BACTERIAL REDUCTION OF MERCURY IN THE HIGH ARCTIC

Annette Klœstrup Møller

Department of Environmental Chemistry and Microbiology
National Environmental Research Institute

Department of Microbiology
University of Copenhagen

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PhD thesis by:

Annette Klœstrup Møller, M. Sc.

Department of Microbiology

University of Copenhagen

&

Department of Environmental Chemistry and Microbiology

National Environmental Research Institute

Supervisors:

Head of Department Niels A Kroer, PhD

Professor Søren J Sørensen, PhD

Senior Scientist Henrik Skov, PhD

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Preface and Acknowledgements

The work presented in this thesis was performed during my three years as a PhD student at the Department of Environmental Chemistry and Microbiology at the National Environmental Research Institute (NERI) in Denmark. The work was carried out as collaboration between NERI and the Department of Microbiology at University of Copenhagen. The work was conducted both at NERI and at the University of Copenhagen and included a two months field trip to Northeastern Greenland in the spring 2007. From February to November 2008 I visited Dr. Tamar Barkay's group at Rutgers University in New Jersey, USA. The PhD scholarship was financed by Danish Agency of Science as an international PhD scholarship.

I am grateful to my supervisors Niels Kroer, Søren J. Sørensen and Henrik Skov for their guidance, support and encouragement throughout the study. I am especially thankful for Niels Kroer's continuous enthusiasm, patience, and for keeping an open mind to my ideas.

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Last but not least, the warmest thanks to my family and friends for their never-ending support, understanding and patience!

1. Summaries

1.1 Dansk Resume

Kviksølv er konstateret i dyr og mennesker i Grønland og andre arktiske områder. Forureningen af kviksølv stammer hovedsageligt fra lavere breddegrader da kviksølv kan transporteres i atmosfæren over lange afstande. Specielt i det arktiske forår deponeres store mængder kviksølv fra atmosfæren ned på sne og havis som et resultat af unikke fotokemiske processer. Hvad der herefter sker med kviksølv som er deponeret er stort set uvis. Til trods for at man ved at bakterier spiller en vigtig rolle i det bio-geokemiske kviksølvkredsløb i tempererede egne, er bakteriers rolle i omsætningen af kviksølv i arktiske miljøer ukendt.

I dette ph.d. studie er de bakterielle samfund i sne, ferskvand og havis i Nordøstgrønland blevet undersøgt med fokus på kviksølvresistente bakterier. Ph.d.-studiet består af tre dele: 1) undersøgelse af bakterielle samfund i sne og ferskvand ved anvendelse af både dyrkningsafhængige og molekylærbiologiske teknikker. 2) Identifikation af kviksølvresistente bakterier fra sne, ferskvand og havis og 3) Identifikation og undersøgelse af kviksølvresistensens genetiske elementer i de arktiske kviksølvresistente isolater.

Dyrkning af bakterieisolater fra tre snedybder og ferskvand viste kun en begrænset overensstemmelse med de bakterielle fyla og slægter der blev identificeret ved den molekylærbiologiske metode. De mikrobielle samfund i alle arktiske lokaliteter var markant forskellige; dog var sammensætningen i de to øverste snelag mest sammenfaldne. Diversiteten i ferskvandsmiljøet var mindre i forhold til alle snemiljøer. Dette afspejler sandsynligvis at ferskvandsmiljøet er mindre ekstremt og et mere stabilt miljø end snemiljøet. For både sne og ferskvand indeholdte de hyppigst forekommende bakterielle fyla et højere antal slægter end de sjældne fyla, hvilket tyder på, at den økologiske succes af et bakteriel fylum afhænger af høj diversitet snarere end dominans af nogle få slægter. De mest dominerende fyla inkluderede *Proteobakterier*, *Aktinobakterier*, *Bacteroidetes*, *Cyanobakterier* og *Firmicutes* i sne og

Proteobakterier, *Bacteroidetes*, *Aktinobakterier* og *Planctomycetes* i ferskvand. Bakterierne identificeret i denne undersøgelse omfattede både fylotyper som er almindeligt forekommende i kolde miljøer samt sjældne fylotyper.

Målinger af total-kviksølv i sne, ferskvand og havis indikerede at kviksølv var deponeret fra atmosfæren i det tidsrum hvor prøvetagning fandt sted. Kviksølvresistente bakterier i sneen blev estimeret til 31% af de dyrkbare bakterier; i ferskvand og havis udgjorde kviksølvresistente bakterier mindre end 2%. De resistente bakterier tilhørte *α*-, *β*- og *γ*-*Proteobakterier*, *Firmicutes*, *Aktinobakterier*, og *Bacteriodetes*. Det blev påvist at 25% af de kviksølv resistente isolater var i stand til at reduceret kviksølv ioner (Hg^{II}) til elemenentær kviksølv (Hg^{0}); dog var der ingen sammenhæng mellem evnen til at reducere Hg^{II}, kviksølvsensitivitet og taksonomisk gruppe. På baggrund af mængden af kviksølvresistente bakterier der blev fundet i sneen blev den potentielle bakterielle reduktion af Hg^{II} i sne estimeret. Disse beregninger tyder på, at bakteriel kviksølvreduktion kan være vigtig i de dybere snelag og indikerer at bakterier kan have en rolle i det bio-geokemiske kviksølv kredsløb i de arktiske områder.

Mens bakteriel kviksølvreduktion, som katalyseres af kviksølvreduktasen MerA, er udbredt i tempererede omgivelser, er der kun en begrænset viden om forekomsten af dette genetiske system i bakterier i Arktis. Sekvenser der koder for *merA* blev identificeret i arktiske kviksølvresistente isolater i seks taksonomiske klasser (*α*-, *β*- og *γ*-*Proteobakterier* *Aktinobakterier*, *Flavobakterier* og *Bacillus*). Otte forskellige *merA* sekvenser blev identificeret; fem sekvenser (fra *α*-, *β*- og *γ*-*Proteobakterier*) viste stor lighed (99-100%) til proteiner i Genbank-databasen, mens de resterende tre sekvenser viste mindre lighed (82-92%) til proteiner i databasen. Af de 71 kviksølvresistente isolater var det kun muligt at påvise *merA* i 26 isolater; der må derfor være endnu ukendte *merA* sekvenser eller andre kviksølvresistensmekanismer i det bakterielle samfund i Arktis. Plasmider blev identificeret i 24% af de kviksølvresistente isolater og *merA* sekvenser blev fundet i to ud af fem forskellige plasmider. Tilstedeværelsen af

plasmider samt tilfældig fordeling af *merA* sekvenser, niveauet af følsomhed og evnen til at reducere Hg inden for de forskellige taksonomiske grupper kunne tyde på horisontal overførsel af *merA* gener. *merA* locus i Flavobakterium isolatet blev identificeret ved genomsekvensering. Dette *merA* locus var en del af en simple *mer* operon og unikt for denne operon var regulator elementet, som viste sig at være *arsR*. I størstedelen af bakterielle *mer* operoner er generne reguleret af *merR*, hvorimod *mer* operoner i arke-bakterier reguleres af *arsR*. Fylogenetisk analyse af MerA sekvenserne viste, at MerA fra Flavobakterium isolatet sammen med andre MerA fra Bacteriodetes var den gruppe af bakterielle MerA sekvenser, der var tættest beslægtet til MerA fra arke-bakterier.

Resultaterne indikerer, at de bakterielle samfund i Arktis, især i sne, kan spille en vigtig rolle i kviksølvtransformation i det arktiske miljø. Desuden viser resultaterne, at der findes en divers og endnu ukendte gruppe af *merA* i arktiske bakterier. og at disse *merA* gener muligvis kan overføres ved horisontal genoverførsel.

1.2 English Summary

It is well-established that mercury (Hg) from lower latitudes is transferred to and pollutes the Arctic environment. One mechanism of Hg transfer is through the atmosphere where Hg is deposited in the Arctic in the spring time during Atmospheric Mercury Depletion Events (AMDE): large amounts of Hg is believed to be depleted from the atmosphere and deposited onto snow and sea-ice through photochemical reactions. The faith of mercury after deposition is poorly understood and while bacteria are known to play an important role in the bio-geochemical Hg cycle in various temperate environments, their role in the dynamics of Hg deposited in the Arctic is unknown. In this PhD study the bacterial communities in snow, freshwater and sea-ice in Northeastern Greenland were examined with focus on Hg resistant bacteria. The PhD study consists of three parts: 1) examination of the bacterial communities in snow and freshwater both by applying culture dependent and independent techniques (pyrosequencing) 2) Identification of Hg resistant bacteria from snow, freshwater and sea-ice and 3) Identification and investigation of Hg resistance genetic elements in arctic Hg resistant isolates.

Cultivation of bacterial isolates from three snow depths and freshwater only showed a scattered representation of the phyla and genera in comparison to strains identified by culture independent methods. The microbial composition of all arctic sample sites was significantly different, with the two uppermost snow layers being most similar to each other. The freshwater environment was less diverse as compared to all snow environments most likely reflecting the freshwater environment as a less extreme and more stable environment than in snow. For both snow and freshwater, abundant bacterial phyla included higher numbers of genera than the rare phyla, suggesting that the ecological success of a bacterial phylum depends on the diversity rather than the dominance of a few genera. The most dominant phyla included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes, Cyanobacteria* and *Firmicutes* in the snow and *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Planctomycetes* in freshwater. The bacteria identified in this

study both included phylotypes commonly found in cold environments as well as rare phylotypes.

During the time of sampling atmospheric ozone measurements and total Hg measurements in the snow indicated that Atmospheric Mercury Depletion Events were taking place, therefore the bacterial resistance to mercury was assesed. In snow, Hg resistant bacteria accounted for up to 31% of the culturable bacteria, but were below 2% in freshwater and sea-ice. The resistant bacteria belonged to the *α*-, *β*- and *γ-Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteriodetes*. It was found that 25% of the isolates resistant to Hg also reduced Hg^{II} to Hg^{0}, although there was no correlation between level of resistance, ability to reduce Hg^{II}, and taxonomic group. An estimation of the potential bacterial reduction of Hg^{II} in snow suggested that this may be important in the deeper snow layers. This highlights the importance of microbial mercury transformation in the biogeochemical mercury cycling in the High Arctic.
While bacterial Hg reduction by the mercuric reductase, MerA, is widespread in temperate environments, its distribution and abundance in the Arctic is largely unexplored. MerA loci were found in six taxonomic classes (*α*-, *β*- and *γ-Proteobacteria*, *Actinobacteria*, *Flavobacteria* and *Bacilli*) among the high Arctic mercury resistant isolates. Eight different *merA* sequences were identified; five of which (from *α*-, *β*- and *γ-Proteobacteria*) showed high similarity (99-100%) to proteins in the Genbank database while the three others were less similar (82-92%) to any protein sequences. Of the 71 mercury resistant isolates, only 26 carried a detectable *merA* and, thus, several other *merA* sequences or Hg reduction mechanisms may be found in the Arctic. Of the Hg resistant isolates, 24% carried plasmids and two out of the five sequenced plasmids contained a *mer*-operon. The presence of plasmids carrying *mer*-operons, an uneven distribution of *merA,* the level of sensitivity and the ability to volatilize Hg within the different taxonomic groups could indicate lateral transfer of the *merA* genes. Whole genome sequencing revealed a simple *mer*-operon in a *Flavobacterium* isolate. Unique for this putative *mer*-operon was the regulatory

element; instead of the common *merR* this operon was initiated by *arsR*, which is common for *mer*-operons in Archaeal species. Clustering of this putative MerA sequence along with other putative MerA sequences from other *Bacteroidetes* showed a closer phylogenetic distance to Archaeal MerA sequences than any other bacterial MerA sequences. The results suggest that bacterial communities in the Arctic, especially in snow covers, may play in important role in the Hg transformation in the Arctic environment. Furthermore, the results indicate that a diverse and yet undiscovered pool of *merA* exists in arctic bacterial assemblages, and these genes may be distributed in the community through lateral gene transfer.

2. List of Abbreviations

Hg – mercury

Hg^0 – elemental mercury

Hg^{II} – mercury compounds in oxidation state two.

MeHg – methyl mercury

AMDE – Atmospheric Mercury Depletion Events

ORF – open reading frame

Mer system – genetic system in bacteria consisting of several genes involved in bacterial Hg resistance

MerA – mercuric reductase, the enzyme that catalyze the reduction of Hg^{II} to Hg^0 in the *mer* system

merA – gene encoding mercuric reductase, MerA

DOC – Dissolved Organic Carbon

hν – light energy (Planck's constant (h) * frequency of photon (ν) = light energy (E))

3. Introduction

The contamination of Polar Regions due to the global distribution of anthropogenic pollutants is of great concern because it leads to the bioaccumulation of toxic substances in Arctic food chains. Especially the neurotoxic, organic methylmercury (MeHg) is accumulated in the food webs. Mercury (Hg) enters the Arctic environments from anthropogenic sources at lower latitudes. A great amount of the Hg entering the Arctic regions is deposited from the atmosphere as mercuric compounds (Hg^{II}) in particular during the polar spring through chemical processes that are dependent on light and bromine released from melting sea-ice. Thus, the site of Hg^{II} deposition is believed to be coastal snow and sea-ice. Hg^{II} is a highly reactive species of Hg yet little is known about the fate of Hg^{II} in snow and sea-ice.

In temperate environments, it is well established that microorganisms are important players in the biogeochemical cycling of Hg that impact the levels of MeHg. Especially in aquatic ecosystems microorganisms contribute significantly to both methylating and demethylating processes; in addition they also control the supply of substrate for the methylation by reducing Hg^{II} to elemental Hg (Hg^{0}). Although the Arctic offers an extreme environment the abundance and diversity of bacteria is relatively high. Thus, bacteria inhabit both sea-ice and snow, and the bacterial diversity includes taxa in which species involved in Hg reduction are common. Yet, the role of bacteria in Hg reduction or other Hg transformations in the Arctic still is remains to be explored.

4. Objectives

The objective of the PhD study was to examine bacterial transformation of Hg in Arctic ecosystems with special emphasis on bacterial Hg^{II}-reduction. Specific objectives were to:

1) Assess the diversity and structure of the microbial communities in coastal snow and a coastal freshwater lake
2) Assess the abundance and diversity of bacteria capable of reducing Hg^{II} in sea-ice, snow and freshwater
3) Identify and characterize genetic elements involved in Hg reduction among arctic bacterial communities.

5. Background

5.1 Hg contamination in the Arctic and effect of human Hg exposure

Mercury (Hg) is a heavy metal with no known biological relevance and is toxic in all existing forms (20). The toxicity of Hg attracted attention and awareness especially after devastating spills in Minamata Bay in Japan and ingestion of bread prepared from MeHg treated seed grains in rural Iraq, where many people and especially children became ill and died (4, 43). In later years, the affect of long term low exposure to Hg - and especially MeHg - has been the focus of studies in populations feeding on natural diets in geographical sites where the wildlife have high concentrations of Hg (36).

The Hg contamination of the wildlife in the High Arctic is of concern due to the bioaccumulation of MeHg in the marine food webs and subsequent exposure of the indigenous human populations, which primarily feed on local food sources (13, 64, 92). Studies of human populations in the Arctic have shown that these populations feed on natural diets containing high levels of Hg and as a result have elevated Hg concentrations in their blood. For example, the average daily intake of MeHg for a population in the Disko Bay in western Greenland was estimated to 38 – 54 µg/day/person (46) which is 3 – 9 times higher than the Tolerable Daily Intake recommendation from European Food Safety Authority (0.23 µg/day/kg body weight) and the US EPA reference dose (0.1 µg/day/person) for a person weighing 60 kg (42). Several studies have indicated neuropsychological dysfunction and impaired development of language, attention and memory skills in children that had prenatal exposure to MeHg exceeding the guidelines from Health agencies (38, 94).

5.2 Hg sources and cycling in the Arctic environment

While most arctic environments are considered pristine, Hg emitted from sources from lower latitudes can reach this extreme environment. One of the major mechanisms by which Hg enters the Arctic is atmospheric deposition. The main sources of atmospheric Hg are geological activities (e.g. volcanoes) and burning of fossil fuels and incineration and disposition of Hg containing devices (e.g. light fixtures, batteries and electrodes utilized in chlor-alkali processes). Hg^0 has a high vapour pressure, is an extremely volatile and non-reactive molecule, which means that Hg^0 can persist in the atmosphere for up to 1 year (87). Modelling studies have estimated that 325 tons of Hg is deposited throughout the arctic over a 1 year period (3) with much of the deposition occurring during the polar sunrise (27, 59, 84). During the polar sunrise Hg^0 is oxidized through photochemical reactions with atomic bromine. Bromine is released from refreezing leads and polynyas (areas or stretches of open water in the sea-ice) (3, 57). This phenomenon of Hg^0 being oxidized and depleted from the atmosphere is referred to as Atmospheric Mercury Depletion Events (AMDE), a simplified description of the chemistry driving the depletion is described in Figure 1.

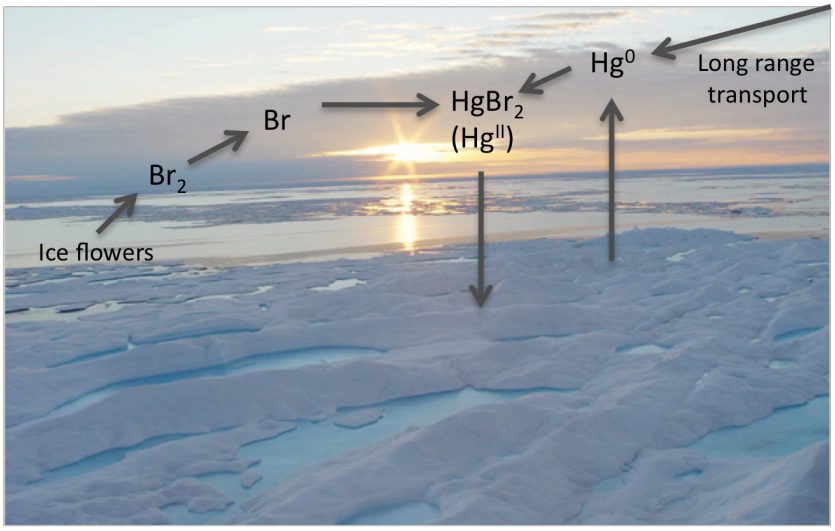

Figure 1. The chemistry of AMDE in the Arctic. The processes driving the atmospheric Hg depletion involve reaction of Hg^0 with bromine radicals:

$Br_2 + hv \rightarrow 2\ Br$ $\quad$ $2\ Br + Hg^0 \rightarrow HgBr_2$

Since the chemistry driving AMDE is dependent on bromine radicals from ice sea water, marine and coastal environments are more susceptible to Hg deposition than inland areas. (58)

Hg^{II} is deposited onto the snow and sea ice either through wet or dry deposition. The Hg concentrations in snow has been shown to increase from about 1 ng/L in the dark season to 55 ng/L when AMDE takes place (58). The highest concentrations of Hg (>90 ng/L) in snow were observed in Barrow, Alaska (57). AMDE is common in the High Arctic (10, 53, 80, 82) but has also been observed in Antarctica (27).

The fate of the Hg deposited onto the snow is poorly understood. It is most likely readily available to the microorganisms and a few studies have shown that a part of the total Hg in snow is bio-available in the form of Hg^{II} (57, 85). These studies have also measured a small amount of MeHg in the snow. Other studies have shown that much of the deposited Hg is quickly reduced to Hg^0 and released back into the atmosphere, and that this process is primarily due to photoreduction (26, 52, 54). Whether microorganisms present in snow also play a role in the reduction is not known.

5.3 Microbial life in cold environments

The Arctic and Antarctica constitute a large part of the cryosphere. Despite the harsh conditions, microbial life has adapted to these environments. Several bacterial species have been identified in several extreme environments including glacial ice, sea-ice, permafrost and polar oceans (40, 47, 51, 63). The biggest obstacles for microbes living in the cryosphere are decreased chemical reaction rates and increased rigidity of the cell membrane that affects the transport of nutrients and metabolites across the membrane (36, 79) Psychrophilic and psychrotrophic bacteria have adapted by producing enzymes having high specific activities at low temperatures (30, 93) and by the introduction of a higher proportion of unsaturated and methyl-branched fatty acids in the membrane lipids (19, 80), which increases the fluidity by changing the packing order of lipids and the number of interaction in the membrane. Other physiological adaptations to low temperatures in bacteria include 1) constitutively expression of cold shock proteins that are involved in cellular vital processes such as transcription, translation and protein folding, 2) expression of exopolysaccharides, which act as cryoprotectants and 3) production of antifreeze proteins (23, 30).

In polar environments, low temperature is not the only harsh environmental factor that the bacteria have to cope with. Other factors such as high osmotic and hydrostatic pressure, high UV-radiation in the summer, and low nutrient availability also affect the living conditions. Cold adaptation is, therefore, often associated with adaption to other factors. For example, bacteria residing in sea-ice are restricted to small amounts of liquid water that form veins in the sea-ice; as the sea-ice freezes the surrounding seawater accumulate salts and eventually form brine veins that consist of liquid water with a very high salinity that prevents it from freezing. Despite these challenges, microbial life has adapted to the environment and the biomass and diversity is often higher than expected.

Microorganisms commonly reported from polar environments are Gram-negative *α-, β-* and *γ-Proteobacteria* and *Bacteriodetes* (*Flavobacteria*) and Gram-positive *Actinobacteria* (*Arthrobacter* and *Micrococcus*) and *Firmicutes*. The very few studies that have been carried out on the diversity of microorganisms in polar snow have reported *Proteobacteria* (*α* and *β*), *Bacteroidetes* (*Flavobacteria* and *Sphingobacteria*) (55) and *Thermus-Deinococcus* (16) to be dominating phyla when using culture-independent methods. Using culturing techniques, isolates belonging to the *Proteobacteria*, *Firmicutes* and *Actinobacteria* have been identified (2). Studies on polar freshwater revealed *Actinobacteria* (*Corynebacteria*), *Bacteriodetes* (*Cytophaga* and *Flavobacteria*) and *Proteobacteria* (*Janthinobacteria* and *Pseudomonas*) (72).

5.4 Bacterial mercury transformation

From studies on Hg cycling in temperate environments there is no doubt that microorganisms play a significant role in the transformation of Hg species. The formation of MeHg which is one of the most toxic forms of Hg and the form that is accumulated in food webs is facilitated by sulphate-reducing and iron-reducing bacteria in anoxic sediments (35, 49). Bacteria also control the supply of MeHg by degradation MeHg and indirectly by reducing Hg^{II}, which is the substrate for biotic Hg methylation. Figure 2 shows an overview of the Hg cycling in temperate environments where anaerobic sulphur-reducing bacteria are a major source of the production of MeHg while aerobic bacteria containing the *mer* operon reduce Hg^{II}

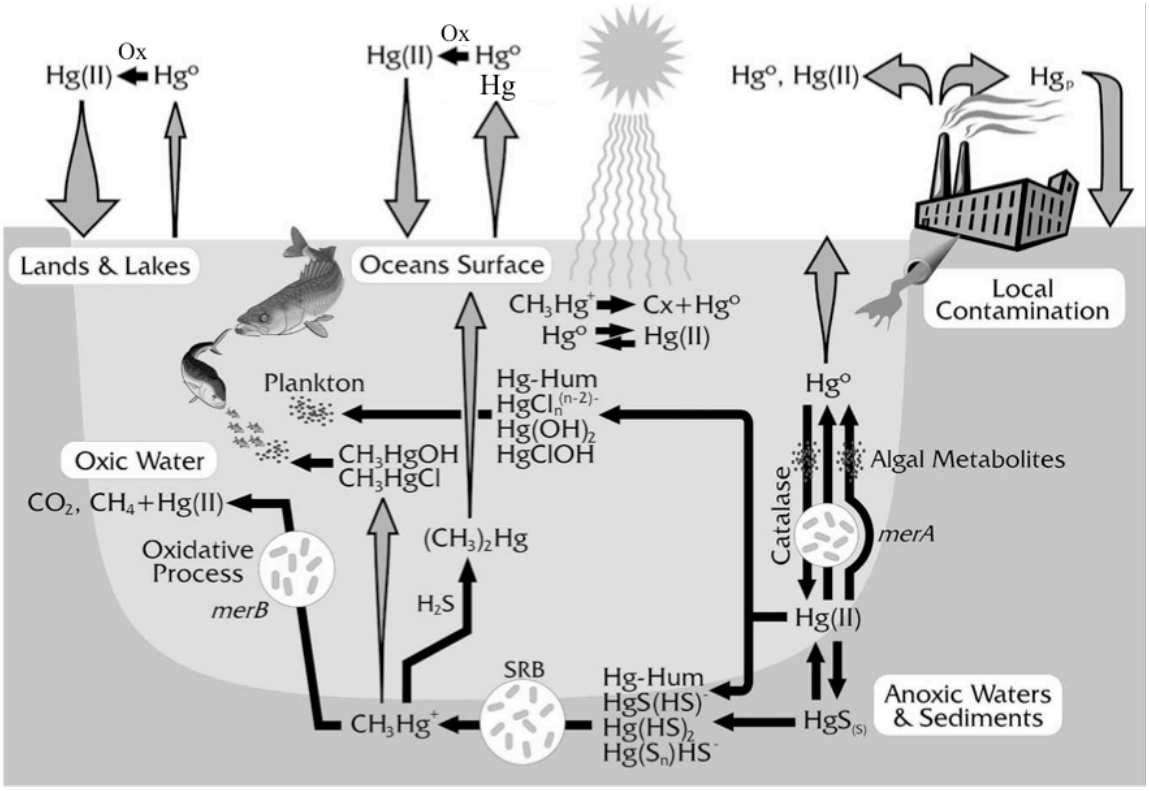

Figure 2: Biochemical Hg cycle. Solid arrows indicate uptake or transformation of Hg while hollow arrows indicate Hg flux between different compartments in the environment. The width of the hollow arrows is approximately proportional to the relative importance of the flux in nature. Processes mediated by bacteria are indicated by circles: MeHg degradation, Hg^{II} methylation and Hg^{II} reduction. (Modified from Barkay et al. 2003(9))

and thereby influence the pool of substrate available for Hg methylation. Note that oxidation of Hg^0 in the atmosphere is the source of Hg^{II} into the ecosystem, and it is important the to keep in mind that atmospheric oxidation of Hg^0 is not strictly a polar phenomenon (44). Nevertheless, AMDE is unique because of the combination of increased solar radiation, the presence of frostflowers and input of bromine radicals from sea-ice that results in large amounts of Hg to be deposited in the springtime in the Arctic.

5.5 Mechanisms of Mercury reduction in microorganisms: The Mer system

Bacteria may respond to Hg^{II} exposure using several strategies, as for example, reduced uptake of Hg^{II} (69), conversion to insoluble mercuric sulphide (68) or dimethylmercury sulphide (5), and reduction to elemental Hg (9). The enzymatic reduction of Hg^{II} to Hg^{0} is by far the best described and widespread mechanism of resistance among many bacterial phyla (reviewed in 9, 67) as well as in Archaeal lineages (83). The enzymatic reduction of Hg^{II} is catalyzed by products of the *mer* operon (*merRTPAD*), which encodes a group of proteins involved in the regulation, transport and reduction of Hg^{II}. The mechanism of the *mer* system is illustrated in Figure 3

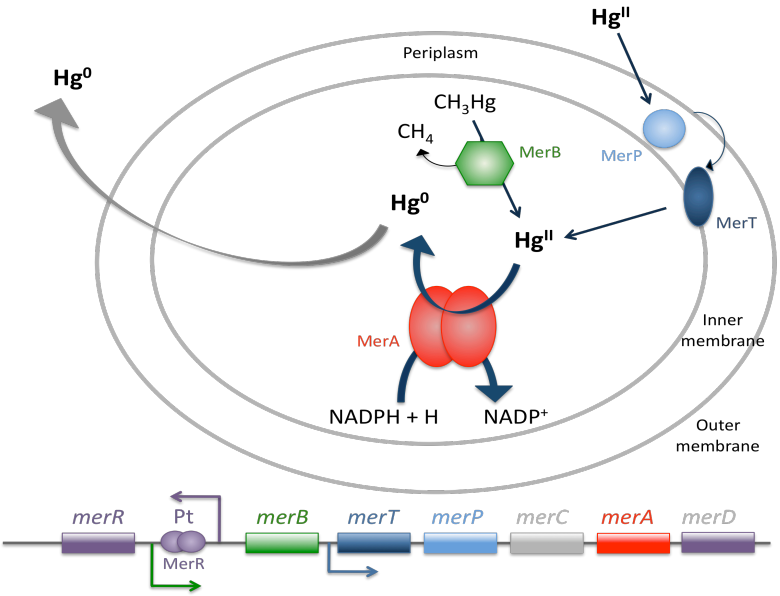

Figure 3. Schematic representation of the Mer system in bacteria and the general genetic organization of the *mer* operon. See the text for details.

The initial sequestration of Hg^{II} involves a pair of cysteine residues on the MerP protein in the periplasm, which is then transferred to MerT via a redox exchange to a pair of cysteine residues. It is proposed that all subsequent transfers occur by this mechanism. The Hg^{II} is transferred to the cysteine pair on the cytosolic side of MerT, prior to being transferred to a cysteine pair on the amino terminal domain of the dimeric MerA (Mercuric Reductase) during a possible transient association between MerT and MerA. Even though MerA is a soluble protein, a fraction of the protein is associated with the inner membrane (45). Hg^{II} is transferred to the carboxyl terminal

cysteine pair where in association with cysteine residues in the active site it is reduced to Hg^0 by electron transfer from NAD(P)H. The less toxic and volatile Hg^0 is released into the cytoplasm and freely diffuses out of the cell.

