BACTERIAL COMMUNITIES IN GLACIER FOREFIELDS OF THE LARSEMANN HILLS, EAST ANTARCTICA:

STRUCTURE, DEVELOPMENT & ADAPTATION

Dissertation von Felizitas Bajerski

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Bacterial Communities in Glacier Forefields of the Larsemann Hills, East Antarctica: Structure, Development & Adaptation

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„Zwei Dinge sind zu unserer Arbeit nötig: Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen.“

(Albert Einstein)

Preface

This study was supported by the Deutsche Forschungsgemeinschaft (DFG) in the framework of the priority programme 1158 “Antarctic Research with Comparative Investigations in Arctic Ice Areas” by a grant to Dirk Wagner (WA 1554/9) and is primarily focused on the structure and development of bacterial communities in deglaciated areas of the Larsemann Hills in East Antarctica.

Two glacier forefields on Broknes Peninsula were chosen for analysis in this study and sampled during the expedition ANT-XXIII/9 of the research vessel "Polarstern" to Antarctica in March 2007. The laboratory work was performed at the Alfred Wegener Institute for Polar and Marine Research, Research Unit Potsdam. This thesis is written in English, organised as a cumulative dissertation and submitted at the Faculty of Mathematics and Natural Science at the University of Potsdam.

The results of this work are presented in four manuscripts with first authorship, whereof three are accepted for publication and one is a manuscript draft. The content of all manuscripts is framed by an overall introduction and synthesis. The introduction states the fundamental hypothesis of the thesis including an overview of the scientific background, a description of the study site and a summary of the manuscripts. The synthesis addresses the questions arisen in the beginning by discussing the combined results of this work and finishes with some conclusions.

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Summary

Antarctic glacier forfields are extreme environments and pioneer sites for ecological succession. The Antarctic continent shows microbial community development as a natural laboratory because of its special environment, geographic isolation and little anthropogenic influence. Increasing temperatures due to global warming lead to enhanced deglaciation processes in cold-affected habitats and new terrain is becoming exposed to soil formation and accessible for microbial colonisation. Taking into account recent reported warming trends in Antarctica and considering the importance of microorganisms for the Antarctic ecosystem, it is essential to get more information about Antarctic terrestrial microbial communities. This study aims to understand the structure and development of glacier forefield bacterial communities, especially how soil parameters impact the microorganisms and how those are adapted to the extreme conditions of the habitat. To this effect, a combination of cultivation experiments, molecular, geophysical and geochemical analysis was applied to examine two glacier forfields of the Larsemann Hills, East Antarctica. Culture-independent molecular tools such as terminal restriction length polymorphism (T-RFLP), clone libraries and quantitative real-time PCR (qPCR) were used to determine bacterial diversity and distribution. Cultivation of yet unknown species was carried out to get insights in the physiology and adaptation of the microorganisms. Adaptation strategies of the microorganisms were studied by determining changes of the cell membrane phospholipid fatty acid (PLFA) inventory of an isolated bacterium in response to temperature and pH fluctuations and by measuring enzyme activity at low temperature in environmental soil samples.

The two studied glacier forefields are extreme habitats characterised by low temperatures, low water availability and small oligotrophic nutrient pools and represent sites of different bacterial succession in relation to soil parameters. The investigated sites showed microbial succession at an early step of soil formation near the ice tongue in comparison to closely located but rather older and more developed soil from the forefield. At the early step the succession is influenced by a deglaciation-dependent areal shift of soil parameters followed by a variable and prevalently depth-related distribution of the soil parameters that is driven by the extreme Antarctic conditions. The Culture-independent community analysis reveals a high bacterial diversity and abundance. The dominant taxa in the glacier forefields are *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Chloroflexi*. The connection of soil characteristics with bacterial community structure showed that soil parameter and soil formation

along the glacier forefield influence the distribution of certain phyla. Several groups like *Cyanobacteria*, *Proteobacteria* or *Gemmatimonadetes* depend on water availability, whereas the most dominant group of the forefields the *Actinobacteria* is related to the presence of trace elements. In the early step of succession the relative undifferentiated bacterial diversity reflects the undifferentiated soil development and has a high potential to shift according to past and present environmental conditions. With progressing development environmental constraints such as water or carbon limitation have a greater influence.

Adapting the culturing conditions to the cold and oligotrophic environment, the number of culturable heterotrophic bacteria reached up to 10^8 colony forming units per gram soil and 148 isolates were obtained. Two new psychrotolerant bacteria, *Herbaspirillum psychrotolerans* PB1^T and *Chryseobacterium frigidisoli* PB4^T, were characterised in detail and described as novel species in the family of *Oxalobacteraceae* and *Flavobacteriaceae*, respectively. The isolates are able to grow at low temperatures tolerating temperature fluctuations and they are not specialised to a certain substrate, therefore they are well-adapted to the cold and oligotrophic environment.

The adaptation strategies of the microorganisms were analysed in environmental samples and cultures focussing on extracellular enzyme activity at low temperature and PLFA analyses. Extracellular phosphatases (pH 11 and pH 6.5), β-glucosidase, invertase and urease activity were detected in the glacier forefield soils at low temperature (14°C) catalysing the conversion of various compounds providing necessary substrates and may further play a role in the soil formation and total carbon turnover of the habitat. The PLFA analysis of the newly isolated species *C. frigidisoli* showed that the cold-adapted strain develops different strategies to maintain the cell membrane function under changing environmental conditions by altering the PLFA inventory at different temperatures and pH values. A newly discovered fatty acid, which was not found in any other microorganism so far, significantly increased at decreasing temperature and low pH and thus plays an important role in the adaption of *C. frigidisoli*.

This work gives insights into the diversity, distribution and adaptation mechanisms of microbial communities in oligotrophic cold-affected soils and shows that Antarctic glacier forefields are suitable model systems to study bacterial colonisation in connection to soil formation.

Zusammenfassung

Gletschervorfelder der Antarktis stellen extreme Habitate dar und sind Pionierstandorte biologischer Sukzession. Insbesondere unter Berücksichtigung zuletzt beobachteter und vorausgesagter Erwärmungstrends in der Antarktis und der Relevanz der Mikroorganismen für das antarktische Ökosystem, ist es essentiell mehr Informationen über die Entwicklung frisch exponierter Gletschervorfelder zu erlangen. Ziel dieser Studie ist es, die Struktur und Entwicklung bakterieller Gletschervorfeldgemeinschaften zu verstehen, insbesondere wie die Mikroorganismen von den Bodenparametern beeinflusst werden und wie diese sich an die extremen Bedingungen des Habitats anpassen. Für die Untersuchung der Proben von zwei Gletschervorfeldern aus den Larsemann Bergen der Ostantarktis diente eine Kombination aus Kultivierungsexperimenten und molekularen, geophysikalischen und geochemischen Analysen. Die untersuchten Gletschervorfelder sind durch extrem niedrige Temperaturen, einer geringen biologischen Wasserverfügbarkeit und oligotrophe Nährstoffgehalte charakterisiert und zeigen unterschiedliche Entwicklungsstufen in Verbindung zu den Bodenparametern. In einem frühen Schritt der Bodenbildung in der Nähe der Gletscherzunge sind die Gemeinschaften undifferenziert, doch mit fortschreitender Entwicklung nimmt der Einfluss von Wasser- und Nährstofflimitationen zu. Nachdem die Kultivierungsbedingungen den kalten und nährstoffarmen Bedingungen des Habitats angepasst wurden, konnten 10^8 koloniebildende Einheiten heterotropher Bakterien pro Gramm Boden angereichert und daraus 148 Isolate gewonnen werden. Zwei neue psychrotolerante Bakterien, *Herbaspirillum psychrotolerans* PB1^T und *Chryseobacterium frigidisoli* PB4^T, wurden detailiert charakterisiert und als jeweils neue Arten beschrieben. Die Anpassungsstrategien der Mikroorganismen an die extremen antarktischen Bedingungen zeigten sich in der Aktivität extrazellulärer Enzyme bei niedriger Temperatur, die mit derer temperierter Habitate vergleichbar ist, und in der Fähigkeit der Mikroorganismen, die Fettsäurezusammensetzung der Zellmembran zu ändern. Eine neue Fettsäure, die bisher in keinen anderen Mikroorganismus gefunden wurde, spielt eine entscheidende Rolle in der Anpassung des neu-beschriebenen Bakteriums *C. frigidisoli* an niedrige Temperaturen und saure pH-Werte.

Diese Arbeit gibt einen Einblick in die Vielfalt, Verteilung und Anpassung mikrobieller Gemeinschaften in nährstoffarmen und Kälte-beeinflussten Habitaten und zeigt, dass antarktische Gletschervorfelder geeignete Modellsysteme, um bakterielle Besiedelung in Verbindung zu Bodenbildung zu untersuchen.

1. Introduction

1.1 Terrestrial habitats under extreme environmental conditions

Polar habitats and high-altitude alpine sites are regions of extreme climatic conditions and predicted environmental changes (Callaghan *et al.*, 1995; Grabherr *et al.*, 2010; Turner *et al.*, 2005). They play an important role in the global climate system and for the understanding of past and future climate change. Interestingly, over 80 % of the terrestrial and marine ecosystems are defined as cold-affected habitats with temperatures around 15 °C or less (Kirby *et al.*, 2012). Permanently frozen ground, called permafrost and defined as a period of two consecutive years with an average temperature below 0 °C (van Everdingen, 1998), is a global phenomenon that makes up to 26 % of the terrestrial soil ecosystem and one quarter of the northern hemisphere landmass (Zhang *et al.*, 2003). In contrast, Antarctic permafrost only occupies 0.36 % of the Antarctic region, but it is predominant in almost all ice-free regions. Permafrost temperatures in continental Antarctica vary from -17.4 °C to -22.5 °C in the McMurdo Dry Valleys, -17.8 °C on nunataks in Queen Maud Land, -9.8 °C at the costal stations at Enderby Land and -8.5 °C at the Progress Station in the Vestfold Hills region on the Larsemann Hills (Vieira *et al.*, 2010). Measured permafrost temperatures for the South Shetland Islands near the seal-level are only slightly below 0 °C. Furthermore, Antarctic permafrost is low in carbon content. In comparison, up to 1672 Pg (10^{15} g) carbon are currently estimated to be stored in soil deposits of northern permafrost regions (Tarnocai *et al.*, 2009). In a global scale, Antarctic permafrost only plays a minor role in green-house gas emissions and has the potential to function as a carbon sink in connection with enhanced microbial and plant colonisation (Convey *et al.*, 2009; Vieira *et al.*, 2010). West Antarctica and the Antarctic Peninsula are going to be highly affected by global warming and recent studies also reported significant warming in East Antarctica but with regional differences and seasonal variations (Fig. 1, Steig *et al.*, 2009; Verleyen *et al.*, 2011). Overall, the continent-wide average near surface temperature trend is positive (Steig *et al.*, 2009; Turner *et al.*, 2005).

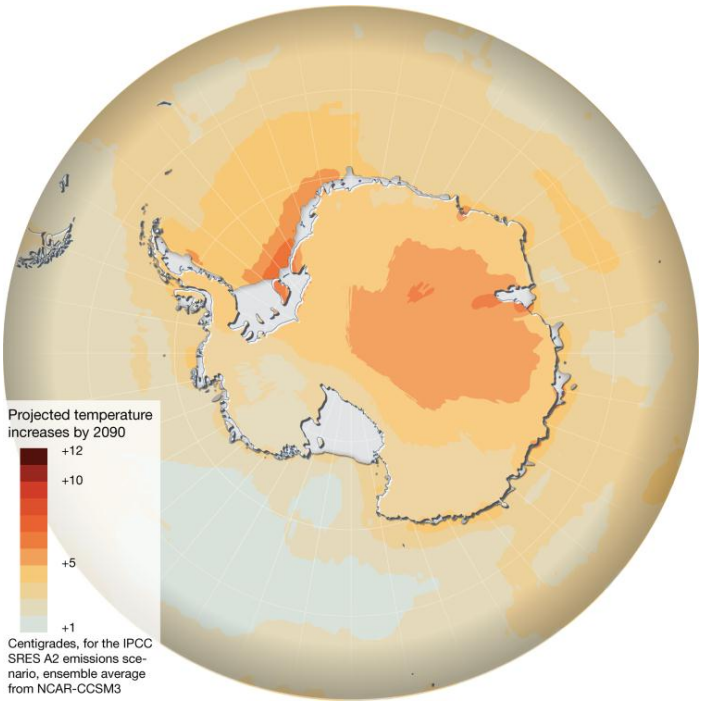

Fig. 1. Temperature increases in the Antarctic due to climate change, 2090 (NCAR-CCM3, SRES A2 experiment; Hugo Ahlenius, 2008).

However, low temperature alone does not make those habitats extreme, but in combination with low atmospheric humidity, low liquid water availability, low nutrient availability, high salinities high elevation, strong winds and periods of high solar radiation that alternate with long periods of darkness they become a hostile environment (Cowan & Ah Tow, 2004). Bird colonies can provide a high nutrient level, whereas most of the Antarctic environments are oligotrophic (Kirby *et al.*, 2012). The Antarctic continent is almost completely covered by ice (> 98 %) with the exception of some ice-free oasis for example the extreme cold Dry Valleys or the Larsemann Hills in Eastern Antarctica (Cary *et al.*, 2010; Hogdson *et al.*, 2001). Glacier forefields and terrestrial Antarctic ecosystems form pioneer sites for soil formation and microbial colonisation. Therefore, unaffected Antarctic glacier forefields, being geographical isolated with little anthropogenic influence, are suitable candidates to study microbial community development in connection to changing environmental conditions.

1.2 Adaption mechanisms of microorganisms in extreme (cold-affected) habitats

Microorganisms in extreme habitats are directly exposed to harsh environmental conditions and therefore need to adapt their metabolic processes, reproduction mechanisms and survival strategies. They are not only able to survive hostile circumstances, but can also optimise their lifestyle with regard to the natural environment evolving to the so-called extremophiles (Margesin & Schinner, 1992). The most challenging parameter of terrestrial Antarctica is low temperature, supporting the colonisation and development of psychrophilic and psychrotolerant microorganisms. According to the definition of Morita (1975) psychrophilic bacteria grow optimally at ≤ 15 °C and are able to withstand temperatures around 0 °C or below. In contrast, psychrotolerant species have optima of 20-40 °C (Madigan, 2012). This definition concentrates on microorganisms living in permanently (temperature-stable) cold environments such as marine habitats, but temperature fluctuations of otherwise extreme cold environments such as Antarctic dry desert soils, are inadequately considered. Applying this restricted definition, psychrotolerant microorganisms are more common than psychrophiles, because they have a broader temperature range for growth and are therefore better adapted to temperature fluctuations of the terrestrial habitat. Low temperatures lead to a deceleration of most biochemical reactions and physiological processes, reduction of the cell membrane fluidity and a cold-denaturation of proteins. They also influence the solubility of salts (decrease) and gas (increase), the viscosity of water (reduction) and the pH values of biological buffers (decrease; Georlette *et al.*, 2004). Thus, (psychrotolerant) microorganisms have developed several mechanisms to overcome the severe conditions and strong fluctuations of their environment: the synthesis of cold-shock proteins and antifreeze molecules, the modification of enzyme kinetics, the regulation of ion channel permeability and the regulation of the cell membrane fluidity (Deming, 2002). The cold shock response of psychrotolerant microorganisms induces the enhanced production of housekeeping gene products and special cold-shock proteins operating at low temperatures. Special antifreeze proteins and small molecules protect the microorganisms against freezing by preventing the formation of ice crystals (Jones & Inouye, 1994). Furthermore, organisms can reduce their metabolism and even form dormant states, but for a real psychrophilic life they have to compensate the lowered reaction rates. Some psychrophiles simply increase the enzyme concentration, have special cold-adapted isotypes of the enzyme or are able to develop enzymes with temperature-independent reaction rates. The majority of the psychrophiles adapt the enzyme kinetics having a higher

specific enzyme activity at lower temperature, whereby the maximal activity is shifted to the temperature minimum (Georlette *et al.*, 2004). Those cold-adapted enzymes open interesting possibilities in biotechnology (Margesin & Schinner, 1994). Another major adaption tool of the microorganisms the ability to adjust the cell membrane phospholipid fatty acid (PLFA) inventory in order to maintain cell membrane fluidity and functionality, which was described as "homeoviscous adaption" (Sinensky, 1974). The cell membrane lipids of psychrophiles are phospholipids with phosphatidylethanolamine, phosphatitylglycerol and diphosphatidylglycerol, whereby the polar head-groups and the acyl chains of the glycerol phospholipids change in response to the environment (Russell & Fukunaga, 1990). At lower temperatures microorganisms incorporate lower melting point PLFAs into the lipids to increase the fluidity of the cell membrane. That is realised by the reduction in chain length, the introduction of cis-double bonds to enhance unsaturation and the branching of the acyl chains by methylation or hydroxylation (Russell, 1984). Here both, anteiso- and iso-branched fatty acids are possible, but some studies reported a higher importance of the anteiso-branched fatty acids in cold adaption (Klein *et al.*, 1999). The incorporation of a cyclisation can support the stability of the cell membrane at low temperature, pH and in slowly growing cultures (Russell, 1989). The same mechanisms are used for the cell membrane adaption of the microorganisms to other environmental stressors for instance changing pH values. Thus, the stress response of an organism is always a combined response to the various constraints of the habitat.

1.3 Diversity and distribution of microorganisms in cold-affected terrestrial environments

Previously, extreme habitats characterised by hostile environmental conditions were thought to contain only a few species that occur in small numbers (Kennedy, 1995; Smith *et al.*, 2006). Meanwhile, microorganisms are known to be widely distributed in permafrost environments, where they can occur as a diverse microbial community and build the basis for an efficient (polar) ecosystem (Aislabie *et al.*, 2006; Wagner *et al.*, 2005; Wynn-Williams, 1996). In maritime Antarctica mineral soils are subjected to initial soil formation processes such as humus accumulation and brownification and some sites are vegetated with mosses and lichens. That leads to a high number of heterotrophic bacteria (up to 10^8 CFU g^{-1} dry soil) and a divers bacterial community consisting of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*,

Firmicutes, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria* and a high proportion of uncultured bacteria (Ganzert *et al.*, 2011a). The nutrient-rich ornithogenic soils associated with penguin colonies are dominated by copiotrophic bacteria with an increased number of culturable heterotrophes (10^{11} CFUs g^{-1} soil) and isolated species such as *Agribacterium*, *Pasteurella*, *Pseudomonas*, *Sphingomonas*, *Arthrobacter* or *Streptomyces* (reviewed in Kirby *et al.*, 2012). Other ice-free "oases" are the Dry Valleys (largest ice-free region) or the Larsemann Hills (ice-free region at the coastline) of Eastern Antarctica. Several early (culture-based) and some recent studies showed a low microbial diversity in mineral soils of the extreme Antarctic Dry Valleys (Kirby *et al.*, 2012; Smith *et al.*, 2006). Recently, the prokaryotic community of Antarctic soils is estimated as highly divers and spatially variable (Barrett *et al.*, 2006; Niederberger *et al.*, 2008). The dominant groups are *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Deinococcus*/*Thermus*, *Acidobacteria*, *Firmicutes* and *Cyanobacteria* (Aislabie *et al.*, 2006). *Verrucomicrobia*, *Planctomycetes Gemmatimonadetes* and *Chloroflexi* are additionally identified in molecular-based studies (Cary *et al.*, 2010; Smith *et al.*, 2006). Taking into account the theory of "everything is everywhere, but the environment selects" (Baas Becking, 1934), it is not surprising that representatives of the phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia* also colonise Arctic permafrost environments (Wagner, 2008). Several culture-independent studies showed that the bacterial community can be highly variable or dominated by certain phyla (Kobabe *et al.*, 2004), e.g. by *Alpha*- and *Deltaproteobacteria* as it was reported in a study of Northern Siberia (Zhou *et al.*, 1997) or by *Actinobacteria* and *Proteobacteria* detected in Canadian permafrost (Steven *et al.*, 2007). Furthermore, methanogenic archaea and methanotrophic bacteria are of special importance in the carbon-rich permafrost environments because of their role in the methane and general carbon cycle. On the one hand, methanogens can make up to 22.4 % of the total microbial cells in the active layer of permafrost (Kobabe *et al.*, 2004) and contribute to greenhouse gas emissions by methanogenesis, on the other hand methanotrophs are the only known sink for methane in these habitats (Barbier *et al.*, 2012; Trotsenko & Khmelenina, 2005). In contrast to arctic permafrost environments, archaea seem to be less relevant in Antarctic dry mineral soils and permafrost, since they could not be detected in several culture-independent studies (Pointing *et al.*, 2009). The oligotrophic freshly-exposed glacial surfaces support the establishment of a diverse microbial community of autotrophs and heterotrophs (Duc *et al.*, 2011; Tscherko *et al.*, 2003). Glacier forefield soils are pioneer sites for microbial succession and soil

formation (Nemergut *et al.*, 2007; Schütte *et al.*, 2009; Sigler *et al.*, 2002). The bacterial succession showing an overall change in the community structure over time strongly influences further plant succession and soil development (Schmalenberger & Noll, 2010; Schütte *et al.*, 2009). Moreover, environmental disturbances such as freeze-thaw cycles, wind or snow can lead to biological heterogeneity within the chronosequence of the glacier forefields (Noll & Wellinger, 2008). Representatives of common soil bacteria e.g. *Acidobacteria*, *Bacteroidetes* and *Verrucomicrobia* and nitrogen-fixing members of *Cyanobacteria* occur in dependence of the age gradient often realised by a replacement of phylotypes (Nemergut *et al.*, 2007; Sigler & Zeyer, 2002). Understanding microbial communities in connection to soil formation and environmental parameters will help to predict how bacterial communities will react to changing environmental conditions.

1.4 Isolation and characterisation of new microorganisms - then and now

"*All bacteria which maintain the characteristics which differentiate one from another when they are cultured on the same medium and under the same conditions should be designated as species, varieties, forms, or other suitable designation.*" Robert Koch (translated from the German)

In 1880 Robert Koch established the basis for the description of pure cultures. He defined criteria that identify certain microorganisms as the trigger of a disease and postulated that microorganisms have to be isolated as a culture (from the infectious organism) and the culture has to be pure. Those requirements are known as Koch´s Postulates (Stanier, 1951). The isolation of a new microorganism starts with an enrichment culture which was first described by Martinus Beijernick in 1901, who enriched and isolated microorganism from soil and water habitats (Madigan, 2012). For an enrichment culture microorganisms are obtained from the natural habitat with selective media, whereby the choice of culture conditions determines the microbial community that is enriched (Figure 2). During that time a main part of the characterisation was based on morphological traits. Colonies can be described macroscopically with colour, shape, margin, odour, profile, surface, consistency and size (Schröder, 1991). Cells differ in shape and size and staining methods reveal additional properties. Staining methods as described by Gram

(1884), Loeffler & Frosch (1898) or Wirtz (1908) are well known since the early days of microbial characterisation, but they are still applied in modern species descriptions.

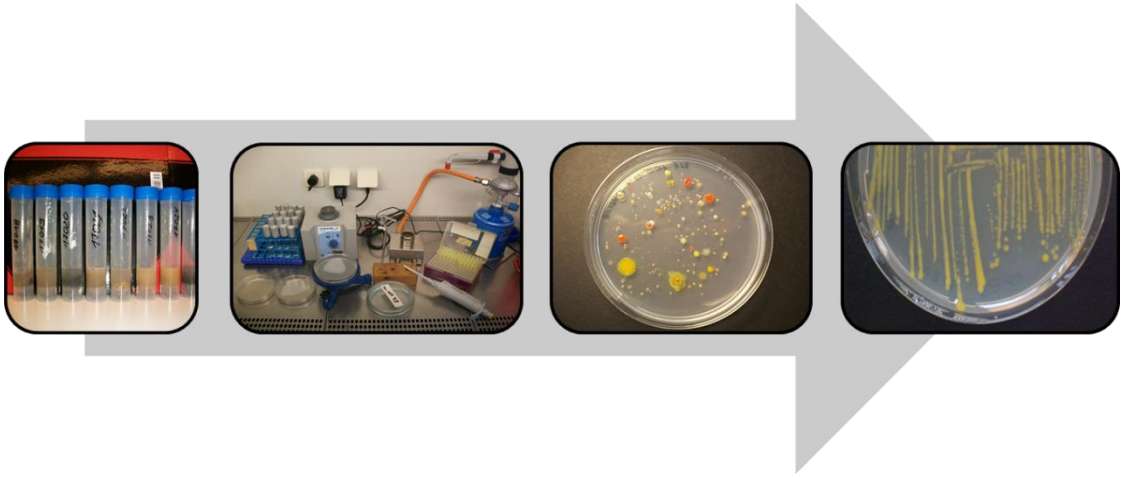

Fig. 2. Flow Chart showing the procedure of the enrichment and isolation of microorganisms. The procedure starts with a sample of the natural environment (here represented by a soil slurry), that is treated with selected media and culture conditions resulting in an enrichment culture from which the pure culture is isolated.

The discovery of desoxyribonucleic acid (DNA) as the carrier for genetic information in 1944 opened completely new perspectives (Avery *et al.*, 1944). In 1977, Sanger *et al.* developed a method for DNA sequencing. That was followed by the Polymerase Chain Reaction (PCR) by Mullis *et al.* in 1986, which build up the basis for modern molecular microbiology and play an important role in the examination of microorganisms. The analysis of ribosomal 16S RNA gene sequences provides a culture-independent method for the identification of new microorganisms, because the 16S rRNA gene is highly conserved and ubiquitous in the genome of all microorganisms. In comparison to the culture-independent approach, classical cultivation became disadvantaged over a long period for some critical reasons. First of all a pure culture has to be isolated, followed by several biochemical and physiological tests, which makes classical cultivation a time and workforce consuming process. The main problem is that the majority of microorganisms are hardly or not at all culturable, because it is not possible to imitate the complex properties and conditions of the microbial ecosystem. Staley and Konopka (1985) reported a so called “great plate count anomaly”, because they count more microorganism under the microscope than with colony forming units. A lot of microorganisms are able to live in the habitat or even in culture, but they do not form any colonies on agar plates, they are “viable but not culturable” (Rollins & Colwell, 1986). Thus, the cultivated microorganisms only represent a small part of the real diversity (Amann *et al.*, 1995) and it is assumed that so far less then 0.1 %

of all microbial species are isolated (Overmann, 2013). Molecular techniques allow identifying a big part of the microbial community, but with only 16S rRNA gene analyses it is not possible to get any information about the physiological characteristics of the microorganisms and their role in the habitat. Since the beginning of the molecular classification of microorganisms different parameters for microbial taxonomy have been defined. Carl Woese´s work on the basis of the 16S rRNA represents the fundament of modern molecular biology. He proposed that the archaea differ in their origin from the prokaryotes (Woese & Fox, 1977), established the three-domain-system of bacteria, eukarya and archaea (Woese *et al.*, 1990) and introduced a new concept for bacterial evolution (Woese, 1987). In 1995, Amann *et al.* referred to the problems of cultivation as well and suggest a molecular-based phylogenetic identification of microbial cells (Amann *et al.*, 1995). Recently, the scientists agree to combine phylogenetic and cultivation techniques in a "polyphasic approach" (Rosselló-Mora & Amann, 2001; Vandamme *et al.*, 1996). Standard values for taxonomic parameters are set and revised frequently to generalise species descriptions. For DNA-DNA-Hybridization (DDH) a value of 70 % or higher has been proposed to comprise one species (Brenner, 1973). Stackebrandt and colleagues compared sequence similarities on 16S rRNA gene basis with DDH experiments and found that organisms with sequence homologies of about 97.5 % do not share more than 60 to 70 % DDH similarity (Stackebrandt & Goebel, 1994). The proposed threshold of 97 % 16S rRNA gene sequence similarity to perform DDH was extended in 2006 to 98.5 % 16S rRNA gene sequence similarity (Stackebrandt & Ebers, 2006). The most recent "notes on the characterisation of prokaryote strains for taxonomic purposes" were given by Tindall *et al.* containing detailed instructions for a genotypic and phenotypic characterisation (2010). They reset the value for DDH to 97 % 16S rRNA gene sequence similarity or suggest analysing genes with a greater resolution, which are typically conserved protein-encoding house-keeping genes. Beneath the general requirements for novel species descriptions, the review summarises "Minimal Standards" for certain groups that should be considered (Tindall *et al.,* 2010). The isolation and characterisation of microorganism from extreme habitats remains important, because representative isolates contribute to the understanding of the role of microorganisms in these habitats and they may posses useful characteristics for biotechnological approaches (Nichols *et al.*, 1999). Thus, several isolates from polar habitats produce cold-adapted enzymes and degrade a wide range of substrates and polymers under extreme conditions (Bajerski *et al.*, 2011; Ganzert *et al.*, 2011b; Vazquez *et al.*, 1995; Whyte *et al.*, 1996).

1.5 Aims and objectives

Increasing temperatures due to climate change lead to enhanced deglaciation processes in cold-affected habitats. It is important to understand how the newly formed ice-free areas in glacier forefields develop with respect to soil formation and primary succession. This work gives an insight on the structure and development of bacterial communities in dependence of soil parameters in two glacier forefields of the Larsemann Hills, East Antarctica. Furthermore adaption strategies of the microorganisms to the extreme environment are addressed to better understand how microorganisms successfully colonise the habitat. In the scope of that the following questions are answered:

- **How do soil parameters change in a glacier forefield?**

Soil parameters such as moisture, pH, conductivity, grain size distribution, the carbon and nitrogen content and the anions and cations of trace elements were determined to understand the habitat formation in the glacier forefield.

- **How is the microbial community composed at different spots in the forefield?**

Terminal Restriction Length Polymorphism (T-RFLP) analysis and clone libraries along the glacier forefield transects were applied to analyse the bacterial community composition. The T-RFs were analysed in a non-metric multivariate scaling analysis (NMDS) to express correlations in the bacterial community composition and to select certain samples for the clone libraries. Bacterial numbers were estimated with quantitative Real-Time PCR.

- **How are habitat formation and bacterial succession linked with each other?**

A redundancy analysis (RDA) was performed with the sedimentological parameters as variables and the T-RF distribution as the supplied data set to understand how bacterial communities change in dependence of soil development. The identification of dominant groups should give a first insight of the role of microorganisms in the habitat.

- **Can novel microorganisms be isolated and how are they characterised?**

Heterotrophic bacteria were analysed as a part of the microbial community. Enrichment cultures were used to determine the number of culturable heterotrophs and to isolate different species.

Selected strains were characterised using a polyphasic approach and two novel species were described in detail.

- **How are microorganisms adapted to the severe conditions of the extreme habitat?**

The potential enzyme activities (protease, β-glucosidase, invertase, acidic and alkaline phosphatase) of the microorganisms in the soil were measured at low temperature (14°C). The potential change in the fatty acid composition of the novel species *Chryseobacterium frigidisoli* PB4^T was analysed at different pH and temperatures using membrane phospholipid fatty acid (PLFA) analysis as an example for the structural adaption of microorganisms in extreme habitats.

1.6 Study Area

The Larsemann Hills are located on the Ingrid Christensen Coast of Princess Elizabeth Land at Prydz Bay, East Antarctica (69°30S, 76°20E; Fig. 3). They are situated between the Rauer Islands and Vestfold Hills in the north-east and Bolingen Island and the Amery ice shelf in west-south-west. The study site comprises an ice-free oasis of approximately 50 km^2 and is the second largest of four major ice-free regions along the East Antarctic coastline. The Larsemann Hills consist of two major peninsulas, which are Stornes and Broknes Peninsula, and several minor peninsulas and islands. The elevation is up to 180 m above sea level and the Larsemann Hills differ in their development compared to other areas of the Prydz Bay (Burgess *et al.*, 1994; Stüwe *et al.*, 1989). The Proterozoic aged rocks are characterised by different gneisses and the absence of shear zones, briffle deformation and mafic dykes. The Larsemann Hills have undergone a series of geomorphologic and deformation events. Due to former glaciation the landscape is formed by erratic boulders, pattered ground and glacial tafoni and parsed by steep valleys and more than 150 partially ice free lakes (Gillieson, 1991; Stüwe *et al.*, 1989). There is only very little development in the geography of the ice sheets surrounding the Larsemann Hills and with a movement of about 0.5 to 2 km over the last 20 kilo annos (ka), the present ice sheet is more or less stagnant. Broknes Peninsula, one of the two main peninsulas of the Larsemann Hills, has been continuously ice free since at least 40 ka BP (Hodgson et al., 2001). The area is characterised by marine influenced continental climate leading to intensive physical weathering processes (Burgess *et al.*, 1994). Air temperatures in the coastal regions are about -18 °C

to -29 °C in Antarctic winter and around 0 °C in summer (Dec-Feb). Precipitation usually occurs as snow and amounts up to about 250 mm a^{-1} (ANARE, 2000).

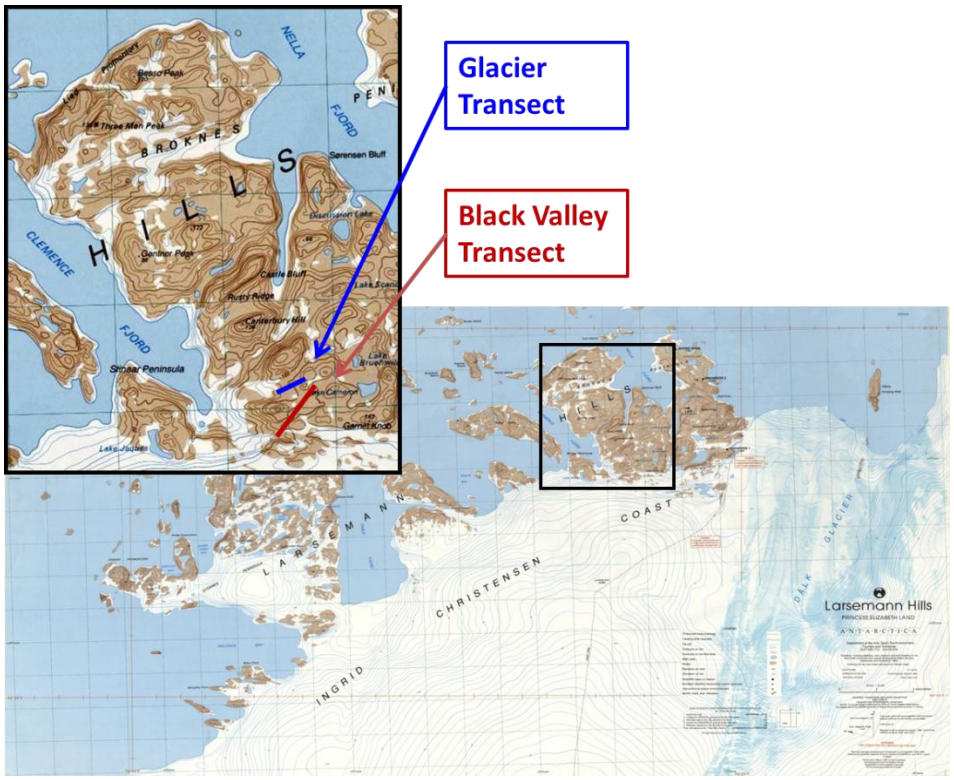

Fig. 3. Two glacier forefields (Glacier and Black Valley Transect) on Broknes Peninsula of the Larsemann Hills in East-Antarctica were chosen for analysis in this study. “Larsemann Hills / Princess Elizabeth Land”, Cat. No: L15 in 'ANARE Catalogue of Maps and Hydrographic Charts 1997' (Division, 1997).

