International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 173–178 Printed in Great Britain

Anaerophaga thermohalophila gen. nov., sp. nov., a moderately thermohalophilic, strictly anaerobic fermentative bacterium

Karin Denger,[1] Rolf Warthmann,[2] Wolfgang Ludwig[3] and Bernhard Schink[1]

[1] Lehrstuhl für Mikrobielle Ökologie, Fakultät für Biologie, Universität Konstanz, Fach M 654, D-78457 Konstanz, Germany

[2] ETH – Geologisches Institut, Sonneggstr. 5, CH-8092 Zürich, Switzerland

[3] Lehrstuhl für Mikrobiologie, Technische Universität München, Arcisstr. 16, D-80290 München, Germany

Author for correspondence: Bernhard Schink. Tel: +49 7531 882140. Fax: +49 7531 882966. e-mail: Bernhard.Schink@uni-konstanz.de

The strictly anaerobic Gram-negative bacterium strain Fru22^T grows at 50 °C in media containing up to 75 g NaCl l^{-1}. Hexoses and pentoses are fermented to equal molar amounts of acetate, propionate and succinate, and no CO_2 is formed. An orange-red pigment similar to flexirubin is produced during stationary phase upon exposure to light for several days. Cells also produce a surface-active extracellular compound which lowers the surface tension of the medium. This tenside is heat-tolerant up to 70 °C and is destroyed by treatment with proteinase K or trypsin, but not by lipase. Comparative 16S rDNA sequence analysis confirmed a phylogenetic affiliation of strain Fru22^T to the phylum *Bacteroides* (*Cytophaga/Flavobacterium/Bacteroides*), moderately related to the genus *Marinilabilia*. Therefore, on the basis of phylogenetic, phenotypic and physiological evidence, a new genus, *Anaerophaga*, is proposed to harbour strain Fru22^T (DSM 12881^T, OCM 798^T) which is described as the type strain of a new species, *Anaerophaga thermohalophila* gen. nov., sp. nov.

Keywords: anaerobic degradation, succinate fermentation, biotensides, carotenoids, microbially improved oil recovery

INTRODUCTION

Thermophilic (for reviews see Brock, 1986; Blöchl *et al.*, 1995; Stetter *et al.*, 1995) or halophilic (Gilmour, 1990; Ventosa & Nieto, 1995) bacteria have been investigated in great detail in the past. Extremes under which life is still possible include temperatures up to 113 °C and salt concentrations up to saturated NaCl brines. Nonetheless, very little is known about bacteria able to thrive upon exposure to both stress factors, elevated temperature and enhanced salt concentration. Such conditions prevail, for example, in many oil reservoirs where elevated temperatures are caused by geogenic heat and salts are dissolved from mineral salt deposits. Abandoned oilfields provide conditions where microbes can multiply under anoxic conditions in the vicinity of oil and salt at elevated temperatures, in the presence of water that had been injected in secondary oil exploitation efforts.

In tertiary oil recovery, microbes are intentionally injected into oil wells to increase oil recovery through effects caused by selective plugging, surface active compounds, change of fluid viscosity and other factors (Bosecker *et al.*, 1991). In an effort to provide suitable organisms for microbially enhanced oil recovery, we isolated strains of moderately thermophilic and halophilic bacteria which could thrive in such environments and be applied to improve oil recovery. Two such strains have been described with respect to their nutritional, morphological and physiological properties (Denger & Schink, 1995).

In the present paper, one of these isolates, strain Fru22^T, is described in more detail, also with respect to the production of a pigment and of an extracellular surface-active agent. Based on 16S rRNA sequence analysis, this strain groups with representatives of the phylum *Bacteroides* (*Cytophaga/Flavobacterium/Bacteroides*) and is moderately related to a sister group, the facultatively anaerobic genus *Marinilabilia*. We therefore describe this strain as the type strain of a new genus and species, *Anaerophaga thermohalophila* gen. nov., sp. nov.

The EMBL accession number for the 16S rDNA sequence of *Anaerophaga thermohalophila* strain Fru22^T is AJ418048.

