Culturable Diversity of Psychrophilic Bacteria from Different Glaciers of Karakoram Mountain Range and Role of Cell Membrane Fatty Acids in Cold Adaptation

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Culturable Diversity of Psychrophilic Bacteria from Different Glaciers of Karakoram Mountain Range and Role of Cell Membrane Fatty Acids in Cold Adaptation

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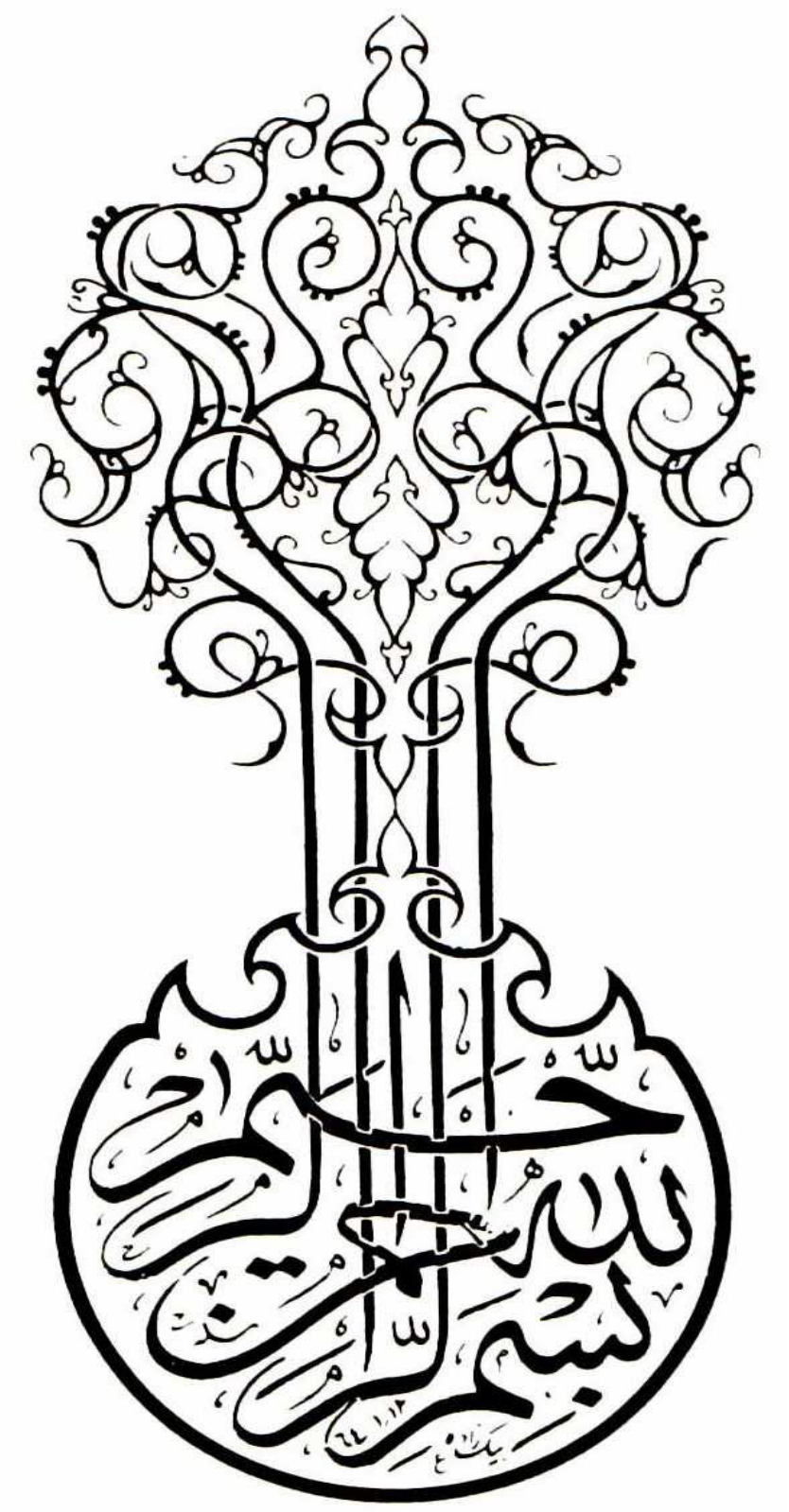

In the name of ALLAH, Most Gracious, Most Merciful.

DEDICATION

Dedicated to

My Beloved Parents

And

Wife

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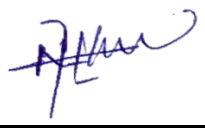
Signature: 

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This is to certify that the research work presented in this thesis, entitled titled ***"Culturable Diversity of Psychrophilic Bacteria from Different Glaciers of Karakoram Mountain Range and Role of Cell Membrane Fatty Acids in Cold Adaptation"*** was conducted by ***Mr. Noor Hassan*** under the supervision of ***Prof. Dr. Fariha Hasan***. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of ***Microbiology***.

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List of Abbreviations

AA	Arachidonic acid
ACP	Acyl carrier protein
α-LA	α-Linolenic acid
ATP	Adenosine tri phosphate
b-FAs	Branched chain fatty acids
CFB	Cytophaga-Flavobacteria-Bacteroides
CFU	Colony Forming Unit
CHS	Cyanobacterium Hopar Sediment
DCGs	Debris-covered glaciers
DHA	Docosahexaenoic acid
DMADP	Dimethylallyl diphosphate
EPA	Eicosapentaenoic acid
EPS	Extracellular polymeric substances
ER	East Rongbuk
FAMEs	Fatty acid methyl esters
FAS	Fatty acid synthases
FTIR	Fourier Transform infrared
GC/MS	Gas chromatography/mass spectroscopy
GC-FID	Gas chromatography flame ionisation detector
GhS	Ghulkin Sediment
GhW	Ghulkin Water
GI	Ghulmit Ice
GLOFs	Glacial lake outburst floods
GS	Ghulmit Sediment
GW	Ghulmit Water
HI	Hopar Ice
HKKH	Hindu Kush-Karakoram-Himalayan
HMG-CoA	Hydroxy-3-methyl glutaryl CoA
HPLC	High-Performance Liquid Chromatography
HS	Hopar Sediment

HW	Hopar Water
LC-PUFAs	Long-chain polyunsaturated fatty acids
MSM	Minimal salts medium
MUFA	Monounsaturated fatty acids
MVA	Mevalonic acid
NCBI	National Center for Biotechnology Information
NJ	Neighbor-Joining
n-MUFAs	Straight chain monounsaturated fatty acids
PCR	Polymerase Chain Reaction
PLFA	Phospholipid fatty acids
PTV	Programmable temperature vaporizing
PUFA	Polyunsaturated fatty acids
qPCR	quantitative Polymerase chain reaction
R2A	Reasoner's 2 Agar
ROS	Reactive oxygen species
rpoB	RNA polymerases β subunit
rRNA	Ribosomal Ribonucleic Acid
R_t	Retention time
SFA	Saturated fatty acids
TN	Total nitrogen
TOC	Total organic carbon
TOC-V	Total organic carbon analyzer
TP	Total phosphorus
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UVR	Ultra Violet Radiations

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Summary

Temperature of approximately 85% of Earth is under 5°C. These low temperature territories are distributed throughout the world ranging from Artic to Antarctica, European Alps to Deep Oceans and Hindu Kush-Karakoram-Himalaya Mountains. Cold environments are accompanied by a wide range of organisms covering all three domains of life including bacteria as well. Bacteria inhabiting such low temperature environments, are exposed to variety of extreme challenges. To thrive under such extreme conditions, bacteria adapt various strategies, most importantly pigment production and alterations of fatty acids exist in cell membranes to maintain normal membrane fluidity. The current study was aimed to study glaciochemistry of Ghulmet, Hopar (Bualtar) and Ghulkin glaciers, located in Karakorum Mountain Range, Pakistan, diversity of pigment producing pyschrotolerant bacteria in these glaciers and to determine the alteration in cell membrane fatty acids of bacterial species in response to different temperature. Glacial samples (ice, sediment and water) were collected from Ghulmet, Hopar and Ghulkin glaciers. Glaciochemistry of these samples was carried out to determine total organic carbon (TOC), total phosphorous (TP) and total nitrogen (TN) as well as major anions and cations. Bacteria were isolated from samples by all glaciers on basis of distinct colony morphology. Bacterial isolates were identified using two biomarkers 16S rRNA and rpoB genes. Pigments were extracted from pigment producing bacteria and identified through High-performance liquid chromatography (HPLC). Effect of temperature (5, 15, 25 and 35°C) on distribution of fatty acids existed in cell membranes of studied bacterial species was assessed. Methanolic HCl was used to extract fatty acid methyl esters (FAMEs) from all bacterial cells which were subsequently identified and quantified via Gas chromatography/mass spectroscopy (GC/MS). Cyanobacterium species CHS1 was recovered from Hopar glacier, Pakistan, identified by 16S-23S sequencing and studied for production of pigment and cell membrane fatty acids.

Sediments of all glaciers were found the most enriched form of sample with all major ions, TOC, TN and TP followed by meltwater and ice. Hopar glacier sediments found to have highest concentrations of Ca^{2+} (10016 µg kg^{-1}), K^{+} (363 µg kg^{-1}), Mg^{2+} (1081 µg kg^{-1}) and PO_4^{2-} (0.7 µg kg^{-1}), Ghulkin glacier sediments with NO^{-} (248 µg kg^{-1}), while sediment of Ghulmet glacier were enriched with Na^{+} (1595 µg kg^{-1}). The highest

TOC, TN and TP observed in sediments of all glaciers followed by meltwater and ice. Based on 16S rRNA and rpoB gene sequencing, all bacterial isolates were found to belong to 5 different bacterial phyla. Proteobacteria was predominantly found bacterial phylum covering 66% of total bacterial isolates followed by Actinobacteria (14% bacterial isolates), Firmicutes (10% isolates), Deinococcus-Thermus (6% bacterial isolates) and Bacteroidetes (4% isolates). In addition, out of 50 bacterial isolates, 39 were confirmed after HPLC to produce 11 different types of pigments including 2 unknown pigments as well. A total of 8 pigments were identified using analytical standards as alloxanthin, astaxanthin, bacterioruberin, β,β-carotene, β,ε-Carotene, 19'-hexanoyloxyfucoxanthin, peridinin, violacein and zeaxanthin.

The fatty acids analysis in cell membranes of bacterial species revealed monounsaturated fatty acids (*n*-MUFAs) and fatty acids with branched chains as priniciple groups of fatty acids of bacterial cell membranes. The *n*-$C_{15:1(cis-10)}$, *n*-$C_{16:1(cis-9)}$ and *n*-$C_{18:1(tr-9)}$ were the main types of individual *n*-MUFAs witnessed in cell membranes of bacterial species, whereas *ai*-$C_{15:0}$ and *ai*-$C_{17:0}$ were the chief types of distinct branched fatty acids (br-FAs). Experiments related to observe effect of different temperature on cell membrane fatty acids of 10 bacterial strains belonged to Gram negative and Gram positive groups, were also carried out in this research work. Results revealed that Gram negative bacterial species have accumulated *n*-MUFAs and Gram positive bacteria produced br-FAs as major groups of cell membrane fatty acids. The quantity of *n*-MUFAs in Gram negative bacteria increased with lowering temperature (5 and 15°C) but decreased with raising temperature (25 and 35°C). Nevertheless, bacterial strains were found constant in production of high quantity of saturated branched chain fatty acids (br-SFAs) in their cell membranes at all temperature. In addition, Gram positive species were observed to accumulate saturated fatty acids in very low quantities in cell membranes at all temperatures. However, br-SFAs in Gram positive group of bacteria were detected in highest quantity in their cell membranes at high temperature (25 and 35°C) as compare to lower temperature (5 and 15°C). Likewise, branched chain monounsaturated fatty acid were observed in lowest concentration at high temperature (25 and 35°C) but in highest concentration at low temperature (5 and 15°C). Similarily, polyunsaturated fatty acids (PUFAs) in very low quantity were found to produce by few bacterial species.

The bacterium species HI6 was recovered from Hopar glacier using Lauria Bertoni (LB) as culture medium and identified as *Serratia marcescens* after 16S rRNA sequencing. Experiments of physiological characterization revealed the ability of isolate HI6 to show optimum growth at neutral pH and 25°C in LB broth. In addition, GC/MS analysis of cell membrane fatty acids of isolate HI6 revealed branched chain fatty acids as most abundant type of fatty acids. Similarly, *n*-$C_{18:2(cis\text{-}9)}$, a polyunsaturated fatty acid, was also detected in cell membrane of isolate HI6.

A cyanobacterium species CHS1 was isolated from the sediment of Hopar glacier using BG_{11} medium and recognized as *Nodularia spumigena* after subjecting to sequencing of 16S-23S intergenetic segment. Physiological characterization of isolate CHS1 showed its ability to grow optimally at pH 7, 2-4/L NaCl, and 15°C. Likewise, chlorohypll-*a* was major pigment produced by isolate CHS1, identified and quantified by High-performance liquid chromatography. In addition, all major types of cell membrane fatty acids including saturated fatty acids (SFAs), br-FAs, MUFAs and PUFAs, were detected by Gas chromatography/mass spectroscopy in isolate CHS1.

This study provides a detailed view about the shift response of cell membrane fatty acids to various temperature in wide range cold-adapted bacterial species for the first time. This would provide basis in future to recognize the utmost potent role of fatty acids to maintain normal fluidity of cell membranes in bacterial species residing outside polar glacial habitats. Likewise, comprehensive outline of fatty acids present in bacterial cell membrane achieved via this study would also be used in upcoming time as biomarker for identification of cold-tolerant bacteria inhabiting cold environments. Furthermore, glaciochemistry would help to monitor any detrimental outcome of global warming on the health of glaciers located in Karakorum Mountain Range, (Pakistan) as they are at high risk to global warming. Similarly, this research work gives an overview of bacterial diversity in the glaciers under study with unique abilities to produce variety of carotenoid pigment that would be helpful in future as an alternative to synthetic and other (e.g. plants) pigment producing sources.

Chapter 1

Introduction

Introduction

Psychrophiles colonize cold environments and are adapted to thrive at 20°C or less but show optimum growth at 15°C. Psychrotrophs also accompany psychrophiles in cold environments because of better nutritional approaches or achieving new genes from mesophiles (Wynn-Williams, 1990; Aislabie *et al.,* 2001). Psychrotrophs demonstrate increased growth rates at temperature more than 15°C and even grow in environments with higher temperatures (Baross and Morita, 1978; Gounot, 1986; 1991). In addition, some other modified definitions are also used for psychrophiles (Stenopsychrophile) and psychrotrophs (Eurypsychrophile) (Cavicchioli, 2006) (Table 1.1). Stenopsychrophiles are capable of thriving in limited or narrow temperature range (<0-20°C) and cannot tolerate higher temperatures. Eurypsychrophiles are capable of thriving in broader temperature range (0-40°C) and can endure mesophilic temperature (Cavicchioli *et al.,* 2002; Raspor and Zupan, 2006). The existence of psychrophiles is evident from various studies in all cold surroundings such as polar, alpine and deep ocean ecosystems (Raspor and Zupan, 2006).

Glaciers provide one of the harshest cold habitat providing unique active biological ecosystem (Anesio *et al.,* 2009). Studies of glaciers located in distinct geographical locations showed great variety of microbial community patterns (Liu *et al.,* 2009; Zhang *et al.,* 2009). Microbial diversity in glaciers related with various factors such as weather patterns of geographical locations and other surrounding conditions such as freeze thaw cycles, intensity of light, speed and direction of wind, nutrients handiness and water availability (Christner *et al.,* 2003; Zhang *et al.,* 2009). Though the variables pouring the community subtleties of microorganisms in glaciers are ambiguous (Xiang *et al.,* 2009) because very limiting reports are available determining the effects of the effects of biogeographic factors on the diversity of microbiota (Christner *et al.,* 2003; Zhang *et al.,* 2009).

The native populations of microbes (especially psychrophilic microorganisms) of glaciers have been proposed to be used as biological indicator to monitor climate change (Raman *et al.,* 2000) by monitoring their growth as they adapt to cold environments and do not populate and survive under mesophilic conditions. The variations within physical and chemical properties among different glaciers could be

reason of novel psychrophilic and psychrotolerant microbes (Perreault *et al.,* 2007; Bottos *et al.,* 2008). Therefore, a huge variation within microbial diversity has been reported from the extreme cold environments of Arctic and Antarctic areas (Bowman *et al.,* 2000). The brief melt season of glacier provides water as a solvent for various biological, geological and chemical reactions that occur at glacier and ice sheets surfaces (Hodson *et al.,* 2008).

Table 1.1. An overview of dissimilarities between psychrophiles and psychrotrophs

S. No	Psychrophiles	Psychrotrophs
1.	They are also known as cold-loving biological entities.	They are also called cold-tolerant or psychrotolerant.
2.	Psychrophiles show optimum growth at 15°C with maximum and minimum limit of growth at 20°C and 0°C or lower, respectively but no growth beyond 20°C.	Psychrotrophs have the ability to show growth over wide range of temperature 0-40°C, but they provide their optimum growth temperature above 15°C.
3.	They are native to cold environments such as polar, non-polar, alpine, permafrost and deep ocean ecosystems.	They are usually introduced to cold environments by anthropogenic activities, wind etc.
4.	Psychrophiles are not exist in cold environments characterized by fluctuating temperatures as they cannot grow in such fluctuation.	Psychrotrophs can also be thrive in cold habitats characterized by fluctuating temperature which is usually caused by seasonal deviation.
5.	Psychrophiles grow more rapidly and actively at low temperatures as they are native to such extreme temperature.	Psychrotrophs grow slowly at low temperatures as compare to psychrophiles.

Diverse groups of psychrophilic bacteria have been reported from glaciers across the world including both polar and non-polar regions because of their abilities to adapt to low temperatures and seasonal desiccation (Simon *et al.,* 2009; Xiang *et al.,* 2009; Edwards *et al.,* 2011) (Table 1.2). Edwards *et al.* (2011) compared microbial diversity

between neighboring glaciers in Svalbard using 16S ribosomal RNA gene analysis. Cyanobacteria are also dominant in these habitats. Distinct microbial diversity in snow samples and melt water of Svalbard glacier indicates the evolution of microbial community in melt season (Larose *et al.,* 2010). Glaciers situated in HKKH mountain range, is not much investigated for bacterial diversity as compared to other cold surroundings around the Earth.

However, Himalayan glaciers have been explored for some cultural studies of bacterial diversity. A group of researchers have studied diversity of bacteria and found Actinobacteria, and Deinococcus-Thermus as most prevalent bacterial phyla in Mt. Qomolangma (Everest) (Liu *et al.,* 2009; Hong *et al.,* 2010). Likewise, Actinobacteria and Proteobacteria were also abundantly documented from ice sample of Rongbuk Glacier, located in Everest (Shen *et al.,* 2012). In addition, Muztagh Ata Glacier, China, has also been studied for bacterial presence and many bacterial phyla were identified such as Actinobacteria, Firmicutes and Proteobacteria have also been reported from (Xiang *et al.,* 2005).

Psychrophilic bacteria existing in glacial habitats have been found actively involved in various nutrient recycling via various processes such as photosynthesis and respiration (Reichardt, 1988). For example, remarkable similarity has been found between the concentration of organic carbon produced by the process of photosynthesis and respiration, for example, Anesio *et al*. (2009) compared Svalbard glaciers and the Greenland Ice Sheet with sediments of temperate latitudes and found almost similar amount of µg of carbon/g of sediment/day on the surfaces of both habitats.

Table 1.2. Distribution of major bacterial phyla in glaciers situated in Polar and non-polar regions

Country/ locations	Glaciers	Source of isolation	Major isolated bacterial phyla	References
Antarctica	Tuva Sørsdal	Cryoconite holes	Actinobacteria, Bacteroidetes, Proteobacteria	Cameron *et al.* (2012)
	Howard Taylor	Cryoconite holes	Cyanobacteria	Porazinska *et al.* (2004)
Greenland	Kronprins Kangerlussuaq	Cryoconite holes	Bacteroidetes, Cyanobacteria, Proteobacteria	Cameron *et al.* (2012)
	Qaanaaq Russel	Snow, ice	Cyanobacteria	Uetake *et al.* (2010)
	Frøya	Cryoconite holes	Cyanobacteria	Anesio *et al.* (2010)
	Larsbreen	Snowpack, slush, ice	Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria	Hell *et al.* (2013)
	Longyearbreen Vestfonna Rieperbreen Foxfonna	Cryoconite holes	Actinobacteria, Bacteroidetes, Cyanobacteia, Proteobacteria	Cameron *et al.* (2012)
European Alps	Rotmoosferner	Cryoconite holes	Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria	Edwards *et al.* (2013)
	Damma	Granite	Actinobacteria, Bacteroidetes, Firmicutes	Frey *et al.* (2010)

Himalaya and Hindu Kush, Pakistan	Tirich Mir Chitral	Ice, sediment, water	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria	Rafiq *et al.* (2019)
	Siachen	Ice, sediment, water	Firmicutes, Flavobacteria, Proteobacteria	Rafiq *et al.* (2017)
Tibetan Plateau, China	Ulugh Muztagh	Ice core	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria	Liu *et al.* (2019), Shen *et al.* (2018)
	Geladandong			
	Noijin Kangsang			
	Yuzhufeng			
Mount Everest, Nepal	Rongbuk	Surface water	Cytophaga-Flavobacteria-Bacteroides (CFB), Actinobacteria, Planctomycetes, Verrucomicrobia, Fibrobacteres	Liu *et al.* (2006)
Himalaya, India	Roopkund	Lake soil and water	Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Nitrospira, Proteobacteria	Reddy *et al.* (2010), Pradhan *et al.* (2010)
	East Rongbuk	Ice core	Actinobacteria, Firmicutes, Proteobacteria	Zhang *et al.* (2007)
	Hamta	Meltwater	Bacteroidetes, Firmicutes	Chaturvedi and Shivaji (2006), Shivaji *et al.* (2005)
	Gangotri	Sediments	Firmicutes, Proteobacteria	Baghel *et al.* (2005)

Moreover, exteriors surfaces of ice sheets and glaciers have been reported with elevated concentration of organic carbon (about 2 to 5 %) (Edwards *et al.*, 2011) as compared to standard concentration of organic carbon reported from the bases of glaciers and peripheral moraines (0.5%) (Kastovska *et al.*, 2005). It has been proved by earlier studies that microbial lives on glaciers' surface play important role in the production of organic carbon either through equilibrium between the process of respiration and photosynthesis (Anesio *et al.*, 2009) or by alteration of external derivatives through the production of EPS (extracellular polymeric substances) (Hodson *et al.*, 2010). Organic carbon synthesized or transformed on the glacial ice surface may be reallocated, as a labile C source to near terrestrial area situated around glaciers (Lafreniere and Sharp, 2004; Hood *et al.*, 2009). Nitrogen fixation on glacial surface has also been observed in recent times (Telling *et al.*, 2011). These researchers reported elevated nitrogen fixation in the late ablation season, but nitrogen sources were seen reduced in the ablation zone.

Glaciers are characterized by bitterly cold temperature, reported as less than -0°C. Psychrophilic bacteria that harbor glacial cold surroundings are exposed to extreme environments of freezing temperature, halophilic conditions, reduced moisture, high Ultra violet radiation and shortage of nutrients (Selbmann *et al.*, 2002; McKenzie *et al.*, 2003). Such extreme environments exert several disastrous effects on bacterial lives include proteins denaturation, disturb normal membrane fluidity, reduced enzymatic activity, increase water viscosity and decline in rate of chemical reaction (Russell, 1990), and also make water inaccessible for hydration of various bio-molecules (Wynn-Williams and Edwards, 2002). In addition, glaciers are accompanied by the high winds responsible for increased evaporation leading to drought and enhanced humidity in upper atmosphere. Increased evaporation leads to the enhanced concentration of salts in glacial lakes, ponds, soil and rocks that cause severe situation of osmotic imbalances for bacteria inhabiting those cold environments (Gunde-Cimerman *et al.*, 2003). Furthermore, psychrophilic bacteria face an extreme challenge of high solar radiation in glacial environments. The solar radiation with short wavelength such as UV-B (280 to 320 nm) has strong capabilities to severely damage bacterial bio-molecules than larger wavelengths (Ross and Vincent, 1998; Cockell and Knowland, 1999). UV-B is a terrible potential active component of solar spectrum, which make it to cause broad

types of harmful consequences. They have abilities to disrupt hydrogen bonding in DNA leading toward mutation, have also been found to destroy lipoprotein of cell membrane and other organelles by oxidizing them (Karentz, 1994) and caused trouble in normal evolution and ecosystems processes (Cockell and Knowland, 1999).

Bacteria inhabiting glacial environments, adapt themselves in order to grow and survive in such lower temperature habitats (Anupama *et al.,* 2011). Despite of presence of extreme factors with numerous consequences, bacteria of both psychrophilic and psychrotolerant nature metabolize efficiently on and within ice sheets and glaciers because of their magnificent adaptability strategies (Armstrong *et al.,* 2010). They protect themselves and actively metabolize in the harsh conditions of extreme low temperature by producing wide range of bio-molecules such as anti-freezing proteins, compatible solutes, cold-active enzymes, carotenoid pigments and exopolymeric substances (Cavicchioli, 2002; Gilbert *et al.,* 2005; Ruisi *et al.,* 2007). Furthermore, an increased dissemination of unsaturated fatty acids, short chain fatty acids and reduced production of saturated fatty acids helped bacteria to maintain proper fluidity of cell membranes (Margesin and Miteva, 2011). Several studies on glacial habitats have revealed that the local bacterial populations are actively involved in copious nutrient recycling e.g. carbon, nitrogen, phosphorous and iron cycling. Likewise, these bacteria especially methanogens participate in methanogenesis contributing its consequence at local and worldwide scales (Hodson *et al.,* 2008).

HKKH is situated in the Asian continent and host approximately 54,000 glaciers (Fig. 1.1) (Bajracharya and Shrestha, 2011). Nearly 37% area of only Karakorum Mountains (KM) within HKKH is covered permanently by ice. Comparatively with other mountain ranges, Karakorum provide different pattern of climate, snow precipitation, glaciers manners and dynamics as it separated from rest of mountains range such as eastern Himalayas by about 2,000 km (Armstrong, 2010). Therefore, consequences of climate changes on Karakoram mountains have been observed very less as compared to eastern Himalayas, because behavior of KM to global warming challenges has been seen totally different than other cold regions of world (Hewitt, 2005; Fowler and Archer, 2006). In addition, an increase in glaciers' mass in the Karakoram and reduction in glaciers' mass in Himalayan mountain have been observed recently (Sarikaya *et al.,* 2013). However, stability, retreating rate and melting of glaciers of KM are still not well understood

(Scherler *et al.*, 2011). Overall, disturbance in these glaciers (HKKH) and ice reserves has been significantly observed by recent weather patterns, although the magnitude of these effects is not yet known (Immerzeel *et al.*, 2012).

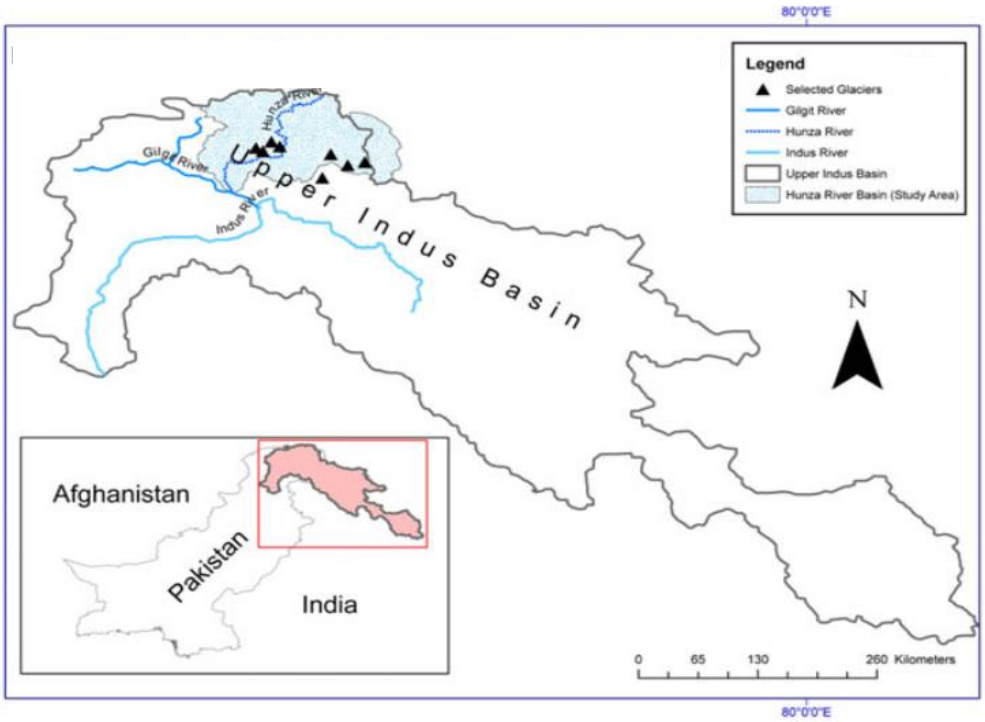

Fig. 1.1. Geographic location of Indus River connected with Gilgit and Hunza Rivers in Karakoram Mountain Range, Pakistan (Baig *et al.*, 2018)

Pakistan constitutes about 5,218 glaciers in HKKH region covering ground area of about 15,041 km^2 (Bajracharya and Shrestha, 2011). Indus River Basin within Karakoram-Hindu Kush comprises major snow regions, glaciers and permafrost, which feeds water to 0.22 billion population of Pakistan. Therefore, Indus River largely depend on meltwater resulted from melting of snow and glaciers. Shrestha *et al*. (2015) has predicted that a decline of 20-30% of glacial area feeding Indus River could occur by 2050 due to climate change. The current studied glaciers (Ghulkin, Ghulmet and Hopar glaciers) are located in the Karakoram mountain range of Pakistan. The Hopar glacier, situated between two heighted peaks Miyar peak (6824 m heighted) and Diran peak (7257 m in altitude), is 18 km long and also known. In addition, Ghulkin glacier is located in Gojal, one of the biggest tehsil of the Gilgit-Baltistan, Pakistan. Its name is derived from two the local Wakhi words, 'Ghulk', meaning 'well' and 'kin', meaning 'whose'. It starts from the Ultar Sar (north-eastern part). Recently, it has been observed

that Ghulkin glacier has severely affected by climatic changes and led to several glacier lake outburst floods (GLOFs), caused severe destructions in one of the three villages and communication tools of Gojal tehsil. Moreover, Ghulmet glacier is situated just below one of the most beautiful peak of the world, Rakaposhi (7788m) that ranked 29th highest mountain in the world. It can clearly be viewed while traveling on the Karakoram Highway between Gilgit and Hunza. Pisan Glacier is situated near to Ghulkin glaciers and also has Bagrot, Minapin and Jaglot at its neighborhood.

This current study explores culture-dependent diversity of cold-adapted bacteria in the Karakoram glaciers; Ghulkin, Ghulmet and Hopar glaciers. Based on this study, a relative psychrophilic bacterial diversity would be compared with the previous studies conducted in polar (cold) environments, and mode of living in these recently investigated cold environments. Hence this study also explores role of cell membrane fatty acids and carotenoid pigments to maintain fluidity of bacterial cell membranes at extremely harsh conditions of low temperatures. These isolates are source of some essential metabolites with industrial application potential such as fatty acids and carotenoids. Glacial ecosystem offers a distinctive foundation for examining microbiota, which is a base for some new innovative applications besides current existing bacterial sources. Glacial surroundings (e.g. glacial ice, water, sediments) seems to be superb medium for durable preservation of microorganisms, with confined wind transported microbes through geological time. Adaptability mechanisms of cold inhabiting bacteria can be implicit by studying their mode of living in glacial cold surroundings, special adaptation and survival strategies which allow them to thrive in cold climates.

Aim and Objectives

This study was aimed to investigate culturable diversity of psychrophilic bacteria from glaciers located in Karakoram mountain range (Pakistan) and the role of cell membrane fatty acids in cold adaptation.

Objectives

- Glaciochemistry of samples collected from Ghulkin, Ghulmet and Hopar glaciers located in Karakoram mountain range (Pakistan).
- Isolation and molecular identification of psychrophilic bacteria based on 16S rRNA and rpoB genes.
- Extraction, identification and quantitative analysis of pigments produced by these psychrophilic bacteria.
- Cell membrane fatty acid analysis of psychrophilic bacteria at different temperatures.
- Molecular identification, physiological characterization and cell membrane fatty acid analysis of bacterial isolate HI6.
- Polyphasic identification, pigments composition and cell membrane fatty acid analysis of cyanobacterial isolate CHS1.

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Chapter 2

Review of Literature

Review of Literature

Glaciers - the extreme habitat

Glaciers are the big physiques of ice that travel under the force of its own weight, formed because of heavily snow bursting and accumulate in an area especially with a temperature that preclude fast snow melting. Snow covers more than 90% of glacial surfaces that ultimately converted into ice or melts during the summer seasons (Lutz *et al.*, 2015). Glaciers are limited only to those areas that prevent snow melting in summer seasons and promote formation of ice grains, also called firns (Rafiq, 2016). Because of deep accumulation of snow every year, the firns' weight enlarge by squeezing most of the air from surroundings that results in formation of a solid glacier ice. Small air bubbles are produced by squeezing of air with ice, which are known for containing small samples of ancient earth atmosphere that merged into ice of glaciers. Researchers use these atmospheric samples to interpret the past climate changes in cold biosphere (Rafiq, 2016). The force of gravity makes glaciers moveable that also results in the transference of a giant amount of sediments. Glaciers are also responsible for producing a unique landscape unlike stream landscape.

Glaciers cover around 10% of the total land surface of Earth. Around 160,000 glaciers (including the polar ice sheets, also known as polar glaciers) have been reported worldwide casing about 15 million km^2 (Paul *et al.*, 2009). Almost 3% of the total glaciers are located in the mountainous area of America, Asia and Europe. Both glaciers and ice sheets contribute to make largest water resources of the world and provide about 70% of fresh water, mostly used for drinking purposes. The Ice Sheets situated in Antarctica and Greenland, store nearly 97% of world's fresh water (Lemke *et al.*, 2007). About 96% out of total glacial ice situated in Polar Regions (Dyurgerov and Meier, 2005). A generalized pattern of reduction in glacier bodies have been observed worldwide as a result of climate change due to global warming (Barry, 2006; Zemp *et al.*, 2006). Effects of climate warming have been witnessed on glacier run-off as an instant response (Chen and Ohmura, 1990). Such fast melting of glaciers promote increased river run-off that often result in massive floods worldwide (Hock *et al.*, 2005).

Besides Polar Regions, the Hindu Kush-Karakoram-Himalayan Mountain Range (HKKH) host the largest reservoirs of glaciers (about 20,000 glaciers) outside Polar Areas (Kulkarni, 1991; 1994) (Fig. 2.1). More than 5,000 glaciers are located in the Karakoram, whereas, 12,000 glaciers are situated just in Himalaya covering a total area of 116×10^3 km^2 (Vohra, 1996; Dyurgerov, 2001; Inman, 2010; Scheel *et al.*, 2011; Kaab *et al.*, 2012). Because of having utmost numbers of glaciers and highest concentration of snow, HKKH region is also called as the Third Pole (Dyurgerov, 2001). The melting of snow and glaciers contribute to supply water to the downstream rivers that plays a pivotal role in making large irrigation systems. Agriculture lands are totally dependent on such large irrigation systems. In addition, melted glaciers and snow provide fresh water for drinking purpose for thousands of millions of inhabitants of Asia Continent (Casassa *et al.*, 2009). Glaciers located in mountain regions play very important role in water storing and cycling as snowing during cold seasons enlarge glacier bodies and melting during summer seasons release water downstream (Fig. 2.2) (Casassa *et al.*, 2009). The mountain glaciers act as proficient flow regulators. Despite of the fact that they make just 0.5% out of total ice of world, mountain glaciers provide freshwater to massive populations living the valleys downstream.

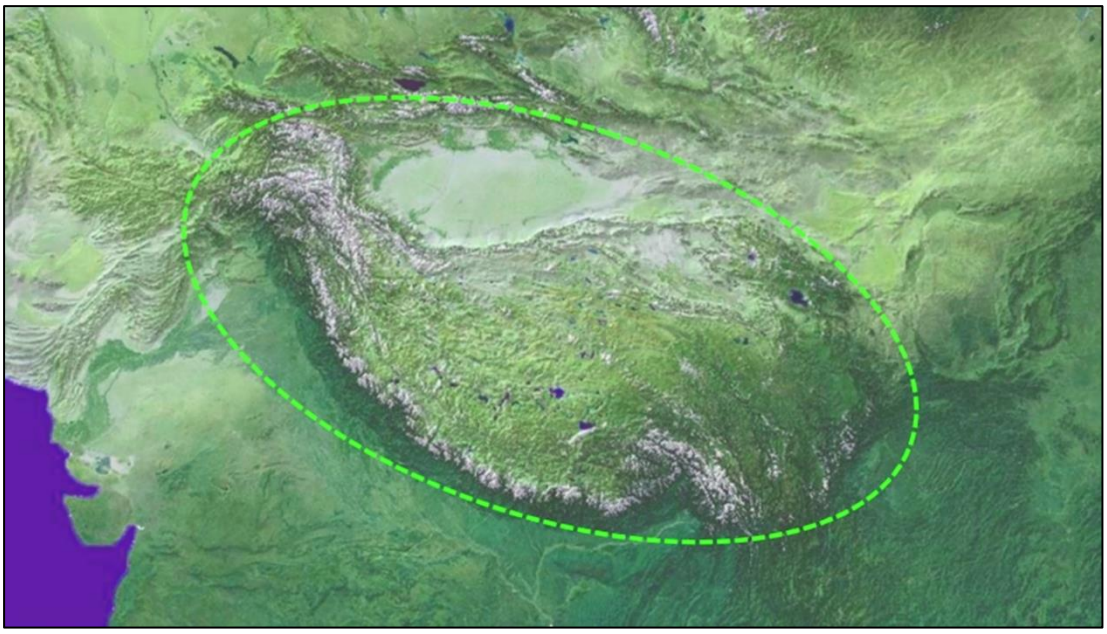

Fig. 2.1. An overview of the HKKH, largest reservoirs of glaciers outside Polar regions. Adopted from (http://www.tpe.ac.cn/)

Mountain glaciers are specifically at risk to global warming, and the HKKH region has no exemption from the adverse effects of global warming. Numerous studies have described many of the conspicuous influences of climate change on the health of glaciers situated in HKKH region, mostly in the form of retreating, shrinking and thinning (Bajracharya *et al.,* 2015). Shrestha *et al.* (1999) reported an increase in temperature between 0.15 and 0.6°C per decade has been observed in Nepalese Himalayas. Creation of glacial lake outburst floods (GLOFs) is one of the most extreme consequence of climate change on glaciers (Kattelmann, 2003; Quincey *et al.,* 2007; Bolch *et al.,* 2008). Several GLOFs reported downstream regions with massive destructions (Yamada, 2000; Bajracharya and Mool, 2007). A continuous persistent trend of GLOFs will lead to reduction of glacial ice that will influence resources of water availability (Barnett *et al.,* 2005; IPCC, 2001). However, glaciers located in the Karakoram mountain range showed a positive behavior in ice bodies' enlargement, perhaps by heavy snow precipitation during winter and limited melting during summer seasons (Archer and Fowler, 2004).

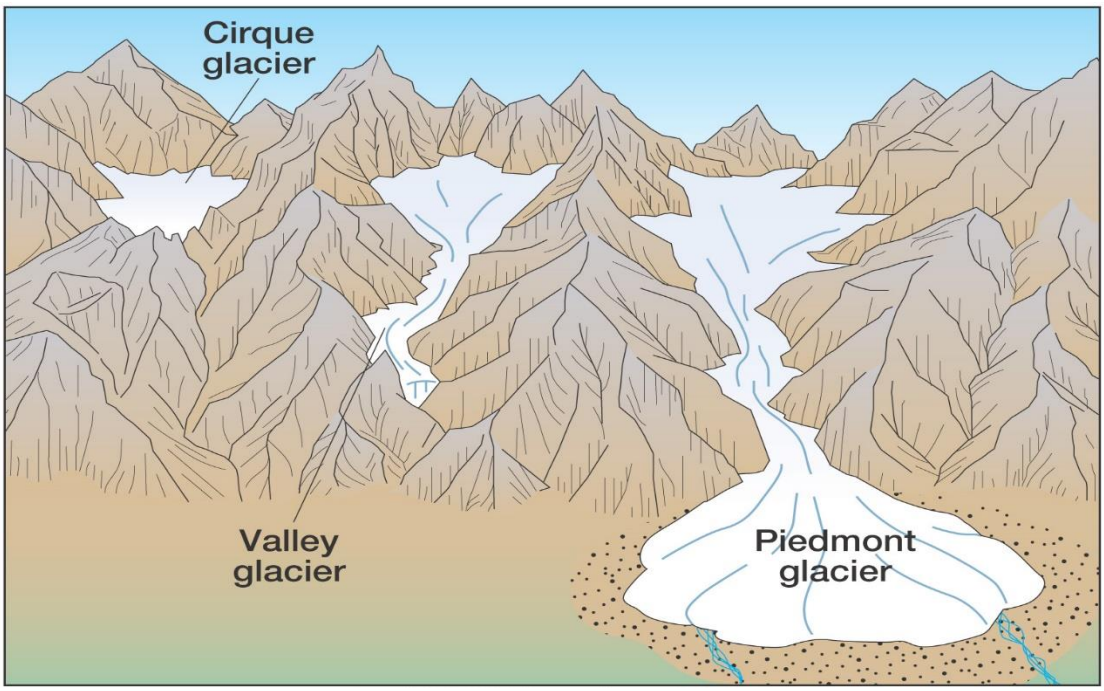

Fig. 2.2. A typical valley glacier with different parts. Adopted from (http://web.gccaz.edu/~lnewman/gph111/topic_units/glacial/glacial2.html)

Pakistan is 36th largest country in the world covers approximately 796,095 km^2 area of Asia continent. The famous HKKH region is positioned in the northern area of Pakistan. They provide world's biggest reservoir of glaciers outside Polar regions. The HKKH glaciers are main source of world's 10 major river systems including the Indus River, also known as the Father River that flows through the Indian subcontinent. It starts from the Tibetan plateau near to Lake Mansarovar in the Tibet Autonomous Region of China, streams via southwest part of Pakistan, named Gilgit Baltistan and continue through south and southwest parts of the country (Bajracharya and Shrestha, 2011). The Indus River covers a total basin area of 1,116,086 km^2 and estimated that it has annual flow rate of about 207 km^3 (Bajracharya and Shrestha, 2011). Some of the famous glaciers located in the HKKH region of Pakistan are Siachen glacier in the Himalaya and Baltoro, Ghulkin, Ghulmet, Hopar, Passu, and Biafo glaciers are located in the Karakoram mountain range of Pakistan.

Microbial diversity of glaciers

Earlier, glacier habitats were thought to be nutrient free and considered as medium of entrapping, collecting and preserving deposited living lives, mainly via atmospheric snow and rain (Butinar *et al.*, 2007), but this conception was proved wrong as glaciers have been identified as the places of biological metabolism and reproduction (Bagshaw *et al.,* 2013). Most interestingly, the psychrophilic and psychrotolerant microbes with chemoorganotrophic properties have been reported from the glaciers situated in polar and non-polar (such as Canada, Europe, Siberia, Alaska, Patagonia) environments that involved in decomposition of the organic matter (Hoover and Pikutab, 2010). Diverse communities of micro and macro organisms inhabit unfavorable living environments of glaciers and perform numerous biological activities (Hodson *et al.,* 2008; Anesio and Laybourn-Parry, 2012). In addition, glacier surfaces provide a big source of meltwater enriched with bioavailable carbon that ultimately activate and assist microbial populations in ecosystems downstream (Lawson *et al.,* 2014).

Local microbial populations (especially psychrophilic microorganisms) of glaciers has gained a lot of attention toward the fact that they are useful to act as bio-markers to screen out climate changes (Raman *et al.,* 2000), because the psychrophilic microorganisms cannot tolerate extreme high temperatures as they eternally adapted to

such cold environments and do not populate and survive under mesophilic conditions. The variations within physical and chemical properties among different glaciers could be reason of novel psychrophilic and psychrotolerant microbes (Bottos *et al.,* 2008). Therefore, a huge variation within microbial diversity has been witnessed in the extreme cold environments of Arctic and Antarctic areas (Bowman *et al.,* 2000).

Several microbial lives covering microbes from all 3 domains of life, have been detected within glacial habitats. Snow algae, also known as Chlorophyta, have been reported as the most common inhabitant of glacial ice surfaces and firstly named by the earliest Greek Aristotle (Gentz-Werner, 2007). They are well-known for their abilities to colonize ice and snow surfaces (Lutz *et al.,* 2014). They have been reported from several glaciers situated throughout the world including non-polar regions such as the Stony Mountains (Thomas and Duval, 1995), the European Alps (Remias *et al.*, 2005), the Himalaya mountain (Yoshimura *et al.,* 2006), Alaska (Takeuchi, 2013) and polar regions such as Svalbard (Leya *et al.*, 2004), Antarctica (Remias *et al.*, 2013) and Greenland (Lutz *et al.*, 2014). In addition, microbes belonged to Archaea domain, have also been reported from the ice and snow deposited on glacial surfaces (Lutz *et al.,* 2015). Comprehensive information about Archaeal biodiversity within glacial habitats are still scarce up to date. However, Archaeal communities associated with ammonium-oxidation have been found on the surface of glaciers (Harding *et al.*, 2011; Lutz *et al.,* 2015).

Presence of fungi in ice and snow of glaciers have been reported very rarely but mostly snow molds have been found in glaciers that were usually associated with the diseases of dormant plants (Matsumoto *et al.,* 2009). Rarer research studies have revealed presence of basidomycetous yeasts (cryophilic nature) in alpine settings, Antarctica and Arctic regions (Buzzini *et al.*, 2005; Vishniac *et al.*, 2006; Edwards *et al.*, 2013; Lutz *et al.,* 2016). Recently, fungi belonged to Chytridiomycota have been found to play major roles in snow food-web dynamics (Naff *et al.*, 2013) and in nutrient cycling by either their parasitic or saprotrophic activities in glacial snow located in alpine and Arctic regions (Lutz *et al.*, 2015; Brown *et al.*, 2015). Chytridiomycota with association with snow algae (also known as symbiotic partners) has been observed in glacier by Brown *et al.* (2015). He proposed that they either assisted each other to tolerate extreme glacial environmental challenges or snow algae were acting as an environmental filter

in fungal populations' assemblage. Moreover, wide range of microbial communities have been reported from glacial environments based on evidences obtained from clone libraries and microscopical researches, including prokaryotes and viruses (Kastovska *et al.*, 2005; Anesio *et al.*, 2007) as well as eukaryotic multicellular organisms (Kohshima, 1984; Kikuchi, 1994; Grongaard *et al.*, 1999).