Two glacier forefields on Broknes Peninsula were chosen for analysis in this study and sampled during the expedition ANT-XXIII/9 of the research vessel "Polarstern" to Antarctica in March 2007 (Fig. 3, Ganzert *et al*., 2008). The first, called “Glacier Transect” (S 69° 24.140; E 76° 20.178 to S 69° 24.135; E 76° 20.296), was about 80 meters long and 13 sediment samples and one surface sample were taken from five profiles (Fig. 4). The second glacier forefield (S 69°24.221; E 76°20.813 to S 69°24.326; E 76°20.273) was named “Black Valley Transect” referring to its surface colour. It was extending over a transect of 500 meter and 11 sediment samples and three surface samples were harvested out of five profiles (Fig. 5). The Black Valley Transect was situated between a small glacial snow cap on one side and the Dalk glacier on the other side (Fig. 3).

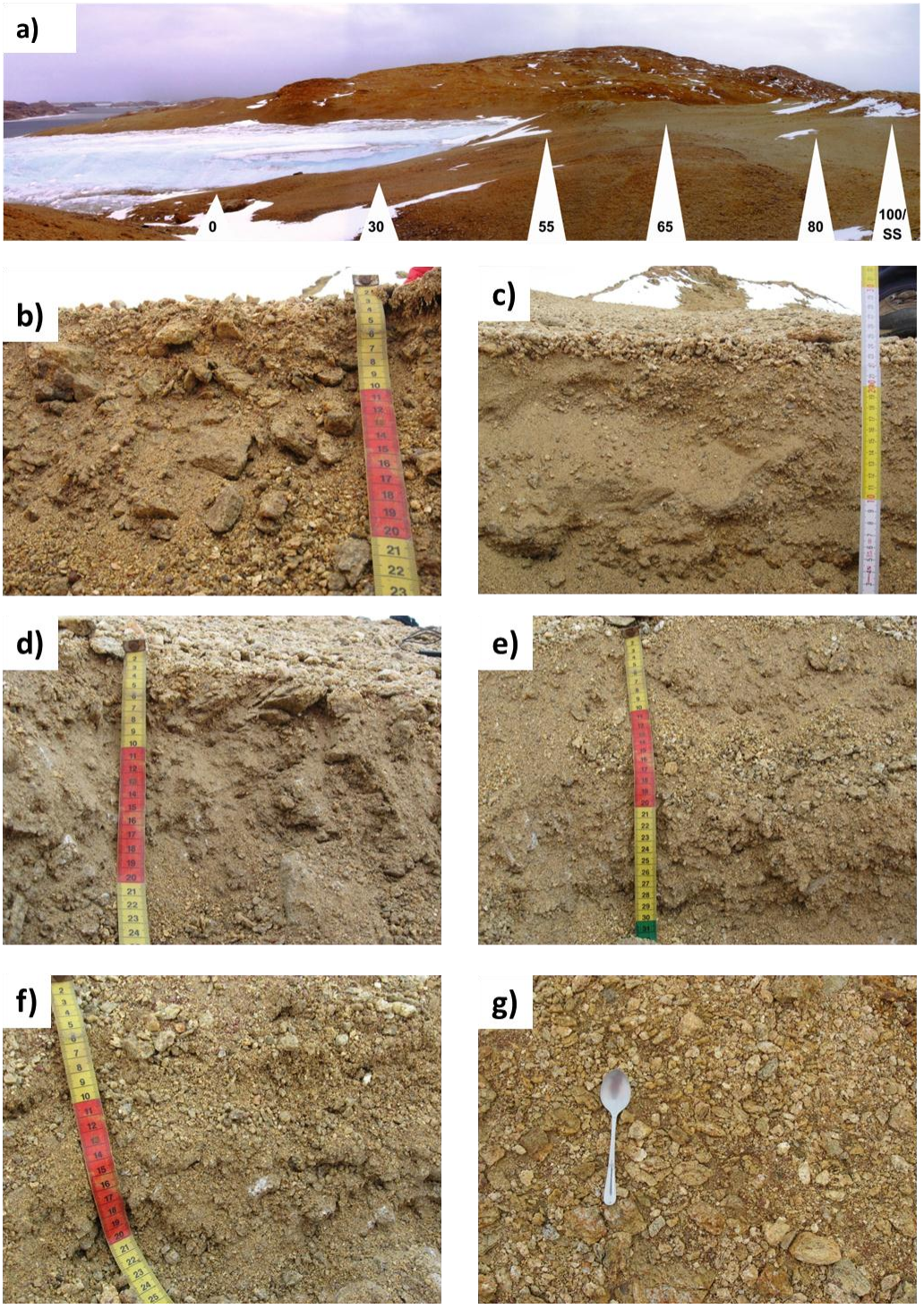

Fig. 4. Glacier Transect; a) Overview of the soil profile 1-5 of the Glacier Transect harvested in 0-80 m distance from the glacier, respectively, and the surface sample (SS) in 100 m distance, b) profile 1 = 0 m, c) profile 2 = 30 m, d) profile 3 = 55 m, e) profile 4 = 65 m, f) profile 5 = 80 m, g) surface sample = 100 m (Picture © Lars Ganzert & Dirk Wagner).

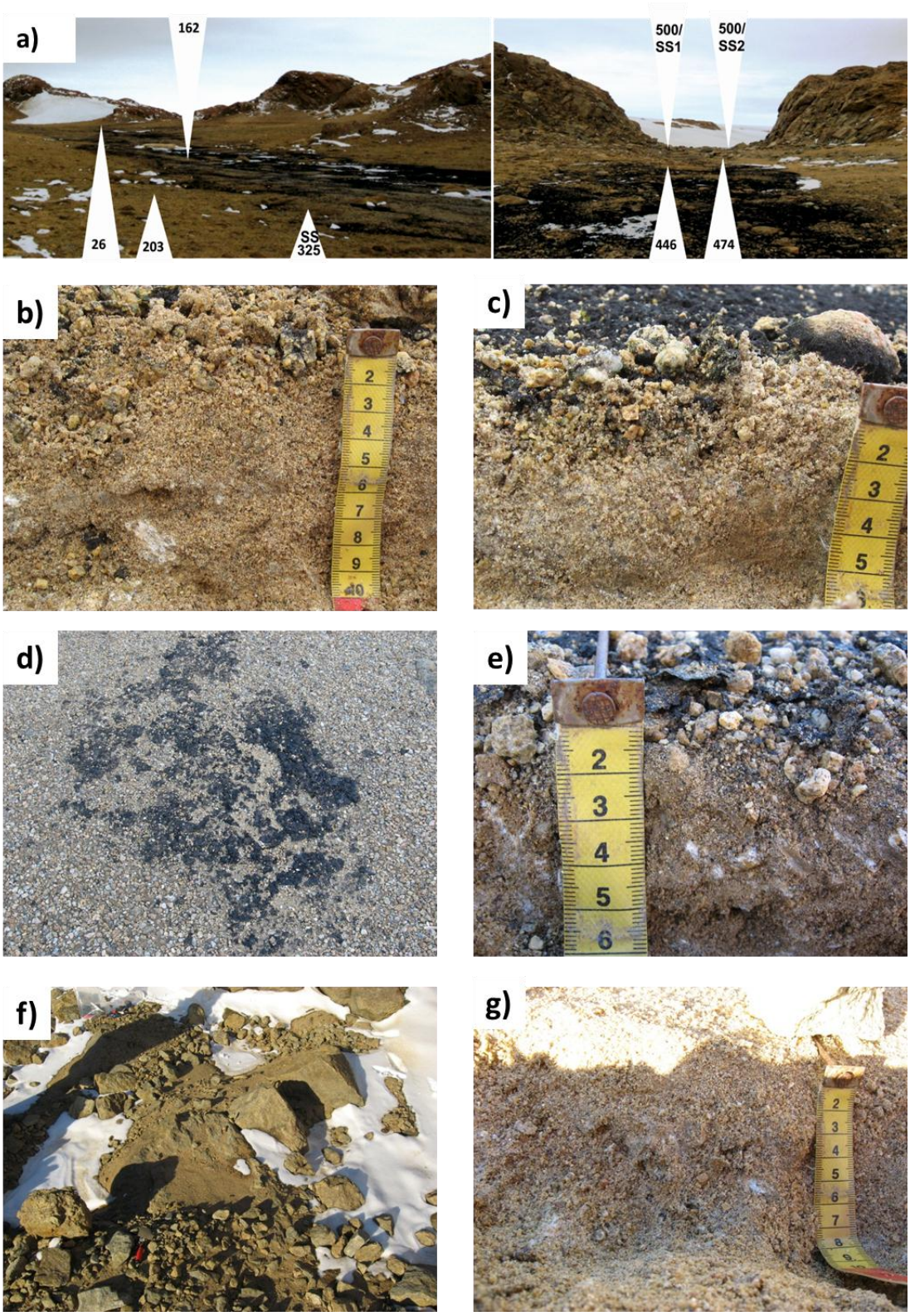

Fig. 5. Black Valley Transect; a) Overview of the soil profiles 1-5 of the Black Valley Transect harvested in 26-474 m distance from the glacier, b) profile 1 = 26 m, c) profile 2 = 162 m, d) overview of profile 4 = 446 m, e) profile 4, f) overview of profile 5 = 474 m, g) profile 5 (Picture © Lars Ganzert & Dirk Wagner).

1.7 Overview of publications and manuscripts

Manuscript I: F. Bajerski & D. Wagner (2013). Bacterial succession in Antarctic soils of two glacier forefields on Larsemann Hills, East Antarctica. FEMS Microbial Ecology. 2013 Jul; **85** (1): 128-42.

Bacterial succession in Antarctic soils of two glacier forefields on Larsemann Hills, East Antarctica.

Authors: Felizitas Bajerski & Dirk Wagner

Aims: The aim of our study was to investigate the structure and development of bacterial communities in dependence of soil parameters along two different glacier forfields in extreme Antarctic environments. We wanted to find out how bacterial communities may respond to changing environmental conditions and how climate change is going to influence habitat and bacterial community succession.

Summary: Antarctic glacier forefields are extreme environments and pioneer sites for ecological succession. Increasing temperatures due to global warming lead to enhanced deglaciation processes in cold-affected habitats and new terrain is becoming exposed to soil formation and microbial colonisation. However, only little is known about the impact of environmental changes on microbial communities and how they develop in connection to shifting habitat characteristics. In this study, using a combination of molecular and geochemical analysis, we determine the structure and development of bacterial communities depending on soil parameters in two different glacier forefields on Larsemann Hills, East Antarctica. Our results demonstrate that deglaciation dependent habitat formation, resulting in a gradient in soil moisture, pH and conductivity, leads to an orderly bacterial succession for some groups, e.g. *Cyanobacteria*, *Bacteroidetes* and *Deltaproteobacteria* in a transect representing "classical" glacier forefields. A variable bacterial distribution and different composed communities were revealed according to soil heterogeneity in a slightly "matured" glacier forefield transect, where *Gemmatimonadetes*, *Flavobacteria*, *Gamma-* and *Deltaproteobacteria* occur depending on water availability and soil depth. *Actinobacteria* are dominant in both sites with dominance connected to certain trace elements in the glacier forefields.

Co-author´s contribution: *Dirk Wagner* carried out the related field work and contributed to the concept, structure and pre-review process of the manuscript.

Manuscript II: Bajerski, F., Ganzert, L., Mangelsdorf, K., Lipski, A., Busse, H.-J., Padur, L. & Wagner, D. (2013b). *Herbaspirillum psychrotolerans* sp. nov., a member of the family *Oxalobacteraceae* from a glacier forefield. International Journal of Systematic and Evolutionary Microbiology. **63** (9), 3197-3203.

***Herbaspirillum psychrotolerans* sp. nov., a member of the family *Oxalobacteraceae* from a glacier forefield.**

Authors: Felizitas Bajerski, Lars Ganzert, Kai Mangelsdorf, André Lipski, Hans-Jürgen Busse, Lisa Padur and Dirk Wagner

Aims: The aim of this study was the isolation and characterisation of a novel species in the family of the *Oxalobacteraceae*, in which some members are known to be able to contribute to biogeochemical cycling with nitrogen fixation.

Summary: A novel psychrotolerant, Gram-negative, shiny white, curved-rod-shaped, facultative anaerobic bacterium PB1^T was isolated from a soil sample collected from a glacier forefield of the Larsemann Hills, East Antarctica. Isolate PB1^T has catalase and low urease activity and hydrolyses gelatine and starch. Strain PB1^T is able to grow between -5 °C and 30 °C with an optimum growth at 14-20 °C. Glycerol, D-/L-arabinose, D-xylose, D-galactose, D-fructose, D-lyxose, D-fucose and potassium gluconate are used as sole carbon sources. The major quinone is ubiquinone Q-8. The major fatty acids (>10 %) for PB1^T are $C_{16:0}$ (19.1 %), $C_{16:1}\omega 7cis$ (44.6 %) and $C_{18:1}\omega 7cis$ (16.2 %). The major polyamines are putrescine (54.9 µmol g^{-1} dry weight) and 2-hydroxyputrescine (18.5 µmol g^{-1} dry weight). DNA base composition is 62.5 mol% G+C. Strain PB1^T is phylogenetically related to species of the genus *Herbaspirillum*, with highest 16S rRNA gene sequence similarities to *Herbaspirillum canariense* (97.3 %), *Herbaspirillum aurantiacum* (97.2 %), *Herbaspirillum soli* (97.2 %) and *Herbaspirillum frisingense* (97.0 %). The DNA-DNA relatedness values were below 30 % between PB1^T and the type strains of *H. canariense*, *H. aurantiacum* and *H. soli*. The different geographical origin of strain PB1^T and its next relatives resulted in a different phenotypic and genotypic specification, whereby strain PB^T represents a

novel species in the genus *Herbaspirillum*, for which the name *Herbaspirillum psychrotolerans* is proposed. The type strain is PB1^T (DSM 26001^T = LMG 27282^T).

Co-author´s contribution: *Lisa Padur* performed most of the physiological and biochemical experiments for the characterisation. *Lars Ganzert* analysed the phylogenetic data and constructed the phylogenetic tree. Fatty acid analysis was carried out by *Kai Mangelsdorf*. Quinones and polar lipids were determined by *André Lipski*. Polyamines were analysed by *Hans-Jürgen Busse*. *Dirk Wagner* contributed to the interpretation of the results and valuable discussion.

Manuscript III: Bajerski, F., Ganzert, L., Mangelsdorf, K., Padur, L., Lipski, A. & Wagner, D. (2013a). *Chryseobacterium frigidisol*i sp. nov., a psychrotolerant species of the family *Flavobacteriaceae* isolated from sandy permafrost from a glacier forefield. International Journal of Systematic and Evolutionary Microbiology **63** (7), 2666-71.

***Chryseobacterium frigidisoli* sp. nov., a psychrotolerant species of the family *Flavobacteriaceae* isolated from sandy permafrost from a glacier forefield.**

Authors: Felizitas Bajerski, Lars Ganzert, Kai Mangelsdorf, Lisa Padur, André Lipski and Dirk Wagner

Aims: This study aimed at the isolation and identification of a novel species from extremely dry and nutrient-poor Antarctic soils. The strain to be characterised is a member of the *Bacteroidetes*, which are known to be present in cold-adapted habitats.

Summary: During diversity studies of the glacier forefields of the Larsemann Hills, East Antarctica, a novel psychrotolerant, non-motile Gram-negative, shiny yellow, rod-shaped, aerobic bacterium, designated strain PB4^T was isolated from a soil sample. Strain PB4^T produces indole from tryptophan and hydrolyses casein. It grows between 0 °C and 25 °C with an optimum growth temperature at 20 °C. A wide range of substrates are used as sole carbon sources and acid is produced from numerous carbohydrates. The major menaquinone is MK-6. Identified polar lipids are ethanolamines and ornithine lipids. Major fatty acids (>10 %) are iso-$C_{15:0}$ (13.0%) and iso-2OH-$C_{15:0}$ (51.2 %). G+C content is 33.7 mol%. The polyamine pattern is composed of sym-homospermidine (25.1 µmol g^{-1} dry weight), minor amounts of cadaverine (0.2 µmol g^{-1} dry

weight) and spermidine (0.4 µmol g^{-1} dry weight) and traces of putrescine and spermin (< 0.1 µmol g^{-1} dry weight). Strain PB4^T had highest 16S rRNA gene similarities with the type strains of *Chryseobacterium humi* (97.0 %) and *Chryseobacterium marinum* (96.5 %). Considering phenotypic and genotypic characterisation, strain PB4^T represents a novel species in the genus *Chryseobacterium* (family *Flavobacteriaceae*), for which the name *Chryseobacterium frigidisoli* sp. nov. is proposed. The type strain is PB4^T (DSM 26000^T = LMG 27025^T).

Co-author´s contribution: *Lisa Padur* performed most of the physiological and biochemical experiments for the characterisation. *Lars Ganzert* analysed the phylogenetic data and constructed the phylogenetic tree. Fatty acid analysis was carried out by *Kai Mangelsdorf*. Quinones and polar lipids were determined by *André Lipski*. *Dirk Wagner* contributed to the interpretation of the results and valuable discussion.

Manuscript IV: Manuscript draft planned to be submitted to Applied and Environmental Microbiology (AEM)

Temperature and pH adaption of the cell membrane fatty acid composition of *Chryseobacterium frigidisoli* PB4^T isolated from glacier forefield soils of the Larsemann Hills East Antarctica.

Authors: Felizitas Bajerski, Dirk Wagner and Kai Mangelsdorf

Aims: Microorganisms from Antarctic glacier forefield are subjected to extreme conditions and temperature fluctuations. In this study we focus on the temperature and pH adaption of the cell membrane structure of *Chryseobacterium frigidisoli* PB4^T, analysing the potential change in the fatty acid composition of the cell membrane.

Summary: Microorganisms in Antarctic glacier forefields are directly exposed to the hostile conditions of their habitat such as a harsh climate with strong temperature fluctuations. A major mechanism of microorganisms to overcome those stress conditions is the change of the cell membrane composition maintaining its structure and functionality is. However, only little is known about the adaptation potential of microorganisms from extreme habitats. In this study, we examined the change of the cell membrane phospholipid fatty acid (PLFA) inventory of *Chryseobacterium frigidisoli* PB4^T, isolated from an Antarctic glacier forefield, in response to

changing temperature (0 °C – 20 °C) and pH (5.5 - 8.5) regimes using gas chromatography-mass spectrometry (GC-MS). The PLFA pattern is dominated by saturated and unsaturated mainly branched fatty acids in the range between 15 and 18 carbon atoms. In the low-temperature adaptation of *C. frigidisoli* the ratios of iso/anteiso-$C_{15:0}$ and iso/anteiso-hydroxy-$C_{15:0}$ fatty acids show a decreasing trend with declining temperature. The relative proportion of an unknown fatty acid, presumably an unsaturated cyclohexyl-C_{17} fatty acid, continuously increases from 20 °C to 0°C and the monounsaturated iso-$C_{17:1}\omega8$ strongly increases from 20 °C to 10 °C. In adaptation to changing pH values most of the dominant fatty acids (iso- and anteiso-C_{15}, anteiso-2OH-C_{15}, iso-$C_{17:1}\omega8$) reveal constant relative proportions around neutral pH (pH 6-8). Strong variations are observed in the relative proportions of hydroxy-fatty acids (low pH) and iso- and anteiso-fatty acids toward the pH extremes (pH 5.5 and 8.5). The study shows the importance of a newly discovered fatty acid in the stress response of a cold-adapted bacterium and thus gives insights in adaptation strategies of microorganisms at low temperature and changing geochemical gradients.

Co-author´s contribution: Kai Mangelsdorf performed the fatty analyses of the strain. Dirk Wagner and Kai Mangelsdorf contributed to the interpretation of the results and valuable discussion.

FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Bacterial succession in Antarctic soils of two glacier forefields on Larsemann Hills, East Antarctica

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Keywords
T-RFLP; microbial communities; Antarctica; 16S rRNA gene; glacier forefield; impact of soil parameters.

Abstract

Antarctic glacier forefields are extreme environments and pioneer sites for ecological succession. Increasing temperatures due to global warming lead to enhanced deglaciation processes in cold-affected habitats, and new terrain is becoming exposed to soil formation and microbial colonization. However, only little is known about the impact of environmental changes on microbial communities and how they develop in connection to shifting habitat characteristics. In this study, using a combination of molecular and geochemical analysis, we determine the structure and development of bacterial communities depending on soil parameters in two different glacier forefields on Larsemann Hills, East Antarctica. Our results demonstrate that deglaciation-dependent habitat formation, resulting in a gradient in soil moisture, pH and conductivity, leads to an orderly bacterial succession for some groups, for example *Cyanobacteria*, *Bacteroidetes* and *Deltaproteobacteria* in a transect representing 'classical' glacier forefields. A variable bacterial distribution and different composed communities were revealed according to soil heterogeneity in a slightly 'matured' glacier forefield transect, where *Gemmatimonadetes*, *Flavobacteria*, *Gamma-* and *Deltaproteobacteria* occur depending on water availability and soil depth. *Actinobacteria* are dominant in both sites with dominance connected to certain trace elements in the glacier forefields.

Introduction

Glacier retreat due to global warming is a phenomenon which can be observed in high mountain ranges like the Alps (Haeberli *et al.*, 2007) as well as in Arctic and Antarctic environments (Rignot, 2001; Bamber *et al.*, 2005; Cook *et al.*, 2005; Oerlemans, 2005). Antarctica plays an important role in the global climate system and helps us to obtain insight into past and future climate changes. It is characterized by extreme conditions and is sensitive to environmental changes (Verleyen *et al.*, 2004; Turner *et al.*, 2005). West Antarctica and the Antarctic Peninsula are known to be strongly affected by global warming, but recent studies also reported significant warming in East Antarctica although with regional differences and seasonal variations (Steig *et al.*, 2009; Verleyen *et al.*, 2011). The continent-wide average near-surface temperature trend is positive (Steig *et al.*, 2009).

Glacier forefields form terrestrial pioneer sites and are influenced by harsh climatic conditions and low nutrient availability (Schütte *et al.*, 2009; Duc *et al.*, 2011). Terrestrial Antarctic ecosystems are characterized by low temperatures, soil moisture and organic matter content and high salinity (Cannone *et al.*, 2008; Niederberger *et al.*, 2008). Antarctica is known as a continent dominated by microbial ecosystems (Wynn-Williams, 1996; Aislabie *et al.*, 2006) as microorganisms play an important role in primary succession, pedogenesis and biogeochemical cycling (Lazzaro *et al.*, 2009; Schütte *et al.*, 2009). Microorganisms support mechanical and chemical bedrock degradation through weathering processes (Frey *et al.*, 2010) and the establishment of more complex microbial communities or higher life like plants (Tscherko *et al.*, 2003). Apparently unaffected glacier forefields provide a unique opportunity as a natural laboratory to study the succession of pioneering plants (Miniaci *et al.*, 2007),

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animals (Kaufmann, 2001) and especially microorganisms (Sigler & Zeyer, 2002; Aislabie *et al.*, 2006; Lazzaro *et al.*, 2009; Duc *et al.*, 2011). Understanding microbial communities in connection to soil formation and environmental parameters will help to predict how bacterial communities will react to changing environmental conditions.

During the last 20 years, microbial communities in extreme habitats became an important research topic and were studied in the Alps (Sigler *et al.*, 2002; Duc *et al.*, 2011), Arctic (Liebner *et al.*, 2009; Barbier *et al.*, 2012) or Antarctic environments (Ganzert *et al.*, 2011a; Yergeau *et al.*, 2012). Former studies have shown the importance of microbial communities in alpine or Arctic glacier forefields and their role in extreme Antarctic environments; however, Antarctic terrestrial microbial communities remain poorly understood and additional knowledge is necessary. With respect to methodology, several studies have used polyphasic approaches consisting of culture-independent methods coupled with culturing techniques and sedimentological analysis (Sigler *et al.*, 2002; Shivaji *et al.*, 2011). Culturing experiments showed the impact of isolated bacteria on granite weathering (Frey *et al.*, 2010). A high abundance of microorganisms with up to 10^9 cells g^{-1} soil could be observed in alpine glacier forefields (Sigler & Zeyer, 2002). In recent years, molecular techniques have become an important tool describing microbial community structures in polar regions (Wagner, 2008). Molecular fingerprints have revealed a difference in communities from younger sites, compared to older sites (Lazzaro *et al.*, 2009), and environmental changes can influence the community structures (Odum, 1970; Tiao *et al.*, 2012). Bacterial communities change along a glacier forefield transect correlated with shifts in pH, soil water or age (Noll & Wellinger, 2008), or they show a patchy distribution according to heterogeneous soil characteristics (Niederberger *et al.*, 2008).

Antarctic glacier forefields are suitable sites to study microbial community development because of its special environment, geographical isolation and little anthropogenic influence. Two different glacier forefields were chosen for analysis not only for comparison, but also to illustrate possible phases of succession. The aim of this study was to investigate the influence of glacier retreat on the development of forefield soil microbial communities in Antarctica. For this approach, a combination of cultivation experiments, molecular, geophysical and geochemical analysis was applied. We used terminal restriction length polymorphism (T-RFLP) and clone libraries to determine bacterial diversity and distribution. Quantitative real-time PCR (qPCR) was carried out to estimate the abundance of bacteria in the soils. Heterotrophic bacteria were examined as a part of the whole community using plate counts.

Materials and methods

Study site

The Larsemann Hills are located on the Ingrid Christensen Coast of Princess Elizabeth Land at Prydz Bay, East Antarctica (69°30′S, 76°20′E). The study site is characterized by an ice-free area of *c.* 50 km^2 and a marine influenced continental climate leading to intensive physical weathering processes (Stüwe *et al.*, 1989; Burgess *et al.*, 1994). There is very little development in the geography of the ice sheets surrounding the Larsemann Hills, and with a movement of about 0.5–2 km over the last 20 ka (kiloannum), the present ice sheet is more or less stagnant. Broknes Peninsula, one of the two main peninsulas of the Larsemann Hills, has been continuously ice free since at least 40 ka BP (Hodgson *et al.*, 2001). Air temperatures in the coastal regions are about −18°C to −29°C in Antarctic winter and around 0°C in summer (December–February). Precipitation usually occurs as snow and amounts up to about 250 mm a^{-1} (ANARE, 2000; Hodgson *et al.*, 2001). Permafrost temperatures measured at the Russian Progress Station are about −8.5°C, and the active layer is 0.7 m deep on average (Vieira *et al.*, 2010).

Sample collection and soil analysis

Two glacier forefields on Broknes Peninsula were chosen for analysis in this study and sampled during the expedition of the research vessel 'Polarstern' to Antarctica in March 2007 (Ganzert *et al.*, 2008). The first, called 'Glacier Transect' (S 69°24.140; E 76°20.178 to S 69°24.135; E 76°20.296), was about 80 m long, and 13 soil samples and one surface sample (0–1 cm) were taken from five profiles (Fig. S1a). In the second glacier forefield (S 69°24.221; E 76°20.813 to S 69°24.326; E 76°20.273), named 'Black Valley Transect' referring to its surface colour and extending over a transect of 500 m, 11 soil samples and three surface samples (0–1 cm) were harvested out of five profiles (Fig. S1b). The Black Valley Transect was situated between a small glacial snow cap on one side and the Dalk Glacier on the other. All 28 soil samples were bulk samples and the sampling depth was chosen according to the graininess and horizonation of the weathering debris (Tables 1 and 2).

For molecular and microbiological analysis, the samples were collected in sterile 250-mL plastic boxes (NALGENE). Samples for geochemical and geophysical analysis were stored in plastic bags. All samples were transported at −25°C on research vessel 'Polarstern' from the Prydz Bay (Antarctica) to Bremerhaven (Germany). The harvested material was mainly composed of weathering debris and soil precursors. According to the definition of

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Table 1. Selected soil properties and bacterial abundance in the glacier forefield called 'Glacier Transect' on Larsemann Hills, East Antarctica. Sample designations refer to distance from the glacier and sample depth. Conductivity and pH values were determined in the field, with exception of the asterisks-marked samples

Site (m)	Depth (cm)	Sample ID	Sand (%)	Silt (%)	Clay (%)	Moisture (%)	Conductivity (µS cm^{-1})	pH value	C (%)	N (%)	CFU g^{-1} soil on R2A	CFU g^{-1} soil on BRII	gene copies g^{-1} soil
0	0–7	GT0/0-7	90.1	7.8	2.1	0.6	12.7	8.3	< 0.1†	0.1	$5.6 \times 10^4 \pm 1.2 \times 10^4$	$7.0 \times 10^4 \pm 9.2 \times 10^3$	$1.2 \times 10^7 \pm 6.2 \times 10^5$
0	7–14	GT0/7-14	89.8	8.1	2.2	1.5	6.2	8.0	< 0.1†	0.1	$1.8 \times 10^4 \pm 6.1 \times 10^3$	$7.8 \times 10^4 \pm 5.6 \times 10^3$	$1.7 \times 10^7 \pm 1.2 \times 10^6$
0	14–25	GT0/14-25	89.2	9.0	1.8	2.4	7.7	7.1	< 0.1†	0.1	$6.5 \times 10^4 \pm 6.5 \times 10^3$	$1.4 \times 10^5 \pm 6.0 \times 10^3$	$3.5 \times 10^6 \pm 2.6 \times 10^5$
30	0–13	GT30/0-13	95.3	3.9	0.8	1.4	11.5	7.9	0.1	0.1	$1.8 \times 10^4 \pm 1.5 \times 10^3$	$2.0 \times 10^5 \pm 1.2 \times 10^4$	$4.2 \times 10^7 \pm 5.5 \times 10^6$
30	13–28	GT30/13-28	84.5	12.5	2.9	3.7	12.6	7.7	< 0.1†	0.1	$5.9 \times 10^4 \pm 1.2 \times 10^4$	$2.8 \times 10^5 \pm 2.4 \times 10^4$	$1.3 \times 10^7 \pm 3.4 \times 10^5$
55	0–10	GT55/0-10	88.3	9.9	1.8	2.1	55.9	8.3	< 0.1†	0.1	$3.8 \times 10^4 \pm 4.4 \times 10^3$	$6.3 \times 10^3 \pm 3.4 \times 10^3$	$6.3 \times 10^7 \pm 8.0 \times 10^6$
55	10–20	GT55/10-20	82.3	14.4	3.3	2.2	9.6	8.5	< 0.1†	0.1	$2.2 \times 10^4 \pm 3.1 \times 10^3$	$1.8 \times 10^4 \pm 6.0 \times 10^3$	$7.4 \times 10^6 \pm 4.0 \times 10^5$
65	0–10	GT65/0-10	90.9	7.2	1.9	0.5	15.5	6.5	< 0.1†	0.1	$1.4 \times 10^4 \pm 1.8 \times 10^3$	$2.3 \times 10^3 \pm 3.5 \times 10^2$	$5.5 \times 10^7 \pm 2.8 \times 10^6$
65	10–20	GT65/10-20	86.7	11.2	2.2	1.2	7.0	6.7	< 0.1†	0.1	$5.5 \times 10^3 \pm 2.1 \times 10^3$	$9.5 \times 10^3 \pm 4.4 \times 10^3$	$4.9 \times 10^6 \pm 2.1 \times 10^5$
65	20–30	GT65/20-30	89.0	8.6	2.3	1.2	7.5	8.7	< 0.1†	0.1	$2.5 \times 10^3 \pm 9.6 \times 10^2$	$4.1 \times 10^3 \pm 2.9 \times 10^3$	$5.6 \times 10^6 \pm 1.4 \times 10^5$
80	0–1	GT80/0-1	92.5	5.9	1.6	0.2	20.1	4.9	0.4	0.1	$1.6 \times 10^4 \pm 2.3 \times 10^3$	$4.2 \times 10^4 \pm 5.2 \times 10^3$	$7.6 \times 10^8 \pm 1.8 \times 10^7$
80	1–10	GT80/1-10	92.4	6.2	1.4	0.3	7.0	6.7	0.1	0.1	$7.4 \times 10^3 \pm 1.8 \times 10^3$	$7.0 \times 10^4 \pm 4.3 \times 10^4$	$5.8 \times 10^7 \pm 6.4 \times 10^6$
80	10–20	GT80/10-20	93.4	5.3	1.2	0.8	4.9	6.8	< 0.1†	0.1	$1.6 \times 10^4 \pm 1.2 \times 10^3$	$4.3 \times 10^4 \pm 3.5 \times 10^3$	$1.1 \times 10^8 \pm 2.2 \times 10^6$
100	0–1	GT100/SS	89.3	9.3	1.5	0.2	142.3*	5.8*	< 0.1†	< 0.05†	$1.7 \times 10^4 \pm 2.4 \times 10^3$	$4.0 \times 10^2 \pm 3.5 \times 10^2$	$1.5 \times 10^8 \pm 1.4 \times 10^7$

*Analysis was performed in the laboratory.
†Below the detection limit, C < 0.1, N < 0.05.
CFU, colony-forming units; GT, Glacier Transect.

Bockheim and for simplicity, all materials in this study are referred to as 'soil' (Bockheim, 1982; Ugolini & Bockheim, 2008).