METHODS

Culture conditions. A pure culture of strain Fru22^T was taken from our laboratory collection. The strain has been deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) under the reference number DSM 12881^T, and with the Oregon Collection of Methanogens under the deposition number OCM 798^T. Strain Fru22^T and a similar strain, Glc12, had been enriched originally from blackish-oily sedimentary residues of an oil separation tank near Hannover, Germany.

The mineral salts medium for enrichment and cultivation was bicarbonate-buffered (50 mM), cysteine-reduced (1 mM) and contained, together with other minerals, 75 g NaCl l^{-1} and 4·0 g $MgCl_2.7H_2O$ l^{-1} (Denger & Schink, 1995). The pH was 6·7–6·8. During the enrichment, the medium received a few drops (50 µl per 25 ml medium) of hexadecane to provide a lipophilic boundary layer. Subcultures were inoculated with oily drops from the surface of the preculture. All details concerning cultivation and physiological characterization have been described by Denger & Schink (1995).

Pigment characterization. Pigments were extracted from stationary-phase cultures exposed to daylight for 3–5 d. After centrifugation at 20000 ***g*** for 20 min at 4 °C, the pellet was extracted in the dark with acetone [3 ml (g wet pellet)$^{-1}$], or with an equal volume of hexane. Extracts were subjected to absorption spectroscopy with a Uvikon 930 scanning spectrophotometer (Kontron).

Preparation and characterization of tensides. For optimal production of a surface-active compound (tenside), cells were grown with 10 mM glucose in the presence or absence of hexadecane. Changes in the surface tension were measured with a tensiometer (Lauda TE1). Possible hydrolysis by enzymes was checked for by incubation experiments with proteinase K (from *Tritirachium album*, 150 µg ml^{-1}; Merck) or trypsin (from pig pancreas, 150 µg ml^{-1}; Merck) in 10 mM Tris/HCl buffer, pH 8·0, or lipase (from *Candida cylindrica*, 100 µg ml^{-1}; Merck) in 10 mM Tris/HCl buffer, pH 7·5. Culture supernatant was incubated at 37 °C for 1 h and surface activity was measured by a test checking for stabilization of an oil–water emulsion (Denger & Schink, 1995).

For enrichment of the surface-active compound, the culture supernatant was titrated to pH 2·0 with 25% (w/v) HCl. After 14 h incubation at 4 °C, a precipitate formed which was separated from the supernatant by filtration through 0·45 µm membrane filters (Sartorius). After washing with 10 mM HCl, the precipitate was redissolved from the filters in 10 mM NaOH and dried by lyophilization. After redissolution in 10 mM NaOH under sonication (10 min in a laboratory equipment sonicator bath), further enrichment was achieved by passage through a Sephadex G25 column in 10 mM NaOH at pH 10·5, with a flow rate of 0·5 ml min^{-1}. Alternatively, the compound could be enriched by FPLC on a reverse-phase column [RPC column pro 5/10 (Pharmacia); eluent A, 0·1%, v/v, trifluoroacetic acid in dist. H_2O; eluent B, 0·1%, v/v, trifluoroacetic acid in methanol; flow rate 0·4 ml min^{-1}). Under these conditions, the tenside eluted at 85–95% eluent B.

Preparations were analysed by separation on SDS-PAGE (12 or 15% acrylamide, sample buffer 60 mM Tris/HCl, pH 6·8, 10% glycerol, 2%, w/v, SDS, 0·025%, w/v, bromophenol blue, with and without 5%, v/v, mercaptoethanol; after Laemmli, 1970). In addition to the usual Coomassie brilliant blue staining, gels were stained also by a technique specific for glycoproteins (Dubray & Bezard, 1982).

