups 4 and 22, which did not grow on liquid minimal medium with hexane reached high biomass on agar plates in a hexane atmosphere. Thus, some of the strains may have lost the ability to grow on hexane or the experimental design might have been suboptimal for some groups. Metabolic functions can be gained or lost quickly under laboratory selection pressures (Clarke, 1984; Mortlock and Gallo, 1992; Lenski, 1995). Population genetics as well as mutation, recombination and even incipient speciation of bacteria in the laboratory have been discussed (Vulic et al., 1999; Cooper and Lenski, 2000). Already 50 years ago, Sergei Winogradsky advised not to use strains from culture collections because they no longer represent the naturally occurring strains (Winogradsky, 1949). Thus, some of the isolated strains may already exhibit a reduced alkane metabolism.

Some strains showed only slight growth on hexane. These strains may be able to utilise hexane more efficiently in combination with other energy or carbon sources and might depend on degradation products of other species in the biofilter.
The strains belonging to *Variovorax* isolated in this study were all originally isolated from colony forming units, which included strains of other species. Strains of *Variovorax paradoxus* have been shown to degrade and grow on acyl-homoserine lactones (acyl-HSLs) as the sole energy and nitrogen source (Leadbetter and Greenberg, 2000). Acyl-HSLs serve as dedicated cell-to-cell signalling molecules in many species of the class *Proteobacteria* (for reviews on quorum-sensing see Fuqua et al., 2001; Whitehead et al., 2001; Whitehead et al., 2002). This could explain the observation that many of the strains of this taxon were isolated from colony-forming units containing other species.

FLUORESCENCE IN SITU HYBRIDISATION AND LABELLING OF BIOFILTER MATERIAL

Because of the well-know assumption that only about 0.1-1% of the bacterial taxa have been isolated to date (Amann, 1995; Pimm et al., 1995; Hugenholtz et al., 1998) it is important to account for a potential bias of isolation procedures. Thus, in addition to the isolation of bacteria from the biofilter samples the samples were also analysed by FISH and the Hamm samples also by labelling of PLFAs.
Based on FISH the full-scale biofilter was dominated by *Actinobacteria* and *Alphaproteobacteria* in 2001, both reaching more than 10% of the DAPI-stained cells. The contribution of the *Actinobacteria* and *Alphaproteobacteria* based on FISH analyses was higher in the Hamm full-scale biofilter in 2001 than in 1997. Even this higher contribution of *Actinobacteria* in 2001 did not reach the numbers obtained for the Alberta biofilter where *Actinobacteria* accounted for almost 38% of the DAPI-stained cells. The biofilter packing material of both biofilters showed relatively high background fluorescence, a complex flake-like structure, and fluorescence signals by FISH were generally weak especially of the Hamm samples, posing difficulties to the microscopic examination. To facilitate analysis an interactive digital image analysis was applied (see Friedrich et al., 2003). Nevertheless, the proportion of DAPI-stained cells that could not be detected with probe EUB338 remained at about 40% for the Hamm biofilter. Bacterial detection rates may have varied due to the physiological state of the cells (DeLong et al., 1989; Whiteley and Bailey, 2000) or due to community shifts with members of different hybridisation properties. Because the pH of the Hamm biofilter was low (pH 2.6-2.8) in 2001, the majority of the cells may have been exposed to suboptimal conditions leading to slow growth rates and thus lower ribosome numbers (Fegatella et al., 1998). Probe-conferred fluorescence intensities were generally lower in the biofilter samples than of the cultivated single strains. Assuming that the

majority of the cells were in a stationary phase in the biofilter samples, they would additionally be expected to confer a lower fluorescence due to a reduced RNA content in comparison with exponentially grown pure cultures (Poulsen et al., 1993). Thus, the hybridised target rRNA molecules may well have been the limiting factor, especially for the strains belonging to the *Proteobacteria*, which generally showed better hybridisation properties than most of the *Actinobacteria* (Table 17).

The analysis of PLFAs discriminates lipids from dead cells or detrital lipids but this method does neither detect LPS bound hydroxy fatty acids nor glycolipids from living cells. Thus some biomarkers are lost during analysis. Nevertheless, the remaining fatty acids provide enough biomarkers to identify dominating taxonomical groups or for example, community changes. The advantage of PLFAs being rapidly degraded (White et al., 1997) and PLFA profiles therefore showing a recent picture of the situation *in situ,* thus outweigh the disadvantages of the PLFA method, which are the decreased spectrum of fatty acids and a reduced comparability with FAME databases.