Conductivity and pH were measured in a soil extract (9 g soil in 45 mL distilled water) directly in the field laboratory. The soil slurry was filtered for pH value determination, whereas conductivity was measured directly in the extract. Moisture content was determined with weighing the moist and dry soil before and after freeze-drying of about 1 kg of soil. Total carbon and nitrogen contents were determined with an automatic element analyser (Elementar Vario EL III). Total organic carbon content was measured after HCl (10%) acid digestion on the analyser Elementar VarioMaxC. For trace element analyses, a soil slurry (5 g soil + 25 mL milli Q water) was mixed in an overhead shaker for 90 min and centrifuged at 3500 *g* for 20 min. Anions were measured with ion chromatography (Dionex-DX320), cations with inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima3000XL) and HCO_3^- with titration (Metrohm Titrino 794). Grain size distribution was determined as described by Biskaborn *et al.* (2012) and measured in a laser particle analyser (Coulter LS 200).

Cultivation and enumeration of culturable heterotrophs

The numbers of culturable heterotrophs were determined by plating serial soil solutions (up to 10^{-4}) on R2A (Reasoner & Geldreich, 1985) and modified BRII medium (Bunt & Rovira, 1955; Ganzert *et al.*, 2011c) as it was described before (Bajerski *et al.*, 2013). Colonies were randomly chosen from all enrichment culture plates to obtain potentially new isolates. Microbial DNA extraction and the amplification of bacterial 16S rRNA genes were performed as described previously (Table S1; Bajerski *et al.*, 2013).

Soil DNA extraction

Soil DNA was extracted in triplicates out of 0.5 g material each with PowerSoil Extraction Kit (MoBio Laboratories, Inc.) according to the manufacturer's protocol. The DNA triplicates were pooled for downstream analysis.

Bacterial 16S rRNA gene copy numbers

Bacterial 16S rRNA gene copy numbers were determined by qPCR to estimate bacterial abundance. A standard was generated out of pure culture, DNA was extracted as described above, and bacterial 16S rRNA genes were amplified using 0.5 µL of each primer Uni 331F and Uni 797R (10 µM, Table S1, Nadkarni *et al.*, 2002), 12.5 µL Syber Green Polymerase Master Mix (2×, Qiagen, Hilden,

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Table 2. Selected soil properties and bacterial abundance in the glacier forefield called 'Black Valley Transect' on Larsemann Hills, East Antarctica. Sample designations refer to distance from the glacier and sample depth. Conductivity and pH values were determined in the field, with exception of the asterisks-marked samples

Site (m)	Depth (cm)	Sample ID	Sand (%)	Silt (%)	Clay (%)	Moisture (%)	Conduc-tivity (µS cm^{-1})	pH value	C (%)	N (%)	CFU g^{-1} soil on RZA	CFU g^{-1} soil on BRII	Gene copies g^{-1} soil
26	0–1	BV26/0-1	89.6	8.6	1.7	1.1	65.6	6.7	1.3	0.2	$4.7 \times 10^5 \pm 7.2 \times 10^4$	$2.8 \times 10^6 \pm 4.2 \times 10^5$	$6.6 \times 10^8 \pm 4.6 \times 10^7$
26	1–9	BV26/1-9	93.8	5.2	0.9	5.4	8.4	6.9	0.3	0.1	$7.8 \times 10^5 \pm 1.6 \times 10^5$	$5.9 \times 10^5 \pm 5.3 \times 10^4$	$1.2 \times 10^9 \pm 7.1 \times 10^7$
162	0–2	BV162/0-2	67.8	26.0	6.2	1.2	31.8	6.8	2.0	0.3	$6.4 \times 10^6 \pm 7.4 \times 10^5$	$1.0 \times 10^8 \pm 5.4 \times 10^6$	$1.1 \times 10^9 \pm 9.2 \times 10^7$
162	2–5	BV162/2-5	91.2	6.8	2.0	14.9	18.9	6.8	0.5	0.1	$7.2 \times 10^6 \pm 7.8 \times 10^5$	$2.0 \times 10^7 \pm 8.7 \times 10^5$	$3.2 \times 10^8 \pm 3.2 \times 10^7$
203	0–7	BV203/0-7	89.6	8.3	2.0	0.6	16.3	6.7	0.2	0.1	$4.4 \times 10^5 \pm 9.2 \times 10^4$	$2.6 \times 10^5 \pm 4.7 \times 10^4$	$1.7 \times 10^8 \pm 1.1 \times 10^7$
203	7–11	BV203/7-11	88.1	9.6	2.2	1.5	5.2	6.9	0.2	0.1	$3.2 \times 10^5 \pm 1.5 \times 10^4$	$2.2 \times 10^6 \pm 3.2 \times 10^5$	$1.6 \times 10^8 \pm 5.3 \times 10^6$
203	11–18	BV203/11-18	82.4	14.2	3.4	4.5	7.3	6.9	0.0	0.1	$7.6 \times 10^5 \pm 2.7 \times 10^5$	$1.1 \times 10^6 \pm 3.8 \times 10^5$	$1.4 \times 10^7 \pm 4.3 \times 10^5$
446	0–1	BV446/0-1	87.5	11.1	1.4	0.9	21.7	6.5	1.5	0.2	$3.6 \times 10^5 \pm 7.1 \times 10^4$	$1.1 \times 10^5 \pm 1.7 \times 10^4$	$1.0 \times 10^9 \pm 2.3 \times 10^7$
446	1–6	BV446/1-6	84.1	12.4	3.5	9.4	7.6	6.8	0.5	0.1	$7.4 \times 10^4 \pm 1.8 \times 10^4$	$3.3 \times 10^5 \pm 1.3 \times 10^5$	$1.8 \times 10^9 \pm 2.6 \times 10^7$
474	0–2	BV474/0-2	95.4	3.7	1.0	1.8	73.2	6.0	0.2	0.1	$4.6 \times 10^6 \pm 3.5 \times 10^5$	$1.2 \times 10^7 \pm 1.4 \times 10^6$	$1.5 \times 10^8 \pm 1.2 \times 10^7$
474	2–6	BV474/2-6	88.1	9.6	2.4	9.4	65.2*	6.5*	0.1	0.1	$1.9 \times 10^6 \pm 2.7 \times 10^5$	$1.6 \times 10^6 \pm 3.1 \times 10^5$	$3.1 \times 10^7 \pm 6.9 \times 10^6$
325	0–1	BV325/SS	91.0	7.5	1.5	0.6	440.0*	6.0*	0.3	< 0.05†	$5.4 \times 10^5 \pm 1.2 \times 10^5$	$3.5 \times 10^5 \pm 1.7 \times 10^4$	$2.0 \times 10^9 \pm 7.8 \times 10^7$
500	0–1	BV500/SS1	90.1	8.6	1.3	0.2	16.4*	6.9*	< 0.1†	< 0.05†	$5.0 \times 10^3 \pm 1.8 \times 10^3$	$1.5 \times 10^4 \pm 1.0 \times 10^4$	$2.8 \times 10^7 \pm 7.7 \times 10^5$
500	0–1	BV500/SS2	87.6	10.9	1.5	0.5	80.2*	6.6*	1.0	0.1	$9.2 \times 10^5 \pm 2.3 \times 10^5$	$1.0 \times 10^5 \pm 3.1 \times 10^4$	$3.4 \times 10^9 \pm 0.9 \times 10^8$

*Analysis was performed in the laboratory.
†Below the detection limit, C < 0.1, N < 0.05.
BV, Black Valley; CFU, colony-forming units.

Germany) and 1 µL DNA template adjusted to 25 µL with PCR clean water. The molarity (nM) of purified PCR products (HiYield PCR Clean-up Kit; SLG) was measured with Agilent 2100 Bioanalyzer (Agilent Biotechnologies, Böblingen, Germany) and multiplied by the Avogadro constant (6.022×10^{23} mol^{-1}) to calculate gene copies µL^{-1}. Quantitative PCR was performed as technical triplicates with primers 338F (Lane *et al.*, 1985) and 518R (Table S1, Muyzer *et al.*, 1995) in a Rotor Gene cycler (Qiagen) to quantify bacterial 16S rRNA gene copies (Fierer & Jackson, 2006). Each 25-µL reaction mixture contained 12.5 µL Syber Green (2×, Qiagen), 0.5 µL of each primer (20 µM), 9.5 µL PCR clean water and 3 µL DNA template in a 10-fold dilution. The inhibition of qPCR was tested and minimized using dilution series (Rasmussen, 2001). Standards and dilutions were measured every run.

Terminal restriction length polymorphism

A semi-nested PCR protocol (Table S1) was applied to enhance PCR product using universal bacterial primers 27F (Lane *et al.*, 1985) and 1492R (reaction I, Dojka *et al.*, 1998) and a 6-carboxyfluorescein-labelled primer FAM-27F and reverse primer 907R (reaction II, Muyzer *et al.*, 1995) to amplify 16S rRNA genes. PCRs were performed as described elsewhere (Barbier *et al.*, 2012). About 150 ng of purified PCR product was used in a 20-µL digestion reaction mixture with 10 U restriction enzyme AluI (New England Biolabs, Frankfurt a. M.) and 2.0 µL corresponding buffer. Amplicons were digested in duplicates at 37 °C for 3 h and the reaction was stopped with incubation at 65 °C for 20 min (UnoCylcer, VWR). Duplicate digestions were pooled and cleaned as described above and run on an ABI 3730xl DNA analyser (Applied Biosystems, Darmstadt, Germany) at GATC Biotech (Konstanz, Germany). GeneScan™ LIZ 500® (Applied Biosystems) was used as an internal size standard.

Data processing

Raw data were analysed with Peak Scanner Software 1.0 (Applied Biosystems), and output T-RF profiles were examined according to the five-step procedure of Dunbar *et al.* (2001). Within one sample, triplicate peaks within the range of 0.5 bp were aligned, but duplicate peaks were allowed and proceeded further as well. Only peaks above the fluorescence threshold of $\geq$ 25 fluorescence units were taken into account.

16S rRNA gene clone libraries

A 16S rRNA gene fragment was amplified according to PCR reaction I of the T-RFLP (Table S1) using Syber

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Green Polymerase Mix (2×, Qiagen). The purified PCR product (1 μL) was cloned using the pGEM®-T-Easy vector system (Promega, Mannheim, Germany) according to the manufacturer's protocol with ligation at 4 °C over night. Reamplification was carried out in a direct colony PCR of the positive clones with each 1.0 μL forward and reverse primers M13 (10 μM, Table S1, Messing *et al.*, 1981) recognizing the vector region, 12.5 μL Mango Polymerase Mix (Bioline, Luckenwalde, Germany) and 1 μL template. Raw sequence data were processed with Sequencher (v4.7; Gene Codes, Ann Arbor, MI) and uploaded as FASTA files to Rdp classifier (Wang *et al.*, 2007) to obtain an overview of the community composition. Cleaned sequences were aligned with Silva Aligner (Pruesse *et al.*, 2007), and a distance matrix was built with Arb (Ludwig *et al.*, 2004) as an infile for Dotur (Schloss & Handelsman, 2005) to determine unique phylotypes ($\geq$ 97% sequence similarity) using nearest neighbour clustering algorithms. A chimera check of the sequences was made with Belerophon 3.0 tool of Greengenes (Huber *et al.*, 2004; DeSantis *et al.*, 2006). Dotur was used to calculate rarefaction curves, Shannon–Weaver (Shannon, 2001) and Simpson diversity (Simpson, 1949) indices and Chao1 richness estimates (Chao, 1984; Chao & Lee, 1992; Chao *et al.*, 1993). GenBank accession numbers are JX171737–JX172264 for sequences of the Glacier Transect and JX172265–JX173055 for sequences of the Black Valley Transect.

Generated clone sequences were digested virtually with TRiFLe (Junier *et al.*, 2008) to identify T-RFs using the same primer set, restriction enzyme and conditions as in the soil T-RFLP analysis.

Statistical analysis

Bacterial community composition was calculated statistically with Primer 6 (Primer-E Ltd, Luton, UK). Relative abundances of all T-RFs (prior to phylogenetic assignment) were fourth-root-transformed, and hierarchal cluster analysis was performed using Bray–Curtis similarity index. A nonmetric multidimensional scaling (NMDS) analysis was applied on the transformed data set.

A redundancy analysis (RDA) was calculated with canoco 4.5 (Ter Braak & Šmilauer, 2002). The geochemical and geophysical soil parameters (distance from the glacier, soil depth, grain size, moisture, carbon and nitrogen content, pH-value, conductivity and trace elements), colony and gene copy numbers built up the species data set. Identified T-RFs were added as a supplied environmental data set. Concentrations and T-RFs were log-transformed, and all data were centred and standardized. The analysis was performed separately for each transect.

Results

Soil properties along two glacier forefield transects

Both glacier transects are characterized by a coarse grain size with a high contingent of sand and gravel (Tables 1 and 2). Soil texture was composed of over 80% sand and < 15% silt and 5% clay in almost all samples.

Although the overall soil moisture was very low, a gradient could be observed along the forefield (Glacier Transect) and in soil depth (both transects). Water content increased with increasing depth in the Glacier Transect, for example from 0.6% (GT0/0-7) in 0–7 cm, over 1.5% (GT0/7-14) in 7–14 cm and up to 2.4% (GT0/14-25) in 14–25 cm depth. It was driest in the upper layer of the most distant profile (0.2%, GT80/0-1) and moistest in the deeper soil closer to the glacier (3.7%, GT30/13-28). Water contents were slightly higher in the Black Valley Transect (up to 14.9%, BV162/2-5) and increased with depth, for example in profile BV203 from 0.6% to 4.5%. For the Glacier Transect, soils furthest from the glacier tended to be more acidic (pH 4.9 in sample GT80/10-20) than in the vicinity of the glacier (pH 8.3 in sample GT0/0-7), while the Black Valley Transect is characterized by slightly acidic to neutral pH values between pH 6.0 (BV474/0-2) and 6.9 (BV203/7-11). The carbon content was low, often below the detection limit (< 0.1, Glacier Transect), and reached a maximum of 0.4% (GT80/0-1) and 2.0% (BV162/0-2) in the surface samples of both transects. Trace elements were distributed heterogeneously in dependence of soil depth and location in the glacier forefield (Tables S2 and S3). Cations of trace elements of the Glacier Transect were increasingly observed from profile GT0 to GT65 and almost all elements reached a maximum in GT65/10-20. Elements of the Black Valley followed different depth trends, but did not shift along the transect.

Bacterial isolates and numbers of culturable heterotrophs

Heterotrophic bacteria were examined as part of the whole bacterial community. We identified 48 isolates out of the Glacier Transect and 100 isolates out of the Black Valley Transect. Representatives of the classes *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria* were isolated from both transects. In addition, the Black Valley Transect included species belonging to *Deinococci* and *Gammaproteobacteria*. Sequence analysis indicated that there were potentially new microorganisms among the isolates (Table S4). The number of culturable heterotrophs ranged between 2.0×10^3 and

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2.8×10^5 cfu g^{-1} dry soil in the Glacier Transect (Table 1) and 5.0×10^2 and 1.0×10^8 cfu g^{-1} dry soil in the Black Valley Transect (Table 2). In general, colony-forming units were higher on BRII than on R2A media, but isolates showed a higher visible diversity on R2A.

Abundance of bacteria

Bacterial abundance was displayed by the number of 16S rRNA gene copies per gram soil using qPCR. In comparison, bacterial numbers in the Black Valley Transect (1.4×0^7 to 3.4×10^9 gene copies g^{-1} soil, Table 2) were higher than in the Glacier Transect (3.5×10^6 to 7.6×10^8 gene copies g^{-1} soil, Table 1). Bacterial gene copy numbers in the Glacier Transect slightly increased with distance from the glacier, but decreased with depth. In the Black Valley Transect, no clear trend could be observed.

Bacterial community composition

Altogether, 79 different terminal restriction fragments (T-RFs) could be observed in the two different glacier forefields, whereby 25 T-RFs were present in both transects. In the Glacier Transect, 21 of 46 T-RFs were unique and in the Black Valley 33 of 58 T-RFs.

With *in silico* digestion of the sequences obtained from the clone libraries, we were able to identify 48 of 76 T-RFs, which were catalogued into 12 phylogenetic groups. Thirteen additional T-RFs could belong to several groups and are highlighted with patterns in the graph (Fig. 1).

Seven samples were chosen for cloning based on the distribution and clustering of T-RFs (as analysed by NMDS, see results of the statistics below). The following clusters and groups refer to the similarities determined with Bray–Curtis cluster analysis. Within the cluster Glacier Transect (Fig. 2), the samples GT0/7-14, GT55/10-20 and GT80/10-20 were analysed via cloning. Altogether, 528 sequences, representing 245 operational taxonomic units (OTUs), were identified from clone libraries. Taking into account rarefaction curves, richness estimators and diversity indices (Table 3, Fig. S2), our study showed a high coverage of species richness of 90% (C_{Chao}) and 88% (C_{ACE}) in GT0/7-14, but did not completely cover estimated species richness in the clone libraries of GT55/10-20 and GT80/10-20.

Clone libraries of the Black Valley Transect were chosen according to the main clusters: group I (BV26/0-1), group III (203/11-18), group IV (BV 446/1-6) and group V (BV474/2-6). Here, 791 sequences generated 374 OTUs (Table 3, Fig. S2). Only 34 OTUs out of 137 clones cover 95% (C_{Chao}) or 87% (C_{ACE}) of the estimated species richness in BV474/0-2, whereas 150 OTUs out of 228 sequences of BV26/0-1 describe < 40% of the whole community. The profiles BV203/11-18 and BV446/1-6 showed a mean coverage of species richness of about 70%.

T-RFs and clone library sequences affiliated to the phyla *Actinobacteria* and *Proteobacteria* were identified in all profiles of the Glacier and Black Valley Transects (Figs 1 and 3). Unique *Acidobacteria* T-RFs were only detected in the profiles GT0 and BV474 and at low abundance in the surface sample BV500/SS1. *Acidobacteria* were present in all clone libraries of the Glacier Transect with similar relative abundances between 4% and 9% and with increasing abundances up to 22% of the sequences in the profiles BV203 and BV446 of the Black Valley Transect. *Actinobacteria* were increasingly observed with increasing distance from the glacier in the Glacier Transect and peak fluores-

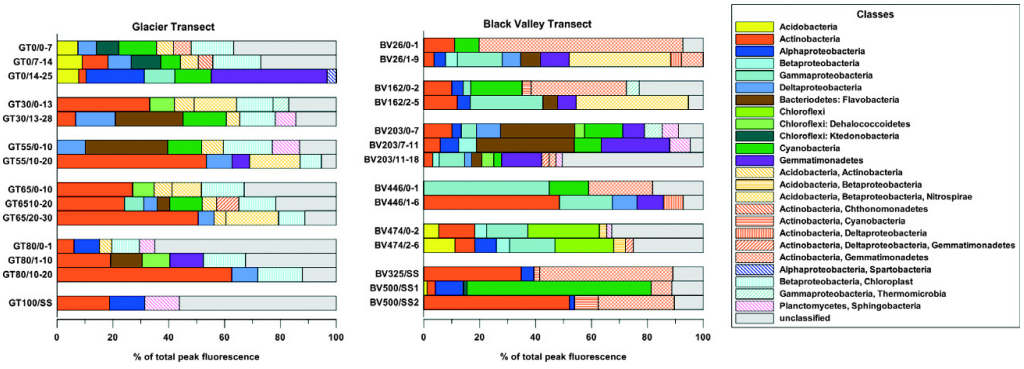

Fig. 1. T-RF-based bacterial community composition after *in silico* identification. Full colours represent 48 of 76 T-RFs, which were catalogued clearly into 12 phylogenetic groups. Patterns show 13 additional T-RFs that could belong to several groups. Sequences were determined on the class level.

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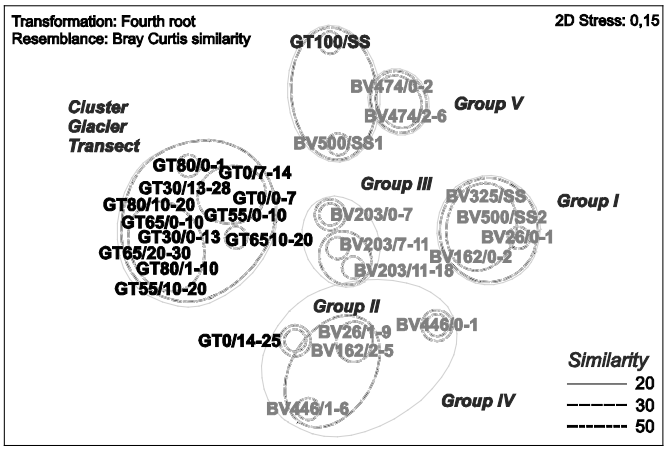

Fig. 2. Plot of the NMDS results, showing the similarities of the T-RFs including the T-RFs of all samples of the Glacier Transect (GT) and the Black Valley Transect (BV) prior to phylogenetic assignment.

Table 3. Characteristics (OTUs, species richness, diversity indexes) of individual and combined bacterial 16S rRNA gene clone libraries of selected samples of the Glacier Transect (GT0/7-14, GT55/10-20, GT80/10-20) and the Black Valley Transect (BV26/0-1, BV203/11-18, BV446/1-6, BV474/0-2)

			Coverage†		Richness estimators (min–max 95% CI)†		Diversity Index (min–max 95% CI)†	
Sample	Clones	OTUs*,†	C_{Chao}	C_{ACE}	Chao1	ACE	Shannon	Simpson
GT0/7-14	167	68	90	88	76 (71–92)	77 (71–92)	4.08 (3.97–4.18)	0.0132
GT55/10-20	183	97	54	53	180 (139–261)	184 (144–260)	4.30 (4.16–4.44)	0.0144
GT80/10-20	178	80	68	65	117 (97–163)	122 (101–164)	4.15 (4.03–4.27)	0.0140
Total	528	245						
BV26/0-1	228	150	38	37	390 (290–562)	411 (305–57)	4.78 (4.66–4.91)	0.0082
BV203/11-18	179	74	70	66	105 (87–147)	113 (93–152)	4 (3.86–4.14)	0.0198
BV446/1-6	247	116	68	67	171 (144–224)	172 (148–216)	4.55 (4.45–4.65)	0.0087
BV474/0-2	137	34	95	87	36 (34–44)	39 (35–52)	3.15 (3.00–3.31)	0.0505
Total	791	374						

*OTUs defined as sequences with 98% similarity.
†Calculated with DOTUR according to confidence interval of 95% (CI).

cence represented over 50% in the deepest layer of the profiles GT55, GT65 and GT80. In the clone libraries, *Actinobacteria* were the most dominant group as well, making up to 51% of all samples. In the Black Valley Transect, *Actinobacteria* occurred in all samples with different relative abundances in T-RFLP profiles and clone libraries (Figs 1 and 3). At a distance of 474 m from the glacier, *Actinobacteria* were the most dominant group in the clone library, making up 66% of the whole community, whereas T-RFs represented only 13%. In the Glacier Transect clone libraries, *Alphaproteobacteria* were the dominant group among the *Proteobacteria* and their relative abundance increased from 2.4% (GT0/7-14) to 15% in the most distant profile (GT80/10-20). Corresponding *Alphaproteobacteria*-T-RFs could only be detected in GT0/14-25, GT80/0-1 and GT100/SS. *Deltaproteobacteria*-T-RFs were explicitly present in most profiles, but only appeared in the clone library of GT80/10-20. *Proteobacteria* were detected in all samples of the Black Valley Transect, except in the T-RFLP profile of BV26/0-1. Within the *Proteobacteria*, *Gammaproteobacteria* were the most abundant group making up a distinctive part in the bacterial communities of BV26/1-9, BV162/2-5, BV446 and BV474. *Bacteroidetes* were detected in all profiles of the Glacier Transect with mainly *Sphingobacteria* in the clone libraries (GT0, GT55) and *Flavobacteria*-related T-RFs (GT30, GT55, GT65, GT80). Clone library results show that *Bacteroidetes* occurred at highest abundance in the vicinity of the glaciers in both transects. They were decreasingly observed in the Glacier Transect and present at both ends of the Black Valley Transect, close to the glacial caps in each case. T-RFs affiliated to *Gemmatimo-*

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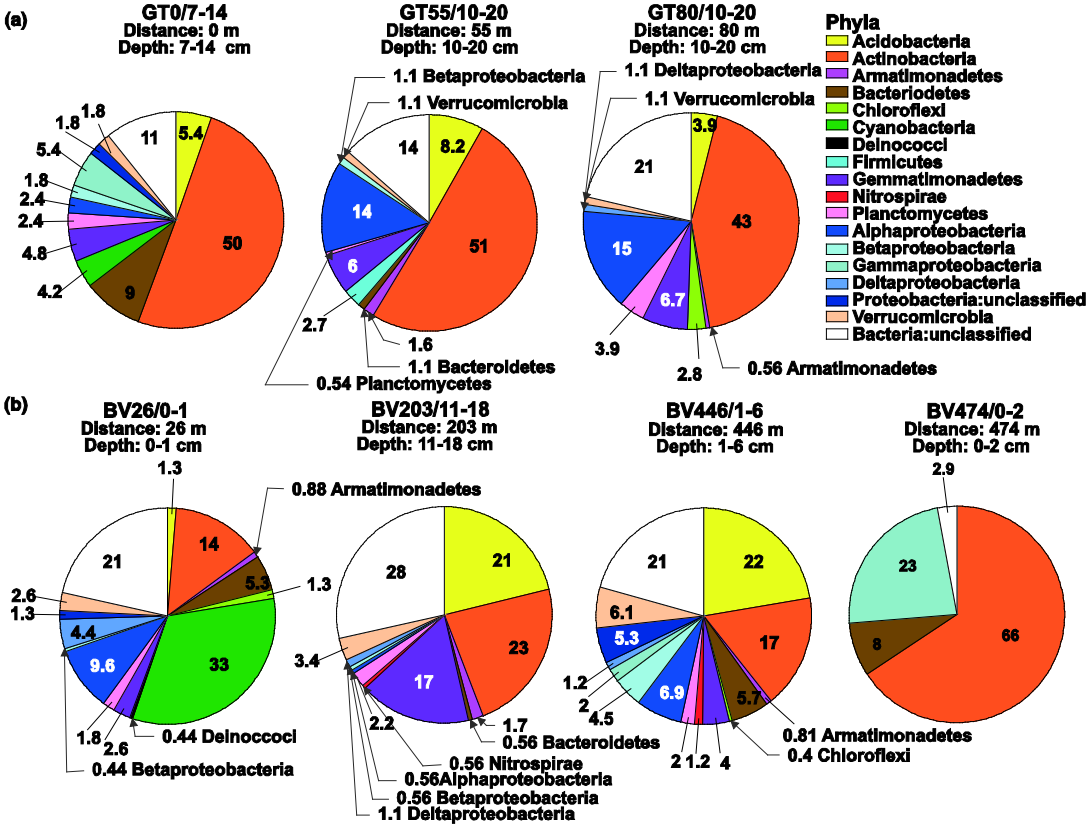

Fig. 3. Relative abundance of phylotypes determined in 16S rRNA gene clone libraries in two glacier forefields on Larsemann Hills, East Antarctica. Number of sequences: a) Glacier Transect (GT) = 167 (GT0/7-14), 183 (GT55/10-20), 178 (GT80/10-10); b) Black Valley Transect (BV) = 228 (BV26/0-1), 179 (BV203/11-18), 247 (BV446/1-6), 137 (BV474/0-2).

nadetes were widely distributed along the transects, more abundant in the deeper layers and became the dominant group in GT0/14-25. A trend implying increasing abundances along the Glacier Transect forefield was found in the clone libraries, but not with T-RFLP. T-RFLP results showed that *Cyanobacteria* and *Chloroflexi* were widely distributed all over the forefields as well, but in contrast to the *Gemmatimonadetes*, they were highly abundant in the surface samples. According to the clone library results, *Cyanobacteria* were present close to the glaciers and at high abundance in BV26/0-1.

T-RFLP profiling revealed a highly diverse and heterogeneous bacterial community of the glacier transects. Clone libraries along the Glacier Transect showed a very similar pattern, whereas the data for the Black Valley Transect revealed a highly variable composition, which indicated different states of development of the forefields. Overall, *Actinobacteria*, *Acidobacteria* and *Proteobacteria* were the dominant classes, but *Bacteroidetes*, *Cyanobacteria* and *Chloroflexi* took a distinct part in the community composition as well. *Armatimonadetes*, *Planctomycetes* and *Verrucomicrobia* could be detected in the clone libraries, but not as unique T-RFs. A trend in community development along both transects was observed for certain groups, but in general, the occurrence of bacteria followed a patchy distribution.

Statistical analysis of environmental and molecular data

T-RFs were compared in a Bray–Curtis similarity and NMDS analysis to identify bacterial community patterns and to select certain samples for cloning (Fig. 2). All T-RFs retrieved from the Glacier Transect, except fragments of GT100/SS and GT0/14-25, clustered together with at least 30% similarity. These T-RFs were clearly

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differentiated from those of the Black Valley Transect. Cluster analysis of the Black Valley Transect T-RFs created five distinct subgroups, connected to the location in the glacier forefield and soil depth. Groups I and II resembled surface and deeper soil samples, respectively, including the profiles BV26, BV162 and BV446. Group IV contained T-RFs of the profile BV446, which shared only 20% similarity to the fragments of the other samples. The T-RFs of BV446/1-6 were also connected to the soil depth group II. T-RFs retrieved from the profiles BV203 and BV474 were assigned location-dependently to groups III and V, respectively. The T-RFs of the surface samples GT100/SS and BV500/SS1 clustered together and were neither related to the cluster Glacier Transect nor to one of the Black Valley subgroups.

The RDA plot showed the distribution of the samples, the orientation and weights of the environmental parameters and the connection to certain microbial taxa (Fig. 4). The first two axes accounted for 38.5% (PC1) and 19.3% (PC2) of the total variance in the Glacier Transect and for 36.4% (PC1) and 21.4% (PC2) of the total variance in the Black Valley Transect. Samples resembling the Glacier Transect were distributed over all quadrants and were not characterized by a certain soil property. Only two clear reverse correlated clusters were formed: GT65 and GT0. Soils of the profile GT0 were negatively correlated with trace elements (Fig. 4a–c, Quadrant I), whereas GT65 depended on cations and anions (nitrate, phosphate and fluoride) of trace elements. A trend in certain environmental parameters could be observed along the glacier forefield. Soil moisture and pH increased with depth and decreased with distance to the glacier, whereas conductivity was inversely correlated and was best explained by sulphate, chloride and sodium concentrations. Bacterial gene copy numbers were higher in surface soils distant from the glacier.

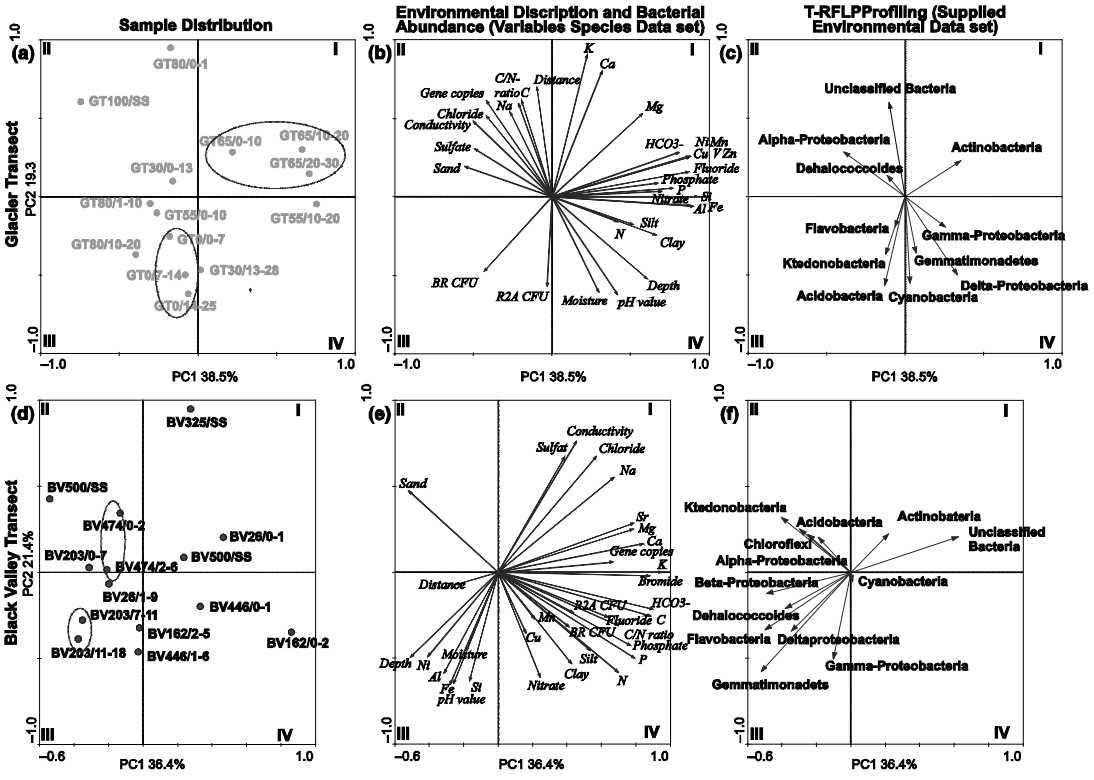

Fig. 4. Ordination Plot of the RDA results with CANOCO (Ter Braak & Šmilauer, 2002), showing the weights and orientation of samples, soil parameters and T-RFs on PC1 and PC2. Soil parameters and bacterial abundances build up the Variables Species Data Set and T-RFs, which were determined without ambiguity, the Supplied Environmental Data Set. One RDA was performed for each glacier transect. For clarity of presentation, the plot has been split into samples (a, d), Variables Species (b, e) and Supplied Environmental (c, f) Data Set. GT, Glacier Transect; BV, Black Valley; Pc, Principle component.

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Samples of the Black Valley Transect were distributed heterogeneously, and the distance from the glacier played a minor role according to the RDA. Position BV474 and surface samples BV500/SS1 and BV203/0-7 were characterized by a coarse grain size (sand, Fig. 4d and e, Quadrant II). On the contrary, surface samples of BV446 and BV162 were influenced by silt, clay, trace elements and salts. Conductivity was linked to sulphate, chloride and sodium and decreased with depth, whereas soil moisture and pH were positively related to soil depth and increased, although only by a small amount. Samples in the deeper soils were characterized through water availability (Fig. 4d and e; Quadrant IV); surface samples depended on conductivity and anions (Fig. 4d and e, Quadrant I). *Gemmatimonadetes* increased with depth and were inversely related to *Actinobacteria* and unclassified bacteria, which all decreased with depth.