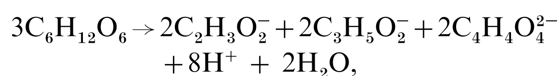
16SrDNA sequencing and phylogenetic analysis. *In vitro* amplification and sequence analysis of rDNA were performed as described by Springer *et al.* (1992). The 16S rRNA sequence of strain Fru22^T (homologous to *Escherichia coli* positions 8–1542) was fitted into an alignment of about 20000 homologous full or partial primary structures available in public databases (Ludwig, 1995) using the respective automated tools of the ARB software package (Ludwig & Strunk, 1997). Distance matrix, maximum-parsimony and maximum-likelihood methods were applied for tree construction as implemented in the ARB software package. Different datasets, varying with respect to the selection of outgroup reference organisms (sequences), as well as alignment positions were analysed.

RESULTS AND DISCUSSION

Physiological properties

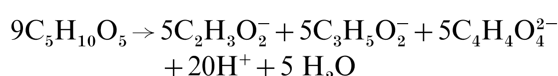
Strains Fru22^T and Glc12 were isolated as possible agents for tenside production in microbially improved oil recovery. Therefore, they were enriched and selected under conditions of elevated temperature (50 °C) and increased salinity (7·5%, w/v). The ability to thrive under the combined influence of both stress factors, heat and salinity, is still unusual within the prokaryotic world and to our knowledge, the combination of both has not yet been reported in the microbiological literature.

The isolated strains were unusual also in their fermentation patterns (Denger & Schink, 1995). Hexoses and pentoses were fermented to equal molar amounts of acetate, propionate and succinate, according to the following equations:



$$3C_6H_{12}O_6 \rightarrow 2C_2H_3O_2^- + 2C_3H_5O_2^- + 2C_4H_4O_4^{2-} + 8H^+ + 2H_2O,$$

or



$$9C_5H_{10}O_5 \rightarrow 5C_2H_3O_2^- + 5C_3H_5O_2^- + 5C_4H_4O_4^{2-} + 20H^+ + 5\ H_2O$$

No CO_2 is released in this type of mixed-acid fermentation.

Upon prolonged exposure of fully grown cultures to daylight, an orange-red pigment was produced which could be extracted from cell pellets by acetone or hexane, indicating that it was a lipophilic component. Absorption spectra of these extracts showed maxima at 488 and 518 nm and a further shoulder around 460 nm, typical of carotenoids (Reichenbach *et al.*, 1974). Carotenoids are known to be produced by several representatives of aerobic gliding bacteria, including *Flexibacter* spp., *Cytophaga* spp. and several myxobacteria (Reichenbach & Dworkin, 1981). The strain described here is the first strict anaerobe producing such pigments after the moderately oxygen-tolerant anaerobe *Cytophaga xylanolytica* (Haack & Breznak, 1993). When pigmented cell material after centrifugation was treated with 10% KOH the colour turned dark-red to brownish, similar to flexirubins of

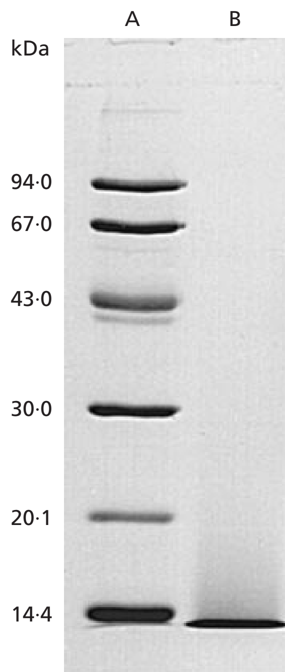


Fig. 1. PAGE of a purified preparation of extracellular tenside produced by strain Fru22ᵀ after periodic acid–silver staining. Lanes: A, calibration proteins; B, tenside preparation.

Cytophaga, *Sporocytophaga* and *Flexibacter* spp. (Reichenbach *et al.*, 1974; Achenbach *et al.*, 1978).

Unfortunately, strain Fru22^T did not grow on agar surfaces, although the media were poured and stored in an oxygen-free glove box under a N_2/CO_2 atmosphere (90:10, v/v). Thus, we could not check for possible gliding motility of strain Fru22^T. Spore-like structures or spheres could be observed in ageing cultures.