The operon is regulated by MerR, a homodimer, which represses the operon in the absence of mercuric ions and enhance transcription when bound to mercuric ions. MerR binds to the operator region, which lies between the -10 and -35 RNA recognition site for the structural genes (Pt) (70). MerR's own promoter overlaps and reads divergently from Pt and thereby prevents transcription of the merR gene when bound to Pt, i.e. MerR is a negative autoregulator (Figure 3). In addition to regulation from MerR, MerD also to binds to the operator region as well and acts an antagonist to MerR. Some bacteria also carries *merB*, a gene encoding a mercurial lyase, which split the C-Hg bond in organomercurials and the released Hg^{II} is then reduced by MerA. Therefore, *mer* operons containing *merB* governs resistance to both inorganic and organic Hg compounds and bacteria carrying the gene are referred to as carrying broad spectrum Hg resistance where as bacteria lacking *merB* carries narrow spectrum Hg resistance. Other *mer* genes have also been recognized, including *merC*, *merF merE* and *merG*, but their function is not fully understood. MerC is membrane-spanning protein that may be involved in transporting mercuric ions (81). MerF also appears to be a membrane protein (96). The merE gene is a predicted ORF but whether it is transcribed is unknown as well as the function of the gene product. MerG appears to be a periplasmic protein that prevents uptake of phenylmercury and is thereby involved in resistance to this organic Hg compound (53).

The *mer* locus is widely distributed in many geographical areas from hot springs (17) to permafrost and from environmental samples to clinical isolates (39). The operon is found on chromosomes, plasmids and transposons and have been identified in Archaea (reviewed in 67), and the bacterial phyla *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Deinococcus-Thermus* and *Aquifae* (8). Few studies indicate the presence of bacteria with the *mer* system in

Arctic environments (61, 74), yet a characterization and enumeration of Hg resistant bacteria from arctic snow, sea-ice and freshwater has not yet been carried out..

6. Methodology

An outline of the experimental work is presented in Figure 4. Snow, freshwater and sea-ice samples ice samples were collected in Northeastern Greenland, Station Nord during the period May 2007 – June 2007. Ozone measurements, measurement of bio-available Hg and bacterial direct counts were performed on site. All bacterial culturing were set up on site and kept at 4-7 C. Samples for DNA extraction and DOC measurements were preserved at – 20 C while samples for total Hg concentration were stored at 4-7 C and transported to Denmark for further processing. Culturing, purification, identification and MIC determination were done at DMU, Roskilde in Denmark. PCR of *merA* genes and Hg volatization assays were performed at Rutgers University, New Jersey in USA. Sequencing of plasmids, whole genome sequencing and pyrosequencing of DNA from environmental samples were performed at the University of Copenhagen, Denmark.

Material and methods are described in detail in the respective manuscripts as indicated in Figure 4. Bio-available Hg was measured with an *E.coli* biosensor as described in Rasmussen *et al* 1997 (77).

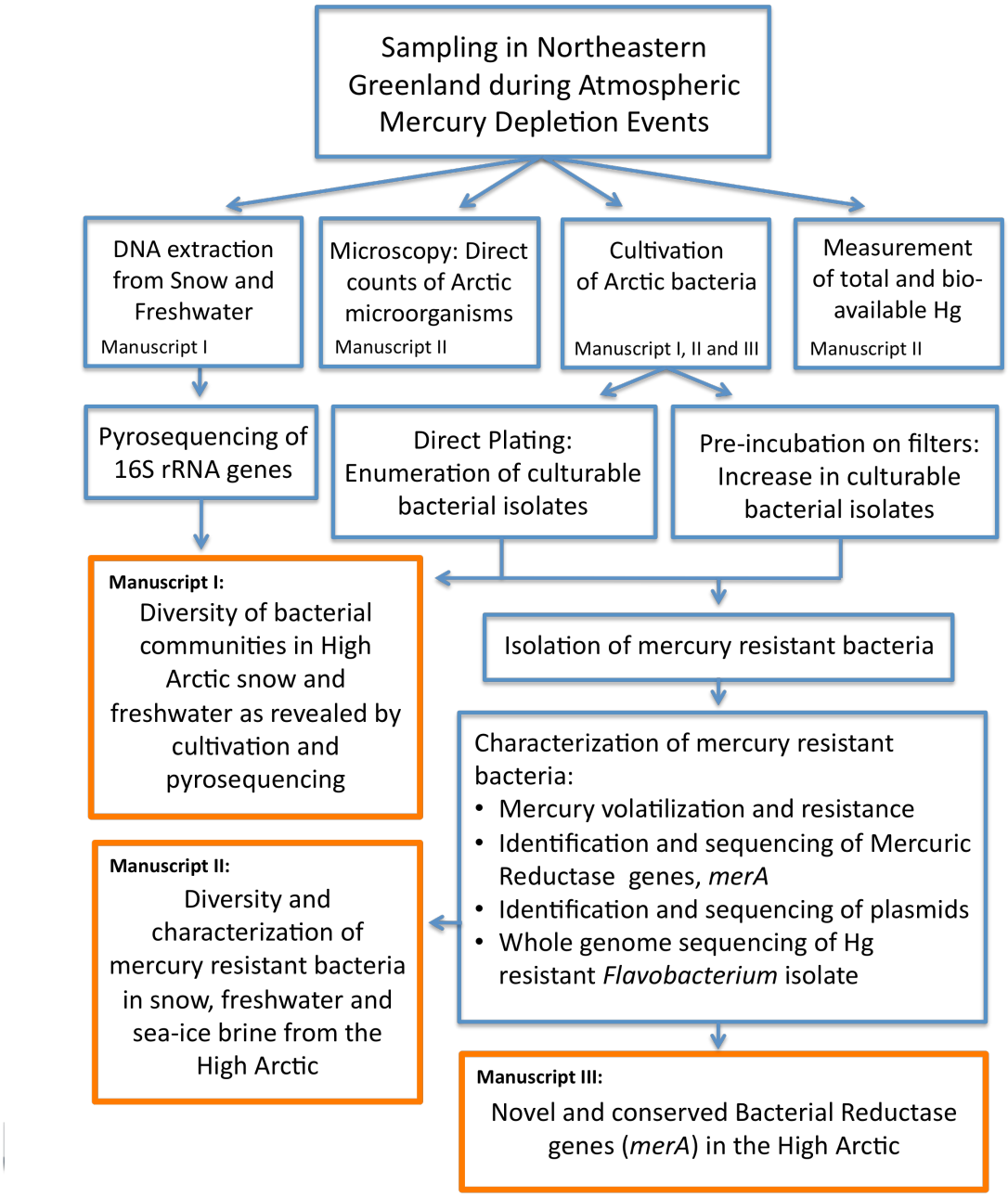

Figure 4. Outline of the experimental work of this PhD thesis.

7. Results and Discussion

1) Aim 1: Assess the diversity and composition of microbial communities found in coastal snow and a coastal fresh water lake (Manuscript I)

Samples were collected in Northeastern Greenland at Dagmar Sund between Danish military base Station Nord and Prinsesse Dagmar Island (snow samples) and 2 km south Station Nord (freshwater samples) in the Spring 2007. Snow samples were taken at three depths (top, middle, bottom) representing layers of different age, hardness and texture. The diversity of bacterial communities were assessed by analyzing 16S rRNA sequences obtained from a) DNA extracted directly from samples (pyrosequencing) and b) from DNA extracted from isolates cultured from snow and freshwater (sanger sequencing).

Figure 5. Sampling in Northeastern Greenland. Left picture, arrow indicates the geographical site of sampling. Right picture shows snow profile from where samples were taken.

Diversity of the bacterial communities

The diversity of the bacterial communities at the three snow depths and the freshwater lake was compared by rarefaction analysis, Shannon Weaver diversity index and Chao1 species

richness estimator. The bacterial diversity in all snow depths was higher than in the freshwater lake, and within the snow, the middle snow layer had the highest diversity. Likewise, when the bacterial communities were compared at the phylum level by Bray-Curtis similarity index, all snow layer communities were similar to each other when compared to the freshwater communities. Even though all four communities were significantly different (χ^2-test, $p<0.001$) the communities in the two top snow layers were most similar (75-95%, see Figure 6).

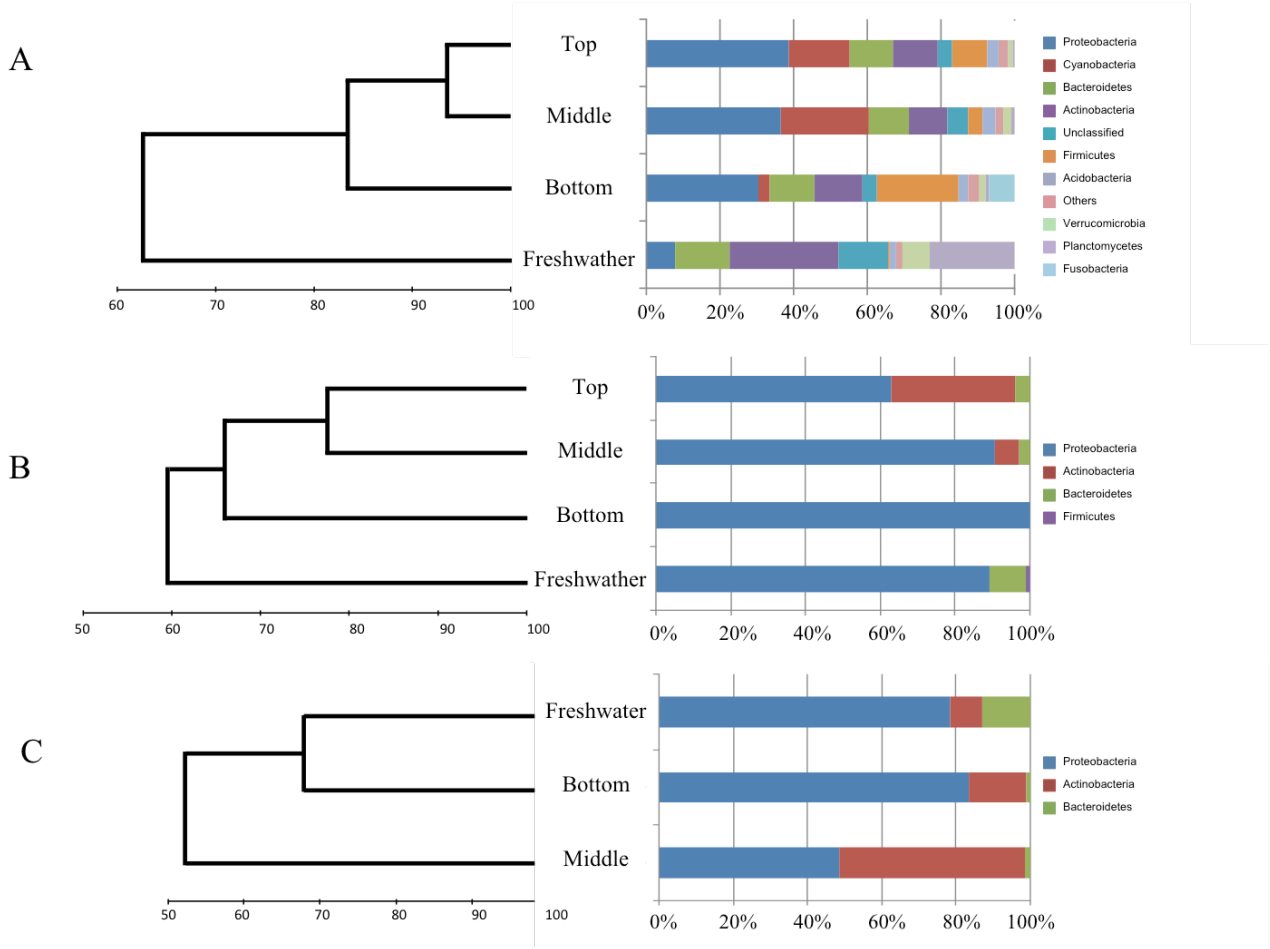

Figure 6. The phylogenetic composition of the Arctic microbial communities. Similarity dendrograms are shown to the right. The bars indicate percent similarity. A: Pyrosequencing data. B: Isolates cultured by filter-incubation method. C: Isolates by direct plating.

The differences in the composition of bacterial communities in snow and freshwater could be due to the number of physico-chemical properties that vary within snow and between snow and freshwater. In snow, for instance, the light intensity decreases with depth, and temperature becomes less variable and extreme. Also, as we sampled snow covering the sea-ice, the deeper layers may have been affected by seawater penetrating through cracks in the ice (in several instances we observed water mixed with snow ('slush ice') up to about 50 cm from the sea ice surface). These varying physical and chemical properties most likely reflect the dissimilarities in the snow bacterial community. Contrary to the variable conditions in snow, the freshwater lake can be characterized by relative high and constant temperatures around 0°C, and most likely by limited light intensity due to the snow and ice coverage. The conditions in the lake, therefore, can be considered as stable and less extreme than in snow and it is very likely that the relatively stable conditions resulted in a lower diversity in the bacterial community.

The most abundant phyla had many more unique OTU's than did the rare phyla in both snow and freshwater, i.e. abundant bacterial groups had higher within-group diversity than rare groups (Fig. 3). The correlation between log (relative abundance) and log (number of unique OTU's) was very high for all communities ($P<0.0001$; $r = 0.969$, 0.944, 0.964 and 0.922 for top, middle and bottom snow and freshwater, respectively). In snow, however, the most abundant phyla had more OTU's than freshwater.

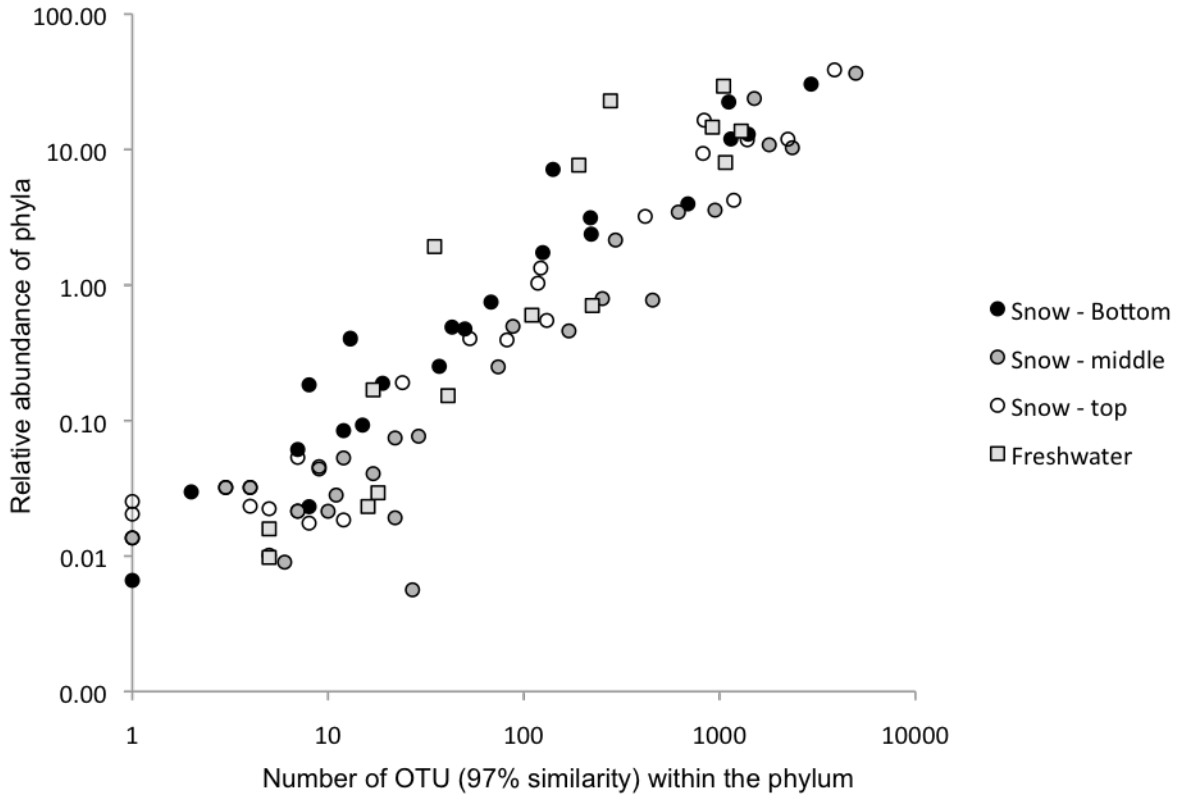

Figure 7. Relative abundance of phyla as a function of the number of OTU (97% similarity) within the phyla

The abundance of the bacterial phyla appeared to be related to their diversity as the abundant phyla had large numbers of unique OTU's rather than few highly abundant ones (Figure 7). A similar observation has been made in the Arctic Ocean (50) and suggests that the ecological success of a bacterial lineage depends upon diversity rather than superior competiveness of a few phylotypes (50).

Phylogenetic composition of bacterial communities in snow

Analysis of snow showed that *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* dominated (frequencies of 30 - 39%, 10 - 13% and 10 -12%, respectively) although in the middle and top snow layers, *Cyanobacteria* also accounted for a substantial fraction (16 - 24%) of the communities (Figure 6A). Another major difference between the bottom and middle/top snow layer was the presence of a large percentage of *Firmicutes* (22%) and *Fusobacteria* (7%) in the bottom layer. Minor frequencies of unclassified bacteria (4 - 6%),

Acidobacteria (2 - 3%) and *Verrucomicrobia* (1 - 2%) were observed in all 3 layers. Several other phyla were observed at frequencies lower than 1%, including the candidate division TM7 (≤ 0.5%), and the Archaean phyla *Euryarchaeota* (≤ 0.4%) and *Crenarchaeota* (≤ 0.1%). Our results combined with other studies it seems that *Proteobacteria* (α, β and γ), *Actinobacteria* and *Bacteriodetes* (especially *Flavobacteria* and *Sphingobacteria*) are commonly found in arctic snow (2, 55).

The structure of the bacterial communities in the different snow layers clearly changed with depth, which may partly be explained by the sources of bacteria into the snow. Bacteria can be transported over long distances with dust particles (48), and Sattler et al. (82) have shown that bacteria may be metabolic active in icy super cooled cloud droplets. Therefore, the atmosphere may be significant as a source of bacteria to the top snow layer, and as the top snow becomes older and eventually covered with fresh snow, the composition of the bacterial communities may gradually change. In this respect, an intense (UV) light intensity undoubtly plays a role as we observed several pigmented isolates (yellow, orange, pink and red colony color) probably as a defense mechanism to high UV radiation. Interestingly, *Cyanobacteria* and chloroplasts* were almost exclusively found in the top and middle layers with the highest density in the middle layer. This shift in *Cyanobacteria* density with depth from high, to very high, to low, suggests that light intensity controlled the distribution of both the *Cyanobacteria* and algae in the snow pack. We primarily found *GpI* and *GpXIII Cyanobacteria,* which are known to include species capable of fixing nitrogen. A likely scenario for explaining the heterotrophic community structure in the different snow layers is, therefore, that some of the nitrogen fixed by the *Cyanobacteria* stimulated the primary production of the algae, while the carbon and nitrogen produced by the autotrophs in turn were feeding the heterotrophic bacteria. Other sources of carbon to the bacterial communities, especially in the top layers, could be aerosols from the

* all chloroplasts were omitted from the community analysis

atmosphere (10). The deepest snow layer did not seem heavily affected by the autrotrophs as their densities were relatively low. Also, the concentration of dissolved organic carbon was about three times lower in this layer (4.0; 3.8 and 1.3 mg carbon ml^{-1} in top, middle and bottom snow layers, respectively, Manuscript II). However, as indicated above, it is likely that seawater microbes may have influenced the bacterial community in the deepest snow layer. Typical Arctic seawater bacteria include *α-Proteobacteria* and especially the *SAR11* family is abundant (50, 59). Indeed, in the bottom snow layer more than 2% of all sequences were classified as *Pelagibacter*, a genus within the *SAR11* family, while sequences classified within *SAR11* were only sporadically observed in the two higher snow layers

Phylogenetic composition of bacterial communities in freshwater

Studies of microbial communities in Arctic and Antarctic freshwater lakes have revealed *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* as the major phyla (22, 72), which all were also well represented in our study. Yet, the most dominant genus in our freshwater samples was *Isophaera*, belonging to the *Planctomycetes* phylum. *Planctomycetes* are commonly found in freshwater environments (33) but have only scarcely been detected in polar environments, e.g. in surface sediments of the Arctic ocean (56). *Cyanobacteria* are found often in freshwater (98) and have been identified in an Antarctic freshwater lake previously (29). In our study they were only detected at very low frequency. However, this is not surprising since the freshwater lake at Station Nord had been ice covered for at least 22 months before our sampling period in 2007 and presumably no light would have maintained a selection for *Cyanobacteria*.

Bacterial culturing

Great efforts were put into culturing bacterial from the Arctic samples using proper conditions and media. Nevertheless, the bacteria isolated from the snow and freshwater did not reflect the composition found by pyrosequencing, illustrating the well-known discrepancy between molecular and cultivation based bacterial community analysis. The discrepancy could be due to

the following reasons; bacteria found in the community could be hard to culture (in snow 0.07-11% of the bacteria were culturable and 0.3% in freshwater were culturable, Manuscript II). For example, *Bacteroidetes,* which constituted a large percentage of the community based on pyrosequencing data, are know to be difficult to culture (65). The other possibility is that the culturable bacteria have been suggested to represent the active fraction of bacterial communities (28, 32). If this is true, then *Proteobacteria*, and especially *Pseudomonas* and *Sphingomonas*, *Actinobacteria* (*Salinibacter*, *Kineococcus, Arthrobacter and Micrococcaceae*) and to lesser extent *Bacteroidetes* and *Firmicutes* were metabolic active during the sampling period.

Aim 2: Assess the abundance and diversity of bacteria capable of reducing Hg^{II} in sea ice, snow and freshwater (Manuscript II)

Under Aim I, bacteria were isolated from Arctic snow and freshwater samples. These isolates were tested for resistance to Hg. Furthermore, 200 bacterial species isolated from sea-ice were also tested. A total of 71 Hg resistant isolates were found, of these, only one originated from the sea-ice. Table 1 summarizes the density, cultivability and percentage of Hg resistant bacteria. Hg concentrations from all sampling sites were measured as well as atmospheric ozone levels that indicated that Atmospheric Mercury Depletion Events (AMDE) took place during the time of sampling.

Sample	Total counts[a] (cells ml^{-1})	Direct plating[a] (cfu ml^{-1})	Cultivability (%)	Hg resistance[b] (%)
Site 1				
Brine	$5.0\times10^{5} \pm 8.3\times10^{4}$	$1.3\times10^{2} \pm 4.0\times10^{1}$	0.03	1.7
Snow (31-52 cm)	$3.1\times10^{3} \pm 1.5\times10^{3}$	$2.0\times10^{0} \pm 2.0\times10^{0}$	0.07	0
Snow (75-90 cm)	$1.4\times10^{3} \pm 1.1\times10^{3}$	$1.7\times10^{2} \pm 1.2\times10^{2}$	11.9	1.7
Snow (96-112 cm)	$8.5\times10^{2} \pm 9.0\times10^{1}$	$6.8\times10^{1} \pm 3.0\times10^{1}$	8.00	31.2
Freshwater	$9.4\times10^{5} \pm 3.8\times10^{4}$	$2.5\times10^{3} \pm 7.1\times10^{1}$	0.3	1.6

Table 1. Bacterial density, cultivability and percent Hg resistant isolates.
a Standard errors (n = 3-5)
b Percentage Hg resistance was calculated based in the CFU from direct plating.

Microorganisms were isolated by two different methods, direct plating and pre-incubation on filters prior to plating. The filters were incubated until micro-colonies were present by microscopic examination (see Figure 8). Direct plating often selects for only a very small fraction of the community, while pre-incubation under conditions that simulate the environment has been shown to increase the cultivability of soil bacteria up to 2800 times (78). The majority

of the Hg resistant isolates from snow originated from direct plating (24 isolates) while only one isolate was obtained using the pre-incubation approach. However, 44 out of 45 of our isolates

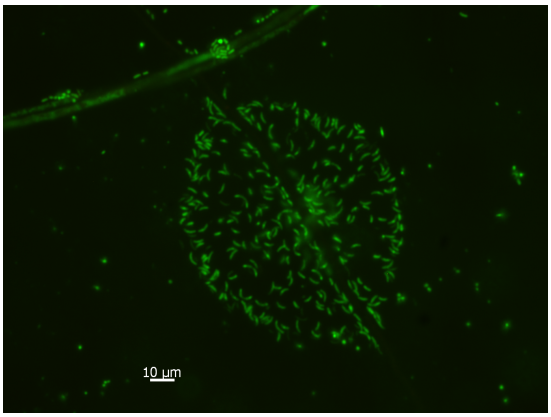

Figure 8. Picture of micro-colony on pre-incubated filters. Cells were washed of the filters and plated onto diluted rich medium when micro-colonies were observed (see Manuscript II for details)

from freshwater were obtained by the pre-incubation method. Thus, pre-incubation on filters prior to plating enabled us to isolate more resistant bacteria than would otherwise have been possible by direct plating on rich medium.

Among the Hg resistant bacterial isolates, 17 different partial 16S rDNA sequences were identified. These were distributed within seven different classes: *α-, β-, γ-Proteobacteria, Actinobacteria, Sphingobacteria, Flavobacteria* and *Bacillus*. The diversity of the bacteria from snow and freshwater communities were similar with the exception of *Flavobacteria* and *Firmicutes* that were only identified in the freshwater and *Actinobacteria* that were unique to the snow (see Figure 9). Within each phylum/class, several sub-groups were observed with identical (99% sequence similarity) partial 16S rDNA sequences. It should be noted that the closest relative for 10 of the sub-groups is either a psychrophile or has been isolated from a cold environment and belongs to phyla and classes that are commonly found in the cold environments. For example *α-*, and *γ-Proteobacteria, Actinobacteria* and isolates belonging to

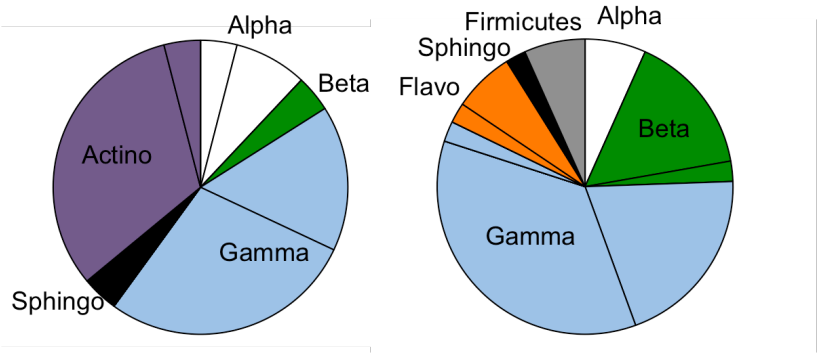

Figure 9. Distribution of the phylogenetic groups in the freshwater with a total of 46 isolates (right) and in snow representing 26 isolates (left). Different colour represent classes as indicated and each pie section represent sub-groups (99% similarity of 16S)

Bacteriodetes are common in sea-ice both in the Arctic and Antarctica (14), and *β-proteobacteria* have been shown to be present in snow covers of an alpine lake (1). Amato et al. (2) isolated *α-*, *β-* and *γ-Proteobacteria*, *Firmicutes* and *Actinobacteria* in snow covers at Spitzbergen, and Larose et al. (55) identified DNA belonging to *α-* and *β-Proteobacteria*, *Sphingobacteria* and *Flavobacteria* from the same area. The Hg resistant bacteria also belong to phyla that represents the most dominant phyla of the bacterial communities at Station Nord (Manuscript I). In addition to bacterial isolates, several Hg resistant fungal colonies were obtained from snow. The closest relative to these were Antarctic yeast belonging to the order *Leucosporidiales* and the psychrophilic species belonging to the genus *Geomyces*.

Sixty-nine Hg resistant isolates were psychrotrophs as they grew at temperatures ranging from 4°C to 20-25°C (room temperature) – the 4 isolates belonging to one of the *Flavobacteria* groups had a maximum temperature for growth at 20°C. Hence, they could be considered true psychrophiles, defined as bacteria with maximum temperature for growth below 20°C (37).

The Hg resistant isolates were characterized by their sensitivity to Hg^{II} (Minimal Inhibitory Concentration, MIC) and their ability to volatilize Hg^{II} to Hg^{0}. In general, the sensitivity to Hg was not temperature dependent, and it therefore did not seem as the resistance were better adapted to cold temperatures. Both the MIC values and the ability to volatilize Hg^{II} varied within

some of the taxonomic groups, suggesting that the level of Hg resistance was acquired at the species level as for instance by horizontal gene transfer. Indeed the best described system for bacterial Hg resistance, the *mer* system, is commonly found on transposon and mobile elements (9, 67).

Total Hg concentrations were measured at different snow depths, in brine and in freshwater. Concentrations (69.2 – 79.6 ng/L) were within previously reported values (55 to 90 ng L^{-1}) during AMDE (57, 58, 89). Attempts to measure bio-available Hg with a live *E.coli* biosensor were made but unfortunately the sensitivity of the sensor were to high (all values were less than 60 ng/L). To investigate if the concentrations in snow and brine were high enough to maintain a selective pressure for Hg resistance, we compared our data with those in other studies reporting both Hg concentrations and percent Hg resistant bacteria (6, 7, 24, 25, 61, 75, 76, 78, 91). A correlation between total or bioavailable Hg with percent Hg resistance was not apparent for the compiled data set (Figure 10). For example, in a coastal marine sample, the total Hg concentration was 1.7×10^{-4} µg ml^{-1} and 23% of the cultured bacteria were Hg resistant (24), whereas only 2.9% of the bacterial isolates were resistant in a soil with 7.6 µg Hg g^{-1} or 8×10^{-4} µg bioavailable Hg g^{-1} (7). Thus, the Hg concentration does not seem to be a sensitive predictor of the population of resistant bacteria. The levels of total Hg in our study were relatively low (70 – 80 ng L^{-1}) when compared to other environments, but nevertheless up to 31% of our isolates were Hg resistant. Others have reported Hg resistant bacteria from cold environments. For instance, Petrova *et al* (73) found up to 2.9% Hg resistant bacteria in permafrost sediments and in Antarctic seawater, frequencies of 1.5 - 4.7% (60) and 68% (25) were found.