In the biofilter samples 19:0 cyclo was significantly more abundant than 18:1 *cis*11 and in contrast to the later it was also increasingly labelled during the experiment (Figures 18 and 19). On the contrary, in the majority of the strains isolated during this study, 18:1 *cis*11 dominated 19:0 cyclo 11-12 when it was present. However, groups 10 (*Bosea* sp.) and 17 (*Pandoraea* sp.) both had about the double amount of 19:0 cyclo compared to 18:1 *cis*11 (Tables 4-9). Among the genus *Bosea* 18:1 *cis*11 dominates 19:0 cyclo (Das et al., 1996; Ouattara et al., 2003). Usually, 18:1 *cis*11 is the precursor to 19:0 cyclo 11-12, thus a larger amount of 18:1 *cis*11 would be expected in growing bacteria. In Gram-negative bacteria the two monoenoic PLFAs 16:1 cis9 and 18:1 cis11 have been shown to increasingly convert to the respective cyclopropyl fatty acids as the microbes move from a logarithmic to a stationary phase of growth (Navarrete et al., 2000). Therefore, the dominance of 19:0 cyclo within the *Bosea* strains of the present study seems to show an aging of these strains, because this group did not grow well on TSB plates and thus slightly older culture material was used except for one strain, which grew faster and could be analysed earlier. In this strain unsurprisingly 19:0 cyclo was dominated by 18:1 *cis*11. All four strains of *Pandoraea*, which were isolated from the biofilter samples in this study, exhibited more 19:0 cyclo than 18:1 *cis*11. Within the genus *Pandoraea* two species also exhibited more 19:0 cyclo than 18:1 *cis*11 (Coenye et al., 2000).

In an attempt to find other fatty acid profiles of bacteria with 19:0 cyclo dominating over 18:1 *cis*11, fatty acid profiles of more than 240 genera were collected from the literature. Only three genera, *Acidiphilium, Acidithiobacillus,* and *Tetragenococcus*, exhibited equal amount or more 19:0 cyclo than 18:1 *cis*11 (Satomi et al., 1997; Knief et al., 2003). The rare abundance of 18:1 *cis*11 in the biofilter profiles of this study can hint at an older ecological

state of the biofilter or the presence of organisms, which have a larger portion of 19:0 cyclo even during an exponential growth phase. A comparison of the fatty acid profile of the biofilter material before and during the incubation experiment did not demonstrate an ageing of the fatty acid 18:1 *cis*11 to 19:0 cyclo 11-12. Apart from gram-negative bacteria increasing the moles percent of cyclopropyl fatty acids as a response to starvation (Guckert et al., 1986; Kieft et al., 1994; Kieft et al., 1997), increased levels of cyclopropane fatty acids seem to enhance the survival of microbial cells exposed to low pH (Brown et al., 1997). Thus, the low pH of the biofilter material in 2001 may have led to the dominance of 19:0 cyclo 11-12 over 18:1 *cis*11.

The presence and labelling of the two fatty acids 16:0 and 18:0 in both, lower and upper, biofilter samples does not allow an assignment to certain taxonomic groups because these fatty acids are common in several families. The fatty acids 16:0 and 18:0 were present in 16 and 12 groups isolated from the industrial biofilter, respectively (Table 19). 18:0 was present in isolates belonging to the *Alpha-, Beta-*, deep branching *Gammaproteobacteria* and the *Actinobacteria.* The fatty acid 16:0 is ubiquitous and was present in isolates of the *Alpha-, Beta-*, deep branching *Gammaproteobacteria, Actinobacteria,* and *Firmicutes*.