Production of a surface-active compound

Surface-active compounds were produced by strain Fru22^T optimally at the end of exponential growth (Denger & Schink, 1995). This tenside efficiently stabilized hexadecane/water emulsions. Production was enhanced in the presence of hexadecane which provides a lipophilic surface in the culture. The surface activity was slightly enhanced at pH 2 as compared to neutral or alkaline pH, indicating that it is an anionic tenside. The surface-active compound(s) was associated partly with the cells and cell surfaces, but was also released into the culture medium; after filtration, about two-thirds of the total tenside present was found in the cell-free filtrate (Denger & Schink, 1995). It was stable during incubation at temperatures up to 70 °C for 20 min, but was destroyed during incubation at 100 °C. Treatment with proteinase K destroyed the activity completely, whereas lipase had no effect. Upon separation of the enriched compound on SDS-PAGE, the compound appeared as a single band in the range of low molecular mass (< 12 kDa, Fig. 1) which stained well with a periodic acid–silver stain for glycoproteins, but only weakly with Coomassie brilliant blue R-250. We conclude that this tenside is an oligopeptide bound to fatty acids and probably also includes sugar residues.

Biosurfactants are produced by several bacteria, especially by those degrading lipophilic substrates. Most biosurfactant producers are strictly aerobic (Parkinson, 1985; Rosenberg, 1986), but also some facultatively aerobic *Bacillus* strains (Jenneman *et al.*, 1983; Javaheri *et al.*, 1985) and even strictly anaerobic bacteria (Cooper *et al.*, 1980) have been reported to produce surface-active compounds. Biosurfactants are typically low-molecular-mass compounds, such as glycolipids, phospholipids or lipopeptides, but polymeric substances which stabilize oil–water emulsions are also excreted by bacteria (Rosenberg, 1986; Desai & Banat, 1997; Rosenberg & Ron, 1999). For technical application in microbially enhanced oil recovery, biosurfactants produced by facultatively or strictly anaerobic bacteria appear to be most promising because such environments are typically entirely anoxic. The best studied systems of this kind are lipopeptides such as surfactin or lichenysin which are produced by *Bacillus subtilis* or *Bacillus licheniformis* (Arima *et al.*, 1968; Eliseev *et al.*, 1991; Kluge *et al.*, 1989; McInerney *et al.*, 1990; Yakimov *et al.*, 1995; Peypoux *et al.*, 1999; Grangemard *et al.*, 1999). The chemical properties of a biosurfactant produced by a strictly anaerobic *Clostridium pasteurianum* strain have not been characterized in detail (Cooper *et al.*, 1980). From the preliminary characterization of the tenside produced by strain Fru22^T, it appears that this compound can also be classified as a lipopeptide of low molecular mass which probably also contains at least one sugar moiety. A more detailed characterization of this compound would require a separate study.

Taxonomy

As indicated by morphological and physiological characteristics described by Denger & Schink (1995) and repeated below in the species description, and corroborated by phylogenetic analysis of 16S rDNA sequences, strain Fru22^T is a member of the phylum *Bacteroides* comprising *Cytophaga*, *Flavobacterium* and *Bacteroides* as representative genera. Based on the similarities of 16S rRNA sequences, the closest relatives of strain Fru22^T are representatives of the genus *Marinilabilia* (formerly *Cytophaga*; Suzuki *et al.*, 1999). However, overall sequence similarities of around 91·6 % indicate an only moderate relationship and justify the proposed establishment of a new genus, *Anaerophaga*. As shown in the phylogenetic tree in Fig. 2, these genera share a common origin with *Cytophaga fermentans* (Bachmann, 1955). The corresponding overall 16S rRNA sequence similarities are 87·9–89·1 %. Strain Fru22^T shares several properties with *Cytophaga* and *Marinilabilia* species, e.g. its morphology (thin, slender rods), production of a flexirubin-like pigment and production of sphere-like struc-