In both transects, *Gemmatimonadetes*, *Gamma-* and *Deltaproteobacteria* were increasingly observed with depth and were influenced by soil moisture and soil pH. The same trend was shown for *Cyanobacteria* in the Glacier Transect and *Betaproteobacteria* and *Bacteroidetes* in the Black Valley Transect. *Actinobacteria* were positively correlated with cations and anions of trace elements that were especially present in profile GT65 (Fig. 4d and e, Quadrant I) and in the surface samples of the Black Valley in Quadrant I (Fig. 4). *Acidobacteria* and *Ktedonobacteria* occurred together, but were not influenced by any particular soil parameter.

Discussion

The two analysed glacier forefields of the Larsemann Hills, East Antarctica, represent areas of different bacterial succession. The results of this study give new insights how soil parameters impact the structure and development of glacier forefield bacterial communities.

The geochemical analysis reveals a deglaciation-dependent habitat formation with very slow primary succession that is still at its beginning, although the area has already been ice free since at least 40 ka BP (Hodgson *et al.*, 2001). In contrast to the Antarctic Peninsula, which is characterized by cold-maritime climatic conditions (Vieira & Ramos, 2003), our study site is influenced by the harsh environmental conditions of continental Antarctica (ANARE, 2000). While initial soil formation processes such as humus accumulation, acidification or brownification were observed in maritime Antarctic terrestrial habitats (Bockheim, 2008; Ganzert *et al.*, 2011a), none of those applied to the studied glacier forefields, which were characterized by very low soil moisture and small oligotrophic nutrient pools, comparable to the extreme Antarctic Dry Valleys (reviewed in Cary *et al.*, 2010).

The Glacier Transect is directly influenced by the glacier tongue and deglaciation. Therefore, water content and pH decrease and conductivity increases along the forefield. Due to its characteristics, the Glacier Transect resembles a 'classical glacier forefield' with a temporary and areal shift as observed previously for pH, organic carbon, sulphate and water content (Noll & Wellinger, 2008).

In contrast to the Glacier Transect, the Black Valley Transect extends over a larger area and is influenced by glacier and snow caps from both sites. All parameters strongly depend on the position in the slope or outflow of the glacier forefield. A trend in soil moisture, pH and conductivity could be observed in soil depth, but not as a shift along the transect. The distribution of soil parameters is influenced by strong winds, snow melting processes or the mechanical movement and downwash of clay, silt and fine particles (Schütte *et al.*, 2009), which lead to soil heterogeneity with no clear trend of the soil characteristics along glacier forefields (Lazzaro *et al.*, 2009; Schütte *et al.*, 2009). Furthermore, Antarctic habitat formation is known to be influenced by local microclimate effects and local-scale variability (Cannone *et al.*, 2008). The Black Valley Transect is characterized by the black material in the outflow, which is suggested to be from biogenic origin because of higher carbon contents at the surface and the fact that several clones and T-RFs were affiliated to *Cyanobacteria*. Similar (black) algae mats have previously been reported for cold deserts (Alger *et al.*, 1997). The higher soil moisture and abundance of microorganisms indicate further that the Black Valley Transect has gone through a longer development process, representing a slightly 'matured' glacier forefield.

We found that the habitat formation of the glacier forefields is influenced by deglaciation processes resulting in an areal shift of soil parameters especially in the 'classical' glacier forefield (Glacier Transect) and it is driven by the extreme Antarctic conditions, leading to a variable and prevalently depth-related distribution of soil parameters in the slightly 'matured' glacier forefield (Black Valley Transect).

Clone library analysis and T-RFLP profiling reveal a high diversity for all studied profiles. The dominant taxa in the glacier forefields are *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Chloroflexi*. Furthermore, clone libraries and the NMDS analysis of T-RFs show a clear difference in the bacterial community composition of 'classical' and 'matured' glacier forefields. Communities of the 'classical' forefield are similarly composed. They are dominated by *Actinobacteria* (*c.* 50% of the clone libraries), supplemented by a forefield-dependent shifting community distribution of *Acidobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Chloroflexi* and *Proteobacteria*.

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Communities of the 'matured' forefield show clearly different compositions at each location in the forefield indicating a specialized bacterial community in dependence on the ecological variations along this transect. Clone library results reveal a location-dependent *Actinobacteria*- or *Cyanobacteria*-dominated community and communities that have a balanced composition without a clear dominance of one group. Some groups show an orderly succession in connection to soil parameters, in agreement with what has been reported for alpine glacier forefields (Sigler *et al.*, 2002; Noll & Wellinger, 2008).

Soil moisture, pH and (trace) elements impact certain microbial taxa and the glacier forefield development. The comparison of environmental and microbiological data shows that several taxa (*Cyanobacteria, Gamma- and Deltaproteobacteria, Bacteroidetes, Gemmatimonadetes*) depend on water availability in both transects; especially *Cyanobacteria* and *Deltaproteobacteria* of the Glacier Transect occur with increasing soil moisture, pH and depth and decrease with distance from the glacier. Nutrient and water limitation have a greater effect on the microbial community structure than changing temperature regimes as shown for dry mineral Antarctic soils (Wynn-Williams, 1996; Yergeau *et al.*, 2012). Several of the identified clones, T-RFs or isolates were affiliated to phototrophic *Cyanobacteria* and *Chloroflexi* or chemolithotrophic/chemoorganotrophic *Nitrosomonas* (*Betaproteobacteria*), *Nitrospira* (*Nitrospirae*) and *Rhizobiales* (*Alphaproteobacteria*), which provide the basis for an efficient ecosystem because of their participation in primary production. Because the glacier forefields lack essential nutrients, primary producers fulfil the tasks of assimilating inorganic carbon or fixing atmospheric nitrogen to provide substrates for other microorganisms. *Bacteroidetes* were most abundant in the vicinity of the glaciers, where constant low temperatures and water availability are most likely to occur. Compared to distant locations in the forefield that are much drier and can reach considerable above-zero temperatures at the soil surface in Antarctic summer, *Bacteroidetes*, comprising several psychrophilic representatives, are well adapted to cold conditions at the glacier tongue (Shivaji *et al.*, 1992; Bajerski *et al.*, 2013). *Bacteroidetes* were detected in various cold-affected and poorly developed habitats (Liebner *et al.*, 2008; Ganzert *et al.*, 2011a) contributing to biological weathering by degrading polymers (Buckley & Schmidt, 2001) and producing extracellular enzymes such as lipases, proteases and phosphatases (Hirsch *et al.*, 1998; Aislabie *et al.*, 2006). In this way, they support initial soil formation, which is an important function in the development of glacier forefields. *Gemmatimonadetes*-affiliated T-RFs in our study were positively related to soil moisture, depth and pH, in contrast to previous studies that did not show significant correlations between *Gemmatimonadetes* (former candidate division BD) and soil organic matter content, inorganic N concentration or soil pH (Mummey & Stahl, 2003). Environmental sequences of the *Gemmatimonadetes* were found to be widespread in different habitats and occurred at different geographical locations, including Antarctic cryoconite holes (Christner *et al.*, 2003) and various soils (Mummey & Stahl, 2003). Their phylogenetic divergence and broad dispersion hint to a diverse metabolic and functional potential, which can allow them to colonize several ecological niches in the glacier forefields. Their exact functions remain unclear as of yet only one isolate has been described (Zhang *et al.*, 2003).

Trace elements (magnesium, calcium, potassium) and salts were found to influence the presence of *Actinobacteria* in both transects. So far, this heterogeneous group is not known to be dependent on a certain salt or nutrient pool, but trace elements play an important role for microbial turnover in general (e.g. electron donors or acceptors, cofactors). *Actinobacteria*, being common in all soils, are the dominant group of the glacier forefields (especially in the Glacier Transect), and they were isolated from cold-affected habitats before (Bajerski *et al.*, 2011; Ganzert *et al.*, 2011b). Their successful colonization of the glacier forefields may be caused by their ability to metabolize a wide range of substrates as sole carbon source. These organic matter turnover and accumulation is also important for the habitat development in the sense of soil formation processes and as a part in the carbon cycle in relation to other microorganisms. Furthermore, they may be adapted to the low temperatures of the study site by releasing simple carbon compounds as compatible solutes that protect the organisms against freezing (Wynn-Williams, 1996; Aislabie *et al.*, 2006).

The oligotrophic conditions of the glacier forefields support the development of an *Acidobacteria*-related community, because they live under extremely low nutrient conditions with very slow metabolic growth rates and tolerate fluctuations in soil hydration (Ward *et al.*, 2009). With this lifestyle, they are well adapted to extreme conditions of the glacier forefields and common in Antarctic terrestrial environments (Saul *et al.*, 2005; Ganzert *et al.*, 2011a), but their concrete function remains unknown due to the lack of isolates.

The comparison of environmental and biological data shows that soil parameters impact certain microbial phyla. There is a clear difference in the community composition of 'classical' and 'matured' glacier forefields, with an undifferentiated and a specialized bacterial community, respectively.

Comparing Antarctic sites and taking into account the poorly developed soils and severe conditions of the studied glacier forefields, a surprisingly high bacterial diversity

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and abundance was observed. Although the communities still remain undersampled in some cases (coverage of species richness estimators < 50%), high diversity indexes (Shannon–Weaver > 3) indicate a diverse bacterial community. Interestingly, the diversity at all sites was higher than in similar Antarctic cold desert soils (Smith *et al.*, 2006) and comparable (although still higher) to the functional diversity of microbial communities of glacier forefields of the Antarctic Peninsula (Pessi *et al.*, 2012). Therefore, glacier forefields are hotspots for microbial diversity among Antarctic soil communities.

In conclusion, we show that glacier forefields are promising model systems to study soil formation along microbial successions. The results of the two studied transects indicate furthermore that microbial successions such as growth/dominance of certain phyla or taxa are not necessarily coupled to soil formation if, for instance, carbon and water are limited. This finding supports the hypothesis that terrestrial ecosystems in the state of initial habitat formation are characterized by highly diverse but undifferentiated microbial communities, which preserve a broad range of genetic potentials. Low metabolic activity seems to be one important aspect to maintain these diverse communities.

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References

The references of all manuscripts are included in the chapter 4. References.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Two glacier forefields on the Larsemann Hills, East Antarctica, were chosen for analysis in this study. a) Glacier Transect; b) Black Valley Transect.

- See Figure 4a and 5a of the chapter 1. Introduction of this thesis.

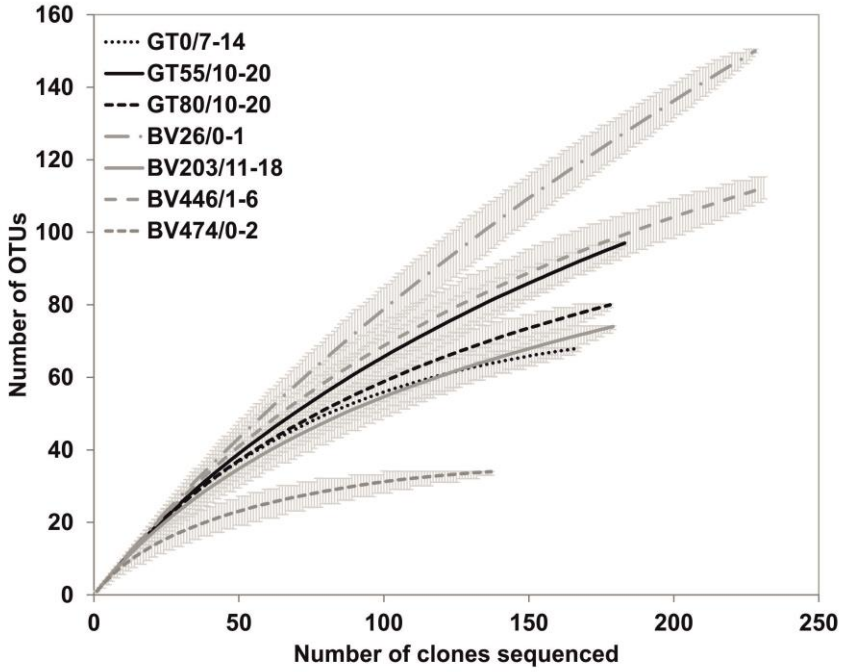

Fig. S2. Rarefaction curves showing phylotype (≥98% similarity) richness of bacterial 16S rRNA gene clone libraries from selected samples of the Glacier Transect (GT0/7-14, GT55/10-20, GT80/10-20) and the Black Valley Transect (BV26/0-1, BV203/11-18, BV446/1-6, BV474/0-2).

Table S1. Summary of PCR primers and protocols used in this study.

Primer	Sequence (5`-3`)	Protocol	Application	Reference
27 F	AGAGTTTGATCMTG GCTCAG	95°C 5min - 30 cycles: 95°C, 56°C and 72°C 1 min each - 72°C 10 min	Identification of isolates, TRFLP PCR I (20 cycles)	(Lane, *et al.*, 1985)
1492 R	GGTTACCTTGTTACG ACTT			(Dojka, *et al.*, 1998)
FAM-27 F	6FAM-AGAGTTTGATCMTG GCTCAG	95°C 5min - 25 cycles: 95°C , 57°C , 72°C 45s each—72°C 10 min	TRFLP PCR II	(Lane, *et al.*, 1985)
907 R	CCGTCAATTCCTTTR AGTTT			(Muyzer, *et al.*, 1995)
27 F	AGAGTTTGATCMTG GCTCAG	95°C 10min - 30 cycles: 95°C , 57°C , 72°C 45s each - 72°C 10 min	Cloning	(Lane, *et al.*, 1985)
907 R	CCGTCAATTCCTTTR AGTTT			(Muyzer, *et al.*, 1995)
M13 R	TGTAAAACGACGGC CAGT	95°C 4min - 28 cycles: 95°C, 56°C, 72°C 1min each - 72°C 10min	Reamplification after cloning	(Messing, *et al.*, 1981)
M13 F	CAGGAAACAGCTAT GACC			
Uni 331F	TCCTACGGGAGGCA GCAGT	95°C 10min - 30 cycles: 95°C 1 min, 53°C 45s, 72°C 45s - 72°C 15 min	Q-RT-PCR-Standard	(Nadkarni, *et al.*, 2002)
Uni 797R	GGACTACCAGGGTA TCTAATCCTGTT			
Eub 338F	ACTCCTACGGGAGG CAGC	95°C 10min - 30 cycles: 95°C 1 min, 53°C 45s, 72°C 45s - 72°C 15 min	Q-RT-PCR	(Lane, *et al.*, 1985, Muyzer, *et al.*, 1995, Fierer & Jackson, 2006)
Eub 518R	ATTACCGCGGCTGCT GG			

Table S2. Cation of trace elements measured in a soil slurry (5 g soil + 25 ml mili Q water) with inductively coupled plasma optical emission spectrometry (ICP-OES, PERKIN ELMER OPTIMA3000XL).

Sample ID	Al mg l^{-1}	Ca mg l^{-1}	Fe mg l^{-1}	K mg l^{-1}	Mg mg l^{-1}	Mn µg l^{-1}	Na mg l^{-1}	P mg l^{-1}	Si mg l^{-1}	Sr µg l^{-1}	V µg l^{-1}	Ni µg l^{-1}	Cu µg l^{-1}	Zn µg l^{-1}
GT0/0-7	3.84	0.12	1.94	0.63	0.48	< 20	2.88	0.10	5.86	< 20	< 20	< 20	< 20	< 20
GT0/7-14	5.36	< 0.10	2.66	0.53	0.42	< 20	1.65	0.15	6.98	< 20	< 20	< 20	< 20	< 20
GT0/14-25	4.47	< 0.10	2.08	0.56	0.39	< 20	1.71	0.15	6.32	< 20	< 20	< 20	< 20	< 20
GT30/0-13	5.61	0.17	2.99	0.90	0.52	< 20	3.03	0.16	7.82	< 20	< 20	< 20	< 20	< 20
GT30/13-28	5.25	< 0.10	2.57	0.70	0.41	< 20	3.19	0.13	7.13	< 20	< 20	< 20	< 20	< 20
GT55/0-10	6.37	< 0.10	2.00	0.98	0.40	< 20	14.10	0.11	8.04	< 20	< 20	< 20	< 20	< 20
GT55/10-20	17.20	0.15	5.77	1.26	1.01	46.80	3.36	0.23	22.20	< 20	27.80	41.79	23.74	21.52
GT65/0-10	23.40	0.16	6.16	1.65	1.31	37.80	4.15	0.22	23.80	< 20	23.67	38.36	20.80	21.94
GT65/10-20	31.70	0.19	7.86	1.68	1.68	43.00	2.64	0.29	29.20	< 20	30.05	49.97	21.32	27.28
GT65/20-30	22.95	0.19	7.15	1.45	1.34	27.60	2.94	0.28	23.35	< 20	24.76	42.58	25.23	21.90
GT80/0-1	2.37	0.30	1.81	2.34	0.77	< 20	5.32	0.17	3.88	< 20	< 20	< 20	< 20	< 20
GT80/1-10	2.24	< 0.10	1.47	0.74	0.25	< 20	1.34	< 0.10	3.82	< 20	< 20	< 20	< 20	< 20
GT80/10-20	0.92	< 0.10	0.42	0.30	< 0.10	< 20	1.07	< 0.10	1.76	< 20	< 20	< 20	< 20	< 20
GT100/SS	0.33	0.24	0.16	2.24	0.88	< 20	16.20	< 0.10	0.98	< 20	< 20	< 20	< 20	< 20
BV26/0-1	2.14	1.91	1.74	4.53	3.62	80.20	15.00	0.84	4.30	23.70	< 20	< 20	< 20	< 20
BV26/1-9	5.87	0.38	3.14	1.21	0.82	22.90	2.94	0.28	8.06	< 20	< 20	< 20	< 20	< 20
BV162/0-2	4.50	2.35	2.00	10.40	3.82	34.20	16.10	2.43	7.90	23.70	< 20	< 20	< 20	< 20
BV162/2-5	14.90	0.72	5.20	1.89	1.40	< 20	6.36	0.56	16.75	< 20	< 20	27.70	< 20	< 20
BV203/0-7	8.44	0.17	3.99	1.28	0.74	< 20	3.38	0.21	10.30	< 20	< 20	22.45	< 20	< 20
BV203/7-11	11.60	0.13	5.27	1.02	0.76	28.90	2.28	0.24	14.00	< 20	< 20	23.62	< 20	< 20
BV203/11-18	13.30	0.13	6.24	1.03	0.84	33.20	2.61	0.26	16.30	< 20	< 20	26.57	< 20	< 20
BV446/0-1	9.90	1.58	4.06	3.32	2.92	< 20	9.59	1.77	15.70	22.40	< 20	27.34	25.84	< 20
BV446/1-6	14.80	0.99	5.51	2.36	2.18	< 20	5.41	0.70	19.50	< 20	< 20	31.59	27.39	< 20
BV474/0-2	9.80	0.90	4.27	1.67	1.98	< 20	12.90	0.19	12.60	< 20	< 20	20.46	< 20	< 20
BV474/2-6	15.90	0.71	6.72	1.89	1.96	20.70	8.93	0.23	20.00	< 20	< 20	30.83	< 20	< 20
BV325/SS	3.65	3.32	1.25	3.99	7.75	< 20	60.10	0.13	5.38	37.20	< 20	< 20	< 20	< 20
BV500/SS1	1.92	< 0.10	0.79	0.42	0.16	< 20	2.60	< 0.10	2.63	< 20	< 20	< 20	< 20	< 20
BV500/SS2	3.51	0.89	1.79	4.71	1.70	23.40	5.84	1.07	6.21	< 20	< 20	< 20	< 20	< 20

Table S3. Anions of trace elements were measured in a soil slurry (5 g soil + 25 ml mili Q water) with ion chromatography (DIONEX-DX320). HCO_3^- was determined with titration (METROHM TITRINO 794).

Sample ID	Fluoride mg l^{-1}	Chloride mg l^{-1}	Sulfate mg l^{-1}	Bromide mg l^{-1}	Nitrate mg l^{-1}	Phosphate mg l^{-1}	HCO_3^- mg l^{-1}
GT0/0-7	< 0.05	4.01	1.33	< 0.05	< 0.15	< 0.10	1.98
GT0/7-14	< 0.05	0.99	0.92	< 0.05	< 0.15	0.15	1.53
GT0/14-25	< 0.05	1.06	0.88	< 0.05	< 0.15	0.18	1.68
GT30/0-13	< 0.05	3.40	1.72	< 0.05	< 0.15	< 0.10	1.83
GT30/13-28	< 0.05	3.56	1.86	< 0.05	< 0.15	< 0.10	2.59
GT55/0-10	< 0.05	21.10	2.66	< 0.05	< 0.15	< 0.10	2.59
GT55/10-20	0.11	2.53	0.66	< 0.05	0.17	0.19	4.12
GT65/0-10	< 0.05	3.96	1.24	< 0.05	< 0.15	< 0.10	3.05
GT65/10-20	0.09	0.81	0.65	< 0.05	< 0.15	0.22	4.12
GT65/20-30	0.08	1.69	0.68	< 0.05	0.08	0.22	2.97
GT80/0-1	< 0.05	9.08	1.16	< 0.05	< 0.15	0.14	3.20
GT80/1-10	< 0.05	1.04	0.50	< 0.05	< 0.15	< 0.10	1.98
GT80/10-20	< 0.05	0.38	0.39	< 0.05	< 0.15	< 0.10	1.83
GT100/SS	< 0.05	29.31	4.95	0.05	< 0.15	< 0.10	1.07
BV26/0-1	0.05	28.83	3.41	0.55	0.78	**1.94**	10.22
BV26/1-9	< 0.05	1.77	0.96	0.08	2.23	0.13	2.59
BV162/0-2	0.16	18.14	3.78	0.74	0.34	**6.40**	29.44
BV162/2-5	0.08	5.69	1.35	0.17	3.24	0.41	4.27
BV203/0-7	< 0.05	4.67	1.42	< 0.05	< 0.15	< 0.10	1.53
BV203/7-11	< 0.05	0.93	1.43	< 0.05	0.23	0.11	1.98
BV203/11-18	< 0.05	1.60	1.33	< 0.05	0.16	0.21	1.98
BV446/0-1	< 0.05	6.48	2.66	1.01	3.97	**2.87**	10.68
BV446/1-6	< 0.05	1.30	1.30	0.23	**12.25**	0.30	4.12
BV474/0-2	< 0.05	22.64	5.51	0.05	< 0.15	< 0.10	1.83
BV474/2-6	< 0.05	13.93	4.96	< 0.05	< 0.15	< 0.10	1.68
BV325/SS	< 0.05	113.17	22.71	0.29	< 0.15	< 0.10	3.20
BV500/SS1	< 0.05	2.52	1.46	< 0.05	< 0.15	< 0.10	1.53
BV500/SS2	< 0.05	6.46	1.13	0.22	0.18	**2.79**	12.66

Table S4. Phylogenetic assignment of isolated strains and 16S rRNA gene similarities to the closest cultured relatives, using GenBank database and BlastN algorithm.

Strain ID	Accession number	Phylogenetic affiliation (order)	Closest cultured relative	Accession number	Simi-larity (%)
05-02PB	JX491397	Sphingobacteriales	Mucilaginibacter daejeonensis strain Jip 10	041505.1	95
05-03PB	JX491398	Sphingobacteriales	Mucilaginibacter daejeonensis strain Jip 10	041505.1	95
05-04PB	JX491399	Burkholderiales	Janthinobacterium agaricidamnosum strain W1r3	026364.1	98
05-05PB	JX491400	Rhizobiales	Methylobacterium organophilum strain ATCC 27886	041027.1	97
05-07PB	JX491401	Sphingomonadales	Sphingomonas melonis strain DAPP-PG 224	028626.1	98
06-01PB	JX491402	Actinobacteridae	Streptomyces psammoticus strain IFO 13971	043372.1	97
07-02PB	JX491404	Actinobacteridae	Streptomyces psammoticus strain IFO 13971	043372.1	98
07-03PB	JX491405	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	99
07-05PB	JX491407	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	99
07-06PB	JX491408	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	99
08-02PB	JX491409	Actinobacteridae	Streptomyces herbaricolor strain NBRC 3838	041212.1	98
08-03PB	JX491410	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	99
09-02PB	JX491412	Actinobacteridae	Streptomyces psammoticus strain IFO 13971	043372.1	98
10-1PB	JX491413	Actinobacteridae	Streptomyces psammoticus strain IFO 13971	043372.1	98
11-01PB	JX491414	Actinobacteridae	Streptomyces herbaricolor strain NBRC 3838	041212.1	97
11-02PB	JX491415	Actinobacteridae	Streptomyces psammoticus strain IFO 13971	043372.1	98
11-03PB	JX491416	Actinobacteridae	Streptomyces clavifer strain NRRL B-2557	043507.1	98
11-04PB	JX491417	Actinobacteridae	Streptomyces clavifer strain NRRL B-2557	043507.1	97
12-01PB	JX491418	Actinobacteridae	Arthrobacter oxydans strain DSM 20119	026236.1	99
12-04PB	JX491421	Actinobacteridae	Streptomyces herbaricolor strain NBRC 3838	041212.1	98
12-05PB	JX491422	Actinobacteridae	Streptomyces psammoticus strain IFO 13971	043372.1	98
13-02PB	JX491479	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	98
13-04PB	JX491423	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	98
13-05PB	JX491424	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	99
13-07PB	JX491425	Actinobacteridae	Demetria terragena strain HK1 0089	026425.1	94

14-01bPB	JX491426	Rhizobiales	Aurantimonas ureilytica strain 5715S-12	043995.1	95
14-04PB	JX491427	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	99
14-05PB	JX491428	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	99
14-07PB	JX491429	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	97
14-08PB	JX491430	Actinobacteridae	Frondihabitans australicus strain E1HC-02	043897.1	98
14-09PB	JX491431	Actinobacteridae	Demetria terragena strain HK1 0089	026425.1	93
15-01bPB	JX491432	Actinobacteridae	Cryobacterium psychrotolerans strain 0549	043892.1	99
15-03PB	JX491433	Actinobacteridae	Salinibacterium xinjiangense strain 0543	043893.1	99
15-04PB	JX491434	Flavobacteriales	Chryseobacterium marinum strain IMCC3228	044280.1	98
15-05PB	JX491435	Flavobacteriales	Chryseobacterium marinum strain IMCC3228	044280.1	98
15-09PB	JX491436	Flavobacteriales	Chryseobacterium jejuense strain JS17-8	044300.1	98
16-02PB	JX491438	Actinobacteridae	Salinibacterium xinjiangense strain 0543	043893.1	97
16-03PB	JX491439	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	97
16-04PB	JX491440	Burkholderiales	Janthinobacterium lividum strain DSM 1522	026365.1	98
16-05PB	JX491441	Flavobacteriales	Chryseobacterium marinum strain IMCC3228	044280.1	98
17-01PB	JX491444	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	99
17-02PB	JX491445	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	98
17-03PB	JX491446	Actinobacteridae	Arthrobacter humicola strain KV-653	041546.1	99
17-05PB	JX491447	Sphingomonadales	Sphingomonas oligophenolica	024685.1	98
17-07PB	JX491449	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	99
17-08PB	JX491450	Actinobacteridae	Arthrobacter ramosus strain DSM 20546	026193.1	99
93-01PB	JX491467	Actinobacteridae	Humicoccus flavidus strain DS-52	043717.1	97
93-03PB	JX491469	Burkholderiales	Massilia niabensis strain 5420S-26	044571.1	98
18-01PB	JX491451	Pseudomonadaceae	Pseudomonas mandelii strain CIP 105273	024902.1	100
18-02PB	JX491452	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
18-03PB	JX491453	Pseudomonadaceae	Pseudomonas thivervalensis strain SBK26	024951.1	99
18-04PB	JX491454	Pseudomonadaceae	Pseudomonas thivervalensis strain SBK26	024951.1	99
18-09PB	JX491455	Comamonadaceae	Polaromonas hydrogenivorans strain DSM 17735	043540.1	99
18-10PB	JX491324	Flavobacteriaceae	Chryseobacterium marinum strain IMCC3228	044280.1	97

18-5PB	JX491320	Actinomycetales	Arthrobacter oxydans strain DSM 20119	026236.1	98
18-6PB	JX491322	Flavobacteriaceae	Sejongia antarctica strain AT1013	025809.1	98
18-8PB	JX491319	Flavobacteriaceae	Sejongia antarctica strain AT1013	025809.1	98
19-11PB	JX491318	Sphingobacteriaceae	Pedobacter terricola strain DS-45	044219.1	95
19-3PB	JX491325	Pseudomonadaceae	Pseudomonas migulae strain CIP 105470	024927.1	98
19-6PB	JX491323	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
19-9PB	JX491321	Flavobacteriaceae	Chryseobacterium marinum strain IMCC3228	044280.1	96
20-02PB	JX491456	Pseudomonadaceae	Pseudomonas migulae strain CIP 105470	024927.1	99
20-04PB	JX491457	Pseudomonadaceae	Pseudomonas mandelii strain CIP 105273	024902.1	100
20-1bPB	JX491458	Pseudomonadaceae	Pseudomonas mandelii strain CIP 105273	024902.1	100
21-03PB	JX491459	Pseudomonadaceae	Pseudomonas thivervalensis strain SBK26	024951.1	99
21-1bPB	JX491460	Pseudomonadaceae	Pseudomonas thivervalensis strain SBK26	024951.1	99
21-1aPB	JX491326	Pseudomonadaceae	Pseudomonas thivervalensis strain SBK26	024951.1	99
21-2PB	JX491327	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99
22-1PB	JX491328	Actinomycetales	Arthrobacter stackebrandtii strain : CCM 2783	042258.1	96
22-3PB	JX491329	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
22-4PB	JX491330	Actinomycetales	Frigoribacterium sp. MSL 08 strain MSL 08	044238.1	98
22-6PB	JX491331	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99
23-1PB	JX491332	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
23-2aPB	JX491333	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
23-3PB	JX491334	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
23-4PB	JX491335	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
23-6PB	JX491336	Actinomycetales	Arthrobacter oxydans strain DSM 20119	026236.1	98
23-7PB	JX491337	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	99
23-8aPB	JX491338	Pseudomonadaceae	Pseudomonas frederiksbergensis strain JAJ28	028906.1	98
24-1PB	JX491339	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
24-2PB	JX491340	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
24-3PB	JX491341	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99
24-4PB	JX491342	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99

24-5PB	JX491343	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99
24-6PB	JX491344	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
25-02PB	JX491461	Oxalobacteraceae	Massilia brevitalea strain byr23-80	044274.1	97
25-11PB	JX491462	Oxalobacteraceae	Undibacterium pigrum strain : CCUG 49009	042557.1	99
25-13PB	JX491463	Pseudomonadaceae	Pseudomonas meridiana strain CMS 38	025587.1	99
25-18PB	JX491464	Oxalobacteraceae	Massilia niabensis strain 5420S-26	044571.1	98
25-21PB	JX491465	Pseudomonadaceae	Pseudomonas meridiana strain CMS 38	025587.1	99
25-1PB	JX491345	Oxalobacteraceae	Janthinobacterium agaricidamnosum strain W1r3	026364.1	98
25-12PB	JX491346	Oxalobacteraceae	Massilia niabensis strain 5420S-26	044571.1	98
25-14PB	JX491347	Actinomycetales	Frigoribacterium sp. MSL 08 strain MSL 08	044238.1	98
25-15aPB	JX491348	Sphingomonadaceae	Sphingomonas oligophenolica	024685.1	99
25-17PB	JX491349	Sphingomonadaceae	Sphingomonas oligophenolica	024685.1	97
25-20PB	JX491350	Comamonadaceae	Polaromonas jejuensis strain JS12-13	044379.1	99
25-3PB	JX491351	Oxalobacteraceae	Herbaspirillum autotrophicum strain IAM 14942	040899.1	97
25-4PB	JX491352	Flavobacteriaceae	Chryseobacterium marinum strain IMCC3228	044280.1	97
25-5PB	JX491353	Sphingomonadaceae	Sphingomonas aquatilis strain JSS-7	024997.1	98
25-7PB	JX491354	Cytophagaceae	Hymenobacter soli strain PB17	041437.1	95
25-8PB	JX491355	Sphingomonadaceae	Sphingomonas aerolata strain NW12	042130.1	99
26-1PB	JX491356	Caulobacteraceae	revundimonas subvibrioides strain CB81	037107.1	97
26-10PB	JX491357	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99
26-11PB	JX491358	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	99
26-12PB	JX491359	Sphingobacteriaceae	Pedobacter composti strain TR6-06	041506.1	96
26-13PB	JX491360	Sphingobacteriaceae	Pedobacter composti strain TR6-06	041506.1	95
26-14PB	JX491361	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
26-16PB	JX491362	Sphingomonadaceae	Sphingomonas aquatilis strain JSS-7	024997.1	98
26-2PB	JX491363	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
26-3PB	JX491364	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
26-4PB	JX491365	Flavobacteriaceae	Chryseobacterium marinum strain IMCC3228	044280.1	96
26-5PB	JX491366	Actinomycetales	Cryobacterium psychrotolerans strain 0549	043892.1	98
26-6PB	JX491367	Actinomycetales	Arthrobacter oryzae strain KV-	041545.1	99

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26-8PB	JX491368	Flavobacteriaceae	Sejongia antarctica strain AT1013	025809.1	98
26-9PB	JX491369	Actinomycetales	Arthrobacter oryzae strain KV-651	041545.1	99
27-02PB	JX491466	Actinomycetales	Arthrobacter humicola strain KV-653	041546.1	96
27-1PB	JX491370	Sphingomonadaceae	Sphingomonas oligophenolica	024685.1	98
27-3PB	JX491371	Actinomycetales	Leifsonia kafniensis strain : KFC-22	042669.1	98
27-4PB	JX491372	Actinomycetales	Frigoribacterium faeni strain 801	026511.1	98
27-5PB	JX491373	Actinomycetales	Cryobacterium psychrotolerans strain 0549	043892.1	99
27-6PB	JX491374	Actinomycetales	Aeromicrobium ginsengisoli strain Gsoil 098	041384.1	99
27-7PB	JX491375	Actinomycetales	Leifsonia kafniensis strain : KFC-22	042669.1	98
28-1PB	JX491376	Deinococcaceae	Deinococcus claudionis strain PO-04-19-125	044331.1	99
28-2PB	JX491377	Actinomycetales	Arthrobacter stackebrandtii strain : CCM 2783	042258.1	97
28-3PB	JX491378	Actinomycetales	Arthrobacter stackebrandtii strain : CCM 2783	042258.1	97
28-4PB	JX491379	Xanthomonadaceae	Rhodanobacter ginsengisoli strain GR17-7	044127.1	99
28-5PB	JX491380	Actinomycetales	Arthrobacter stackebrandtii strain : CCM 2783	042258.1	97
28-6PB	JX491381	Actinomycetales	Cryobacterium psychrotolerans strain 0549	043892.1	99
28-7PB	JX491382	Actinomycetales	Salinibacterium xinjiangense strain 0543	043893.1	98
28-8PB	JX491383	Actinomycetales	Arthrobacter humicola strain KV-653	041546.1	97
94-07PB	JX491470	Caulobacteraceae	Brevundimonas subvibrioides strain CB81	037107.1	99
94-1PB	JX491384	Caulobacteraceae	Brevundimonas subvibrioides strain CB81	037107.1	99
94-10PB	JX491385	Cytophagaceae	Hymenobacter actinosclerus strain 1187	026470.1	97
94-2PB	JX491386	Oxalobacteraceae	Herbaspirillum frisingense strain GSF30	025353.1	97
94-5PB	JX491387	Flavobacteriaceae	Sejongia antarctica strain AT1013	025809.1	98
94-6PB	JX491388	Moraxellaceae	Psychrobacter glacincola strain DSM 12194	042076.1	98
94-8PB	JX491389	Flavobacteriaceae	Sejongia antarctica strain AT1013	025809.1	98
94-9PB	JX491390	Actinomycetales	Arthrobacter agilis strain DSM 20550	026198.1	98
95-1PB	JX491391	Actinomycetales	Arthrobacter ramosus strain DSM 20546	026193.1	98
95-3PB	JX491392	Actinomycetales	Arthrobacter polychromogenes strain DSM 20136	026192.1	98
96-06PB	JX491471	Oxalobacteraceae	Janthinobacterium lividum strain DSM 1522	026365.1	98
96-1PB	JX491393	Comamonadaceae	Polaromonas jejuensis strain	044379.1	98

			JS12-13		
96-10PB	JX491472	Cytophagaceae	Hymenobacter soli strain PB17	041437.1	93
96-2PB	JX491394	Oxalobacteraceae	Massilia brevitalea strain byr23-80	044274.1	98
96-3PB	JX491395	Flavobacteriaceae	Sejongia antarctica strain AT1013	025809.1	98
96-4BPB	JX491396	Oxalobacteraceae	Janthinobacterium lividum strain DSM 1522	026365.1	99
96-8PB	JX491473	Cytophagaceae	Hymenobacter soli strain PB17	041437.1	94

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Herbaspirillum psychrotolerans **sp. nov., a member of the family Oxalobacteraceae from a glacier forefield.**

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Key words: *Herbaspirillum*, Antarctic soil, psychrotolerant

New Taxa - Proteobacteria

The GenBank accession number of the 16S rRNA gene sequence of *Herbaspirillum psychrotolerans* PB1^T is JN390675.

