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Biodiversity of bacterial isolates from Antarctic lakes and polar seas

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‘If Antarctica were music it would be Mozart. Art, and it would be Michelangelo. Literature, and it would be Shakespeare. And yet it is something even greater; the only place on earth that is still as it should be. May we never tame it.’

Andrew Denton

Dankwoord

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Anyway the water turns
You're always on my mind
You made all this
Because you made all this

Stefanie
Gent, 31 maart 2004

Contents

List of non-standard abbreviations

AA	Arachidonic Acid
ACAM	Australian Collection of Antarctic Microorganisms, University of Tasmania, Hobart, Australia
AFLP	Amplified Fragment Length Polymorphism
ARDRA	Amplified rDNA Restriction Analysis
ATCC	American Type Culture Collection, Manassas, VA, USA
AWI	Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven, Germany
BCCM/ LMG	Belgian Co-ordinated Collections of Microorganisms/ Laboratorium voor Microbiologie Gent, University of Ghent, Belgium
CFU	Colony Forming Units
CIP	Collection de l'Institut Pasteur, Paris, France
CM-cellulose	Carboxymethylcellulose
DCM	Deep Chlorophyl Maximum
DGGE	Denaturing Gradient Gel Electrophoresis
DHA	Docosahexaenic Acid
DOM	Dissolved Organic Matter
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany
EMBL	European Molecular Biology Laboratory, European Bioinformatics Institute, Cambridge, UK
EPA	Eicosapentaeonic Acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
FAA	Fatty Acid Analysis
HPLC	High-performance Liquid Chromatography
HTL	Higher Trophic Levels
IAM	Institute of Applied Microbiology, University of Tokyo, Japan
IFO	Institute for Fermentation, Osaka, Japan
JCM	Japanese Collection of Microorganims, Tokyo, Japan
KMM	Collection of Marine Microorganisms of Pacific Institute of Bio-organic Chemistry of Far-Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia

NCIMB	National Collection of Industrial and Marine Bacteria, Aberdeen, UK
PAR	Photosynthetically Available Radiation
PCA	Principal Component Analysis
PUFA	Polyunsaturated Fatty Acids
PYG	Peptone Yeast-extract Glucose agar
RAPD	Random Amplified Polymorphism DNA
RCA	Reinforced Clostridial Agar
REP	Repetitive Extragenic Palindrome
RFLP	Restriction Fragment Length Polymorphism
S_{canb}	Canberra metric similarities
S_D	Standard deviation
SIMCO	Sea-ice Microbial Community
TSA	Trypticase Soy Agar
UPGMA	Unweighted Pair Group Method with arithmetic Averages

PREFACE

1. The polar regions

The earth's position within the solar system, its shape, orientation and rotation determine the climate of our planet. The global thermal budget shows a strong negative gradient from the equator towards the poles. As a result from their location, polar regions are exposed to similarly low and strongly variable light conditions and exhibit a series of extreme features which characterise these zones as some of the most inhospitable places on earth. Due to these conditions, Northern and Southern polar ecosystems are unique and apparently very similar. The Arctic and Antarctic zones show very low average temperatures and apart from a few high-altitude areas at lower latitudes, they are the coldest places on earth. Overall light levels are particularly low and show a strong seasonality, with twenty-four hour daylight in summer and continuous darkness during winter. Due to natural and anthropogenic thinning of the ozone layer above the poles, UV light levels are abnormally high at these latitudes. Polar regions are also very dry and the limited precipitation accumulates almost entirely as ice, which permanently covers most land areas and a large proportion of the sea surface in winter. In addition, this extensive ice cover modifies the albedo of these surfaces and enhances the cold climate. In both regions extremely strong winds are regularly recorded. Despite these extreme conditions, the poles harbour a wide variety of different terrestrial and aquatic biotopes, ranging from the surrounding oceans, sea-ice and marine sediments to continental lakes, the ice sheet, soils and rocks.

However, major differences between these two polar zones exist (Grémillet & Le Maho, 2003). The distribution of land and sea is completely different in the Arctic and Antarctic, with Antarctica being a continent surrounded by water, whereas the Arctic is actually an ocean surrounded by land (high latitude regions of the Northern hemisphere continents) (see Fig. 1.1 and 1.2). Antarctica is more isolated than the Arctic and this is not only the result of the large distance to the next continent (South America; the Drake Passage between the Antarctic Peninsula and Tierra del Fuego is 1800 km wide), but also because of the strong circumpolar

currents of the Southern Ocean, which make the Antarctic continent even more isolated. Due to this Antarctic Polar Front, the climatic features of the Antarctic waters are more extreme and constant than those of the Arctic. In the Arctic isolation is less stringent and the range of temperature variations is wider. The Antarctic as a whole is also older than the Arctic. The Antarctic continent drifted away some 140 million years ago, and has been subjected to complete isolation and a cold climate for at least 15 My. This is not the case for the Arctic which is a younger system and the Northern polar ecosystems as we know them today evolved only during the last 15 000 years.

Another example of these differences between the polar regions can be found in their lake environments (Laybourn-Parry, 2003). Both have freshwater and saline lakes but in Antarctica the spectrum spans from brackish to hypersaline, whereas in the Arctic, saline lakes are less common and do not reach the salinity levels seen in Antarctic lakes. Arctic lakes also have more complex plankton communities than the Antarctic lakes. Generally, primary production is higher in the Arctic and this is largely due to higher temperatures in these systems and a longer growing season. In Antarctica the productivity continues during the winter months whereas evidence suggests that in the Arctic lakes, organisms shut down their activity during winter.

2. History of polar research

The polar regions are extreme environments and are of key importance for our understanding of how the world functions. The processes taking place now in the Arctic and Antarctic affect the world's climate and its oceans, linking these regions to what we experience thousands of kilometres away. In understanding the global change, the poles also play a crucial role. Locked up in the thick ice sheets is a record for past climate for the last 500 000 years. Trapped bubbles in the ice hold an archive of atmospheric gasses and evidence for levels of global pollution by industry, agriculture and atomic bombs is frozen into the ice. For all these reasons, the poles are an extremely interesting and important subject for scientific research. For the early explorers the Artic and Antarctic were the ultimate survival contest. For scientists it remains a place of intellectual challenge whilst for the modern tourist it is simply a wilderness of great beauty.

The history of polar research starts with the great explorers, heading to the North and South poles to map out the world's unknown continents, or the mysterious

'Terra Incognita' like the Antarctic continent was named. William Barents led several expeditions to the Arctic in search for a connection between the White Sea and the Bering Sea and in 1596 he claimed Spitsbergen. In the 18th and 19th centuries, the Russians conducted several expeditions to map and describe the coasts of Siberia and North-America. James Cook was the first in 1773 to cross the Antarctic Polar Circle and John Davis was the first man in 1821 to set foot on the Antarctic Peninsula. In 1831 James Ross discovered the Northern magnetic pole and in 1841 he found the Antarctic Ross Sea. Captain Koldewey and his crew reached a northerly latitude of 81° in 1868 and Alfred Wegener led numerous expeditions to Greenland. In the period between 1877 and 1884, captain Dallman played a key role in improving access to Siberia and a sea route to the estuaries of the rivers Ob and Yenisey in Siberia was established. The first scientific expedition to the South Pole was conducted by a Belgian marine officer, Adrien de Gerlache de Gommery in 1897 and on the 16th of August they left with the three-master the Belgica. In March 1898 they got locked in by the ice and had to pass winter on Antarctic pack-ice, but despite this disaster, meteorological observations could be made for the first time during a whole year. Several others followed the example of De Gerlache and in 1901 the Discovery, conducted by Robert Scott, left England to stay during winter on McMurdo Sound. Ernest Shakleton (1907-1909) got close to the geographical South Pole (180 km) and other members of this expedition reached the magnetic South Pole. But the honour was for Roald Amundsen who reached in 1911 the geographical South Pole for the first time. In 1914, Shakleton wanted to cross the Antarctic continent but his ship the Endurance sank in the Weddel Sea. Wilkins was the first to fly over the Antarctic continent (1928) and in 1946, the US Navy organised a large scientific expedition to explore the Antarctic coastal areas. Overall, the importance of scientific research in Antarctica (and in the poles in general) was understood during the International Geophysical Year (1957) and led to the Antarctic Treaty in 1959.

In the beginning the scientific expeditions were focused on geographical, geomagnetic and meteorological questions, but the huge unexplored polar regions also awakened the curiosity of geologists and biologists. Especially the Southern ocean is biologically very rich but also the Arctic and Antarctic continents harbour life, dominated by microorganisms, mosses, lichens and relatively few groups of invertebrates. As such, these polar regions, where life approaches its environmental limits are not only of great interest to zoologists, geologists and other scientists but also for the microbiologists, a true paradise.

3. Objectives of this work

This work will focus on the bacterial diversity in microbial mats from Antarctic lakes and from polar seas, and several challenges regarding this research area exist:

1. Polar areas contain extreme habitats where microorganisms are the most abundant and often the only form of life. Research on microbial diversity in these regions is still in its infancy and there is little information about the bacteria that inhabit these extreme environments.
2. Several polar habitats, have not been studied into detail and only specific areas have already been investigated, leaving a large part unexplored. Additionally, most of this information is limited to certain seasons and due to logistic constraints, most of these habitats have not been sampled during winter.
3. The extreme environmental conditions in the polar habitats have led to the origin of novel, endogenous species and only recently the information on these new taxa is increasing, with the description of novel species.
4. These novel species also have new biochemical adaptations, like anti-freeze proteins, cold-adapted enzymes, desiccation and salt tolerance, etc. with potentially a large amount of unexplored biotechnological applications.

This work on the taxonomical research of polar prokaryotes was started in the frame of the MICROMAT-project 'Biodiversity of microbial mats in Antarctica' (Project N° BIO4-CT98-0040), funded by the European Commission under the Biotech Programme (see Annex III), and in the frame of the cooperation with T. L. Tan from the Alfred Wegener Institut für Polar- und Meeresforschung (AWI, Bremerhaven). The major aim of this thesis was to handle especially the first three of these problems by characterising the bacterial diversity in Antarctic lakes and polar seas. Firstly, this study focused on heterotrophic bacteria in the Antarctic lakes and oligotrophic bacteria in the polar seas, and addresses the first problem of a lack of information on bacterial functioning in polar environments.

Secondly, special attention was given to rather unexplored areas of these polar habitats, with regard to the microbial Antarctic mats and several expeditions in the Arctic and Antarctic seas. Samples from microbial mats in Antarctic lakes were only taken during austral summer (from November to March 1999 & 2000), whereas the samples from the Arctic Ocean were gathered during an expedition

in June 1987 (Tan & Rüger, 1991) and samples from the Antarctic Ocean are from March/ April 1990 (Tan *et al.*, 1999). The information about the microbial diversity in these polar regions is of huge importance for the better understanding of the composition and functioning of microbial communities in extreme environments.

Through a polyphasic taxonomic approach this work should lead to the description of several novel taxa, handling the third challenge. It will allow generating unique collections of samples, isolates and genomic materials and databases with genotypic and phenotypic properties of polar bacterial strains, by using up to date techniques. These can be used in the future to identify new isolates from similar habitats and to develop genomic primers for *in situ* detection.

The fourth challenge was also addressed during the MICROMAT-project, since almost 800 bacterial isolates were made available for several industrial partners (e.g. Genencor Holland, Merck Sharp and Dohme Madrid and Vicuron Pharmaceuticals, formerly BioSearch Italia) who screened them for potentially novel compounds such as cold-adapted enzymes (like proteases, cellulases and lipases) and compounds with antimicrobial activity. Additionally, by describing several of these new taxa from Antarctic lakes and polar seas, reference strains will be deposited in different culture collections and as such these well characterised strains will become publicly available for the scientific community, which will make the search for novel biochemical adaptations easier.

PART 1

Overview of the literature

CHAPTER 1

Bacterial diversity in Antarctic lakes and polar seas

1.1 Antarctic lakes and polar seas

Antarctica is characterised by its geographical and climatic isolation and most of the continent has experienced little or no anthropogenic influence. Antarctica is a very unique and extreme environment since only 2% of its surface is ice-free, the lowest temperatures on earth occur here and the continent also has the lowest precipitation and one of the lowest relative humidity levels. On the contrary, the continent contains 70-90% of the world's freshwater but most of the time the water is frozen and the Antarctic lakes are either covered by perennial ice of variable thickness, completely frozen or so saline they rarely freeze (Simmons *et al.*, 1993). Most lakes with thick ice covers thaw along their margins to form moats during summer and are exposed to the atmosphere and running water for only a few weeks of the year. Although most of the Antarctic continent is covered by ice, desert like ice-free areas exist and these are often called 'oases'. In these areas the Antarctic lakes are located and there are several hypotheses to explain the origin of these oases. Solopov (1969) stated that ice-free areas are formed when the ice sheet thins sufficiently, because of global climatic change (the warming trends of the Holocene) so areas with some degree of elevation become ice-free. The positive radiation balance (associated with dark soils and rocks) maintains these ice-free areas, basins in these areas would collect melt water and lakes are formed. According to Priddle & Heywood (1980) the origin of Antarctic lakes can be found in the accumulation of blown sand on the ice sheet. The sand will act as a solar collector and the surrounding ice will melt. The formed depression will collect more sand, enlarges and a proglacial lake is formed. These features are often found on ice structures and are called 'cryoconite holes' (Wharton *et al.*, 1981). In section 1.2, more details are given about the lakes in three different

Antarctic regions where samples were taken during the MICROMAT-project.

Polar oceans are cold and oligotrophic habitats, where most of the microorganisms are found in the water column and in sediments and polar cooling and the formation of sea-ice renders the water mass of the polar seas unique characteristics. The annual cycle of sea-ice formation and melting, the exclusion of salts during ice formation and the absence of wind mixing, result in a very stable and highly stratified water column (Aagaard *et al.*, 1981). The sea-ice itself harbours a unique community dominated by microorganisms, often referred to as the SIMCO (sea-ice microbial community) (Karl, 1993).

The Arctic Ocean is perennially ice covered, surrounded by continents (see Fig. 1.1) and receives 10% of the freshwater flowing into the world's ocean. The Arctic Ocean communicates with the North Atlantic and the North Pacific only via relatively narrow straits (Bering and Fram Straits, Norwegian Sea) and sea-ice tends to accumulate here even in summer.

The Southern Ocean is quite different from the Arctic Ocean. It surrounds the continent Antarctica (see Fig. 1.2) and when Antarctica and Australia separated, the Antarctic Circumpolar Current originated, isolating the waters south of the Polar Front from the southern parts of the Atlantic, Pacific and Indian Oceans.

Figure 1.1. Location map of the Arctic Region with the Arctic Ocean and the surrounding continents. The sampling site is indicated by a red star.

These strong circumpolar currents dispatch a large fraction of the Antarctic sea-ice as soon as it breaks up in springtime. The Southern Ocean receives almost no freshwater inflow and no terrestrially derived nutrients (Kumar *et al.*, 1995). A large diversity of microbial habitats exists in this cold Antarctic Ocean ranging from hypersaline and cold sea-ice environments to the open ocean habitats of the Antarctic Circumpolar Current and the geothermally heated waters of the Scotia Arc.

Figure 1.2. Map of Antarctica with the surrounding ocean. Sampling sites are indicated by red stars.

1.2 The Antarctic ice-free areas

1.2.1 McMurdo Dry Valleys

The McMurdo Dry Valleys of Southern Victoria Land represent the largest ice-free area in Antarctica (about 4800 km^2), located on the western coast of Ross Sea (77°0′5″S-162°52′5″E) and are ice-free for approximately the last 4 million years. They belong to the most extreme and cold deserts of the world with temperatures ranging from –55°C to 5°C and a precipitation of less than 10 cm per year. The Dry Valleys were formed by the advances and retreats of glaciers through the coastal areas of the Transantarctic Mountains, which act as a barrier to the flow of ice from the polar plateau. The McMurdo Dry Valleys presently contain more than 20 permanent lakes and ponds, which vary in character and are considered to be very old, probably hundreds of thousands of years. One of the Dry Valleys, the Taylor Valley, has a few major lakes, Lake Fryxell, Lake Bonney and Lake Hoare (see Fig. 1.3) and these are fed by 15 glaciers and are the remnants of a large glacial lake, Lake Washburn, which existed 10 000-20 000 years ago (Doran *et al.*, 1994). Some glaciers are in direct contact with the lakes, for example, the Taylor Glacier for Lake Bonney and the Canada Glacier for Lake Hoare. The Taylor Valley is a mosaic of ice-covered lakes, streams, arid soil, permafrost and surrounding glaciers. Wind and water are the two forces responsible for the transport of materials between different sites (Lyons *et al.,* 2000).

The lakes of the Taylor valley are different in many aspects (Roberts & Laybourn-Parry, 1999; Takacs & Priscu, 1998; Roberts *et al.*, 2000).

Lake Fryxell has a maximum depth of 20 m, a surface area of 7 km^2 and is a permanently stratified meromictic lake[1] with a brackish monimolimnion. Water temperatures during the summer range between 0.01 and 2.7 °C, with temperature increasing with depth. The chemocline is situated at 9.5 m with an anoxic layer below.

In contrast, Lake Hoare is effectively a freshwater, amictic system[2]. It has a maximum depth of 34 m and a surface area of 3 km^2. Summer temperatures in the water column range between 0.01 and 1.0 °C. Some physico-chemical properties

[1] In meromictic lakes the water is seasonally and partially mixed. These lakes have a mixed upper oxic layer (mixolimnion), a lower stagnant anoxic layer (monimolimnion), which never mixes with the upper layer and a pycnocline (density gradient) which forms a physical barrier to the mixing of the water (Bowman *et al.*, 2000b).

[2] An amictic system is a lake where no mixing occurs.

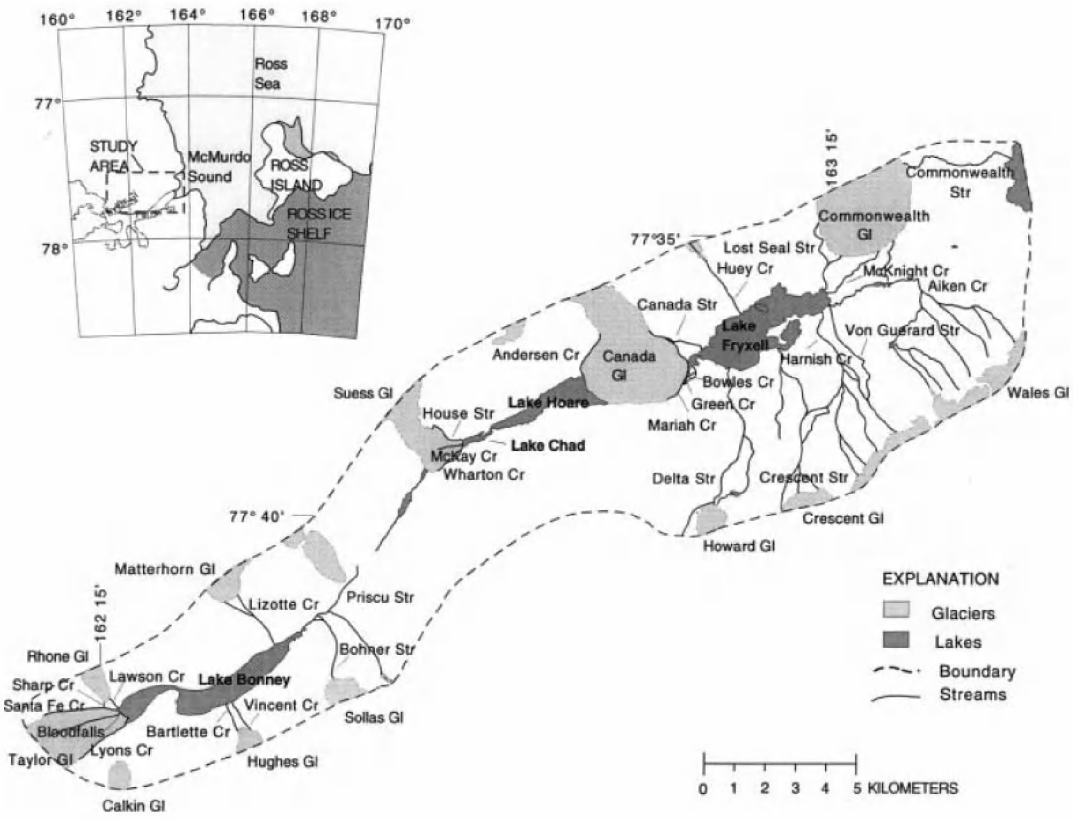

Figure 1.3. Location map of lakes Fryxell, Hoare and Bonney in the Taylor Valley, Southern Victoria Land, Antarctica (from Lyons *et al.*, 2000).

of lakes Fryxell and Hoare (sampled during the MICROMAT-project) are given in Table 1.1.

Lake Bonney has a surface area of approximately 4 km^2 and a maximum depth of 40 m. The monimolimnia of both lobes of Lake Bonney are hypersaline.

Lakes in the Dry Valleys are perennially ice-covered. The ice is typically 3 to 6 m thick and contains a layer of sand and organic matter of aeolian[3] origin below the surface (Priscu *et al.*, 1998). This rock dust and debris renders the ice opaque and as a consequence, the light climate in the water column is poor. Strong conductivity, nutrient and oxygen gradients and the presence of an ice cover, create distinct layers in the water column in which the plankton lives.

[3] The term 'aeolian' means wind-born and is applicable for deposits.

Table 1.1. Physico-chemical properties for the investigated lakes of the Vestfold Hills, McMurdo Dry Valleys and Larsemann Hills.

Lake	Antarctic region	Latitude	Longitude	Elevation	Mixing status	Lake category	Maximum depth
Ace	Vestfold Hills	68° 28'	78° 11'	8,8 m	meromictic [1]	hyposaline-marine [1]	25 m [1]
Druzhby	Vestfold Hills	68° 35'	78° 18'	8,1 m	ND	fresh [1]	25 m [1]
Grace	Vestfold Hills	68° 25'	78° 27'	3 m	holomictic [1]	fresh [1]	3 m [1]
Highway	Vestfold Hills	68° 27'	78° 13'	8,1 m	holomictic [1]	hyposaline [1]	17,4 m [1]
Pendant	Vestfold Hills	68° 27'	78° 14'	2,9 m	meromictic [1]	hyposaline [1]	18,4 m [1]
Organic	Vestfold Hills	68° 27'	78° 11'	2 m	meromictic [1]	hypersaline [1]	7 m [1]
Watts	Vestfold Hills	68° 36'	78° 13'	ND	holomictic [1]	fresh [1]	29,5 m [1]
Hoare	Dry Valleys	77° 38'	162° 55'	73 m	unstratified [2]	fresh [3]	34 m [3]
Fryxell	Dry Valleys	77° 37'	163° 09'	18 m	meromictic [2]	fresh-brackish [3]	20 m [3]
Reid	Larsemann Hills	76° 22'	69° 23'	33 m	meromictic [4]	fresh-brackish [4]	3,8 m [4]

Data are from the following references: Roberts & McMinn (1999) [1], Roberts & Laybourn-Parry (1999) [2], Doran *et al.* (1994) [3] and personal communication (W. Vyverman) [4]. ND, no data available.

1.2.2 Vestfold Hills

The Vestfold Hills is an ice-free oasis of about 400 km^2, which lies on the eastern side of Prydz Bay on the Ingrid Christiansen Coast, East Antarctica (68°40′ S, 78°35′ E). The lakes of the Vestfold Hills are relatively young, about 8000-10 000 years old and were formed during isostatic uplift (Adamson & Pickard, 1986). The climate of this area is cold, dry and windy, due to the dry winds from the continental plateau and moist oceanic winds from the north-east. The resulting landscape is seeded with hundreds of lakes ranging in size from small ponds to large lakes up to 140 m deep. The lakes closer to the ice sheet are typically freshwater (Laybourn-Parry & Marchant, 1992). In contrast, the lakes closer to the coast are often saline to hypersaline and result from the entrapment of seawater in depressions as the land rose out of the sea or from fjords cut off from the sea. The salt of some of these marine lakes has subsequently been flushed out by glacial meltwater and the lakes ultimately became freshwater (Bird *et al.*, 1991).

The lakes of the Vestfold Hills range from large and deep freshwater ultra-oligotrophic systems (Crooked Lake) to smaller oligotrophic freshwater lakes, brackish lakes, saline meromictic lakes and hypersaline monomictic[4] lakes. These can be divided into different geographical groups (Gibson, 1999), see Fig. 1.4.

Long Peninsula has the greatest concentration of lakes in the Vestfold Hills and these lakes are generally small and shallow. The largest lakes are *Ace, Pendant* and *Abraxas* and are surrounded by marine terraces. *Pendant Lake* is a lake with freshwater on top and salty underneath and a great deal of biological activity. *Organic Lake* is a shallow meromictic lake with unusually high levels of dimethylsulfide in its bottom waters (Franzmann *et al.*, 1987). This hypersaline lake is richly served by organic inputs from penguins. *Highway Lake* is a long, narrow freshwater lake between Long Fjord and Taynaya Bay. A lake at the northern part of Long Peninsula, near the ice plateau is *Grace Lake*.

Broad Peninsula contains two groups of meromictic lakes and the first group of lakes (*Ekho, Shield, Oval* and *Farrell*) lies in depressions that were once part of a fjord-like system, which was isolated from the ocean. All these lakes are surrounded by marine terraces and are hypersaline, suggesting the evaporation of water after the connection with the sea was disrupted and probably a limited input of freshwater.

[4] A monomictic (or holomictic) lake is completely mixed in contrast to a meromictic system where only the upper water layer is mixed.

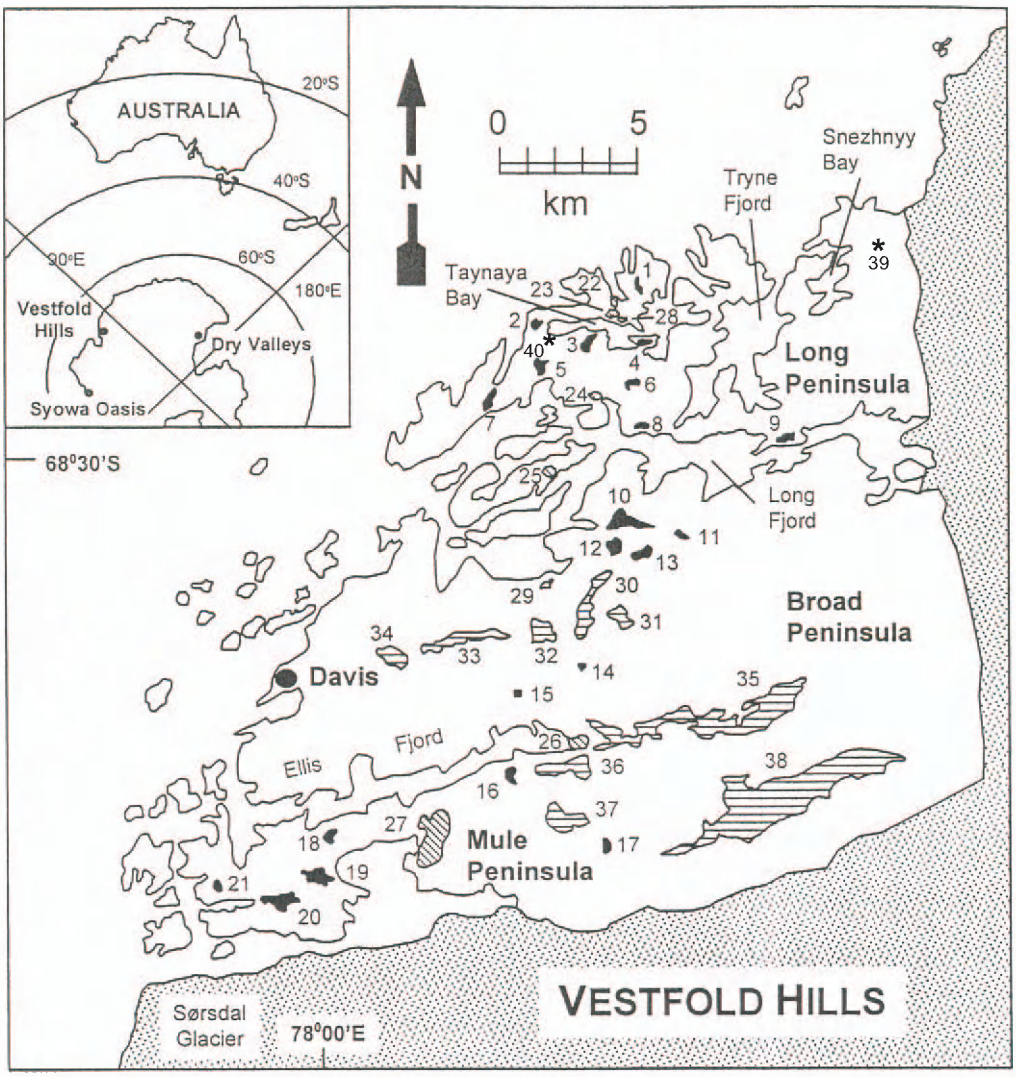

Figure 1.4. Locations of the meromictic lakes of the Vestfold Hills, Antarctica (from Gibson, 1999). The lakes and basins are: 1. unnamed lake; 2. **Organic Lake**; 3. **Pendant Lake**; 4. Glider Lake; 5. **Ace Lake**; 6. unnamed lake; 7. Williams lake; 8. Abraxas Lake; 9. Johnstone Lake; 10. Ekho Lake; 11. Lake Farrell; 12. Shield Lake; 13. Oval Lake; 14. Ephyra Lake; 15. Scale Lake; 16. Lake Anderson; 17. Oblong Lake; 18. Lake McCallum; 19. Clear Lake; 20. Laternula Lake; 21. South Angle Lake; 22. Bayly Bay; 23. Lake Fletcher; 24. Franzmann Lake; 25. Deprez Basin; 26. 'Small Meromictic Basin', Ellis Fjord; 27. Burton Lake; 28. Burch Lake; 29. Tassie Lake; 30. Club Lake; 31. Lake Jabs; 32. Deep Lake; 33. Lake Stinear; 34. Lake Dingle; 35. **Lake Druzhby**; 36. **Watts Lake**; 37. Lebed' Lake; 38. Crooked Lake; 39. **Grace Lake**; 40. **Highway Lake**. All lakes and basin names are official except 'Small Meromictic Basin' and the unnamed lakes. The stippling indicates continental ice. Lakes 39 and 40, indicated by a star (from Roberts & McMinn, 1999). In bold face: lakes investigated during the MICROMAT project.

The second group of meromictic lakes on Broad Peninsula (*Scale* and *Ephyra*) is characterised by relatively fresh surface water and salinity sharply increases towards the base of the water column. Probably the salt in these lakes was blown into them from hypersaline lakes located nearby.

The fourth group of lakes (*Anderson* and *Oblong*) was isolated from Ellis Fjord during isostatic rebound and these lakes are hypersaline.

The last group of lakes is located at the western end of Mule Peninsula and these lakes are quite diverse in salinity, depth and surface. There are six SIMBs (Seasonally Isolated Marine Basins) located in the Vestfold Hills and these are connected to the ocean during summer and isolated for the rest of the year when they effectively become lakes. Environmental change leads to stratified lakes losing their meromictic status and vice versa. The lakes in the Vestfold Hills are fed by relatively small snow banks, which generally melt completely during summer. The loading of the lake is thus a function of precipitation and the frequency of storm events which transport the snow into the basins.

Some physico-chemical properties of the lakes sampled during the MICROMAT-project (Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts) of the Vestfold Hills are given in Table 1.1.

1.2.3 Larsemann Hills

The Larsemann Hills are a series of ice-free peninsulas and islands along the coast of East-Antarctica, about 100 km northwest of the Vestfold Hills (between 69°20′ S, 76°00′ E and 69°30′ S, 76°30′ E). The total ice-free area covers about 200 km^2 and the highest elevations are around 180 m above sea level. There are over 150 freshwater lakes in the hills, ranging from small ponds less than 1 m deep to glacial lakes up to 10 ha and 38 m deep. The lakes are young with the oldest basins being about 9000 years old. The characteristics of the lakes vary and reflect their deglaciation history, proximity to the continental ice margin and exposure to the ocean. The main source of water is snowmelt and for the more exposed lakes, seaspray. The waters are well mixed by katabatic[5] winds. The lakes normally thaw fully or partially for up to 8 weeks during summer, but some are permanently frozen. The ice cover can reach 2 m in thickness and make up

[5] Katabatic winds are winds that flow from the high elevations of mountains, plateaus and hills down their slopes to the valleys or plains below. These winds are observed at every latitude of the globe, but nowhere are they as strong as they are in Antarctica.

more than 50% of the lake volume in shallow lakes at the end of the winter (Gillieson *et al.*, 1990).

One of the lakes of the Larsemann Hills is the shallow, meromictic Lake Reid, with a sharply stratified water column under a 1.6 m thick ice cover. It is an oval lake about 0.4 km north of Law Base and it drains southwards into Lake Scandrett. The water is heavily mineralised. Physico-chemical properties of Lake Reid (sampled during the MICROMAT-project) are given in Table 1.1.

1.3 Polar microbial habitats

1.3.1 Heterotrophic bacteria in Antarctic lakes

1.3.1.1 The plankton in Antarctic lakes

Life on the Antarctic continent is well adapted to aquatic habitats, since the aquatic communities are better protected against the extreme environmental conditions than the terrestrial communities. This is why most of the Antarctic non-marine biomass is found in the lakes. Antarctic lakes are pristine biotopes and include freshwater and saline systems that are subject to long periods of ice and snow cover, low temperatures and low levels of annual photosynthetically available radiation (PAR). The presence of an ice cover reduces light penetration into the water column and limits the interaction between the atmosphere and the lake, creating unusual gas concentrations found in these lakes. Usually Antarctic lakes are nutrient poor because the input of minerals is low during the short austral summer, when glacial melt-streams and snowmelt occur. In combination, these harsh environmental conditions and the isolation of the Antarctic continent, render such lakes among the most unproductive in the world and impose a considerable physiological stress on the organisms that inhabit them.

The survival of the organisms in these cold, dark aquatic environments is enhanced by a variety of strategies. A large proportion of Protozoa in Antarctic lakes is mixotrophic[6] (Roberts & Laybourn-Parry, 1999) and photoautotrophs are capable of extremely efficient photosynthesis at low levels of PAR. During winter, the microbial activity continues, using for example endogenous energy reserves and species enter the austral summer with relatively large, actively growing populations (Bell & Laybourn-Parry, 1999). Many life forms and interactions

[6] Mixotrophy is a combination of autotrophy and heterotrophy.

normally found in temperate lakes and streams, do not occur in the Antarctic lakes (Ellis-Evans, 1996). Their planktonic community is species poor and dominated by microbial loop organisms, including bacteria, protozoa and phytoplankton and little or no metazoans are present (Laybourn-Parry & Marchant, 1992; Laybourn-Parry *et al.*, 1997). Many of the planktonic populations occur in well-defined depth ranges within the water column (Spaulding *et al.*, 1994), indicating that stratification of food resources and environmental conditions may control the position of the plankton in the water. Motility or buoyancy are important properties of organisms living in these stable waters, enabling them to maintain their position at the most appropriate level in the water column for physiological functioning.

1.3.1.2 The benthic community in Antarctic lakes

The benthic areas of Antarctic lakes receive sufficient solar radiation and are covered by microbial mats composed primarily of cyanobacteria, diatoms and eubacteria (Vincent, 1988). These microbial mats not only differ in the relative abundance of the species, which compose the mat, but they also belong to four different morphological categories (Simmons *et al.*, 1993; Doran *et al.*, 1994).

Prostrate mats are the first category and their upper surfaces are smooth and flocculous in texture and either highly pigmented (aerobic mats) or black with a distinct H_2S odour (anaerobic mats). Prostrate mats are formed by the gliding of the filaments of the cyanobacteria over the lake bottom and form a cohesive tissue-like structure. Local environmental parameters determine whether a mat remains prostrate or develops into a different type.

Lift-off mats are the result of a combination of physical and biological processes (Wharton *et al.*, 1983). Parts of these mats tear lose from the lake bottom because of elevated gas levels within the shallow parts of the lake, and in some cases they tear completely lose and float to the ice cover where the material freezes into the ice and makes its way out through ablation. Most of this mat material is still alive and as such this is an important mechanism for the distribution of microorganisms between lakes and other environmental areas.

Some of the lift-off mats remain in place and form vertically, stable sheets and columnar structures (the third category of microbial mats). Calcite crystals are observed and may have a stabilising effect.

Pinnacle mats have solid structures consisting of super-imposed mat layers

without hollow central areas as observed in lift-off and columnar mats. These pinnacles are formed when the gliding filaments get entrapped in each other and move upward over one another towards the light (positive phototaxis).

Additionally, the mats are trapping and binding carbonates and various other minerals and these organosedimentary structures can be classified as modern stromatolites[7]. Sediment that is deposited through the perennial ice cover or carried in glacial meltwater, will settle to the lake bottom and buries parts of the microbial mats. Recolonization of these areas will probably be from cells of adjacent, unburied parts of the mat. The absence of metazoans that would disrupt the mats, the continuous influx of sediment, the availability of carbonate and the lack of strong internal currents, promote the formation and preservation of these stromatolitic structures in Antarctic lakes.

1.3.1.3 The food web in Antarctic lakes

In the Antarctic lakes, bacterial growth and production seems to mirror those of the phytoplankton (Vincent, 1981) and is limited to zones of maximum photosynthesis, suggesting nutrient cycling between these two groups. The majority of the biomass and biological activity of plankton is found at the bottom of the water column at the oxic-anoxic interface and this planktonic layer is often referred to as the 'Deep Chlorophyl Maximum' or DCM. Total bacterial counts also increase near the sediment-water interface and this is probably due to the presence of dissolved organic matter from the microbial mat on the sediment surface (Mikell *et al.*, 1984).

In Ace Lake, stable stratification and the resulting physico-chemical conditions have led to the development of two distinct communities: an aerobic mixolimnion community of prokaryotic and eukaryotic microorganisms, with a small number of metazoans, and an anaerobic community dominated by prokaryotes in the anoxic waters of the monimolimnion (Bell & Laybourn-Parry, 1999). This pattern is typical for Antarctic meromictic lakes, like for example lakes in the Vestfold Hills and McMurdo Dry Valleys (Gibson, 1999; Laybourn-Parry *et al.*, 1997; Roberts *et al.*, 2000). Winter is a period of reduced but sustained microbial activity in Antarctic lakes and bacterial populations remain active and are not grazed to extinction during the winter (Tacaks & Priscu, 1998). But the limited sampling season, due

[7] Stromatolites are layered structures, sometimes of considerable size, normally formed in warm, shallow waters by mats of cyanobacteria and fossils of similar structure have been found in Precambrian rocks.

to logistical constraints, has left the autumn and winter largely unstudied and more has to be focused on winter microbial dynamics in the future to come to a more complete understanding of these lakes.

1.3.2 Oligotrophic bacteria in polar seas

1.3.2.1 Different polar sea habitats

Bacteria and other microorganisms are ubiquitous in the oligotrophic, marine environment, regardless of latitude, water depth, or distance from the coast and Arctic and Antarctic waters are no exception. There are several habitats in the polar oceans and a lot of microorganisms in these seas live in microenvironments, frequently associated with suspended particles, plants or animal surfaces, or discontinuities of the seawater and sea-ice column (Karl, 1982). In these diffusion-controlled microenvironments, which have different properties than the surrounding open waters, there is an enrichment of specialised groups of microorganisms. Bacteria attached to these surfaces are generally larger and more metabolically active than those that live free in the water column. In the sea-ice microbial community (SIMCO) bacteria concentrate in diatom assemblages, which occur either as surface populations, internal band assemblages or at the sea-ice/ seawater interface (Palmisano & Garrison, 1993).

Sea-ice is one of the most extreme environments for life on earth with temperatures ranging from 0 to -12°C and salinities from 0,1 to five times normal seawater concentration in the brine channels and pockets. Several sea-ice bacteria produce vacuoles and these structures allow them to position themselves at certain depths in the water column.

1.3.2.2 The food web in polar seas

Polar oceans suffer from extreme seasonality, since the daily light flux can exceed that in the tropics during austral summer, as a result of the length of the solar day, while this flux is significantly reduced during winter. This seasonal increase of radiant energy is not only providing PAR for the growth of phototrophic microorganisms, but is also responsible for the heating of the upper ocean, which stabilises the surface waters, especially in coastal areas, protected from deep,

wind-driven mixing and for the annual cycle of sea-ice formation. The ablation of sea-ice further stabilises the water column through the addition of low-density meltwater. As a result, during spring and summer, a phytoplankton bloom is formed, which provides carbon and energy sources to sustain the entire polar food web.

Karl *et al.* (1993) formulated a hypothetical Antarctic trophic model, which rejects the formerly, generally accepted concept of a highly efficient and simple Antarctic marine food chain where the energy is transferred from large phytoplankton cells to krill to higher trophic levels. This model however, still needs a thorough and quantitative field evaluation and at least four different phases can be hypothesised (Fig. 1.5), see Box.

Phase A (early spring) corresponds to the initiation of the phytoplankton bloom. Dissolved inorganic nutrients are removed and converted to algal biomass, the microbial loop is absent and benthic processes are dormant. Phase B (early summer) corresponds to the maximum grazing by macrozooplankton and as a response, the algal biomass shifts to smaller cells. The production of dissolved organic matter (DOM) by excretion, grazing, death and autolysis is high, bacterial and microzooplankton populations expand and benthic metabolism is high. Phase C (late summer) corresponds to the postbloom period. The phytoplankton standing stocks and production are low, bacterial and protozoan productions are high and benthic metabolism continues. Phase D corresponds to the austral winter period. Photoautotrophy is low and supplemented by chemolitho-autotrophy, bacterial cells exhibit a 'starvation-survival' response (see 1.3.2.3) in absence of DOM, microzooplankton populations are low and particle flux and benthic processes are at their annual minima. In other regions of the southern ocean, where phytoplankton standing stocks are low throughout the year and where this spring bloom does not develop, the microbial loop processes (phase C) may be more important. The heterotrophic bacteria in this Antarctic marine microbial loop rely upon the availability of low- and high-molecular-weight DOM for their carbon and energy demands. Bacterial biomass is removed by the combined effects of grazing by protozoa and higher trophic levels (HTL), death, autolysis and viral infections.

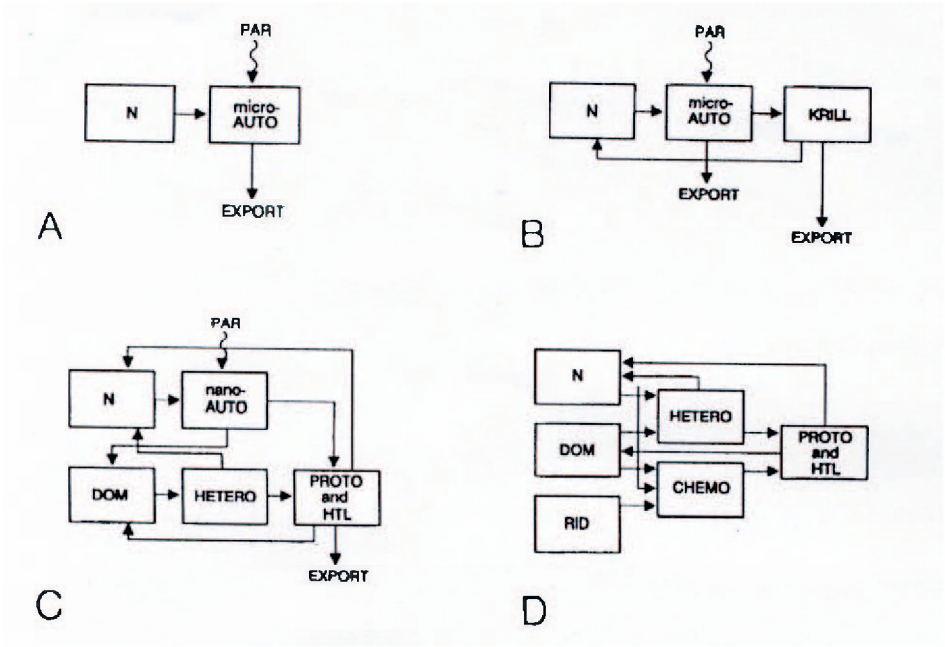

Figure 1.5. Hypothetical Antarctic trophic model (from Karl *et al.*, 1993). Phase A: early spring; phase B: late spring- early summer; phase C: late summer and phase D: winter. Micro-AUTO: autotrophic cells, primarly diatoms; PAR: photosynthetically available radiance; N: nutrients; nano-AUTO: autotrophic cells, primarly flagellates; DOM: dissolved organic matter; HETERO: heterotrophic bacteria; PROTO: protozoans; HTL: higher trophic levels; CHEMO: chemolitho-autotrophic bacterial processes and RID: reduced inorganic detritus.

1.3.2.3 The starvation-survival response

The starvation-survival response of bacteria consists of two independent processes: the need to maintain cellular integrity and to cope with low nutrient concentrations without losing the ability to respond quickly when nutrients become available again, and the need to maintain themselves against all forms of cell loss (death, predation, parasitism, etc.). The starved, non-growing cells are metabolically active and during the first days of this response, cells change their morphology from rod-shaped to coccoid and the cell diameter and optical density decreases. The net result of these changes is an increase in the surface-to-volume ratio, which increases the cells' ability to take up substrates from nutrient-limited environments (Morita, 1982) and may allow a better avoidance of predators, as protozoa prefer consuming larger bacteria (Gonzalez *et al.*, 1990). Because of the lack of energy in this low-nutrient, marine environments, the normal state of most of the bacteria, living free in these habitats, is the starvation mode and ultramicrocells are formed (Morita, 1985).

It is assumed that bacteria in most polar marine environments do not grow very rapidly and during austral winter may not grow at all. However, for members of this bacterial community, starvation is not a permanent state and unbalanced growth may be normal, with periods of growth at various rates, alternated with periods of non-growth, starvation, recovery and regrowth (Kjelleberg *et al.*, 1993), depending on the availability of nutrients.

1.3.2.4 Psychrophily and oligotrophy

The active microbial components of most of the polar environments are either psychrophilic or psychrotrophic. Psychrophilic bacteria have an optimal growth temperature of 15-20°C or lower, a maximal growth temperature at about 20-25°C and a minimal temperature at 0°C or lower. Psychrotrophic (also termed psychrotolerant) bacteria have the ability to grow at low temperatures but have their optimal and maximal growth temperatures above 15-20°C (Morita, 1975). The seasonal process of sea-ice formation with the catchment of microorganisms in the winter, exposure to severe winter conditions and release again in summertime, is thought to be responsible for the seeding of the ocean with psychrophiles.

Heterotrophic bacteria constitute the major biomass component of marine ecosystems and most of them are oligotrophic, because of the low nutrient concentrations and availability in these environments. Oligotrophic bacteria (also called oligocarbophilic or low-nutrient bacteria) are those organisms able to grow in low-nutrient media with 1-15 mg C l^{-1} or 10-50 mg C l^{-1} (Morita, 1992). Oligotrophs can be divided in two categories: facultative oligotrophs are capable of being adapted to grow at higher concentrations than the definition permits, while the obligate oligotrophs cannot be adapted to grow at higher organic carbon concentrations. Marine psychrophilic and oligotrophic bacteria are difficult to isolate and cultivate but it became clear that their oligotrophic way of life is probably a transient characteristic (Schut *et al.*, 1997).

1.4 Bacterial diversity in polar habitats

1.4.1 Diversity of heterotrophic bacteria in Antarctic lakes

Antarctic limnology has focused largely on processes and not on taxonomic investigations. Only recently this has changed with the application of molecular techniques and during the last years, bacterial diversity and taxonomy studies are being published. Most of the studies on Antarctic lakes also focus on other planktonic species than bacteria (primarily on phototrophs) and are often restricted to bacterial counts and production measurements in the water column (Laybourn-Parry *et al.*, 1995; Laybourn-Parry *et al.*, 1997; Takacs & Priscu, 1998; etc.). The first detailed study of aerobic bacteria and yeasts in Antarctic freshwater lakes was that reported for three Signy Island lakes by Ellis-Evans (1981a, b, 1982) and Ellis-Evans & Sanders (1988). Volkman *et al.* (1988) reported that the major species of bacterioplankton in Ace Lake were green sulfur bacteria and purple, methanogenic and sulfate reducing bacteria were also identified. McMeekin (1988) used culture and isolation techniques to study psychrotrophic and psychrophilic bacteria from five habitats in the Vestfold Hills area. The majority of these isolates were *Pseudomonas* spp., pigmented *Flavobacterium* spp. and non-pigmented *Moraxella* spp. Some studies focused on specialised groups of bacteria in Antarctic lakes, for example the ammonia-oxidizing bacteria, methanotrophs and cyanobacteria (Galchenko, 1994; Fritsen & Priscu, 1998; Voytek *et al.*, 1999). Several of the novel microbes cultivated from lakes of the Vestfold Hills, containing lakes of marine salinity, were found to be closely related to known marine bacteria (Franzmann & Dobson, 1993; Franzmann, 1996).

The microbial composition within the sediments of three hypersaline Antarctic lakes (Vestfold Hills) has been studied by Bowman *et al.* (2000a) using 16S rRNA clone library analysis and compared to sediments of low to moderate saline Antarctic lakes, the diversity of the hypersaline lake sediments was significantly lower. The community of Deep Lake was almost entirely made up of halophilic Archaea (*Halobacteriales*), while the sediment communities of Organic and Ekho Lake were more complex, with phylotypes clustering within the *Proteobacteria* (*Sulfitobacter*, *Silicibacter*, *Roseovarius*, *Halomonas*), *Cytophagales* (*Psychroflexus*, *Gelidibacter*) and algal chloroplasts. Several phylotypes of these lakes were related to taxa more adapted to marine-like salinity and perhaps these bacteria derive from the lower salinity surface waters and were exported into the sediment. In another study of Bowman *et al.* (2000b), the diversity and community

structure within anoxic sediment from marine saline meromictic lakes and a coastal meromictic marine basin in the Vestfold Hills was investigated. It was expected that the cold, anoxic meromictic sediments would have a relatively limited diversity but results indicate that diversity in these sediments is surprisingly high. 16S rDNA clone library analysis revealed that the abundant phylotypes were related to the low G+C Gram-positives, cyanobacteria, diatom chloroplasts, δ-*Proteobacteria* and the orders *Chlamydiales* and *Spirochaetales*. Most of the archaeal clones belonged to a group of *Euryarchaeota* and libraries of Burton Lake and Taynaya Bay contained a high diversity of *Cytophagales* phylotypes. However, it should be noticed that benthic sediments not only contain species living naturally in the sediment and anoxic water layers of the lake, but also biomass that has sunk from the upper mixing waters. Karr *et al.* (2003) reported a remarkable diversity of phototrophic purple bacteria in Lake Fryxell by analysis of a photosynthesis-specific gene, *pufM*. The distribution of these purple bacteria was highly stratified and the isolates also contained gas vesicles, structures that may be necessary for the organisms to position themselves in the water column at certain depths. Sjoling & Cowan (2003) investigated the 16S rDNA bacterial diversity in maritime meltwater lake sediments from Bratina Island and found that the bacterial population was highly diverse. Sequenced clones fell into seven major lineages of the Bacteria (α-, γ- and δ-*Proteobacteria*, *Bacteroidetes*, *Spirochaetaceae* and *Actinobacteria*), and archaeal clones belonged to the group of *Crenarchaeota*.

The conclusion of these diversity studies in Antarctic lakes, and especially in their sediments, is that the bacterial diversity in these extreme and cold environments is surprisingly high. However the more saline these lakes, the less diverse their bacterial communities are and phylogenetically they can be assigned to a few major lineages (*Proteobacteria*, *Bacteroidetes* and Gram-positives).

The extreme environmental conditions in the polar habitats have led to a high selection pressure on the organisms that live there and as a consequence, to the evolution of novel, endogenous species. The recent increase in taxonomic studies on Antarctic lakes has lead to an enormous expansion of the description of new species and in Table 1.2 this burst of new polar taxa, belonging to the Eubacteria, during the last decade has been summarised. It is clear that most of the novel taxa derive from sea-ice habitats or coastal areas, while 38 come from Antarctic lakes and 22 of these have been isolated from the benthic microbial

mats or sediments in the lakes. The list of bacteria isolated from these extreme environments will undoubtedly continue to grow.

1.4.2 Bacterial diversity in polar seas

1.4.2.1 Diversity in the sea-ice community

Polar seas are perennially cold and in some locations permanently ice covered and there is a large diversity of microbial habitats and assemblages, ranging from the sea-ice community to the open-ocean waters and the deep, anoxic sediments. Iizuka *et al.* (1966) first reported that Antarctic sea-ice contained a variety of bacteria and Sullivan & Palmisano (1984) found that a variety of morphological types of bacteria was associated with sea-ice, including rods, cocci, straight and branching filaments and fusiform and prosthecate bacteria. Of these bacteria 70% were free-living, while the other 30% were attached to living algal cells or to detritus. Staley *et al.* (1989) isolated pigmented and gas vacuolate bacteria from both sea-ice and underlaying water samples from McMurdo Sound and the bacterial isolates from the sea-ice were filamentous and pigmented, while those isolated from the water column were unicellular and non-pigmented. The highest concentrations of bacteria in sea-ice were found in conjunction with the highest algal concentrations. Phylogenetical analysis of these bacteria revealed that they belong to the *Proteobacteria* and the *Bacteroidetes* (Gosink & Staley, 1995). The sea-ice microbial community (SIMCO) contains algae (mostly diatoms), protozoa, and bacteria and recent investigations of Arctic and Antarctic sea-ice samples (Bowman *et al.*, 1997a; Brown & Bowman, 2001) indicate that these bacteria belong to a few major phylogenetic groups: the α- and γ-*Proteobacteria*, the *Bacteroidetes*, the high and low mol % G+C Gram-positives and the orders *Chlamydiales* and *Verrucomicrobiales*. Archaea associated with the SIMCO have also been reported and in this SIMCO several novel bacterial genera and species have been discovered, including *Polaromonas*, *Polaribacter*, *Psychroflexus*, *Gelidibacter*, *Octadecabacter*, etc., see Table 1.2. Junge *et al.* (2002) performed a culture-based survey of cold-adapted oligotrophs in Arctic sea-ice in order to assess the phylogenetic diversity of heterotrophic bacteria that are numerically abundant in sea-ice. The results indicate close relationships exclusively to known marine psychrophiles within two bacterial divisions: the *Proteobacteria* (the genera *Alteromonas*, *Colwellia*, *Glaciecola*, *Octadecabacter*, *Pseudoalteromonas* and

Table 1.2. The description of novel taxa from polar habitats, belonging to the *Eubacteria*, during the last decade.

Phylogenetic branch	Novel Taxa	Isolation source	Reference
Bacteroidetes	*Aequorivita antarctica*	Seawater, sea-ice, quartz stone subliths, Antarctica	Bowman & Nichols, 2002
Bacteroidetes	*Aequorivita lipolytica*	Seawater, sea-ice, quartz stone subliths, Antarctica	Bowman & Nichols, 2002
Bacteroidetes	*Aequorivita crocea*	Seawater, sea-ice, Antarctica	Bowman & Nichols, 2002
Bacteroidetes	*Aequorivita sublithincola*	Quartz stone subliths, Antarctica	Bowman & Nichols, 2002
Bacteroidetes	*Algoriphagus ratkowskyi*	Sea-ice, cyanobacterial mat, Antarctica	Bowman *et al.*, 2003c
Bacteroidetes	*Brumimicrobium glaciale*	Sea-ice, continental shelf sediment, Antarctica	Bowman *et al.*, 2003c
Bacteroidetes	*Cellulophaga algicola*	Sea ice, algal assemblage, Antarctica	Bowman, 2000
Bacteroidetes	*Crocinitomix catalasitica*	Under frozen sand, Auke Bay, Alaska, USA	Bowman *et al.*, 2003c
Bacteroidetes	*Cryomorpha ignava*	Seawater, quartz stone subliths, Antarctica	Bowman *et al.*, 2003c
Bacteroidetes	*Flavobacterium frigidarium*	Marine sediment, Adelaide Island, Antarctica	Humphry *et al.*, 2001
Bacteroidetes	*Flavobacterium gillisiae*	Sea-ice, Antarctica	McCammon & Bowman, 2000
Bacteroidetes	*Flavobacterium hibernum*	Freshwater lakes, Antarctica	McCammon *et al.*, 1998
Bacteroidetes	*Flavobacterium tegetincola*	Microbial mat, Antarctica	McCammon & Bowman, 2000
Bacteroidetes	*Flavobacterium xanthum* nom. rev.	Pool mud, Syowa Station, Antarctica	McCammon & Bowman, 2000
Bacteroidetes	*Gelidibacter algens*	Sea-ice, Ellis Fjord, Vestfold Hills, Antarctica	Bowman *et al.*, 1997b
Bacteroidetes	*Hymenobacter roseosalivarius*	Continental soils and sandstone, Dry Valleys, Antarctica	Hirsch *et al.*, 1998
Bacteroidetes	*Polaribacter filamentus*	Seawater sample, Arctica	Gosink *et al.*, 1998
Bacteroidetes	*Polaribacter franzmanii*	Sea-ice, McMurdo Sound, Antarctica	Gosink *et al.*, 1998
Bacteroidetes	*Polaribacter glomeratus* comb. nov.	Marine environment, Antarctica	Gosink *et al.*, 1998
Bacteroidetes	*Polaribacter irgensii*	Seawater sample, Antarctica	Gosink *et al.*, 1998
Bacteroidetes	*Psychroflexus gondwanense* comb. nov.	Organic Lake, Vestfold Hills, Antarctica	Bowman *et al.*, 1998d
Bacteroidetes	*Psychroflexus torquis*	Sea-ice, Prydz Bay, Vestfold Hills, Antarctica	Bowman *et al.*, 1998d
Bacteroidetes	*Psychroserpens burtonensis*	Burton Lake, Vestfold Hills, Antarctica	Bowman *et al.*, 1997b
Bacteroidetes	*Salegentibacter salegens* comb. nov.	Organic Lake, Vestfold Hills, Antarctica	McCammon & Bowman, 2000
Gram-positives	*Alicyclobacillus acidocaldarius* subsp. *rittmanni*	Soil, Mount Rittmann, Antarctica	Nicolaus *et al.*, 1998
Gram-positives	*Arthrobacter flavus*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2000
Gram-positives	*Arthrobacter roseus*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2002a
Gram-positives	*Bacillus thermantarcticus* corrig.	Geothermal soil, Mount Melbourne, Antarctica	Nicolaus *et al.*, 1996*
Gram-positives	*Clostridium bowmanii*	Microbial mat, Lake Fryxell, Antarctica	Spring *et al.*, 2003
Gram-positives	*Clostridium frigoris*	Microbial mat, Lake Fryxell, Antarctica	Spring *et al.*, 2003
Gram-positives	*Clostridium lacusfryxellense*	Microbial mat, Lake Fryxell, Antarctica	Spring *et al.*, 2003
Gram-positives	*Clostridium psychrophilum*	Microbial mat, Lake Fryxell, Antarctica	Spring *et al.*, 2003
Gram-positives	*Clostridium vincentii*	Pond sediment, McMurdo Ice Shelf, Antarctica	Mountfort *et al.*, 1997
Gram-positives	*Exiguobacterium antarcticum*	Microbial mat, Lake Fryxell, Antarctica	Frühling *et al.*, 2002
Gram-positives	*Exiguobacterium undae*	Microbial mat, Lake Fryxell, Antarctica	Frühling *et al.*, 2002

Phylogenetic branch	Novel Taxa	Isolation source	Reference
Gram-positives	*Friedmanniella antarctica*	Sandstone, Linnaeus Terrace, Dry Valleys, Antarctica	Schumann *et al.*, 1997
Gram-positives	*Friedmanniella lacustris*	Ekho Lake, Vestfold Hills, Antarctica	Lawson *et al.*, 2000
Gram-positives	*Kocuria polaris*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2003b
Gram-positives	*Leifsonia aurea*	Pond, Antarctica	Reddy *et al.*, 2003c
Gram-positives	*Leifsonia rubra*	Pond, Antarctica	Reddy *et al.*, 2003c
Gram-positives	*Micrococcus antarcticus*	Chinese Great-Wall station, Antarctica	Liu *et al.*, 2000
Gram-positives	*Modestobacter multiseptatus*	Soils from Linnaeus Terrace, Dry Valleys, Antarctica	Mevs *et al.*, 2000
Gram-positives	*Nesterenkonia lacusekhoensis*	Ekho Lake, Vestfold Hills, Antarctica	Collins *et al.*, 2002
Gram-positives	*Nocardioides aquaticus*	Ekho Lake, Vestfold Hills, Antarctica	Lawson *et al.*, 2000
Gram-positives	*Paenibacillus antarcticus*	Lake sediment, South Shetland Islands, Antarctica	Montes *et al.*, in press
Gram-positives	*Paenibacillus cineris*	Volcanic soils, Antarctica	Logan *et al.*, in press
Gram-positives	*Paenibacillus cookii*	Volcanic soils, Antarctica	Logan *et al.*, in press
Gram-positives	*Planococcus antarcticus*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2002b
Gram-positives	*Planococcus maitriensis*	Cyanobacterial mat, Schirmacher Oasis, Antarctica	Alam *et al.*, 2003
Gram-positives	*Planococcus psychrophilus*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2002b
Gram-positives	*Planomicrobium mcmeekinii* comb. nov.	Sea-ice, McMurdo Sound, Antarctica	Yoon *et al.*, 2001
Gram-positives	*Rhodoglobus vestalii*	Lake, Dry Valleys, Antarctica	Sheridan *et al.*, 2003
Gram-positives	*Sporosarcina macmurdoensis*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2003a
α-*Proteobacteria*	*Antarctobacter heliothermus*	Ekho Lake, Vestfold Hills, Antarctica	Labrenz *et al.*, 1998
α-*Proteobacteria*	*Octadecabacter antarcticus*	Sea-ice, Antarctic water samples	Gosink *et al.*, 1997
α-*Proteobacteria*	*Octadecabacter arcticus*	Sea-ice, Arctic water samples	Gosink *et al.*, 1997
α-*Proteobacteria*	*Roseovarius tolerans*	Ekho Lake, Vestfold Hills, Antarctica	Labrenz *et al.*, 1999
α-*Proteobacteria*	*Sphingomonas aerolata*	Ice of Taylor Dome, soil around Scott Base, Antarctica	Büsse *et al.*, 2003
α-*Proteobacteria*	*Staleya guttiformis*	Ekho Lake, Vestfold Hills, Antarctica	Labrenz *et al.*, 2000
α-*Proteobacteria*	*Sulfitobacter brevis*	Ekho Lake, Vestfold Hills, Antarctica	Labrenz *et al.*, 2000
β-*Proteobacteria*	*Polaromonas vacuolata*	Marine waters, Antarctica	Irgens *et al.*, 1996
β-*Proteobacteria*	*Rhodoferax antarcticus*	Microbial mat, Ross Island, Antarctica	Madigan *et al.*, 2000
γ-*Proteobacteria*	*Colwellia demingiae*	Sea-ice, diatom assemblages, Antarctica	Bowman *et al.*, 1998b
γ-*Proteobacteria*	*Colwellia hornerae*	Sea-ice, diatom assemblages, Antarctica	Bowman *et al.*, 1998b
γ-*Proteobacteria*	*Colwellia psychrotropica*	Sea-ice, diatom assemblages, Antarctica	Bowman *et al.*, 1998b
γ-*Proteobacteria*	*Colwellia rossensis*	Sea-ice, diatom assemblages, Antarctica	Bowman *et al.*, 1998b
γ-*Proteobacteria*	*Glaciecola pallidula*	Sea-ice, Antarctica	Bowman *et al.*, 1998c
γ-*Proteobacteria*	*Glaciecola punicea*	Sea-ice, Antarctica	Bowman *et al.*, 1998c
γ-*Proteobacteria*	*Methylosphaera hansonii*	Meromictic lakes, Vestfold Hills, Antarctica	Bowman *et al.*, 1997d
γ-*Proteobacteria*	*Oleispira antarctica*	Coastal seawater, Ross Sea, Antarctica	Yakimov *et al.*, 2003
γ-*Proteobacteria*	*Pseudoalteromonas antarctica*	Coastal areas, Antarctica	Bozal *et al.*, 1997

Phylogenetic branch	Novel Taxa	Isolation source	Reference
γ-*Proteobacteria*	*Pseudoalteromonas prydzensis*	Sea-ice, Antarctica	Bowman, 1998a
γ-*Proteobacteria*	*Pseudomonas antarctica*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2004
γ-*Proteobacteria*	*Pseudomonas meridiana*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2004
γ-*Proteobacteria*	*Pseudomonas proteolytica*	Microbial mat, pond, Dry Valleys, Antarctica	Reddy *et al.*, 2004
γ-*Proteobacteria*	*Psychrobacter fozii*	Coastal marine environments, Antarctica	Bozal *et al.*, 2003
γ-*Proteobacteria*	*Psychrobacter frigidicola*	Ornithogenic soils, Antarctica	Bowman *et al.*, 1996
γ-*Proteobacteria*	*Psychrobacter glacincola*	Sea-ice, Vestfold Hills area; Amery Ice Shelf, Antarctica	Bowman *et al.*, 1997e
γ-*Proteobacteria*	*Psychrobacter immobilis*	Ornithogenic soils, Antarctica	Bowman *et al.*, 1996
γ-*Proteobacteria*	*Psychrobacter luti*	Coastal marine environments, Antarctica	Bozal *et al.*, 2003
γ-*Proteobacteria*	*Psychrobacter proteolyticus*	Antarctic krill (*Euphausia superba*)	Denner *et al.*, 2001
γ-*Proteobacteria*	*Psychrobacter urativorans*	Ornithogenic soils, Antarctica	Bowman *et al.*, 1996
γ-*Proteobacteria*	*Psychromonas antarcticus*	Pond sediment, McMurdo Ice Shelf, Antarctica	Mountfort *et al.*, 1998
γ-*Proteobacteria*	*Psychromonas arctica*	Arctic seawater, Spitzbergen	Groudieva *et al.*, 2003
γ-*Proteobacteria*	*Saccharophilum impatiens*	Ekho Lake, Vestfold Hills, Antarctica	Labrenz *et al.*, 2003
γ-*Proteobacteria*	*Shewanella frigidimarina*	Sea-ice and coastal areas, Antarctica	Bowman *et al.*, 1997c; Bozal et al., 2002
γ-*Proteobacteria*	*Shewanella gelidimarina*	Sea-ice, Antarctica	Bowman *et al.*, 1997c
γ-*Proteobacteria*	*Shewanella livingstonensis*	Coastal areas, Antarctica	Bozal *et al.*, 2002
δ-*Proteobacteria*	*Desulfofaba gelida*	Arctic marine sediments, Svalbard	Knoblauch *et al.*, 1999
δ-*Proteobacteria*	*Desulfofrigus fragile*	Arctic marine sediments, Svalbard	Knoblauch *et al.*, 1999
δ-*Proteobacteria*	*Desulfofrigus oceanense*	Arctic marine sediments, Svalbard	Knoblauch *et al.*, 1999
δ-*Proteobacteria*	*Desulfotalea arctica*	Arctic marine sediments, Svalbard	Knoblauch *et al.*, 1999
δ-*Proteobacteria*	*Desulfotalea psychrophila*	Arctic marine sediments, Svalbard	Knoblauch *et al.*, 1999

* *Bacillus thermantarcticus* corrig.: validated in 2002 (*Int J Syst Evol Microbiol*, 52: 3-4).