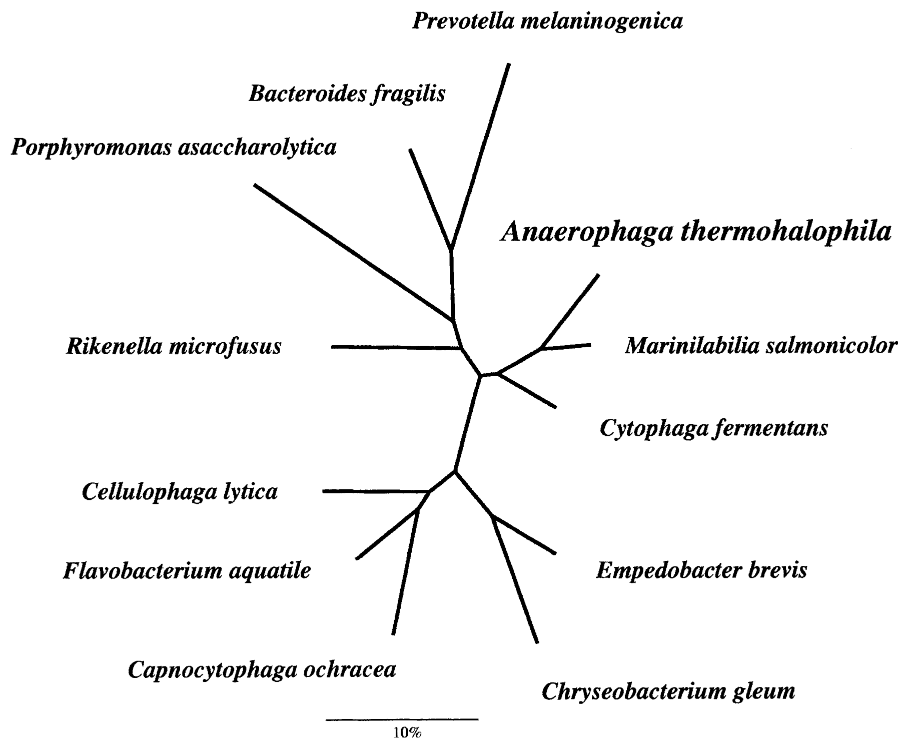


Fig. 2. Maximum-parsimony tree reflecting the phylogenetic position of *Anaerophaga thermohalophila* strain Fru22^T and type species representing a selection of related genera of the *Bacteroides* phylum. The tree is based upon the results of an optimized maximum-parsimony analysis of a dataset of about 20000 small subunit rRNA sequences. The tree topology was supported by performing tree evaluations based on maximum-parsimony, maximum-likelihood and distance matrix analyses of various datasets applying the software tools of the ARB program package (Ludwig & Strunk, 1997). The bar indicates 10% estimated sequence divergence. The EBI (European Bioinformatics Institute, Hinxton, Cambridge; http://www.ebi.ac.uk) accession numbers for the reference organisms are: *Bacteroides fragilis* ATCC 25285^T, M11656; *Capnocytophaga ochracea* ATCC 27842^T, U41350; *Cellulophaga lytica* ATCC 23178^T, M28058, M62796; *Chryseobacterium gleum* ATCC 35910^T, M58772; *Cytophaga fermentans* ATCC 19072^T, M58766; *Empedobacter brevis* ATCC 14234, M59052; *Flavobacterium aquatile* ATCC 11947^T, M28236, M62797; *Marinilabilia salmonicolor* ATCC 19041^T, D12672; *Porphyromonas asaccharolytica* ATCC 25260^T, L16490; *Prevotella melaninogenica*, L16469; *Rikenella microfusus* ATCC 29728^T, L16498.