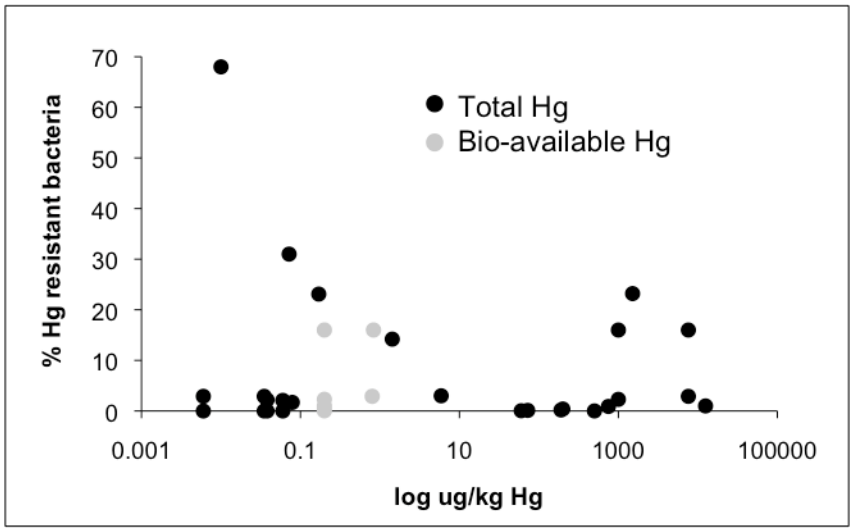

Figure 10. Plot of percentage of Hg resistant bacteria as a function of the concentration of Hg present in the environment the bacteria were isolated from.

Although Hg concentrations in polar environments are relatively low, it is likely that in polar environments such as snow, the Hg is highly available to microorganisms, thus selecting and maintaining Hg resistance in the communities.

Hg^0 measured in snow fluctuates during AMDE (26, 52) largely due to volatilization when Hg^{II} is reduced Hg^0. Emissions from snow take place simultaneously with increasing solar radiation and reduction rates decline drastically with snow depth and when snow samples are placed in the dark. Thus, the emission of Hg^0 from light exposed snow is believed to be largely driven by photoreduction (26, 52, 54).

Biological Hg reduction *in situ* is difficult to measure as Hg^{II} may be reduced by chemical redox processes under dark conditions (31). Hence, measurements of formation of Hg^0 in the dark would not be indicative of biological reduction. To assess the potential for *merA*-mediated reduction in snow we, therefore, made an estimate based on our own data and on literature values (see Manuscript II for details) and found the estimated bacterial reduction rates increased with snow depth (Table 2). In the uppermost layer no resistant cells were observed and, hence, no reduction could be estimated. However, reduction appeared to increase by a factor of almost 20

when going from ~ 83 cm to ~ 105 cm depth. Dommergue *et al* (26) measured total Hg reduction in interstitial air at different depths in the snow pack in Kuujjuarapik, Quebec, Canada. They found that rates decreased with increasing depth and suggested photoreduction to be the primary mechanism for the Hg reduction. A comparison with our data shows that 6 - 38% of the total reduction may potentially be attributed to bacterial reduction at the deeper snow depths (Table 2). Thus, in deeper snow layers, where light attenuation limits photoreduction, bacterial reduction may become an important contributor to the emission of volatile Hg^0 from snow to the atmosphere. The light penetrating the two top layers in this study is consistent with the presence of a large fraction of *Cyanobacteria* in these layers and not in the bottom layer (Manuscript I). The conclusion that bacterial Hg^{II} reduction may be important in layers were light is absent agrees with a study of coastal waters in the Canadian High Arctic (74), in which the relative contribution of bacterial reduction appeared to increase with depth, accounting for up to 94% of the total production of Hg^0 at the greater depths.

	Bacterial reduction rate Station Nord		**Total reduction rate Kuujjuarapik, Canada (26)**		**Bacterial/ Total**
	1% enzyme activity	100% enzyme activity			
Depth (cm)	Reduction Rate (nmol/hour/m^3)	Reduction Rate (nmol/hour/m^3)	Snow depth (cm)	Reduction Rate (nmol/hour/m^3)	(%)[1]
31-52	0	0	54	$1.0x10^{-3}$	0
75-90	$5.4x10^{-9}$	$5.4x10^{-7}$	80	$2.6x10^{-4}$	0.2
96-112	$9.8x10^{-8}$	$9.8.x10^{-6}$	102	$1.7x10^{-4}$	5.8
Average	$3.2x10^{-8}$	$3.2x10^{-6}$	Average	$4.9x10^{-4}$	0.7

Table 2: Estimation of Bacterial Hg reduction rates in snow in the High Arctic (see details for calculation in Manuscript II)

Aim 3: Identification and abundance of Hg reduction genetic elements in the arctic bacterial communities (Manuscript III)

The presence of *merA* genes in the Hg resistant isolates from Aim II were examined by PCR with primers designed from previously described *merA* sequences. All Hg resistant isolates were screened for plasmids and 5 distinctive plasmids were sequenced. Furthermore, one isolate belonging to *Bacteroidetes* with no detectable *merA* gene was fully sequenced.

Combined with the study described in manuscript II it seems that the *mer* system is ubiquitous in the Arctic environment. The presence of *mer* genes in the Arctic Hg resistant isolates from St. Nord in Northeastern Greenland was examined by PCR with degenerate primers designed from known *merA* sequences, sequencing of plasmids found in the Hg resistant isolates and whole genome sequencing of one of the isolates. The examination revealed 3 major observations: 1) Indication of high diversity of common and novel *merA* sequences distributed in isolates representing 4 bacterial phyla, 2) indication of lateral gene transfer of the mer operon and 3) the *mer*-operon in Bacteroidetes resembles an early lineage of the *mer* operon based on the clustering of the MerA sequence and the organization of the operon in the *Bacteroidetes* isolates.

merA sequences was identified in Hg resistant isolates that belonged to *Proteobacteria* (α, β and γ), *Actinobacteria*, *Firmicutes* and *Bacteroidetes*) and represented 7 distinctive *merA* types (based on 99% similarity at the DNA sequences), for overview, see Figure 11.

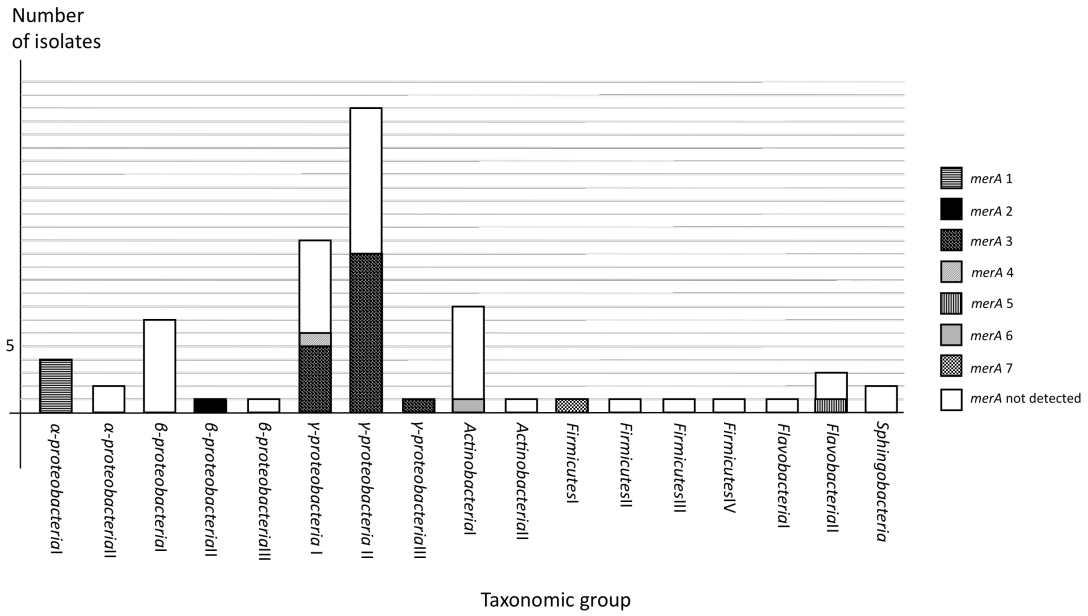

Figure 11. Distribution of *merA* sequence types within the taxonomic sub-groups (each group at least 97% similarity of the 16S rDNA gene) of Arctic Hg resistant isolates

Seven different *merA* sequences from 27 of the 71 Hg resistant High Arctic bacteria were identified (see figure 11). The *merA* sequences were identified in isolates belonging to *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Four of the MerA sequences was identified in *Proteobacteria* isolates and showed high sequence similarity (99-100%) to known MerA sequences while the other three MerA sequences showed low sequence similarity (82-92%) to known MerA sequences and originated from isolates belonging to *Actinobacteria*, *Bacteroidetes* and *Firmicutes*.

While *merA* sequences were only identified in 37% of the Hg resistant bacteria, 13 of the remaining 45 Hg resistant isolates were able to volatilize Hg^{II} to Hg^{0} (Manuscript II), which strongly suggests the presence of a *mer* system. It is very likely that the isolates apparently lacking *merA*, but were able to volatilize Hg^{II} to Hg^{0} carry yet unidentified *merA* genes that would not be recognized with primers based on already-known *merA* sequences. Thus the diversity of *merA* represented in the arctic microbial community at St. Nord is most likely underestimated. The sequences were most closely related to other MerA proteins; *merA* type 1-4

were all isolated from *Proteobacteria* and were almost identical (99% similar at the amino acid level) to MerA sequences previously identified and originating from bacteria found in diverse environments such as sugar beet (90), Hg contaminated soil in USA (66), river sediments in Kazakhstan (88) and Siberian permafrost (61). Interestingly, the *merA* sequence from the *α-Proteobacterium* was most closely related to a sequence in the filamentous fungi *Sordaria macrospora* – no *mer* system has been observed in Eukayria, eventhough Hg resistance is recognized in fungi (20). Regardless, it is worth noting that this sequence also showed weak resemblance (59% similarity at the amino acid) to a putative MerA in the marine psychrotroph *Sphingopyxis alaskensis*. *merA* types 5-7 were identified in *Bacteriodetes*, *Actinobacteria* and *Firmicutes* respectively and were less similar to any public available MerA sequences (68-93%).

The un-even distribution of *merA* sequences within the taxonomic groups indicated lateral gene transfer. For example, *merA* type 3 was present within 3 taxonomic groups (*γ-Proteobacteria* group I, II and III) and only some of the isolates within *γ-Proteobacteria* group I and II and *Actinobacteria* group I had a detectable *merA* sequence. Indeed, 60% of all the Hg resistant bacteria carried plasmids and sequencing of 5 representative distinctive plasmids revealed that two *merA* genes were located in an operon on a plasmid (*merA* type 2 and 7). In addition, the *mer*-operons on both plasmids were found in close proximity to genes involved in transposition or plasmid transfer. *Mer* genes are often found on mobile and transposable elements (67), for example the majority of the *merA* sequences identified in this study were almost identical to sequences located on transposons (*merA* type 2, 3 and 4). Whether lateral gene transfer is occurring in the Arctic is uncertain, since little is known about plasmid transfer in cold environments. Plasmids are commonly found in Arctic and Antarctic isolates and at least one plasmid has been shown to have a broad host range (62), but plasmid transfer in situ has not yet been demonstrated. Bacterial plasmid transfer requires direct contact between donor and recipient and considering the relatively low concentration of bacteria in snow this may be a rare

event unless the distribution of bacteria are clustered together in for example pockets of liquid water. Another possibility of horizontal gene transfer in the Arctic is transduction; for example in sea ice and arctic sea water the viral load have been found to be relatively high (71, 95) and bacterial phage-hosts have been isolated from sea-ice in Svalbard as well (12). Regardless, one of the sequenced plasmids originating from a *γ-Proteobacterium* belonged to IncP-1*β*, a group of plasmids that are conjugative and have a broad host range.

Hg resistance in *Bacteroidetes* is not well described and only one study from 1997 reports Hg resistance in a *Flavobacterium* isolate (34). Originating from Artic freshwater we isolated six *Bacteroidetes* strains, four belonging to *Flavobacteria* and two belonging to *Sphingobacteria*. All six isolates showed high resistance to Hg^{II} (25-50 μM in dilute media, Manuscript II) and the four *Flavobacteria* isolates had the ability to volatilize Hg^{II} to Hg^{0}. Since Hg resistance is not well described in this phylum, attempts to amplify *merA* was done both with degenerate primers and with primers designed on the basis of the putative *merA* in the genome sequence of *Loewenhokiella blandensis*, which is the closest related species to *Flavobacteria* with a putative MerA. Since PCR efforts to identify any *merA* in *Bacteroidetes* were unsuccessful, the genome of one of the isolates belonging to *Flavobacteria*, was fully sequenced. A *mer* operon was identified in isolate SOK62 using amino acid sequences of any putative MerA sequence found in *Bacteroidetes* in the database (total of 4 sequences resembled MerA) and the MerA sequence of Tn*501* as queries in blast searches of the SOK62 genome sequence. The *merA* locus of SOK62 showed little resemblance to any *merA* or other ORF's at the DNA level, which would explain the negative PCR results. The amino acid sequence of the MerA was most similar to the MerA loci of *Sphingobacterium spiritivorum* and *Chryseobacterium gleum*, two other isolates belonging to *Bacteroidetes*. The identified *merA* in SOK62 was located in a putative simple *mer*-operon which also and shared similarities in the operon structure to both *S. spiritivorum* and *C. gleum*; all three operons consisted of ArsR, a putative Hg^{II} transport protein and MerR. The

operons of *S. spiritivorum* and *C. gleum,* which were almost identical, also had a region with coppertranslocating ATPase, a hypothetical protein and Hg^{II} lyase (*merB*) inserted between the Hg^{II} transport protein and *merA*. Both operons from *S. spiritivorum* and *C. gleum* were surrounded by genes involved in transposition whereas the *mer*-operon of SOK62 was located in close proximity to other genes that appeared to encode proteins involved in metal transformation: multicopperoxidase, ferrodoxin, a high affinity Fe^{2+}/Pb^{2+} permease and a heavy metal transporting ATPase

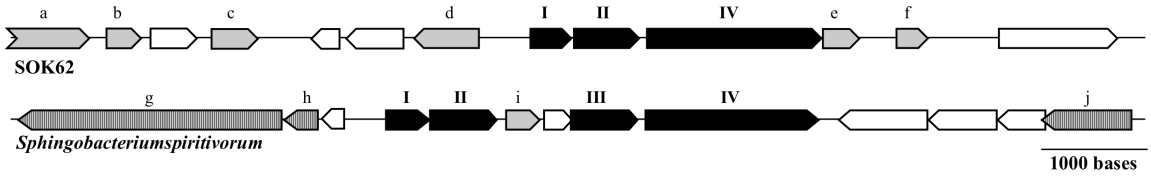

Figure 12: Genetic irganization of ther mer-operon in Flavobacterium isolate SOK62 and S. spiritivorum. Black arrows indicate open reading frames encoding putative Hg resistance genes (I – ArsR family transcriptional regulator, II – Hg^{II} transport protein, III – Mercurial lyase (merB), IV - Hg^{II} reductase (merA)); grey/stribed arrows indicate ORF's encoding genes involved in conjugation or transposition (g – protein found in conjugative transposition, h – TraF, j – protein found in conjugative transposition); grey arrows indicate ORF's encoding other genes (a – multicopperoxidase, b- ferrodoxin, c – universal stress protein, d – high affinity Fe^{2+}/Pb^{2+} permease, e Heavy metal transporting ATPase, f – protein with DNA binding domain excisionase, i – Copper-translocation P family ATPase); white arrows indicate ORF's encoding hypothetical or conserved proteins.

The putative MerA in SOK62 showed 42-83% similarity to all the other four putative MerA loci in Bacteroidetes and all five MerA loci formed a cluster that were basal to bacterial MerA and may represent an early lineage in the MerA phylogeny (Figure 13).

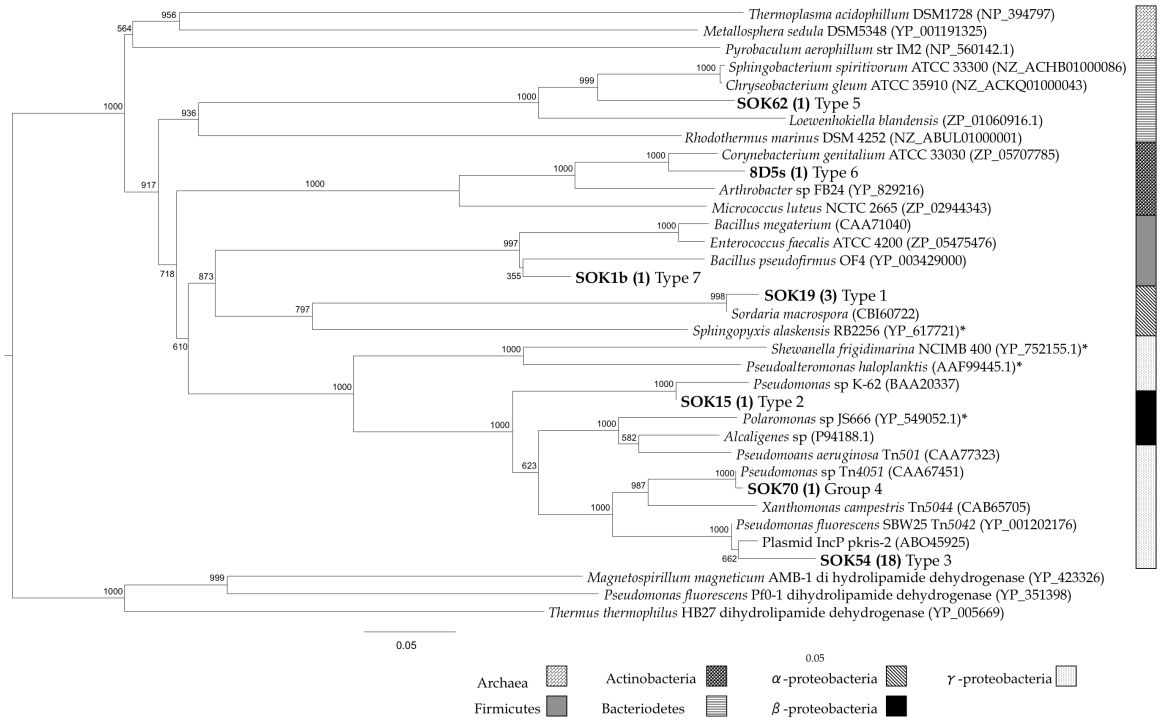

Figure 13. Neighbor-joining tree showing the phylogeny of MerA protein sequences. The tree is arbitrarily rooted in the enzyme Dihydrolipoamide dehydrogenase. Representative of each merA type (translated nucleotide sequence) identified in the Arctic Hg resistant isolates are shown in Bold, number in parenthsis indicates the number of isolates carrying this merA sequences. Values to the left reflect the bootstrap value

MerR, the regulatory element in most *mer*-operons, was not identified in the SOK62 *mer*-operon; instead ArsR appeared to be the regulatory element of the operon. MerR was not present in either of the other Bacteroidetes mer-operons, instead, like SOK62, an ArsR gene was also found in the putative mer-operons in *S. spiritivorum*, *C. gleum* and *R. marinus*. ArsR regulators belong to a group of metal-binding transcriptional inhibitors; in contrast to MerR that both inhibits transcription in the absence of the metal-ligand but remains bound to the operator upon binding to the metal-ligand and induce transcription (15), ArsR acts exclusively as repressors that dissociate from the DNA when bound to the metal-ligand (18, 97). ArsR also regulates Archaeal *mer*-operons and is sporadically present in *mer*-operons in gram-positive bacteria, while *merR* is found in all *mer*-operons in gram-negative and in most *mer*-operons in gram-positive bacteria (8). It may be that MerR is more efficient regulator and represents a late

development in the mer-operon that has replaced regulation from AsrR. This further supports the theory that the *Bacteroidetes mer*-operon is an early lineage in the *mer* phylogeny.

8. Conclusions

The bacterial community structure of high Arctic habitats as well as the bacterial resistance to Hg was assessed. High Arctic snow and freshwater were found to have a high bacterial diversity, with the highest diversity in snow. The bacterial composition in the snow differed with depth, reflecting the heterogeneity of the snow pack and influences from exposure to the atmosphere in the top layers and the underlying sea-ice and sea water for the deepest layer. In the top snow layers, a high frequency of carbon and nitrogen-fixing organisms were present suggesting that carbon sources in the snow include production from primary producers.

Hg resistant bacteria seem to be ubiquitous in High Arctic snow and freshwater since Hg resistant isolates were found in each of the culturable phyla: *Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria*. In the deep snow layers Hg resistant isolates were especially abundant and it is very likely that Hg resistant bacteria contribute to Hg reduction where light cannot penetrate and drive photoreduction as in the upper snow layers. We estimated that bacteria in the snow may contribute up to 2% of the total reduction in the deep snow layers; this number may be underestimated since we did not factor in the effect of local heterogeneities that could cause much higher local Hg^{II} concentrations and thus, increase the reduction rates.

Both previously described as well as novel *merA* genes were identified in a relatively small fraction of the Hg resistant isolates; other mechanisms of Hg resistance than *merA*-mediated resistance may therefore be present in the High Arctic bacterial communities. Several other novel *merA* genes, which were not detectable with the primers we used, are also very likely to be present. Indeed, we identified one novel *merA* gene by whole genome sequencing in a *Bacteroidetes* isolate that was not detectable with primers targeting known sequences. This *merA* locus along with other four putative *merA* loci in the Bacteroidetes phylum may represent an early lineage of *merA* genes.

The distribution of the *merA* genes that was found and the distribution of isolates able to reduce $Hg^{(II)}$ to $Hg^{(0)}$ strongly suggests that *merA* genes in the Arctic environment are transferred horizontally. While we did identify plasmids carrying *merA* sequences, transfer through conjugation has not been documented in arctic environments.

The heterogenousity of bacterial communites in snow indicates that the microbes found in these habitats are metabolically active and well adapted to their environment. A high percentage of Hg resistant isolates suggests that the biological aspect of the Hg cycle in arctic environments and should be considered when investigating Hg transformation in the High Arctic. Bacterial Hg reduction in the Arctic is at least partly, occurring through the *mer* system. However, the pool of *merA* genes seem diverse and both commonly known as well as novel *merA* sequences are present in the arctic bacterial community

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10 Other work included in this PhD work

10.1 Presentations:

Annette K. Møller, Søren J. Sørensen, Henrik Skov, Tamar Barkay. *Identification and characterization of Hg resistant bacteria from the High Arctic.* Invited speaker at the Polar Research Institute of China, Beijing. June 2009

Annette K. Møller, Søren J. Sørensen, Henrik Skov, Tamar Barkay. *Identification and characterization of Hg resistant bacteria from the High Arctic.* Oral presentation at the 9th International Conference on Hg as a global pollutant. Guiyang, China. June 2009.

Annette K. Møller, Søren J. Sørensen, Henrik Skov, Tamar Barkay. *Identification and characterization of Hg resistant bacteria from the High Arctic.* Poster and oral presentation at Symposium on microbial degradation of soil pollutants, process and impact. KU LIFE, Copenhagen, Denmark. October 2009

Annette K. Møller, Søren J. Sørensen, Henrik Skov, Tamar Barkay. *Mercury resistant bacteria isolated from snow, freshwater and sea-ice during atmospheric depletion events in Northeastern Greenland.* Poster presentiation at the 3rd Annual Conference on polar and alpine microbiology. Banff, Canada. May, 2008

10.3 Article in 'Dansk Kemi'

Annette K. Møller, Niels Kroer and Henrik Skov (2007) Kviksølvets Kolde Gåde – fra atmosfære til den arktiske fødekæde. Dansk Kemi, vol. 88. no. 9, p 14-17.

Kviksølvets kolde gåde – fra atmosfære til den arktiske fødekæde

I de arktiske egne findes kviksølv i alarmerende høje mængder i nogle populationer af grønlændere og inuitter. Det skyldes, at disse befolkningsgrupper hovedsageligt lever af fisk og havpattedyr, som er forurenet med kviksølv

Af Anette K. Møller, Niels Kroer and Henrik Skov, Danmarks Miljøundersøgelser, Aarhus Universitet

Kviksølv er et metal med usædvanlige egenskaber, og det findes hovedsageligt som elementært kviksølv i atmosfæren; andre tungmetaller findes kun i forbindelse med partikler. Ved stuetemperatur er stoffet en sølvfarvet viskøs væske, hvorfra navnet kviksølv stammer. Det latinske navn, *hydrargyrum*, som også er ophav til det kemiske tegn, Hg, betyder direkte oversat vandsølv. Kviksølv har et betydeligt damptryk og afgiver kviksølvdampe til atmosfæren. Dette er en af de vigtigste grunde til, at kviksølv er så farlig en miljøgift. Kviksølv optages desuden let i kroppen, og fordi det er en monoatomisk, neutral gas er det fedtopløseligt. Det betyder, at det kan diffundere let over cellemembraner og komplekse membraner som f.eks. blodbarrierer og dermed kan transporteres rundt i kroppen til de forskellige organer. Efter at kviksølvdampe er optaget i kroppen og transporteret til de forskellige væv, er det dog den gængse opfattelse, at det indgår i en ligevægt mellem elementært kviksølv (Hg^0) og Hg(II), og det er netop kviksølv i oxidationstrin 2+, der kan reagere med biologisk væv og derfor har den toksiske effekt. Mekanismerne for disse ligevægte i kroppen er endnu ikke præcist fastlagte.

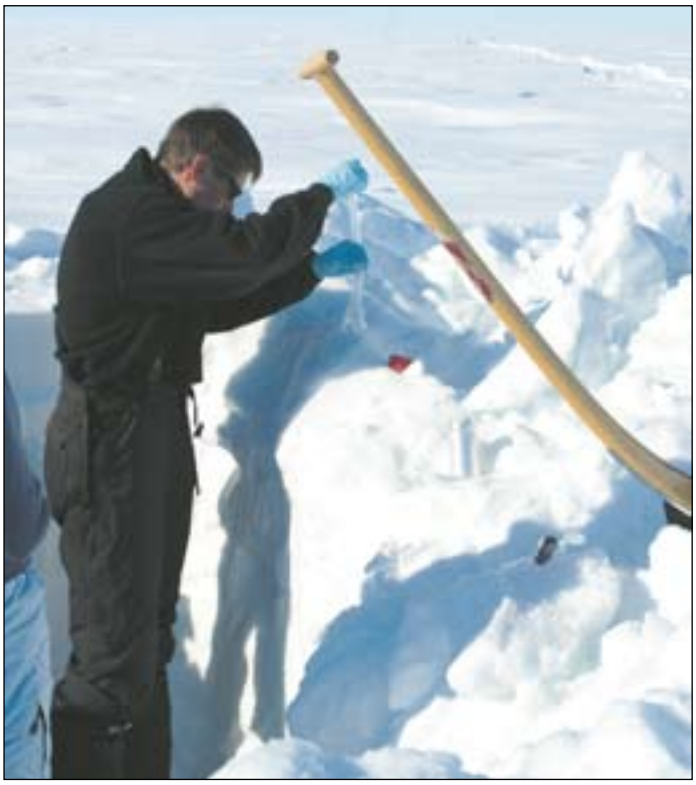
Farlig methylkviksølv

De organiske forbindelser, som f.eks. methylkviksølv, er de giftigste kviksølvforbindelser, og de ophobes i de marine fødekæder. De er uhyre giftige, og kan i selv meget små mængder være fatale for sunde og raske voksne mennesker. Dog er der indtil nu kun rapporteret methylkviksølvforgiftninger med dødelig udgang som følge af arbejdsuheld eller ulykker med spild af methylkviksølv. En af grundene til at methylkviksølv er endnu mere giftigt end kviksølvdampe er, at methylkviksølv kan reagere med thiolgrupper på aminosyren cystein. Derved minder denne »kviksølv-aminosyre« utroligt meget om den neutrale aminosyre methionin. Så i modsætning til Hg^0, som passivt bliver transporteret rundt i kroppen, kan methylkviksølv transporteres aktivt rundt i kroppen via methionin-carriers.

Kviksølvniveauet i befolkningsgrupper, der lever i arktiske egne, har været målt til helt op til 600 µg methylkviksølv pr. L blod. World Health Organization (WHO) har sat en grænseværdi på 20 µg/L. Selvom mængderne ikke nødvendigvis er livstruende, har studier vist en korrelation mellem methylkviksølvkoncentration i navlestrenge og nedsat intellektuel udvikling hos børn, hvis mødre har været udsat for ophopning af methylkviksølv [1].

Kviksølv er et globalt problem

Ifølge USA's »Miljøstyrelse«, US-EPA, udledes der i dag op



Annette Møller og Niels Kroer under feltarbejde ved Station Nord i Nordøstgrønland.

Bakterier isoleret fra sneen i Nordøstgrønland – potentielle kviksølvstransformere?

til 7500 tons kviksølv om året. Det er estimeret, at ca. 50-75% stammer fra antropogene kilder, som afbrænding af kul og fra deponering af kviksølvholdigt affald som batterier, og elektroder brugt i klor-alkali-processer. En del stammer også fra naturlige kilder som f.eks. vulkansk aktivitet. Kviksølv langtransporteres i atmosfæren og er derfor et globalt problem. Hovedsageligt findes kviksølv i atmosfæren som gasformigt elementært kviksølv (Gaseous Elementary Mercury - GEM), der har en estimeret levetid på ca. et år.