Shewanella) and the *Bacteroidetes* (*Cytophaga*, *Flavobacterium*, *Gelidibacter* and *Polaribacter*). A comprehensive assessment of bacterial diversity and community composition in Arctic and Antarctic pack ice was conducted through cultivation and cultivation-independent molecular techniques (Brinkmeyer *et al.*, 2003). Results confirmed that at both poles the α- and γ-*Proteobacteria* and the *Cytophaga-Flavobacterium* group were the dominant taxonomic bacterial groups.

Overall, these results indicate a limited bacterial diversity for the numerically important microorganisms in sea-ice compared to the water column and there are several reasons to explain this lower genetic diversity. Firstly, the sea-ice environments are geologically recent developments on Earth so relatively little time has been available for the evolution of highly diverse sea-ice bacteria. Secondly, the sea-ice environment is an extreme physical habitat and as a consequence, the bacterial communities are dominated by a few populations uniquely adapted to survive and grow under these extreme conditions. Finally, sea-ice is also a porous habitat with many attachment sites that may select for specific types of bacteria (Junge *et al.*, 2002).

North and South Pole sea-ice communities also provide a special test case for bacterial dispersal between the poles in a biogeographical study and finding the same species at both poles would indicate that these bacteria are cosmopolitan in distribution. However, several constraints exist on the dispersal between the poles since the long distance between the polar regions makes the transport of the microorganisms very difficult and the psychrophilic bacteria of the SIMCO would probably not survive the warmer temperatures (>20°C) at the equator. The polar oceans also evolved independently and exhibit differences in environmental conditions. Until now, a bipolar species has not been found in sea-ice (Staley & Gosink, 1999), supporting the polar endemism theory, however this is only applicable at the species level and some psychrophilic, sea-ice genera are present at both poles (for example *Polaribacter* and *Octadecabacter*). Only recently, a bipolar distribution of a bacterial species (*Shewanella frigidimarina*) has been suggested by Junge *et al.* (2002). Arguments against this polar endemism theory are related to the efficient dispersal abilities of certain organisms (bacteria that produce spores or cysts) and several aerobiological studies have demonstrated the transport of a wide range of propagules from South America through Drake Passage under ideal weather conditions (Clarke, 2003). Another possibility for transequatorial passage are the cold, deep underwater currents and deep bottom water produced in the Antarctic Weddel Sea for example, which may act as a cold

corridor driving northwards into the Atlantic Ocean. However, these currents take hundreds of years to carry water from one pole to another and it is extremely doubtful that these bacteria, which have been removed from their normal habitat, could survive such a long transit. Alternatively, the passage across the equator in ice crystals in the upper atmosphere could be another mechanism, but there is no evidence to indicate that this occurs.

1.4.2.2 Diversity in the Southern ocean

There is little information on the phylogenetic composition of bacterial assemblages in polar oceans (except for the sea-ice communities) and an important question is whether the composition of bacterial communities in cold polar oceans has diverged substantially from those in temperate and tropical waters. For example, certain cyanobacteria are ubiquitous and important members of plankton communities in temperate and tropical oceans, yet they are not found in polar seas. Similarly, gas vacuolate bacteria are important in sea-ice communities, but they have not been reported in temperate or tropical seas. Also, the question whether the bacterioplankton communities in polar oceans are the same or different, with important biogeographical information, still needs to be addressed. In 1998, Murray *et al.* investigated the seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. Results revealed that the bacterial assemblage composition may reflect changes in water column stability, depth or season. Lopez-Garcia *et al.* (2001) investigated the diversity of free-living prokaryotes from a deep-sea site at the Antarctic Polar Front using molecular techniques. This deep-sea planktonic community is phylogenetically related to α- (SAR11), γ- and δ- (SAR324) *Proteobacteria*, *Cytophagales*, *Planctomyces*, Gram-positives and the group of environmental sequences SAR406. Among them γ-proteobacterial sequences were the most abundant and diverse and within the Archaea, euryarchaeotal sequences were retrieved. The sequences of uncultured SAR-groups are evolutionarily distant from all known isolates and several of these groups appear to be widely distributed in the world ocean. SAR11 for example, initially found in the Sargasso Sea, has been identified in different seas and at different depths, including Antarctic surface waters. Hollibaugh *et al.* (2002) analysed the phylogenetic compositions of ammonia-oxidizing bacteria of the β-*Proteobacteria* from Southern Ocean samples. They found a *Nitrosospira*-like 16S rRNA gene sequence in all samples and this

sequence was also found in Arctic Ocean samples (Bano & Hollibaugh, 2000), indicating a transpolar (if not global) distribution. However slight differences between Arctic and Antarctic sequences may be evidence of polar endemism. Bowman *et al.* (2003a; b) reported about the prokaryotic activity and community structure in continental shelf sediments, located off eastern Antarctica. Biomass and activity were maximal within the 0- to 3-cm depth range and declined rapidly with sediment depths below 5 cm. The culturable bacterial population was predominantly psychrophilic and many of the identified isolates belonged to genera characteristic of deep-sea habitats, although most appear to be novel species. Sequencing of DGGE bands, 16S rDNA clone library analysis and rRNA probe hybridization analysis revealed that the major community members belonged to δ-*Proteobacteria*, putative sulphide oxidizers of the γ-*Proteobacteria*, flavobacteria, *Planctomycetales* and *Archaea*.

1.4.2.3 Diversity in the Arctic Ocean

As data are emerging for the Southern Ocean, the number of comparable studies of the Arctic Ocean is also increasing. Ferrari & Hollibaugh (1999) used DGGE banding patterns to compare the composition of bacterial assemblages in the Arctic Ocean, however no sequence information was provided. Yager *et al.* (2001) showed that the composition and physiological properties of bacterial assemblages in the Chukchi Sea changed in response to an algal bloom and a few 16S rDNA sequences were provided. Ravenschlag *et al.* (2001) investigated the microbial community in marine Arctic sediments (Svalbard) through quantitative molecular analysis and found that high fractions of Bacteria were present and phylogenetically these belong to the β-, γ- and δ-*Proteobacteria*, the *Bacteroidetes*, the *Planctomycetales* and Gram-positive bacteria. Besides δ-proteobacterial sulphate-reducing bacteria, members of the *Bacteroidetes* were the most abundant group detected in this sediment and these results are comparable with those of a previous study of Ravenschlag *et al.* (1999) where cold, Arctic sediments were investigated near Spitsbergen. They found a predominance of bacteria of the sulphur cycle, of which several belonged to the γ-*Proteobacteria*. In a study of Bano & Hollibaugh (2002) where Arctic Ocean samples collected over three seasons were investigated by sequencing clones, evidence was found that the Arctic bacterioplankton assemblage was composed of a mixture of uniquely polar and cosmopolitan phylotypes. All clones fell into the α-, γ-, δ- and ε-*Proteobacteria*,

the *Bacteroidetes*, the *Verrucomicrobiales* and the green non-sulfur bacteria. The majority of clones belonged to the α- and γ-*Proteobacteria* while none of the clones grouped with the β-*Proteobacteria*. Some of the phylotypes were similar to isolate sequences but the majority were most closely related to uncultured, environmental sequences. Prominent among these were members of the SAR11 group. DGGE fingerprints showed that most of the bands were common to all samples in all three seasons, but additional bands, representing sequences related to *Cytophaga* and *Polaribacter* appeared in samples collected during summer and fall.

In conclusion, the continued diversity studies of bacterial communities in polar seas suggest that the diversity of bacteria is high, with phylogenetic lineages in the *Proteobacteria*, the *Bacteroidetes*, Gram-positives, *Verrucomicrobiales* and *Planctomycetales*. However, more work on these extremely cold habitats has to be done to allow comparison with studies about bacterial diversity of habitats in moderate temperature regions.

1.5 Importance of polar microorganisms

1.5.1 Industrial applications

Prokaryotes dominate many polar ecosystems and play major roles in food chains and biogeochemical cycles. The availability of novel Arctic and Antarctic species, isolated from these extreme habitats, opens perspectives for possible biotechnological exploration and these unique environments represent a biodiversity resource of huge dimensions, of which relatively little is known. Culture collections are important for the long-term availability of these strains and their genes, and for the preservation of the strains and organisms for biotechnological research. However, only few publicly accessible collections exist which hold microorganisms isolated from Arctic and Antarctic habitats, for example ACAM (Australian Collection of Antarctic Microorganisms), BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Belgium), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), etc.

1.5.1.1 PUFA-production

It is well known that lipid composition of the membrane changes in response to temperature and psychrophilic and psychrotrophic bacteria contain more (poly) unsaturated, branched and/or cyclic fatty acids (Rotert *et al.*, 1993). Research with polyunsaturated fatty acids (PUFA) producing, Antarctic strains revealed undescribed new taxa within the genera *Shewanella* and *Colwellia* (Bowman *et al.*, 1997c; Bowman *et al.*, 1998b). Several *Shewanella* species contained proportions of eicosapentaenoic acid (EPA; 20:5ω3) and members of the genus *Colwellia* produced docosahexaenic acid (DHA; 22:6ω3). Nichols *et al.* (1997) reported about an Antarctic bacterium that produced both EPA and arachidonic acid (AA; 20:4ω6) in response to the growth temperature, whereas Jøstensen & Landfald (1997) found a high prevalence of PUFA producing bacteria in Arctic invertebrates. It is considered that the benefit of PUFA to cold-adapted organisms derives from their stabilisation of the lipid phase at low temperatures, in addition to their fluidising effect in the membrane (Russell & Nichols, 1999). Nichols *et al.* (1996) found a novel $C_{31:9}$ polyene in sea-ice microbial communities and Helmke *et al.* (2000) reported about a nearly symmetric polyene in cell extracts from some psychrophilic and barophilic bacterial strains, isolated from sea-ice and deep-sea samples. The polyene is considered to play a role in primary metabolism with a possible function in temperature and pressure adaptation.

Provision of dietary PUFA, especially the fatty acids EPA and DHA, is essential for normal growth and development of the larvae of many aquaculture species. In addition to microalgae, PUFA producing bacteria can be used in aquaculture diets, either as extracts or by direct addition to feed and this is an expanding area of interest (Nichols *et al.*, 1999). PUFA producing bacteria have been used to enrich rotifers, a food organism for larval fish and PUFA's are also added to human diets since they proved to be beneficial for human health. Normally, most of these PUFA's derive from fish oils but since fish stocks are diminishing world wide, PUFA producing bacteria may be an alternative solution. Several important stages in the optimization of PUFA production and storage still need to be investigated.

1.5.1.2 Cold-adapted enzymes

Cold-adapted enzymes are produced by organisms living in permanently cold habitats located in polar zones, at high altitudes or in the deep-sea, sea-ice,

seawater, sediments, snow and permafrost. Low temperatures have a strong negative effect on biochemical reactions, but organisms living in these cold conditions can survive through adaptations in their membranes, proteins and enzymes. These enzymes can be used to study the adaptations of life to low temperatures and have potential biotechnological applications (McMeekin *et al.*, 1993; Nichols *et al.*, 1999). A range of industries and products can benefit from these enzymes, like for example cleaning agents and detergents, leather processing, textile industry, food processing (fermentation, cheese manufacture, meat industry and bakery), and molecular biology (heterologous gene expression) with potential biomedical products.

Psychrophilic enzymes have maximal catalytic activity at temperatures below 30-50°C and usually display some degree of thermolability. Recently, much research has focused on the protein structural characteristics of this cold adaptation (Feller & Gerday, 1997). As temperature decreases, enzymes demonstrate a decline of their catalytic rate due to the reduction of structural flexibility and eventually they undergo cold denaturation. The tertiary and quaternary structures of psychrophilic enzymes are more open and flexible with better access of substrates to the active site at lower temperatures and show a high catalytic efficiency (Gerday *et al.*, 2000). Psychrophilic enzymes are not only useful for their high specific activity, thereby reducing the amount of enzyme needed, but also for their easy inactivation, which can prevent the prolonged action of some enzymes.

Antarctic bacteria, especially those derived from ice, are good sources of psychrophilic enzymes and the presence of protease, β-galactosidase, phosphatase and amylase enzymes with strong cold adaptations has been found in several of these strains. McCammon *et al.* (1998) isolated a lactose utilizing bacterium from a freshwater Antarctic lake. The use of this cold-adapted β-galactosidase can help in the processing of dairy foods to solve the problem of lactose intolerance. Indeed, the optimal temperature for hydrolysis of lactose by conventionally used β-galactosidases ranges between 30 and 40°C, which is also the ideal temperature for mesophiles, contaminating and spoiling the dairy products.

1.5.1.3 Bioremediation

Antarctica is generally considered one of the last remaining pristine environments, however over the past decade, a number of fuel spills have occurred and this has resulted in research about the hydrocarbon degradation by Antarctic microorganisms (Delille *et al.*, 1997; Cavanagh *et al.*, 1998). Bacteria capable of degrading n-alkanes and aromatics were isolated and novel intermediate products suggest that these bacteria harbour novel degradation pathways. Organic Lake is nutrient-rich and contains naturally occurring hydrocarbons. From this lake, strains have been isolated with the ability to degrade various types of hydrocarbons, like hexadecane and phenanthrene (McMeekin *et al.*, 1993). The degradation of xenobiotic compounds, more specific poly-chlorinated biphenyls in Arctic soil has been demonstrated by Master & Mohn (1998). The high specificity and catalytic activity of these cold-adapted enzymes, capable of hydrocarbon degradation, makes them ideal candidates for the bioremediation of recalcitrant chemicals and offers a feasible alternative to physicochemical methods.

1.5.1.4 Biocatalysis under low water conditions

The commercial synthesis of several valuable compounds (fatty acid esters, peptides, oligosaccharide derivates) are often obtained from substrates with poor solubility in aqueous media and this process might be improved by using enzymes operating under low water conditions. In these systems, the level and distribution of residual water is important because the catalytic efficiency of enzymes is often a strong function of the hydration state and when the associated water falls below a certain level, the enzymes become more rigid. Overall, under very low hydration conditions, enzyme efficiency is generally poor and reaction kinetics are too slow. Psychrophilic enzymes might therefore have a potential advantage for application under low water conditions, because of their inherent greater flexibility. This will be particularly useful in conditions wherein the activity of mesophilic and thermophilic enzymes is severely impaired by an excess of rigidity (Gerday *et al.*, 2000).

1.5.1.5 Anti-freeze proteins

The presence of anti-freeze proteins in many cold-adapted organisms prevents the formation of ice and these proteins offer significant potential for biotechnological exploitation. The exogenous addition of anti-freeze proteins in the manufacture of frozen food stuffs to enhance freeze-thaw properties has been considered and the in vivo expression of anti-freeze protein genes in transgenic plants or animals offers opportunities for expanding crop-production or food storage properties (Cowan, 1997). The functionally related ice-nucleation proteins from psychrotrophic and phytopathogenic bacteria (Swings *et al.*, 1990) have already commercially been used in the process of snow-making, but also have potential applications in the production of ice-cream and similar food stuffs and via transgenic expression, in the prevention of frost damage in economic crops.

1.5.1.6 Pigments

Several microorganisms living in polar habitats produce pigments (for example cyanobacteria which produce carotenoids, scytonemin, fucoxanthin, etc.) and these pigments protect the microbial communities against the damaging effect of UV-radiation on living cells. This is especially true for the poles, where solar radiation is continuous in summer and the UV-dose is very high, due to the ozone hole. However, the production of pigments may also be an adaptation to other forms of environmental challenges experienced at low temperatures, such as salt stress and research on carotenoids (and perhaps pigments in general) in bacteria from the briny pockets of sea-ice probably will reveal new links between psychrophily and halophily (or halotolerance) (Fong *et al.*, 2001). The production of these novel pigments opens perspectives for several industries like the food and textile industry.

1.5.2 Exobiology

Exobiology considers the question of the origin and distribution of life in the universe and the most likely candidates for harbouring microbial life in our solar system, now and in the past, are Mars and Europa. Mars is a cold and dry planet with a thin atmosphere and there are indications of present polar ice caps and past water. The recent Mars exploration mission in search of answers about the

history of water and the presence of life on Mars shows that this subject is still very up to date. Antarctic ecosystems are relevant for Mars' exobiology in two ways: they provide models for possible Martian habitats and the study of microorganisms in Antarctic environments can be used for the development of methods to locate and identify microbial forms on Mars (Wynn-Williams & Edwards, 2000). Not only the Antarctic ice-covered lakes provide possible Martian analogs, but also the cryptoendolithic communities in Antarctic rocks and the volcanoes on the Antarctic continent may act as relevant models.

Europa, one of the moons of Jupiter, is also of interest to exobiology, because of the possibility of a liquid water ocean under an outer shell of ice. The subglacial Antarctic lake Vostok possesses a perennially thick ice cover of 3 km and provides a good model for the potential europan biosphere (McKay, 1993).

The theory of 'panspermia' holds that reproductive bodies of living organisms can exist throughout the universe and develop wherever the environment is favourable with transport of life from one planet to the other. Since space is extremely cold and suffers from severe radiation and other extreme conditions, research in analogous extreme terrestrial habitats may help to test this theory (Rothschild & Mancinelli, 2001).

1.6 Conceptual framework

The first section of this thesis will focus on the diversity of heterotrophic bacteria of the mat communities in diverse freshwater and saline Antarctic lakes. With the MICROMAT-project almost 800 bacterial strains were isolated in the lab of Microbiology (Ghent) from mats collected from 10 different lakes from the McMurdo Dry Valleys (lakes Hoare and Fryxell), the Vestfold Hills (lakes Ace, Pendant, Druzhby, Organic, Grace, and Watts) and the Larsemann Hills (Lake Reid) (Van Trappen *et al.*, 2002). These strains could be assigned to 41 clusters by numerical analysis of their fatty acid profiles and 31 strains formed single branches. 16S rDNA sequence analysis of representative strains revealed that they belong to the α-, β- and γ- *Proteobacteria*, the high and low percent G+C Gram-positives and to the *Bacteroidetes* and many clusters represent as yet unnamed new taxa (see chapter 2). More detailed analysis is needed to determine the species diversity within each of the FAA clusters and several novel taxa can be described using a polyphasic taxonomic approach (Vandamme *et al.*, 1996), which combines different genotypic and phenotypic methods.

My PhD-work started with the investigation of the genomic diversity of these bacterial strains from Antarctic microbial mats, belonging to the fatty acid clusters 1 to 15 (phylogenetically related to the *Bacteroidetes*) and fatty acid cluster 41 (related to the α-subclass of the *Proteobacteria*), by using the repetitive extragenic palindromic DNA (rep)-PCR fingerprinting technique (Rademaker & de Bruijn, 1997). Rep-PCR fingerprinting of the isolates allowed a further subclustering at the genotypic level and it is clear, through studies that compare rep-PCR genomic fingerprint analysis with DNA-DNA relatedness methods, that both techniques yield results that are in close agreement (Nick *et al.*, 1999; Rademaker *et al.*, 2000). Therefore, rep-PCR fingerprinting was used as a genomic screening method to differentiate at the species- to subspecies-level and to select representatives for additional 16S rDNA sequence analysis, to obtain a phylogenetic allocation of the different rep-groups, and DNA-DNA hybridizations. The rep-PCR results illustrate that the diversity of the heterotrophic bacterial strains in Antarctic microbial mats is much higher than estimated by fatty acid analysis and preliminary 16S rDNA sequencing. In total, eight new species could be delineated belonging to the *Bacteroidetes* (with six new *Flavobacterium* species, one new *Algoriphagus* species and a new genus, *Gillisia* of the family *Flavobacteriaceae*). These results are presented in chapter 3. From FAA-cluster 41, related to the α-*Proteobacteria*, three new species of a new genus *Loktanella* were described and results are presented in chapter 4.

The second section of this work will handle about the diversity of oligotrophic bacteria in polar seas and a collection of 173 bacterial strains, which were isolated after enrichment under oligotrophic, psychrophilic conditions from Arctic (98 strains) and Antarctic (75 strains) seawater (Tan & Rüger, 1991; Tan *et al.*, 1999), was available. These strains have been previously analysed by their substrate utilization patterns using the Biolog system (Tan, 1997; Tan & Rüger, 1999) and by fatty acid analysis and 16S rDNA sequence analysis of representatives (Mergaert *et al.*, 2001b). They belong to six metabolic groups and eight FAA-clusters, containing two to 59 strains, could be delineated, while eight strains formed separate branches. Results of the 16S rDNA sequence analysis indicate that they belong to the α- and γ- *Proteobacteria*, the high percent G+C Gram-positives and to the *Bacteroidetes*. Additionally, several clusters represent as yet unnamed, new taxa, since they show less than 97% 16S rDNA sequence similarity to their nearest named neighbours.

In the meantime, 56 additional strains, isolated using the same methods, were also analysed using the Biolog system. For my PhD-work these additional strains were included in fatty acid analysis and they belong to FAA-clusters B, C, D, E and F (as delineated in Mergaert *et al.*, 2001b) and three new clusters (I, J and K) were found. The genomic diversity of 19 strains from clusters E and F and two related, unclustered strains, was further investigated by rep-PCR genomic fingerprinting and, using a polyphasic taxonomic approach, seven Antarctic strains could be assigned to a novel species within the genus *Alteromonas*, while another four strains could be assigned to the genus *Glaciecola*, with the description of a novel *Glaciecola* species. These results are presented in chapter 5.

PART 2

Experimental work

CHAPTER 2

Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes

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Microbial mats, growing in Antarctic lakes constitute unique and very diverse habitats. In these mats microorganisms are confronted with extreme life conditions. We isolated 746 bacterial strains from mats collected from ten lakes in the Dry Valleys (lakes Hoare and Fryxell), the Vestfold Hills (lakes Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts) and the Larsemann Hills (lake Reid), using heterotrophic growth conditions. These strains were investigated by fatty acid analysis, and by numerical analysis, 41 clusters, containing 2 to 77 strains, could be delineated, whereas 31 strains formed single branches. Several fatty acid groups consisted of strains from different lakes from the same region, or from different regions. The 16S rRNA genes from 40 strains, representing 35 different fatty acid groups were sequenced. The strains belonged to the alpha, beta and gamma subclasses of the *Proteobacteria*, the high and low percent G+C Gram-positives, and to the *Bacteroidetes*. For strains representing 16 fatty acid clusters, validly named nearest phylogenetic neighbours showed pairwise sequence similarities of less than 97%. This indicates that the clusters they represent, belong to taxa that have not been sequenced yet or as yet unnamed new taxa, related to *Alteromonas, Bacillus, Clavibacter, Cyclobacterium, Flavobacterium, Marinobacter, Mesorhizobium, Microbacterium, Pseudomonas, Salegentibacter, Sphingomonas* and *Sulfitobacter*.

Introduction

Antarctica is characterised by its geographical and climatic isolation, and most of the continent has experienced little or no anthropogenic influence. Antarctic lakes harbour pristine biotopes and include freshwater and saline systems that are subject to long periods of ice and snow cover, low temperatures and low levels of photosynthetically active radiation. As such these oligotrophic lakes are among the most unproductive in the world. Their planktonic community is dominated by microbial loop organisms, including bacteria, protozoa and phytoplankton, and little or no metazoans are present (Ellis-Evans, 1996; Laybourn-Parry *et al.*, 1997; Laybourn-Parry & Marchant, 1992). The benthic areas that receive sufficient solar radiation are covered by microbial mats composed primarily of cyanobacteria, diatoms and bacteria. These complex microbial communities have accumulated during thousands of years and the microorganisms are confronted with extreme life conditions, such as low temperatures, freezing-thawing cycli, UV-irradiation, desiccation and varying light conditions, salinities and nutrient concentrations. As a consequence they have been under a high selection pressure and are potentially belonging to endogenous, as yet undescribed new taxa (Ellis-Evans *et al.*, 1998) with potential novel biochemical adaptations like anti-freeze proteins, cold-adapted enzymes, desiccation and salt tolerance. Indeed, several new bacterial species have been isolated from these Antarctic benthic microbial communities, e.g. *Flavobacterium tegetincola* (McCammon & Bowman, 2000), *Arthrobacter flavus* (Reddy *et al.*, 2000), *Rhodoferax antarcticus* (Madigan *et al.*, 2000), and the anaerobes *Psychromonas antarcticus* (Mountfort *et al.*, 1998) and *Clostridium vincentii* (Mountfort *et al.*, 1997).

During expeditions on the Antarctic continent (MICROMAT project, http://www.nerc-bas.ac.uk/public/mlsd/micromat) mat samples were collected from lakes in three Antarctic regions (Vestfold Hills, McMurdo Dry Valleys and Larsemann Hills). One of these samples, taken from Lake Fryxell, McMurdo Dry Valleys, has been investigated by Tindall *et al.* (2000) and Brambilla *et al.* (2001), using culturing and culture independent methods. Their results show that a high phylogenetic diversity of bacteria is present in the mat, including partial 16S rDNA sequences related to anaerobes, *Proteobacteria*, Gram-positives, *Verrucomicrobiales*, and the *Bacteroidetes*.

Although it is established that only part of the community can be isolated (Spring *et al.*, 2000), the obvious advantage of the culturing technique is that

strains of new taxa can be preserved for detailed taxonomic analysis, physiological characterization, as well as for screening for potential applications, such as the production of cold-adapted enzymes, pigments, antibiotics and other bioactive compounds. Brambilla *et al.* (2001) also demonstrated the incongruence between the results obtained by culturing and culture independent methods. Indeed, both approaches yielded complementary results with almost no overlap. Similar conclusions were drawn from a study on the diversity of bacteria involved in the biodeterioration of mural paintings (Gurtner *et al.*, 2000).

We extended the study of the bacterial diversity in Antarctic microbial mats by investigating 17 samples from ten different lakes in three regions of the Antarctic, using direct cultivation under heterotrophic conditions, chemotaxonomic characterization by fatty acid analysis (FAA) and numerical grouping of 746 isolates, and phylogenetic analysis by 16S rDNA sequencing of 40 representative strains.

Materials and Methods

Source of samples

Samples were taken from two lakes in the Dry Valleys (Lake Fryxell and Lake Hoare), seven lakes in the Vestfold Hills (Ace Lake, Grace Lake, Organic Lake, Pendant Lake, Watts Lake, Lake Druzhby, and Highway Lake) and from Lake Reid in the Larsemann Hills, and dispatched to Belgium in ice-cooled sterile tubes. Sample designations, date of sampling and date of processing are given in Table 2.1. Several lakes were sampled twice. The samples FR1 and FR2 were duplicate samples, collected from the littoral zone in the moated area of the lake. The sample PE2 was taken from the littoral zone at a water depth of 30 cm. All other samples were taken at a water depth of 3-4m. Sample RE1 of lake Reid, that had been preserved frozen since sampling, was processed in January 2000.

Dry weight of the mat samples ranged from 7 to 81 %. The higher dry weights were obtained with samples having a soily aspect, the lower ones from more flocculous mat samples. The salinity from the lakes ranges from fresh (Druzhby, Grace, Watts and Hoare) over hyposaline-saline (Ace, Highway, Pendant, Fryxell and Reid) to hypersaline (Organic).

Enumeration and isolation of heterotrophic bacteria

Per sample, 1 g (wet weight) was aseptically weighed and homogenized in 9 ml sterile physiological water (0.86 % NaCl) during 1 minute in a Stomacher apparatus and subsamples were taken for the preparation of ten-fold dilution series. A first subsample was diluted in sterile physiological water and plated on R2A (Oxoid) and Reinforced Clostridial Agar (RCA, Oxoid), a second subsample was diluted in sterile, filtered seawater and plated on Marine Agar 2216 (Difco). All media were inoculated in duplo using a Whitley Automatic Spiral Plater (Don Whitley Scientific Ltd, Shipley, England), and were incubated either in an anaerobic chamber at room temperature (about 20°C, RCA plates), or aerobically at 4°C or 20°C (all other plates). Colony forming units (CFU) were counted, and selected colonies grown on the most diluted plates over a period of four weeks were isolated and purified on the same media. Pure cultures were cryopreserved using the MicroBank system (PRO-LAB Diagnostics, Ontario, Canada).

Fatty acid analysis

The strains were investigated by fatty acid analysis (FAA), according to the methods described by Mergaert *et al.* (1993), with the following modifications. Cells were cultivated at 20°C on R2A or Marine Agar. Preliminary results showed that differences between extracts prepared from cells of the same strains grown on these two media were negligible. After preparation, gas-liquid chromatographic separation of fatty acid methyl esters was achieved using the MIDI system (MICROBIAL ID Inc., Newark, Delaware, USA) and fatty acid methyl esters were identified by comparison to the peak library version 4.00. The fatty acid profiles were grouped according to their Canberra metric similarities (S_{canb}) with the UPGMA clustering method, using the Bionumerics software package (Applied Maths, Sint Martens-Latem, Belgium).

16S rDNA sequencing

DNA preparations and almost complete 16S rDNA sequences (1388-1550 bp) were obtained using the methods described by Mergaert *et al.* (2001b). The sequencing primers were those described by Coenye *et al.* (1999). Sequence assembly was performed using the program AutoAssembler 1.4.0 (Perkin-Elmer

Applied Biosystems). The closest related sequences in the EMBL database were found using the FASTA programme (http://www2.ebi.ac.uk/fasta3/). Phylogenetic analysis was performed using the Bionumerics sofware package (Applied Maths, Sint Martens-Latem, Belgium), taking into account the homologous nucleotide positions after discarding all unknown bases and gaps. Using the same software package, a neighbour joining dendrogram (Saitou & Nei, 1987) was constructed based on global alignment of the sequences.

Nucleotide sequence accession numbers

The 16S rDNA sequences determined in this study have been deposited in the EMBL data base and the accession numbers are given in Table 2.5.

Results and Discussion

Enumeration and isolation of heterotrophic aerobic bacteria from Antarctic microbial mats

Seventeen microbial mat samples from ten Antarctic lakes from three different regions (McMurdo Dry Valleys, Vestfold Hills and Larsemann Hills) were investigated using culturing techniques. Colonies grown at 20°C or 4°C were enumerated after 4-5 days or 12-14 days, respectively, when the count curves reached the asymptote. Log numbers of CFU/g (dry weight) ranged between 5 and 10. The data obtained with incubations at 20°C are shown in Table 2.1. For most mats, counts on Marine Agar, a medium rich in salt, were similar to those on R2A, a medium poor in salt. The exceptions are the mat sample from the hypersaline Organic Lake and the sample AC2 from the saline Ace Lake, which showed much higher counts on Marine Agar, and from the freshwater lakes Grace and Druzhby and the sample RE2 from the hyposaline lake Reid, which showed higher counts on R2A. Plates incubated at 4 °C contained similar or slightly lower numbers of colonies than the plates incubated at 20 °C (data not shown). In general, prolonged incubation resulted in additional colonies showing up. None of the samples yielded growth of colonies after 14 days anaerobic incubation on RCA (detection limit 4.3 Log CFU/g). In total, 746 colonies, grown on the most diluted plates were isolated. The number of isolates per sample is given in Table 2.1.

Table 2.1. Source, labelling and dry weight of the mat samples, number of colony forming aerobic, heterotrophic bacteria, and number of strains isolated per sample.

Region & Lake	Sample	Sampling date	Processing date	% Dry weight	Log CFU/g (dry weigth) [a] on medium		Number of isolates
					Marine Agar	R2A	
McMurdo Dry Valleys							
Fryxell	FR1	February 1999	February 1999	32	5.9	6.9	98
	FR2	February 1999	February 1999	27	6.4	6.8	90
Hoare	HO	February 1999	February 1999	81	8.1	8.1	34
Vestfold Hills							
Organic	OR	March 1999	May 1999	80	8.4	5.8	31
Ace	AC1	March 1999	May 1999	10	8.9	9.2	107
	AC2	Nov/Dec 1999	February 2000	12	8.9	6.1	15
Grace	GR	March 1999	May 1999	7	8.7	10.0	34
Druzhby	DR	March 1999	May 1999	34	5.2	8.6	34
Pendant	PE1	March 1999	May 1999	14	10.1	9.3	29
	PE2	Nov/Dec 1999	February 2000	16	9.6	9.3	30
	PE3	Nov/Dec 1999	February 2000	28	7.2	6.3	18
Watts	WA1	March 1999	May 1999	14	10.2	10.1	42
	WA2	Nov/Dec 1999	February 2000	15	8.4	8.6	12
Highway	HI1	Nov/Dec 1999	February 2000	15	8.9	8.9	6
	HI2	Nov/Dec 1999	February 2000	19	7.1	7.1	41
Larsemann Hills							
Reid	RE1	December 1997	January 2000	39	7.5	8.3	63
	RE2	February 2000	April 2000	5	5.8	8.9	62

[a] CFU, colony forming units after 4-5 days incubation at 20 °C. Averages of duplicate plates. Standard deviations were less than 0.1 log CFU/g.

Chemotaxonomic and phylogenetic diversity of the isolates

Gas-liquid chromatographic analysis of the whole-cell fatty acid compositions was used to characterize all isolates. Indeed, fatty acid analysis has been widely applied for the characterization of bacteria from polar environments (Bowman *et al.*, 1997c; Bozal *et al.*, 1997; Franzman & Tindal, 1990; Gosink & Staley, 1995; Mergaert *et al.*, 2001b; Pukall *et al.*, 1999), and provides a suitable method for rapidly grouping large numbers of strains into chemotaxonomically similar entities, to form a basis for the selection of representative strains for phylogenetic analysis (Heyrman *et al.*, 1999; Mergaert *et al.*, 2001a, b). A dendrogram was constructed based on the fatty acid compositions of the strains. At $S_{canb} \geq 75\%$, 41 FAA clusters, containing 2 to 77 strains, could be delineated, and 31 strains formed single branches (Fig. 2.1). Twenty-eight clusters (clusters 1 to 21, 32 to 34, 36, 37, and 40) consisted of strains forming pigmented colonies (mainly yellow or orange, some pink, red, or bordeau red), the remaining clusters consisted mainly of unpigmented strains.

The lakes from which the strains were isolated are indicated in Fig. 2.1. Most clusters contained strains from different lakes, and often from different regions. Several clusters contained strains isolated from almost all samples, suggesting that taxa showing these fatty acid compositions might be ubiquitous in Antarctic lakes. In clusters 38, 40 and 41 strains isolated from the hypersaline lake Organic grouped with strains from freshwater lakes Hoare and Watts, indicating that these strains show a broad salinity tolerance. Highly similar FAA cluster composition was observed in the duplicate samples FR1 and FR2, from each of which a large and comparable number of isolates was investigated. Indeed, 13 out of 16 clusters, with at least two strains from Lake Fryxell, were in common for both samples. For the two samples of Lake Reid there was a significant difference in composition (4 out of 18 clusters, with at least two strains from that lake, in common), and this can be explained by their different times of sampling, the presumably different sampling location in the lake and the fact that the frozen sample RE1 was processed almost two years after sampling. For other lakes, too low numbers of strains (Ace, Pendant, Highway, Watts) were isolated for at least one sample to allow comparison between samples.

A wide variety of different fatty acid profiles were obtained and a total of 90 different fatty acids were detected among the strains. The results are summarized in Tables 2.2, 2.3 and 2.4. The extracts of the strains from the clusters 1 to 15

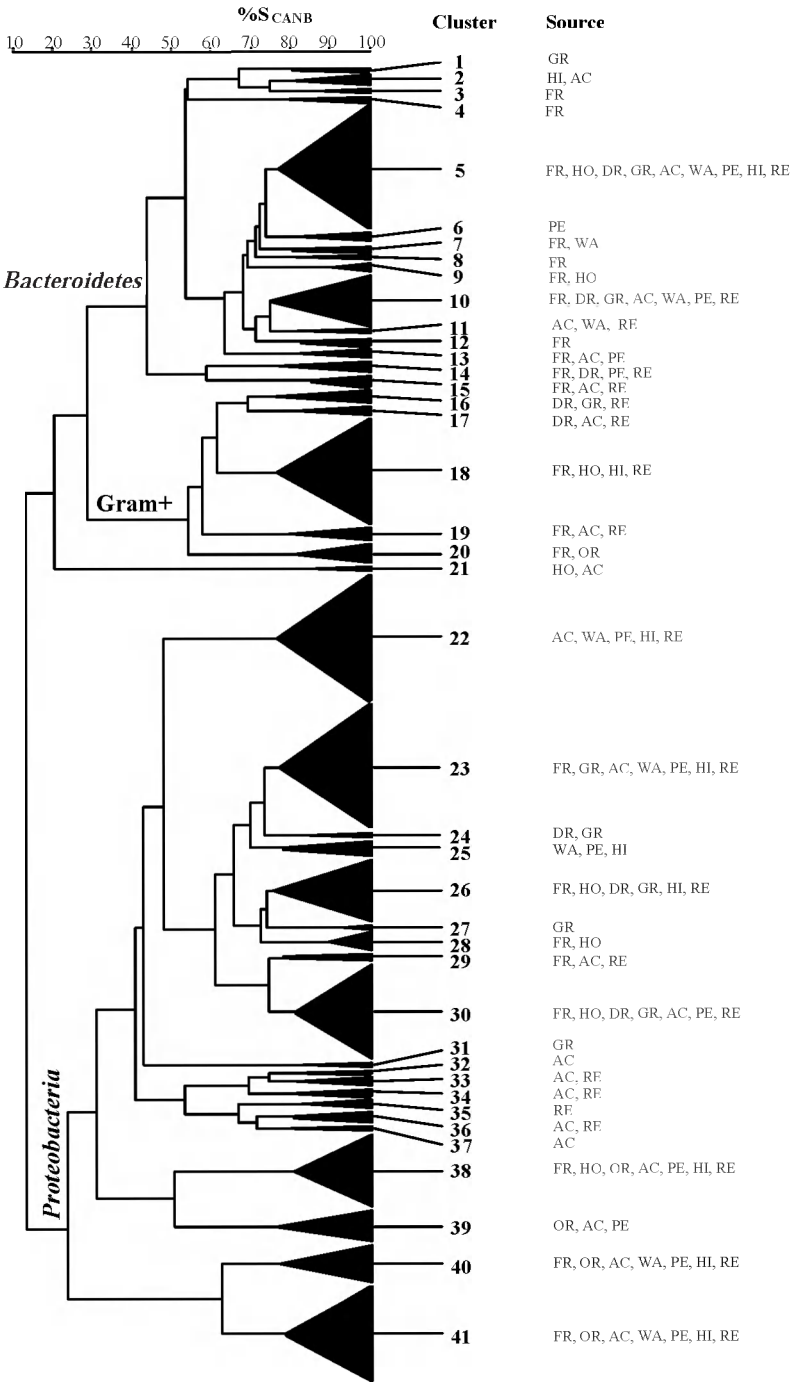


Figure 2.1. Abridged dendrogram obtained by numerical analysis of the fatty acid compositions of 746 strains, isolated from microbial mats from Antarctic lakes, using the Canberra metric similarity coefficient (S_{canb}) and UPGMA clustering. Single strain branches are not shown. The branch of the Gram-positive bacteria is designated "Gram+". The abbreviations FR, HO, DR, OR, GR, AC, WA, PE, HI and RE stand for lakes Fryxell, Hoare (in the McMurdo Dry Valleys), Druzhby, Organic, Grace, Ace, Watts, Pendant, Highway (in the Vestfold Hills) and Reid (in the Larsemann Hills).

(affiliated with the *Bacteroidetes*, see below) mainly contained branched fatty acids. Straight chain saturated and unsaturated fatty acids were also present. Within the unidentified fatty acids, summed feature 3 (consisting of either 15:0 iso 2OH or 16:1ω7c, or both) predominated in the extracts from clusters 14 and 15.

The extracts of the strains from clusters 16 to 20 (affiliated with the Gram-positives, see below) contained mainly saturated branched fatty acids and no hydroxylated fatty acids were detected. The alcohol derivate of 16:1ω7c was found in the extracts from the strains from cluster 20 in relatively high amounts.

The extracts of the strains from clusters 21 to 41 (affiliated with the *Proteobacteria*, see below) contained high amounts of straight chain fatty acids and summed feature 3. The straight chain fatty acids were mainly unsaturated in the extracts of the strains from clusters 21 and 32 to 41, while the saturated straight chain fatty acids dominated in the extracts from clusters 22 to 31. The extracts from the strains from cluster 41 primarily contained one fatty acid, 18:1ω7c (83.7%). In the extracts from the clusters 21, 22 and 31 a relatively high amount of saturated branched fatty acids were found.

To determine their phylogenetic affiliation, 16S rDNA sequence analysis was performed on 40 strains representing 35 clusters obtained by fatty acid analysis. These sequences were compared to each other and to related sequences from the EMBL database. The results are shown in Table 2.5. The strains belonged to the α-, β- and γ-*Proteobacteria*, the *Bacteroidetes* and the high and low percent G+C Gram-positives.

The nearest validly named phylogenetic neighbours of the strains often belong to taxa isolated from cold, aquatic environments, such as *Shewanella baltica, Psychrobacter glacincola, Sulfitobacter pontiacus, Flavobacterium frigidarium, Flavobacterium gillisiae, Salegentibacter salegens* and *Gelidibacter algens*. Sequences from sixteen strains showed pairwise sequence similarities of less than 97% to their nearest validly named neighbours, indicating that they represent as yet unnamed new taxa or belong to species for which no sequences are yet available (Table 2.5) (Stackebrandt & Goebel, 1994). The latter authors, as well as many others, also demonstrated that strains showing sequence similarities of more than 97% may show low DNA-DNA reassociation values and thus constitute different species.

For FAA clusters 5 (related to *Flavobacterium*), 29 and 30 (β-*Proteobacteria*) and 41 (α-*Proteobacteria*) we sequenced two or three representatives, selected on the basis of their remote positions within the FAA clusters in the dendrogram,

Table 2.2. Fatty acid composition of isolates belonging to the *Bacteroidetes*, expressed as mean percentages of total.

Fatty acid class	Fatty acid cluster (number of isolates) [a]											
	2 (6)	4 (3)	5 (75)	6 (5)	7 (5)	9 (4)	10 (31)	11 (4)	12 (4)	13 (4)	14 (7)	15 (8)
saturated, straight [b]	9.0	3.6	9.9	5.1	12.6	9.5	11.7	10.0	20.1	7.7	4.1	TR
unsaturated, straight	2.4	3.1	16.0	18.3	14.3	8.9	10.0	3.9	14.0	5.1	15.3	6.4
saturated, branched	21.8	24.2	25.9	18.0	31.8	23.9	30.1	32.7	18.1	26.9	23.7	30.0
unsaturated, branched	33.7	28.7	11.6	16.5	14.6	28.9	17.7	16.8	12.5	18.2	11.5	16.5
saturated, straight, hydroxylated	5.6	17.1	4.2	6.2	5.2	2.8	3.8	4.4	7.7	8.4	2.6	2.6
saturated, branched, hydroxylated	26.2	14.3	18.4	17.5	17.6	12.9	23.1	24.5	21.4	29.1	6.0	18.3
not classified [c]	1.2	8.9	14.1	18.2	3.8	12.9	3.4	7.6	6.1	4.6	36.5	25.4

[a] See also Fig. 2.1. Clusters with 2 strains or less and unclustered strains were not included. Fatty acid classes which account for less than 1% of the total fatty acids in all clusters are not shown.

[b] Mainly odd numbered.

[c] Not classified: Summed feature 1, 2, 3 and unknown fatty acids 11.543, 13.565, 14.959 and 16.582. Summed feature 1 comprises any combination of 15:1 iso H, 15:1 iso I and 13:0 3OH. Summed feature 2 comprises any combination of 12:0 aldehyde, unknown 10.928, 16:1 iso I and 14:0 3OH. Summed feature 3 comprises 15:0 iso 2OH, 16:1 ω7c, or both. Unknown fatty acids are designated with their equivalent chain length, relative to the chain lengths of known straight chain, saturated fatty acids.

Table 2.3. Fatty acid composition of isolates belonging to the Gram-positives, expressed as mean percentages of total. [a]

Fatty acid class	Fatty acid cluster (number of isolates)				
	16 (9)	17 (5)	18 (64)	19 (10)	20 (12)
saturated, straight	1.4	1.1	4.4	1.6	2.2
unsaturated, straight	TR	-	TR	-	4.6
saturated, branched	92.0	92.1	76.2	89.3	64.1
unsaturated, branched	2.2	5.1	9.9	9.0	12.2
alcohol derivate of 16:1ω7c	2.4	-	-	-	16.8
not classified [b]	TR	1.5	8.9	-	-

[a] See footnotes in Table 2.2. Symbols: - , not detected; TR, trace amounts (≤ 1% of total).

[b] Not classified: Summed feature 1, 3 and unknown fatty acids 13.565, 14.959 and 15.669. Summed feature 1 comprises any combination of 15:1 iso H, 15:1 iso I and 13:0 3OH. Summed feature 3 comprises 15:0 iso 2OH, 16:1ω7c, or both.

and the sequence similarity between the strains within these clusters was 96.5 to 97.9%, 95.5%, 90.3%, and 90.8%, respectively. This indicates that these, and most probably also other FAA clusters, contain multiple taxa with similar fatty acid profiles. On the other hand, in some cases more than 97% similarity was found between representatives from different FAA clusters, i.e. clusters 1, 5, 6 and 7 (related to *Flavobacterium*), clusters 2 and 10 (related to *Flavobacterium*), clusters 28, 29 and 30 (β-*Proteobacteria*), clusters 26 and 31 (related to *Pseudomonas*), and clusters 33 and 34 (related to *Porphyrobacter*), demonstrating that phylogenetically closely related taxa are sometimes quite different in their fatty acid compositions. These observations were also made by Mergaert *et al.* (2001b), who characterized polar marine bacteria using the same methods. Although fatty acid analysis has been proven a convenient method for rapid screening of large numbers of bacteria from different phylogenetic affiliation, our results indicate that higher resolution techniques are to be applied to investigate the genomic diversity within each fatty acid cluster in more detail. Indeed, when a higher similarity level (80%) for the delineation of clusters in the FAA dendrogram, shown in Fig. 2.1, is used, and which is comparable to the level used by Mergaert *et al.* (2001b), 13 additional clusters and 20 additional singles were found that potentially belong to additional phylogenetic lineages.

Brambilla *et al.* (2001) and Tindall *et al.* (2000) focussed on a sample taken from Lake Fryxell, which was derived from the same sampling master batch as our samples FR1 and FR2. Their results show that a high phylogenetic diversity

Table 2.4. Fatty acid composition of isolates belonging to the *Proteobacteria*, expressed as mean percentages of total. [a]

Fatty acid class	Fatty acid cluster (number of isolates)																
	22 (77)	23 (77)	24 (4)	25 (8)	26 (37)	28 (13)	29 (4)	30 (58)	33 (5)	34 (4)	35 (5)	36 (7)	37 (3)	38 (43)	39 (19)	40 (22)	41 (59)
saturated, straight [b]	25.2	25.6	26.3	27.1	26.2	30.6	18.2	22.9	4.8	10.0	17.0	9.6	11.4	7.3	24.3	14.0	5.0
unsaturated, straight	15.5	21.5	14.4	16.3	16.2	5.8	19.3	5.2	44.3	36.1	32.1	39.1	42.3	66.3	37.6	62.9	84.2
saturated, branched	21.8	TR	-	1.3	TR	TR	-	TR	4.7	2.0	-	TR	1.0	1.1	TR	-	TR
unsaturated, branched	TR	TR	4.3	TR	TR	TR	TR	TR	-	TR	10.7	TR	3.0	TR	-	TR	1.1
saturated, straight, hydroxylated	2.3	12.0	7.4	4.1	18.5	9.9	3.3	5.6	25.8	26.9	12.0	12.7	10.7	6.2	11.9	3.9	3.5
unsaturated, straight, hydroxylated	-	TR	-	2.6	TR	-	-	-	6.5	1.1	-	TR	TR	-	-	TR	TR
saturated, branched, hydroxylated	3.6	TR	-	TR	TR	TR	-	-	5.7	1.4	-	1.6	TR	TR	-	-	TR
cyclic	-	TR	-	-	12.2	10.7	-	TR	-	-	-	-	-	TR	TR	TR	TR
not classified [c]	31.4	40.2	47.4	47.8	25.8	42.4	58.6	65.5	8.1	22.3	28.0	34.9	30.0	19.1	24.3	18.8	5.1

[a] See footnotes in Table 2.2. Symbols: - , not detected; TR, trace amounts (≤ 1% of total).

[b] Mainly even numbered.

[c] Not classified: Summed feature 1, 2, 3, 5, 7 and unknown fatty acids 11.799, 12.484, 13.957, 14.263, 14.502, 14.959, 18.814 and 9.531. Summed feature 5 comprises 18:2 ω6,9c, 18:0 anteiso, or both. Summed feature 7 comprises any combination of unknown 18.846, 19:1 ω6c and 19:0 cyclo ω10c.

Table 2.5. Phylogenetic relationships of strains representative for the different FAA clusters delineated in Fig. 2.1.

FAA cluster	Strain No. [a]	LMG No. [b]	Sequence accession No. [c]	Source (sample) [d]	Validly named nearest phylogenetic neighbour	16S rDNA similarity (%) [e]	Phylogenetic branch
1	R-9003	21468	AJ441000	GR	*Flavobacterium frigidarium*	98.0	*Bacteroidetes*
2	R-9033	21469	AJ441001	AC1	*Flavobacterium aquatile, F. tegetincola*	95.7	*Bacteroidetes*
4	R-8282	21470	AJ440991	FR1	*Salegentibacter salegens*	92.9	*Bacteroidetes*
5	R-8022	21471	AJ440988	FR2	*Flavobacterium gillisiae*	98.4	*Bacteroidetes*
5	R-7933	21472	AJ440987	FR1	*Flavobacterium hydatis*	97.3	*Bacteroidetes*
5	R-7550	21473	AJ440979	FR2	*Flavobacterium frigidarium*	97.5	*Bacteroidetes*
6	R-9122	21474	AJ441005	PE1	*Flavobacterium gillisiae*	98.4	*Bacteroidetes*
7	R-7579	21475	AJ440981	FR2	*Flavobacterium hydatis*	96.9	*Bacteroidetes*
9	R-7515	21476	AJ440977	FR2	*Flavobacterium gillisiae*	94.6	*Bacteroidetes*
10	R-8899	21477	AJ440996	AC1	*Flavobacterium flevense*	95.3	*Bacteroidetes*
11	R-8963	21478	AJ440998	AC1	*Flavobacterium tegetincola*	98.2	*Bacteroidetes*
12	R-7666	21479	AJ440984	FR1	*Flavobacterium tegetincola*	95.7	*Bacteroidetes*
13	R-9217	21480	AJ441008	AC1	*Gelidibacter algens*	99.8	*Bacteroidetes*
14	R-7572	21481	AJ440980	FR1	*Hymenobacter actinosclerus*	97.3	*Bacteroidetes*
15	R-9286	21482	AJ441012	AC1	*Cyclobacterium marinum*	92.5	*Bacteroidetes*
16	R-9112	21483	AJ441003	GR	*Microbacterium keratanolyticum*	96.0	Gram+ (high % GC)
17	R-9183	21484	AJ441006	AC1	*Micrococcus luteus*	99.7	Gram+ (high % GC)
18	R-7549	21485	AJ440978	FR2	*Arthrobacter agilis*	99.6	Gram+ (high % GC)
19	R-8287	21486	AJ440992	FR1	*Clavibacter michiganensis*	96.1	Gram+ (high % GC)
20	R-8161	21487	AJ440990	FR1	*Bacillus oleronius*	93.2	Gram+ (low % GC)
22	R-12605	21488	AJ440976	RE2	*Shewanella baltica*	98.7	γ-*Proteobacteria*
23	R-8971	21489	AJ440999	AC1	*Pseudomonas anguilliseptica, P. migulae*	95.8	γ-*Proteobacteria*

24	R-8875	21490	AJ440994	DR	*Pseudomonas saccharophila*	97.2	β-*Proteobacteria*
25	R-11381	21491	AJ440975	HI2	*Alteromonas macleodii*	93.9	γ-*Proteobacteria*
26	R-7616	21492	AJ440983	HO	*Pseudomonas orientalis*	99.5	γ-*Proteobacteria*
28	R-7687	21493	AJ440985	HO	*Janthinobacterium lividum*	99.7	β-*Proteobacteria*
29	R-9284	21494	AJ441011	AC1	*Hydrogenophaga palleronii*	98.4	β-*Proteobacteria*
29	R-7724	21495	AJ440986	FR2	*Aquaspirillum delicatum*	97.8	β-*Proteobacteria*
30	R-8890	21496	AJ440995	AC1	*Hydrogenophaga palleronii*	97.7	β-*Proteobacteria*
30	R-7614	21497	AJ440982	HO	*Janthinobacterium lividum*	99.6	β-*Proteobacteria*
31	R-9113	21498	AJ441004	GR	*Pseudomonas syringae*	98.6	γ-*Proteobacteria*
33	R-9478	21499	AJ441013	AC1	*Porphyrobacter neustonensis*	97.7	α-*Proteobacteria*
34	R-9216	21500	AJ441007	AC1	*Porphyrobacter neustonensis*	97.6	α-*Proteobacteria*
35	R-10753	21501	AJ440974	RE1	*Devosia riboflavina*	97.2	α-*Proteobacteria*
36	R-9221	21502	AJ441010	AC1	*Sphingomonas natatoria*	94.5	α-*Proteobacteria*
38	R-8160	21503	AJ440989	FR1	*Psychrobacter glacincola*	97.5	γ-*Proteobacteria*
39	R-9035	21504	AJ441002	AC1	*Marinobacter hydrocarbonoclasticus*	95.6	γ-*Proteobacteria*
40	R-8358	21505	AJ440993	FR2	*Brevundimonas subvibrioides*	99.1	α-*Proteobacteria*
41	R-9219	21506	AJ441009	AC1	*Mesorhizobium loti*	96.9	α-*Proteobacteria*
41	R-8904	21507	AJ440997	AC1	*Sulfitobacter pontiacus*	93.5	α-*Proteobacteria*

[a] As preserved in the research collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

[b] As deposited in the BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

[c] As deposited in the EMBL data base.

[d] For abbreviations: see Table 2.1.

[e] The 16S rDNA sequence similarities are based on pairwise alignments.

of bacteria is present in the mats and that the results from the culturing and culture independent methods they used, showed almost no overlap. We compared the complete sequences of our strains to the 7 almost complete and 126 partial sequences (320 nucleotides from the 5' terminus) of 12 cultured and 121 uncultered bacteria, reported by these authors, by a FASTA search. Only five of our sequences, four of which were from isolates from Lake Fryxell, showed a significant similarity to the sequences determined for the clones and isolates by Tindall *et al.* (2000) and Brambilla *et al.* (2001). The partial sequence of clone 391 ev (AJ287642) is identical to the corresponding part of the sequence of our strain R-7724 (cluster 29), and the partial sequence of clone 204 ev (AJ287671) showed a sequence similarity of 99.7% to the corresponding part of the full sequence of our strain R-7933 (cluster 5). Strain R-8160 (cluster 38), showed a sequence similarity of 99.3% to the full sequence of isolate A1/C-aer/OII (AJ297439). Strains R-7550 (cluster 5) and R-9003 (cluster 1; isolated from Grace Lake) showed a sequence similarity of respectively 97.5% and 97.1% to the full sequence of isolate A1/C-aer/OIV (AJ297440) of Brambilla *et al.* (2001).

Although a high amount of bias on the assessment of the diversity of the heterotrophic isolates was introduced due to the limited number of samplings and culturing procedures, and the limited number of strains isolated from several samples, our results combined with those reported by Tindall *et al.* (2000) and Brambilla *et al.* (2001), demonstrate that the numbers and diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is extremely high and that the strains isolated constitute a unique collection for further taxonomic analysis, physiological characterization and screening. More detailed genomic analysis will be needed to determine the species diversity within each of the FAA clusters delineated in this paper.

CHAPTER 3

New taxa from Antarctic lakes within the *Bacteroidetes*

3.1 Polyphasic taxonomy of FAA clusters 1 to 15

Fatty acid clusters 1 to 15 (as delineated in Van Trappen *et al.* (2002), see chapter 2), which belong to the *Bacteroidetes*, were further investigated using a polyphasic taxonomic approach. The phylum of the *Bacteroidetes*, can be subdivided into 3 different classes, the '*Bacteroidetes*', the '*Flavobacteria*' and the '*Sphingobacteria*' (as illustrated in Fig. 3A) with the families *Bacteroidaceae*, *Sphingobacteriaceae*, '*Flexibacteriaceae*' and *Flavobacteriaceae* (Bernardet *et al.*, 2002).

The genomic diversity of the strains of fatty acid clusters 1 to 15 (as delineated in Van Trappen *et al.* (2002), see chapter 2), which belong to the *Bacteroidetes*, was investigated by rep-PCR fingerprinting, using the GTG_5-primer (Rademaker & de Bruijn, 1997). In total, 161 fingerprinting patterns were obtained (for strain R-9191 of FAA cluster 5, no DNA could be extracted after several attempts) and 27 clusters could be delineated, whereas 38 strains formed single branches, at a cut-off value of 70% (Pearson correlation coefficient) (see Fig. 3.1). These results illustrate that the diversity of heterotrophic bacteria in Antarctic microbial mats is extremely high, and strains showing the same pattern are often isolated from different lakes (rep-clusters II, VI, XXII, XXVII) and even from different Antarctic regions (rep-clusters I, IX, X, XII, XVII, XVIII, XXI, XXIV). Reference strains of nine related *Flavobacterium* species were also included in this rep-clustering, but it is clear that none of their fingerprinting patterns, showing only a few bands, is closely related to the patterns of the Antarctic strains (see Fig. 3.1).

Overall this rep-clustering is consistent with the delineation of fatty acid clusters with strains from the same rep-cluster belonging to the same FAA cluster (see Fig. 3.1). However, a few exceptions can be found. For example, strains of

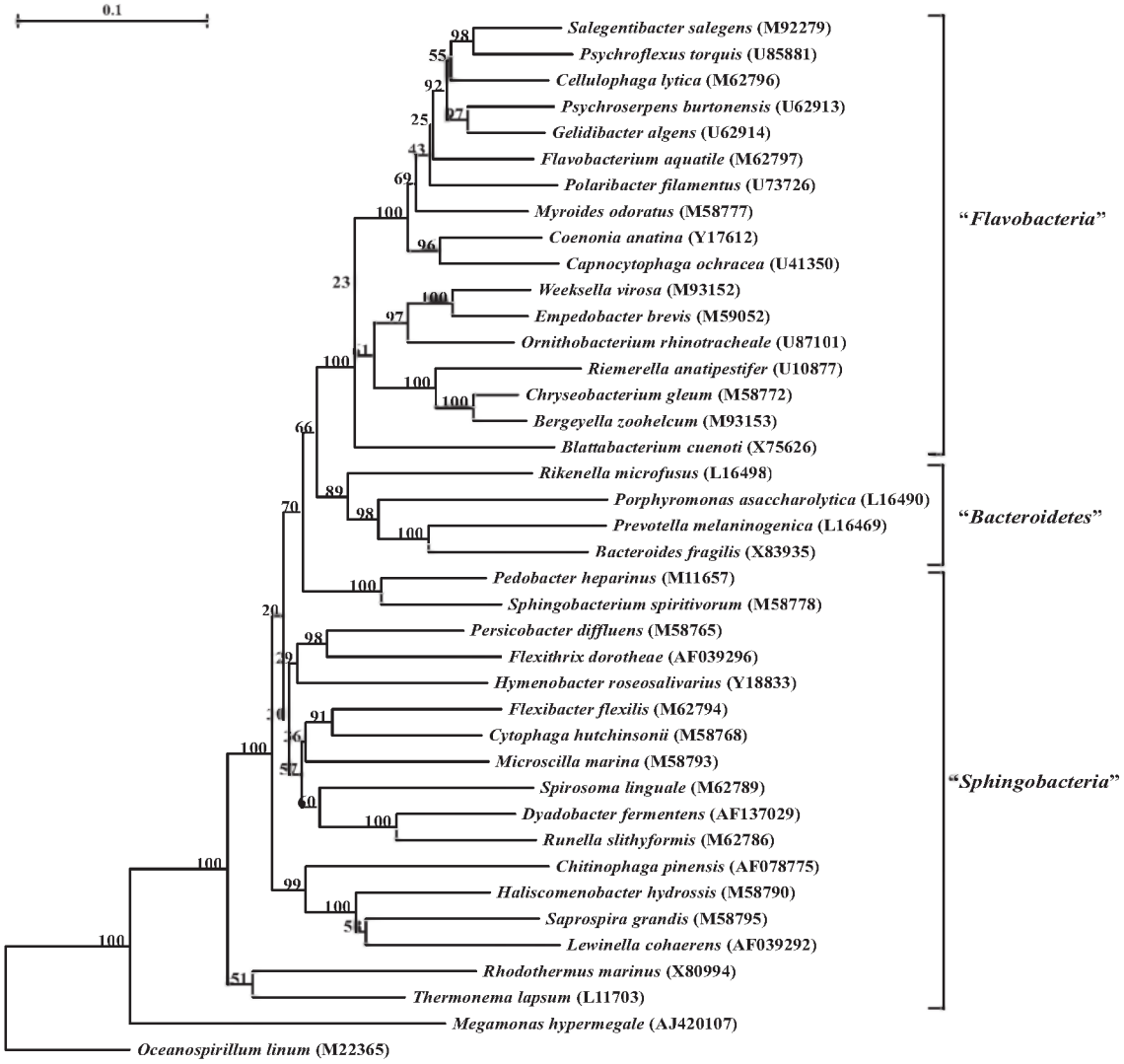
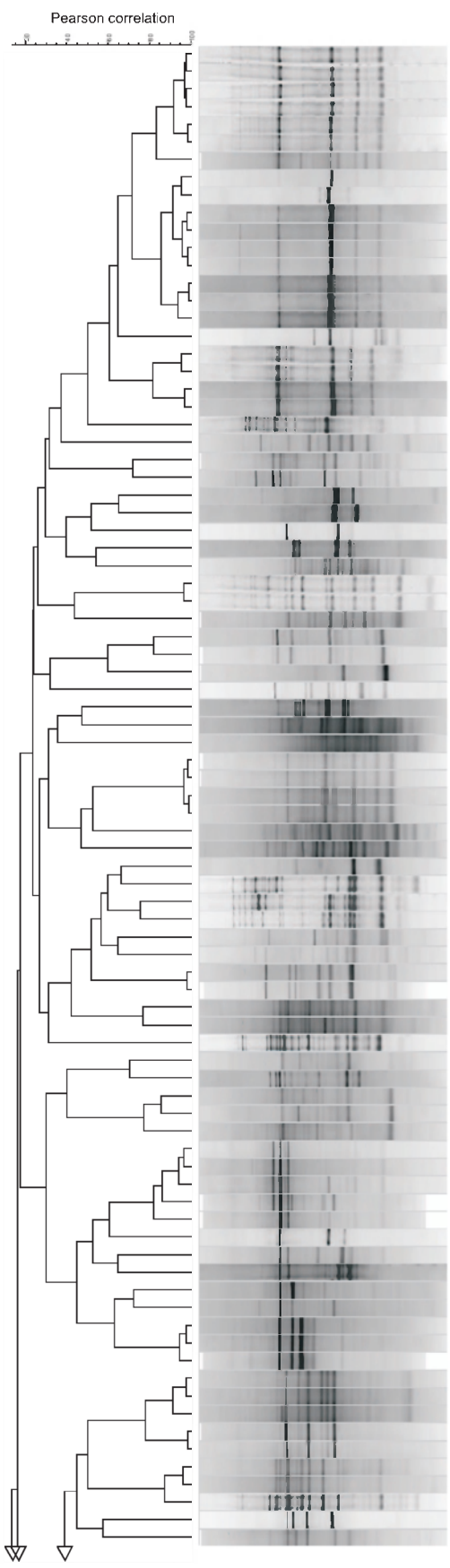


Figure 3A. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of representatives of the *Bacteroidetes* phylum on the basis of 16S rRNA gene sequences. *Oceanospirillum linum* was choosen as outgroup and the different classes of the *Bacteroidetes* phylum (*Flavobacteria*, *Bacteroidetes* and *Sphingobacteria*) are indicated. Bootstrap values (percentages of 100 replicates) are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

fatty acid clusters 5 and 6 group within the same rep-cluster (I) and show almost identical profiles. This can be explained by the fact that their fatty acid compositions are very similar and differences are largely due to different amounts of fatty acids $C_{15:0}$, iso $C_{15:0}$, anteiso $C_{15:1}$ and summed feature 3. Indeed, principal component analysis (PCA) confirms that there is no clear separation between FAA clusters 5 and 6. Rep-cluster X (FAA clusters 3 and 5) and rep-cluster XVI (FAA clusters 1 and 5) for example also contain strains from different FAA clusters but clear differences can be found in their rep-profiles after visual comparison. Cluster



Strain n°	Lake	FAA cluster	Rep cluster
R-8991	Pe	5	I
R-8993	Pe	5	I
R-8988	Pe	5	I
R-8992	Pe	5	I
R-9106	Ac	5	I
R-9119	Pe	5	I
R-9125	Pe	6	I
R-9123	Pe	6	I
R-9132	Dr	5	I
R-12608	Re	5	I
R-9122	Pe	6	I
R-9118	Pe	6	I
R-9124	Pe	6	I
R-11356	Pe	5	I
R-11563	Hi	5	I
R-8982	Pe	5	I
R-8134	Fr	10	
R-7582	Fr	5	II
R-7681	Ho	5	II
R-8023	Fr	5	II
R-8283	Fr	5	II
R-7577	Fr	5	
LMG 4031^T			
R-9026	Wa	7	III
R-9280	Wa	7	III
LMG 8328^T			
R-7550	Fr	5	
LMG 1341^T			
R-7933	Fr	5	
R-8104	Fr	14	
R-7581	Fr	5	IV
R-7587	Fr	5	IV
LMG 21424^T			
R-9046	Gr	10	V
R-9101	Gr	10	V
R-12626	Re	10	
R-9051	Dr	10	
R-8297	Ho	5	
R-8973	Dr	14	
R-7722	Fr	14	
R-7515	Fr	9	VI
R-7720	Ho	9	VI
R-7571	Fr	9	VI
R-7932	Fr	9	VI
R-11111	Pe	14	
R-12582	Re	14	
LMG 8385^T			
R-8016	Fr	5	
R-9192	Gr	5	VII
R-9193	Gr	5	VII
R-8101	Fr	8	
R-8102	Fr	8	
R-9217	Ac	13	VIII
R-9220	Ac	13	VIII
R-10750	Re	15	IX
R-9286	Ac	15	IX
R-8018	Fr	5	
R-10674	Re	3	X
R-7509	Fr	5	X
R-10672	Re	10	XI
R-10676	Re	3	XI
R-10847	Re	10	XI
R-10710	Re	15	XII
R-10752	Re	15	XII
R-10749	Re	15	XII
R-8282	Fr	4	XII
R-11427	Re	15	XII
R-9151	Gr	5	
R-8290	Fr	15	
R-9029	Wa	5	
R-7610	Fr	4	XIII
R-7730	Fr	4	XIII
R-7666	Fr	12	XIV
R-7721	Fr	12	XIV
R-7665	Fr	12	XIV
R-11272	Hi	2	XV
R-11383	Hi	2	XV
R-11608	Hi	2	XV
R-11271	Hi	2	XV
R-11274	Hi	2	XV
R-9003	Gr	1	XVI
R-9229	Gr	1	XVI
R-9143	Gr	5	XVI
LMG 12010^T			
R-7723	Fr	12	

Figure 3.1. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of 161 strains, belonging to FAA clusters 1 to 15 and nine related *Flavobacterium* species. Dendrogram derived from UPGMA-clustering of the profiles with the Pearson correlation coefficient and rep-clusters were delineated at a cut-off value of 70%. *Flavobacterium* species: LMG 1341 (*F. johnsoniae*), LMG 4031 (*F. pectinovorum*), LMG 8328 (*F. flevense*), LMG 8372 (*F. xanthum*), LMG 8384 (*F. saccharophilum*), LMG 8385 (*F. hydatis*), LMG 12010 (*F. frigidarium*), LMG 21422 (*F. gillisiae*), LMG 21424 (*F. hibernum*).