tures in ageing cultures (Staley *et al.*, 1989; Reichenbach, 1992). Moreover, the facultatively anaerobic *Cytophaga fermentans*, *Cytophaga xylanolytica*, *Marinilabilia salmonicolor* and *Marinilabilia agarovorans* all have been described to produce acetate together with propionate and succinate as main products of sugar fermentation (Staley *et al.*, 1989; Haack & Breznak, 1993). The G+C contents of the DNA of *Cytophaga fermentans* and *Marinilabilia* spp. is in the range of 30–42 mol%; strain Fru22^T (41·8 mol% G+C) would be at the upper limit of this range. Nonetheless, there are substantial differences between strain Fru22^T, *C. fermentans* and the described species of the genus *Marinilabilia*. First of all, strain Fru22^T is strictly anaerobic. Moreover, the growth parameters of strain Fru22^T, especially its growth up to 55 °C and its salt tolerance up to 12% (w/v) salt with an optimum around 6% clearly separate this strain from all described *Cytophaga* and *Marinilabilia* species. So far, only a few *Cytophaga* species show temperature maxima at 40–45 °C ('*Cytophaga aprica*', '*Cytophaga lytica*') or 45 °C ('*Cytophaga diffluens*'); all three strains also tolerate NaCl concentrations up to 6% and all are strict aerobes. '*Cytophaga aprica*' and '*Cytophaga diffluens*' have been reclassified as *Flammeovirga aprica* and *Persicobacter diffluens*, respectively (Nakagawa *et al.*, 1997). '*Cytophaga lytica*' has been reclassified as *Cellulophaga lytica* (Johansen *et al.*, 1999).

The genus *Capnocytophaga* has been separated from other *Cytophaga* species as a genus of facultatively anaerobic bacteria that need CO_2 at enhanced concentrations in the atmosphere for efficient growth. Also, *Capnocytophaga* species produce acetate, propionate and succinate during sugar fermentation, but all have been described as facultatively aerobic organisms. Moreover, all species described so far were found to be associated with higher animals, especially the oral cavity of man (Leadbetter *et al.*, 1979). No thermophilic or halophilic representatives of this genus are known.

On the basis of this comparison, it appears necessary to establish a new genus and species for strain Fru22^T.

A new genus, *Anaerophaga* gen. nov., is proposed to comprise strictly anaerobic, non-photosynthetic, non-fruiting-body-forming bacteria. The type species is *Anaerophaga thermohalophila* gen. nov., sp. nov., with strain Fru22^T (= DSM 12881^T = OCM 798^T) as type strain.

Description of *Anaerophaga* gen. nov.

Anaerophaga (An.a.e.ro.pha′ga. Gr. suff. *an* non-; Gr. n. *aer* air; Gr. (aor.) v. *phagein* to eat; M.L. fem. n. *Anaerophaga* an anaerobic eater).

Strictly anaerobic, chemo-organotrophic bacteria of fermentative metabolism. Non-photosynthetic. External electron acceptors not used. No fruiting bodies formed.

Description of *Anaerophaga thermohalophila* sp. nov.

Anaerophaga thermohalophila (ther.mo.ha.lo′phi.la. Gr. adj. *thermos* warm, hot; Gr. masc. n. *hals*, *halos* salt; Gr. masc. n. *philos* friend, loving; M.L. fem. adj. *thermohalophila* heat and salt loving).

Slender flexible rods with rounded ends, 0·3 × 3–8 µm in size. Formation of spheres and spore-like structures in ageing cultures. Strict anaerobe, catalase and oxidase-negative. Cytochromes of *b*-type present. Glucose, fructose, arabinose, xylose, cellobiose, mannose, trehalose, raffinose, galactose, starch and (contrary to a previous report) lactose used for growth. Hexoses and pentoses fermented to equal molar amounts of acetate, propionate and succinate. No growth with ribose, sorbose, rhamnose, dulcitol, mannitol, glycerol, glycerate, tartrate, malate, glycolate, lactate, pyruvate, succinate, fumarate, methanol, ethanol, ethylene glycol, acetoin, alanine, serine, threonine, glutamate, aspartate, proline, cellulose, arabinogalactan, chitin, yeast extract or peptone. Growth requires media with enhanced CO_2/bicarbonate content and salt concentrations of at least 2 % (w/v). Salt tolerance between 2 and 12 % (w/v), optimum at 2–6 %. Growth possible at 37–55 °C with an optimum at 50 °C; no growth at 30 and 60 °C. Ageing cultures exposed to daylight form an orange-red carotenoid pigment similar to flexirubins. G + C content of the DNA 41·8 ± 0·7 mol % (HPLC determination). Habitat is anoxic subsurface sites of enhanced temperature and salt content. Type strain Fru22^T, deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig as DSM 12881^T, and with the Oregon Collection of Methanogens under the deposition number OCM 798^T.