Kviksølv i Arktis

Der er de senere år kommet øget fokus på kviksølvdeponering i de arktiske egne, hvor det har vist sig, at GEM kan forsvinde fra atmosfæren på få timer i det arktiske forår under de såkaldte »Atmospheric Mercury Depletion Events« (AMDE) [2]. Mekanismen for AMDE er ikke fuldt ud klarlagt, men forskningen antyder, at brom frigivet fra havis ved genfrysning af våger reagerer med ozon i atmosfæren eller alternativt med GEM og derved danner det såkaldte Reactive Gasous Mercury (RGM) som hurtigt afsættes på isen og sneen.

$$O_3 + Br \rightarrow O_2 + BrO$$
$$Br + Hg \rightarrow HgBr \rightarrow$$
$$HgBr \rightarrow HgBr_2$$

hvor $HgBr_2$ er foreslået at være det RGM, man måler i Arktis [3,4].

Hvad der sker med det afsatte kviksølv, ved man ikke ret meget om. Man ved blot, at store mængder kviksølv forsvinder fra atmosfæren i det arktiske forår, og at store mængder methylkviksølv igen er at finde i fisk og havpattedyr. Hvorledes RGM bliver omdannet til methylkviksølv i de arktiske områder er endnu ikke beskrevet, og forskning til at belyse dette manglende link er stort set ikke eksisterende.

I de tempererede områder ved man dog, at mikroorganismer spiller en afgørende rolle i kviksølvs geokemi. Nogle bakterier er således involveret i methyleringen af kviksølvioner ($Hg^{2+} \rightarrow$ methylHg), mens andre grupper af mikroorganismer er i stand til at reducere kviksølvioner ($Hg^{2+} \rightarrow Hg^0$). Det er derfor en

nærliggende mulighed, at netop bakterier i havisen og sneen er involveret i omdannelsen af det deponerede kviksølv. Desuden har en enkelt forskergruppe netop demonstreret, at nogle af de bakterielle gener, der er involveret i kviksølvtransformation, faktisk er til stede i biomasse isoleret fra havvand og fra kystområder i Arktis [5].

Deponeret kviksølv

En vigtig brik i puslespillet vil derfor være at påvise, om deponeret kviksølv er at finde i Arktis under AMDE. Derudover vil det være af endnu større interesse at undersøge, om der findes kviksølv i en form, der er tilgængelig for de biologiske systemer. Dette er muligt at undersøge ved brug af en såkaldt kviksølvbiosensor (se boks 1).

Hvis man samtidig kan finde bakterier i sneen og isen, der er i stand til at methylere Hg^{2+}, er man kommet et stort skridt videre i mysteriet om, hvorledes kviksølv finder vej fra atmosfæren til de arktiske befolkningsgrupper.

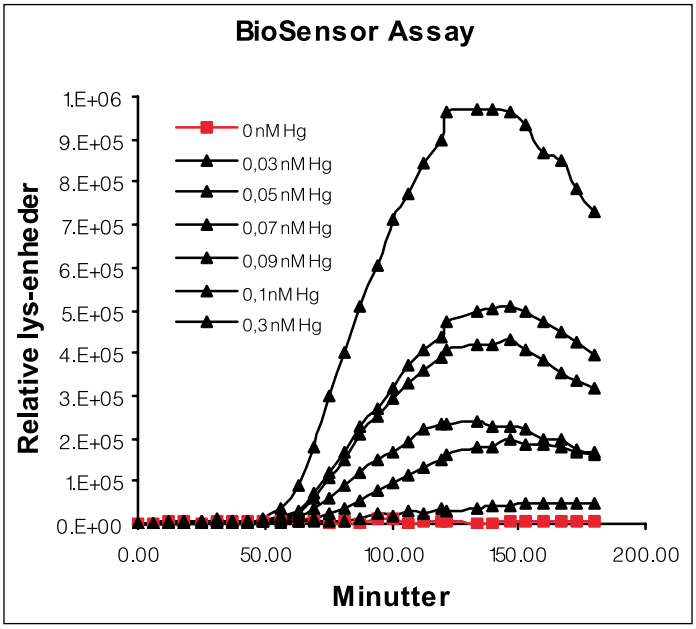
Et forskerhold fra Danmarks Miljøundersøgelser, Aarhus Universitet, er netop vendt hjem fra et ophold ved Station Nord på Nordøstgrønland, hvor de har indsamlet prøver af sneen og havisen. Overraskende var det biotilgængelige kviksølv i sneen og havisen under detektionsgrænsen på 10,0 ng/L i hele undersøgelsesperioden. Også mængden af total kviksølv lader til at være overraskende lav, under 50 ng/L. (Endelige analyser af total kviksølv er for tiden under behandling, se også boks 2).

Boks 1 – Kviksølv-biosensor

Grundlaget for udviklingen af kviksølv-biosensoren er bakteriers kviksølvresistensgener. Bakteriel kviksølvresistens kan opdeles i 2 hovedgrupper: bredspektret kviksølvresistens og smalspektret kviksølvresistens. Ved bredspektret kviksølvresistens er bakterierne resistente over for både organisk og uorganisk kviksølv, mens bakterier med smalspektret resistens kun kan modstå uorganisk kviksølv. Det funktionelle protein for smalspektret resistens er en Hg^{2+}-reduktase, dvs. et enzym, der reducerer Hg^{2+} til elementært kviksølv, som så frit diffunderer over cellemembranen og frigives til luften. De bredspektrede resistente bakterier indeholder desuden et gen, som koder et enzym, der kan spalte kulstofatomer fra kviksølvatomet og derved gøre Hg^{2+}-ionen tilgængelig for Hg^{2+}-reduktasen. For at bakterierne ikke bruger unødvendig energi på at syntetisere kviksølvresistensproteinerne, når der ikke er kviksølv til stede, er resistensgenerne under en meget effektiv kontrol. De bliver således kun udtrykt, når der er kviksølv til stede i miljøet, eller med andre ord, kviksølvresistente bakterier er meget effektive til at »føle«, om der er kviksølv til stede i miljøet omgivelser. Denne kviksølvkontrol er placeret foran de funktionelle resistensgener og sikrer, at resistensgenerne kun bliver udtrykt, hvis kviksølv binder sig til kontrolregionen.

I kviksølvbiosensoren udnytter man denne kontrolsekvens, men i stedet for det funktionelle gen, der koder for kviksølvresistens har man indsat et gen, der udtrykker et protein, der udsender lys. Lysmængden udsendt af bakterierne er proportional med mængden af kviksølv og kan let måles i et luminometer.

Biosensoren er meget følsom og kan måle helt ned til 10,0 ng/L biotilgængelig kviksølv. Dog skal man være opmærksom på, at prøven man vil undersøge ikke må indeholde stoffer, der har en bakteriehæmmende effekt, som f.eks. stærke syrer der ofte bruges som fikseringsmiddel for kviksølv.



Data fra biosensorforsøg. Prøver med kendte mængder kviksølv, hvor mængden er proportional med hældningen og maks.værdien for lysproduktionen fra biosensoren. Sne- og havisprøver viste kurver, der var på samme niveau som baggrundskurven.

I Alaska har man målt helt op til 135 ng/L total kviksølv under AMDE [6]. Om de lave værdier målt i Nordøstgrønland skyldes generelt lave kviksølvmængder dette forår, eller om processerne foregår inden for en meget kort tidshorisont og i et afgrænset geografisk område vides endnu ikke. Det vides heller ikke, om det er helt andre mekanismer, der ligger til grund for kviksølvs indgang til den arktiske biota.

Sne- og isprøver fra Station Nord er for tiden til undersøgelse for den bakterielle sammensætning. Når den mikrobielle diversitet og bakteriernes fysiologi er nærmere undersøgt,

Boks 2 - Måling af total kviksølv

Total kviksølv bliver målt i en Tekran 2537, A Mercury Vapour Analyzer Instrument. Dette instrument måler elementært kviksølv på luftform, Hg^0. Derfor er det nødvendigt, at alle kviksølvforbindelser i prøverne bliver omdannet til Hg^0. Først behandles prøven med bromchlorid, BrCl, i mindst 24 timer. BrCl er stærkt oxiderende og forskellige kviksølvforbindelser i prøven bliver omdannet til Hg^{2+}. Under al behandling af prøverne er det vigtigt, at det foregår i surt miljø for at stabilisere Hg^{2+}, så de ikke spontant omdannes til kviksølvdampe og derved fordamper fra prøven. Efter oxidering af prøven skal overskydende BrCl fjernes, da det kan skade den guldfælde, der anvendes til at opsamle og fokusere prøverne i Tekran-instrumentet. Prøverne behandles med hydroxylamin, NH_2OH, der reducerer overskydende BrCl. Umiddelbart før måling i Tekranen omdannes Hg^{2+} til Hg^0 ved at tilsætte tin(II)chlorid, $SnCl_2$. Herefter måles prøven på Tekran-instrumentet, der suger de dannede Hg^0 dampe ind i instrumentet. Her opfanges de på en guldfælde og danner guldamalgam. Hg^0 frigives igen, når guldfælden efterfølgende opvarmes til 500°C i en strøm af argon. Herefter måles mængden af kviksølv med fluorescensspektroskopi. Kviksølvmængden i prøven bestemmes ved at sammenligne den målte værdi fra Tekranen med værdier fra en standardkurve, der laves ved at måle på kendte mængder Hg^{2+}. Denne metode kan måle helt ned til 2 ng Hg pr. L.

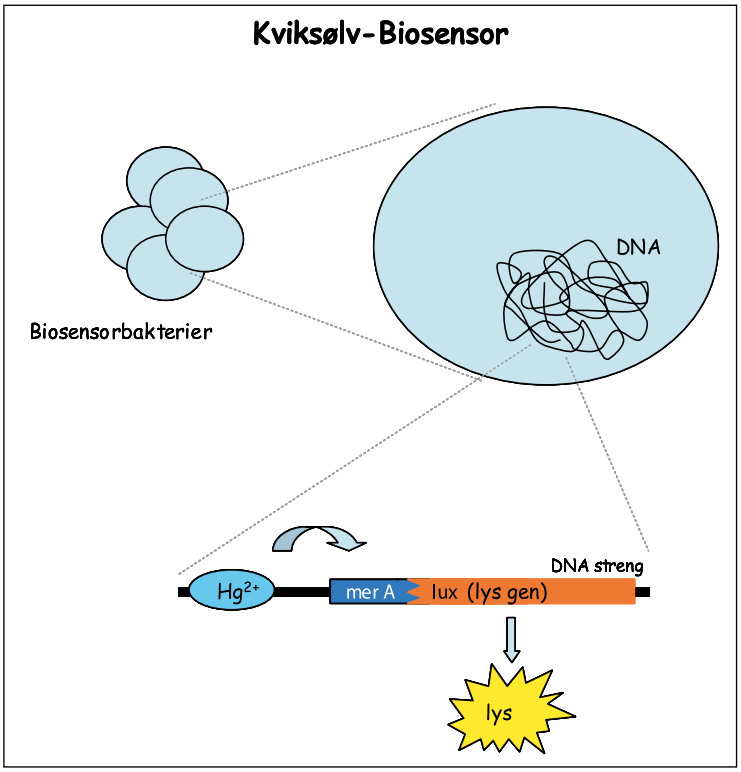


Illustration af mekanismen i biosensoren. Bakterier har indsat en DNA-konstruktion, hvorved kviksølv kan binde til en DNA-sekvens og aktivere et gen, der udtrykker et enzym, der udskiller lys (se også boks 1).

vil det forhåbentlig kaste lys over deres eventuelle rolle i historien om kviksølvs rute fra atmosfære til de arktiske befolkningsgrupper.

E-mail-adresser
Anette K. Møller: annm@dmu.dk
Niels Kroer: nk@dmu.dk
Henrik Skov: hsk@dmu.dk

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11. Manuscripts included in this Thesis:

Manuscript I

Annette K. Møller, Ditte A. Søborg, Waleed Abu Al-Soud, Søren J. Sørensen and Niels Kroer. *Diversity of bacterial communities in High Arctic snow and freshwater as revealed by pyrosequencing of 16S rRNA genes and cultivation.* Manuscript in preparation for submission to Environmental Microbiology

Manuscript II:

Annette K. Møller, Tamar Barkay, Waleed Abu Al-Soud, Søren J. Sørensen, Henrik Skov and Niels Kroer. *Diversity and characterization of mercury resistant bacteria in snow, freshwater and sea-ice brine from the High Arctic.* Submitted to FEMS Microbiology Ecology.

Manuscript III

Annette K. Møller, Tamar Barkay, Martin A. Hansen, Anders Norman, Lars H. Hansen, Søren J. Sørensen and Niels Kroer. *Novel and Conserved bacterial mercuric reductase genes (merA) in the High Arctic.* Manuscript in preparation for submission to The ISME Journal.

Diversity of bacterial communities in High Arctic snow and freshwater as revealed by pyrosequencing of 16S rRNA genes and cultivation

Annette K. Møller[1,2], Ditte A. Søborg[1], Waleed Abu Al-Soud[2], Søren J. Sørensen[2] and Niels Kroer[1]

[1]National Environmental Research Institute, University of Aarhus, Frederiksborgvej 399, DK-4000 Roskilde, Denmark

[2]Department of Biology, University of Copenhagen, Sølvgade 83H, DK-1307K Copenhagen, Denmark

Corresponding author:

Niels Kroer

National Environmental Research Institute, Aarhus University, Frederiksborgvej 399, 4000 Roskilde, Denmark

E-mail: nk@dmu.dk; Fax: +45 46 30 11 14; Phone: +45 46 30 13 88

Running title:

Diversity of bacterial communities in High Arctic snow and freshwater

Summary

Bacterial community structures in High Arctic snow and an ice covered freshwater lake were examined by pyrosequencing of 16S rDNA and sequencing of 16S rDNA from cultivated isolates. The phylogenetic composition of the microbial assemblages differed among snow layers and between snow and freshwater. The highest diversity was seen in snow. The *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* phyla dominated in snow, although in the middle and top snow layers, *Cyanobacteria* (and chloroplasts) were also abundant. In the deepest snow layer, large percentages of *Firmicutes* and *Fusobacteria* were observed. In freshwater, *Planctomycetes* and *Actinobacteria* were the most abundant phyla while relatively few *Proteobacteria* and *Cyanobacteria* were observed. Possibly, light intensity controlled the distribution of the *Cyanobacteria* and algae in the snow while carbon and nitrogen fixed by these autotrophs in turn fed the heterotrophic bacteria. In the lake, the temperature regime was stable and the light input lower than in snow. Furthermore, since numbers of *Cyanobacteria* and chloroplasts were low, the input of organic carbon and nitrogen to the heterotrophic bacteria probably was limited. Thus, differences in physico/chemical conditions may play an important role in the processes leading to distinctive bacterial community structures in High Arctic snow and freshwater.

Introduction

The Arctic and the Antarctic continents constitute up to 14% of the biosphere (Priscu and Christner, 2004) and offer the coldest and most arid environments on Earth (Onofri et al., 2004). Snow is an important component of the polar regions (Jones, 1999) and recent reports suggest that microorganisms in the snow may impact the dynamics, composition and abundance of nutrients (Hodson et al., 2008) as well as the surface albedo of snow (Thomas and Duval, 1995).

Various physiological adaptations, including increased membrane fluidity(Kumar et al., 2002), production of enzymes active at cold temperatures (Groudieva et al., 2004), production of cold shock proteins (Cloutier et al., 1992) and antifreeze proteins (Gilbert et al., 2005) , enable bacteria to be active under cold conditions, and bacterial activity has been detected at subzero temperatures in sea-ice and snow (Carpenter et al., 2000);(Junge et al., 2004); (Panikov and Sizova, 2006).

Most studies on microbial diversity in polar environments has focused on ice, permafrost and marine environments, while the microbial community structure in snow has only been scarcely examined. *Proteobacteria* (*alpha* and *beta*), *Bacteroidetes* (*Flavobacteria* and *Sphingobacteria*) and *Thermus-Deinococcus* have been found to be dominating phyla when using culture-independent approaches (Carpenter et al., 2000); (Larose et al., 2010), whereas *Proteobacteria*, *Firmicutes* and *Actinobacteria* have been isolated by cultivation-based methods (Amato et al., 2007).

16S rRNA gene clone-libraries are routinely used to examine microbial community diversity. However in highly diverse ecosystems, clone libraries, typically consisting of a few hundred clones, only recognize the most frequent members of a community. On the contrary, pyrosequencing of 16S rDNA include thousands of sequences and, hence, may detect the rare members and more accurately estimate the community diversity. A few Arctic environments have currently been examined by pyrosequencing including glacial ice (Simon et al., 2009), permafrost (Yergeau et al., 2010) and the Arctic Ocean (Kirchman et al., 2010).

The goal of this study was to explore questions about the bacterial diversity in different snow layers and an ice-covered freshwater lake in the High Arctic. We hypothesized that due to differences in the physico/chemical conditions, different bacterial community structures would be observed. The study is the first to assess the taxonomic diversity of High Arctic snow and freshwater microbial assemblages by analysis of pyrosequencing-derived data sets. To complement this approach, we also used cultivation-dependent approaches, i.e. direct cultivation on rich medium and pre-incubation under simulated natural conditions prior to plating on rich medium, because cultivable bacteria probably represent the active fraction of the communities (Ellis et al., 2003); (Frette et al., 2004).

Results

We examined 16S rRNA genes of cultivated bacterial isolates and of metagenomic DNA from microbial communities in snow and freshwater in the High Arctic. Snow samples were taken at three depths (top, middle, bottom) representing layers of different age, hardness and texture. Total bacterial numbers were 1×10^3 cells ml^{-1} for snow and ~ 1×10^6 cells ml^{-1} for freshwater.

Cultivation of the snow and freshwater microorganisms resulted in a total of 791 bacterial isolates. About 2/3 of these (570) originated from a pre-incubation approach, in which the bacteria under simulated natural conditions were grown to micro-colony size on polycarbonate membranes before being plating on rich medium, while 221 resulted from direct plating. In addition to the bacteria, several yeasts were found. Partial sequencing of the 16S rRNA genes was done for all bacterial isolates; however, 28 sequences had to be discharged due to poor quality. Numbers of different culturable OTU's (≥ 97% sequence similarity; Table 1) were slightly higher in snow (20 OTU's) than in freshwater (17 OTU's). By application of the pre-incubation approach, 25 different OTU's were obtained. Direct plating resulted in 15 different OTU's.

A total of 35 polycarbonate membranes, onto which bacteria in snow meltwater and freshwater were collected, were pyrosequenced. When combining the up to 10 replicate filters per environment, the total number of 16 rRNA gene fragments (tags) obtained for each bacterial community ranged between 60 000 and 103 000 (Table 2). The number of different bacterial OTU's (≥ 97% sequence similarity) per community varied between 5 300 and 13 800 (Table 2). Several chloroplast (primarily *Streptophyta* but also some *Chlorophyta*) were identified in snow and excluded from the analysis. The highest number of chloroplast tags was observed in the middle snow layer (21 855) followed by the top (6 114) and the bottom (2 640) layers. In freshwater, the number of chloroplast tags was low (14).

Diversity of the microbial communities

The diversity of the bacterial communities at the three snow depths and the freshwater lake was compared by rarefaction analysis (Fig. 1) and by other measures of diversity (Table 2). Rarefaction curves indicated that among the snow communities, the diversity was highest in the middle snow layer. However, diversity at all snow depths seemed higher than in the freshwater lake (Fig. 1A). Rarefaction analysis based on the culturable bacteria did not indicate notable differences between the snow and lake communities (Fig. 1B). The Chao1 species richness estimator and the Shannon-Weaver diversity index for OTU's sharing ≥ 97% sequence similarity supported the conclusion that the middle snow layer showed the highest diversity and that the snow microbial communities were generally more diverse than the freshwater community (Table 2). The Shannon-Weaver diversity index calculated for culturable bacteria (Table 1) did not indicate a difference in the diversity between snow and freshwater. The rarefaction curves of both the meta-genomic and the cultivation approaches suggested that the entire diversity of the communities was not captured, as the curves did not reach a plateau with increasing sample size. Based on the Chao1 species richness estimator, an estimated 52-54% of all OTU's were identified in snow while 65% were identified in freshwater.

Overall similarity of the microbial communities was examined at the phylum level (distribution of tags within each phylum compared between the four environments) by a χ^2-test and by the Bray-Curtis similarity measure (Fig. 2). Although the communities were significantly different as determined by the χ^2-test ($P < 0.001$), the Bray-Curtis similarity measure, based on the pyrosequencing data and the sequence data from the cultivated bacteria obtained by the pre-incubation technique, indicated that the three snow layers were more similar to each other (65 – 85% similarity) than to freshwater (60 – 65%). Within the snow, the top and middle layers were most related (75 – 95%) (Fig. 2A & B). Only one isolate belonging to the *Burkholderiales* was obtained from the top layer of the snow by direct plating. Hence, it was not possible to include this layer in the Bray-Curtis similarity measure, and the similarity analysis was consequently different from the analyses based on the pyrosequencing data and the cultivated bacteria isolated from the pre-incubated filters (Fig. 2C).

The most abundant phyla had many more unique OTU's than did the rare phyla in both snow and freshwater, i.e. abundant bacterial groups had higher within-group diversity than rare groups (Fig. 3). The correlation between log [relative abundance] and log [number of unique OTU's] was very high for all communities ($P < 0.0001$; $r = 0.969$, 0.944, 0.964 and 0.922 for top, middle and bottom snow and freshwater, respectively). In snow, however, the most abundant phyla had more OTU's than freshwater.

Phylogenetic composition of microbial communities

The phylogenetic composition of the microbial assemblages varied within the snow layers and between snow and freshwater (Fig. 2). In snow, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* dominated (frequencies of 30 - 39%, 10 - 13% and 10 -12%, respectively) although in the middle and top snow layers, *Cyanobacteria* also accounted for a substantial fraction (16 - 24%) of the communities (Fig. 2A). Another major difference between the bottom and middle/top snow layer was the presence of a large percentage of *Firmicutes* (22%) and

Fusobacteria (7%) in the bottom layer. Minor frequencies of unclassified bacteria (4 - 6%), *Acidobacteria* (2 - 3%) and *Verrucomicrobia* (1 - 2%) were observed in all 3 layers. Several other phyla were observed at frequencies lower than 1%, including the candidate division TM7 (≤ 0.5%), and the Archaean phyla *Euryarchaeota* (≤ 0.4%) and *Crenarchaeota* (≤ 0.1%). Contrary to snow, the freshwater community was characterized by a relatively large fraction of *Planctomycetes* (23%) and *Actinobacteria* (28%), several unclassified bacteria (15%) and a relatively infrequent population of *Proteobacteria* (8%) (Fig. 2A). The frequency of TM7 and *Archaea* was less than 0.05%.

Among the cultivable bacteria, the *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* phyla dominated (Fig. 2B & C). Depending on cultivation method (direct vs. pre-incubation), the *Proteobacteria* accounted for 48 – 100% of the snow communities while the *Actinobacteria* constituted 0 - 50% and *Bacteroidetes* 1 - 4%. Contrary to snow, the freshwater community contained relatively few *Actinobacteria* (0 – 9% depending on cultivation method) whereas the *Bacteroidetes* phylum was more abundant (9 - 13%). Also, *Firmicutes* was only seen in freshwater (0 - 1%) (Fig. 2B & C). When pre-cultivating the bacteria on polycarbonate membranes before plating on rich medium, the membranes were floated on sample water supplemented with Tryptic Soy Broth (TSB) at concentrations of 0, 0.01% or 0.1%. These amendments, however, had no effect on the composition of the isolated bacteria (data not shown).

At the genus level, *Sphingomonas* was the most frequent genus among the *Proteobacteria* in snow, accounting for 7 - 8% of all sequences in the top and middle snow layers and 3% in the bottom layer based on the pyrosequencing data (Table 3). GpI *Cyanobacteria* were also abundant in the top and middle snow layers constituting 13% and 15% of all sequences, respectively. In addition to GpI, GpXIII *Cyanobacteria* were present in the middle layer (4%). In the bottom layer, the frequency of GpI *Cyanobacteria* was only about 2%. *Actinobacteria*, which constituted 8 – 13% of the snow communities (Fig. 2A) were represented by several genera (Supplementary

material, Table S1) and, hence, only in top and middle snow was *Actinomycetales* among the 10 most abundant (≥ 97%) clusters (Table 3), accounting for less than 2% of all sequences at both depths. Similarly, *Fusobacteria*, which represented 7% of the community of the bottom snow layer (Fig. 2A), were comprised of several genera each accounting for less than 1% of the observed sequences. The by far most dominating genus in the bottom snow layer was *Streptococcus* as it constituted 14% of all the observed sequences in this layer. *Streptococcus* was also represented in the top snow, where 2% of the sequences belonged to this genus. In freshwater, the dominating genus was *Isophaera* (22% of all sequences) followed by the order *Actinobacteridae* (12%). Surprisingly, *Isophaera* constituted more than 96% of all the *Planctomycetes* found in freshwater (Table S1).

The dominance of individual genera amongst the cultivable bacteria differed from what was observed by pyrosequencing of extracted DNA as especially *gamma-Proteobacteria* were highly represented (Table 4). *gamma-Proteobacteria* were dominant in freshwater and in the bottom and middle snow layers accounting for up to 90% of the isolates of this phylum (Table 4). In top snow, on the other hand, *alpha* and *beta* classes each represented about 47 - 50% of the *Proteobacteria*. In freshwater and bottom snow, *Actinobacteria* were only observed by direct plating. *Micrococcaceae* and *Microbacteriaceae* dominated in freshwater, while *Arthrobacter* were dominant in bottom snow (Table 4). In the two other snow layers, *Salinibacter* and *Kineococcus* dominated in the middle snow layer, whereas *Rhodococcus* was only found in top snow. Among the *Bacteroidetes*, which primarily were observed in freshwater (Fig. 2A), *Flavobacteria* constituted more than 90% of the isolated bacteria within this phylum.

Discussion

The structure of High Arctic snow and freshwater bacterial communities was assessed by pyrosequencing of extracted DNA and by sequencing of DNA from bacteria isolated by two different cultivation approaches. The cultivated bacteria did not reflect the composition found by

pyrosequencing, illustrating the well-known discrepancy between molecular and cultivation based techniques. However, we decided to include cultivation in the study because culturable bacteria have been suggested to represent the active fraction of bacterial communities (Ellis et al., 2003); (Frette et al., 2004). If this is the case, then *Proteobacteria* (*Pseudomonas* and *Sphingomonas*), *Actinobacteria* (*Salinibacter*, *Kineococcus, Arthrobacter and Micrococcaceae*) and to lesser extent *Bacteroidetes* and *Firmicutes* were metabolic active during the sampling period.

The abundance of the bacterial phyla appeared to be related to their diversity as the abundant phyla had large numbers of unique OTU's rather than few highly abundant ones (Fig. 3). A similar observation has been made in the Arctic Ocean (Kirchman et al., 2010) and suggests that the ecological success of a bacterial lineage depends upon diversity rather than superior competiveness of a few phylotypes (Kirchman et al., 2010).

The most abundant phyla identified in snow varied with depth but included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Fusobacteria*. Freshwater was characterized by abundant *Planctomycetes*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia*, while *Proteobacteria* were less frequent relative to snow. Larose et al. (Larose et al., 2010) established clone libraries of DNA extracted from snow and a meltwater river and found *Proteobacteria* (*alpha*, *beta* and *gamma*), *Bacteroidetes* (*Sphingomonas* and *Flavobacteria*), *Cyanobacteria* and eukaryotic chloroplasts to be dominating. In Antarctic snow, clones belonging to *Deinococcus*, *beta-Proteobacteria* and *Bacteroidetes* have been identified(Carpenter et al., 2000). Amato et al. (Amato et al., 2007) cultivated bacteria directly from snow in Svalbard and isolates belonging to *alpha-*, *beta-* and *gamma-Proteobacteria*, *Actinobacteria* and *Firmicutes* were found. Thus, *Proteobacteria* (*alpha beta* and *gamma*), *Actinobacteria* and *Bacteriodetes* (especially *Flavobacteria* and *Sphingobacteria*) seem to be commonly found in polar snow.

Several of the most abundant genera that we observed have previously been associated with polar or cold environments, e.g. *Sphingomonas*, *Pseudomonas*, *Rhodobacter*, *Hymenobacter*, *Pelagibacter*, *Flavobacterium* and *Actinotobacter* (Brinkmeyer et al., 2003); (Pearce et al., 2003); (Brinkmeyer et al., 2004); (Malmstrom et al., 2007). Surprisingly, however, streptococci were highly frequent in the deepest snow layer (9% of all OUT's). *Streptococcus* is mainly recognized as a potential pathogen and most commonly associated with a host organism or environments influenced by fecal contamination (Lopez-Benavides et al., 2007). Another unexpected genus in the deepest snow layer was *Fusobacterium* which also is considered a human pathogen. However, a newly recognized genus within the family of *Fusobacteriaceae* is based on a *Fusobacterium*-like marine, psychrotrophic isolate *Psychrilyobacter atlanticus* from the Arctic ocean (Zhao et al., 2009). The presence of large numbers of genera normally associated with clinical microbiology is most likely due to wildlife droppings which may have persisted for a prolonged period of time as the sampled snow at Station Nord was 2-3 years old.

Previous investigations of microbial communities in Arctic and Antarctic freshwater lakes have revealed *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* as the major phyla(Pearce et al., 2003);(Crump et al., 2007). These phyla were also represented in our study, however, the most dominant genus in our freshwater samples was *Isophaera*, belonging to the *Planctomycetes*. *Planctomycetes* are commonly found in freshwater environments (Fuerst, 1995) but have only scarcely been detected in polar environments, e.g. in surface sediments of the Arctic ocean (Li et al., 2009). *Cyanobacteria* are often common in freshwater (Zwart et al., 2002) and have been identified in an Antarctic freshwater lake (Ellis-Evans, 1996). In the freshwater lake at Station Nord, *Cyanobacteria* were only detected at a very low frequency. However, since the lake had been ice covered for at least 22 months, the resulting low light intensity probably was not suitable for growth of these microorganisms.