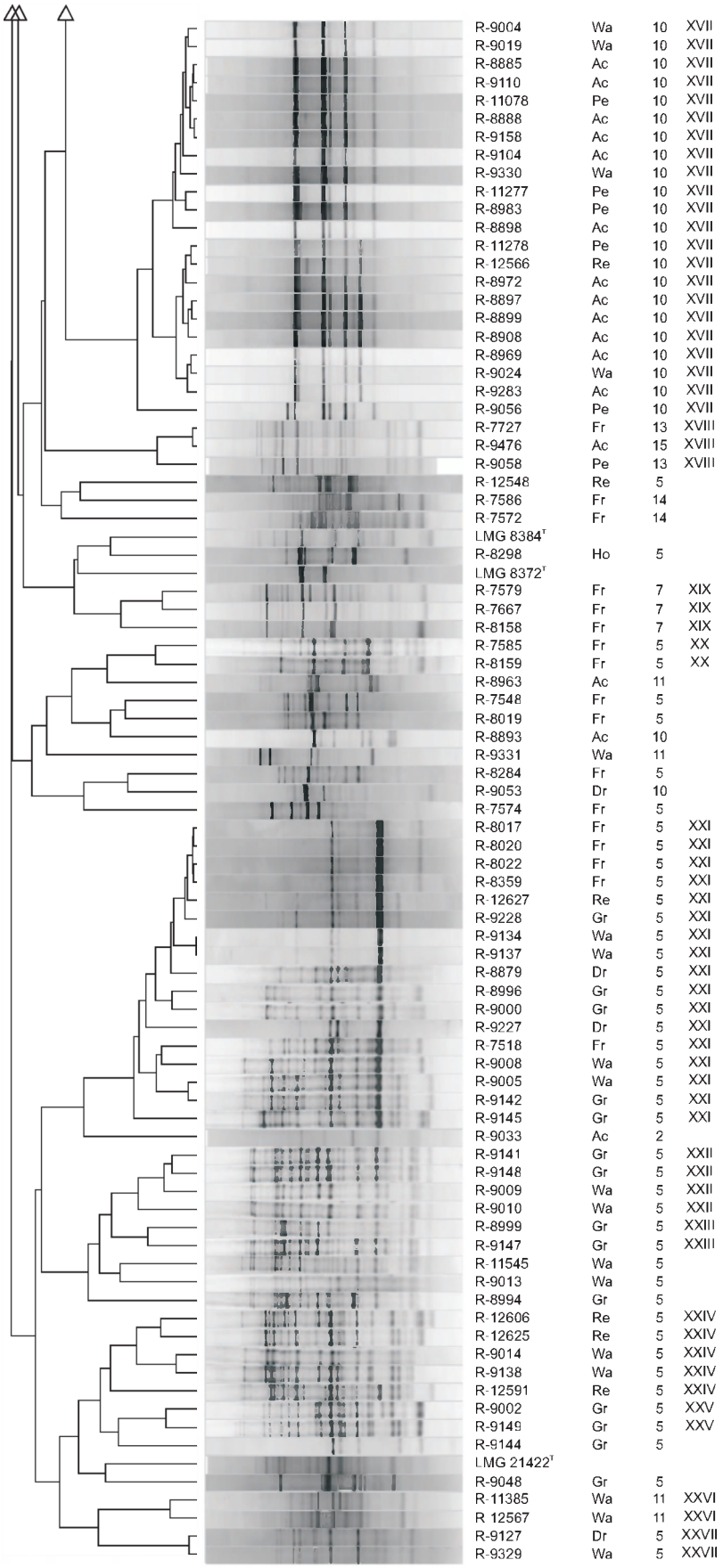


Figure 3.1. (continued)

analysis is mostly used to present data in an organized way but when the number of fingerprints is high, the situation gets complex and it is more difficult to assign reliable groups (Rademaker & de Bruijn, 1997). Especially rep-profiles with very few clear bands, concentrated in a specific area of the rep-profile can lead to anomalies in the clustering (Versalovic *et al.*, 1994).

The sub-clustering on the basis of rep-PCR patterns, allowed us to select representatives for additional 16S rDNA sequence analysis and DNA-DNA hybridization. In Table 3.1, 16S rDNA sequence similarities of representative strains of the different rep-clusters, with their nearest phylogenetic neighbours are given. For several of these strains, validly named nearest phylogenetic neighbours showed sequence similarities of less than 97%, indicating that the clusters they represent belong to unnamed new taxa (Stackebrandt & Goebel, 1994). We focused on rep-clusters with minimum three strains and with clear and a sufficient number of bands in their rep-patterns.

For 14 reference strains of eight different rep-clusters belonging to the large FAA cluster 5, DNA-DNA hybridizations were performed, to get a first glimpse of their relatedness at the species level (see Table 3.2). The hybridization values between strains of the same rep-cluster (rep-clusters I, II and XXII) are high (> 70%) and according to Wayne *et al.* (1987), these strains belong to the same species. Indeed, Versalovic *et al.* (1994) have shown that strains with the same rep-PCR profile are always closely related, and this has been confirmed by several authors (e.g. Rademaker & de Bruijn, 1997; Rademaker *et al.*, 2000). However, hybridization values between strains of different clusters are sometimes high (> 70%), indicating that the rep-clusters they represent belong to the same species (rep-clusters XXII and XXVII; clusters XXI and XXIV). Indeed, when looking at these patterns into more detail, similarities between the different rep-profiles can be found (see Fig. 3.1). For several of these rep-clusters, additional hybridizations were performed, to confirm their genomic relatedness:

- Rep-cluster I contains 16 strains which belong to FAA clusters 5 and 6. Hybridizations were performed between strains R-9106 (FAA cluster 5), R-9122 (FAA cluster 6) and R-9123 (FAA cluster 6; showing, together with strain R-9132 of FAA cluster 5, a different pattern than the other strains of rep-cluster I, with only one clear band instead of four). A low hybridization level is obtained between R-9123 and the other strains (19.6%), so strains R-9123 and R-9132 were omitted from further experiments. A high hybridization value

Table 3.1. Phylogenetic relationship of strains representative for FAA clusters 1 to 15 belonging to the *Bacteroidetes*.

Strain No.	Rep-cluster [a]	FAA cluster [b]	16S rDNA similarity (%) [c]	Validly named nearest phylogenetic neighbour
R-9003	XVI	1	98.0	*Flavobacterium frigidarium*
R-11271	XV	2	98.7	*Flavobacterium limicola*
R-9033	NC	2	95.3	*Flavobacterium tegetincola*
R-8282	XII	4	92.8	*Salegentibacter salegens*
R-9192	VII	5	97.4	*F. saccharophilum, F. pectinovorum*
R-9106	I	5	98.5	*Flavobacterium gillisiae*
R-7582	II	5	98.5	*Flavobacterium limicola*
R-8023	II	5	98.7	*Flavobacterium limicola*
R-7585	XX	5	96.0	*Flavobacterium limicola*
R-7581	IV	5	95.3	*Flavobacterium tegetincola*
R-9014	XXIV	5	98.4	*Flavobacterium gillisiae*
R-8022	XXI	5	98.4	*Flavobacterium gillisiae*
R-7518	XXI	5	98.1	*Flavobacterium gillisiae*
R-9010	XXII	5	99.0	*Flavobacterium xanthum*
R-7548	NC	5	97.9	*Flavobacterium tegetincola*
R-7933	NC	5	97.7	*Flavobacterium limicola*
R-7550	NC	5	98.9	*Flavobacterium limicola*
R-9122	I	6	98.4	*Flavobacterium gillisiae*
R-7579	XIX	7	97.3	*Flavobacterium limicola*
R-7515	VI	9	95.0	*Flavobacterium limicola*
R-9046	V	10	98.6	*Algoriphagus chordae*
R-10847	XI	10	98.5	*Flavobacterium limicola*
R-8899	XVII	10	95.1	*Flavobacterium flevense*
R-8885	XVII	10	95.1	*Flavobacterium flevense*
R-8893	NC	10	94.5	*Flavobacterium aquatile*
R-11385	XXVI	11	98.5	*Flavobacterium omnivorum*
R-8963	NC	11	98.2	*Flavobacterium tegetincola*
R-9331	NC	11	98.2	*Flavobacterium tegetincola*
R-7666	XIV	12	95.7	*Flavobacterium limicola*
R-9217	VIII	13	99.7	*Gelidibacter algens*
R-7572	NC	14	97.3	*Hymenobacter actinosclerus*
R-9286	IX	15	98.7	*Algoriphagus chordae, A. ratkowskyi*
R-10710	XII	15	98.7	*Algoriphagus chordae, A. ratkowskyi*
R-11427	XII	15	98.7	*Algoriphagus chordae, A. ratkowskyi*
R-9476	XVIII	15	96.4	*Flavobacterium limicola*

[a] Rep-clusters are as delineated in Fig. 3.1.

[b] FAA clusters are as delineated in Van Trappen *et al.* (2002), see chapter 2. NC, not clustered.

[c] The 16S rDNA sequence similarities are based on pairwise alignments.

Table 3.2. DNA-DNA relatedness (%) of representative strains of FAA cluster 5.

Strain No.	Rep-cluster [a]	R-9010	R-9141	R-9127	R-7581	R-7582	R-8023	R-9000	R-12606	R-9014	R-12627	R-8982	R-9106	R-8988	R-9192
R-9010	XXII	**100**													
R-9141	XXII	**95**	**100**												
R-9127	XXVII	**91**		**100**											
R-7581	IV	35	38		**100**										
R-7582	II	35	36		59	**100**									
R-8023	II	37	36	47	60	**96**	**100**								
R-9000	XXI	23	23		24	22	22	**100**							
R-12606	XXIV		24		28	25		**92**	**100**						
R-9014	XXIV	24	24	31	27	23	28	**82**		**100**					
R-12627	XXI	22		23			25			**91**	**100**				
R-8982	I	18	18		18	24	20	27	28	27		**100**			
R-9106	I	19	20	21	24	22	25	30		29	26	**100**	**100**		
R-8988	I	25		24			28			35	32		**94**	**100**	
R-9192	VII	30	27	29	27	26	29	25		23	19	19	20	20	**100**

[a] Rep-clusters are as delineated in Fig. 3.1. In bold face: values of more than 70%.

between strains R-9106 and R-9122 of rep-cluster I (97.6%) shows that the strains they represent are genotypically closely related and most probably belong to the same species, which is also novel since hybridization values with nearest phylogenetic neighbours are low. Phenotypic results confirm this species delineation and the name *Flavobacterium degerlachei* sp. nov. is proposed (see section 3.3).

- For rep-cluster VII, hybridizations were performed between the two strains (R-9192 and R-9193) and strain R-8016, with a very similar rep-profile, and type strains of nearest phylogenetic neighbours. The results show clearly that these three strains belong to a single and novel species, for which the name *Flavobacterium micromati* sp. nov. is proposed (see section 3.3).

- For rep-clusters XXI and XXIV, additional hybridization results between strains of these clusters and nearest phylogenetic neighbours, show that they represent a new species within the genus *Flavobacterium* for which the name *F. frigoris* sp. nov. is proposed (see section 3.3 and 3.4).

- The four strains of rep-cluster II belong to another new *Flavobacterium* species, with the name *F. psychrolimnae* sp. nov., according to additional hybridization results with nearest phylogenetic neighbours (see section 3.4).

- The three strains R-8284, R-8019 and R-7548 show similar rep-profiles and high hybridization values, and represent a novel species for which the name *Flavobacterium fryxellicola* sp. nov. is proposed (see section 3.4).

- Strain R-9010 shows a high sequence similarity (99.0%) with the type strain of *Flavobacterium xanthum* and hybridization results (91.0%) confirm that the strains of rep-cluster XXII and related clusters XXIII and XXVII most probably belong to this validly described *Flavobacterium* species (see section 3.4).

- For the 22 strains of rep-cluster XVII, belonging to FAA cluster 10, the low sequence similarity (95.1%) with *Flavobacterium flevense* indicates that they belong to a novel species (Stackebrandt & Goebel, 1994), for which the name *Flavobacterium gelidilacus* sp. nov. is proposed (see section 3.2).

- The three strains of FAA cluster 4 (of which two belong to rep-cluster XIII) showed only 92.8% sequence similarity to *Salegentibacter salegens*, their nearest phylogenetic neighbour, and hybridization results together with phenotypic features confirm that they belong to a single species within a novel genus, for which the name *Gillisia limnaea* gen. nov., sp. nov. is proposed (see section 3.5).

- The eight strains of FAA cluster 15 belong to three different rep-clusters (IX, XII and XVIII) and 16S rDNA sequences show that rep-clusters IX and XII are phylogenetically related to *Cyclobacterium marinum* (with only 92% similarity) while rep-cluster XVIII is related to *Flavobacterium limicola* (96.4% similarity). Only the strains related to *Cyclobacterium* were investigated further and hybridizations between six of them (from strain R-10750, no DNA could be extracted after several attempts) show that they belong to a single species of the recently described genus *Algoriphagus*. They are classified as *Algoriphagus antarcticus* sp. nov. (see section 3.6).

3.2 *Flavobacterium gelidilacus* sp. nov., isolated from microbial mats in Antarctic lakes

Stefanie Van Trappen, Joris Mergaert and Jean Swings

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Twenty-two isolates from microbial mats in eastern Antarctic lakes showed similar fatty acid compositions and were investigated further using a polyphasic taxonomic approach. Repetitive extragenic palindromic DNA - PCR fingerprinting of the 22 strains revealed three groups, and DNA-DNA hybridizations between representatives showed more than 87 % DNA-DNA reassociation with each other. 16S rRNA gene sequence analysis placed two representative strains, LMG 21477^T and LMG 21619 within the genus *Flavobacterium*, with 95.1 % sequence similarity to *Flavobacterium flevense*, 95.0 % to *Flavobacterium tegetincola*, less than 95 % to other *Flavobacterium* species and less than 90 % to representatives of other genera. The name *Flavobacterium gelidilacus* sp. nov. is proposed, with LMG 21477^T (= DSM 15343^T) as the type strain, and a description of the species is given on the basis of morphological, biochemical and physiological characteristics and fatty acid composition. The G+C content of the genomic DNA is 30.0-30.4 mol%.

Introduction

Members of the genus *Flavobacterium* have been isolated from diverse habitats such as freshwater (*Flavobacterium aquatile, Flavobacterium flevense, Flavobacterium hibernum, Flavobacterium saccharophilum*), soil (*Flavobacterium johnsoniae, Flavobacterium pectinovorum*, *Flavobacterium xanthum*) and sea-ice (*Flavobacterium gillisiae*); some are known as important fish pathogens (*Flavobacterium branchiophilum, Flavobacterium columnare, Flavobacterium psychrophilum*). They are abundant in freshwater and marine ecosystems, and these heterotrophic bacteria may have a specialized role in the uptake and degradation of the high-molecular-mass fraction of dissolved organic matter in these environments (Kirchman, 2002).

Several novel species, added to the genus since 1996, were derived from Antarctic habitats, and several new genera containing polar organisms have recently been described within the family *Flavobacteriaceae* (*Gelidibacter, Psychroserpens, Polaribacter, Psychroflexus, Salegentibacter*). So far, only one species, *Flavobacterium tegetincola,* has been isolated from a cyanobacterial mat, collected from the Antarctic saline Ace Lake located in the Vestfold Hills (McCammon & Bowman, 2000).

During the MICROMAT project (November 1998 to February 2001), 746 bacterial strains were isolated under heterotrophic conditions from microbial mat samples, collected from 10 Antarctic lakes in the Vestfold Hills (lakes Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts), the Larsemann Hills (lake Reid) and the McMurdo Dry Valleys (lakes Hoare and Fryxell) (Van Trappen *et al.*, 2002). Numerical analysis of their fatty acid composition revealed 41 clusters, and 16S rRNA gene sequence analysis, performed on representative strains, showed that they belong to the α-, β- and γ-subclasses of the *Proteobacteria*, the high- and low-G+C-content Gram-positives and to the *Bacteroidetes* (Van Trappen *et al.*, 2002). The results of the fatty acid analysis and 16S rRNA gene sequence analysis showed that the diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is very high. Moreover, many fatty acid clusters contain multiple taxa, as defined by repetitive extragenic palindromic DNA-PCR (rep-PCR) fingerprinting, a technique used to investigate the genomic diversity of each fatty acid cluster in more detail (Van Trappen *et al.*, 2001).

In the present work, we studied further the taxonomic relationships of 22 strains from fatty acid cluster 10 (as delineated by Van Trappen *et al.*, 2002),

related to the genus *Flavobacterium*, by genomic and phenotypic characterization. Van Trappen *et al.* (2002) found less than 96 % 16S rRNA gene sequence similarity to the closest relatives within the genus *Flavobacterium*, indicating that these strains constitute a new species (Stackebrandt & Goebel, 1994).

Materials and Methods

The isolates investigated, together with their sources, are listed in Table 3.3. The strains were routinely cultivated on R2A medium (Difco) at 20 °C for 48 h or, for strain LMG 8328^T on TSA medium (BBL) at 20 °C for 48 h, except where mentioned otherwise.

DNA was prepared according to the method of Pitcher *et al.* (1989), and rep-PCR fingerprinting was performed on all strains of fatty acid analysis cluster 10 of Van Trappen *et al.* (2002), using the primer GTG_5 (Versalovic *et al.*, 1991), as described by Rademaker & de Bruijn (1997) and Rademaker *et al.* (2000). Numerical analysis was carried out using the BIONUMERICS software package (Applied Maths), as described by the same authors.

DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C and reciprocal experiments were performed for every pair of strains.

The G+C content of the DNA's from reference strains was determined using a HPLC method. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$, pH 4.0, with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference.

Almost complete 16S rRNA gene sequences of two reference strains were obtained as described previously (Mergaert *et al.*, 2001b). The most closely related sequences were found using the FASTA program. Phylogenetic analysis was performed using the BIONUMERICS software package, taking into account homologous nucleotide positions after discarding all unknown bases and gaps. A neighbour-joining dendrogram (Saitou & Nei, 1987) with the nearest phylogenetic relatives was constructed on the basis of global alignment of the sequences, using the same software package.

The fatty acid compositions are based on the data generated by Van Trappen *et al.* (2002), or were determined as described by the same authors.

The following morphological, physiological and biochemical tests were performed. Colony morphology was determined on R2A medium after 6 days. In addition, growth and adherence of colonies on marine and nutrient agars, TSA and Anacker and Ordal's agar (Anacker & Ordal, 1955) were tested after 14 days growth. Cells were tested for their reaction to the Gram stain and for catalase and oxidase activity. Tests in the commercial systems API ZYM, API 20NE and API 20E (bioMérieux) were performed according to the instructions of the manufacturer. API ZYM tests were read after 4 h incubation at 20 °C; other API tests were read after 48 h at 20 °C. Congo red absorption (Bernardet *et al.*, 2002), production of flexirubine-type pigments (Reichenbach, 1989), the presence of gliding motility, degradation of casein and chitin (Reichenbach & Dworkin, 1981), alginate (West & Colwell, 1984), DNA (using DNA agar from Difco, supplemented with 0.01 % toluidine blue from Merck), pectin (Paton, 1959), starch and L-tyrosine (Barrow & Feltham, 1993), the production of a brown diffusible pigment on L-tyrosine agar and the precipitation of egg-yolk agar (Barrow & Feltham, 1993) were also investigated; reactions were read after 5 days. Hydrolysis of carboxy-methylcellulose was tested in Anacker & Ordal's broth gelified with 3 % carboxymethylcellulose sodium salt (high viscosity; Sigma). This medium was stab-inoculated, and liquefaction of the medium within 7 days was scored as a positive reaction. Growth at different temperatures was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1-10 % NaCl after 14 days incubation.

Results and discussion

Twenty-two strains of fatty acid analysis cluster 10, listed in Table 3.3, showed similar rep-PCR profiles (see also Fig. 3.2) and they could be divided into three clusters according to their profile type, hereafter referred to as rep-PCR profile type I (with 9 strains), rep-PCR profile type II (with 12 strains), and rep-PCR profile type III (containing the single strain LMG 21620). Versalovic *et al.* (1994) have shown that strains with the same rep-PCR profile are always closely related, and this has been confirmed by several authors (e.g. Rademaker & de Bruijn, 1997).

Five strains (LMG 21477^T, LMG 21618, LMG 21619, LMG 21620 and LMG

Table 3.3. Strains investigated in this study.

Strain	Isolation source
Rep-PCR profile type I	
LMG 21477^T (= DSM 15343^T = R-8899^T), R-8897, R-8908, R-8969, R-8972, R-9283	Ace Lake, Vestfold Hills
R-9024	Lake Watts, Vestfold Hills
R-11278	Pendant Lake, Vestfold Hills
LMG 21618 (= R-12566)	Lake Reid, Larsemann Hills
Rep-PCR profile type II	
LMG 21619 (= R-8885), R-8888, R-8898, R-9104, R-9110, R-9158	Ace Lake, Vestfold Hills
LMG 21621 (= R-9330), R-9004, R-9019	Lake Watts, Vestfold Hills
R-11078, R-11277, R-8983	Pendant Lake, Vestfold Hills
Rep-PCR profile type III	
LMG 21620 (= R-9056)	Pendant Lake, Vestfold Hills

Numbers with an 'R-'prefix refer to strains from the research collection of the LMG, as used by Van Trappen *et al.* (2002).

21621) representing the three rep-PCR profile types and chosen on the basis of their isolation source were used for DNA-DNA hybridizations to investigate their genomic relatedness. The DNA-DNA binding values among the five strains were high, ranging from 87 % to 97 %, and differences between reciprocal experiments were less than 13 %. These DNA-DNA binding values confirm that the 22 strains belong to a single species (Wayne *et al.*, 1987).

The G+C content of the DNA's from strains LMG 21477^T, LMG 21618, LMG 21619, LMG 21620 and LMG 21621 was determined and the G+C contents of the novel strains were 30.0-30.4 mol%, which is slightly below the range (32-37 mol % G+C) mentioned by Bernardet *et al.* (1996) for the genus *Flavobacterium*.

Almost complete 16S rRNA gene sequences (1467-1468 base pairs) of strains LMG 21477^T (rep-profile type I) and LMG 21619 (rep-profile type II) were obtained. A neighbour-joining dendrogram with the nearest phylogenetic relatives is shown in Fig. 3.3. Dendrograms obtained using maximum-parsimony and maximum-likelihood analyses showed essentially the same topography. The 16S rRNA gene sequences of strains LMG 21477^T and LMG 21619 differed by only one base, and showed 95.1 % similarity to that of *F. flevense*, 95.0 % to that of *F. tegetincola*, less than 95 % to sequences of other *Flavobacterium* species and less than 90 % to sequences of other genera, indicating that they belong to a novel *Flavobacterium* species.

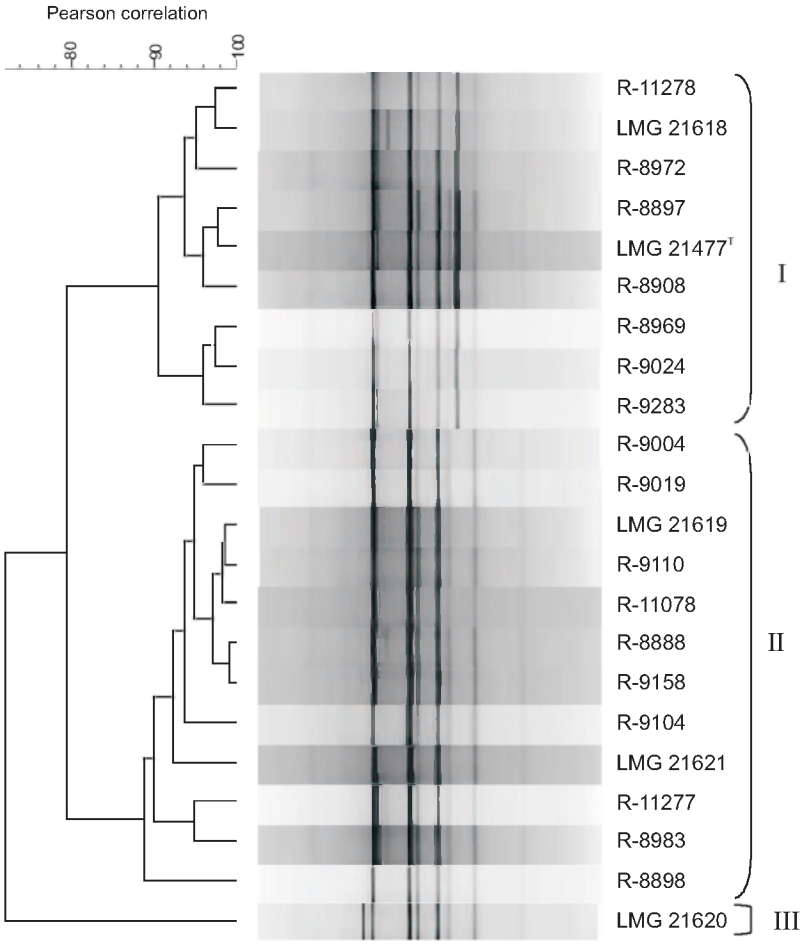

Figure 3.2. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of 22 strains belonging to fatty acid cluster 10. Dendrogram derived from the UPGMA-clustering of the profiles with the Pearson correlation coefficient.

The 22 novel strains yielded very similar fatty acid profiles. The mean composition was 4% 14:0 iso, 10% 15:0, 1% 15:0 3-OH, 8% 15:0 anteiso, 12% 15:0 iso, 6% 15:0 iso 3-OH, 1% 15:1 anteiso, 10% 15:1 iso, 6% 15:1ω6c, 8% 16:0 iso, 10% 16:0 iso 3-OH, 4% 16:1 iso, 6% 17:0 iso 3-OH, 3% 17:1ω6c, 2% 17:1 iso ω9c, 1% 18:1ω5c and 2% 15:0 iso 2-OH and/or 16:1ω6c. Other fatty acids each accounted for less than 1%. The fatty acid profiles of the novel strains resemble those determined for other *Flavobacterium* species (Bernardet *et al.*, 1996), but differ in terms of the relative amounts of 15:0 anteiso, 15:0 iso, 16:0 iso and 16:0 iso 3-OH.

The strains showed morphological characteristics typical of *Flavobacterium* (Bernardet *et al.,* 2002) and were almost identical in their physiological and biochemical characteristics (see Description). The novel species can be clearly

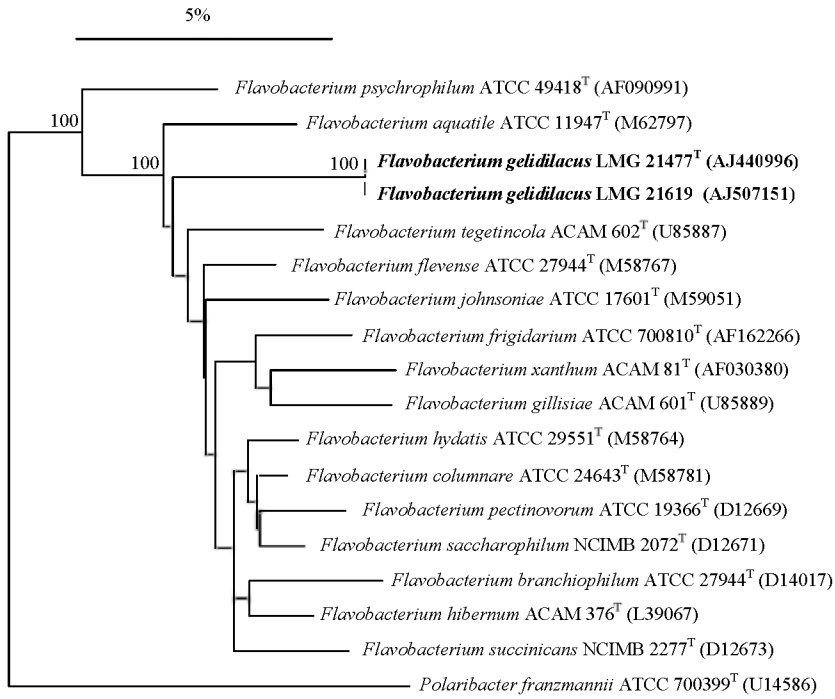

Figure 3.3. Neighbour-joining dendrogram based on 16S rDNA sequences showing the estimated phylogenetic relationships of *Flavobacterium gelidilacus* sp. nov., other *Flavobacterium* species and *Polaribacter franzmannii* (outgroup). Bootstrap values are shown as percentages of 1000 replicates, if higher than 95 %. Bar, 5 % sequence divergence.

differentiated from other *Flavobacterium* species by several phenotypic characteristics (Table 3.4).

The results of the polyphasic analysis support the recognition of a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium gelidilacus* sp. nov. is proposed.

Description of *Flavobacterium gelidilacus* sp. nov.

Flavobacterium gelidilacus (ge.li.di.la'cus. L. adj. *gelidus* ice-cold; L. n. *lacus* lake; N. L. gen. n. *gelidilacus* of the ice-cold lake, referring to the isolation source, microbial mats in Antarctic lakes).

Gram-negative rods, <1 x 2-4 µm, that exhibit gliding motility on nutrient-poor medium (R2A), except for strains LMG 21477^T and LMG 21619, for which no gliding motility is detected. The strains grow at 5-25°C, with optimal growth at 20

Table 3.4. Characteristics that differentiate between *F. gelidilacus* sp. nov. and other *Flavobacterium* species.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Growth on:																
TSA	+	+	+	+	+	+	+	(+)	-	-	-	+	+	+	+	+
Nutrient agar	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+
Growth at 25°C on agar	+	-	+	(+)	(+)	(+)	+	+	+	+	-	+	+	+	+	+
Flexirubin-type pigment	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	-
Congo red absorption	-	+	-	-	-	-	-	-	-	+	-	-	v	-	-	-
Glucose utilization	-	+	+	+	+	+	+	ND	ND	-	-	+	+	+	+	+
Acid from carbohydrates	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+
Degradation of:																
Casein	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Starch	+	-	+	+	+	-	ND	v	+	-	-	+	+	+	+	+
Carboxymethylcellulose	-	-	-	-	-	-	-	-	-	-	-	ND	+	+	+	ND
Agar	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
Alginate	-	-	-	-	-	-	-	ND	ND	ND	-	-	+	+	ND	ND
Pectin	-	-	-	-	-	-	+	ND	ND	ND	-	+	+	+	+	ND
Chitin	-	-	-	-	+	-	-	-	-	-	-	(+)	+	+	-	-
Aesculin	-	+	+	+	+	-	+	v	-	-	-	+	+	+	+	+
DNA	-	-	+	-	-	-	-	-	-	+	(+)	+	+	+	-	+
Tyrosine	-	-	+	-	-	-	-	v	+	-	v	+	+	+	+	-
Precipitate on egg-yolk agar	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
β-Galactosidase activity	-	-	+	-	-	-	+	ND	+	-	-	+	+	+	+	+
H_2S production	-	-	-	+	-	-	-	-	-	+	-	-	-	ND	+	+
Nitrate reduction	-	-	+	+	-	-	ND	ND	-	ND	-	+	+	+	+	v
Mean G+C content (mol%)	30	35	36	36	32	32	35	33	33	32	35	34	34	35	33	36

Species: 1, *F. gelidilacus* sp. nov.; 2, *F. frigidarium*; 3, *F. hibernum*; 4, *F. xanthum*; 5, *F. gillisiae*; 6, *F. tegetincola*; 7, *F. flevense*; 8, *F. aquatile*; 9, *F. branchiophilum*; 10, *F. columnare*; 11, *F. psychrophilum*; 12, *F. hydatis*; 13, *F. johnsoniae*; 14, *F. pectinovorum*; 15, *F. saccharophilum*; 16, *F. succinicans*. Data were taken from Bernardet *et al.* (1996), McCammon *et al.* (2000), Humphry *et al.* (2001) and this study. +, Positive; (+), positive, weak or delayed response; -, negative; v, results vary between strains of species or between references; ND, no data available.

°C; no growth at 30 °C. Yellow to orange, convex, translucent colonies, 1–4 mm in diameter and with entire margins, are formed on R2A plates after 6 days at 20 °C. Colonies on Anacker & Ordal's agar are flat, round with entire margins and 0.5-1 mm in diameter after 14 days incubation. Growth also occurs on TSA, nutrient agar and marine agar, and colonies do not adhere to the agar. Degrades casein and starch. Gelatinase activity is observed, except in the case of strain LMG 21619. Catalase- and oxidase-positive. No growth is observed on glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate. Acid is not produced from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melobiose, amygdalin, arabinose. Agar, alginate, pectin, chitin, aesculin, carboxymethylcellulose, DNA, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. There is no production of a brown diffusible pigment on L-tyrosine agar and no precipitate is formed on egg-yolk agar. The Voges-Proskauer reaction and tests for indole production, citrate utilization, nitrate reduction and hydrogen sulfide production are negative. None of the strains shows activity for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (C14), α-chymotrypsine, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, and α-fucosidase. Weak enzymic activity is observed for cystine arylamidase, medium activity is found for acid phosphatase, esterase lipase (C8), phosphohydrolase and α-glucosidase, and strong activity is found for alkaline phophatase, leucine arylamidase and valine arylamidase. No β-glucosidase or *N*-acetyl-β-glucosaminidase activity is detected, except for strain LMG 21621. Different reactions are obtained for esterase (C4) and trypsin. The cells contain the fatty acids 15:0 iso, 16:0 iso 3-OH, 15:1 iso, 15:0, 15:0 anteiso and 16:0 iso as the main constituents. Growth occurs in the absence of NaCl and in the presence of 1-5 %NaCl, but not 10% NaCl, indicating that the strains are not halophilic but merely halotolerant. The G+C content is 30.0-30.4 mol%.

The type strain is LMG 21477^T (= DSM 15343^T). Twenty-two strains were isolated from microbial mats from freshwater and saline lakes in eastern Antarctica (Table 3.3).

3.3 *Flavobacterium degerlachei* sp. nov., *Flavobacterium frigoris* sp. nov. and *Flavobacterium micromati* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes

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Taxonomic studies were performed on thirty-six strains that were isolated from microbial mats in Antarctic lakes of the Vestfold Hills, the Larsemann Hills and the McMurdo Dry Valleys. Phylogenetic analyses based on 16S rRNA gene sequences indicated that these strains are related to members of the genus *Flavobacterium*; sequence similarity values with their nearest phylogenetic neighbours ranged from 96.8% to 98.5%. Results of DNA-DNA hybridization and comparison of repetitive extragenic palindromic DNA-PCR fingerprinting patterns revealed that these strains are members of three distinct species. Genotypic results, together with phenotypic characteristics, allowed the differentiation of these species from related *Flavobacterium* species with validly published names. The isolates are Gram-negative, chemoheterotrophic, rod-shaped cells that are psychrophilic and moderately halotolerant; their DNA G + C contents range from 33.1 to 34.5 mol%. Their whole-cell fatty acid profiles are similar and include $C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$, iso-$C_{16:0}$3-OH and summed feature 3 (which comprises iso-$C_{15:0}$ 2-OH, $C_{16:1}\omega7c$, or both) as major fatty acid components. On the basis of these results, three novel species are proposed, namely *Flavobacterium degerlachei* sp. nov. (consisting of 14 strains, with LMG 21915^T = DSM 15718^T as the type strain), *Flavobacterium micromati* sp. nov. (consisting of 3 strains, with LMG 21919^T = CIP 108161^T as the type strain) and *Flavobacterium frigoris* sp. nov. (consisting of 19 strains, with LMG 21922^T = DSM 15719^T as the type strain).

Introduction

The genus *Flavobacterium* belongs to the *Bacteroidetes* and was proposed by Frankland in 1889. Since then, the description of this genus has been revised several times (Bernardet *et al.*, 1996). *Flavobacterium* species have been isolated from diverse habitats such as fresh- and salt water, soil, sediment, sea-ice, diseased fish and microbial mats. Members of the *Bacteroidetes* are highly abundant in freshwater and marine ecosystems and became dominant in response to the input of organic substrates (Höfle, 1992; Rossello-Mora *et al.*, 1999). These findings suggest that these bacteria may have a specialized role in the uptake and degradation of organic matter in cold, aquatic environments (Kirchman, 2002). Indeed, many species of the genus *Flavobacterium* are capable of the hydrolysis of organic polymers such as complex polysaccharides (Bernardet *et al.*, 1996).

Several novel species that have been added to the genus *Flavobacterium* since 1996 originated from Antarctic habitats, e.g. *Flavobacterium hibernum* (McCammon *et al.*, 1998), *Flavobacterium gillisiae* (McCammon & Bowman, 2000) and *Flavobacterium frigidarium* (Humphry *et al.*, 2001), but only two species have so far been isolated from cyanobacterial mats: *Flavobacterium tegetincola* (McCammon & Bowman, 2000) and *Flavobacterium gelidilacus* (Van Trappen *et al.*, 2003), which were collected from Antarctic lakes. Recently, three novel psychrophilic *Flavobacterium* species have been described: *Flavobacterium limicola* from freshwater sediments (Tamaki *et al.*, 2003) and *Flavobacterium xinjiangense* and *Flavobacterium omnivorum* from the China No.1 glacier (Zhu *et al.*, 2003).

During the MICROMAT project (November 1998 - February 2001), 746 bacterial strains were isolated under heterotrophic conditions from microbial mat samples that were collected from 10 Antarctic lakes in the Vestfold Hills (lakes Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts), the Larsemann Hills (lake Reid) and the McMurdo Dry Valleys (lakes Hoare and Fryxell) (Van Trappen *et al.*, 2002). Salinity of these lakes ranges from fresh (Druzhby, Grace, Watts and Hoare) over hyposaline/saline (Ace, Highway, Pendant, Fryxell and Reid) to hypersaline (Organic). Numerical analysis of the fatty acid composition of the isolates revealed 41 clusters and 16S rRNA gene sequence analysis, performed on representative strains, showed that they belong to the α-, β- and γ-subclasses of the *Proteobacteria*, the high and low percent G+C Gram-positives and to the *Bacteroidetes* (Van Trappen *et al.*, 2002). Results of fatty acid and 16S

rRNA gene sequence analyses showed that the diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is very high. Moreover, many fatty acid clusters were shown to contain multiple taxa when tested by repetitive extragenic palindromic DNA (rep)-PCR fingerprinting, a technique used to investigate the genomic diversity of each fatty acid cluster more in detail (Van Trappen *et al.*, 2001). Twenty-two isolates from fatty acid cluster 10 have already been described as a novel species, *F. gelidilacus* (Van Trappen *et al.*, 2003).

In the present work, we have studied the taxonomic relationships of 36 strains from fatty acid clusters 5 and 6 (as delineated by Van Trappen *et al.*, 2002) that are related to the genus *Flavobacterium* by polyphasic taxonomic characterization.

Materials and Methods

The isolates investigated are listed in Table 3.5. Strains were cultivated routinely on R2A medium (Difco) at 20 °C for 48 h or longer (LMG 21919^T) or [for strains LMG 4031^T (*Flavobacterium pectinovorum*) and LMG 8384^T (*Flavobacterium saccharophilum*)] on TSA medium (BBL) at 20 °C for 48 h, except when mentioned otherwise.

DNA was prepared according to the method of Pitcher *et al.* (1989) and rep-PCR fingerprinting (based on primers that targeted the repetitive extragenic palindromic sequence) was performed on all strains of fatty acid clusters 5 (75 strains) and 6 (five strains) of Van Trappen *et al.* (2002) using the primer GTG_5 (Versalovic *et al.*, 1991), as described by Rademaker & de Bruijn (1997) and Rademaker *et al.* (2000). Numerical analysis was carried out by using the Bionumerics software package (Applied Maths; available at http://www.applied-maths.com), as described by the same authors.

Small-scale DNA extracts were prepared by using the method of Pitcher *et al.* (1989) and the almost-complete 16S rRNA gene sequences of reference strains were amplified by PCR with conserved primers (Coenye *et al.*, 1999). PCR products were purified by using a QIAquick PCR Purification kit (Qiagen) according to the instructions of the manufacturer. Sequence analysis was performed by using an ABI Prism 3100 automatic DNA sequencer (Applied Biosystems), applying a BigDye Terminator Cycle Sequencing Ready Reaction kit (version 2.0; PerkinElmer Applied Biosystems), following the protocols of the manufacturer. Sequence

assembly was performed by using the program AutoAssembler 1.4.0 (PerkinElmer Applied Biosystems). The most closely related sequences were found by using the FASTA program; sequences from reference strains were aligned and editing of the alignment and reformatting were performed with the BIOEDIT program (Hall, 1999) and ForCon (Raes & Van de Peer, 1999). Evolutionary distances were calculated using the Jukes-Cantor evolutionary model and a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with the TREECON program (Van de Peer & De Wachter, 1994).

DNA was prepared according to the method of Pitcher *et al.* (1989) and DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 BioAssay reader (PerkinElmer) for fluorescence measurements. The hybridization temperature was 32 °C and reciprocal experiments were performed for every pair of strains.

DNA G+C contents of the Antarctic strains were determined by using an HPLC-based method as described by Van Trappen *et al.* (2003).

The following morphological, physiological and biochemical tests were performed. Colony morphology was determined on R2A medium after 6 days. In addition, growth and adherence of colonies on marine, nutrient and trypticase soy agars and on Anacker and Ordal's agar (Anacker & Ordal, 1955) after 14 days were tested. Cells were tested for their Gram-stain reaction and for catalase and oxidase activities. Tests in the commercial API ZYM, API 20NE and API 20E systems (bioMérieux) were generally performed according to the instructions of the manufacturer. The API ZYM tests were read after 4 h incubation at 20 °C and other API tests were read after 48 h at 20 °C. Congo red absorption (Bernardet *et al.*, 2002), production of flexirubine-type pigments (Reichenbach, 1989), presence of gliding motility, degradation of casein and chitin (Reichenbach & Dworkin, 1981), alginate (West & Colwell, 1984), DNA [using DNA agar (Difco), supplemented with 0.01 % toluidine blue (Merck)], pectin (Paton, 1959), starch and L-tyrosine (Barrow & Feltham, 1993), production of brown diffusible pigment on L-tyrosine agar and precipitation of egg-yolk agar (Barrow & Feltham, 1993) were also investigated; reactions were read after 5 days. Hydrolysis of CM-cellulose was tested in Anacker & Ordal's broth (Anacker & Ordal, 1955) gelidified with 3 % high-viscosity CM-cellulose sodium salt (Sigma). This medium was stab-inoculated and liquefaction of the medium within 7 days was scored as a positive reaction. Growth at different temperatures was assessed after 5 days incubation. Salt

tolerance was tested on R2A medium supplemented with 1-10 % NaCl after 14 days incubation.

Results and discussion

Thirty-six strains of fatty acid clusters 5 and 6, listed in Table 3.5, showed similar rep-PCR profiles (see also Fig. 3.4), and they could be divided into four different clusters according to their profile type, hereafter referred to as rep-PCR profile type I (which comprises 14 strains), II (with three strains), III (with eight strains) and IV (with 11 strains). Versalovic *et al.* (1994) have shown that strains with the same rep-PCR profile are always closely related and this has been confirmed by several authors (e.g. Rademaker & De Bruijn, 1997).

Almost-complete 16S rRNA gene sequences (1457-1480 nt) of strains LMG 21915^T, LMG 21474, LMG 21919^T, LMG 21922^T and LMG 21471 were obtained. A neighbour-joining dendrogram with the nearest phylogenetic relatives within the genus *Flavobacterium* is shown (Fig. 3.5). Dendrograms obtained by maximum-parsimony and maximum-likelihood analyses showed essentially the same topography (data not shown).

The novel Antarctic strains form three distinct branches within the genus *Flavobacterium*, which are supported by high bootstrap values, and they belong to a clade of the phylogenetic tree that consists only of recently described *Flavobacterium* species from cold environments, such as *F. gillisiae, F. xinjiangense, F. xanthum, F. omnivorum, F. frigidarium*, *F. gelidilacus* and *F. limicola*. However, other Antarctic *Flavobacterium* species, *F. hibernum* and *F. tegetincola*, do not belong to this clade and form separate branches.

The 16S rRNA gene sequences of the two representative strains of rep-PCR profile type I (LMG 21915^T and LMG 21474) were almost identical (99.9% sequence similarity) and showed 98.5 % similarity to *F. gillisiae*, 97.7 % to *F. xinjiangense*, 97.5 % to *F. limicola*, 96.9 % to *F. omnivorum* and 96.8 % to *F. xanthum*. The sequence of strain LMG 21919^T, which belongs to rep-PCR profile type II, showed 97.4 % similarity to *F. saccharophilum*, 97.4 % to *F. pectinovorum*, 97.2 % to *F. limicola* and 96.9 % to *F. omnivorum*. The 16S rRNA gene sequences of the two representative strains of rep-PCR profile types III and IV (LMG 21922^T and LMG 21471, respectively) show 99.1 % sequence similarity to each other and 98.4 % to *F. gillisiae*, 97.4 % to *F. xinjiangense*, 97.3 % to *F. xanthum*, 97.2%

Table 3.5. Strains investigated, isolation site, fatty acid cluster and rep-PCR profile type.

Species and strain	Fatty acid cluster	Rep-PCR cluster	Isolation site
***F. degerlachei* sp. nov.:**			
LMG 21915 ^{T} = R-9106	5	I	Lake Ace, Vestfold Hills
LMG 21916 = R-8982	5	I	Pendant Lake, Vestfold Hills
LMG 21917 = R-8988	5	I	Pendant Lake, Vestfold Hills
LMG 21474 = R-9122	6	I	Pendant Lake, Vestfold Hills
LMG 21918 = R-9125	6	I	Pendant Lake, Vestfold Hills
R-8991, R-8992, R-8993, R-9119, R-11356	5	I	Pendant Lake, Vestfold Hills
R-12608	5	I	Lake Reid, Larsemann Hills
R-11563	5	I	Highway Lake, Vestfold Hills
R-9118, R-9124	6	I	Pendant Lake, Vestfold Hills
***F. micromati* sp. nov.:**			
LMG 21919 ^{T} = R-9192	5	II	Grace Lake, Vestfold Hills
LMG 21920 = R-9193	5	II	Grace Lake, Vestfold Hills
LMG 21921 = R-8016	5	II	Lake Fryxell, Dry Valleys
***F. frigoris* sp. nov.:**			
LMG 21922^{T} = R-9014	5	III	Watts Lake, Vestfold Hills
LMG 21924 = R-12606	5	III	Lake Reid, Larsemann Hills
LMG 21471 = R-8022	5	IV	Lake Fryxell, Dry Valleys
LMG 21923 = R-9000	5	IV	Grace Lake, Vestfold Hills
LMG 21925 = R-12627	5	IV	Lake Reid, Larsemann Hills
R-9002, R-9144, R-9149	5	III	Grace Lake, Vestfold Hills
R-9138	5	III	Watts Lake, Vestfold Hills
R-12591, R-12625	5	III	Lake Reid, Larsemann Hills
R-8017, R-8020, R-8359	5	IV	Lake Fryxell, Dry Valleys
R-8996	5	IV	Grace Lake, Vestfold Hills
R-9134, R-9137	5	IV	Watts Lake, Vestfold Hills
R-9227	5	IV	Lake Druzhby, Vestfold Hills
R-9228	5	IV	Grace Lake, Vestfold Hills

Abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; R-, strain numbers from the research collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium, and as used by Van Trappen *et al.* (2002); fatty acid clusters are as delineated by Van Trappen *et al.* (2002).

to *F. omnivorum* and 97.1 % to *F. limicola*. Strains LMG 21922^{T} and LMG 21915^{T} showed 98.7% sequence similarity to each other and only 96.4% to strain LMG 21919^{T}.

Genomic relatedness between the novel Antarctic strains (representing the four different rep-PCR profile types) and their most closely related phylogenetic

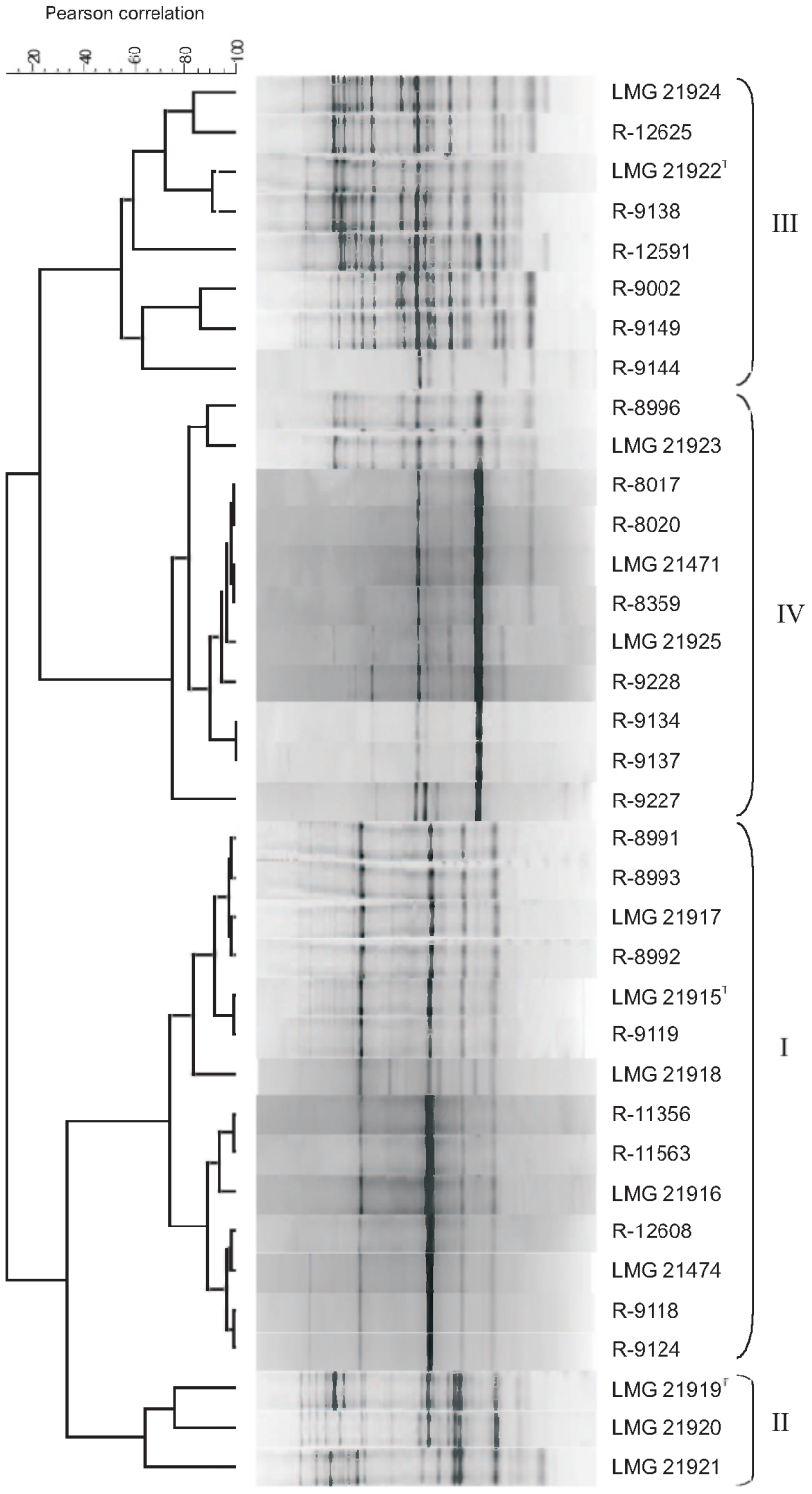

Figure 3.4. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of 36 strains belonging to fatty acid clusters 5 and 6. Dendrogram derived from the UPGMA-clustering of the profiles with the Pearson correlation coefficient and rep-clusters were delineated at a cut-off value of 50%. Rep-cluster I, *F. degerlachei* sp. nov.; rep-cluster II, *F. micromati* sp. nov.; rep-cluster III and IV, *F. frigoris* sp. nov.

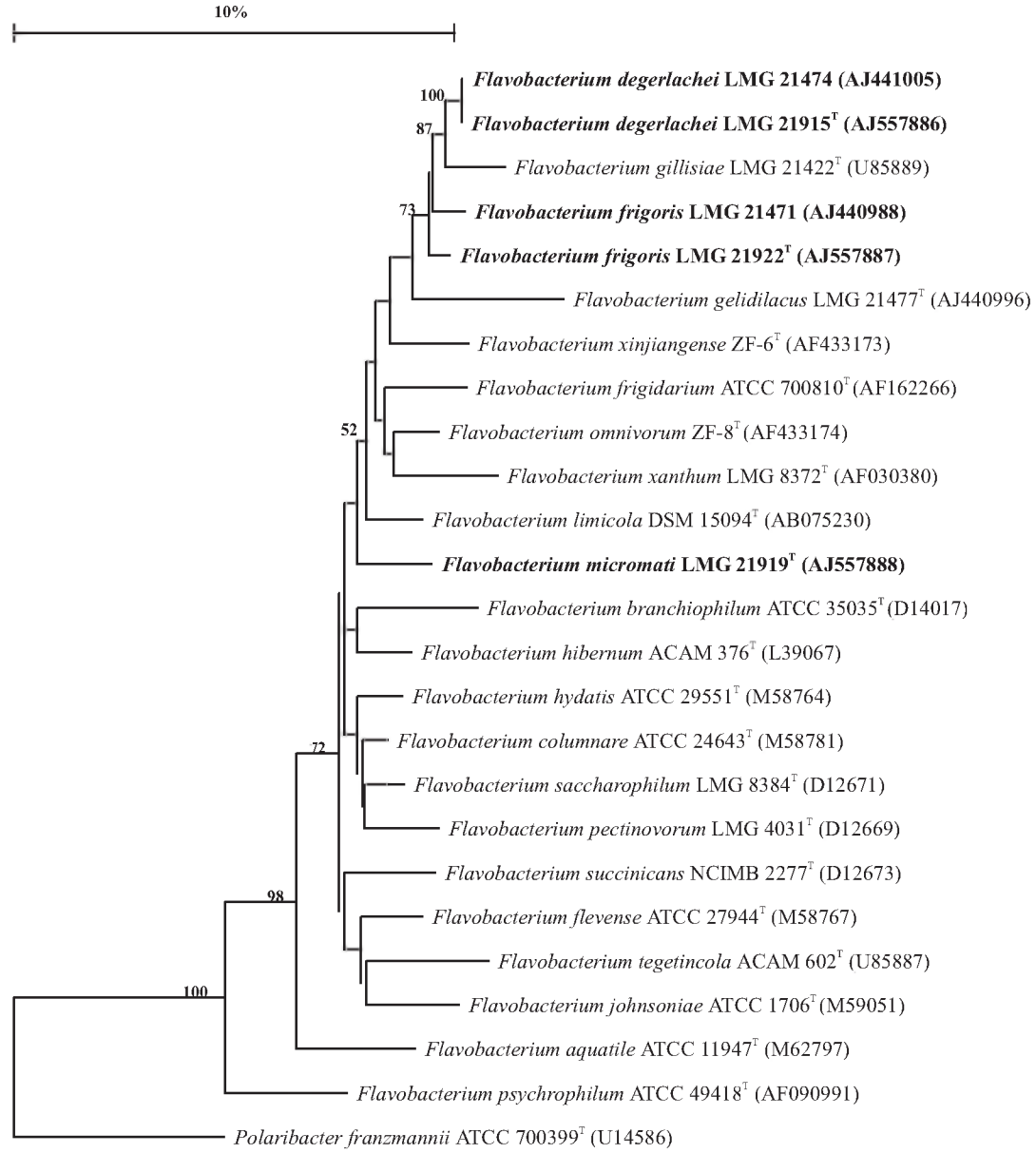

Figure 3.5. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of *Flavobacterium degerlachei* sp. nov., *Flavobacterium micromati* sp. nov., *Flavobacterium frigoris* sp. nov. and other *Flavobacterium* species on the basis of 16S rRNA gene sequences. *Polaribacter franzmannii* was choosen as outgroup. Bootstrap values (percentages of 500 replicates) of > 50 % are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 10% sequence divergence.

neighbours (*Flavobacterium gillisiae* for rep-PCR profile types I, III and IV and *F. pectinovorum* and *F. saccharophilum* for rep-PCR profile type II) was determined by DNA-DNA hybridization. The hybridization level between strains LMG 21915^T, LMG 21916, LMG 21917 and LMG 21474 of rep-PCR profile type I was ranging between 93.6-97.7 %, indicating that the 14 strains of rep-PCR profile type I belong

to one single species (Wayne *et al.*, 1987). Hybridization values of LMG 21915^T with its nearest phylogenetic neighbours, *F. gillisiae* (LMG 21422^T), *F. xanthum* (LMG 8372^T) and LMG 21922^T, were respectively 28.9, 18.4 and 28.4%, indicating that the strains of rep-PCR profile type I represent a novel *Flavobacterium* species, for which the name *Flavobacterium degerlachei* sp. nov. is proposed.

High hybridization values (81.1%-84.7%) were obtained between strains LMG 21919^T, LMG 21920 and LMG 21921 of rep-PCR profile type II. The low hybridization level (13.2-16.1%) between LMG 21919^T and its nearest phylogenetic neighbours *F. pectinovorum* (LMG 4031^T) and *F. saccharophilum* (LMG 8384^T) reveals that the three strains of rep-PCR profile type II constitute a new species, for which the name *Flavobacterium micromati* sp. nov. is proposed.

Hybridization results between strains LMG 21922^T, LMG 21923, LMG 21924 and LMG 21925 of rep-PCR profile types III and IV (82.5 - 91.2 %) showed that the strains of these two different rep-PCR profile types represent a single species that is clearly different from related *Flavobacterium* species. LMG 21922^T showed only 52% hybridization with *F. gillisiae* (LMG 21422^T) and 4.1% with *F. xanthum* (LMG 8372^T); the name *Flavobacterium frigoris* sp. nov. is proposed for this species.

Differences between reciprocal experiments were < 14 %. These results show clearly that the novel Antarctic isolates are genotypically distinct from related *Flavobacterium* species, although the isolates share > 97% (up to 98.7%) 16S rRNA gene sequence similarity with their closest phylogenetic neighbours, and that they constitute three novel species within the genus *Flavobacterium*.

The DNA G+C contents of strains LMG 21915^T, LMG 21916, LMG 21917, LMG 21474 and LMG 21918 of *F. degerlachei* sp. nov. are 34.2, 34.2, 34.1, 33.8 and 34.2 mol%, respectively. The DNA G+C contents of strains LMG 21919^T, LMG 21920 and LMG 21921 of *F. micromati* sp. nov. are 34.4, 33.1 and 33.1 mol%, respectively and those of strains LMG 21922^T, LMG 21923, LMG 21924 and LMG 21925 of *F. frigoris* sp. nov. are 34.5, 34.2, 34.4 and 33.8 mol%, respectively. These values are consistent with the DNA G+C contents of members of the genus *Flavobacterium*, which range from 30 to 37 mol % G+C (Bernardet *et al.*, 1996; Van Trappen *et al.*, 2003).

Cellular fatty acid patterns of the Antarctic strains are based on the data generated by Van Trappen *et al.* (2002). The strains showed similar fatty acid

Table 3.6. Fatty acid compositions of the novel Antarctic species: *Flavobacterium degerlachei* sp. nov., *Flavobacterium micromati* sp. nov. and *Flavobacterium frigoris* sp. nov.

Fatty acid	*F. degerlachei* (n = 14)	*F. micromati* (n = 3)	*F. frigoris* (n = 19)
iso-$C_{14:0}$	2.7 ± 0.5	2.2 ± 0.5	3.3 ± 1.0
$C_{15:0}$	7.2 ± 2.3	7.7 ± 0.9	6.9 ± 1.2
$C_{15:0}$3-OH	1.5 ± 0.5	TR	1.2 ± 0.5
anteiso-$C_{15:0}$	7.7 ± 1.4	5.9 ± 1.1	10.2 ± 2.7
iso-$C_{15:0}$	5.8 ± 2.2	6.7 ± 1.8	7.4 ± 1.4
iso-$C_{15:0}$3-OH	5.1 ± 1.1	3.7 ± 1.0	3.9 ± 0.9
anteiso-$C_{15:1}$	1.3 ± 1.6	TR	TR
iso-$C_{15:1}$	5.1 ± 1.2	3.6 ± 1.2	2.7 ± 0.7
$C_{15:1}\omega6c$	10.5 ± 2.0	6.4 ± 0.4	11.3 ± 2.2
$C_{16:0}$	1.3 ± 0.7	4.5 ± 0.4	1.8 ± 0.5
$C_{16:0}$3-OH	1.9 ± 0.8	2.9 ± 0.2	1.8 ± 0.5
iso-$C_{16:0}$	3.9 ± 0.9	9.1 ± 2.9	6.9 ± 1.6
iso-$C_{16:0}$3-OH	9.7 ± 1.8	10.5 ± 1.4	7.8 ± 2.0
iso-$C_{16:1}$	3.9 ± 0.9	4.2 ± 1.2	4.4 ± 1.2
Iso-$C_{17:0}$3-OH	4.7 ± 1.2	4.5 ± 1.4	3.4 ± 1.3
$C_{17:1}\omega6c$	6.8 ± 2.0	4.9 ± 0.3	3.7 ± 1.2
$C_{17:1}\omega8c$	1.0 ± 0.4	1.0 ± 0.1	1.1 ± 0.3
iso-$C_{17:1}\omega9c$	1.3 ± 0.4	1.3 ± 0.6	2.0 ± 0.5
Summed feature 3	13.2 ± 2.5	15.7 ± 0.9	14.5 ± 3.0

Mean percentages ± S_D of total fatty acids are given. Other fatty acids accounted for < 1% each. Summed feature 3 comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both. TR, Trace, < 1% of total.

profiles (Table 3.6); major constituents included $C_{15:0}$, iso- $C_{15:0}$, iso-$C_{16:0}$3-OH and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both). Strains of *F. degerlachei* sp. nov. and *F. frigoris* sp. nov. also possessed relatively large amounts of anteiso-$C_{15:0}$ and $C_{15:1}\omega6c$, whilst strains of *F. micromati* sp. nov. showed relatively large amounts of iso-$C_{16:0}$. Hydroxylated fatty acids and iso- and anteiso-branched fatty acids were also present as minor components. Their fatty acid profiles resemble those determined for other *Flavobacterium* species (Bernardet *et al.*, 1996), but differ in the relative amounts of anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{17:0}$3-OH.

The strains showed typical morphological characteristics of the genus *Flavobacterium* (Bernardet *et al.*, 2002) and their physiological and biochemical

characteristics are given in the species descriptions. *F. degerlachei* sp. nov., *F. micromati* sp. nov. and *F. frigoris* sp. nov. can be differentiated clearly from each other and from related *Flavobacterium* species by several phenotypic characteristics (Table 3.7).

The results of the polyphasic analysis support the recognition of three novel Antarctic species within the genus *Flavobacterium*, for which the names *Flavobacterium degerlachei* sp. nov., *Flavobacterium micromati* sp. nov. and *Flavobacterium frigoris* sp. nov. are proposed.

Description of *Flavobacterium degerlachei* sp. nov.

Flavobacterium degerlachei (de.ger.lach'e.i. N. L. gen. n. *degerlachei* of Adrien de Gerlache, in honour of the Belgian pioneer who conducted the first scientific expedition to Antarctica in 1897-1899).

Cells are Gram-negative, short rods (<1 x 3-4 µm), that often form pairs or short chains. Gliding motility is not observed. Growth occurs at 5-30°C with an optimal growth temperature of 20 °C, whereas no growth occurs at 37 °C. Yellow, convex, translucent colonies with entire margins and a diameter of 1–3 mm are formed on R2A plates after 6 days incubation. Colonies on Anacker & Ordal's agar are flat, round with entire margins and 0.5-1 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar, nutrient agar and marine agar; colonies do not adhere to the agar. Aesculin and starch are degraded. Catalase and oxidase tests are positive. Growth is observed (API 20NE) on glucose, mannose and maltose, whereas no growth is detected on arabinose, mannitol, *N*-acetyl-glucosamine, gluconate, caprate, adipate, malate, citrate or phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, casein, CM-cellulose, DNA, gelatin, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. No production of brown diffusible pigment occurs on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction, Voges-Proskauer reaction and hydrogen sulfide production are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), α-chymotrypsine, trypsin, α-galactosidase, β-

Table 3.7. Phenotypic characteristics that differentiate *Flavobacterium degerlachei* sp. nov., *Flavobacterium micromati* sp. nov. and *Flavobacterium frigoris* sp. nov. from related *Flavobacterium* species.

Characteristic	1	2	3	4	5	6
Growth on:						
Trypticase soy agar	+	(+)	+	+	+	+
Nutrient agar	+	+	-	+	+	+
Growth at 25°C on agar	+	(+)	(+)	(+)	+	+
Flexirubin-type pigment	-	-	-	-	+	+
Voges-Proskauer reaction	-	+	-	-	ND	ND
Glucose utilization	+	-	+	+	+	+
Acid from carbohydrates	-	-	-	+	+	+
Degradation of:						
Gelatin	-	-	-	-	+	+
Casein	-	-	+	+	+	+
Starch	+	-	+	+	+	+
CM-cellulose	-	-	-	-	+	+
Agar	-	-	-	-	-	+
Alginate	-	-	-	-	+	ND
Pectin	-	-	-	-	+	+
Chitin	-	-	-	+	+	-
DNA	-	-	-	-	+	-
Tyrosine	-	-	+	-	+	+
β-Galactosidase activity	-	-	-	-	+	+
H_2S production	-	-	-	-	ND	+
Nitrate reduction	-	-	v	-	+	+
Mean G+C content (mol%)	34	33	34	32	35	33

Flavobacterium species: 1, *F. degerlachei*; 2, *F. micromati*; 3, *F. frigoris;* 4, *F. gillisiae*; 5, *F. pectinovorum*; 6, *F. saccharophilum*. Data from Bernardet *et al.* (1996), McCammon & Bowman (2000) and this study. Symbols: +, positive test; (+), positive test, weak or delayed response; -, negative test; v, test results vary between strains of species; ND, no available data. All species shown here are negative for Congo red absorption and precipitate formation on egg-yolk agar and are positive for degradation of aesculin.

galactosidase, β-glucuronidase, α-mannosidase, and α-fucosidase (API ZYM). Weak enzymic activity is observed for cystine arylamidase, medium activity is observed for esterase (C4), esterase lipase (C8), α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase, and strong activity is observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API ZYM). Cells contain the fatty acids $C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$3-OH, $C_{17:1}\omega6c$ and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as the main constituents. Growth occurs in 0-5% NaCl but not in 10% NaCl, indicating that the strains are not halophilic, but are moderately halotolerant. DNA G+C content is 33.8-34.2 mol%.