The fatty acid 16:0 iso was fairly abundant and significantly labelled especially in the lower biofilter samples. Among the isolated strains this fatty acid was present in members of the *Firmicutes* and *Actinobacteria*. Additionally, it was also present in group 22, belonging to the deep branching *Gammaproteobacteria.* This fatty acid was not present in any of the isolated strains belonging to the *Alpha-* or *Betaproteobacteria*. The fatty acids 16:1 *cis*10 and 18:0 10 methyl were only present in *i*solates of the *Actinobacteria* and the fatty acid 18:1 *cis*9 was predominantly present in the isolated *Actinobacteria* (Table 19). The occurrence of 18:0 10 methyl, contributing at least 8% to the total fatty acid profile in all Hamm biofilter samples and its high degree of labelling with up to 23% indicates a quantitative importance of genera such as *Gordonia, Rhodococcus, Noacardia, Mycobacterium, Aeromicrobium, Nocardiopsis*, and *Actinomadura*, all of which can contain major amounts of 18:0 10 methyl (Bousfield et al., 1983; Grund and Kroppenstedt, 1990; Kroppenstedt et al., 1990; Von Graevenitz et al., 1991; Klatte et al., 1996; Yoon et al., 1997; Yoon et al., 1997). 18:0 10 methyl is generally widespread within the *Actinobacteria*. One of the most abundantly isolated groups of the Hamm biofilter also is a member of the *Actinobacteria* and this group belonging to *Gordonia* is characterised by large amounts of 18:0 10 methyl (Table 9). Four of the most abundant fatty acids and five of the seven labelled fatty acids of the fatty acid profile of the microbial community of the biofilter are

the five most common fatty acids of *Gordonia* isolates of the Hamm biofilter. Thus, it is most likely that group 16, which also belongs to the best hexane degraders in lab-scale experiments, plays an important role in the hexane degradation within the full-scale biofilter. Additionally, two other groups (27 and 28) isolated from Hamm belonging to the *Corynebacterineae* included, apart from 16:0 iso and 19:0 cyclo 11-12, all labelled fatty acids from the incubated biofilter samples. Therefore, these groups could also play an important role in the hexane degradation in the industrial biofilter. Group 27 was assigned to the genus *Rhodococcus* and was similar to group 5 of the experimental biofilter which showed very good growth on hexane. The fatty acid 19:0 cyclo 11-12 occurred only in proteobacterial isolates, therefore its labelling suggests a contribution of proteobacterial strains in the hexane degradation. The proteobacterial groups 20 and 21 exhibited good growth on hexane and group 22, which was the only group containing both 19:0 cyclo 11-12 and 16:0 iso, grew well on minimal medium solid broth with hexane in the airspace. Thus, either one group exhibiting both labelled fatty acids, for example group 22, or at least two other taxonomical groups exhibiting either 19:0 cyclo 11-12 or 16:0 iso also contributed to the hexane degradation in the Hamm biofilter. Apart from the proteobacterial group 22, 16:0 iso also occurred in groups 8, 9, 18 and 25. The fatty acid profiles of the later four did not correspond to the fatty acids of the PLFA profile of the Hamm biofilter. 16:0 iso was the only branched fatty acid, which reached more than 5% in the PLFA profile of the biofilter. Apart from 16:0 iso none of the major fatty acids of these groups contributed significantly to the PLFAs of the biofilter samples and these groups only contained 2 of the 7 labelled fatty acids. Thus, of the isolated groups containing 16:0 iso, the proteobacterial group 22 corresponded best to the PLFA profile of the biofilter. Nevertheless, the presence of cells belonging to groups 8, 9, 18 or 25 can explain the presence of several branched fatty acids in the biofilter profile, but the rare abundance and lack of labelling of branched fatty acids except for 16:0 iso showed their irrelevance in the hexane degradation of this biofilter.

The absence of a fatty acid or the non-labelling of it in biofilter samples can be more informative than the presence or labelling of a certain fatty acid, especially when considering the previously mentioned assumption that until now only 0.1 – 1% of all *Bacteria* have been isolated (Amann, 1995; Pimm et al., 1995; Hugenholtz et al., 1998). A lack of fatty acids points towards the absence of organisms with those fatty acids. Additionally, the absence of specific labelled fatty acids allows conclusions that certain taxa are not involved in particular

substrate transformations. In contrast, the presence of fatty acids suggests the occurrence of known organisms with these fatty acids but possibly also of unknown taxa with the same fatty acids.