Abstract

A novel psychrotolerant, Gram-negative, shiny white, curved-rod-shaped, facultative anaerobic bacterium PB1^T was isolated from a soil sample collected from a glacier forefield of the Larsemann Hills, East Antarctica. Isolate PB1^T has catalase and low urease activity and hydrolyses gelatine and starch. Strain PB1^T is able to grow between -5°C and 30°C with an optimum growth at 14-20°C. Glycerol, D-/L-arabinose, D-xylose, D-galactose, D-fructose, D-lyxose, D-fucose and potassium gluconate are used as sole carbon sources. The major quinone is ubiquinone Q-8. The major fatty acids (>10 %) for PB1^T are $C_{16:0}$ (19.1 %), $C_{16:1}\omega 7cis$ (44.6 %) and $C_{18:1}\omega 7cis$ (16.2 %). The major polyamines are putrescine (54.9 µmol g^{-1} dry weight) and 2-hydroxyputrescine (18.5 µmol g^{-1} dry weight). DNA base composition is 62.5 mol% G+C. Strain PB1^T is phylogenetically related to species of the genus *Herbaspirillum*, with highest 16S rRNA gene sequence similarities to *Herbaspirillum canariense* (97.3%), *Herbaspirillum aurantiacum* (97.2 %), *Herbaspirillum soli* (97.2 %) and *Herbaspirillum frisingense* (97.0 %). The DNA-DNA relatedness values were below 30 % between PB1^T and the type strains of *H. canariense*, *H. aurantiacum* and *H. soli*. The different geographical origin of strain PB1^T and its next relatives resulted in a different phenotypic and genotypic specification, whereby strain PB^T represents a novel species in the genus *Herbaspirillum*, for which the name *Herbaspirillum psychrotolerans* is proposed. The type strain is PB1^T (DSM 26001^T = LMG 27282^T).

The genus *Herbaspirillum* includes 13 species and two sub-species and belongs to the family *Oxalobacteraceae*. The type species *Herbaspirillum seropedicae* was described as a root-associated nitrogen-fixing bacterium by Baldani *et al.* (1986) with an emended description in 1996 (Baldani *et al.*, 1996). *Herbaspirillum* isolates were derived from different plant material (e.g. Jung *et al.*, 2007; Kirchhof *et al.*, 2001), well water (Ding & Yokota, 2004) and soils (Im *et al.*, 2004). The most recently discovered species *Herbaspirillum canarinense*, *H. aurantiacum*

and *H. soli* were isolated from soil in Tenerife (Canary Islands) and the authors added the presence of (slow and low) urease activity and the utilisation of several carbon compounds to the genus characterisation (Carro *et al.*, 2012). The isolation and characterisation of novel microorganisms from extreme habitats uncloses new possibilities in biotechnology for example in bioremediation or medicine (Nichols *et al.*, 1999). Several isolates from polar habitats produce cold-adapted enzymes and degrade a wide range of substrates and polymers under extreme conditions, as it was reported for *Arthrobacter* species from Antarctic (Ganzert *et al.*, 2011b) or *Cryobacteria* from Arctic soil (Bajerski *et al.*, 2011).

Within the scope of environmental studies of microbial communities in glacier forefields of the Larsemann Hills, East Antarctica, several cold-adapted bacteria were isolated from extremely dry and nutrient-poor soils. In this study, we report the isolation and classification of strain PB1^T. Considering morphological, physiological, biochemical and chemotaxonomic characterisation as well as phylogenetic analysis we propose strain PB1^T as a novel psychrotolerant species in the genus *Herbaspirillum*.

Strain PB1^T was isolated from a surface soil sample of a glacier forefield transect of the Larsemann Hills, East Antarctica (S 69°24, E 76°20). The soil material was suspended in a physiological salt solution (0.9 % NaCl, w/v), plated on R2A medium (0.05 % proteose peptone, 0.05 % casamino acids, 0.05 % yeast extract, 0.05 % dextrose, 0.05 % soluble starch, 0.03 % dipotassium phosphate, 0.005 % magnesium sulfate 7×H_2O, 0.03 % sodium pyruvate, 1.5 % agar for solid media (w/v), Reasoner & Geldreich, 1985) and incubated at 10°C for three weeks. A pure culture was derived by separating the colonies from the enrichment culture. The isolated bacteria were cultivated at 18°C on R2A and nutrient medium (w/v: 0.5 % tryptone, 0.3 % meat extract, for solid media 1.5 % agar, pH 7.2). The same conditions and media were used for all tests, unless otherwise noted.

Colony characteristics were determined visually on agar plates after seven days of bacterial growth. Cell morphology was examined by light microscopy (Zeiss Axioskop 2 plus, Jena, Germany) of cells in the exponential growth phase. Gram staining (using Gram staining set, Merck, Germany), spore detection and flagella presence was carried out by classical procedure as described by Gram, Wirtz and Loeffler, respectively (Schröder, 1991). The temperature and pH range for growth and salt tolerance were examined visually by checking turbidity and the formation of cell aggregates in liquid nutrient medium over 14 days. For pH determination the

medium was buffered with glycine (pH 4.0-5.0 and pH 10.0), MES [2-(*N*-morpholino) ethanesulfonic acid**;** pH 5.0-6.5], HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.0-8.0] and BTP [1.3-bis(tris(hydroxymethyl)methylamino)propane; pH 8.5-9.5; (Hoaki *et al.*, 1994)]. Anaerobic growth was tested on nutrient agar plates incubated at 22°C for 14 days under a N_2/CO_2 (80:20 v/v) atmosphere. Susceptibility to 12 different antibiotics (1 mg ml^{-1}) and lysozyme (1 mg ml^{-1}) was examined by measuring the inhibition areola in a filter disc test. API® 50 CH and API® 50 CHB/E medium (bioMérieux, Nürtingen, Germany) were used to identify acid production from carbohydrates. API® 50 CH strips and a minimal medium (Bajerski *et al.*, 2011), modified with 0.1% (w/v) yeast extract, were applied to check for the utilisation of carbon compounds as sole carbon sources. The stripes were incubated at 18°C for 14 days and the results were documented daily. The methyl-red test was performed using a glucose containing medium (v/w: 1 % tryptone, 0.5 % NaCl, 1 % glucose-monohydrate) and methyl-red as indicator. The same medium was used to determine urease activity adding 1 % Urea (w/v) as substrate and 1 % ethanolic phenolphthalein-solution (Merck) as indicator. Catalase activity was determined by bubble production in a 10 % hydrogen peroxide solution. Oxidase activity was analysed with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) as a redox indicator as described by Kovacs (1956). Lead acetate impregnated filter stripes were used to determine the production of hydrogen sulphide in a modified nutrient medium with 0.1% sodium thiosulfate-pentahydrate. The production of indole from tryptophan could be observed as a red-coloured organic phase in tryptophan medium (w/v: 1 % tryptone, 0.5 % NaCl, 0.1 % tryptophan) after adding Kovacs reagent (Fluka, Germany). A calcium caseinate agar (Merck) was used to determine the hydrolysis of casein, observing clearing zones around colonies in the opaque medium. Starch hydrolysis was analysed by flooding agar plates containing starch (w/v: 2.5 % nutrient agar DEV, Germany, 2 % soluble starch) with Lugol's iodine after incubation. Hydrolysis of gelatine was tested by flooding gelatine agar plates (w/v: 1 % tryptone, 1 % meat extract, 1 % gelatine, 0.5 % NaCl, 1 % agar) with 1 % tannin solution after incubation, in which opaque zones occur in the clear agar for a positive reaction.

Isolation of DNA from strain $PB1^T$ was done using a microbial DNA isolation kit (MoBio Laboratories) according to the manufacturer's protocol. For 16S rRNA gene amplification, general bacterial primers 27F (Lane *et al.*, 1985) and 1492R (Dojka *et al.*, 1998) were used. Sequencing (by GATC Biotech, Konstanz, Germany) resulted in a 1303 bp gene product.

Alignments were performed with closely related sequences obtained from GenBank using the integrated SINA alignment tool from the ARB-SILVA website (Pruesse *et al.*, 2007). After manually checking the alignment using the ARB program (Ludwig *et al.*, 2004), evolutionary distances were calculated and a phylogenetic tree (Figure 1) was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with a correction of Jukes & Cantor (1969) and a termini filter that is implemented in the ARB program. To evaluate the tree topologies, a bootstrap analysis with 1000 replications was performed. For strain PB1^T, highest 16S rRNA gene sequence similarity was found to the type strains of *Herbaspirillum canariense* (97.3 %), *Herbaspirillum aurantiacum* (97.2 %), *Herbaspirillum soli* (97.2 %) and *Herbaspirillum frisingense* (97.0 %).

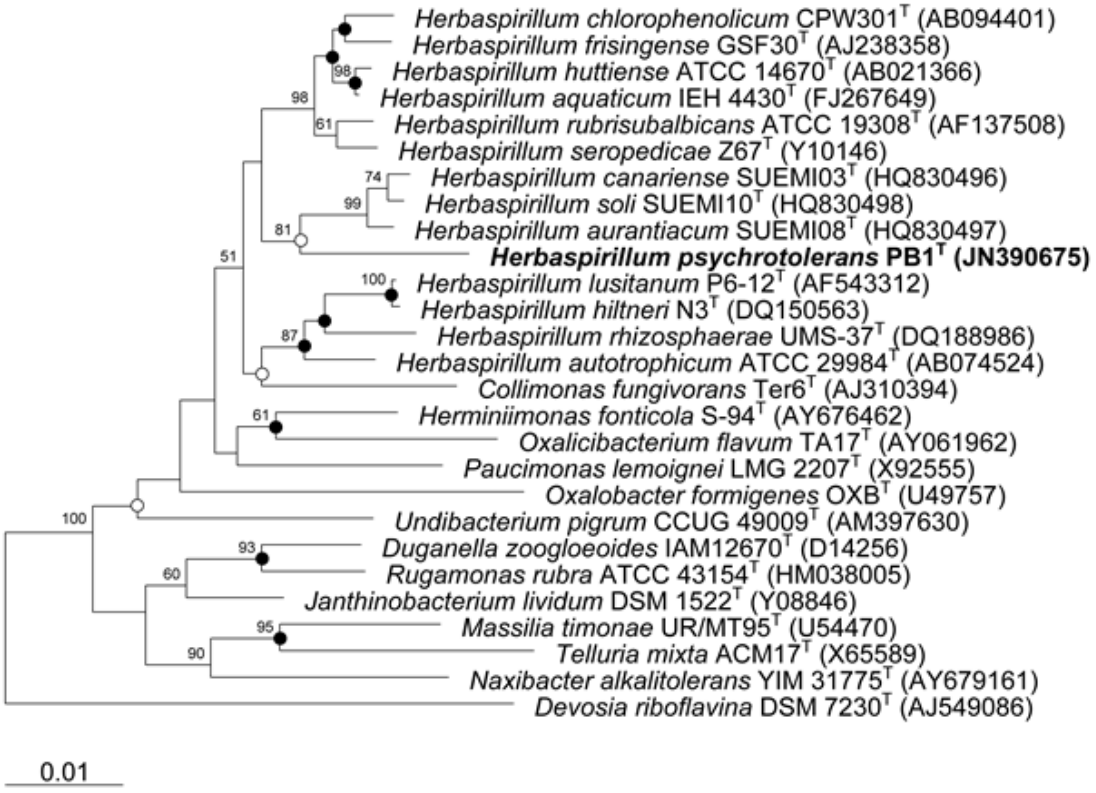

Figure 1: Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain PB1^T within the genus *Herbaspirillum* and the relation to type strains of closely related genera of the family *Oxalobacteriaceae*. Open circles indicate branches that were also found in maximum-likelihood trees (Fitch, 1971); black circles indicate branches found in both. maximum-likelihood and maximum-parsimony trees (Felsenstein, 1981). Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values ≥50% are shown. Bar: 0.01 substitutions per nucleotide position.

DNA-DNA hybridization experiments between strain PB1^T and the type strains of *H. canariense* LMG 26151^T, *H. aurantiacum* LMG 26150^T and *H. soli* LMG 26149^T have been performed by the Belgian Co-ordinated Collections of Microorganisms (BCCM™/ LMG, Gent, Belgium).

The hybridizations were performed in the presence of 48 % formamide at 43°C according to a modification (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998) of the method described by Ezaki *et al.* (1989). The DNA-DNA relatedness percentage is a mean of 5 hybridizations and reciprocal reactions were performed. The DNA-DNA relatedness values for strain PB1^T and each type strain were the following: 26 % with *H. canariense*, 26 % with *H. aurantiacum* and 27 % with *H. soli*, which is clearly below 70 %, generally accepted for species delineation (Wayne *et al.*, 1987). The interstrain relatedness of the type strains of the closest relatives of PB1^T was 56% between *H. canariense* LMG 26151^T and *H. aurantiacum* LMG 26150^T, 65% between *H. canariense* LMG 26151^T and *H. soli* LMG 26149^T and 63% between *H. aurantiacum* LMG 26150^T and *H. soli* LMG 26149^T. Carro *et al.* (2012) reported similar values of 58 %, 59 % and 59 % DNA-DNA relatedness, respectively. Determination of G+C content of DNA was done by HPLC (Tamaoka & Komagata, 1984) and calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah *et al.* (1989). The analysis was performed by the DSMZ (Braunschweig, Germany).

The presence of nitrogenase metalloprotein-encoding genes was analysed using specific primer pairs FdB261/ FdB260 (nifD; Stoltzfus *et al.*, 1997) and PolF/ PolR (nifH; Poly *et al.*, 2001). Both nitrogenase genes were amplified from the positive controls *H. frisingense* DSM 13128^T and *H. seropedicae* DSM 6445^T, which are known to fix nitrogen (Baldani *et al.*, 1986; Kirchhof *et al.*, 2001), but not from strain PB1^T or any of the type strains of *H. canariense* LMG 26151^T, *H. aurantiacum* LMG 26150^T and *H. soli* LMG 26149^T.

Cells of strain PB1^T were grown in R2A medium (pH 7.2) at 18°C until they reached the end of the exponential growth phase to analyse fatty acid composition, polyamine pattern, polar lipid profile and the quinone system. Extraction and analysis of fatty acid methyl esters was conducted according to the protocol of Zink & Mangelsdorf (2004). Cells of the type strains of *H. canariense* LMG 26151^T, *H. aurantiacum* LMG 26150^T and *H. soli* LMG 26149^T were treated equally and analysed comparatively. Polyamines were extracted as described by Busse & Auling (1988), analysed using the HPLC equipment described by Stolz *et al.* (2007) and applying the slight modification in the gradient as reported by Busse *et al.* (1997). Isoprenoid quinones and polar lipids were extracted using the small-scale integrated procedure of Minnikin *et al.* (1984).

Quinones were analysed by a Hewlett Packard series 1050 HPLC equipped with an ODS Hypersil column (Agilent Technologies) and a diode-array detector. Methanol and isopropyl ether (9:2, v/v) were used as mobile phase with a flow rate of 1.0 ml min^{-1} and a column temperature of 30°C (Hu *et al.*, 1999). Completely hydrolysed cells were analysed by a thin layer chromatography to determine isomers of 2.6-diaminopimelic acid (Dpm) and of 2.6-diamino-3-hydroxypimelic acid (OH-Dpm) by using the solvent system of Rhuland *et al.* (1955; Schumann, 2011).

Identified fatty acids for PB1^T were as follows: $C_{12:0}$ (0.3 %), $C_{14:0}$ (0.6 %), $C_{15:0}$ (0.4 %), $C_{16:0}$ (19.1 %), $C_{17:0}$ (0.4 %), $C_{18:0}$ (6.1%), $C_{19:0}$ (0.2%), $C_{20:0}$ (0.4 %), $C_{22:0}$ (0.5 %), $C_{24:0}$ (0.3 %), iso-$C_{16:0}$ (0.5 %), anteiso-$C_{17:0}$ (0.4 %), C17:0 cyclo (1.0 %), $C_{16:1}\omega7cis$ (44.6 %), $C_{16:1}\omega7trans$ (3.1%), $C_{18:1}\omega7cis$ (16.2 %), $C_{18:1}\omega7trans$ (1.1 %), $C_{18:1}\omega9$ (1.5 %) and $C_{19:1a}$ (3.4 %). The fatty acids $C_{19:1a}$ is an unsaturated fatty acid with unknown double bond position.

The polyamine pattern consisted of major amounts of putrescine (54.9 µmol g^{-1} dry weight), 2-hydroxy-putrescine (18.5 µmol g^{-1} dry weight) and minor amounts of cadaverine (0.7 µmol g^{-1} dry weight) and spermidine (0.2 µmol g^{-1} dry weight). This polyamine pattern is very similar to that of the type species of the genus *Herbaspirillum seropedicae* ATCC 35892^T which was reported to contain predominately putrescine and as a second major compound 2-hydroxy-putrescine (Hamana & Takeuchi, 1998).

The polar lipid pattern of isolate PB1^T was characterised by phosphatidylethanolamine, phosphatidyl glycerol, diphosphatidylglycerol, two unknown phospholipids (PL1 and PL2) and two unknown polar lipids (L1 and L2, Supplementary Material Figure S1). This polar lipid pattern is in accord with those of the type strains of *H. soli, H. canariense,* and *H. aurantiacum* (Orthova & Busse, unpublished results) quite similar to close relatives of the genus *Herbaspirillum* such as *Unibacterium pigrum* (Kämpfer et al., 2007) and *Herminiimonas contaminans* (Kämpfer *et al.*, 2012). The identified quinone was ubichinon-8 (Q-8). Due to the cell wall composition of gram-negative bacteria, the amount of peptidoglycan of strain PB1^T was below the detection limit for Dpm or OH-Dpm. Considering morphological, physiological and biochemical differences and comparative phylogenetic analyses (Table 1 and 2, Supplementary Material Table 1), strain PB1^T can be clearly differentiated from its most closely related neighbours and we propose strain PB1^T as a novel species within the genus *Herbaspirillum*, named *Herbaspirillum psychrotolerans*.

Table 1: Physiological and biochemical characteristics of strain PB1^T and related species of the genus *Herbaspirillum*. Strains: 1) *H. psychrotolerans* sp. nov. PB1^T; 2) *H. canariense* SUEM103^T (data from Carro *et al.,* 2012); 3) *H. aurantiacum* SUEMI08^T (data from Carro *et al.,* 2012); 4) *H. soli* SUEMI10^T (data from Carro *et al.*, 2012); 5) *H. seropedicae* Z67^T (data from Baldani *et al.*, 1996). * data from this study, + positive; +/- weakly positive; - negative; nd not determined.

	1*	**2**	**3**	**4**	**5**
Isolation source	glacier forefield soil	soil in Tenerife (Canary Islands			associated with cereal roots
Colony color	white	white-cream	orange opaque	pale orange opaque	brownish
Cell form	curved rods		slightly curved rods		vibroid, rods
Cell dimensions (µm)					
Length	3.5-4.5	1.9-1.6	1.7–1.9	1.9	1.5-5.0
Width	0.8-1.2	1.0-1.3	0.8–1.1	1.2	0.6-0.7
Motility	+		1 polar flagellum		1-3, mono- or bipolar
Temperature for growth (°C)					
Range	-5 to <30	15-34	15-34	15-37	22>T<38
Optimum	14-20	28	28	28	nd
pH value					
Range	4.5-8	4.5-8	>4.5-8	>4.5-8	5.3-8.0
Optimum	6.5-7	6-8	6-8	6-8	nd
Relation to O2	facultative anaerobic	aerobic	aerobic	aerobic	micro aerobic
Oxidase	-	+	+	+	+
Resistance to antibiotics					
Ampicillin	+	+	-	+	nd
Erythromycin	+	-	-	-	-
Oxytetracycline	-	-	-	-	-
Penicillin	+	+	-	-	+
Hydrolysis of gelatine	+	-	-	-	nd
Urease activity	+	-/+	-	-	+
GC content (mol%)	62.5	61.6	60.4	61.9	66-67

Description of *Herbaspirillum psychrotolerans* sp. nov.

Herbaspirillum psychrotolerans (psy.chro.to`le.rans. Gr. adj. *psychros* cold; L. part. adj. *tolerans* tolerating; N.L. neut. part. adj. *psychrotolerans* cold-tolerating).

Colonies of PB1^T are circular, smooth, friable convex, shiny, white and about 2 mm in diameter after 7 days of growth on nutrient agar. Cells are Gram-negative, facultative-anaerobic, non-spore-forming, motile, curved rods and about 3.5-4.5 µm in length and 0.8-1.2 µm in width. Growth was observed between -5°C and 30°C, but not at -10°C or 36°C. The optimum temperature for growth is 14 -20°C. Strain PB1^T was able to grow at pH values from 4.5 to 8.0 with optimum growth between pH 6.5 and 7.0 and tolerates salt concentrations up to 2.5% NaCl with slow growth at 2.5% and optimum growth at 0-0.5% NaCl. It is sensitive to gentamycin, kanamycin-sulphate, oxytetracycline and rifampicin, but resistant to ampicillin, cephalosporin, erythromycin, metronidazole, novobiocin, penicillin, fosfomycin and troleandomycin and not inhibited by lysozyme. Acid is produced from glycerol, D-arabinose and D-ribose and weak from L-arabinose and L-xylose (Supplementary Material Table 1). An alkalinisation is reported for potassium gluconate. Glycerol, D-/L-arabinose, D-Xylose, D-galactose, D-fructose, D-lyxose, D-fucose and potassium gluconate are used as sole carbon sources. PB1^T is catalase positive and oxidase-negative and is able to hydrolyse starch and gelatine but not casein. Methyl-red test is negative and neither indole nor hydrogen sulphide are produced. Urease activity is low. G+C content was 62.5 mol%. The quinone system contains predominately ubiquinone Q-8 and the polyamine pattern is predominated by putrescine and 2-hydroxy-putrescine. The polar lipid profile contains the major lipids phosphatidylglycerol, diphosphaditylglycerol, and phosphatidylethanolamine. Minor amounts of a phospholipid and polar lipids not containing a sugar moiety, a phosphate- or an aminogroup are present, as well (Supplementary Figure S1). The fatty acid profile is listed in Table 2.

The type strain of *Herbaspirillum psychrotolerans* sp. nov. is PB1^T (DSM 26001^T = LMG 27282^T), isolated from a soil sample of the Larsemann Hills East Antarctica.

Table 2: Fatty acid composition of strain PB1^T and the closest relatives. Strains: 1) *H. psychrotolerans* sp. nov. PB1^T; 2) *H. canariense* LMG 26151^T; 3) *H. aurantiacum* LMG 26150^T; 4) *H. soli* LMG 26149^T 5) *H. frisingense* strain DSM 13128^T; 6) *H. seropedicae* DSM 6445^T, * data from Carro *et al.*, 2012. nd not detected, summed feature 3: $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, summed feature 8: $C_{18:1}\omega6c$ and /or $C_{18:1}\omega7c$, a,b unsaturated fatty acid with unknown double bond position.

Fatty Acid	1	2	3	4	5	6*
$C_{10:0}$	nd	nd	nd	nd	nd	0.2
$C_{12:0}$	0.3	nd	nd	nd	nd	0.8
$C_{14:0}$	0.6	2.0	2.1	1.2	0.3	4.1
$C_{15:0}$	0.4	1.4	1.6	0.9	0.4	nd
$C_{16:0}$	19.1	45.5	29.3	24.0	19.6	23.3
$C_{17:0}$	0.4	2.1	1.8	0.6	0.5	0.2
$C_{18:0}$	6.1	14.5	22.6	7.6	6.1	1.2
$C_{19:0}$	0.2	nd	nd	nd	nd	nd
$C_{20:0}$	0.4	nd	nd	nd	nd	nd
$C_{22:0}$	0.5	nd	nd	nd	nd	nd
$C_{24:0}$	0.3	nd	nd	nd	nd	nd
iso-$C_{16:0}$	0.5	1.0	nd	nd	nd	nd
anteiso-$C_{15:0}$	nd	0.8	nd	nd	nd	nd
anteiso-$C_{17:0}$	0.4	3.1	nd	nd	nd	nd
$C_{17:0}$ cyclo	1.0	8.4	5.0	2.6	30.1	0.4
$C_{10:0}$ 3-OH	nd	nd	nd	nd	nd	1.9
$C_{12:0}$ 2-OH	nd	nd	nd	nd	nd	1.8
$C_{12:0}$ 3-OH	nd	nd	nd	nd	nd	4.6
$C_{14:0}$ 2-OH	nd	nd	nd	nd	nd	1.4
$C_{16:1}\omega7cis$	44.6	6.8	16.4	33.0	12.2	nd
$C_{16:1}\omega7trans$	3.1	2.4	3.3	2.3	3.1	nd
$C_{18:1}\omega7cis$	16.2	6.0	11.9	18.5	18.8	nd
$C_{18:1}\omega7trans$	1.1	2.5	3.0	1.8	6.2	nd
$C_{18:1}\omega9$	1.5	3.6	3.2	2.0	1.1	nd
$C_{19:1a}$	3.4	nd	nd	5.6	nd	nd
$C_{19:1b}$	nd	nd	nd	nd	1.4	nd
summed feature 3	nd	nd	nd	nd	nd	38.9
summed feature 8	nd	nd	nd	nd	nd	21.2

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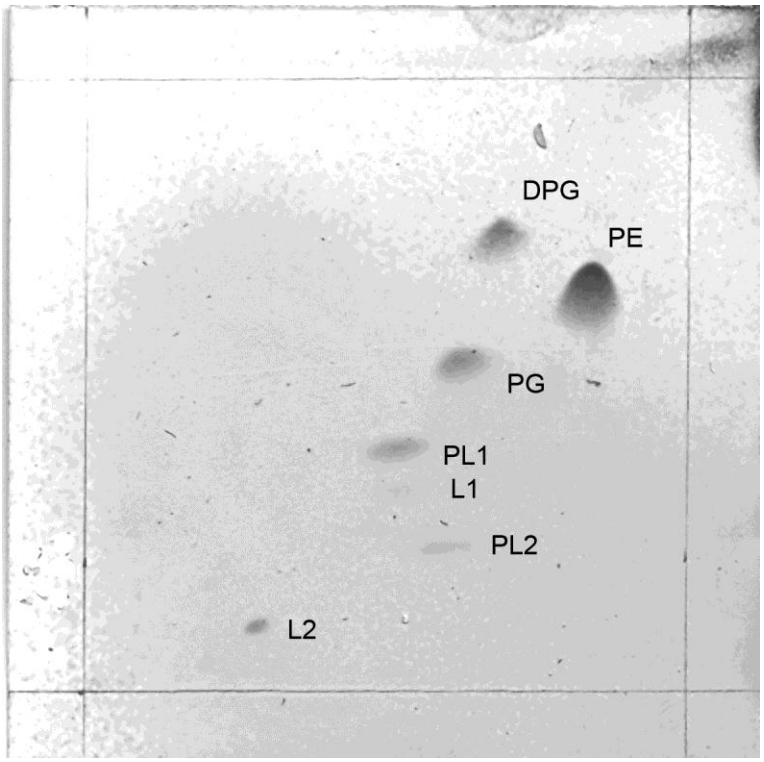
Supporting Material

Supplementary Material Table 1: Acid production from carbohydrates and the use of carbohydrates as sole carbon sources. Strains: 1) *H. psychrotolerans* sp. nov. PB1^T, 2) *H. canariense* LMG 26151^T, 3) *H. aurantiacum* LMG 26150^T, 4) *H. soli* LMG 26149^T, 5) *H. frisingense* strain DSM 13128^T. All data was determined in this study with api ®50CH. + positive; w weak positive; - negative. a alkalinisation. CH Carbohydrate.

Carbohydrate	Acid production from CH					CH as sole carbon source				
	1	2	3	4	5	1	2	3	4	5
Control	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	w	+	+	+	+	+	-	-
Erythritol	-	-	-	-	-	-	-	w	-	-
D-Arabinose	+	w	w	+	+	+	+	+	-	-
L-Arabinose	w	w	+	+	+	+	w	+	-	-
D-Ribose	+	-	+	+	+	-	w	+	-	-
D-Xylose	-	w	-	w	+	+	w	+	-	-
L-Xylose	w	w	-	+	+	-	w	+	-	-
D-Adonitol	-	-	-	-	+	-	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-	w	-	-	-	-	-
D-Galactose	-	-	-	-	+	+	-	+	-	-
D-Glucose	-	-	-	-	+	-	-	w	-	-
D-Fructose	-	-	-	-	+	+	-	+	w	-
D-Mannose	-	-	-	-	+	-	-	w	-	-
L-Sorbose	-	-	-	-	-	-	-	w	-	-
L-Rhamnose	-	-	-	-	-	-	-	w	-	-
Dulcitol	-	-	-	-	-	-	-	w	-	-
Inositol	-	-	-	-	-	-	-	w	-	-
D-Mannitol	-	-	-	-	+	-	-	w	-	-
D-Sorbitol	-	-	-	-	+	-	-	w	-	-
Methyl-αD-Mannopyranoside	-	-	-	-	-	-	-	w	-	-
Methyl-αD-Glucopyranoside	-	-	-	-	-	-	-	w	-	-
N-Acetyl-Glucosamine	-	-	-	-	-	-	-	w	-	-
Amygdalin	-	-	-	-	-	-	-	w	-	-

Arbutin	-	-	-	-	-	-	-	w	-	-
Esculin ferric citrate	-	-	-	-	-	-	-	w	-	-
Salicin	-	-	-	-	-	-	-	w	-	-
D-Cellobiose	-	-	-	-	-	-	-	w	-	-
D-Maltose	-	-	-	-	-	-	-	w	-	-
D-Lactose	-	-	-	-	+	-	-	w	-	-
D-Melibiose	-	-	-	-	-	-	-	w	w	-
D-Saccharose	-	-	-	-	-	-	-	w	w	+
D-Trehalose	-	-	-	-	-	-	-	w	w	-
Inulin	-	-	-	-	-	-	-	+	w	-
D-Melezitose	-	-	-	-	-	-	-	w	-	-
D-Raffinose	-	-	-	-	-	-	-	w	-	-
Amidon (Starch)	-	-	-	-	-	-	-	w	-	-
Glycogen	-	-	-	-	-	-	-	w	-	-
Xylitol	-	-	-	-	+	-	-	w	-	-
Gentiobiose	-	-	-	-	-	-	-	w	-	-
D-Turanose	-	-	-	-	-	-	-	w	w	+
D-Lyxose	-	+	+	+	+	+	-	+	w	-
D-Tagatose	-	-	-	-	-	-	-	w	w	-
D-Fucose	-	-	-	-	+	+	-	+	w	-
L-Fucose	-	-	-	-	+	-	-	+	w	-
D-Arabitol	-	-	-	-	+	-	-	-	-	+
L-Arabitol	-	-	-	-	+	-	-	-	-	-
potassium Gluconate	a	a	a	a	a	+	+	+	w	-
potassium 2-Ketogluconate	-	-	-	-	a	-	+	+	w	-
potassium 5-Ketogluconate	-	-	-	-	a	-	+	+	w	-



Supplementary Figure S1. Two-dimensional TLC of polar lipids of *Herbaspirillum psychrotolerans* PB1^{T} with sulfuric acid staining. PE phosphatidylethanolamine; PG phosphatidylglcerol; DPG diphosphatidylglycerol; PL1 and PL2 Phospholipids; L1 and L2 other unidentified polar lipids, not containing a sugar moiety, a phosphate- or an amino-group.

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Chryseobacterium frigidisoli sp. nov., a psychrotolerant species of the family _Flavobacteriaceae_ isolated from sandy permafrost from a glacier forefield.