The type strain is LMG 21915^T (= DSM 15718^T). Isolated from microbial mats from Lakes Ace and Pendant in the Vestfold Hills and lake Reid in the Larsemann Hills, Antarctica.

Description of *Flavobacterium micromati* sp. nov.

Flavobacterium micromati (mi.cro.mat'i. N.L. gen. n. *micromati* referring to the MICROMAT project).

Cells are Gram-negative, short rods (<1 x 3-4 µm); gliding motility is not observed. Growth occurs at 5°C to 20°C, very weak growth is observed at 25°C and no growth occurs at 30 °C. Orange-red, convex, translucent colonies with entire margins and a diameter of 1–3 mm are formed on R2A plates after 6 days of incubation. Colonies on Anacker & Ordal's agar are flat, round with entire margins and 0.5-1 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar (weak), nutrient agar and marine agar (weak). Colonies do not adhere to the agar. Aesculin is degraded. Catalase and oxidase tests are positive. Growth on carbohydrates (API 20NE) is not observed and acids from carbohydrates are not produced (API 20E). Voges-Proskauer reaction is positive for all strains. Agar, alginate, pectin, chitin, casein, CM-cellulose, DNA, gelatin, tyrosine, starch and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. No production of brown diffusible pigment occurs on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction and hydrogen sulfide production are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine

decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), cystine arylamidase, α-chymotrypsine, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase (API ZYM). Medium enzymic activity is observed for esterase (C4) and esterase lipase (C8) and strong activity is observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase (API ZYM). Cells contain the fatty acids $C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$, iso-$C_{16:0}$3-OH and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as the main constituents. Growth occurs in 0-2 % NaCl, but not in 5 % NaCl. DNA G+C content is 33.1-34.4 mol%.

The type strain is LMG 21919^T (= CIP 108161^T). Isolated from microbial mats from Lake Grace in the Vestfold Hills and Lake Fryxell in the McMurdo Dry Valleys, Antarctica.

Description of *Flavobacterium frigoris* sp. nov.

Flavobacterium frigoris (fri'go.ris. L. gen. n. *frigoris* of the cold).

Cells are Gram-negative, short rods (<1 x 4-6 µm); gliding motility is not observed. Growth occurs at 5-20°C, weak growth is observed at 25°C and no growth occurs at 37 °C. Yellow, convex, translucent colonies with entire margins and a diameter of 2–5 mm are formed on R2A plates after 6 days incubation. Colonies on Anacker & Ordal's agar are flat, round with entire margins and 0.5-1 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar and marine agar, but not on nutrient agar. Colonies do not adhere to the agar. Aesculin, casein, tyrosine and starch are degraded. Catalase and oxidase tests are positive. Growth on carbohydrates (API 20NE) is observed for glucose, mannose and maltose; acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, CM-cellulose, DNA, gelatin and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. No production of brown diffusible pigment occurs on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, Voges Proskauer reaction and hydrogen sulfide production are negative. Strain LMG 21924 is able to reduce nitrate to nitrite. None of the strains shows activity for the enzymes arginine

dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), α-chymotrypsin, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase (API ZYM). Weak enzymic activity is observed for cystine arylamidase, medium activity is observed for esterase (C4), esterase lipase (C8) and *N*-acetyl-β-glucosaminidase, and strong activity is observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase (API ZYM). Only strain LMG 21924 showed medium activity for β-glucosidase and strain LMG 21922 for lipase (C14). Cells contain the fatty acids $C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$, iso-$C_{16:0}$3-OH and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as the main constituents. Growth occurs in 0-5 % NaCl, but not in 10% NaCl, indicating that the strains are not halophilic, but moderately halotolerant. DNA G+C content is 33.8-34.5 mol%.

The type strain is LMG 21922^T (= DSM 15719^T). Isolated from microbial mats from Lakes Watts, Grace and Druzhby in the Vestfold Hills, Lake Fryxell in the McMurdo Dry Valleys and Lake Reid in the Larsemann Hills, Antarctica.

3.4 *Flavobacterium fryxellicola* sp. nov. and *Flavobacterium psychrolimnae* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes

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Taxonomic studies were performed on seven strains isolated from microbial mats in Antarctic lakes of the McMurdo Dry Valleys. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains are related to the genus *Flavobacterium*; sequence similarity values with their nearest phylogenetic neighbours ranged from 97.0 to 98.7%. The results of DNA-DNA hybridization and comparison of repetitive extragenic palindromic DNA-PCR fingerprinting patterns revealed that these strains are members of two distinct species. Genotypic results, together with phenotypic characteristics, allowed the differentiation of these species from related *Flavobacterium* species with validly published names. The isolates are Gram-negative, chemoheterotrophic, rod-shaped cells that are psychrophilic. Their whole-cell fatty acid profiles are similar and include $C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$, iso-$C_{16:0}$3-OH, iso-$C_{16:1}$ and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as major fatty acid components. On the basis of these results, two new species are proposed, namely *Flavobacterium fryxellicola* sp. nov. (consisting of 3 strains with LMG 22022^T = CIP 108325^T as type strain) and *Flavobacterium psychrolimnae* sp. nov. (consisting of 4 strains with LMG 22018^T = CIP 108326^T as type strain). DNA G + C contents of *Flavobacterium fryxellicola* and *Flavobacterium psychrolimnae* are 35.5 and 34.1 mol%, respectively.

Introduction

Members of the *Bacteroidetes* show a high abundance in freshwater and marine ecosystems and these bacteria may have a specialized role in the uptake and degradation of organic matter in cold, aquatic environments (Kirchman, 2002). Several new species, added to the genus *Flavobacterium* since 1996, originated from Antarctic habitats (Van Trappen *et al.*, 2003; 2004a; and references cited therein).

During the MICROMAT project (November 1998 - February 2001), 746 bacterial strains were isolated under heterotrophic conditions from microbial mat samples that were collected from ten Antarctic lakes (Van Trappen *et al.*, 2002). Numerical analysis of the fatty acid composition of the isolates and 16S rRNA gene sequence analysis, performed on representative strains, showed that the diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is very high. Moreover, many fatty acid clusters were shown to contain multiple taxa when tested by repetitive extragenic palindromic DNA-PCR (rep-PCR) fingerprinting, a technique used to investigate the genomic diversity of each fatty acid cluster more in detail (Van Trappen *et al.*, 2003; 2004a). Several of these strains belonging to fatty acid clusters 5, 6 and 10 were already described as new *Flavobacterium* species: *Flavobacterium gelidilacus* sp. nov., *Flavobacterium degerlachei* sp. nov., *Flavobacterium frigoris* sp. nov. and *Flavobacterium micromati* sp. nov. (Van Trappen *et al.*, 2003; 2004a).

In the present work, we studied further the taxonomic relationships of twenty-two additional strains from fatty acid cluster 5 (as delineated by Van Trappen *et al.*, 2002). A group of eleven of these strains was identified as *Flavobacterium xanthum*, while another rep-cluster of four strains was identified as the recently described *Flavobacterium frigoris* (Van Trappen *et al.*, 2004a), based on 16S rDNA sequence analysis (Fig. 3.7) and DNA-DNA hybridizations (S. Van Trappen, unpublished results). These strains were not further investigated, and are listed in Table 3.8. Seven strains, also listed in Table 3.8, proved to belong to new taxa, and were studied by polyphasic taxonomic analysis.

Materials and Methods

Strains were cultivated routinely on R2A medium (Difco) at 20 °C for 48 h or [for strains LMG 4031^T (*Flavobacterium pectinovorum*) and LMG 8384^T (*F. saccharophilum*)] on TSA medium (BBL) at 20 °C for 48 h, and [for strains LMG 21985^T (*F. xinjiangense*) and LMG 21986^T (*F. omnivorum*)] on R2A medium at 11°C for 5 days, except when mentioned otherwise.

DNA was prepared according to the method of Pitcher *et al.* (1989) and rep-PCR fingerprinting (based on primers targeting the repetitive extragenic palindromic sequence) was performed on all strains of FAA clusters 5 (75 strains) of Van Trappen *et al.* (2002), using the primer GTG_5 (Versalovic *et al.*, 1991), as described previously (Van Trappen *et al.*, 2003). Numerical analysis was carried using the Bionumerics software package (Applied Maths), as described by the same authors.

Almost-complete 16S rRNA gene sequences of reference strains were determined as described earlier (Van Trappen *et al.*, 2004a). The most closely related sequences were found using the FASTA program; sequences were aligned and editing of the alignment and reformatting was performed with the BIOEDIT program (Hall, 1999) and ForCon (Raes & Van de Peer, 1999). Evolutionary distances were calculated by using the Jukes-Cantor evolutionary model and a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with the TREECON program (Van de Peer & De Wachter, 1994).

DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using a HTS7000 BioAssay reader (PerkinElmer) for the fluorescence measurements. The hybridization temperature was 30 °C and reciprocal experiments were performed for every pair of strains.

DNA G+C contents of the Antarctic strains were determined using an HPLC method as described by Van Trappen *et al.* (2003).

Morphological, physiological and biochemical tests were performed, as described previously (Van Trappen *et al.*, 2003).

Results and discussion

Seven strains of fatty acid cluster 5, listed in Table 3.8, showed similar rep-PCR profiles (see also Fig. 3.6), and could be divided into two different clusters according to their profile type, hereafter referred to as rep-PCR profile type I (comprising 3 strains) and type II (with 4 strains).

Almost-complete 16S rRNA gene sequences (1466-1479 nt) of strains LMG 22022^T, LMG 22018^T, LMG 22020, R-9010 and R-7518 were determined and a phylogenetic tree is shown (Fig. 3.7). The seven novel Antarctic strains form two distinct branches within the genus *Flavobacterium*, which are supported by high bootstrap values, and they belong to a clade of the phylogenetic tree that consists almost exclusively of recently described *Flavobacterium* species from cold environments, such as *F. gillisiae, F. degerlachei*, *F. frigoris*, *F. xinjiangense, F. xanthum, F. omnivorum, F. frigidarium*, *F. gelidilacus, F. limicola*, *F. tegetincola* and *F. micromati*. Other psychrophilic *Flavobacterium* species, like *F. hibernum* and *F. psychrophilum*, do not belong to this clade and form separate branches.

The 16S rRNA gene sequence of the representative strain of rep-PCR profile type I (LMG 22022^T) showed 97.9 % similarity to *F. tegetincola*, 97.2 % to *F. flevense*, 96.0% to *F. johnsoniae* and less than 96.0 % to other *Flavobacterium* species. The sequences of the representative strains of rep-PCR profile type II (LMG 22018^T and LMG 22020), are identical and showed 98.7 %sequence similarity to *F. limicola*, 98.4 % to *F. omnivorum*, 97.9% to *F. xinjiangense*, 97.7 % to *F. degerlachei*, 97.6% to *F. frigoris*, 97.5% to *F. gillisiae*, 97.3 % to *F. xanthum* and less than 97.0% to other *Flavobacterium* species.

Genomic relatedness between the novel Antarctic strains (representing the two different rep-PCR profile types) and their most closely related phylogenetic neighbours was determined by DNA-DNA hybridization. The hybridization level between strains LMG 22022^T, LMG 22023 and LMG 22024 of rep-PCR profile type I was 79.0-93.3 %, indicating that these three strains belong to one single species (Wayne *et al.*, 1987).

Hybridization values of LMG 22022^T with its nearest phylogenetic neighbours, *F. tegetincola* (LMG 21423^T) and *F. flevense* (LMG 8328^T), were less than 19%, indicating that the strains from rep-PCR profile type I represent a new *Flavobacterium* species, for which the name *Flavobacterium fryxellicola* sp. nov.

Table 3.8. Strains investigated, isolation site, and rep-PCR profile type.

Species and strain	Isolation site
***F. xanthum*:**	
R-8994, R-8999, R-9141, R-9147, R-9148	Grace lake, Vestfold Hills
R-9009, R-9010, R-9013, R-9329, R-11545	Watts lake, Vestfold Hills
R-9127	Lake Druzhby, Vestfold Hills
***F. frigoris*:**	
R-9005	Watts lake, Vestfold Hills
R-9142, R-9145	Grace lake, Vestfold Hills
R-7518	Lake Fryxell, Dry Valleys
***F. fryxellicola* sp. nov. (rep-PCR cluster I):**	
LMG 22022^T = R-7548	Lake Fryxell, Dry Valleys
LMG 22023 = R-8019	Lake Fryxell, Dry Valleys
LMG 22024 = R-8284	Lake Fryxell, Dry Valleys
***F. psychrolimnae* sp. nov. (rep-PCR cluster II):**	
LMG 22018^T = R-7582	Lake Fryxell, Dry Valleys
LMG 22019 = R-7681	Lake Hoare, Dry Valleys
LMG 22020 = R-8023	Lake Fryxell, Dry Valleys
LMG 22021 = R-8283	Lake Fryxell, Dry Valleys

Abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; R-, strain numbers from the research collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium, and as used by Van Trappen *et al.* (2002).

is proposed. Hybridization results between strains LMG 22018^T and LMG 22020 of rep-PCR profile type II (96.5%) showed that the strains of rep-PCR profile type II belong to a single species. It is now well established that similar rep-PCR profiles are correlated to high genomic DNA-DNA hybridization values (Versalovic *et al.*, 1994; Rademaker & De Bruijn, 1997; Rademaker *et al.*, 2000; Van Trappen *et al.*, 2003; 2004a). The low hybridization level (21.9-48.8%) between LMG 22018^T and the nearest phylogenetic neighbours *F. limicola* (LMG 21930^T), *F. omnivorum* (LMG 21986^T), *F. xinjiangense* (LMG 21985^T), *F. degerlachei* (LMG 21915^T), *F. frigoris* (LMG 21922^T), *F. gillisiae* (LMG 21422^T) and *F. xanthum* (LMG 8372^T), reveals that the four strains of rep-PCR profile type II constitute a new species, for which the name *Flavobacterium psychrolimnae* sp. nov. is proposed. Differences between reciprocal experiments were less than 11 %. These results clearly show

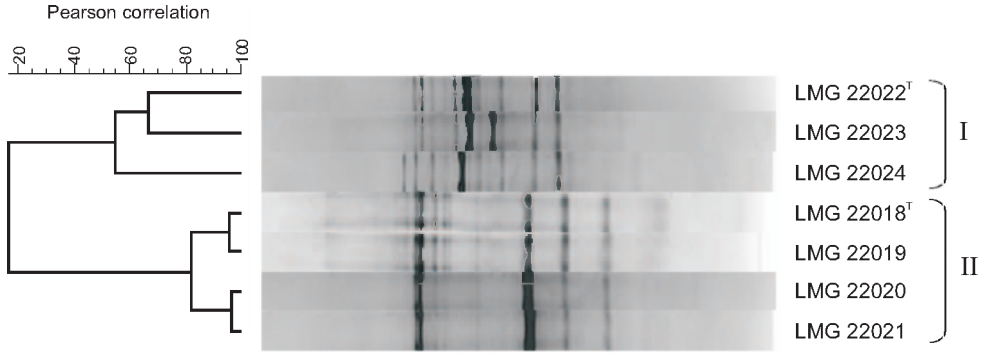

Figure 3.6. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of seven strains belonging to fatty acid cluster 5. Dendrogram derived from the UPGMA clustering of the profiles with the Pearson correlation coefficient and rep-clusters were delineated at a cut-off value of 50%. Rep-cluster I, *F. fryxellicola* sp. nov.; rep-cluster II, *F. psychrolimnae* sp. nov..

that the novel Antarctic isolates are genotypically distinct from related *Flavobacterium* species, although the new isolates share more then 97% (up to 98.7%) 16S rRNA gene sequence similarity with their closest phylogenetic neighbours.

The DNA G+C contents of strains LMG 22022^T, LMG 22023 and LMG 22024 of *F. fryxellicola* sp. nov. are 35.2, 35.9 and 35.5 mol%, respectively. The DNA G + C contents of strains LMG 22018^T, LMG 22019, LMG 22020 and LMG 22021 of *F. psychrolimnae* sp. nov. are 34.5, 33.9, 34.1 and 33.8 mol%, respectively. These values are consistent with the G+C contents of members the genus *Flavobacterium*, which range from 30 to 37 mol % (Bernardet *et al.*, 1996; Van Trappen *et al.*, 2003).

Cellular fatty acid patterns of the Antarctic strains are based on the data generated by Van Trappen *et al.* (2002) and are very similar (Table 3.9). The major constituents include $C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$3-OH and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both). Hydroxylated fatty acids and iso- and anteiso-branched fatty acids were present as minor components. The strains of *F. psychrolimnae* sp. nov. also possessed relatively large amounts of iso-$C_{16:0}$. Their fatty acid profiles resemble those determined for other *Flavobacterium* species (Bernardet *et al.*, 1996).

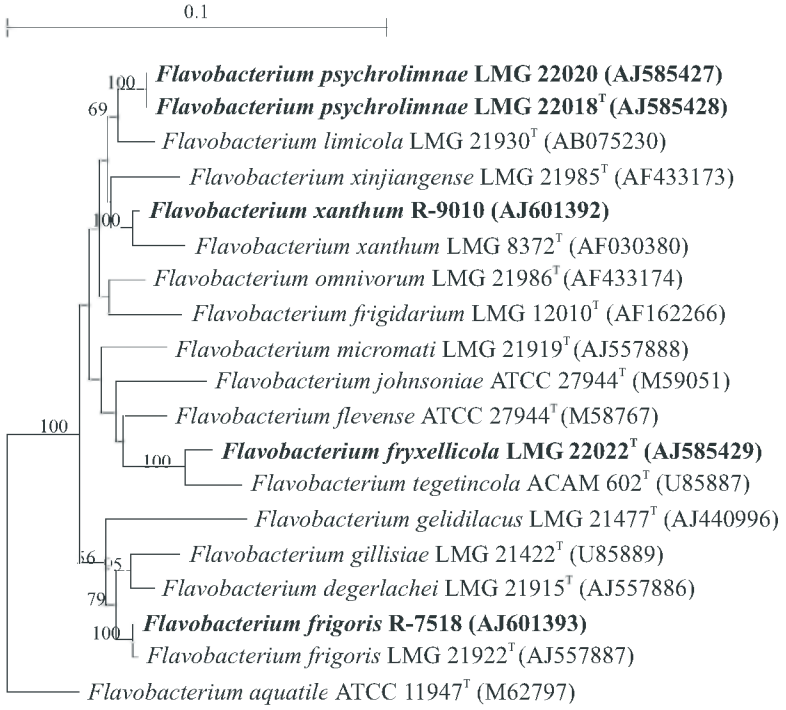

Figure 3.7. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of *Flavobacterium fryxellicola* sp. nov., *Flavobacterium psychrolimnae* sp. nov. and nearest phylogenetic neighbours on the basis of 16S rRNA gene sequences. Bootstrap values (percentages of 500 replicates) of > 50 % are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

The strains showed typical morphological characteristics of the genus *Flavobacterium* (Bernardet *et al.,* 2002). Their physiological and biochemical characteristics are given in the species descriptions. *F. fryxellicola* sp. nov. and *F. psychrolimnae* sp. nov. can be differentiated clearly from each other and from related *Flavobacterium* species by several phenotypic characteristics (Table 3.10); *Flavobacterium* species not mentioned in the table are also different from these novel species.

Description of *Flavobacterium fryxellicola* sp. nov.

Flavobacterium fryxellicola (fry.xel.li'co.la. N.L. n. *Fryxellum* or *Fryxellus* Lake Fryxell; L. suffix *-cola* an inhabitant; N.L. n. *fryxellicola* inhabitant of Lake Fryxell).

Cells are Gram-negative, short rods (1-1.5 x 3-4 µm), that often form short chains. Gliding motility was not observed. Growth at 5-25°C with an optimal growth

Table 3.9. Fatty acid composition of the novel Antarctic species *Flavobacterium fryxellicola* sp. nov. and *F. psychrolimnae* sp. nov.

Fatty acid	*F. fryxellicola* (n = 3)	*F. psychrolimnae* (n = 4)
iso-$C_{14:0}$	3.4 ± 0.3	3.8 ± 0.2
$C_{15:0}$	6.9 ± 1.7	5.4 ± 0.3
$C_{15:0}$3-OH	1.0 ± 0.1	1.4 ± 0.1
anteiso-$C_{15:0}$	3.5 ± 0.2	5.1 ± 0.3
iso-$C_{15:0}$	8.1 ± 0.1	6.9 ± 0.5
iso-$C_{15:0}$3-OH	4.6 ± 1.0	4.1 ± 0.4
iso-$C_{15:1}$	3.4 ± 0.3	3.9 ± 0.2
$C_{15:1}$ ω6c	7.9 ± 1.3	7.8 ± 0.5
$C_{16:0}$	3.3 ± 0.3	1.6 ± 0.2
$C_{16:0}$3-OH	1.9 ± 0.4	TR
iso-$C_{16:0}$	9.8 ± 1.5	9.7 ± 0.6
iso-$C_{16:0}$ 3-OH	10.7 ± 1.9	10.4 ± 1.5
iso-$C_{16:1}$	4.5 ± 0.5	8.2 ± 0.6
iso-$C_{17:0}$3-OH	4.9 ± 0.5	4.0 ± 0.7
$C_{17:1}$ω6c	5.3 ± 0.8	4.8 ± 0.4
$C_{17:1}$ω8c	1.0 ± 0.2	TR
$C_{18:1}$ω5c	TR	1.2 ± 0.2
iso-$C_{17:1}$ω9c	1.6 ± 0.03	3.5 ± 0.1
Summed feature 3	13.9 ± 2.2	13.1 ± 0.7

Mean percentages ± S_D of total fatty acids are given. Other fatty acids accounted for < 1% each. Summed feature 3 comprises iso-$C_{15:0}$2-OH, $C_{16:1}$ω7c, or both. TR, Trace, < 1% of total.

temperature of 20 °C, whereas no growth occurs at 30 °C. Yellow-orange, convex, translucent colonies with entire margins and a diameter of 1–3 mm are formed on R2A plates after 6 days incubation. Colonies on Anacker & Ordal's agar (Anacker & Ordal, 1955) are flat, round with entire margins and 0.5-1 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar (weak) and nutrient agar; there is no growth on marine agar. Colonies do not adhere to the agar. Growth occurs in 0-2% NaCl but not in 5-10% NaCl. Aesculin is degraded. Catalase and oxidase tests are positive. Growth is observed (API 20NE) on glucose and maltose, whereas no growth is detected on arabinose, mannitol, mannose, *N*-

acetyl-glucosamine, gluconate, caprate, adipate, malate, citrate and phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, casein, CM-cellulose, DNA, gelatin, starch, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. No production of brown diffusible pigment occurs on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction and hydrogen sulfide production are negative. Voges-Proskauer reaction is positive for all strains. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), α-chymotrypsin, trypsin, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase (API ZYM). Weak enzymic activity is observed for esterase lipase (C8), cystine arylamidase and α-galactosidase, medium activity is observed for esterase (C4) and α-glucosidase, and strong activity is observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM). Cells contain the fatty acids $C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$, iso-$C_{16:0}$3-OH and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as the main constituents. DNA G+C content is 35.2-35.9 mol%.

The type strain is LMG 22022^T (= CIP 108325^T). Isolated from microbial mats from Lake Fryxell (fresh/brackish) in the McMurdo Dry Valleys, Antarctica.

Description of *Flavobacterium psychrolimnae* sp. nov.

Flavobacterium psychrolimnae (psy.chro'lim.nae. Gr. adj. *psychros* cold; Gr. f. n. *limna* lake; M.L. gen. n. *psychrolimnae* of the cold lake).

Cells are Gram-negative, short rods (0.5 x 2 µm); gliding motility is not observed. Growth occurs at 5-25°C, whereas weak growth is observed at 30°C and no growth occurs at 37 °C. The optimal growth temperature is 20°C. Yellow, convex, translucent colonies with entire margins and a diameter of 1–3 mm are formed on R2A plates after 6 days incubation. Colonies on Anacker & Ordal's agar (Anacker & Ordal, 1955) are flat, round with entire margins and 0.5-1 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar and nutrient agar, whereas no growth is detected on marine agar. Colonies do not

Table 3.10. Phenotypic characteristics that differentiate *Flavobacterium fryxellicola* sp. nov. and *F. psychrolimnae* sp. nov. from other *Flavobacterium* species.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Growth on:															
Trypticase soy agar	(+)	+	+	+	+	-	+	+	+	+	+	+	+	+	(+)
Nutrient agar	+	+	+	-	+	(+)	+	+	+	+	+	+	+	+	+
Growth at 25°C on agar	(+)	+	+	(+)	+	-	-	(+)	+	+	(+)	(+)	-	+	(+)
Flexirubin pigment type	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Congo red absorption	-	-	-	-	+	ND	ND	-	-	V	-	-	+	-	-
Glucose utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Acid from carbohydrates	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-
Degradation of:															
Gelatin	-	-	-	-	+	+	-	-	-	+	-	+	+	V	-
Casein	-	+	-	+	+	+	+	-	-	+	+	+	+	+	-
Starch	-	+	+	+	+	-	+	-	ND	+	+	+	-	+	-
CM-cellulose	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
Agar	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Alginate	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
Pectin	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-
Chitin	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-
Aesculin	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+
DNA	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Tyrosine	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-
Precipitate on egg-yolk agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-galactosidase activity	-	+	-	-	-	-	+	-	+	+	-	-	-	-	-
H_2S production	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Nitrate reduction	-	-	-	v	-	-	+	-	ND	+	-	+	-	-	-
Mol% G+C (mean)	35	34	34	34	35	34	35	32	35	34	32	36	35	30	33

Flavobacterium species: 1, *F. fryxellicola*; 2, *F. psychrolimnae*; 3, *F. degerlachei*; 4, *F. frigoris;* 5, *F. limicola*; 6, *F. xinjiangense*; 7, *F. omnivorum*; 8, *F. tegetincola*; 9, *F. flevense*; 10, *F. johnsoniae*; 11, *F. gillisiae*; 12, *F. xanthum*, 13, *F. frigidarium*; 14, *F. gelidilacus*; 15, *F. micromati*. * Data from Bernardet *et al.* (1996), McCammon & Bowman (2000), Humphry *et al.* (2001), Zhu *et al.* (2003), Tamaki *et al.* (2003), Van Trappen *et al.* (2004a) and this study. Symbols: +, positive test; (+), positive test, weak or delayed response; -, negative test; V, test results are variable; ND, no available data.

adhere to the agar. Growth occurs in 0-2 % NaCl, but not in 5-10 % NaCl. Aesculin, casein and starch are degraded. Catalase and oxidase tests are positive. Growth is observed (API 20NE) on glucose, mannose and maltose, whereas no growth is detected on arabinose, mannitol, *N*-acetyl-glucosamine, gluconate, caprate, adipate, malate, citrate and phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, CM-cellulose, DNA, gelatin, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-

type pigments are present. No production of brown diffusible pigment occurs on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction, Voges-Proskauer reaction and hydrogen sulfide production are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase (API ZYM). Weak enzymic activity is observed for esterase (C4), esterase lipase (C8), cystine arylamidase, α-chymotrypsin, trypsin and β-glucosidase, medium activity is observed for *N*-acetyl-β-glucosaminidase, acid phosphatase, α-glucosidase and naphthol-AS-BI-phosphohydrolase, and strong activity is observed for alkaline phosphatase, leucine arylamidase and valine arylamidase (API ZYM). Cells contain the fatty acids $C_{15:0}$, iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{16:0}$, iso-$C_{16:0}$3-OH, iso-$C_{16:1}$ and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as the main constituents. DNA G+C content is 33.8-34.5 mol%.

The type strain is LMG 22018^T (= CIP 108326^T). Isolated from microbial mats from the freshwater lakes Fryxell (fresh/ brackish) and Hoare in the McMurdo Dry Valleys, Antarctica.

3.5 *Gillisia limnaea* gen. nov., sp. nov., a new member of the family *Flavobacteriaceae* isolated from a microbial mat in Lake Fryxell, Antarctica

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A taxonomic study was performed on three strains isolated from microbial mats in Lake Fryxell, McMurdo Dry Valleys, Antarctica. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains belong to the family *Flavobacteriaceae*, in which they form a distinct lineage. The isolates are Gram-negative, chemoheterotrophic, aerobic, rod-shaped cells. They are psychrophilic and yellow-pigmented, with DNA G + C contents in the range of 37.8-38.9 mol%. Whole-cell fatty acid profiles revealed mainly branched fatty acids and $C_{17:0}$2-OH. On the basis of genotypic, phenotypic, chemotaxonomic and phylogenetic results, it is proposed that the isolates represent a novel species in a new genus, *Gillisia limnaea* gen. nov., sp. nov. The type strain is LMG 21470^T (= DSM 15749^T).

Introduction

Members of the *Cytophaga-Flavobacterium* cluster constitute one of the dominant bacterial groups in marine and freshwater environments (Bowman *et al.*, 1997a; Pinhassi *et al.*, 1997; Glöckner *et al.*, 1999). In addition, it is now thought that flavobacteria play an important role in the uptake and degradation of complex dissolved and particulate organic matter (Kirchman, 2002). Therefore, this group has an important and central role in remineralization processes in aquatic systems. Recently, several new genera of the family *Flavobacteriaceae* have been described, i.e. *Cellulophaga*, *Zobellia*, *Muricauda*, *Arenibacter*, *Tenacibaculum*, *Vitellibacter, Mesonia* and *Ulvibacter* (Johansen *et al.*, 1999; Barbeyron *et al.*, 2001; Bruns *et al.*, 2001; Ivanova *et al.*, 2001; Suzuki *et al.*; 2001; Nedashkovskaya *et al.*, 2003c, 2003a, 2004). Members of several of these genera, i.e. *Gelidibacter*, *Psychroserpens*, *Psychroflexus, Polaribacter*, and *Salegentibacter* (Bowman *et al.*, 1997b, 1998d; Gosink *et al.*, 1998; McCammon & Bowman, 2000), were originally isolated from Antarctic maritime lakes and the surrounding Southern Ocean, whereas isolates of the genus *Aequorivita* were found in terrestrial and marine Antarctic habitats (Bowman & Nichols, 2002).

During the MICROMAT project (November 1998 - February 2001), 746 bacterial strains were isolated under heterotrophic conditions from microbial mat samples collected from 10 Antarctic lakes in the Vestfold Hills (lakes Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts), the Larsemann Hills (lake Reid) and the McMurdo Dry Valleys (lakes Hoare and Fryxell) (Van Trappen *et al.*, 2002). Numerical analysis of their fatty acid composition revealed 41 clusters and 16S rRNA gene sequence analysis, performed on representative strains, showed that they belong to the α-, β- and γ-subclasses of the *Proteobacteria*, the high- and low-G+C-containing Gram-positives and the phylum *Bacteroidetes* (Van Trappen *et al.*, 2002).

In the present work, the taxonomic relationship between the three strains from fatty acid cluster 4 (as delineated by Van Trappen *et al.*, 2002) was studied by a polyphasic taxonomic approach. A novel genus of the family *Flavobacteriaceae* is described, *Gillisia* gen. nov., with *Gillisia limnaea* sp. nov. as the type species.

Materials and Methods

The strains investigated were LMG 21470^T (= DSM 15749^T = R-8282^T), LMG 21966 (= R-7730) and LMG 21965 (= R-7610), isolated as described by Van Trappen *et al.* (2002) from microbial mat samples (FR1 and FR2) taken from Lake Fryxell, McMurdo Dry Valleys, Antarctica. The strains were routinely cultivated on marine agar 2216 (Difco) at 20 °C for 48 h, except when mentioned otherwise.

DNA extracts were prepared using the method of Pitcher *et al.* (1989). DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Willems *et al.* (2001), using an HTS7000 BioAssay reader (Perkin Elmer) for fluorescence measurements. The hybridization temperature was 30 °C and reciprocal experiments were performed for every pair of strains.

The almost complete 16S rRNA gene sequence of one representative strain of fatty acid cluster 4 was obtained as described earlier (Van Trappen *et al.*, 2002). The closest related sequences were found using the program FASTA. Sequences from reference strains were aligned and editing of the alignment and reformatting was performed with BIOEDIT (Hall, 1999) and FORCON (Raes & Van de Peer, 1999). Evolutionary distances were calculated using the Jukes & Cantor evolutionary model and a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with TREECON (Van de Peer & De Wachter, 1994).

The G+C content of DNA from the Antarctic strains was determined using an HPLC method, as described by Van Trappen *et al.* (2003).

Morphological, physiological and biochemical tests were performed, as described previously (Van Trappen *et al.*, 2003).

Results and discussion

Genomic relatedness between the novel strains was determined by DNA-DNA hybridizations. The mean hybridization level between strains LMG 21470^T, LMG 21966 and LMG 21965 was 81-91 %, indicating that the strains belong to a single species (Wayne *et al.*, 1987). Differences between reciprocal experiments were less than 14 %.

The almost complete 16S rRNA gene sequence (1483 nt) of strain LMG 21470^T was obtained and a phylogenetic tree is shown in Fig. 3.8. Dendrograms obtained by maximum-parsimony and maximum-likelihood analyses showed essentially the

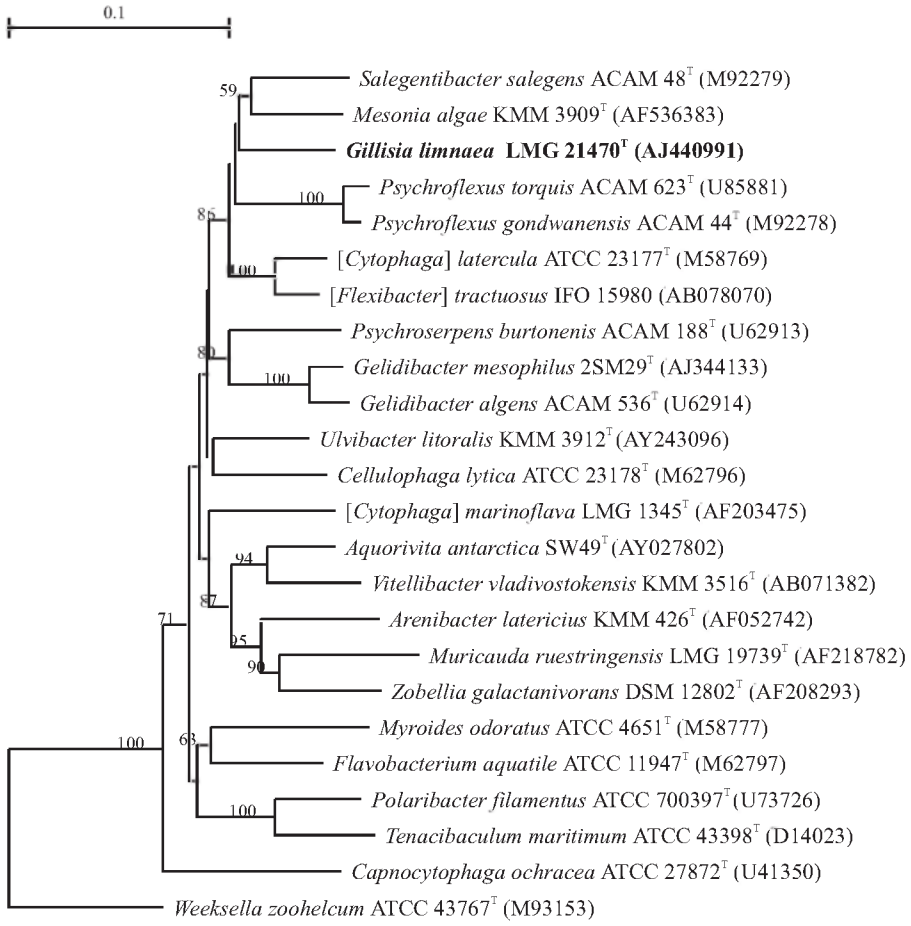

Figure 3.8. Neighbour-joining dendrogram showing the estimated phylogenetic relationship of *Gillisia limnaea* gen. nov., sp. nov. and related members of the family *Flavobacteriaceae* on the basis of 16S rRNA gene sequences. *Weeksella zoohelcum* was choosen as the outgroup. Bootstrap values over 50% are shown (percentages of 500 replicates). Bar, 1 nt substitution per 10 nt. EMBL accession numbers for reference strains are shown in parentheses.

same topography (data not shown). Results of the phylogenetic analysis revealed that the novel strains form a distinct lineage within the family *Flavobacteriaceae* (Bernardet *et al.*, 2002) and belong to a cluster of species: *Salegentibacter salegens*, *Mesonia algae, Psychroflexus torquis*, *Psychroflexus gondwanensis*, *Gelidibacter algens, Gelidibacter mesophilus, Psychroserpens burtonensis* and the misclassified strains [*Flexibacter*] *tractuosus* IFO 15980 and [*Cytophaga*] *latercula* ATCC 23177^T (see Fig. 3.8). The 16S rDNA sequence similarity values between strain LMG 21470^T and its closest relatives [*F.*] *tractuosus*, *S. salegens* and *Psychroflexus gondwanensis*, were 93.0, 92.8 and 92.0%, respectively. The 16S rDNA sequence of the recently described *M. algae* (Nedashkovskaya *et al.*, 2003a) showed only 91.5% similarity with that of strain LMG 21470^T. The low

level of sequence similarity between the novel strains and other bacteria belonging to the *Flavobacteriaceae* (87.4-93.0%) clearly demonstrates that they represent a new genus.

The G+C contents of strains LMG 21470^T, LMG 21966 and LMG 21965 were 37.8, 38.7 and 38.9 mol%, respectively. These values are consistent with G+C contents observed in the family *Flavobacteriaceae* (27-44 mol %) (Bernardet *et al.*, 2002).

Cellular fatty acid patterns of the novel strains have been published previously (Van Trappen *et al.*, 2002; cluster 4). The strains showed similar fatty acid profiles and the major constituents were branched fatty acids (<65 % of total), which is typical for members of the *Flavobacteriaceae* (Bernardet *et al.*, 2002). Significant differences in the fatty acid compositions of the novel strains and related taxa were found, e.g. extracts of *Gillisia limnaea* strains contained considerable amounts of $C_{17:0}$2-OH (13.1% of total), iso-$C_{17:1}\omega$9c (7.1%), anteiso-$C_{17:1}\omega$9c (7.4 %) and summed feature 3 (8.2 %; comprises iso-$C_{15:0}$2-OH and/or $C_{16:1}\omega$7c or both), whereas these fatty acids were not detected in *S. salegens*, *Psychroflexus gondwanensis* and [*C.*] *latercula* (Bowman *et al.*, 1998d).

The strains show the typical morphological characteristics of members of the *Flavobacteriaceae* (Bernardet *et al.*, 2002) and their physiological and biochemical characteristics are given in the species description. Results of the polyphasic analysis support the formation of a new genus within the family *Flavobacteriaceae*, *Gillisia* gen. nov., with *Gillisia limnaea* sp. nov. as the type species. The new genus can be clearly differentiated from related members of the *Flavobacteriaceae* by several phenotypic characteristics (Table 3.11).

Description of *Gillisia* gen. nov.

Gillisia (Gil.lis'i.a. N.L. fem. n. *Gillisia* after Monique Gillis, a Belgian bacteriologist who has made major contributions to bacterial taxonomy).

Gram-negative, rod-shaped cells which are strictly aerobic, moderately halotolerant, psychrophilic and chemoheterotrophic. Produces yellow pigments. No flexirubins are formed. Gliding motility is not detected. Does not form endospores. Positive for cytochrome oxidase, catalase and β-galactosidase. The main cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:1}$, iso-$C_{16:0}$, $C_{17:0}$2-OH, iso-$C_{17:0}$3-OH, iso-$C_{17:1}\omega$9c, anteiso-$C_{17:1}\omega$9c and summed feature 3 (comprising

Table 3.11. Phenotypic characteristics that differentiate *Gillisia* gen. nov. from related members of the *Flavobacteriaceae*.

Characteristic	1	2	3	4	5	6	7	8
Gliding motility	-	-	-	V	-	-	+	+
Oxidase	+	+	+	+	+	-	-	+
Catalase	+	+	+	+	-	+	+	+
Pigments	Y	Y	Y	O	O-R	Y	Y	O-Y
Growth in > 10% NaCl	-	+	+	V	-	ND	ND	-
Acid from carbohydrates	-	-	-	+	+	-	+	+
Hydrolysis of:								
Agar	-	-	-	-	+	-	-	+
Casein	-	-	+	-	+	+	V	V
Gelatin	+	+	+	+	-	V	V	+
Starch	-	+	-	+	+	-	+	+
DNA	-	+	-	+	+	-	+	+
Nitrate reduction	-	+	-	-	+	-	-	V
H_2S production	-	+	+	-	+	ND	ND	-
G+C content (mol%)	37-39	37-38	32-34	32-36	34	27-29	36-38	33-38

Genera/ species: 1, *Gillisia* gen. nov.; 2, *Salegentibacter*; 3, *Mesonia*; 4, *Psychroflexus*; 5, [*Cytophaga*] *latercula*; 6, *Psychroserpens*; 7, *Gelidibacter*; 8, *Cellulophaga*. Abbreviations: -, negative; +, positive; V, variable; ND, not determined; O, orange; Y, yellow; O-R, orange-red. Data for *Gillisia* are from this study; data for the other genera/ species shown are from Bowman *et al.* (1997a, 1998), Reichenbach (1989), Johansen *et al.* (1999), McCammon & Bowman (2000) and Nedashkovskaya *et al.* (2003a).

iso-$C_{15:0}$2-OH and/or $C_{16:1}\omega7c$, or both). 16S rRNA gene sequence analysis reveals that the genus *Gillisia* belongs to the family *Flavobacteriaceae* of the phylum *Bacteroidetes*. The type species is *Gillisia limnaea*.

Description of *Gillisia limnaea* sp. nov.

Gillisia limnaea (lim.nae'a. Gr. adj. *limnaeos* pertaining to, living in lakes; N.L. fem. adj. *limnaea* living in the water, referring to the isolation source, microbial mats in Lake Fryxell).

The main characteristics are the same as given for the genus. In addition, cells are 3 x 0.7 µm. Grows at 5-25°C; optimal growth at 20 °C. Weak growth is observed at 30°C and no growth occurs at 37 °C. Yellow, convex, translucent colonies with diameters of 1–3 mm and entire margins are formed on marine agar plates after 6 days incubation. Colonies on Anacker & Ordal's agar are flat, round

with entire margins and 0.7-0.9 mm in diameter after 14 days incubation. Growth also occurs on nutrient agar and R2A and colonies do not adhere to the agar. No growth on trypticase soy agar. Degrades aesculin and gelatin. Growth is not observed (API 20NE) on glucose, mannose, maltose, L-arabinose, mannitol, *N*-acetyl-glucosamine, gluconate, caprate, adipate, malate, citrate and phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, casein, carboxymethylcellulose, DNA, starch, Tween 80, tyrosine and urea are not degraded. Congo red is not absorbed. No brown diffusible pigment is produced on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction, the Voges-Proskauer reaction and hydrogen sulfide production are negative. None of the strains has the following enzyme activities: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), α-galactosidase, β-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase (API ZYM). Weak enzymic activity is observed for cystine arylamidase, β-glucuronidase and α-glucosidase, medium activity for esterase (C4), esterase lipase (C8) and trypsin and strong activity for alkaline and acid phophatases, leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase. Variable results are observed for α-chymotrypsin activity. Growth occurs in up to 5% NaCl, but not in 10% NaCl, indicating that strains are moderately halotolerant but not halophilic. DNA G+C content is 37.8-38.9 mol%.

The type strain is LMG 21470^T (= DSM 15749^T). Isolated from microbial mats from Lake Fryxell in the McMurdo Dry Valleys, Antarctica.

3.6 *Algoriphagus antarcticus* sp. nov., a novel psychrophile from microbial mats in Antarctic lakes

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A taxonomic study was performed on six strains isolated from microbial mats of lakes Reid, Fryxell and Ace in Antarctica. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains belong to the family '*Flexibacteriaceae*' and are closely related to the recently described genera *Algoriphagus* and *Hongiella*. The isolates are Gram-negative, chemoheterotrophic, aerobic, psychrophilic and orange-red-pigmented bacteria; their DNA G + C contents range from 39.9 to 41.0 mol%. Their whole-cell fatty acid profiles include mainly branched fatty acids and summed feature 3 (which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both). On the basis of genotypic, phenotypic, chemotaxonomic and phylogenetic results, the novel bacteria are classified as *Algoriphagus antarcticus* sp. nov. The type strain is LMG 21980^T (= DSM 15986^T).

Introduction

Members of the *Cytophaga-Flavobacterium* cluster constitute one of the dominant bacterial groups in the marine environment (Bowman *et al.*, 1997a; Pinhassi *et al.*, 1997; Glöckner *et al.*, 1999) and it is now thought that they play an important role in remineralization processes in aquatic systems (Kirchman, 2002). Recently, new genera of the '*Flexibacteriaceae*' have been described like *Reichenbachia*, *Algoriphagus*, *Hongiella* and *Belliella* (Nedashkovskaya *et al.*, 2003b; Bowman *et al.*, 2003c; Yi & Chun, 2004; Brettar *et al.*, 2004) isolated from seawater, sea-ice, algal mats of saline lakes and tidal flat sediment. Only one strain (strain A230 of *Algoriphagus ratkowskyi*) was isolated from a cyanobacterial mat sample from Ace Lake, Antarctica.

During the MICROMAT project (November 1998 - February 2001), 746 bacterial strains were isolated under heterotrophic conditions from microbial mat samples that were collected from 10 Antarctic lakes in the Vestfold Hills (lakes Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts), the Larsemann Hills (lake Reid) and the McMurdo Dry Valleys (lakes Hoare and Fryxell) (Van Trappen *et al.*, 2002). Numerical analysis of their fatty acid composition revealed 41 clusters, and 16S rRNA gene sequence analysis, performed on representative strains, showed that they belong to the α-, β- and γ-subclasses of the *Proteobacteria*, the high and low percent G+C Gram-positives and to the *Bacteroidetes* phylum (Van Trappen *et al.*, 2002).

In the present work we studied the taxonomic relationships of six strains from fatty acid cluster 15 (as delineated by Van Trappen *et al.*, 2002), by a polyphasic taxonomic approach. A novel species of the genus *Algoriphagus* is described as *Algoriphagus antarcticus* sp. nov.

Materials and Methods

The investigated strains are LMG 21980^T (= DSM 15986^T = R-10710^T), LMG 21981 (= R-10749), LMG 21982 (= R-10752), LMG 21983 (= R-11427), from Lake Reid, Larsemann Hills, Antarctica; LMG 21984 (= R-8290), from Lake Fryxell, McMurdo Dry Valleys, Antarctica and LMG 21482 (= R-9286), from Ace Lake, Vestfold Hills, Antarctica. The strains were isolated as described by Van Trappen *et al.* (2002). They were cultivated routinely on marine agar 2216 (Difco) at 20 °C for 4 days, except when mentioned otherwise. Strains LMG 21435^T (*Algoriphagus*

ratkowskyi), LMG 21969^T (*Algoriphagus winogradskyi*), LMG 21970^T (*Algoriphagus chordae*) and LMG 21971^T (*Algoriphagus aquimarinus*) were cultivated routinely on marine agar 2216 (Difco) at 20°C, whilst strain LMG 22067^T (*Algoriphagus halophilus*) was cultivated on marine agar 2216 (Difco) at 28°C.

DNA extracts were prepared by using the method of Pitcher *et al.* (1989). Almost complete 16S rRNA gene sequences of representative strains were amplified by PCR with conserved primers (Coenye *et al.*, 1999). PCR products were purified by using a QIAquick PCR Purification kit (Qiagen) according to the instructions of the manufacturer. Sequence analysis was performed by using an ABI Prism 3100 DNA sequencer (Applied Biosystems), applying a BigDye Terminator Cycle Sequencing Ready Reaction kit (version 2.0; PerkinElmer Applied Biosystems), following the protocols of the manufacturer. Sequence assembly was performed by using the program AutoAssembler (version1.4.0; PerkinElmer Applied Biosystems). The most closely related sequences were found by using the FASTA program; sequences were aligned and editing of the alignment and reformatting was performed with the BioEdit program (Hall, 1999) and ForCon (Raes & Van de Peer, 1999). Evolutionary distances were calculated using the Jukes-Cantor evolutionary model and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the TREECON program (Van de Peer & De Wachter, 1994).

DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Willems *et al.* (2001), using an HTS7000 BioAssay reader (PerkinElmer) for the fluorescence measurements. The hybridization temperature was 34 °C and reciprocal experiments were performed for every pair of strains.

DNA G+C contents of the novel strains were determined using an HPLC method, as described by Van Trappen *et al.* (2003).

Morphological, physiological and biochemical tests were performed, as described earlier (Van Trappen *et al.*, 2003).

Results and discussion

Almost complete 16S rRNA gene sequences (1462-1491 nt) of strains LMG 21482, LMG 21980^T and LMG 21983 were obtained and a phylogenetic tree is shown in Fig. 3.9. Dendrograms obtained by maximum parsimony and maximum likelihood analyses showed essentially the same topography (data not shown). The results of the phylogenetic analysis reveal that the novel strains belong to the recently described genus *Algoriphagus* within the family '*Flexibacteriaceae*' (Bowman *et al.*, 2003c; Nedashkovskaya *et al.*, in press), which is most closely related to the genera *Hongiella*, *Belliella* and *Cyclobacterium* (see Fig. 3.9). The Antarctic strains form a robust branch, supported by a high bootstrap value (all methods, 100% of the bootstrap replications).

The 16S rRNA gene sequences of strains LMG 21980^T and LMG 21983 are identical to each other, whilst the sequence of LMG 21482 differs by only one base from these sequences (99.9% similarity). The 16S rRNA gene sequences of the novel strains show 98.7% sequence similarity to *Algoriphagus chordae*, 98.7% to *A. ratkowskyi*, 98.6% to *A. winogradskyi*, 98.5% to *A. aquimarinus*, 97.4% to *A. halophilus*, 94.7% to *H. ornithinivorans*, 93.8% to *H. mannitolivorans*, 92.9% to *Belliella baltica,* 92.9% to *Cyclobacterium marinum* and less than 90% to sequences of other related genera.

Genomic relatedness between the novel strains and their most closely related phylogenetic neighbours (*Algoriphagus ratkowskyi, A. chordae*, *A. aquimarinus*, *A. winogradskyi* and *A. halophilus*) was determined by DNA-DNA hybridization. The hybridization level between strains LMG 21980^T, LMG 21981, LMG 21982, LMG 21983 and LMG 21984 was 89.0-98.7 %, whereas strain LMG 21482 showed a hybridization value of only 74.2 $\pm$ 3.9% to strain LMG 21980^T and 72.7 $\pm$ 0.5% to strain LMG 21983. Hybridization values of LMG 21980^T and LMG 21983 with their nearest phylogenetic neighbours *Algoriphagus ratkowskyi* (LMG 21435^T), *A. chordae* (LMG 21970^T), *A. aquimarinus* (LMG 21971^T) and *A. winogradskyi* (LMG 21969^T), were 20.5-38.6%. The hybridization value between strains LMG 21983 and LMG 22067^T (*A. halophilus*) was only 7.2%. Differences between reciprocal experiments were less than 8 %. These results show clearly that the Antarctic strains represent a new species within the genus *Algoriphagus*.

The DNA G+C contents of strains LMG 21482, LMG 21980^T, LMG 21981, LMG 21982, LMG 21983 and LMG 21984 are 40.8, 40.6, 40.6, 40.6, 39.9 and

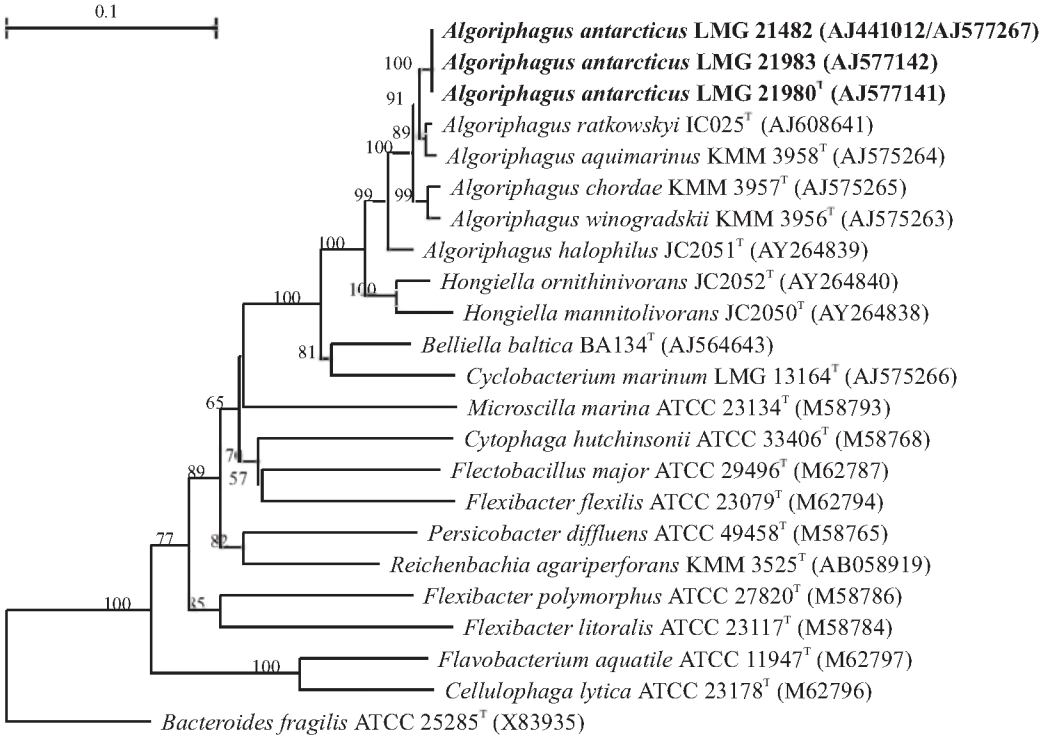

Figure 3.9. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of *Algoriphagus antarcticus* sp. nov. and related members of the family '*Flexibacteriaceae*' on the basis of 16S rRNA gene sequences. *Bacteroides fragilis* was choosen as outgroup. Bootstrap values (percentages of 500 replicates) of > 50 % are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

41.0 mol%, respectively. These values are consistent with the DNA G+C contents of members of the genus *Algoriphagus*, which range from 35 to 42 mol % (Bowman *et al.*, 2003c; Nedashkovskaya *et al.*, in press).

Cellular fatty acid patterns of the novel strains are based on the data generated by Van Trappen *et al.* (2002). The strains show similar fatty acid profiles and the mean fatty acid composition includes 3.4% anteiso-$C_{15:0}$, 23.1% iso-$C_{15:0}$, 4.1% iso-$C_{15:0}$3-OH, 9.3% iso-$C_{15:1}$, 1.8% $C_{16:0}$3-OH, 1.9% iso-$C_{16:0}$, 2.5% iso-$C_{16:0}$3-OH, 2.9% iso-$C_{16:1}$, 4.9% $C_{16:1}\omega5c$, 12.5% iso-$C_{17:0}$3-OH, 3.4% iso-$C_{17:1}\omega9c$, 24.3% iso-$C_{15:0}$2-OH and/or $C_{16:1}\omega6c$. Other fatty acids each account for less than 1%. The fatty acid profiles of the novel strains resemble those determined for the other *Algoriphagus* species but differ in terms of relative amounts of iso-$C_{15:0}$, iso-$C_{15:1}$, iso-$C_{17:0}$3-OH and iso-$C_{15:0}$2-OH/$C_{16:1}\omega6c$ (Bowman *et al.*, 2003c; Nedashkovskaya *et al.*, in press).

The strains showed typical morphological characteristics of the genus *Algoriphagus* (Bowman *et al.*, 2003c; Nedashkovskaya *et al.*, in press) and their

physiological and biochemical characteristics are given in the species description. The results of the polyphasic analysis support the recognition of a new species within the genus *Algoriphagus*, for which the name *Algoriphagus antarcticus* sp. nov. is proposed. The new species can be clearly differentiated from related *Algoriphagus* and *Hongiella* species by several phenotypic characteristics (Table 3.12).

Description of *Algoriphagus antarcticus* sp. nov.

Algoriphagus antarcticus (ant.arc'ti.cus. L. masc. adj. *antarcticus* of the Antarctic environment, from where the strains were isolated).

Cells are Gram-negative, short rods (2-3 µm x 0.5 µm); motility was not detected. Growth occurs at 5-20°C, with weak growth at 25°C and an optimal growth temperature of 20 °C. No growth occurs at 30 °C. Orange-red pigmented, convex, opaque colonies with entire margins and a diameter of 0.5–3 mm are formed on marine agar plates after 6 days incubation. Colonies on Anacker & Ordal's agar are flat, round with entire margins and 0.5-0.7 mm in diameter after 14 days incubation. Growth also occurs on nutrient agar and R2A; colonies do not adhere to the agar. No growth occurs on trypticase soy agar. Catalase and oxidase tests are positive. Aesculin is degraded. Growth is not observed (API 20NE) on glucose, mannose, maltose, L-arabinose, mannitol, *N*-acetyl-glucosamine, gluconate, caprate, adipate, malate, citrate and phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, casein, CM-cellulose, DNA, starch, gelatin, tyrosine and urea are not degraded. Congo red is not absorbed. No production of brown diffusible pigment occurs on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction, Voges-Proskauer reaction and hydrogen sulfide production are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), cystine arylamidase (except strain LMG 21983), α-galactosidase, β-glucuronidase, α-mannosidase, and α-fucosidase (API ZYM).

Table 3.12. Phenotypic characteristics that differentiate between *Algoriphagus antarcticus* sp. nov. and related species.

Characteristic	1	2	3	4	5	6	7	8	9	10
Coiled, ring-shaped, spiral cells	-	-	-	-	-	-	-	-	-	+
Color of cell mass	O-R	O-R	O-R	O-R	P	O-R	O	O	P-O	P-R
Growth on										
10% NaCl	-	-	+	+	-	+	-	+	-	+
Growth at										
25°C	+	+	+	+	+	+	+	+	+	+
30°C	-	+	+	+	-	+	+	+	+	+
Nitrate reduction	-	+	-	-	-	-	+	-	+	-
Production of										
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	-	+	+	+	+	+
Acid from carbohydrates	-	+	+	+	+	-	-	-	v	+
Hydrolysis of:										
Agar	-	+	+	+	-	-	-	-	ND	-
Aesculin	+	+	+	+	ND	+	+	+	+	ND
Alginate	-	+	+	+	ND	-	-	-	ND	ND
Casein	-	-	-	+	+	-	-	-	-	-
Chitin	-	-	-	-	v	-	-	-	-	-
CM-cellulose	-	-	-	-	-	-	-	-	-	ND
DNA	-	-	-	+	-	-	+	+	+	-
Gelatin	-	+	-	+	-	+	+	+	-	-
Starch	-	+	-	-	v	-	+	+	+	-
Tween 80	ND	-	-	+	-	+	-	+	ND	-
Urea	-	-	-	-	-	-	-	-	ND	-
Mean G+C content (mol%)	40	41	40	41	35	37	42	38	35	36

Species: 1, *Algoriphagus antarcticus*; 2, *A. winogradskyi*; 3, *A. chordae*; 4, *A. aquimarinus*; 5, *A. ratkowskyi*; 6, *A. halophilus;* 7, *H. mannitolivorans*; 8, *H. ornithinivorans*; 9, *Belliella baltica*; 10, *Cyclobacterium marinum*. Data from Bowman *et al.* (2003c), Yi & Chun (2004), Brettar *et al.* (2004), Nedashkovskaya *et al.* (in press) and this study. Symbols: +, positive test; -, negative test; v, variable between strains; ND, no available data; O, orange; R, red; P, pink.

Weak enzymic activity is observed for esterase (C4), esterase lipase (C8), α-glucosidase, and β-glucosidase, medium activity is observed for valine arylamidase, and β-galactosidase, and strong activity is observed for alkaline and acid phophatases, leucine arylamidase, trypsin, α-chymotrypsin, and naphthol-AS-BI-phosphohydrolase. Variable results were obtained for *N*-acetyl-β-glucosaminidase. Growth occurs in up to 5% NaCl but not in 10% NaCl, indicating that the strains are not halophilic but moderately halotolerant. DNA G+C content is 39.9-41.0 mol%.

The type strain is LMG 21980^T (= DSM 15986^T). Isolated from microbial mats from lakes Reid, Fryxell and Ace, Antarctica.

CHAPTER 4

New taxa from Antarctic lakes within the α-*Proteobacteria*

4.1 Polyphasic taxonomy of FAA cluster 41

Fatty acid cluster 41 (as delineated in Van Trappen *et al.* (2002), see chapter 2), belonging to the α-*Proteobacteria*, was further investigated using a polyphasic taxonomic approach. The phylogenetic position of the α-*Proteobacteria* with different families (e. g. *Acetobacteriaceae*, *Rhodospirillaceae*, *Sphingomonadaceae*, the *Rhodobacter* group, *Caulobacteriaceae*, *Rhizobiaceae* and *Hyphomicrobiaceae*) is illustrated in Fig. 4A and 4B.

The genomic diversity of the 59 strains of fatty acid cluster 41 (as delineated in Van Trappen *et al.* (2002), see chapter 2), belonging to the α-*Proteobacteria*, was investigated by rep-PCR fingerprinting, using REP1R-I and REP2-I primers and the GTG_5-primer (Rademaker & de Bruijn, 1997). In total, 57 combined fingerprinting patterns were obtained (from strains R-9063 and R-9178, no DNA could be extracted because of poor growth) and 10 clusters could be delineated, whereas 12 strains formed single branches, at a cut off value of 70% (Pearson correlation coefficient) (see Fig. 4.1). Looking at the REP- and GTG_5-clustering separately, it is clear that, although the GTG_5-primer leads to profiles with much more bands, the GTG_5-clustering is often too detailed. For example at a cut-off value of 70% (Pearson correlation), 11 different GTG_5-groups can be delineated and only seven REP-groups (data not shown). For rep-clusters I and IX there is a good correlation between the clustering based on the two different primers (see Fig. 4.1). However, for rep-clusters IV, V, VI and VII, the clustering based on the REP-primers is more suitable, since less and more dense clusters are obtained, with overall higher Pearson correlations compared to the GTG_5-derived clusters. Hybridization results between representatives of the rep-clusters IV, V and VII confirmed that they are very closely related (see later).

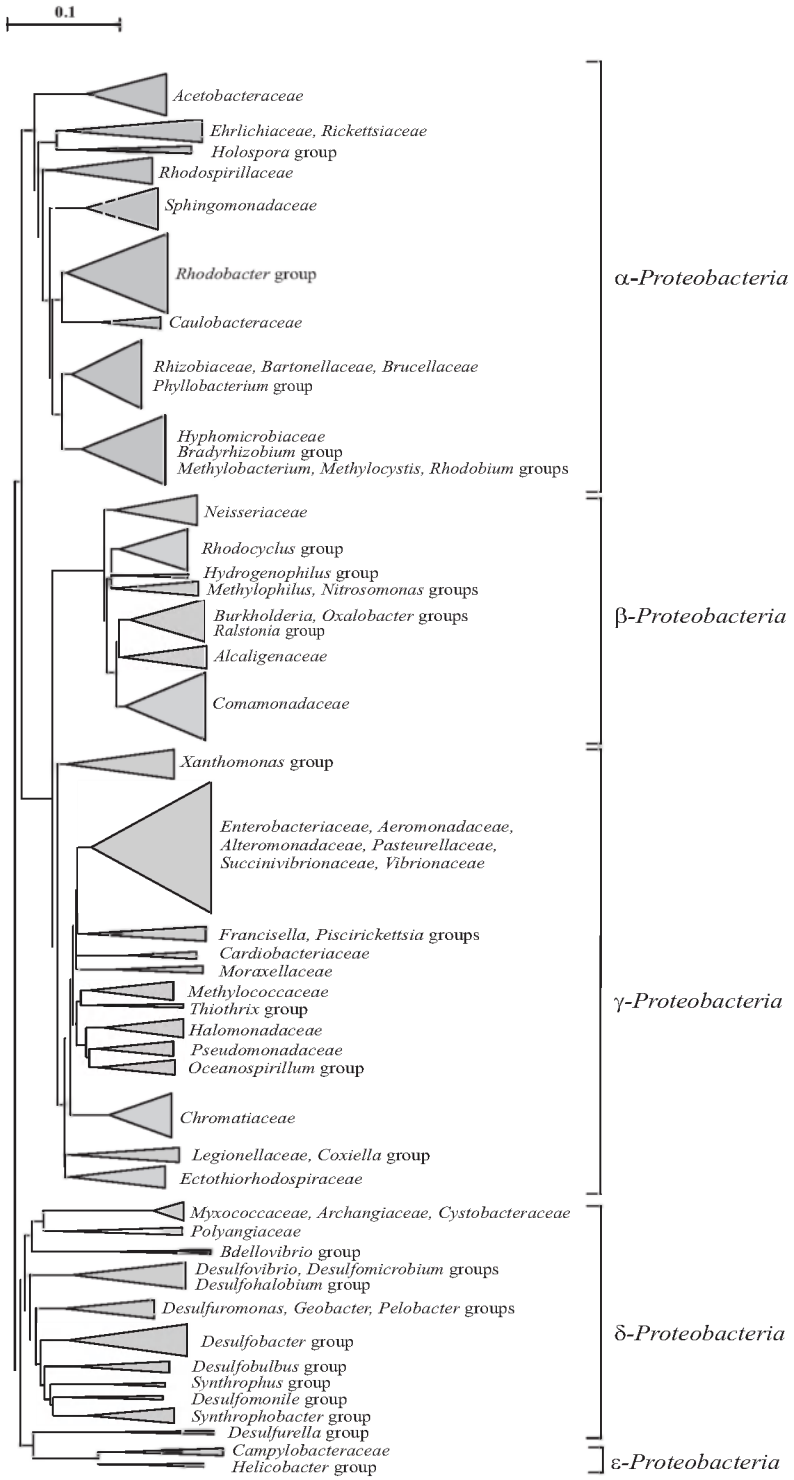

Figure 4A. Simplified neigbour-joining phylogenetic tree of the *Proteobacteria* based on the 16S rDNA sequences of the type strains of the proteobacterial genera. Distances were calculated using the substitution rate calibration method in TREECON 3.1 (Van de Peer and De Wachter, 1994). The bar indicates 10% estimated sequence divergence. *Bacillus subtilis* was used as outgroup (not shown). The width of the triangles is proportional to the number of genera within each cluster (from Kersters *et al.*, 2002).