The fatty acid 18:2 *cis, cis*9,12 has been found in almost all Eukarya (Lechevalier and Lechevalier, 1988; Stahl and Klug, 1996). Thus, its absence or low abundance represents an absence or minor importance of Eukarya in the analysed biotechnological systems. Additionally, 18:2 *cis, cis*9,12 occurs not only in Eukarya but also in some *Actinobacteria* (Bernard et al., 1991; Bendinger et al., 1992), *Alpha-* (Guckert et al., 1991; Bowman et al., 1993) and *Gammaproteobacteria* (Dees and Moss, 1975; Schlater et al., 1989; Bowman et al., 1997; Makemson et al., 1997), in several phototrophic bacteria (Caudales et al., 2000) as well as in *Bacillales* (Bernard et al., 1991; Von Graevenitz et al., 1991) and *Sphingobacteriales* (Bowman et al., 1998). None of the bacterial isolates from the biofilters analysed during this study contained 18:2 *cis, cis*9,12. However, microscopic analysis did not reveal unicellular eukaryotes being of numeric importance in the Hamm biofilter in 2001. Additionally, at this time the percentage of 18:2 *cis*, *cis*9,12 was below 1 and 4% in the upper and lower samples, respectively (Fig. 18 and 19). Interestingly, the contribution of the fatty acid 18:2 *cis*, *cis*9,12 to the total fatty acid profile was significantly higher in the lower than in the upper sample. Thus, eukaryotes or bacterial cells with this fatty acid seemed to play a larger role in the lower biofilter samples. Compared with fatty acid profiles of some other biofilters, in which the contribution of 18:2 *cis*, *cis*9,12 reached up to 22% (Alexandrino et al., 2001; Knief et al., 2003), 18:2 *cis*, *cis*9,12 was of minor importance in the Hamm biofilter samples. The percentages of 18:1 *cis*9 were also higher in the lower than in the upper samples, with 16.3% (SD = 1.5) and 9.8% (SD = 0.6), respectively. However, apart from the eukaryotic microorganisms with their characteristic products of the aerobic fatty acid synthesis pathway, 18:2 *cis, cis*9,12 and 18:1 *cis*9, these aerobic synthesis products are also formed by *Actinobacteria* and some families of the *Proteobacteria* such as *Pasteurellaceae* and *Moraxellaceae* (Erwin and Bloch, 1964; Kroppenstedt, 1985; Schlater et al., 1989; Vestal and White, 1989). Among the strains isolated from Hamm, 18:1 *cis*9 occurred only in 3 groups belonging to the *Actinobacteria* and group 21, forming a new genus within the deep-branching *Gammaproteobacteria* (Table 19). Based on the fatty acids the Hamm biofilter seemed to be dominated by *Actinobacteria* or by bacteria with similar fatty acid profiles. Especially the comparison of the fatty acid profiles with other fatty acid

profiles of biofilters or biotechnological sampling sites showed the strong contribution of 18:0 10 methyl in this biofilter. With more than 8 and 12% in the lower and upper samples, respectively, 18:0 10 methyl contributed more to the total fatty acid profile than in other sites analysed in our lab where this fatty acid generally lay in the range of 0-1% (Alexandrino et al., 2001; Knief et al., 2003) with exceptions of up to 4% (Piehl, 2002).

In sum, the labelling experiment, PLFA profile analysis of non-incubated biofilter samples and the FISH technique all underline a strong contribution of *Actinobacteria* in the Hamm biofilter in 2001. A higher proportion of *Actinobacteria* of the DAPI-stained cells in the upper biofilter sample may be reflected by the higher percentage of 18:0 10 methyl in this biofilter sample. However, because some other predominantly actinobacterial fatty acids were not more abundant in the upper than in the lower sample it cannot be concluded that *Actinobacteria* were more predominant in the upper than in the lower biofilter layer. Additionally, all fatty acids that accounted for more than 5% of the total fatty acids in the labelled samples were labelled. Therefore, the organisms quantitatively important in the biofilter also belonged to the hexane degrading community. This finding suggests a tight link between the microbial community and the waste gas compound hexane in the studied industrial biofilter. In a previous culture-independent study on a biofilter of an animal rendering plant no significant correlations were found between waste gas and microbial parameters (Friedrich et al., 2003). Evidently various ubiquitously occurring volatiles such as the ones found in animal rendering plants may be degraded by various bacterial taxa, which may explain the lack of significant correlations (Friedrich et al., 2003). However, the high labelling ratio in the present study suggests that hexane was indeed a main C-source for the microbial community in the industrial hexane biofilter.