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New Taxa - Bacteriodetes

The GenBank accession number of the 16S rRNA gene sequence of *Chryseobacterium frigidisoli* PB4^T is JN390676.

Abstract

During diversity studies of the glacier forefields of the Larsemann Hills, East Antarctica, a novel psychrotolerant, non-motile Gram-negative, shiny yellow, rod-shaped, aerobic bacterium, designated strain PB4^T was isolated from a soil sample. Strain PB4^T produces indole from tryptophan and hydrolyses casein. It grows between 0°C and 25°C with an optimum growth temperature at 20°C. A wide range of substrates are used as sole carbon sources and acid is produced from numerous carbohydrates. The major menaquinone is MK-6. Identified polar lipids are ethanolamines and ornithine lipids. Major fatty acids (>10%) are iso-$C_{15:0}$ (13.0%) and iso-2OH-$C_{15:0}$ (51.2%). G+C content is 33.7 mol%. The polyamine pattern is composed of sym-homospermidine (25.1 µmol g^{-1} dry weight), minor amounts of cadaverine (0.2 µmol g^{-1} dry weight) and spermidine (0.4 µmol g^{-1} dry weight) and traces of putrescine and spermin (< 0.1 µmol g^{-1} dry weight). Strain PB4^T had highest 16S rRNA gene similarities with the type strains of *Chryseobacterium humi* (97.0%) and *Chryseobacterium marinum* (96.5%). Considering phenotypic and genotypic characterisation, strain PB4^T represents a novel species in the genus *Chryseobacterium* (family *Flavobacteriaceae*), for which the name *Chryseobacterium frigidisoli* sp. nov. is proposed. The type strain is PB4^T (DSM 26000^T = LMG 27025^T).

In 1994, Vandamme *et al.* (1994) introduced new perspectives in the classification of the genus *Flavobacterium*. Six strains in the family *Flavobacteriaceae* were proposed as new species in the genus *Chryseobacterium* with former *Flavobacterium gleum* (Holmes *et al.*, 1984), renamed as *Chryseobacterium gleum*, as the type species. Today, the genus *Chryseobacterium* comprises over 60 different species isolated from various environments such as clinical sources (Kämpfer *et al.*, 2009b), water reservoir (Kim *et al.*, 2008) or soils (Benmalek *et al.*, 2010; Weon *et al.*, 2008; Zhou *et al.*, 2007). Several isolates have been derived from polar habitats (Loveland-Curtze *et al.*, 2010; Yi *et al.*, 2005).

The isolation and characterisation of microorganism from extreme habitats has become more and more important in the last years, because representative isolates can help identify functions of microorganisms in these habitats. Members of the phylum *Actinobacteria* for example are known to use a wide range of substrates or degrade polymers at low temperatures as it was reported for *Arthrobacter* and *Leifsonia* species from Livingston Island in Antarctica (Ganzert *et al.*, 2011a, b) or *Cryobacterium arcticum* from Greenland (Bajerski *et al.*, 2011).

In the context of microbial community analysis in extreme environments, several psychrotolerant bacteria were isolated from Antarctic soil. In this study, we report the isolation and identification of strain PB4^T which is considered to be a new cold-adapted representative in the genus *Chryseobacterium*.

Strain PB4^T was isolated from sandy dry permafrost in 1-6 cm depth from a glacier forefield transect located in the Larsemann Hills region, East Antarctica (S 69°24, E 76°20). Soil material was suspended in a physiological salt solution (0.9% NaCl, w/v), plated on R2A medium (0.05% proteose peptone, 0.05% casamino acids, 0.05% yeast extract, 0.05% dextrose, 0.05% soluble starch, 0.03% dipotassium phosphate, 0.005% magnesium sulfate 7×H_2O, 0.03% sodium pyruvate, 1.5% agar for solid media (w/v); Reasoner & Geldreich, 1985) and incubated at 10°C for three weeks. Isolates were separated from enrichment cultures and cultivated at 18°C on R2A and the same conditions and media were used for all tests, unless otherwise noted.

Colony properties were observed on agar plates after seven days of bacterial growth. Cell characteristics were determined by light microscopy (Zeiss Axioskop 2 plus, Jena, Germany) using cells being in the end of the exponential growth phase. Gram staining (using Gram staining set, Merck, Germany), spore detection and flagella presence was examined with classical methods as proposed by Gram (1884), Wirtz (1908) and Loeffler (1898), respectively. Physiological parameters (temperature, pH range of growth, salt tolerance) were studied in liquid R2A medium for 14 days by photometric determination of turbidity. For pH measurements, the medium was modified with the following buffers: glycine (pH 4.0-5.0 and pH 10.0), MES [2-(*N*-morpholino)ethanesulfonic acid**;** pH 5.0-6.5], HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.0-8.0] and BTP [1,3-bis(tris(hydroxymethyl)methylamino)propane; pH 8.5-9.5; Hoaki *et al.*, 1994)]. Bacterial growth in the absence of oxygen was determined on R2A agar plates incubated at 22°C for 14 days under a N_2/CO_2 (80:20 v/v) atmosphere. A filter disc test with 12 different antibiotics (1 mg ml^{-1}) and lysozyme (1 mg ml^{-1}) was carried out by measuring the inhibition areola. Acid production from carbohydrates was tested with api®50CH and api ®50CHB/E medium (bioMérieux, Nürtingen, Germany) following the manufacture's protocol. Results were documented daily over a period of 10 days with incubation at 18°C. The api®50CH test stripes were also used to determine the utilisation of carbohydrates as sole carbon source, with a minimal media as described previously (Bajerski *et al.*, 2011), that was modified with the addition of 0.1% yeast extract (w/v). Methyl-

red was used as an indicator to examine bacterial induced medium acidification in a glucose containing medium (w/v: 1% tryptone, 0.5% NaCl, 1% glucose-monohydrate). Urease activity was determined with the same basis medium, Urea (1% w/v) as substrate and indicator phenolphthalein (1% ethanolic solution, Merck). Catalase activity was observed by bubble production after adding a 10% hydrogen peroxide solution to the grown culture. Oxidase activity was examined with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) as a redox indicator as described by Kovacs (Kovacs, 1956). The production of hydrogen sulphide was determined in a modified nutrient medium with 0.1% sodium thiosulfate-pentahydrate and test filter stripes impregnated with lead acetate. The ability to produce indole from tryptophan was analysed with tryptophan medium (1% tryptone, 0.5% NaCl, 0.1% tryptophan, w/v). A positive result appeared as a red-coloured organic phase after adding Kovacs reagent (Fluka, Germany). The hydrolysis of casein was determined on calcium caseinate agar plates (Merck) by observation of clearing zones around colonies on the opaque medium. Starch hydrolysis was examined by flooding starch containing agar plates (2.5% nutrient agar DEV, Germany, 2% soluble starch, w/v) with Lugol's iodine after incubation. Gelatine agar plates (1% tryptone, 1% meat extract, 1% gelatine, 0.5% NaCl, 1% agar, w/v) were flooded with 1% tannin solution after incubation to analyse gelatine degradation, in which turbid zones occur in the clear agar for a positive reaction.

Microbial DNA from strain PB4^T was isolated with a Microbial DNA isolation kit (MoBio Laboratories) following the manufacturer's protocol. Bacterial 16S rRNA gene was amplified with primers 27F (Lane *et al.*, 1985) and 1492R (Dojka *et al.*, 1998). Sequencing was performed by GATC Biotech (Konstanz, Germany) and resulted in a 1301 bp gene product. Closely related sequences derived from GenBank were aligned with the integrated SINA alignment tool from the ARB-SILVA website (Pruesse *et al.*, 2007). Evolutionary distances were calculated with ARB (Ludwig *et al.*, 2004) and a phylogenetic tree (Figure 1) was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with a correction of Jukes & Cantor (1969) and a termini filter that is implemented in the ARB program. A bootstrap analysis with 1000 replications was performed to evaluate the tree topologies. Strain PB4^T had highest 16S rRNA gene similarities with the type strains of *Chryseobacterium humi* (97.0%) and *Chryseobacterium marinum* (96.5%). DNA-DNA hybridization experiments were not carried out as the sequence similarities to the nearest relatives were clearly below 98.5%, which was proposed by Stackebrandt & Ebers (2006). Determination of G+C content of DNA was performed by HPLC (Tamaoka & Komagata,

1984) and calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah (1989).

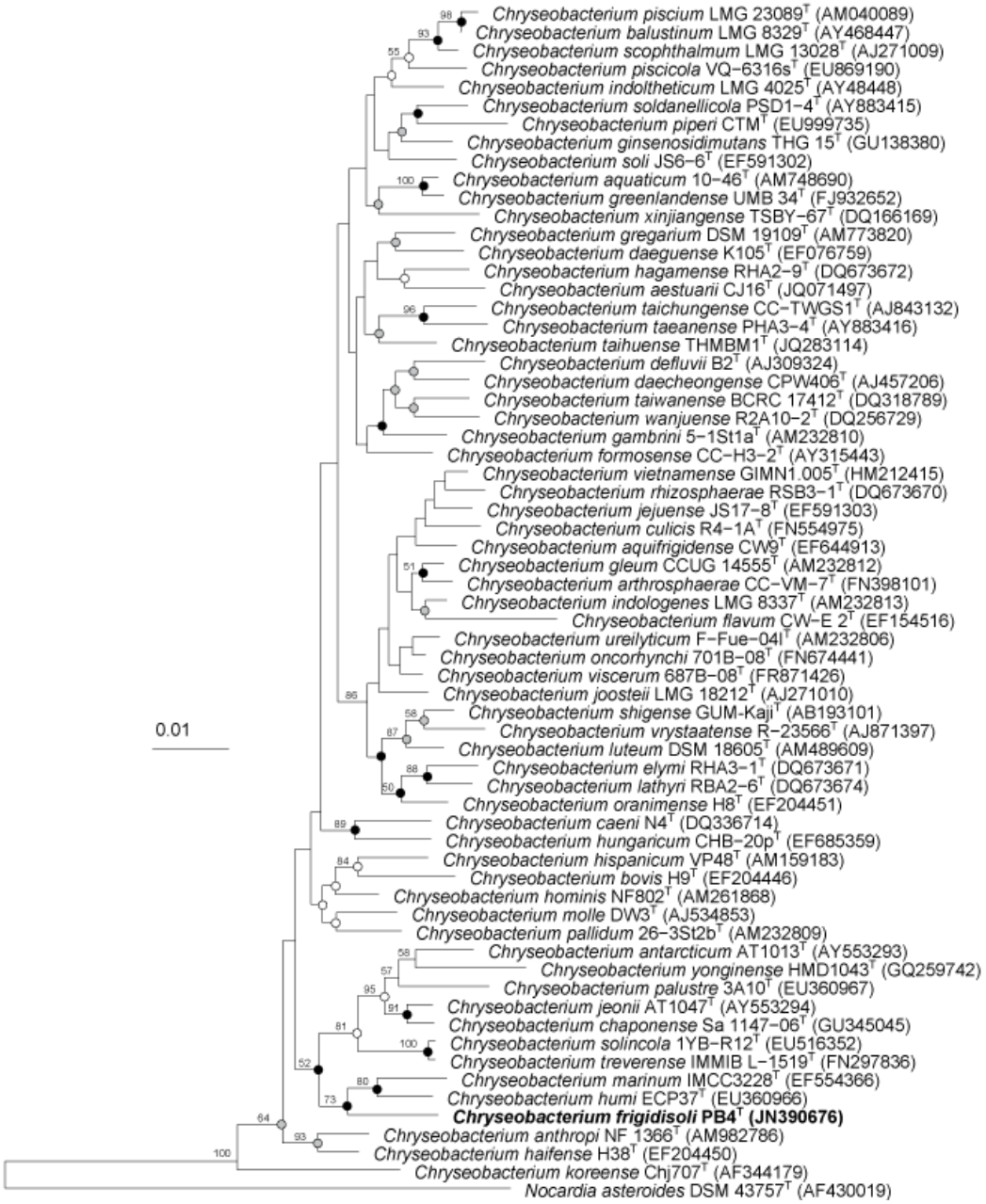

Figure 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain PB4^T within the genus *Chryseobacterium*. Open circles indicate branches that were also found in maximum-likelihood trees (Fitch, 1971) and grey circles indicate branches that were found in maximum-parsimony trees (Felsenstein, 1981); black circles indicate branches found in both. Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values ≥ 50% are shown. Bar: 0.01 substitutions per nucleotide position.

For chemotaxonomic analysis (fatty acid composition, polyamine pattern, polar lipid profile, quinone system) cells of strain PB4^T were grown in R2A medium (pH 7.2) at 18°C until they reached the end of the exponential phase. Extraction of fatty acid methyl esters was conducted according to the protocol of Zink & Mangelsdorf (2004). Cells of *Chryseobacterium humi* as the closest relative of strain PB4^T were treated equally and analysed comparatively. Polyamines were extracted as described by Busse & Auling (1988), analysed using the HPLC equipment described by Stolz *et al*. (2007) and applying the slight modification in the gradient as reported by Busse *et al.* (1997). Polar lipids and isoprenoid quinones were extracted and analysed using the small-scale integrated procedure of Minnikin *et al*. (1984). The characterization of polar lipids was carried out in a two-dimensional thin layer chromatography (2D-TLC) with molybdophosphoric acid staining and a second chromatography with sulfuric acid staining. Additionally, intact polar lipids were measured using a liquid chromatography-electrospray interface-mass spectrometry (HPLC-ESI-MS) system as described in Zink & Mangelsdorf (2004). Menaquinones (MK) were analysed by a Hewlett Packard series 1050 HPLC equipped with an ODS Hypersil column (Agilent Technologies) and a diode-array detector. Methanol and isopropyl ether (9:2, v/v) was used as mobile phase with a flow rate of 1.0 ml min^{-1} and a column temperature of 30°C (Hu *et al.*, 1999). Completely hydrolysed cells were analysed by a TLC to determine isomers of 2,6-diaminopimelic acid (Dpm) and of 2,6-diamino-3-hydroxypimelic acid (OH-Dpm) by using the solvent system of Rhuland *et al.* (1955; Schumann, 2011).

Identified fatty acids for PB4^T were as follows (Table 1): $C_{14:0}$ (0.6%), iso-$C_{15:0}$ (13.0%), anteiso-$C_{15:0}$ (6.7%), $C_{15:0}$ (0.9%), iso-$C_{16:0}$ (2.8%), iso-2OH-$C_{15:0}$ (51.2%), anteiso-2OH-$C_{15:0}$ (6.4%), $C_{16:0}$ (8.5%), iso-$C_{17:1}\omega8$ (3.0%), $C_{18:1}\omega9$ (1.4%) and $C_{18:0}$ (5.5%).

Sym-homospermidine (25.1 µmol g^{-1} dry weight) was detected as the major polyamine of strain PB4^T as it was indicated for *Chryseobacteria* in the emended genus description (Kämpfer *et al.*, 2009b). Additionally the polyamine pattern consisted of minor amounts of cadaverine (0.7 µmol g^{-1} dry weight) and spermidine (0.2 µmol g^{-1} dry weight) and traces of putrescine and spermine (< 0.1 µmol g^{-1} dry weight), which is consistent with other species of the genus (Kämpfer *et al.*, 2009b; Vandamme *et al.*, 1994).

Table 1: Fatty acid composition of strain PB4^T and the closest related type strain *Chryseobacterium humi* DSM 21580^T. Data was determined in this study.

Fatty Acid	PB4^T	*Chryseobacterium humi*
$C_{14:0}$	0.58	0.00
$C_{15:0}$	0.85	0.34
$C_{16:0}$	8.52	4.78
$C_{17:0}$	0.00	0.20
$C_{18:0}$	5.53	2.89
anteiso-$C_{15:0}$ 2OH	6.38	3.12
anteiso-$C_{15:0}$	6.69	12.18
anteiso-$C_{17:0}$	0.00	0.19
iso-$C_{15:0}$ 2OH	51.23	41.47
iso-$C_{15:0}$	13.03	29.52
iso-$C_{16:0}$	2.83	0.44
iso-$C_{17:0}$	0.00	0.29
iso-$C_{17:1}\omega 8$	2.97	3.14
$C_{18:1}\omega 9$	1.39	1.10
unknown	0.00	0.33

The polar lipid pattern of isolate PB4^T consisted of phosphatidylethanolamines, unknown aminophospholipids, unknown amino lipids and several other unknown lipids determined by 2D-TLC (supplementary material Figure S1). The polar lipid pattern is in accordance with those described for other type strains of this genus (Herzog *et al.*, 2008; Kämpfer *et al.*, 2009b). The HPLC-ESI-MS measurements confirmed the occurrence of phosphatidylethanolamines and allowed the identification of ornithine lipids among the series of unknown polar lipids.

The identified quinone was menaquinone-6 (MK-6). As a gram-negative bacterium strain PB4^T only contains small amounts of peptidoglycan. The amount of peptidoglycan was below the detection limit for isomers of Dpm or OH-Dpm.

Morphological, physiological and biochemical properties differentiate strain PB4^T from its most closely related neighbours and we propose strain PB4^T as a novel species within the genus *Chryseobacterium* (Table 2), named *Chryseobacterium frigidisoli*.

Table 2: Physiological and biochemical characteristics of strain PB4^T and related *Chryseobacterium* species. Strains: 1) *Chryseobacterium frigidisoli* sp. nov. PB4^T; 2) *Chryseobacterium humi* strain DSM 21580^T, data from Pires *et al. (2010);* 3) *Chryseobacterium marinum* strain IMCC 3228^T, data from Lee *et al.* (2007) and Kämpfer *et al.* (2009a); 4) *Chryseobacterium gleum* F93^T, data from Holmes *et al.* (1984) and Vandamme *et al.* (1994). * data from this study, + positive; +/- weak positive; - negative; MK: menaquinone; nd: not detected.

	1*	**2**	**3**	**4**
Isolation source	glacier forefield soil	polluted soil	antarctic surface seawater	nd
Cell dimensions (µm)				
length	2.0-3.5	1.6-2.5	0.6-1.4	2.0-3.0
width	0.4-0.5	0.5-0.6	0.5-0.8	nd
Temperature (°C)				
range	0-25	4-37	3-25	18-22, 37
optimum	20	25-30	15	nd
pH				
range	5.5-8.5	6-9	6-10	nd
optimum	6.5	7-8	7	nd
NaCl tolerance	0-5%	0-7%	0-3,5%	nd
Resistence to antibiotics				
Ampicillin	+	-	+	nd
Gentamicin-sulfat	-	+	+	nd
Rifampicin	+	-	-	nd
Production of indole	+	-	-	negative (+ with Ehrlich reagent)
Hydrolysis of casein	-	+	-	+
GC-content (mol%)	33.7	34	35	37.6
Quinone	MK-6	MK-6	MK-6	nd

Description of *Chryseobacterium frigidisoli* sp. nov.

Chryseobacterium frigidisoli (fri.gi.di.so.li. L. adj. *frigidus* cold, cool, chilled; L. n. *solum,* soil; N.L. neut. adj. *frigidisoli,* pertaining to cold soil, as the strain was isolated from a cold Antarctic soil).

Colonies of PB4^T are circular with entire margins, convex, shiny, mucilaginous, yellow and about 5 mm in diameter after 7 days on R2A agar. Cells are gram-negative, obligate aerobic, non-spore-forming, non-motile, rods and are about 0.4 to 0.5 µm x 2.0 to 3.5 µm in size. Temperature range for growth is between 0°C and 25°C with an optimum at 20°C. Strain PB4^T grows at pH values from pH 5.5 to 8.5 with optimum at pH 6.5 and can tolerate salt concentrations up to 5% NaCl with optimum growth at 0.5% NaCl. The isolate is sensitive to erythromycin, gentamycin-sulphate, kanamycin-sulphate, oxytetracycline, fosfomycin and troleandomycin, resistant to ampicillin, cephalosporin, metronidazole, novobiocin, penicillin, and rifampicin and not inhibited by lysozyme. Acid is produced from esculin ferric citrate, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, D-melizitose, glycogen, amidon (starch) and gentibiose and weakly from D-glucose, amygdalin, salicin and D-turanose (supplementary material Table S1). Strain PB4^T is able to use a wide range of substrates as sole carbon sources and can even grow weakly on minimal media without additional carbon source. The following carbohydrates are clearly used as sole carbon sources: glycerol, erythritol, D-ribose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-glucopyranoside, N-acetyl-glucosamine, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol, gentiobiose and D-arabitol. PB4^T is catalase and oxidase-positive and can hydrolyse starch and gelatine but not casein. Methyl-red test and urease activity are negative. Isolate PB4^T produces indole from tryptophan and hydrogen sulphide. G+C content is 33.7mol%.

The type strain of *Chryseobacterium frigidisoli* sp. nov is PB4^T (DSM 26000^T = LMG 27025^T), isolated from a soil sample from Larsemann Hills, East Antarctica.

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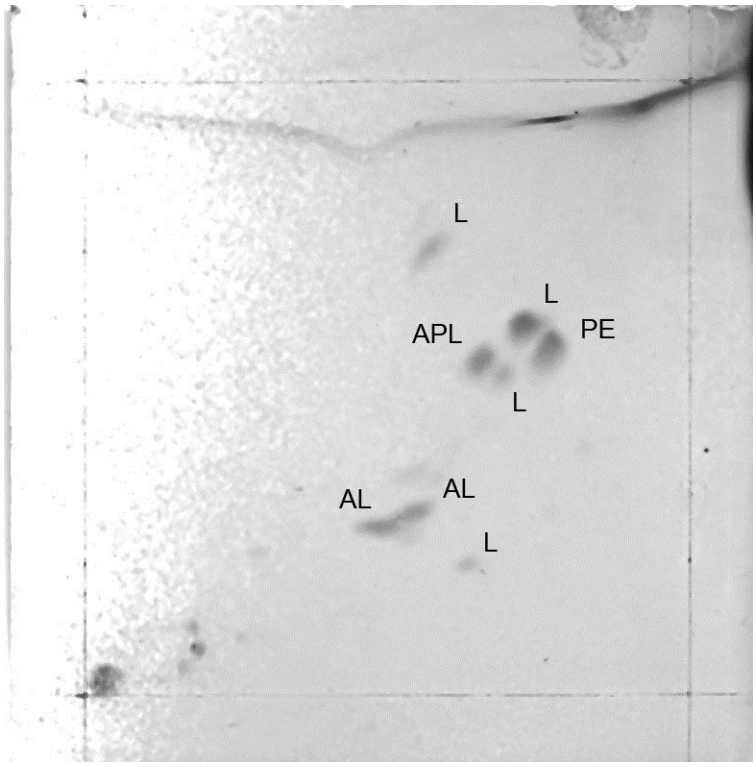
Supplementary Material Table S1: Acid production from carbohydrates and the use of carbohydrates as sole carbon sources. Strains: 1) *Chryseobacterium frigidisoli* sp. nov. PB4^T; 2) *Chryseobacterium humi* strain DSM 21580^T. Data was determined in this study with api ®50CH. + positive; w weak positive; - negative, CH Carbohydrate.

Carbohydrates	Acid production		CH as sole carbon source	
	1	2	1	2
Control	-	-	w	-
Glycerol	-	-	+	-
Erythritol	-	-	+	-
D-Arabinose	-	-	w	w
L-Arabinose	-	-	-	-
D-Ribose	-	-	+	-
D-Xylose	-	-	+	-
L-Xylose	-	-	-	-
D-Adonitol	-	-	+	-
Methyl-ßD-Xylopyranoside	-	-	w	-
D-Galactose	-	-	+	-
D-Glucose	w	+	+	+
D-Fructose	-	-	+	-
D-Mannose	-	-	+	+
L-Sorbose	-	-	+	-
L-Rhamnose	-	-	+	-
Dulcitol	-	-	+	-
Inositol	-	-	+	-
D-Mannitol	-	-	+	-
D-Sorbitol	-	-	+	-
Methyl-aD-Mannopyranoside	-	-	w	-
Methyl-aD-Glucopyranoside	-	-	+	-
N-Acetyl-Glucosamine	-	-	+	w
Amygdalin	w	-	w	w
Arbutin	-	-	+	w
Esculin ferric citrate	+	+	+	+
Salicin	w	-	+	-
D-Cellobiose	+	+	+	w
D-Maltose	+	+	+	+
D-Lactose	+	-	+	-
D-Melibiose	-	-	+	-
D-Saccharose	+	-	+	+

D-Trehalose	+	-	+	-
Inulin	-	-	+	-
D-Melezitose	+	-	+	-
D-Raffinose	-	-	+	-
Amidon (Starch)	+	+	+	+
Glycogen	+	+	+	+
Xylitol	-	-	+	-
Gentiobiose	+	+	+	w
D-Turanose	w	-	w	+
D-Lyxose	-	-	w	-
D-Tagatose	-	-	w	-
D-Fucose	-	-	w	-
L-Fucose	-	-	w	-
D-Arabitol	-	-	+	-
L-Arabitol	-	-	w	-
potassium Gluconate	-	-	-	-
potassium 2-Ketogluconate	-	-	w	-
potassium 5-Ketogluconate	-	w	-	-



Supplemental Material Figure S1. Two-dimensional TLC of polar lipids of *Chryseobacterium frigidisoli* PB4^{T} with sulfuric acid staining. AL, aminolipid; L, other lipids; PE, phosphatidylethanolamine; APL, aminophospholipid.

Cell membrane fatty acid composition of *Chryseobacterium frigidisoli* PB4^T, isolated from Antarctic glacier forefield soils, in response to changing temperature and pH conditions.

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Working title

Low temperature and pH adaption of psychrotolerant bacteria

Abstract

Microorganisms in Antarctic glacier forefields are directly exposed to the hostile conditions of their habitat such as a harsh climate with strong temperature fluctuations. A major mechanism of microorganisms to overcome those stress conditions is the change of the cell membrane composition maintaining its structure and functionality. However, only little is known about the adaptation potential of microorganisms from extreme habitats. In this study, we examined the

change of the cell membrane phospholipid fatty acid (PLFA) inventory of *Chryseobacterium frigidisoli* PB4^T, isolated from an Antarctic glacier forefield, in response to changing temperature (0 °C – 20 °C) and pH (5.5 - 8.5) regimes using gas chromatography-mass spectrometry (GC-MS). The PLFA pattern is dominated by saturated and unsaturated mainly branched fatty acids in the range between 15 and 18 carbon atoms. In the low-temperature adaptation of *C. frigidisoli*, the ratios of iso-/anteiso-$C_{15:0}$ and iso-/anteiso-hydroxy-$C_{15:0}$ fatty acids show a decreasing trend with declining temperature. The relative proportion of an unknown fatty acid, presumably an unsaturated cyclohexyl-C_{17} fatty acid, continuously increases from 20 °C to 0°C and the monounsaturated iso-$C_{17:1}\omega8$ strongly increases from 20 °C to 10 °C. In adaptation to changing pH values, most of the dominant fatty acids (iso- and anteiso-C_{15}, anteiso-2OH-C_{15}, iso-$C_{17:1}\omega8$) reveal constant relative proportions around neutral pH (6 - 8). Strong variations are observed in the relative proportions of hydroxy-fatty acids (low pH) and iso and anteiso fatty acids toward the pH extremes (5.5 - 8.5). The study shows the importance of a newly discovered fatty acid in the stress response of a cold-adapted bacterium and thus gives insights in adaptation strategies of microorganisms at low temperature and changing geochemical gradients.

Introduction

Microorganisms successfully colonise almost all existing ecological niches, including hostile environments such as hot springs, the deep sea, hot or polar deserts (Rothschild & Mancinelli, 2001). The polar regions are microbial-dominated ecosystems and create perfect conditions for extremophiles (Wynn-Williams, 1996). The Antarctic continent is characterised by extreme climatic conditions, oligotrophic nutrient pools, high salinity, low temperatures and low water availability (Cannone *et al.*, 2008). Soil microbial communities are able to respond and adapt to severe environmental conditions such as changing temperature and pH regimes (Bajerski & Wagner, 2013; Bakermans *et al.*, 2012; Ganzert *et al.*, 2011a). In general, there are several ways of stress response such as the induction of special heat/cold shock proteins, the production and release of protective compatible solutes or an enhanced/reduced metabolism (Georlette *et al.*, 2004). Another crucial adaptation process is the ability to adjust the cell membrane structure, because a fluid cell membrane is essential for microorganisms to maintain the function of important metabolic systems such as the electron transport chain (Denich *et al.*, 2003). Bacterial cell membranes are mainly formed by phospholipid bilayers best described in the "Mosaic Model" by Singer (1972). Microorganisms have developed several mechanisms to change their

cell membrane composition in order to maintain cell membrane fluidity and functionality in response to shifting environmental conditions, which is known as homeoviscous adaption (Sinensky, 1974). The membrane phospholipid composition can change regarding the polar head groups or the acyl side chains (Boggs, 1986; Russell, 1984). Microbial phospholipid fatty acid (PLFA) side chains are saturated or monounsaturated (rarely polyunsaturated) fatty acids with 12 to 24 carbon atoms and the acyl side chains can contain branches or ring structures such as cyclopropane, -pentane and –hexane rings. Thus, the phenotypic adaption of the cell membrane structure can be regulated by the degree of unsaturation, the chain length, branching or cyclisation of bacterial membrane fatty acids (Denich *et al.*, 2003). The adaption to low temperature is realised by a higher proportion of unsaturated fatty acids and a shift to more short chain fatty acids (Suutari & Laakso, 1994). Another adaptation process is the change of iso (i) and anteiso (ai) branched fatty acids, whereas the proportion of anteiso fatty acids increases with decreasing temperature (Kaneda, 1991). The introduction of cis-unsaturation, short-chained fatty acids and branching reduce the melting temperature of the cell membrane leading to an increase of fluidity. Several studies indicate different and variable adaptations of the PLFA inventory in response to changing pH regimes: A decrease of i-$C_{15:0}$ and i-$C_{16:0}$ at increasing pH and no significant effect of the anteiso branched fatty acids was reported (Bååth & Anderson, 2003) as well as an increase of ai-$C_{15:0}$ and no change of i-$C_{15:0}$ at higher pH (Männistö *et al.*, 2007). Furthermore, the proportion of unsaturated fatty acid may increase with rising pH (Männistö *et al.*, 2007). Thus, additional knowledge on the pH adaption of the microbial cell membrane is necessary. The role of cyclic compounds in the adaptation process is not completely understood and needs further investigation as well. In general a cyclisation disrupts the cell membrane packing, lowering the melting temperature of the cell membrane comparable to the effects of short chained, branched or unsaturated fatty acids. In contrast, it was suggested that the incorporation of a cyclopropane ring may enhance the stability of the cell membrane at low temperature, low pH or in slowly growing cells (Brown *et al.*, 1997; Russell, 1989). While desaturation is induced post-synthesis as a rapid answer system, other adaptions like chain length or branching need de novo synthesis and thereby require bacterial growth under extreme conditions. Former studies provide a general idea of the mechanisms involved in bacterial cell membrane adaptation to changing environmental condition (reviewed in Denich *et al.*, 2003), but the structural adaptation of microorganism from extreme habitats such as Antarctic soils still remains poorly understood. It is not convenient to simply conclude from one studied species to

other genera, because the change in the fatty acid composition varies between different bacterial genera. Therefore, more species need to be investigated. Studying the effects of different environmental parameters on the cell membrane structure could give new insights in the adaptation mechanisms of the microorganism to the extreme environment, because microorganisms can apply several mechanisms to maintain the fluidity and functionality of the cell membrane under different conditions.

In this study we focus on the temperature and pH adaption of the cell membrane structure of *Chryseobacterium frigidisoli* PB4^{T}, a cold-adapted representative of the *Bacteroidetes*, which was isolated from a mineral soil of a glacier forefield of the Larsemann Hills, East Antarctica. In the natural environment the strain is subjected to extreme geochemical gradients and temperature fluctuations. The potential change in the fatty acid composition was analysed after an incubation of the cells between 0 °C and 20°C and in a pH range of 4.5 to 8.5. Changes of the membrane phospholipid fatty acid (PLFA) were analysed by gas chromatography-mass spectrometry (GC-MS) experiments.

Methods

Study Site

The Larsemann Hills are an ice-free oasis situated in the Prydz Bay region in East Antarctica (69°30'S, 76°20'E; Stüwe *et al.*, 1989). The study site is characterised by severe climatic conditions with low temperatures and little precipitation (Hodgson *et al.*, 2001). Soil formation processes could not be observed and the studied material can be classified as dry and oligotrophic weathering debris. A detailed site description is given in an environmental study dealing with bacterial succession in two glacier forefields of the Larsemann Hills (Bajerski & Wagner, 2013). The studied bacterial strain was isolated from the weathering debris of the glacier forefield called "Black Valley Transect" in 446 m distance from the glacier (S 69°24.315; E 76°20.295). The harvested profile was characterised by a black organic mat mixed with coarse-grained sandy material at the surface and sandy permanently frozen ground in 1-6 cm depth. The soil pH of the study site ranged from acidic (pH 4.9) to alkaline (8.3), whereby the studied strain originated from neutral environment (pH 6.8).

Bacterial strains

Chryseobacterium frigidisoli strain PB4^T was chosen to examine the effect of changing temperature and pH on the fatty acid composition of the bacterial cell membrane. This strain was described as a novel psychrotolerant bacterium being able to grow between 0 °C and 25 °C with an optimum growth at 20 °C (Fig. S1). The gram-negative bacterium grows at pH values from pH 5.5 to 8.5 with optimum at pH 6.5 (Fig. S2; Bajerski *et al.*, 2013a). To study temperature adaption the strain was incubated at 0, 5, 10, 14 and 20°C in R2A medium (0.05 % Proteose peptone, 0.05 % Casamino acids, 0.05 % yeast extract, 0.05 % dextrose, 0.05 % soluble starch, 0.03 % dipotassium phosphate, 0.005 % magnesium sulfate 7×H_2O, 0.03 % sodium pyruvate [w/v], pH 7.2; Reasoner & Geldreich, 1985). The impact of changing pH values on the membrane composition was analysed using a medium modified with the following buffers: glycine (pH 4.0-5.0 and pH 10.0), MES [2-(*N*-morpholino)ethanesulfonic acid**;** pH 5.0-6.5], HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.0-8.0] and BTP [1,3-bis(tris(hydroxymethyl)methylamino)propane; pH 8.5-9.5; Hoaki *et al.*, 1994]. Growth in dependence of pH was detected at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 after an incubation at 18°C. The cells for phospholipid extraction were harvested at the transition from exponential to stationary growth phase by centrifuging for 5 min at 10.000 g, washing with sterilised tab water and centrifuging again.