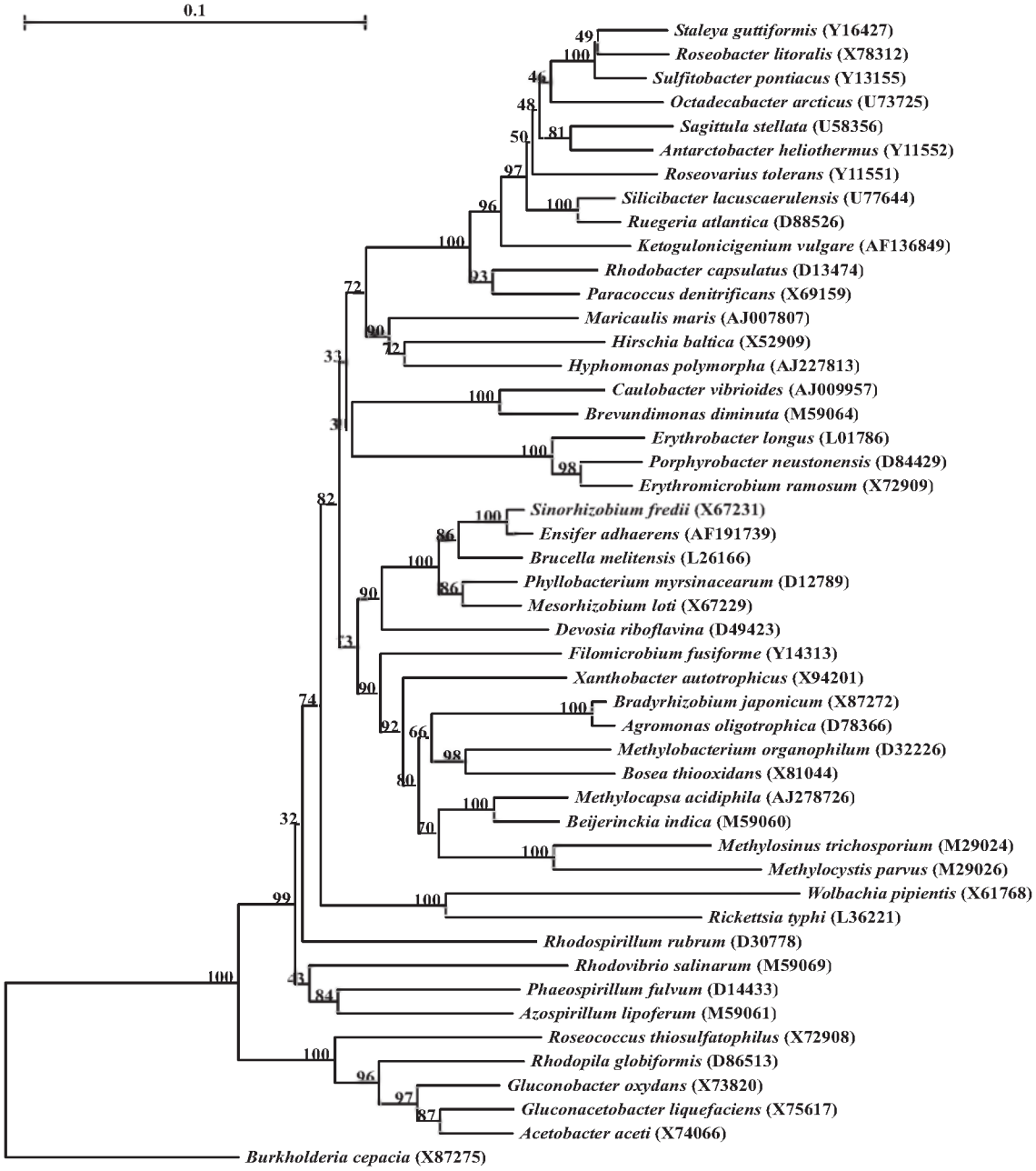

Figure 4B. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of representatives of the α-*Proteobacteria* on the basis of 16S rRNA gene sequences. *Burkholderia cepacia* was choosen as outgroup. Bootstrap values (percentages of 100 replicates) are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

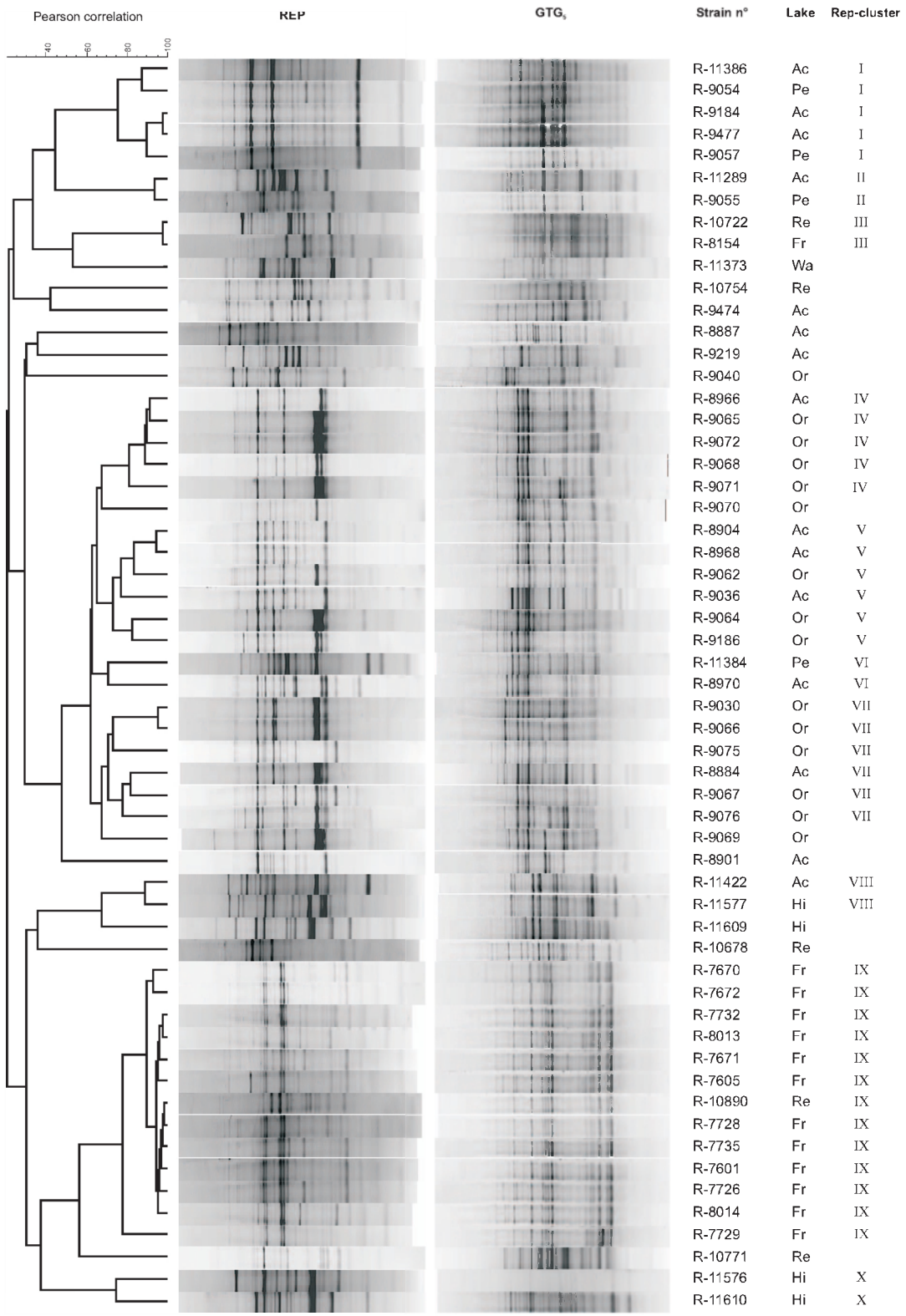

Figure 4.1. Digitized representation of normalized and combined rep-PCR profiles (REP1R-I and REP2-I primers and the GTG_5-primer) of 57 strains belonging to FAA cluster 41. Dendrogram derived from the UPGMA-clustering of the profiles with the Pearson correlation coefficient and rep-clusters were delineated at a cut-off value of 70%.

Figure 4.2. Digitized representation of normalized rep-PCR profiles (REP1R-I and REP2-I primers) of type strains of related species of the '*Roseobacter- Sulfitobacter- Silicibacter*' group within the α-*Proteobacteria*. Dendrogram derived from UPGMA-clustering of the profiles with the Pearson correlation coefficient.

These results illustrate that the diversity of heterotrophic bacteria in Antarctic microbial mats is much higher than estimated by fatty acid and 16S rDNA sequence analyses. In contrast to the *Bacteroidetes*, the different profile types correlate well with the geographical origin of the strains. Strains showing the same rep-PCR profile are often isolated from the same or geographically close lakes (for rep-clusters I and II lakes Ace and Pendant; for rep-clusters IV, V, VI and VII lakes Ace and Organic) and strains from rep-cluster IX are almost exclusively originating from Lake Fryxell (except strain R-10890, isolated from Lake Reid). Reference strains of related species were also included in this rep-clustering (only REP-primers), but it is clear that none of their fingerprinting patterns is similar to the patterns of the Antarctic strains (see Fig. 4.2).

16S rDNA sequences of two representative strains from FAA cluster 41 were obtained from a previous study (see chapter 2). Strain R-9219 (unclustered) showed a sequence similarity of 96.9% to *Mesorhizobium loti* and strain R-8904 (rep-cluster V) showed a sequence similarity of 93.5% to *Sulfitobacter pontiacus*. Additional sequences were determined for representative strains of rep-clusters I, IV, VII and IX and results show that they are phylogenetically related to the *Rhodobacter* group within the α-*Proteobacteria*, showing low similarities (94,2-95,8%) to *Jannaschia helgolandensis*, *Octadecabacter antarcticus* and *Ketogulonicigenium vulgare*, their nearest phylogenetic neighbours.

We focused on 26 strains belonging to five different rep-groups: four strains from rep-cluster I, 12 strains from rep-cluster IX and a selection of 10 strains from rep-clusters IV, V and VII, with the most similar rep-profiles. Hybridization values between representative strains of the three different rep-clusters I, V and IX were low (10.5-17.6%), indicating that they belong to three different species (Wayne *et al.*, 1987). Hybridization results between representative strains from rep-clusters IV, V and VII indicated that these rep-clusters are closely related (showing hybridization values of 78.2-85.5%) and they constitute a single species of a

novel genus for which the name *Loktanella salsilacus* sp. nov. is proposed. 16S rDNA sequence analysis and phenotypic results, showed that the strains from rep-cluster I also belong to a single species of this novel genus, for which the name *L. vestfoldensis* sp. nov. is proposed, whereas the strains from rep-cluster IX constitute a new species within this genus, for which the name *Loktanella fryxellensis* sp. nov. is proposed (see section 4.2).

4.2 *Loktanella fryxellensis* gen. nov., sp. nov., *Loktanella vestfoldensis* sp. nov. and *Loktanella salsilacus* sp. nov., new members of the *Rhodobacter* group, isolated from microbial mats in Antarctic lakes

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A taxonomic study was performed on twenty-six strains isolated from microbial mats in Antarctic lakes of the Vestfold Hills and the McMurdo Dry Valleys. Phylogenetic analysis based on 16S rRNA gene sequences placed these strains within the *Rhodobacter* group of the α-subclass of the *Proteobacteria*; sequence similarity values with their nearest phylogenetic neighbours (*Jannaschia, Octadecabacter* and *Ketogulonicigenium*) ranged from 94.0 to 95.8%. Results of DNA-DNA hybridization and comparison of repetitive extragenic palindromic DNA-PCR fingerprinting patterns revealed that these strains are members of three distinct species. The isolates are Gram-negative, chemoheterotrophic, non-motile rods; their DNA G + C contents range from 59.4 to 66.4 mol%. Their whole-cell fatty acid profiles are similar and the primary fatty acid in all the strains is $C_{18:1}\omega7c$ (74.1-87.7% of total content). Genotypic results, together with phenotypic characteristics, allowed the differentiation of these species from related species of the α-subclass of the *Proteobacteria* with validly published names. The strains are assigned to a new genus with three new species: *Loktanella salsilacus* sp. nov., which is the type species (consisting of 10 strains with LMG 21507^T = CIP 108322^T as type strain), *Loktanella fryxellensis* sp. nov. (consisting of 12 strains with LMG 22007^T = CIP 108323^T as type strain), and *Loktanella vestfoldensis* sp. nov. (consisting of 4 strains with LMG 22003^T = CIP 108321^T as type strain).

Introduction

During the last few years, there has been an increase in the isolation and description of novel marine and freshwater bacteria and several of these new isolates are members of the α-subclass of the *Proteobacteria*, in which they are phylogenetically related to the genus *Rhodobacter*. The abundance of some members of the *Rhodobacter* group (like *Sulfitobacter*) in these aquatic environments has been correlated with the presence of algal blooms and it has been suggested that they play an important role in sulfur cycling (Gonzalez *et al.*, 1999; 2000).

Several of these novel members originate from Antarctic habitats: *Antarctobacter heliothermus* (Labrenz *et al.*, 1998), *Roseovarius tolerans* (Labrenz *et al.*, 1999), *Staleya guttiformis* and *Sulfitobacter brevis* (Labrenz *et al.*, 2000) from Ekho Lake, and *Octadecabacter arcticus* and *O. antarcticus* (Gosink *et al.*, 1997) from polar sea-ice and seawater. Recently, two new genera have been added to this *Rhodobacter* group: *Ketogulonicigenium* (Urbance *et al.*, 2001), isolated from soil, which oxidizes L-sorbose to 2-keto-L-gulonic acid, and *Jannaschia helgolandensis* (Wagner-Döbler *et al.*, 2003), isolated from the North Sea.

During the MICROMAT project (November 1998 - February 2001), 746 heterotrophic bacterial strains were isolated from microbial mat samples that were collected from 10 Antarctic lakes (Van Trappen *et al.*, 2002). Numerical analysis of the fatty acid composition of the isolates revealed 41 clusters and 16S rRNA gene sequence analysis, performed on representative strains, showed that they belong to the α-, β- and γ-subclasses of the *Proteobacteria*, the Gram-positives, and the *Bacteroidetes* (Van Trappen *et al.*, 2002). Results of fatty acid and 16S rRNA gene sequence analyses showed that the diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is very high. Moreover, many fatty acid clusters were shown to contain multiple taxa when tested by repetitive extragenic palindromic DNA-PCR fingerprinting, a technique used to investigate the genomic diversity of each fatty acid cluster more in detail, especially those belonging to the *Bacteroidetes* group (Van Trappen *et al.*, 2003; 2004a, b).

In the present work, we studied the relationship of 26 strains from fatty acid cluster 41 (as delineated by Van Trappen *et al.*, 2002; belonging to the α-subclass of the *Proteobacteria*), by polyphasic taxonomic characterization.

Materials and Methods

The investigated isolates, their origin and genomic profile grouping are listed in Table 4.1. Strains were cultivated routinely on marine agar 2216 (Difco) at 25 °C for 48 h, except when mentioned otherwise.

DNA was prepared according to the method of Pitcher *et al.* (1989) and rep-PCR fingerprinting (based on primers targeting the repetitive extragenic palindromic sequence) was performed on all the strains of fatty acid cluster 41 (59 strains) of Van Trappen *et al.* (2002), using the primers GTG_5 and REP1R-I and REP2-I (Versalovic *et al.*, 1991), as described by Rademaker & de Bruijn (1997) and Rademaker *et al.* (2000). Numerical analysis was carried out using the Bionumerics software package (Applied Maths).

Almost complete 16S rRNA gene sequences of representative strains were determined as described earlier (Van Trappen *et al.*, 2004a). The most closely related sequences were found using the FASTA program; sequences were aligned and editing of the alignment and reformatting was performed with the BIOEDIT program (Hall, 1999) and ForCon (Raes & Van de Peer, 1999). Evolutionary distances were calculated using the Jukes-Cantor evolutionary model and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the TREECON program (Van de Peer & De Wachter, 1994).

DNA was prepared according to the method of Marmur (1961) and DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 BioAssay reader (PerkinElmer) for the fluorescence measurements. The hybridization temperature was 45°C and reciprocal experiments were performed for every pair of strains.

DNA G+C contents of the Antarctic strains were determined using an HPLC method, as described by Van Trappen *et al.* (2003).

The following morphological, physiological and biochemical tests were performed. Growth at different temperatures (5-45°C) was tested on marine agar, whereas salt tolerance was tested on R2A agar (composition per liter: 0.5 g yeast extract, 0.5 g proteose peptone No.3, 0.5 g casamino acids, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g dipotassium phosphate, 0.05 g magnesium sulfate and 15.0 g agar), supplemented with 1 to 20% NaCl at 25°C. Colony morphology was determined on marine agar after 7 days incubation. In addition, growth and adherence of colonies on R2A, nutrient and trypticase soy agars were tested. Cells were tested for their reaction to the Gram stain and for

catalase and oxidase activity. Tests in the commercial systems API ZYM, API 20NE and API 20E (bioMérieux) were generally performed according to the instructions of the manufacturer. The API ZYM tests were read after 4 h incubation at 25 °C, other API tests after 48 h incubation at 25 °C. Degradation of casein (Reichenbach & Dworkin, 1981), DNA [using DNA agar (Difco), supplemented with 0.01 % toluidine blue from Merck], starch, Tween 80 and L-tyrosine (Barrow & Feltham, 1993) were tested; reactions were read after 5 days.

Results and discussion

Twenty-six strains of fatty acid cluster 41 (Table 4.1), showed similar rep-PCR profiles and they could be divided into three different clusters according to their combined profile type (Fig. 4.3), and these clusters were delineated by numerical analysis at a Pearson correlation coefficient level of 50%. They are hereafter referred to as rep-PCR profile type I (comprising 12 strains), type II (with 4 strains) and type III (with 10 strains). It is now well established that similar rep-PCR profiles are correlated to high total genomic DNA-DNA hybridization values (Versalovic *et al.*, 1994; Rademaker & De Bruijn, 1997; Rademaker *et al.*, 2000; Van Trappen *et al.*, 2003; 2004a).

Almost complete 16S rRNA gene sequences (1404-1449 nt) of strains LMG 22003^T, LMG 22006, LMG 22007^T, LMG 21507^T, LMG 22000 and LMG 22002 were obtained and a phylogenetic tree is shown in Fig. 4.4. Dendrograms obtained by maximum parsimony and maximum likelihood analyses showed essentially the same topography (data not shown).

The novel Antarctic strains form a distinct evolutionary clade, supported by high bootstrap values, within the α-subclass of the *Proteobacteria* and are associated with the *Rhodobacter* group. The 16S rRNA gene sequence of strain LMG 22007^T (representative for the strains of rep-PCR profile type I) revealed 98.6% similarity to strain LMG 21507^T (identical to LMG 22000 and LMG 22002; representing rep-PCR profile type III) and 95.4% to strain LMG 22003^T (representing rep-PCR profile type II and which sequence is identical to that of strain LMG 22006). The strains with nearest related sequences to that of strain LMG 22007^T (rep-PCR profile I) are *Jannaschia helgolandensis* Hel10^T (95.8%), *Octadecabacter antarcticus* 307^T (94.5%) and the currently unclassified marine alpha proteobacterium strain QSSC9-5 (97.3%). The 16S rRNA gene sequence

Table 4.1. Strains investigated, source of isolation and rep-PCR profile type.

Species	Strain No.	Isolation site
***Loktanella fryxellensis* sp. nov.**	LMG 22007 ^{T} (= R-7670)	Lake Fryxell, Dry Valleys
(rep-PCR cluster I)	LMG 22008 (= R-7672)	Lake Fryxell, Dry Valleys
	LMG 22009 (= R-7726)	Lake Fryxell, Dry Valleys
	LMG 22010 (= R-7728)	Lake Fryxell, Dry Valleys
	R-7601, R-7605, R-7671, R-7729, R-7732, R-7735, R-8013, R-8014	Lake Fryxell, Dry Valleys
***Loktanella vestfoldensis* sp. nov.**	LMG 22003 ^{T} (= R-9477)	Ace Lake, Vestfold Hills
(rep-PCR cluster II)	LMG 22006 (= R-9184)	Ace Lake, Vestfold Hills
	LMG 22004 (= R-9054)	Pendant Lake, Vestfold Hills
	LMG 22005 (= R-9057)	Pendant Lake, Vestfold Hills
***Loktanella salsilacus* sp. nov.**	LMG 21507 ^{T} (= R-8904)	Ace Lake, Vestfold Hills
(rep -PCR cluster III)	LMG 21999 (= R-8968)	Ace Lake, Vestfold Hills
	R-8884, R-8901, R-9036	Ace Lake, Vestfold Hills
	LMG 22000 (= R-9030)	Organic Lake, Vestfold Hills
	LMG 22001 (= R-9066)	Organic Lake, Vestfold Hills
	LMG 22002 (= R-9068)	Organic Lake, Vestfold Hills
	R-9064, R-9186	Organic Lake, Vestfold Hills

Abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium; R-, strain numbers from the research collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium, and as used by Van Trappen *et al.* (2002).

of strain LMG 22003^{T} (rep-PCR profile type II) showed 95.4% sequence similarity to *Jannaschia helgolandensis* Hel10^{T}, 94.2% to *Ketogulonicigenium vulgare* DSM 4025^{T}, 94.3% to *Ruegeria algicola* DSM 10251^{T} and 96.2% to the currently unclassified strain AS-26. The 16S rRNA gene sequence of strain LMG 21507^{T} (rep-PCR profile type III) showed 95.7% similarity to *Jannaschia helgolandensis* Hel10^{T}, 94.2% to *Octadecabacter antarcticus* 307^{T}, 94.2% to *Ketogulonicigenium vulgare* DSM 4025^{T} and 98.4% to strain QSSC9. The low level of sequence similarities of the novel strains with other to date described bacteria belonging to the *Rhodobacter* group of the α-subclass of the *Proteobacteria* (91.0-95.8%), clearly demonstrates that they represent a new genus.

Genomic relatedness between the novel Antarctic strains, representing the three different rep-PCR profile types was determined by DNA-DNA hybridization. The hybridization level between strains LMG 22007^{T} (rep-PCR profile type I), LMG 22003^{T} (rep-PCR profile type II) and LMG 21507^{T} (rep-PCR profile type III)

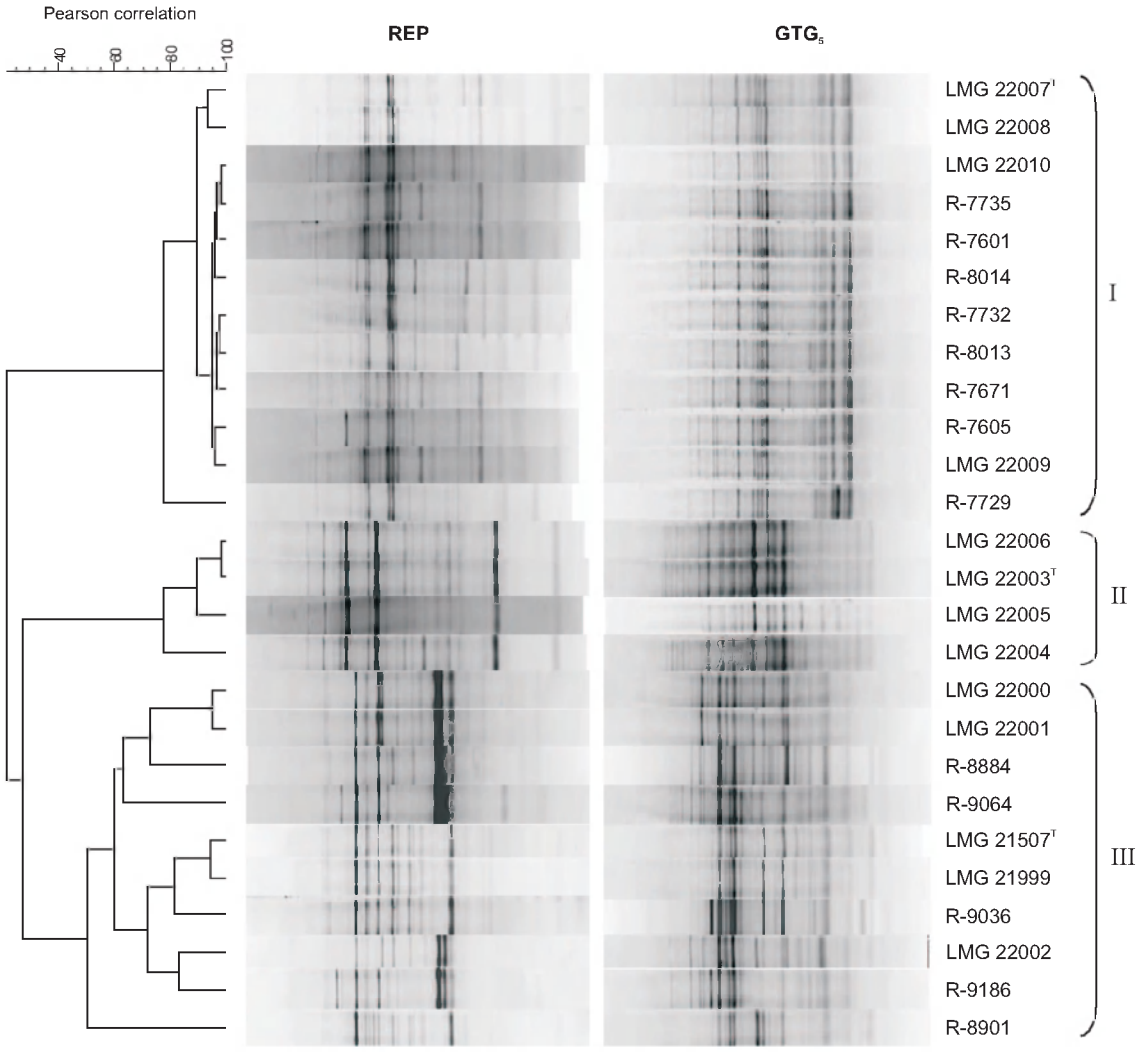

Figure 4.3. Digitized representation of normalized rep-PCR profiles (combined profiles of REP- and GTG_5-primers) of 26 strains from fatty acid cluster 41. Dendrogram derived from the UPGMA clustering of the profiles with the Pearson correlation coefficient and rep-clusters were delineated at a cut-off value of 50%. Rep-cluster I, *Loktanella fryxellensis* sp. nov.; rep-cluster II, *L. vestfoldensis* sp. nov.; rep-cluster III and IV, *L. salsilacus* sp. nov.

was 10.5-17.6 %, indicating that they belong to three different species (Wayne *et al.*, 1987). Differences between reciprocal experiments were less than 10 %. The rep-PCR profiles within each of the clusters I and II were almost identical (see Fig. 4.3), indicating that within each of these clusters, strains belong to a single species (Versalovic *et al.*, 1994). Indeed the 16S rRNA gene sequences of two strains of rep-PCR group II are identical. The hybridization values of the three representative strains (LMG 21507^T, LMG 22000 and LMG 22002) of rep-PCR profile type III, showing slight differences in their rep-PCR profiles, were 78.2% and 85.5% respectively, proving that they constitute a single new species, as

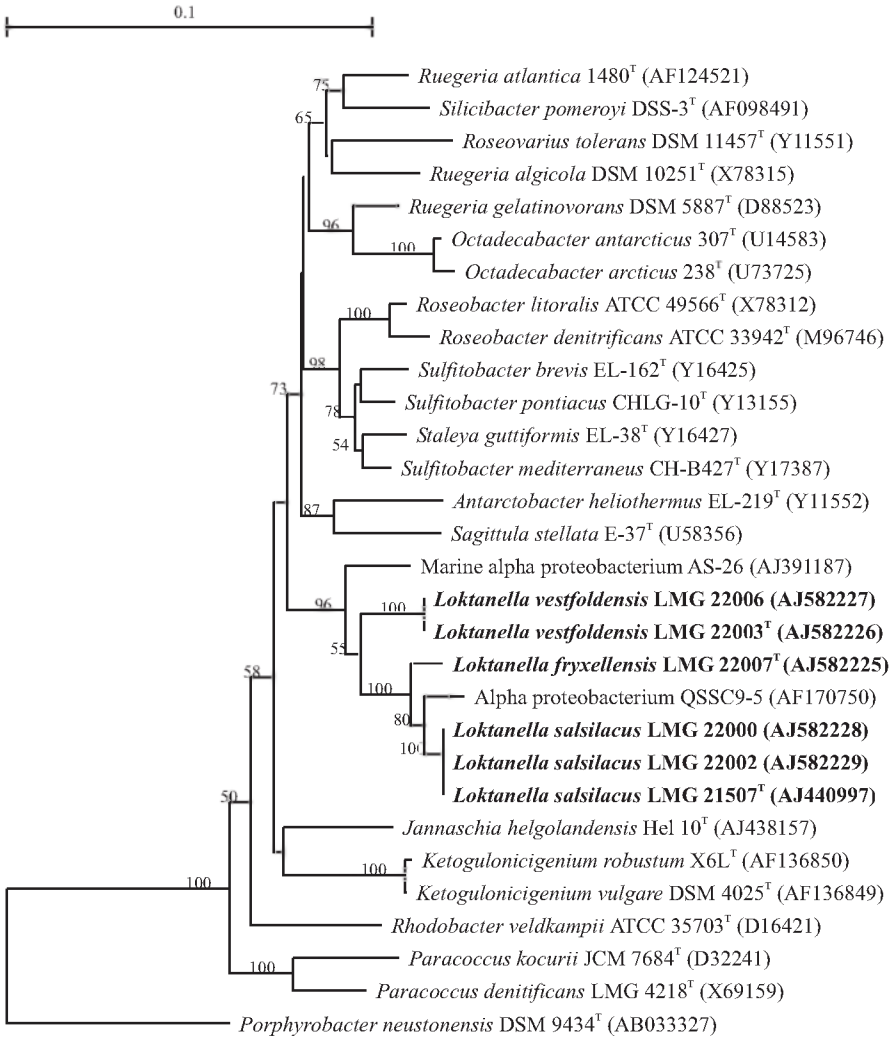

Figure 4.4. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of *Loktanella salsilacus* sp. nov., *Loktanella fryxellensis* sp. nov., *Loktanella vestfoldensis* sp. nov. and other related genera of the α-subclass of the *Proteobacteria* on the basis of 16S rRNA gene sequences. *Porphyrobacter neustonensis* was choosen as outgroup. Bootstrap values (percentages of 500 replicates) of > 50 % are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

would be expected from their identical 16S rRNA gene sequences.

DNA G+C values of strains LMG 22007T, LMG 22008, LMG 22009 and LMG 22010 from rep-PCR cluster I are 65.7, 66.2, 66.4 and 66.3 mol%, respectively. The values of the strains LMG 22003T, LMG 22004, LMG 22005 and LMG 22006 from rep-PCR cluster II are 62.1, 62.6, 62.3 and 63.1 mol%, respectively and those of strains LMG 21507T, LMG 21999, LMG 22000, LMG 22001 and LMG 22002 of rep-PCR cluster III are 60.4, 60.3, 59.7, 60.1 and 59.4 mol%, respectively.

Table 4.2. Fatty acid composition of the three novel species within the genus *Loktanella*.

Fatty acid	*L. salsilacus* (n = 10)	*L. fryxellensis* (n = 12)	*L. vestfoldensis* (n = 4)
$C_{10:0}$ 3-OH	2.4 ± 0.7	3.7 ± 1.1	6.1 ± 1.5
$C_{12:1}$3-OH	-	-	5.6 ± 1.4
$C_{16:0}$	2.9 ± 0.9	2.7 ± 1.1	2.9 ± 0.7
$C_{18:0}$	1.4 ± 0.8	1.6 ± 0.9	1.8 ± 0.3
$C_{18:1}\omega7c$	87.7 ± 1.9	84.9 ± 3.7	74.1 ± 3.1
$C_{18:1}\omega7c$-11 methyl	TR	-	1.9 ± 0.8
Summed feature 2	TR	1.7 ± 0.7	-
Summed feature 3	2.8 ± 0.9	-	-
Summed feature 7	1.2 ± 1.0	4.7 ± 2.0	4.7 ± 0.7
Unknown 11.799	-	-	2.3 ± 1.2

Mean percentages ± S_D of total fatty acids are given. -, Not detected; TR, trace (<1% of total). Other fatty acids accounted for < 1% each. Summed feature 2 comprises any combination of $C_{12:0}$ aldehyde, unknown 10.928, iso I-$C_{16:1}$ and $C_{14:0}$3-OH. Summed feature 3 comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both. Summed feature 7 comprises any combination of unknown 18.846, $C_{19:1}\omega6c$ and cyclo-$C_{19:0}$ $\omega10c$. Unknown fatty acids are designated by their equivalent chain lengths, relative to the chain lengths of known straight chain saturated fatty acids.

These values are consistent with the DNA G+C contents of members of the *Rhodobacter* group, which range from 52.1 to 65 mol % (Labrenz *et al.*, 2000; Urbance *et al.*, 2001; Wagner-Döbler *et al.*, 2003; Gonzalez *et al.*, 2003).

Cellular fatty acid patterns of the Antarctic strains are based on the data generated by Van Trappen *et al.* (2002). The strains show similar fatty acid profiles (Table 4.2) and the most abundant fatty acid is $C_{18:1}\omega7c$, accounting for 74.1-87.7 % of the total fatty acids. This feature is characteristic for several major phylogenetic groups of the α-subclass of the *Proteobacteria*. Other fatty acids, in lower proportions, are $C_{10:0}$3-OH, $C_{16:0}$, $C_{18:0}$ and summed feature 7 (comprising the unknown fatty acid 18.846, $C_{19:1}\omega6c$ and cyclo-$C_{19:0}\omega10c$). The Antarctic strains can be differentiated from their phylogenetic neighbours *Jannaschia helgolandensis* by the relative amount of $C_{18:1}\omega7c$ (45-52%) and cyclo-$C_{19:0}$ (20-25%), and from *Ketogulonicigenium* by the relative amount of $C_{16:0}$ (32-39%) and $C_{18:1}\omega7c$ (41-55%). The strains belonging to the different rep-PCR clusters can be differentiated from each other by the presence or absence of e.g. summed feature 2 (comprising any combination of $C_{12:0}$ aldehyde, unknown 10.928, iso I-$C_{16:1}$ and $C_{14:0}$3-OH), 11 methyl-$C_{18:1}\omega7c$ and the unknown fatty acid 11.799.

The strains are aerobic and chemoheterotrophic, and there is no growth under anaerobic conditions. Strains of rep-PCR cluster III and rep-PCR cluster I are able to grow between 5°C and 30°C, and 5°C and 25°C respectively, whereas strains of rep-PCR cluster II tolerate temperatures up to 37°C. None of the strains grows at 40°C. Growth appears on R2A agar with up to 10% NaCl for the strains of rep-PCR cluster III and rep-PCR cluster II, whereas strains of rep-PCR cluster I only grow with up to 5% NaCl.

The strains show the typical morphological characteristics of the *Rhodobacter* group (Labrenz *et al.*, 2000; Urbance *et al.*, 2001; Wagner-Döbler *et al.*, 2003; Gonzalez *et al.*, 2003) and their physiological and biochemical characteristics are given in the species descriptions. The strains of rep-PCR clusters I, II and III can be differentiated from each other and related genera by several phenotypic characteristics (Table 4.3 and 4.4).

On the basis of these results a new genus with the name *Loktanella* gen. nov. is proposed with three species, *Loktanella salsilacus* sp. nov. (rep-PCR cluster III,

Table 4.3: Phenotypic characteristics that differentiate the three species of the genus *Loktanella*.

Characteristic	***L. salsilacus***	***L. fryxellensis***	***L. vestfoldensis***
Pigmentation	Beige	Pink-beige	Pink
Growth on :			
Trypticase soy agar	-	-	(+)
Nutrient agar	-	-	(+)
Salinity range (% NaCl)	0-10	0-5	0-10
Temperature range (°C)	5-30	5-25	5-37
Hydrolysis of:			
Urea	-	-	+
Production of:			
Trypsin	-	-	+
α-Galactosidase	+	-	-
Mean G+C content (mol%)	59.4-60.4	65.7-66.4	62.1-63.1

Symbols: +, positive test; (+), positive test, weak or delayed response; -, negative test.

Table 4.4. Phenotypic characteristics that differentiate *Loktanella* species from other related members of the family *Rhodobacteriaceae*.

Characteristic	*Loktanella*	*Ketogulonicigenium*	*Jannaschia*	*Octadecabacter*	*Antarctobacter*	*Sulfitobacter*	*Roseobacter*
Rosettes formed	-	-	-	-	+	+	V
Colony colour	Pink-beige	Brown	White	White	Brown-yellow	V	V
Motility	-	+	-	-	+/-	+	+
Temp optimum (°C)	25	25-31	25-30	4-15	16-26	17-28	20-30
Oxidase	+	+	(+)	-	+	+	+
Carbon utilization	-	+	+	+	+	+	+
Hydrolysis of:							
Aesculin	+	ND	-	ND	ND	ND	+
Gelatin	-	ND	-	-	+	-	+
DNA	-	ND	ND	ND	+	-	ND
Tween 80	+	ND	-	ND	-	+	ND
Nitrate reduction	-	ND	-	-	+	-	V
Mean G+C content (mol%)	59-66	52-54	63	56-57	62	57-63	56-60

Symbols: -, negative; +, positive; V, variable results; ND, no available data. Data for *Ketogulonicigenium*, *Jannaschia, Octadecabacter, Antarctobacter, Sulfitobacter* and *Roseobacter* are from the literature (Urbance *et al.*, 2001; Wagner-Döbler, 2003; Gosink *et al.*, 1997; Labrenz *et al.*, 1998; Labrenz *et al.*, 2000; Pukall *et al.*, 1999; Shiba, 1991; Lafay *et al.*, 1995; Ruiz-Ponte *et al.*, 1998; Labrenz *et al.*, 1999).

type species), *Loktanella fryxellensis* sp. nov. (rep-PCR cluster I), and *Loktanella vestfoldensis* sp. nov. (rep-PCR cluster II).

Description of *Loktanella* gen. nov.

Loktanella (Lok.tan.el.la. N.L. fem. n. *Loktanella* named after Tjhing-Lok Tan from the Alfred Wegener Institute in Bremerhaven, who contributed to our understanding of marine and polar bacteriology and ecology).

Gram-negative, rod-shaped cells which are strictly aerobic, moderately halotolerant and chemoheterotrophic. They do not form spores and the optimal growth temperature is 25°C. Motility was not observed. The catalase test was positive and activities for cytochrome oxidase and β-galactosidase were detected. The dominant fatty acid is $C_{18:1}\omega7c$ and other characteristic fatty acids are $C_{10:0}$3-OH, $C_{16:0}$, $C_{18:0}$ and summed feature 7 (which comprises the unknown fatty acid 18.846, $C_{19:1}\omega6c$ and cyclo-$C_{19:0}\omega10c$). DNA G+C contents range from 59.4-66.4%. As determined by 16S rRNA gene sequence analysis, the genus *Loktanella* belongs to the *Rhodobacter* group of the α-subclass of the *Proteobacteria*. The type species is *Loktanella salsilacus* sp. nov.

Description of *Loktanella salsilacus* sp. nov.

Loktanella salsilacus (sal.si.la'cus. L. adj. *salsus* salt, salty; L. gen. n. *lacus* of a lake; N. L. gen. n. *salsilacus* of a salt lake, referring to the isolation source, Ace Lake and Organic Lake, Vestfold Hills, Antarctica).

Cells are Gram-negative, short rods (<1µm x 3-4 µm), that often form pairs or short chains. Growth occurs at 5-30°C, whereas a weak growth is observed at 37°C and no growth occurs at 45°C. Beige, convex, translucent colonies with entire margins and a diameter of 1-2 mm are formed on marine agar plates. Growth also occurs on R2A, while no growth is observed on trypticase soy agar and nutrient agar. Colonies do not adhere to the agar. Aesculin, Tween 80 and citrate are degraded. Growth on carbohydrates (API 20NE) is not observed and acids from carbohydrates are not produced (API 20E). Agar, casein, DNA, gelatin, starch, tyrosine and urea are not degraded. Tests for indole production, nitrate reduction,

Voges Proskauer reaction and hydrogen sulfide production are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, trypsin, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase (API ZYM). Weak enzymic activity was observed for alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase, medium activity is observed for esterase (C4), esterase lipase (C8) and leucine arylamidase, and strong activity is observed for α-galactosidase (API ZYM). Growth occurs in 0-5 % NaCl, with a weak growth in 10% NaCl, indicating that the strains are not halophilic but moderately halotolerant. DNA G+C content is 59.4-60.4 mol%.

The type strain is LMG 21507^T (= CIP 108322^T). Isolated from microbial mats from lakes Ace and Organic in the Vestfold Hills, Antarctica.

Description of *Loktanella fryxellensis* sp. nov.

Loktanella fryxellensis (fry.xell.en'sis. N. L. fem. adj. *fryxellensis*, referring to the isolation source, Lake Fryxell, Antarctica).

Cells are Gram-negative, short rods (<1µm x 2-3 µm), that often form pairs or short chains. Growth occurs at 5-25°C, with an optimal growth temperature of 25 °C, whereas a weak growth occurs at 30 °C. Pale pink, convex, translucent colonies with entire margins and a diameter of 1 mm are formed on marine agar plates after 6 days incubation. Strain LMG 22007^T forms beige colonies on marine agar. Growth also occurs on R2A while the strains do not grow on nutrient agar and trypticase soy agar; colonies do not adhere to the agar. Aesculin, Tween 80 and citrate (weak reaction) are degraded. No growth is observed (API 20NE) on carbohydrates and acids are not produced from carbohydrates (API 20E). Agar, casein, DNA, gelatin, tyrosine and urea are not degraded. Tests for indole production, nitrate reduction, Voges-Proskauer reaction and hydrogen sulfide production are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), cystine arylamidase, α-chymotrypsine, trypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and

α-fucosidase (API ZYM). Weak enzymic activity is observed for valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase, medium activity is observed for alkaline phosphatase, esterase (C4), esterase lipase (C8), β-galactosidase and leucine arylamidase, and strong activity is observed for β-glucosidase (API ZYM). Growth occurs in 0-5% NaCl but not in 10% NaCl, indicating that the strains are not halophilic but moderately halotolerant. DNA G+C content is 65.7-66.4 mol%.

The type strain is LMG 22007^T (= CIP 108323^T). Isolated from microbial mats from Lake Fryxell, in the McMurdo Dry Valleys, Antarctica.

Description of *Loktanella vestfoldensis* sp. nov.

Loktanella vestfoldensis (vest.fold.en'sis. N. L. fem. adj. *vestfoldensis*, referring to the isolation source, lakes Ace & Pendant, Vestfold Hills, Antarctica).

Cells are Gram-negative, short rods (<1µm x 3-4 µm), that often form pairs or short chains. Growth occurs at 5-37°C, whereas no growth is observed at 45 °C. Pale pink, convex, translucent colonies with entire margins and a diameter of <1 mm are formed on marine agar plates. Growth also occurs on trypticase soy agar (weak), nutrient agar (weak) and R2A. Colonies do not adhere to the agar. Aesculin, Tween 80, citrate and urea are degraded. No growth is observed (API 20NE) on carbohydrates and acids are not produced from carbohydrates (API 20E). Agar, casein, DNA, gelatin, tyrosine and starch are not degraded. Tests for indole production, nitrate reduction, hydrogen sulfide production and Voges-Proskauer reaction are negative. None of the strains shows activity for the enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsine, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase (API ZYM). Weak enzymic activity is observed for alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase, medium activity is observed for esterase (C4), esterase lipase (C8) and acid phosphatase, and strong activity is observed for trypsin (API ZYM). Growth occurs in 0-5 % NaCl and a weak growth in 10% NaCl. DNA G+C content is 62.1-63.1 mol%.

The type strain is LMG 22003^T (= CIP 108321^T). Isolated from microbial mats from lakes Ace and Pendant in the Vestfold Hills, Antarctica.

CHAPTER 5

New taxa from polar seas within the γ-*Proteobacteria*

5.1 Polyphasic taxonomy of FAA clusters E, F and related strains

In a previous study the diversity of oligotrophic bacteria in polar seas was investigated (Mergaert *et al.*, 2001b). After enrichment under oligotrophic and psychrophilic conditions, 173 bacterial strains were isolated from Arctic (98 strains) and Antarctic (75 strains) seawater (Tan & Rüger, 1991; Tan *et al.*, 1999). These strains had been previously analysed by their substrate utilization patterns using the Biolog system (Tan, 1997; Tan & Rüger, 1999) and they belong to six metabolic groups. The strains were included in fatty acid analysis and 16S rDNA sequence analysis of representatives (Mergaert *et al.*, 2001b) and eight FAA-clusters, containing two to 59 strains, could be delineated, whereas eight strains formed separate branches (see Fig. 5.1). The clusters A, C, D, E and H contained isolates both from Arctica as well as Antarctica. Clusters B and F contained only Arctic strains, cluster G, only Antarctic strains. Results of the 16S rDNA sequence analysis indicate that they belong to the α- and γ- *Proteobacteria* (FAA clusters A and B belong to the α- *Proteobacteria* and clusters C, D, E and F to the γ- *Proteobacteria*) the high percent G+C Gram-positives (cluster H) and to the *Bacteroidetes* (cluster G). The sequences from four clusters and seven unclustered strains were closely related (with sequence similarities above 97%) to reference sequences of *Sulfitobacter*, *Halomonas*, *Alteromonas*, *Pseudoaltermonas*, *Shewanella* and *Rhodococcus*. The other four clusters and one unclustered strain showed sequence similarities below 97% with nearest named neighbours, including *Rhizobium*, *Glaciecola*, *Pseudomonas* and *Alteromonas*, indicating that they represent as yet unnamed, new taxa.

In the meantime, 56 additional strains, isolated using the same methods,

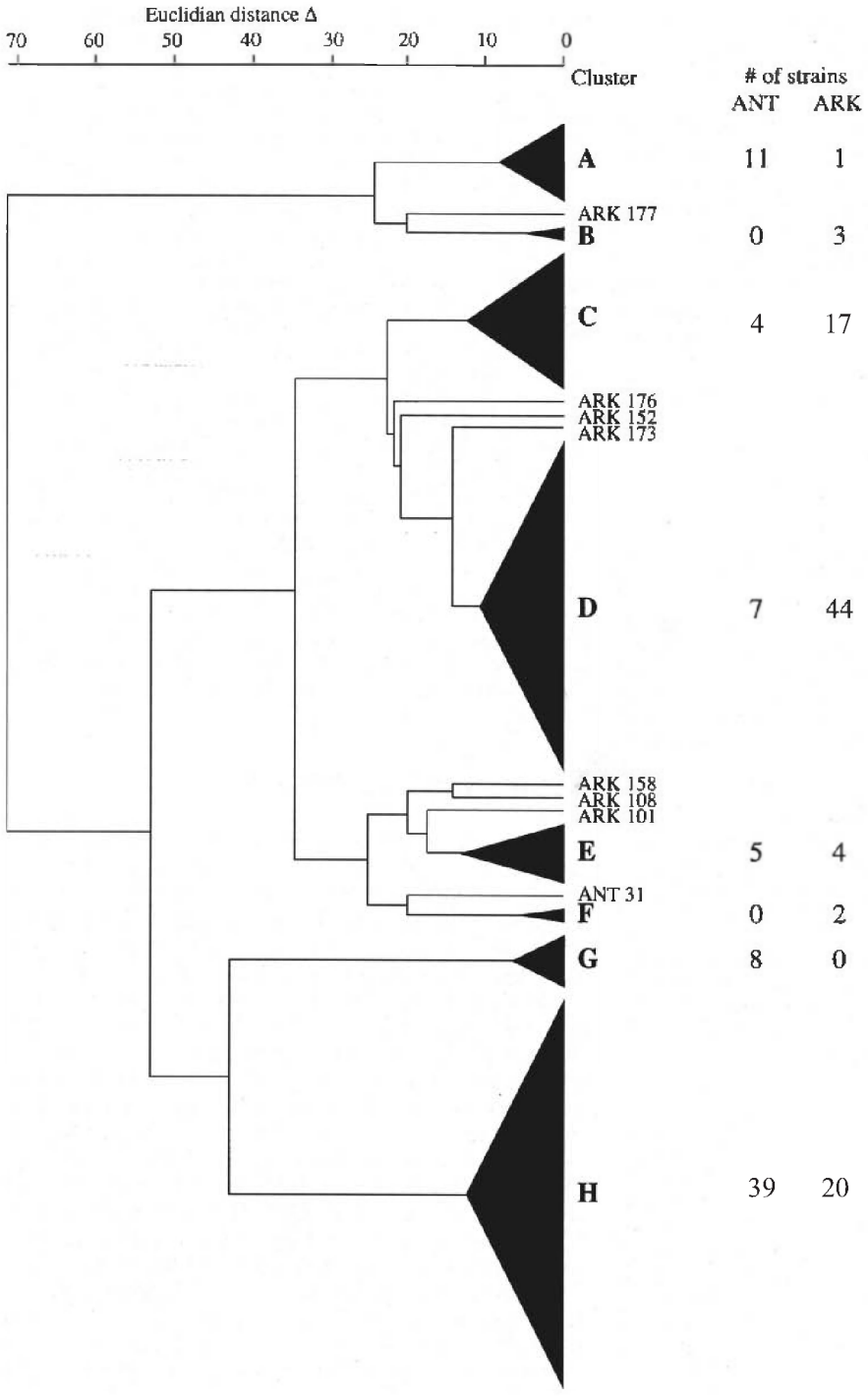

Figure 5.1. Abridged dendrogram obtained by numerical analysis of the fatty acid compositions of 173 strains from Arctic (ARK) and Antarctic (ANT) seawater using the Euclidian distance coefficient and UPGMA clustering. Clusters were delineated at a Euclidian distance of $\Delta \leq 14$ (from Mergaert *et al.*, 2001b).

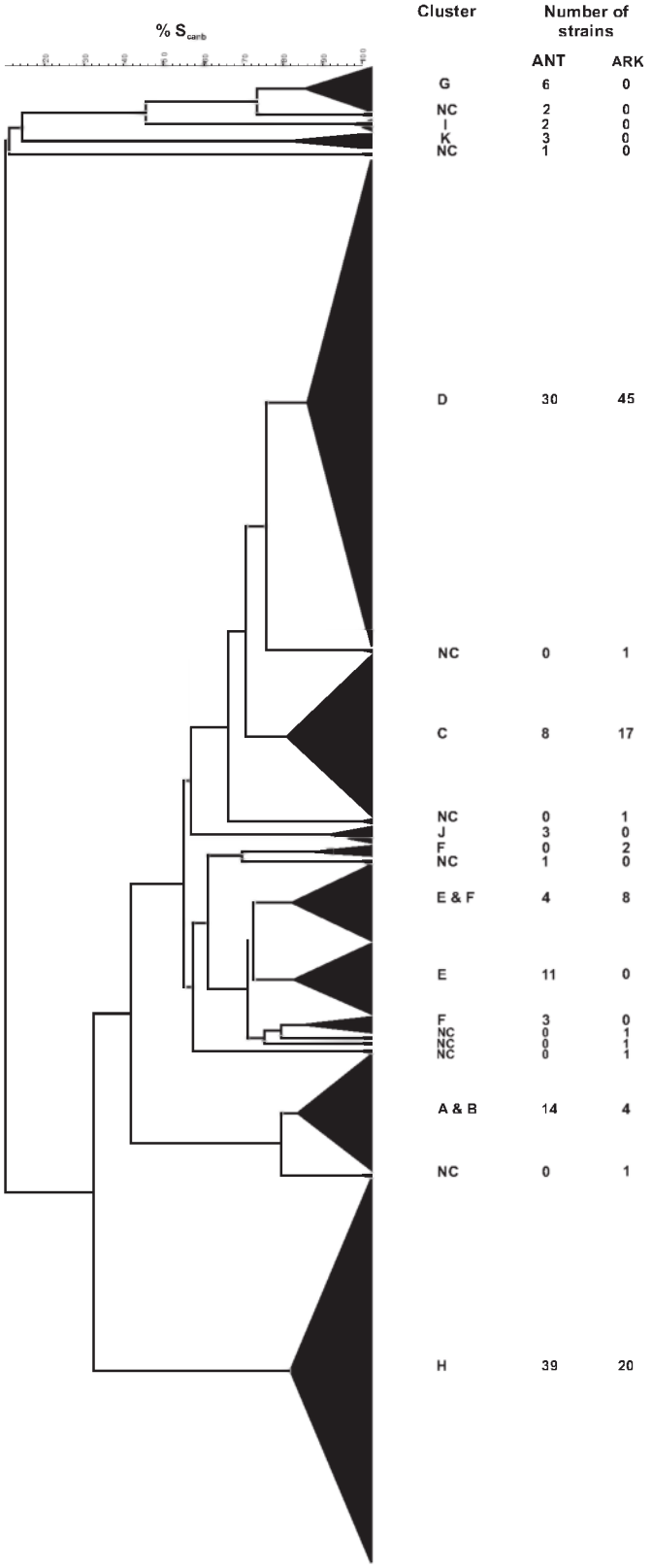

Figure 5.2. Abridged dendrogram obtained by numerical analysis of the fatty acid compositions of 229 strains from Arctic (ARK) and Antarctic (ANT) seawater using the Canberra metric similarity coefficient (S_{canb}) and UPGMA clustering. Clusters were delineated at a cut-off value of 80%.

were also analysed using the Biolog system. In this study, these additional strains were included in fatty acid analysis and they belong to FAA-clusters B, C, D, E and F (as delineated in Mergaert *et al.*, 2001b) and three new clusters (I, J and K) were found (see Fig. 5.2 and Annex II). All clusters contain strains from both Arctica as well as Antarctica, except clusters G, I, J and K, which solely consist of Antarctic strains.

The fatty acid clusters E and F and two similar (in fatty acid analysis), unclustered strains, phylogenetically allocated to the γ-*Proteobacteria*, were further investigated using a polyphasic taxonomic approach. The phylogenetic position of the γ-*Proteobacteria* with different families (e.g. *Enterobacteriaceae*, *Aeromonadaceae*, *Alteromonadaceae*, the '*Xanthomonas* group', the '*Oceanospirillum* group', *Pseudomonaceae*, *Legionellaceae* and *Halomonadeaceae*) is illustrated in Fig. 4A and 5A.

The genomic diversity of 19 strains from clusters E and F and two unclustered strains, was further investigated by rep-PCR genomic fingerprinting using the GTG_5-primer (Rademaker & de Bruijn, 1997). In total, 21 fingerprinting patterns were obtained (for strain ARK 101 no rep-profile could be obtained since this culture was not viable anymore and strain ANT 31 proved to be phylogenetically related to the Gram-positives) (Mergaert *et al.*, 2001b). Five clusters could be delineated, whereas four strains formed single branches, at a cut-off value of 70% (Pearson correlation coefficient) (see Fig. 5.3). These results illustrate that the genomic diversity of these two FAA clusters (E and F) is higher than estimated by fatty acid and 16S rDNA sequence analyses. Most of these rep-clusters contain strains isolated from only one pole, either Arctic for rep-clusters I and V, or Antarctic for rep-clusters II and III, whereas rep-cluster IV contains strains from both poles.

16S rDNA sequence analysis of strains from rep-clusters I and IV (FAA cluster E and F) showed that they are related to the genus *Pseudoalteromonas*, with similarities of 97.7-99.7% (Mergaert *et al.*, 2001b) and buds and prosthecate formations were observed in strains ARK 140, ARK 142 and ARK 102 from rep-cluster I and strains ANT 224, ARK 108 and ANT 223 from rep-cluster IV (personal communication, T.-L. Tan). Rep-cluster II (FAA cluster E) is phylogenetically related to *Alteromonas*, with strain ANT 69a showing 98.3% sequence similarity to *A. macleodii*. The unclustered strain ARK 158 is related to *Shewanella frigidimarina* (99.9%). Strains from rep-clusters III and V (FAA cluster F), are related to *Glaciecola*, with sequence similarities of 98.0-99.7% to *G. mesophila*. Hybridizations

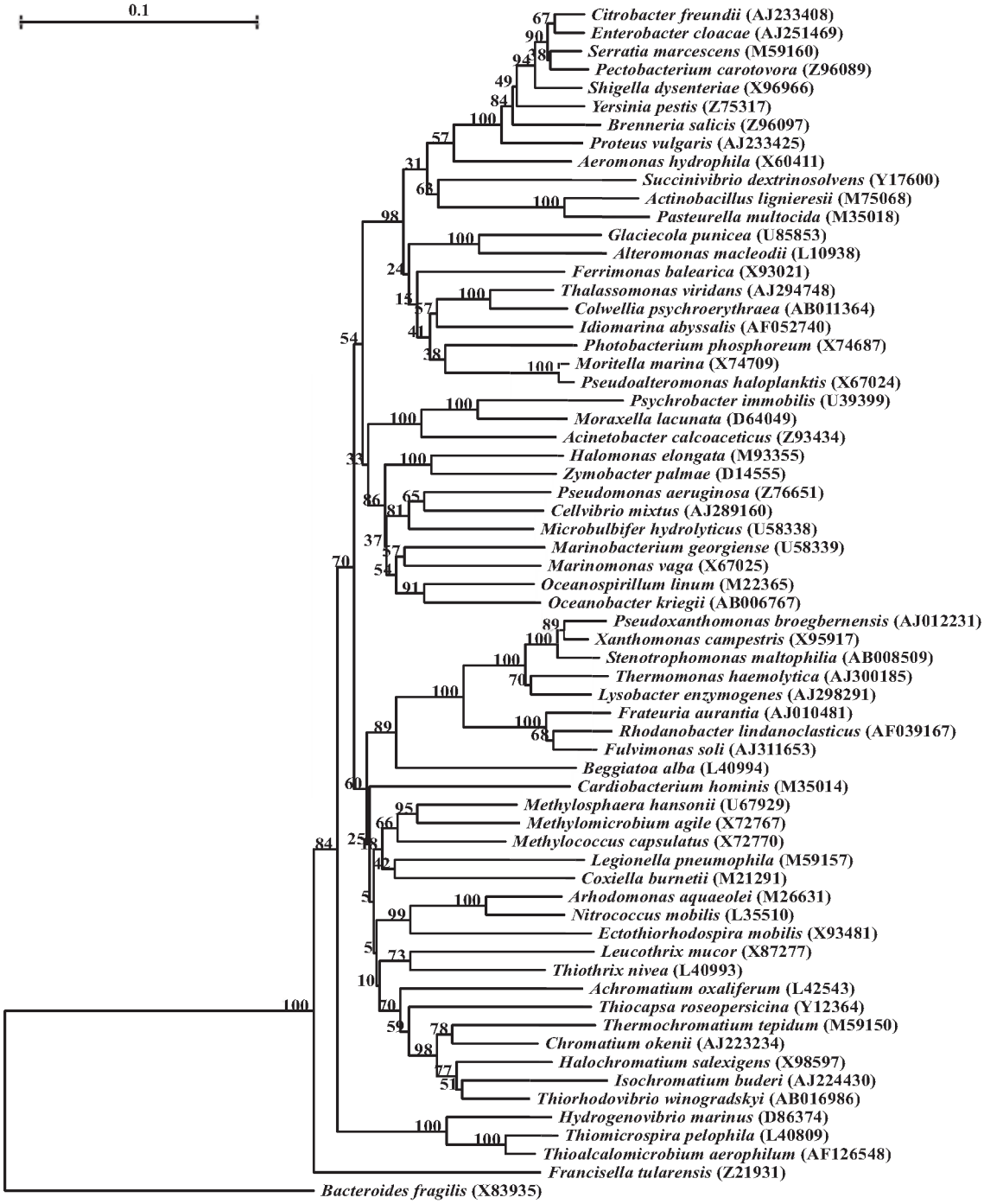

Figure 5A. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of representatives of the γ-*Proteobacteria* on the basis of 16S rRNA gene sequences. *Bacteroides fragilis* was choosen as outgroup. Bootstrap values (percentages of 100 replicates) are shown. GenBank accession numbers for each reference strain are shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

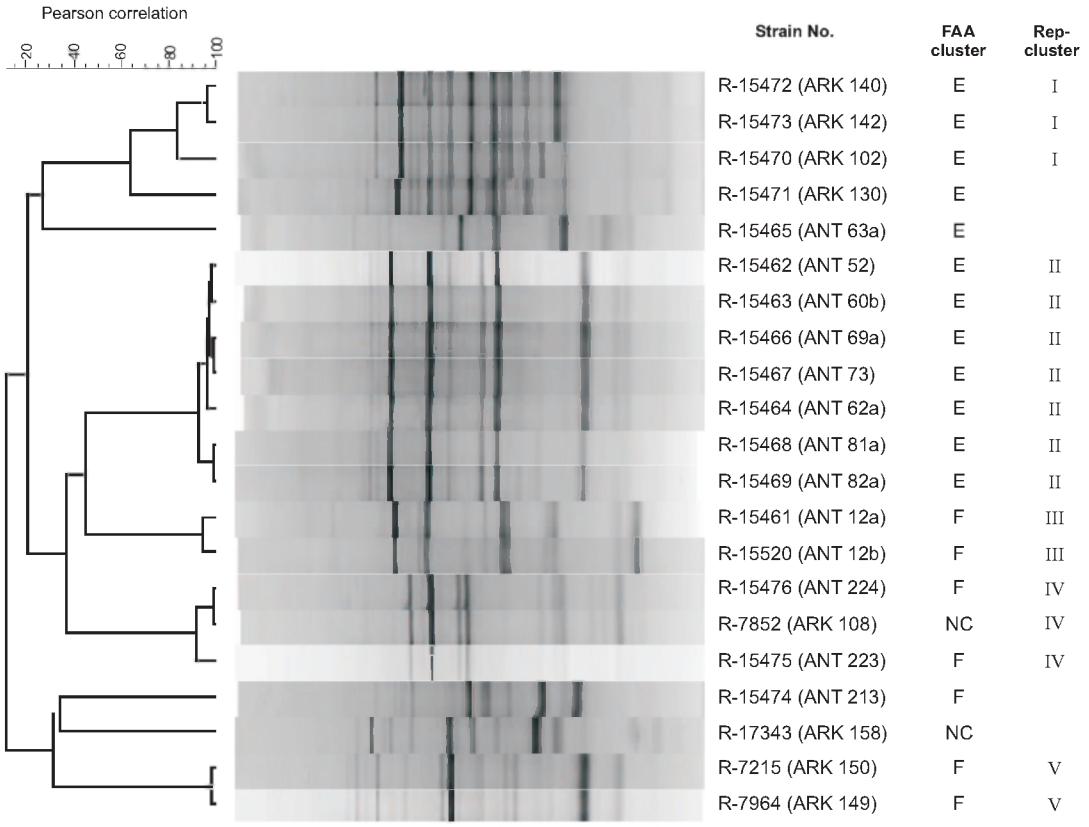

Figure 5.3. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of 21 strains belonging to FAA cluster E, F and similar, unclustered strains (NC: not clustered). Dendrogram derived from the UPGMA-clustering of the profiles with the Pearson correlation coefficient and rep-clusters were delineated at a cut-off value of 70%.

and additional 16S rDNA sequence analysis were performed for the strains of rep-clusters II, III and V.

Hybridization results together with phenotypic characteristics showed that the seven strains of rep-cluster II belong to a novel *Alteromonas* species that produces buds and prosthecae and for which the name *Alteromonas stellipolaris* is proposed (see section 5.2).

The two strains from rep-cluster V also belong to novel budding and prosthecate bacteria, phylogenetically related to *Glaciecola* and the name *G. polaris* is proposed. The two strains from rep-cluster III were proven to belong to the validly described species *Glaciecola mesophila* (see section 5.3).

5.2 *Alteromonas stellipolaris* sp. nov.: novel budding and prosthecate bacteria from Antarctic seas

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Seven novel strains of cold-adapted, strictly aerobic and facultative oligotrophic bacteria, isolated from Antarctic seawater, were investigated using a polyphasic taxonomic approach. The isolates are Gram-negative, chemoheterotrophic, motile, rod-shaped cells which are psychrotrophic and moderately halophilic. Buds can be produced on mother and daughter cells and on prosthecae. Prostheca formation is peritrich and prosthecae can be branched. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains belong to the γ-subclass of the *Proteobacteria* and are related to the genus *Alteromonas*, with 98.3% sequence similarity to *Alteromonas macleodii* and 98.0% to *Alteromonas marina*, their nearest phylogenetic neighbours. Their whole-cell fatty acid profiles are very similar and include $C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:1}\omega8c$ and $C_{18:1}\omega8c$ as major fatty acid components. These results support the affiliation of the new isolates to the genus *Alteromonas*. DNA-DNA hybridization results and differences in phenotypic characteristics show that the strains represent a new species within the genus *Alteromonas*. Their DNA G+C content ranges from 43 to 45 mol%. The name *Alteromonas stellipolaris* sp. nov. (with the isolate ANT $69a^T$ = LMG 21861^T = DSM 15691^T as type strain), is proposed. An emended description of the genus *Alteromonas* is given.

Introduction

The genus *Alteromonas* belongs to the γ-subclass of the *Proteobacteria* and was created by Baumann *et al.* (1972) for marine Gram-negative heterotrophic bacteria, motile by a single polar flagellum. On the basis of 16S rDNA sequence analysis, the genus was revised in 1995 to contain a single species, *Alteromonas macleodii* and the remaining species were reclassified as *Pseudoalteromonas* (Gauthier *et al.*, 1995). In 1993, the yellow-gray pigmented '*Alteromonas rava*' which is able to produce novel antibiotics, was described (Kodama *et al.*, 1993), but the species has not been validated yet. A mesophilic, heterotrophic bacterium, isolated from seawater collected near a deep-sea hydrothermal vent, was identified as *Alteromonas macleodii* but the authors classified it as a new subspecies, '*A. macleodii* subsp. *fijiensis*' on the basis of a relatively low DNA-DNA hybridization level (lower than 90%, but higher than 70%), metabolic differences between the type strain and the new strain, the ability of the new bacterium to produce a novel exopolysaccharide and the isolation source (Raguénès *et al.*, 1996). The subspecies name *fijiensis* has not yet been validated. In 1997, Raguénès *et al.* proposed a new *Alteromonas* species, '*Alteromonas infernus*', for a polysaccharide-producing bacterium, isolated from the surface of the vestimentiferan worm *Riftia pachyptila*, which inhabits sites near hydrothermal vents. This new species, however, has also not been validated. In 1994, Romanenko *et al.* described a new species, *Alteromonas fuligenea* but phylogenetic analysis based on 16S rDNA sequence data, pointed out that it is more closely related to *Pseudoalteromonas haloplanktis* and therefore needs to be reclassified as a member of the genus *Pseudoalteromonas* (Yoon *et al.*, 2003). Recently, a new species *Alteromonas marina,* isolated from the East Sea in Korea (Yoon *et al.*, 2003), has been validly described. As such, there are only two validly described species within the genus *Alteromonas*, namely *A. macleodii* (the type species) and *A. marina*.