Based on FISH *Alphaproteobacteria* were the second most dominant bacteria in the Hamm biofilter in 2001 (Fig. 16). The comparison of the PLFAs and the fatty acid profiles of the three alphaproteobacterial groups isolated from the Hamm biofilter allowed no clear assignment. In the lab group 20 exhibited high growth rates on hexane. This group may also contribute to the hexane degradation in the biofilter. However, especially the absence of 18:1 *cis*11 11methyl in the biofilter does not underline a strong contribution of groups 10 or 20. Two groups, for example one belonging either to *Gordonia* (group 16) or *Rhodococcus* (group 27) and the gammaproteobacterial group 22 are sufficient to contribute all of the fatty acids labelled in the Hamm samples. Neither group 21 nor group 22 hybridised well with the probe to detect *Gammaproteobacteria* in the biofilter. Thus, their contribution in the Hamm biofilter based on FISH may be underestimated in this study. Both groups are new genera within the deep-branching *Gammaproteobacteria* of which only few taxa have been described so far. Their isolation and further characterisation may well help to understand other habitats, especially because based on their fatty acid profiles and physiological tests

these two groups may also contribute to the hexane degradation in the Hamm biofilter. Thus, this work provides valuable biotechnological, ecological, and new microbiological insights also by presenting four new species, which are capable of hexane degradation.

V. Summary

Biofiltration is used for the abatement of numerous waste gas compounds. Whereas thorough studies on biofilters of animal-rendering plants were already conducted (Bendinger et al., 1992; Lipski et al., 1992; Ahrens et al., 1997; Friedrich et al., 2003) little is known about the microbial ecology of biofilters used for hexane elimination. Previous studies were especially concerned with the technical performance of these biofilters (Morgenroth et al., 1996; Budwill and Coleman, 1997; Kastner et al., 1999; Zhu et al., 2004). This study focused on the microorganisms of two biofilters used for hexane abatement. A thorough isolation approach led to 30 bacterial groups, which were members of the *Alpha-, Beta-,* and *Gammaproteobacteria*, as well as the *Flavobacteria, Actinobacteria*, and *Firmicutes*. A polyphasic classification of the isolates revealed two new genera and at least four new species. Two new species of *Sphingomonas* sensu stricto belonging to the *Alphaproteobacteria* and two new genera within the deep-branching *Gammaproteobacteria* are proposed.

Several isolates did not grow well in standard commercial growth media. Tests with different pH-values suggested different pH-optima for the two new *Sphingomonas* species, which had been isolated from two biofilters with different pH-values (<4.2 or 7). Growth experiments on a variety of short- and medium-chain-length *n*-alkanes were conducted for 16 groups. In tests with hexane as the sole carbon source four groups exhibited high growth yields. Two of the groups belonged to the genera *Rhodococcus* and *Gordonia*, of which members have previously been shown to convert alkanes (Kummer et al., 1999; Saadoun et al., 1999). The two other groups were one of the new *Sphingomonas* species and one of the deep-branching *Gammaproteobacteria*. The other deep-branching *Gammaproteobacterium* as well as the *Flavobacteria* group could grow on hexane on solid medium but not in broth. Two different pH-values were chosen for the enrichment samples of the industrial biofilter. None of the isolated groups was found at both different pH-value treatments, emphasizing the importance of selected enrichment and isolation parameters. To account for the possible bias caused by isolation procedures also isolation-independent techniques were applied.

The laboratory-scale biofilter was clearly dominated by *Actinobacteria* based on fluorescence *in situ* hybridisation (FISH). In 2001 the full-scale biofilter exhibited more than

10% of each the *Actinobacteria* and *Alphaproteobacteria* based on FISH. Nevertheless the full-scale biofilter exhibited a large proportion of DAPI-stained cells that could not be detected with probes. One reason for these difficulties of FISH analysis could be the low pH-value of the full-scale biofilter, which may have impaired bacterial growth and as such led to rRNA molecules being limited for FISH-based detection. The low pH of the biofilter material in 2001 may also have led to the dominance of 19:0 cyclo 11-12 over 18:1 *cis*11. The increased percentage of cyclopropyl fatty acids has previously been shown to be a response to exposure of cells to low pH (Brown et al., 1997). Together with the fatty acids 16:0 iso, 16:0, 18:1 *cis*9, and 18:0 10 methyl, 19:0 cyclo 11-12 belonged to the fatty acids that dominated the industrial biofilter.

The occurrence of the fatty acid 18:0 10 methyl and its strong degree of [^{2}H]-labelling suggests a quantitative importance of actinobacterial taxa such as the genera *Gordonia* and *Rhodococcus* in terms of hexane degradation. Additionally, four of the most abundant fatty acids and five of the seven labelled fatty acids of the biofilter community profile were the most common fatty acids of the copiously isolated *Gordonia* strains of this biofilter. The isolated *Gordonia* strains also belonged to the best hexane degraders in the lab-scale experiments and thus seem to play a central role in the hexane degradation within the full-scale biofilter. Because the fatty acid 19:0 cyclo 11-12 was also labelled proteobacterial strains appear to also contribute to the degradation of hexane. The new *Sphingomonas* species isolated from this industrial biofilter also belonged to the best hexane utilisers in the lab experiments. But the lack of the fatty acid 18:1 *cis*11 11methyl of this alphaproteobacterial species in the biofilter does not suggest a dominant role in the biofilter. The representatives of the new genera belonging to the deep-branching *Gammaproteobacteria* isolated from the full-scale biofilter also exhibited growth on hexane.