Bacterial diversity in glacial habitats

Glaciers of Antarctica

Antarctic continent is situated at the world's South Pole. According to Fox *et al.* (1994), nearly 99% of the total Antarctic area is roofed by ice persistently. Antarctica provides one of the driest and coolest climate on the Earth (Ovstedal and Smith, 2001). Several ecologically active habitats including cryoconite holes, subglacial lakes water and glacial melt water, are present in Antarctic glaciers. Among these habitats, cryoconite holes are most important ones. They form on glacier surfaces and look like dark spots. When a significant quantity of the solar radiations absorb and dark dust accumulates on the surfaces of glaciers, which speed up the ice melting, leading towards the formation of a hole that is packed with dust particles and water, called as a cryoconite hole (Anesio *et al.*, 2010). Cryoconite holes have slightly high temperature as compared to the surroundings and this characteristic makes them remain in fine and stable form for even decades (Fountain *et al.*, 2004). In addition, this property also makes cryoconite holes a favorable home for vast microbial populations including prokaryotes (e.g. autotrophic and heterotrophic bacteria) (Christner *et al.*, 2003).

The cryoconite holes have widely been investigated for the presence of bacteria (Christner *et al.*, 2003). Various cynobacterial genera including *Chlorococcus*, *Chroococcus*, *Crinalium*, *Oscillatoria*, *Nostoc*, and *Sprirulin* have been reported from the Taylor and Hughes glaciers, McMurdo Dry Valley, Antarctica (Porazinska *et al.*, 2004). In addition, Oscillatoriaceae (a family of cyanobacteria) has been studied in the Qaanaaq and Russel glaciers (Uetake *et al.*, 2010).

Autotrophic and heterotrophic bacterial communities, identified through 16S rRNA biomarker as members of Alphaproteobacteria, Betaproteobacteria and Deltaproteobacteria as well as Actinobacteria, Bacteroidetes, Cyanobacteria, have been

also reported from Antarctica (Cameron *et al.,* 2012). In another study, Bajerski and Wagner (2013) have reported different bacterial species from glacier forefields situated in East Antarctica which were found to belong to various major phyla including Actinobacteria, Bacteroidetes, Cyanobacteria, Flavobacteria, and Proteobacteria. A new psychrotolerant bacterial species *Chryseobacterium frigidisoli* belonging to the family Flavobacteriaceae, has been isolated from Larsemann Hills by Bajerski *et al.* (2013).

Glaciers of Arctic and Svalbard

Biologists described Arctic as those regions that are free from trees existence (Bliss and Matveyeva, 1992). The Arctic region is partitioned into 5 different zones ranging from A (the coolest) to E (the warmest) (Walker *et al.,* 2005). These five zones include various islands such as Ellesmere, Franz Joseph, New Siberian Islands, Novaya Zemlya and Svalbard, all in the region of 80-85°N. Billings (1992) has observed that the living communities of Arctic face extreme conditions at higher latitude because of lower air and soil temperatures. Biogeochemical cycling of various nutrient in the Arctic, is primarily carried out by microbial communities (Nemergut *et al.,* 2005). It has been observed that increased amount of gases such as CH_4, CO_2 and N_2O freed into the atmosphere due to heighten microbial activities that perhaps put negative effects on the climate system (Fierer *et al.,* 2007; Mackelprang *et al.,* 2011).

The Arctic warming imposed potential changes in ecosystem which are central to understand the variety, structure and constancy of microbial populations as well as their alteration with environmental change in Arctic habitat (Malard and Pearce, 2018). Very rare studies have been carried out exploring soil bacterial diversity in Arctic and Svalbard regions. Malard and Pearce (2018) have concluded that Proteobacteria was the predominant inhabitants of Arctic soil. However, various orders of cyanobacteria such as Nostocales, Oscillatoriales and Synechococcales, have been found in the soil of Arctic crusts (Steven *et al.,* 2013; Pushkareva *et al.,* 2015). In addition, several species of bacterial phyla were reported from ice, sediment and waters samples of John Evans glacier, Canadian high Arctic (Cheng *et al.,* 2007). Amato *et al.* (2007) identified various bacterial species belonging to Alphaproteobacteria, Betaproteobacteria,

Gammaproteobacteria, Actinobacteria and Firmicutes from the Kongsvegen glacier, Spitzberg, Svalbard.

Edwards *et al.* (2011) has comprehensively reported the diversity of psychrophilic bacteria from Austre Brøggerbreen, Midtre Lovenbreen and Vestre Brøggerbreen, Svalbard. He used TRFLP of the 16S rRNA locus as biomarker. His findings revealed that 46% of clones were of Proteobacteria, 12% Cyanobacteria, 11% Bacteroidetes, while 10% of bacterial clones were found to belong to Acidobacteria, Actinobacteria, Chloroflexus and Planctomycetes. Likewise, Cameron *et al.* (2012) showed Actinomycetales (Actinobacteria), Burkholderiales (Proteobacteria), Sphingobacteriales (Bacteriodetes) as the most abundant bacterial members in cryoconite holes taken from 39 different sites across 10 glacial locations of Arctic and Svalbard regions. Bacteroidetes, Chloroflexi, Cyanobacteria, Planctomycetes and Proteobacteria were the common inhabitants of small valley glaciers, Midtre Lovenbreen and Vestre Brøggerbreen, Svalbard (Edwards *et al.,* 2013). Hell *et al.* (2013) used various techniques to analyze 16S rRNA genes such as T-RFLP, qPCR and 454 pyrosequencing to observe effect of snow melt on bacterial diversity and community structure present on the surface of glaciers located in Svalbard. The ammonia-oxidizing bacterial genus *Polaromonas* was the most predominant in all glacial samples.

Glaciers of European Alps

The European Alps are extended through eight European countries, known as the biggest range of mountain of Europe providing approximately 200 km width and 800 km length (Casty *et al.,* 2005). This mountain range extends from nearly 44-48°N to 3-16.5°E (Casty *et al.,* 2005). They provide mountains with average peak height of about 2500 m, while hosting also several elevated peaks of 4400-4800 m. It has been reported that mean summer temperature of these mountain range between 15°C and 24°C as well as the mean winter season temperature reach up to −5°C and up to 8°C (especially in the mountains adjoining the Mediterranean). The Atlantic atmosphere is the key player to greatly influence the climate of European Alps (Wanner *et al.,* 1997; Begert *et al.,* 2005; Auer *et al.,* 2005). Most importantly, these Alps range have plenty of glaciers

that contribute greatly to provide the drinking water source to the locals living downstream.

The glaciers situated in the European Alps have been studied by many researchers for presence of bacterial communities. Presence of wide range of photosynthetic and non-photosynthetic bacteria in dolomite rocks, the Piora Valley in the Swiss Alps, have been reported by using small subunit ribosomal RNA gene sequences (Horath and Bachofen, 2009). The genus *Leptolyngbya* was the most commonly found endolithic cyanobacterial community in these rocks, whereas 64 clones of chemotrophic bacteria belonged to Acidobacteria, Actinobacteria, Bacteroidetes and Proteobacteria were also identified in this study. Frey *et al.* (2010) reported various bacterial genera including *Arthrobacter*, *Frigoribacter*, *Janthinobacterium*, *Paenibacillus*, *Pedobacter*, *Pseudomonas* and *Variovorax* from granite samples collected from slope of the Winterstock Mountain, the Damma glacier, situated in the Central Alps of Switzerland. In addition, several bacterial species representing various bacterial families and orders (such as Acidimicrobidae, Actinobacteridae, Gemmatimonadales, Nitrospirales, Rubrobacteridae, Sphingobacteriales and Sphingomonadales) were reported from two Italian Debris-covered glaciers (DCGs), the Miage and the Belvedere glaciers (Franzetti *et al.*, 2013). Various bacterial phyla such as Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria from cryoconite holes, Rotmoosferner glacier, Austria, have been reported by Edwards *et al.* (2013).

Glaciers of Hindukush-Karakoram-Himalayas (HKKH)

The Hindu Kush-Karakoram-Himalayan Mountain Range (HKKH) host the largest reservoirs of glaciers (about 20,000 glaciers) outside Polar Areas (Kulkarni, 1991; 1994). More than 5,000 glaciers are located in the Karakoram, whereas, 12,000 glaciers are situated just in Himalaya covering a total area of 116×10^3 km^2 (Vohra, 1996; Dyurgerov, 2001; Inman, 2010; Scheel *et al.*, 2011; Kaab *et al.*, 2012). In addition, glaciers situated in the Tibetan Plateau shielded 104,850 km of total area in the whole mountain rang covering 49,873 km in China while 40000 km in Pakistan and India. Because of having utmost numbers of glaciers and highest concentration of snow, HKKH region is also called the Third Pole (Dyurgerov, 2001). The HKKH region is famous as reservoir of the freshwater in South Asia. The melting of snow and glaciers

contribute to supply water to the downstream rivers that play a pivotal role in making large irrigation systems. The Indus in Pakistan, and Ganges, Indus and Brahmaputra in India, are famous rivers providing drinking water to millions of people. These are originating from Karakoram-Himalayan Mountain Range.

The Hindu Kush-Karakoram-Himalayan Mountain Range (HKKH) has scarcely investigated for existence of bacterial communities. However, Rafiq *et al.* (2017) has reported various bacterial genera from Siachen glacier, Pakistan. The commonly found genus was *Pseudomonas* followed by *Alcaligenes*, *Janthinobacterium*, *Rhodococcus*, *Carnobacterium*, *Arthrobacter*, *Bacillus*, *Lysinibacillus*, *Staphylococcus* and *Planomicrobium*. Members of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria have also been reported different glacial samples of Tirich Mir glacier, Chitral, Pakistan (Rafiq *et al.,* 2019). Four groups of bacteria including Actinobacteria, Firmicutes, Alpha-Proteobacteria and Gamma-Proteobacteria were also reported from Rongbuk Glacier (Shen *et al.*, 2012). Several bacterial phyla were reported from soil samples of Pindari glacier, Himalaya and identified by construction and analysis of 16S rRNA gene clone libraries (Shivaji *et al.*, 2011).

Bacterial species belonged to the genera *Arthrobacter*, *Bacillus* and *Pseudomonas* were identified using partial 16S rRNA gene sequences in soil samples taken from the western Himalayas (Gangwar *et al.,* 2009). Firmicutes and Proteobacteria were reported from ice core collected from the East Rongbuk (ER) Glacier by Zhang *et al.* (2008). Liu *et al.* (2006) examined glacial meltwaters, collected from the remote Mount Everest region via flow cytometry and 16S rRNA gene clone library techniques and identified Actinobacteria, Cytophaga-Flavobacteria-Bacteroides (CFB), Fibrobacteres, Planctomycetes, Proteobacteria and Verrucomicrobia in samples. Bacterial species, *Bacillus licheniformis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* were also reported from glacial soil of Gangotri glacier, Western Himalaya, India (Baghel *et al.,* 2005). Likewise, many individual novel bacterial species have been isolated from different glaciers located in the Himalayas of Indian side, such as *Exiguobacterium indicum* and *Dyadobacter hamtensis* (Hamta glacier), *Leifsonia pindariensis* and *Cryobacterium Pindariense* (Pindari glacier), *Cryobacterium roopkundense* (Roopkund glacier), *Paenibacillus glacialos* (kafni glacier) (Chaturvedi *et al.,* 2005; 2006; Reddy *et al.,* 2008; 2009; 2010; Kishore *et al.,* 2010).

Bacterial isolates representing 53 genera belonged to Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria were isolated and identified via culture-dependent and culture-independent approaches from glacial ice collected from the Tibetan Plateau (Liu *et al.,* 2019). Different bacterial genera *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Pseudomonas*, *Stenotrophomonas* and *Yersinia* reported from glacial ice recovered from Yuzhufeng Glacier, Tibetan Plateau (Shen *et al.,* 2018). *Pseudomonas* was the most commonly found bacterial genus of this study. In addition, Sun *et al.* (2018) reported pigment producing bacterial species related to 19 genera and 4 phyla from soil of Mingyong glacier, China. An *et al.* (2010) has established bacterial clone libraries using 16S rRNA gene and identified *Acinetobacter*, *Enterobacter*, *Flexibacter*, *Planococcus* and *Propionibacter* as major bacterial genera from the Muztagh Ata Glacier, China. Moreover, bacterial phyla Cytophaga-Flavobacterium-Bacteroides and Proteobacteria have been reported from glacial ice recovered from glacier of Pamirs Plateau (China) (Xiang *et al.,* 2005).

Challenges for bacteria in glacial habitats

Bacteria living in cold environments are facing extreme stressful conditions of low temperatures, reduced moisture, increased desiccation, reduced nutrients, elevated salinity and higher UV radiation. These impending challenging conditions might differ noticeably from glacier to glacier but bacteria must restrained them. A temperature up to -70°C, which do not support any life survival or growth is termed as coldness (Smith, 1993; Rivkina *et al.,* 2000). Glacial habitats are also commonly accompanied by frequently occurring freeze thaw cycles (Montiel, 2000). The detrimental effects of extreme low temperatures on bacterial lives include proteins denaturation, disturb normal membrane fluidity, reduced enzymatic activity, increase water viscosity and decline rate of chemical reaction (Crowe *et al.,* 1992: Russell, 1990), and also make water inaccessible for hydration of various bio-molecules (Wynn-Williams and Edwards, 2000).

Temperature below zero encourages freezing that plays central role in crystallization process. Crystallization is a process of seed crystals formation at subzero temperature that cause serious cell injuries eventually leading to death (Lee *et al.,* 1993). Extreme dryness of cold habitats such as in Antarctica (provides about 70% of world total fresh

water but existed in ice form) also promote low water availability and high salinity. In addition, glaciers are accompanied by the high winds responsible for increased evaporation leading to drought and enhanced humidity in upper atmosphere. Higher evaporation rate leads to increased concentration of salts in glacial lakes, ponds, soil and rocks that cause the severe situation of osmotic imbalances for bacteria inhabiting those cold environments (Gunde-Cimerman *et al.*, 2003).

Psychrophilic bacteria face an extreme challenge of high solar radiation in glacial environments. The solar radiation with short wavelength such as UV-B (280 to 320 nm) has strong capabilities to severely damage bacterial bio-molecules than the larger wavelengths (Ross and Vincent, 1998; Cockell and Knowland, 1999). UV-B is a terrible potential active component of solar spectrum, which make it to cause different types of harmful consequences (Gröniger, 2000: Sinha, 2000). It has been reported that UV-B was more lethal during earlier summer and late austral spring (Frederick *et al.*, 1994). Several direct and indirect detrimental injuries of UV-B radiation have been well reported by Agogue *et al.* (2005) and Hader and Sinha (2005). Various bacterial vital molecules have been reported to be damaged by UV-B radiation. They have abilities to disrupt hydrogen bonding in DNA leading toward mutation, have also found to destroy lipoprotein of cell membrane and other organelles by oxidizing them (Karentz, 1994) and caused trouble in normal evolution and ecosystem processes (Cockell and Blaustein, 2001).

Adaptability mechanisms of bacteria

Bacteria (living in glacial cold habitats) have found ways to use multiple adaptive toolkits developed on sophisticated molecular, structural and physiological levels (Fig. 2.3). Such adaptive toolkits have been used by bacteria to respond to the challenges and their consequences exerted by low-temperatures in cold environments (Collins and Margesin, 2019). Several adaptive mechanisms still need to be fully understood. Essentially, several of these strategies perform overlying functions to cope with the multiple defies or their combinations faced by bacteria in a glacial habitat. The response of bacteria to any cold exerted challenge varies depending on the type of bacteria and the particular glacial habitat. Notably, bacteria do not use all of adaptive strategies, which they have in their cold adaptive toolkits (Collins and Margesin, 2019). It has

been found that bacteria either use a specific strategy or mixtures of adaptive strategies depending on the type of cold environments, types/groups of bacteria and other environmental parameters (Collins and Margesin, 2019).

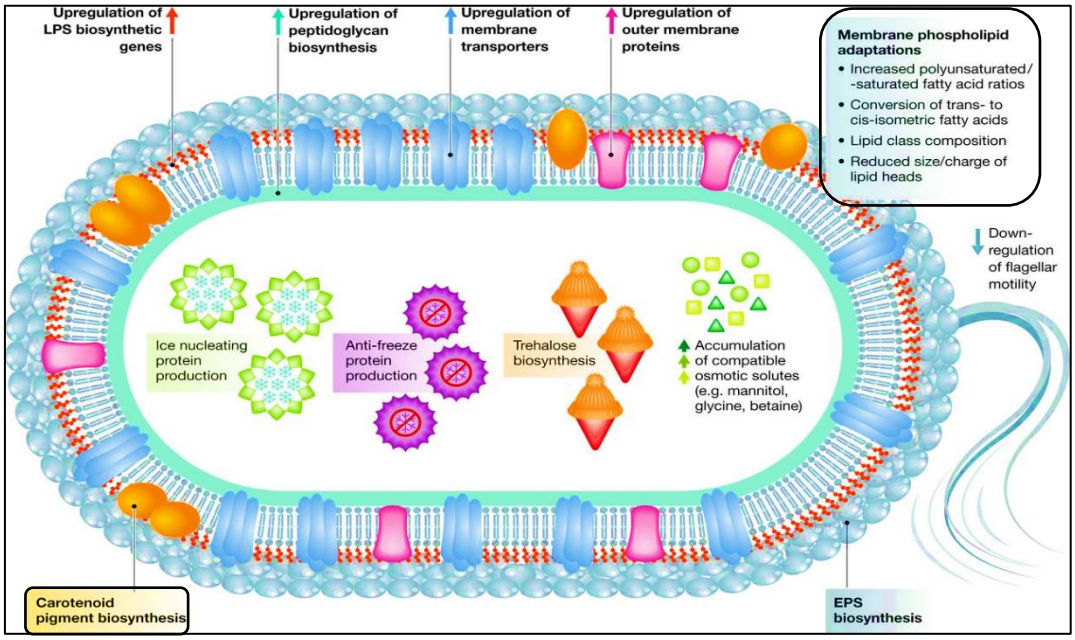

Fig. 2.3. An overview of adaptation mechanisms in psychrophilic bacteria (De Maayer *et al.*, 2014)

Most commonly used strategies by bacteria include an increased production of antifreeze proteins, enzymes, compatible solutes, and enhanced unsaturation of fatty acids in cell membranes. Compatible solutes are the strong osmoprotectants, that stabilize the cell membrane and proteins, protect cell from osmotic imbalances created by freezing and desiccation (Goordial *et al.*, 2016). Antifreeze proteins promote cell membrane stabilization and prevent ice adhesion by inhibiting ice growth (thermal hysteresis) and stopping ice recrystallization process (Bar Dolev *et al.*, 2016). Moreover, psychrophilic bacteria produced ice-nucleating proteins, also known as extracellular ice crystal nucleation in cold habitats. They have the capability to prevent formation of intracellular ice crystals as well as discharge of latent heat of crystallisation (Lorv *et al.*, 2014).

Extracellular polymeric substances avoid the process of ice-recrystallisation. They have been found to stabilize the cell membrane and proteins, protect cell from osmotic imbalances created by freezing and desiccation (Caruso *et al.*, 2018). Cold active

enzymes have the most important role to maintain the proper stable metabolic rate within bacterial cells (Collins and Gerday, 2017). Chaperones enhance the stability of protein folding as well as secondary structures of RNA/DNA (Lim *et al.,* 2000). Many other storage molecules are also produced by microbes in cold habitats such polyhydroxyalkanoates and cyanophycins. Such molecules fulfil carbon and nitrogen shortages of bacterial cells (Ciesielski *et al.,* 2014). Different types of pigments (carotenoids) have been found to accumulate in cell membranes by bacteria in order to maintain functions of cell membranes (Shen *et al.,* 2018). An increase in production of polyunsaturated of fatty acids and decrease in saturated fatty acids in bacterial cell membranes inhabiting cold environments have been observed by many researchers (Siddiqui *et al.,* 2013).

Cell membrane fatty acids

Introduction to Fatty acids - types and structures

Fatty acids are hydrocarbon chain molecules possessing a carboxyl group and a methyl group at both ends (Fig. 2.4). The methyl group is designated as "ω" based on the closeness of carbon-carbon double bond position to methyl group (Rustan and Drevon, 2000). Moreover, the carbon atom present next to -COOH is termed as α-carbon followed by another carbon atom called β-carbon. All of fatty acids are generally classified into 3 major groups based on presence or absence of double bonds in their basic carbon chains such as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).

SFAs with several types are abundantly present in microbes including bacteria. For example, palmitic acid, a $C_{16:0}$ fatty acid, is the most commonly found type of fatty acid in microbes, plants and animals. In addition, some fungi also accumulate stearic acid ($C_{18:0}$) as major fatty acid but found very rarely in plant sources. Myristic acid ($C_{14:0}$) is present as key fatty acid in cell membrane of bacteria and many other microbes. Monounsaturated fatty acids containing one double bond, are found in cell membranes of bacteria but also often derived from plant sources. Oleic acid, a $C_{18:1}$, ω-9 MUFA fatty acids, has widespread occurrence in microbial sources. In addition, palmitoleic

acid ($C_{16:1}$, ω -7) acts as a chief fatty acid component present in lipids of microbes and plants (e.g. seed oil).

Likewise, polyunsaturated fatty acids which posses more than one double bonds have been found as key players to help bacteria to withstand high pressure and low temperatures. Different examples of SFAs, MUFAs and PUFAs are given in (Fig. 2.4). α-Linolenic acid is mainly derived from higher plants sources such as soybean oil and rape seed oils as well as from algae. In addition, docosahexaenoic acid and eicosapentaenoic acid majorly present in the cell membrane of marine algae and Gram negative bacteria.

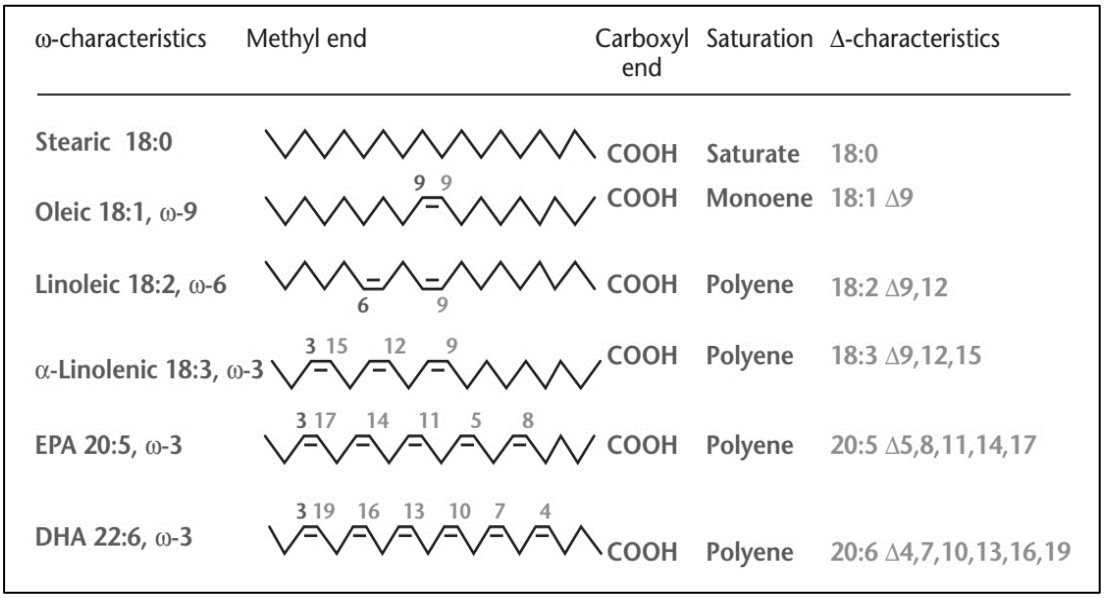

Fig. 2.4. This figure represents structures of important fatty acids produced by psychrophilic bacteria (Rustan and Drevon, 2000)

Biosynthesis of fatty acids

Fatty acids synthesis is the utmost pervasive corridors in microbes. Fatty acids synthesized inside cells act as building molecules for variety of lipids e.g. phospholipids, sphingolipids, sterols, important part of cell signaling systems and many essential proteins and do exist as secondary metabolite in bacterial cells (Janßen and chel, 2014). The biosynthesis of fatty acids in bacteria is carried out by an enzyme complex termed as type-II fatty acid synthases. However, this enzyme system also exists in the plant plastids as well (a place of process of de novo fatty acids synthesis in

plants) (Liedvogel and Kleinig, 1980; Kachroo and Kachroo, 2009). Though, some Gram positive bacteria possess type-I fatty acid synthases, mainly as single polypeptide chain, to synthesize mycolic acid (Kikuchi *et al.,* 1992; Fernandes and Kolattukudy, 1996). In addition, type-II fatty acid synthases are also present in Gram positive bacteria, which are usually used for the medium chain fatty acid elongation but do not have the ability to initiate de novo biosynthesis of fatty acid (Kolattukudy *et al.,* 1997). The main dissimilarity between type-I and type-II FAS is that type-II FAS is not encoded by a single gene or operon but type-I FAS is organized as single operon or gene. For example, cluster of genes encode type-II fatty acid synthases complex of *E. coli*.

The genes involved in the biosynthesis of fatty acids are highly conserved within the kingdoms of organisms including bacteria as well. The summary of bacterial fatty acids biosynthesis (Fig. 2.5) as it starts when acetyl-CoA reacted with hydrogen carbonate in carboxylation step led towards the formation of malonyl-CoA by burning one molecule of adenosine triphosphate (ATP) (Janßen and Chel, 2014). In next step, malonyl-ACP is formed by replacing coenzyme A with the acyl carrier protein (ACP). The upcoming degradation threats during synthesis process then prevented by the formation of acyl carrier protein. Acetoacetyl-ACP, free coenzyme A and hydrogen carbonate produced by the process of condensation of acetyl-CoA with malonyl-ACP in next step. After that, reduction of previous step product to 3-hydroxybutyryl-ACP and then to butyryl-ACP occurs. Again malonyl-ACP is formed as a result of condensation of butyryl-ACP. Once the anticipated chain length of fatty acid is achieved, the whole process of bio-synthesis stops and the produced fatty acid is then incorporated into cell membrane (Janßen and Chel, 2014).

Role of fatty acids in cold adaptation

Alteration to the composition of fatty acid present in phospholipid bilayer by psychrophilic bacteria at low temperatures, has been seen as an important tool of homeoviscous adaptation of plasma membranes (D'Amico *et al.,* 2001; Siddiqui *et al.,* 2013). Chintalapati *et al.* (2004) and Russell (2008) have observed enhanced production of unsaturated fatty acids in cell membranes accompanied by high presence of methyl branched (iso/antiso configurations) and short chain fatty acids at low temperatures.

These changes in fatty acids composition prevent the conversion of the phospholipid bilayer from liquid phase to gel phase at low temperatures by decreasing the packing density and interrupting the packing order of lipid bilayer.

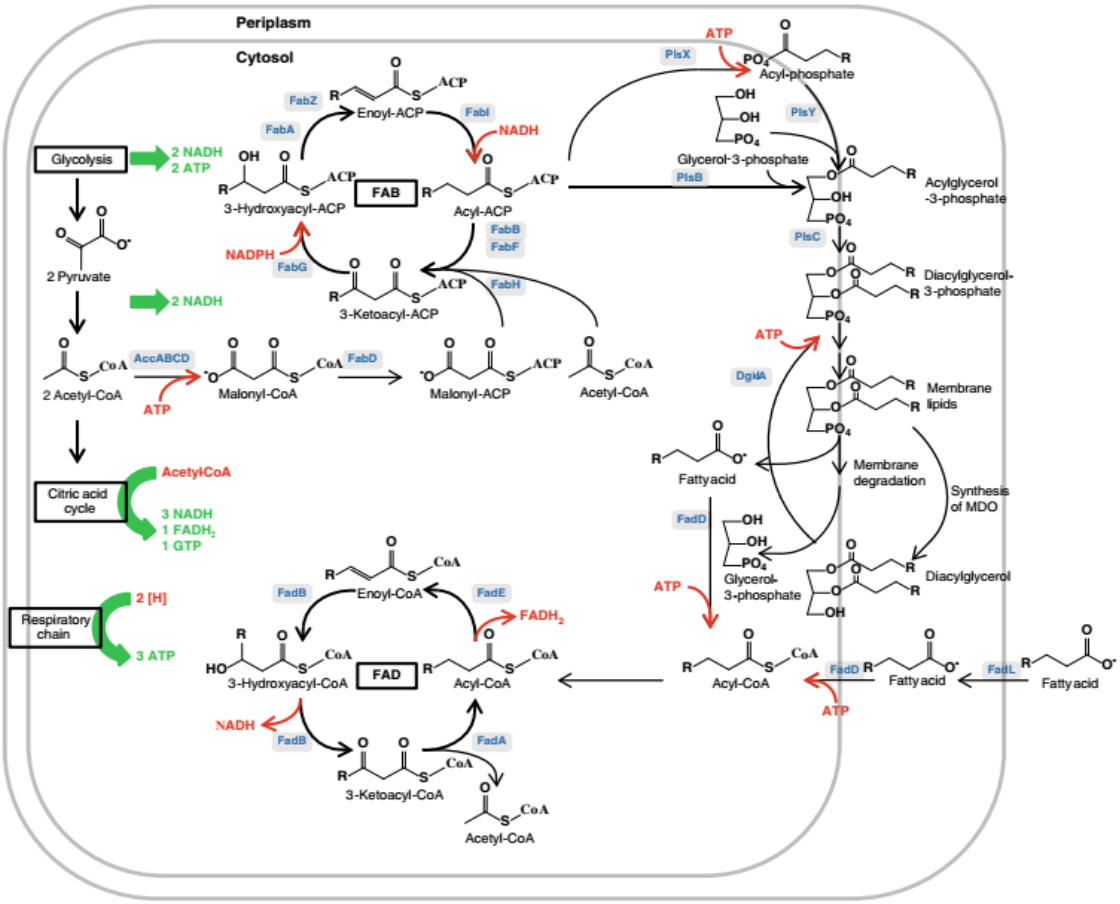

Fig. 2.5. Biosynthetic pathway of fatty acids proposed for *E. coli* (Janßen and Chel, 2014)

The unsaturated fatty acids possessing limited number of double bonds had a clear role in maintenance. The proper functions of cell membrane in cold habitats were proved by many studies but long-chain polyunsaturated fatty acids (LC-PUFAs) with their suitable role in membrane fluidity still needs to be further explore (Collins and Margesin, 2019). The long-chain polyunsaturated fatty acids especially EPA, DHA and ARA (Fig. 2.4), have been observed in several bacteria inhabiting marine environments, which are thought to be playing central role to maintain normal fluidity of cell membranes at both elevated pressure and lowest temperature (Feng *et al.*, 2014; Yoshida *et al.*, 2016). Many comparative genomic studies of marine bacteria have

revealed that the polyketide synthase gene cluster (pfaA, B, C, D and E) encoded polyketide synthases complex that were responsible for synthesis of polyunsaturated fatty acids and such gene clusters were highly conserve to marine inhibiting bacterial species (Shulse and Allen, 2011).

In addition, low temperatures act as inducers to upregulate expression of several genes involved in maintenance of cell membrane fluidity at molecular level. These genes encode multiple enzymes (KAS-II, KAS-III and fatty acid cis/trans isomerases) that participate in fatty acid biosynthesis and unsaturation as well as in membrane biogenesis (He *et al.,* 2015). They also have key role in protection of cell membrane from reactive oxygen species (ROS) (Goordial *et al.,* 2016). Different genes encoded proteins participated in itemization of certain molecules that promoted membrane rigidity, has also been observed with enhanced expression, thus helpful in reducing the rigidity of cell membrane at lower temperatures (Medigue *et al.,* 2005). Furthermore, Bakermans *et al.* (2007) and De Maayer *et al.* (2014) have reported upregulation of many other proteins (situated in phospholipid bilayers) from psychrophiles that were involved in reducing diffusion rates as well as managing transport of unwanted molecules imposed by lower temperatures.

Cell membrane pigments (carotenoids)

Carotenoids types and structures

Carotenoids belonging to isoprenoid family, also known as terpenes, represent utmost widely occurring natural biomolecules. Natural colors of birds, crustaceans, fish, insects microbes and plants such as orange, red, purple and yellow, are because of carotenoids as they can absorb light of wavelength between 300-600 nm. The ability of carotenoids to absorb light of different wavelengths is dependent on the presence of conjugated double bonds and functional groups in their basic structures (Liaaen-Jensen *et al.,* 1985). According to Britton *et al.* (2004) and Jackson *et al.* (2008), more than 750 different types of carotenoids have yet been extracted and identified from various biological sources, whereas new carotenoids still continue to be explored (Osawa *et al.,* 2011). The carotenes and xanthophylls are the two main classes of carotenoids. Based on the chemical structures, xanthophylls possess hydroxyl (-OH) groups, whereas

carotenes does not (Britton *et al.*, 2004). Some carotenoids also have sugar units, known as glycosylated carotenoids and the first glycosylated carotenoid was reported from saffron by Aschoff *et al*. (1818). Numerous glycosylated carotenoids have been reported from variety of biological sources but most often from bacterial species of both marine and terrestrial environments (Britton, 1993; Krubasik *et al.,* 2001).

As approximately 750 different types of carotenoids have been extracted and identified from natural biological sources (most important microbial sources and lower plants), the most important among them were α-carotene, β-carotene, and lycopene (carotenes) as well as alloxanthin, astaxanthin, β-cryptoxanthin, canthaxanthin, lutein and zeaxanthin (xanthophylls) (Fig. 2.6) (Britton *et al.,* 2004). These carotenoids were of great importance in pharmaceutical and medical fields. However, due to their huge demand in different commercial sectors, the natural sources are not enough to supply adequately. Therefore, multiple of carotenoids have also been synthesized chemically as well as isolated from higher plants. Several combinatorial biosynthetic strategies including genetic and metabolic engineering tools, have been employed to produce structurally diverse and plenty of carotenoids to fulfil the requirements of carotenoids for multiple purposes.

Biosynthesis of carotenoids

Biosynthesis of microbial carotenoids is finely structured and mainly regulated by environmental conditions such as cultural stress (Bhosale *et al.,* 2004). Multiple carotenogenic genes (about 25) have been identified to be involved in the biosynthesis of carotenoids and the first carotenogenic biosynthetic gene along with its function and product was reported from *Escherichia coli* in 1990 (Misawa *et al.,* 1990; Armstrong *et al.,* 1996; Sandmann, 2002). These carotenogenic genes perform different catalytic functions and synthesize various carotenoids such as β-carotene desaturase is encoded by crtU, β-carotene ketolase encoded by crtW, crtZ encodes β-carotene hydoroxylase, β-carotene mono-ketolase encoded by crtO and crtX encodes zeaxanthin glucosyltransferase (Schmidt-Dannert, 2000) (Fig. 2.6). In addition, these genes involved in biosynthesis of carotenoids have also been transferred and functionally expressed in many bacterial species, most important *Escherichia coli,* that generated

new paths to produce novel or rarely occurring carotenoids in large quantities (Schmidt-Dannert, 2000).

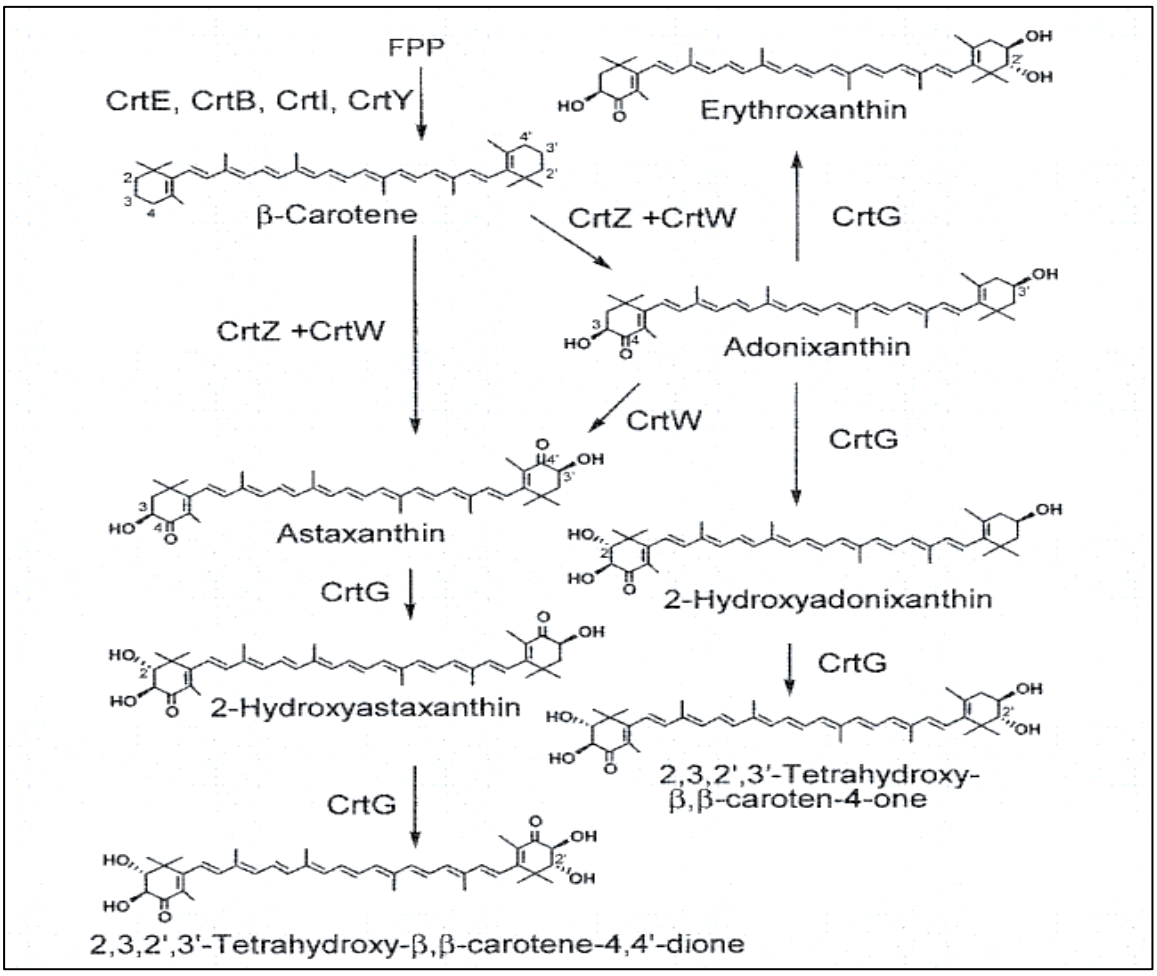

Fig. 2.6. This figure represents structures of carotenoid pigments, biosynthetic pathway and genes involved in the synthesis of carotenoids (Britton *et al.*, 2004)

Many researchers have studied biosynthetic pathways of carotenoids in microbes during the last five decades (Schmidt, 2000; Sandmann *et al.*, 2001; Lee and Schmidt, 2002) (Fig. 2.6). The isopentenyl pyrophosphate (IPP) is used as a bioprecursor for the initiation of carotenoid biosynthesis (Goodwin *et al.*, 1980), whereas isopentenyl pyrophosphate is synthesized from key precursor acetyl-CoA during a pathway termed as the mevalonic acid (MVA) pathway. During the first step of MVA pathway, conversion of acetyl-CoA to 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) is carried by HMG-CoA synthase which is further transformed to mevalonic acid (a C6 compound). In the next step, isopentenyl pyrophosphate (a C5 compound) is formed

from mevalonic acid as result of phosphorylation carried out by MVA kinase and decarboxylation (Bloch, 1983). After that three molecules of isopentenyl pyrophosphate and one molecule of dimethylallyl diphosphate (DMADP) condensed with each other by the action of pyrenyltransferase and give rise diterpene GGDP (a C20 compound), which then combines with one other diterpene GGDP and produce phytoene (the first colorless carotenoid of biosynthesis pathway) (Vachali *et al.,* 2012).

Phytoene get desaturated and converted into neurosporene or lycopene (a C40 acyclic carotenoid) (Takaichi, 1999). The neurosporene or lycopene is then used as a precursor to synthesize multiple of commercially important carotenoids such as acyclic, cyclic and oxygenated (xanthophylls) carotenoids. The β-carotene or α-carotene (cyclic carotenoid) is formed after the subsequent desaturation of lycopene at both ends. The xanthophylls are produced from post-carotene molecules after a series of oxidation reactions leading to production of -epoxy, -hydroxy and -oxo groups (Vachali *et al.,* 2012). A zeaxanthin molecule (a C3,C3'-dihydroxy derivative of β,β-carotene) is formed when hydroxyl (-OH) groups are introduced at C3 of β,β-carotene molecule (Britton, 1993). Many other carotenoids having keto (C=O) as functional groups such as canthaxanthin and astaxanthin, are also produced with or without hydroxylation reactions during the biosynthetic pathway of carotenoids when C=O groups introduced at C4 of β-carotene. The usage of β-carotene as precursor for production of keto-carotenoids have been properly investigated in algae, yeast and non-photosynthetic bacteria (Britton, 1993).

Role of pigments in cold adaptation

Carotenoid pigments (polyisoprenoid hydrocarbons) play pivotal role a role in the maintenance of fluidity of bacterial cell membranes. These pigments have been extracted and identified from multiple psychrophilic microorganisms inhabiting several cold habitats such as high altitude soils, ice cores, glaciers and marine surface waters (Dieser *et al.,* 2010; Pandey *et al.,* 2018; Shen *et al.,* 2018). In addition, an increase in production of pigments especially polar carotenoids with decreasing temperatures have also been seen in many psychrophilic bacteria (Jagannadham *et al.,* 2000), furthermore, some of the recent studies on Arctic bacteria also show that production of pigments is greatly affected by reducing the temperature (Singh *et al.,* 2017). As it has already been

proven by many studies that unsaturated fatty acids played important role in homeoviscous adaptation of psychrophilic bacterial cell membranes by providing stability and maintain fluidity, similar role of pigments have also been suggested by many researchers (Jagannadham *et al.,* 2000).

Likewise, carotenoid pigments posses many other protective roles in cold environments, most important is the photoprotection from damaging UV radiations. These carotenoids perform the function of photoprotection by making conjunction with other molecules e.g. mycosporine like amino acids and scytonemin, which are also known for their abilities to neutralize the detrimental effects of high light and UV radiation commonly found in several cold habitats (Pandey *et al.,* 2018). The formation of reactive oxygen species (ROS) enhances greatly in cold environments exerting severe consequences, carotenoids act as antioxidants thus protect the bacterial species from the consequences of ROS. These pigments are also very strong light harvesters and aid process of photosynthesis. They even observed to have sufficient antimicrobials activities (Pandey *et al.,* 2018). In addition, carotenoids have also been reported to protect bacteria from frequently occurring freeze-thaw cycles by acting as strong cryoprotectant (Dieser *et al.,* 2010). However, further studies are need to be carried out in order to unravel the specific and true functions as well as various types, different structures, suitable effects of carotenoid pigments in psychrophilic bacteria living in cold environments.

Applications of psychrophilic bacteria - fatty acids and pigments

The diverse significance of psychrophilic bacteria makes them the most potential contestants for copious applications in numerous fields ranging from cosmetics, food, pharmaceuticals to environment. Psychrophilic bacteria have been reported to produce fatty acids, pigments, antibiotics, cold-active enzymes, anti-freeze proteins and several other industrially important products (Collins and Margesin, 2019).

Fatty acids

It is indicated by some recent studies that despite having their role in cold adaptation, long-chain monounsaturated fatty acids (LC-MUFAs) along with PUFA (e.g. arachidonic, docosahexaenoic and eicosapentaenoic acids) also act as scavengers of

reactive oxygen species (ROS) at low temperatures (Nishida *et al.,* 2007; Okuyama *et al.,* 2008). They provide antioxidative functions for bacterial species inhabiting cold and marine environments as these habitats are widely accompanied by high level of ROS. One of the suggested basic mechanism of antioxidant activities of LC-MUFAs are involved in providing membrane shields, promote excessive hydrophobic interfaces within phospholipid bilayers and thus block entrance of ROS e.g. H_2O_2, into the microbial cells (Yoshida *et al.,* 2016). It is also suggested that presence of LC-MUFAs and PUFA in bacterial cells, especially in marine bacteria, in huge amount is not just because of maintaining the fluidity of cell membranes but they also contribute to the protection of cells against oxidative stress. Some other functions have also been proposed for LC-MUFAs and PUFA in addition to their antioxidant activities such as their role in cell division, efflux processes and act as chaperones for membrane proteins (Okuyama *et al.,* 2008; Yoshida *et al.,* 2016).

Carotenoid pigments

Numerous plants (such as vegetables and fruits), animals (e.g. edible fish and shellfish) and microbes including psychrophilic bacteria produce multiple types of carotenoid pigments that possess several advantageous impacts on human health (Kirti *et al.,* 2014) e.g. prevention against age-related macular degeneration, cancers and cardiovascular ailments (Bertram, 1999; Nishino *et al.,* 2002; Fraser and Bramley, 2004). Copious studies on carotenoids including clinical, epidemiological and experimental (using animals) studies have revealed the characteristic and specific individuality function of carotenoid related to health benefits. Based on epidemiological studies, sufficient intake of β-cryptoxanthin (3-hydroxy-β-carotene) and β-carotene reduced risks of lung cancer (Yuan *et al.,* 2003; Mannisto *et al.,* 2004), however, lycopene was observed in an inverse proportion with prostate cancer risk (Giovannucci *et al.,* 1995; Vogt *et al.,* 2012).

In addition, several studies have shown lutein and zeaxanthin were effective against eye related disorders such as progress or development of age-related macular degeneration (Mazaffarieh *et al.,* 2003; Semba and Dagnelie, 2003). Astaxanthin proved to be useful in prevention of low-density lipoprotein oxidation (Iwamoto *et al.,* 2000). Glycosylated carotenoids of bacterial origin were observed with prodigious potential to use as natural

surfactants that would be helpful to prepare compounds in future with antimicrobial and antitumor activities (Dembitsky, 2004). One of the most important function of carotenoids is its antioxidant activities as they are strong antioxidant agents. They are used as precursors of vitamin A. Likewise, carotenoid pigments provide wide range of other applications including their usage in skin care and sunscreen products, used to enhance coloration of natural foods, also used in animal/aquaculture feed as well as used as building materials for the manufacturing of fragrance products (Collins and Margesin, 2019).