Analytic procedure

The cell pellets of *Chryseobacterium frigidisoli* cultivated under different temperature and pH conditions were extracted with a flow blending system using a solvent mixture of methanol-dichloromethane (DCM)-ammonium acetate buffer with a ratio of 2:1:0.8 (v/v) for 5 min (modified after Bligh & Dyer, 1959). Afterwards, the solvent ratio of the extract was changed to 1:1:0.9 by adding DCM and ammonium acetate buffer resulting into a phase separation of an organic and an aqueous phase. Subsequently, the aqueous phase was re-extracted three times with DCM. The combined organic phases were concentrated using a Turbo Vap system (Zymark). Afterwards, the extract was chromatographically separated into fractions of different polarity resulting among other fractions into a phospholipid fraction as described in Zink & Mangelsdorf (2004). An aliquot of the phospholipid fraction was used for trans-esterification to obtain the methylated formerly phospholipid linked fatty acids (PLFA) by following the method described by Müller *et al.* (1990). The aliquot of the PL fraction is dissolved in 50 µl of

dichloromethane/methanol (9:1, v/v) in a 2 ml vial. Afterwards 50 µl of trimethylsulfoniumhydroxid (TMSH) was added and the vial was sealed before placed into an oven for 2 h at 70 °C. The trans-esterified (methylated) PLFAs were dried and subsequently solved in 50 µl dichloromethane before analysed on a gas chromatographic system (Trace GC Ultra, Thermo Electron) coupled to a DSQ Thermo Electron Quadrupole mass spectrometer. The GC was equipped with a cold injection system operating in the splitless mode and a SGE BPX 5 fused silica capillary column (50 m length, 0.22 mm ID, 0.25 µm film thickness) using the following temperature program: initial temperature 50 °C, 1 min isotherm, heating rate 3 °C min^{-1} to 310 °C, held isothermal for 30 min. Helium was used as a carrier gas with a constant flow rate of 1 ml min^{-1}. The injector temperature was programmed from 50 to 300 °C at a rate of 10 °C s^{-1}. Full scan mass spectra were recorded from m/z 50 to 600 at a scan rate of 2.5 scans s^{-1}.

Statistics

Correlation and significance of the derived data was calculated with IBM SPSS Statistics 21 analysing bivariate correlation using the Pearson correlation coefficient and testing two-tailed significance.

Results & Disussion

Fatty acid inventory of Chryseobacterium frigidisoli PB4^T

The major fatty acids (> 10 %, Tab. 1 and 2) determined in this study are iso- and anteiso-$C_{15:0}$, iso-2OH-$C_{15:0}$, iso-$C_{17:1}\omega 8$ and an unknown fatty acid, which was not found in any other microorganism so far. The newly discovered fatty acid is most probably characterised by 17 carbon atoms (molecular mass m/z 280, which means a C_{17} fatty acid with two double bond equivalences), an unsaturation and a cyclisation (cyclohexyl m/z 83; Fig. S3). The identified major fatty acids are in accordance with the fatty acid composition of the strain description that identified iso-2OH-$C_{15:0}$ and iso-$C_{15:0}$ as the major fatty acids (Bajerski *et al.*, 2013a). Other fatty acids such as iso-$C_{13:0}$, iso-$C_{14:0}$, another branched $C_{14:0}$ (presumably anteiso-$C_{14:0}$), $C_{15:1}\omega 6$, $C_{15:0}$, branched $C_{16:1}$ (presumably iso-and anteiso-$C_{16:1}$), iso-$C_{16:0}$, $C_{16:1}\omega 7$, a branched 2OH-$C_{16:0}$, a branched 2OH-$C_{15:0}$ (presumably anteiso-2OH-$C_{15:0}$), $C_{16:1}$, a branched $C_{16:0}$ (presumably anteiso-$C_{16:0}$), branched $C_{17:1}$ (presumably iso- and anteiso-$C_{17:1}$), 2OH-$C_{16:0}$, $C_{17:1}$, 2OH-$C_{16:1}$, $C_{18:1}\omega 9$, $C_{18:1}\omega 7$ and $C_{18:0}$ occur only in minor to trace amounts accounting in total for less than 10 % of

the total PLFA pattern (Tab.1 and 2). In some of the minor components the exact branching position could not be assigned to a standard, but due to the retention time in the mass spectra (Fig. 1 and 2) they are presumably iso- and anteiso-branched fatty acids, labelled with a and b in Tab. 1 and 2, respectively.

Tab. 1. Changes in the fatty acid inventory of *Chryseobacterium frigidisoli* PB4^T in response to different temperatures. a branched fatty acid with unknown branching position, probably iso-branched. b branched fatty acid with unknown branching position, probably anteiso-branched. + positively correlated. - negatively correlated. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). nd not detected. FA fatty acid.

Fatty Acid (FA) Composition at different Temperatures	**0 °C**	**5 °C**	**10 °C**	**14 °C**	**20 °C**
iso-$C_{13:0}$	nd	0.30	0.18	0.32	0.48
iso-$C_{14:0}$	nd	0.27	0.29	0.65	1.18
$C_{14:0b}$	0.29	0.12	0.19	0.46	0.60
iso-$C_{15:0}$	**12.28**	**13.67**	**12.09**	**21.46**	**21.28**
ai-$C_{15:0}$	**14.89**	**11.47**	**8.23**	**11.38**	**10.98**
ratio: iso/ai-$C_{15:0}$	**0.82**	**1.19**	**1.47**	**1.89**	**1.94**
$C_{15:1}\omega6^{+*}$	0.30	0.42	0.38	0.68	0.66
$C_{15:0}^{+*}$	0.19	0.35	0.25	0.96	1.07
br-$C_{16:1}$	1.26	1.47	1.08	0.61	0.85
iso-$C_{16:0}$	0.37	1.24	1.23	1.60	5.78
$C_{16:1}\omega7$ & br-2OH-$C_{16:0}$	2.27	3.05	3.20	2.40	1.39
iso-2OH-$C_{15:0}^{+*}$	**15.14**	**18.51**	**19.46**	**20.34**	**20.92**
anteiso-2OH-$C_{15:0}$	3.73	2.78	2.27	2.92	2.43
ratio: iso/ai-br-OH-$C_{15:0}$	**4.06**	**6.65**	**8.58**	**6.97**	**8.60**
$C_{16:1}$	nd	0.42	0.40	1.48	1.89
$C_{16:0b}$	1.19	0.36	0.50	2.95	1.88
iso-$C_{17:1}\omega8$	**17.86**	**23.41**	**27.79**	**17.73**	**14.40**
br-$C_{17:1a}$	0.50	0.39	0.44	nd	nd
br-$C_{17:1b}^{-*}$	2.68	1.44	1.60	1.16	0.91
unknown FA^{-**}	**25.73**	**18.58**	**16.75**	**10.20**	**8.82**
2OH-$C_{16:0}$	nd	0.47	0.62	0.39	1.11
$C_{17:1}$	nd	0.26	0.26	0.32	0.58
2OH-$C_{16:1}$	0.40	0.75	0.70	0.49	1.27
$C_{18:1}\omega9$	0.29	0.09	0.13	0.58	0.72
$C_{18:1}\omega7$	nd	nd	1.70	nd	nd
$C_{18:0}$	0.64	0.19	0.26	0.92	0.79

Tab. 2. Changes in the fatty acid inventory of *Chryseobacterium frigidisoli* PB4^T in response to different pH values. a branched fatty acid with unknown branching position, probably iso-branched. b branched fatty acid with unknown branching position, probably anteiso-branched. + positively correlated. -negatively correlated. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). nd not detected. FA fatty acid.

Fatty Acid (FA) Composition at different pH	**pH 5.5**	**pH 6**	**pH 6.5**	**pH 7**	**pH 7.5**	**pH 8**	**pH 8.5**
iso-$C_{13:0}^{+*}$	0.29	0.21	0.21	0.21	0.28	0.62	0.97
iso-$C_{14:0}$	0.24	0.33	0.46	0.38	nd	0.30	0.78
$C_{14:0b}$	0.51	0.15	0.12	0.10	0.27	0.37	0.34
iso-$C_{15:0}^{+*}$	**11.01**	**21.32**	**23.12**	**23.43**	**20.76**	**23.38**	**31.01**
anteiso-$C_{15:0}^{+*}$	**6.74**	**10.72**	**10.18**	**10.95**	**10.41**	**12.67**	**24.08**
ratio: iso/ai-$C_{15:0}$	**1.63**	**1.99**	**2.27**	**2.14**	**1.99**	**1.85**	**1.29**
$C_{15:1}\omega6^{+*}$	0.31	0.57	0.71	0.66	0.55	0.69	0.85
$C_{15:0}^{-*}$	0.59	0.31	0.30	0.37	0.26	0.25	nd
br-$C_{16:1a}$	0.56	nd	nd	nd	nd	nd	nd
br-$C_{16:1b}$	1.27	0.90	0.68	0.56	0.54	1.20	1.24
iso-$C_{16:0}$	0.86	1.14	1.21	0.97	0.37	1.19	1.16
$C_{16:1}\omega7$ & br-2OH-$C_{16:0}$	2.39	2.18	1.33	1.19	0.88	1.85	2.76
iso-2OH-$C_{15:0}$	**15.59**	**17.16**	**16.58**	**19.29**	**24.34**	**15.97**	**6.57**
anteiso-2OH-$C_{15:0}^{-*}$	**3.03**	**2.61**	**1.70**	**1.96**	**2.30**	**1.73**	**nd**
ratio: iso/ai-br-2OH-$C_{15:0}$	5.14	6.57	9.74	9.86	10.59	9.24	-
$C_{16:1}$	nd	0.71	0.76	0.70	nd	nd	nd
$C_{16:0b}$	4.36	0.41	0.36	0.59	1.53	1.81	1.29
iso-$C_{17:1}\omega8$	**23.08**	**20.85**	**24.04**	**22.94**	**21.96**	**21.11**	**18.20**
br-$C_{17:1a}$	0.89	0.31	0.44	0.47	0.57	0.57	1.31
br-$C_{17:1b}$	2.33	1.53	1.05	0.89	0.83	0.55	1.26
unknown FA^{-**}	**18.93**	**17.33**	**15.46**	**12.85**	**11.88**	**13.62**	**6.41**
2OH-$C_{16:0}$	0.57	0.26	0.21	0.26	0.22	0.50	nd
$C_{17:1}^{-*}$	0.27	0.42	0.48	0.41	0.15	nd	nd
2OH-$C_{16:1}$	0.37	0.27	0.24	0.35	0.25	0.47	nd
$C_{18:1}\omega9$	0.97	0.12	0.14	0.20	0.41	0.46	0.89
$C_{18:0}$	4.86	0.19	0.20	0.27	1.26	0.70	0.88

Additionally, the fatty acid inventory of *C. frigidisoli* is characterised by short chained and branched fatty acids, especially iso-/anteiso-C_{15} fatty acids and the iso-hydroxy-C_{15}-fatty acid. Both features are used for instance by cells that are able to grow under disturbed conditions to maintain membrane fluidity and functionality (Denich *et al.*, 2003). By those means *C. frigidisoli* is well adapted to a psychrotolerant life style, because short chain fatty acids and branching

increase the fluidity of the cell membrane at low temperatures (Russell, 1984). Furthermore, the whole genus *Chryseobacterium* is characterised by the presence of branched fatty acids and especially ai-$C_{15:0}$ is distinctive in psychrotolerant species of this genus (Hantsis-Zacharov & Halpern, 2007; Yi *et al.*, 2005). The incorporation of anteiso fatty acids lowers the melting temperature of the cell membrane enhancing motion ability, because for instance ai-$C_{15:0}$ (25.5 °C) has a critical lower main phase transition temperature than i-$C_{15:0}$ (52.2 °C; Suutari & Laakso, 1994). In general, the genus comprises several psychrotolerant species isolated from cold-affected habitats indicating that *Chryseobacteria* have the suitable settings for a life at low temperature.

Changes in the fatty acid inventory in adaptation to different temperatures

As discussed in the previous paragraph the cell membrane of *C. frigidisoli* predominately contains branched short chain fatty acids as an adaptation to psychrotolerant life. The ratio of iso to anteiso branched fatty acids of the major fatty acids i-/ai-$C_{15:0}$ and i-/ai-2OH-$C_{15:0}$ (Tab. 1) shows an increasing trend with rising cultivation temperatures. The relative proportion of the ai-2OH-$C_{15:0}$ fatty acids remains comparatively constant with an overall slight decrease with increasing temperature (Fig. 3a), whereas the relative proportion of ai-$C_{15:0}$ shows a clear initial decrease between 0 °C and 10 °C and remains constant on a slightly higher content between 14 °C and 20 °C. In contrast, the iso branched congeners show a general increase with increasing temperatures (Tab. 1, Fig. 1 and 3). The change of the iso-/anteiso ratio allows the organism to adapt the cell membrane melting temperature in response to different ambient temperatures. The importance of anteiso branched fatty acids at low temperatures was also reported in the temperature adaptation of *Bacillus subtilis* by Klein *et al.* (1999), which observed a relative dominance of ai-$C_{15:0}$ and ai-$C_{17:0}$ in the fatty acid inventory in cold shock experiments with *Bacillus subtilis*.

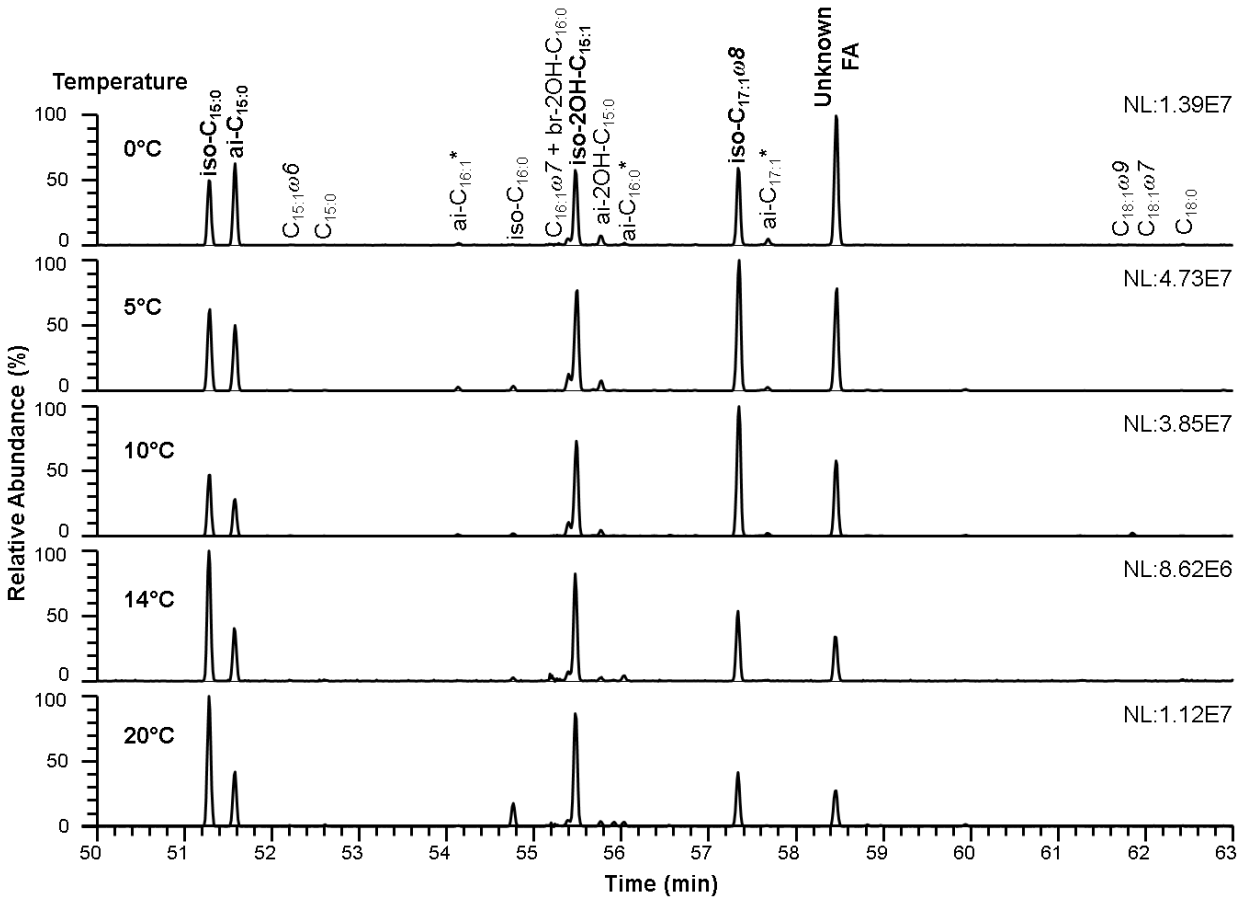

Fig. 1. The fatty acid inventory of *Chryseobacterium frigidisoli* PB4^T at different temperatures. The process of adaption is displayed between 5 °C and 20 °C. The major fatty acids (> 10 %) are printed in bold type. * tentatively identified.

Another adaptation to ambient temperature can be the chain length of the PLFAs, which can be reduced with decreasing temperatures, since the melting temperature of the respective membrane phospholipid fatty acids decreases with shortening chain length (Russell, 1989; Suutari & Laakso, 1994). However, in our study on *C. frigidisoli* the chain length does not seem to be a regulation mechanism (Tab. 1) in response to changing temperature regimes, because the strain uses other mechanisms to maintain the cell membrane fluidity at low temperature.

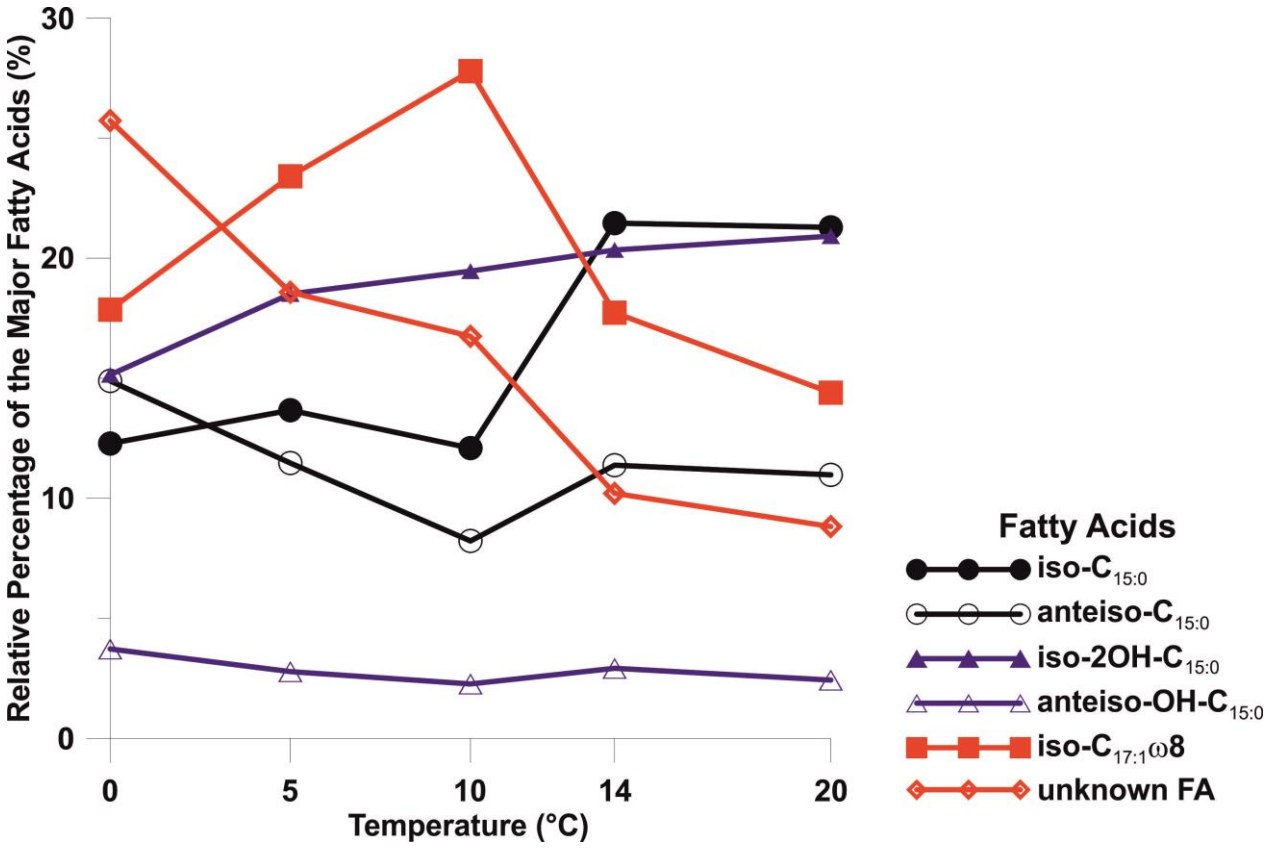

Fig. 3. The gradient of the major fatty acids of *Chryseobacterium frigidisoli* PB4^T at different temperatures.

In contrast to the chain length, temperature adaptation via the relative abundance of unsaturated fatty acids seems to play a role. The high abundant monounsaturated i-$C_{17:1}\omega8$ fatty acid shows a strong increase from 20 °C to 10 °C, which can be interpreted as a temperature adaptation, since a higher relative proportion of unsaturated fatty acids lowers the membrane solid to liquid phase transition temperature (Russell, 1989) in strain PB4^T (Fig.1 and 3). The relative amount of iso-$C_{17:1}\omega8$ decreases again between 10 °C and 0 °C. In contrast, the unknown fatty acid, presumably a cyclo-$C_{17:1}$ fatty acid, continuously increases (Fig. 1 and 3) from 20 °C to 0 °C. As mentioned above, the ai-$C_{15:0}$ fatty acid also increases from 10 °C to 0 °C and so also slightly does the ai-2OH-$C_{15:0}$ (Fig. 3). Therefore, the data indicate that there is an interchange in the adaptation mechanisms in the cold range (0 °C to 10 °C) between the unknown fatty acid and the ai-$C_{15:0}$ fatty acid both increasing and the i-$C_{17:1}\omega8$, which is decreasing again in the cold range. The statistical analysis shows that the unknown fatty acid strongly correlated with temperature ($p < 0.01$). Its relative proportion decreases with increasing temperature and accounts for the

highest amount of all fatty acids (25.73 %) at 0 °C (Tab.1). Thus, the unknown fatty acid seems to be an important and efficient regulation component for temperature adaption in *C. frigidisoli*. Detailed studies on changes in the PFLA profiles of microorganism especially from extreme habitats are still rare and the discovery of the new fatty acid shows that the microorganisms develop various ways of structural stress response. However, there are several studies reporting the critical role of reducing the fatty acid chain length and altering the branching from iso to anteiso in reaction to low temperatures (Annous *et al.*, 1997; Paton *et al.*, 1978). In 1984, Russell stated that unsaturation is more effective than chain length reduction in low temperature adaptation, but Mangelsdorf *et al.* (2009) showed that in microbial communities from Siberian permafrost the temperature regulation was mainly realised by chain length and the trend in the relative proportion of saturated and unsaturated fatty acids did not change significantly. Chain length and desaturation played a minor role in the cold shock response of *Bacillus subtilis* and evidence for the dominance of anteiso branched fatty acids in the adaptation to low temperature was given (Klein *et al.*, 1999). In our study, we report a high importance of iso and anteiso branched fatty acids in cold adaption. Additionally, high and variable relative amounts of i-$C_{17:1}\omega 8$ and the new discovered fatty acid are distinctive in the temperature adaption of *C. frigidisoli*.

Changes in the fatty acid inventory in response to shifting pH values

For most of the major fatty acids of *C. frigidisoli* the relative abundance is constant at neutral pH with a plateau phase between pH 6.5 and 7.5 (Fig. 4). At the same time fatty acids at pH minima and maxima show different compositions and variable adaptation mechanisms. The ratio of iso and anteiso branched fatty acids plays an important role in the maintenance of the cell membrane fluidity and functionality at pH stress (Tab. 2). The ratio of iso to anteiso fatty acids is high at neutral pH and becomes lower towards the pH extremes indicating an increasing importance of the anteiso branched fatty acids in response to high and low pH. The introduction of more anteiso branched fatty acids supports a higher flexibility of the cell membrane (Russell, 1989). The main challenge for the microorganism in an acidic or alkaline environment is the change of the ion strength that influences proton motive force of the cell membrane. The maintenance of the transmembrane electrical potential ($\Delta\psi$) and the transmembrane ΔpH is mainly realised using transporters including active proton transport (Krulwich *et al.*, 2011). A higher flexibility of the cell membrane could enable the incorporation of new membrane channels generating the ion

transport through the cell membrane in reaction to the changing ion strength at different pH values. Furthermore both, the relative amounts of iso- and ai-$C_{15:0}$ increases at increasing pH, whereas i-2OH-$C_{15:0}$ decreases (Tab. 2, Fig. 2 and 4).

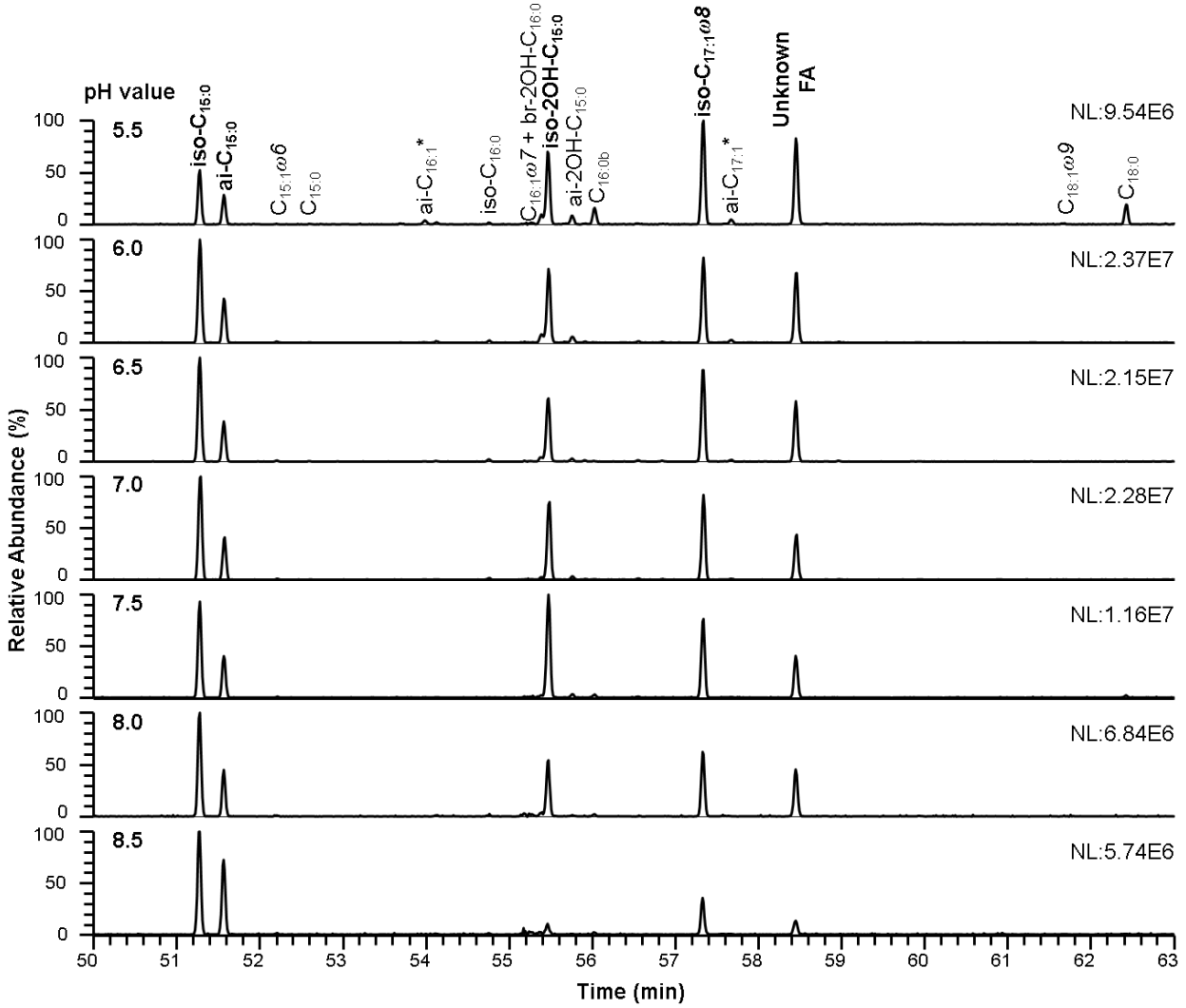

Fig. 2. The fatty acid inventory of *Chryseobacterium frigidisoli* PB4^T at different pH values.
The process of adaption is displayed between pH 5.5 and pH 8.5. The major fatty acids (> 10 %) are printed in bold type. * tentatively identified.

Different branching patterns in reaction to changing pH were discussed in literature before. Bååth and Anderson (2003) reported a decrease of i-$C_{15:0}$ and i-$C_{16:0}$ at increasing pH and no significant effect of the anteiso branched fatty acids, while Männistro *et al.* (2007) observed an increase of ai-$C_{15:0}$ and no change of i-$C_{15:0}$ at higher pH in bacterial communities in Arctic Fjelds of Finnish Lapland. Furthermore, an increase of branched (iso, anteiso) fatty acids was observed in response

to low pH (Russell, 1984). Interestingly, the relative abundance of the iso- and anteiso-C_{15} fatty acids with or without a hydroxyl group differs. The amount of fatty acids with a hydroxyl-side chain was low at alkaline pH of 8.5 with a significant decrease of iso- and ai-2-OH-$C_{15:0}$ (Tab.2, Fig. 4), whereas i-$C_{15:0}$ and ai-$C_{15:0}$ fatty acids increase significantly. At pH 5.5 the iso-$C_{15:0}$ and ai-$C_{15:0}$ fatty acids decrease while the hydroxyl congeners remain relatively constant. To our knowledge, this is the first study showing a pH dependent change in the relative amount of methyl and hydroxyl-side chains of the fatty acids and we can only assume that a methyl group is favoured at alkaline pH. For acidophilic archaea it was shown that hydroxyl groups on sugar moieties prevent the protons from penetrating the cell membrane at low pH (Wang *et al.*, 2012). That indicates that a hydroxylation is a suitable adaptation mechanism in an acidic environment, which would introduce a possible explanation for the incorporation of hydroxyl groups in the cell membrane.

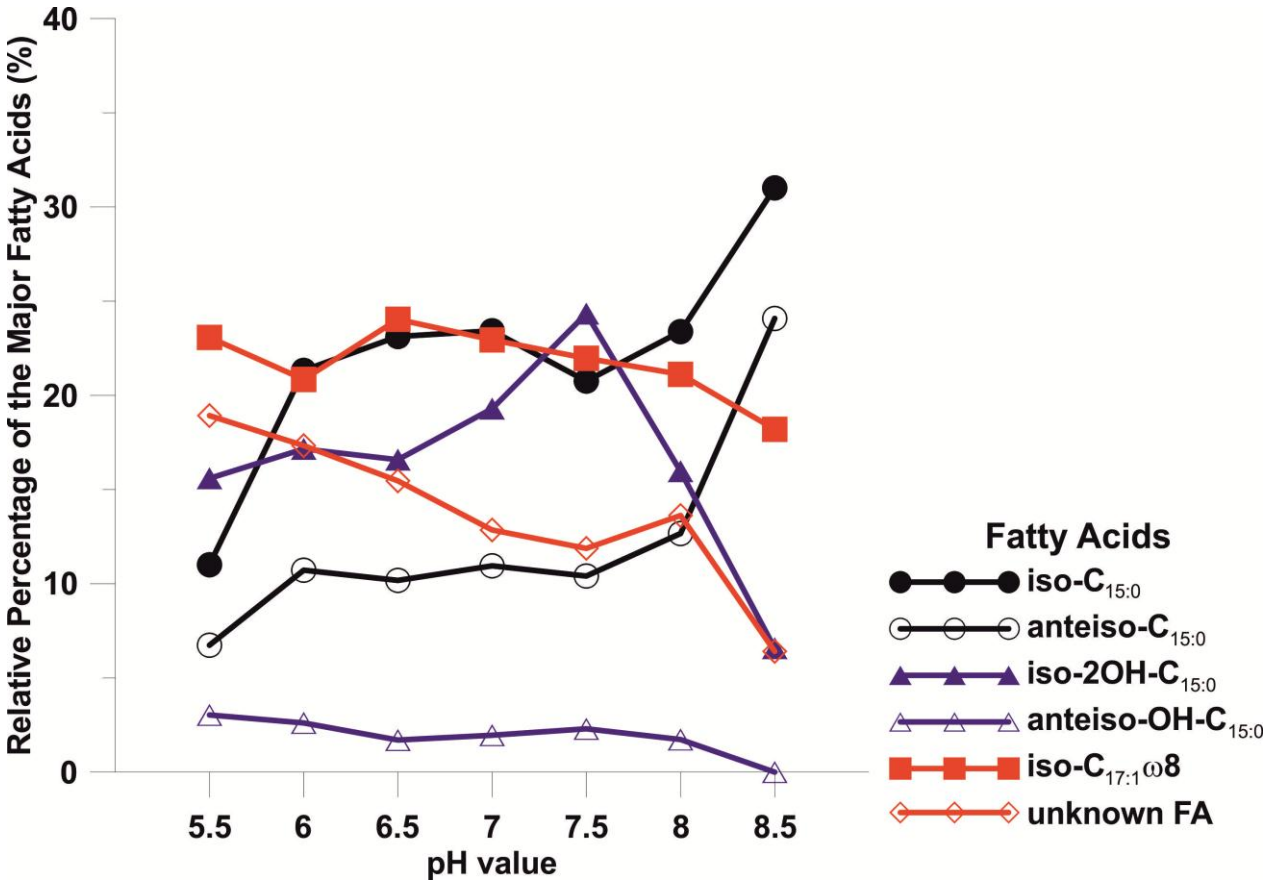

Fig. 4. The gradient of the major fatty acids of *Chryseobacterium frigidisoli* PB4^T at different pH values.