The novel species *Alteromonas stellipolaris* sp. nov. described here, belongs to novel budding and prosthecate bacteria from the γ-subclass of the *Proteobacteria*. New strains of marine prosthecate and budding bacteria belonging to the genus *Hyphomonas*, a taxon of the α-*Proteobacteria*, have been described (Weiner et al., 2000), and this is the first report of budding and prosthecate bacteria from the γ-subclass of the *Proteobacteria*. It is evident now that budding and prosthecate bacteria are abundant in marine and polar environments (Weiner *et al.*, 2000; Labrenz *et al.*, 1998; Labrenz *et al.*, 1999). Moreover, bud and prosthecate

formations are a common strategy for rod-shaped bacteria to enhance their surface to volume ratio, thus enabling the organisms for efficient substrate uptakes in oligotrophic habitats (van Gemerden & Kuenen, 1984).

During expeditions in the Arctic (Tan & Rüger, 1991) and Antarctic seas (Tan & Rüger, 1999), facultatively oligotrophic and psychrotrophic bacteria were isolated. These 173 strains have been previously analysed by their substrate utilization patterns using the Biolog system (Tan, 1997; Tan & Rüger, 1999), and by fatty acid and 16S rDNA sequence analyses of representatives (Mergaert *et al.*, 2001b). They belong to six metabolic groups and eight different fatty acid clusters containing two to 59 strains. In the meantime, 56 additional strains were isolated using the same methods and were also analysed using the Biolog system and fatty acid analysis. These additional strains belong to clusters B, C, D, E and F (as delineated in Mergaert *et al.*, 2001b) and three new clusters (I, J and K) were found (S. Van Trappen, unpublished results). The genomic diversity of 19 strains from clusters E and F and two related, unclustered strains, was further investigated and, using a polyphasic taxonomic approach, seven Antarctic strains could be assigned to a novel species within the genus *Alteromonas*, named *A. stellipolaris* sp. nov.

Materials and Methods

Antarctic strains were isolated from seawater after an enrichment technique in dialysis chambers as previously described (Tan & Rüger, 1999; Tan, 1997; Tan, 1986). The seven Antarctic strains (with the prefix 'ANT') are listed in Table 5.1, together with their source of isolation. The reference strains LMG 2843^T (*Alteromonas macleodii*) and LMG 22057^T (*A. marina*) were included in some experiments. The strains were cultivated routinely on marine agar 2216 (Difco) at 20 °C for 48 h, except when mentioned otherwise.

Strains were arranged in similarity groups based upon the results of repetitive extragenic palindromic DNA-PCR fingerprinting using the GTG_5 primer (Versalovic *et al.*, 1991; Rademaker & de Bruijn, 1997; Rademaker *et al.*, 2000). Numerical analysis was carried out using the Bionumerics software package, as described by the same authors.

The almost complete 16S rRNA gene sequence of one strain was determined as previously described by Mergaert *et al.* (2001b). Partial 16S rRNA gene sequences of the other strains were determined by QIAGEN, 40724 Hilden, Germany, using the forward primer 8F (AGA GTT TGA TCC TGG CTC AG) and

the reverse primer 1492R (TAC GGY TAC CTT GTT ACG ACT T). The most closely related sequences were found by using the FASTA program; sequences were aligned and editing of the alignment and reformatting was performed with the BIOEDIT program (Hall, 1999) and ForCon (Raes & Van de Peer, 1999). Evolutionary distances were calculated by using the Jukes-Cantor evolutionary model and a phylogenetic tree was constructed by using the neighbour-joining method with the TREECON program (Van de Peer & De Wachter, 1994).

DNA was prepared according to the method of Pitcher *et al.* (1989) and DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 BioAssay reader (PerkinElmer) for the fluorescence measurements. The hybridization temperature was 37 °C and reciprocal experiments were performed for every pair of strains.

DNA G+C contents of the Antarctic strains were determined using an HPLC method. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$, pH 4.0, with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference.

Growth of the strains at different temperatures (5-40°C) was tested on marine agar 2216 (Difco), whereas salt tolerance was tested on R2A agar (Oxoid), supplemented with 1 to 20% NaCl at 20°C. The effect of the pH on the growth rate was determined from 5.0 to 10 (with an interval of 0.5 pH unit), using tubes with 10 ml of 2216E liquid medium, incubated at 20°C after inoculation. The turbidity was measured by spectrophotometry at 590 nm (Vitalab 10, Vital Scientific, The Netherlands). The biochemical characteristics were determined using standard protocols (Smibert & Krieg, 1994; West & Colwell, 1984; Reichenbach & Dworkin, 1981; Bowman *et al.*, 1998c; Van Trappen *et al.*, 2003), and API kits (API 20E, API 20NE, API ZYM and API32 ID, bioMérieux). Bacterial suspensions were made in sterile, chilled artificial seawater (Instant Ocean, synthetic sea salt, Aquarium Systems) and marine agar 2216 (Difco) was used as the basal medium. For BIOLOG GN2 microplates, the bacteria were grown on PYG agar at 20°C for 5 d; the cells were harvested and suspended in "Inoculating Fluid" (IF). The salinity of the IF was adjusted to 26 ‰ with NaCl. The microplates were incubated at 20°C and substrate utilizations were measured after 3, 5, 7, 14, 21, and 28 d at 590 nm with an eight-canal-photometer (Spectra 2, SLT Labinstruments). Methylpyruvate, L-asparagine, L-aspartic acid and glycyl-L-aspartic acid had been utilized by the

seven strains, if the microplates were incubated at 12°C (see cluster 3 in Tan & Rüger, 1999).

Results and discussion

The nineteen strains of FAA clusters E and F and two related, unclustered strains were arranged in similarity groups based upon the results of rep-PCR fingerprinting using the GTG_5 primer. One cluster of seven Antarctic strains (LMG 21861^T, LMG 21856, LMG 21859, LMG 21860, LMG 21862, LMG 21863, LMG 21864) belonging to FAA cluster E, with almost identical rep-PCR-profiles could be delineated (Fig. 5.4), of which 16S rRNA gene sequence analysis revealed that they belong to the genus *Alteromonas* within the γ-subclass of the *Proteobacteria*.

The almost complete 16S rRNA gene sequence (1482 nucleotides) of strain LMG 21861^T was determined and has accession number AJ295715. Partial 16S rRNA gene sequences (735-766 nucleotides long) of strains LMG 21856, LMG 21859, LMG 21860, LMG 21862, LMG 21863 and LMG 21864 were also obtained

Table 5.1. Strains investigated in this study and their isolation source.

Strain	Isolation source
***Alteromonas stellipolaris* sp. nov.**	
LMG 21 861^T = DSM 15691^T = ANT 69a^T	Seawater, 25 m: 66°20.0'S; 08°53.4'E
LMG 21 856 = DSM 15672 = ANT 52	Seawater, 25 m: 66°21.9'S; 33°46.7'E
LMG 21 859 = ANT 60b	Seawater, 25 m: 67°03.9'S; 37°27.6'E
LMG 21 860 = ANT 62a	Seawater, 25 m: 66°55.1'S; 34°18.2'E
LMG 21 862 = ANT 73	Seawater, 25 m: 65°01.6'S; 09°11.2'E
LMG 21 863 = ANT 81a	Seawater, 25 m: 65°49.3'S; 14°08.5'E
LMG 21 864 = ANT 82a	Seawater, 25 m: 65°44.7'S; 13°39.6'E
Alteromonas macleodii* subsp. *macleodii	
IAM 12920^T = LMG 2843^T	Seawater
Alteromonas marina	
JCM 11804^T = LMG 22057^T	Seawater, East Sea, Korea

Abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Figure 5.4. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of seven strains from fatty acid cluster E. Dendrogram derived from UPGMA clustering of the profiles with the Pearson correlation coefficient.

and have accession numbers AJ564723, AJ564724, AJ564725, AJ564726, AJ564727, AJ564728 respectively. A phylogenetic tree is shown in Fig. 5.5. Dendrograms obtained by maximum parsimony and maximum likelihood analyses showed essentially the same topography (data not shown).

The 16S rRNA gene sequence of strain LMG 21861^T showed 98.3 % similarity to *Alteromonas macleodii*, 98.0% to *Alteromonas marina* and 97.9 % to *'Alteromonas infernus'*, whereas the partial sequences of the Antarctic strains (LMG 21856, LMG 21859, LMG 21860, LMG 21862, LMG 21863 and LMG 21864) were almost identical to each other and to the according sequence of strain LMG 21861^T (99.5% - 99.8%). The phylogenetic tree in Fig. 5.5 illustrates that the new Antarctic isolates form a distinct branch within the genus *Alteromonas*, supported by high bootstrap values.

Genomic relatedness between strains LMG 21861^T, LMG 21863 and most closely related strains LMG 2843^T (*Alteromonas macleodii*) and LMG 22057^T (*Alteromonas marina*), was determined by DNA-DNA hybridization. The DNA hybridization level between both strains LMG 21861^T and LMG 21863, and *Alteromonas macleodii* (LMG 2843^T) and *Alteromonas marina* (LMG 22057^T), was very low (12.4-15.6%, respectively). The DNA-DNA binding value between LMG 21861^T and LMG 21863 was high, namely 93.9%, indicating that the strains they represent belong to a single species. Indeed, Versalovic *et al.* (1994) have shown that strains with the same rep-PCR profile are always closely related and this has been confirmed by several authors (e.g. Rademaker & De Bruijn, 1997). Differences between reciprocal experiments were less than 12 %. From these hybridization

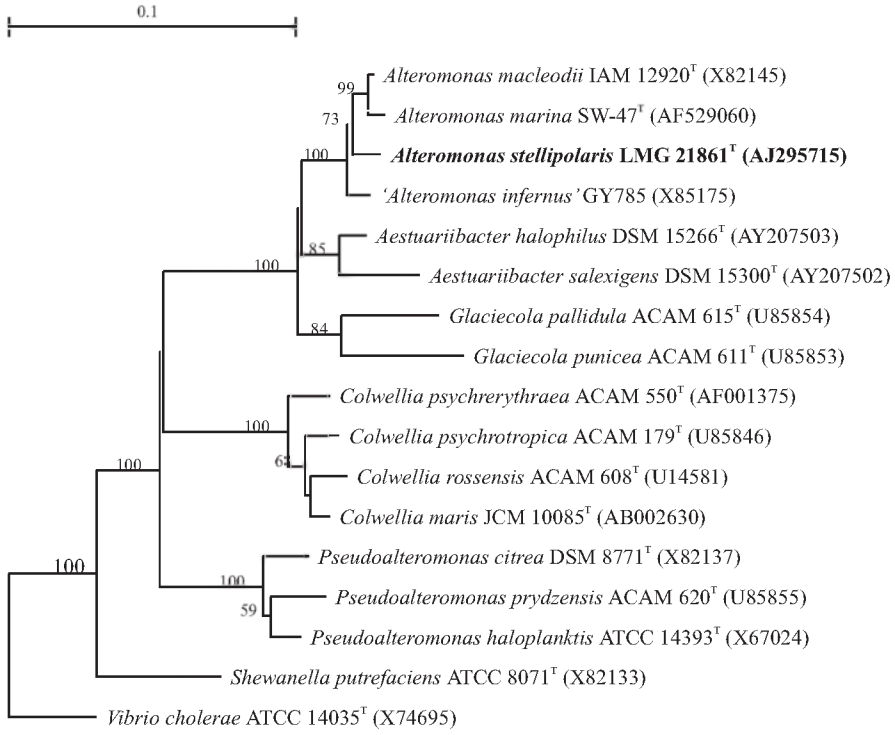

Figure 5.5. Neighbour-joining dendrogram showing the estimated phylogenetic relationship of *Alteromonas stellipolaris* sp. nov. and related marine chemoheterotrophs of the γ-subclass of the *Proteobacteria*. Bootstrap values (percentages of 500 replicates) of > 50 % are shown. The GenBank accession number for each reference strain is shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

results it can be concluded that the seven Antarctic isolates are genotypically distinct from *Alteromonas macleodii* and *A. marina*, their phylogenetically nearest neighbours and thus constitute a new species within the genus *Alteromonas* (Wayne *et al.*, 1987).

DNA G+C contents of strains LMG 21861^T, LMG 21856, LMG 21859, LMG 21860, LMG 21862, LMG 21863 and LMG 21864 are 43.3%, 44.0%, 44.8%, 44.7%, 44.7%, 43.3% and 44.7%, respectively. These values are consistent with the DNA G+C content of the genus *Alteromonas*, which ranges between 44 and 46 mol% (Baumann *et al.*, 1972; Yoon *et al.*, 2003).

Cellular fatty acid patterns of the Antarctic strains are based on the data generated by Mergaert *et al.* (2001b) or were determined as described by the same authors. The Antarctic strains showed very similar fatty acid patterns and the major constituents include $C_{16:0}$ (12.6 ± 1.3), $C_{17:1}\omega8c$ (9.4 ± 2.3), $C_{18:1}\omega7c$

(18.0 ± 2.1) and summed feature 3 (27.3 ± 3.0) which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both. Hydroxylated fatty acids and alcohol derivatives of fatty acids $C_{16:0}$ and $C_{16:1}\omega7c$ were also present as minor components or at trace levels. The fatty acid profiles of the Antarctic strains clearly resemble those determined for other marine genera of the γ-subclass of the *Proteobacteria*, such as *Alteromonas*, *Pseudoalteromonas* and *Glaciecola* (Ivanova *et al.*, 2000; Mikhailov *et al.*, 2002).

The polar strains are Gram-negative, rod-shaped, small cells (0.4µm in width and 2-7 µm in length), possessing a single polar flagellum (Fig. 5.6). Prosthecae are formed peritrichously, and can be branched. Buds can be produced on mother and daughter cells, but also at the end of the prosthecae when grown on Peptone-Yeast extract-Glucose agar (PYG according to Tan & Rüger, 1999) at 12°C for 7 days (Fig. 5.6 and 5.7). Strain LMG 21856 releases a brown, diffusible pigment in the medium. This property is shared by several reclassified *Alteromonas* species (Gauthier & Breittmayer, 1992) and this brown-black pigment on solid media is often characterized as melanin, a high molecular weight amorphous polymer of indole quinone. The first biosynthesis step involves the hydroxylation of L-tyrosine to form L-3, 4-dihydroxyphenylalanine (L-dopa), which is used in the treatment of Parkinson´s disease. Attempts have therefore been made to adapt melanin-producing microorganisms for the commercial production of L-dopa.

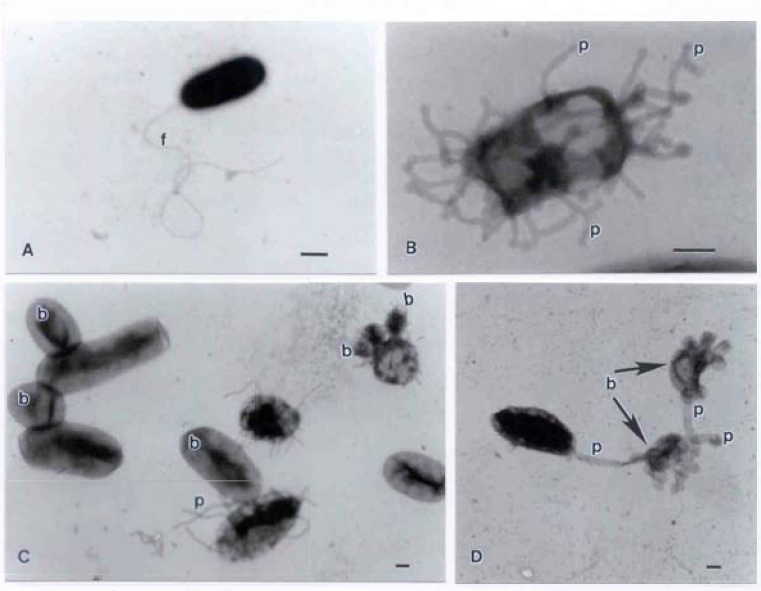

Figure 5.6. Electron micrographs of negatively stained preparations of strains LMG 21859 (A), LMG 21863 (B), LMG 21856 (C) and LMG 21861^T (D) cells, showing a polar flagellum (f), prosthecae (p), and buds (b). Colonies used for analysis were grown on PYG agar at 12°C for 7 d. Cells were stained with 1% uranyl acetate in 0.4% sucrose. Bars, 300 nm.

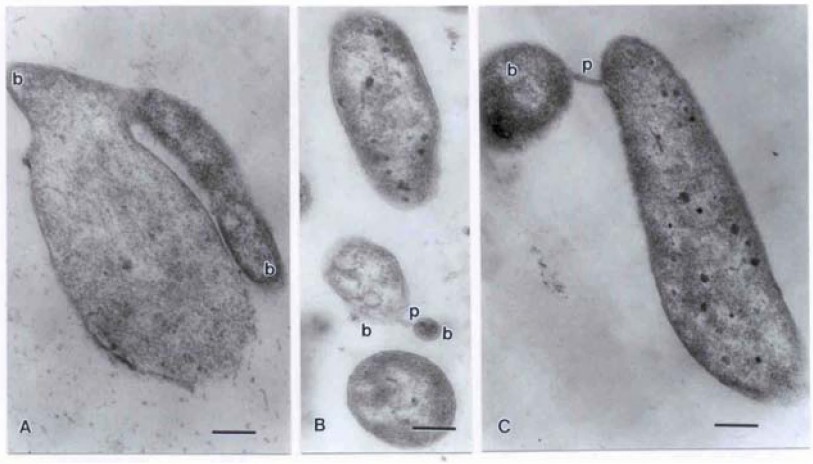

Figure 5.7. Electron micrographs of thin section preparations of strains LMG 21861^T (A + C) and LMG 21860 (B) cells, showing bud (b) formations not only on the cell surface but also at the end of the prostheca (p). Colonies used for analysis were grown on PYG agar at 12°C for 7 d. Thin section preparations were stained with lead citrate and 1% uranyl acetate. Bars, 300 nm.

For most of the phenotypic characteristics, all the strains are identical (see description), and these properties are typical for species of the genus *Alteromonas* and *Pseudoalteromonas* of the γ-subclass of the *Proteobacteria* (Baumann *et al.*, 1972).

The Antarctic strains can be differentiated from their nearest phylogenetic neighbours, *Alteromonas macleodii* and *Alteromonas marina* by several phenotypic characteristics (Table 5.2). On the basis of this polyphasic taxonomic study the Antarctic strains can be assigned to a new species for which the name *Alteromonas stellipolaris* sp. nov. is proposed. Our results also require the emendation of the genus *Alteromonas* with regard to the cell morphology.

Emended description of the genus *Alteromonas* (Gauthier *et al.*, 1995), emend. Van Trappen *et al.*

The description is as described by Gauthier *et al.* (1995) with the following additional morphological features. When grown on marine or PYG agar at low temperatures (12-20°C) for three days or more, cells of *Alteromonas macleodii* (LMG 2843^T), *Alteromonas marina* (LMG 22057^T) and *Alteromonas stellipolaris* (LMG 21861^T, LMG 21856, LMG 21859, LMG 21860, LMG 21863) produce buds and prosthecae (see Fig. 5.6 -5.7 -5.8). Cells of *Alteromonas macleodii* and *A.*

Table 5.2. Differential phenotypic characteristics of *Alteromonas* species.

Characteristic	*A. stellipolaris*	*A. marina*	*A. macleodii**
Branching of prosthecae	+	-	-
Utilization of:			
D-Mannitol	+	-	v (-§)
Acid production from:			
D-Mannitol	w	-	+
Enzyme activity (API ZYM)			
Valine arylamidase	+	-	+
Growth at 4°C	+	+	-
Growth at 40°C	-	+	v (-§)
Mean G+C content (mol%)	43-45	44-45	45-46

Symbols: +, positive; -, negative; w, weakly positive; v, variable. All the strains are straight and rod-shaped cells with polar flagella. * Data from Baumann *et al.* (1972). § Data are for the type strain (Yoon *et al.*, 2003, and this study). Tests positive for all strains: motility, oxidase, catalase, hydrolysis of Tween 80, acid production from sucrose and utilization of D-galactose, D-fructose, sucrose, maltose and acetate. Tests negative for all strains: Gram stain, spore formation, growth at 45°C, acid production from L-arabinose and L-rhamnose and utilization of D-sorbitol, succinate, citrate and L-malate.

marina only form short and straight prosthecae; branching was not observed (Fig. 5.8).

Description of *Alteromonas stellipolaris* sp. nov.

Alteromonas stellipolaris (stel.li.po.la.ris. L. fem. n. *stella* star; L. adj. *polaris* polar; M.L. gen. n. *stellipolaris*, referring to the POLARSTERN (AWI, Bremerhaven), the name of the vessel used to collect the samples from which the organisms were isolated).

Cells are Gram-negative, short rods (0.4 x 2-7 µm), having a single polar flagellum. Prosthecae are produced peritrichously, and can be branched. Buds can be formed on mother and daughter cells, also at the end of the prostheca. They form creamy-white, circular, flat to low convex, shiny, opaque and slimy colonies that are slightly adherent to agar, with entire margins and a diameter of 2-5 mm on marine agar plates after 3 days incubation at 20 °C. Growth occurs on

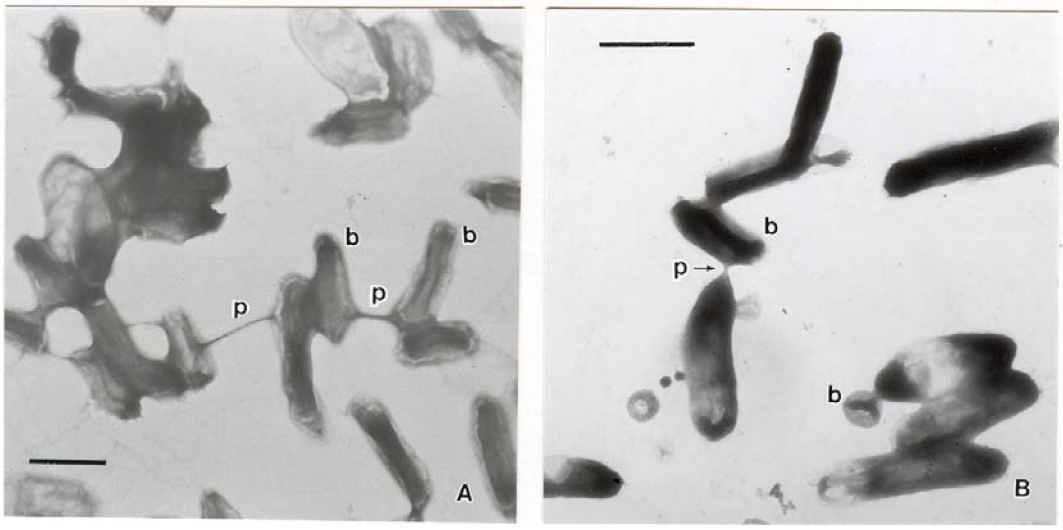

Figure 5.8. Electron micrographs of negatively stained preparations of *Alteromonas macleodii* (A) and *Alteromonas marina* (B) cells, showing prosthecae (p) and buds (b). Colonies used for analysis were grown on marine agar at 20°C for 12 d, or on PYG agar at 20°C for 3 d. Cells were stained with 1% uranyl acetate in 0.4% sucrose. Bars, 1000 nm.

marine agar and PYG agar, and a slight growth on nutrient agar; there is no growth on TSA and R2A agar. Strain LMG 21856 releases a brown, diffusible pigment in the medium. The range of growth temperature is 5- 37°C, while no growth occurs at 40°C or higher temperatures. Growth is supported on R2A agar with up to 10% NaCl. These results indicate that they are moderately halophilic and psychrotrophic. The strains can grow between pH 6 and 9, while the optimum pH is 7-8.5. There is no evidence for growth under anaerobic conditions and the catalase and cytochrome oxidase tests are positive. No polyhydroxybutyrate is accumulated and spores are not formed. Precipitation on egg-yolk agar is positive for some strains (LMG 21861^T, LMG 21856, LMG 21860, LMG 21862, LMG 21863). Strains are negative for indole and acetoine production, Voges-Proskauer test, citrate utilization, hydrolysis of urea, nitrate reduction and production of hydrogen sulfide. Degradation of starch, aesculin, gelatin and DNA is positive for all strains, and β-galactosidase activity is detected. All the strains are able to utilize Tween 40, Tween 80, D-fructose, D-galactose, gentiobiose, α-D-glucose, maltose, D-mannitol, D-melibiose, D-trehalose, furanose, acetic acid, propionic acid, alaninamide, L-alanyl-glycine, L-glutamic acid and glycyl-L-glutamic acid; all the strains except strain LMG 21862 are able to utilize dextrin, α-D-lactose, lactulose, D-raffinose, sucrose, D-galacturonic acid and β-hydroxybutyric acid; all the strains except strain LMG 21864 are able to utilize glycogen; all the strains except LMG

21863 are able to utilize cellobiose; all the strains except strain LMG 21860 are able to utilize D-mannose; all the strains except strain LMG 21859 are able to utilize D-psicose. Variable results are obtained for α-cyclodextrin, β-methyl-D-glucose, D-gluconic acid, α-keto-butyric acid, succinic acid, L-alanine, L-leucine, L-proline, L-serine, L-threonine, inosine, uridine and glycerol. No metabolic activity is observed on adonitol, L-arabinose, D-arabitol, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, iso-erythritol, L-fucose, meso-inositol, L-rhamnose, D-sorbitol, xylitol, methylpyruvate, mono-methyl-succinate, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-keto-glutaric acid, α-keto-valeric acid, D,L-lactic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-phenylalanine, L-pyro-glutamic acid, D-serine, D,L-carnitine, γ-amino-butyric acid, urocanic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D,L-α-glycerolphosphate, glucose-1-phosphate, and glucose-6-phosphate. For all strains, acids are produced for amygdaline in a clear positive reaction, whereas an intermediate-positive reaction is detected for mannitol, sucrose and melibiose. No acids are produced from glucose, inositol, sorbitol, rhamnose and arabinose, and the degradation tests of alginate and chitin are negative. There is no activity for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, cystine arylamidase, α-chymotrypsine, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. For all strains, low activity (score 1) or no activity is obtained for lipase (C14), medium activity (score 2 or 3) is observed for esterase (C4), esterase lipase (C8), valine arylamidase, trypsine, α-galactosidase, and high activity (score 4 or 5) is observed for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase. Cells contain fatty acids $C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:1}\omega8c$ and $C_{18:1}\omega8c$ as the main constituents. DNA G+C content is 43-45 %. The type strain is LMG 21861^T (= DSM 15691^T).

5.3 *Glaciecola polaris* sp. nov., novel budding and prosthecate bacteria from the Arctic Ocean, and emended description of the genus *Glaciecola*

Stefanie Van Trappen, Tjhing-Lok Tan, Jifang Yang, Joris Mergaert and Jean Swings

Redrafted from: *International Journal of Systematic and Evolutionary Microbiology*, in press (DOI10.1099/ijs.0.63123-0)

Four strains of cold-adapted, strictly aerobic and facultative oligotrophic bacteria were isolated from polar seas and investigated using a polyphasic taxonomic approach. Two strains (LMG 21857^T and LMG 21854) derive from Arctic seawater whereas the other two strains (LMG 21855 and LMG 21858) were isolated from Antarctic seawater. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains belong to the γ-subclass of the *Proteobacteria* and are related to the genus *Glaciecola*, with 98.0-99.7% sequence similarity to *Glaciecola mesophila* and 94.2-95.3% to *Glaciecola pallidula*, their nearest phylogenetic neighbours. Two strains (LMG 21855 and LMG 21858) are identified as *Glaciecola mesophila*, whereas DNA-DNA hybridization results and differences in phenotypic characteristics show that the other two strains (LMG 21857^T and LMG 21854) constitute a new species within the genus *Glaciecola*, with a DNA G+C content of 44.0 mol%. The isolates are Gram-negative, chemoheterotrophic, motile, rod-shaped cells which are psychrotrophic and moderately halophilic. Buds can be produced on mother cells and on prosthecae. Branch formation of prosthecae occurs. Whole-cell fatty acid profiles of the isolates are very similar and include $C_{16:0}$ and $C_{16:1}\omega7c$ as the major fatty acid components. On the basis of genotypic and phenotypic properties, a novel species of the genus *Glaciecola* is described as *Glaciecola polaris* sp. nov. with the isolate LMG 21857^T (CIP 108324^T = ARK 150^T) as type strain. An emended description of the genus Glaciecola is given.

Introduction

The genus *Glaciecola* was proposed by Bowman *et al.* (1998c) for two groups of psychrophilic bacteria isolated from sea-ice diatom assemblages from the coastal areas of eastern Antarctica and forms a separate lineage within the γ-subclass of the *Proteobacteria*, distantly related to *Alteromonas macleodii*. Recently, another species of the genus *Glaciecola* has been described, *Glaciecola mesophila* isolated from marine invertebrate specimens (Romanenko *et al.*, 2003). Many genera of this class of *Proteobacteria* (*Alteromonas, Pseudoalteromonas, Glaciecola, Idiomarina* and *Colwellia*) are common inhabitants of the marine part of the biosphere and have very diverse habitats like coastal and open water areas, deep-sea and hydrothermal vents, marine sediments and sea-ice (Mikhailov *et al.*, 2002).

In another study, we reported that seven Antarctic strains belong to a novel species within the genus *Alteromonas*, i.e. *A. stellipolaris* (Van Trappen *et al.*, in press). Together with the new *Glaciecola* species described here, they all belong to novel budding and prosthecate bacteria from the γ-subclass of the *Proteobacteria*. It is evident now that budding and prosthecate bacteria are abundant in marine and polar environments (Weiner *et al.*, 2000; Labrenz *et al.*, 1998; Labrenz *et al.*, 1999). Moreover, bud and prosthecate formations are a common strategy for rod-shaped bacteria to enhance their surface to volume ratio, thus enabling the organisms for efficient substrate uptakes in oligotrophic habitats (van Gemerden & Kuenen, 1984).

During expeditions in the Arctic (Tan & Rüger, 1991) and Antarctic seas (Tan & Rüger, 1999), facultative oligotrophic and psychrotrophic bacteria were isolated. These strains (173) have been previously analysed by their substrate utilization patterns using the Biolog system (Tan, 1997; Tan & Rüger, 1999) and by fatty acid and 16S rDNA sequence analyses of representatives (Mergaert *et al.*, 2001b). They belong to six metabolic groups and eight different fatty acid clusters containing two to 59 strains. In the meantime, additional strains (56) were isolated using the same methods and were also analysed using the Biolog system and fatty acid analysis. The new strains belong to fatty acid clusters B, C, D, E and F (as delineated in Mergaert *et al.*, 2001b) and three new fatty acid clusters (I, J and K; S. Van Trappen, unpublished results) were found. The genomic diversity of the 19 strains from fatty acid clusters E and F and two related, unclustered strains, was

further investigated (see also Van Trappen *et al.*, in press). Using a polyphasic taxonomic approach, four strains (two Arctic and two Antarctic) could be assigned to the genus *Glaciecola*.

Materials and Methods

Strains were isolated from seawater after enrichment in dialysis chambers as previously described (Tan, 1986; Tan, 1997). The investigated strains are LMG 21857^T = CIP 108324^T = ARK 150^T and LMG 21854 = ARK 149 isolated from Arctic seawater and LMG 21855 = ANT 12a and LMG 21858 = ANT 12b from Antarctic seawater. The reference strains LMG 21426^T *Glaciecola punicea*, LMG 21427^T *Glaciecola pallidula* and LMG 22017^T *Glaciecola mesophila* were included in some experiments. Strains were routinely cultivated on marine agar 2216 (Difco) at 20°C for 48 h, or for strains LMG 21426^T and LMG 21427^T on marine agar at 10°C for 6 days, and for strain LMG 22017^T on marine agar at 28°C for 24 h, except when mentioned otherwise.

Strains were arranged in similarity groups based upon the results of repetitive extragenic palindromic DNA-PCR fingerprinting using the GTG_5 primer (Versalovic *et al.*, 1991; Rademaker & de Bruijn, 1997; Rademaker *et al.*, 2000). Numerical analysis was carried out using the Bionumerics software package, as described by the same authors.

Small scale DNA extracts were prepared using the method of Pitcher *et al.* (1989) and almost complete 16S rRNA gene sequences of strains were amplified by PCR using conserved primers (Coenye *et al.*, 1999). PCR products were purified using a QIAquick PCR Purification kit (Qiagen) according to the instructions of the manufacturer. Sequence analysis was performed as described earlier (Van Trappen *et al.*, 2004a). Evolutionary distances were calculated using the algorithm of Jukes-Cantor and a phylogenetic tree was constructed using the neighbour-joining method with the TREECON program (Van de Peer & De Wachter, 1994).

DNA was prepared according to the method of Pitcher *et al.* (1989) and DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 BioAssay reader (PerkinElmer) for the fluorescence measurements. The hybridization temperature was 35 °C and reciprocal experiments were performed for every pair of strains.

DNA G+C contents of the Arctic and Antarctic strains were determined using

an HPLC method as described by Van Trappen *et al.* (2003).

The growth of the strains at different temperatures (5-37 °C) was tested on marine agar, whereas salt tolerance was tested on R2A agar, supplemented with 1-20 % NaCl at 20 °C. Biochemical characteristics were determined using standard protocols (Smibert & Krieg, 1994; West & Colwell, 1984; Reichenbach & Dworkin, 1981; Bowman *et al.*, 1998c; Van Trappen *et al.*, 2003) and API kits (API 20E, API 20NE, API ZYM and API32 ID, bioMérieux). Bacterial suspensions were made in sterile, chilled seawater and marine agar was used as the basal medium. For BIOLOG GN2 microplates, the bacteria were grown on PYG agar at 20°C for 5 d; the cells were harvested and suspended in "Inoculating Fluid" (IF). The salinity of the IF was adjusted to 26 parts per thousand with NaCl. The microplates were incubated at 20°C and substrate utilizations were measured after 3-28 d at 590 nm with an eight-canal-photometer (Spectra 2, SLT Labinstruments).

Results and discussion

Strains of fatty acid clusters E and F and two related, unclustered strains were arranged in similarity groups based upon the results of rep-PCR fingerprinting using the GTG_5 primer. One Arctic (LMG 21857^T, LMG 21854) and one Antarctic (LMG 21855, LMG 21858) cluster of each two strains, belonging to FAA cluster F, with almost identical rep-PCR-profiles could be delineated (Fig. 5.9), of which 16S rRNA gene sequence analysis revealed that they belong to the genus *Glaciecola* within the γ-subclass of the *Proteobacteria*.

Almost complete 16S rRNA gene sequences (1485 nucleotides) of strains LMG 21857^T, LMG 21854, LMG 21855 and LMG 21858 were obtained and a phylogenetic tree is shown in Fig. 5.10. Dendrograms obtained by maximum parsimony and maximum likelihood analyses showed essentially the same topography (data not shown).

The 16S rRNA gene sequences of the two Arctic strains (LMG 21857^T and LMG 21854) are identical (100 % sequence similarity) and showed 98.0 % similarity to *G. mesophila*, 94.2 % to *G. punicea* and 93.5 % to *G. pallidula*, whereas the sequences of the Antarctic strains (LMG 21855 and LMG 21858), which are also identical to each other, showed 99.7% sequence similarity to *G. mesophila*, 95.3 % to *G. punicea* and 94.9 % to *G. pallidula*. The sequence similarity between the Arctic and Antarctic strains is 98.4 %. The phylogenetic tree in Fig. 5.10 illustrates

Figure 5.9. Digitized representation of normalized rep-PCR profiles (GTG_5-primer) of four strains from fatty acid cluster F and dendrogram derived from the UPGMA clustering of the profiles with the Pearson correlation coefficient.

the phylogenetic relationships of the polar isolates within the genus *Glaciecola*. Strains LMG 21855 and LMG 21858 are very closely related to *G. mesophila* whilst strains LMG 21857^T and LMG 21854 form a distinct branch supported by a high bootstrap value.

The genomic relatedness between the strains LMG 21857^T, LMG 21855 and the most closely related strains *G. mesophila* LMG 22017^T and *G. punicea* LMG 21426^T, was determined by DNA-DNA hybridizations. The hybridization level between strain LMG 21857^T and *G. mesophila* LMG 22017^T and *G. punicea* LMG 21426^T was 17.2 % and 4.0 % respectively, whereas the DNA-DNA binding value between LMG 21857^T and LMG 21855 was 23.4%. The hybridization level between strain LMG 21855 and *G. mesophila* LMG 22017^T and *G. punicea* LMG 21426^T was 67.7 % and 6.0 % respectively. Differences between reciprocal experiments were less than 10 %. DNA-DNA hybridizations between strains of the same rep-PCR-cluster were not performed since Versalovic *et al.* (1994) have shown that strains with the same rep-PCR profile are always closely related and this has been confirmed by several authors (e.g. Rademaker & De Bruijn, 1997). These results suggest that the two Arctic isolates are genotypically distinct from *G. mesophila* and *G. punicea*, their phylogenetically nearest neighbours and constitute a new species within the genus *Glaciecola*. The two Antarctic isolates are closely related to *G. mesophila*, showing a DNA-DNA reassociation value near 70 %, which is generally accepted as the borderline for species delineation (Wayne *et al.*, 1987).

DNA G+C contents of strains LMG 21857^T, LMG 21854, LMG 21855 and LMG 21858 are 44.2%, 43.6%, 43.9% and 44.2%, respectively. These values are

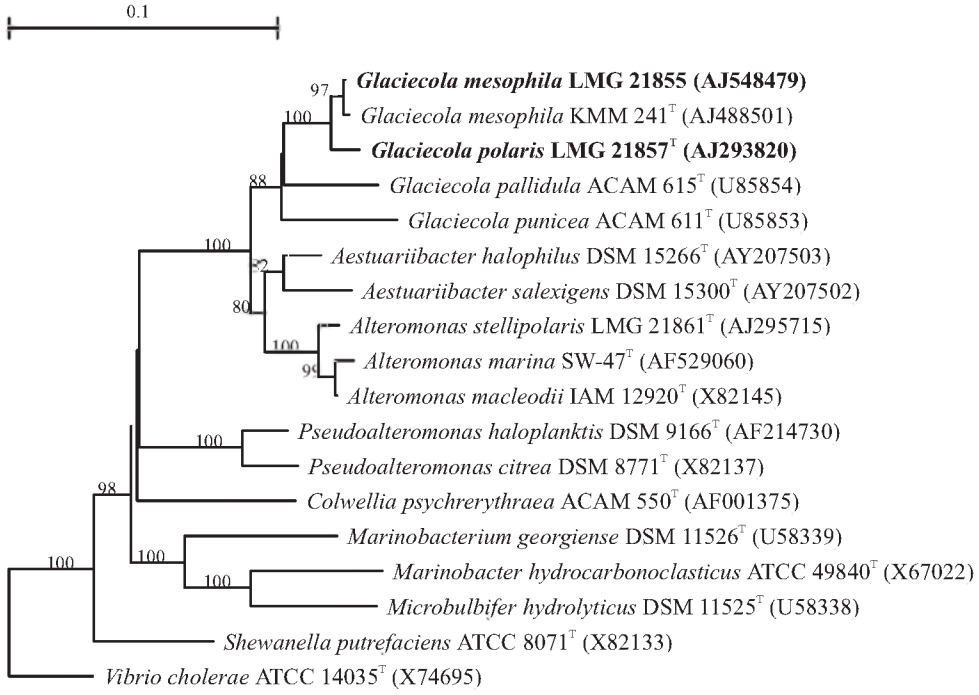

Figure 5.10. Neighbour-joining dendrogram showing the estimated phylogenetic relationships of the Arctic and Antarctic isolates, and other marine chemoheterotrophs of the γ-subclass of the *Proteobacteria*. Bootstrap values (percentages of 500 replicates) of > 70 % are shown. The GenBank accession number for each reference strain is shown in parentheses. Bar, 1 nucleotide substitution per 10 nucleotides.

consistent with the G+C content of the genus *Glaciecola*, which ranges between 40-46 mol% (Bowman *et al.*, 1998c).

Cellular fatty acid patterns of the polar strains are based on the data generated by Mergaert *et al.* (2001b) or were determined as described by the same authors. The Arctic strains show very similar fatty acid profiles and the mean composition is 3.1% $C_{12:0}$, 5.5 % $C_{12:0}$3-OH, 4.1% $C_{14:0}$, 2.0% $C_{15:0}$, 23.3% $C_{16:0}$, 1.7 % $C_{16:0}$2-OH, 2.0 % $C_{16:1}$2-OH, 2.6 % $C_{17:1}\omega8c$, 4.8 % $C_{18:1}\omega7c$, 1.4 % 10 Me-$C_{18:0}$ and 41.7 % summed feature 3 which comprises iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both. The Antarctic strains show very similar fatty acid patterns to strains KMM 241^T and KMM 642 (*G. mesophila*), with $C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:1}\omega8c$ and $C_{18:1}\omega7c$ as the dominant fatty acids. Hydroxylated fatty acids and iso-branched fatty acids are also present as minor components or at trace levels in the Arctic strains. The fatty acid profiles of the polar strains clearly resemble those determined for other marine genera of the γ-subclass of the *Proteobacteria* like *Alteromonas*, *Pseudoalteromonas* and *Glaciecola* (Ivanova *et al.*, 2000).

The Antarctic strains are Gram-negative, rod-shaped cells (0.4 µm in width and 2-3 µm in length), which are flagellated. Buds and prosthecae can be produced (see Fig.5.11). The strains are able to grow between 5-30 °C, whereas no growth occurs at 37 °C; growth is supported on R2A agar with up to 10 % NaCl. Strains possess a mucoid consistency and show very similar reactions to strains KMM 241^T and KMM 642 of *G. mesophila* and reduce nitrate. They differ from these strains in the degradation of agar, the utilization of D-fructose, D-trehalose, L-glutamate and L-proline, and the Antarctic strains can grow at 4 °C and in 10 % NaCl (see Table 5.3). The Antarctic strains LMG 21855 and LMG 21858 are identified as *G. mesophila* since there are only a few phenotypic differences and these could be due to the different protocols used, and DNA-DNA hybridization results together with the 16S rRNA gene sequence similarities also support that the Antarctic isolates are very closely related to *G. mesophila*.

The Arctic strains are Gram-negative, rod-shaped cells (0.4 µm in width and 2-3 µm in length), which are polarly or subpolarly flagellated. Buds can be produced on mother cells or on prosthecae (see Fig.5.12). Prostheca formation is peritrich; prostheca can be branched. The strains are able to grow between 5-30 °C, whereas no growth occurs at 37 °C; growth is supported on R2A agar with up to 10 % NaCl, indicating that they are moderately halophilic and psychrotropic. This is in contrast to *G. punicea* and *G. pallidula* which are psychrophilic and have an absolute requirement for seawater (Bowman *et al.*, 1998c), and *G. mesophila* which is slightly halophilic and mesophilic (Romanenko *et al.*, 2003). The strains are aerobic, chemoheterotrophic bacteria and there is no evidence for growth under anaerobic conditions. Only strain LMG 21854 possesses a mucoid consistency. The Arctic strains are positive for precipitation on egg-yolk agar and show the typical properties of the genus *Glaciecola* (see species description).

On the basis of this polyphasic taxonomic analysis, the Arctic strains can be clearly differentiated from the other species within the genus *Glaciecola* (see Table 5.3) and can be assigned to a new species for which the name *Glaciecola polaris* sp. nov. is proposed.

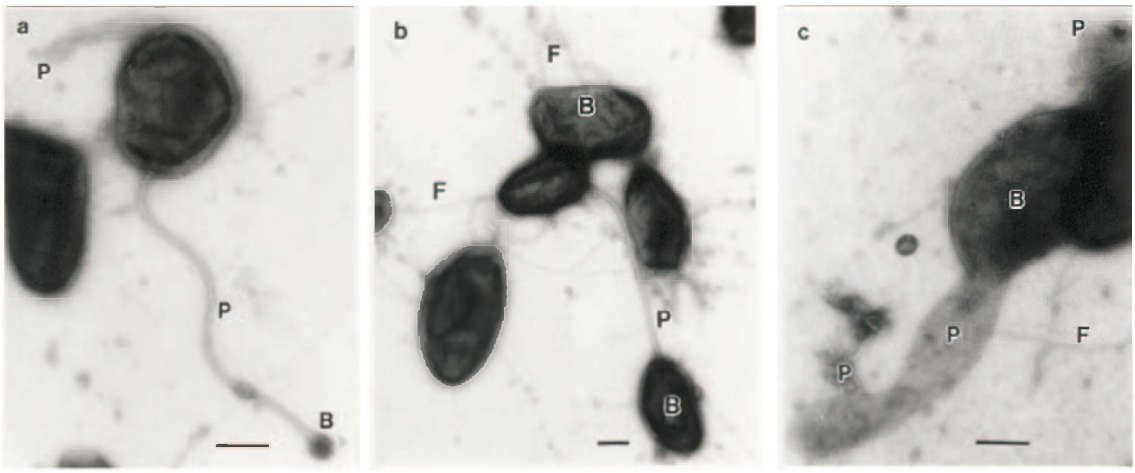

Figure 5.11. Electron micrographs of negatively stained preparations of strain LMG 21857^T (a-c) cells, showing flagella (F), prosthecae (P) and buds (B). Colonies used for analysis were grown on PYG-agar at 12°C for 7 d. Cells were stained with 1% uranyl acetate in 0.4% sucrose. Bars, 300 nm.

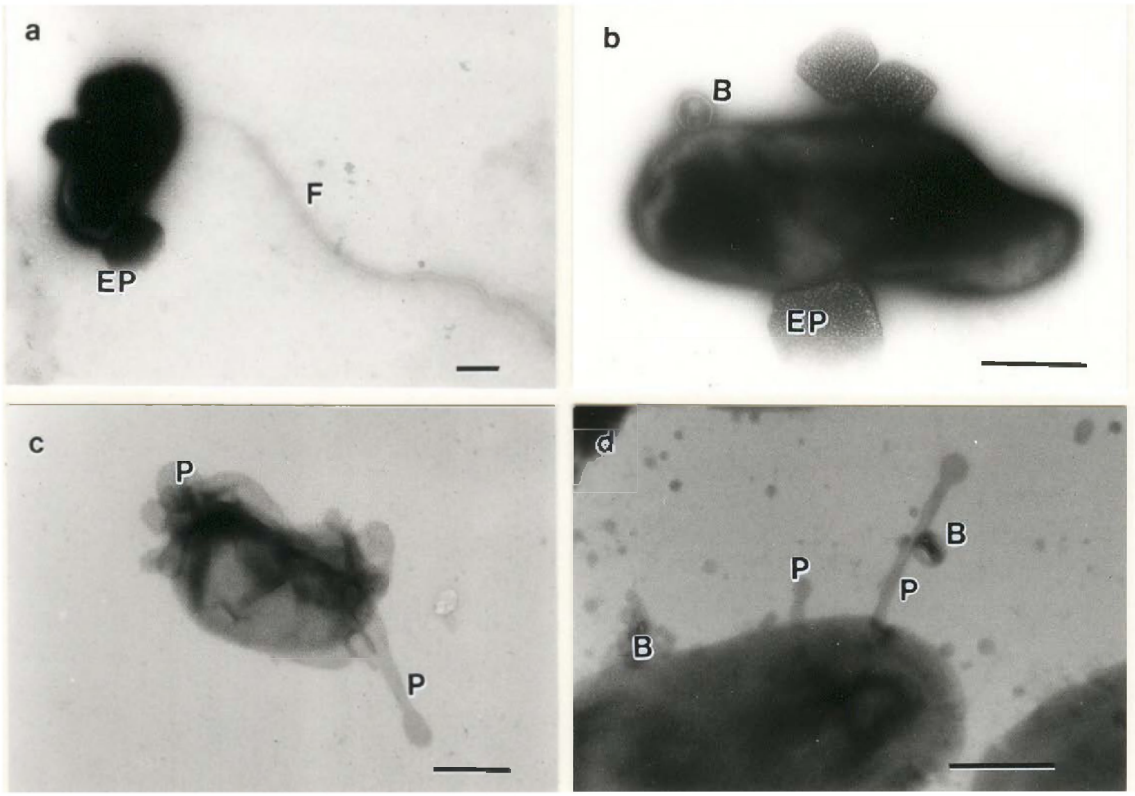

Figure 5.12. Electron micrographs of negatively stained preparations of strains LMG 21855 (a-c) and LMG 21858 (d) cells, showing flagella (F), prosthecae (P), buds (B) and extracellular products (EP). Methods, see legend to Fig. 5.11.

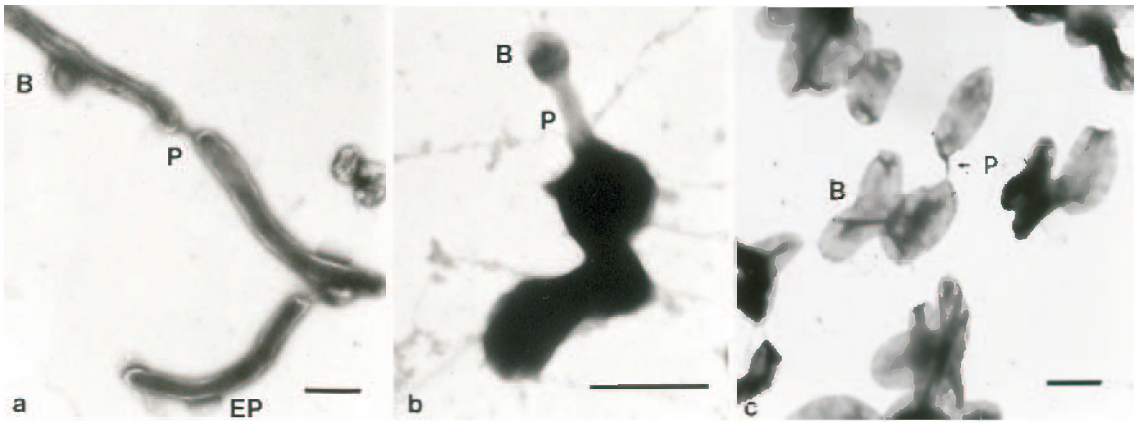

Figure 5.13. Electron micrographs of negatively stained preparations of strains *Glaciecola punicea* LMG 21426^T (a), *Glaciecola pallidula* LMG 21427^T (b) and *Glaciecola mesophila* LMG 22017^T (c) cells showing prosthecae (P), buds (B), and extracellular products (EP). Colonies used for analysis were grown on PYG agar at 20 °C for 3 d (*G. mesophila*), or on Marine Agar at 12 °C for 21 d (*G. punicea*) and 12 d (*G. pallidula*), respectively. Cells were stained with 1% uranyl acetate in 0.4% sucrose. Bars, 1000 nm.

Emended description of the genus *Glaciecola* (Bowman *et al.*, 1998), emend. Van Trappen *et al.*

The description is as described by Bowman *et al.* (1998c) with the following additional morphological features. When grown on marine or PYG agar at low temperatures (12-20°C) for three days or more, some strains can produce buds and prosthecae (see Fig. 5.11-5.13).

Description of *Glaciecola polaris* sp. nov.

Glaciecola polaris (po.la.ris. N. L. fem. adj. *polaris* polar; referring to the origin of the strains, the Arctic Ocean).

Cells are Gram-negative, short rods (0.4 x 2-3 µm) and motile by the presence of a polar or subpolar flagellum. Buds can be produced on mother cells or on prosthecae. Prostheca formation is peritrich and prostheca can be branched (Fig.5.11-5.13). They form non-pigmented, circular, low convex, shiny and opaque colonies that are not adherent to agar, with entire margins and a diameter of 1-4

Table 5.3. Phenotypic characteristics that differentiate *Glaciecola polaris* sp. nov. from its nearest phylogenetic neighbours.

Characteristic	**1**	**2**	**3**	**4**
Pigmentation	-	-	pink-red	pale pink
Growth at 25°C	+	+	+	-
Growth in 10% NaCl	+	v-	-	-
Hydrolysis of:				
Egg yolk	+	-	-	-
Starch	+	+	-	v +
Aesculin	+	+	v +	-
DNA	+	+	-	-
β-Galactosidase	+	+	+	-
Nitrate reduction	-	+	-	-
Utilization of:				
D-Glucose, D-mannitol, cellobiose	+	+	-	-
Sucrose, maltose	+	v+	-	-
D-Galactose	+	v+	-	-
D-Fructose, D-trehalose	+	v+	-	-
D-Mannose	+	v+	-	-
Glycerol	-	-	-	+
Acetate	+	-	-	+
Glycogen, dextrin	+	+	-	+
DL-Lactate	-	-	-	+
Propionate	+	-	-	-
L-Glutamate	+	v+	-	+
L-Malate	-	-	+	-
Mean G+C content (mol%)	44	44	44-46	40

Glaciecola species: 1, *G. polaris*; 2, *G. mesophila*; 3, *G. punicea*; 4, *G. pallidula*. Data from Bowman *et al.* (1998c), Romanenko *et al.* (2003) and this study. Symbols: +, positive test; -, negative test; v +, variable between strains, type strain positive; v -, variable between strains, type strain negative. All strains were positive for the following tests: motility, sodium ion requirement for growth, oxidase, catalase, growth at 7-20°C and growth in 1-6% NaCl. All strains are negative for growth at 37-40°C, indole reaction, arginine dihydrolase, chitin hydrolase and utilization of L-arabinose, citrate, L-histidine, L-ornithine, L-threonine and *N*-acetylglucosamine.

mm on marine agar plates after 7 days incubation at 20 °C. Growth occurs on marine and PYG agar, and a slight growth on nutrient agar; there's no growth on TSA and R2A agar. The range of growth temperature is 5-30°C, whereas no growth occurs at 37°C or higher temperatures; growth is observed on R2A agar with up to 10% NaCl, indicating that they are moderately halophilic and psychrotrophic. There is no evidence for growth under anaerobic conditions and the catalase and cytochrome oxidase tests are positive. The degradation of starch, aesculin and DNA is positive and precipitation on egg-yolk agar occurs. β-Galactosidase activity is detected for both strains. They are able to utilize α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, D-arabitol, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, sucrose, D-trehalose, D-saccharose, furanose, methylpyruvate, acetic acid, β-hydroxybutyric acid, propionic acid, L-alanine, L-alanyl-glycine, L-glutamic acid, glycyl-L-glutamic acid, L-leucine, L-pyro-glutamic acid and salicin. Both strains are negative for indole and acetoine production, Voges-Proskauer test, citrate utilization, nitrate reduction and production of hydrogen sulfide. No growth is observed on arabinose, *N*-acetyl-glucosamine, caprate, adipate, malate, citrate, phenylacetate, L-fucose, D-sorbitol, valerate, histidine, 2-keto-gluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, rhamnose, D-ribose, inositol, itaconate, suberate, malonate, DL-lactate, 5-keto-gluconate, 3-hydroxy-benzoate, L-serine, alaninamide, L-threonine and glycerol. No acids are produced from the carbohydrates glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melobiose, amygdalin, arabinose and the degradation tests of alginate, chitin, casein, gelatin and urea are negative. There is no activity of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophane deaminase. There is no activity for the enzymes lipase (C14), cystine arylamidase, α-chymotrypsine, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. For both strains low activity (score 1) is observed for valine arylamidase, trypsine, α-glucosidase and β-glucosidase, medium activity (score 2 or 3) is observed for esterase (C4), esterase lipase (C8), acid phosphatase, naphtol-AS-BI-phosphohydrolase and α-galactosidase, and high activity (score 4 or 5) is observed for alkaline phophatase and leucine arylamidase. Cells contain fatty acids $C_{16:0}$ and summed feature 3 (iso-$C_{15:0}$2-OH, $C_{16:1}\omega7c$, or both) as main constituents. DNA G+C content is 44.0 %. The type strain is LMG 21857^T (= CIP 108324^T).

PART 3

Concluding remarks

CHAPTER 6

Conclusions & future perspectives

In this chapter the research challenges regarding bacterial diversity in Antarctic lakes and polar seas as stated in the Objectives of this work (see Preface), are discussed again in the light of the obtained results (chapters 2 to 5). Also, the strategy followed and the techniques used in this study, are evaluated. Finally, future perspectives for the bacterial diversity studies in different polar habitats are discussed.

6.1 Polyphasic taxonomy of polar bacteria

6.1.1 Fatty acid analysis

In this work a polyphasic taxonomic approach, combining different genotypic (rep-PCR genomic fingerprinting, 16S rDNA sequence analysis, DNA-DNA hybridization, % G + C determination) and phenotypic methods (fatty acid analysis, study of morphological, biochemical and physiological characteristics) was applied to study bacterial diversity in Antarctic lakes and polar seas. In previous studies (Mergaert *et al.*, 2001b; Van Trappen *et al.*, 2002), fatty acid analysis (FAA) was used to obtain a first grouping of the isolates into different clusters. For the Antarctic lake isolates, 41 clusters were delineated at 75 % Canberra metric similarity and 31 strains formed single branches. For the polar sea strains, eight clusters were found and 8 strains formed separate branches at a Euclidian distance of $\leq$ 14, which is comparable to 80% Canberra metric similarity. Representatives of these clusters were analysed by 16S rDNA sequencing. Additional polar sea isolates (56) were also included in fatty acid analysis and they proved to belong to FAA-clusters B, C, D, E and F (as delineated in Mergaert *et al.*, 2001b) and three new clusters (I, J and K) (see Fig. 5.2). An overall clustering of the fatty acid profiles (see Annex VI) based on Canberra metric similarities of the Antarctic lake and

polar sea isolates, showed that the different FAA-clusters were maintained. Some clusters were further subdivided (e.g. 5, 10, 16, 18, 22, 23, 25, 40, 41, see chapter 2; A, C, E, F, G, see section 5.1) and the new clusters I and K grouped together with FAA clusters 1 to 15 (phylogenetically related to the *Bacteroidetes*), whereas cluster J formed a branch together with FAA clusters 35 and 36, which belong to the α-*Proteobacteria*.

Several of the FAA clusters were found to contain multiple taxa with similar fatty acid profiles, whereas phylogenetically closely related taxa are sometimes quite different in their fatty acid compositions and belong to different FAA clusters. These results indicate that a dendrogram based on fatty acid profiles does not allow a straightforward taxonomic interpretation. Indeed, when a higher similarity level for the cluster delineation in the dendrogram is used, additional clusters and singles show up, which potentially belong to additional phylogenetic lineages. Our fatty acid profiles also didn't allow identification with the TSBA4.0 database, since different growth conditions were used (for example incubation time and temperature, see chapter 2), than the standard conditions. However, despite the restraints of this technique, fatty acid analysis is a convenient method for the rapid screening of a large number of bacteria, belonging to different phylogenetic groups. But techniques with a higher resolution had to be applied to investigate the genomic diversity of each fatty acid cluster in more detail.

6.1.2 Rep-PCR fingerprinting

Several fatty acid clusters were further investigated by rep-PCR genomic fingerprinting (using GTG_5- and REP-primers, depending on the group) to assess their genomic variability. These FAA clusters were mainly chosen on the basis of a low 16S rDNA sequence similarity (< 97%) of representative strains with their nearest phylogenetic neighbours, indicating the novelty of these bacteria. Rep-PCR fingerprinting of the isolates allowed a further sub-clustering at the genotypic level and overall this sub-clustering was consistent with the delineation of fatty acid clusters (see chapter 2 and sections 3.1, 4.1 and 5.1). One exception was found (see 3.3) where strains of fatty acid clusters 5 and 6 belonged to the same rep-cluster and proved to be members of the same species, namely *Flavobacterium degerlachei*. The differences in fatty acid compositions between strains from FAA clusters 5 and 6 are largely due to different amounts of certain fatty acids (see section 3.1). For the three strains of FAA cluster 4, different rep-profiles were

obtained with only few bands (1-3 clear bands) and strain R-8282 clustered together with strains of FAA cluster 15 (see Fig. 3.1). However, DNA-DNA hybridization results confirmed the delineation on the basis of fatty acid composition, since the strains of FAA cluster 4 constitute one species within a novel genus (*Gillisia limnaea*) whereas members of FAA cluster 15 belong to another genus (*Algoriphagus antarcticus*). These results illustrate that only rep-profiles with a sufficient number of clear bands spread over the low and high molecular weight areas of the rep-profile, are reliable. Therefore, the most dominant rep-clusters with at least three strains and clear profiles with a sufficient number of different bands were investigated further.

Several DNA-based typing methods exist (e.g. RFLP, AFLP, ARDRA and RAPD) but the major advantage of rep-PCR, besides its high reproducibility and taxonomic resolution, is that it is a very rapid method (Versalovic *et al.*, 1994; Rademaker & de Bruijn, 1997), ideal to investigate a large number of strains. Rep-PCR fingerprinting allows phylogenetically closely related strains to be further sub-divided into different groups and no prior genotypic knowledge on the bacterial strains is required. Several rep-primers can be used (REP, GTG_5, ERIC, BOX) depending on the bacterial group investigated. REP-primers for example, yielded no clear profiles with a sufficient number of bands for the strains belonging to the *Bacteroidetes*, in contrast to the GTG_5-primer. Rep-profiles obtained by using different primers can also be combined, resulting in more information about the reliability of clusters delineated with a certain primer (see section 4.1).

6.1.3 16S rDNA sequence analysis

Representative strains were analysed by using additional 16S rDNA sequencing to allow a phylogenetic allocation of the different rep-clusters. The complete 16S rDNA sequence of 35 representative strains belonging to FAA clusters 1 to 15 (see Table 3.1) was determined and of these, ten showed a sequence similarity of less than 97% with their nearest phylogenetic neighbours. For FAA cluster 41, seven strains were sequenced and similarities below 97% were found with *Mesorhizobium loti* and members of the *Rhodobacter* group as closest relatives. For strains of FAA clusters E, F and two related unclustered strains, 16S rDNA sequence similarities of more than 97% were found with *Alteromonas*, *Pseudoalteromonas* and *Glaciecola* species. An overall dendrogram (see Annex VI) based on the 16S rDNA sequence analysis of both the Antarctic

lake isolates and polar sea isolates, shows that no high sequence similarities can be found between strains isolated from these different habitats, indicating that they belong to separate species. However, they have a few genera in common. For the γ-*Proteobacteria* the genera *Glaciecola*, *Shewanella* and *Pseudomonas* were found in both Antarctic lake and polar sea isolates and for the α-*Proteobacteria* representatives of the *Rhodobacter* group and the genera *Rhizobium*/*Mesorhizobium* were found in both habitats. The genera belonging to the *Bacteroidetes* are clearly different, with only a few FAA clusters (G, I and K) related to *Cytophaga* in the polar sea isolates, and a lot of FAA clusters (1 to 15) related to the genera *Flavobacterium*, *Algoriphagus*, *Gelidibacter*, *Hymenobacter* and *Gillisia* for the Antarctic lake isolates.

According to Stackebrandt & Goebel (1994), 97% sequence similarity is considered as a threshold value below which two strains are expected to belong to different bacterial species. However, the latter authors, as well as many others, also demonstrated that strains with more than 97% sequence similarity may show low DNA-DNA reassociation values and thus constitute different species. This has been confirmed by our results within the genus *Flavobacterium*, where isolates sharing more then 97% (up to 98.7%) 16S rDNA sequence similarity with their closest relatives, proved to be genotypically distinct from these related *Flavobacterium* species on the basis of hybridization results (see chapter 3). The type strains of *Flavobacterium frigoris* and *Flavobacterium degerlachei* for example share a 98.7% homology of their 16S rRNA sequence and proved to belong to seperate species. This is another clear example of the fact that 16S rDNA sequence similarity higher than 97 % is not sufficient to demonstrate that two strains belong to the same species. As such, much more of these polar bacterial strains possibly belong to as yet undescribed new species as can be predicted on the basis of this 97% threshold value.

16S rDNA sequence analysis is a technique that has been widely applied in bacterial taxonomy and is used to study the phylogenetic relationships among bacteria. The major advantage of this technique is its high reproducibility and use in comparative studies because of the availability of large publicly accessible databases (EMBL) of bacterial rDNA sequences.

6.1.4 DNA-DNA hybridizations

It is clear, through studies that compare rep-PCR genomic fingerprint analysis with DNA-DNA relatedness methods that both techniques yield results that are in close agreement (Nick *et al.*, 1999; Rademaker *et al.*, 2000), suggesting that genomic fingerprinting techniques truly reveal genotypic relationships of organisms. Indeed, for the different investigated rep-clusters with identical profiles, DNA-relatedness values were found above 70%, the level recommended for species delineation (Wayne *et al.*, 1987) and 16S rDNA sequence similarities between strains of these rep-clusters were always very high (99-100%). However, strains which showed a different rep-profile sometimes proved to belong to the same species, showing hybridization values of more than 70% (e.g. *Flavobacterium frigoris*, *Flavobacterium micromati* and *Flavobacterium fryxellicola*).

These results indicate that, although the resolution of rep-PCR fingerprinting is sometimes too high with clustering at the subspecies level, this technique is useful to reduce the number of strains needed for the labourious DNA-DNA hybridization studies. This rapid and highly discriminatory screening technique, together with 16S rDNA sequence analysis of representative strains, can be used to determine the taxonomic diversity and phylogenetic structure of large bacterial collections. Several novel genospecies could be delineated and phenotypic analysis resulted in a final description of 13 new species (see chapters 3, 4 and 5).

6.2 Bacterial diversity in Antarctic lakes and polar seas

One of the main objectives of this work was to explore the general taxonomic diversity of bacterial isolates from microbial mats in Antarctic lakes and polar seas. Therefore, samples were taken from very different lakes instead of multiple samples from a single lake and water samples were collected from different stations in the polar seas and at different water depths, in order to obtain an as much diverse collection of strains as possible. For FAA clusters 1 to 15 (161 strains) belonging to the *Bacteroidetes*, rep-PCR fingerprinting revealed 27 clusters and 38 singles, and for strains of FAA cluster 41 (57 strains) belonging to the α-*Proteobacteria*, 10 clusters and 12 singles could be delineated (cut off value of 70% Pearson correlation coefficient). This wealth of different fingerprinting patterns

demonstrates that the bacterial genomic diversity in microbial mats from Antarctic lakes is extremely high and is much higher than previously estimated by fatty acid and 16S rDNA sequence analyses (see chapter 2). Similar results were found for the polar sea isolates and for the investigated strains (21, belonging to FAA clusters E, F and two related unclustered strains; see section 5.1) five clusters could be delineated and four strains formed separate branches.

Only part of the bacterial diversity in Antarctic lakes and polar seas has been investigated into depth (15 FAA clusters related to the *Bacteroidetes*, one cluster belonging to the α-*Proteobacteria* and 21 polar sea isolates of two FAA clusters related to the γ-*Proteobacteria*) with the identification of some isolates to previously known species (*Flavobacterium xanthum* and *Glaciecola mesophila*) and the description of several novel bacterial species [six new *Flavobacterium* species, one new *Algoriphagus* species and a new genus, *Gillisia* of the family *Flavobacteriaceae* (see Chapter 3); three species of a new genus *Loktanella* of the α-*Proteobacteria* (see Chapter 4) and a new *Alteromonas* and *Glaciecola* species (see Chapter 5)] and already the estimated diversity is very high. Further investigations within the other phylogenetic groups (Gram-positives, α-, β- and γ-*Proteobacteria*) would provide a more complete picture of the bacterial diversity in these polar habitats. Through the description of these novel species, unique databases with several of their genotypic and phenotypic properties, using highly reproducible techniques (rep-PCR fingerprinting, FAA analysis, 16S rDNA sequencing), are available and will make the characterization and identification of other polar isolates much easier.