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Chapter 3

Psychrophilic bacterial diversity and pigment analysis

Abstract

The Karakoram Mountain Range (KMR) is one of the largest mountain ranges in the world, with ~ 37% of its area glaciated. Here, we present the geochemistry of ice, sediment and meltwaters sampled from Ghulmet, Ghulkin and Hopar glaciers of the Karakoram Range, Pakistan, in addition to the first information on the diversity of pigmented bacteria evaluated using culture-dependent techniques. Geochemical analyses revealed Ca^{2+} and SO_4^{2-} to be the most abundant cation and anion species across all glacial samples, respectively. Total organic carbon (TOC), total nitrogen (TN) and total phosphorus (TP) were found in large quantity in the sediments of all glaciers studied in current research. Bacterial species were capable of producing a variety of different pigments, including alloxanthin, astaxanthin, bacterioruberin, β–carotene, 19'–hexanoyloxyfucoxanthin, peridinin, violacein and zeaxanthin. In addition, unknown pigments were also found to be produced by bacterial isolates. Culturable bacterial diversity was studied using two molecular biomarkers, 16S rRNA and rpoB gene, with a total of 82 bacterial strains representing 25 genera identified across all glacial samples. This study provides the first characterization of glacier-associated, pigment-producing bacterial communities from the KMR. Findings are important for considerations of alternative sources of conventional pigment production in industrial fields.

Keywords: Bacterial diversity, carotenoid pigments, geochemistry, glaciers, Karakoram

Introduction

Ice sheets and glaciers cover around 15 million km^2 (10%) of the Earth's surface which are characterized by low temperatures (Stibal *et al.,* 2015). In recent times, both ice sheets and glaciers have been recognized as ecosystems harboring microorganisms that contribute to important biogeochemical processes globally (Hodson *et al.,* 2008; Anesio and Laybourn-Parry, 2012). Based on the study of Castello and Rogers (2005), glacier melt releases approximately 1×10^{17} to 1×10^{21} viable microbes at the global scale annually. Glaciers become habitable during summers as water availability leads to occurrence of microflora in various habitats including snow, ice, biofilms and cryoconite holes (Lutz *et al.,* 2014). According to Cameron *et al.* (2012) and Edwards *et al.* (2014), cryoconite holes have been the most widely investigated habitat up to now. They are formed on glacial surfaces because of melting of inorganic and organic debris into ice driven by solar radiation (Anesio *et al.,* 2009).

Extremophiles existence has been observed in almost all ice phases described from glacial environments (Priscu and Christner, 2004), such as atmospheric particles (Sattler *et al.,* 2001), snow (Takeuchi *et al.,* 1998), glacier ice (Priscu *et al.,* 2006), accretion ice (Priscu *et al.,* 2006), and basal ice/till mixtures (Skidmore *et al.,* 2000; Foght *et al.,* 2004). Pressure, solar radiation, rock-water contact, pH, ionic strength, reduction potential, moisture and nutrient content are some of the parameters that may differ immensely in supraglacial, englacial, and subglacial environments (Hodson *et al.,* 2017). Such variety of abiotic conditions characteristic of glacial environments likely provides a wealth of niches inhabitable by a diversity of microorganisms.

A combination of adaptation strategies for cellular metabolism has been observed in these organisms (Cavicchioli *et al.,* 2002), such as metabolically active enzymes, increased production of antifreeze proteins and exopolymeric substances to cope with cold tolerance (Margesin *et al.,* 2011; Hassan *et al.,* 2016). Cell membrane fluidity and integrity is vital for normal functioning of plasma membrane at reduced temperatures. It is known from previous studies that organisms cope with cold conditions by increasing production of unsaturated fatty acids and decreasing long chain fatty acids which leads toward the maintenance of cell membrane fluidity (Anesio *et al.,* 2009; Hood *et al.,* 2009; Hassan *et al.,* 2016).

Likewise, elevated cold habitats are exposed to higher solar radiation especially between 280 and 400 nm (UV radiation) (Zagarese *et al.,* 1998; Rothschild, 1999). Increased UV radiation has extreme detrimental effects on microbial life inhabiting cold environments by directly damaging enzymes, lipids, proteins, DNA and RNA (Cockell and Knowland, 1999; Rothschild, 1999). Detrimental impacts of UV radiation can be evident following direct absorption by biomolecules or via indirect mechanisms, for example, by stimulating the production of reactive oxygen species (ROS) that ultimately cause oxidation of essential cell components (Moline *et al.,* 2013). Accordingly, microbial adaptations have evolved in order to minimize such effects, including pigment production (Libkind *et al.,* 2009).

The higher occurrence of pigment production observed in bacteria isolated from ice cores, glaciers and marine surface waters (Foght *et al.,* 2004; Agogue *et al.,* 2005; Zhang *et al.,* 2008) proposes the role of pigment in bacteria adaptation against UV radiation in cold habitats. Most importantly, carotenoid pigments have been found to modulate fluidity of cell membrane in bacteria residing cold environments by UV radiation (Cockell and Knowland, 1999; Jagannadham *et al.,* 2000). In addition, studies also reported carotenoid pigments as a protective tool for phytoplankton and non-photosynthetic bacteria in low temperature against UV radiation (Cockell and Knowland, 1999; Zenoff *et al.,* 2006).

Bacterial diversity in extreme cold settings ranging from the Arctic to Antarctica and Himalayas has been previously examined by numerous studies (Prabagaran *et al.,* 2007; Niederberger *et al.,* 2008; Hell *et al.,* 2013). In addition, Lutz *et al.* (2015) reported Proteobacteria from Icelandic glaciers and ice caps as a predominant bacterial phylum. Similarly, glacial cryoconite holes located in the Arctic and the Antarctic regions, have been reported with presence of phyla Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria (Cameron *et al.,* 2012). Ammonia-oxidizing bacteria belonged to order Nitrosomonadales have been identified from Robertson Glacier by Boyd *et al.* (2011). Mikucki and Priscu (2007) found Betaproteobacteria predominantly in samples collected from Taylor Glacier.

Glaciers located in non-polar habitats most famously of the HKKH mountains range, have also been studied for the presence of bacterial populations (Branda *et al.,* 2010;

Baghel *et al.,* 2005; Gupta *et al.,* 2015; Rafiq *et al.,* 2017; 2019). Many researchers reported occurrence of bacteria belonged to genera *Bacillus*, *Cryobacterium*, *Dyadobacter*, *Exiguobacterium*, *Leifsonia*, *Paenibacillus* and *Pedobacter* (Shivaji *et al.*, 2005; Chaturvedi *et al.*, 2005; 2006; Reddy *et al.*, 2008; 2009; 2010; Kishore *et al.*, 2010) from various samples of glacial soil, snow, water and sediments of HKKH mountain range.

The current research work was aimed to study geochemical properties of glacial samples which were not studied before and the culturable diversity of pyschro-tolerant bacteria from glaciers of Karakoram Mountain range, Pakistan, for the first time. Moreover, these bacteria were studied for pigments production and analyzed for the effects of temperature on selected pigments production.

Material and methods

Sampling sites and processing

Three different glaciers, named Ghulmet glacier (36°12.474 N, 74°29.035 E), Hopar (Bualtar) glacier (36.2108228 N, 74.7724664 E) and Ghulkin glacier (36.42791 N, 74.80659 E), located in Karakorum Mountain Range, Pakistan, were selected for this study (Fig. 3.1).

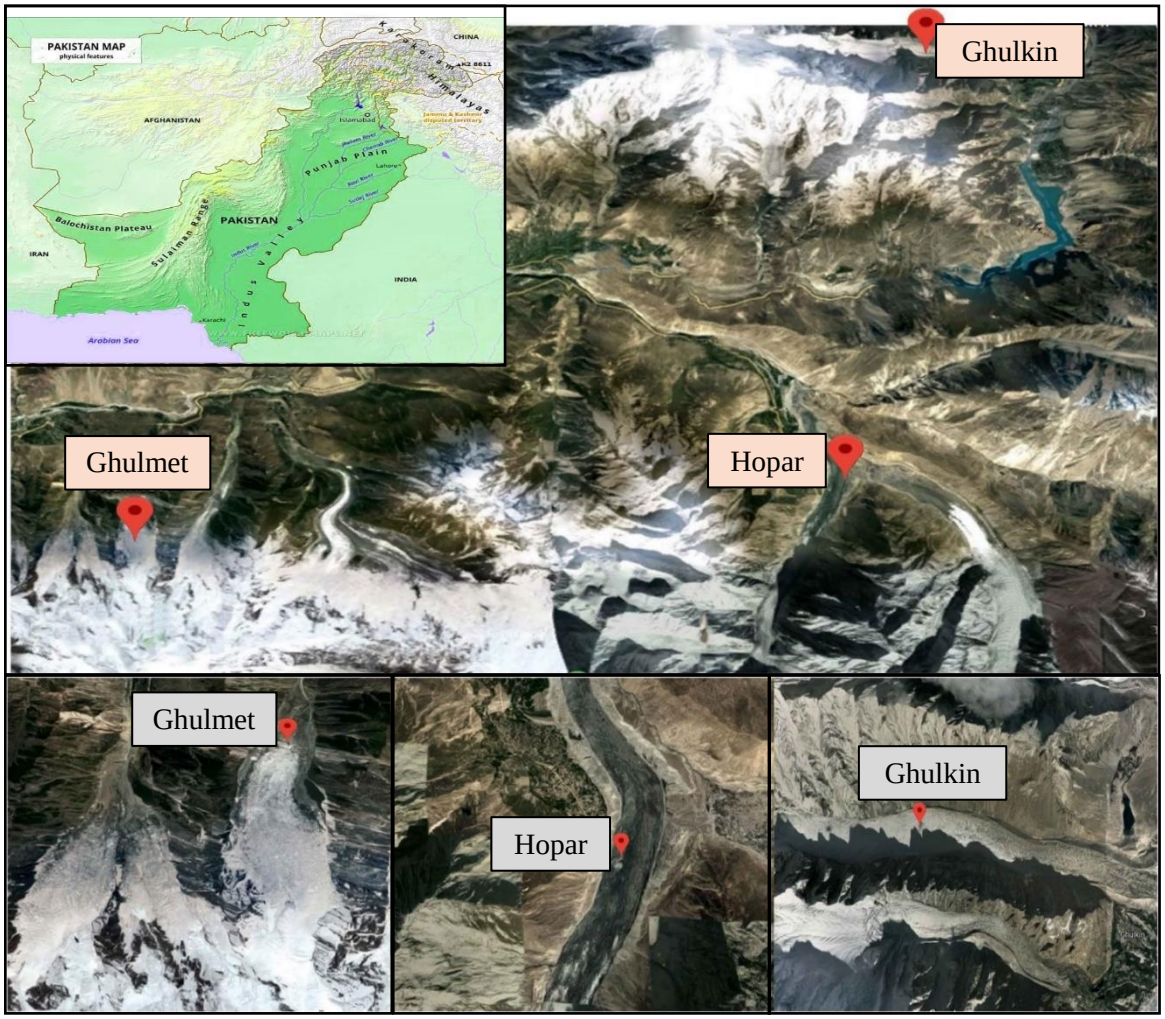

Fig. 3.1. Topographic representation of sampling sites located in Karakoram mountain range, Pakistan

The Hopar glacier, situated between two heighted peaks Miyar peak (6824 m heighted) and Diran peak (7257 m in altitude), is 18 km long and also known as Baultar glacier. Its total length is 18 km. In addition, Ghulkin glacier is located in Gojal, one of the

biggest tehsil of the Gilgit-Baltistan, Pakistan. Its name is derived from two the local Wakhi words, 'Ghulk', meaning 'well' and 'kin', meaning 'whose'. It starts from the Ultar Sar (north-eastern part). Ghulkin glacier has a beautiful peak in the background, called Shispare. Moreover, Ghulmet glacier is situated just below one of the most beautiful peak of the world, Rakaposhi (7788 m) that ranked 29th highest mountain in the world. Pisan Glacier is situated near to Ghulkin glaciers and also has Bagrot, Minapin and Jaglot at its neighborhood.

Glacial ice, sediments and meltwater were taken from each glacier following the standard microbiological sampling protocol. Nasco Whirl-Pak bags and bottles (Fisher Scientific) were used to place individual sample. Temperature and pH were recorded at the time of sampling (Table 3.1). All the glacial samples were transported on ice to Department of Microbiology, Quaid-i-Azam University Islamabad and stored at -20°C for subsequent use.

Determination of TOC, TN, TP and major ions content

Concentrations of major anions (Cl^- and SO_4^{2-}) and cations (Ca^{2+}, K^+, Mg^{2+}, Na^+ and NH_4^+) were conducted in all samples using a Dionex ICS-5000 (Thermo Scientific), while NH_3, NO_3^- and PO_4^{2-} were analyzed using a Gallery Plus automated photometric analyzer (Thermo Scientific). Water and ice after melting, were filtered prior all analyses processes. Specific retention times (40 min), an eluent gradient (KOH, MSA, K_2CO_3, and LiOH) as well as a limit of detection between 0.05-10 ppm were used for detection of ions. In addition, for sediment analysis, sediment was digested using aqua-regia (HCl: HNO3 = 3:1) method.

Briefly, 15 mL aqua-regia reagent was mixed with 1 g of sediment and allowed to heating at 150°C. After appearance of brown fumes, 3 mL of 5% hypochloric acid were added and kept again at 150°C. Once the white fumes were appeared, mixture was finally filtered and diluted using double deionized water.

Total organic carbon (TOC) and total nitrogen (TN) were measured through FlastEA 1112 nitrogen and carbon elemental analyzer following procedure proposed by Hedges and Stern (1984), whereas, total phosphorus (TP) was determined according the protocol described by Koroleff (1976). Briefly, for TP analysis, samples were added

into a solution of oxidizing reagent (g/L: 50 g potassium peroxodisulphate, 30 g boric acid and 15 g sodium hydroxide) and autoclaved at 121°C for 30 minutes and then analyzed by gallery™ plus automated photometric analyzer (Thermo Scientific). Prior to autoclaving, ratio of oxidizing reagent and samples was adjusted as 5:50, respectively. For TN and TOC analysis, 0.1g of sediment was mixed with 2ml of 1M HCL, agitated for 5 min using ultrasonic agitator and the dried at 50°C for 12 hrs. Lastly, dried samples were shifted to tin vials for analysis.

Determination of colony forming units

Colony forming units (CFU) per mL or g for each glacial sample was determined following protocol described by Rafiq *et al.* (2017). Approximately 100 µL of glacial ice, sediment and meltwater were taken and spread on petri plates containing bacterial culture medium. A 1:10 ratio was adjusted by adding 1 mL (ice and meltwater) in sterile glass tubes containing 9 mL of normal saline, while 1 g of sediment was added in 10 ml of sterile normal saline. 5 and 25˚C were used for incubation of plates.

After 30 days of initial incubation, the average colony formation units (CFU/mL or g) was counted in order to determine number of culturable bacteria. Bacterial cultures displaying visually different morphologies were selected and further sub-cultured to obtain pure bacterial cultures.

Culturing condition and isolation of bacterial cultures

Bacterial cultures were isolated by using three different culture media such as Nutrient agar (NA), Minimal salts medium (MSM) and Reasoner's 2A (R2A) (for media composition, see Appendices).

After initial incubation for 30 days, the distinct bacterial colonies were taken and sub-cultured using NA, R2A and MSM media. In order to examine the degree of true psychrophilic nature of isolates that were isolated at 5˚C, all bacterial isolates were grown at 25˚C for 10 days. Bacterial isolates were preserved in 30% glycerol using Nutrient Broth (NB) (see Appendices) for subsequent analyses.

Molecular identification of bacterial isolates

DNA extraction and PCR amplification of target gene

The Invitrogen PureLink Microbiome DNA Kit (Invitrogen) was used to extract genomic DNA of all the bacterial isolates following the manufacturer's guidelines. The amplification of target genes of all the bacterial isolates was carried out using T100 Thermal Cycler (Bio-Rad Laboratories, Inc). An already prepared PCR Master Mix (2X) (Thermo Fisher Scientific) was purchased which contained all PCR reagents (0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP) except templet DNA and primers. In brief, a reaction mixture of 100 µL volume was prepared comprising of 10 µM of each primers, 0.1 µL of templet DNA (20 ng), 50 µL of 2X PCR Master Mix was mixed with 50 µL nuclease-free water (Thermofisher scientific) to amplify target bacterial genes.

16S rRNA amplification

The universal bacterial primers 27F and 1492R were used to amplify 16S rRNA gene of all the bacterial isolates (Table 3.2). The PCR conditions were adjusted as primary denaturation at 94°C for 5 min, then 40 cycles of 94, 56 and 72°C each for 30s and a final step of extension at 72°C for 8 min. The amplified PCR products were confirmed via 1.2% (w/v) agarose gel electrophoresis.

rpoB gene *amplification*

Only those bacterial isolates who were not identified to the species level by 16S rRNA gene sequencing, were subjected to rpoB gene sequencing. Failure of 16S rRNA gene to identify bacterial isolates at species level could be possible of its presence as multiple intragenomic copies that could lead to differences in sequences, which ultimately result in multiple ribotypes identified for a single bacterium (Case *et al.,* 2007). For example, 16S rRNA gene based identification has been found inadequate to differentiate among *Acinetobacter* species (Alvarez-Buylla *et al.,* 2012).

Therefore, many researchers have found the RNA polymerase β-subunit (rpoB) gene sequencing as a powerful tool to identify and classify taxonomically numerous bacterial

species (Gundi *et al.,* 2009; Alvarez-Buylla *et al.,* 2012). Due to the property of rpoB gene existed as a single copy in genomes of bacteria, distinguishing power of rpoB gene has found higher than *16S* rRNA gene sequencing (Adekambi *et al.,* 2006; 2009). Amplification of rpoB gene was done using primers rpoB1698F, rpoB2041R, rpoB1531F, rpoB2760R, rpoB2491F and rpoB3554R (Table 3.2). The rpoB gene was amplified using PCR program set as 94°C for 90s for initial denaturation, followed by 45 cycles of 94°C for 10s, 50°C for 20s and 72°C for 50s, as well as a final extension step at 72°C for 5 min.

Sequencing and phylogenetic tree analysis

The QIAquick PCR Purification Kit (QIAGEN) was used for purification of PCR products. Purified PCR products were then sent for sequencing to MRC PPU DNA sequencing and services, University of Dundee, Scotland, UK. The resultant sequences were trimmed and filtered using BioEdit software (see Appendices) (Hall, 1999). After quality filtering, remaining sequences were subjected to BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for similarity index. The most similar sequences, related to the bacterial isolates of this study, were downloaded from National Center for Biotechnology Information (NCBI) for phylogenetic tree construction. Mega 6.0 software was used for construction of the phylogenetic tree of all bacterial isolates (Tamura *et al.,* 2013). In addition, the maximum likelihood method with pairwise deletion was used to construct phylogenetic tree for all studied bacterial species.

Table 3.1. Colony forming unit (CFU)/mL or g and coordinates of samples

Glaciers	**Samples**	**pH**	**Temperatures** (˚C)	CFU/g or mL at different temperatures		**Elevation in meters (m)**	**GPS coordinates**
				5˚C	25˚C		
Ghulkin	Sediment	7	1	3.73×10^5	2.36×10^7	3083	36.42791 N 74.80659 E
	Meltwater	7	1	3.95×10^3	3.87×10^5		
Ghulmit	Ice	7	-13	1.34×10^2	6.50×10^4	3072	36°12.474 N 74°29.035 E
	Sediment	7	3	2.73×10^6	1.36×10^9		
	Meltwater	7	1	4.73×10^4	2.85×10^6		
Hopar	Ice	7	-12	3.50×10^2	7.60×10^4	2963	36.2108228 N 74.7724664 E
	Sediment	7	2	3.55×10^7	1.65×10^9		
	Meltwater	7	1	4.50×10^3	2.65×10^6		

Quantitative pigment analysis

Pigment extraction

Pigment producing bacterial isolates were grown in NB for 7 days in 250 mL sterile flasks and incubated at 15°C. In addition, two isolates (GS_1 and GW_1) were incubated at four different temperature (5, 15, 25 and 35°C) in order to examine the effects of temperature on pigment production. Furthermore, their pigment were more intense in colors and found to cover whole petri plates as compare to others. Bacterial cultures grown on solid media, were transferred to 15 mL sterile falcon tubes and weighed. Before this, an empty similar falcon tube was weighed and the subtracted its weight from falcon tubes containing bacterial cultures and so on. Approximately 25 mg of cell biomass was mixed with 5 mL of ice cold acetone and vortexed for 2 min.

The falcon tubes were placed into chilled sonicator bath (<10°C) for 20 min. Following sonication, tubes were placed into fridge for 24 hours to allow extraction. After 24 hours, tubes were vortexed for 2 min and sonicated in chilled sonicator for 20 min. The extracts were filtered through 0.2 µm syringe filter into new 15 mL falcon tubes. Using a glass Pasteur pipette, ~ 1-2 mL of extracts were filtered into 2 mL amber glass screw vails (Thermo Scientific), and capped under nitrogen for storage at -20 until measurement. In parallel, n=3 blanks were prepared as above using extraction solvent.

Table 3.2. The primers used for the PCR amplification of 16S rRNA and rpoB genes of bacterial isolates

Primers	[1]Target position	Biomarker genes	Sequence (5′-3′)	References
[2]27F	7-27	16S rRNA	AGAGTTTGATCMTGGC TCAG	Paju *et al.* (2003)
[3]1492R	1492-1510	16S rRNA	GGTTACCTTGTTACGA CTT	Paju *et al.* (2003)
rpoB1698F	1698-1715	rpoB	AACATCGGTTTGATCA AC	Dahllof *et al.* (2000)

rpoB2041R	2041-2060	rpoB	CGTTGCATGTTGGTAC CCAT	Dahllof *et al.* (2000)
rpoB1531F[2]	1531-1552	rpoB	TGGCCGAGAACCAGTT CCGCGT	Tayeb *et al.* (2005)
rpoB2760R[3]	2760-2781	rpoB	CGGCTTCGTCCAGCTT GTTCAG	Tayeb *et al.* (2005)
rpoB2491F[2]	2491-2511	rpoB	AACCAATTCCGTATIG GTTT	Michel and Raoult (2002)
rpoB3554R[3]	3554-3573	rpoB	CCGTCCCA AGTCATGAAAC	Michel and Raoult (2002)

[1] rpoB and 16S nucleotides numbered used *E*.coli, *Pseudomonas aeruginosa* and *Pseudomonas putida* as reference.

[2,3]F (forward) and R (Reverse) demonstrating primers trend linking to the rRNA.

High-performance liquid chromatography (HPLC) analysis

A revised version of the HPLC method described by Van Heukelem and Thomas (2001) was used to analyze pigment extracts. An Agilent 1100 HPLC (HP, Waldbronn, Germany) equipped with photo-diode array detector (wavelength was set between 350 to 700 nm), C_8 column, chilled auto-sampler/injector slot with 900 µL syringe head, quaternary pump with in-line vacuum degasser and thermo-statted column slot, were used for pigment analysis. Identification and quantification of analyzed pigment was carried out by considering in-line photo-diode array spectra ranging from 350 to 700 nm and compared with analytical standards purchased from Sigma and using R_t (retention time) (Fig. 3.2). In the temperature experiment, the correlation between pigment and temperature were calculated using GraphPad Prism 5.0 software.

Results

Geochemistry of glacial samples

The measured concentrations of major ions (anions and cations) as well as TOC, TN and TP content of studied glaciers are given in (Tables 3.3a and 3.4b). Sediments of all glaciers were found the most enriched form of sample with all major ions, TOC, TN and TP followed by meltwater and ice. Hopar glacier sediments was observed with highest concentrations of Ca^{2+} (10016 µg kg^{-1}), K^{+} (363 µg kg^{-1}), Mg^{2+} (1081 µg kg^{-1}) and PO_4^{2-} (0.7 µg kg^{-1}), whereas Ghulkin glacier sediments with NO_3^{-} (248 µg kg^{-1}), while sediments of Ghulmet glacier was enriched with Na^{+} (1595 µg kg^{-1}). K^{+} and PO_4^{2-} were not detected in all glacial samples collected from Ghulmet and Ghulkin glacier, respectively. Similarly, ice sample taken from Ghulmet glacier was found lacking Mg^{2+} and SO_4^{2-}. PO_4^{2-} was not found in glacial ice taken from Hopar and Ghulmet glaciers.

In addition, Ca^{2+} and SO_4^{2-} were seen as a predominant cation and anion in all glacial samples, respectively. NH_3 was dominated in glacial sediments followed by water and ice. Comparatively, glacial samples were more enriched with TOC and least with TP content. The highest TOC, TN and TP were observed in sediments of all glaciers followed by meltwater and ice. Ice from both Hopar and Ghulmet glaciers as well as meltwater of Ghulkin glacier were lacking TP content. However, sediments and meltwater collected from all glaciers were observed with low TP content. TN content of glacial ice (28 µg g^{-1}) was very low as compared to glacial sediments and meltwater.

Table 3.3a. Major ion content of glacial samples

Glaciers	Samples	Concentrations (µg l^{-1} kg^{-1})									
		Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	NH_4^{+}	Cl^{-}	NO_3^{-}	PO_4^{2-}	SO_4^{2-}	NH_3
Hopar	Ice	2598	79	180	390	197	143	36	[1]ND	112	280
	Sediment	10016	363	1081	1015	378	28	145	0.7	760	625
	Meltwater	7076	260	809	734	284	224	103	0.2	996	95
Ghulkin	Sediment	9009	225	856	1324	464	76	248	ND	645	741
	Meltwater	5008	204	707	946	308	100	81	ND	884	310
Ghulmet	Ice	2257	ND	ND	441	221	121	58	ND	ND	51
	Sediment	8348	ND	469	1595	398	163	207	0.5	558	569
	Meltwater	6229	ND	929	990	271	ND	94	0.4	852	276

[1]Not detected

Molecular identification of bacterial isolates

Bacterial isolates with their homologous bacterial species and accession numbers (16S rRNA and rpoB genes) are given in (Tables 3.4a, 3.4b and 3.4c). In the current study, a total of 82 bacterial isolates were isolated (30 isolates from Ghulmet glacier, 30 from Hopar glacier and 22 bacterial isolates from Ghulkin glacier). All isolates bacteria were found to belonged 5 different bacterial phyla. Proteobacteria was predominantly found bacterial phylum covering 66% of total bacterial isolates followed by Actinobacteria (14%), Firmicutes (10%), Deinococcus-Thermus (6%) and Bacteroidetes (4%).

Table 3.3b. Total organic carbon, total nitrogen and total phosphorus content of glacial samples

Glaciers	Samples	Concentrations (µg ml^{-1} g^{-1})		
		TOC	**TN**	**TP**
Hopar	Ice	486	57	[1]ND
	[2]**SD**	1.00	1.00	-
	Sediment	7543	993	5.2
	SD	1.30	1.00	0.40
	Meltwater	2771	125	3.4
	SD	1.00	1.00	0.30
Ghulkin	Sediment	6897	1139	1.5
	SD	1.10	1.00	0.10
	Meltwater	1799	101	ND
	SD	1.50	1.1	-
Ghulmet	Ice	557	28	ND
	SD	1.00	0.25	-
	Sediment	9174	1040	4.1
	SD	1.5	1.00	0.60
	Meltwater	2005	141	3.8
	SD	1.80	0.85	0.40

[1]Not detected

[2]Standard deviation

Based on 16S rRNA and rpoB genes sequencing results, all bacterial isolates were found to represent 7 bacterial classes, 12 orders, 18 bacterial families and 25 bacterial genera as well as most predominant genus was *Pseudomonas* (19% of isolates).

Table 3.4a. The studied bacterial species with corresponding homologous strains and their accession numbers isolated from Ghulmet glacier

Isolates	Accession No (16S rRNA)	Homologous species	Accession No (rpoB)	Homologous species
[1]GI_1	MK456529	*Massilia oculi*	-	-
GI_2	MK456530	*Enterobacter hormaechei*	-	-
GI_3	MK456531	*Massilia* sp.	MK606636	*Massilia aurea*
GI_4	MK456532	Uncultured bacterium	MK606637	*Massilia aurea*
[2]GS_1	MK456533	*Arthrobacter agilis*	-	-
GS_2	MK456534	*Pseudomonas* sp.	MK606638	*Pseudomonas brassicacearum*
GS_3	MK456535	*Pseudomonas* sp.	MK606639	*Pseudomonas migulae*
GS_4	MK456536	*Pseudomonas* sp.	MK606640	*Pseudomonas frederiksbergensis*
GS_5	MK456537	*Flavobacterium xinjiangense*	-	-
GS_6	MK456538	*Janthinobacterium agaricidamnosum*	-	-
GS_7	MK456539	*Flavobacterium sinopsychrotolerans*	-	-
GS_9	MK456541	*Paracoccus hibiscisoli*	-	-
GS_{11}	MK456543	*Brevundimonas vesicularis*	-	-

GS_{12}	MK456544	*Pseudomonas* sp.	MK606643	*Pseudomonas mandelii*
GS_{13}	MK456545	*Arthrobacter nitroguajacolicus*	-	-
GS_{14}	MK456546	*Rhizobium* sp.	MK606644	*Rhizobium herbae*
GS_{15}	MK456547	*Sporosarcina psychrophila*	-	-
GS_{17}	MK456549	*Paenisporosarcina macmurdoensis*	-	-
GS_{18}	MK456550	*Brevundimonas mediterranea*	-	-
GS_{19}	MK456551	*Paracoccus carotinifaciens*	-	-
GS_{20}	MK456552	*Arthrobacter nitroguajacolicus*	-	-
GS_{21}	MK456553	*Brevundimonas intermedia*	-	-
$^{3}GW_{1}$	MK456554	*Janthinobacterium lividum*	-	-
GW_{2}	MK456555	*Pseudomonas* sp.	MK606646	*Pseudomonas frederiksbergensis*
GW_{4}	MK456557	*Brevundimonas* sp.	MK606648	*Brevundimonas vesicularis*
GW_{5}	MK456558	*Staphylococcus equorum*	-	-
GW_{6}	MK456559	*Janthinobacterium agaricidamnosum*	-	-
GW_{7}	MK456560	*Deinococcus aquaticus*	-	-
GW_{8}	MK456561	*Sphingomonas faeni*	-	-
GW_{9}	MK456562	*Acidovorax radices*	-	-
Keys: ^{1}GI (Ghulmit Ice), ^{2}GS (Ghulmit Sediment), ^{3}GW (Ghulmit Water)				

The bacterial isolates were found to belonged *Pseudomonas*, *Arthrobacter*, *Brevundimonas*, *Massilia*, *Flavobacterium*, *Deinococcus*, *Enterobacter*, *Staphylococcus*, *Janthinobacterium*, *Paracoccus*, *Rhizobium*, *Bacillus*, *Sphingobium*, *Sphingomonas*, *Sporosarcina*, *Acidovorax*, *Acinetobacter*, *Delftia*, *Paenisporosarcina*, *Plantibacter*, *Pseudarthrobacte*, *Pseudorhodobacter*, *Sanguibacter*, *Serratia* and *Stenotrophomonas*.

Table 3.4b. The studied bacterial species with corresponding homologous strains and their accession numbers isolated from Ghulkin glacier

Isolates	Accession No (16S rRNA)	Homologous species
$^{1}GhS_1$	MK456506	*Deinococcus depolymerans*
GhS_3	MK456508	*Pseudomonas frederiksbergensis*
GhS_4	MK456509	*Sphingobium xenophagum*
GhS_5	MK456510	*Staphylococcus equorum*
GhS_6	MK456511	*Deinococcus aquaticus*
GhS_7	MK456512	*Pseudomonas frederiksbergensis*
GhS_8	MK456513	*Acinetobacter radioresistens*
GhS_9	MK456514	*Arthrobacter sulfureus*
GhS_{10}	MK456515	*Sphingomonas faeni*
GhS_{11}	MK456516	*Enterobacter cloacae*
GhS_{12}	MK456517	*Enterobacter mori*
GhS_{13}	MK456518	*Pseudomonas frederiksbergensis*
GhS_{14}	MK456519	*Stenotrophomonas maltophilia*
$^{2}GhW_1$	MK456520	*Deinococcus aquaticus*
GhW_2	MK456521	*Acinetobacter radioresistens*
GhW_3	MK456522	*Brevundimonas vesicularis*
GhW_4	MK456523	*Pseudomonas frederiksbergensis*
GhW_5	MK456524	*Staphylococcus equorum*
GhW_6	MK456525	*Brevundimonas nasdae*
GhW_7	MK45652	*Sanguibacter antarcticus*
GhW_8	MK456527	*Deinococcus aquaticus*

GhW_9	MK456528	*Pseudomonas frederiksbergensis*
Keys: [1]GhS (Ghulkin Sediment), [2]GhW (Ghulkin Water). [3]Isolates showed 100% similarity with the homologous species after BLAST search in NCBI.		

In addition, the complete identification of bacterial isolates GI_1, GI_4, GS_2, GS_3, GS_4, GS_{12}, GS_{14}, GW_2, GW_4, HI_2 and HS_{14} to the species level was achieved by using rpoB gene as biomarker (Tables 3.4a and 3.4c).

Table 3.4c. The studied bacterial species with corresponding homologous strains and their accession numbers isolated from Hopar glacier

Isolates	Accession No (16S rRNA)	Homologous species	Accession No (rpoB)	Homologous species
[1]HI_1	MK456563	*Massilia aurea*	-	-
HI_2	MK456564	Uncultured bacterium	MK606652	*Massilia oculi*
HI_3	MK456565	*Massilia aurea*	-	-
HI_4	MK456566	*Plantibacter auratus*	-	-
HI_5	MK456567	*Rhizobium giardinii*	-	-
HI_6	MG641443	*Serratia marcescens*	-	-
HI_7	MK456568	*Massilia timonae*	-	-
[2]HS_1	MK456569	*Arthrobacter psychrolactophilus*	-	-
HS_2	MK456570	*Pseudarthrobacter scleromae*	-	-
HS_3	MK456571	*Flavobacterium sinopsychrotolerans*	-	-
HS_4	MK456572	*Bacillus butanolivorans*	-	-
HS_5	MK456573	*Pseudomonas frederiksbergensis*	-	-
HS_6	MK456574	*Arthrobacter psychrolactophilus*	-	-

HS_7	MK456575	*Bacillus simplex*	-	-
HS_8	MK456576	*Pseudomonas extremaustralis*	-	-
HS_9	MK456577	*Pseudomonas veronii*	-	-
HS_{10}	MK456578	*Pseudomonas fluorescens*	-	-
HS_{13}	MK456581	*Delftia acidovorans*	-	-
HS_{14}	MK456582	*Pseudomonas* sp.	-	-
HS_{15}	MK456583	*Pseudarthrobacter sulfonivorans*	-	-
HS_{16}	MK456584	*Paracoccus carotinifaciens*	-	-
HS_{17}	MK456585	*Flavobacterium xinjiangense*	-	-
HS_{18}	MK456586	*Arthrobacter nitroguajacolicus*	-	-
HS_{19}	MK456587	*Pseudorhodobacter collinsensis*	-	-
$^2HW_1$	MK456588	*Paenisporosarcina quisquiliarum*	-	-
HW_2	MK456589	*Pseudomonas frederiksbergensis*	-	-
HW_3	MK456590	*Arthrobacter ginsengisoli*	-	-
HW_4	MK456591	*Rhizobium soli*	-	-
HW_5	MK456592	*Staphylococcus equorum*	-	-
HW_6	MK456593	*Brevundimonas vesicularis*	-	-
Keys: ^{1}HI (Hopar Ice), ^{2}HS (Hopar Sediment), ^{3}HW (Hopar Water).				

Phylogenetic analysis of all bacterial isolates done by maximum-likelihood method are given in (Fig. 3.3a, 3.3b and 3.3c). Analysis of phylogenetic relationships of bacterial isolates with proposed homologous bacterial species further confirmed that bacterial isolates were resembled to the same bacterial species as given in (Tables 3.4a, 3.4b and 3.4c).

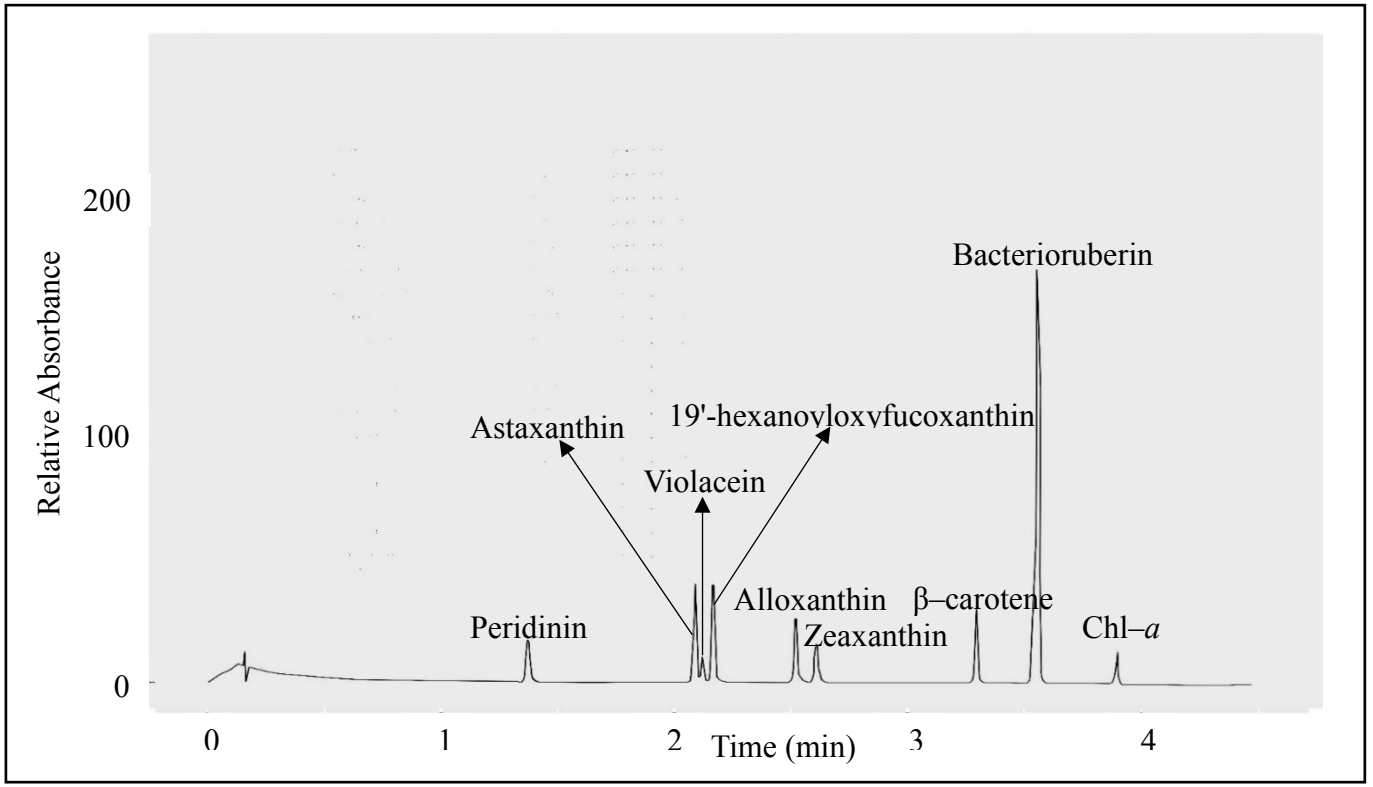

Fig. 3.2. HPLC chromatograph/profile of known standards of pigme

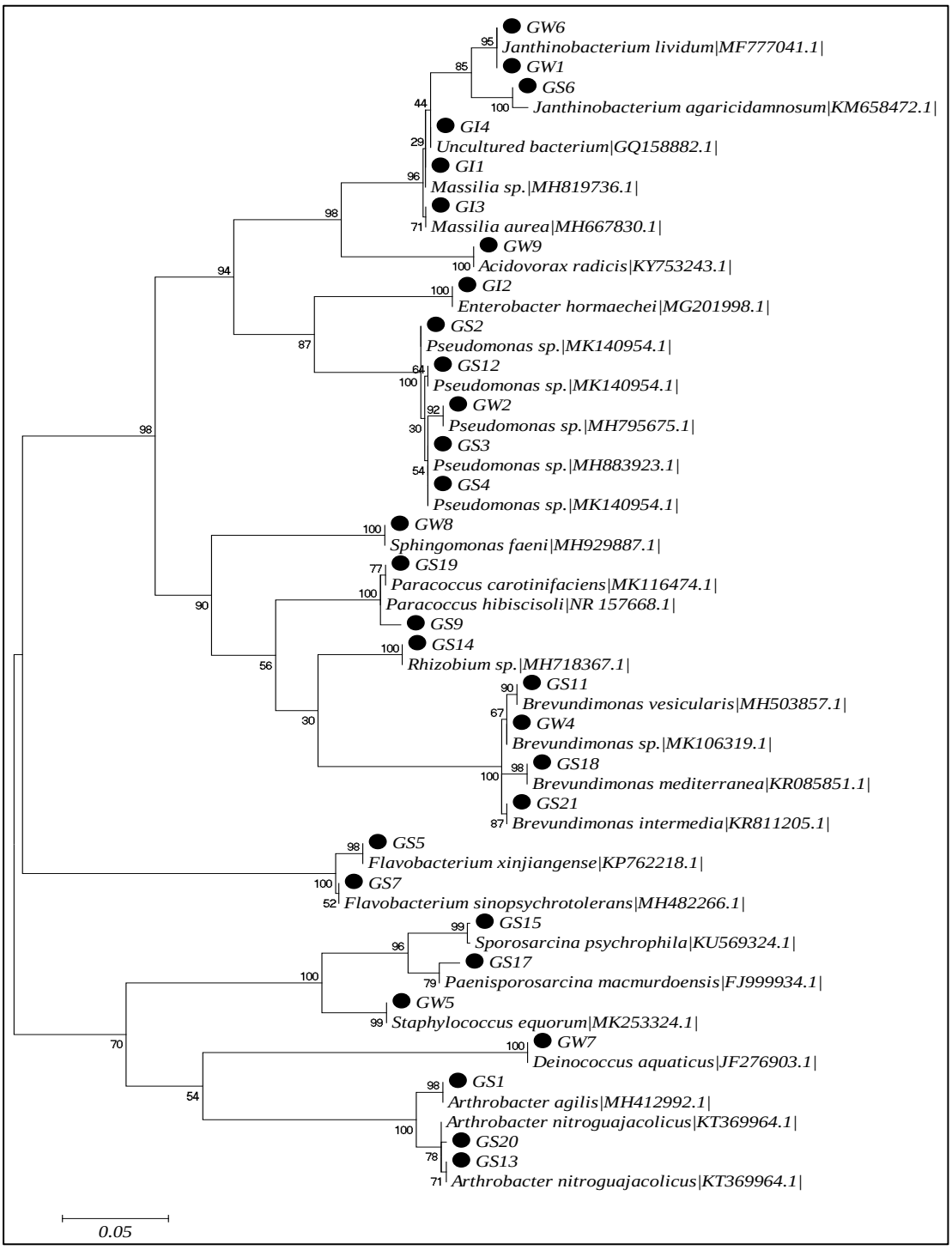

Fig. 3.3a. This figure is representing phylogenetic association of studied bacterial species isolated from Ghulmet glacier with their homologous bacteria made by the Maximum Likelihood method using bootstrap values (1000 replicates)

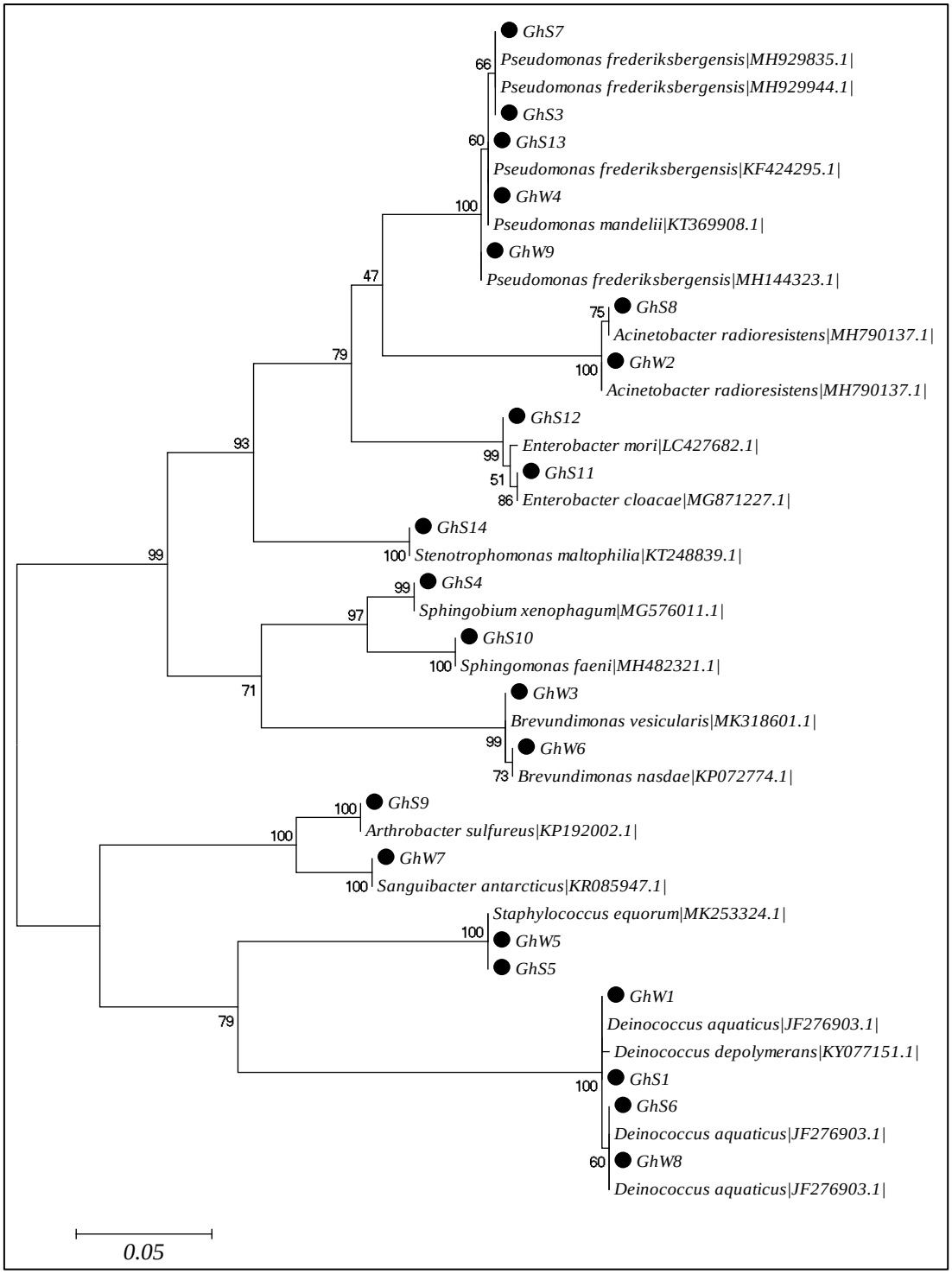

Fig. 3.3b. This figure is representing phylogenetic association of studied bacterial species isolated from Ghulkin glacier with their homologous bacteria made by the Maximum Likelihood method using bootstrap values (1000 replicates)

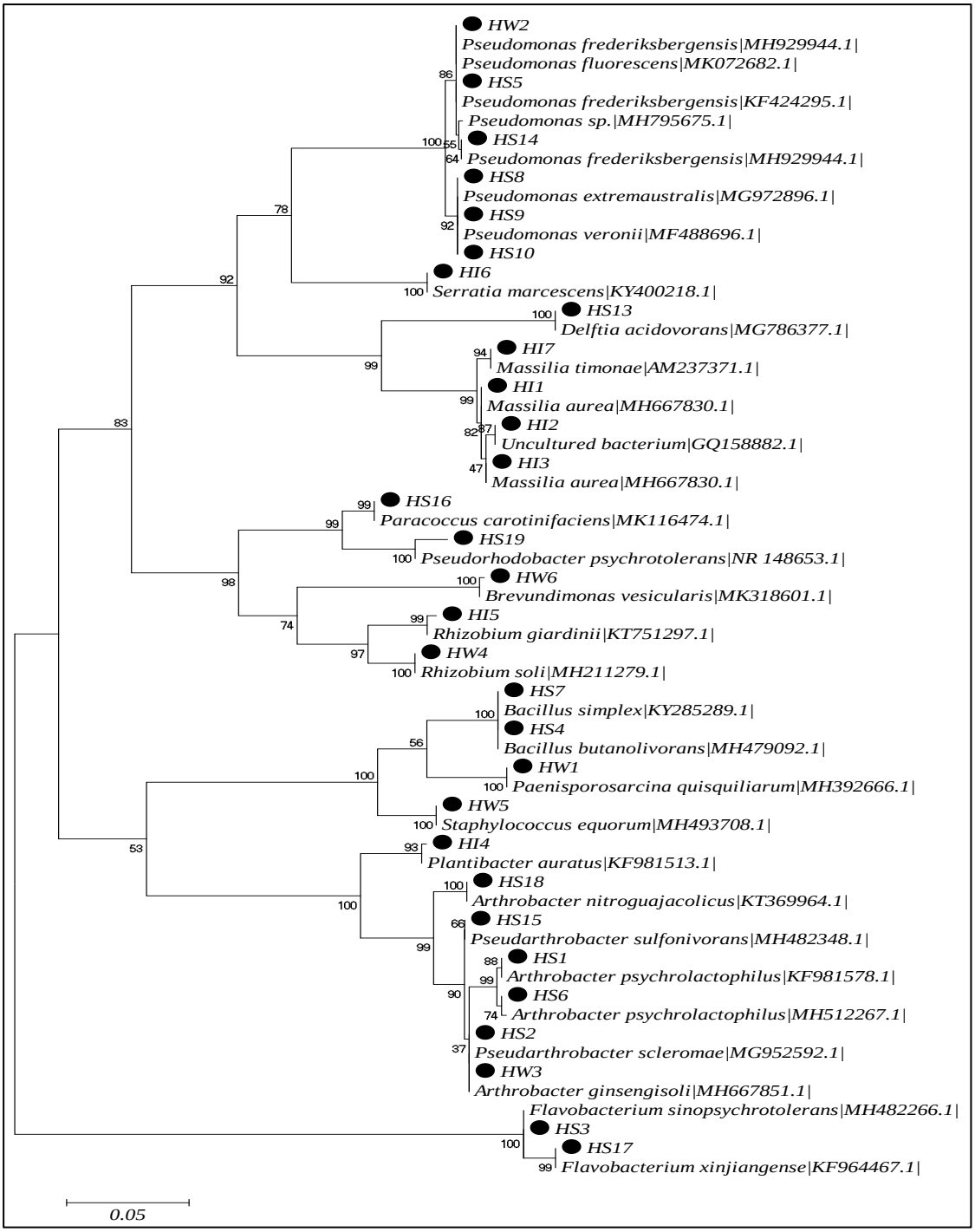

Fig. 3.3c. This figure is representing phylogenetic association of studied bacterial species isolated from Hopar glacier with their homologous bacteria made by the Maximum Likelihood method using bootstrap values (1000 replicates)

Quantitative pigment analysis

An overview of quantified and identified pigment produced by bacterial isolates are listed in (Table 3.5). Out of 50 bacterial isolates, 39 isolates were confirmed after HPLC to produce 11 different types of pigment including 2 unknown pigment as well. Eight pigment (including carotenes and xanthophylls) were identified using analytical standards as alloxanthin, astaxanthin, bacterioruberin, β,β-carotene, β,ε-Carotene, 19'-hexanoyloxyfucoxanthin, peridinin, violacein and zeaxanthin. Twenty-three percentage of bacterial isolates were able to produce astaxanthin, 48% β,β-carotene, 15% β,ε-Carotene, 23% 19'-hexanoyloxyfucoxanthin, 23% peridinin and 23% bacterial isolates were producing zeaxanthin. Alloxanthin was produced only by bacterial isolates GhS_2 and HS_8. Only bacterial isolate GS_1 and GW_1 were observed to produce bacterioruberin (138.42 µg g^{-1}) and violacein (352 µg g^{-1}), respectively.