A number of physico-chemical properties vary within snow and between snow and freshwater. In snow, for instance, the light intensity decreases with depth, and the temperature becomes less

variable and extreme. Also, as we sampled snow on sea-ice, the deeper layers may have been affected by seawater penetrating through cracks in the ice (in a few instances we observed water mixed with snow ('slush ice') up to about 50 cm from the snow surface). Contrary to the variable conditions in snow, the freshwater lake can be characterized by relative high and constant temperatures around 0°C, and most likely by limited light intensity due to the snow and ice coverage.

The structure of the bacterial communities in the different snow layers changed with depth. Since bacteria may be transported over long distances with dust particles (Kellogg and Griffin, 2006), and have been demonstrated to be metabolic active in icy super cooled cloud droplets (Sattler et al., 2001), the atmosphere may be significant as a source of bacteria to the top snow layer. As the top snow gets older and eventually becomes covered with fresh snow, the composition of the bacterial communities may gradually change. An intense (UV) light intensity undoubtly plays a role in this respect as we observed several pigmented isolates (yellow, orange, pink and red colony color) probably as a defense mechanism to high UV radiation. Interestingly, both *Cyanobacteria* and chloroplasts were almost exclusively found in the top and middle layers with the highest density in the middle layer. This shift in *Cyanobacteria* density with depth from high, to very high, to low, suggests that light intensity controlled the distribution of the *Cyanobacteria* and the algae in the snow pack. We primarily found GpI and GpXIII *Cyanobacteria* which are known to include species capable of fixing nitrogen. A likely scenario for explaining the heterotrophic community structure in the different snow layers is, therefore, that some of the nitrogen fixed by the *Cyanobacteria* stimulated the primary production of the algae, while the carbon and nitrogen produced by the autotrophs in turn were feeding the heterotrophic bacteria. Other sources of carbon to the bacterial communities, especially in the top layers, could be aerosols from the atmosphere (Bauer et al., 2002). The deepest snow layer did not seem heavily affected by the autrotrophs as their densities were relatively low. Also, the concentration of dissolved organic carbon was about three times lower in this layer (unpublished

data). However, as indicated above, it is likely that seawater microbes may have influenced the structure of the community. Typical Arctic seawater bacteria include *alpha-Proteobacteria* and especially the SAR11 family is abundant (Malmstrom et al., 2007); (Kirchman et al., 2010). Indeed, in the bottom snow layer more than 2% of all sequences were classified as *Pelagibacter* (Table 3), a genus within the SAR11 family. Sequences classified within SAR11 were only sporadically found in the upper snow layers.

The bacterial diversity in freshwater was low compared to snow. The source(s) of the bacteria to the lake may have been snow meltwater and soil. Since several phyla were identical for the snow and freshwater, this suggests that meltwater was a significant source. Contrary to the snow environment, the temperature regime in the lake was relatively stable around 0°C. Furthermore, since the lake was covered by ice and snow, the light input probably was lower than in snow. The conditions in the lake, therefore, can be considered more constant and less extreme than in snow and it is very likely that the relatively constant conditions affected the bacterial community structure. Furthermore, since numbers of *Cyanobacteria* were low and chloroplasts were virtually absent, the input of organic carbon and nitrogen to the heterotrophic bacteria must have been limited.

In conclusion, we carried out an in-depth analysis of the bacterial communities in High Arctic snow and freshwater by pyrosequencing of extracted DNA and by sequencing of DNA from bacteria isolated by two different cultivation approaches. Cultivation of snow and freshwater isolates showed only a scattered representation of the phyla and genera identified by the pyrosequencing and confirm that culture independent methods are important when describing microbial communities. The diversity was higher in snow as compared to freshwater, which may reflect that the environmental/climatic conditions of the freshwater ecosystem were less extreme. Regardless, a strong overlap between the genera in snow and freshwater indicates that snow meltwater may have been a significant source of microorganisms to the freshwater lake. The phylogenetic composition in the three snow layers was significantly different, yet the two upper

snow layers were more similar to each other than to the deepest snow layer. For instance, the two top layers were inhabited by large numbers of *Cyanobacteria* and chloroplasts probably feeding the heterotrophic members of the microbial communities. This suggests that snow, from a microbial perspective, is a heterogenous habitat and that the communities within the snow are metabolically active.

Experimental procedures

Study area and sampling

Snow and freshwater were collected in Spring 2007 at Station Nord in North-eastern Greenland. Snow was sampled in Dagmar Sund (81° 36.58'N; 16°42.83'W) between Station Nord and Prinsesse Dagmar Island, while the freshwater samples were taken from a small ice and snow covered lake about two km south of the station (81°34.48'N; 16°37.46'W).

To collect the snow samples, a vertical snow profile of 120 cm was made by digging with a sterile shovel. Immediately prior to sampling, the outermost one cm of snow was removed with a sterile knife. Sampling was done using a sterile Plexiglas corer (internal diameter: 14 cm, length: 25 cm) at 31-52 cm (top), 75-90 cm (middle) and 96-112 cm (bottom) depths. Sampling depths were determined on basis of observations of the snow hardness and texture to insure that different snow layers were sampled. Six to seven cores were taken at each depth and transferred to sterile plastic buckets covered with a lid. The snow was slowly melted at 5-7 °C for up to 48 h to avoid stressing of the bacteria during melting.

Freshwater samples were collected by pumping from approximately 70 cm depth below the ice. The hose from the pump was flushed with 3000 L of water before 2 L were collected in sterile glass bottles.

Isolation and cultivation of bacteria

Bacteria were isolated by two different procedures: i) direct plating and ii) pre-incubation under simulated natural conditions using polycarbonate membranes as a growth support before plating on rich medium (Rasmussen et al., 2008). Briefly, subsamples of melted snow or freshwater were plated onto 10% strength Tryptic Soy Agar (TSA) and incubated at 4-10°C until a constant number of CFU's was reached. The pre-incubation procedure involved filtration onto 0.2 µm pore-size polycarbonate membranes (25 mm diameter). The polycarbonate membranes, with the bacterial cells facing upward, were placed on the fixed 0.22-µm Anopore disc of 25-mm Nunc tissue culture inserts, and the tissue culture inserts placed in 6 wells plates containing one ml sample water supplemented with Tryptic Soy Broth (TSB) at concentrations of 0, 0.01% or 0.1%. The membranes were incubated at 4-10°C and the growth medium replaced with fresh medium every 7 days. Formation of micro-colonies was followed by microscopy. After 77 days of incubation, bacteria from the membranes were extracted in salt buffer (KH_2PO_4 (0.25 g L^{-1}), $MgSO_4 \cdot 7\ H_2O$ (0.125 g L^{-1}), NaCl (0.12 5 g L^{-1}), $(NH_4)_2SO_4$ (0.2 g g L^{-1})) by vigorous vortexing for one minute. Appropriate dilutions were plated on 10% TSA plates and incubated at 6°C until numbers of colonies were constant. Between 140 and 200 colonies from each location were randomly picked. All isolates were re-streaked on TSA at least two times to insure purity.

Partial sequencing of 16S rRNA genes of isolated bacteria

DNA was extracted from the isolated mercury resistant bacteria by boiling (Fricker et al., 2007). For some of the isolates extraction by boiling was not applicable. Instead the PowerMax DNA soil kit (MoBio Laboratories, Inc.) was used following the manufacturers instructions. All DNA preparations were stored at - 20°C.

The 16S rDNA of the bacteria was amplified by PCR with universal bacterial primers 27f (5'AGA GTT TGA TCM TGG CTC AG) and 519r (5'GWA TTA CCG CGG CKG CTG). The PCR mixture (25 µl) consisted of 2 µl DNA template, 1 U Taq DNA polymerase (Fermentas),

0.4 µM of each primer, 400 µM dNTP's and 2 mM $MgCl_2$ (0.5 mM $MgCl_2$ for 27f and 519r). PCR incubation conditions were 2 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C sec followed by final extension for 5 min at 72°C). The PCR products were analyzed on agarose gels stained with Ethidium Bromide. Purification and sequencing of the PCR products in one direction with primer 27f was performed by MACROGEN (South Korea).

Pyrosequencing

Meltwater or freshwater (200-500 ml) were filtered through 0.2 µm polycarbonate filters and filters stored in 1 ml RNAlater (Ambion) at -20°C until extraction. A total of 35 filters were collected (10 from freshwater, 10 from the top snow layer, 9 from the middle snow layer and 6 from the bottom snow layer). DNA was extracted from both the filters and from the pellets of centrifuged RNAlater (to recover cells detached from the filters during storage). Extractions were performed with the Genomic Mini Kit from A&A Biotechnologies (Poland) according to the manufacturers instructions with the following modifications: filters and pellets were combined in 1 ml extraction buffer (50 mM Tris-HCl, 5 mM EDTA. 3% SDS) and beadbeated for 30 sec on a mini beadbeater (Glen Mills Inc) and the supernatant added to 465 µl 5 M ammonium acetate. After centrifugation at 16000 × *g* for 10 min, two volumes of 7 M Guanidine-HCl were added to the supernatant and the mixture applied to the spin column provided in the kit and the DNA purified following the manufactures instructions.

The V3 and V4 regions of the 16S rRNA gene were amplified by PCR with primers 341F (5'CCTACGGGRBGCASCAG-3') and 806R (5'GGACTACNNGGGTATCTAAT-3'). The PCR amplification (25 µl) were done in 1 x Phusion HF buffer, 0.2 mM dNTP mixture, 0.5 U Phusion Hot Start DNA Polymerase (Finnzymes Oy, Espoo, Finland), 0.5 µM of each primer and one µl DNA. PCR conditions were 30 sec at 98°C followed by 30 cycles of 5 sec at 98°C, 20 sec at 56°C and 20 sec at 72°C sec followed by final extension for 5 min at 72°C. After the PCR

amplification, samples were held at 70°C for 3 minutes and then moved directly on ice to prevent hybridization between PCR products and short nonspecific amplicons. The PCR products were analyzed on 1% agarose gel and purified with QIAEX II Gel Extraction Kit (QIAGEN). The purified PCR products were tagged by another PCR (15 cycles) using primers 341F and 806R with adapters and tags (Supplementary material; Table S2). The tagged PCR amplicons were gel purified with Montage Gel Extraction Kit (Millipore) and the fragments quantified using a Qubit™ fluorometer (Invitrogen) and mixed in approximately equal amounts ($4x10^5$ copies per µl) to ensure equal representation of each sample. DNA samples were sequenced on one of two-regions of 70x75 GS PicoTiterPlate (PTP) by using a GS FLX pyrosequencing system (Roche) according to manufactures instructions.

Sequence analyses

Sequences of more than 150 bp derived from the pyrosequencing were sorted and trimmed by the Pipeline Initial Process at the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://rdp.cme.msu.edu) (Cole et al., 2009). The RDP Classifier of the RDP's Pyrosequencing Pipeline was used to assign 16S rRNA gene sequences to higher-order bacterial taxonomy with a confidence threshold of 50% as recommended for sequences shorter than 250 bp. Alignments and clustering (maximum distance of 3%) of the sequence libraries was done using the Aligner and Complete Linkage Clustering tool of the RDP's Pyrosequencing Pipeline. Diversity indexes were calculated using the Shannon & Chao1 index analysis tool in RDP. Bray Curtis similarity analysis between different samples at the phylum-level was done with PRIMER 5 for Windows version 5.2.9 (http://www.primer-e.com).

The quality of the partial 16S rDNA sequences of the isolates were manually checked and sorted. All analysis and diversity calculations were performed as for the pyrosequences.

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences and the sequences derived from pyrosequencing have been deposited in Genbank.

Acknowledgements

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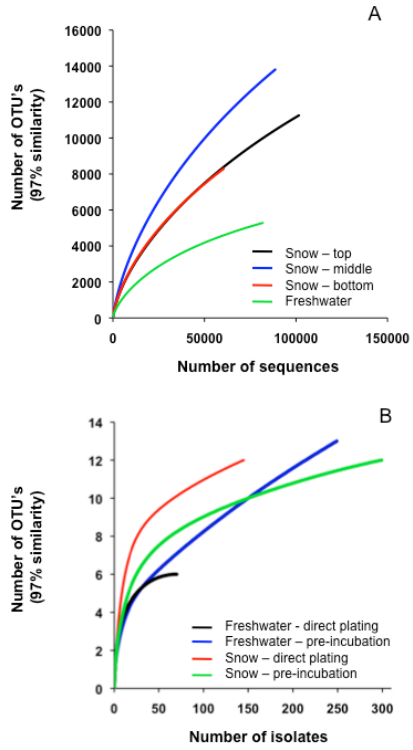

Fig. 1

Fig. 1. Rarefaction curves of OTU's (≥ 97% sequence similarity) of the 16S rDNA meta-genomic community libraries (A) and of the cultivated bacteria (B). Note that in (B), OTU's of the three snow depths have been combined.

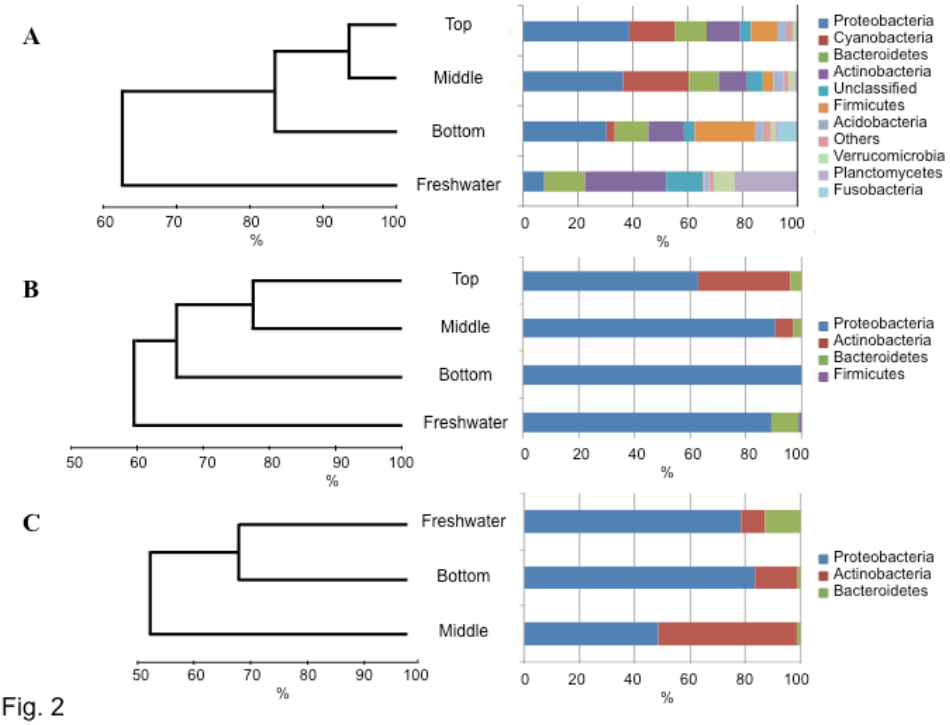

Fig. 2

Fig. 2. Dendrograms based on the Bray-Curtis similarity measure showing the relatedness of the microbial communities in snow and freshwater. Shown is also the distribution of the most frequent phylogenetic groups within each community. A – pyrosequencing; B – cultivation/pre-incubation on filters; C – cultivation/direct plating.

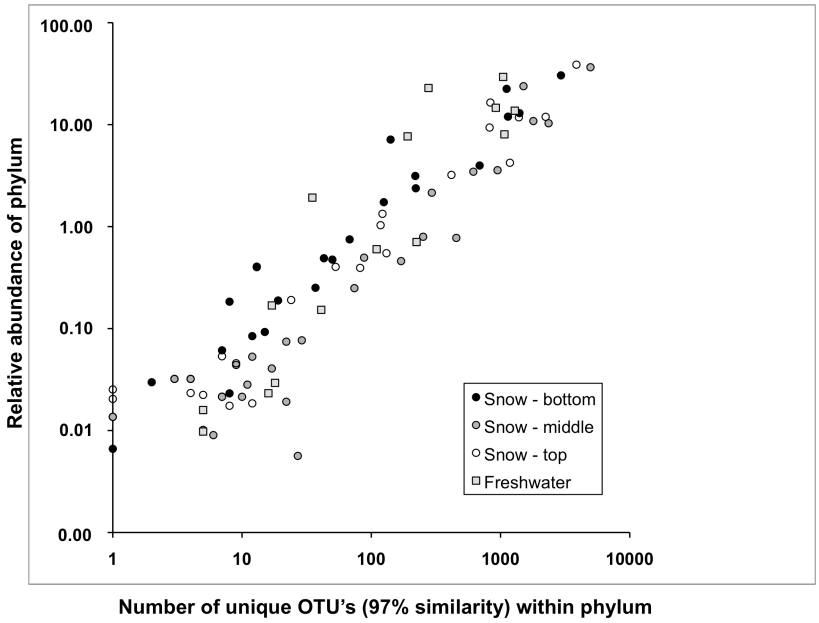

Fig. 3

Fig. 3. Relative abundance of phyla as function of number of unique OTU's.

Table 1. Summary of number of obtained 16S rRNA partial gene sequences and number of OTU's (≥ 97% sequences similarity) from cultivated bacterial isolates.

Sample	Pre-incubation on filters		Direct plating		Shannon-Weaver
	Sequences	OUT's	Sequences	OUT's	
Freshwater	249	13	70	6	1.34
Snow	299	12	145	12	1.39

Table 2. Summary of pyrosequence numbers (tags), numbers of OTU's (≥ 97% sequence similarity) and diversity estimates. To allow comparison of the Shannon-Weaver diversity index between samples, an equal number of sequences (60000) from each community were randomly selected and used for the analysis.

Sample	Number of sequences	OUT's	Chao1	Shannon-Weaver
Freshwater	81865	5283	8057	5.44
Snow - Bottom	60468	8295	15852	6.71
Snow - Middle	88712	13809	25655	7.46
Snow - Top	102824	11330	20863	6.85

Table 3. Phylogenetic classification of the 10 most abundant phylo-types within each microbial community as determined by pyrosequencing.

Assemblage	Phylum	Class	Subclass/Order/Genus	Frequency
Freshwater	*Planctomycetes*	*Plantomycetecia*	*Isophaera*	0.218
	Unclass. Bacteria			0.137
	Actinobacteria	*Actinobacteria*	Unclass. *Actinobacteridae*	0.124
	Actinobacteria	*Actinobacteria*	Unclass. *Actinomycetales*	0.072
	Actinobacteria	*Actinobacteria*	*Ilumatobacter*	0.068
	Bacteroidetes	*Flavobacteria*	*Flavobacterium*	0.068
	Verrucomicrobia	Unclass. *Verrucomicrobia*		0.051
	Acidobacteria	*Acidobacteria Gp3*	Gp3	0.016
	Actinobacteria	*Sphingobacteria*	Unclass. *Sphingobacteriaece*	0.016
	Bacteroidetes	*Actinobacteria*	Unclass. *Actinobacteria*	0.015
Snow - Top	*Cyanobacteria*	*Cyanobacteria*	GpI	0.127
	Proteobacteria	*Alpha*	*Sphingomonas*	0.068
	Proteobacteria	*Gamma*	*Acinetobacter*	0.042
	Unclass. Bacteria			0.042
	Bacteroidetes	*Flavobacteria*	*Cloacibacterium*	0.033
	Proteobacteria	*Gamma*	*Pseudomonas*	0.030
	Proteobacteria	*Alpha*	*Rhodobacter*	0.025
	Firmicutes	*Bacilli*	*Streptococcus*	0.021
	Actinobacteria	*Actinobacteria*	Unclass. *Actinomycetales*	0.018
	Cyanobacteria	*Cyanobacteria*	Unclass. *Cyanobacteria*	0.018
Snow - Middle	*Cyanobacteria*	*Cyanobacteria*	GpI	0.145
	Proteobacteria	*Alpha*	*Sphingomonas*	0.082
	Unclass. Bacteria			0.060
	Cyanobacteria	Unclass. *Cyanobacteria*		0.046
	Proteobacteria	*Alpha*	Unclass. *Acetobacteraceae*	0.038
	Cyanobacteria	*Cyanobacteria*	GpXIII	0.035
	Proteobacteria	*Alpha*	*Roseomonas*	0.030
	Bacteroidetes	*Sphingobacteria*	Unclass. *Cytophagaceae*	0.017
	Bacteroidetes	*Sphingobacteria*	*Hymenobacter*	0.017
	Actinobacteria	*Actinobacteria*	Unclass. *Actinomycetales*	0.015
Snow - Bottom	*Firmicutes*	*Bacilli*	*Streptococcus*	0.142
	Fusobacteria	*Fusobacteria*	*Fusobacterium*	0.069
	Unclass. Bacteria			0.040
	Proteobacteria	*Alpha*	*Sphingomonas*	0.031
	Bacteroidetes	*Bacteroidia*	*Prevotella*	0.024
	Cyanobacteria	*Cyanobacteria*	GpI	0.023
	Proteobacteria	*Alpha*	*Pelagibacter*	0.022
	Firmicutes	*Bacilli*	*Staphylococcus*	0.020
	Proteobacteria	Unclass. *Proteobacteria*		0.019
	Proteobacteria	*Gamma*	*Pseudomonas*	0.018

Table 4. Distribution of phylo-types of isolated bacteria. The first number in the columns represent data from pre-incubating the cells on filters, while the second number represents data from direct plating.

Phylum Class	Order/Family/Genus	% of phylum			
		Freshwater	Snow Bottom	Snow Middle	Snow Top
Proteobacteria					
Alpha	*Sphingomonas*	1.3 - 0	1.2 - 0	21.1 - 0	47.8 - nd
Alpha	Unclass. *Sphingomonadaceae*	0 - 0	0 - 0	0 - 3.3	0 - nd
Beta	*Herminiimonas*	3.5 - 0	0 - 0	0 - 0	0 - nd
Beta	*Janthinobacter*	4.4 - 56.4	1.2 - 18.3	0 - 3.3	0 - nd
Beta	*Variovorax*	1.3 - 0	0 - 0	0 - 0	0 - nd
Beta	Unclass. *Oxalobacteriaceae*	0 - 0	5.9 - 0	0.8 - 0	0 - nd
Beta	*Duganella*	0 - 0	1.2 - 0	0.8 - 0	10.9 - nd
Beta	*Unclass. Bradyrhizobiaceae*	0 - 0	0 - 0	6.3 - 0	34.8 - nd
Beta	*Massilia*	0 - 0	0 - 0	0.8 - 0	0 - nd
Beta	Unclass. *Burkholderiaceae*	0 - 0	0 - 0	0 - 0	2.2 - nd
Beta	Unclass. *Burkholderiales*	0 - 0	0 - 0	0 - 0	2.2 - nd
Gamma	*Pseudomonas*	88.9 - 40.0	90.6 - 66.2	69.5 - 6.7	0 - nd
Gamma	*Psychrobacterbacter*	0 - 3.6	0 - 12.7	0 - 3.3	2.2 - nd
Gamma	Unclass. *Pseudomonadaceae*	0.4 - 0	0 - 0	0 - 0	0 - nd
Actinobacteria					
Actino	*Salinibacter*	0 - 0	0 - 0	88.9 - 0	0 - nd
Actino	*Kineococcus*	0 - 0	0 - 0	11.1 - 71.0	0 - nd
Actino	*Rhodococcus*	0 - 0	0 - 0	0 - 0	100 - nd
Actino	*Arthrobacter*	0 - 0	0 - 100	0 - 29.0	0 - nd
Actino	Unclass. *Micrococcaceae*	0 - 66.7	0 - 0	0 - 0	0 - nd
Actino	Unclass. *Microbacteriaceae*	0 - 33.3	0 - 0	0 - 0	0 - nd
Bacteroidetes					
Flavo	*Flavobacterium*	91.7 - 0	0 - 0	0 - 0	0 - nd
Sphingo	*Dyadobacter*	4.2 - 0	0 - 100	0 - 0	0 - nd
Sphingo	*Mucilaginibacter*	0 - 0	0 - 0	100 - 100	100 - nd
Sphingo	*Pedobacter*	4.2 - 0	0 - 0	0 - 0	0 - nd
Firmicutes					
Bacilli	*Bacillus*	100 - 0	0 - 0	0 - 0	0 - nd

nd = no data available.

Table S1. Distribution of most abundant genera within selected phyla from snow and freshwater.

Phylum Class	Genus	% of phylum			
		Fresh-water	Snow Bottom	Snow Middle	Snow Top
Proteobacteria					
	Unclassified_"Proteobacteria"	8.6	1.4	2.8	1.2
Alpha	*Rhodobacter*	1.5	2.5	4.0	6.4
Alpha	*Roseomonas*	0.1	1.6	8.1	3.5
Alpha	*Sphingomonas*	0.1	10.1	22.3	17.7
Alpha	*Unclassified_Acetobacteraceae*	4.5	3.7	10.3	4.7
Alpha	*Unclassified_Alphaproteobacteria*	10.4	2.3	3.6	1.7
Alpha	*Unclassified_Sphingomonadaceae*	0.5	1.0	4.2	2.7
Beta	*Janthinobacterium*	8.5	0.9	0.9	0.3
Beta	*Rhodoferax*	7.5	0.1	0	0
Beta	*Unclassified_Comamonadaceae*	14.2	2.1	0.3	0.5
Gamma	*Acinetobacter*	0.2	0.8	0.4	10.9
Gamma	*Pelagibacter*	0	7.1	0.2	0.3
Gamma	*Pseudomonas*	5.6	6.1	2.9	7.8
Gamma	*Sulfitobacter*	0	5.2	0.7	3.4
Gamma	*Unclassified_Gammaproteobacteria*	1.3	6.3	0.5	0.6
Actinobacteria					
Actino	*Actinomyces*	0	9.0	0.1	0.5
Actino	*Ilumatobacter*	23.2	6.0	11.3	6.9
Actino	*Rothia*	0	12.6	0.1	1.0
Actino	*Rubrobacter*	0	0.2	2.5	7.2
Actino	*Unclassified_Actinomycetales*	24.4	9.8	15.0	13.9
Actino	*Unclassified_Actinobacteria*	5.2	6.6	7.9	8.5
Actino	*Unclassified_Actinobacteridae*	42.3	4.5	1.4	1.9
Actino	*Unclassified_Microbacteriaceae*	0.1	9.8	5.2	3.1
Actino	*Unclassified_Frankineae*	1.9	2.4	5.6	7.2
Bacteroidetes					
Bacteroidia	*Prevotella*	0	20.4	0.1	1.7
Flavobacteria	*Cloacibacterium*	0.1	9.2	10.2	28.3
Flavobacteria	*Flavobacterium*	46.4	8.5	5.6	5.1
Flavobacteria	*Ornithobacterium*	5.7	0.2	0	0
Flavobacteria	*Unclassified_Flavobacteriaceae*	6.2	5.0	7.3	9.9
Sphingobacteria	*Hymenobacter*	0	4.6	15.6	7.7
Sphingobacteria	*Pedobacter*	5.8	6.2	4.2	3.0
Sphingobacteria	*Unclassified_"Chitinophagaceae"*	3.7	3.1	6.1	5.4
Sphingobacteria	*Unclassified_Cytophagaceae*	3.2	4.3	16.0	7.7
Sphingobacteria	*Unclassified_Sphingobacteriaceae*	10.7	2.6	2.0	8.8
Cyanobacteria					
Cyanobacteria	*GpI*	100	73.9	60.9	57.4
Cyanobacteria	*GpIV*	0	1.4	2.5	1.2
Cyanobacteria	*GpVI*	0	3.4	1.5	0.5
Cyanobacteria	*GpXIII*	0	6.1	14.6	6.9
Cyanobacteria	*Unclassified_Cyanobacteria*	0	12.9	19.2	8.0
Firmicutes					
	Unclassified_"Firmicutes"	30.4	0.9	7.7	4.6
Bacilli	*Bacillus*	0	0.9	2.6	6.0
Bacilli	*Exiguobacterium*	0	0	0.8	11.4
Bacilli	*Gemella*	0	2.6	0.5	1.4
Bacilli	*Granulicatella*	0	2.0	0.9	0.1
Bacilli	*Staphylococcus*	1.6	8.9	6.9	7.8
Bacilli	*Streptococcus*	2.4	63.4	12.3	22.0
Bacilli	*Lactococcus*	0	0.4	5.6	7.6
Clostridia	*Geosporobacter*	1.6	0	0	0
Clostridia	*Unclassified_"Clostridia"*	15.2	0.4	2.9	0.7
Clostridia	*Unclassified_Clostridiales*	40.0	1.9	9.3	2.6
Clostridia	*Unclassified_"Lachnospiraceae"*	1.6	2.5	8.0	2.1
Clostridia	*Unclassified_"Ruminococcaceae"*	1.0	1.3	8.0	1.8
Planctomycetes					
Planctomycetacia	*Isophaera*	95.5	42.6	15.7	31.0
Planctomycetacia	*Planctomyces*	0	20.5	4.1	3.4
Planctomycetacia	*Singulisphaera*	0	12.4	39.7	42.4
Planctomycetacia	*Blastopirellula*	0	0.2	0.3	0.2
Planctomycetacia	*Unclassified_Planctomycetaceae*	4.5	14.6	24.4	15.4

Table S2. Primers with tags and adapters used in pyrosequencing.