ACKNOWLEDGEMENTS

This study was supported by a grant of the Bundesministerium für Bildung und Forschung, Bonn, in a programme on Microbially Enhanced Oil Recovery, and by the Fonds der Chemischen Industrie, Frankfurt/M.

REFERENCES

Achenbach, H., Kohl, W. & Reichenbach, H. (1978). The flexirubin-type pigments – a novel class of natural pigments from gliding bacteria. *Rev Latinoam Quim* **9**, 111–124.

Arima, K., Kakinuma, A. & Tamura, G. (1968). Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem Biophys Res Commun* **31**, 488–494.

Bachmann, B. J. (1955). Studies on *Cytophaga fermentans*, nov. sp., a facultatively anaerobic lower *Myxobacterium*. *J Gen Microbiol* **13**, 541–551.

Blöchl, E., Burggraf, S., Fiala, G., Lauerer, G., Huber, G., Huber, R., Rachel, R., Segerer, A., Stetter, K. O. & Völkl, P. (1995). Isolation, taxonomy and phylogeny of hyperthermophilic microorganisms. *World J Microbiol Biotechnol* **11**, 9–16.

Bosecker, K., Frederickson, H., Hoffmann, G. G., Kessel, D., Näveke, R., Schink, B., Siemianowski, K., Timmis, K. N., Widdel, F. & Zabka, S. (1991). Möglichkeiten einer verbesserten Erdölförderung. In *Bedingungen Deutscher Lagerstätten und Relevante Erkenntnisse aus der Mikrobiologie*. Edited by DGMK Deutsche Wissenschaftliche Gesellschaft für Erdöl, Erdgas und Kohle eV, Hamburg.

Brock, T. D. (1986). Thermophiles. In *General*, *Molecular*, *and Applied Microbiology*. Edited by T. D. Brock. New York: Wiley Interscience.

Cooper, D. G., Zajic, J. E., Gerson, D. F. & Manninen, K. I. (1980). Isolation and identification of biosurfactants produced during anaerobic growth of *Clostridium pasteurianum*. *J Ferment Technol* **58**, 83–86.

Denger, K. & Schink, B. (1995). New halo- and thermotolerant fermenting bacteria producing surface-active compounds. *Appl Microbiol Biotechnol* **44**, 161–166.

Desai, J. D. & Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev* **61**, 47–64.

Dubray, G. & Bezard, G. (1982). A highly sensitive periodic acid–silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 325–329.

Eliseev, S. A., Vildanova-Martsishin, R. I., Shulga, A. N., Shabo, Z. V. & Turovsky, A. A. (1991). Oil-washing bioemulsifier produced by *Bacillus* species – potential application of bio-emulsifier in oil removal for sand decontamination. *Mikrobiol Zh* **53**, 61–66 (in Russian).

Gilmour, D. (1990). Halotolerant and halophilic microorganisms. In *Microbiology of Extreme Environments*, pp. 147–177. Edited by C. Edwards. New York: McGraw-Hill.

Grangemard, I., Bonmatin, J. M., Bernillon, J., Das, B. C. & Peypoux, F. (1999). Lichenysins G, a novel family of lipopeptide biosurfactants from *Bacillus licheniformis* IM 1307: production, isolation and structural evaluation by NMR and mass spectrometry. *J Antibiot* **52**, 363–373.

Haack, S. K. & Breznak, J. (1993). *Cytophaga xylanolytica* sp. nov., a xylan-degrading, anaerobic gliding bacterium. *Arch Microbiol* **159**, 6–15.

Javaheri, M., Jenneman, G. E., McInerney, M. J. & Knapp, R. M. (1985). Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. *Appl Environ Microbiol* **50**, 698–700.

Jenneman, G. E., McInerney, M. J., Knapp, R. M., Clark, J. B., Feero, J. M., Revus, D. E. & Menzie, D. E. (1983). A halotolerant,

biosurfactant-producing *Bacillus* species potentially useful for enhanced oil recovery. *Dev Ind Microbiol* **24**, 485–492.