Bacterial isolates GS_{15}, GS_{20}, GhS_9, GhS_{13}, HS_1, HS_6, HS_{13} and HS_{18} were found to produce completely unknown pigment. Moreover, it has been observed in quantitative analysis that xanthophylls were produced in higher quantities by bacterial isolates as compare to carotenes. Among xanthophylls, 19'-hexanoyloxyfucoxanthin was produced predominantly by GhS_2 (710.40 µg g^{-1}) followed by GS_7 (340.40 µg g^{-1}), zeaxanthin by HS_3 (351.67 µg g^{-1}) followed by HW_6 (108.26 µg g^{-1}), astaxanthin by GhS_1 (56.28 µg g^{-1}) followed by HW_4 (54.48 µg g^{-1}). On other hand, β,β-carotene was produced in high quantity than β,ε-Carotene, comparatively as β,β-carotene by GS_9 (191.82 µg g^{-1}) followed by HS_3 (50.77 µg g^{-1}) and β,ε-Carotene by GW_8 (140.68 µg g^{-1}) followed by GW_9 (101.32 µg g^{-1}).

In addition, effects of different temperatures on pigment produced by GS_1 and GW_1 are given in (Fig. 3.4). A significant reduction in pigment quantity was observed in both bacteria isolates with increase of temperature. Pigment produced by GW_1 was 352 µg g^{-1} at 5°C and 81 µg g^{-1} at 35°C. Similarly, a 66% (81 µg g^{-1}) reduction in pigment production was observed when bacterial isolate GS_1 was grown at 35°C, compared to 66% (352 µg g^{-1}) increase production when it was grown at 5°C.

Table 3.5. An overview of quantified and identified pigments produced by bacterial isolates

Isolates	Pigments (μg g^{-1})								
	Alloxanthin	**Astaxanthin**	**β,β-Carotene**	**β,ε-Carotene**	**19'-Hexanoyloxy fucoxanthin**	**Peridinin**	**Zeaxanthin**	**Unknown 1**	**Unknown 2**
GI_1	-	-	4.04	-	8.51	-	-	23.39	-
GI_4	-	-	7.84	-	11.23	-	2.99	182.03	-
GS_5	-	-	4.14	-	110.5	-	9.70	-	-
GS_7	-	-	12.04	-	350.40	7.45	10.69	-	-
GS_9	-	8.13	191.82	-	-	-	30.49	60.43	199.75
GS_{14}	-	-	-	21.32	-	-	-	-	-
GS_{15}	-	-	-	-	-	-	-	20.83	-
GS_{18}	-	38.43	4.38	-	61.68	124.03	-	-	-
GS_{19}	-	16.87	29.66	-	-	23.91	24.08	5.72	-
GS_{20}	-	-	-	-	-	-	-	8.60	-
GS_{21}	-	39.59	2.95	-	-	49.05	-	185.23	-
GW_4	-	54.45	5.39	-	-	178	-	74.32	-
GW_7	-	31.03	-	-	-	-	-	-	-
GW_8	-	140.68	-	-	-	-	-	28.42	15.53
GW_9	-	-	-	101.32	-	-	-	15.85	-
GhS_1	-	56.28	-	-	-	-	-	-	-

GhS_4	-	-	2.65	-	34.74	151.54	-	-	-
GhS_8	-	36.10	4.12	-	-	103.64	-	-	-
GhS_9	-	-	-	-	-	-	-	2.71	-
GhS_{13}	-	-	-	-	-	-	-	6.99	-
GhW_6	-	21.15	5.64	-	-	312.96	-	-	-
GhW_7	-	8.88	3.01	-	-	128.06	-	-	-
HI_1	-	-	-	-	-	-	-	126.40	-
HI_2	-	-	5.55	-	-	-	-	228.27	-
HI_3	-	-	-	31.42	-	-		20.40	30.39
HI_7	-	-	-	-	-	-	-	12.95	-
HS_1	-	-	-	-	-	-	-	6.99	-
HS_3	-	-	3.17	-	-	-	351.67	-	-
HS_6	-	-	-	-	-	-	-	106.78	-
HS_8	48.46	-	50.77	-	-	-	72.01	29.32	-
HS_{13}	-	-	-	-	-	-	-	17.75	-
HS_{14}	-	-	-	10.37	-	-	-	94.30	30.65
HS_{16}	-	14.54	45.03	-	-	-	14.12	14.67	-
HS_{18}	-	-	-	-	-	-	-	12.51	-
HW_6	-	6.59	4.14	-	-	108.26	-	-	-

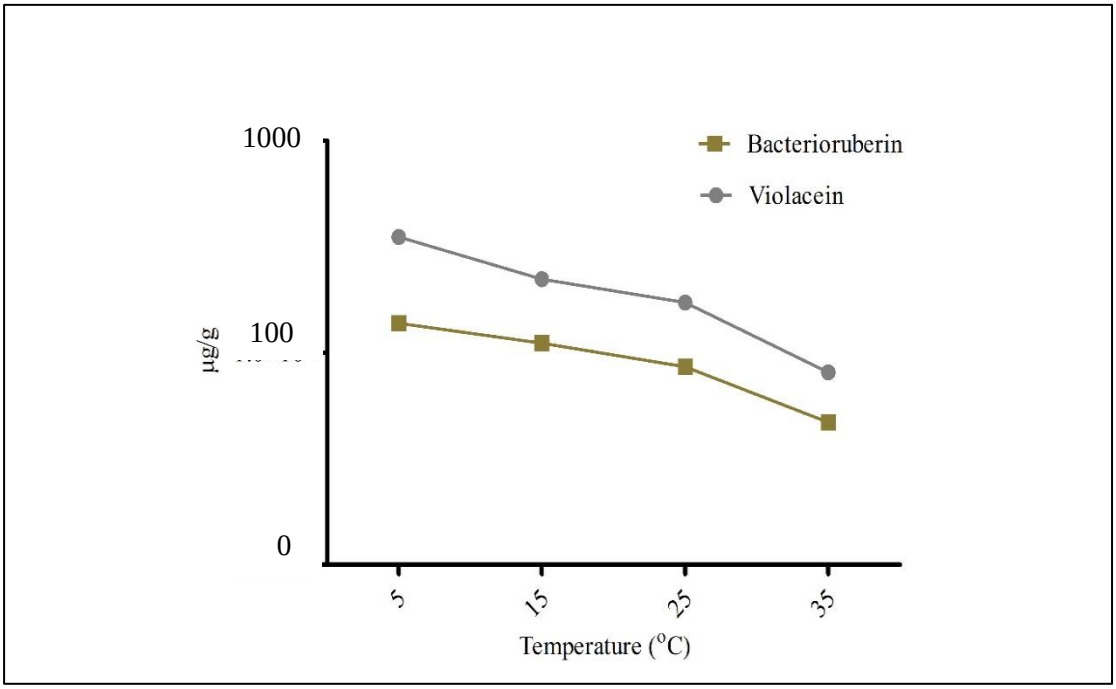

Fig. 3.4. Pearson correlation of violacein ($r = 0.980$, $P = 0.021$) and bacterioruberin ($r = 0.979$, $P = 0.021$) produced by *Arthrobacter agilis* GS1 and *Janthinobacterium lividum* GW1, respectively, at different temperature

Discussion

In the current study, we are reporting first study of quantitative analysis of major anions and cations of glacial samples collected from Ghulkin, Ghulmit and Hopar glaciers situated in Karakoram Mountain Range (KMR), Pakistan. To the best of our knowledge, only Hodson *et al.* (2002) has examined major ions and minor elements content of meltwater originating from Batura glacier located in same KMR, Pakistan. In the present research work, NH_4^+ and NO_3^- have been detected in all glacial samples suggesting presence of active nitrogen fixer bacterial communities. Hodson *et al.* (2005) has shown that an active population of nitrifiers involved in conversion of NH_4^+ to NO_3^- on glacial surfaces. In addition, Ca^{2+} was found as a major cation in all glacial samples, unanimously. Hodson *et al.* (2002) research work supported our study as Ca^{2+} was the most abundant cation in their findings as well. Hasnain and Thayyen (1999) have also reported Ca^{2+} as a major cation from Dokriani glacier meltwaters, Himalaya. The excessiveness of Ca^{2+} in rocky glaciers could be possible of carbonate weathering coupled by one or all of followings; gypsum dissolution, acid hydrolysis accompanied by sulphide oxidation or neutralization of acid aerosols containing NO_3^- and SO_4^{2-} (Hodson *et al.,* 2002). Cations (K^+, Na^+ and Mg^{2+}) were observed in higher concentration as compare to anions (Cl^-, PO_4^{2-} and SO_4^{2-}) in studied glacial samples. The present studied glaciers are situated in valLey area and glaciers located in valleys, were also observed with higher quantities of cations (most importantly Ca^{2+}) but lesser with anions quantities (most importantly SO_4^{2-}) in previous geochemical studies of glaciers (Tranter *et al.,* 1994; 1997; Brown and Fuge, 1998a; b). Interestingly, similar results with higher Ca^{2+} and SO_4^{2-} content were also reported from glaciers located in Polar Regions (Fortner *et al.*, 2005; Yde *et al.*, 2005).

Measurement of total organic carbon, total nitrogen and total phosphorus content of all glacial samples in the current study revealed that glacial sediments were enriched with C, N and P as compare to glacial ice and water. To the best of our knowledge, none of the study has been carried out to asses TOC, TN and TP content of glaciers located in Karakoram Mountain Range. TOC, TN and TP content of the studied glaciers were not as much as observed in Artic glaciers (Nash *et al.*, 2018). However, the observed values matched with generally observed C, N and P content of glaciers forefields (Bradley *et al.*, 2014).

In present research work, bacterial isolates were identified using 16S rRNA and rpoB genes sequencing. 16S rRNA gene based identification is commonly used to detect and measure diversity of bacteria and archaea in variety of habitats (Peixoto *et al.,* 2002; Vos *et al.,* 2012). However, identification of many bacterial isolates at species level were not achieved with 16S rRNA sequencing in the current study but done with rpoB gene sequencing. The bacterial isolates that isolated and identified in the present research work, have been reported from various cold habitats in previous studies. Bacterial isolates were recovered dominantly from glacial sediments, which could be possible of having numerous nutrient and slightly higher temperature as compared to glacial ice and water (Parnell *et al.,* 2016). It is well understood that bacteria belonged to Proteobacteria are predominantly exist in glacial habitats throughout world (Lutz *et al.,* 2015; Anesio *et al.,* 2017; Rafiq *et al.,* 2017) as seen in this study as well.

Genus *Pseudomonas* has been reported from a wide range of habitats covering from aquatic to desert as well as cold to warm environments (Lee *et al.,* 2017). It has been found in Antarctica (Lee *et al.,* 2017), Arctic (Prasad *et al.,* 2013), cryoconite holes (Boetius *et al.,* 2015) and glaciers (Baghel *et al.,* 2005). Likewise, bacterial genus *Arthrobacter* has recovered from Antarctica (Leiva *et al.,* 2015), Permafrost (Zhang *et al.,* 2013) and Himalayan glaciers (Rafiq *et al.,* 2017). Bacterial genera *Deinococcus*, *Massilia* and *Sporosarcina* have been studied from the McMurdo Dry Valleys, Antarctica (Antibus *et al.,* 2012). Liu *et al.* (2009) and Zhang *et al.* (2007) have isolated bacteria belonged to genus *Acidovorax*, *Acinetobacter*, *Enterobacter*, *Rhizobium* and *Sphingomonas* from various glaciers located in Tibetan Plateau. The genus *Staphylococcus* has been reported from Antarctica (Leiva *et al.,* 2015), Himalayan glacier (Rafiq *et al.,* 2017) and cold marine habitat (Villarreal-Gomez *et al.,* 2010).

To the best of our knowledge, bacterial isolates *Sphingobium xenophagum*, *Pseudarthrobacter scleromae* and *Stenotrophomonas maltophilia* have been reported for the first time from glacier situated either in Polar and non-polar territories. In addition, genera *Paenisporosarcina*, *Pseudorhodobacter*, *Sanguibacter* have been studied in Antarctica (Reddy *et al.,* 2013; Leiva *et al.,* 2015; Lee *et al.,* 2016). Similarly, bacteria belonged to *Brevundimonas*, *Plantibacter* and *Paracoccus* have been reported from Greenland, deserts of Himalayas and cold marine habitats (Miteva *et al.,* 2004; Misawa *et al.,* 2011; Yadav *et al.,* 2015). *Delftia* has been reported from Svalbard,

Arctic (Cuthbertson *et al.,* 2017). Genus *Bacillus* has studied by many researchers in glaciers located Himalaya and Tibetan Plateau (Baghel *et al.,* 2005; Liu *et al.,* 2009; Pradhan *et al.,* 2010). Likewise, *Flavobacterium* and *Janthinobacterium* genera have been reported from Antarctica (Xu *et al.,* 2011; Prasad *et al.,* 2013) and Himalayan glaciers (Rafiq *et al.,* 2017).

In current research work, bacterial isolates isolated from glacial habitats were thoroughly screened for pigment production. Quantitative identification of pigment produced by bacterial isolates revealed that a large number of isolates were able to produced various types of pigment such as astaxanthin and β-carotene that act as a super antioxidants by preventing ROS formation resultant from UV radiation (Asker *et al.,* 2018). It is well reported that cold environments are exposed to higher UV radiation and detrimental effects of UVR especially with short wave lengths such as UV-B (280-320 nm) and UV-A (320-400 nm) on the microbial life inhabiting cold habitats are well documented (Jeffrey *et al.,* 2000; Hader and Sinha, 2005). To cope with the damaging effect of UVR, microbes evolved various resistant mechanisms. Pigment production by microbial communities in cold environments is one of the strategy adapted by microbes to withstand increased UV radiation. Higher pigment production by microbial populations recovered from glaciers, ice cores and sea surfaces clearly indicating role of pigment in adaptation to elevated UV radiation (Foght *et al.,* 2004; Agogue *et al.,* 2005; Zhang *et al.,* 2008). Interestingly, a large number of unknown pigment produced by studied bacterial isolates, with almost similar retention time and UV absorption spectra were observed in the contemporary research work. We believed that such similar looking unknown pigment perhaps central to cope with damaging effects of low temperatures that would need further investigation.

In addition, it has been observed that bacterial isolates (GS_1 and GW_1 identified as *Arthrobacter agilis* and *Janthinobacterium lividum*, respectively) have reduced quantity of produced pigment with raising temperature but enhanced pigments production at lower temperature. The bacterial isolate GW_1 has produced high pigment at 5°C (352 µg g^{-1}) as compare to 35°C (81 µg g^{-1}). Similarly, GS_1 isolates also shown same pattern. These results highlight the possible role of pigment to stabilize the cytomembrane at lower temperature as cell membrane faces fluidity maintenance at reduced temperature. It has been previously linked to a relationship between increased

pigment production and decreased temperature, probably related to the maintenance and increased firmness of the cytomembrane (Foght *et al.*, 2004). Few researchers have also suggested psychrophilic microbes that produce pigments in order to protect organelles from oxidation resultant from extreme UV radiation such as reported from the glaciers of the Tibetan Plateau (Remias *et al.*, 2010: Boric *et al.*, 2011). Gorton and Vogelmann (2003) reported carotenoid pigment protecting cytoplasm of *Chlamydomonas nivalis* from UV-B but also suggested role of integral parts of the cell in protection against UV radiation.

In current study, GI_4, HI_1, HI_3 (identified as *Massilia aurea*), GS_8 and HI_7 (*Massilia timonae*) have been observed to produce β-carotene and 19' hexanoyloxyfucoxanthin. *Massilia timonae* and *Massilia aurea* has been reported with yellow pigment in previous studies but composition of pigment of both bacteria have not yet been identified (Gallego *et al.*, 2006). Bacterial isolates GS_5 (identified as *Flavobacterium xinjiangense*), GS_7 and HS_3 (*Flavobacterium sinopsychrotolerans*) were able to produce β-carotene and zeaxanthin. Both *Flavobacterium xinjiangense* and *Flavobacterium sinopsychrotolerans* producing pale yellow pigment were isolated from China No 1 glacier (Zhu *et al.*, 2003; Xu *et al.*, 2011) but with no exploration of pigment components. In addition, GS_9 (identified as *Paracoccus hibiscisoli*), HS_{16} and GS_{19} (*Paracoccus carotinifaciens*) were found to produce β-carotene, astaxanthin and zeaxanthin. To the best of our knowledge, none of *Paracoccus hibiscisoli* or *Paracoccus carotinifaciens* has been found to produce β-carotene and zeaxanthin. However, astaxanthin pigment has been reported from *Paracoccus carotinifaciens* (Tsubokura *et al.*, 1999).

Brevundimonas mediterranea GS_{18}, *Brevundimonas intermedia* GS_{21}, *Brevundimonas nasdae* GhW_6, Brevundimonas *vesicularis* GW_4 and *B. vesicularis* HW_6 were able to produce astaxanthin, β-carotene and 19'hexanoyloxyfucoxanthin. To the best of our knowledge, β-carotene and 19'hexanoyloxyfucoxanthin have not yet been identified from above mentioned bacterial species but astaxanthin producing *Brevundimonas vesicularis* has recovered from marine haunts (Nishida *et al.*, 2005; Asker, 2017; Asker *et al.*, 2018). In addition, *Janthinobacterium lividum* GW_6 was able to produce purple colored pigment violacein. Production of violacein from *Janthinobacterium lividum* has been reported by many researchers in previous studies (Masuelli *et al.*, 2016; Kanelli

et al., 2018). *Arthrobacter agilis* GS_1 produced dark red rose like pigment as reported earlier from *Arthrobacter agilis* isolated from Pony Lake, Antarctica by Dieser *et al.* (2010).

In addition, *Deinococcus depolymerans* GhS_1, *Deinococcus aquaticus* GhS_8, *D. aquaticus* GW_7 and *Sphingomonas faeni* GW_8 produced astaxanthin in the present study. It has already been reported that *Deinococcus* could produce similar reddish to pink pigment (Im *et al.,* 2008; Asker *et al.,* 2011). Recently, Sajjad *et al.* (2017) isolated *Deinococcus* sp. that produced astaxanthin with antioxidative activities. Astaxanthin producing psychrotrophic *Sphingomonas faeni* has been reported by Mageswari *et al.* (2015). *Sanguibacter antarcticus* GhW_7 produced astaxanthin and β-carotene. Hong *et al.* (2008) isolated yellow pigment producing *Sanguibacter antarcticus* producing yellow pigment from Antarctic sea sand.

Conclusion

It is concluded from the current study that 82 different types of bacterial strains were isolated from Ghulmet, Hopar and Ghulkin glaciers, located in Karakorum mountain range, Pakistan. Glaciochemistry of samples showed Ca^{+2} and SO_4^- to be present in higher concentrations in all glacial samples. 16S rRNA and rpoB gene sequencing revealed most of the bacterial isolates belonged to genus *Pseudomonas*. In addition, bacterial isolates were found to produce both types of pigments including carotenes and xanthophylls but most of bacterial isolates produced β-carotene followed by zeaxanthin. Glaciochemistry would help in future to monitor any detrimental effect of climate change on the health of glaciers located in Karakoram mountain range, Pakistan, as they are at high risk to global warming. Likewise, this research work reported bacterial diversity in glaciers with enhanced ability to produce variety of carotenoid pigments that would be an alternative to the pigments extracted from synthetic and other (e.g. plants).

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Chapter 4

Cell membrane fatty acids of psychrophilic bacteria and their role in cold adaptation

Abstract

Bacteria colonizing non-polar glaciers are vulnerable to lots of fluctuations in temperatures which distress their cell membrane fluidity. Psychrophilic bacteria vary composition of cell membrane fatty acids in order to overcome with consequences resulted from such variations in temperatures. Knowledge about the exact pattern of cell membranes adaptation in bacteria residing cold habitats of non-polar glaciers is insufficient. In the contemporary research study, 42 species of bacteria have recovered from the glaciers of Hunza Nagar valley, Karakorum mountains range, Pakistan and studied fatty acid distribution in their cell membranes using chromatographic techniques such as GC/MS. Fatty acid methyl esters (FAMEs) were analysed through GC-MS which were extracted from bacteria by acid catalyzed methanolysis process. Moreover, effects of diverse range of temperature (5, 15, 25 and 35°C) on fatty acid distribution in cell membranes of a number of bacterial species belonged to gram positive and gram negative groups were also carried out in this study. This analyses revealed presence of *n*-MUFAs and br-FAs as a key classes of fatty acids which accounted more than seventy percent (70%) out of total analysed fatty acids. Temperature directly affected distribution of fatty acids including both branched chains and non-branched chains fatty acids as their production in cell membranes was either decreased or increased with lowering or raising temperature. Furthermore, accumulation of polyunsaturated fatty acids in bacterial cell membranes have also been seen in this study but only at lower temperature (5 and 15°C). The role of br-FAs in upkeeping cell membrane flexibility of bacteria inhabiting non-polar habitats is highlighted for the first time through this study.

Keywords: Cold adaptation, cold-adapted bacteria, fatty acid methyl esters, low temperature, membrane fluidity

Introduction

Bacterial internal temperature varies greatly as they are poikilotherms and cannot maintain thermal homeostasis (Russell, 1983). The most abundantly found phospholipids within bacteria are phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol (also known as cardiolipin) (Goldfine, 1982). Phosphatidylethanolamine is found to be the most abundant in Gram negative bacteria, while phosphatidylglycerol and cardiolipin are the principal phospholipids in Gram positive bacteria (Russell, 1983). Previous studies have shown the presence of phospholipids in both cytoplasmic membranes and outer membranes of Gram negative bacteria in addition to their lipopolysaccharide in their outer membrane (Bajerski *et al.,* 2017). Sterols are not present in bacterial cell membranes but they might have glycolipid in smaller quantity, such as Streptococci contain glycolipids (majorly in the form of glucosyldiacylglycerol) as predominant constituent of lipids (Russell, 1989). Any modification in nature of the polar head group or the acyl side chains is associated with promoting alteration of bacterial membrane composition (Russell, 1984; Boggs, 1986). Acyl side chains of microbial SFAs or MUFAs with 12 to 24 carbon atoms consist of branches and various ring structures like cyclopropyl, -pentyl, and -hexyl rings. Straight-chain fatty acids lipids of Gram negative bacteria have even-number while Gram positive bacterial lipids consist of odd numbered fatty acids (Russell, 1983).

Up till now, biggest part of biosphere on planet earth including oceans, is wide-open to low temperatures which is underneath 5°C either temporarily season based or permanently (Margesin *et al.,* 2007; Hoshino and Matsumoto, 2012). Cold habitations such as Arctic and Antarctica, which is deprived of direct sunlight, also high peak mountains and deep oceans experience freezing temperatures. Amid of these cold territories, deep sea represents major component of cryosphere which covers 71% of our planet earth with almost 90% of ocean that has temperature below 5°C. Likewise, cold soils (superficial soil), caves, lakes and other large cold habitats do also exist covering large portion of earth e.g. just glacial habitats are found to be cover about 10% of the earth surface (Singh *et al.,* 2006). Cold-adapted bacteria present in cold settings experience elongated frozen temperatures and diurnal freeze thaw cycles (Montiel, 2000). They have to manage and survive under special challenges of freezing

temperatures which are also accompanied by slow chemical reactions, very little or limited activity of enzymes, denaturation of protein structure and decrease in fluidity of cell membrane (Hassan *et al.,* 2016) as well as limited water availability which acts as universal solvent for bio chemical reactions (Wynn-Williams, 1990). Under such punitive conditions, bacteria have evolved versatile adaptabilities to cope with harsh environmental conditions such as variations in pH and temperature (Ganzert *et al.,* 2011; Bajerski and Wagner, 2013). These adaptive strategies include formation of compatible solutes as well as expression of shock (both hot and cold) proteins (Georlette *et al.,* 2004). In addition, the utmost important strategy, which bacteria employed in extreme low temperatures, is to bring variation in cell plasma membrane arrangement as it is involved in metabolism of all vital products and acts as a major interconnection in electron transport chain. The important phenomena in bacterial communities is homeoviscous adaptation i.e. changing in fatty acids composition of cell membranes to maintain the fluidity of cell membranes in response to external shift in environmental conditions (Sinensky, 1974). Psychrophilic bacteria tend to synthesize those fatty acids offering long range of melting points which play central role to keep membranes lipids in their right transition states (Mansilla *et al.,* 2004). For example, br-FAs, MUFAs and PUFAs are fatty acids with low melting points which have been found to provide the requisite fluidity of cell membrane in more or less all living entities (Hazel *et al.,* 1990; Suutari and Laakso, 1994).

Presence of low temperatures and elevated pressures are central to bring alteration in integrity of cell membranes by introducing irreversible changes e.g. shifting from fluid state to rigid state (Cossins *et al.,* 1984; Hazel *et al.,* 1990). Bacteria, which adapted themselves to low temperatures and high pressures, basically involve to alter membrane phospholipids composition by increasing content of br-FAs and unsaturated fatty acids (Delong *et al.,* 1986; Wirsen *et al.,* 1986; Nichols *et al.,* 1998). Nonetheless, these findings have been acquired by working on only bacteria of gram negative group that resided abysmal sea and Antarctica habitats. The existent study aims for exploring profiles of cell membrane fatty acids of psychrotolerant gram positive and gram negative bacteria, isolated from non-polar glaciers as well as to determine role of saturated and unsaturated fatty acids (SFAs and USAs), straight chains as well as branched chains fatty acids in adaptability of bacteria to low temperatures.

Material and methods

Selection of bacterial isolates and culturing conditions

A total of 42 bacterial strains representing 23 different genera were selected for this study, described in detail in chapter 3 (Table. 4.1a and 4.1b). Briefly, cultures of every selected species were inoculated in 50 mL Nutrient Broth (NB) and kept at 15°C for 5-10 days. The bacterial cultures were centrifuged at centrifuging speed of 45,00 g in 50 mL germ free tubes for half hour. The harvested bacterial cultures were then preserved in 35% glycerol in small 2,000 µL stowage vials and stored at -20°C for further use.

Production media for bacterial growth

Fatty acids of bacterial cell membranes were analysed using Nutrient Agar (NA) and Reasoner's 2A (R2A) as production media. The selected bacteria were grown in 250 mL Erlenmeyer flasks (Fisher Scientific) containing 50 mL growth media and incubated at 15°C for 5-7 days. The biomass of selected bacterial species was collected by centrifugation of cells culture at 45,00 g for half hour. The resultant pellets of cell cultures were kept in 2 mL small storage tubes and freeze-dried for later usage.

Preparation of fatty acid methyl esters (FAMEs)

Extraction of FAMEs from all bacterial species was carried out by using 5% methanolic HCl. In short, about 100 mg biomass of each bacteria was mixed with 2,000 µL of 5% methanolic HCl and kept in water bath at 70°C for 2 hrs in tightly closed sterile glass tubes. Then tubes were cooled down by placing at room temperature for half hour.

After that 1 mL milli-Q water and 2 mL hexane were added to glass tubes and tightly closed their seals. The mixture was stoutly vortexed and the fatty acid methyl esters were extracted. After some time, two layers were formed in the tubes and then upper clean layer was removed and stored in a clean tube. Finally, the transferred clean layer in tube was dried under nitrogen.

Analysis of cell membrane fatty acid on different temperature

In this study, 10 different bacterial species representing various genera were selected and analysed for similarity and dissimilarity in distribution of fatty acids in cell membranes on different temperature. In addition, the studied bacteria were belonged to both Gram negative and positive groups. Bacteria were selected on their capability to show massive growth on a varied array of temperature and also their selection for the first time for such studies. Concisely, cultures of bacterial species were inoculated in NB and placed at 5, 15, 25 and 35°C for 7 days.

The 100 mg biomass of selected bacterial species was collected by centrifugation of cells culture at 45,00 g for half hour. FAMEs were then extracted from the resultant cell pellets. The same procedure was used as described above for extraction of FAMEs using 5% methanolic HCl. After extraction, FAMEs were subjected GC/MS analysis for identification and quantification purposes.

Gas chromatography/mass spectroscopy (GC/MS) analysis

After adjusting the FAMEs concentration suitable for GC/MS analysis, extracted FAMEs of all the bacterial isolates were subjected to GC/MS using procedure narrated by Abd El Razak *et al.* (2014) with few modifications. The GC-MS instrument was consist of a ThermoScientific Trace 1300 gas chromatograph combined with Thermo Scientific ISQ LT single quadrupole mass spectrometer. An injector, programmable temperature vaporising (PTV), was fixed with GC. In addition, an Agilent-HP 1 capillary column covered with a 100% dimethylpolysiloxane stationary phase (50 m × 0.32 mm internal diameter × 0.17 mm film thickness) was also fitted with GC. The flow rate of carrier gas, helium, was 2 mL min^{-1}. The analytes was programmed to transfer onto the column via heating up PTV up to 300°C at 14°C sec^{-1}. The temperatures were programmed as starting temperature set at 50°C, then increased to 100°C at 10°C/min, 250°C at 4°C/min and finally to 300°C at 20°C/min.

Identification of extracted FAMEs was carried out by determination of their molecular weights and comparing their retention times with purchased commercial standards (Sigma Aldrich C_4-C_{24} FAMEs mix, including unsaturated fatty acids). Likewise, quantification of all FAMEs was done by calculating their peak areas and using methyl

tetracosanoate (Sigma-Aldrich) as an internal standard (Fig. 4.1). Finally, GraphPad Prism 5.00 software was used to calculate the correlations between different temperature (5, 15, 25 and 35°C) and identified fatty acids produced by selected bacteria species.

Results

In the present research work, *n*-MUFAs were found major group of fatty acids in cell membranes of 24 bacterial species, whereas 18 isolates accumulated br-FAs as a key group of fatty acids. The major group and individual fatty acids in various psychrotrophic bacterial species are given in (Table. 4.1a and 4.1b). The chief type of *n*-MUFAs observed in cell membranes were *n*-$C_{15:1(cis\text{-}10)}$, *n*-$C_{16:1(cis\text{-}9)}$ and *n*-$C_{18:1(tr\text{-}9)}$, whereas *i*-$C_{15:0}$, *a*-$C_{15:0}$ and *a*-$C_{17:0}$ were the dominantly found types of br-FAs. Generally, *a*-$C_{15:0}$ was the most common fatty acid which was found in cell membranes of 15 bacteria, followed by *n*-$C_{16:1(cis\text{-}9)}$ in 12 species and *n*-$C_{18:1(tr\text{-}9)}$ was detected in cell membranes of 10 bacterial species. In addition, *i*-$C_{15:0}$ was observed in just one species as a foremost type of fatty acid. There were four species having *ai*-$C_{17:0}$ and *n*-$C_{15:1(cis\text{-}10)}$, as principal fatty acid moieties in their cell membranes, respectively.

Table 4.1a. List of bacterial strains producing *n*-MUFAs as main group of cell membrane fatty acids

Bacterial species	% of *n*-MUFAs /CM FA	Major fatty acids (%/cell membrane FA)	
Deinococcus aquaticus GW7	78.3	*****n-$C_{15:1(cis\text{-}10)}$	32.9
Massilia aurea HI1	84.3	n-$C_{15:1(cis\text{-}10)}$	37.1
Massilia oculi GI1	58.5	n-$C_{16:1(cis\text{-}9)}$	50.8
Pseudomonas brassicacearum GS2	69.4	n-$C_{16:1(cis\text{-}9)}$	35.8
Pseudomonas migulae GS3	49.0	n-$C_{16:1(cis\text{-}9)}$	23.2
Pseudomonas mandelii GS12	72.0	n-$C_{16:1(cis\text{-}9)}$	32.0
Arthrobacter nitroguajacolicus GS13	89.6	n-$C_{16:1(cis\text{-}9)}$	42.0
Paenisporosarcina macmurdoensis GS17	42.0	n-$C_{16:1(cis\text{-}9)}$	15.8
Janthinobacterium lividum GW1	67.1	n-$C_{16:1(cis\text{-}9)}$	52.9
Pseudomonas extremaustralis HS8	60.1	n-$C_{16:1(cis\text{-}9)}$	35.1
Pseudomonas veronii HS9	72.2	n-$C_{16:1(cis\text{-}9)}$	25.8
Pseudomonas fluorescens HS10	71.7	n-$C_{16:1(cis\text{-}9)}$	29.3
Pseudarthrobacter sulfonivorans HS14	70.6	n-$C_{16:1(cis\text{-}9)}$	34.1

Massilia timonae HI7	66.0	*n*-$C_{16:1(cis\text{-}9)}$	65.7
Flavobacterium sinopsychrotolerans GS7	89.0	*n*-$C_{18:1(*tr\text{-}9)}$	50.8
Paracoccus hibiscisoli GS9	94.4	*n*-$C_{18:1(tr\text{-}9)}$	93.2
Brevundimonas vesicularis GS11	72.0	*n*-$C_{18:1(tr\text{-}9)}$	35.9
Brevundimonas mediterranea GS18	77.2	*n*-$C_{18:1(tr\text{-}9)}$	58.6
Brevundimonas intermedia GS21	83.7	*n*-$C_{18:1(tr\text{-}9)}$	57.5
Sphingobium xenophagum GhS4	75.1	*n*-$C_{18:1(tr\text{-}9)}$	60.0
Acinetobacter radioresistens GhS8	78.0	*n*-$C_{18:1(tr\text{-}9)}$	41.0
Brevundimonas nasdae GhW6	77.5	*n*-$C_{18:1(tr\text{-}9)}$	58.2
Sanguibacter antarcticus GhW7	53.6	*n*-$C_{18:1(tr\text{-}9)}$	45.5
Rhizobium giardinii HI5	72.8	*n*-$C_{18:1(tr\text{-}9)}$	61.7

The polyunsaturated fatty acids were detected in plasma membranes of four bacterial strains but just at reduced temperature (5 and 15°C). For example, *Flavobacterium sinopsychrotolerans* GS7 was observed to produce $C_{18:3(cis\text{-}6)}$ and $C_{18:3(cis\text{-}9)}$. Moreover, $C_{18:2(cis\text{-}9)}$, $C_{18:2(tr\text{-}9)}$ and $C_{18:3(cis\text{-}6)}$ were detected in cell membranes of *Janthinobacterium lividum* GW1, $C_{18:2(cis\text{-}9)}$, $C_{18:2(tr\text{-}9)}$, $C_{20:3(cis\text{-}8)}$ and $C_{20:3(cis\text{-}11)}$ in *Brevundimonas nasdae* GhW6 and $C_{15:2(cis)}$, $C_{15:2(tr)}$, $C_{18:2(cis\text{-}9)}$ and $C_{18:2(tr\text{-}9)}$ were detected in plasma membranes of *Sphingomonas faeni* GW8.

Table 4.1b. List of bacterial strains producing br-FAs as main group of cell membrane fatty acids

Bacterial species	**% of br-FA /CM FA**	**Major fatty acids (%/cell membrane FA)**	
Enterobacter cloacae GhS11	73.4	**i*-$C_{15:0}$	34.4
Stenotrophomonas maltophilia GhS14	73.3	*i*-$C_{15:0}$	32.3
Arthrobacter agilis GS1	97.9	**a*-$C_{15:0}$	58.2
Rhizobium herbae GS14	96.6	*a*-$C_{15:0}$	42.3
Sporosarcina psychrophila GS15	82.2	*a*-$C_{15:0}$	59.6
Deinococcus depolymerans GhS1	50.6	*a*-$C_{15:0}$	28.5
Staphylococcus equorum GhS5	90.2	*a*-$C_{15:0}$	55.4

Arthrobacter sulfureus GhS9	98.5	*a*-$C_{15:0}$	56.4
Enterobacter mori GhS12	95.2	*a*-$C_{15:0}$	58.9
Plantibacter auratus HI4	89.0	*a*-$C_{15:0}$	51.9
Arthrobacter psychrolactophilus HS1	93.8	*a*-$C_{15:0}$	60.3
Pseudarthrobacter scleromae HS2	91.1	*a*-$C_{15:0}$	47.7
Bacillus butanolivorans HS4	84.0	*a*-$C_{15:0}$	40.1
Bacillus simplex HS7	93.8	*a*-$C_{15:0}$	49.4
Delftia acidovorans HS13	89.0	*a*-$C_{15:0}$	59.3
Pseudomonas frederiksbergensis HW2	88.4	*a*-$C_{15:0}$	44.0
Sphingomonas faeni GW8	96.6	*a*-$C_{17:0}$	38.5
Acidovorax radices GW9	98.3	*a*-$C_{17:0}$	43.6

In addition, either br-FAs or *n*-MUFAs were covering above 70% of total plasma membranes fatty acids in 32 bacterial isolates. For example, 94% of identified fatty acids in *Paracoccus hibiscisoli* GS9 were found to belong *n*-MUFAs, 89.6% in *Arthrobacter nitroguajacolicus* GS13, 89.0% in *Flavobacterium sinopsychrotolerans* GS7, 84.3% in *Massilia aurea* HI1 and 83.7% fatty acids in *Brevundimonas intermedia* GS21 were recognized as *n*-MUFAs in this study.

Furthermore, the br-FAs in 10 bacteria species covered more than 90% out of total fatty acids such as 98.3% of fatty acids in cell membrane of *Arthrobacter sulfureus* GhS9 was br-FAs. Similarly, 97.9% fatty acids in *Arthrobacter agilis* GS1, 96.7% in *Sphingomonas faeni* GW8, 96.6% in *Rhizobium herbae* GS14, 95.2% fatty acids in *Enterobacter mori* GhS12, 93.8% in *Bacillus simplex* HS7, 93.7% fatty acids in *Arthrobacter psychrolactophilus* HS1, 91.1% in *Pseudarthrobacter scleromae* HS2 and 90.2% fatty acids in cell membrane of *Staphylococcus equorum* GhS5 were br-FAs. In addition, few bacteria produced a single fatty acid compound abundantly such as *Paracoccus hibiscisoli* GS9 and *Rhizobium giardinii* HI5 have accumulated *n*-$C_{18:1(tr\text{-}9)}$ covering 93.3% and 61.7% out of total fatty acids, respectively.

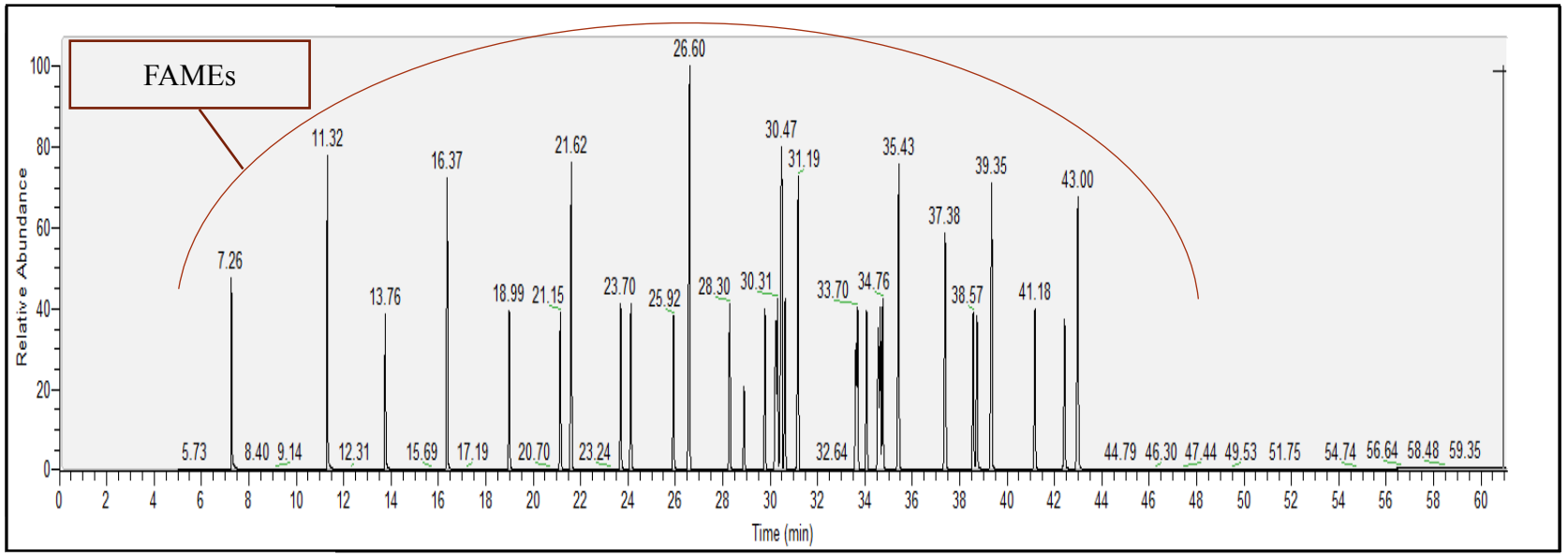


Fig. 4.1. (a) Gas chromatography/mass spectroscopy chromatograph/profile of external FAMEs standards with retention times. **(b)** Fatty acids methyl acids (FAMEs) and retention time (min); $C_{8:0}$ = 7.26, $C_{10:0}$ = 11.32, $C_{11:0}$ = 13.76, $C_{12:0}$ = 16.37, $C_{13:0}$ = 18.99, $C_{14:1(cis\text{-}9)}$ = 21.15, $C_{14:0}$ = 21.62, $C_{15:1(cis\text{-}10)}$ = 23.70, $C_{15:0}$ = 24.08, $C_{16:1(cis\text{-}9)}$ = 25.92, $C_{16:0}$ = 26.60, $C_{17:1(cis\text{-}9)}$ = 28.30, $C_{17:0}$ = 28.85, $C_{18:3(cis\text{-}6)}$ = 29.74, $C_{18:2(cis\text{-}9)}$ = 30.19, $C_{18:3(cis\text{-}9)}$ = 30.21, $C_{18:2(tr\text{-}9)}$ = 30.43, $C_{18:1(cis\text{-}9)}$ = 30.47, $C_{18:1(tr\text{-}9)}$ = 30.59, $C_{18:0}$ = 31.19, $C_{19:0}$ = 33.35, $C_{20:4(cis\text{-}5)}$ = 33.58, $C_{20:5(cis\text{-}8)}$ = 33.64, $C_{20:3(cis\text{-}8)}$ = 34.04, $C_{20:3(cis\text{-}11)}$ = 34.52, $C_{20:2(cis\text{-}11)}$ = 34.60, $C_{20:1(cis\text{-}11)}$ = 34.76, $C_{20:0}$ = 35.43, $C_{21:0}$ = 37.34, $C_{22:5(cis\text{-}9)}$ = 37.38, $C_{22:2(cis\text{-}13)}$ = 38.57, $C_{22:1(cis\text{-}13)}$ = 38.69, $C_{22:0}$ = 39.35, $C_{23:0}$ = 41.18, $C_{24:1(cis\text{-}15)}$ = 42.39, $C_{24:0}$ = 43.00.