Primer	Sequence
LinA_341F_1	GCCTCCCTCGCGCCATCAG-ACGAGTGCGT-CCTAYGGGRBGCASCAG
LinA_341F_2	GCCTCCCTCGCGCCATCAG-ACGCTCGACA-CCTAYGGGRBGCASCAG
LinA_341F_3	GCCTCCCTCGCGCCATCAG-AGACGCACTC-CCTAYGGGRBGCASCAG
LinA_341F_4	GCCTCCCTCGCGCCATCAG-AGCACTGTAG-CCTAYGGGRBGCASCAG
LinA_341F_5	GCCTCCCTCGCGCCATCAG-ATCAGACACG-CCTAYGGGRBGCASCAG
LinA_341F_6	GCCTCCCTCGCGCCATCAG-ATATCGCGAG-CCTAYGGGRBGCASCAG
LinA_341F_7	GCCTCCCTCGCGCCATCAG-CGTGTCTCTA-CCTAYGGGRBGCASCAG
LinA_341F_8	GCCTCCCTCGCGCCATCAG-CTCGCGTGTC-CCTAYGGGRBGCASCAG
LinA_341F_9	GCCTCCCTCGCGCCATCAG-TAGTATCAGC-CCTAYGGGRBGCASCAG
LinA_341F_10	GCCTCCCTCGCGCCATCAG-TCTCTATGCG-CCTAYGGGRBGCASCAG
LinA_341F_11	GCCTCCCTCGCGCCATCAG-TGATACGTCT-CCTAYGGGRBGCASCAG
LinA_341F_13	GCCTCCCTCGCGCCATCAG-CATAGTAGTG-CCTAYGGGRBGCASCAG
LinA_341F_14	GCCTCCCTCGCGCCATCAG-CGAGAGATAC-CCTAYGGGRBGCASCAG
LinA_341F_15	GCCTCCCTCGCGCCATCAG-ATACGACGTA-CCTAYGGGRBGCASCAG
LinA_341F_16	GCCTCCCTCGCGCCATCAG-TCACGTACTA-CCTAYGGGRBGCASCAG
LinA_341F_17	GCCTCCCTCGCGCCATCAG-CGTCTAGTAC-CCTAYGGGRBGCASCAG
LinA_341F_18	GCCTCCCTCGCGCCATCAG-TCTACGTAGC-CCTAYGGGRBGCASCAG
LinA_341F_19	GCCTCCCTCGCGCCATCAG-TGTACTACTC-CCTAYGGGRBGCASCAG
LinA_341F_20	GCCTCCCTCGCGCCATCAG-ACGACTACAG-CCTAYGGGRBGCASCAG
LinA_341F_21	GCCTCCCTCGCGCCATCAG-CGTAGACTAG-CCTAYGGGRBGCASCAG
LinA_341F_22	GCCTCCCTCGCGCCATCAG-TACGAGTATG-CCTAYGGGRBGCASCAG
LinA_341F_23	GCCTCCCTCGCGCCATCAG-TACTCTCGTG-CCTAYGGGRBGCASCAG
LinA_341F_24	GCCTCCCTCGCGCCATCAG-TAGAGACGAG-CCTAYGGGRBGCASCAG
LinA_341F_25	GCCTCCCTCGCGCCATCAG-TCGTCGCTCG-CCTAYGGGRBGCASCAG
LinA_341F_26	GCCTCCCTCGCGCCATCAG-ACATACGCGT-CCTAYGGGRBGCASCAG
LinA_341F_27	GCCTCCCTCGCGCCATCAG-ACGCGAGTAT-CCTAYGGGRBGCASCAG
LinA_341F_28	GCCTCCCTCGCGCCATCAG-ACTACTATGT-CCTAYGGGRBGCASCAG
LinA_341F_29	GCCTCCCTCGCGCCATCAG-ACTGTACAGT-CCTAYGGGRBGCASCAG
LinA_341F_30	GCCTCCCTCGCGCCATCAG-AGACTATACT-CCTAYGGGRBGCASCAG
LinA_341F_31	GCCTCCCTCGCGCCATCAG-AGCGTCGTCT-CCTAYGGGRBGCASCAG
LinA_341F_32	GCCTCCCTCGCGCCATCAG-AGTACGCTAT-CCTAYGGGRBGCASCAG
LinA_341F_33	GCCTCCCTCGCGCCATCAG-ATAGAGTACT-CCTAYGGGRBGCASCAG
LinA_341F_34	GCCTCCCTCGCGCCATCAG-CACGCTACGT-CCTAYGGGRBGCASCAG
LinA_341F_35	GCCTCCCTCGCGCCATCAG-CAGTAGACGT-CCTAYGGGRBGCASCAG
LinB_806R	GCCTTGCCAGCCCGCTCAG-GGACTACNNGGGTATCTAAT

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Diversity and characterization of mercury resistant bacteria in snow, freshwater and sea-ice brine from the High Arctic

Annette K. Møller[1,2], Tamar Barkay[1,3], Waleed Abu Al-Soud[2], Søren J. Sørensen[2], Henrik Skov[1] & Niels Kroer[1]*

[1] National Environmental Research Institute, Aarhus University, Frederiksborgvej 399, DK-4000 Roskilde, Denmark

[2] Department of Biology, University of Copenhagen, Sølvgade 83H, DK-1307K Copenhagen, Denmark

[3] Department of Biochemistry and Microbiology, Rutgers, The State University of New Jersey, Cook College, 76 Lipman Dr., New Brunswick, NJ 08901-8525, USA

* Corresponding author: nk@dmu.dk

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Abstract

It is well-established that atmospheric deposition transports mercury from lower latitudes to the polar regions. However, the role of bacteria in the dynamics of the deposited Hg is unknown. We characterized mercury resistant bacteria from High Arctic snow, freshwater and sea-ice brine. Mercury concentration in brine was ~80 ngL^{-1} and 69-76 ngL^{-1} in snow. The bacterial density was 9.4×10^{5} cells mL^{-1} in freshwater, 5×10^{5} cells mL^{-1} in brine and 0.9-3.1×10^{3} cells mL^{-1} in snow melt water. Highest cultivability was observed in snow (11.9%) followed by freshwater (0.3%) and brine (0.03%). In snow, the mercury resistant bacteria accounted for up to 31% of the culturable bacteria, but were below 2% in freshwater and brine. The resistant bacteria belonged to the α-, β- and γ-*Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteriodetes.* Resistance of most isolates was not temperature dependent between 4-20°C. Twenty-five percent of the resistant isolates reduced Hg(II) to Hg(0). No relation between resistance level, ability to reduce Hg(II), and taxonomic group was observed. An estimation of the potential bacterial reduction of Hg(II) in snow suggested that it may be important in the deeper snow layers. This highlights the importance of microbial mercury transformation in the biogeochemical mercury cycling in the High Arctic.

Introduction

A growing body of evidence implies atmospheric mercury to be the main source of mercury entering the arctic environment (Schroeder*, et al.*, 1998; Skov*, et al.*, 2004; Steffen*, et al.*, 2008). Since mercury can persist in the atmosphere as elemental mercury Hg(0) for a prolonged period of time, mercury emitted in other parts of the world find its way to the Arctic (Steffen*, et al.*, 2008). During atmospheric mercury depletion events (AMDEs), Hg(0) in the atmosphere is oxidized to Hg(II) through reaction with Br-atoms, released from refreezing leads, when exposed to solar radiation (Skov*, et al.*, 2004; Steffen*, et al.*, 2008). Hg(II) is then deposited onto the snow and sea ice thereby entering the ecosystem. Although atmospheric mercury is deposited year round in the Arctic, the deposition of mercury is doubled in the Spring due to AMDE (Skov*, et al.*, 2004).

Studies have shown that part of the deposited mercury is reduced within the snow and emitted back to the atmosphere as volatile mercury (St Louis*, et al.*, 2005), and that the half-life of Hg(II) in the snow is short (Dommergue*, et al.*, 2007). Evidence suggests that this reduction is light dependent (Dommergue*, et al.*, 2007; Poulain*, et al.*, 2004) but it is not known to which extent biological reduction by microorganisms play a role. In temperate environments, it is well established that microorganisms are important players in the biogeochemical cycling of mercury that impact the levels of methylmercury by both methylating and demethylating processes (Morel*, et al.*, 1998; Pak & Bartha, 1998). Also, in a more indirect manner, microorganisms may control the supply of substrate for the methylation, by reducing Hg(II) to Hg(0) (reviewed in Barkay*, et al.*, 2003). Whether this is the case in high arctic environments remains to be elucidated.

Mindlin *et al.* (2005) isolated mercury resistant bacteria from sediments in permafrost, and Poulain *et al.* (2007) showed that the *merA* gene, specifying the reduction of Hg(II) to Hg(0), was present and actively transcribed in coastal lagoons and sea-ice leads in the Canadian High

Arctic. Mercury resistant bacteria from snow and brine, however, have not been enumerated and characterized.

Here we investigated the potential for biological mercury reduction in High Arctic environments during atmospheric mercury depletion events (AMDE's). We isolated and identified mercury resistant microorganisms from three different habitats in the High Arctic: snow, freshwater and sea-ice brine water. The isolates were characterized by the level of resistance to mercury at different temperatures and by ability to reduce Hg(II) to Hg(0). In addition, we estimated potential microbial mercury reduction rates and found that microbiological reduction may potentially contribute up to 2% of the total reduction.

Materials and methods

Study area

Samples of snow, freshwater, and sea-ice brine water were collected in Spring 2007 at Station Nord in Northeastern Greenland. Snow and brine water were collected at two sites in Dagmar Sund (Site 1: 81° 36.58'N; 16°42.83'W; Site 2: 81°35.46'N; 16°45.91'W) between Station Nord and Prinsesse Dagmar Island, while the freshwater samples were taken from a small ice covered lake 2 km south of Station Nord (81°34.48'N; 16°37.46'W).

Measurement of atmospheric ozone

Ozone was measured with an UV absorption monitor (Teledyne Technologies Inc., CA, USA) with a detection limit of 1 part per billion volume (ppbv) and an uncertainty of 3% for concentrations above 10 ppbv and 6% for concentrations below 10 ppbv (all uncertainties are at 95% confidence interval) (Skov*, et al.*, 2004).

Sampling of snow, brine and freshwater

Sampling of snow and brine at Site 1 was done on May 12 and at Site 2 on May 25. Total mercury concentrations were measured at both sites, while microbiology sampling was only done at Site 1.

A vertical snow profile was created by digging with a sterile shovel. The snow depth from the surface to the sea ice was 120 cm (Site 1) and 105 cm (Site 2). Sampling of the snow layers was based on observations of the snow texture to insure that different snow layers were sampled. Immediately prior to sampling, the outermost one cm snow of the profile was removed with a sterile knife. Snow for determination of dissolved organic carbon (DOC), bacterial numbers and enumeration and isolation of culturable bacteria was collected by use of a sterile Plexiglas corer (internal diameter: 14 cm, length: 25 cm). Six to seven cores were taken at each depth (31-52 cm, 75-90 cm, 96-112 cm) and transferred to sterile plastic buckets covered with a lid. The snow was melted slowly at 5-7 °C (up to 48 h) to avoid stressing of the bacteria during melting. Depending on the snow texture, one core of snow resulted in 850-1250 ml melt water (average snow density of 0.28 g ml^{-1} which is comparable to previous observations at Station Nord (Ferrari*, et al.*, 2004). Sampling for determination of the total mercury concentration was done using a glass tube (length: 11.5 cm, internal diameter: 2.1 cm). Samples were transferred to triplicate 100 ml glass bottles, containing 200 µl conc. HNO_3 (final pH = 2), and sealed with a lid with a Teflon insert. All glassware was pre-rinsed with 4 M H_2SO_4. Samples were stored a 4-5°C until analysis.

Brine water was collected by removing the snow cover and drilling 3 replicate holes (40 cm deep and 20 cm wide) with an ethanol sterilized ice drill. Slush ice in the bore holes was removed with a sterile spoon and brine water seeping into the holes collected using a sterile pipette and pooled. The salinity of the brine was measured with a refractometer (Atago). Subsamples (50 ml) for total mercury determination were treated as described for the snow samples.

Freshwater samples were collected on June 13 by pumping from approximately 70 cm depth below the ice. The hose from the pump was flushed with 3000 liters of lake water before 2 L were collected in a sterile glass bottle. Subsamples (50 mL) for total mercury determination were treated as described for the snow samples.

Total mercury concentration

Samples (50 mL) were treated for 24 hours with 500 µL of 2 mM $BrCl_2$ to convert all mercury species to Hg(II). Excess $BrCl_2$ was neutralized with addition of 500 µl of 30% NH_2OH. The volumes were adjusted to a total of 100 mL and poured into 1 L Teflon flasks containing 1 mL of 20% $SnCl_2$. The flasks were immediately capped, shaken vigorously for 30 seconds, and mercury in the head space measured on a Tekran Mercury Vapor Analyzer 2537A. Three independent samples from each site were analyzed and the concentration of Hg(II) determined against a 6-point standard curve (0-100 ng L^{-1} $HgCl_2$). Measurements that were not significantly different from the blanks were not included in the study (students t-test, $p<0.05$). All glassware used for the mercury measurements was rinsed three times in 4 M H_2SO_4 and rinsed several times in MilliQ water before use.

Dissolved organic carbon

Triplicate samples of 10-15 mL melt water and brine water were filtered through 0,2 µm Sartorius Minisart syringe filters into acid rinsed (10% HCl) glass scintillations vials and stored frozen at – 15-18 °C. The syringe filters were rinsed in 5 mL sample water before collecting the sample. The content of organic carbon was measured on a Shimadzu TOC5000 analyzer (Kroer, 1993). Prior to analysis, samples were acidified and purged with O_2 for 5 min to remove inorganic carbon.

Total bacterial abundance

Total bacterial abundance was determined by direct counts with an Olympus BH2 microscope. Bacteria were collected on 0.1 µm (brine samples) or 0.2 µm pore-size black polycarbonate membranes (Osmonics/Nucleopore) and stained with a 1:1000 × dilution of SYBR Gold (Invitrogen). Samples were analyzed immediately after sampling or as soon as snow samples were melted.

Enumeration and isolation of culturable bacteria

Bacteria were isolated by two different procedures: i) direct plating and ii) pre-incubation under simulated natural conditions using polycarbonate membranes as a growth support before plating on standard medium (Rasmussen*, et al.*, 2008).

i) Direct plating. Five × 100 µL of melted snow, brine and freshwater were plated onto 10% strength of Tryptic Soy Agar (TSA) (Tryptic Soy Broth, Sharlauf Microbiology, Denmark and Noble agar, Difco, Denmark) plates. The medium was prepared using autoclaved water from the appropriate sampling location. The plates were incubated at 4-10°C and the colony forming units counted at successive intervals until a constant count was obtained. All colonies appearing on the plates were re-streaked for purity on 10% TSA plates prepared with distilled water (for isolates originating from snow and freshwater) or 10% TSA supplemented with sea salts (SIGMA-Aldrich) adjusted to the same salinity as the sea-ice brine (4.5%).

The isolates were streaked on 10% TSA plates supplemented with 10 µM $HgCl_2$ to test for mercury resistance. Mercury resistance was scored as positive if single colonies grew on the plates. Isolates growing on the mercury plates were re-streaked on fresh plates of appropriate mercury containing medium at least three times to confirm purity and mercury resistance.

ii) Pre-incubation on polycarbonate membranes. Five ml brine water, 50 mL melted snow and 5 mL freshwater were filtered through 0.1 µm (brine) or 0.2 µm pore-size polycarbonate membranes (25 mm diameter, Nucleopore). The polycarbonate membranes were placed on fixed 0.22 µm Anopore discs of 25-mm Nunc tissue culture inserts (Nunc A/S Roskilde), Denmark) with the bacterial cells facing upward. Each of the assembled membrane-tissue culture inserts were placed in a separate well of 6-well Costar plates, each well containing 1 mL of sample water supplemented with Tryptic Soy Broth (TSB) at concentrations of 0, 0.1% or 0.01%. The membranes were incubated at 4-10°C. The growth medium was replaced with fresh medium every 7 days and the formation of micro-colonies followed by microscopy. After 77 days of incubation, microcolonies on three replicate filter

membranes from each location were dislodged by placing them in an Eppendorf tube with 1 ml salt buffer ([KH_2PO_4 (0.25 g L^{-1}), $MgSO_4 \cdot 7\ H_2O$ (0.125 g L^{-1}), NaCl (0.12 5 g L^{-1}), $(NH_4)_2SO_4$ (0.2 g g L^{-1})]) and vortexed vigorously for one minute. Appropriate dilutions were prepared in salt buffer and 100 µL plated on 10% TSA plates (prepared as described above). Plates were incubated at 6°C. Between 140 and 200 colonies from each location were randomly picked and the colonies re-streaked for purity on 10% TSA plates prepared with distilled water (for snow melt water and freshwater samples) or 10% TSA supplemented with sea salts (SIGMA-Aldrich) adjusted to the same salinity as the brine water (4.5%). Mercury resistant isolates were identified as described above.

After 90 days, cells from one filter from each sampling location were extracted and plated onto 10% TSA plates supplemented with 10 µM $HgCl_2$. All isolates growing on mercury plates were re-streaked on fresh mercury plates at least three times to confirm their purity and mercury resistance.

Identification of isolated mercury resistant bacteria. DNA was extracted from the isolated mercury resistant bacteria by boiling (Fricker*, et al.*, 2007). For some of the isolates (8D5s, 8D7, 8D12, 8D12b, SOK1b, SOK 15, SOK17a, SOK17b, SOK19, SOK19y, SOK27, SOK32, SOK33, SOK35, SOK38, SOK43, SOK48, SOK52, SOK57) extraction by boiling was not applicable. Instead the PowerMax DNA soil kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) was used following the manufacturers instructions.

The 16S rDNA of the bacteria was amplified by PCR with universal bacterial primer sets GM3F (AGA GTT TGA TCM TGG) and GM4R (TAC CTT GTT ACG ACT T) (27) (5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 46°C and 3 min at 72°C, the last cycle was followed by 10 min at 72°C), and 27f (AGA GTT TGA TCM TGG CTC AG) and 519r (GWA TTA CCG CGG CKG CTG) (19) (2 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C sec followed by final extension for 5 min at 72°C).

The PCR mixture (25 µl) consisted of 2 µL DNA template, 1 U taq polymerase, 0.4 µM of each primer, 400 µM dNTP's and 2 mM $MgCl_2$ (0.5 mM $MgCl_2$ for 27f and 519r).

No PCR products were obtained from three of the isolates using the bacterial primers. Therefore, a new PCR (2 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C sec followed by final extension for 5 min at 72°C) was set up in which the internal transcribed spacer region ITS1 - 5.8S rDNA - ITS2 between the fungal 18S and 28S rDNA genes was amplified using primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (48). The PCR mixture consisted of 2 µL DNA template, 1 U taq polymerase, 0.4 µM of each primer, 400 µM dNTP's and 0.5 mM $MgCl_2$.

PCR products were gel purified using Qiagen Gel Extraction Kit (Qiagen, Denmark). Sequencing of the purified PCR products in one direction with primer GM3F (or ITF1 or 27f) was performed by GENEWIZ (South Plainfields, NJ, USA). The quality of the sequences was manually checked and the closest know relative was determined by using BLAST 2.2.1 (http>//www.ncbi.nlm.hih.gov/BLAST/). The closest related sequences in GenBank together with one representative sequence from each subgroup (99% sequence similarity) were aligned and a phylogenetic tree constructed using the *neighbor-joining method* using MEGA4 (Tamura*, et al.*, 2007).

Determination of the minimal inhibitory mercury concentration (MIC). MIC was defined for at least one representative isolate from each of the 16S rDNA groups. Cell material of freshly grown isolates on 10% TSA plates supplemented with 5 µM $HgCl_2$ was resuspended in 2 mL TBS and the optical density (A_{600nm}) adjusted to 0.100. From appropriate dilutions, 100 µL was plated on 10% TSA supplemented with 0, 10, 25, 50, 100 and 200 µM $HgCl_2$. Plates were incubated at 4°C, 12°C and 20°C, or at 4°C and 15°C, and numbers of CFU's counted. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of $HgCl_2$ that resulted in a more than 80% reduction in numbers of CFU's relative to control plates with no added $HgCl_2$.

Mercury volatilization assay. The ability of the isolates to volatilize Hg(II) to Hg(0) was determined using the assay of Nakamura & Nakahara (Nakamura & Nakahara, 1988). The assay is based on the darkening of an x-ray film when Hg(0) is produced. Isolates were grown on 10% TSA supplemented with 5 μM $HgCl_2$ and resuspended into assay buffer (0.07 M phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 0.2 mM magnesium acetate and 5 mM sodium thioglycolate). $HgCl_2$ was added to the cell suspensions to a final concentration of 250 μM. Microtiter plates containing the cell suspensions were covered with x-ray film (KODAK Scientific Imaging, Ready Pack, Rochester, NY) and incubated at room temperature for two hours in the dark. Alternatively, for cells producing a negative result, cells were resuspended in 10% TSB supplemented with 5 μM $HgCl_2$ to insure induction of the mercuric reductase. After 20 min incubation at room temperature, the cells were washed and resuspended in assay buffer and set up in microtiter plates with 250 μM $HgCl_2$. Isolate SOK32 showed very strong mercury reduction and was routinely used as positive control. Assay buffer with no cells served as negative control.

Accession numbers. The 71 bacterial 16S rRNA sequences and the 3 fungal internal transcribed spacers regions have been submitted to Genbank and given the accession numbers GU932907-GU932980.

Results

Physical/chemical characterization of the sampling site

The atmospheric temperature during the sampling period ranged between -22.5ºC and -2.9ºC in May and between -12.8ºC and 7.8ºC in June. The temperature range on the two sampling days was -15.9 to -6.2 (May 12) and -10.7 to -6.3 (May 25), respectively. Continuous ozone measurements showed that atmospheric ozone was highly variable with periods close to zero (Fig. 1A). Fluctuating ozone concentrations are usually observed during AMDE's (Fig. 1B

and (21, 42)) and, therefore, AMDE's were likely to have occurred during our sampling period.

Measured mercury concentrations in brine and snow were in the range of 70 – 80 ng L^{-1} (Table 1). Several of the samples had mercury concentrations that were not significantly different from the blanks (not included).

The DOC concentration in brine was almost 40 mg L^{-1}. In snow, the concentration was in the range of 1 - 4 mg L^{-1} (Table 1).

Bacterial abundance

The bacterial abundance in snow ranged from 8×10^2 to 3×10^3 cells mL^{-1} (Table 2). In fresh water and brine, densities were up to two orders of magnitude higher. The cultivability of the bacteria was highly variable ranging from less than 0.1% in brine to 12% in snow (Table 2). The highest cultivability was observed in the two deepest snow layers. It should be noted that only one colony was isolated from the uppermost snow layer making the estimate of the cultivability somewhat uncertain. Among the culturable bacteria, the highest percentage of mercury resistance (31.2%) was observed for the deepest snow layer, where the fraction of mercury resistant bacteria was more than 15 times higher than at the other snow depths (0 - 1.7%) (Table 2).

Diversity of isolated mercury resistant isolates

A total of 1100 bacteria were isolated and among these, 71 were mercury resistant as indicated by growth on 10% TSA plates amended with 10 μM $HgCl_2$. To ensure true mercury resistance, isolates were re-streaked on TSA plates supplemented with 10 μM $HgCl_2$ at least three times. Isolates were classified as mercury resistant only if they showed good growth, and were growing on the plates as single colonies. Most of the mercury resistant isolates originated from freshwater (47 isolates) and the deepest snow layer (24 isolates) while only one isolate originated from brine and one from each of the upper snow layers.

Among the mercury resistant bacterial isolates, 17 different partial 16S rDNA sequences were identified. These were distributed within seven different phyla: *α-, β-, γ-Proteobacteria, Actinobacteria, Sphingobacteria, Flavobacteria* and *Firmicutes*. Within each phylum/class, several sub-groups were observed with identical (99% sequence similarity) partial 16S rDNA sequences. A phylogenetic tree with representatives from each of these sub-groups and their closest relatives is shown in Fig. 2. It should be noted that the closest relative for 10 of the isolates is either a psychrophile or has been isolated from a cold environment.

In addition to bacterial isolates, 150 mercury resistant fungal colonies were obtained from snow (depth 75-90 cm) after pre-incubation on polycarbonate membranes. The colony color (pink) and morphology of all these isolates were identical. Sequencing of the ITS1 - 5.8S rDNA - ITS2 region of two representative isolates showed that they were identical and that the closest relative was an Antarctic yeast belonging to the order *Leucosporidiales*. Another isolate with white colony color from snow (96-112 cm) was also sequenced with fungal specific primers. It was most closely related to a psychrophilic species belonging to the genus *Geomyces*.

The diversity of the isolated mercury resistant bacteria from freshwater and snow was almost similar (Fig. 3). Both habitats were dominated by *γ-Proteobacteria* which constituted 56% and 42% of the isolates in freshwater and snow, respectively. There were, however, differences between the two habitats as *Flavobacteria* and *Firmicutes* were only found in freshwater while *Actinobacteria* were only found in snow (31%). In brine, only one mercury resistant bacterial isolate, belonging to the *Firmicutes*, was identified.

All 71 mercury resistant isolates were psychrotrophs as they grew at temperatures ranging from 4°C to 20-25°C (room temperature). The maximal temperature for growth of the three isolates representing *Flavobacterium* group II was 20°C. Hence, they could be considered true psychrophiles, defined as bacteria with maximum temperature for growth below 20°C ((Gounot, 1986)).

Mercury resistance of the bacterial isolates

Minimal inhibitory concentrations (MIC) generally ranged from 5 to 50 μM (Table 3). One isolate belonging to *γ-Proteobacteria* sub-group I, however, showed a MIC value of 100 μM. Although some isolates belonging to the *β*-and *γ-Proteobacteria*, and *Flavobacteria* showed higher MIC's at higher temperatures, the resistance of most isolates did not show a temperature dependency (Table 3). Isolates SOK79 and SOK70s, belonging to *Flavobacteria* and *Sphingobacteria*, showed highest MIC values at middle temperatures (Table 3 & Fig. 4). This could be explained by impaired growth at 20°C, which would also affect mercury resistance.

MIC was determined for several isolates of *γ-Proteobacteria* sub-group I and II and *Flavobacteria* sub-group II. Interestingly, the values varied greatly (from 5-100 μM, 5-50 μM and 12-50 μM, respectively) even though these isolates were 99% identical with respect to their 16S rDNA sequence.

Volatilization of mercury

All the *Flavobacteria*, several of the *γ-Proteobacteria*, and single isolates of the *α*- and *β*-*Proteobacteria* reduced Hg(II) to Hg(0) (Table 3 and Fig. 5). Within the individual sub-groups there were discrepancies as not all isolates within the groups were positive with the exception of *Flavobacteria* (Table 3).

Discussion

We isolated and identified 74 mercury resistant microorganisms representing seven bacterial and two fungal phyla/classes from the High Arctic during atmospheric mercury depletion events. The isolates were highly resistant to mercury and some were demonstrated to be able to reduce Hg(II) to Hg(0).

Microorganisms were isolated by two different methods, direct plating and pre-incubation on filters prior to plating. Direct plating often selects for only a very small fraction of the

community, while pre-incubation under conditions that simulate the environment has been shown to increase the cultivability of soil bacteria up to 2800 times (Rasmussen*, et al.*, 2008). The majority of the mercury resistant isolates from snow originated from direct plating (24 isolates) while only one isolate was obtained using the pre-incubation approach. However, 44 out of 45 of our isolates from freshwater were obtained by the pre-incubation method. Thus, pre-incubation on filters prior to plating enabled us to isolate more resistant bacteria than would otherwise have been possible by direct plating on rich medium.

The bacterial density in snow was on the average 1.8×10^3 cells mL^{-1} melt water. This is comparable to concentrations in snow covers of glaciers in the Tibetan plateau (Liu*, et al.*, 2009) but about one order of magnitude lower than concentrations found in snow cover at Spitsbergen in Svalbard (Amato*, et al.*, 2007) and at alpine sites (Bauer*, et al.*, 2002; Sattler*, et al.*, 2001). The observed bacterial density in brine (5×10^5 cell mL^{-1}) was comparable to densities in sea-ice in the Arctic and Antarctic (Brinkmeyer*, et al.*, 2003).

The mercury resistant isolates belonged to phyla and classes commonly found in cold environments. α-, and *γ-Proteobacteria, Actinobacteria* and isolates belonging to *Bacteriodetes* are common in sea ice both in the Arctic and Antarctica (Brinkmeyer*, et al.*, 2003), and *β-proteobacteria* have been shown to be present in snow covers of an alpine lake (Alfreider*, et al.*, 1996). Amato *et al.* (2007) isolated α-, β- and *γ-Proteobacteria*, *Firmicutes* and *Actinobacteria* in snow covers at Spitzbergen, and Larose *et al.* (Larose*, et al.*, 2010) identified DNA belonging to α- and *β-Proteobacteria*, *Sphingobacteria*, *Flavobacteria* and *Acidobacteria* from the same area. We did not, however, identify any mercury resistant isolates belonging to the *Acidobacteria*.

Mercury resistance is well characterized in β- and *γ-Proteobacteria*, *Firmicutes* and *Actinobacteria*, while resistance in *Bacteroidetes* has only been sparsely reported (Oregaard & Sørensen, 2007; Rasmussen*, et al.*, 2008). We isolated mercury resistant *Sphingobacteria* and *Flavobacteria* both belonging to the *Bacteroidetes*. Since *Flavobacteria* are well represented

in polar communities, the isolation of mercury resistant flavobacteria could indicate a potentially important role of these bacteria in the cycling of mercury in the Arctic.

Minimal inhibitory concentrations of Hg ranged from 5 μM to 100 μM and were comparable to values of mercury resistant isolates of temperate soils (de Lipthay*, et al.*, 2008). MIC values varied within some of the taxonomic groups, suggesting that the level of mercury resistance was isolate-specific. The best described mechanism of mercury resistance in bacteria is the reduction of Hg(II) to Hg(0) by the mercuric reductase, encoded by the *merA* gene (reviewed in Barkay*, et al.*, 2003). This system is commonly found in wide range of bacteria often encoded by plasmids or associated with transposable elements (Barkay*, et al.*, 2003; Olson*, et al.*, 1979). It is therefore likely that the isolate-specific mercury resistance patterns of our Arctic isolates may be the result of horizontal transfer of a mercury resistance gene. This is further supported by the fact that no correlation between the ability to reduce Hg(II) to Hg(0) and the taxonomic groups was observed. It should be noted, however, that the X-ray film darkening method is a relative rough method for detecting mercury reduction. Negative results should, consequently, be interpreted with caution.