Our results are consistent with the data found by the other partners of the MICROMAT-project. Brambilla *et al.* (2001) investigated the diversity of aerobic and anaerobic bacterial isolates, together with the bacterial and archaeal 16S rDNA clones of a mat sample of Lake Fryxell, and results indicate that the diversity is very high. The cyanobacterial diversity in microbial mats from Lake Fryxell was studied by Taton *et al.* (2003) and evidence was provided that the molecular diversity of cyanobacteria is quite high with a few Antarctic endemic species. In contrast, the fungal flora in these biomats was dominated by a relatively small number of species belonging to a few genera (Göttlich *et al.*, 2003) and this is unexpected since fungi are among the organisms with highest tolerance of extreme conditions. The (cultivated and 'yet-to-be' cultivated) diversity of protists and eukaryotic photosynthetic microorganisms from these Antarctic mats, investigated by several other partners, was also very high and regional differences can be

seen. The diversity of eukaryotes seems lower than in less extreme biotopes. Overall, the surprisingly high diversity of the different microorganisms in the mats demonstrates an enormous complexity of the microbial communities in Antarctic lakes and this is more than would be expected from an extreme environment.

6.3 Geographical distribution of bacterial taxa

Although a high amount of bias on the assessment of the diversity of the heterotrophic isolates from Antarctic lakes was introduced due to the limited number of samplings and culturing procedures, and the limited number of strains isolated from several samples (see chapter 2), results allow to make, to some extent, observations on the geographical distribution of some taxa. Table 6.1 summarizes the sources of the strains from each of the FAA clusters (see Fig. 2.1) and all samples contained representatives of the γ-*Proteobacteria*, whereas all samples, except the one from Organic Lake, yielded members of the *Bacteroidetes*. Gram-positives were not found in the samples from lakes Watts and Pendant. No α-*Proteobacteria* were isolated from samples from lakes Hoare and Grace while no β-*Proteobacteria* were isolated from samples from lakes Watts, Highway and Organic Lake. Several major FAA clusters (with more than 30 isolates) contained strains isolated from almost all samples, suggesting that taxa showing these fatty acid compositions might be ubiquitous in Antarctic lakes, e.g. cluster 5 and 10, related to *Flavobacterium* (see Fig. 2.1). Members of other FAA clusters (with more than 10 isolates) were not detected in the McMurdo Dry Valley lakes, e.g. cluster 22, related to *Shewanella*, or were almost exclusively detected in these lakes, such as cluster 18, related to *Arthrobacter*, and cluster 28, related to *Janthinobacterium*. Other clusters (with more than 4 strains) were only detected in samples from a single lake, like cluster 6, related to *Flavobacterium,* isolated from Pendant Lake.

It is clear that the bacterial diversity in polar habitats and in Antarctic lakes is extremely high and due to the unique and harsh conditions that prevail here, microorganisms are potentially belonging to endogenous, as yet undescribed new taxa. Through a polyphasic approach, several of these novel taxa could be described (see Table 6.2). Some general assumptions can be made about the relationship between these novel taxa and the lakes from which they were isolated since it is impossible to draw firm conclusions on the ecological implications of these lakes due to the constraints on the sampling and culturing methods used.

Table 6.1. Source, FAA clustering (see Fig. 2.1) and phylogenetical affiliation of the strains isolated from Antarctic lakes.

FAA cluster	Phylogenetic group	Number of strains isolated from mat samples from lake (number of samples) [a]										
		FR (2)	HO (1)	DR (1)	OR (1)	GR (1)	AC (2)	WA (2)	PE (3)	HI (2)	RE (2)	Totals (17)
1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15	*Bacteroidetes*	++++	++	++	-	++++	+++	+++	+++	++	+++	158
16, 17, 18, 19	Gram+ (high % GC)	++++	++	++	-	+	+	-	-	+	+++	88
20	Gram+ (low % GC)	+++	-	-	+	-	-	-	-	-	-	12
32, 33, 34, 35, 36, 40, 41	α-*Proteobacteria*	+++	-	++	+++	-	++++	+	++	++	++++	104
24, 28, 29, 30	β-*Proteobacteria*	++++	+++	+++	-	+	+++	-	+	-	++	79
22, 23, 25, 26, 31, 38, 39	γ-*Proteobacteria*	++++	+++	+	+++	++	++++	++++	++++	++++	++++	263
3, 8, 21, 27, 37	Not determined	+	+	-	-	+	++	-	-	-	+	11
Not clustered	Not determined	++	-	-	+	+	++	-	++	+	++	31
Totals		188	34	34	31	34	122	54	77	47	125	746

[a] Samples are pooled per lake. Abbreviations are as given in Table 2.1. Symbols: -, none; +, 1-3; ++, 4-10; +++, 11-20; ++++, ≥21.

Table 6.2. Source, FAA clustering, phylogenetical affiliation and physiological characteristics of the novel taxa described in this study.

New taxon	Phylogenetic branch	FAA cluster [a]	Rep-cluster [b]	Isolation source	Salinity range	Temperature range
Flavobacterium gelidilacus	*Bacteroidetes*	10	XVII	Antarctic lakes: AC, PE, WA, RE	0-5% NaCl	5-25°C (20°C)
Flavobacterium degerlachei	*Bacteroidetes*	5, 6	I	Antarctic lakes: AC, PE, HI, RE	0-5% NaCl	5-30°C (20°C)
Flavobacterium micromati	*Bacteroidetes*	5	VII	Antarctic lakes: GR, FR	0-2% NaCl	5-25°C (20°C)
Flavobacterium frigoris	*Bacteroidetes*	5	XXI, XXIV	Antarctic lakes: GR, WA, FR, RE, DR	0-5% NaCl	5-25°C (20°C)
Flavobacterium fryxellicola	*Bacteroidetes*	5	NC	Antarctic lakes: FR	0-2% NaCl	5-25°C (20°C)
Flavobacterium psychrolimnae	*Bacteroidetes*	5	II	Antarctic lakes: FR, HO	0-2% NaCl	5-30°C (20°C)
Gillisia limnaea	*Bacteroidetes*	4	XIII, NC	Antarctic lakes: FR	0-5% NaCl	5-30°C (20°C)
Algoriphagus antarcticus	*Bacteroidetes*	15	IX, XII, XVIII	Antarctic lakes: RE, FR, AC	0-5% NaCl	5-25°C (20°C)
Loktanella fryxellensis	α-*Proteobacteria*	41	IX	Antarctic lakes: FR	0-5% NaCl	5-30°C (25°C)
Loktanella vestfoldensis	α-*Proteobacteria*	41	I	Antarctic lakes: AC, PE	0-10% NaCl	5-37°C (25°C)
Loktanella salsilacus	α-*Proteobacteria*	41	IV, V, VII	Antarctic lakes: AC, OR	0-10% NaCl	5-37°C (25°C)
Alteromonas stellipolaris	γ-*Proteobacteria*	E	II	Antarctic sea	1-10% NaCl	5-37°C (20°C)
Glaciecola polaris	γ-*Proteobacteria*	F	V	Arctic sea	1-10% NaCl	5-30°C (20°C)

[a] The FAA clusters are as delineated in Van Trappen *et al.* (2002) for strains originating from Antarctic lakes and in Mergaert *et al.* (2001b) for strains from polar seas.

[b] The rep-clusters are as delineated in section 3.1 for the strains belonging to the *Bacteroidetes*, in section 4.1 for the strains belonging to the α-*Proteobacteria* and in section 5.1 for the strains belonging to the γ-*Proteobacteria*.

Firstly, most of these novel taxa derive from a small number of lakes, namely lakes Ace, Pendant, Watts, Reid and Fryxell. Only two species come from Grace Lake (*Flavobacterium micromati* and *F. frigoris*), whereas separate single species *Loktanella salsilacus*, *F. degerlachei*, *F. psychrolimnae* and *F. frigoris*, originate from lakes Organic, Highway, Hoare and Druzhby, respectively. The potential new species (see Table 6.3) also derive from a small number of lakes and they mainly come from lakes Ace and Fryxell. The reason for this can be found in the fact that from lakes Fryxell and Ace, a higher number of bacterial strains were isolated (see chapter 2; 188 and 122 isolates, respectively) compared to lakes Hoare, Organic, Grace and Druzhby (31-34 isolates). As a consequence, strains from the latter lakes are likely to belong to fewer phylogenetic groups and to less of these novel taxa than isolates from lakes Fryxell and Ace. However, another explanation for the striking fact that a lot of these novel species (seven out of thirteen) derive from Lake Fryxell in the Dry Valleys, can also be found in the age of these lakes. Indeed, Dry Valley lakes are very old (hundreds of thousands of years compared to only 8000-10 000 years old for the lakes of the Vestfold Hills and the Larsemann Hills) so they have had more time for the evolution of potentially novel taxa to occur.

Some of these new species were isolated from a certain Antarctic region (*Flavobacterium fryxellicola*, *F. psychrolimnae*, *Gillisia limnaea* and *Loktanella* sp.), whereas others derive from different Antarctic regions, suggesting that they might be more or less ubiquitous on the Antarctic continent (*F. gelidilacus*, *F. degerlachei*, *F. micromati*, *F. frigoris* and *Algoriphagus antarcticus*). Again, this ubiquity is probably due to the larger number of isolates belonging to the latter species.

Some Antarctic lake isolates were identified as *Flavobacterium xanthum*, originally isolated from Antarctic soil, whereas two polar sea isolates (Antarctic sea) were identified as the recently described *Glaciecola mesophila* from the Sea of Japan in Russia. Another two polar sea isolates (Arctic sea) belonged to a novel species within the genus *Glaciecola*, *Glaciecola polaris*, showing that this genus has a bipolar distribution. For the polar sea isolates most of the FAA clusters contain isolates both from Antarctica as well as Arctica and only clusters G, I, J and K consist solely of Antarctic strains.

Table 6.3. Source, FAA clustering and phylogenetical affiliation of potential novel taxa from Antarctic lakes and polar seas.

Strain No.	FAA cluster [a]	Phylogenetic branch	Nearest phylogenetic neighbour	Sequence similarity	Isolation source
Antarctic lakes					
R-9033	2	*Bacteroidetes*	*Flavobacterium tegetincola*	95.3	AC
R-7581	5	*Bacteroidetes*	*Flavobacterium tegetincola*	95.3	FR
R-7585	5	*Bacteroidetes*	*Flavobacterium limicola*	96.0	FR
R-7515	9	*Bacteroidetes*	*Flavobacterium limicola*	95.0	FR
R-8893	10	*Bacteroidetes*	*Flavobacterium aquatile*	94.5	AC
R-7666	12	*Bacteroidetes*	*Flavobacterium limicola*	95.7	FR
R-9476	15	*Bacteroidetes*	*Flavobacterium limicola*	96.4	AC
R-9112	16	Gram-positives	*Microbacterium keratanolyticum*	96.0	GR
R-8287	19	Gram-positives	*Clavibacter michiganensis*	96.1	FR
R-8161	20	Gram-positives	*Bacillus oleronius*	93.2	FR
R-8971	23	γ-*Proteobacteria*	*Pseudomonas migulae*	95.8	AC
R-11381	25	γ-*Proteobacteria*	*Alteromonas macleodii*	93.9	HI
R-9221	36	α-*Proteobacteria*	*Sphingomonas natatoria*	94.5	AC
R-9035	39	γ-*Proteobacteria*	*Marinobacter hydrocarbonoclasticus*	95.6	AC
R-9219	41	α-*Proteobacteria*	*Mesorizhobium loti*	96.9	AC
Polar seas					
ARK 177	NC	α-*Proteobacteria*	*Roseobacter litoralis*	93.8	Arctic sea
ARK 126	B	α-*Proteobacteria*	*Rhizobium mediterraneum*	95.0	Arctic sea
ANT43	C	γ-*Proteobacteria*	*Pseudomonas migulae*	96.5	Antarctic sea
ARK 104	C	γ-*Proteobacteria*	*Pseudomonas migulae*	96.5	Antarctic sea
ARK 161	C	γ-*Proteobacteria*	*Pseudomonas migulae*	96.5	Antarctic sea

[a] The FAA clusters are as delineated in Van Trappen *et al.* (2002) for strains originating from Antarctic lakes and in Mergaert *et al.* (2001) for strains from polar seas.

6.4 Ecological considerations

It was not our objective to compare the Antarctic lake samples from a physicochemical point of view and the salinity of the samples was not determined. However, information on the salinity class of the lakes is known and some temptative reflexions about the correlation between the lakes and the salt tolerance of these novel taxa can also be made. Generally, strains from Organic Lake phylogenetically belong to the *Proteobacteria* and the Gram-positives (*Bacillus*), based on fatty acid and 16S rDNA sequence analyses (see chapter 2). From the novel taxa, only *Loktanella salsilacus* consists of strains from the saline/ hypersaline lakes Organic and Ace. When looking at the physiological characteristics of these strains, they are moderately halotolerant and are able to grow with up to 10% NaCl. Moreover, there is a clear link between the salinity of the lakes from which strains were isolated and their salt tolerance (see Table 6.2). Strains that are only able to grow with up to 2% salt derive from fresh/ brackish lakes (*F. micromati*, *F. fryxellicola* and *F. psychrolimnae* from lakes Grace, Hoare and Fryxell), whereas strains that tolerate up to 5-10% NaCl come from more saline lakes (e.g. *F. gelidilacus* and *Loktanella vestfoldensis* from lakes Ace, Pendant and Reid). The new species from the polar seas are moderately halophilic (no growth without NaCl) in contrast to the Antarctic lake isolates, which are moderately halotolerant (able to grow without NaCl). A possible explanation might be their different way of isolation by using enrichment methods with seawater for the sea isolates, compared to the more classical isolation methods used for the Antarctic lake isolates.

From all the novel taxa, the ones that are psychrophilic (with an optimal growth temperature of 20°C and a maximum of 25-30°C) belong to the *Bacteroidetes*, whereas the psychrotrophic taxa rather belong to the *Proteobacteria* (with an optimal growth temperature of 20-25°C and a maximum of 30-37°C). Several new psychrophilic *Flavobacterium* species have been recently described (e.g. *F. limicola*, *F. omnivorum* and *F. xinjiangense*) and a lot of strains of the *Bacteroidetes* group have been characterized by their adaptability to low temperatures (Bernardet *et al.*, 1996; Bowman *et al.*, 1997a). Their counterparts of the *Proteobacteria* seem to grow in a broader range of temperatures and this is certainly true for the genus *Glaciecola* with psychrophilic species (*G. punicea*, *G. pallidula*) isolated from sea-ice assemblages and the recently described, mesophilic *G. mesophila*, isolated from the Sea of Japan in Russia. In the MICROMAT-project, artificial mats from Lake Fryxell were cultivated to get a first glimpse of the ecology of the mats and evidence was found that the microbial community was cold-adapted (Buffan-Dubau

et al., 2001; Pringault *et al.*, 2001). However, it remains unknown whether psychrophiles dominate in Antarctic mat communities or whether they only constitute a minority.

In Organic Lake, high levels of dimethylsulfide were detected and it may not be a coincidence that the new species we described (*Loktanella salsilacus*) with strains isolated from this lake, belongs to the *Rhodobacter* group, of which certain members (for example *Sulfitobacter*) potentially play an important role in the sulphur cycle. However for *L. salsilacus*, evidence for its participation in sulphur cycling has not been found (there is no production of hydrogen sulphide) and the oxidation of reduced sulphur still needs to be investigated.

6.5 Biotechnological applications

In the last decade, the discovery of novel structural classes of different pharmaceuticals has declined and therefore, there is a renewed interest in examining microorganisms for the production of these novel compounds, especially the ones that live in unexplored ecological niches. The polar areas and their surrounding marine sites for example, offer a unique opportunity to investigate the unexplored microbial diversity since the extreme conditions that thrive here, have led to the evolution of new endogenous taxa with potentially novel biochemical adaptations. In these extreme environments the production of several metabolic compounds against bacteria and fungi, can confer a competitive survival advantage, just like the production of pigments offers a protection against strong UV irradiation and the production of resistant spores in fungi may represent a survival tool to desiccation, low temperatures and high salinities often found in Antarctic lakes.

In the context of the MICROMAT-project, the biotechnological exploitation of the microbial richness in microbial mats from Antarctic lakes, particularly of bacteria and fungi, was investigated for the production of novel cold-adapted enzymes and antimicrobial compounds of interest against human pathogens of clinical relevance. Data about the extra-cellular hydrolytic activity of the Antarctic bacterial and fungal isolates indicated a relatively low frequency of extra-cellular enzyme activities. Results show that every type of enzyme activity screened for was represented in the investigated strains. For the bacteria, amylase, cellulase, esterase, lipase and protease activity was observed in 17%, 20%, 48%, 17% and 34% of the strains, respectively (see Table 6.4). For the fungi, esterases were

Table 6.4. Extra-cellular enzyme activities of Antarctic bacterial strains.

FAA cluster [a]	Tested strains [b]	Positive strains [c]	Number of strains with enzyme activity:				
			Amylase	Cellulase	Esterase	Lipase	Protease
1	2	2	0	2	0	0	2
5	17	10	5	2	3	4	6
6	2	1	1	0	0	0	0
10	5	2	2	0	1	0	0
11	2	1	ND	1	ND	ND	ND
14	1	1	0	1	1	1	1
16	8	3	1	1	1	1	1
17	5	4	0	2	1	2	1
18	63	54	20	37	38	9	13
19	5	1	ND	1	ND	ND	ND
20	12	6	0	1	0	1	5
21	2	2	0	1	1	2	1
22	73	66	22	1	39	7	61
23	64	42	3	3	39	12	8
25	6	5	1	4	1	0	1
26	37	30	4	4	26	3	22
27	2	1	1	0	1	0	0
28	12	6	1	1	4	2	5
30	13	7	0	1	5	3	3
36	5	4	ND	3	1	ND	0
38	42	34	3	16	29	19	3
39	1	1	0	0	1	1	0
40	4	1	0	0	1	0	0
41	11	5	1	1	3	2	3
NC	11	5	4	2	3	0	4
Total	**405**	**294**	**69**	**84**	**200**	**69**	**140**

[a] FAA clusters are as delineated in Van Trappen *et al.* (2002).

[b] Total number of tested strains of the FAA clusters containing positive isolates.

[c] Number of strains that are positive for at least one enzyme. For FAA clusters 2, 3, 4, 7, 8, 9, 12, 13, 15, 24, 29, 31, 32, 33, 34, 35 and 37 no positive isolates were found. ND: enzyme activity was not detectable because strains were unable to grow under standard incubation conditions.

observed in 93% of the strains, whereas lipase was found in 37% of the strains. Production of amylase, cellulase and protease was detected in 59%, 62% and 75% of the strains, respectively. No single genus could be especially linked to any one particular enzyme activity, due probably to the low number of strains in the test sample (Ciciliato *et al.*, 2001).

Marinelli *et al.* (personal communication, see Annex IV) reported about the screening of heterotrophic bacteria and fungi from Antarctic lakes for the production of new antibiotics. They found unexpected high antimicrobial activity rates for the Antarctic isolates (29% from fungi and 17% from bacteria) and the frequency of antibacterial activity is particularly high against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli*, both among bacterial and fungal isolates. Most of the antifungal activities against the fungi *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* were obtained from fungi. Further studies were performed with a subset of the bacterial hits exhibiting potential activities against bacterial human pathogens and two isolates from the McMurdo Dry Valleys (R-7513 and R-7941) were studied in more detail. The isolates correspond to coccoid high % G + C Gram-positives with antibacterial activities against *Bacillus subtilis*, *S. aureus* and *Enterococcus faecium* and are phylogenetically related to *Arthrobacter agilis*. They produce similar compounds which belong to the cyclic thiazolyl peptide antibiotics with activities against Gram-positive bacteria. Further work is now in progress to study the chemical and biological profiling of the metabolites produced by the bacterial and fungal hits and the assessment of their novelty will help to understand to which extent the chemical diversity correlates with the taxonomic diversity, so far discovered in these microbial communities of Antarctic lakes.

6.6 Future perspectives

Although polar regions have been regarded as inhospitable and isolated environments, inhabited by simple and species-poor communities, several studies have revealed a large microbial diversity and should encourage scientists to look in more detail at these extreme environments. With the MICROMAT-project, the cultivated and 'not-yet-cultivated' microbial diversity in microbial mats from Antarctic lakes was studied. However, our study on the bacterial diversity was exploratory rather than thoroughly and samples of ten lakes in three different Antarctic regions were investigated. In most cases, comparisons about the diversity in different samples from the same lake could not be made because too low numbers of strains were isolated for at least one sample (see chapter 2). To get a more complete picture about this microbial diversity in Antarctic lakes, and in polar habitats in general, much more samples would be needed and additional areas should be investigated. More samples will also allow a better understanding of the variation

in diversity and composition between the different lakes and may shed light on how these communities have evolved and how dispersal affects local biodiversity. The same is true for the polar oceans where microbial processes are beginning to be understood and more comprehensive field studies need to be conducted to get a better idea of the functioning of the microbial communities in these habitats, especially in the deep-sea, mesopelagic and benthic areas, that have been virtually ignored. The spatial and temporal variability that exists in polar environments needs to be carefully documented before any conclusions can be drawn about the precise role of bacteria in the food web. Recently, research about the microbial taxonomy in polar regions has become better focused by using molecular genetic approaches and combining culture-independent molecular techniques with the results from culturing studies, will allow a better understanding of these microbial communities.

Our results will not only help to understand the composition and functioning of cold extreme environments, but also have implications for fundamental and applied microbiology. Firstly, studying polar regions is important to have a clearer view on the response of these ecosystems to environmental changes, especially in the form of global warming since the polar areas act as a beacon for this change. Indeed, many lakes of the McMurdo Dry Valleys in Antarctica have risen significantly during the last century and evidence has been found that this phenomenon is a direct result of an increase in summertime air temperatures. The polar regions of the Earth, especially the Arctic, are undergoing relatively rapid environmental changes on a global perspective, such that inhabitants of sea-ice communities for example become threatened with extinction in this century. The particularly diverse and rich microbial mat communities are one of the most complex ecological systems known on the Antarctic continent and may serve as useful monitors of past and present climatic change.

Secondly, another area of interest regarding the study of the poles is the increasing effect of human activities on these pristine ecosystems. The potential environmental impact of pollution from research vessels, scientific bases and tourism on the polar communities must be carefully monitored, since the magnitude of research activities on the poles will certainly accelerate in the near future.

Thirdly, the novel taxa found in these polar environments potentially have new biochemical adaptations which can be used to find novel biotechnological and pharmaceutical applications. Screening research on our bacterial isolates has already indicated interesting activities in several strains and has lead to a

renewed interest in the microbial exploitation of polar habitats (see section 6.5).

Finally, a better understanding of the complexity of microbial communities under extreme conditions, might be relevant for the search for life in similar extreme environments, like Lake Vostok (3 km underneath the ice sheet) and other planets (for example Mars and Europa, moon of Jupiter). Polar habitats provide models for possible extraterrestrial habitats and the study of microorganisms in these cold, extreme environments can be used for the development of methods to locate and identify microbial forms of life elsewhere.

In conclusion, bacterial diversity in polar seas and microbial mats from Antarctic lakes is very high and these unique habitats harbour a wealth of potentially endogenous, new taxa, which offer great promise for future research.

SUMMARY

The polar regions suffer from extreme environmental conditions and as such, these areas are some of the most inhospitable places on earth. However, the poles harbour a wide variety of different terrestrial and aquatic biotopes, where microorganisms are the most abundant and often the only form of life. Several polar habitats have not been explored into detail and especially the bacterial component of the microbial food web has been poorly investigated. Studies on the bacterial diversity in polar habitats were performed in the framework of the European research project MICROMAT (see Annex III), which addresses the microbial diversity in the mat communities of Antarctic lakes, and the study of oligotrophic bacteria in polar seas in cooperation with T. L. Tan from the Alfred Wegener Institut für Polar- und Meeresforschung (AWI, Bremerhaven).

During the MICROMAT-project, the diversity of heterotrophic bacteria in microbial mats from diverse freshwater and saline Antarctic lakes was investigated and almost 800 bacterial strains were isolated from mats collected from 10 different lakes from the McMurdo Dry Valleys (lakes Hoare and Fryxell), the Vestfold Hills (lakes Ace, Pendant, Druzhby, Organic, Grace, and Watts) and the Larsemann Hills (Lake Reid). Fatty acid analysis was used to obtain a first grouping of the large amount of isolates into different clusters and 41 clusters could be delineated, whereas 31 strains formed single branches. Representative strains were chosen for further study by 16S rDNA sequence analysis and results revealed that they belong to the α-, β- and γ- *Proteobacteria*, the high and low percent G+C Gram-positives and to the *Bacteroidetes* and many sequences showed a sequence similarity below 97% with their nearest phylogenetic neighbours, indicating that they represent as yet unnamed new taxa.

Techniques with a higher resolution had to be applied to investigate the genomic diversity of each fatty acid cluster in more detail and in a first part of this study, several FAA clusters belonging to the *Bacteroidetes* and the α-*Proteobacteria* were further investigated by repetitive extragenic palindromic (rep)-PCR genomic fingerprinting. A wealth of different fingerprinting patterns was obtained and results

demonstrate that the genomic diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is extremely high. Strains showing the same rep-PCR pattern are often isolated from different lakes and even from different Antarctic regions for the FAA clusters belonging to the *Bacteroidetes*, whereas for the FAA cluster related to the α-*Proteobacteria*, the different rep-PCR profile types correlated well with the geographical origin of the strains. Rep-PCR fingerprinting of the isolates allowed a further subclustering at the genotypic level and was used to select representatives for additional 16S rDNA sequence analysis and DNA-DNA hybridizations.

Several of the additional 16S rDNA sequences showed similarity values of less than 97% to the closest described species in the EMBL database, indicating their novelty, but during the last years it has been demonstrated that strains with more than 97% sequence similarity may show low DNA-DNA reassociation values and thus constitute different species. This has been confirmed by our results within the genus *Flavobacterium*, where isolates sharing more than 97% (up to 98.7%) 16S rDNA sequence similarity with their closest relatives, proved to be genotypically distinct from these related species. As such, much more of these Antarctic bacteria possibly belong to as yet undescribed new species as can be predicted on the basis of their 16S rDNA sequence.

For the different investigated rep-clusters with identical profiles, DNA-relatedness values were found above 70% and 16S rDNA sequence similarities between strains of these rep-clusters were always very high (99-100%). It is clear, through studies that compare rep-PCR genomic fingerprint analysis with DNA-DNA relatedness methods that both techniques yield results that are in close agreement (Nick *et al.*, 1999; Rademaker *et al.*, 2000). However, strains which show a different rep-profile sometimes proved to belong to the same species, showing hybridization values of more than 70%. These results indicate that, although the resolution of rep-PCR fingerprinting is sometimes too high, this technique is useful to reduce the number of strains needed for the laborious DNA-DNA hybridization studies. Through this polyphasic taxonomic approach, different genospecies could be delineated and phenotypic analysis resulted in a final description of several novel species:

- ***Flavobacterium degerlachei* (14 strains)**, ***Flavobacterium micromati* (3)**, ***Flavobacterium frigoris* (23)**, ***Flavobacterium psychrolimnae* (4)**, ***Flavobacterium fryxellicola* (3)** and ***Flavobacterium gelidilacus* (22)**.

Strains belonging to these six novel *Flavobacterium* species derive from lakes in the three different Antarctic regions.

- ***Gillisia limnaea***. The three strains of this new genus were isolated from Lake Fryxell in the McMurdo Dry Valleys. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains belong to the family *Flavobacteriaceae*.

- ***Algoriphagus antarcticus* (6 strains)**. Strains belonging to this species were isolated from microbial mats in lakes Reid, Fryxell and Ace. Phylogenetic analysis based on 16S rDNA sequences indicated that these strains belong to the family '*Flexibacteriaceae*'.

- ***Loktanella salsilacus* (10 strains)**, ***Loktanella fryxellensis* (12)** and ***Loktanella vestfoldensis* (4)**. Strains of this novel genus were isolated from lakes Ace, Pendant, Organic and Fryxell. Phylogenetic analysis based on 16S rDNA sequences placed these strains within the *Rhodobacter* group of the α-subclass of the *Proteobacteria*.

The second part of this work handles about the diversity of oligotrophic bacteria in polar seas and a collection of 173 bacterial strains, which were isolated after enrichment under oligotrophic, psychrophilic conditions from Arctic (98 strains) and Antarctic (75 strains) seawater, was available. These strains have been previously analysed by their substrate utilization patterns using the Biolog system (Tan, 1997; Tan & Rüger, 1999) and by fatty acid and 16S rDNA sequence analyses (Mergaert *et al.*, 2001b). They belong to six metabolic groups and eight FAA-clusters could be delineated, whereas eight strains formed separate branches. Results of the 16S rDNA sequence analysis indicate that they belong to the α- and γ- *Proteobacteria*, the high percent G+C Gram-positives and to the *Bacteroidetes*. Additionally, several clusters represent as yet unnamed, new taxa, since they show less than 97% 16S rDNA sequence similarity to their nearest named neighbours.

In the meantime, additional strains isolated using the same methods, were included in fatty acid analysis during this study and the genomic diversity of 21 strains was further investigated by rep-PCR genomic fingerprinting. Using a polyphasic taxonomic approach, two novel species within the γ- *Proteobacteria*

could be described:

- ***Alteromonas stellipolaris***. Seven Antarctic strains could be assigned to a novel species within the genus *Alteromonas* and buds can be produced on mother and daughter cells and on prosthecae. Prostheca formation is peritrich and prosthecae can be branched.

- ***Glaciecola polaris***. Two Arctic strains constitute a new species within the genus *Glaciecola* and buds can be produced on mother cells and on prosthecae. Branch formation of prosthecae occurs.

Overall, the bacterial diversity in polar seas and microbial mats from Antarctic lakes is very high and these unique habitats harbour a wealth of endogenous, new taxa, with several potential industrial applications. In the context of the MICROMAT-project, the production of novel cold-adapted enzymes and antimicrobial compounds by bacterial strains was investigated and unexpected high antimicrobial activity rates were found for the Antarctic isolates.

SAMENVATTING

In de Noord- (Arctica) en Zuidpool (Antarctica) heersen extreme omgevingscondities en bijgevolg behoren deze regio's tot de meest onbewoonbare plaatsen op aarde. De poolgebieden bezitten echter wel een grote variëteit aan verschillende terrestrische en aquatische habitats, waar micro-organismen de meest abundante en vaak enige levensvorm uitmaken. Verschillende polaire habitats werden nog niet in detail bestudeerd en vooral de bacteriële component van de microbiële voedselketen is slechts in beperkte mate onderzocht. Studies over de bacteriële diversiteit in de poolgebieden werden uitgevoerd in het kader van het Europees Onderzoeksproject MICROMAT (zie Annex III), dat zich toespitste op de microbiële diversiteit in de matgemeenschappen van Antarctische meren, en de studie over oligotrofe bacteriën in poolzeeën in samenwerking met T. L. Tan van het Alfred Wegener Institut für Polar- und Meeresforschung (AWI, Bremerhaven).

Tijdens het MICROMAT-project werd de diversiteit van heterotrofe bacteriën in microbiële matten van verschillende Antarctische zoetwater- en zoute meren onderzocht en bijna 800 bacteriële stammen werden geïsoleerd uit matten, verzameld vanuit 10 verschillende meren van de McMurdo Dry Valleys (de meren Hoare en Fryxell), de Vestfold Hills (de meren Ace, Pendant, Druzhby, Organic, Grace en Watts) en de Larsemann Hills (het meer Reid). Vetzuuranalyse werd gebruikt om een eerste clustering te bekomen van het grote aantal isolaten en 41 clusters konden afgebakend worden, terwijl 31 stammen apart vielen. Representatieve stammen werden gekozen om verder te bestuderen aan de hand van 16S rDNA sequentie-analyse en de resultaten tonen aan dat ze tot de α-, β- en γ- *Proteobacteria*, de hoog- en laag-percent G+C Gram-positieven en tot de *Bacteroidetes* behoren. Verschillende sequenties vertonen een lagere similariteit dan 97% met hun nauwste fylogenetische verwanten en dit toont aan dat ze tot nieuwe, nog niet beschreven taxa behoren.

Technieken met een hogere resolutie werden gebruikt om de genomische diversiteit van elk vetzuurcluster in detail te bestuderen en in een eerste deel van deze studie werden verschillende vetzuurclusters die behoren tot de *Bacteroidetes*

en de α-*Proteobacteria* verder onderzocht aan de hand van de repetitieve extragenische palindromische (rep)-PCR genomische fingerprintingtechniek. Een groot aantal verschillende fingerprintingpatronen werd bekomen en de resultaten tonen aan dat de genomische diversiteit van heterotrofe bacteriën in microbiële matten van Antarctische meren uitzonderlijk groot is. Stammen die eenzelfde rep-patroon vertonen werden vaak geïsoleerd uit verschillende meren en zelfs uit verschillende Antarctische regio's voor de vetzuurclusters die behoren tot de *Bacteroidetes*, terwijl voor het vetzuurcluster behorende tot de α-*Proteobacteria*, de verschillende rep-profielen goed overeenkomen met de geografische oorsprong van de stammen. Rep-PCR-fingerprinting van de isolaten liet een verdere subclustering op genotypisch niveau toe en werd gebruikt om representatieven te selecteren voor additionele 16S rDNA sequentie-analyse en DNA-DNA hybridisaties.

Verschillende van de additionele 16S rDNA sequenties vertonen een similariteit lager dan 97% met de meest verwante species in de EMBL-databank, wat er op wijst dat ze tot nieuwe taxa behoren, maar gedurende de laatste jaren werd aangetoond dat stammen met meer dan 97% sequentiesimilariteit eveneens lage DNA-DNA reassociatiewaarden kunnen vertonen en bijgevolg tot verschillende species behoren. Dit werd bevestigd door onze resultaten binnen het genus *Flavobacterium*, waar isolaten met meer dan 97% (tot 98.7%) 16S rDNA sequentiesimilariteit met hun nauwste verwanten, tot genotypisch verschillende species bleken te behoren. Bijgevolg kunnen veel meer van deze Antarctische bacteriën mogelijk nog niet beschreven, nieuwe species vormen, dan kan voorspeld worden op basis van hun 16S rDNA sequentie.

Voor de verschillende onderzochte rep-clusters met identieke profielen, werden DNA-DNA verwantschapswaarden boven 70% bekomen en 16S rDNA sequentiesimilariteiten tussen stammen van deze rep-clusters waren altijd zeer hoog (99-100%). Het is duidelijk vanuit studies die rep-PCR genomische fingerprintanalyse vergelijken met DNA-DNA verwantschapsmethoden, dat beide technieken resultaten opleveren die goed overeenstemmen (Nick *et al.*, 1999; Rademaker *et al.*, 2000). Maar stammen die een verschillend rep-profiel vertonen, bleken soms tot hetzelfde species te behoren met hybridisatiewaarden van meer dan 70%. Deze resultaten tonen aan dat, hoewel de resolutie van rep-PCR fingerprinting soms te hoog is, deze techniek bruikbaar is om het aantal stammen, nodig voor de arbeidsintensieve DNA-DNA hybridisaties, te reduceren. Door deze polyfasische taxonomische aanpak konden verschillende genospecies afgebakend

worden en fenotypische analyses leidden tot een finale beschrijving van verschillende nieuwe species:

- ***Flavobacterium degerlachei* (14 stammen)**, ***Flavobacterium micromati* (3)**, ***Flavobacterium frigoris* (23)**, ***Flavobacterium psychrolimnae* (4)**, ***Flavobacterium fryxellicola* (3)** and ***Flavobacterium gelidilacus* (22)**. Stammen behorende tot deze zes nieuwe *Flavobacterium* species komen uit meren in de drie verschillende Antarctische regio's.

- ***Gillisia limnaea***. De drie stammen van dit nieuwe genus werden geïsoleerd uit het meer Fryxell in de McMurdo Dry Valleys. Phylogenetische analyse gebaseerd op 16S rDNA sequenties toont aan dat deze stammen tot de familie van de *Flavobacteriaceae* behoren.

- ***Algoriphagus antarcticus* (6 stammen)**. Stammen van dit species werden geïsoleerd uit microbiële matten van de meren Reid, Fryxell en Ace. Phylogenetische analyse gebaseerd op 16S rDNA sequenties toont aan dat deze stammen tot de famile van de '*Flexibacteriaceae*' behoren.

- ***Loktanella salsilacus* (10 stammen)**, ***Loktanella fryxellensis* (12)** and ***Loktanella vestfoldensis* (4)**. Stammen van dit nieuwe genus werden geïsoleerd uit de meren Ace, Pendant, Organic en Fryxell. Phylogenetische analyse gebaseerd op 16S rDNA sequenties plaatst deze stammen binnen de *Rhodobacter* groep van de α-subklasse van de *Proteobacteria*.

Het tweede deel van dit werk behandelt de diversiteit van oligotrofe bacteriën in polaire zeeën en een verzameling van 173 bacteriële stammen, die geïsoleerd werden na aanrijking onder oligotrofe, psychrofiele condities uit Arctisch (98 stammen) en Antarctisch (75 stammen) zeewater, was beschikbaar. Deze stammen werden eerder geanalyseerd aan de hand van hun Biolog-patronen waarbij het gebruik van verschillende substraten werd uitgetest (Tan, 1997; Tan & Rüger, 1999) en aan de hand van vetzuur- en 16S rDNA sequentie-analyses (Mergaert *et al.*, 2001b). Ze behoren tot zes metabolische groepen en acht vetzuurclusters konden afgebakend worden, terwijl acht stammen apart vielen. De resultaten van de 16S rDNA sequentie-analyse tonen aan dat ze tot de α- en γ- *Proteobacteria*, de hoog-percent G+C Gram-positieven en tot de *Bacteroidetes* behoren.

Verschillende clusters vertegenwoordigen nog niet beschreven, nieuwe taxa, aangezien ze een 16S rDNA sequentiesimilariteit van minder dan 97% met hun nauwste verwanten vertonen.

Intussen werden additionele stammen geïsoleerd aan de hand van dezelfde methoden en deze werden ingesloten in vetzuuranalyse tijdens deze studie en de genomische diversiteit van 21 stammen werd verder onderzocht aan de hand van rep-PCR genomische fingerprinting. Aan de hand van deze polyfasische taxonomische aanpak konden twee nieuwe species behorende tot de γ-*Proteobacteria* beschreven worden:

- ***Alteromonas stellipolaris***. Zeven Antarctische stammen konden toegekend worden aan een nieuw species van het genus *Alteromonas* en knoppen kunnen gevormd worden op moeder- en dochtercellen en op prostheca. De vorming van prostheca is peritrich en prostheca kunnen vertakt zijn.

- ***Glaciecola polaris***. Twee Arctische stammen behoren tot een nieuw species binnen het genus *Glaciecola* en knoppen kunnen gevormd worden op moedercellen en op prostheca. Vertakkingen op prostheca kunnen voorkomen.

De bacteriële diversiteit in poolzeeën en microbiële matten van Antarctische meren is zeer groot en deze unieke habitats bezitten een enorme rijkdom aan endogene, nieuwe taxa, met verschillende potentiële industriële toepassingen. In de context van het MICROMAT-project, werd de produktie van nieuwe, aan koude aangepaste enzymes en antimicrobiële componenten door bacteriële stammen onderzocht en een onverwacht hoge antimicrobiële activiteit werd aangetoond voor deze Antarctische isolaten.

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Annex I

List of strains from Antarctic lakes

R-No.	LMG-No.	Sample [a]	FAA cluster [b]	Accession-No. [c]	Chapter [d]	R-No.	LMG-No.	Sample [a]	FAA cluster [b]	Accession-No. [c]	Chapter [d]
9003	21468	GR	1	AJ441000	2	8283	22021	FR1	5		3.4
9229		GR	1			8284	22024	FR1	5		3.4
9033	21469	AC1	2	AJ441001	2	8297		HO	5		
11271		HI2	2		3.1	8298		HO	5		
11272		HI2	2			8359		FR1	5		3.3
11274		HI2	2			8879		DR	5		
11383		HI2	2			8982	21916	PE1	5		3.3
11608		HI2	2			8988	21917	PE1	5		3.3
10674		RE1	3			8991		PE1	5		3.3
10676		RE1	3			8992		PE1	5		3.3
7610	21965	FR2	4		3.5	8993		PE1	5		3.3
7730	21966	FR1	4		3.5	8994		GR	5		3.4
8282	21470	FR1	4	AJ440991	3.5	8996		GR	5		3.3
7509		FR1	5			8999		GR	5		3.4
7518		FR2	5	AJ601393	3.4	9000	21923	GR	5		3.3
7548	22022	FR2	5	AJ585429	3.4	9002		GR	5		3.3
7550	21473	FR2	5	AJ440979	2	9005		WA1	5		3.4
7574		FR1	5			9008		WA1	5		
7577		FR1	5			9009		WA1	5		3.4
7581		FR2	5		3.1	9010		WA1	5	AJ601392	3.4
7582	22018	FR2	5	AJ585428	3.4	9013		WA1	5		3.4
7585		FR2	5		3.1	9014	21922	WA1	5	AJ557887	3.3
7587		FR2	5			9029		WA1	5		
7681	22019	HO	5		3.4	9048		GR	5		
7933	21472	FR1	5	AJ440987	2	9106	21915	AC1	5	AJ557886	3.3
8016	21921	FR1	5		3.3	9119		PE1	5		3.3
8017		FR1	5		3.3	9127		DR	5		3.4
8018		FR2	5			9132		DR	5		
8019	22023	FR2	5		3.4	9134		WA1	5		3.3
8020		FR2	5		3.3	9137		WA1	5		3.3
8022	21471	FR2	5	AJ440988	3.3	9138		WA1	5		3.3
8023	22020	FR2	5	AJ585427	3.4	9141		GR	5		3.4
8159		FR2	5			9142		GR	5		3.4

9143		GR	5		
9144		GR	5		3.3
9145		GR	5		3.4
9147		GR	5		3.4
9148		GR	5		3.4
9149		GR	5		3.3
9151		GR	5		
9191		GR	5		
9192	21919	GR	5	AJ557888	3.3
9193	21920	GR	5		3.3
9227		DR	5		3.3
9228		GR	5		3.3
9329		WA1	5		3.4
11356		PE3	5		3.3
11545		WA2	5		3.4
11563		HI2	5		3.3
12548		RE2	5		
12591		RE2	5		3.3
12606	21924	RE2	5		3.3
12608		RE2	5		3.3
12625		RE2	5		3.3
12627	21925	RE2	5		3.3
9118		PE1	6		3.3
9122	21474	PE1	6	AJ441005	3.3
9123		PE1	6		
9124		PE1	6		3.3
9125	21918	PE1	6		3.3
7579	21475	FR2	7	AJ440981	2
7667		FR2	7		
8158		FR2	7		
9026		WA1	7		
9280		WA1	7		
8101		FR1	8		
8102		FR2	8		
7515	21476	FR2	9	AJ440977	2
7571		FR1	9		
7720		HO	9		

7932		FR1	9		
8134		FR1	10		
8885	21619	AC1	10	AJ507151	3.2
8888		AC1	10		3.2
8893		AC1	10		3.1
8897		AC1	10		3.2
8898		AC1	10		3.2
8899	21477	AC1	10	AJ440996	3.2
8908		AC1	10		3.2
8969		AC1	10		3.2
8972		AC1	10		3.2
8983		PE1	10		3.2
9004		WA1	10		3.2
9019		WA1	10		3.2
9024		WA1	10		3.2
9046		GR	10		3.1
9051		DR	10		
9053		DR	10		
9056	21620	PE1	10		3.2
9101		GR	10		
9104		AC1	10		3.2
9110		AC1	10		3.2
9158		AC1	10		3.2
9283		AC1	10		3.2
9330	21621	WA1	10		3.2
10672		RE1	10		
10847		RE1	10		3.1
11078		PE2	10		3.2
11277		PE2	10		3.2
11278		PE2	10		3.2
12566	21618	RE2	10		3.2
12626		RE2	10		
8963	21478	AC1	11	AJ440998	2
9331		WA1	11		3.1
11385		WA2	11		3.1
12567		RE2	11		
7665		FR1	12		

7666	21479	FR1	12	AJ440984	2
7721		FR1	12		
7723		FR1	12		
7727		FR1	13		
9058		PE1	13		
9217	21480	AC1	13	AJ441008	2
9220		AC1	13		
7572	21481	FR1	14	AJ440980	2
7586		FR2	14		
7722		FR1	14		
8104		FR1	14		
8973		DR	14		
11111		PE3	14		
12582		RE2	14		
8290	21984	FR2	15		3.6
9286	21482	AC1	15	AJ441012/ AJ577267	3.6
9476		AC1	15		3.1
10710	21980	RE1	15	AJ577141	3.6
10749	21981	RE1	15		3.6
10750		RE1	15		
10752	21982	RE1	15		3.6
11427	21983	RE1	15	AJ577142	3.6
8980		DR	16		
9112	21483	GR	16	AJ441003	2
10744		RE1	16		
10745		RE1	16		
10772		RE1	16		
10848		RE1	16		
10891		RE1	16		
12529		RE2	16		
12575		RE2	16		
8880		DR	17		
9183	21484	AC1	17	AJ441006	2
10747		RE1	17		
11715		AC2	17		
12571		RE2	17		

7508		FR1	18		
7513		FR2	18		
7514		FR2	18		
7517		FR2	18		
7519		FR2	18		
7521		FR2	18		
7523		FR1	18		
7524		FR1	18		
7525		FR1	18		
7526		FR1	18		
7527		FR1	18		
7528		FR1	18		
7529		FR1	18		
7530		FR2	18		
7531		FR2	18		
7532		FR2	18		
7533		FR2	18		
7534		FR2	18		
7535		FR2	18		
7536		FR2	18		
7537		FR2	18		
7538		FR2	18		
7539		FR2	18		
7540		FR2	18		
7541		FR2	18		
7543		FR1	18		
7544		FR1	18		
7546		FR2	18		
7547		FR2	18		
7549	21485	FR2	18	AJ440978	2
7570		FR1	18		
7573		FR1	18		
7578		FR1	18		
7580		FR2	18		
7588		FR1	18		
7589		FR1	18		

7590		FR1	18		
7591		FR1	18		
7592		FR1	18		
7593		FR1	18		
7594		FR1	18		
7598		FR1	18		
7599		FR1	18		
7600		FR1	18		
7604		FR1	18		
7608		FR2	18		
7609		FR2	18		
7673		FR1	18		
7674		FR1	18		
7683		HO	18		
7685		HO	18		
7688		HO	18		
7731		FR1	18		
7939		FR1	18		
7941		HO	18		
7942		HO	18		
7943		HO	18		
8011		FR1	18		
8012		FR1	18		
8015		FR2	18		
8979		DR	18		
8981		DR	18		
10774		RE1	18		
11619		HI1	18		
8139		FR2	19		
8141		FR2	19		
8156		FR1	19		
8287	21486	FR1	19	AJ440992	2
8291		FR2	19		
9100		AC1	19		
10675		RE1	19		
10679		RE1	19		
10887		RE1	19		

11578		RE1	19		
7512		FR1	20		
7551		FR2	20		
7552		FR1	20		
7575		FR1	20		
7606		FR2	20		
7611		FR2	20		
7612		FR2	20		
8135		FR1	20		
8161	21487	FR1	20	AJ440990	2
8163		FR2	20		
8164		FR2	20		
9116		OR	20		
7686		HO	21		
11374		AC2	21		
8895		AC1	22		
8896		AC1	22		
8900		AC1	22		
8902		AC1	22		
8905		AC1	22		
8990		PE1	22		
9016		WA1	22		
9018		WA1	22		
9020		WA1	22		
9140		WA1	22		
9153		AC1	22		
9154		AC1	22		
9157		AC1	22		
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9190		AC1	22		
9281		PE1	22		
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11076		HI2	22		
11077		PE2	22		
11079		PE2	22		

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11294		AC2	22		
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11398		WA2	22		
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11564		HI2	22		
11569		AC2	22		
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7522		FR2	23		
7669		FR2	23		
7675		FR1	23		
7725		FR2	23		
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7736		FR2	23		
7937		FR2	23		
8021		FR2	23		
8100		FR1	23		
8149		FR2	23		
8883		AC1	23		
8886		AC1	23		
8891		AC1	23		
8906		AC1	23		
8971	21489	AC1	23	AJ440999	2
8984		PE1	23		
9006		WA1	23		

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11091	HI2	22
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11096	HI2	22
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11099	PE2	22
11104	PE3	22
11105	PE3	22
11106	PE2	22
11108	PE3	22
11109	PE2	22
11113	PE2	22
11115	AC2	22
11116	WA2	22
11117	WA2	22
11118	WA2	22
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11284	PE2	22
11285	PE2	22
11287	AC2	22
11288	AC2	22
11290	WA2	22
11292	AC2	22

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11380		HI2	25			
11381	21491	HI2	25	AJ440975	2	
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11547		PE3	25			
11548		PE2	25			
7463		FR2	26			
7616	21492	HO	26	AJ440983	2	
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7620		HO	26			

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9012	WA1	23
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9017	WA1	23
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9023	WA1	23
9025	WA1	23
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9121	PE1	23
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9135	WA1	23
9136	WA1	23
9139	WA1	23
9146	GR	23
9152	PE1	23
9156	AC1	23
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9182	AC1	23
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9223	AC1	23
9282	AC1	23
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11081	PE2	23
11097	HI2	23
11279	PE2	23
11286	PE2	23

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8152		FR2	28		
8293		HO	28		
8295		HO	28		
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7516		FR2	30		
7520		FR2	30		
7545		FR1	30		
7583		FR2	30		
7584		FR2	30		
7614	21497	HO	30	AJ440982	2
7664		FR1	30		
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7938		FR1	30		
8046		FR2	30		
8105		HO	30		
8155		FR1	30		
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7680	HO	26
7682	HO	26
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8882	DR	26
8995	GR	26
9279	DR	26
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12530	RE2	26
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12601	RE2	26
9114	GR	27
9150	GR	27
7462	FR2	28
7464	FR1	28

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8964	AC1	30
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8976	DR	30
8977	DR	30
8985	PE1	30
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9034	AC1	30
9037	AC1	30
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9102	DR	30
9105	AC1	30
9107	AC1	30
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10892		RE1	33		
9216	21500	AC1	34	AJ441007	2
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10889		RE1	34		
10894		RE1	34		
10707		RE1	35		
10753	21501	RE1	35	AJ440974	2
10775		RE1	35		
10803		RE1	35		
10886		RE1	35		
9221	21502	AC1	36	AJ441010	2
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10668		RE1	36		
10706		RE1	36		
10712		RE1	36		
10721		RE1	36		
11429		RE1	36		
9326		AC1	37		
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7553		FR2	38		
7595		FR1	38		
7596		FR1	38		
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7602		FR1	38		
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7607		FR2	38		
7676		FR1	38		
7678		FR2	38		
7934		FR1	38		

7940		FR2	38		
8138		FR2	38		
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9042		OR	39		
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9045		OR1	39		
9077		OR1	39		
9103		OR1	39		
9109		AC1	39		
9115		OR1	39		
9177		AC1	39		
9212		AC1	39		
9213		AC1	39		
9479		PE1	39		
7668		FR2	40		
8157		FR2	40		
8358	21505	FR2	40	AJ440993	2
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8876		DR	40		
8975		DR	40		
9050		DR	40		
9211		DR	40		
9225		OR	40		
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10667		RE1	40		
10670		RE1	40		
10671		RE1	40		
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10718		RE1	40		
10720		RE1	40		
10777		RE1	40		
10804		RE1	40		
11276		PE2	40		
11399		RE1	40		
12569		RE2	40		

7601		FR1	41		4.2	9075		OR	41			
7605		FR2	41		4.2	9076		OR	41			
7670	22007	FR1	41	AJ582225	4.2	9178		AC1	41			
7671		FR1	41		4.2	9184	22006	AC1	41	AJ582227	4.2	
7672	22008	FR1	41		4.2	9186		OR1	41		4.2	
7726	22009	FR1	41		4.2	9219	21506	AC1	41	AJ441009	2	
7728	22010	FR1	41		4.2	9474		AC1	41			
7729		FR1	41		4.2	9477	22003	AC1	41	AJ582226	4.2	
7732		FR2	41		4.2	10678		RE1	41			
7735		FR2	41		4.2	10722		RE1	41			
8013		FR1	41		4.2	10754		RE1	41			
8014		FR1	41		4.2	10771		RE1	41			
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8904	21507	AC1	41	AJ440997	4.2	11386		PE2	41			
8966		AC1	41			11422		AC2	41			
8968	21999	AC1	41		4.2	11576		HI2	41			
8970		AC1	41			11577		HI2	41			
9030	22000	OR1	41	AJ582228	4.2	11309		HI2	41			
9036		AC1	41		4.2	11610		HI2	41			
9040		CR	41			7677		FR2	NC			
9054	22004	PE1	41		4.2	8045		FR1	NC			
9055		PE1	41			8142		FR1	NC			
9057	22005	PE1	41		4.2	8146		FR1	NC			
9062		OR	41			8147		FR2	NC			
9063		OR	41			8148		FR2	NC			
9064		OR	41		4.2	8281		FR1	NC			
9065		OR	41			8286		FR1	NC			
9066	22001	OR	41		4.2	8288		FR1	NC			
9067		OR	41			8878		OR	NC			
9068	22002	OR	41	AJ582229	4.2	8986		PE1	NC			
9069		OR	41			8987		PE1	NC			
9070		OR	41			8998		GR	NC			
9071		OR	41			9099		AC1	NC			
9072		OR	41			9175		AC1	NC			

9176	AC1	NC	
9222	AC1	NC	
9224	AC1	NC	
9287	AC1	NC	
9289	AC1	NC	
9328	OR1	NC	
10677	RE1	NC	
10714	RE1	NC	
10778	RE1	NC	
11114	AC2	NC	
11366	PE2	NC	
11579	RE1	NC	
11628	PE3	NC	
11850	HI2	NC	
12583	RE2	NC	
12584	RE2	NC	

[a] Samples from which the strains were isolated are as indicated in Van Trappen *et al.* (2002), see chapter 2.

[b] Fatty acid clusters are as delineated in van Trappen *et al.* (2002), see chapter 2. NC: not clustered.

[c] The accession numbers refer to the 16S rDNA sequences as deposited in the EMBL database.

[d] The numbers refer to the chapters and sections in which the isolates were analysed and discussed.

Annex II

List of strains from polar seas

R-No.	Original No.	Station [a]	Water depth [b]	FAA cluster [c]	Accession-No.[d]	Chapter [e]	R-No.	Original No.	Station [a]	Water depth [b]	FAA cluster [c]	Accession-No.[d]	Chapter [e]
7072	ANT 18c	16/509	100	A			7948	ARK 123	235/DP	25	C		
7244	ANT 19c	16/509	100	A			7965	ARK 151a	242/D	25	C		
7371	ANT 8	16/507	25	A	AJ293822		7966	ARK 154	242/D	25	C		
7694	ANT 13	16/509	25	A			7968	ARK 183	223/D	25	C		
7703	ANT 30	16/518	25	A			7972	ARK 223	223/D	25	C		
7706	ANT 34	16/518	100	A	AJ278782		7973	ARK 224	223/D	25	C		
7709	ANT 40	16/526	25	A			**15536**	**ANT 195**	**28/301**	**30**	**C**		**5.1**
7710	ANT 44	16/526	25	A			**15539**	**ANT 198**	**28/301**	**30**	**C**		**5.1**
7843	ANT 81b	16/554	25	A			**15540**	**ANT 199**	**28/301**	**30**	**C**		**5.1**
7844	ANT 82b	16/557	25	A			**15542**	**ANT 201**	**28/301**	**30**	**C**		**5.1**
7848	ANT 84b	16/557	25	A			7070	ANT 7	16/507	25	D		
7971	ARK 218	223/D	25	A			7071	ANT 11	16/509	25	D		
7946	ARK 121	235/DP	25	B			7073	ANT 54a	16/530	25	D		
7951	ARK 126	235/DP	25	B	AJ278784		7074	ANT 41	16/526	25	D	AJ278781	
7953	ARK 128	235/DP	25	B			7075	ANT 58	16/534	25	D		
15521	**ANT 177**	**28/301**	**30**	**B**		**5.1**	7079	ARK 105	235/DP	25	D	AJ293821	
15531	**ANT 190**	**28/301**	**30**	**B**		**5.1**	7080	ARK 109	235/DP	25	D		
15556	**ANT 225**	**28/301**	**200**	**B**		**5.1**	7081	ARK 111	235/DP	25	D		
7077	ARK 103	223/DP	25	C			7082	ARK 112	235/DP	25	D		
7078	ARK 104	223/DP	25	C	AJ293824		7083	ARK 113	235/DP	25	D		
7217	ARK 185	223/D	25	C			7084	ARK 115	235/DP	25	D		
7226	ARK 200	223/D	25	C			7085	ARK 116	235/DP	26	D		
7228	ARK 202	223/D	25	C			7087	ARK 137	235/D	25	D		
7235	ARK 216	223/D	25	C			7088	ARK 138	235/D	25	D		
7238	ARK 220	223/D	25	C			7089	ARK 139	235/D	25	D		
7365	ARK 151b	242/D	25	C			7092	ARK 155	242/D	25	D		
7366	ARK 161	223/D	200	C	AJ293826		7093	ARK 164	223/D	200	D		
7367	ARK 181	223/D	25	C			7094	ARK 165	223/D	200	D		
7368	ARK 187	223/D	25	C			7095	ARK 166	223/D	200	D		
7372	ANT 18b	16/509	100	C			7096	ARK 167	223/D	200	D		
7375	ANT 43	16/526	25	C	AJ293825		7097	ARK 168	223/D	200	D		
7376	ANT 53	16/530	25	C			7098	ARK 169	223/D	200	D		
7697	ANT 17	16/509	100	C			7099	ARK 170	223/D	200	D		

7100	ARK 171	223/D	200	D		
7101	ARK 172	223/D	1000	D		
7102	ARK 173	223/D	1000	D(ex-NC)	AJ293827	
7104	ARK 179	223/D	25	D		
7105	ARK 180	223/D	25	D		
7106	ARK 184	223/D	25	D		
7107	ARK 188	223/D	25	D		
7218	ARK 189	223/D	25	D		
7219	ARK 190	223/D	25	D		
7220	ARK 193	223/D	25	D		
7221	ARK 194	223/D	25	D		
7222	ARK 195	223/D	25	D		
7223	ARK 196	223/D	25	D		
7224	ARK 197	223/D	25	D		
7225	ARK 199	223/D	25	D		
7227	ARK 201	223/D	25	D		
7229	ARK 204	223/D	25	D		
7230	ARK 205	223/D	25	D		
7231	ARK 206	223/D	25	D		
7232	ARK 212	223/D	25	D		
7233	ARK 213	223/D	25	D		
7234	ARK 215	223/D	25	D		
7236	ARK 217	223/D	25	D		
7237	ARK 219	223/D	25	D		
7239	ARK 222	223/D	25	D		
7240	ARK 225	235/DP	25	D		
7690	ANT 4a	16/507	25	D		
7696	ANT 15	16/509	25	D		
7969	ARK 186	223/D	25	D		
15522	**ANT 180**	**28/301**	**30**	**D**		**5.1**
15523	**ANT 181**	**28/301**	**30**	**D**		**5.1**
15525	**ANT 184**	**28/301**	**30**	**D**		**5.1**
15526	**ANT 185**	**28/301**	**30**	**D**		**5.1**
15529	**ANT 188**	**28/301**	**30**	**D**		**5.1**
15530	**ANT 189**	**28/301**	**30**	**D**		**5.1**
15533	**ANT 192**	**28/301**	**30**	**D**		**5.1**
15534	**ANT 193**	**28/301**	**30**	**D**		**5.1**
15535	**ANT 194**	**28/301**	**30**	**D**		**5.1**

15537	**ANT 196**	**28/301**	**30**	**D**		**5.1**
15538	**ANT 197**	**28/301**	**30**	**D**		**5.1**
15541	**ANT 200**	**28/301**	**30**	**D**		**5.1**
15545	**ANT 205**	**28/301**	**30**	**D**		**5.1**
15547	**ANT 210**	**28/301**	**30**	**D**		**5.1**
15548	**ANT 212**	**28/301**	**30**	**D**		**5.1**
15549	**ANT 214**	**28/301**	**100**	**D**		**5.1**
15550	**ANT 215**	**28/301**	**100**	**D**		**5.1**
15551	**ANT 216**	**28/301**	**100**	**D**		**5.1**
15552	**ANT 218**	**28/301**	**100**	**D**		**5.1**
15553	**ANT 219**	**28/301**	**100**	**D**		**5.1**
15554	**ANT 221**	**28/301**	**100**	**D**		**5.1**
15557	**ANT 226**	**28/301**	**200**	**D**		**5.1**
15558	**ANT 227**	**28/301**	**200**	**D**		**5.1**
7076	ARK 102	223/DP	25	E	AJ295713	
7086	ARK 130	242/DP	25	E		
7090	ARK 140	242/D	200	E		
7214	ARK 142	242/D	200	E		
9870	ANT 52	16/530	25	E		
9872	ANT 60b	16/534	25	E		
9873	ANT 63a/2	16/535	25	E		
9875	ANT 69a	16/546	25	E	AJ295715	
9876	ANT 73	16/549	25	E		
15462	**ANT 52/2**	**16/530**	**25**	**E**		**5.2**
15463	**ANT 60b/2**	**16/534**	**25**	**E**		**5.2**
15464	**ANT 62a**	**16/535**	**25**	**E**		**5.2**
15465	**ANT 63a/3**	**16/535**	**25**	**E**		**5.1**
15466	**ANT 69a/2**	**16/546**	**25**	**E**		**5.2**
15467	**ANT 73/2**	**16/549**	**25**	**E**		**5.2**
15468	**ANT 81a**	**16/554**	**25**	**E**		**5.2**
15469	**ANT 82a**	**16/557**	**25**	**E**		**5.2**
15470	**ARK 102/2**	**223**	**25**	**E**		**5.1**
15471	**ARK 130/2**	**242**	**25**	**E**		**5.1**
15472	**ARK 140/2**	**242**	**200**	**E**		**5.1**
15473	**ARK 142/2**	**242**	**200**	**E**		**5.1**
7215	ARK 150	242/D	25	F	AJ293820	5.3
7964	ARK 149	242/D	200	F		5.3
15461	**ANT 12a**	**16/509**	**25**	**F**	**AJ548479**	**5.3**

15474	**ANT 213**	**28/301**	**100**	**F**		**5.1**
15475	**ANT 223**	**28/301**	**100**	**F**		**5.1**
15476	**ANT 224**	**28/301**	**100**	**F**		**5.1**
15520	**ANT 12b**	**16/509**	**25**	**F**		**5.3**
7695	ANT 14	16/509	25	G	AJ278780	
7698	ANT 18d	16/509	100	G		
7702	ANT 26b	16/518	25	G		
7707	ANT 35	16/518	100	G		
7717	ANT 54b	16/530	25	G		
7830	ANT 65	16/540	25	G		
7069	ANT 2	16/507	25	H		
7211	ARK 129	242/DP	25	H		
7212	ARK 131	242/DP	25	H	AJ278779	
7213	ARK 132	242/DP	25	H		
7241	ANT 1	16/507	25	H	AJ293818	
7242	ANT 4b	16/507	25	H		
7243	ANT 10	16/509	25	H		
7245	ANT 20	16/515	25	H		
7246	ANT 21	16/515	25	H		
7364	ARK 124	235/DP	25	H		
7369	ARK 214	223/D	25	H		
7370	ANT 6	16/507	25	H		
7373	ANT 23	16/515	25	H		
7374	ANT 28	16/518	25	H		
7689	ANT 3	16/507	25	H		
7692	ANT 9	16/507	25	H		
7700	ANT 24	16/518	25	H		
7705	ANT 32	16/518	100	H		
7708	ANT 39	16/526	25	H		
7711	ANT 45	16/526	25	H		
7712	ANT 46	16/526	25	H		
7713	ANT 47	16/526	25	H		
7714	ANT 49	16/526	100	H		
7715	ANT 50	16/526	100	H		
7716	ANT 51	16/526	100	H		
7718	ANT 57	16/530	200	H		
7827	ANT 62b	16/535	25	H		

7829	ANT 64	16/540	25	H		
7831	ANT 66	16/540	25	H		
7832	ANT 67	16/547	25	H		
7833	ANT 68	16/547	25	H		
7834	ANT 69b	16/547	25	H		
7835	ANT 69c	16/547	25	H		
7836	ANT 71	16/549	25	H		
7837	ANT 74	16/549	25	H		
7838	ANT 76a	16/552	25	H		
7839	ANT 76b	16/552	25	H		
7840	ANT 79	16/554	25	H		
7841	ANT 80a	16/554	25	H		
7842	ANT80b	16/554	25	H		
7845	ANT 83a	16/557	25	H		
7846	ANT 83b	16/557	25	H		
7847	ANT 84a	16/557	25	H		
7849	ANT 84c	16/557	25	H		
7850	ARK 106	235/DP	25	H		
7851	ARK 107	235/DP	25	H	AJ293819	
7853	ARK 119	235/DP	25	H		
7944	ARK 118	235/DP	25	H		
7945	ARK 120	235/DP	25	H		
7947	ARK 122	235/DP	25	H		
7950	ARK 125	235/DP	25	H		
7952	ARK 127	235/DP	25	H		
7957	ARK 133	235/D	25	H		
7958	ARK 141	242/D	200	H		
7959	ARK 143	242/D	200	H		
7960	ARK 144	242/D	200	H		
7961	ARK 145	242/D	200	H		
7962	ARK 147	242/D	200	H		
7963	ARK 148	242/D	200	H		
15527	**ANT 186**	**28/301**	**30**	**I**		**5.1**
15528	**ANT 187**	**28/301**	**30**	**I**		**5.1**
15532	**ANT 191**	**28/301**	**30**	**J**		**5.1**
15555	**ANT 222**	**28/301**	**100**	**J**		**5.1**
15559	**ANT 228**	**28/301**	**200**	**J**		**5.1**

15543	**ANT 203**	**28/301**	**30**	**K**		**5.1**	
15544	**ANT 204**	**28/301**	**30**	**K**		**5.1**	
15546	**ANT 209**	**28/301**	**30**	**K**		**5.1**	
7091	ARK 152	242/D	25	NC	AJ278783		
7103	ARK 176	223/D	25	NC	AJ293828		
7216	ARK 158	223/D	200	NC	AJ295714	5.1	
7247	ANT 27a	16/518	25	NC (ex-G)			
7704	ANT 31	16/518	100	NC	AJ295711		
7701	ANT 26a	16/518	25	NC (ex-G)			
7852	ARK 108	235/DP	25	NC	AJ295712	5.1	
7967	ARK 177	223/D	25	NC	AJ293823		
9879	ARK 101	223/DP	25	NC	AJ295716		
15524	**ANT 182**	**28/301**	**30**	**NC**		**5.1**	

[a] Polarstern station numbers are as described in Tan & Rüger (1991), Tan (1997) and Tan *et al.* (1999), see also Annex V. Cruise no. 16: ANT VIII/6, 1990; Cruise no. 28: ANT XI/2, 1993/94. From the Arktis-Expedition, two different enrichment techniques were applied, i.e. in Dialysis chambers or in Double Petri-dishes, abbreviates as D and DP respectively (Tan, 1997).