In the current study, the effect of different temperatures on the distribution of fatty acids in the cell membranes of 10 bacterial species have been comprehensively analyzed (Fig. 4.2a and 4.2b). The selected bacteria belong to Gram-negative (e.g. *Flavobacterium sinopsychrotolerans* GS7, *Paracoccus hibiscisoli* GS9, *Janthinobacterium lividum* GW1, *Sphingomonas faeni* GW8, *Brevundimonas nasdae*-GhW6, *Rhizobium giardinii* HI5 and *Pseudomonas extremaustralis*-HS8) and Gram-positive groups (e.g. *Sporosarcina psychrophila* GS15, *Staphylococcus equorum* GhS5 and *Arthrobacter psychrolactophilus* HS1). In addition, correlations between various temperature and single type of cell membrane fatty acid is enlisted in (Table 4.2a and 4.2b).

Table 4.2a. Correlations between various temperature and single type of cell membrane fatty acid (Gram-negative bacteria)

Correlations					
Fatty acids (mg/g)	**Temperature (°C)**				**Correlation (R^2)**
	5	**15**	**25**	**35**	
***Rhizobium giardinii* HI5**					
***n*-C$_{16:0}$**	0.034	0.135	0.253	0.404	*0.991***
***n*-C$_{18:0}$**	0.085	0.163	0.252	0.369	*0.991***
***ai*-C$_{19:1}$**	0.315	0.606	0.803	0.961	*0.980**
***n*-C$_{16:1(cis\text{-}9)}$**	1.313	0.872	0.531	0.122	*0.997***
***n*-C$_{18:1(tr\text{-}9)}$**	1.116	1.248	1.501	1.694	*0.987***
***Brevundimonas nasdae* GhW6**					
***n*-C$_{15:0}$**	0.072	0.149	0.174	0.212	*0.943**
***n*-C$_{16:0}$**	0.195	0.423	0.520	0.724	*0.979**
***n*-C$_{17:1(tr\text{-}10)}$**	0.328	0.189	0.153	0.071	*0.944**
***n*-C$_{16:1(cis\text{-}9)}$**	0.643	0.371	0.114	0.070	*0.928*
***n*-C$_{18:1(tr\text{-}9)}$**	1.716	1.765	1.923	2.045	*0.964**
***Flavobacterium sinopsychrotolerans* GS7**					
***n*-C$_{16:0}$**	0.215	0.378	0.542	0.923	*0.948**
***n*-C$_{17:0}$**	0.036	0.046	0.150	0.233	*0.923*
***n*-C$_{16:1(cis\text{-}9)}$**	1.073	0.930	0.422	0.291	*0.936*
***n*-C$_{17:1(tr\text{-}10)}$**	0.757	0.536	0.145	0.115	*0.920*

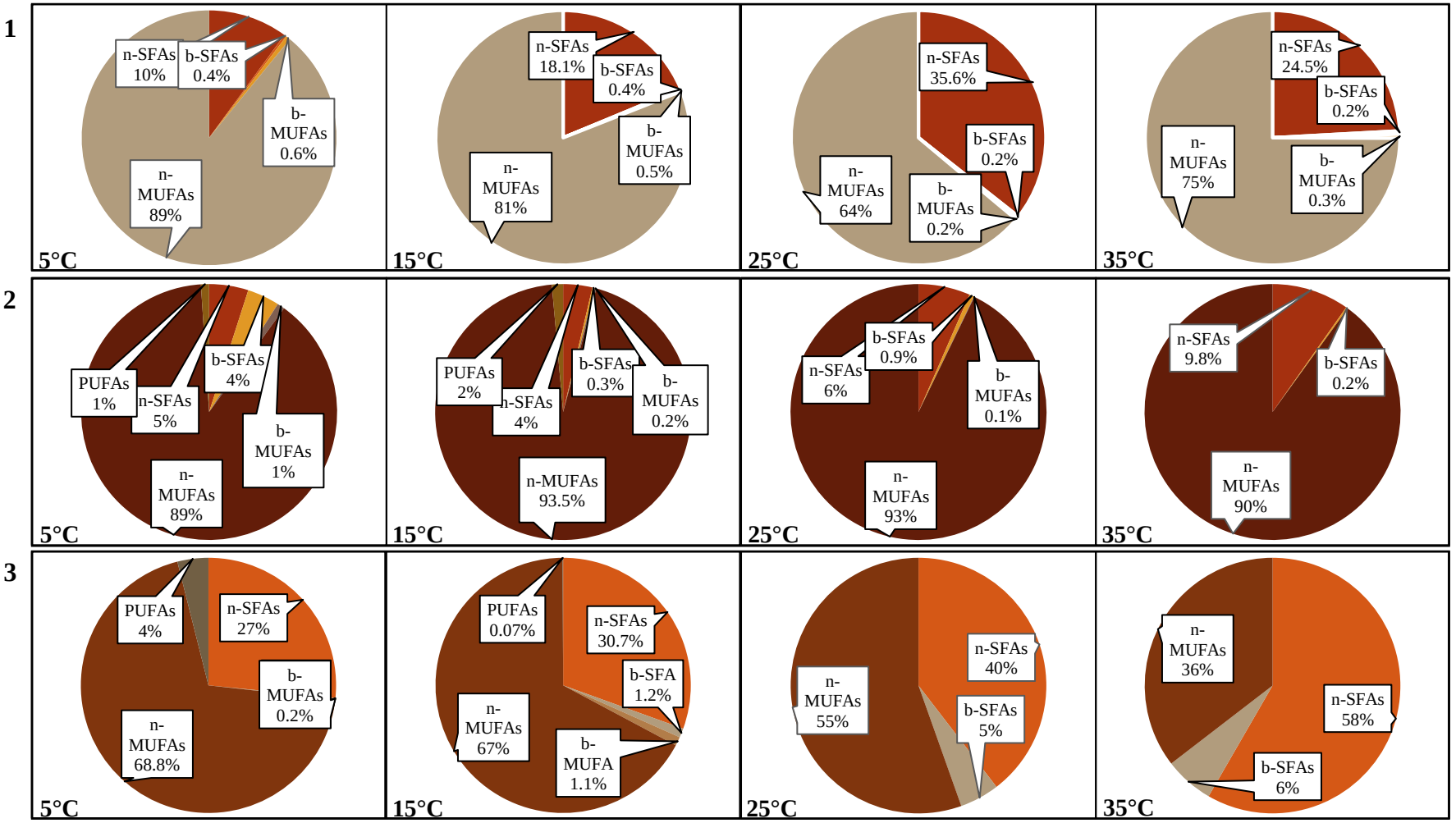
***n*-C$_{18:1(tr\text{-}9)}$**	1.247	1.762	1.82	2.032	*0.874*
***Paracoccus hibiscisoli* GS9**					
***n*-C$_{16:0}$**	0.0003	0.001	0.004	0.010	*0.879*
***n*-C$_{18:0}$**	0.046	0.051	0.092	0.140	*0.911*
***ai*-C$_{15:0}$**	0.012	0.015	0.022	0.028	*0.977**
***i*-C$_{17:1}$**	1.200	0.946	0.03	0.014	*0.880*
***n*-C$_{18:1(tr\text{-}9)}$**	0.043	0.720	1.201	1.576	*0.982***
***Janthinobacterium lividum* GW1**					
***n*-C$_{16:0}$**	0.491	0.521	0.739	0.845	*0.932*
***n*-C$_{18:0}$**	0.003	0.005	0.04	0.059	*0.914*
***n*-C$_{16:1(cis\text{-}9)}$**	0.831	0.636	0.015	0.007	*0.884*
***n*-C$_{16:1(tr\text{-}9)}$**	0.015	0.058	0.268	0.404	*0.949**
***i*-C$_{17:0}$**	0.0006	0.001	0.023	0.017	*0.665*
***Sphingomonas faeni* GW8**					
***n*-C$_{16:0}$**	0.059	0.251	0.478	0.580	*0.979**
***n*-C$_{18:0}$**	0.015	0.401	0.540	0.613	*0.876*
***ai*-C$_{15:0}$**	0.573	0.279	0.124	0.063	*0.911*
***i*-C$_{16:0}$**	0.290	0.177	0.091	0.043	*0.969**
***ai*-C$_{17:0}$**	0.477	0.225	0.076	0.056	*0.880*
***Pseudomonas extremaustralis* HS8**					
***n*-C$_{16:0}$**	0.154	0.329	0.551	0.888	*0.977**
***i*-C$_{15:0}$**	0.228	0.161	0.129	0.004	*0.937*
***ai*-C$_{15:0}$**	0.432	0.231	0.094	0.0002	*0.972**
***ai*-C$_{17:1}$**	0.084	0.057	0.004	0.000	*0.918*
***n*-C$_{16:1(cis\text{-}9)}$**	0.961	0.751	0.676	0.618	*0.904*
Keys; **P* < 0.05 level ***P* < 0.01 level					

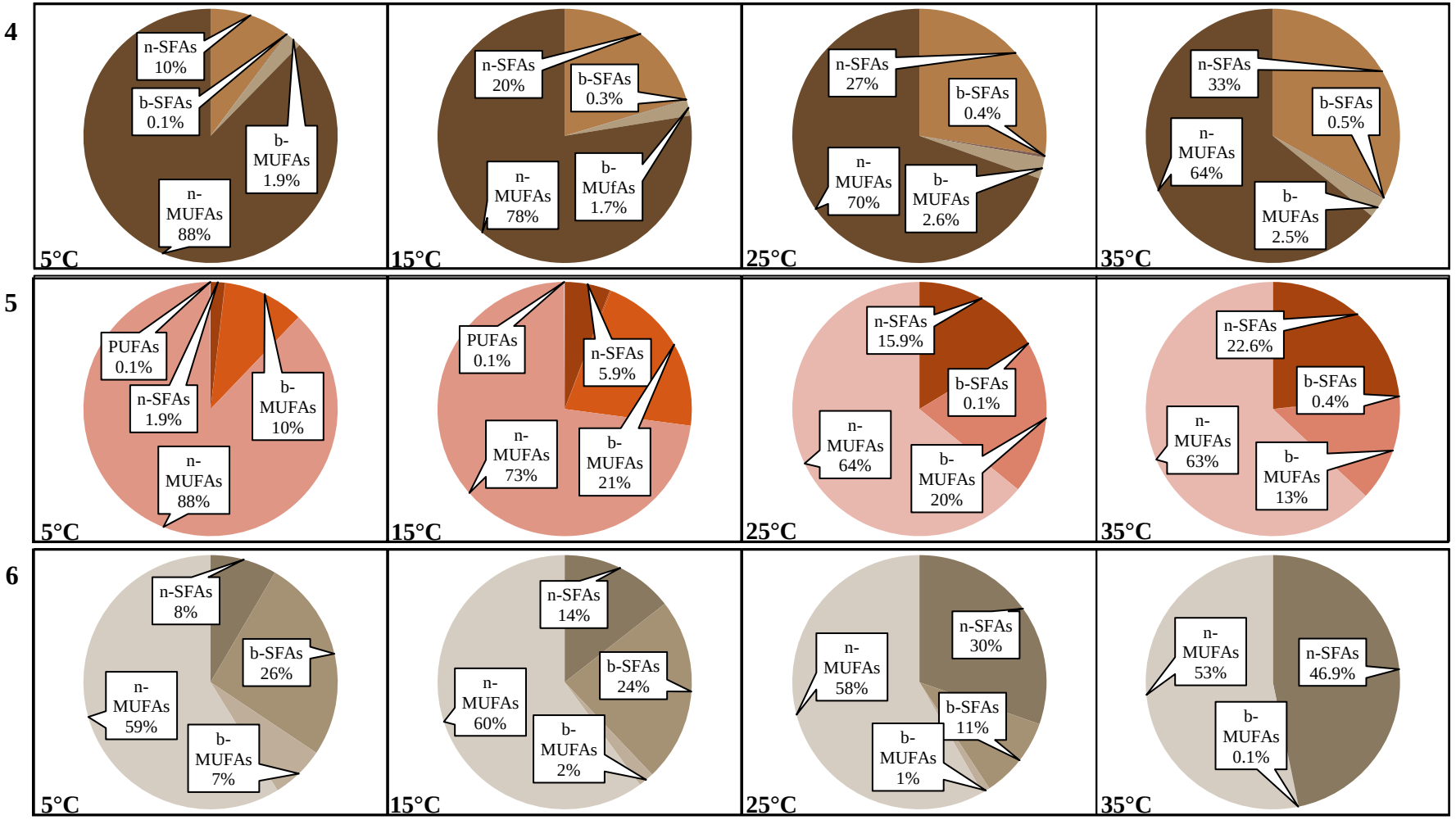
Comparatively, Gram-negative group of bacteria were found to accumulate *n*-MUFAs, whereas Gram positive bacteria were tended to produce br-FAs as key groups of fatty acids (Fig. 4.2a and 4.2b). However, *Sphingomonas faeni* GW8 (Gram-negative

bacterium) was the only species producing br-FAs as a principle fatty acids among other Gram-negative bacteria. Overall, Gram negative bacteria were observed to distribute highest *n*-MUFAs in their plasma membranes at decreased temperature (5 and 15°C) than elevated temperature (25 and 35°C).

Table 4.2b. Correlations between various temperature and single type of cell membrane fatty acid (Gram-positive bacteria)

Correlations					
Fatty acids (mg/g)	**Temperature (°C)**				**Correlation (R^2)**
	5	**15**	**25**	**35**	
***Staphylococcus equorum* GhS5**					
***n*-C$_{16:0}$**	0.005	0.009	0.034	0.062	*0.923*
***i*-C$_{15:0}$**	0.132	0.251	0.323	0.438	*0.991***
***ai*-C$_{15:0}$**	0.742	0.920	1.130	1.390	*0.992***
***i*-C$_{19:1}$**	0.165	0.094	0.032	0.009	*0.957**
***ai*-C$_{19:1}$**	0.255	0.177	0.061	0.002	*0.986***
***Sporosarcina psychrophila* GS15**					
***i*-C$_{15:0}$**	0.037	0.103	0.254	0.305	*0.962**
***ai*-C$_{15:0}$**	1.055	1.145	1.438	1.605	*0.964**
***i*-C$_{15:1}$**	0.342	0.279	0.102	0.0001	*0.970**
***ai*-C$_{15:1}$**	0.867	0.771	0.214	0.0004	*0.932*
***Arthrobacter psychrochitiniphilus* HS1**					
***n*-C$_{16:0}$**	0.002	0.011	0.02	0.041	*0.948**
***ai*-C$_{15:0}$**	0.805	0.994	1.101	1.186	*0.964**
***ai*-C$_{17:0}$**	0.635	0.635	0.794	0.874	*0.997***
***ai*-C$_{16:1}$**	0.014	0.008	0.004	0.0006	*0.982**
Keys; *P < 0.05 level **P < 0.01 level					


1
n-SFAs 10%
b-SFAs 0.4%
b-MUFAs 0.6%
n-MUFAs 89%
5°C
n-SFAs 18.1%
b-SFAs 0.4%
b-MUFAs 0.5%
n-MUFAs 81%
15°C
n-SFAs 35.6%
b-SFAs 0.2%
n-MUFAs 64%
b-MUFAs 0.2%
25°C
n-SFAs 24.5%
b-SFAs 0.2%
n-MUFAs 75%
b-MUFAs 0.3%
35°C
2
PUFAs 1%
n-SFAs 5%
b-SFAs 4%
b-MUFAs 1%
n-MUFAs 89%
5°C
PUFAs 2%
n-SFAs 4%
b-SFAs 0.3%
b-MUFAs 0.2%
n-MUFAs 93.5%
15°C
n-SFAs 6%
b-SFAs 0.9%
b-MUFAs 0.1%
n-MUFAs 93%
25°C
n-SFAs 9.8%
b-SFAs 0.2%
n-MUFAs 90%
35°C
3
PUFAs 4%
n-SFAs 27%
b-MUFAs 0.2%
n-MUFAs 68.8%
5°C
PUFAs 0.07%
n-SFAs 30.7%
b-SFA 1.2%
n-MUFAs 67%
b-MUFA 1.1%
15°C
n-SFAs 40%
n-MUFAs 55%
b-SFAs 5%
25°C
n-MUFAs 36%
n-SFAs 58%
b-SFAs 6%
35°C


4
n-SFAs 10%
b-SFAs 0.1%
b-MUFAs 1.9%
n-MUFAs 88%
5°C
n-SFAs 20%
b-SFAs 0.3%
n-MUFAs 78%
b-MUfAs 1.7%
15°C
n-SFAs 27%
b-SFAs 0.4%
n-MUFAs 70%
b-MUFAs 2.6%
25°C
n-SFAs 33%
b-SFAs 0.5%
n-MUFAs 64%
b-MUFAs 2.5%
35°C
5
PUFAs 0.1%
n-SFAs 1.9%
b-MUFAs 10%
n-MUFAs 88%
5°C
PUFAs 0.1%
n-SFAs 5.9%
n-MUFAs 73%
b-MUFAs 21%
15°C
n-SFAs 15.9%
b-SFAs 0.1%
n-MUFAs 64%
b-MUFAs 20%
25°C
n-SFAs 22.6%
b-SFAs 0.4%
n-MUFAs 63%
b-MUFAs 13%
35°C
6
n-SFAs 8%
b-SFAs 26%
n-MUFAs 59%
b-MUFAs 7%
5°C
n-SFAs 14%
b-SFAs 24%
n-MUFAs 60%
b-MUFAs 2%
15°C
n-SFAs 30%
n-MUFAs 58%
b-SFAs 11%
b-MUFAs 1%
25°C
n-MUFAs 53%
n-SFAs 46.9%
b-MUFAs 0.1%
35°C

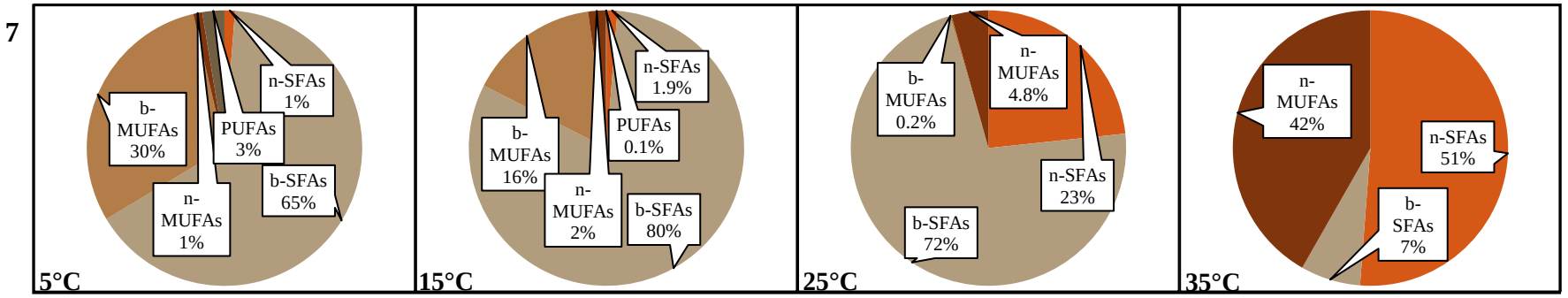


Fig. 4.2a. Temperature derived distributions of the major groups of fatty acids in cell membranes of gram-negative bacteria species: (1) *Flavobacterium sinopsychrotolerans* GS7, (2) *Paracoccus hibiscisoli* GS9, (3) *Janthinobacterium lividum* GW1, (4) *Brevundimonas nasdae* GhW6, (5) *Rhizobium giardinii* HI5, (6) *Pseudomonas extremaustralis* HS8 and (7) *Sphingomonas faeni* GW8.

Both br-SFAs and br-MUFAs (branched chain MUFAs) were produced by Gram positive group of bacteria in elevated quantities in their cell membranes (Fig. 4.2a and 4.2b). However, the ratio of producing br-SFAs was higher in bacterial strains than producing br-MUFAs. Importantly, saturated fatty acids were greatly affected by all temperature as their production in cell membranes were increased with raising temperature and decreased with lowering temperature.

In addition, the quantities of br-SFAs in Gram positive bacteria were also affected by various temperature. Their distribution in plasma membranes elevated by elevating temperature (25 and 35°C) but decreased by decreasing temperature (5 and 15°C) except *Sphingomonas faeni* GW8, which has produced straight chain saturated fatty acids about 51% at 35°C as compared to b-SFAs (65%) at 5°C. Conversely, br-MUFAs production in cell membranes of selected bacteria were witnessed to increase with lowering temperature but decreased with raising temperature.

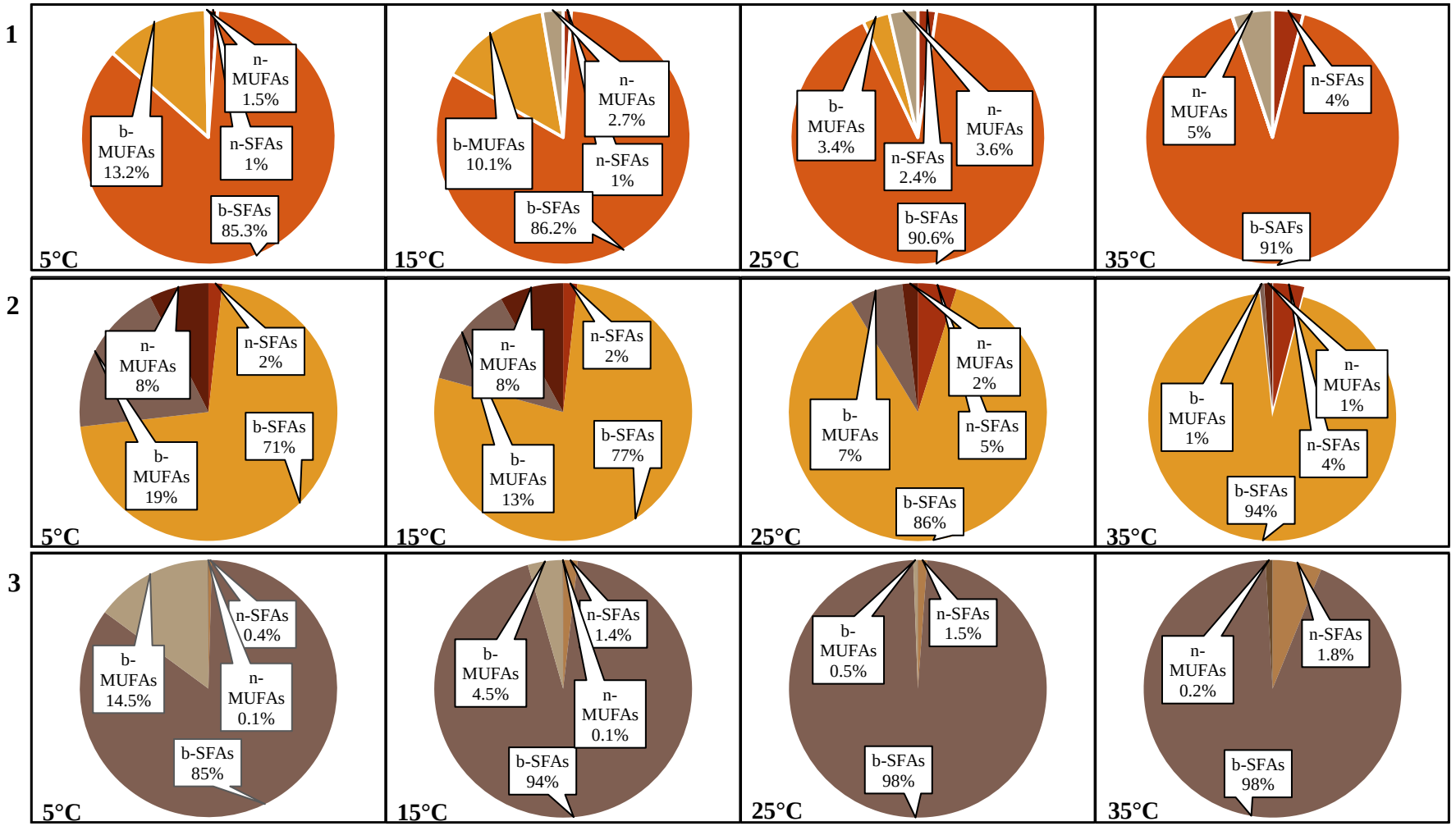

1
b-MUFAs 13.2%
n-MUFAs 1.5%
n-SFAs 1%
b-SFAs 85.3%
5°C
b-MUFAs 10.1%
n-MUFAs 2.7%
n-SFAs 1%
b-SFAs 86.2%
15°C
b-MUFAs 3.4%
n-SFAs 2.4%
n-MUFAs 3.6%
b-SFAs 90.6%
25°C
n-MUFAs 5%
n-SFAs 4%
b-SAFs 91%
35°C
2
n-MUFAs 8%
n-SFAs 2%
b-SFAs 71%
b-MUFAs 19%
5°C
n-MUFAs 8%
n-SFAs 2%
b-SFAs 77%
b-MUFAs 13%
15°C
b-MUFAs 7%
n-MUFAs 2%
n-SFAs 5%
b-SFAs 86%
25°C
b-MUFAs 1%
n-MUFAs 1%
n-SFAs 4%
b-SFAs 94%
35°C
3
n-SFAs 0.4%
b-MUFAs 14.5%
n-MUFAs 0.1%
b-SFAs 85%
5°C
n-SFAs 1.4%
b-MUFAs 4.5%
n-MUFAs 0.1%
b-SFAs 94%
15°C
b-MUFAs 0.5%
n-SFAs 1.5%
b-SFAs 98%
25°C
n-MUFAs 0.2%
n-SFAs 1.8%
b-SFAs 98%
35°C

Fig. 4.2b. Temperature derived distributions of the major groups of fatty acids in cell membranes of gram-positive bacteria species: (1) *Sporosarcina psychrophile* GS15, (2) *Staphylococcus equorum* GhS5, and (3) *Arthrobacter psychrolactophilus* HS1.

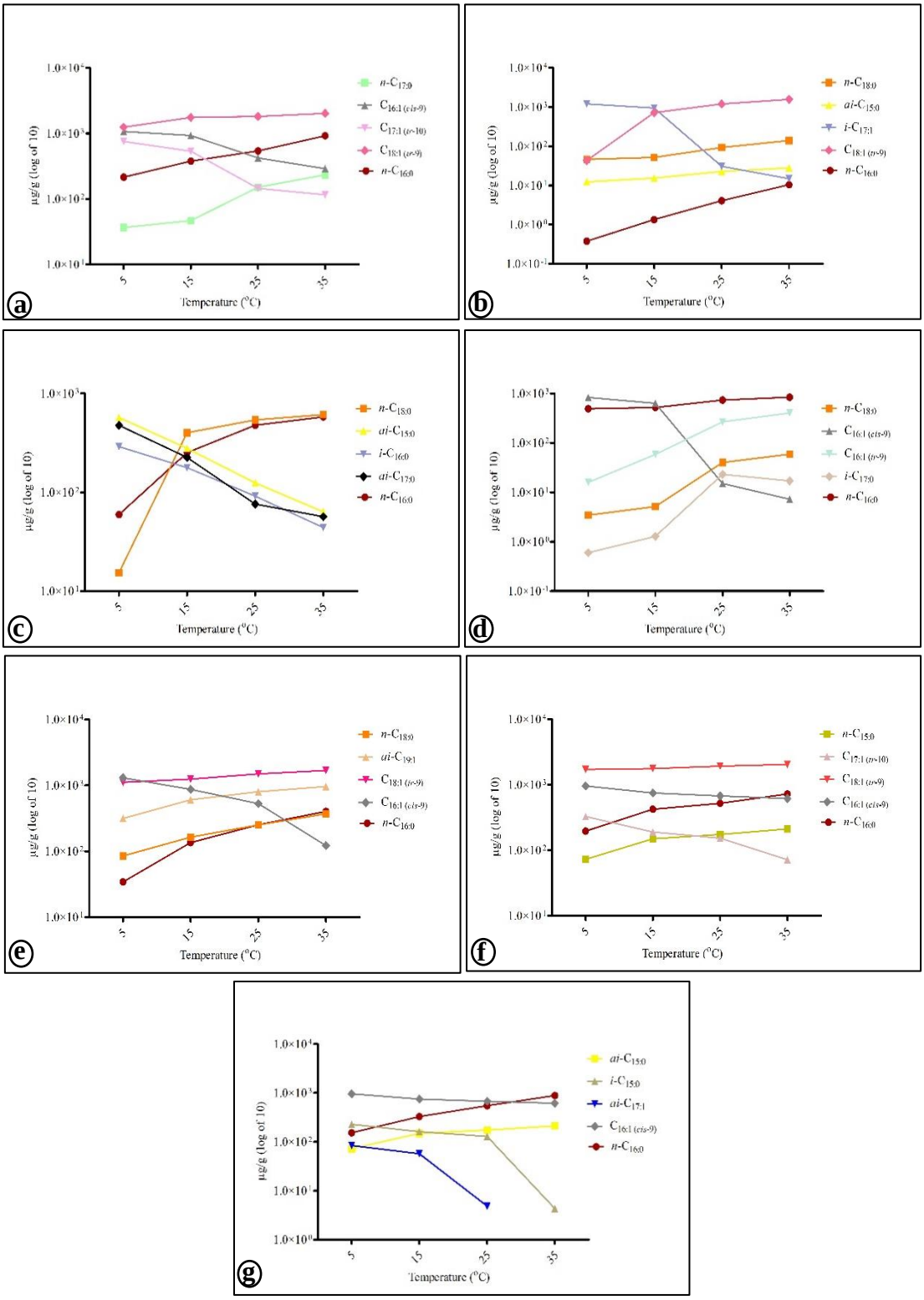


Fig. 4.3a. Distribution of major fatty acids in cell membranes of gram-negative bacteria species at different temperatures: (a) *Flavobacterium sinopsychrotolerans* GS7, (b) *Paracoccus hibiscisoli* GS9, (c) Sphingomonas faeni GW8 (d)

Janthinobacterium lividum GW1, (e) *Rhizobium giardinii* HI5, (f) *Brevundimonas nasdae* GhW6 and (g) *Pseudomonas extremaustralis* HS8

Distribution of distinct fatty acids were either increased or decreased with lowering or raising temperature (Fig. 4.3a and 4.3b). Overall, higher accumulation of *n*-SFAs such as *n*-$C_{15:0}$, *n*-$C_{16:0}$, *n*-$C_{17:0}$ and *n*-$C_{18:0}$ were detected at higher temperatures (25 and 35°C) than at lower temperatures (5 and 15°C). The fatty acids e.g. *n*-$C_{16:1(cis\text{-}9)}$, *n*-$C_{16:1(tr\text{-}9)}$ and *n*-$C_{17:1(tr\text{-}10)}$ and *n*-$C_{18:1(tr\text{-}9)}$ in Gram negative bacteria were found in highest concentration at elevated temperature but observed in low quantities at decreased temperature. However, br-FA *i*-$C_{16:0}$ in *Sphingomonas faeni* GW8 showed upward trend in production at lower temperatures (5 and 15°C).

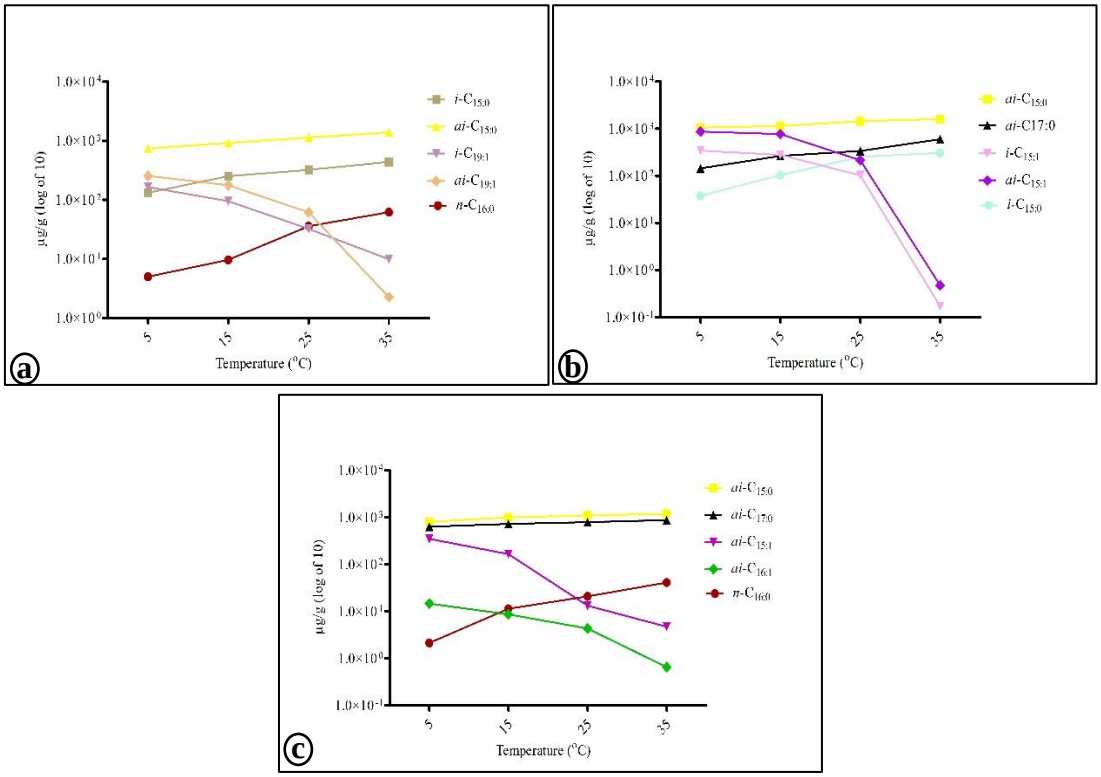


Fig. 4.3b. Distribution of major types of fatty acids in cell membranes of gram-positive bacteria species at different temperatures: (a) *Staphylococcus equorum* GhS5, (b) *Sporosarcina psychrophile* GS15 and (c) *Arthrobacter psychrolactophilus* HS1.

Furthermore, the single br-SFA, *a*-$C_{15:0}$, was the most predominant fatty acid in Gram positive bacteria accumulated in elevated quantity in their cell membranes. Both *i*-$C_{15:0}$ and *a*-$C_{15:0}$ were observed to be progressively produced in high concentrations with

raising temperature but with no adequate difference. But br-MUFAs (such as *i*-$C_{15:1}$, *a*-$C_{15:1}$, *i*-$C_{19:1}$ and *a*-$C_{19:1}$) showed an opposite trend as compare to br-SFAs as they were produced in elevated levels at low temperature (5 and 15°C). Contrariwise, *n*-SFAs e.g. *n*-$C_{16:0}$ and *n*-$C_{18:0}$ were those fatty acids which greatly increased in production at higher temperature.

Discussion

The current research work was aimed to study accumulation and dissemination of phospholipid fatty acids (PLFAs) in plasma membranes of a range of gram negative and positive cold-adapted bacterial strains. The extensive PLFAs profile of bacterial species obtained from Pakistani glaciers could be used to spot and quantify the microbial biomass present in other frozen haunts as every bacterium unveil a unique profile of PLFAs. Among PLFAs, $C_{16:0}$ is commonly found and reported in highest quantities in bacteria of all kinds. PLFAs usage could act as bio-marker for quantification and identification of bacteria which is also getting a popular attention in scientific community (Willers *et al.,* 2015). Moreover, quickest degradation of PLFAs upon bacterial death, makes PLFAs an authentic tool in quantification process. Hence, they are very symbolic to bacterial living biomass and have more accuracy than DNA based approaches because phospholipid fatty acids are readily transform following death of bacteria than DNA (Feinstein *et al.*, 2009; Balasooriya *et al.*, 2014).

PLFA profiling technique based quantification of bacteria helped many researchers to understand basic functions of genes that were involved in different processes of metabolism, screening of pathogenic bacterial strains as well as for studying community structures and their diversity (Buhring *et al.,* 2012; Naeher *et al.,* 2012). However, basic comparison of fatty acids between bacterial groups residing cold as well as from warm habitats for purpose of identification, could be misguided as composition of PLFAs vary greatly with any change in environmental conditions (Willers *et al.,* 2015). Phospholipid fatty acids could be useful in linking the physiological behavior and changes in bacterial population.

Frostegard and Baath (1996) have reported unique characteristic pattern of fatty acids distribution in cell membranes of both gram negative and gram positive bacteria. For example, gram positive bacteria are characterized by presence of br-SFAs, while gram negative bacteria are dominated by *n*-MUFAs in their cell membranes. Therefore, findings of the present study is also validated by literature as *a*-$C_{15:0}$, *n*-$C_{16:0}$ and *n*-$C_{18:1}$ were the most prevailing fatty acids in Gram positive species in current research work (Dong *et al.*, 2014; Fichtner *et al.*, 2014; Reinsch *et al.,* 2014). The main types of cell membrane fatty acids of studied bacterial species were especially *n*-$C_{16:1}$ and *n*-$C_{18:1.}$

Previous studies have also revealed *n*-$C_{16:1}$ and *n*-$C_{18:1}$ as principal phospholipid fatty acids which have also been used as bio signature of Gram negative bacteria in various environments (Tavi *et al.,* 2013; Zheng *et al.,* 2013; Banks *et al.,* 2014; Lange *et al.,* 2014; Reinsch *et al.,* 2014).

Apart from general distribution of phospholipids fatty acids in studied species, we have checked direct effect of growth temperature on their relative abundance and fatty acids composition in membranes of gram positive as well as gram negative bacteria. An increase in temperature caused a considerable reduction of br-SFAs and br-MUFAs from 90% (5 and 15°C) to less than 1% (35°C), whereas there was an increase in production of saturated fatty acids from 0.4-2% to 1.8-51%, especially in gram positive bacteria. Hence these observations could verify the key role of branched fatty acids in adaptability of these bacteria towards the change in growth temperature.

The branched fatty acids maintain normal transition state of bacterial cell membrane by providing reduced melting points, thus empower plasma membranes to execute usual activities at very low temperature (Sun *et al.,* 2012). Low temperatures associated with elevated production of br-FAs in psychrophilic bacteria has been reported formerly in many bacteria e.g. *Listeria monocytogenes* (Annous *et al.,* 1997). To the best of our knowledge, such temperature dependent adaptation has been reported for the first time for bacterial species *Sporosarcina psychrophila*, *Sphingomonas faeni*, *Staphylococcus equorum* and *Arthrobacter psychrolactophilus*. Though, other gram positive bacteria such as *Staphylococci*, have been observed with addition of high amount of br-MUFAs to their plasma membranes at lower temperature (Laura *et al.,* 2018). It has been reported for *bacillus subtilis* and *Bacillus megaterium* that they have accumulated an increased concentrations of alpha fatty acids at very low temperature (Suutari and Laakso, 1992). Likewise, *Listeria monocytogenes* has tended to increase production of alpha branched fatty acids and short chain fatty acids in plasma membrane when temperature dropped from 45°C to 5°C (Annous *et al.,* 1997).

Shift responses in fatty acids composition at varying growth temperatures were noticeable for gram negative bacterial species such as *Rhizobium giardinii* HI5 and *Pseudomonas extremaustralis* HS8. But levels of saturated fatty acids such as $C_{15:0}$, $C_{16:0}$, $C_{17:0}$ and $C_{18:0}$ particularly varied considerable. SFAs could minimize fluidity of

cell membrane and they help bacteria to maintain high temperature (Knothe and Dunn, 2009). Among investigated gram negative bacterial strains, *n*-MUFAs were the basic group of plasma membrane fatty acids present at all temperature but respective level of *n*-$C_{16:1(cis-9)}$ fatty acid decreased with increasing temperature. It has been observed that the presence of MUFAs and their *cis* configuration contributed to lowering melting point of fatty acids at low temperature and enhanced membrane fluidity (Mangelsdorf *et al.,* 2009). For example, SFA *n*-$C_{16:0}$ melts at about 63°C, but addition of one double bond (conversion of *n*-$C_{16:0}$ to *n*-$C_{16:1}$) lowers melting point to almost -1°C (Knothe and Dunn, 2009).

So far, fatty acids based adaptation of cell membranes in response to growth temperature has not yet been reported for above gram negative species. Theberge *et al.* (1996) has made observation for fatty acid distribution in *Rhizobium leguminosarum* at various temperatures (10, 15, 22 and 30°C) containing higher levels of MUFAs and $C_{18:1}$ at reduced temperatures. Likewise, a bacterium, *Chryseobacterium frigidisoli*, was found with high proportions of *i*-$C_{17:1}$ at low temperature i.e. 10°C (Bajerski *et al.*, 2017). MUFAs with *cis*/*trans* configuration were observed in high quantities in plasma membranes of *Pseudomonas syringae*, isolated from Antarctica ice sample (Kiran *et al.,* 2005). Thus all these data endorsing the key role of UFAs, which provide low melting point, in cold adaptation of bacteria (Russell, 1984; 1989).

Overall, the witnessed transferences in distributions of SFAs, MUFAs, br-FAs and PUFAs with various temperature, seemed constant throughout all 10 studied species of Gram-positive and Gram-negative bacteria. This highlights the prospective of developing temperature-sensitive molecular ratios for (non-polar) glacial sceneries e.g. glacial ice, melted water and sediments which accompanied by high extents of bacterial biomass. But such approaches are remain to be done *in situ*.

Conclusion

In conclusion, fatty acids of 42 cold-tolerant bacterial species, belonged to gram positive and gram negative groups, isolated from various glaciers of the Karakorum mountains range, have been analysed for presence of *n*-MUFAs, PUFAs and br-FAs in their respective cell membranes. Besides, fatty acids in bacterial membrane have been greatly affected by shifting in temperature. A profound increase from saturated fatty acids and *n*-MUFAs to br-SFAs with raising temperature has been noticed in bacteria of gram negative group, whereas saturated fatty acids were observed to increase with increasing temperature in gram positive bacterial species. This study signifies fatty acids role in preservation of normal fluid nature of cell membranes in bacterial species from non-polar glaciers. These temperature dependent shifts in fatty acids composition may further adds up to development of temperature sensitive biomolecular agents to track variations of temperature in sub glacial habitats which contain highest proportion of bacterial mass. Ultimately, bio-desirable fatty acids such as polyunsaturated fatty acids, seems to be produce from heterotrophic microbes in glacial habitats as alternative source in future to existed conventional sources.

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Chapter 5

Physiological characterization and cell membrane fatty acid analysis of *Serratia marcescens HI6*

Abstract

In this study, the bacterial isolate HI6 was recovered from ice of Hopar (Bualtar) glacier, Karakoram Mountain Range, Pakistan. Isolate HI6 was identified as *Serratia marcescens* through microscopic and molecular analysis (16S rRNA sequencing). *Serratia marcescens* HI6 analyzed for different physiological characteristics including temperature, pH and culture media. *Serratia marcescens* HI6 showed best growth at 25°C, on LB medium and pH 7. In addition, the Fatty acid methyl ester (FAMEs) was extracted and subjected to Fourier Transform infrared (FTIR) and Gas chromatography/mass spectroscopy (GC/MS) analysis. FTIR and GC/MS analysis has revealed that cell membrane of *Serratia marcescens* HI6 accompanied by various types of fatty acids (FA). *i*-$C_{16:1}$ (35.3% out of total cell membrane fatty acids) was found most predominantly followed by *i*-$C_{15:1}$ (18.5%) and *n*-$C_{16:0}$ (13.2%). Moreover, branched fatty acids (62%) including monounsaturated fatty acids (MUFA) (e.g. *i*-$C_{15:1}$ and *i*-$C_{16:1}$) and saturated fatty acids (SFA) were the dominated types of fatty acids in the cell membrane. A polyunsaturated fatty acid (PUFA), n-$C_{18:2(cis\text{-}9)}$ (known as linoleic acid) was also detected among analyzed fatty acids. This study would be helpful to understand the maintenance of cell membrane fluidity in glacial environments by *Serratia marcescens*.

Keywords: *Serratia marcescens* HI6, Hopar glacier, FAME, linoleic acid, fatty acids

Introduction

The genus *Serratia* belongs to family Enterobacteriaceae and species of genus *Serratia* share very close genotypic and phenotypic characteristics with each other (Grimont and Grimont, 2006; Karkey *et al.,* 2018). A bacterium with ability to produce red pigments was first observed on polenta by Bizio (1823), which was later identified and named as *Serratia marcescens* (Martinec and Kocur, 1961a). Williams and Qadri (1980) have observed many strains of *Serratia marcescens* that were producing a non-diffusible red pigment prodigiosin. The prodigiosin (red colour pigment) produced by these strains appeared akin to a blood stain with quite devastating consequences on various human consumable items such as consecrated wafers, bread, and polenta (Grimont and Grimont, 2006). In this perspective, many researchers had linked the genus *Serratia*'s history, hundreds of years back (Gaughran, 1969; Harrison, 1924; Reid, 1982). Although, prodigiosin or prodigiosin-like pigments or various types of pigments with red colour, have also been observed produced by some bacterial species other than *Serratia* (Williams and Qadri, 1980), thus characteristics of microbes concerning these prominent phenomena can only be deduced.

Serratia marcescens, an Enterobacteriaceae family member, is a Gram-negative rod that inhabits soil and water (Szewzyk *et al.,* 1993; Sandner-Miranda *et al.,* 2018; Yeung *et al.,* 2018). It is a saprophytic bacterium, and has been identified in food, notably in starchy variants which offer a marvelous growth environment. Previously it was known as *Chromobacterium prodigiosum* (Wheat *et al.,* 1951; Sleigh, 1983; Ghaith *et al.,* 2018), Bizio proposed the name *Serratia marcescens* in 1823, which was later used by Gaughran, in 1969. Some environmental isolates were reported to produce characteristic red pigment prodigiosin, and formerly it was confused with fresh blood (Gaughran, 1969). It is an omnipresent bacterium and widely found in natural habitats such as soil, honeybee gut, water, air, plants and animals (Grimont and Grimont, 2006; Raymann *et al.,* 2017; Purkayastha *et al.,* 2018; Yeung *et al.,* 2018). The biosynthesis of prodigiosin is an important attribute of *Serratia marcescens* (Grimont and Grimont, 2006). Over past few years, it has been known as main causative agent of hospital acquired infections (Mahlen, 2011; Montagnani *et al.,* 2015).