Total mercury concentrations were measured at different snow depths, in brine and in freshwater. Concentrations were within previously reported values (60 to 600 ng L^{-1}) during AMDE (Lindberg*, et al.*, 2002; Lu*, et al.*, 2001; Steffen*, et al.*, 2008). To investigate if the concentrations in snow and brine were high enough to maintain a selective pressure for mercury resistance, we compared our data with those in other studies reporting both mercury concentrations and percent mercury resistant bacteria (Ball*, et al.*, 2007; Barkay, 1987; de Lipthay*, et al.*, 2008; De Souza*, et al.*, 2006; Mindlin*, et al.*, 2005; Ranjard*, et al.*, 2000; Rasmussen & Sørensen, 2001; Rasmussen*, et al.*, 2008; Timoney*, et al.*, 1978). A correlation between total or bioavailable mercury with percent mercury resistance was not apparent for the compiled data set (not shown). For example, in a coastal marine sample, the total mercury concentration was 1.7×10^{-4} μg mL^{-1} and 23% of the cultured bacteria were mercury resistant

(de Lipthay*, et al.*, 2008), whereas only 2.9% of the bacterial isolates were resistant in a soil with 7.6 μg Hg g^{-1} or 8×10^{-4} μg bioavailable Hg g^{-1} (Barkay, 1987). Thus, the mercury concentration does not seem to be a sensitive predictor of the population of resistant bacteria. The levels of total mercury in our study were relatively low (70 – 80 ng L^{-1}) when compared to other environments, but nevertheless up to 31% of our isolates were mercury resistant. Others have reported mercury resistant bacteria from cold environments. For instance, Petrova *et al.* (Petrova*, et al.*, 2002) found up to 2.9% mercury resistant bacteria in permafrost sediments, and in Antarctic seawater frequencies of 1.5 - 4.7% (Miller*, et al.*, 2009) and 68% (De Souza*, et al.*, 2006) were observed. Although mercury concentrations in polar environments are relatively low, it is likely to be highly bioavailable, thus selecting and maintaining mercury resistance in the microbial communities.

Hg(0) measured in snow fluctuates during AMDE (Dommergue*, et al.*, 2003; Kirk*, et al.*, 2006) largely due to volatilization when Hg(II) is reduced Hg(0). Emissions from snow take place simultaneously with increasing solar radiation and reduction rates decline drastically with snow depth and when snow samples are placed in the dark. Thus, the emission of Hg(0) from light exposed snow is considered to be largely driven by photoreduction (Dommergue*, et al.*, 2003; Kirk*, et al.*, 2006; Lalonde*, et al.*, 2002).

Biological mercury reduction *in situ* is difficult to measure as Hg(II) may be reduced by chemical redox processes under dark conditions (Ferrari*, et al.*, 2004). Hence, measurements of formation of Hg(0) in the dark would not be indicative of biological reduction. To assess the potential for *merA*-mediated reduction in snow we, therefore, made an estimate based on our own data and on literature values. The average total mercury concentration in snow was 3.6×10^{-4} μM, and assuming that 15% was bioavailable (Lindberg*, et al.*, 2002), the available mercury concentration can be estimated to have been 5.4×10^{-5} μM. MerA mediated reduction is known to follow Michaelis-Menten kinetics (Philippidis*, et al.*, 1991), i.e. the reduction rate can be calculated as $V = (V_{max} \times [Hg])/([Hg] \times K_m)$. Philippidis *et al.* (1991)

determined V_{max} and K_m of the mercuric reductase to be 8.2 nmol min^{-1} mg $protein^{-1}$ and 3.8 μM, respectively. Thus, assuming a per-cell protein content of 2.4×10^{-11} mg $cell^{-1}$ (Zubkov*, et al.*, 1999), the per-cell reduction rate can be calculated (1.7×10^{-13} nmol Hg h^{-1} $cell^{-1}$). Numbers of resistant culturable bacteria in snow varied between 1.7 and 31.2 % depending on depth (Table 2). If the culturable bacteria were representative for the total bacterial communities, numbers of resistant bacteria can be calculated to have been 6.4×10^6 cells m^{-3} (75 – 90 cm) and 1.2×10^8 cell m^{-3} (96 – 112 cm) using a snow-to-melt water correction factor of 0.28 (see above). Based on these numbers and the per-cell reduction rate, bacterial reduction rates per m^3 snow were calculated (Table 4).

Estimated bacterial reduction rates increased with snow depth (Table 4). In the uppermost layer no resistant cells were observed and, hence, no reduction could be estimated. However, when going from ~ 83 cm to ~ 105 cm depth, reduction appeared to increase by a factor of almost 20. Very little information is available in the literature on reduction rates of Hg(II) in snow. Dommergue *et al.* (2003) measured total mercury reduction in interstitial air at different depths in the snow pack in Kuujjuarapik, Quebec, Canada. They found that day-time release rates of Hg(0) decreased with increasing depth and suggested photoreduction to be the primary mechanism for the mercury reduction. A comparison with our data indicates that an average of up to 2% of the total reduction may potentially be bacterial and that bacterial volatilization of mercury becomes relatively more important in the deeper snow layers (Table 4). This agrees with a study of coastal waters in the Canadian High Arctic (Poulain*, et al.*, 2007), in which the relative contribution of bacterial reduction appeared to increase with depth, accounting for up to 94% of the total production of Hg(0) at the greater depths. Ferrari *et al.* (2004) estimated the night-time production of elemental gaseous Hg in snow at Station Nord prior to the onset of AMDEs and found fluxes that were 6 – 25 times lower than those reported by Dommergue *et al.* (2003). A comparison with our estimated potential bacterial

reduction rates implies that most of the night-time production of Hg(0) may be attributed to bacterial activity.

In summary, we isolated a diverse group of mercury resistant bacteria from snow, freshwater and sea-ice brine from the high Arctic during AMDE. The isolates showed a high level of resistance to mercury and some were shown to be able to reduce Hg(II) to Hg(0). Although photoreduction probably is the major mechanism of mercury re-emission to the atmosphere, mercury resistant bacteria have the potential to contribute to the reduction of mercury in snow, especially in deeper snow layers where light attenuation is limiting photoreduction. Thus, by reducing Hg(II) to Hg(0), the mercury resistant bacteria may limit the supply of substrate for the methylation processes.

Acknowledgements

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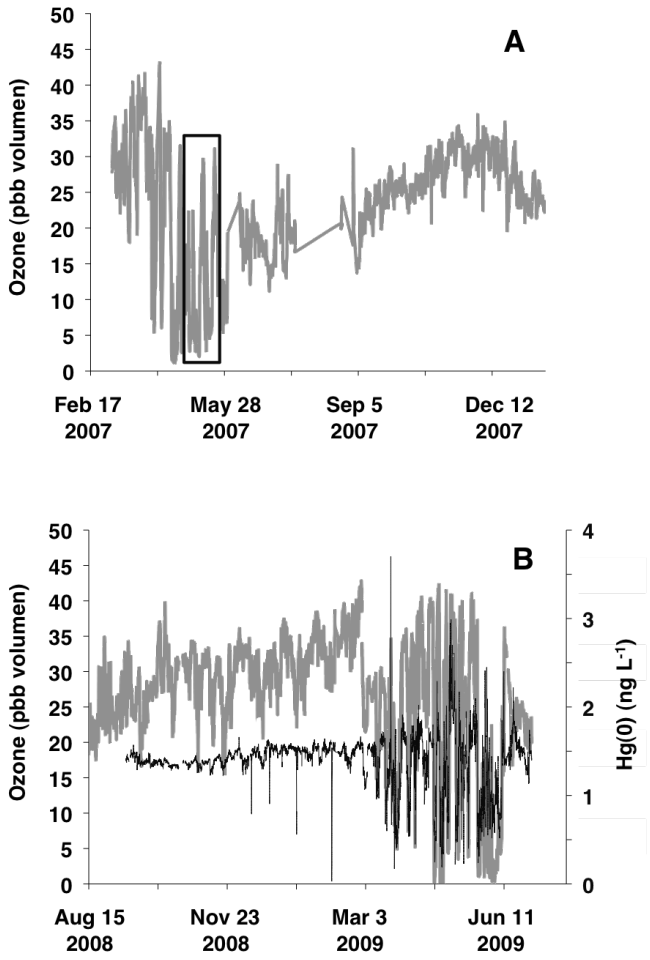

Figure 1

Atmospheric ozone concentrations (grey line) and atmospheric mercury concentrations (black line). Panel A shows the ozone concentration between March and December 2007. The sampling period is indicated by the rectangle. No mercury measurements were available in 2007. Panel B shows ozone and mercury concentrations in 2008 - 2009. As seen on the graphs, low ozone concentrations were indicative of low mercury concentrations, i.e. of atmospheric mercury depletion events.

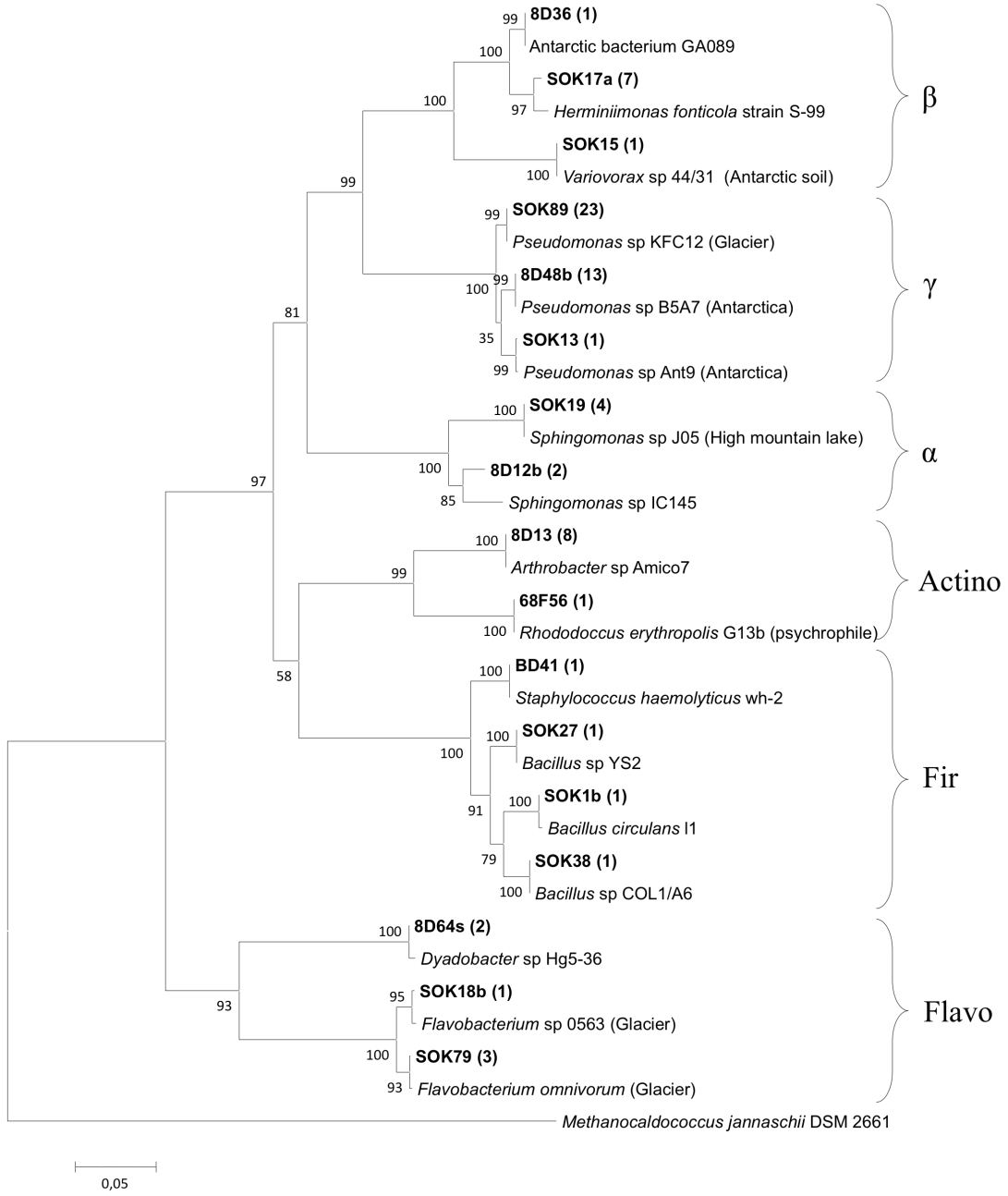

Figure 2

Neighbor-joining tree showing the phylogeny of representative 16S rRNA gene sequences of the mercury resistant isolates. The tree has arbitrarily been rooted in the archaeon *Methanocaldococcus jannashii*. Values to the left of the nodes reflect the bootstrap values (percent out of 1000 replicates). Numbers in parentheses indicate the number of isolates within each sub-group having 99% sequence similarity. The tree is drawn to scale.

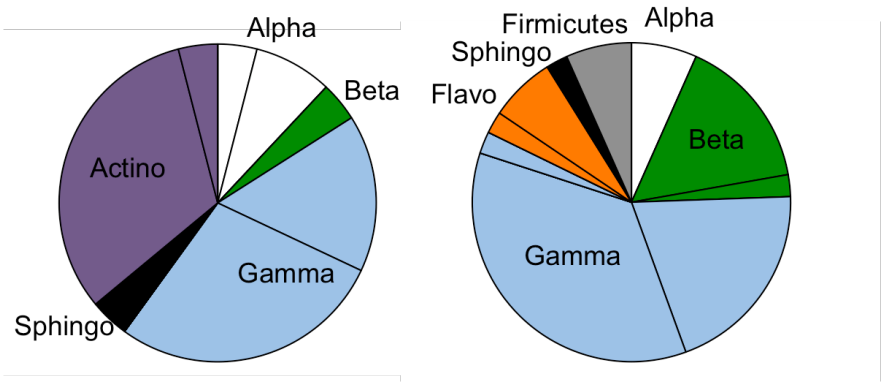

Figure 3

Distribution of the phylogenetic groups in snow (A) and freshwater (B). The colors indicate the different phyla/classes. Twenty-six isolates are represented in (A) and 46 isolates represented in (B).

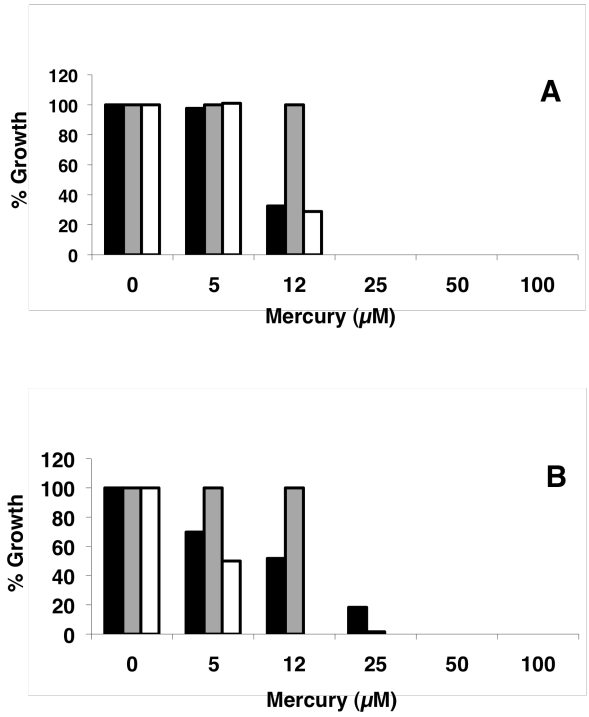

Figure 4

Effect of temperature on MIC of *Sphingomonas* (A) and *Flavobacterium* I (B). Black bars indicate cells grown at 20°C, dark grey bars cells grown at 12°C, and light grey bars cells grown at 4°C. Percent growth is relative to growth on plates without added Hg.

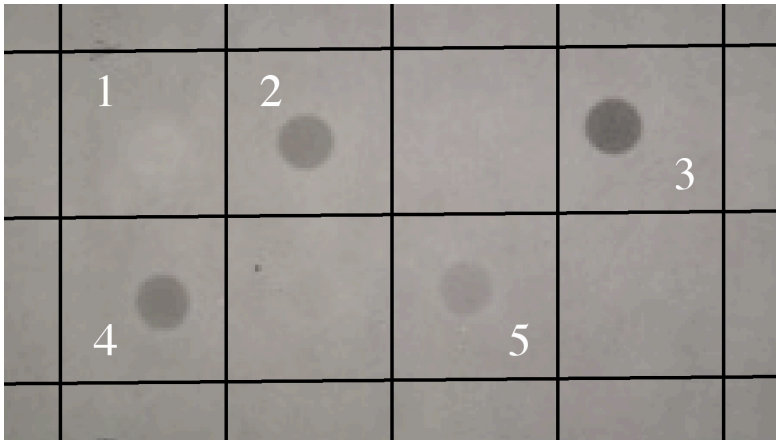

Figure 5

Mercury volatilization. Darkening of the x-ray film indicates formation of Hg(0). 1: negative control (mercury sensitive bacterium). 2: positive control (SOK32; γ-*Proteobacteria* group I). 3: SOK33 (freshwater isolate, γ *Proteobacteria* group I). 4:SOK79 (freshwater isolate, *Flavobacteria* group II). 5: SOK5 (freshwater isolate, α-*Proteobacteria* group I).

Table 1. Dissolved organic carbon (DOC) and total mercury concentration.

Sample	DOC (mg C L^{-1})	Total Hg (ng L^{-1})
Site 1		
Brine	37.5 ± 11.1	NS
Snow (31-52 cm)	4.0 ± 0.7	NS
Snow (75-90 cm)	3.8 ± 0.8	72.2 ± 4.2
Snow (96-112 cm)	1.3 ± 0.02	NS
Site 2		
Brine	ND	79.6 ± 26.8
Snow (7-24 cm)	ND	76.5 ± 6.3
Snow (42-58 cm)	ND	69.2 ± 13.1
Snow (81-95 cm)	ND	NS
Freshwater	ND	NS

Standard errors (n=3).
NS – not significantly different from blanks (Students t-test, $p<0.05$).
ND – Not determined.

Table 2. Bacterial densities, cultivability and proportion of mercury resistant isolates.

Sample	Total counts[a] (cells ml^{-1})	Direct plating[a] (cfu ml^{-1})	Cultivability (%)	Hg resistance[b] (%)
Site 1				
Brine	$5.0\times10^5 \pm 8.3\times10^4$	$1.3\times10^2 \pm 4.0\times10^1$	0.03	1.7
Snow (31-52 cm)	$3.1\times10^3 \pm 1.5\times10^3$	$2.0\times10^0 \pm 2.0\times10^0$	0.07	0
Snow (75-90 cm)	$1.4\times10^3 \pm 1.1\times10^3$	$1.7\times10^2 \pm 1.2\times10^2$	11.9	1.7
Snow (96-112 cm)	$8.5\times10^2 \pm 9.0\times10^1$	$6.8\times10^1 \pm 3.0\times10^1$	8.00	31.2
Freshwater	$9.4\times10^5 \pm 3.8\times10^4$	$2.5\times10^3 \pm 7.1\times10^1$	0.3	1.6

[a]Standard errors (n = 3 - 5).
[b]Percentage Hg resistance calculated on basis of numbers of CFU's from the direct plating

Table 3: Hg inhibition (MIC) of bacterial isolates and their Hg-volatilizing ability.

Bacterial group	Isolate	MIC (µM)				Hg volatilization[a]
		3°C	12°C	15°C	20°C	
Alpha I	SOK5	50	-	50	-	-
	SOK19, SOK19y	-	-	-	-	-
	8D1	-	-	-	-	+
Alpha II	30D12	5	5	5	5	-
	8D12b	-	-	-	-	-
Beta I	SOK35	50	50	-	50	-
	SOK17a,SOK17b, SOK18t, SOK48, SOK51, SOK57	-	-	-	-	-
Beta II	SOK15	12	12	-	25	-
Beta III	8D36	12	12	-	25	+
Gamma I	SOK32	50	-	50	50	+
	SOK33, SOK43, SOK52	-	-	-	-	+
	SOK61, SOK67	5	-	25	-	+
	SOK71	12	-	25	-	+
	SOK70b	100	-	100	-	-
	SOK80	50	50	-	50	-
	8D32, 8D45	5	-	5	-	-
	8D48b, 8D48s	-	-	-	-	-
Gamma II	SOK19w	-	-	-	-	+
	SOK75	50	50		50	-
	SOK41, SOK44, SOK50, SOK54, SOK59, SOK65, SOK68, SOK73, SOK84, SOK85, SOK89	-	-	-	-	-
	SOK84s, 8D26	-	-	-	-	-
	SOK90	25	25	-	25	-
	SOD12	5	-	12	-	+
	8D21, 8D41	-	-	-	-	-
	8D55s, 8D55t	5	-	5	-	+
	8D56	12	12	-	12	+
	8D64b	12	12	-	12	+
Gamma III	SOK13	50	50	-	50	-
Flavo I	SOK18b	5	12	-	12	+
Flavo II	SOK79	12	25	-	12	+
	SOK62	50	-	50	-	+
	SOK29	-	-	-	-	+
Sphingo	SOK70s	5	25	-	12	-
	8D64s	-	-	-	-	-
Actino I	8D5s	25	25	-	25	-
	8D5b, 8D7, 8D8, 8D10, 8D11, 8D12, 8D13	-	-	-	-	-
Actino II	68F56	5	5	-	5	-
Firmicutes	SOK1b, BD41	50	50	-	50	-
	SOK27	5	5	-	5	-
	SOK38	-	-	-	-	-

[a] Volatilization assay: + positive; – negative

Nomenclature of the isolates indicate their origin: SOK – freshwater; 8D – snow depth 96-112 cm; 30D – snow depth 75-90 cm; 68F – snow depth 31-52 cm; BD – brine.

Table 4. Estimation of bacterial mercury reduction rates and their contribution to observed total reduction rates in snow.

Station Nord			**Kuujjuarapik, Canada**[1]		**Bacterial/Total**
Snow depth (cm)	Bacterial reduction rate (nmol h^{-1} m^{-3})		Snow depth (cm)	Total reduction rate (nmol h^{-1} m^{-3})	(%)
	100% of cells active	15% of cells active[2]			
31-52	0	0	54	1.0×10^{-3}	0
75-90	1.1×10^{-6}	1.6×10^{-7}	80	2.6×10^{-4}	0.1 – 0.4
96-112	2.0×10^{-5}	3.0×10^{-6}	102	1.7×10^{-4}	1.8 – 11.8
Average	1.1×10^{-5}	1.6×10^{-6}	Average	4.9×10^{-4}	0.3 – 2.2

[1] Data from Dommergue *et al.* (2003)

[2] Average % active cells in melted sea-ice (Junge*, et al.*, 2004) and a high mountain lake during winter (Posch*, et al.*, 1997).

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Novel and Conserved Bacterial Mercuric Reductase Genes (*merA*) in the High Arctic

Annette K. Møller[1,2], Tamar Barkay[1,3], Martin A. Hansen[2], Anders Norman[2], Lars H. Hansen[2], Søren J. Sørensen[2] and Niels Kroer[1]*

[1]National Environmental Research Institute, University of Aarhus, Frederiksborgvej 399, DK-4000 Roskilde, Denmark

[2]Department of Biology, University of Copenhagen, Sølvgade 83H, DK-1307K Copenhagen, Denmark

[3]Department of Biochemistry and Microbiology, Rutgers, The State University of New Jersey, Cook College, 76 Lipman Drive, New Brunswick, NJ 08901-8525, USA

*Corresponding author: nk@dmu.dk

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Key words: Mercury Reductase (merA) – arctic bacteria — whole genome sequencing – plasmids

Abstract

Bacterial mercury (Hg) reduction mediated by the enzyme mercury reductase (MerA) plays a significant role in the bio-geochemical Hg cycle in temperate environments but is largely unexplored in polar regions. Here we present the identification of MerA loci from high Arctic mercury resistant isolates belonging to 6 taxonomic classes (*α*-, *β*- and *γ*-*Proteobacteria*, *Actinobacteria*, *Flavobacteria* and *Bacilli*) by PCR amplification with degenerate *merA* primers and by whole genome sequencing. We identified 10 different *merA* sequences; 7 of which (from *α*-, *β*- and *γ*-*Proteobacteria*) showed high similarity (99-100%) to proteins in the Genbank database while the 3 others were less similar (82-92%) to any protein sequences. Of the 71 mercury resistant isolates, only 26 carried a detectable *merA* and, thus, several other *merA* sequences or Hg reduction mechanisms may be found in the Arctic. Thus, our study illustrates the limitations in the use of degenerate primers in PCR based detection of specific genes and an application of next generation sequencing technology as a tool for identification of target genes. Of the Hg resistant isolates, 24% carried plasmids and 2 out of the 5 sequenced plasmids contained a *mer*-operon. The presence of plasmids carrying *mer*-operons, and an uneven distribution of *merA* within the different taxonomic groups indicate lateral transfer of the *merA* genes. Whole genome sequencing revealed a simple *mer*-operon in a *Flavobacterium* isolate. Unique for this putative *mer*-operon was the regulatory element; instead of the common *merR* this operon was initiated by *arsR*, which is common for *mer*-operons in Archaeal species. Clustering of the putative MerA sequence along with other putative MerA sequences from other *Bacteroidetes* showed closer phylogenetic distance to Archaeal MerA sequences than to bacterial MerA sequences. Our results suggest that a diverse and undiscovered pool of *merA* exist in arctic bacterial assemblages, and that the bacterial communities may play an important role in the Hg transformation in arctic environments.

Introduction

Mercury (Hg) is a toxic heavy metal that occurs naturally as elemental (Hg^0), oxidized (Hg^{II}) and organic Hg (typically methyl-Hg). Both Hg^{II} and Methyl-Hg have strong affinities for sulphur atoms in cysteine residues and, thus, interfere with protein structure and function (Carty and Malone 1979, Philbert et al, 2000).

Bacterial reduction of Hg^{II} plays a significant role in the global biogeochemical cycle of Hg (Barkay and Wagner-Dobler, 2005). Bacteria may respond to Hg^{II} by using different strategies (Pan-Hou et al. 1981a, Pan-Hou et al. 1981b), however, the enzymatic reduction of Hg^{II} to Hg^0 is the most widespread mechanism of mercury resistance (reviewed in Barkay et al. 2004 and Osborn et al. 1997). The reduction is catalyzed by products of the Hg resistance (*mer*) operon, which encodes a group of proteins involved in the detection, scavenging, transport and reduction of Hg^{II} (Barkay et al. 2003).

Hg pollution of Arctic food webs is increasing (Muir et al. 1999, Braune et al. 2005) and so is the exposure of indigenous human populations (Van Oostdam 2005). The sources of the Hg entering the Arctic are emissions from industrialized locations at lower latitudes. The emitted Hg is transported globally and is deposited from the atmosphere in polar regions. Modelling studies have estimated that more than 300 tons of Hg are deposited annually in the Arctic (Ariya et al. 2004). Much of this deposition occurs during the polar sunrise due to the oxidation of Hg^0 in the atmosphere by reactive halogen radicals from sea salt aerosols. (Skov et al. 2004, Steffen et al. 2007) How the deposited Hg^{II} enters the Arctic food chain, and the role of microbial transformations in the Hg bio-geochemistry, are at present unknown. Within this context, mercury resistant microbes may limit the amount of Hg^{II} that is available for methylation (Poulain et al. 2007).

Contrary to temperate environments very little is known about the presence, diversity and distribution of *merA* genes (encoding the mercuric reductase, MerA) amongst arctic bacterial assemblages. Also, the knowledge on the potential for bacterial reduction of Hg^{II} is limited. We

have previously isolated and characterized Hg resistant bacteria from snow, freshwater and sea-ice brine in the High Arctic (Møller et al. submitted). We showed that 0-31% of the culturable bacteria were Hg resistant and estimated that up to 6% of the Hg^{II} reduction in snow was bacterial.

Here we report the identification of *merA* sequences in Hg resistant bacteria isolated from the High Arctic. In addition to already known *merA* sequences identified in *Proteobacteria*, we also report the identification of novel *merA* sequences from isolates belonging to the *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. The *merA* genes were identified both by PCR, using primers specific of *merA*, as well as by whole genome sequencing. Furthermore, we isolated several plasmids some of which carried the *mer*-operon.

Material and Methods

Isolation, identification and cultivation of mercury resistant bacteria

Bacteria in snow, sea-ice brine and freshwater from North-eastern Greenland (81°36.58'N; 16°42.83'W) were isolated as described in Møller et al. (Møller et al. 2010 submitted). Briefly, cells were plated directly on 10% strength Tryptic Soy Agar (TSA) (Difco) or pre-incubated under simulated natural conditions, using polycarbonate membranes as a growth support, before plating on 10% TSA plates. Hg resistance was tested by streaking on 10% TSA plates supplemented with 10 µM $HgCl_2$. Resistant isolates were identified by partial 16S rDNA sequencing according to Møller et al. (Møller et al. 2010 submitted).