[b] The water depth where samples were taken are given in m.

[c] FAA clusters are as delineated in Mergaert *et al.* (2001b).

[d] The accession numbers refer to the 16S rDNA sequences as deposited in the EMBL database.

[e] The numbers refer to the chapters and sections in which the isolates were analysed and discussed.

In bold face: the 56 additional strains investigated in this study.

Annex III

Project descriptions

MICROMAT (BIO4-CT98-0040)

Title	Biodiversity of microbial mats in Antarctica
Participants	Dr. A. Wilmotte, University of Liège (coordinator) Dr. D. Hodgson, NERC British Antarctic Survey (BAS) Prof. Dr. J. Laybourn-Parry, University of Nottingham Prof. Dr. J. Swings, University of Ghent Prof. Dr. W. Vyverman, University of Ghent Prof. Dr. E. Stackebrandt, DSMZ Dr. R. De Wit, University of Bordeaux Dr. E. Göttlich, IWW Rheinish-Westfälisches Institut für Wasserforschung Gemeinnützige Prof. Dr. S. de Hoog, Institute of Royal Netherlands Academy of Arts and Sciences Dr. F. Marinelli, BioSearch Italia S. P. A., Microbial Isolation & Fermentation Prof. Dr. B. Jones, Genencor International B. V. Dr. O. Genilloud, Merck Sharp & Dohme de Espana
Description	In order to assess and improve the characterisation of the cultivated and 'yet-to-be' cultivated diversity of the bacteria, protists and fungi in the mats of Antarctica and to test this biodiversity for its novelty and potential biotechnological use, two objectives are being pursued during this EC Biotech project : 1. **The biodiversity of mat communities from diverse freshwater and saline lakes will be studied.** For the cultivated biodiversity, classical and novel isolation methods will be used. For example the Benthic Gradient Chamber will be used to mimic some of the gradients present in the mats with the purpose to enrich for some organisms and try to maintain the mats. Phenotypic and genotypic characteristics of the strains will be determined. Modern molecular strategies, based on SSU rDNA will be used for genotypic characterisation of all types of microorganisms, in order to establish a standard taxonomic approach. The diversity of pigments and light-protective compounds will be assessed. In parallel to the isolation of strains, the 'yet-to-be' cultivated biodiversity of all groups will be estimated for representative samples using molecular approaches based on rDNA sequences and involving clone libraries and DGGE-like techniques. 2. **Biotechnological use of the biodiversity.** Isolated strains of bacteria, fungi and protists will be screened for novel cold-tolerant enzymes and bio-active compounds. The nucleic acids extracted from the samples will also be submitted to screening for genes coding for proteases, cellulases and peptide synthetases.
Duration	01/11/1998- 24/02/2001

BOF-project (Bijzonder Onderzoeksfonds, Universiteit Gent)

Title	Prokaryotische diversiteit in poolzeeën en Antarctische meren
Promotor	Prof. Dr. Ir. J. Swings, Vakgroep Biochemie, Fysiologie & Microbiologie, Universiteit Gent
Contact person	Dr. J. Mergaert, Vakgroep Biochemie, Fysiologie & Microbiologie, Universiteit Gent
Description	De onderzoeksgroep is betrokken bij het taxonomisch onderzoek van polaire prokaryoten via twee kanalen, enerzijds door haar deelname aan het Europees BIOTECH project "Biodiversity of microbial mats in Antarctica", ("MICROMAT"; 01-11-98 tot 30-10-00; http://www.nerc-bas.ac.uk/public/mlsd/micromat/), en anderzijds door haar samenwerking met T. L. Tan van het Alfred Wegener Institut für Polar- und Meeresforschung ("AWI", Bremerhaven). Met het MICROMAT project voert de onderzoeksgroep een, zij het eerder verkennend, onderzoek uit naar de biodiversiteit van heterotrofe bacteriën uit microbiële matten die groeien in Antarctische meren, en zij beschikt over een unieke verzameling van zo'n 500 bacteriënkulturen uit microbiële matten (uit 10 verschillende Antarctische meren uit drie regio's) die tijdens verschillende expedities door de British Antarctic Survey en de Australian Antarctic Division werden verzameld. Deze matstalen werden gecryopreserveerd in het laboratorium en zijn beschikbaar voor verder onderzoek. In de Arctische en Antarctische zeeën zijn bacteriën actief onder oligotrofe, psychrotrofe omstandigheden, als plankton (Tan, 1997; Tan & Rüger, 1999), of in zee-ijs (Bowman et al., 1997a), die slechts na langdurige aanrijking en adaptatie kunnen geïsoleerd worden. Het AWI bezit een unieke verzameling van zo'n 500 facultatief oligotrofe prokaryotische isolaten die aangerijkt en geadapteerd werden uit waterstalen genomen tijdens verschillende expedities van het onderzoeksschip POLARSTERN in Arctische en Antarctische zeeën, en de onderzoeksgroep heeft een 150-tal stammen onderzocht in het kader van een stagescriptie. Uit onze voorlopige resultaten (Verhelst, 1999) en deze van Tan (1997) en Tan & Rüger (1999) blijkt dat ze behoren tot potentieel nieuwe taxa. De doelstelling van het beoogde project is de biodiversiteit te bestuderen van polaire prokaryoten door isolatie en polyfasische taxonomische analyse (Vandamme et al., 1996). Verschillende indices van biodiversiteit zullen hierbij onderzocht worden: Arctische versus Antarctische zeeën, isolaten uit zeeën versus isolaten uit Antarctische microbiële matten, vergelijking van matten uit verschillende Antarctische regio's, physicochemische diversiteit van de meren, nieuwe isolaten versus reeds bekende prokaryoten uit Antarctica.
Duration	01/01/2001- 31/12/2003

Annex IV

Biotechnological exploitation of heterotrophic bacteria and filamentous fungi isolated from benthic mats of Antarctic lakes

Flavia Marinelli, Mara Brunati, Federica Sponga, Ismaela Ciciliato, Daniele Losi, Stefanie Van Trappen, Joris Mergaert, Jean Swings, Elke Göttlich, Sybren de Hoog, Jose Luis Rojas and Olga Genilloud

Written for *Microbial Genetic Resources and Biodiscovery*,
eds. J. Swings en I. Kurtboke

Antarctic lakes represent a unique undisturbed environment for exploring microbial diversity. The MICROMAT project, an academic and industrial joint research effort funded by the EC to study microbial mats growing in Antarctic lakes, has shown the enormous richness of taxa inhabiting these ecosystems and their biotechnological potential. 723 heterotrophic bacteria and 158 fungi were isolated from 13 lakes in the McMurdo Dry Valleys, the Vestfold Hills and the Larsemann Hills and screened for the production of antimicrobial compounds of interest against human pathogens of clinical relevance. High and unexpected antimicrobial activity rates were obtained from these Antarctic isolates (29 % from fungi and 17% from bacteria). The frequency of antibacterial activity is particularly high against the Gram-positive *S. aureus* and the Gram-negative *E. coli* both among bacterial and fungal isolates. Most antifungal activities against the fungi *C. albicans, A. fumigatus* and *C. neoformans* were obtained from fungi.

The MICROMAT project

Natural products have been a critically important source of clinically relevant therapeutic molecules. However, the discovery rate of novel structural classes of antimicrobial molecules has declined in the last decade (MacNeil et al. 2001). Recent progress in molecular microbial ecology shows that the extent of microbial diversity in nature is far greater than previously thought, as the number of known species is less than 1 % (Rondon et al. 1999). A renewed interest in examining microorganisms for novel pharmaceuticals has stimulated the development of integrated approaches combining specific isolation methods and the access to geographically diverse sample sources and to different ecological niches (Peláez and Genilloud, 2002). Metabolic potential is also being exploited by cloning microbial genes in environmental libraries without undergoing the step of culturing microbes (MacNeil et al. 2001).

In this context, the academic and industrial joint research of the EC project MICROMAT (http://www.nerc-bas.ac.uk/public/mlsd/micromat) has focused on the study of the culturable and uncultivable – or, better, the "not-yet-culturable" – diversity in microbial mats occurring in Antarctic lakes. The Antarctic continent and its surrounding marine sites offer a unique opportunity to investigate an unexplored microbial biodiversity (Bernan et al. 1997; Brambilla et al. 2001). Antarctica is in fact characterized by its geographical and climatic isolation. The extreme climate has led to evolution of novel biochemical adaptations to severe low temperatures and the possibility of indigenous species. Moreover most of the continent has experienced little or no anthropogenic influence. Antarctic lakes include both freshwater and hyper-saline systems and some of them are covered by perennial ice (Wharton et al. 1993; Doran et al. 1994). Their benthic areas receive sufficient solar radiation to be covered by microbial mats. These benthic mats have accumulated for thousands of years and are virtually undisturbed due to the particular climatic conditions and the absence of higher metazoans. Results from the MICROMAT project have pointed out the extremely high microbial diversity in mats where numerous novel phylotypes have and are being described (Van Trappen et al. 2002; Van Trappen et al. 2003; Tindall et al. 2000; Brambilla et al. 2001; and unpublished results).

Sampling

Mats were sampled from 13 lakes of different ages and physico-chemical characteristics located in three distinct regions of the Antarctic continent (Fig.1). The Larsemann Hills are a series of granite and gneiss peninsulas into Prydz Bay (Eastern Antarctica) with fjords and lakes directly (currents, inlets) or indirectly (sea spray) subjected to marine influences; most of them thaw for up to 2 months in summer and during this time are subjected to considerable wind driven mixing. The Vestfold Hills constitute a low-lying area situated South of the Larsemann Hills, where hundreds of water bodies are found in the valleys, with salinities ranging from fresh to hypersaline (ten times seawater) (Bowman et al. 2000). In contrast to the Larsemann and Vestfold Hills, the lakes in the McMurdo Dry Valleys of South Victoria Land are very old (hundreds of thousand of years). They do not loose their ice-cover and thus lack any turbulence. They vary from dilute meltwaters to hypersaline lakes (Wharton et al. 1993; Doran et al. 1994; Laybourn-Parry et

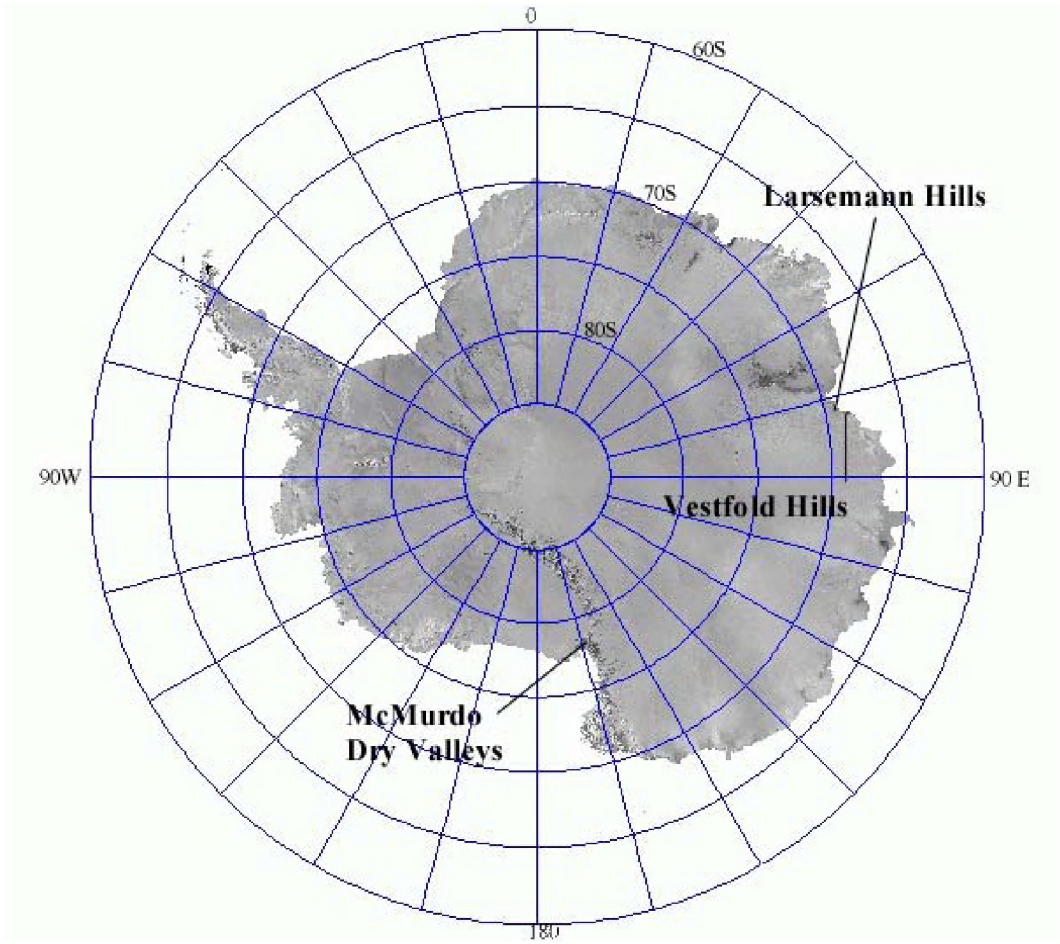

Figure 1. Map of the Antarctic continent showing the three sampling fields.

al. 1997).

Samples of the mats were taken either at a water depth of 3-4 m or from the littoral zone of the lakes (Van Trappen et al., 2002). The diversity of bacteria, cyanobacteria, algae, protozoans and fungi in the microbial mat samples of several Antarctic lakes was studied by conventional (direct microscopy, cultivation) and molecular methods (clone libraries and DGGE based on the SSU rDNA from the samples).

More than 1500 strains were isolated and part of them was screened for the production of new cold-adapted enzymes and antimicrobial compounds. In this report we describe our combined efforts to screen 158 filamentous fungi and 723 heterotrophic bacteria isolated from mats collected in five lakes in the Larsemann Hills, six in the Vestfold Hills and two in the McMurdo Dry Valleys. Our main objective was to assess the potential of this microbial diversity with the aim of discovering new anti-infective producers among these Antarctic microorganisms.

Diversity of Antarctic isolates

Table 1 and Table 2 show the geographical distribution and the lake of origin of the bacteria and fungi isolated from thirteen lakes located in the three distinct Antarctic regions sampled, i.e. Larsemann Hills, Vestfold Hills and McMurdo Dry Valley. Taxonomical diversity among 746 Antarctic bacterial isolates, including the strains (723) tested in this work, was previously studied by fatty acid clustering analysis and by 16S rDNA sequencing of cluster representative strains: a dendrogram with 41 different fatty acid clusters and 31 strains forming single branches was described by Van Trappen et al. (2002). Table 1 reports the taxonomical distribution for 675 of 723 bacterial strains tested in this paper. The 675 isolates were distributed in all the 41 different fatty acid clusters previously identified and they also included 28 strains forming single branches. Fatty acid profiles on the rest of the screened strains (48 out of 723, not listed in Table 1) were not interpretable due to their low resolution or their strain cultivation conditions were unsuitable for general comparison (data not shown). The clustered bacteria were phylogenetically affiliated on the basis of their 16S rDNA sequences with several lineages in the alpha, beta and gamma subclasses of the *Proteobacteria*, the *Bacteroidetes*, and the high and low percentage G+C Gram-positives. As shown in Table 1, most clusters (28) contained strains (614) isolated from different lakes, and often from different regions, suggesting that taxa showing these fatty acid

Table 1. Diversity of bacterial taxa isolated in different Antarctic areas.

FAA cluster [a]	Number of isolates tested [b]	Phylogenetic branch	Nearest phylogenetic neighbour [c]	Lake of origin [d]	Area [e]
1	1	*Bacteroidetes*	*Flavobacterium frigidarium* (98.0%)	GR	VH
2	5	*Bacteroidetes*	*Flavobacterium aquatile, F. tegetincola* (95.7%)	HI	VH
3	1	Not determined	Not determined	RE	LH
4	3	*Bacteroidetes*	*Saligentibacter salegens* (92.9%)	FR	DV
5	69	*Bacteroidetes*	*Flavobacterium gillisiae* (98.4%), *F. hydatis*	FR (21), HO (2)	DV
			(97.3%), *F. frigidarium* (97.5%)	PE (7), GR (17), WA (12), AC (1), DR (3), HI (1)	VH
				RE (5)	LH
6	5	*Bacteroidetes*	*Flavobacterium gillisiae* (98.4%)	PE	VH
7	4	*Bacteroidetes*	*Flavobacterium hydatis* (96.9%)	FR (2)	DV
				WA (2)	VH
8	1	Not determined	Not determined	FR	DV
9	2	*Bacteroidetes*	*Flavobacterium gillisiae* (94.6%)	HO (1), FR (1)	DV
10	25	*Bacteroidetes*	*Flavobacterium flevense* (95.3%)	AC (12), PE (4), WA (3), GR (2), DR (1)	VH
				RE (3)	LH
11	4	*Bacteroidetes*	*Flavobacterium tegetincola* (98.2%)	AC (1), WA (2)	VH
				RE (1)	LH
12	1	*Bacteroidetes*	*Flavobacterium tegetincola* (95.7%)	FR	DV
13	4	*Bacteroidetes*	*Gelidibacter algens* (99.8%)	FR (1)	DV
				PE (1), AC (2)	VH
14	6	*Bacteroidetes*	*Hymenobacter actinosclerus* (97.3%)	FR (4)	DV
				PE (1)	VH
				RE (1)	LH
15	7	*Bacteroidetes*	*Cyclobacterium marinum* 92.5%)	FR (1)	DV
				AC (2)	VH
				RE (4)	LH
16	9	Gram + (high %GC)	*Microbacterium keratanolyticum* (96.0%)	DR (1), GR (1)	VH
				RE (7)	LH
17	5	Gram + (high %GC)	*Micrococcus luteus* (99.7%)	DR (1), AC (2)	VH
				RE (2)	LH
18	64	Gram + (high %GC)	*Arthrobacter agilis* (99.6%)	FR (54), HO (6)	DV
				DR (2), HI (1)	VH
				RE (1)	LH
19	7	Gram + (high %GC)	*Clavibacter michiganensis* (96.1%)	FR (3)	DV
				AC (1)	VH
				RE (3)	LH
20	12	Gram + (low %GC)	*Bacillus oleronius* (93.2%)	FR (11)	DV
				OR (1)	VH
21	2	Not determined	Not determined	HO (1)	DV
				AC (1)	VH

22	74	*γ-Proteobacteria*	*Shewanella baltica* (98.7%)	AC (18), PE (20), WA (11), HI (20)	VH
				RE (5)	LH
23	74	*γ-Proteobacteria*	*Pseudomonas anguilliseptica, P. migulae* (95.8%)	FR (11)	DV
				AC (13), PE (13), WA (22), GR (1), HI (5)	VH
				RE (9)	LH
24	2	*β-Proteobacteria*	*Pseudomonas saccharophila* (97.2%)	DR	VH
25	6	*γ-Proteobacteria*	*Alteromonas macleodii* (93.9%)	PE (4), WA (1), HI (1)	VH
26	37	*γ-Proteobacteria*	*Pseudomonas orientalis* (99.5%)	FR (1), HO (10)	DH
				DR (3), GR (1), HI (5)	VH
				RE (17)	LH
27	2	Not determined	Not determined	GR	VH
28	13	*β-Proteobacteria*	*Janthinobacterium lividum* (99.7%)	FR (5), HO (8)	DV
29	3	*β-Proteobacteria*	*Hydrogenophaga palleronii* (98.4%), *Aquaspirillum delicatum* (97.8%)	FR (1)	DV
				AC (1)	VH
				RE (1)	LH
30	42	*β-Proteobacteria*	*Hydrogenophaga palleronii* (97.7%), *Janthinobacterium lividum* (99.6%)	FR (10), HO (3)	DV
				DR (8), AC (12), GR (1), PE (2)	VH
				RE (6)	LH
31	2	*γ-Proteobacteria*	*Pseudomonas syringae* (98.6%)	GR	VH
32	2	Not determined	Not determined	AC	VH
33	5	*α-Proteobacteria*	*Porphyrobacter neustonensis* (97.7%)	AC (3)	VH
				RE (2)	LH
34	3	*α-Proteobacteria*	*Porphyrobacter neustonensis* (97.6%)	AC (1)	VH
				RE (2)	LH
35	5	*α-Proteobacteria*	*Devosia riboflavina* (97.2)	RE	LH
36	8	*α-Proteobacteria*	*Sphingomonas natatoria* (94.5%)	AC (1)	VH
				RE (7)	LH
37	3	Not determined	Not determined	AC	VH
38	43	*γ-Proteobacteria*	*Psychrobacter glacincola* (97.5%)	FR (15), HO (1)	DV
				AC (3), OR (1), PE (5), HI (3)	VH
				RE (15)	LH
39	18	*γ-Proteobacteria*	*Marinobacter hydrocarbonoclasticus* (95.6%)	AC (7), OR (10), PE (1)	VH
40	16	*α-Proteobacteria*	*Brevundimonas subvibrioides* (99.1%)	FR (2)	DV
				DR (3), OR (1), PE (1)	VH
				RE (9)	LH
41	52	*α-Proteobacteria*	*Mesorhizobium loti* (96.9%), *Sulfitobacter pontiacus* (93.5%)	FR (10)	DV
				AC (15), OR (15), PE (4), WA (1), HI (2)	VH
				RE (5)	LH
NC	28	Not determined	Not determined	FR (8)	DV
				OR (2), PE (3), GR (1), AC (8), HI (1)	VH
				RE (5)	LH
Total	675				

[a] The 41 fatty acid clusters were delineated in Van Trappen *et al.* (2002). 28 of the tested strains (NC means not clustered) formed single branches.

[b] Data of cluster affiliation are reported on 675 bacterial strains out of the 723 screened in this paper. The fatty acid profiles of the remaining 48 strains -not listed in this table- were not clearly interpretable.

[c] The nearest phylogenetic neighbour was identified as described in Van Trappen et al (2002); (%) indicates the 16S rDNA sequence similarities based on pairwise alignments.

[d] Number of strains isolated from each lake is indicated in parenthesis. The abbreviations FR, HO, RE, AC, DR, GR, HI, PE, WA and OR stand for lakes Fryxell, Hoare, Reid, Ace, Druzby, Grace, Highway, Pendant, Watts and Organic.

[e] The abbreviations LH, VH, DV stand for Areas Larsemann Hills, Vestfold Hills and McMurdo Dry Valleys.

Table 2. Diversity of fungal taxa isolated in the different Antarctic areas.

Number of isolates tested	Order	Genus	Lake of origin [a]	Area [b]
71	*Erysiphales*	*Thelebolus*	AC (7), DR (6), HI (4), WA (1)	VH
			FR (9), HO (2)	DV
			MA (5), OR (17), RE (20)	LH
2	*Eurotiales*	*Aspergillus*	PE (1)	VH
			ST (1)	LH
16	*Eurotiales*	*Penicillium*	PE (12), WA (1), HI (1), AC (1)	LH
			OR (1)	VH
1	*Helotiales*	*Botrytis*	PE	VH
3	*Hypocreales*	*Beauveria*	PE (1), WA (2)	VH
1	*Mytosporic Ascomycotina*	*Acremonium*	PE	VH
1	*Mytosporic Ascomycotina*	*Arthrinium*	PE	VH
17	*Mytosporic Ascomycotina*	*Cladosporium*	PE (8), AC (1)	VH
			RE (6), MA (2)	LH
2	*Mytosporic Ascomycotina*	*Phialophora*	WA	VH
9	*Mytosporic Ascomycotina*	*Phoma*	DR (3)	VH
			MA (3), RE (3)	LH
4	*Onygenales*	*Geomyces*	PE (2), AC (2)	VH
1	*Pleosporales*	*Alternaria*	PE	VH
5	*Pleosporales*	*Curvularia*	PE (1)	VH
			MA (4)	LH
1	*Pleosporales*	*Embellisia*	FR	DV
1	*Onychophora* (velvet worm)	*Onychophora*	FR	DR
23		Non-identified	PE (4), HI (2), AC (1), WA (4)	VH
			RE (15)	LH
Total 158				

[a] Number of strains isolated from each lake is indicated in parenthesis. The abbreviations FR, HO, RE, AC, DR, HI, PE, WA, MA, OR and ST stand for lakes Fryxell, Hoare, Reid, Ace, Druzby, Highway, Pendant, Watts, Manning, Organic and Sarah Tarn.

[b] The abbreviations LH, VH, DV stand for Areas Larsemann Hills, Vestfold Hills and McMurdo Dry Valleys.

compositions might be ubiquitous in Antarctic lakes.

The diversity of fungal Antarctic strains was determined on the basis of the taxonomical description provided by Göttlich et al. (2003). Table 2 shows the taxonomic distribution of fungi isolated from the three geographic areas. *Thelebolus* sp. was the predominant species in all the three regions sampled. *Thelebolus* was almost the only genus recovered from the old and isolated meromictic lakes of McMurdo Dry Valleys. Members of the most widely distributed genera such as *Penicillium*, *Aspergillus*, *Phoma*, *Cladosporium, Curvularia* were isolated in the Larsemann and Vestfold Hills areas. In terms of distribution of the isolated fungi among different genera, lakes in the Vestfold Hills turned out to be the most productive, as some representatives of genera *Botrytis*, *Beauveria*, *Acremonium*, *Arthrinium*, *Phialophora, Geomyces, Alternaria* were isolated only from these sources.

Cultivation of Antarctic isolates

The cultivation of Antarctic heterotrophic bacteria and filamentous fungi was optimized in terms of medium composition and incubation temperature by a screening of different nutrients in pre-culture and fermentative media and by varying growth and production temperatures (data not shown). Major changes in the fermentation conditions were the introduction of less concentrated media and the addition of marine salts, which are common adaptations of classical medium composition to the cultivation of marine microbes (Sponga et al. 1999; Bernan et al. 1997). About half of the bacteria grew better in marine derived media whereas for the other half rich classical media were suitable. In any cultivation condition used, all the bacteria and fungi produced a high biomass. When growth was monitored at different temperatures (4°C, 10°C, 22°C, 28°C), optimal temperatures were in the range of 22 to 28°C, suggesting that all these isolates are psychrotolerant rather than psychrophilic. However, *Thelebolus* strains produced more biomass around 20°C but sporulated at 10°C, indicating true psychrophily.

Antimicrobial activity

A total of 6,348 and 1,422 samples were prepared from the fermentation of the 723 bacteria and 158 fungi, respectively. Up to four different sample preparation methods - three based on the extraction by/with solvents with different polarity and another one consisting in the adsorption/elution to polystyrenic resin - were used to widen/favour the recovery of secondary metabolites with different molecular weights and lipo/hydrophilic properties/polarities. These samples were tested for growth inhibition against a panel of human pathogenic microorganisms (*Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*) either in solid or liquid assay formats.

Table 3 reports the frequency of microbiological activity against each test pathogen among the heterotrophic bacteria. From 124 bacteria out of 723 that were active, 110 produced only antibacterial activities and 14 showed some inhibition of the tested fungal strains. The frequency of antibacterial activity was normal against the Gram-positive *S. aureus* (13.2%) and high against the Gram-negative *E. coli* (7.4%). The active strains were distributed among 21 clusters out of the 41 above described and in several lineages in the alpha, beta and gamma subclasses of the *Proteobacteria*, the *Bacteroidetes*, and the high and low percentage G+C Gram-positives. Almost 64 % of the active bacteria (79 isolates) were members of the 6 major fatty acid clusters (5, 22, 23, 26, 30, 41) that contained isolates widely distributed in the different sampling areas (see Table 1). In contrast, 39 active cultures were associated to 15 minor clusters; 4 strains formed single branches and 2 belonged to the not clustered group.

Further studies were performed with a subset of these strains (hits) that exhibited some relatively potent antibacterial activities against bacterial human pathogens (Table 4). None of the selected strains showed antifungal activity in the production conditions tested (data not shown). Two isolates (R-7513 and R-7941) obtained in the McMurdo Dry Valleys, specifically in the lakes Fryxell and Hoare, were studied more in detail. The isolates corresponded to coccoid high %GC Gram positives, which exhibited antibacterial activity against *B. subtilis*, *S. aureus* and *E. faecium*. Both strains, although isolated in different lakes, turned out to have similar fatty acid composition and 99.6 % 16SrDNA sequence similarity to *Arthrobacter agilis.* They apparently produced similar compounds. Preliminary mass spectrometry of purified extracts suggests these compounds to be cyclic

Table 3. Distribution of isolates producing antimicrobial activities among bacterial taxa.

Phylogenetic branch	FAA Cluster [a]	Nearest phylogenetic neighbour [b]	Strains tested [c]	Active Strains	N° of Strains producing antibacterial activities on:			N°of Strains producing antifungal activities on:		
					S. aureus	*E. faecum*	*E. coli*	*C. albicans*	*C. neoformans*	*A. fumigatus*
Bacteroidetes	**5**	*Flavobacterium gillisiae* (98.4%), *F. hydatis* (97.3%), *F. frigidarium* (97.5%)	69	17	13	2	12	0	0	1
Bacteroidetes	**7**	*Flavobacterium hydatis* (96.9%)	4	2	2	0	1	0	0	0
Bacteroidetes	**9**	*Flavobacterium gillisiae* (94.6%)	2	1	1	1	0	0	0	0
Bacteroidetes	**10**	*Flavobacterium flevense* (95.3%)	25	4	3	1	1	0	0	0
Bacteroidetes	**14**	*Hymenobacter actinosclerus* (97.3%)	6	2	2	1	0	0	0	1
Gram + (high %GC)	**16**	*Microbacterium keratanolyticum* (96.0%)	9	1	1	0	0	0	0	0
Gram + (high %GC)	**17**	*Micrococcus luteus* (99.7%)	5	1	1	0	0	0	0	0
Gram + (high %GC)	**18**	*Arthrobacter agilis* (99.6%)	64	5	3	3	3	0	0	0
Gram + (low %GC)	**20**	*Bacillus oleronius* (93.2%)	12	3	1	1	3	0	0	0
α-Proteobacteria	**40**	*Brevundimonas subvibrioides* (99.1%)	16	3	3	0	2	0	0	0
α-Proteobacteria	**41**	*Mesorhizobium loti* (96.9%), *Sulfitobacter pontiacus* (93.5%)	52	9	9	2	5	0	0	0
β-Proteobacteria	**24**	*Pseudomonas saccharophila* (97.2%)	2	2	2	0	2	0	0	0
β-Proteobacteria	**28**	*Janthinobacterium lividum* (99.7%)	13	6	6	1	0	1	5	0
β-Proteobacteria	**29**	*Hydrogenophaga palleronii* (98.4%), *Aquaspirillum delicatum* (97.8%)	3	1	1	0	0	0	0	0
β-Proteobacteria	**30**	*Hydrogenophaga palleronii* (97.7%), *Janthinobacterium lividum* (99.6%)	42	11	11	1	3	1	1	1
γ-Proteobacteria	**22**	*Shewanella baltica* (98.7%)	74	19	17	0	7	0	2	0
γ-Proteobacteria	**23**	*Pseudomonas anguilliseptica, P. migulae* (95.8%)	74	10	5	1	8	0	0	0
γ-Proteobacteria	**26**	*Pseudomonas orientalis* (99.5%)	37	13	6	7	7	0	0	3
γ-Proteobacteria	**38**	*Psychrobacter glacincola* (97.5%)	43	5	2	3	4	0	0	0
γ-Proteobacteria	**39**	*Marinobacter hydrocarbonoclasticus* (95.6%)	18	2	2	0	0	0	0	0
Not determined	**8**	Not determined	1	1	1	0	0	0	0	0
Not determined	**NC**	Not determined	28	4	3	0	2	0	0	0
Not determined	**N I**	Not determined	48	2	1	0	1	0	0	0
	Total		647	**124**	**96**	**24**	**61**	**2**	**8**	**6**

[a] The fatty acid clusters were delineated in Van Trappen *et al.* (2002). In this table, only those fatty acid clusters comprising active isolates are listed, i.e. 21 out of the 41 previously described. 28 of the tested strains (NC) formed single branches. The fatty acid profiles of the remaining 48 strains were not clearly interpretable (NI). See also Table 1.

[b] The nearest phylogenetic neighbour was identified as described in Van Trappen et al (2002); (%) indicates the 16S rDNA sequence similarities based on pairwise alignments.

[c] Total strains tested per each of the 21clusters comprising active isolates.

Table 4. Origin, taxonomical affiliation and antibacterial activities of selected bacterial isolates (hits).

Area	Lake	Strain Code [a]	FAA Cluster	Phylogenetic branch	Nearest phylogenetic neighbour	Antibacterial activities in agar diffusion assays (mm) on: [b]		
						S. aureus	*E. faecium*	*E. coli*
Dry Valleys	Fryxell	R-7513	18	Gram + (high %GC)	*Arthrobacter agilis* (99.6%)	10	11	0
Dry Valleys	Hoare	R-7687	28	*β-Proteobacteria*	*Janthinobacterium lividum* (99.7%)	7	8	6
Dry Valleys	Hoare	R-7941	18	Gram + (high %GC)	*Arthrobacter agilis* (99.6%)	7	8	0
Vestfold Hills	Pendant	R-8990	22	*γ-Proteobacteria*	*Shewanella baltica* (98.7%)	7	0	7
Larsemann Hills	Reid	R-12565	26	*γ-Proteobacteria*	*Pseudomonas orientalis* (99.5%)	7	6	7
Larsemann Hills	Reid	R-12533	26	*γ-Proteobacteria*	*Pseudomonas orientalis* (99.5%)	6	8	6
Larsemann Hills	Reid	R-12535	26	*γ-Proteobacteria*	*Pseudomonas orientalis* (99.5%)	5	0	8
Larsemann Hills	Reid	R-12605	22	*γ-Proteobacteria*	*Shewanella baltica* (98.7%)	7	0	9
Larsemann Hills	Reid	R-12597	38	*γ-Proteobacteria*	*Psychrobacter glacincola* (97.5%)	5	0	6
Larsemann Hills	Reid	R-12583	NC	Not determined	Not determined	9	5	9

[a] As preserved in the research collection of the Laboratory of Microbiology, University of Ghent, Belgium.

[b] Antibacterial activities were indicated as the diameter in mm of the inhibition zone obtained in agar diffusion assays.

thiazolyl peptide antibiotics with similar antibacterial spectrum of activity against Gram positive bacteria (Z. Guan, personal communication). The other isolate (R-7687) from Lake Hoare in the McMurdo Dry Valleys belonged to the beta-*Proteobacteria* and differs from the previous ones for the activity on *E. coli*. Seven other isolates, six from Lake Reid and one from Pendant, were also particularly active against Gram-positive and Gram-negative pathogens. Three of them are similar to *Pseudomonas orientalis,* whereas the others belonged to taxa isolated from cold, aquatic environments such as *Shewanella baltica* and *Psychrobacter glacincola.*

Table 5 shows the distribution of the antimicrobial activities among filamentous fungi belonging to different species, genera and orders. The frequency of antimicrobial activities was more than 29 % in total. As in the case of bacteria, the frequency of antibacterial activity was high against the Gram-positive *S. aureus* (14 %) and the Gram-negative *E. coli* (10 %). In contrast to bacteria, high antifungal activities against *C. albicans, A. fumigatus* and *C. neoformans* were obtained from these fungal isolates. It is worth noting that although *Thelebolus* was the taxon most frequently isolated (71 strains tested originated from nine lakes in the three different areas), it was one of the less productive (only 11 active isolates, 15 %) in contrast to *Penicillium* and *Cladosporium*, two of the most active groups that were isolated from the Vestfold Hills and Larsemann Hills (see Table 2). *Penicillium* spp. were among the most active species with a rate of 93 % active strains, while lower percentages were obtained with *Cladosporium* spp. (35 %), two genera where comparable numbers of strains were tested.

Since many fungi are good producers of toxins, a cytotoxicity test based on the inhibition of labeled thymidine uptake in HeLa was introduced to select molecules not toxic to mammalian cells among those active extracts coming from fungi. Two thirds of the fungal extracts showed a high cytotoxicity against eukaryotic cells (data not shown). Table 6 reports the cytotoxicity test results and the antimicrobial spectrum of activity (expressed as end point inhibition) for eight selected fungi (hits). *Aspergillus clavatus* (IWW447) showed potent antibacterial and antifungal properties associated with a marked cytotoxic effect. This spectrum is typical of toxins produced by aspergilli and other fungi. The five selected *Penicillium* strains showed different antimicrobial profiles associated with lower values in the cytotoxicity test, demonstrating that although isolated in the same region, they may produce diverse metabolic compounds. More interesting for the discovery of new specifically acting anti-infectives are those metabolites showing

Table 5. Distribution of isolates producing antimicrobial activities among fungal taxa.

Order	Genus & species	Strains tested	Active strains	N° of Strains producing antibacterial activities on:			N° of Strains producing antifungal activities on:		
				S. aureus	*E. faecium*	*E. coli*	*C. albicans*	*C. neoformans*	*A. fumigatus*
Leotiales	*Thelebolus* sp.	71	11	6	0	0	5	4	1
Eurotiales	*Aspergillus clavatus*	1	1	1	1	1	1	1	0
Eurotiales	*Aspergillus niger*	1	0	0	0	0	0	0	0
Eurotiales	*Penicillium chrysogenum*	6	6	6	0	1	0	0	1
Eurotiales	*Penicillium crustosum*	2	2	0	0	1	1	0	0
Eurotiales	*Penicillium rugulosum*	1	1	1	1	1	0	0	0
Eurotiales	*Penicillium* sp.	7	6	1	0	5	3	3	0
Helotiales	*Botrytis* sp.	1	1	0	0	1	0	0	0
Hypocreales	*Beauveria* sp.	3	2	0	0	0	1	1	1
Mitosporic Ascomycotina	*Acremonium* sp.	1	0	0	0	0	0	0	0
Mitosporic Ascomycotina	*Arthrinium* sp.	1	0	0	0	0	0	0	0
Dothideales	*Cladosporium* sp.	8	6	4	0	2	1	1	0
Dothideales	*Cladosporium herbarum*	9	0	0	0	0	0	0	0
Mitosporic Ascomycotina	*Phialophora* sp.	2	0	0	0	0	0	0	0
Mitosporic Ascomycotina	*Phoma* sp.	9	3	3	0	2	0	1	0
Onygenales	*Geomyces pannorum*	3	1	0	0	0	1	1	0
Onygenales	*Geomyces* sp.	1	0	0	0	0	0	0	0
Pleosporales	*Alternaria* sp.	1	0	0	0	0	0	0	0
Pleosporales	*Curvularia* sp.	5	1	0	0	0	1	0	0
Pleosporales	*Embellisia* sp.	1	0	0	0	0	0	0	0
Onychophora (velvet worm)	*Onychophora* sp.	1	0	0	0	0	0	0	0
	Non identified	23	6	0	0	2	3	0	4
	Total	**158**	**47**	**22**	**2**	**16**	**17**	**12**	**7**
	%		29	14	1	10	11	8	4

Table 6. Origin, taxonomical affiliation, cytotoxicity and antimicrobial activities of selected fungal isolates (hits).

Area	Lake	Strain Code [a]	Strain	Cytotoxi-city test [b]	Antibacterial activities in liquid microtiter plate assay on: [c]			Antifungal activities in liquid microtiter plate assay on: [c]		
					S. aureus	*E. faecium*	*E. coli*	*C. albicans*	*C. neoformans*	*A. fumigatus*
Larsemann Hills	Sarah Tarn	IWW447	*Aspergillus clavatus*	160	2048	512	1024	128	2048	ND
Vestfold Hills	Highway	IWW1035	*Penicillium chrysogenum*	10	32	8	8192	0	0	0
Vestfold Hills	Watts	IWW1023	*Penicillium crustosum*	10	0	0	16	0	0	0
Vestfold Hills	Pendant	IWW1054	*Penicillium* sp.	10	0	0	8	8	16	0
Vestfold Hills	Pendant	IWW1055	*Penicillium* sp.	20	32	0	8	0	0	16
Vestfold Hills	Ace	IWW1059	*Penicillium* sp.	20	0	0	8	16	16	0
Vestfold Hills	Pendant	IWW1017	*Beauveria* sp.	<10	0	0	0	0	8	0
Vestfold Hills	Pendant	IWW1019	*Cladosporium* sp.	<10	0	0	16	0	0	0

[a] As preserved in the IWW Rheinisch Westfalisches Institut fur Wasserforschung Gemeinnutzige.

[b] Cytotoxicity was measured as endpoint in microdilution method, i.e. the highest dilution which inhibits 40% of HeLa cell thymidine uptake.

[c] Antibacterial and antifungal activities were measured as endpoints in microdilution method, i.e. the highest dilution which inhibits 80% of test strain growth.

lower but more specific and not cytotoxic activities, such as the antifungal characteristics of *Beauveria* (IWW1017) or the specific *E. coli* inhibition effect of *Cladosporium* extract (IWW1019).

Discussion and general considerations

Although Antarctic lakes have been for a long time considered as inhospitable and isolated environments inhabited by species-poor communities, studies funded by the EC project MICROMAT revealed the presence of a large microbial diversity, especially for prokaryotes. Results from this interdisciplinary and polyphasic approach, of which part has been published up to now (Van Trappen et al. 2002; Van Trappen et al. 2003; Tindall et al. 2000; Brambilla et al. 2001; Göttlich et al. 2003) showed that bacterial diversity in the sampled mats from Antarctic lakes is extremely high and novel phylotypes were discovered. The observed lower eukaryotic diversity was indeed dominated by a few but highly specialized and often endemic taxa. The overall evidence supported the strategy for industrial screening of these fresh isolates as unexplored source of biotechnologically valuable bioactive molecules.

The main purpose of the studies reported in this paper was to evaluate and compare the ability of Antarctic bacteria and fungi to produce anti-microbial molecules. The frequency of the antimicrobial activities produced by these isolates was considered to be an indicator of their capability to produce anti-infective procedures already in place at Biosearch Italia S.p.A. (now Vicuron Pharmaceuticals) and Merck Research Centers and the resulting frequencies of antimicrobial activities were thus comparable with those routinely achieved by "in house" screening of thousands actinomycetes and fungi isolated from different sources.

Antarctic bacteria have been extensively isolated by using enrichment methods under oligotrophic and psychrotrophic conditions, from freshwater lakes, saline and hypersaline lakes and ponds, soil, sandstone and sea ice (Friedmann 1993; Tan et al. 1996; Tan et al. 1999; Bowman et al. 1997; Bowman et al. 2000; Gosink and Staley 1995; Mergaert et al. 2001b; Murray 1998; Tindall et al. 2000; Brambilla et al. 2001; Staley and Gosink 1999; Wery et al. 2003). Several new species have been isolated from Antarctic benthic microbial communities, e.g. *Flavobacterium gelidilacus* (Van Trappen et al. 2003), *Flavobacterium degerlachei,*

Flavobacterium frigoris, *Flavobacterium micromati* (Van Trappen et al. 2004a), *Gillisia limnaea* (Van Trappen et al. 2004b), *Flavobacterium tegetincola* (McCammon and Bowman 2000), *Arthrobacter flavus* (Reddy et al 2000), *Rhodoferax antarcticus* (Madigan et al. 2000), and the anaerobes *Psychromonas antarcticus* (Mountfort et al. 1998) and *Clostridium vincentii* (Mountfort et al. 1997). Indeed, only few studies were devoted to the high-throughput cultivation and screening of Antarctic microbial isolates (Ashbolt 1990; Bull et al. 2000). In this work, the 723 Antarctic bacteria belonging to 41 fatty acid clusters and 28 single branches and phylogenetically affiliated with 24 lineages in the alpha, beta and gamma *Proteobacteria*, the *Bacteroidetes*, and the high and low percentage G+C Gram-positives, were massively cultivated and screened for their antimicrobial activities. The nearest validly named phylogenetic neighbours of these strains often belonged to taxa isolated from cold, aquatic environments, such as *Shewanella baltica*, *Psychrobacter glacincola*, *Sulfitobacter pontiacus, Flavobacterium frigidarium, Flavobacterium gillisiae, Salegentibacter salegens, Gelidibacter algens.* Sequences from many strains showed pairwise sequence similarities of less than 97 % to their nearest validly named neighbours (Van Trappen et al. 2002), indicating that they represent taxa that have not been sequenced yet or as yet unnamed new taxa, related to *Alteromonas*, *Bacillus, Clavibacter*, *Cyclobacterium*, *Flavobacterium*, *Marinobacter*, *Mesorhizobium*, *Microbacterium*, *Pseudomonas*, *Salegentibacter*, *Sphingomonas* and *Sulfitobacter*. These results on the taxonomic diversity and novelty of these isolates supported that they constitute a unique biotechnologically exploitable collection.

From the screening results we observed a high percentage of antibacterial activities (*ca.* 15 %) that contrasts with the few producers of antifungal metabolites (almost 2 %). These frequencies were comparable to the ones observed in the screening of soil actinomycetes, which are considered the most prolific and versatile microbial source of antibiotics (Waksman and Lechevalier 1962; Axelrood et al. 1996; Lazzarini et al. 2000; Sponga et al. 1999), supporting the idea that mats from these different lakes contain a rich prokaryotic diversity where the antibiotic production can confer survival advantage. This is indeed not surprising if we consider that these complex microbial communities dominated by prokaryotes have accumulated during thousands of years and bacteria were confronted with extreme conditions, such as low temperatures, freezing-thawing cycles, UV-irradiation, desiccation and varying light conditions, salinities and nutrient concentrations. As a consequence they have been under a high selective pressure

and may belong to indigenous new taxa with potentially novel biochemical adaptations.

Distribution of fungi in Antarctica was previously studied mainly in environments such as mosses, lichen communities or in relation to the distribution of "hosts" such as birds, penguins and invertebrates (Del Frate and Caretta 1990; Tosi et al. 2002; Vishniac 1993). Most of the filamentous fungi and yeasts described by these authors are cosmopolitan and cold tolerant, but some such as *Thelebolus* appear to be indigenous species (Del Frate and Caretta 1990). Our investigations (Göttlich et al. 2003) revealed that benthic mats are dominated by a relatively small number of fungal species, given the high diversity in eubacteria in the same lakes and compared to the number of species known in the fungal kingdom. None of the filamentous fungi proved to be truly psychrophilic, except *Thelebolus* strains. They often produced markedly pigmented mycelia, probably to protect themselves from strong UV irradiation (Hughes et al. 2003). Also the production of abundant and resistant spores typical of some of these genera such as *Cladosporium* and *Geomyces* may represent a survival tool to desiccation, to low temperatures or to the presence of high saline concentrations such as it occurs in the lakes of the Vestfold Hills. Finally some species such as *Thelebolus* and *Geomyces* previously isolated by other authors from Antarctic soils or other material such as mosses (Del Frate and Caretta 1990; Tosi et al. 2002) showed a high tolerance to low temperatures.

The frequency of antibacterial and antifungal activities detected among fungal isolates (29 % active isolates) was higher than the one usually detected in the screening of other ecological groups of fungal isolates (Suay et al. 2000, Sponga et al. 1999). Valuable activities were noted against *C. albicans*, *C. neoformans* or *A. fumigatus* and it is interesting to note that many yeast-like organisms were isolated from Antarctic samples: they often were true psychrophiles and among them were the most frequently isolated genera such as *Candida* and particularly *Cryptococcus* (E. Göttlich and G.S.de Hoog, personal communication; Tosi et al. 2002). Possessing metabolic potential to produce active molecules against yeasts can confer a competitive advantage to local filamentous fungi, which turned out to be a further appealing aspect for the biotechnological exploitation of such isolates.

Further work is now in progress on the chemical and biological profiling of metabolites produced by the selected "hits" among these Antarctic fungi and bacteria, which show an interesting antimicrobial spectrum of activities. The

assessment of their novelty will help to understand to which extent chemical diversity correlates with the taxonomical diversity so far discovered in these Antarctic benthic microbial communities.

Annex V

Origin of bacterial isolates from Arctic and Antarctic seas

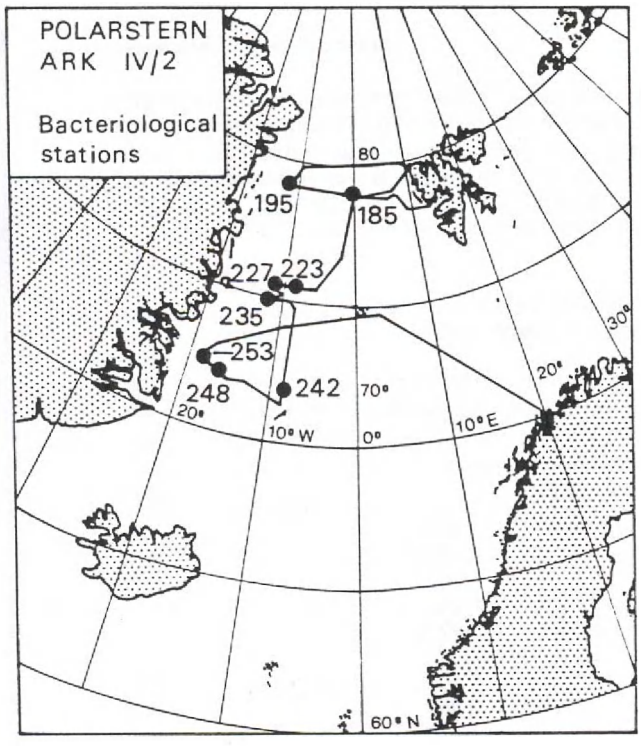

Figure 1. Bacteriological stations in Fram Strait and the Western Greenland Sea (from Tan & Rüger, 1991).

Station No.	Water depth [m]	Position of Station	Enrichment Technique	Strain No. ARK
223	25	75°33.3'N; 08°48.8'W	Dialysis	176; 179; 180; 184; 186; 188; 189; 190; 193; 194; 195; 196; 197; 199; 201; 204; 205; 206; 212; 213; 215; 217; 219; 222
223	200		Dialysis	164; 165; 166; 167; 168; 169; 170; 171
223	1000		Dialysis	172; 173
235	25	75°09.4'N; 12°27.6'W	DoublePetri	105; 107; 109; 111; 112; 113; 115; 116; 119; 120; 122; 124
235	25		Dialysis	133; 137; 138
242	25	71°56.1'N; 08°21.1'W	DoublePetri	129; 131
242	200		Dialysis	145

Table 1. Origin of bacterial strains from the Western Greenland Sea (from Tan, 1997).

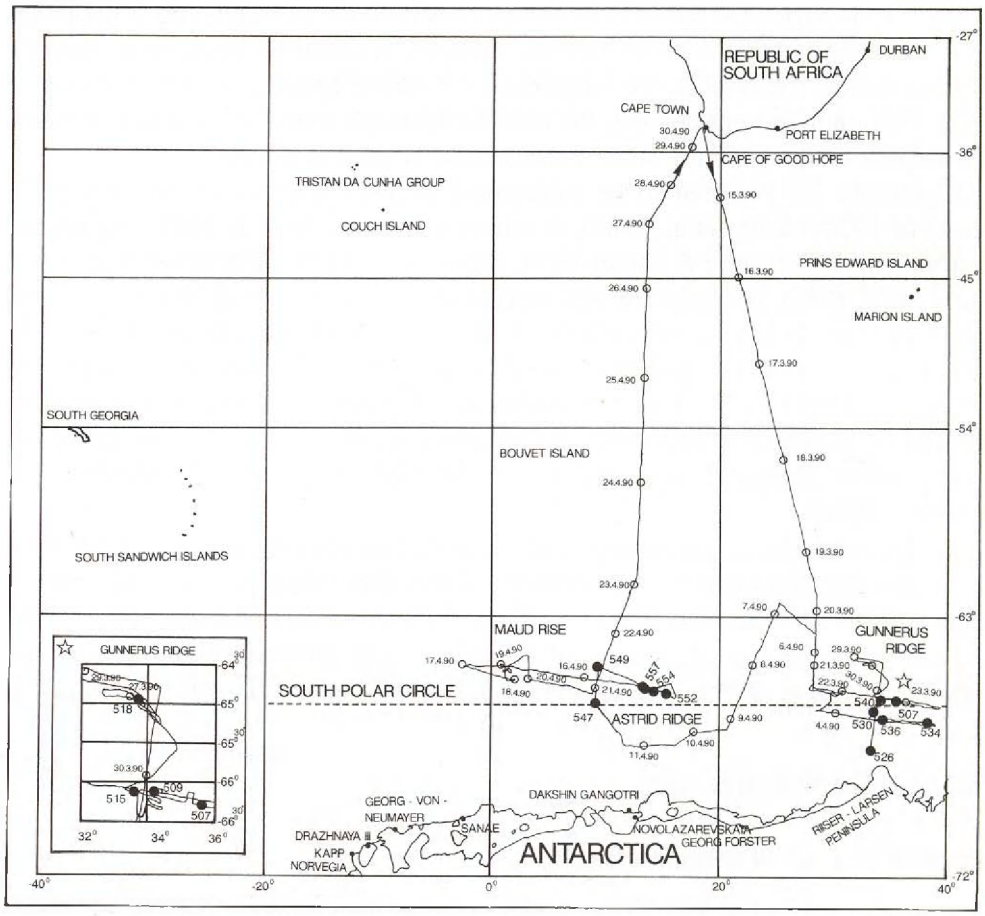

Figure 2. Cruise track of RV POLARSTERN during Leg ANT-VIII/6 and positions of bacteriological stations (from Tan *et al.*, 1999).

Station no. 16/	Sampling date	Water depth, m	Position of station	Strain no. AN
507	03.24.90	025	66°11.2´S; 35°18.5´E	011; 021; 031; 041; 042; 051; 061; 071; 081; 091
509	03.25.90	025 100	66°06.7´S; 34°17.8´E	101; 111; 121; 122; 131; 141; 151 161; 184; 193
518	03.28.90	025 100	64°57.4´S; 33°37.4´E	251; 261; 262; 271; 273; 281; 291 331; 341; 351
526	03.31.90	025	67°44.1´S; 33°17.9´E	401; 411; 441; 451; 461; 481
530	04.01.90	025	66°21.9´S; 33°46.7´E	521; 541; 542
534	04.02.90	025	67°03.9´S; 37°27.6´E	581; 602
535	04.03.90	025	66°55.1´S; 34°18.2´E	621
547	04.12.90	025	66°20.0´S; 08°53.4´E	691; 701; 702
549	04.13.90	025	65°01.6´S; 09°11.2´E	731; 741
554	04.15.90	025	65°49.3´S; 14°08.5´E	802; 811; 812
557	04.15.90	025	65°44.7´S; 13°39.6´E	821; 822; 832; 841; 843

Table 2. Origin of oligotrophic Antarctic strains from enrichment cultures in dialysis chambers (from Tan & Rüger, 1999).

Annex VI

Dendrograms of Antarctic lake and polar sea isolates

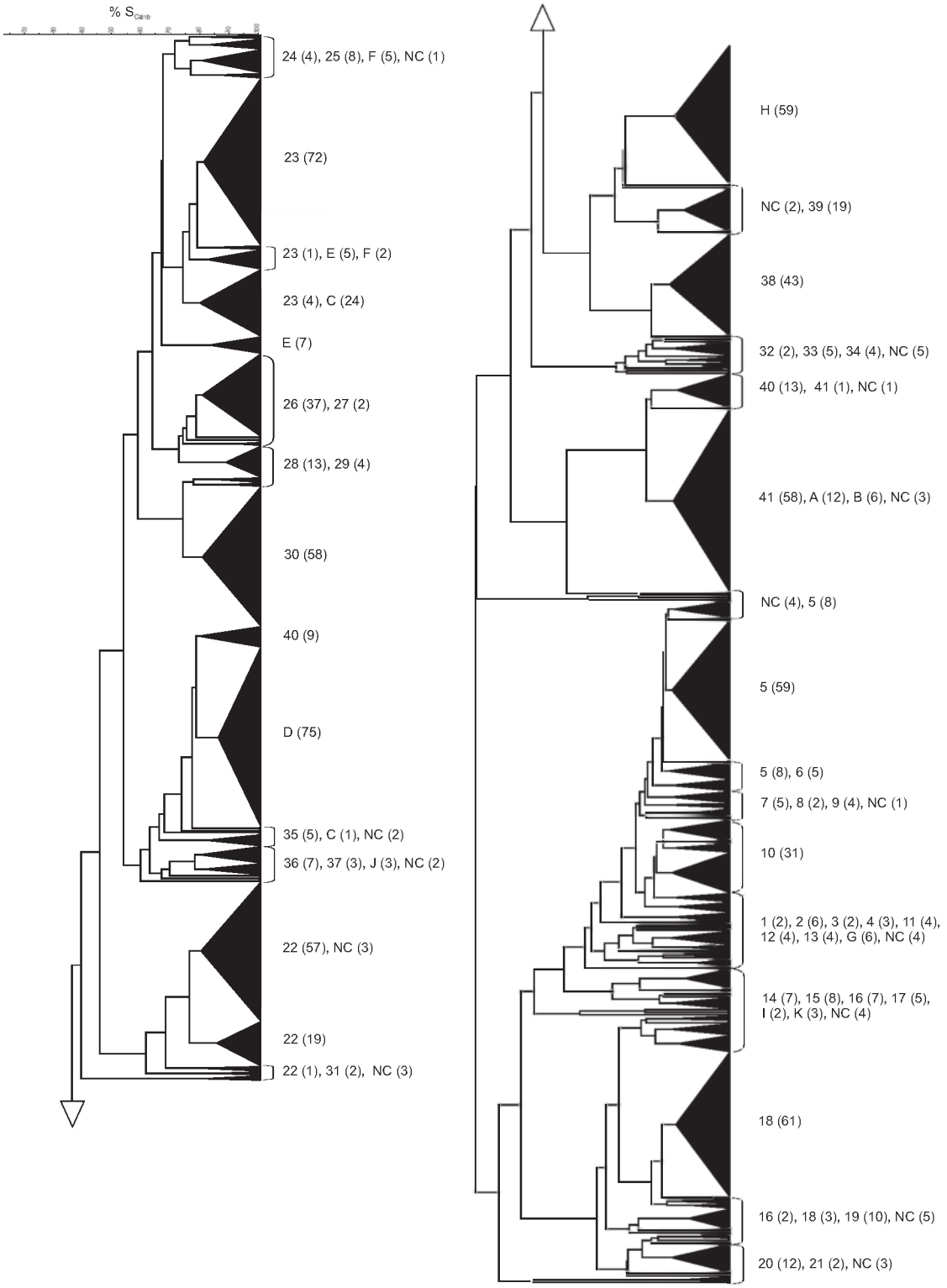

Figure 1. Abridged dendrogram obtained by numerical analysis of the fatty acid compositions of all Antarctic lake and polar sea isolates (975) using the Canberra metric similarity coefficient (S_{canb}) and UPGMA clustering. Clusters were delineated at a cut-off value of 80% and numbered as described in Van Trappen *et al.* (2002) and Mergaert *et al.* (2001b). Number of strains per cluster are indicated between brackets.

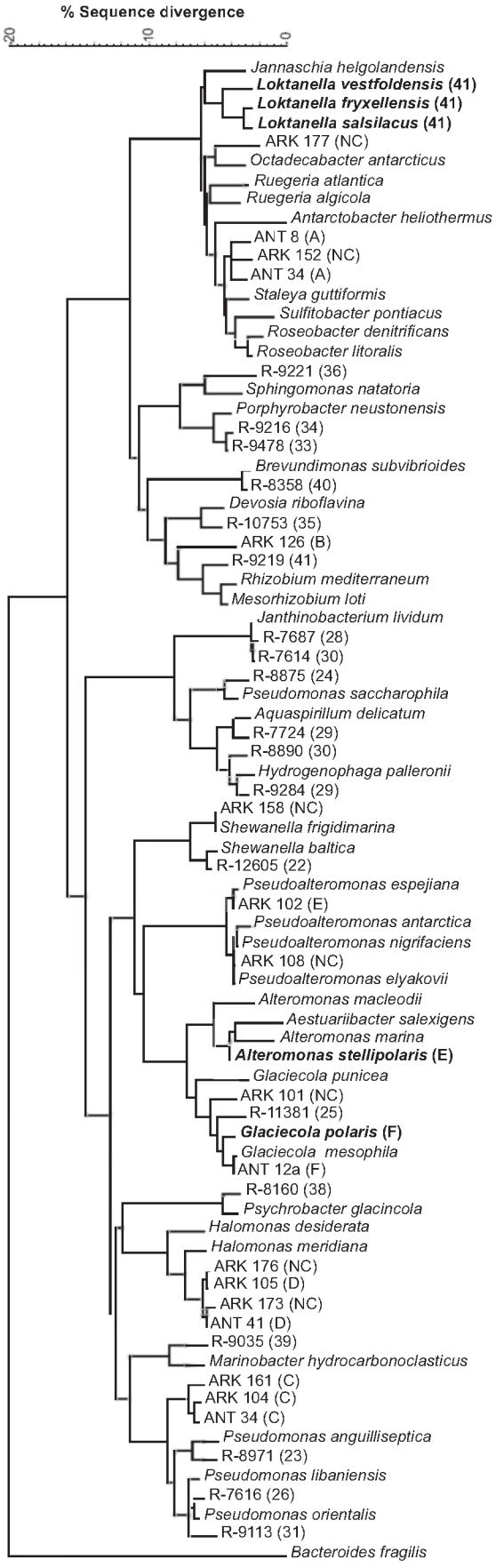

Figure 2. Neighbour-joining dendrogram based on all 16S rDNA sequences of the Antarctic lake and polar sea isolates belonging to the *Proteobacteria* using *Bacteroides fragilis* as an outgroup. Bar, 20% sequence divergence. Between brackets: the clusters as delineated in Van Trappen *et al.* (2002) and Mergaert *et al.* (2001b). In bold face: the novel taxa described in this study.

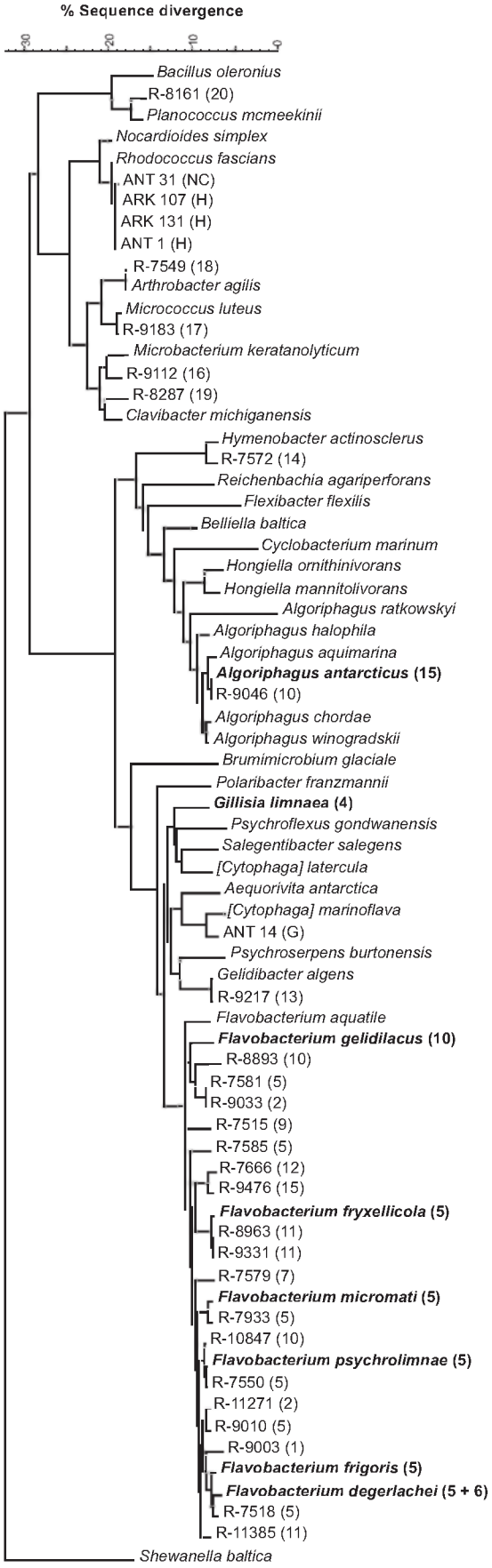

Figure 3. Neighbour-joining dendrogram based on all 16S rDNA sequences of the Antarctic lake and polar sea isolates belonging to the *Bacteroidetes* and Gram-positives using *Shewanella baltica* as an outgroup. Bar, 30% sequence divergence. Between brackets: the clusters as delineated in Van Trappen *et al.* (2002) and Mergaert *et al.* (2001b). In bold face: the novel taxa described in this study.

Curriculum vitae

Personalia

Van Trappen Stefanie
Born on 19 September 1978, Ronse

Education

1990-1996
O.-L.-Vrouwinstituut zusters Bernardinnen, Oudenaarde, Sciences (Latin-Sciences)

1996-2000
Ghent University, licentiate in the Biotechnology
Thesis: Identificatie van *Burkholderia cepacia*-achtige bacteriën geïsoleerd uit mucoviscidosepatiënten en de omgeving d.m.v. PCR-gesteunde technieken.

Professional Employment

2000- present
Ghent University, PhD student. Involved in the following research projects:

MICROMAT-project (BIO4-CT98-0040) within the EU Framework programme IV: Biodiversity of microbial mats in Antarctica, 1 November 1998- 24 February 2001.

Study of oligotrophic bacteria in polar seas in cooperation with T. L. Tan from the Alfred Wegener Institut für Polar- und Meeresforschung ("AWI", Bremerhaven).

BOF-project (Bijzonder Onderzoeksfonds, Universiteit Gent): Prokaryotische diversiteit in polaire zeeën en Antarctische meren, 1 januari 2001- 31 december 2003.

Publications

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Oral presentations

Mergaert, J., Van Trappen, S., Van Eygen, S., Cnockaert, M. & Swings, J. (2001). Diversity of culturable heterotrophic bacteria in Antarctic microbial mats. MICROMAT meeting, 22- 23 February, Gerenzano.

Van Trappen, S., Mergaert, J. & Swings, J. (2001). Genomic diversity of heterotrophic bacteria in microbial mats from Antarctic lakes. VIII SCAR International Biology Symposium, Antarctic Biology in a Global Context, Amsterdam.

Poster presentations

Van Trappen, S., Mergaert, J., & Swings, J. (2001). Biodiversity of heterotrophic bacteria in microbial mats from Antarctic Lakes. Abstract European Platform for Biodiversity Strategy, Scientific tools for biodiversity conservation: monitoring, modelling and experiments, 2-4 December, Brussels, Belgium.

Van Trappen, S., Mergaert, J., & Swings, J. (2001). Diversity of heterotrophic bacteria in microbial mats from Antarctic Lakes. Abstract 5th Symposium Belgian Society for Microbiology, Towards Understanding Microbial Interactions, 9 December, Louvain-la-Neuve, Belgium.

Van Trappen, S., Mergaert, J. & Swings, J. (2002). Diversity of Flavobacteria in microbial mats from Antarctic lakes. Xth International Congress of Bacteriology and Applied Microbiology, Joint Meeting of the three divisions of the IUMS, 27 July - 1 August; Paris, France, Abstract B-304.

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