Membrane lipid homeostasis has been shown to play a significant role in bacterial physiology. *Escherichia coli* was used as a study model bacterium for research in lipid biosynthesis for ages (Parsons and Rock, 2013). Biosynthesis of unsaturated fatty acids is a key factor in bacterial membrane homeostasis (Zhang and Rock, 2008). Combination of fatty acids with different melting points was integrated into phospholipids. Bacteria cope with low temperature by increasing the ratio of UFAs incorporated in phospholipids, which leads to high membrane fluidity (Parsons and Rock, 2013). In addition, retaining of membrane fluidity at lower temperatures is achieved in bacteria by integrating fatty acids with lower melting points into lipid bilayer with decrease of outer temperatures that help bacteria to keep balance the order-disorder transition of temperature of the membranes (Suutari and Laakso, 1994; Annous *et al.*, 1997; Bajerski *et al.*, 2017). It is evident from previous studies that high temperature leads to high membrane rigidity, which can be handle by decrease in degree of unsaturation of fatty acids (UFAs) (Ganzert *et al.*, 2011; Bakermans *et al.*, 2012; Bajerski and Wagner, 2013).

A variety of mechanisms are used by bacteria to sustain membrane integrity to cope with temperature changes (Parsons and Rock, 2013). In previous studies, *Serratia marcescens* has been well documented for occurrence of fatty acids in its general cell structure but not specifically in the cell membrane (Bishop and Still 1963; Bergan *et al.*, 1983). Bermingham *et al.* (1971) has reported stimulation of growth and production of pigments at temperatures higher than normal by long-chain unsaturated fatty acids in *Serratia marcescens*. The current research work was aimed to study and characterize cell membrane fatty acid profile of psychrotolerant bacterial isolate from Hopar glacier also known as Bualtar glacier in Pakistan.

Materials and Methods

Selection criteria of the isolate HI6

In the current PhD research work, during initial isolation and before molecular identification of all isolates, isolate HI6 was found to produce pigment in large quantity covering whole petri plate as compare to all other isolates. Therefore, isolate HI6 was selected on priority basis for molecular identification, physiological characterization and to determine the nature of pigment. Isolate HI6 (Hopar ice) (selected for this study) was isolated from glacial ice, Hopar glacier (36°12'54.77 N, 74°46'9.49 E) using Luria Bertani (LB) agar and incubated at 15˚C. After initial isolation, the isolate HI6 was preserved in 30% glycerol using Luria Bertani broth (LB) (see Appendices) as culture medium and stored at -20°C for future use.

Morphology of the isolate HI6

The colony morphology of the isolate HI6 was observed and recorded in terms of shape, production of pigments, size, opacity, margin, elevation and texture. Gram staining of the isolate HI6 was done following the protocol described by Beveridge (2001) and microscopic features were recorded using Laxco™ LMC-3000 Series Bright field Compound Microscope System.

Molecular identification of the isolate HI6

DNA extraction, PCR amplification

The DNA of the isolate HI6 was extracted using the protocol described by Zhou *et al.* (1996). The thermocycler (T100^{TM} Thermal cycler, Bio-Rad Laboratories, Inc.) was used to amplify the extracted DNA of isolate HI6. In addition, the selection of bacterial primers for amplification purposes and PCR conditions were adjusted same as used in Chapter 3 for various cold-tolerant bacteria.

Sequencing and phylogenetic analysis

Sequencing (16S rRNA) of amplified PCR product of the isolate HI6 was done by Macrogen Inc. Seoul, Korea. Trimming and filtering of obtained sequences was done using BioEdit (v7.0.5). The trimmed sequences were used to find the homologous bacterial strain by searching in the National Centre for Biotechnology Information (NCBI) (Thompson *et al.,* 1994). MEGA 6.0 software was used to construct phylogenetic tree at the bootstrap value of 1000 (Tamura and Nei, 1993).

Characterization of physiological parameters of the isolate HI6

Effect of different pH, media and temperatures on growth of the isolate HI6 was evaluated in this study. Four different temperatures, 5, 15, 25 and 35°C, five different media, Minimal salt medium (MSM), Luria Bertani (LB), Nutrient broth (NB), Peptone-yeast-meat extracts (PYM) and Artificial salt media (ASM) as well as five different pH, 3, 5, 7, 9 and 11 were selected for this experiment. Nutrient broth was used as culture medium for all temperatures and pH experiments, whereas, 15°C as incubation temperature was used for pH research work. 7 days old culture of the isolate HI6 was inoculated in 250 mL Erlenmeyer flask containing 100 mL of broth medium and incubated in shaking incubator with 120 rpm for 7 days. To check the growth, optical density (600 nm) of the isolate HI6 was recorded after every 24 hrs via spectrophotometer (Shimadzu). In addition, two-way ANOVA was applied to find the statistical significant of this experiment.

Cell membrane fatty acids analysis

Fatty acids methyl ester preparation (FAME)

The isolate HI6 was grown in LB broth and incubated at 15°C for seven days. About 100 mg culture of the isolate HI6 was taken in 20 mL glass vial and subjected to FAME extraction. Added 2 mL of 5% methanolic HCl to glass vial containing bacterial culture and heated at 70°C for 2 hours. After heating, glass vial was placed at room temperature for 30 minutes to cool down. In next step, distilled water (1 mL) was added and then vortexed well. For extraction of FAME, 1 mL of hexane was poured and vigorously

vortexed for about 10 minutes. After formation of two layers in glass tube, upper layer was transferred to new 2 mL clean glass vail and stored under nitrogen at -20°C.

Fourier Transform infrared (FTIR) spectroscopy

The extracted FAME extracted from the isolate HI6 was analysed through by FTIR spectrophotometer (Bruker Tensor 27, equipped with ZnSe ATR) along with the standard obtained through (The Vitamin Company USA). The spectra of FAME from the isolate HI6 were evaluated and recorded by keeping the range in between 4000-600 cm^{-1}. About 35 µL (in triplicate) of extracted FAME was placed on the FTIR spectrophotometer tray and recorded infrared spectrum of sample. The software Opus 65 was used to compare spectrum of extracted FAME with the spectrum of known compounds present in library.

Gas chromatography/mass spectroscopy (GC/MS) analysis

FAMEs analysis was done via GC (Agilent 7890A GC) linked to MS (Agilent 5975C MSD) and controlled by a HP Compaq computer using ChemStation software by following protocol described by Abd El Razak *et al.* (2014). Helium used as a carrier gas (flow rate of 1 ml/min, initial pressure of 50 kPa, split at 10 ml/min). Moreover, an auto-sampler (HP7683B) was used to inject about 1 µL of sample in hexane. In addition, FAMEs separation was carried with column (30 m× 0.25 mm) (An Agilent-fused silica capillary column) coated with 0.25 µm dimethyl poly-siloxane (HP-5). The temperature of GC was set initially from 30 to 130°C at 5°C/min then to 300°C at 20°C/min and final temperature held for 5 min. FAME Mix (C_4-C_{24}) (Sigma-Aldrich) was used to identify the resultant peaks by comparing mass spectra and R_t (retention time).

Results

Morphology and molecular identification

The isolate HI6 produced red pigment, with round, smooth, convex (3-6 mm in diameter) colonies (Fig. 5.1). The isolate HI6 was Gram negative short rod (0.7-2.3 µm in length and 0.3-0.8 µm in diameter) bacterium revealed by microscopic analysis.

Fig. 5.1. The colony morphology of the isolate HI6 with production of red pigment. **(a)** Uncovered petri plate **(b)** Covered petri plate

Based on 16S rRNA sequencing, the bacterial isolate HI6 was identified as *Serratia marcescens* [GenBank accession number MG641443]. In addition, the isolate HI6 showed 100% similarity with *Serratia marcescens* after searching in National Centre for Biotechnology Information database. Phylogenetic analysis based on partial 16S gene sequences, clearly shows that the isolate *Serratia marcescens* HI6 grouped within clusters of closely related species of *Serratia marcescens* in phylogenetic tree (Fig. 5.2).

Physiological characteristics

Serratia marcescens HI6 responded differently to various physiological parameters. In this experiment, it has been found that *S. marcescens* HI6 grow optimally at 25°C and pH 7 in LB broth (Table 5.1). The *Serratia marcescens* HI6 showed decline in its growth after 120 hrs of initial incubation in about all physiological parameters. It

seemed that it has entered in exponential growth phase after 48 hrs of its starting incubation. *S. marcescens* HI6 was unable to show any growth at pH 3 and 45°C.

Table 5.1. Growth characteristics of the *Serratia marcescens* HI6 on different physiological parameters

Isolate	**Physiological parameters**								
	Temp (°C)	**Time of incubation in hrs and OD[1]**							
HI6		**0**	**24**	**48**	**72**	**96**	**120**	**144**	**168**
	5	0.243	0.578	0.710	0.989	1.219	1.487	1.398	1.387
	15	0.123	0.256	0.794	1.091	1.883	1.980	2.106	1.970
	25	0.473	0.869	1.420	1.756	1.999	2.209	2.439	2.429
	35	0.490	0.898	1.321	1.580	1.784	1.985	2.001	1.973
	45	0.123	0.077	–	–	–	–	–	–
	pH	**Time of incubation in hrs and OD**							
		0	**24**	**48**	**72**	**96**	**120**	**144**	**168**
	3	0.143	0.131	–	–	–	–	–	–
	5	0.113	0.148	0.213	0.199	0.0722	0.063	0.060	0.058
	7	0.123	0.256	0.794	1.091	1.883	1.980	2.106	1.970
	9	0.134	0.230	0.753	0.963	1.352	1.103	1.102	1.090
	11	0.135	0.174	0.213	0.219	0.190	0.179	0.169	0.157
	Media	**Time of incubation in hrs and OD**							
		0	**24**	**48**	**72**	**96**	**120**	**144**	**168**
	ASW	0.020	0.0311	1.092	1.568	1.578	1.597	1.558	1.497
	LB	0.123	0.256	0.794	1.091	1.883	1.980	2.106	1.970
	MSM	0.041	0.052	0.059	0.890	1.480	1.501	1.450	1.399
	NA	0.023	0.032	0.052	1.129	1.068	1.204	1.189	1.179
	PYM	0.146	0.246	0.240	0.994	1.758	1.854	1.869	1.778

Keys: [1]Optical density

**P* values for the pH and media experiments were *>0.05*

**P* value for the for temperature experiment was *<0.05*

The LB broth was reported as best growth medium followed by PYM, ASW, MSM and NA. In addition, optimum growth was at pH 7 followed by pH 9, 11 and 5. Moreover, best growth was observed at 25°C followed by 35, 15 and 5°C.

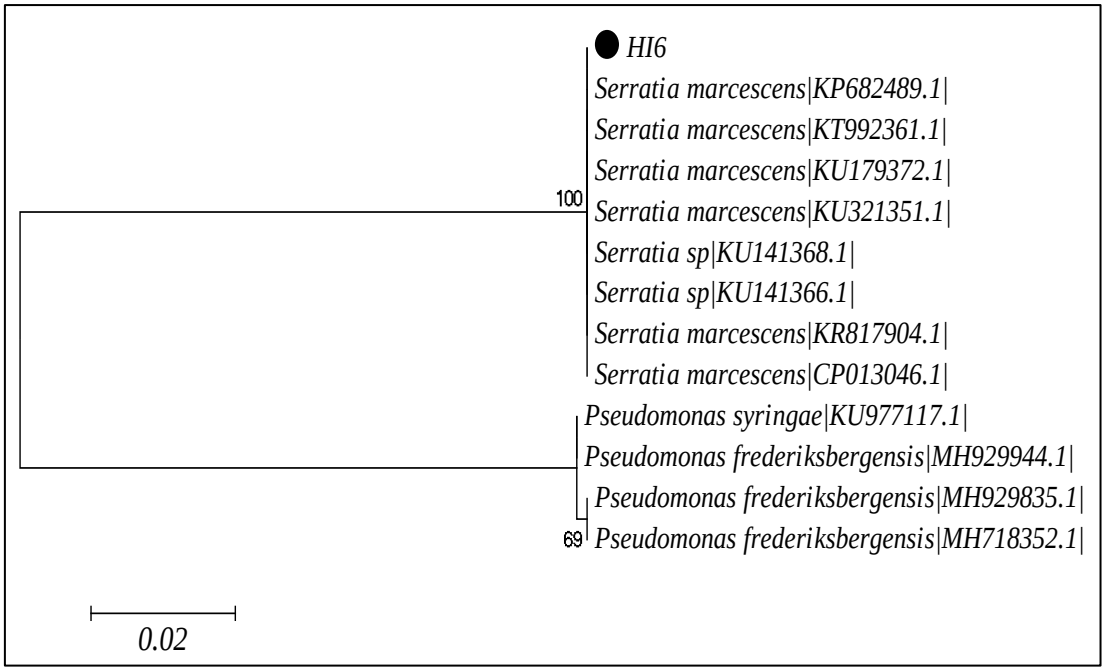

Fig. 5.2. Analysis of evolutionary relationship and relatedness of *Serratia marcescens* HI6 based on partial 16S gene sequences with closely related species by Maximum Likelihood method. Bootstrap values (1000 replicates) for node values from 50% are indicated. The species belonging to genus *Pseudomonas* were used as an outgroup

Fourier Transform infrared (FTIR) analysis

Results of FTIR analysis revealed different functional groups present in the FAME extracted from *Serratia marcescens* HI6 (Fig. 5.3). The spectrum of the analysed FAME was compared with already online available databases as well as with standard and found that typical functional groups that are exist in fatty acids (FA) (such as saturated FA, monounsaturated FA and polyunsaturated FA), have also been presented in analysed FAME. For example, peak value 2812-3012 cm^{-1} region (which represent =C-H stretching vibration bond), 1710-1766 cm^{-1} (associated with C=O carbonyl bond) and multiple C=C double bond at 1400-1600 cm^{-1}, have clearly indicated that *Serratia*

marcescens HI6 had various types of saturated FA, monounsaturated FA and polyunsaturated FA in its cell membrane (Fig. 5.3).

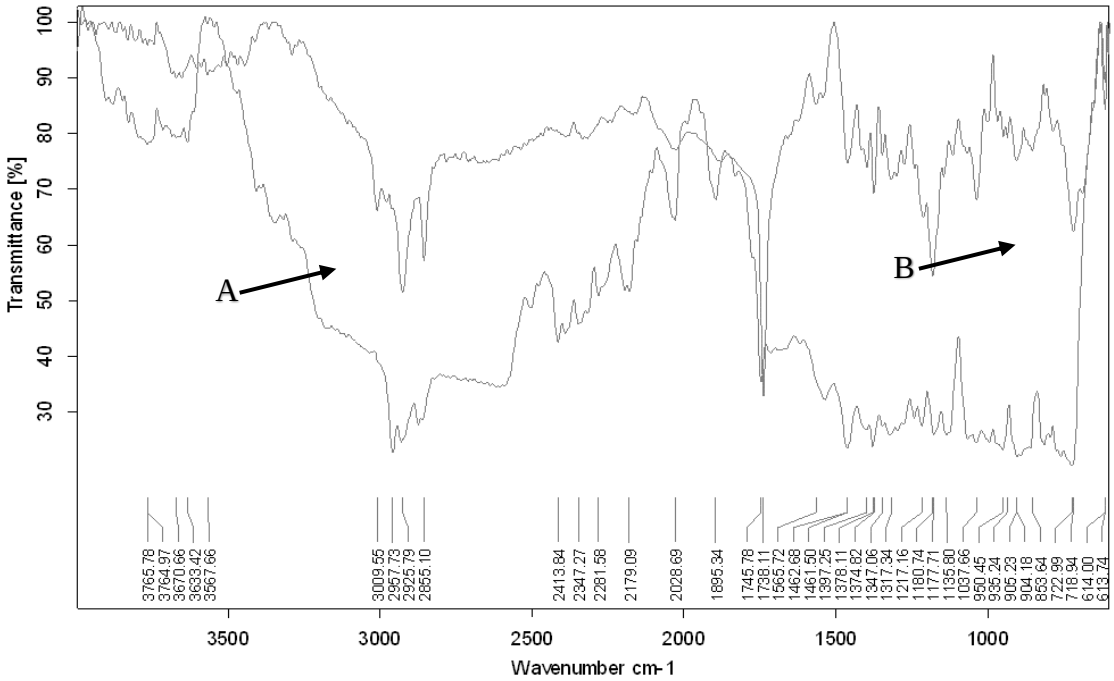

Fig. 5.3. Fourier Transform infrared (FTIR) spectrum of FAME extracted from the *Serratia marcescens* HI6. **(A)** Extracted FAME of the *Serratia marcescens* HI6 **(B)** Standard

Gas chromatography/mass spectroscopy (GC/MS) analysis

The results of GC/MS has revealed that most copious types of fatty acids were branched fatty acids followed by monounsaturated fatty acids, n-chain saturated fatty acids, hydroxyl fatty acids and polyunsaturated fatty acids (Fig. 5.4a, b) (Table 5.2). In addition, *i*-$C_{16:1}$ was found most abundant type of fatty acid followed by *i*-$C_{15:1}$, *n*-$C_{18:2(cis\text{-}9)}$, *n*-$C_{16:0}$, *ai*-$C_{17:0}$, *3-OH*-$C_{14:0}$, *n*-$C_{12:0}$, *n*-$C_{14:0}$, *i*-$C_{17:0}$, *n*-$C_{18:0}$, *n*-$C_{18:1(trans\text{-}9)}$, n-$C_{18:2(cis\text{-}9)}$, *n*-$C_{15:0}$ and *n*-$C_{17:0}$ (Table 5.2).

Moreover, *Serratia marcescens* HI6 was able to produce only one type of polyunsaturated fatty acids, *n*-$C_{18:2(cis\text{-}9)}$ (also known as linoleic acid). Overall, branched fatty acids (62%) and *i*-$C_{16:1}$ (35.2% out of total membrane fatty acids) were the main types of fatty acids produced by *Serratia marcescens* HI6 predominantly.

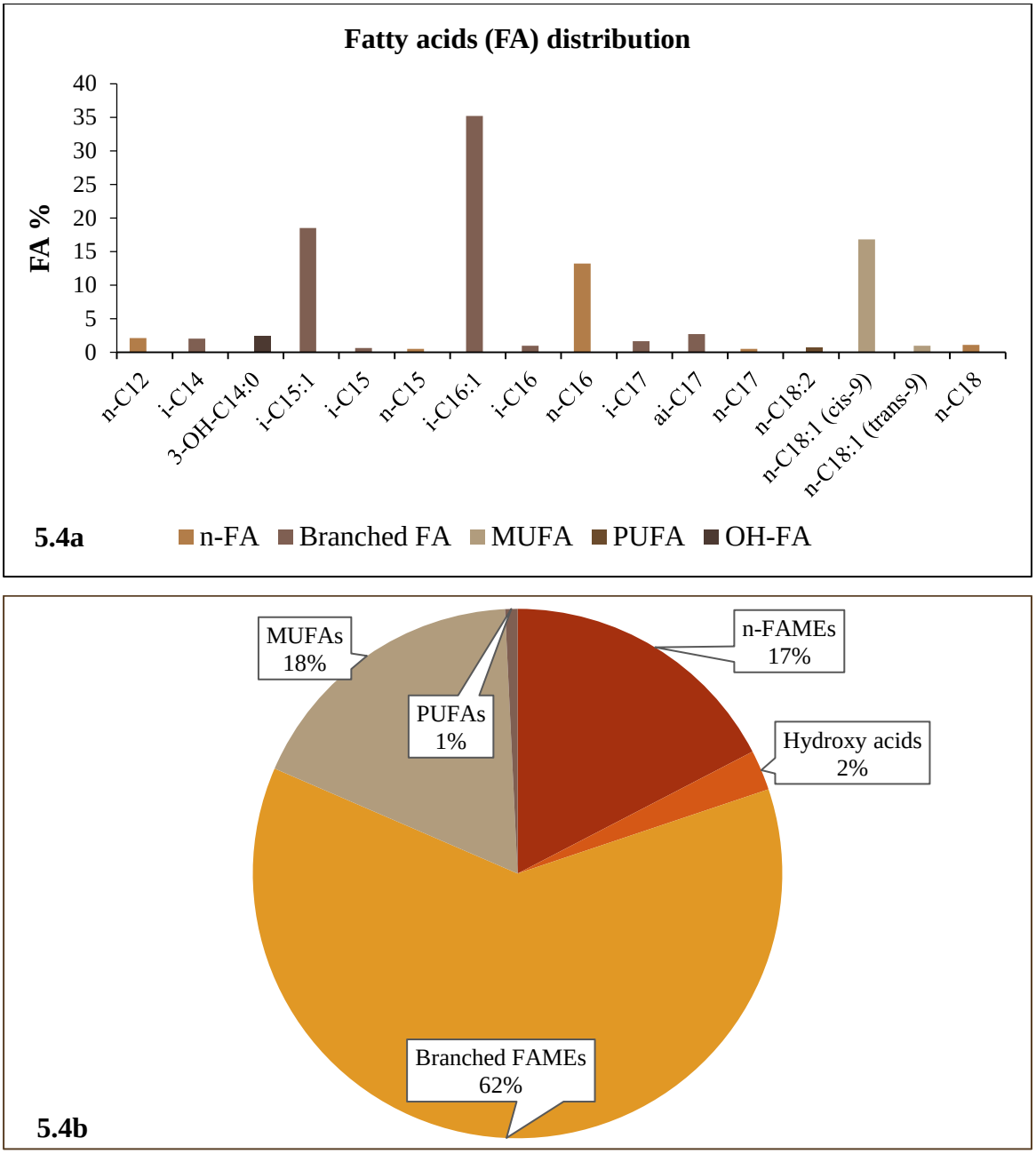

Fig. 5.4a, b. (a) Distribution of individual fatty acids in the cell membrane of *Serratia marcescens* HI6 (**b)** Percentage of the main types of fatty acids in the cell membrane of *Serratia marcescens* HI6

Table 5.2. Distribution and percentage of fatty acids in the cell membrane of the *Serratia marcescens* HI6

Isolate	Carbon Chain length	Retention time	IUPIC names	%/total CM fatty acids
***Serratia marcescens* HI6**	*n*-$C_{12:0}$	16.34	Dodecanoate	2.1
	i-$C_{14:0}$	20.72	(Z)-tetradec-9-enoate	2.0
	3-OH-$C_{14:0}$	25.18	3-Hydroxytetradecanoate	2.46
	i-$C_{15:1}$	20.46	*iso*-10-Pentadecenoate	18.5
	i-$C_{15:0}$	23.20	*iso*-Pentadecanoate	0.6
	n-$C_{15:0}$	24.08	Pentadecanoate	0.5
	i-$C_{16:1}$	24.89	(Z)-hexadec-9-enoate	35.2
	i-$C_{16:0}$	25.67	*iso*-Hexadecanoate	1.0
	n-$C_{16:0}$	26.57	Hexadecanoate	13.2
	i-$C_{17:0}$	27.28	*iso*-Heptadecanoate	1.6
	ai-$C_{17:0}$	28.05	*anteiso*-Heptadecanoate	2.7
	n-$C_{17:0}$	28.85	Heptadecanoate	0.5
	n-$C_{18:2(cis\text{-}9)}$	30.43	(9Z,12Z)-octadeca-9,12-dienoate	0.76
	n-$C_{18:1(cis\text{-}9)}$	30.45	(Z)-octadec-9-enoate	16.8
	n-$C_{18:1(trans\text{-}9)}$	30.59	(E)-octadec-9-enoate	1.0
	n-$C_{18:0}$	31.15	Octadecanoate	1.1

Keys; *i*= iso, *ai*=anteiso, CM= cell membrane, IUPIC= International Union of Pure and Applied Chemistry

Table 5.3. The quantitative overview (with triplicates data and standard deviations) of cell membrane fatty acids of *Serratia marcescens* HI6

Isolate	**Fatty acids (carbon Chain length)**										
Nodularia spumigena CHS1	**Saturated fatty acids**										
	Triplicates (µg/g)	***n*-C$_{12:0}$**	***i*-C$_{14:0}$**	***3-OH*-C$_{14:0}$**	***i*-C$_{15:0}$**	***n*-C$_{15:0}$**	***i*-C$_{16:0}$**	***n*-C$_{16:0}$**	***i*-C$_{17:0}$**	***ai*-C$_{17:0}$**	***n*-C$_{17:0}$**	***n*-C$_{18:0}$**
		166.3	158.5	194.8	47.5	40.1	79.2	1045.5	126.7	213.8	39.6	87.1
		166.7	157.8	194.5	47.6	40.6	79.0	1045.8	127.4	212.9	40.1	87.5
		166.1	157.9	193.7	46.8	40.3	78.4	1046.1	125.1	213.1	40.3	86.9
	SD*	0.30	0.37	0.56	0.43	0.25	0.41	0.30	1.17	0.47	0.36	0.30
	Monounsaturated fatty acids											
	Triplicates (µg/g)	***i*-C$_{15:1}$**	***i*-C$_{16:1}$**	***n*-C$_{18:1}$ (*cis*-9)**	***n*-C$_{18:1}$ (*trans*-9)**							
		1465.2	2772.4	1330.6	79.2							
		1465.8	2771.8	1331.7	78.9							
		1464.3	2771.9	1330.8	78.8							
	SD	0.75	0.32	0.58	0.20							
		Polyunsaturated fatty acids										
		***n*-C$_{18:2(cis–9)}$**										
		60.1										

	Triplicates (µg/g)	61.0										
		60.3										
	SD	0.47										
*SD: Standard deviation												

Discussion

This study was aimed to characterize physiological parameters and analyzed cell membrane fatty acids of the bacterium isolate *Serratia marcescens* HI6. In our knowledge, we are reporting for the first time *Serratia marcescens* from any glacial environment of Pakistan. Although in one study, it has been studied for laccase production isolated from glacial site in Indian Himalayan Region (IHR) (Kaira *et al.,* 2015). *Serratia marcescens* has been known for its abilities to infect insects and spoils various types of food (Flyg and Xanthopoulos, 1983; Abdour, 2003; Bahar and Demirbag, 2007). In addition, its association with promotion of plant growth by combating with plant pathogens has also been observed (Kloepper *et al.,* 1993). Moreover, *Serratia marcescens* has been found to fertile soils by producing phosphatases and unfriendly substances for plant pathogens (Tripura *et al.,* 2007). Likewise, Pares (1964) has observed in soil that *Serratia marcescens* had role in the nutrient recycling by solubilizing organic iron and dissolving gold and copper. In another research, Janota-Bassalik (1963) has reported mineralization role of psychrotolerant *Serratia marcescens* accompanying by low-moor peat.

The genus *Serratia* is usually found widely in water, plants, mammals and hospitalized human patients (Grimont and Grimont, 2006) but studies about its presence and activities in both Polar and Non-polar cold regions are very rare. The primary habitat for genus *Serratia* is water as plenty of species belonged to *Serratia* has been reported from water (Grimont and Grimont, 2006). Gavini *et al.* (1979) has isolated *Serratia fonticola* well waters and springs. In another study conducted by Grimont and Grimont (2006), a total of 150 bacterial species belonged to genus *Serratia* including *Serratia marcescens* were reported from river water. In addition, Groscop and Brent (1964) has observed toxicity of non-diffusible red pigment prodigiosin to protozoa, produced by genus *Serratia*, which perhaps a promising factor important for its survival in water and soil habitats. Based on such research, Grimont and Grimont (2006) has concluded that *Serratia* sp. with pigmentation abilities are existed in non-polluted rather than from polluted water.

Tolerance of *Serratia marcescens* HI6 to different extreme temperatures, pH and media was assessed. *Serratia marcescens* HI6 was able to grow on temperature between 5-35°C (25°C optimum growth temp), pH between 5-11 (7 optimum growth pH) as well as on various media including ASW, LB, MSM, NB and PYM (LB optimum growth medium). Recently, Kaira *et al.* (2015) has reported similar findings for *Serratia marcescens* isolated from glacial site in Indian Himalayan Region (IHR). They observed psychrotolerant *Serratia marcescens* to show optimum growth at 25°C. Many other researchers have also used various temperatures (5-37°C) for the growth of *Serratia marcescens* and they have found that it had the abilities to grow over a wide range of temperatures (Bishop and Still, 1963; Selvakumar *et al.,* 2008). In our knowledge, none of study has been carried out relating to tolerance of *Serratia marcescens* to extreme pH and various media.

In the present study, the fatty acids present in the cell membrane of *Serratia marcescens* HI6 were assessed using Fourier Transform Infrared (FTIR) spectroscopy. FTIR has the efficiency to recognize major parts of biological material (lipids, proteins, nucleic acids and carbohydrates) in any solution (Forfang *et al.*, 2017). Basically, specific absorbance frequencies of FTIR are central to detect fatty acids (Forfang *et al.*, 2017). In our study, the bands between 1710-1766 cm^{-1} were observed after analysis of extracted FAMEs by FTIR indicating the presence of C=O that associated with fatty acids. According to Dean *et al.* (2010), presence of the band at 1740 cm^{-1} indicated existence of C=O of ester groups, principally from FA.

Moreover, Shurvell (2002) has described the bands obtained from lipids after Fourier Transform Infrared spectroscopy analysis in detail. According to them, the lipid content present in any sample can easily detected by getting peaks associated to C-O-C stretching in esters (1070-1250 cm^{-1}), CH_2 bending (1460 cm^{-1}), C=O stretching in esters (1745 cm^{-1}), C-H stretching vibrations (=C-H stretch at 3010 cm^{-1}). The FTIR has been used by several researcher to identify the lipids and fatty acids content in many microbes such as *Chlamydomonas reinhardti, Scenedesmus subspicatus, Mucor plumbeus*, *Mucor hiemalis*, *Mucor circinelloides*, *Mortierella alpine* (Dean *et al.*, 2010; Forfang *et al.*, 2017).

Furthermore, the fatty acids profile of cell membrane of the isolate *Serratia marcescens* HI6 was properly studied using Gas chromatography/mass spectroscopy (GC/MS) in the current study. Findings of this analysis showed that C16:1 was present abundantly in cell membrane of *Serratia marcescens* HI6. None of such study has been carried out in past. Although Bergan *et al.* (1983) and Bishop and Still (1963) have analyzed the whole cell fatty acids of *Serratia marcescens*, whereas, current study is focused on cell membrane fatty acids of *Serratia marcescens*, so it is very challenging to exactly compare and discuss current study (cell membrane fatty acids analysis) with previous studies (whole cell fatty acids analysis) of *Serratia marcescens* because whole cell fatty acids could significantly differ in type and function from fatty acids present in cell membrane. As distribution of various types of fatty acids in cell membrane play significant role in bacterial adaptability in cold habitats, therefore, present research work specifically focused on it rather than to study whole cell fatty acids.

However, current study has generally been discussed with other related bacterial strains of *Serratia marcescens* (Jantzen *et al.*, 1974a, 1974b; Jantzen *et al.*, 1975). Our results has supported by research work of many other researchers that observed foremost amount of *n*-$C_{16:0}$, $C_{16:1}$ and *n*-$C_{18:1}$ in some strains of *Serratia marcescens* (Kates *et al.*, 1964; Kates and Hagen, 1964). Although the contribution of the two last-mentioned fatty acids may also be small from the same strain Kates and Hagen (1964) has reported that amount of $C_{16:1}$ and *n*-$C_{18:1}$ varied depending upon culture conditions of growth of *Serratia marcescens*. It has been observed by many researcher that gram negative bacterial species possessed higher quantities of *n*-$C_{16:0}$, $C_{16:1}$ and *n*-$C_{18:1}$ than gram positive bacteria in their cell membranes (Jantzen *et al.*, 1974a; 1974b; Jantzen *et al.*, 1975; Zhao *et al.*, 2011). Moreover, Bergan *et al.* (1983) has observed that family Enterobacteriaceae had high amount of *n*-$C_{16:0}$ than $C_{16:1}$ in their cells and has also reported in few *Acinetobacter* strains (Jantzen *et al.*, 1975).

Likewise, the branched fatty acids were witnessed predominately in cell membrane the isolate *Serratia marcescens* HI6. The branched *iso* and *anteiso* pattern was observed in both saturated fatty acids and monounsaturated fatty acids in the current results. The incorporation of *iso* and *anteiso* branches in the fatty acids chains of cell membrane, is perhaps a strategy to cope with devastating effects of low temperature by maintaining

the fluidity of cell membrane (Bajerski *et al.*, 2013; 2017). In few studies, it has been shown that *anteiso* branches pattern of fatty acids of cell membranes in psychrotolerant bacterial species (such as *Chryseobacterium frigidum* and *Chryseobacterium haifense*) reduced the melting temperature of the plasma membrane increasing motion capability and made it possible to freely move molecules across the cell membrane (Hantsis-Zacharov and Halpern, 2007; Kim *et al.,* 2016).

Conclusion

In conclusion, the bacterium isolate HI6 was identified as *Serratia marcescens* after 16S rRNA sequencing. The bacterium isolate *Serratia marcescens* HI6 showed a good potential to grow over a wide range of extreme physiological conditions. Results of FTIR and GC/MS has showed that the isolate HI6 had the ability to produce and accumulate various types of fatty acids in its cell membrane but predominantly *i*-$C_{16:1}$ and other branched chain fatty acids. This study is important because it gives a clear idea about Fatty acids distribution in the cell membrane of *Serratia marcescens* and their possible role in adaptation in glacial habitats. In addition, this study would be helpful to understand the maintenance of cell membrane fluidity in glacial environments by *Serratia marcescens*. Finally, the bacterium isolate *Serratia marcescens*-HI6 would be used as source of polyunsaturated fatty acids (e.g. linoleic acid) as alternative to the conventional plant sources of PUFA, thus would be helpful to prevent deforestation.

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Chapter 6

Polyphasic identification, pigment composition and cell membrane fatty acid analysis of *Nodularia spumigena CHS1*

Abstract

In the present study, cyanobacterium isolate CHS1 isolated from Hopar glacier, Pakistan, was analyzed for the first time for cell membrane fatty acids and production of pigments. Sequencing of the 16S-23S intergenetic region confirmed identification of the isolate CHS1 as *Nodularia spumigena*. All chlorophyll and carotenoid pigments were quantified using high-performance liquid chromatography (HPLC) and experiments to test tolerance against a range of physico-chemical conditions were conducted. Likewise, the fatty acid (FA) profile of the cell membrane CHS1 was analyzed using gas chromatography and mass spectroscopy. The cyanobacterium isolate CHS1 demonstrated tolerance to 8% NaCl, 35°C and pH 5-9. The characteristic polyunsaturated fatty acid (PUFA) of isolate CHS1, *n*-$C_{18:4}$, was observed in fatty acid methyl esters (FAMEs) extracted from the cell membrane. CHS1 was capable of producing saturated fatty acids (SFA) (e.g. *n*-$C_{16:0}$), monounsaturated fatty acids (MUFA) (e.g. *n*-$C_{18:1}$) and polyunsaturated fatty acids (e.g. *n*-$C_{20:5}$) in the cell membrane. In this study, we hypothesize that one mechanism of cold adaptation displayed by isolate CHS1 is the accumulation of high amounts of PUFA in the cell membrane.

Keywords: *Nodularia spumigena* CHS1, cyanobacteria, cell membrane, FAMEs, Hopar glacier

Introduction

Members of the genus *Nodularia* (Order Nostocales) are filamentous heterocystous nitrogen-fixing cyanobacteria that mainly thrive in aquatic environments, such as planktonic and benthic habitats (Nordin and Stein, 1980; Baker, 1992), though can also be found in moist soils. *N. spumigena*, the most common species, has been recognized for production of widespread hepatotoxic blooms along coastlines globally (da Silveiraa *et al.*, 2017). The *Nodularia* genus has been classified through morphological (variation in cell types), physiological (production of gas vesicles and nodularin) and ultrastructural features, as well as ecological characteristics (Laamanen *et al.*, 2001). The genus *Nodularia* has been found phylogenetically closer to genera *Anabaena*, *Nostoc*, and *Cylindrospermum*, based on 16S rRNA gene sequencing (Main *et al.*, 1977; Wilmotte, 1994; Turner, 1997). Recently, seven species were distinguished by Komarek *et al.* (1993), including four planktonic species that have the potential to produce gas vesicles (*N. spumigena*, *N. baltica*, *N. litorea*, and *N. crassa*), and three species devoid of gas vesicles that thrive in benthic, periphytic, or soil habitats (*N. harveyana*, *N. sphaerocarpa*, and *N. willei*).

The filamentous, nitrogen fixing, *N. spumigena* forms extensive blooms in the Baltic Sea (Laamanen *et al.*, 2001; Congestri *et al.*, 2003), with previous studies suggesting the co-existence of different phenotypes with variable biochemical and physiological traits (Barker *et al.*, 2000; Laamanen *et al.*, 2001; Reakova *et al.*, 2014). Production of cyclic pentapeptide nodularin (NOD) results in the toxicity of blooms that can impact ecosystems and water quality (Karjalainen *et al.*, 2007; Sotton *et al.*, 2015). Besides nodularin, various non-ribosomal peptides (NRPs) with a high degree of chemical diversity are also produced by *N. spumigena* (Mazur-Marzec *et al.*, 2013).

It has been observed that *N. spumigena* can tolerate phosphorus starvation without affecting its ability of nitrogen fixation (Gronlund *et al.*, 1996). As *N. spumigena* grows on the surface of sea, therefore exposed more to photosynthetic active radiation (PAR) (400-700 nm) and ultraviolet radiation (UVR) (280-400 nm) (Paerl, 1988). UVR is known to adversely affect marine primary producers (Villafane *et al.*, 2003), but with high PAR and UVR generally favoring phytoplankton with well-developed strategies

of photo-protection. The adaptive strategies employed by phytoplankton to reduce the magnitude of photo-damage induced by UVR exposure differ between lineages (Karentz, 1994).

In cyanobacteria, mycosporine-like amino acids (MAAs) are produced to reduce UVR induced photo-damage (Sinha and Hader, 2008). The photoprotective functions of various MAAs have been investigated, demonstrating absorption maxima between 310-362 nm (Mohlin and Wulff, 2009). MAAs and carotenoids with high resistivity towards UVR were reported in *Nodularia* sp. (Wulff *et al.,* 2007; Mohlin and Wulff, 2009), as well as accumulation/aggregation of thin layers of *Nodularia* for self-shading at the surface of water along with highly productive microenvironments with steep O_2 and pH gradients during light as well as during darkness (Ploug, 2008).

Cyanobacteria are known to dominate extreme environments including those associated with extreme cold, arid, high salinity and UV conditions (Chrismas *et al.,* 2015). In the cryosphere, i.e. the frozen water component of the Earth system, Cyanobacteria are recognized as a dominant member of the biota, playing central roles in biogeochemical cycling (Vincent, 2000). Cyanobacteria have been reported from both Antarctic and Arctic Polar Regions (Taton *et al.,* 2006a, b; 2008; Zakhia *et al.,* 2007), with 16S rRNA gene analysis demonstrating a cosmopolitan assemblage structure (Harding *et al.,* 2011; Jungblut *et al.,* 2014).

Glaciers are one of the most important cryospheric habitats, well known for their poor nutrient availability and very low temperatures (Edwards *et al.,* 2014). Previously thought to be free from life (Uetake *et al.,* 2010), glaciers are now know to host multiple groups of psychrophilic macro and microorganisms belonging to all three domains of life (DeSmet and Rompu, 1994; Takeuchi, 2001; Segawa *et al.,* 2005; Turchetti *et al.,* 2008; Rafiq *et al.,* 2017). Recent studies employing both culture dependent and independent approaches have confirmed the presence of cyanobacteria in many glaciers located around the Arctic, Antarctica and Asia (Takeuchi, 2001; Segawa *et al.,* 2017), with cyanobacteria often reported to be associated with the formation of cryoconites holes (Takeuchi *et al.,* 2001a, b) and thus important for both glacier melt and carbon cycling (Uetake *et al.,* 2010).

In addition, Vincent *et al.* (2004) proposed that cyanobacteria could be the possible survivor against the widespread Antarctic glaciation. To the best of our knowledge, *N. spumigena* has not yet been reported from glaciers located in Antarctica and Asia. Therefore, the current study aimed to identify, characterize and analyze the cell membrane fatty acids and pigments of the psychrotolerant isolate CHS1 isolated from Hopar glacier, Pakistan.

Material and methods

Sampling of glacial sediments and processing

Hopar glacier (18 km in length), also known as Baultar glacier, is situated in Hunza valley, Karakorum Mountains Range, Pakistan. It is located between the two highest peaks, Diran peak (7257 m high) and Miyar peak (6824 m high). It has been observed that Hopar glacier is one of the fastest melting glaciers in Pakistan. For the present study, glacial sediments (~ 200 - 400 g) were collected from Hopar glacier (36.2108228 N, 74.7724664 E) into sterilized Whirl−pack bags (Nasco, Fort Atkinson, WI) using a pre-sterlized plastic/metal scoop following standard microbiological protocol. Temperature (using portable thermometer) and pH (using pH strips) were recorded as 7.0 and 1°C, respectively. Sediments samples were transported under chilled conditions to the Department of Microbiology, Quaid-i-Azam University, Islamabad, and stored at -20°C until further analysis.

Solidified BG11 medium (Gibco) (boric acid 28.7 mg l^{-1}, calcium chloride 27 mg l^{-1}, cupric sulfate 0.8 mg l^{-1}, ferric ammonium citrate 12 mg l^{-1}, magnesium sulfate 75 mg l^{-1}, manganese chloride 18.1 mg l^{-1}, potassium phosphate dibasic 39 mg l^{-1}, sodium molybdate 3.9 mg l^{-1}, sodium nitrate 1500 mg l^{-1}, sodium carbonate 20 mg l^{-1}, zinc sulfate 2.2 mg l^{-1}, ethylenediaminetetraacetic acid 1 mg l^{-1}, agar 15000) was used for the isolation of the cyanobacterium isolate CHS1. Small amount of sediments dissolved in sterilized mili-Q water were streaked on solidified BG11 plates and incubated at 15°C with 30 µmol m^{-2} s^{-1} of light for 60 days. After appearance of visible colonies, the cyanobacterium colonies were transferred to a shaking incubator at 15°C.

Morphology of the isolate CHS1

The morphology of the isolate CHS1 was assessed via microscopy following the procedure of Lehtimaki *et al.* (2000). Cyanobacterial isolate CHS1 was cultured in liquid BG11 medium and incubated at 15°C in a shaking incubator for 14 days. Vegetative cells and intercalary heterocytes of the axenic culture were observed using

an Olympus CX 40 light microscope with an attached Nikon Digital Camera DXM 1200.

Molecular identification of the isolate CHS1

Extraction of genomic DNA

Genomic DNA of the isolate CHS1 was extracted using the Invitrogen PureLink Microbiome DNA Kit following the manufactures procedure with a few modifications; approximately 5 mg of cell culture was suspended in 800 µL lysis buffer (provided by the manufacture), supplemented with 100 µL lysis enhancer buffer (provided by the manufacture), 30 µL lysozyme (≥40,000 units/mg), 10 µL sodium sarcosyl (2% w/v), 10 µL proteinase K (~20 mg/mL) and 10 µL sucrose (25% w/v) and incubated for 60 min at 37°C. Extracted DNA was dissolved in elution buffer and stored at -20°C until future use.

PCR amplification, sequencing and phylogenetic analysis

The extracted genomic DNA was subjected to PCR amplification using T100 Thermal Cycler (Bio-Rad Laboratories, Inc). The genomic regions including 16S rRNA gene and ITS (16S-23S intergenetic segment) were amplified using four different primers including 27F, 359F, 781R and 23S30R (Table 6.1). 25 µM of each primer, 0.5 µL of template DNA (15 ng), 2X PCR Master Mix (Thermofisher Scientific) with a volume of 25 µL, were mixed with 25 µL of nuclease-free water (Thermofisher Scientific) to prepare a reaction mixture of 50 µL volume. PCR amplification was performed using two different reactions. The first PCR reaction involved using 27F and 23S30R primers with the PCR settings including preliminary denaturalization step at 94°C for 5 min, 35 cycles of 94 and 53°C for 30s, 3 min at 72°C, and a final elongation step at 72°C for 7 min. In the second PCR reaction, 359F and 781R primers was used by adjusting the PCR settings to starting denaturation for 5 min at 94°C, 35 cycles of 94, 60 and 72°C for 3 min each, followed by an elongation step at 72 °C for 7 min.

Amplified PCR products were purified prior to sequencing using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions and sent for

sequencing at the MRC PPU (Protein Phosphorylation and Ubiquitylation) DNA sequencing and services, University of Dundee, Scotland, UK. The sequencing of 16S-23S intergenetic segment was performed with four primers 27F, 359F, 781R and 23S30R. After sequencing, the obtained sequences were filtered and low quality sequences removed using BioEdit software (Hall, 1999). The filtered sequences were searched for similarity index in BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the most similar sequences downloaded and subjected to phylogenetic analysis. The phylogenetic tree for the isolate CHS1 was constructed using Mega 6.0 (Tamura *et al.*, 2013).

Table 6.1. Details of the primers used for PCR amplification of 16S-23S intergenetic segment of the isolate CHS1

Primers	**Target position**[1]	**Target gene**	**Sequence (5′-3′)**	**References**
27F[2]	7-27	ITS-16S rRNA	AGAGTTTGATCMTGGCTCAG	Wilmotte *et al.* (1993)
359F[2]	359-378	16S rRNA	CTTCGCCTCTGTGTGCCTAGG	Nubel *et al.* (1997)
781R[3]	781-805	16S rRNA	GGGGGATCTTCCGCAATGGG	Nubel *et al.* (1997)
23S30R[3]	30-52	ITS-16S rRNA	GACTACTGGGGTATCTAATCCCAT	Lepere *et al.* (2000)
[1]16S rRNA nucleotides numbering used *E.coli* as reference. [2,3]F (forward) and R (Reverse) representing primers direction relating to the rRNA.				

Physiological characterization

The isolate CHS1 was subjected to various NaCl concentrations (2, 4, 6, 8 and 10 g/L), temperatures (5, 15, 35 and 45°C) and pH regimes (3, 5, 7, 9 and 11) in order to assess its tolerance to extreme conditions. Liquid BG11 medium was used for all experiments. The isolate CHS1 was cultured in 50 mL BG11 medium and incubated at 15°C for 15 days in a shaker incubator during NaCl and pH experiments, whereas incubation

temperatures were modified to 5, 25, 35 and 45°C during temperature response experiments with all other parameters maintained as during experiments with NaCl and pH.

Importantly, just one factor was investigated at a time and the rest kept constant during the physiological characterization. Finally, one-way ANOVA was applied to find the statistically significance of recorded data.

Pigment analysis

Approximately 5 mg of CHS1 culture (grown in liquid BG11 medium) was extracted in 5 mL of chilled acetone for 24 hours in the dark following vortexing and sonication steps to aid cell lysis. All extracts were stored at -20^{o}C until analysis by high performance liquid chromatography (HPLC) using a modified method of Van Heukelem and Thomas (2001) on an Agilent series 1100 HPLC system equipped with a C_8 column and diode array detector (350 - 750 nm) (Hewlett-Packard, Waldbronn, Germany). Pigments were identified and quantified against analytical standards from DHI and Sigma using both retention time and spectral analysis.

Cell membrane fatty acids analysis of the isolate CHS1

Fatty acids methyl ester (FAME) preparation

Fatty acids methyl ester of cell membrane of the isolate CHS1 was prepared by following the procedure of Abd El Razak *et al.* (2014). Approximately 5 mg of isolate culture was suspended with 5% methanolic HCL in a sterile 15 mL glass vial, vortexed for 2 min and incubated at 70°C for 120 min. After incubation, the glass vail was placed at room temperature for 30 min, then 2 mL hexane added and vortexed vigorously in order to extract FAMEs. After formation of two clear layers, the upper layer containing FAME was transferred to a new 2 mL glass vail, capped under nitrogen and stored at -20°C for subsequent analysis. Three copies of FAMEs were extracted. Finally, standard deviation of triplicate data was calculated using Statistix 8.1.