Regarding chain length, *C. frigidisoli* incorporates more short chained fatty acids, predominantly C_{15}, at higher pH. The mono-unsaturated i-$C_{17:1}\omega 8$ fatty acid and the unknown fatty acid significantly decrease at increasing pH (Tab.2, Fig. 2). A similar shift from short-chained saturated to long-chained mono-saturated fatty acids with decreasing pH was observed in *Streptococcus mutans* and other oral bacteria as a survival strategy in acidic environment, but the function of the shift is currently not understood (Fozo *et al.*, 2004; Quivey *et al.*, 2000). The unknown fatty acid seems to play an important role in the cell membrane adaption at low pH value, but the concrete structural function remains unclear because the exact structure could not be clarified so far. Nevertheless, a ring-structure, as suggested, could increase the membrane rigidity by decreasing the permeability of the cell membrane to protons, therefore an increased level of cyclo fatty acids may enhance the survival of microorganism at low pH (Brown *et al.*, 1997). The ability to produce different membrane fatty acid profiles, e. g. via the incorporation of longer-chained mono-saturated fatty or cyclo fatty acids may increase the survival of the strain at low pH. In general, the pH adaptation of the PLFA inventory seems to be a balanced combination of stabilisation and enhanced flexibility of the cell membrane to either prevent or enable protons passing the cell membrane.

Conclusion

In its natural habitat *Chryeobacterium frigidisoli* PB4^T is exposed to extreme low temperatures and changing geochemical gradients. In this study we discussed how *C. frigidisoli* develops different strategies to maintain the cell membrane function under changing environmental conditions by adapting the fatty acid inventory at different temperatures and pH values. In the low-temperature adaptation of *C. frigidisoli*, the ratio of iso-/anteiso-$C_{15:0}$ and iso-/anteiso-hydroxy-$C_{15:0}$ fatty acids complemented by a high amount of the monounsaturated iso-$C_{17:1}\omega 8$ and another unknown C_{17} fatty acid play a critical role. The pH adaption displays a complex combination of the fatty acids and was not linear but realised by a plateau phase at neutral pH and a difference in hydroxylation (low pH) and methylation (high pH) of the side chains at the pH extremes. In this study, we report the discovery of a new fatty acid that is an important compound for the adaption of *C. frigidisoli* to low temperature and pH stress. Therefore, it would be interesting to search for the new fatty acid in other bacteria being exposed to environmental stressors for instance in psychrotolerant *Chryseobacteria*.

Acknowledgements

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Supplementary Material

Fig. S1. Temperature range for growth of strain *Chryseobacterium frigidisoli* PB4^T. The specific growth rate μ was determined by measuring turbidity photometrical at 600 nm during an incubation in R2A media.

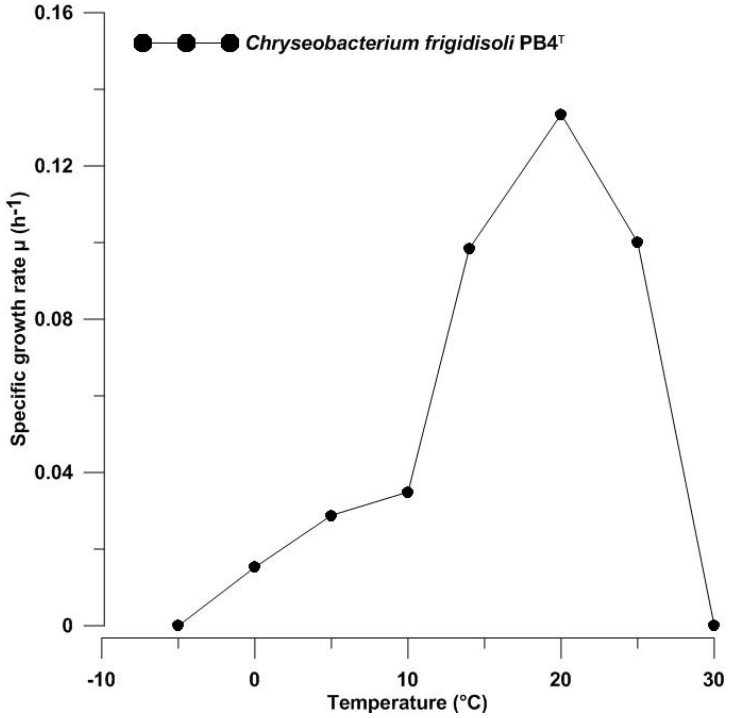


Fig. S2. PH range for growth of strain *Chryseobacterium frigidisoli* PB4^T. The specific growth rate μ was determined by measuring turbidity photometrical at 600 nm during an incubation in R2A media.

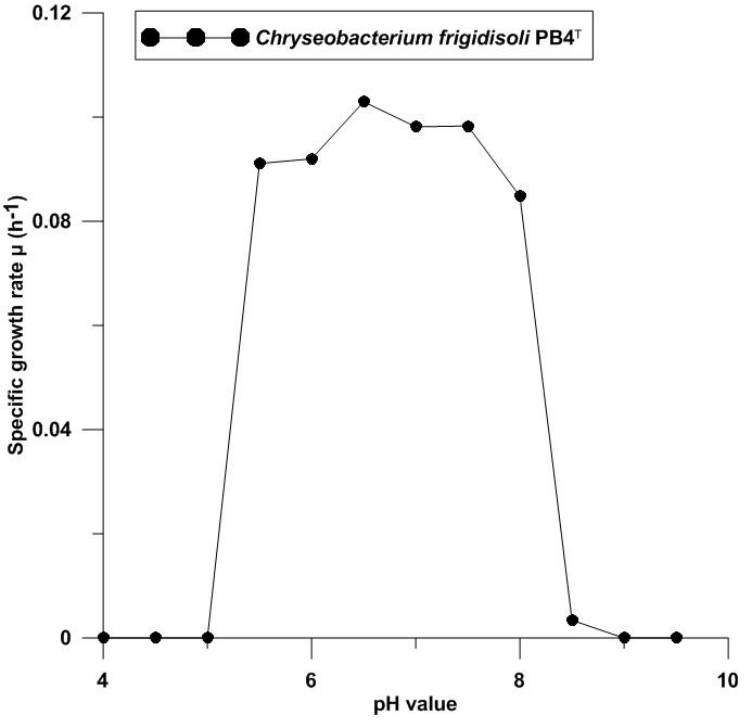
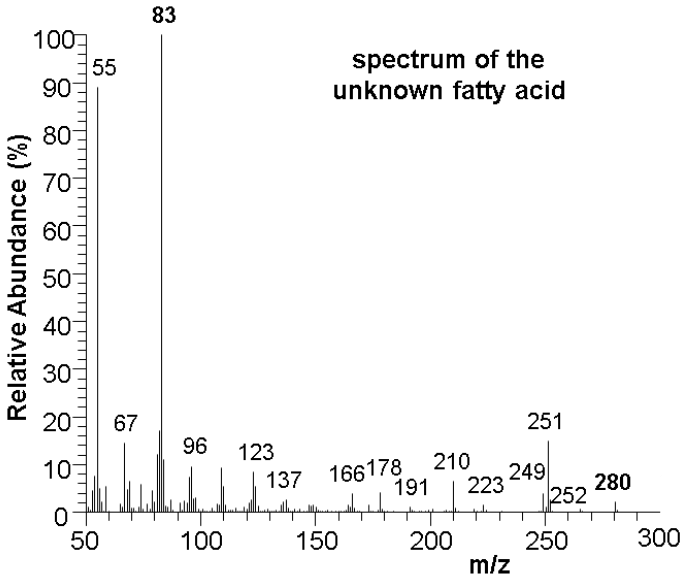


Fig. S3. Spectrum of the unknown fatty acid in the fatty acid profile of *Chryseobacterium frigidisoli*. The newly discovered fatty acid is most probably characterised by 17 carbon atoms (molecular mass m/z 280, which means a C_{17} fatty acid with two double bond equivalences), an unsaturation and a cyclisation (cyclohexyl m/z).



3. Synthesis

3.1 Discussion

The combined approach of culturing experiments, molecular community studies and geochemical and geophysical analysis in Antarctic glacier forefields provides additional knowledge on microbial communities in cold-affected terrestrial habitats. This study gives insights how soil characteristics influence the structure and development of bacterial communities and identifies adaptation strategies of the microorganisms to overcome the constraints of the extreme environment. The understanding of Antarctic terrestrial microbial communities is of great importance, because the Antarctic continent is a microbial-dominated ecosystem (Wynn-Williams, 1996) and a site of predicted environmental changes (Turner *et al.*, 2005). Thus, this work shows that Antarctic glacier forefields are a suitable model system to study bacterial colonisation in connection to soil formation.

The studied glacier forefields are extreme habitats characterised by low temperatures, low water availability and small oligotrophic nutrient pools comparable to the harsh conditions of the Antarctic Dry Valleys (Cary *et al.*, 2010). In contrast to studies of Alpine or Arctic glacier forefields, that showed a fast primary succession within several years (Hodkinson *et al.*, 2003; Sigler *et al.*, 2002), soil formation processes are not observed in the studied Antarctic glacier forefields, although Broknes Peninsula of the Larsemann Hills is ice-free since at least 40 ka BP (Hogdson *et al.*, 2001). The glacier forefield bedrock consists of weathering debris and coarse-grained unconsolidated deposits that can be referred to as "soil" according Bockheim´s definition for Antarctic soils (Bockheim, 1982; Ugolini & Bockheim, 2008). The two glacier forefield transects of this study represent sites of different soil development and bacterial succession. The glacier forefield "Glacier Transect" is directly influenced by the deglaciation-dependent areal shift of soil parameters such as water, pH and conductivity. According to similar reported shifts for Alpine glacier forefields (Noll & Wellinger, 2008), it is defined as "classical" glacier forefield. Additionally, strong winds, freeze-thaw cycles, snow-melting processes and mechanical translation lead to a variable and prevalently depth-related distribution of the soil parameters, especially in the second studied glacier forefield "Black Valley Transect". In this study, the Black Valley Transect resembles a "slightly matured" glacier forefield, because it is characterised by

larger amounts of soil moisture and organic carbon and a higher microbial abundance and activity in comparison to the "Glacier Transect".

The culture-independent, molecular-based analysis of the bacterial community revealed distinct differences in the composition of "classical" and "matured" glacier forefields. The communities of the less developed "classical" glacier forefield are similarly composed, not very differentiated and they are dominated by generalists of soil microbial communities, such as *Actinobacteria*. Furthermore, *Acidobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Chloroflexi* and *Proteobacteria* were distributed according to the deglaciation-dependent trend of the soil parameters. In contrast, communities of the "matured" forefield are variably composed at each spot in the glacier forefield depending on the proximity of the glacial or snow caps and the location at the slope or in the outflow of the glacier. The variation of the habitat supports the development of specialised bacterial communities that are either dominated by one group, e.g. *Cyanobacteria* or *Actinobacteria*, or they have a balanced composition without a clear dominance of one group.

In the glacier forefield transects, several of the bacterial phyla are influenced by the soil characteristics, either following an orderly succession in agreement with what has been reported for some Alpine glacier forefields (Noll & Wellinger, 2008; Sigler *et al.*, 2002), or mirroring soil heterogeneity and local variability of the extreme habitat (Cannone *et al.*, 2008; Schütte *et al.*, 2009). *Bacteroidetes*, for example, occur predominately in the vicinity of the glaciers, because the psychrophilic representatives of this group are well adapted to the low temperatures at the glacier tongue. The production of cold-adapted extracellular enzymes and the degradation of polymeric substances contribute to biological weathering of the rocks and thus support soil formation processes in the glacier forefields (Aislabie *et al.*, 2006; Buckley & Schmidt, 2001). *Cyanobacteria*, *Gamma-* and *Deltaproteobacteria*, *Bacteroidetes* and *Gemmatimonadetes* strongly depend on water availability; whereas the most dominant group, the *Actinobacteria*, are correlated to the distribution of trace elements showing that nutrient and water limitation have a great effect on the distribution of these groups. Those limitations may stronger influence microbial community structure than changing temperature regimes (Cannone *et al.*, 2008). In the contrary, other groups, for instance the *Acidobacteria*, are not that much influenced by a certain soil parameter, because they tolerate the extremely low nutrient conditions and strong fluctuation of soil hydration in the glacier forefields (Ward *et al.*, 2009). In comparison to similar Antarctic cold desert soils (Smith *et al.*, 2006), a surprisingly high bacterial abundance and diversity was

observed, even higher than the functional diversity of microbial communities of the Antarctic Peninsula (Pessi *et al.*, 2012). This high abundance and diversity is the key to microbial dominance and importance in the habitat, because the microorganisms take part in the primary production, nutrient cycling and biological weathering: Several autotrophic bacteria, for instance representatives of *Cyanobacteria* or *Chloroflexi*, are involved in the primary production by fixing atmospheric nitrogen or inorganic carbon (Madigan, 2012) and thus support the very early succession phase of the habitat. The assimilated products are substrates for heterotrophic bacteria, e.g. representatives of the groups of *Actinobacteria*, *Alpha-*/ *Beta-*/ *Gammproteobacteria*, *Deinococci*, *Flavobacteria* and *Sphingobacteria* that have been isolated in this study. The exchange of organic and inorganic compounds via the microorganisms may generate the nutrient turnover and biogeochemical cycling of the habitat. Thus, the autotrophic assimilation is followed by a more complex and organic-depended second succession step. Furthermore, members of the classes *Actinobacteria, Bateriodetes* and *Beta-/ Gammaproteobacteria* are associated with weathering processes by dissolving silicates, e.g. via the formation of oxalic acids or extracellular enzymes (Frey *et al.*, 2010). Thus, the production of extracellular enzymes, as it was found in the soil and isolates of this study, can contribute to soil formation processes of the habitat.

In both, culture-dependent and -independent methods, sequences of cultured strains or of environmental samples are classified as "unknown", because a majority of the species is not culturable at all (Rollins & Colwell, 1986) and recent studies assume that less than 0.1 % of all bacterial species are isolated (Overmann, 2013). These circumstances especially apply to extreme habitats (Ganzert *et al.*, 2011a) and in this study up to 28 % of the clone sequences could not be assigned to a known phyla. To extend the existing database and to identify some of the unknown candidates, isolation, cultivation and characterisation experiments were carried out focusing on the community of heterotrophic bacteria. The enrichment and cultivation conditions were adapted to the environmental conditions using nutrient-poor media and low incubation temperatures. The choice of selective media and conditions leads to the enrichment of communities that differ in abundance (up to 10^8 colony forming units per gram soil) and visible diversity (148 identified isolates). Out of all isolates six potentially new species are characterised in detail and strain PB1^T and PB4^T are described as novel psychrotolerant strains *Herbaspirillum psychrotolerans* (Bajerski *et al.*, 2013b) and *Chryseobacterium frigidisoli* (Bajerski *et al.*, 2013a), respectively.

Strain PB1^T is a gram-negative, aerobic bacterium growing between -5 °C and 30 °C with an optimum temperature for growth at 14-20 °C. Isolate PB1^T has catalase and low urease activity and hydrolyses gelatine and starch. Glycerol, D-/L-arabinose, D-xylose, D-galactose, D-fructose, D-lyxose, D-fucose and potassium gluconate are used as sole carbon sources. Strain PB4^T is a gram-negative, obligate aerobic bacterium growing between 0 °C and 25 °C with an optimum temperature for growth at 20 °C. Strain PB4^T produces indole from tryptophan, hydrolyses casein and can use numerous carbohydrates as sole carbon sources. The isolates are able to grow at low temperatures tolerating temperature fluctuations and they are not specialised to a certain substrate, therefore they are well-adapted to the cold and oligotrophic environment.

In general, microorganisms have developed several adaptation mechanisms such as the synthesis of special cold-shock proteins or antifreeze molecules, metabolic adaptations and structural modifications of enzymes and cell membrane components (Deming, 2002). Another aspect of the study was focused on cold-adapted extracellular enzymes and the change of the cell membrane phospholipid fatty acid (PFLA) inventory in response to changing temperature and pH regimes. The characterisation of selected psychrotolerant strains shows that microorganisms colonising Antarctic glacier forefields have specialised cold-adapted enzymes. The microbial production of extracellular phosphatase (pH 11 and pH 6.5), β-glucosidase, invertase and urease was measured in the glacier forefield soils (Appendix 5.1 Methods). Although the activity of invertase and urease was low (Appendix 5.2 Results, Tab. 1), the low-temperature activity of the phosphatases and to some extent also the β-glucosidase is comparable to enzyme activities of temperate soils measured at optimum enzyme activity temperature (Eivazi & Tabatabai, 1977; Ma *et al.*, 2010). Overall, enzyme activities of the slightly "matured" glacier forefield are higher than in the less developed "classical" forefield, confirming the discussed different development phases. Again, a deglaciation-dependent trend (activities increased at increasing distance from the glacier) is shown in the "classical" glacier forefield, whereas enzyme activities of the "matured" forefield followed the heterogeneous bacterial succession (Appendix 5.2 Results, Fig. 1 and 2). Thus, the detection of enzyme activities at low temperature provides a good example for microbial adaptation strategies to the extreme environment.

The PLFA analysis shows that the novel isolated species *C. frigidisoli*, which was used as a model organism, develops different strategies to maintain the cell membrane function under changing environmental conditions by altering the cell membrane composition at different

temperatures and pH values. First, psychrotolerant microorganisms could have a general genotypic-based adaptation to the low temperature, e.g. by the incorporation of a majority of branched or short chain fatty acids into the cell membrane to enhance its fluidity in the cold (Mangelsdorf *et al.*, 2009). Second, the adaptation of the cell membrane is not realised by a single mechanism, but by a combination of structural changes, that can vary in response to different parameters or even within one parameter (Russell, 1984). In the low-temperature adaptation of *C. frigidisoli*, the ratio of iso/anteiso-$C_{15:0}$ and iso/anteiso-hydroxy-$C_{15:0}$ fatty acids complemented by a high amount of the monounsaturated iso-$C_{17:1}\omega 8$ and another unknown C_{17} fatty acid play a critical role. The analysis shows that the unknown fatty acid consists out of 17 carbon atoms, an unsaturation and cyclisation. The incorporation of branched, unsaturated and cyclic fatty acids lowers the melting temperature of the cell membrane to generate its fluidity at low temperatures. The newly discovered fatty acid significantly increased at decreasing temperature and pH and thus plays an important role in the adaption of *C. frigidisoli*. The pH adaption of *C. frigidisoli* displays a complex combination of the fatty acids and was not linear but constant at neutral pH with differences in hydroxylation (low pH) and methylation (high pH) of the side chains at the pH extremes. The introduction of more anteiso-branched fatty acids at the pH extremes may allow for the incorporation of new membrane channels generating the ion transport through the cell membrane in reaction to the changing ion strength at different pH values (Krulwich *et al.*, 2011). Furthermore, a hydroxylation seems to be a suitable cell membrane alteration in an acidic environment by preventing the protons from penetrating the cell membrane as it was described for hydroxyl groups on sugar moieties in the cell membrane of acidophilic archaea (Wang *et al.*, 2012).

3.2 Conclusion

This work, presented in four manuscripts, gives insights into the diversity, distribution and adaptation mechanisms of microbial communities in oligotrophic cold-affected soils. In the background of global climate change the understanding of microbial succession during changing environmental conditions e.g. elevated temperature and soil formation is from a paramount interest. By characterising the microbial population in habitats like the two glacier forefields of the Larsemann Hills, which are reflecting different development steps in the meaning of soil

formation, important parameters for climate and geochemical modelling are provided. The investigated sites allowed studying the microbial succession at an early step of soil formation near the ice tongue in comparison to closely located but rather older and more developed soil from the forefield. The connection of geochemical and geophysical data with bacterial community structure showed that soil parameter and soil formation along the glacier forefield influence the distribution of certain phyla, but environmental constraints such as water or carbon limitation similarly effect the distribution. It was found that the relative undifferentiated bacterial diversity reflects the undifferentiated soil development. In this step of succession the microbial community has a high potential to shift according to past and present environmental conditions e.g. temperature, availability water and substrates. The observed high amount of yet unknown species in these soils and the description of two new isolated bacteria strengthen the concept that cultivation based studies and pure cultures are required to understand the physiology and adaptation mechanisms of microorganisms. The adaptation strategies of the microorganisms were analysed in environmental samples and cultures focussing on extracellular enzyme activity at low temperature and PLFA analyses. The results showed that extracellular enzymes catalyse the conversion of various compounds and thereby provide necessary substrates even in the low temperature regime. It is discussed that these enzymes play further on a role in the soil formation and total carbon turnover. The formation of cold-adapted enzymes may also carry an interesting biotechnological potential. There are only a few data on the PLFA profiles of microorganism from extreme habitats, with this study the importance of a new identified fatty acid, involved in the stress response of the novel characterised bacterium is highlighted.

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5. Appendix

5.1 Methods: "**Enzyme activity at low temperatures: A view on bacterial activity in terrestrial Antarctic environments.**"

Study Site

The Larsemann Hills are situated at the Ingrid Christensen Coast in the Prydz Bay area, East Antarctica. They consist of two main peninsulas and several smaller peninsulas and islands being ice-free since at least 20 ka (kilo annum). The study site is characterized by the cold and dry continental Antarctic climate with low mean temperatures and little precipitation (250 mm a^{-1}). Neither vegetation nor initial soil formation could be observed at the study site. The studied material can be described as gravel and coarse-grained soil precursors. During the expedition of the research vessel "Polarstern" to Antarctica in March 2007 altogether 28 bulk soil samples were taken from two different glacier forefield transects. A detailed description of the sampling procedure and soil characteristics was given before. Samples for the enzyme activity tests were collected in sterile 250 ml plastic boxes (Nalgene) and transported at -25°C on research vessel "Polarstern" from the Prydz Bay (Antarctica) to Bremerhaven (Germany).

Enzyme activity tests

An aliquot of the samples was thawed at 4°C and the naturally moist soil samples were sieved for a grain size smaller than 2 mm.

Phosphatase

The alkaline (pH 11) and acidic (pH 6.5) phosphatase activity was determined using the p-nitrophenyl phosphate assay as described by Tabatabai and Bremner (1969). The release of p-nitrophenyl from provided p-nitropenyl phosphate solution was measured colorimetrical at 400 nm after an incubation of 3 h at 15°C on a horizontal shaker. The experiment was performed with 1 g moist and sieved soil in triplicates and with one blank value. Phosphatase activity was stated in µg p-nitrophenyl* g dry $soil^{-1}$* 3 h^{-1}.

Glucosidase

The activity of β-glucosidase in soils was analysed according to the protocol of Eivazi and Tabatabei (1988). The assay is based on the release of p-nitrophenyl from provided p-nitrophenyl-β-D-glucoside (PNG) solution in the presence of toluene after an incubation of 3 h at 15°C on a horizontal shaker. All soil samples were analysed in triplicates and one blank value

with 1 g moist sieved soil. The extinction of the filtrates was measured colorimetrical at 430 nm and β-glucosidase activity was expressed as µg p-nitrophenyl* g dry soil^{-1}* 3 h^{-1}.

Invertase

To determine invertase activity in soils the released reduced decomposition products of the hydrolysis of provided sucrose were measured after an incubation of 3 h at 15°C on a horizontal shaker following the principle of Schinner and von Mersi (1990). The experiment was carried out with 5 g of moist sieved soil with triplicate samples and one blank value. After the development of the Prussian Blue stain the extinction was measured colorimetrical within 30 min at 690 nm. Invertase activity is denoted as µg glucose-monohydrate* g dry soil^{-1}* 3 h^{-1}.

Urease

Urease activity was determined via the release of ammonia from provided urea solution as described for the unbuffered procedure by Kandeler and Gerber (1988). The determination was carried out in triplicates and one blank value with 5 g moist and sieved soil respectively. All samples were incubated at 15°C for 2 h on a horizontal shaker and measured colorimetrical at 690 nm. Urease activity was displayed as µg N hydrolysed* g dry soil^{-1}* 2 h^{-1} at 15°C.

5.2 Results: "**Enzyme activity at low temperatures: A view on bacterial activity in terrestrial Antarctic environments.**"

Tab. 1. Calorimetric measured enzyme activities of the glacier transects. GT - Glacier Transect, BV - Black Valley Transect. ID - identification

[1]Alkaline Phosphatase in µg p-nitrophenyl* g dry soil^{-1}* 3 h^{-1}

[2]Acidic Phosphatase in in µg p-nitrophenyl* g dry soil^{-1}* 3 h^{-1}

[3] β-Glucosidas in µg p-nitrophenyl* g dry soil^{-1}* 3 h^{-1}

[4]Invertase in µg glucose-monohydrate* g dry soil^{-1}* 3 h^{-1}

[5] Urease in µg N hydrolysed* g dry soil-1* 2 h^{-1}

*Standard Deviation, nd - not detected

Sample ID	ALKP[1]	StDv ALKP*	ACP[2]	StDv ACP*	b-Glu[3]	StDv b-Glu*	Inv[4]	StDv Inv*	Ure[5]	StDv Ure*
GT0/ 0-7	1.93	1.39	27.82	12.68	6.41	5.96	nd	nd	nd	nd
GT0/ 7-14	2.83	1.13	22.45	6.15	nd	nd	nd	nd	nd	nd
GT0/	2.29	1.68	4.53	5.70	6.14	10.63	nd	nd	nd	nd

14-25										
GT30/ 0-13	1.25	1.17	29.85	2.17	nd	nd	0.01	0.01	0.07	0.11
GT30/1 3-28	4.56	2.06	19.72	3.11	7.92	10.66	0.05	0.08	nd	nd
GT55/ 0-10	2.74	1.71	22.10	7.90	2.68	4.42	0.05	0.09	0.19	0.12
GT55/ 10-20	5.78	1.06	13.71	3.46	nd	nd	nd	nd	0.03	0.05
GT65/0 -10	4.93	1.78	37.81	3.29	5.80	10.05	0.01	0.01	0.03	0.05
GT65/ 10-20	4.02	2.92	6.73	3.01	13.15	10.48	nd	nd	0.04	0.04
GT65/ 20-30	1.17	0.93	20.95	12.42	31.47	10.00	nd	nd	0.06	0.05
GT80/ 0-1	15.27	2.30	234.61	109.94	107.20	5.31	0.05	0.08	1.70	0.54
GT80/ 1-10	0.24	0.33	47.08	8.88	7.23	4.92	0.02	0.04	0.23	0.10
GT80/ 10-20	5.68	3.42	19.60	4.32	16.56	5.49	0.08	0.11	0.19	0.06
GT100/ SS	33.24	4.27	397.82	38.24	38.96	5.30	nd	nd	0.27	0.25
BV26/ 0-1	109.90	4.31	668.18	203.13	102.98	17.77	1.16	0.67	9.00	3.35
BV26/ 1-9	48.07	5.89	494.28	84.43	19.22	10.70	nd	nd	0.75	0.06
BV162/ 0-2	95.00	12.18	787.29	188.18	25.94	3.78	0.67	0.42	16.39	5.83
BV162/ 2-5	26.16	3.69	337.32	136.45	14.55	3.61	nd	nd	0.98	0.30
BV203/ 0-7	10.82	1.65	226.10	12.62	26.94	8.54	0.05	0.05	nd	nd
BV203/ 7-11	3.83	1.24	49.60	1.26	7.70	3.13	0.18	0.14	0.22	0.18
BV203/ 11-18	1.66	2.34	8.54	0.24	1.89	3.05	nd	nd	0.30	0.29
BV446/ 0-1	374.21	68.45	706.79	204.17	33.52	5.93	0.42	0.14	2.87	0.61
BV446/ 1-6	130.21	66.61	727.56	74.30	30.12	2.31	0.29	0.51	1.01	0.17
BV474/ 0-2	5.94	0.26	63.67	22.36	24.09	8.72	nd	nd	0.04	0.07
BV474/ 2-6	0.93	0.71	37.29	5.62	6.27	5.81	0.05	0.05	nd	nd
BV325/ SS	256.36	23.60	164.08	13.18	27.39	5.27	nd	nd	2.03	1.37
BV500/ SS1	9.64	0.93	169.92	43.68	6.85	7.50	0.04	0.04	0.49	0.08
BV500/ SS2	5338.13	815.21	769.82	90.06	74.94	18.18	nd	nd	13.18	0.70

Fig. 1. Colorimetric measured enzyme activities of the glacier transects in dependence of distance from the glacier. The depths of the profiles are summed up.

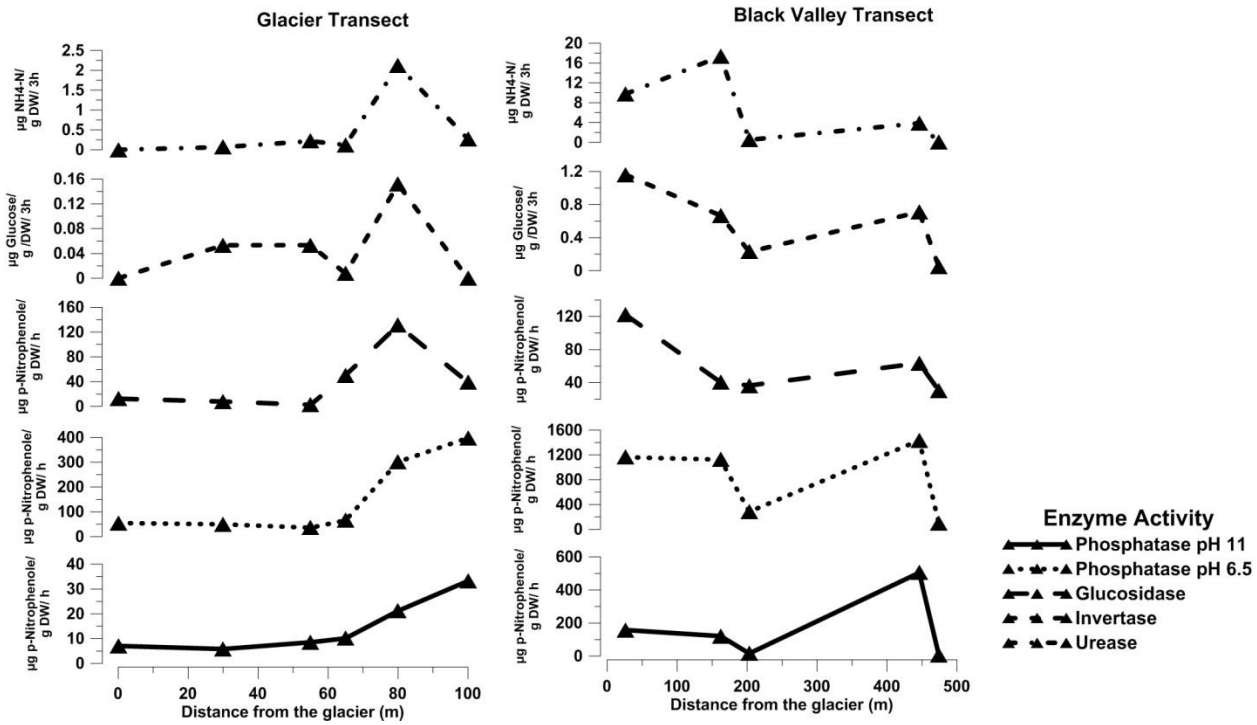
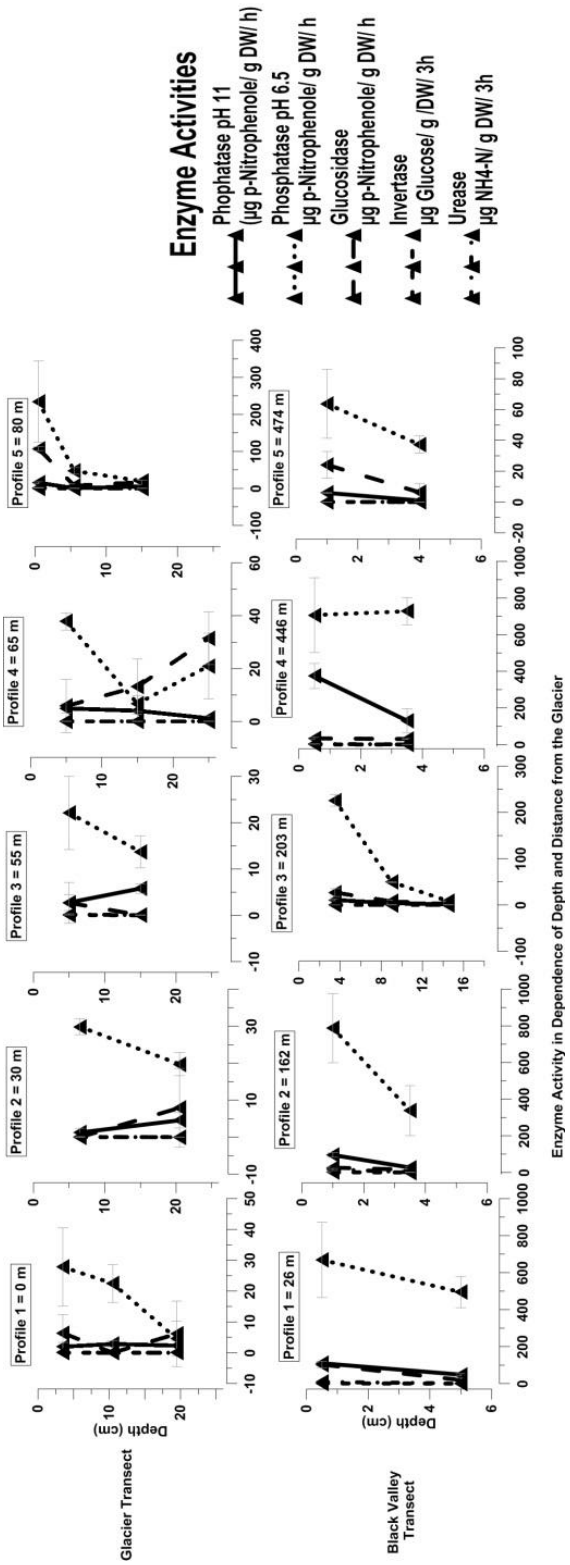


Fig. 2. Colorimetric measured enzyme activities of the glacier transects in dependence of depth. Each profile along the glacier transect is shown separately. Standard deviations are displayed as grey bars.



Selbständigkeitserklärung

Die vorliegende Dissertation wurde von mir am Alfred-Wegener-Institut für Polar- und Meeresforschung in Potsdam bearbeitet und wurde in englischer Sprache geschrieben.

Hiermit versichere ich an Eides statt, dass die vorliegende Arbeit selbständig und unter Verwendung keiner anderen als der angegebenen Hilfsmittel verfasst wurde.

F. Bajerski

(Felizitas Bajerski)
Unterschrift

Potsdam, 29.4.2013

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Ort, Datum