Johansen, J. E., Nielsen, P. & Sjøholm, C. (1999). Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 1231–1240.

Kluge, B., Vater, J., Salnikow, J. & Eckart, K. (1989). Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. *FEBS Lett* **231**, 107–110.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Leadbetter, E. R., Holt, S. C. & Socransky, S. S. (1979). *Capnocytophaga*: new genus of gram-negative bacteria. II. General characteristics, taxonomic considerations and significance. *Arch Microbiol* **122**, 9–16.

Ludwig, W. (1995). Sequence databases. In *Molecular Microbial Ecology Manual*, chapter 3.3.5, pp. 1–22. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. Dordrecht: Kluwer Academic Publishers.

Ludwig, W. & Strunk, O. (1997). ARB: a software environment for sequence data. http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/ARB.ps.

McInerney, M., Javaheri, M. & Nagle, D. P. (1990). Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. *J Ind Microbiol* **5**, 95–102.

Nakagawa, Y., Hamana, K., Sakane, T. & Yamasato, K. (1997). Reclassification of *Cytophaga aprica* (Lewin 1969) Reichenbach 1989 in *Flammeovirga* gen. nov. as *Flammeovirga aprica* comb. nov. and of *Cytophaga diffluens* (ex Stanier 1940; emend. Lewin 1969) Reichenbach 1989 in *Persicobacter* gen. nov. as *Persicobacter diffluens* comb. nov. *Int J Syst Bacteriol* **47**, 220–223.

Parkinson, M. E. (1985). Biosurfactants. *Biotechnol Adv* **3**, 65–83.

Peypoux, F., Bonmatin, J. M. & Wallach, J. (1999). Recent trends in the biochemistry of surfactin. *Appl Microbiol Biotechnol* **51**, 53–63.

Reichenbach, H. (1992). The order *Cytophagales*. In *The Prokaryotes*, 2nd edn, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Reichenbach, H. & Dworkin, M. (1981). The order *Cytophagales* (with addenda on the genera *Herpetosiphon*, *Saprospira*, and *Flexithrix*). In *The Prokaryotes*, pp. 356–379. Edited by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows & H. G. Schlegel. New York: Springer.

Reichenbach, H., Kleinig, H. & Achenbach, H. (1974). The pigments of *Flexibacter elegans*: Novel and chemosystematically useful compounds. *Arch Microbiol* **101**, 131–144.

Rosenberg, E. (1986). Microbial surfactants. *Crit Rev Biotechnol* **3**, 109–132.

Rosenberg, E. & Ron, E. Z. (1999). High- and low-molecular-mass microbial surfactants. *Appl Microbiol Biotechnol* **52**, 154–162.

Springer, N., Ludwig, W., Drozanski, V., Amann, R. & Schleifer, K. H. (1992). The phylogenetic status of *Sarcobium lyticum*, an obligate intracellular parasite of small amoebae. *FEMS Microbiol Lett* **96**, 199–202.

Staley, J. T., Bryant, M. P., Pfennig, N. & Holt, J. G. (editors). (1989). *Bergey's Manual of Systematic Bacteriology*, Vol. 3. Baltimore: Williams & Wilkins.

Stetter, K. O., Fiala, G., Huber, G., Huber, R. & Segerer, G. (1990). Hyperthermophilic microorganisms. *FEMS Microbiol Rev* **75**, 117–124.

Suzuki, M., Nakagawa, Y., Harayama, S. & Yamamoto, S. (1999). Phylogenetic analysis of genus *Marinilabilia* and related bacteria based on the amino acid sequences of GyrB and emended description of *Marinilabilia salmonicolor* with *Marinilabilia agarovorans* as its subjective synonym. *Int J Syst Bacteriol* **49**, 1551–1557.

Ventosa, A. & Nieto, J. J. (1995). Biotechnological applications and potentialities of halophilic microorganisms. *World J Microbiol Biotechnol* **11**, 85–94.

Yakimov, M. M., Timmis, K. N., Wray, V. & Fredrickson, H. L. (1995). Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS 50. *Appl Environ Microbiol* **61**, 1706–1713.