Moreover, all fatty acids that always accounted for more than 5% of the total fatty acids in the labelled samples were labelled. Thus, the organisms quantitatively important in the biofilter also belonged to the hexane degrading community. This finding suggests a tight link between the microbial community and the waste gas compound hexane in the studied industrial biofilter. In a previous study on a biofilter of an animal rendering plant no significant correlations were found between waste gas and microbial parameters (Friedrich et al., 2003). Obviously various ubiquitously occurring volatiles such as the ones found in animal rendering plants may be degraded by various bacterial taxa, which may explain the

lack of significant correlations (Friedrich et al., 2003). However, the high labelling ratio in the present study suggests that hexane was indeed a main C-source for the microbial community in the industrial hexane biofilter.

In sum, the present study revealed a large diversity of bacteria capable of growing on short- and medium-chain alkanes. Furthermore, stable isotope probing using deuterated hexane was shown to be a valuable tool for the identification of actively degrading microorganisms and revealing microbial structure-function relationships *in situ.*

VI. Zusammenfassung

Die Biofiltration kommt bei der Eliminierung einer Vielzahl von Abluftkomponenten zum Einsatz. Im Gegensatz zu Biofiltern von Tierkörperverwertungsanlagen, die bereits gründlich untersucht wurden (Bendinger et al., 1992; Lipski et al., 1992; Ahrens et al., 1997; Friedrich et al., 2003) ist vergleichsweise wenig über die mikrobielle Ökologie von Hexan-eliminierenden Biofilter bekannt, da frühere Studien einen Fokus auf den technischen Betrieb dieser Biofilter legten (Morgenroth et al., 1996; Budwill and Coleman, 1997; Kastner et al., 1999; Zhu et al., 2004). Diese Studie konzentrierte sich auf die Mikroorganismen von zwei Biofiltern, die der Eliminierung von Hexan dienen. Ein umfassender Isolierungsansatz führte zur Differenzierung von 30 bakteriellen Gruppen, die den *Alpha-, Beta-,* und *Gammaproteobacteria*, sowie den *Flavobacteria, Actinobacteria*, und *Firmicutes* zugeordnet wurden. Eine polyphasische Klassifizierung der Isolate wies auf zwei neue Gattungen und mindestens vier neue Arten hin. Zwei neue Arten von *Sphingomonas* sensu stricto (*Alphaproteobacteria*) und zwei neue Gattungen innerhalb der tiefabzweigenden *Gammaproteobacteria* werden vorgeschlagen.

Einige Isolate wuchsen nur eingeschränkt in kommerziell erhältlichen Wachstumsmedien. Die Ergebnisse der Wachstumstests bei verschiedenen pH-Werten wiesen auf verschiedene pH-Optima der beiden neuen *Sphingomonas*-Arten hin. Diese beiden Arten wurden auch von zwei Biofiltern mit unterschiedlichen pH-Werten (<4,2 bzw. 7) isoliert. Eine Reihe von Wachstumstests mit verschiedenen kurz- und mittelkettigen Alkanen wurde für 16 Gruppen durchgeführt. Bei den Tests mit Hexan als alleiniger Kohlenstoffquelle wiesen vier Gruppen hohe Wachstumserträge auf. Zwei der Gruppen gehören zu den Gattungen *Rhodococcus* und *Gordonia,* von denen bereits Alkan-abbauende Vertreter bekannt sind (Kummer et al., 1999; Saadoun et al., 1999). Die anderen beiden Gruppen waren eine der neuen *Sphingomonas*-Arten und eine Art der tiefabzweigenden *Gammaproteobacteria*. Das andere tiefabzweigende *Gammaproteobacterium* und die isolierten *Flavobacteria* konnten zwar auf Hexan in Verbindung mit minimalem Festmedium aber nicht in Flüssigkultur wachsen. Die Beobachtung, dass keine Gruppe aus beiden pH-Bedingungen der Anreicherungen des Industriebiofilters isoliert wurde verdeutlicht die Bedeutung der Auswahl geeigneter Anreicherungs- und Isolierungsparameter. Um möglichen Verfälschungen Kultivierungs-abhängiger Methoden Rechnung zu tragen, wurden in der vorliegenden Arbeit zusätzlich Kultivierungs-unabhängige Techniken eingesetzt.