Gas chromatography/mass spectroscopy (GC/MS) analysis

Extracted FAMEs of the isolate CHS1 were analyzed through GC (Agilent 7890A)/MS (Agilent 5975C) linked with an auto-sampler (HP7683B) and a HP Compaq computer using ChemStation software following the protocol of Abd El Razak *et al.* (2014) with a few modifications. 1 µL of FAME sample was injected by the auto-sampler. A column (50 × 0.32 × 0.17 mm) was used for the purposes of FAMEs separation. The starting temperature of GC/MS was 50°C, temperature was increased to 100°C at 10°C/min, to 250°C at 4°C/min, and finally to 300°C at 20°C/min. The final temperature was held with a flow rate of 1 mL/min, initial pressure of 50 kPa, split at 10 mL/min for total 5 min with helium as the carrier gas. The resultant peaks of FAMEs were quantified and identified by comparing their R^t and mass spectra with FAME Mix, C_4-C_{24} (external standards) (Sigma-Aldrich) and C_{24} (internal standard) (Sigma-Aldrich).

Results

Morphology and molecular identification of the isolate CHS1

After morphological observations and sequencing of 16S-23S intergenetic segment of the isolate CHS1, it was identified as *Nodularia spumigena* (Table 6.2). In the morphological analysis, vegetative cells (shorter, disc like in shape, wide 4.5-5.5 µm and length 5.0-7.0 µm) and heterocysts (shorter, length 5.5-7.5 µm and wide 6.5-9.5 µm) were observed, whereas akinetes (common, disc shaped) were not observed under optical microscopy (Fig. 6.1).

Table 6.2. The resemblance directory of the isolate CHS1 with respective homologous species

Isolate	Primers names	Accession No.	Homologous strain with accession No.	Identity (%)
[1]CHS_1	359F	MH745043	*Nodularia spumigena* |CP020114.1|	100
	781R	MH745044	*Nodularia spumigena* |CP020114.1|	100
	27F	MH745045	*Nodularia spumigena* |CP020114.1|	100
	23S30R	MH752041	*Nodularia spumigena* | NR_115707.1|	100
[1]CHS (Cyanobacteria Hoper Sediment)				

The sequences of the isolate CHS1 showed 100% similarity with *N. spumigena* using BLAST (Basic Local Alignment Search Tool) search in NCBI (National Center for Biotechnology Information) database (Table 6.2). In addition, after downloading closet sequences from NCBI and constructing phylogenetic tree of the isolate CHS1, it was also revealed that the isolate CHS1 shares a close evolutionary history with *N.spumigena* (Fig. 6.2).

Characterization of physiological parameters

The isolate CHS1 was able to grow across the range of 2-8 g/L NaCl, 5-35°C and pH 5-9 (Table 6.3). Optimal growth was observed at 15°C, pH 7 and 2-4/L NaCl, whereas

the slowest growth was apparent at 35°C, pH 9 and 10 g/L NaCl, with no growth at 45°C.

Fig. 6.1. Microscopic observation of isolate CHS1 with light microscope Olympus CX 40. **(A)** Vegetative cells **(B)** Heterocysts

Pigment analysis

HPLC analysis confirmed the production of chlorohypll-*a* in addition to several types of carotenoids including canthaxanthin, β-β-carotene and echinenone by the isolate CHS1.

Table 6.3. Growth characteristics of the cyanobacterium isolate CHS1 on different physiological parameters

Isolate		**Physiological parameters**				
	Temp*	**Biomass mg/5 days**				
	(°C)	**5**	**10**	**15**	**20**	**25**
***Nodularia spumigena* CHS1**	**5**	4.92	18.13	37.03	42.57	41.23
	15	19.91	63.87	120.98	119.7	117.9
	25	14.31	47.32	103.95	102.9	101.3
	35	6.01	20.41	31.91	35.6	34.2
	pH*	**Biomass mg/5 days**				
		5	**10**	**15**	**20**	**25**
	5	2.45	7.54	10.34	12.45	12.01
	7	18.34	60.41	117.76	116.37	115.76
	9	4.12	13.36	19.61	22.18	20.23
	NaCl*	**Biomass mg/5 days**				
		5	**10**	**15**	**20**	**25**
	2	12.12	47.41	98.16	97.78	97.61
	4	7.12	33.21	78.32	77.21	76.89
	6	5.61	20.43	41.32	43.96	42.93
	8	3.65	14.90	22.45	25.71	25.10

Keys;

**P* values for the pH and Temperature experiments were < 0.01

**P* value NaCl experiment was < *0.04*

Chlorophyll-*a* was the most abundant pigment produced (1061 µg g^{-1}) followed by β-β-carotene (341 µg g^{-1}), echinenone (121 µg g^{-1}) and canthaxanthin (31 µg g^{-1}) (Table 6.4). No unknown pigment was identified during pigment analysis.

Table 6.4. Different pigments produced by the *Nodularia spumigen*a CHS1

Strain	Pigments	µg g^{-1}	R^t (min)	Pigments	µg g^{-1}	R^t (min)
CHS1	Canthaxanthin	31	28.56	Chlorophyll-*a*	1061	35.43
	Echinenone	121	34.75	β-β-carotene	341	38.87
Keys; R^t = Retention time						

Cell membrane fatty acids analysis

The isolate CHS1 was found to produce both straight chain fatty acids including saturated, monounsaturated and polyunsaturated fatty acids, as well as branched chain fatty acids such as saturated and monounsaturated fatty acids in its cell membrane (Table 6.5). Major fatty acids produced by CHS1 are shown in (Table 6.5 and Table 6.6).

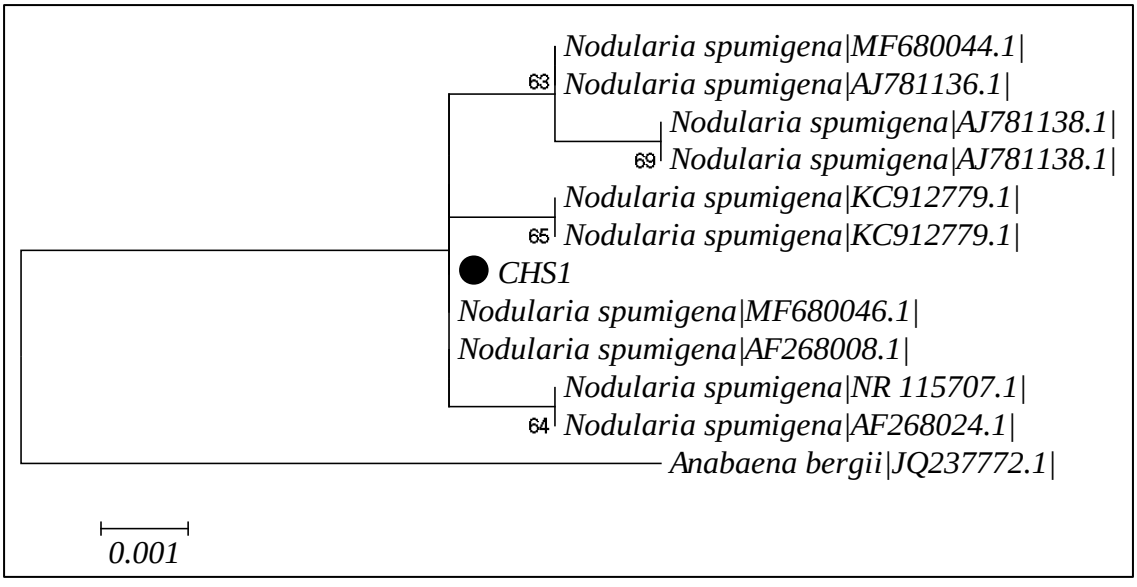
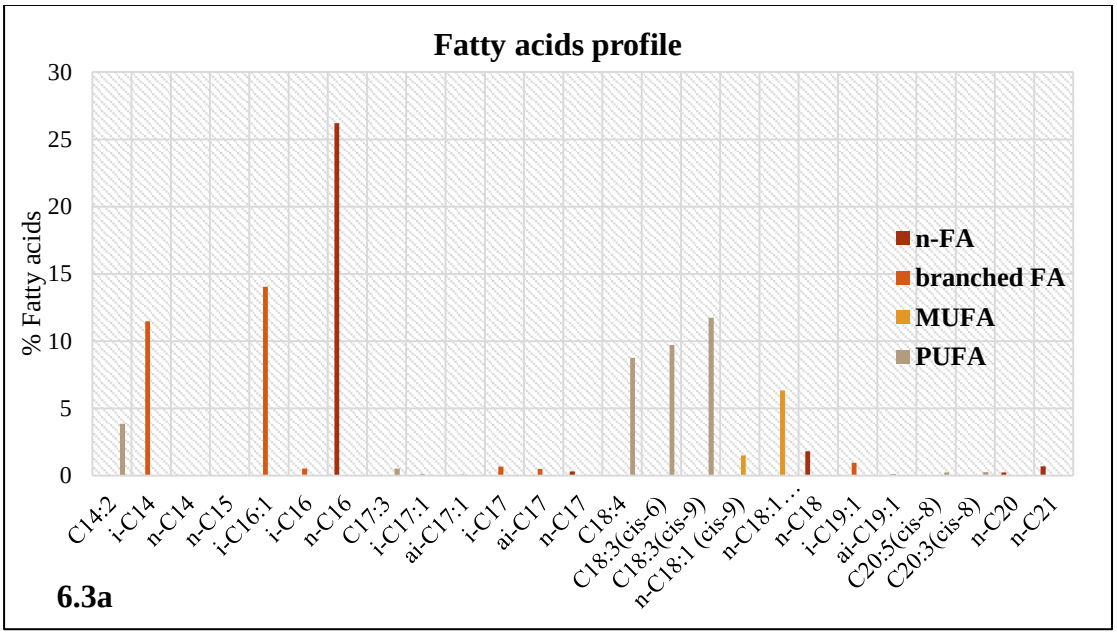


Fig. 6.2. Phylogenetic analysis of the *Nodularia spumigen*a CHS1 based on 16S-23S intergenetic region by Maximum Likelihood method

The most abundant fatty acids observed after GC/MS analysis was polyunsaturated fatty acids (35%) followed by straight chain fatty acids (29%), branched chain fatty acids (27%) and monounsaturated fatty acids (8%) (Fig. 6.3a, b). In addition, Omega 3 and 6 polyunsaturated fatty acids, most importantly *n*-$C1_{8:3}$(n-3) (Alpha-linolenic acid), *n*-$C_{20:5}$(n-3) (Eicosapentaenoic Acid) and *n*-$C_{20:3}$(n-6) (Dihomo-gamma-linolenic acid), were found in the cell membrane of the isolate CHS1. Likewise, Omega 9 fatty acids (with single double bonds) such as *n*-$C_{18:1(cis\text{-}9)}$ (n-9) (Oleic acid) and *n*-$C_{18:1(tr\text{-}9)}$ (n-9) (Elaidic acid), were present.



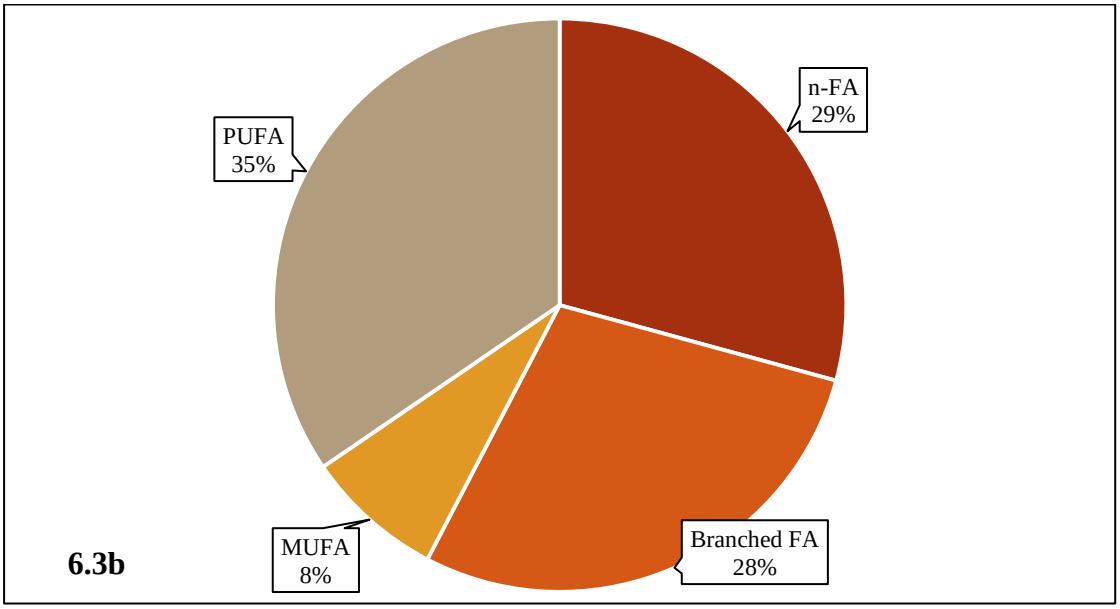


Fig. 6.3a, b. (a) Major fatty acids components of cell membrane of the *Nodularia spumigena* CHS1 **(b)** Percentage of main types of fatty acids in cell membrane of the *Nodularia spumigena* CHS1.

Table 6.5. The profile of cell membrane fatty acids of *Nodularia spumigen*a CHS1

Strain	Carbon Chain length	R^t (min)	Quantity µg g^{-1}	%/total CM fatty acids
Nodularia spumigena CHS1	*n*-$C_{14:2}$	18.51	485	4.0
	i-$C_{14:0}$	20.72	1440	11.5
	n-$C_{14:0}$	21.58	0.06	0.001
	n-$C_{15:0}$	24.08	0.06	0.01
	i-$C_{16:1}$	24.89	1763.7	14.0
	i-$C_{16:0}$	25.67	64.5	0.50
	n-$C_{16:0}$	26.57	3291	26.0
	i-$C_{17:1}$	27.28	10.7	0.10
	ai-$C_{17:1}$	27.45	9.46	0.10
	i-$C_{17:0}$	28.05	83.3	0.70
	ai-$C_{17:0}$	28.23	63.7	0.50
	n-$C_{17:0}$	28.85	37.3	0.30
	n-$C_{18:4}$	29.64	1099	8.0
	n-$C_{18:3(cis\text{-}6)}$	29.74	1219	9.0
	n-$C_{18:3(cis\text{-}9)}$	30.27	1472	11.0
	n-$C_{18:1(cis\text{-}9)}$	30.45	187.2	1.5
	n-$C_{18:1(trans\text{-}9)}$	30.59	795.3	6.3
	n-$C_{18:0}$	31.15	226.6	2.0
	i-$C_{19:1}$	31.21	119.8	1.0
	ai-$C_{19:1}$	32.00	10.6	0.10
	n-$C_{20:5(cis\text{-}8)}$	30.65	30.1	0.24
	n-$C_{20:3(cis\text{-}8)}$	34.03	31.6	0.25
	n-$C_{20:0}$	35.39	29.9	0.20
	n-$C_{21:0}$	37.34	87.3	0.70
Keys; *i*= iso, *ai*=anteiso, CM= cell membrane, *n* (straight chain).				

Table 6.6. The quantitative overview (with triplicates data and standard deviations) of cell membrane fatty acids of cyanobacterium CHS1

Isolate	**Fatty acids (carbon Chain length)**											
Nodularia spumigena CHS1	**Saturated fatty acids**											
	Triplicates (µg/g)	***i*-C$_{14:0}$**	***n*-C$_{14:0}$**	***n*-C$_{15:0}$**	***i*-C$_{16:0}$**	***n*-C$_{16:0}$**	***i*-C$_{17:0}$**	***ai*-C$_{17:0}$**	***n*-C$_{17:0}$**	***n*-C$_{18:0}$**	***n*-C$_{20:0}$**	***n*-C$_{20:1}$**
		1439	0.08	0.6	65.9	3290	84.2	63.4	37.1	226.5	23.8	87.1
		1438	0.05	0.7	64.7	3290	83.0	63.1	37.9	225.4	24.9	87.0
		1443	0.07	0.5	63.0	3294	83.2	64.6	38.1	228.0	23.0	87.8
	[1]SD	2.64	0.01	0.10	1.45	2.30	0.64	0.79	0.59	1.30	0.95	0.43
	Monounsaturated fatty acids											
	Triplicates (µg/g)	***i*-C$_{16:1}$**	***i*-C$_{17:1}$**	***ai*-C$_{17:1}$**	***n*-C$_{18:1}$ (*cis-9*)**	***n*-C$_{18:1}$ (*tr-9*)**	***i*-C$_{19:1}$**	***ai*-C$_{19:1}$**				
		1762.9	9.9	9.1	187.5	796.6	119.2	9.9				
		1762.6	10.5	9.8	186.2	795.5	118.9	10.8				
		1765.6	11.7	9.2	188.2	793.7	121.3	11.1				
	SD	1.65	0.91	0.37	1.02	1.45	1.30	0.62				
		Polyunsaturated fatty acids										
		***n*-C$_{14:2}$**	***n*-C$_{18:4}$**	***n*-C$_{18:3}$ (*cis-6*)**	***n*-C$_{18:3}$ (*cis-9*)**	***n*-C$_{20:5}$ (*cis-8*)**	***n*-C$_{20:3}$ (*cis-8*)**					

	Triplicates (µg/g)	486.1	1100	1219.6	1472.1	31.0	32.1					
		484.1	1101	1217.8	1475.4	30.2	30.9					
		486.2	1096	1221.7	1472.3	29.1	31.8					
	SD	1.18	2.64	1.95	1.85	0.95	0.62					
Keys; [1]SD: Standard deviation												

Discussion

In the current study, the cyanobacterium isolate CHS1 was isolated from glacial habitat, physiological characterized and analyzed for pigment composition as well as cell membrane fatty acids. In the present research study, the isolate CHS1 was observed with heterocysts, but akinetes were not seen during microscopical observation. *N. spumigen*a is known for morphological complexity as it can differentiate into different morphological forms such as akinetes, heterocysts or hormogonia (Rippka *et al.*, 1979). Akinetes are formed during harsh and unfavorable conditions and aid in the survival of the species. The formation of akinetes by *N. spumigena* has been documented in the Baltic Sea during autumn, where these resting stages sink and settle at the bottom through the winter season. During spring, blooms initiate from akinetes (Suikkanen *et al.,* 2010).

So far, we are reporting *N. spumigena* for the first time from a non-polar glacier. However, *N. spumigena* has been reported world-wide in a range of aquatic environments, such as from mesozooplankton grazers (Gorokhova and Engström-Öst, 2009), estuaries (Huber, 1985; 1986: Runnegar *et al.,* 1988; John and Kemp, 2006), phytoplankton, Baltic Sea (Salomon *et al.,* 2003), farm dams (Main *et al.,* 1977; Aplin, 1983), natural Australian blooms (Bolch *et al.,* 1999), shallow brackish and coastal water (Lehtimaki *et al.,* 2000; Oliva *et al.,* 2009; McGregor *et al.,* 2012; Mazur-Marzec *et al.,* 2012). Moreover, *N. spumigena* has been documented at very high concentrations in Baltic Sea habitats (Suikkanen *et al.,* 2007; Mohlin and Wulff, 2009: Ploug *et al.,* 2011) . *N. spumigena* has been observed and reported from some of the areas located in Antarctica such as Taylor Valley, southern Victoria Land, Antarctica (Broady, 1982; McKnight *et al.,* 1998), McMurdo Dry Valleys, Antarctica (Taton *et al.,* 2003), and Svalbard, Arctic (Hong *et al.,* 2010). Likewise, the genus *Nodularia* has been reported from microbial mats in lakes of Eastern Antarctica (Taton *et al.,* 2006).

The current study revealed that the isolate CHS1 was capable to survive and grow across a wide of physiological conditions, such as 5-35°C, 5-9 (pH) and 2-8 g/L NaCl. CHS1 tolerance to 8 g/L NaCl demonstrated during the present study reflects the conditions apparent in the range of habitats that *N. spumigena* has been found. In a few

studies *Nodularia sp.* growth has been observed at salt range between 7-13 g/L (Lehtimaki *et al.,* 1994; Wasmund, 1997; Moisander *et al.,* 2002), though beyond this threshold it is likely to adversely impact nitrogenase activity (Mazur-Marzec *et al.,* 2005). In addition, Hong *et al.* (2010) has reported that *N. spumigena* was able to grow at temperatures ranging between 15-20°C. To the best of our knowledge, no previous data is available on the physiological tolerance of *N. spumigena* to extreme pH conditions as tested in this study. We assumed that the isolate CHS1 had some protective mechanisms that helped it to tolerate such diverse ranges of pH. Most probably, proton pumping, modifications of cell surfaces and lipid composition of cell membrane could be the possible protective mechanisms used by the isolate CHS1 in order to cope with low and high pH. It has been observed in previous studies that microbes triggered proton pumping, enhance ammonia production, increase acids production and proton-consuming decarboxylation as well as modify cell surfaces and lipid composition of cell membrane in order to cope with low and high pH (Padan *et al.,* 2005; Lund *et al.,* 2014).

In the current study, it has been found that the isolate CHS1 had chlorophyll-*a* in adequate quantity and various types of the carotenoid most important β,β-carotene. Similar results were also reported from the blooms of *N. spumigena* isolated from Baltic Sea by Henriksen (2005). However, it is well established that chlorophyll-*a*, carotene and attached phycobilisomes constitute the main components of cyanobacterial thylakoid membranes (Omata and Murata, 1984), whereas xanthophylls dominate the outer and plasma membrane (Omata and Murata, 1984; Resch and Gibson, 1983; Jüirgens and Weckesser, 1985). Both of the membranes are of equal importance as they are the places where photosynthesis and respiratory electron transportation occur (Omata and Murata, 1985).

In the current research work, we present the first quantification of the isolate N. spumigena CHS1 cell membrane fatty acid composition from glacial environments. The maintenance of membrane fluidity by cyanobacteria in cold habitats is important as it provides a platform for cell energy generation such as occurs in the photosystems (Murata and Los, 1997). The cyanobacterium *N. spumigena* CHS1 was observed to

produce polyunsaturated fatty acids (e.g. $C_{14:2}$, α-$C_{18:3}$, γ-$C_{18:3}$, $C_{18:4}$, $C_{20:3}$ and $C_{20:5}$) as a predominate type of fatty acids in its cell membrane, consistent with cold adaptation apparent across cyanobacterial species that inhabit cold environments in order to maintain cell membrane fluidity in low temperatures (Los, 2004). We assumed that the isolate CHS1 had actively expressing genes accountable for the expression of fatty acid desaturases enzymes that responsible for fatty acids unsaturation. The cyanobacterium *Synechocystis* has been found to maintain the membrane fluidity by adding double bonds into acyl chains of lipid-bound fatty acids, mediated by fatty acid desaturases enzymes (Los, 2004). A study, conducted by Stanier and Cohen-Bazire (1977) and Sato and Murata (1980), has showed that the degree of unsaturation of fatty acids increased when temperature decreased. They also observed that induction of genes responsible for the expression of fatty acid desaturases enzymes increased when temperature decreased. The lipid and fatty acid composition of chloroplasts and cell membrane of cyanobacteria has been found similar to that of eukaryotic plants (Murata and Nishida, 1987; Wada and Murata, 1990).

However, the effects of low temperatures on fatty acid composition and fluidity of cell membranes of cyanobacteria have been studied very rarely. A few studies have been conducted to observe changes to cell membranes fluidity and other physiological functions induced by low temperatures in cyanobacterial strains such as *Anacystis nidulans*, *Anabaena variabilis*, *Synechococcus lividus* and *Synechocystis* sp. (Murata, 1989). Best to our knowledge, the fatty acids composition of cell membrane of the cyanobacterium CHS1 has been studied in this research work for the first time from glacial environments. Based on the fatty acids composition of cell membranes, cyanobacteria group into four major classes (Kenyon *et al.*, 1972: Los and Mironov, 2015). The first group contains only SFA and MUFA (such as $C_{16:0}$ and $C_{18:1}$), while the other three groups have also PUFA in addition to SFA and MUFA (such as α-$C_{18:3}$, γ-$C_{18:3}$, and $C_{18:4}$) (Los and Mironov, 2015). Our data identify the isolate CHS1 to belong to the fourth group as we have demonstrated production of $C_{18:4}$ in the cell membrane.

Conclusion

In conclusion, the cyanobacterium isolate CHS1 was isolated for the first time from the Hopar glacier, situated in Pakistan. HPLC and GC/MS analysis have confirmed that isolate CHS1 contained chlorophyll-*a* and PUFAs were the main constituents of cell membrane fatty acids, respectively. This research is important because it is providing important information about the fatty acids (especially PUFA) present in the cell membrane of isolate CHS1 that would be helpful in future as well to understand the possible role of PUFA in adaptation of *N. spumigena* in cold habitats. In addition, *Nodularia spumigena* would be a main topic of interest in future research regarding its ability to produce cyclic pentapeptide nodularin (NOD) and toxify freshwater originating from glaciers. Production of nodularin results in the toxicity of blooms that can impact ecosystems and water quality. While on other hand, glaciers situated in Karakoram mountains are the main source of freshwater to massive populations living the valleys downstream.

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Overall Conclusions

Conclusions

The current study explored geochemistry and diversity of psychrophilic bacteria of Ghulkin, Ghulmet and Hopar glaciers, located in Karakoram mountains, Pakistan, as well as role of cell membrane fatty acid and pigments in bacterial cold adaptation.

- The highest TOC, TN and TP were observed in sediments of all glaciers followed by meltwater and ice.
- Ca^{2+} and SO_4^{2-} were present in higher concentrations in all glacial samples.
- Highest concentrations of Ca^{2+}, K^{2+}, Mg^{2+} and PO_4^{2-} detected in sediment of Hopar glacier. Sediment of Ghulmet glacier was rich in Na^{+}, while that of Ghulkin glacier in NO_3^{-}.
- In this study, 82 bacterial species were isolated from Ghulmet, Hopar and Ghulkin glaciers, located in Karakoram mountain range.
- All bacterial isolates were found to represent 7 classes, 12 orders, 18 families and 25 genera after 16S rRNA and rpoB gene sequencing analysis.
- Proteobacteria was reported as predominant bacterial phylum covering 66% of total bacterial strains followed by Actinobacteria, Firmicutes, Deinococcus-Thermus and Bacteroidetes.
- The strains belonging to genus *Pseudomonas* (19%) were the most abundant genus identified in the current research work.
- Out of 82 bacterial strains, 39 were confirmed to produce 10 different types of pigments including 2 unknown pigments as well via HPLC analysis.
- A total of 39 bacterial strains were found to produce both type of pigments including carotenes and xanthophylls however, most of them produced β-carotene followed by zeaxanthin.
- Xanthophylls were produced in higher quantities as compared to carotenes.
- Bacterial strains were able to produce astaxanthin, β,β-carotene, β,ε-carotene, 19'-hexanoyloxyfucoxanthin and zeaxanthin.
- Branched chain and straight- chain monounsaturated fatty acids were observed as chief types of cell membrane fatty acid of studied bacteria, revealed after cell membrane fatty acid analysis. Both lower and higher temperatures were

markedly effected composition of cell membranes fatty acids in bacterial species.

- An increasing and decreasing shift of saturated fatty acids and n-MUFAs from lower (5°C) to higher temperature (35°C) observed.
- Branched chain saturated fatty acids and b-MUFAs observed in higher concentrations at higher (25 and 35°C) and lower temperature (5 and 15°C), respectively.
- Bacterial strains were found to produce polyunsaturated fatty acids at low temperature only.
- Red pigment producing isolate HI6 was identified as *Serratia marcescens* through molecular approach.
- *Serratia marcescens* HI6 capable to bear a varied array of pH (5-11°C) and temperature (5-35°C).
- The principle cell membrane fatty acids of *Serratia marcescens* HI6 were *i*-$C_{16:1}$ and other branched chain fatty acids.
- After sequencing of 16S-23S intergenetic region, isolate CHS1 was identified as *Nodularia spumigena*.
- *Nodularia spumigena* CHS1 was found to produce Chlorophyll-*a* as a major pigment.
- *Nodularia spumigena* CHS1 was found to endure extreme physiological conditions of NaCl, temperature and pH.
- The characteristic PUFA of *Nodularia spumigena* CHS1 was *n*-$C_{18:4}$. However, *n*-$C_{16:0}$, *n*-$C_{18:1}$ and *n*-$C_{20:5}$ were also abundantly found in the plasma membrane of the isolate *Nodularia spumigena* CHS1 in this study.

This study reported geochemistry, culturable diversity of psychrophilic bacteria and presence of *Nodularia spumigena* in the Ghulkin, Ghulmet and Hopar glaciers for the first time. In addition, cell membrane fatty acid and pigment analysis as well as their role in cold adaptation have been conducted for the first time in bacteria isolated from any glacier of Pakistan as well as outside Polar Regions.

Future prospects

Future prospects

- Trace elements present in water such as iron, manganese, zinc etc. have key importance for metabolic activities of both animals and plants. While glacier situated in Karakoram mountains are the main source of freshwater to massive populations (including both animals and plants) living the valleys downstream. Hence, studied glaciers need to be studied properly for trace elements in order to determine their concentrations and their impact on lives residing downstream.
- The studied glaciers have explored for the presence of psychrophilic bacteria via culture-dependent methods which could not give complete picture of bacteria present in these glaciers. Therefore, deep metagenomic approaches would be best for the detection of more potent unculturable bacteria in samples collected from studied glaciers. In fact, there are more than 5000 glaciers in HKKH which need to be explored microbiologically via both culture-dependent and independent techniques.
- Many unknown pigments produced by bacteria have been detected in the current research work. As pigments have multiple use in different fields, these unknown pigments require to be identified appropriately with the aim to find novel pigment having multiple applications.
- Psychrophilic bacteria have been recognized worldwide to possess several utilities in various industrial and pharmaceutical arena. Therefore, there is immense need to investigate properly in future the numerous applications of the current isolated psychrophilic bacteria including production of psychrophilic compounds e.g. enzymes, antifreeze proteins, antibiotics, biofertilizers, pigments and fatty acids (especially eicosapentenoic acids).
- Climate change is no longer just a myth as its happening in real due to rise in temperature. While glaciers located in HKKH have affected massively due to this phenomenon in last few years. Microbes residing in these glaciers would be used as bio-indicators to monitor the effects of climate change on these glaciers.

Appendices

Media composition

Nutrient agar

Contents	Amount (g/L)
Peptone	15
Sodium chloride	6
Yeast extract	3
D(+)-glucose	1
Agar	15

Reasoner's 2 Agar

Contents	Amount (g/L)
Casein acid hydrolysate	0.5
Dextrose	0.5
Protease peptone	0.5
Sodium pyruvate	0.3
Starch soluble	0.5
Yeast extract	0.5
Dipotassium phosphate	0.3
Magnesium sulfate	0.024
Agar	15

Minimal salts medium

Contents	Amount (g/L)
Sodium phosphate dibasic	6
Yeast extract	3
Potassium phosphate monobasic	3
Magnesium sulphate, anhydrous	0.12
Calcium chloride dihydrate 0.05	1
Agar	15

Luria Bertani Agar

Contents	Amount (g/L)
Tryptone	10
Sodium chloride	5
Yeast extract	5
Agar	15

16S ribosomal RNA gene, partial sequences

>GhS_1 (*Deinococcus depolymerans*)

ATCGCTAATACGTGATGTGCTGCTCCCTCCTGTGGGATCAGTAAAGATTTA
TTGCTTTGGGATGGGGTTGCGTTCCATCAGCTAGTTGGTAGGGTAAAGGCC
TACCAAGGCGACGACGGATAGCCGGCCTGAGAGGGTGGCCGGCCACAGG
GGCACTGAGACACGGGCCCCACTCCTACGGGAGGCAGCAGTTAGGAATCT
TCCACAATGGGCGAAAGCCTGATGGAGCGACGCCGCGTGAGGGATGAAG
GTTTTCGGATCGTAAACCTCTGAATCAGGGACGAAAGACGCTTTATGCGG
GATGACGGTACCTGAGTAATAGCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCAAGCGTTACCCGGAATCACTGGGCGTAAAGGG
CGTGTAGGCGGGATGTTAAGTCTGGTTTTAAAGACTGCGGCTCAACCGCA
GGGATGGACTGGATACTGGCATTCTTGACCTCTGGAGAGAGAACTGGAAT
TCCTGGTGTAGCGGTGGAATGCGTAGATACCAGGAGGAACACCAATGGCG
AAGGCAGGTTCTTGGACAGAAGGTGACGCTGAGGCGCGAAAGTGTGGGG
AGCGAACCGGATTAGATACCCGGGTAGTCCACACCCTAAACGATGTACGT
TGGCTAATCGCAGGATGCTGTGATTGGCGAAGCTAACGCGATAAACGTAC
CGCCTGGGAAGTACGGCCCGCAAGGTTGAAACTCAAAGGAATTGAC

>GhS_3 (*Pseudomonas frederiksbergensis*)

AACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC
GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG
TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG
TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTT
ACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTAGAGTATG
GTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGG
AAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCG
CAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA
ACTCAAATGAATTGACGGGGGGCCCGCACAAGCGGTGGG

>GhS_4 (*Sphingobium xenophagum*)

ATACCGGATGATGACGAAAGTCCAAAGATTTATCGCCCAGGGATGAGCCC
GCGTAGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCC
TTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGACAATGGGCGAAAG
CCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGC
TCTTTTACCCGAGATGATAATGACAGTATCGGGAGAATAAGCTCCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGA
ATTACTGGGCGTAAAGCGCACGTAGGCGGCGATTTAAGTCAGAGGTGAAA
GCCCGGGGCTCAACCCCGGAACTGCCTTTGAGACTGGATTGCTAGAATCT
TGGAGAGGCGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTC
GGAAGAACACCAGTGGCGAAGGCGGCTCGCTGGACAAGTATTGACGCTG
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGATAACTAGCTGCTGGGGCACATGGTGTTTCGGTGGC
GCAGCTAACGCATTAAGTTATCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCAAAGGAATTGACGGGGGGCCTGCACAAGCGGTGGG

>GhS_5 (*Staphylococcus equorum*)

TTTGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATA
AGACTGGAATAACTTCGGGAAACCGGAGCTAATGCCGGATAACATTTGGA
ACCGCATGGTTCTAAAGTAAAAGATGGTTTTGCTATCACTTATAGATGGAC
CCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGAT
ACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAA
CTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGAC
GGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTA
GGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT
CATTGGAAACTGGGAAACTTGAGTACAGAAGAGGAAAGTGGAATTCCAT
GTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGG
CGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAA
ACAGGATTAGATACCCTGGTAGTCCA

>GhS_6 (*Deinococcus aquaticus*)

GTGATGTGCTGCTCCCTCCTGTGGGATCAGTAAAGATTTATTGCTTTGGGA
TGGGGTTGCGTTCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCG
ACGACGGATAGCCGGCCTGAGAGGGTGGCCGGCCACAGGGGCACTGAGA
CACGGGCCCCACTCCTACGGGAGGCAGCAGTTAGGAATCTTCCACAATGG

GCGAAAGCCTGATGGAGCGACGCCGCGTGAGGGATGAAGGTTTTCGGATC
GTAAACCTCTGAATCAGGGACGAAAGACGCTTTATGCGGGATGACGGTAC
CTGAGTAATAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA
GGGTGCAAGCGTTACCCGGAATCACTGGGCGTAAAGGGCGTGTAGGCGG
GATGTTAAGTCTGGTTTTAAAGACTGCGGCTCAACCGCAGGGATGGACTG
GATACTGGCATTCTTGACCTCTGGAGAGAGAACTGGAATTCCTGGTGTAG
CGGTGGAATGCGTAGATACCAGGAGGAACACCAATGGCGAAGGCAGGTT
CTTGGACAGAAGGTGACGCTGAGGCGCGAAAGTGTGGGGAGCGAACCGG
ATTAGATACCCGGGTAGTCCACACCCTAAACGATGTACGTTGGCTAATCG
CAGGATGCTGTGATTGGCGAAGCTAACGCGATAA

>GhS$_7$ (*Pseudomonas frederiksbergensis*)

AGAAGTGAAAACAAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGGAC
GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGA
AACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC
GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG
TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG
TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTT
ACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAT
CCCCGGGCTCAACCTGG

>GhS$_8$ (*Acinetobacter radioresistens*)

TCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGG
ACCTTTGGGCCTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGG
TAGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATG
ATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCT
ACCTAGATTAATACTTTAGGATAGTGGACGTTACTCGCAGAATAAGCACC
GGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAA
TCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCCAATTAAGTCAAATG
TGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTGGCTAGA
GTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA
GATCTGGAGGAATACCGATGGCGAAGGCAGCTATCTGGCCTAATACTGAC
GCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCCTTGAGGCTTTAGT

GGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCCGCAAG
AACTAAAA

>GhS$_9$ (*Arthrobacter sulfureus*)

GATAAGCCTGGGAAACTGGGTCTAATACCGGATATGCACCGTGGACCGCA
TGGTTCTTGGTGGAAAGAATTTTGGTCAGGGATGGACTCGCGGCCTATCA
GCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAG
CGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTA
GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTATCGTGAAAGTCC
GAGGCTCAACCTCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTA
GGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAG
CGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGT
AAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAG
CTAA

>GhS$_{10}$ (*Sphingomonas faeni*)

AAGATTTATCGCCGAGGGATGAGCCCGCGTAGGATTAGGTAGTTGGTGTG
GTAAAGGCGCACCAAGCCGACGATCCTTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGA
GTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTACCCGGGATGATAATGA
CAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGAGCTAGCGTTATTCGGAATTACTGGGCGTAAAGCGCACGT
AGGCGGCTTTGTAAGTAAGAGGTGAAAGCCCAGAGCTCAACTCTGGAATT
GCCTTTTAGACTGCATCGCTTGAATCATGGAGAGGTCAGTGGAATTCCGA
GTGTAGAGGTGAAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGC
GGCTGACTGGACATGTATTGACGCTGAGGTGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCCGTAAACGATGATAACTAGC
TGTCCGGACACTTGGTGTTTGGGTGGCGCAGCTAACGCATTAAGTTTATCC
GCCTGGGGGAGTACGGCCGCCAGGTTAAAACTCAGATGAATTGGACGGG

>GhS$_{11}$ (*Enterobacter cloacae*)

TGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGA
CCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGT

GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT
GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGT
GTTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCG
GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGT
GAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAG
ATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCC
TTCCGGAGCTAACGCGTTAAGTTCGACCGCCCTGGGGG

>GhS$_{12}$ (*Enterobacter mori*)

GGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAA
AGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTA
GCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG
GGGAGGAAGGTGTTGAGGTTAATAACCTCAGCGATTGACGTTACCCGCAG
AATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC
AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACT
GGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
CAAAGACTGACGCTCAAGTGCGAAAGCGTGAGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTT
GAGGCGTGGCTTCC

>GhS$_{13}$ (*Pseudomonas frederiksbergensis*)

GCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGG
GGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGA
TGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCAGTTACCTAATACGTATCTGTTTTGACGTTACCGACAGAATAAGCACC
GGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAA

TCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATG
TGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTAG
AGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGA
CACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTA
GTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCA

>GhS$_{14}$ (*Stenotrophomonas maltophilia*)

ATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAG
CAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGC
TAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGA
GAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCAT
ACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAA
AGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATA
AGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAG
CGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGT
CCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGC
GACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATG
CGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAA
CACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTG
GCACGCGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGGAGTACGG
TCGCCAGGACTGAAAACTCAAAAGGAATTGACGGGGGGCCCCCCACAAG
CGGGGGGAGTATGTGGTTTTA

>GhW$_1$ (*Deinococcus aquaticus*)

AATACGTGATGTGCTGCTCCCTCCTGTGGGATCAGTAAAGATTTATTGCTT
TGGGATGGGGTTGCGTTCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCA
AGGCGACGACGGATAGCCGGCCTGAGAGGGTGGCCGGCCACAGGGGCAC
TGAGACACGGGCCCCACTCCTACGGGAGGCAGCAGTTAGGAATCTTCCAC
AATGGGCGAAAGCCTGATGGAGCGACGCCGCGTGAGGGATGAAGGTTTTC
GGATCGTAAACCTCTGAATCAGGGACGAAAGACGCTTTATGCGGGATGAC
GGTACCTGAGTAATAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTACCCGGAATCACTGGGCGTAAAGGGCGTGTA
GGCGGGATGTTAAGTCTGGTTTTAAAGACTGCGGCTCAACCGCAGGGATG
GACTGGATACTGGCATTCTTGACCTCTGGAGAGAGAACTGGAATTCCTGG
TGTAGCGGTGGAATGCGTAGATACCAGGAGGAACACCAATGGCGAAGGC
AGGTTCTTGGACAGAAGGTGACGCTGAGGCGCGAAAGTGTGGGGAGCGA

ACCGGATTAGATACCCGGGTAGTCCACACCCTAAACGATGTACGTTGGCT
AATCGCAGGATGCTGTGATTGGCGAAGCTAACGCGATAAACGTACCGCCT
GGGAAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTTAATTCGAAGCAACGCGAAGAAACC
TTACCAGGTCTTGACATGCACAGAACCTTTGAGAGATCAGAGGGTGCCC

>GhW$_2$ (*Acinetobacter radioresistens*)

GAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACC
TTTGGGCCTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTAG
GGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATC
CGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTAC
CTAGATTAATACTTTAGGATAGTGGACGTTACTCGCAGAATAAGCACCGG
CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATC
GGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCCAATTAAGTCAAATGTG
AAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTGGCTAGAGT
ATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
TCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGC
TGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCCTTGAGGCTTTAGTGGC
GCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAG

>GhW$_3$ (*Brevundimonas vesicularis*)

AAACTTGTGCTAATACCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCTT
TAGAGCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAA
GGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTG
AGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCA
ATGGGCGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAG
GATTGTAAAATTCTTTCACCGGGGACGATAATGACGGTACCCGGAGAAGA
AGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAG
CGTTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGT
CAGGGGTGAAATCCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGT
GTCTTGAGTATGAGAGAGGTGTGTGGAACTCCGAGTGTAGAGGTGAAATT
CGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACACACTGGCTCATT
ACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGATTGCTAGTTGTCGGGATGCATGCATTT
CGGTGACGCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCA
AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG

>GhW$_4$ (*Pseudomonas frederiksbergensis*)

GCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGG
GGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGA
TGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCAC
CGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGAT
GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTA
GAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAG
ATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTG
ACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTT
AGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGGAGTACGGCCGCA
AGGTTAAAACTCAAATGAATTGACGGGGGCCCG

>GhW$_5$ (*Staphylococcus equorum*)

TTCGGGAAACCGGAGCTAATGCCGGATAACATTTGGAACCGCATGGTTCT
AAAGTAAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATT
AGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG
CAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTATTA
GGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTT
AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGAAACTTGAGTACAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGT
CTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTT
CCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCCACTCCG

>GhW$_6$ (*Brevundimonas nasdae*)

TGCTAATACCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGC
GGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGAC
GATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACAC

GGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCG
AAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTA
AAATTCTTTCACCGGGGACGATAATGACGGTACCCGGAGAAGAAGCCCCG
GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCT
CGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGT
GAAATCCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAG
TATGAGAGAGGTGTGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGATA
TTCGGAAGAACACCAGTGGCGAAGGCGACACACTGGCTCATTACTGACGC
TGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGATTGCTAGTTGTCGGGATGCATGCATTTCGGTGAC
GCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGT

>GhW$_7$ (*Sanguibacter antarcticus*)

TACTGGATACGAAGCGCCTCGGCATCGGGTGCGCTTGGAAAGTTTTTCGG
TCAAGGATGGACTCGCGGCCTATCAGCTTGTTGGCGGGGTAACGGCCCAC
CAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAAGGCC
TTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACC
TGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGT
TTGTCGCGTCTGGTGTGAAAACTCAAGGCTCAACCTTGAGCTTGCATCGGG
TACGGGCAGACTAGAGTGCGGTAGGGGTGACTGGAATTCCTGGTGTAGCG
GTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCAC
TGGGCCGCAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGCCCTGGGGC
TCATTCCACGAGTTCCGTGCCGCAGCAAACGCATTAAGTGCCCCCGCCTG
GGGGAGTACGGCCCGTA

>GhW$_8$ (*Deinococcus aquaticus*)

GGCGTCTTAGAGACTGTAGTGGCGCACGGGTGAGTAACGCGTAACTGACC
TACCCCAAAGTCGCGGATAACGGTTCAGAAAGAATCGCTAATACGTGATG
TGCTGCTCCCTCCTGTGGGATCAGTAAAGATTTATTGCTTTGGGATGGGGT
TGCGTTCCATCAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGACG
GATAGCCGGCCTGAGAGGGTGGCCGGCCACAGGGGCACTGAGACACGGG
CCCCACTCCTACGGGAGGCAGCAGTTAGGAATCTTCCACAATGGGCGAAA
GCCTGATGGAGCGACGCCGCGTGAGGGATGAAGGTTTTCGGATCGTAAAC
CTCTGAATCAGGGACGAAAGACGCTTTATGCGGGATGACGGTACCTGAGT
AATAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC

AAGCGTTACCCGGAATCACTGGGCGTAAAGGGCGTGTAGGCGGGATGTTA
AGTCTGGTTTTAAAGACTGCGGCTCAACCGCAGGGATGGACTGGATACTG
GCATTCTTGACCTCTGGAGAGAGAACTGGAATTCCTGGTGTAGCGGTGGA
ATGCGTAGATACC

>GhW$_9$ (*Pseudomonas frederiksbergensis*)

GGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGAC
CTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT
CAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGC
ATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGG
CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTAGAG
TATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATA
TAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACA
CTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTG
GCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTT
AAAACTCAAATGAATTGACG

>GI$_1$ (*Massilia oculi*)

ATATATCGGAACGTACCCAAGAGTGGGGGATAACGTAGCGAAAGTTACGC
TAATACCGCATACGATCTAAGGATGAAAGCAGGGGATCTTCGGACCTTGT
GCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTGAGGTAAAGGCTC
ACCAAGGCTACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTG
GACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGC
CTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGGGCTAATA
TCCTTCGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGG
CGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGACGTGAAATCCCCGGGC
TCAACCTGGGAATTGCGTTGGAGACTGCAAGGCTAGAATCTGGCAGAGGG
GGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAAC
ACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC
GATGTCTACTAGTTGTCGGGTTTTAATTAACTTGGTAACGCAGCTAACGCG
TGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG

>GI_2 (*Enterobacter hormaechei*)

TAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTA
GCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC
TTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGC
TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG
GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAA
TAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAG
GGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTA
ACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAA
ATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTACTCTT

>GI_3 (*Massilia aurea*)

AAGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCT
AAGGATGAAAGCAGGGGATCTTCGGACCTTGTGCTCCTGGAGCGGCCGAT
ATCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCCACGATCAGT
AGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGC
CTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCT
CTTTTGTCAGGGAAGAAACGGTGAGGGCTAATATCCCTCGCTAATGACGG
TACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGG
CGGTTTTGTAAGTCTGACGTGAAATCCCCGGGCTCAACCTGGGAATTGCGT
TGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAATTCCACGTGT
AGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGGCAGC
CCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGGAGCAAACA
GGATTAGATACCCTGGGTAGTCCACGCCCCTAAACGATGTCTACTAGGTT
GTCGGGGTTT

>GI_4 (*Massilia aurea*)

GAGTAATATATCGGAACGTACCCAAGAGTGGGGGATAACGTAGCGAAAG
TTACGCTAATACCGCATACGATCTAAGGATGAAAGCAGGGGATCTTCGGA
CCTTGTGCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTGAGGTAAA
GGCTCACCAAGGCTACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAG
AAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGGGC
TAATATCCTTCGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTA
CTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGACGTGAAATCCC
CGGGCTCAACCTGGGAATTGCGTTGGAGACTGCAAGGCTAGAATCTGGCA
GAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGA
GGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGC
ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCC
TAAACGATGTCTACTAGTTGTCGGGTTTTAATTAACTTGGTAACGCAGCTA
ACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGG

>GS_1 (*Arthrobacter agilis*)

AACCGGGTCTAATACTGGATACGACCTTCTGGCGCATGCCATGTTGGTGG
AAAGCTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGG
GTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGA
GGGATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCCGG
CCTTTCGGGGTTGGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGG
GGCTTAACTCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGCAGTAGG
GGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
ACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCG
AAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGGTA
AACGTTGGGGCACTAGGTGTGGGGGGACATTCCACGTTTTTCCGCGCCGG
TAGCTA

>GS_2 (*Pseudomonas brassicacearum*)

CGCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAG
GGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAG

TTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG
GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCC
GCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGA
AGGGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGC
ACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGG
ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGC
TAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGT
AGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATAC
TGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTC
TTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGGAGTACGGC

>GS_3 (*Pseudomonas migulae*)

GTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGA
CGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCT
TGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGG
CTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACAC
TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAA
GGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTA
ATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGT
GCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAG
GGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAA
CACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAAC
GCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAA

>GS_4 (*Pseudomonas frederiksbergensis*)

GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGA
AACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC
GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG
TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG
TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTT

ACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATG
GTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGG
AAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCG
CAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCC

>GS_5 (*Flavobacterium xinjiangense*)

GAGAAATTTGGATTAATACCTTATAGTAATACGACTTGGCATCAAGATGT
ATTTAAAGATTTATCGGTGAAAGATGAGCATGCGTCCCATTAGCTTGTTGG
TAAGGTAACGGCTTACCAAGGCAACGATGGGTAGGGGTCCTGAGAGGGA
GATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAG
CAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCG
TGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGTACAGGAAGAAA
CCCTTTCACGTGTGGAAGATTGACGGTACTGTAAGAATAAGGATCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGA
ATCATTGGGTTTAAAGGGTCCGTAGGCGGTCAAGTAAGTCAGTGGTGAAA
GCCCATCGCTCAACGGTGGAACGGCCATTGATACTGCTTGACTTGAATTAT
TAGGAAGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTAC
ATGGAATACCAATTGCGAAGGCAGGTTACTACTAATGGATTGACGCTGAT
GGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGGATACTAGCTGTTGGGAGCAATCTCAGTGGCTAAGCGAA
AGTGATAAGTATCCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
GATACGCGAGGAACCTTACCAAG

>GS_6 (*Janthinobacterium agaricidamnosum*)

GAGAAATTTGGATTAATACCTTATAGTAATACGACTTGGCATCAAGATGT
ATTTAAAGATTTATCGGTGAAAGATGAGCATGCGTCCCATTAGCTTGTTGG
TAAGGTAACGGCTTACCAAGGCAACGATGGGTAGGGGTCCTGAGAGGGA
GATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAG
CAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCG
TGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGTACAGGAAGAAA
CCCTTTCACGTGTGGAAGATTGACGGTACTGTAAGAATAAGGATCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGA
ATCATTGGGTTTAAAGGGTCCGTAGGCGGTCAAGTAAGTCAGTGGTGAAA
GCCCATCGCTCAACGGTGGAACGGCCATTGATACTGCTTGACTTGAATTAT

TAGGAAGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTAC
ATGGAATACCAATTGCGAAGGCAGGTTACTACTAATGGATTGACGCTGAT
GGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGGATACTAGCTGTTGGGAGCAATCTCAGTGGCTAAGCGAA
AGTGATAAGTATCCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
GATACGCGAGGAACCTTACCAAG

>GS_7 (*Flavobacterium sinopsychrotolerans*)

GAGAAATTTGGATTAATACCTTATAGTAATACGACTTGGCATCAAGATGT
ATTTAAAGATTTATCGGTGAAAGATGAGCATGCGTCCCATTAGCTAGTTG
GTAAGGTAACGGCTTACCAAGGCAACGATGGGTAGGGGTCCTGAGAGGG
AGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCA
GCAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGC
GTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTATACAGGAAGAA
ACAGTTCTACGTGTAGAACCTTGACGGTACTGTAAGAATAAGGATCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGG
AATCATTGGGTTTAAAGGGTCCGTAGGCGGTCAGATAAGTCAGTGGTGAA
AGCCCATCGCTCAACGGTGGAACGGCCATTGATACTGTCTGACTTGAATT
ATTAGGAAGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATT
ACATGGAATACCAATTGCGAAGGCAGGTTACTACTAATTGATTGACGCTG
ATGGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGGATACTAGCTGTTGGGAGCAATCTCAGTGGCTAAGCG
AAAGTGATAAGTATCCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGATACGCGAGGAACCTTACCAAG

>GS_9 (*Paracoccus hibiscisoli*)

CGAGCGAGACCTTCGGGTCTAGCGGCGGACGGGTGAGTAACGCGTGGGA
ACGTGCCCTTCTCTACGGAATAGCCCCGGGAAACTGGGAGTAATACCGTA
TACGCCCTTTGGGGGAAAGATTTATCGGAGAAGGATCGGCCCGCGTTGGA
TTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCCATAGCTGG
TTTGAGAGGATGATCAGCCACACTGGGACTGAGAAACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATCTTAGACAATGGGGGCAACCCTGATCT
AGCCATGCCGCGTGAGTGATGAAGCCCCTTAGGGTTGTAAAGCTCTTTCA
GCTGGGAAGCATAATGACGGTACCAGCAGAAGAAGCCCCGGCTAACTCC
GTGCCAGCAGCCGCGGTAATACGGAGGGGGCTAGCGTTGTTCGGAATTAC
TGGGCGTAAAGCGCACGTAGGCGGACTGGAAAGTCATGGGTGAAATCCC
AGGGCTCAACCTTGGAACTGCCTTTGAAACTATCAGTCTGGAGTTCGAGA
GAGGTGAGTGGAATTCCCGAGTGTAGAGGTGAATGTCGTAGATATTCGGA

GGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC

>GS_{11} (*Brevundimonas vesicularis*)

TACCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCGGCCCG
CGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAG
TAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCA
AACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGC
CTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATT
CTTTCACCGGGGACGATAATGACGGTACCCGGAGAAGAAGCCCCGGCTAA
CTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAA
TTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAAT
CCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAGTATGA
GAGAGGTGTGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGG
AAGAACACCAGTGGCGAAGGCGACACACTGGCTCATTACTGACGCTGAGG
CTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGATTGCTAGTTGTCGGGATGCATGCATTTCGGTGACGCAGC
TAACGCATTAAGCAATCCGCCTGGGGGAGTACGGGTCGCCAGGATTAAAA
CTCAAAGGAATT

>GS_{12} (*Pseudomonas mandelii*)

AAATGAACGGGTACTTGTACCTGGTGGCGAGCGGCGGACGGGTGAGTAAT
GCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTA
ATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGC
TATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCAC
CAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT
CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTTACCTAATACGT
AAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAG
CAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAGGG

>GS_{13} (*Arthrobacter nitroguajacolicus*)

TGGGAAACTGGGTCTAATACCGGATATGACCATCTGACGCATGTCATGGT
GGTGGAAAGCTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTG
GTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
TGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC

AGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCC
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGA
AGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCG
TAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCTC
AACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGA
CTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAG
CATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGT
TGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACG
CATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAGAGG
AATTGACGGGGGCCCG

>GS$_{14}$ (*Rhizobium herbae*)

CTAATACCGTATACGCCCTTCGGGGGAAAGATTTATCGGAGTTGGATGAG
CCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGA
TCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGG
CCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGC
AAGCCTGATCCAGCCATGCCGCGTGTGTGATGAAGGCCTTAGGGTTGTAA
AGCACTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCG
GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTT
CGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATCGATCAGTCAGGGGT
GAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATACTGTCGGTCTAGA
GTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGAT
ATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACG
CTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAATGTTAGCCGTCGGGTAGTTTACTATTCGGTGG
CGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTA
AA

>GS$_{15}$ (*Sporosarcina psychrophila*)

CAGATGGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCAGTTT
GCCCGCATGGGCGAACTCTGAAAGACGGTTTCGGCTGTCACTGTAGGATG
GGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCAAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAATGCCGCGTGAGCGAAGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTAAGGGAAGAACAAGTACGGGAGTAACTGCCCGTGCCAT
GACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC

GCAGGCGGTTCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG
GGTCATTGGAAACTGGAGAACTTGAGTACAGAAGAGGAAAGCGGAATTC
CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA
AGGCGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACGCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGGGGG

>GS_{17} (*Paenisporosarcina macmurdoensis*)

AGTCGAGCGGAATGATGAAGAAGCTTGCTTCTTCTGATTTTAGCGGCGGA
CGGGTGAGTAACACGTGGGCAACCTACCTTGTAGATTGGGATAACTCCGG
GAAACCGGGGCTAATACCGAATAATCCATTTTGCTTCATGGCAGAATGTT
GAAAGACGGTTTCGGCTGTCACTACGAGATGGGCCCGCGGCGTATTAGCT
AGTTGGTAGGGTAATGGCCTACCAAGGCGACGATACGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAA
CGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGG
AAGAACACGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTTATTAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAG
TCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
GGACTTGAG

>GS_{18} (*Brevundimonas mediterranea*)

AACTTGTGCTAATACCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCATT
AGAGCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAG
GCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGA
GACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAAT
GGGCGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGA
TTGTAAAATTCTTTCAGTAGGGACGATAATGACGGTACCTACAGAAGAAG
CCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCG
TTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGTCA
GGGGTGAAATCCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGTGT
CTTGAGTATGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTGAAATTCG
TAGATATTCGGAAGAACACCAGTGGCGAAGGCGACATACTGGCTCATTAC
TGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGATTGCTAGTTGTCGGGATGCATGCATTTC
GGTGACGCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCAA
GATTAAAACTCAAAGGAATTGACGGGGGC

>GS_{19} (*Paracoccus carotinifaciens*)

GCAAGTCGAGCGAGACCTTCGAGGTCTAGCGGCGGACGGGTGAGTAACG
CGTGGGAACGTGCCCTTCTCTACGGAATAGCCCCGGGAAACTGGGAGTAA
TACCGTATACGCCCTTTGGGGGAAAGATTTATCGGAGAAGGATCGGCCCG
CGTTGGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCCA
TAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAATGGGGGCAACC
CTGATCTAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCT
CTTTCAGCTGGGAAGATAATGACGGTACCAGCAGAAGAAGCCCCGGCTAA
CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCTAGCGTTGTTCGGAA
TTACTGGGCGTAAAGCGCACGTAGGCGGACTGGAAAGTCAGAGGTGAAA
TCCCAGGGCTCAACCTTGGAACTGCCTTTGAAACTATCAGTCTGGAGTTCG
AGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCG
GAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAG
GTGCGAAAGCGTGGGGAGCAAACAGGATT

>GS_{20} (*Arthrobacter nitroguajacolicus*)

AGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTGACTCTGGGA
TAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCATCTGACGCATGT
CATGGTGGTGGAAAGCTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAGC
TTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGA
GAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCG
ACCCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGG
GAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGG
GGCTCAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGCAGTGGG
GGAGAGGGAATTCCTGGTGTAGCGGTAAAGTGCGCATATATCGGGAGGA
ACACGGTGG

>GS_{21} (*Brevundimonas intermedia*)

GGAAACTTGTGCTAATACCGAATGTGCCCTTCGGGGGAAAGATTTATCGC
CATTAGAGCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCAC
CAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGA
CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCG
CAATGGGCGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTT
AGGATTGTAAAATTCTTTCACCGGGGACGATAATGACGGTACCCGGAGAA
GAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCT

AGCGTTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAA
GTCAGGGGTGAAATCCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGG
GTGTCTTGAGTATGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTGAAA
TTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACATACTGGCTCA
TTACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGATTGC

>GW_1 (*Janthinobacterium lividum*)

CGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGGGGGATC
GCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTA
GGGTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTG
AGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGG
TGAGAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTG
AAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAA
TCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATA
TGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGC
TCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACG
CAGCCTAA

>GW_2 (*Pseudomonas frederiksbergensis*)

GGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAA
AGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA
GCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCT
GAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGC
CATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTG
GGAGGAAGGGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGA
ATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTA
AGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTG
TCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAA
ATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGAC
TGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTT

GAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTAC
GGCCCGCAA

>GW_4 (*Brevundimonas vesicularis*)

ATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCGGCCCGCGTCTG
ATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTG
GTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGACG
CAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCAC
CGGGGACGATAATGACGGTACCCGGAGAAGAAGCCCCGGCTAACTTCGTG
CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGG
GCGTAAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCGGG
GCTCAACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAGTATGAGAGAG
GTGTGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAAGAA
CACCAGTGGCGAAGGCGACACACTGGCTCATTACTGACGCTGAGGCTCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGATTGCTAGTTGTCGGGATGCATGCATTTCGGTGACGCAGCTAACG
CATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTG

>GW_5 (*Staphylococcus equorum*)

CGGGAAACCGGAGCTAATGCCGGATAACATTTGGAACCGCATGGTTCTAA
AGTAAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGC
TAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTATTAGGG
AAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
AAACTTGAGTACAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAAT
GCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGT
AACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAAC

>GW_6 (*Janthinobacterium agaricidamnosum*)

CCTAGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGAT
CTAAGGATGAAAGTGGGGGATCGCAAGACCTCATGCTCGTGGAGCGGCCG

ATATCTGATTAGCTAGTTGGTAGGGTAAAAGCCTACCAAGGCATCGATCA
GTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG
CCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGC
TCTTTTGTCAGGGAAGAAACGGTGAGAGCTAATATCTCTTGCTAATGACG
GTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAG
GCGGTTTTGTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGC
ATTGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAATTCCACGT
GTAGCAGTGAAATGCGTAGATATGTGGAGGAACACCGATGGCGAAGGCA
GCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCTACTAGTTGTC
GGGTCTTAATTGACTTGGTAACGCAGCCTAACGCGTTGAAGTAGACCGCC
TGGGGGAGTACGGGTCGCCAGGATTAAAAACTTCAAAGGAAATTGA

>GW_7 (*Deinococcus aquaticus*)

TCGCTAATACGTGATGTGCTGCTCCCTCCTGTGGGATCAGTAAAGATTTAT
TGCTTTGGGATGGGGTTGCGTTCCATCAGCTAGTTGGTAGGGTAAAGGCCT
ACCAAGGCGACGACGGATAGCCGGCCTGAGAGGGTGGCCGGCCACAGGG
GCACTGAGACACGGGCCCCACTCCTACGGGAGGCAGCAGTTAGGAATCTT
CCACAATGGGCGAAAGCCTGATGGAGCGACGCCGCGTGAGGGATGAAGG
TTTTCGGATCGTAAACCTCTGAATCAGGGACGAAAGACGCTTTATGCGGG
ATGACGGTACCTGAGTAATAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGGAGGGTGCAAGCGTTACCCGGAATCACTGGGCGTAAAGGGC
GTGTAGGCGGGATGTTAAGTCTGGTTTTAAAGACTGCGGCTCAACCGCAG
GGATGGACTGGATACTGGCATTCTTGACCTCTGGAGAGAGAACTGGAATT
CCTGGTGTAGCGGTGGAATGCGTAGATACCAGGAGGAACACCAATGGCG
AAGGCAGGTTCTTGGACAGAAGGTGACGCTGAGGCGCGAAAGTGTGGGG
AGCGAACCGGATTAGATACCCGGGTAGTCCACACCCTAAACGATGTACGT
TGGCTAATCGCAGGATGCTGTGATTGGCGAAGCTAACGCGATAAACGTAC
CGCCTGGGAAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
GGCCCGCACAAGCGGTGGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACC

>GW_8 (*Sphingomonas faeni*)

CGACTGCTAATACCGGATGATGACGTAAGTCCAAAGATTTATCGCCGAGG
GATGAGCCCGCGTAGGATTAGGTAGTTGGTGTGGTAAAGGCGCACCAAGC
CGACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT
GGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGG

TTGTAAAGCTCTTTTACCCGGGATGATAATGACAGTACCGGGAGAATAAG
CTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGCG
TTATTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCTTTGTAAGTAA
GAGGTGAAAGCCCAGAGCTCAACTCTGGAATTGCCTTTTAGACTGCATCG
CTTGAATCATGGAGAGGTCAGTGGAATTCCGAGTGTAGAGGTGAAATTCG
TAGATATTCGGAAGAACACCAGTGGCGAAGGCGGCTGACTGGACATGTAT
TGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGCTAAACGATGATAACTAGCTGTCCGGGACACTTGGTG
TTTTGGGTGGCGCAGCTAACGCATTTAAGTTATCCGGCCTGGGGGAGTAC
GGG

>GW_9 (*Acidovorax radicis*)

ATACATCGGAACGTGCCCGATCGTGGGGGATAACGGAGCGAAAGCTTTGC
TAATACCGCATACGATCTACGGATGAAAGCAGGGGACCGCAAGGCCTTGC
GCGGACGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTT
ACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTG
GACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGC
CTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGACCTCTTCTAATAA
AGGGGGTCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGTGCGCAGGCGGTTATGTAAGACAGATGTGAAATCCCCGGGCT
CAACCTGGGAACTGCATTTGTGACTGCATAGCTAGAGTACGGTAGAGGGG
GATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACAC
CGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGAAAG
CGTGGA

>HI_1 (*Massilia aurea*)

AATATATCGGAACGTACCCAAGAGTGGGGGATAACGTAGCGAAAGTTAC
GCTAATACCGCATACGATCTAAGGATGAAAGCAGGGGATCTTCGGACCTT
GTGCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTGAGGTAAAGGC
TCACCAAGGCCACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTT
TGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAG
GCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGGGCTAA
TATCCCTCGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGACGTGAAATCCCCGG
GCTCAACCTGGGAATTGCGTTGGAGACTGCAAGGCTAGAATCTGGCAGAG
GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGA

ACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAA
ACGATGTCTACTAGTTGTCGGGTTTTAATTAACTTGGTAACGCAGCTAACG
CGTGAAGTAGACCGCCTGGGGAGTACG

>HI$_2$ (*Massilia oculi*)

CATACGATCTAAGGATGAAAGCAGGGGATCTTCGGACCTTGTGCTCCTGG
AGCGGCCGATATCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGC
TACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGG
GCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTT
GTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGGGCTAATATCCTTCGCT
AATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
TGCGCAGGCGGTTTTGTAAGTCTGACGTGAAATCCCCGGGCTCAACCTGG
GAATTGCGTTGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAA
TTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGC
GAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCTAC
TAGTTGTCGGGTTTTAATTAACTTGGTAACGCAGCTAACGCGTGAAGTAG
ACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGG
GGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAA
AAAC

>HI$_3$ (*Massilia aurea*)

TAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGC
AGGGGATCTTCGGACCTTGTGCTCCTGGAGCGGCCGATATCTGATTAGCTA
GTTGGTGAGGTAAAGGCTCACCAAGGCCACGATCAGTAGCTGGTCTGAGA
GGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATG
CCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAA
GAAACGGTGAGGGCTAATATCCCTCGCTAATGACGGTACCTGAAGAATAA
GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTC
TGACGTGAAATCCCCGGGCTCAACCTGGGAATTGCGTTGGAGACTGCAAG
GCTAGAATCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGC
GTAGAGATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAG
ATTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCCCTAAACG

>HI_4 (*Plantibacter auratus*)

GAGTAACACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCGTTGGAAAC
GACGTCTAATACCGGATACGAGCTTCAGCCGCATGGCTAGGAGCTGGAAA
GAATTTCGGTCAAGGATGGACTCGCGGCCTATCAGCTAGTTGGTGAGGTA
ATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGG
GACGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAG
TGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCT
CGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGT
CTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCC
TGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAA
GGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAG
CAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGCGCTA
GATGTGGGGACCATTCCACGGTTTCCGTGTCGTA

>HI_5 (*Rhizobium giardinii*)

GAAAGATTTATCGGAGTTGGATGAGCCCGCGTTGGATTAGCTAGTTGGTG
GGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGAT
CAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTG
TGTGATGAAGGCCTTAGGGTTGTAAAGCACTTTCACCGGAGAAGATAATG
ACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGT
AATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACG
TAGGCGGATCGATCAGTCAGGGGTGAAATCCCGCAGCTCAACTGCGGAAC
TGCCTTTGATACTGTCGGTCTAGAGTATGGAAGAGGTGAGTGGAATTCCG
AGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAG
GCGGCTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGC
CGTCGGGCAGTTTACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGC

>HI_7 (*Massilia timonae*)

GCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGCAGGGGAT
CTTCGGACCTTGTGCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTG
AGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGT
GAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACG

GTGAAGGCTAATATCCTTCGCTAATGACGGTACCTGAAGAATAAGCACCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGTCGT
GAAATCCCCGGGCTCAACCTGGGAATTGCGATGGAGACTGCAAGGCTAGA
ATCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAG
ATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGAC
GCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCTACTAGTTGTCGGGTTTTAATTAACTTGGTAA
CGCAGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTA
AAACTC

>HS_1 (*Arthrobacter psychrolactophilus*)

GTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAA
ACGGGGTCTAATACTGGATATTGACTTTTCCTCGCATGGGGATTGGTTGAA
AGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGT
AATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAACAAGGCCAG
TGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGG
CTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGG
AGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC
ACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAA
AGCATGGGGAGCGAACAGGATTAGATACCCTGGAAGTCCATGCCGAAAA
CGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAACTA
ACGCATTAAGTGCCCCGCCTGGGGAGTAC

>HS_2 (*Pseudarthrobacter scleromae*)

GGGAAACTGGGTCTAATACCGGATATGACTCCTCATCGCATGGTGGGGGG
TGGAAAGCTTTATTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTG
GTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
TGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGA
AGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCG
TAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTC
AACTCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAG

ACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC
CGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAA
GCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG
TTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAAC
GCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAAC

>HS_3 (*Flavobacterium sinopsychrotolerans*)

ACGGGTGCGTAACGCGTATGCAATCTACCTTTCACAAAGGGATAGCCCAG
AGAAATTTGGATTAATACCTTATAGTAATATGACTTGGCATCAAGATGTAT
TTAAAGATTTATCGGTGAAAGATGAGCATGCGTCCCATTAGCTAGTTGGT
AAGGTAACGGCTTACCAAGGCAACGATGGGTAGGGGTCCTGAGAGGGAG
ATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGC
AGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGT
GCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTATACAGGAAGAAAC
ACTACTTCGTGAAGTAGCTTGACGGTACTGTAAGAATAAGGATCGGCTAA
CTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAA
TCATTGGGTTTAAAGGGTCCGTAGGCGGTCAGATAAGTCAGTGGTGAAAG
CCCATCGCTCAACGGTGGAACGGCCATTGATACTGTCTGACTTGAATTATT
AGGAAGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTACA
TGGAAATACCAATTGCGAAGGCAGGTTACTACTAATTGATTGACGCTGAT
GGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGGTAGTCCAC
GCCGTAAACGATG

>HS_4 (*Bacillus butanolivorans*)

GCTAATACCGGATACGTTCTTTTCTCGCATGAGAGAAGATGGAAAGACGG
TTTCGGCTGTCACTTATAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAG
GTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
ACGAAGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGT
ACCAGAGTAACTGCTGGTACCTTGACGGTACCTAACCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCG
GAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCTTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGA
TTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACAC
TGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGG

>HS_5 (*Pseudomonas frederiksbergensis*)

GCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGG
GGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGA
TGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCAC
CGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGAT
GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTA
GAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAG
ATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTG
ACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTT
AGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGGAGTACGGCCGCA
AGGTTAAAACTCAAATGAAATTGACGG

>HS_6 (*Arthrobacter psychrolactophilus*)

GGTCTAATACTGGATATTGACATTTCACCGCATGGTGGTTTGTTGAAAGAT
TTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATG
GCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATG
ACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAACAAGGCCAGCATTTT
TGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTA
AAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAA
CCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACT
GGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGA
TGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCAT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTCGG
GGCACTAGGTG

>HS_7 (*Bacillus simplex*)

GACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATACGTTCTTTTC
TCGCATGAGAGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGG
CCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG

CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAA
AGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTTCGGGTCGTAA
AGTTCTGTTGTTAGGGAAGAACAAGTACCAGAGTAACTGCTGGTACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCG
CAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGG
GTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCC
AAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGGAAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAG

>HS_8 (*Pseudomonas extremaustralis*)

GGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGAC
CTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT
CAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGC
AGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGG
CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAG
TATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATA
TAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACA
CTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTG
GCGCAGCTAACGCAT

>HS_9 (*Pseudomonas veronii*)

GCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTT
GCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGC
TCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG
GTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAA
TACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTG
CCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGG
CTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGG

GTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC
ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACG
CATTAAGTTGACCGCCTGGGGAGTACGGCCCGCAAGGTT

>HS_{10} (*Pseudomonas fluorescens*)

GACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGC
CTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACC
TAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCT
GTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCC
GGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAG
AGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG
AACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTA
ACG

>HS_{13} (*Delftia acidovorans*)

AGCTAATACCGCATACGATCTGAGGATGAAAGCGGGGGACCTTCGGGCCT
CGCGCGATTGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAG
CTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
TTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGCAGGATGAA
GGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAAGCTTCTCCTAA
TACGAGAGGCCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGTGCGCAGGCGGTTATGTAAGACAGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTTGTGACTGCATGGCTAGAGTACGGTAGAG
GGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGA
ACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAA
ACGATGTCAACTGGTTGTTGGGAATTAGTTTTCTCAGTAACGAAGCTAACG
CGTGAAG

>HS_{14} (*Pseudomonas frederiksbergensis*)

ATAACGCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAG
CAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGC
TAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGA
GAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCA
TGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGG
AGGAAGGGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAAT
AAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAG
TTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTC
GAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAAT
GCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTG
ATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGA
GCTCT

>HS_{15} (*Pseudarthrobacter sulfonivorans*)

AGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATAC
CGGATATGACTCCTCATCGCATGGTGGGGGGTGGAAAGCTTTATTGTGGTT
TTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCA
AGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGC
AGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTT
GTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTGGGT
ACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCT
GGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGA
CATTCCACGTTT

>HS_{16} (*Paracoccus carotinifaciens*)

ACGGAATAGCCCCGGGAAACTGGGAGTAATACCGTATACGCCCTTTGGGG
GAAAGATTTATCGGAGAAGGATCGGCCCGCGTTGGATTAGGTAGTTGGTG
AGGTAACGGCTCACCAAGCCGACGATCCATAGCTGGTTTGAGAGGATGAT
CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA

GTGGGGAATCTTAGACAATGGGGGCAACCCTGATCTAGCCATGCCGCGTG
AGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCAGCTGGGAAGATAATG
ACGGTACCAGCAGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACG
TAGGCGGACTGGAAAGTCAGAGGTGAAATCCCAGGGCTCAACCTTGGAAC
TGCCTTTGAAACTATCAGTCTGGAGTTCGAGAGAGGTGAGTGGAATTCCG
AGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAG
GCGGCTCACTGGCTCGATACTGACGCTGAGGTGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGA
CGTCGGCAAGCATGCTTGTCGGTGTCACACCTAACGGATTAAGCATTCCG
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGC
CCGCAC

>HS$_{17}$ (*Flavobacterium xinjiangense*)

GATTAATACCTTATAGTAATACGACTTGGCATCAAGATGTATTTAAAGATT
TATCGGTGAAAGATGAGCATGCGTCCCATTAGCTTGTTGGTAAGGTAACG
GCTTACCAAGGCAACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACA
CTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAA
TATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGA
CGGTCCTATGGATTGTAAACTGCTTTTGTACAGGAAGAAACCCTTTCACGT
GTGGAAGATTGACGGTACTGTAAGAATAAGGATCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTT
TAAAGGGTCCGTAGGCGGTCAAGTAAGTCAGTGGTGAAAGCCCATCGCTC
AACGGTGGAACGGCCATTGATACTGCTTGACTTGAATTATTAGGAAGTAA
CTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTACATGGAATACC
AATTGCGAAGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAGC
GTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GGATACTA

>HS$_{18}$ (*Arthrobacter nitroguajacolicus*)

ATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCATCTGACGCATG
TCATGGTGGTGGAAAGCTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAG
CTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTG
AGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAG
GGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCG
GGGCTCAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGCAGTAG

GGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGC
GAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGC
TAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGC

>HS_{19} (*Pseudorhodobacter collinsensis*)

CTGAGAGTAATACCGTATGTGCCCTTCGGGGGAAAGATTTATCGCCAAAG
GATTGGCCCGCGTTGGATTAGGTAGTTGGTGGGGTAATGGCCTACCAAGC
CGACGATCCATAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAATG
GGGGAAACCCTGATCTAGCCATGCCGCGTGATCGATGAAGGCCTTAGGGT
TGTAAAGATCTTTCAGATGGGAAGATAATGACGGTACCATCAGAAGAAGC
CCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACCGAGGGGGCTAGCGT
TATTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATTAGAAAGTCAG
AGGTGAAATCCCAGGGCTCAACCTTGGAACTGCCTTTGAAACTCCTAGTCT
TGACGTCGAGAGAGGTGAGTGGAATTCCAAGTGTATAGGTGAAATTCGTA
GATATTCTGAGGAACACCAGTGGCGAAAGCGGCTCACTGGCTCGATACTG
ACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG

>HW_1 (*Paenisporosarcina quisquiliarum*)

TAACACGTGGGCAACCTACCTTGTAGATTGGGATAACTCCGGGAAACCGG
GGCTAATACCAAATAATCCATTTTGCTTCATGGCGAAATGTTGAAAGGCG
GCTTCGGCTGTCACTACGAGATGGGCCCGCGGCGCATTAGCTAGTTGGTA
GGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGT
GAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAC
GTACGAGAGTAACTGCTCGTACCTTGACGGTACCTTATTAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGT
GAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGA
GTACAGAAGAGGAAAGCGGAATTCCAAGTGTAGCGGTGAAATGCGTAGA
GATTTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGAC
GCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTA
GTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAG
GCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG

TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCA
CTGACCGGTTTAGAGATAAGCC

>HW_2 (*Pseudomonas frederiksbergensis*)

GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCT
CGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGA
CCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGT
GAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGA
TCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGC
ATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGG
CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGTCGAGCTAGAG
TATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATA
TAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACA
CTGAGGTGCGAAAGCGTGGGGAGCAA

>HW_3 (*Arthrobacter ginsengisoli*)

GGGTCTAATACCGGATATGACTCCTCATCGCATGGTGGGGGGTGGAAAGC
TTTATTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTA
ATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAG
TGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCT
CGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGA
TCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTC
CTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGA
AGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGA
GCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACT
AGGTGTGGGGGACATTC

>HW_4 (*Rhizobium soli*)

TATGTGCCCTTCGGGGGAAAGATTTATCGGTAAAGGATCGGCCCGCGTTG
GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCT
GGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTC

CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT
CCAGCCATGCCGCGTGTGTGATGAAGGCCTTAGGGTTGTAAAGCACTTTC
ACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTC
GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTAC
TGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCA
GAGCTCAACTCTGGAACTGCCTTTGATACTGGGTATCTTGAGTATGGAAG
AGGTAAGTGGAATTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAGG
AACACCAGTGGCGAAGGCGGCTTACTGGTCCATAACTGACGCTGAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAATGTTAGCCGTCGGGCAGTTTACTGTTCGGTGGCGCAGCTAA
CGCATTAAACAT

>HW$_5$ (*Staphylococcus equorum*)

ACGTGGGTAACCTACCTATAAGACTGGAATAACTTCGGGAAACCGGAGCT
AATGCCGGATAACATTTGGAACCGCATGGTTCTAAAGTAAAAGATGGTTT
TGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAA
CGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTA
AGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATT
ATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTACAGA
AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGG
AGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATG
TGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA
GCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGACCGCAAGGTTGAAA
CTCAAAGGAATTG

>HW$_6$ (*Brevundimonas vesicularis*)

ATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCGGCCCGCGTCTG
ATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTG
GTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGACG
CAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCAC
CGGGGACGATAATGACGGTACCCGGAGAAGAAGCCCCGGCTAACTTCGTG
CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGG
GCGTAAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCGGG

GCTCAACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAGTATGAGAGAG
GTGTGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAAGAA
CACCAGTGGCGAAGGCGACACACTGGCTCATTACTGACGCTGAGGCTCGA
GAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGATTGCTAGTTGTCGGGATGCATGCATTTCGGTGACGCAGCTAACG
CATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG

DNA-directed RNA polymerase beta subunit (rpoB) gene, partial sequence

>GI_3 (*Massilia aurea*)

CAATACGGCTTCCTGGAACCCCGTACCGCAAGGTCGACGGTTCGAAGGTC
ACCGATCAGATCGATTATCTGTCGGCCATCGAAGAAGGCCGCTACATCAT
TGCCCAGGCAAACGCCGCGATCAATGAAAACGGCCAACTGATCGACGAG
CTGGTCTCGTCGCGTGAAGCCGGCGAAACGATCCTGGTCTCGCCAGAGCG
CGTCCAGTACATGGACGTTGCACCAGGCCAGATCGTGTCGGTCGCTGCCT
CGCTGATTCCGTTCCTGGAACACGATGATGCGAACCGTGCACTGATGGGT
ACAGCAATGCAAC

>GI_4 (*Massilia aurea*)

CCCCCCTGACAATACGGCTTCCTGGAACCCCGTACCGCAAGGTCGACGGT
TCGAAGGTCACCGATCAGATCGATTATCTGTCGGCCATCGAAGAAGGCCG
CTACATCATTGCCCAGGCAAACGCCGCGATCAATGAAAACGGCCAACTGA
TCGACGAGCTGGTCTCGTCGCGTGAAGCCGGCGAAACGATCCTGGTCTCG
CCAGAGCGCGTCCAGTACATGGACGTTGCACCAGGCCAGATCGTGTCGGT
CGCTGCCTCGCTGATTCCGTTCCTGGAACACGATGATGCGAACCGTGCACT
GATGGGTACAGCAATGCAACGAAA

>GS_2 (*Pseudomonas brassicacearum*)

GCGTCTGTCGATGGCTGAAAGCGAAGGCCTGATGCCGCAAGACCTGATCA
ACGCCAAGCCAGTGGCTGCGGCGGTGAAAGAGTTCTTCGGTTCCAGCCAG
CTTTCCCAGTTCATGGACCAGAACAACCCGCTGTCCGAGATCACCCACAA
GCGTCGTGTTTCTGCACTCGGCCCTGGCGGTTTGACTCGTGAGCGTGCGGG
CTTTGAAGTTCGTGACGTACACCCGACTCACTACGGTCGTGTATGCCCGAT
TGAAACGCCGGAAGGTCCGAACATCGGTCTGATCAACTCCCTGGCCGCTT
ATGCGCGCACCAACCAGTACGGCTTCCTCGAAAGCCCGTACCGTGTGGTG
AAAGACGCTCTGGTCACCGACGAGATCGTGTTCCTGTCCGCCATCGAAGA
AGCTGATCACGTGATCGCTCAGGCTTCGGCCACGATGAACGACAAGAAAA
TGCTGATCGACGAGCTGGTAGCTGTTCGTCACTTGAACGAGTTCACTGTCA

AGGCGCCGGAAGACGTCACCTTGATGGACGTTTCTCCGAAGCAGGTAGTT
TCGGTTGCAGCGTCGCTGATCCCGTTCCTAGAGCACGATGACGCCAACCG
TGCGTTGATGGGTTCGAACATGCAGCGTCAAGCTGTACCAACCCTGCGCG
CTGACAAGCCGCTGGTCGGTACTGGCATGGAGCGTAACGTAGCTCGTGAC
TCCGGTGTTTGCGTCGTGGCTCGTCGTGGTGGCGTTATCGATTCCGTCGAC
GCCAGCCGTATCGTGGTTCGTGTTGCTGATGATGAAGTTGAAACCGGTGA
AGCTGGTGTCGACATCTACAACCTGACCAAATACACCCGCTCCAAC

>GS_3 (*Pseudomonas migulae*)

TCTGTCGATGGCTGAAAGCGAAGGCCTGATGCCGCAAGACCTGATCAACG
CCAAGCCAGTGGCTGCGGCGGTGAAGGAGTTCTTCGGTTCCAGCCAGCTT
TCCCAGTTCATGGACCAGAACAACCCGCTGTCCGAGATCACCCACAAGCG
TCGTGTTTCTGCACTCGGCCCTGGCGGTTTGACTCGTGAGCGTGCTGGCTT
TGAAGTTCGTGACGTACACCCGACTCACTATGGTCGTGTATGCCCGATTGA
AACGCCGGAAGGTCCGAACATCGGTCTGATCAACTCCCTGGCTGCCTATG
CGCGCACCAACCAGTACGGCTTCCTCGAGAGCCCGTACCGTGTGGTTAAA
GACGCTCTGGTTACCGACGAGATCGTGTTCCTGTCCGCCATCGAAGAAGC
TGATCACGTGATCGCTCAGGCTTCGGCCACGATGAACGACAAGAAAATGC
TGATCGACGAGCTGGTAGCTGTTCGTCACTTGAACGAGTTCACCGTCAAG
GCGCCGGAAGACGTCACCTTGATGGACGTATCGCCGAAGCAGGTAGTTTC
GGTTGCAGCGTCGCTGATCCCGTTCCTCGAGCACGATGACGCCAACCGTG
CGTTGATGGGTTCGAACATGCAGCGTCAAGCTGTACCAACCCTGCGTGCT
GACAAGCCGCTGGTCGGTACCGGCATGGAGCGTAACGTAGCTCGTGACTC
CGGCGTTTGCGTCGTGGCTCGTCGTGGTGGCGTTATCGATTCCGTCGACGC
CAGCCGTATCGTGGTTCGTGTTGCTGATGATGAAGTTGAAACCGGTGAAG
CTGGTGTCGACATCTACAAC

>GS_4 (*Pseudomonas frederiksbergensis*)

GAGCGTCTGTCGATGGCTGAAAGCGAAGGCCTGATGCCGCAAGACCTGAT
CAACGCCAAGCCAGTGGCTGCGGCGGTGAAAGAGTTCTTCGGTTCCAGCC
AGCTTTCCCAGTTCATGGACCAGAACAACCCGCTGTCCGAGATCACCCAC
AAGCGTCGTGTTTCTGCACTCGGCCCTGGCGGTTTGACTCGTGAGCGTGCG
GGCTTTGAAGTTCGTGACGTACACCCGACTCACTACGGTCGTGTATGCCCG
ATTGAAACGCCGGAAGGTCCGAACATCGGTCTGATCAACTCCCTGGCCGC
TTATGCGCGCACCAACCAGTACGGCTTCCTCGAAAGCCCGTACCGTGTGG
TGAAAGACGCTCTGGTCACCGACGAGATCGTGTTCCTGTCCGCCATCGAA
GAAGCTGATCACGTGATCGCTCAGGCTTCGGCCACGATGAACGACAAGAA
AATGCTGATCGACGAGCTGGTAGCTGTTCGTCACTTGAACGAGTTCACTGT
CAAGGCGCCGGAAGACGTCACCTTGATGGACGTTTCTCCGAAGCAGGTAG
TTTCGGTTGCAGCGTCGCTGATCCCGTTCCTAGAGCACGATGACGCCAACC

GTGCGTTGATGGGTTCGAACATGCAGCGTCAAGCTGTACCAACCCTGCGC
GCTGACAAGCCGCTGGTCGGTACTGGCATGGAGCGTAACGTAGCTCGTGA
CTCCGGTGTTTGCGTCGTGGCTCGTCGTGGTGGCGTTATCGATTCCGTCGA
CGCCAGCCGTATCGTGGTTCGTGTTGCTGATGATGAAGTTGAAACCGGTG
AAGCTGGTGT

>GS_{12} (*Pseudomonas mandelii*)

CGTCTGTCGATGGCTGAAAGCGAAGGCCTGATGCCGCAAGACCTGATCAA
CGCCAAGCCAGTGGCTGCGGCGGTGAAAGAGTTCTTCGGTTCCAGCCAGC
TTTCCCAGTTCATGGACCAGAACAACCCGCTGTCCGAGATCACCCACAAG
CGTCGTGTTTCTGCACTCGGCCCTGGCGGTTTGACTCGTGAGCGTGCGGGC
TTTGAAGTTCGTGACGTACACCCGACTCACTACGGTCGTGTATGCCCGATT
GAAACGCCGGAAGGTCCGAACATCGGTCTGATCAACTCCCTGGCCGCTTA
TGCGCGCACCAACCAGTACGGCTTCCTCGAAAGCCCGTACCGTGTGGTGA
AAGACGCTCTGGTCACCGACGAGATCGTGTTCCTGTCCGCCATCGAAGAA
GCTGATCACGTGATCGCTCAGGCTTCGGCCACGATGAACGACAAGAAAAT
GCTGATCGACGAGCTGGTAGCTGTTCGTCACTTGAACGAGTTCACTGTCAA
GGCGCCGGAAGACGTCACCTTGATGGACGTTTCTCCGAAGCAGGTAGTTT
CGGTTGCAGCGTCGCTGATCCCGTTCCTAGAGCACGATGACGCCAACCGT
GCGTTGATGGGTTCGAACATGCAGCGTCAAGCTGTACCAACCCTGCGCGC
TGACAAGCCGCTGGTCGGTACTGGCATGGAGCGTAACGTAGCTCGTGACT
CCGGTGTTTGCGTCGTGGCTCGTCGTGGTGGCGTTATCGATTCCGTCGACG
CCAGCCGTATCGTGGTTCGTGTTGCTGATGATGAAGTTGAAACCGGTGAA
GCTGGTGTCGACA

>GS_{14} (*Rhizobium herbae*)

GGCTTCATCGAAAGCCCTTACCGCAAGATCGTTGACGGTAAGGTGACCAA
GGATGTCGTCTACCTGTCGGCGATGGAAGAAGCCAAGTATCACGTGGCCC
AGGCCAACTCCGAACTCAACGAAGACCAGTCCTTCGTTGAAGAATTCGTT
GTTTGCCGTCACGCCGGCGACGTTATGCTCGCCCCGCGCGACATCGTGAA
CCTGATGGACGTTTCGCCCAAGCAGCTCGTGTCTGTGGCGGCAGCGCTTAT
CCCGTTCCTGGAAAACGACGACGCCAACCGCGCCCTCATGGGTACCAGAC
ATGCAACGAA

>GW_2 (*Pseudomonas frederiksbergensis*)

GTCTGTCGATGGCTGAAAGCGAAGGCTTGATGCCGCAAGACTTGATCAAC
GCCAAGCCAGTGGCTGCGGCAGTGAAAGAGTTCTTCGGTTCCAGCCAGCT
TTCCCAGTTCATGGACCAGAACAACCCGCTGTCCGAGATCACCCACAAGC
GTCGTGTATCTGCACTCGGCCCTGGCGGTTTGACTCGTGAGCGTGCCGGCT

TTGAAGTTCGTGACGTACACCCGACTCACTACGGTCGTGTATGCCCGATTG
AAACGCCGGAAGGTCCGAACATTGGCCTGATCAACTCCTTGGCCGCCTAT
GCGCGCACCAACCAGTACGGCTTCCTCGAAAGCCCGTACCGTGTGGTGAA
AGATGCTCTGGTCACCGACGAGATCGTGTTCCTGTCCGCCATCGAAGAAG
CTGATCACGTGATCGCTCAGGCTTCGGCCACGATGAACGACAAGAAAATG
CTGATCGACGAGCTGGTAGCTGTTCGTCACTTGAACGAGTTCACCGTCAA
GGCGCCGGAAGACGTCACCTTGATGGACGTTTCTCCGAAGCAGGTAGTTT
CGGTTGCAGCGTCGTTGATCCCGTTCCTGGAGCACGATGACGCCAACCGT
GCGTTGATGGGTTCGAACATGCAGCGTCAAGCTGTACCAACCCTGCGCGC
TGACAAGCCGCTGGTCGGTACCGGCATGGAGCGTAACGTAGCTCGTGACT
CCGGCGTTTGCGTCGTGGCTCGTCGTGGCGGCGTGATCGACTCCGTTGATG
CCAGCCGTATCGTGGTTCGTGTTGCTGATGACGAAGTTGAAACCGGCGAA
GCTGGTGTCG

>GW_4 (*Brevundimonas vesicularis*)

ACAGTTACGGCTTCATCGAGAGCCCTTACCGTCGCGTGAAGGACGGCCAG
GCCCAGGGCGAGGTGGTCTACATCTCGGCCATGGAAGAGTCGAAATACAC
GATCGCTCAGGCCAACATCGAACTGAAGAATGGTCAGATCGTCGAGGACC
TGGTCCCCGGCCGGATCAACGGTGAATCCCAGCTCCTGAACAAGGACGCC
GTGGACATGATGGACGTGTCGCCGAAACAGGTCGTTTCGGTCGCTGCGGC
CCTGATCCCGTTCCTGGAAAACGACGACGCCAACCGCGCGCTGATGGGTA
CCACCATGCAACG

>HI_2 (*Massilia oculi*)

GACAATACGGCTTCCTGGAACCCCGTACCGCAAGGTCGACGGTTCGAAGG
TCACCGATCAGATCGATTATCTGTCGGCCATCGAAGAAGGCCGCTACATC
ATTGCCCAGGCAAACGCCGCGATCAATGAAAACGGCCAACTGATCGACG
AGCTGGTCTCGTCGCGTGAAGCCGGCGAAACGATCCTGGTCTCGCCAGAG
CGCGTCCAGTACATGGACGTTGCACCAGGCCAGATCGTGTCGGTCGCTGC
CTCGCTGATTCCGTTCCTGGAACACGATGATGCGAACCGTGCACTGATGG
GTACCAGCCCATGCAACGAA