Der experimentelle Biofilter wurde auf Grundlage der Ergebnisse der Fluoreszenz *in situ* Hybridisierung (FISH) deutlich von Vertretern der Actinobacteria dominiert. Im Jahr 2001 wies der Industriebiofilter jeweils mehr als 10% *Actinobacteria* und *Alphaproteobacteria* auf. Dennoch gab es in den Proben des Industriebiofilters weiterhin einen großen Anteil von

DAPI-markierten Zellen, die durch keine FISH-Sonde markiert wurden. Ein möglicher Grund für diese Schwierigkeiten bei der Anwendung der FISH-Technik könnte der niedrige pH-Wert des Industriebiofilters sein. Dieser könnte das bakterielle Wachstum insoweit beeinträchtigt haben, dass es zu einer Limitierung der Anzahl von rRNS-Molekülen für die FISH-basierte Detektion kam. Der niedrige pH-Wert des Biofiltermaterials könnte 2001 auch zu der Dominanz der Fettsäure 19:0 cyclo 11-12 über 18:1 *cis*11 geführt haben. Eine Erhöhung des Anteils der Zyklofettsäuren als Antwort der Zellen auf niedrige pH-Werte ist bekannt (Brown et al., 1997). Die Fettsäure 19:0 cyclo 11-12 gehörte zusammen mit 16:0 iso, 16:0, 18:1 *cis*9 und 18:0 10 methyl zu den Fettsäuren, die im Industriebiofilter dominierten.

Das Auftreten von 18:0 10 methyl und dessen starke [^{2}H]-Markierung weisen auf eine quantitativ bedeutende Rolle von einigen Vertretern der *Actinobacteria,* wie *Gordonia* oder *Rhodococcus,* bei der Hexan-Eliminierung hin. Die fünf häufigsten Fettsäuren der mehrfach von beiden Probestellen aus dem Industriebiofilter isolierten *Gordonia*-Stämme stellten zudem fünf der sieben markierten Fettsäuren der Biofilterpopulation dar. Die isolierten *Gordonia*-Stämme gehörten außerdem zu den besten Hexanverwertern in den Laborversuchen und scheinen daher eine zentrale Rolle im Hexanabbau des Industriebiofilters zu spielen.

Da die Fettsäure 19:0 cyclo 11-12 ebenfalls markiert war, tragen offensichtlich aber auch Vertreter der *Protoeobacteria* zu dem Abbau von Hexan bei. Die aus diesem Industriebiofilter isolierte neue *Sphingomonas*-Art gehörte zu den besten Hexanverwertern. Das Fehlen der bei dieser Art vorhandenen Fettsäure 18:1 *cis*11 11methyl im Biofilter deutet auf eine quantitativ nicht dominierende Rolle beim Hexanabbau in diesem Biofilter hin. Die Vertreter der beiden neuen Gattungen der tiefabzweigenden *Gammaproteobacteria* wiesen ebenfalls gutes Wachstum auf Hexan auf.

Alle Fettsäuren, die stets mehr als 5% des Fettsäureprofils der Biofiltergemeinschaft ausmachten, wurden im Markierungsexperiment markiert. Daher gehörten alle quantitativ wichtigen Organismen im Biofilter auch zu den Hexan-Abbauern. Diese Beobachtung weist auf eine enge Verknüpfung der mikrobiellen Gemeinschaft mit der Abluftkomponente Hexan in dem untersuchten Industriebiofilter hin. In einer früheren Studie eines Biofilters einer Tierkörperverwertungsanlage konnten keine signifikanten Korrelationen von Abluft- und mikrobiellen Parametern gefunden werden (Friedrich et al., 2003). Möglicherweise können ubiquitär verbreitete, flüchtige Abluftinhaltsstoffe wie die aus Tierkörperverwertungsanlagen von diversen bakteriellen Taxa abgebaut werden und dies kann das Fehlen signifikanter Korrelationen erklären (Friedrich et al., 2003). Dagegen deutet der hohe Grad der Markierung der Hauptfettsäuren in der vorliegenden Arbeit auf die Hauptrolle des Hexans als Kohlenstoffquelle für die mikrobielle Gemeinschaft in dem Industriebiofilter hin.

Zusammenfassend wurde in der vorliegenden Arbeit eine Vielzahl von Bakterien verschiedener Taxa beschrieben, die auf kurz- und mittelkettigen Alkanen wachsen können. Darüber hinaus wurde deuteriertes Hexan in Kombination mit massenspektrometrischen Untersuchungen von Phospholipid-Fettsäuren erfolgreich eingesetzt, um aktiv am Hexanabbau beteiligte Mikroorganismen zu detektieren und dabei mikrobielle Struktur-Funktions-Beziehungen *in situ* aufzudecken.

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Appendix

List of publications and manuscripts

Friedrich, U., **Naismith, M.M.**, Altendorf, K., and Lipski, A. (1999) Community analysis of biofilters using fluorescence in situ hybridization including a new probe for the *Xanthomonas* branch of the class *Proteobacteria*. *Appl Environ Microbiol* **65**: 3547-3554.

Friedrich, M.M., Altendorf, K., and Lipski, A. (2000) Poster: Taxonomical and functional characterisation of bacteria isolated from hexane-degrading biofilters *Microbiology 2000, VAAM Annual Meeting,* Technical University of Munich, Germany

Friedrich, M.M. (2005) Poster: Jugend forscht in Schule und Labor, *Genlabor und Schule II*, XLAB, Göttingen, Germany

Lipski, A. Piehl, J. and **Friedrich, M.M**. (2005) Poster: Stable Isotope Probing as an Effective Tool for Direct Identification of Active Microorgansims in Biofilters. *VAAM Jahrestagung*, Universität Göttingen, Germany

Friedrich, M.M. Lorenz, D., Erdmann, H. and Friedrich, U. (2005) Poster: Extensive LAB strain diversity of PROBAT™ 505. *8th Symposium on Lactic Acid Bacteria*, Egmond aan Zee, The Netherlands.

Lipski, A. Piehl, J. and **Friedrich, M.M**. (2005) Stable Isotope Probing as an Effective Tool for Direct Identification of Active Microorgansims in Biofilters. In: Biotechniques for Air Pollution Control. Proceedings of the International Congress Biotechniques for Air Pollution Control (C. Kennes and M. C. Biega, eds.).p. 31-37. A Coruna, Spain, Universidade da Coruna.

Friedrich, M.M., Altendorf, K., and Lipski, A. Characterisation of hexane-degrading microorganisms from waste gas biofilters. Manuscript in preparation.

Friedrich, M.M., Altendorf, K., and Lipski, A. *Sphingomonas alkanivorans* sp. nov. and *Sphingomonas purgata* sp. nov., isolated from biofilters for waste gas treatment. Manuscript in preparation.

Friedrich, M.M., Altendorf, K., and Lipski, A. *Alkanibacter difficilis* gen. nov., sp. nov. and *Imprimimonas variabilis,* gen. nov., sp. nov., two new genera belonging to the deep-branching *Gammaproteobacteria.* Manuscript in preparation.

Curriculum vitae

Name:	Michèle Martine Friedrich, nee Naismith
Date/place of birth:	01.11.1971, Osnabrück, Germany
Marital status:	married to Dr. Udo Friedich

Scientific career:

2002 - present	Scientist at the University of Applied Sciences Flensburg, Germany
1997 - 2001	PhD student and scientist at the Department of Microbiology (Prof. Dr. K. Altendorf), University Osnabrück, Germany
1996 - 1997	Diploma thesis at the Max-Planck-Institute of Limnology, Plön, Germany
1995 - 1996	Biology studies at the University Osnabrück, Germany
1994 - 1995	Postgraduate Diploma in Science at the University of Otago, Dunedin, New Zealand
1991 - 1994	Undergraduate biology studies at the University Osnabrück, Germany

School education:

1989 - 1991	Gymnasium Carolinum in Osnabrück, Allgemeine Hochschulreife
1988 - 1989	Honorary Diploma at Roseburg Senior High School, Oregon, USA
1983 - 1988	Gymnasium Carolinum in Osnabrück
1981 - 1983	Orientierungsstufe Dom in Osnabrück
1977 - 1981	Rückertschule in Osnabrück

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfsmittel verfasst und die benutzten Hilfsmittel vollständig angegeben habe.

Osnabrück, November 2005

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(Michèle M. Friedrich)