Subsequent cultivation of the isolates was done at room temperature in 10% Tryptic Soy Broth (TSB), or in the case of three *Bacteroidetes* isolates (SOK29, SOK62 and SOK79) at 15 °C in PYG medium (5 g polypeptone, 5 g tryptone, 10 g yeast extract, 10 g glucose and 40 ml salt solution in 1 L. The salt solution, pH 7.2, contained per L: 0.2 g $CaCl_2$, 0.4 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 10.0 $NaHCO_3$ and 2.0 NaCl). Both media were supplemented with 10 µM $HgCl_2$.

merA sequencing, analysis and phylogeny

DNA was extracted from the isolated mercury resistant bacteria by boiling. For some of the isolates (8D5s, 8D7, 8D12, 8D12b, SOK1b, SOK 15, SOK17a, SOK17b, SOK19, SOK19y, SOK27, SOK32, SOK33, SOK35, SOK38, SOK43, SOK48, SOK52, SOK57) extraction by boiling was not applicable. Instead the PowerMax DNA soil kit (MoBio Laboratories, Inc.) was used following the manufacturers instructions. All DNA preparations were stored at - 20°C.

Detection of *merA* sequences was carried out by PCR using 10 phylum-specific *merA* primer sets. The primers, primer targets, annealing temperatures, PCR extension times and product sizes are listed in Table S1 (online supplementary material). The following PCR conditions were used: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 50 – 64°C (see Table S1) for 30 sec, 72°C for 1-3 min (see Table S1) and one cycle of final extension at 72°C for 10 minutes. The PCR mixtures contained 0.4 µM of each primer, 400 µM dNTP, 2.5 mM MgCl2 and 2 U Taq DNA polymerase (Fermentas, Germany). PCR products could not be obtained from all isolates, hence, for one of these isolates (SOK62), the *merA* sequence was retrieved by whole genome sequencing (see below). The PCR products were gel purified using Qiagen Gel Extraction Kit. Sequencing of the purified PCR products in both directions with the same primers as used in the PCR was performed by GENEWIZ, South Plainsfield, NJ, USA, or by Eurofins MWG Operon, Germany.

Sequences were assembled in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and manually trimmed for primer sequences. The sequences were blasted in NCBI genbank using BlastN and BlastX (DNA sequences were translated with the translate tool at Swiss Institute of BioInformatics: http://www.expasy.org/tools/dna.html (Gasteiger E. et al, 2003)). The sequences were also aligned in BioEdit and 99% identical sequences were grouped. One representative from each group, the most closely related published sequence, and selected MerA protein sequences were aligned in ClustalX (Thompson et al 1997) and a phylogenetic tree constructed using the

neighbor joining method. FigTree (http://tree.bio.ed.ac.uk/software/figtree/) was used to illustrate the tree.

Plasmid DNA preparation, restriction analysis and sequencing

Plasmid DNA was isolated from cell material from freshly grown colonies using QIAprep Spin Miniprep Kit (QIAGEN). Cells of isolates belonging to the *Firmicutes*, *Actinobacteria* and *Bacteriodetes* were treated with lysozyme for 1 h at room temperature prior to the plasmid DNA isolation. Plasmids were gel electrophoresed (0.8% agarose) and grouped according to their migration patterns.

Representative plasmids from the five different groups were sequenced. Plasmid DNA was prepared from exponentially growing cells of isolates SOK1b, SOK15, SOK19, SOK65 and SOK71 using QIAGEN Midi Prep Kit. SOK1b was treated with lysozyme for one h prior to DNA isolation. Plasmid DNA was treated with DNAase (Plasmid Safe, Epicentre Biotechnologies) overnight at 37°C to digest any contaminating chromosomal DNA and the DNA was subsequently ethanol precipitated. Between two and five µg of plasmid DNA was fragmented by nebulization and libraries were build according to the standard protocol except for preparations of SOK15. Here an additional DNA amplification was done using REPLI-g UltraFast Mini Kit (Qiagen) to increase the input DNA. Each library was tagged with a standard MID tag (1-5) to allow mixing of the different libraries in one region of the plate. Following library build, the DNA was amplified by PCR by using FLX fw: GCCTCCCTCGCGCCATCAG and FLXrev: GCCTTGCCAGCCCGCTCAG. Amplified DNA fragments were then separated by gel-electrophoresis and bands between 350 and 650 bp were cut from the gel and purified using QIAEX II kit (Qiagen, Hilden, Germany). DNA was then quantified using a Qubit™ fluorometer (Invitrogen) and pooled in equimolar concentrations. Sequencing of the pooled libraries was done on a Genome Sequencer FLX instrument using a emPCR kit II and a standard FLX sequencing Kit.

The depth values from the 454 Alignmentinfo.tsv file, generated by the Newbler assembler software of the Genome Sequences FLX instrument, were used to calculate the average sequencing coverage (i.e., the average number of times a given DNA nucleotide was represented in sequence reads) of each contig. Based on a frequency distribution of the depth values, a cut-off value was set to distinguish between plasmid and chromosomal contigs in each assembly. Thus, contigs with an average coverage above the cut-off value were considered to be part of the plasmid(s), while contigs with an average coverage less than the cut-off value were considered chromosomal and excluded from the assembly. Contigs less than 500 bases in size were also excluded from the assembly.

Open reading frames of contigs were determined with the Prodigal gene finding program (Hyatt et al. 2010) and loaded into the Biopieces bioinformatics tool (www.biopieces.org) for further analysis. Here the contigs were blasted against the *merA* sequences we identified in this study as well as with all known bacterial MerA proteins sequences. MerA and MerP both contain a conserved heavy-metal-associated domain, thus, *merP* was also identified in the MerA blast. In addition, the contigs were blasted against several genes specific for plasmid replication or transfer to identify the Inc group of the plasmids. The fragments blasted were the following genes/sites: IncN: *rep*, *kikA*, *oriT*. IncP: *oriT*, *trfA1*, *trfA2*, *korA*, *traG*. IncQ: *repB*, *oriV*, *oriT*. IncW: *oriV*, *oriT*, *trwAB* (Gotz et al. 1996).

Whole genome sequencing of isolate SOK62

DNA from isolate SOK62 for whole genome sequencing was prepared from exponentially growing cells with DNeasy Blood and Tissue Kit (QIAGEN). A paired end library with an insert size of 500 bp was prepared from 5 µg of high molecular weight DNA according to the Illumina GA II paired-end library preparation protocol. Sequencing on the lllumina GA II instrument for 2 x 32 cycles using the paired-end settings resulted in 17.3M paired-end reads. Assembly was performed using Velvet version 0.7.59 (Zerbino and Birney 2008) with parameters obtained

using VelvetOptimizer.pl (Zerbino et al, 2008) and were as follows: velveth: 23 -fastq -shortPaired. velvetg: -ins_length 500 -exp_cov auto -min_contig_lgth 500 -cov_cutoff 35.9458254230799. The assembly yielded a total of 48 contigs (longest contig 505,321 nucleotides, n50 = 193,975) indicating a chromosome size of approximately 3.9 Mbp with an average GC content of 34%.

Automatic annotation was performed using the RAST annotation system (Aziz et al. 2008), which identified 237 subsystems (network of metabolites and enzymes that comprise e.g. a metabolism or signal transduction pathway), 3,502 coding sequences and 46 RNAs. 933 features (coding sequences) were located in subsystems (26 hypotheticals) while 2,569 features were not in subsystems (1,476 hypotheticals).

MerA *sequence accession numbers*

The *merA* sequences, the fragments of the *mer*-operon obtained from the isolated plasmids and the whole genome of isolate SOK62 have been submitted to Genbank.

Results

Diversity of mercury resistant bacteria

Among a total of 1,100 bacterial isolates, 71 were Hg resistant. The resistant isolates belonged to the *α-Proteobacteria* (6 isolates), *β-Proteobacteria* (9 isolates), *γ-Proteobacteria* (37 isolates), *Flavobacteria* (4 isolates), *Sphingobacteria* (2 isolates), *Actinobacteria* (9 isolates) and *Firmicutes* (4 isolates). Within each phylum/class, several sub-groups were observed with identical (97% sequence similarity) partial 16S rDNA sequences (Table 1).

Number and diversity of merA *sequences*

Partial *merA* gene sequences were found by PCR amplification or whole genome sequencing in 38% of the mercury resistant isolates representing all four observed bacterial phyla (Table 1).

Surprisingly, we did not amplify any *merA* sequences from 45 isolates in spite of the fact that 13 were positive for Hg volatization (Møller et al. submitted). The majority (76%) of the *merA* sequences were found in isolates belonging to the *γ-Proteobacteria*, while 12% belonged to the *α-Proteobacteria* and 4% to each of the *β-Proteobacteria*, *Firmicutes* and *Actinobacteria*. In some phylogenetic sub-groups, *merA* was not detected (e.g. Beta I), while in others, only some of the isolates had *merA*. In sub-group Gamma II, for instance, only 44% of the isolates had *merA* (Table 2).

Sequencing of the *merA* genes revealed 7 different loci ('types') as defined by 99% sequence similarity, with two types (type 1 and 3) found in more than one isolate (Table 2). Among the *γ-Proteobacteria*, more than one *merA* type was found within taxonomic sub-group Gamma I. In all other sub-groups, only one *merA* type was observed.

merA type 3 and *merA* type 4 from the *γ-Proteobacteria* were 99-100% identical to MerA of Tn*5042* and Tn*4051,* respectively (Fig 1 & Table 2). *merA* type 2 was only found in an isolate belonging to the *β-Proteobacteria* but was identical to a MerA previously found in a γ-proteobacterium (Fig. 1 & Table 2). Three type 1 *merA* sequences from isolates belonging to the *α-Proteobacteria* were 100% identical at the amino acid level to a hypothetical protein in the filamentous fungi, *Sordaria macrospore* (Fig. 1 & Table 2).

merA found in the *Bacteroidetes*, *Actinobacteria* and *Firmicutes* isolates had a lower degree of similarity to *merA* sequences in the data bases relative to those from the *Proteobacteria* (Table 2 & Fig. 1). *merA* type 5 from strain SKO62 was 83% similar to its most closely related amino acids sequence, while no hit came up in the DNA blast (BlastN). *merA* type 5 was identified by whole genome sequencing since it was not possible to amplify it with degenerate primers nor with primers designed from the putative *merA* from *Loewenhokiella blandensis* (Bact primers in Table S1), which is the most closely related species with a putative *merA*. The poor similarity of the *merA* loci in SOK62 to that of *L. blandensis* and any other *merA* loci would explain the negative PCR result. Type 6 merA, representing an Actinobacterium isolate (taxonomic group

Actino I), was 80% and 92% similar to literature gene and protein sequences, respectively. Finally, the type 7 *merA* sequence of an isolate belonging to *Firmicutes* was 68% similar to a *merA* gene from an *Enterococcus faecium* isolate (blastN) and 86% similar at the amino acid level (blastX) to a MerA from *Bacillus pseudofirmus* OF4 (Table 2).

Sequencing of plasmids and identification of mer-*operon genes on plasmid contigs*

Plasmids were present in 24 of the mercury resistant isolates, and 68% of the isolates with *merA* also had at least one plasmid (Table 1). Based on gel electrophoresis, 5 plasmid patterns were identified (Fig. 2). Sequencing of representative plasmids from each of the different patterns resulted in several contigs, and based on the average depth of coverage of the individual contigs they were classified as either plasmid DNA or contaminating chromosomal DNA. Also, depending on the sequence coverage, the plasmid DNA contigs were determined to belong to one or more plasmids.

Plasmids from *Firmicutes* isolate SOK1b (plasmid pattern I) resulted in 6 contigs and a total of 39,798 bases. The coverage of the contigs suggested the presence of two plasmids with sizes of ~23.7 kb (3 contigs) and ~16.1 kb (3 contigs). On the largest contig (16.1 kb) of the 23.7 kb plasmid, a *merA* sequence was identified along with *merB* and 3 regulator sequences (*merR*, *merR2* and an *arsR* family regulator) (Fig. 3). On the same contig, several genes involved in transposition were also present: two transposases, a relaxase, a resolvase and a mobilization protein. Immediately upstream *merA*, an ORF with highest similarity to a cytochrome C biosynthesis gene was present.

Five contigs of a total of 87.2 kb from *γ-Proteobacterium* isolate SOK65 (plasmid pattern IV) were identified. The sequence coverage of the contigs suggested presence of only one plasmid. On a 6.9 kb contig, the entire *mer*-operon was present, including *merA*, *merB*, *merC*, *merP*, *merT* and *merR* (Fig. 3). In addition, three transposases were identified.

No *merA* sequences were seen on pattern V plasmids isolated from *γ-Proteobacterium* isolate SOK71, (6 contigs, total of 22.8 kb, 2 plasmids predicted of 14.2 kb and 8.6 kb). It should be noted that we were not able to amplify *merA* from the boiling lysate from this isolate either. Regardless, we did identify 3 other *mer* genes, *merR*, *merT* and *merP* on a 6.3 kb contig belonging to the 14.2 kb plasmid (Fig. 3). Downstream the incomplete *mer*-operon, we identified two transposases and one resolvase.

None of the 9 contigs (total of 57.5 kb, 2 plasmids of 48.6 kb and 8.9 kb) originating from the plasmid DNA of the *β-Proteobacterium* isolate SOK15 (plasmid pattern II) contained *mer* genes. However, on two small contigs, *merA* and other *mer* genes (*merP*, *merB*, *merD* and *merE*) were present (Fig. 3), but since these contigs had a relatively low sequencing coverage, it is uncertain if they originated from plasmid or chromosomal DNA.

No *merA* on any of the contigs from plasmids isolated from the *α-Proteobacterium* isolate SOK19 (plasmid pattern III, 16 contigs, total of 100.3 kb, 3 plasmids of 20.5 kb, 51.7 kb and 28.1 kb) was observed.

We searched all contigs for DNA sequences specific to incompatibility groups IncP, IncN, IncW and IncQ. Four of the 5 contigs of one of the plasmids from SOK15 (plasmid pattern II, 48.6 kb) contained sequences identical to *traG*, *trfA1*, *trfA2*, *korA* and *oriT* from IncP-alpha (Gotz et al. 1996). No other Inc-specific gene sequences were found in any of the contigs from the other plasmid patterns.

Whole genome sequencing of isolate SOK62 and identification of mer-*operon genes*

The whole genome of SOK62 (group Flavo II) was sequenced as we were not able to amplify *merA* by PCR although all isolates of this group volatilized ionic mercury (Møller et al. submitted). The chromosome of SOK62 was determined to be ~3.9 Mb. We identified an open reading frame with similarities to *merA* on a 275 kb contig by blasting *merA* from Tn*501* and 4 putative MerA sequences from *Bacteroidetes* isolates. Several hits appeared from the blast

searches but only one open reading frame was identified as *merA* based on conserved amino acid sites characteristic for mercury reductases (Barkay et al. 2010) These include the Redox Active Site and vicinal CC pair at the C-terminus. The genetic organization of part of the 275 kb contig including this *merA* and surrounding genes is shown in Fig. 4. Genes of interest in close proximity of *merA* are two genes upstream that together resemble a *mer* operon. The first gene, *arsR*, is a transcriptional regulator and the second gene a putative mercuric transport protein. Other homologous genes proximal to the putative *merA* in SOK62 include some that are involved in metal metabolism such as a multicopperoxidase, ferrodxin, a high affinity Fe^{2+}/Pb^{2+} permease, and a heavy metal transporting ATPase.

Discussion

We identified 7 different *merA* determinants in bacteria from the High Arctic. Three of these determinants had low sequence similarity to known *merA* and were found among isolates belonging to the *Bacteriodetes*, *Firmicutes* and *Actinobacteria*. The remaining 4 *merA* were obtained from *Proteobacteria* (*α*-, *β*- and *γ*-subclasses). At least 65% of the isolates, in which we detected *merA*, also contained plasmids and *merA* was clearly identified on two – possibly three - plasmids.

We only detected *merA* in 37% of the 71 resistant isolates. This is relatively low percentage considering that the *mer* system is the most common mode of mercury resistance in bacteria (Osborn et al. 1997). In a previous study (Møller et al. submitted), we determined the ability of the resistant isolates to reduce ionic mercury. Of 18 isolates shown to reduce Hg^{II} to Hg^{0}, only 5 carried a *merA* that we were able to detect using degenerate PCR primers and whole genome sequencing. The isolates that apparently lacked *merA*, but were able to volatilize mercury, belonged to the *β*- and *γ*-*Proteobacteria* and to the *Flavobacteria*. This suggests that in the arctic, yet unidentified *merA* genes or resistance mechanisms may be present.

The majority of the sequenced *merA* genes were most similar at both the DNA and protein levels to those of Tn*5042*, Tn*5058* and Tn*4051* (Fig. 1) which have been found in environments as diverse as sugar beet leaves in the UK (Tett et al. 2007), Hg contaminated soil in Tennessee (Oregaard et al. årstal?), 120 000 year old Siberian permafrost samples (Mindlin et al. 2005), a Hg mine in central Asia (Kholodii et al. 1997), phenyl-Hg polluted soil (Kiyono et al. 1997) and Hg polluted river sediment in Kasakhstan (Smalla et al. 2006). Lateral gene transfer alone cannot explain this extreme spread of almost identical MerA proteins but supports the hypothesis of Osborn et al (1997) that the distribution of MerA is a combination of highly conserved proteins, localised selective pressure and lateral gene transfers.

The presence of two types of *merA* (type 3 & 4) within one sub-group of the *γ-Proteobacteria* isolates, the lack of *merA* in several of the isolates within the same *γ-Proteobacteria* sub-group, and the presence of identical *merA*'s in more than one sub-group suggests that the *merA* were localized on mobile genetic elements. Indeed, in 66% of the isolates that carried *merA*, we also identified one or more plasmids. We identified a total of 5 different plasmid patterns and sequencing of one representative of each plasmid pattern showed that two types of plasmids carried an entire *mer* operon. In addition, in close proximity to the operons, several genes involved in conjugation or transposition were identified, too. These results strongly suggest that *merA* in bacteria isolated from the High Arctic have been subjected to lateral gene transfer possibly in the Arctic environment. This is not surprising as *mer*-operons are often laterally transferred as indicated by their location on plasmids and transposable element (Osborn et al. 1997, Liebert et al. 1997, Kholodii et al. 2002, Bogdanova et al. 1998).

The plasmid isolated from strain SOK15, a β-proteobacterium, belonged to IncP-α, a group of plasmids, which are known to have a broad host range (Thomas and Smith 1987, Bathe et al. 2004). This may explain why the *merA* on this plasmid was most similar to a *merA* originally isolated from the γ-proteobacterium, *Pseudomonas* sp. K62. Even though the *merA* in strain SOK15 seemed to have been located on the chromosome, the IncP-α plasmid may have been the

shuttle for a transposon that carried *merA*. Indeed, a high similarity of the chromosomal *merA* in SOK15 (99%) to a transposon (Tn*50580*)-borne *merA* from an IncP plasmid (Smalla et al. 2006) was noted. Considering the broad host range of IncP plasmids (Thomas and Smith 1987, Bathe et al. 2004, Musovic et al. 2006) and their transfer frequencies especially under selective pressure (Pukal et al. 1996, Musovic et al. 2006), the presence of these plasmids in the arctic community further implies lateral gene transfer of mercury resistant genes as an efficient mechanism for the microbial community to rapidly adapt to increasing mercury concentrations as for example in the spring time during atmospheric mercury depletion events. Plasmid-carrying isolates are common in both the Arctic (Miteva et al 2004) and Antarctic (Kobori et al 1984, Miller et al 2009) and at least one arctic plasmid has been shown to have a broad host range (Miteva et al 2008). Only one of our sequenced plasmids belonged to a known Inc group. Therefore, we can only speculate if the other plasmids are also highly conjugative and have a broad host range.

Surprisingly, a *merA* from three α-Proteobacteria was 99% similar to an unnamed protein in the genome of the filamentous fungus, *Sordaria marcrospora* (Nowrousian et al, 2010) and only 94% similar to a *merA* in a mercury resistant α-Proteobacterium isolated from contaminated soil in Oak Ridge, Tennessee (Rasmussen et al, 2008). The unnamed protein in *S. macrospore* was identified on a 7.3 kb contig of a whole genome sequencing assembly, and was flanked by protein homologues resembling MerT and MerR as well as transposable elements. Even though mercury resistance in fungi has recently been described (Crane et al., in revision), a functional MerA has to the best of our knowledge not been identified among the Eukarya.

Whole genome sequencing identified a *merA* sequence with low similarity to other *merA* in strain SOK62, which belongs to the *Flavobacteria*. In the phylum *Bacteriodetes* only 4 putative MerA proteins have been identified, and MerA in SOK62 is only partially similar (42-83%) to these putative MerA loci in *Sphingobacterium spiritivorum* ATCC 33300, *Chryseobacterium gleum* ATCC 35910, *Loewenhokiella blandensis* and *Rhodothermus marinus* DSM 4252 (4 first hits in BlastX analysis against non-redundant proteins). The 5 *Bacteriodetes* MerA form a cluster

that is basal to all bacterial MerA (Fig. 1) suggesting that these proteins represent an early lineage in the MerA phylogeny. The novel MerA in strain SOK62 was a part of a simple operon consisting, in addition to the MerA homolog, of an ArsR-like regulator and a mercury transport protein. The putative *mer* operon of SOK62 shared some characteristics with those in *S. spiritivorum* and *C. gleum*, which were almost identical to each other (Fig. 4). All three operons contained an ArsR-like family regulator and a mercuric transport protein in the same reading direction as the rest of the putative operons and upstream of the *merA* homologues. However, contrary to the operon in SOK62, the other two operons carried a hypothetical protein and *merB* upstream of *merA*. It should also be noted that in *S. spiritivorum* and *C. gleum*, the operons were surrounded by several genes involved in transposition while in SOK62, the functions encoded by genes proximal to the *mer* system were associated with metal transformation. We did not identify *merR*, the universal regulator of the *mer* operon, in any of the *Bacteroidetes* operons (including *L. blandensis* and *R. marinus*). Rather, the ArsR family regulator was the first gene in all the *Bacteroidetes* operons. MerA and ArsR families represent two general classes of metal-binding transcriptional regulatory proteins thus responding to heavy metal stress and toxicity (Busenlehner et al 2003, Brown et al 2003). ArsR acts exclusively as a transcriptional repressor that dissociates from the DNA upon interaction with metal ligands (Chen and Rosen, 1997, Xu et al, 1996), while MerR-like regulators repress transcription in the absence of metal ions and induce transcription upon metal binding (Brown et al, 2003). Thereby, MerR may facilitate a higher level of resistance to mercury as compared to ArsR. ArsR regulators are often associated with early MerA lineages and are the only regulators observed in archael *mer* operons as well as in some *mer*-operons in *Actinobacteria* and *Firmicutes* (Barkay et al., 2010). Thus, it is possible that *merR* is a late development in the evolution of the *mer* operon replacing the ArsR family regulators and resulting in more efficient *mer* systems. The presence of ArsR regulators further supports the antiquity of the *Bacteroidetes mer* system among the Bacteria. Regardless, the Hg

resistant *Flavobacteria* that we isolated showed a high level of resistance (Møller et al) illustrating that this simple mer operon may be still be efficient.

In conclusion, our study suggests that a high diversity of *merA* genes exists in bacteria in the High Arctic. In addition to the 10 different *merA* loci we identified within 4 different phyla, several other *merA* loci may be present in the mercury resistant bacteria that cannot be PCR amplified using primers based on known sequences. Our data also indicate lateral transfer of the *merA* loci, as at least two *mer*-operons were located on plasmids. By use of whole genome sequencing we identified a putative *merA* sequence in an isolate belonging to Bacteroidetes, a phylum where mercury resistance is not well described. Observations on the clustering of this sequence along with 4 other putative *merA* loci from *Bacteroidetes* isolates, and the genetic organization of the regulatory element of the operon, suggests these loci may represent early lineages in the *mer* phylogeny.

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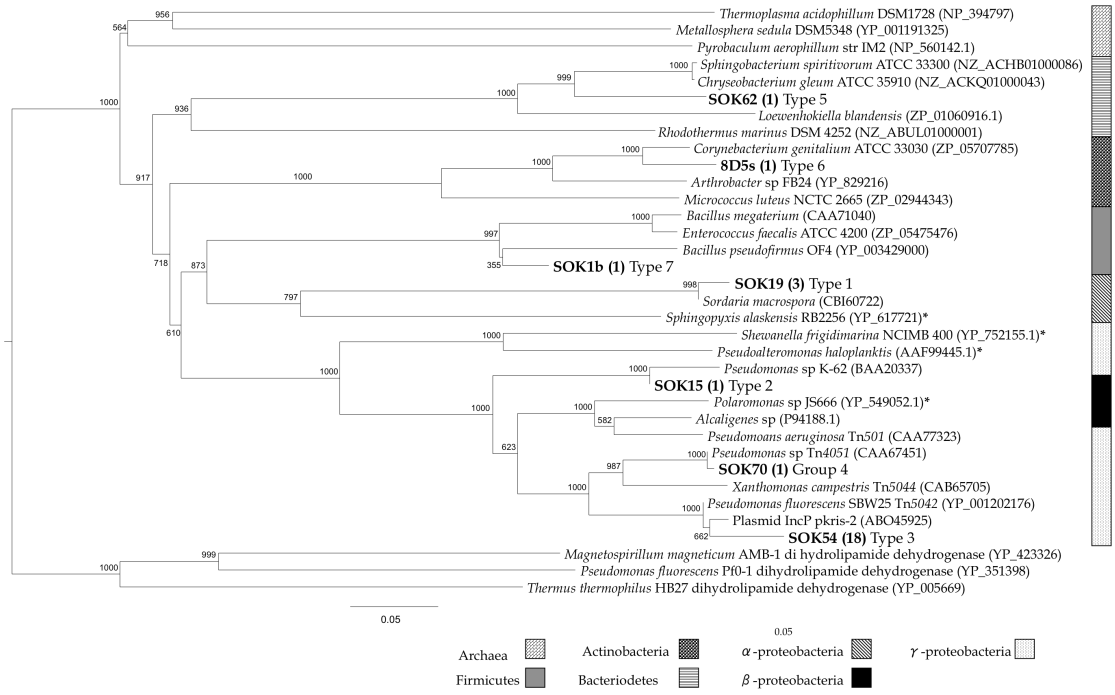

Fig. 1. Neighbor-joining tree showing the phylogeny of MerA protein sequences. The tree has arbitrarily been rooted in the enzyme Dihydrolipamide dehydrogenase. Representative of each merA type (translated nucleotide sequence) identified in the Arctic mercury resistant isolates are shown in bold. Values to the left of the nodes reflect the bootstrap values (percent out of 1000 replicates). Numbers in parentheses indicate the number of merA sequenceses within each type having 99% sequence similarity. The tree is drawn to scale.

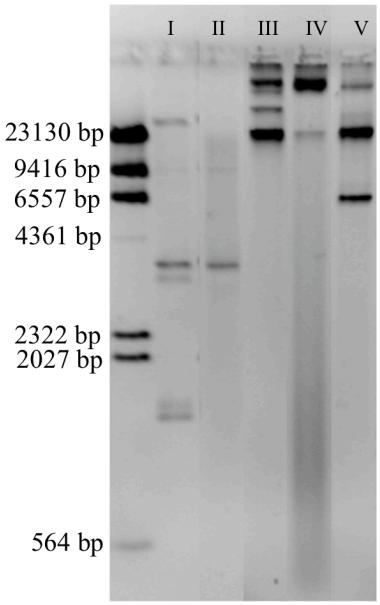

Fig. 2. Representative plasmids isolated from isolates SOK1b SOK15 SOK19 SOK65 SOK71 (lanes 2 – 6). Roman letters indicate the different plasmid groups.

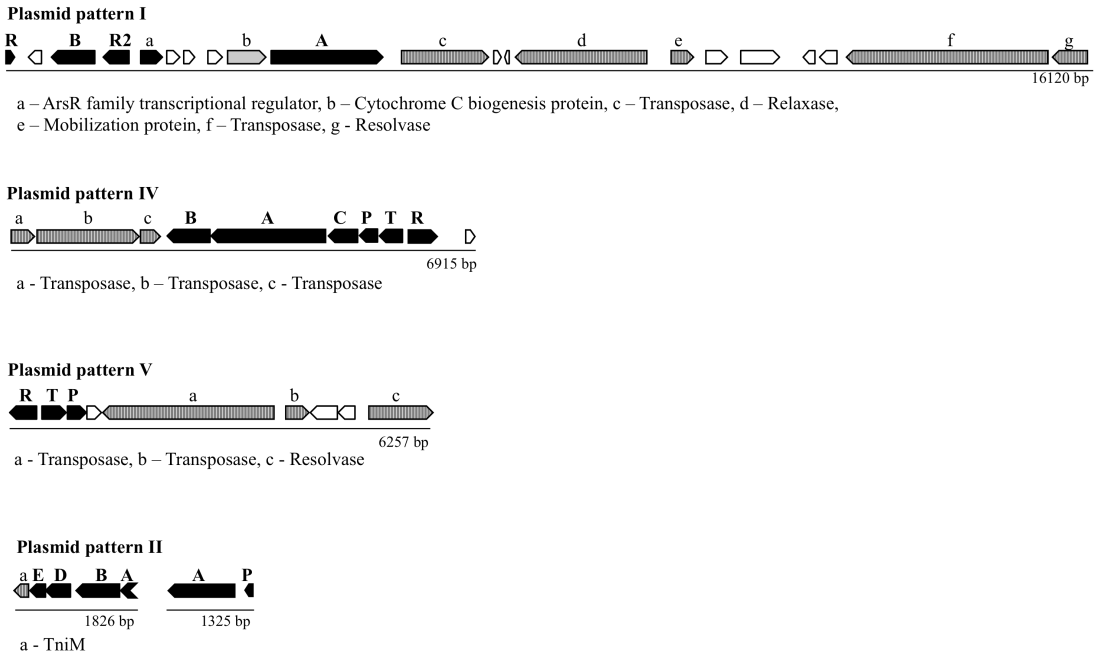

Fig. 3. Maps of plasmid contigs containing genes of the *mer*-operon. Black reading frames indicate genes of the *mer*-operon; grey/stribed reading frames indicate genes encoding proteins involved in plasmid transfer or transposition; grey reading frames indicate genes encoding other functions, while white reading frames indicate genes encoding hypothetical proteins or proteins with unknown function.

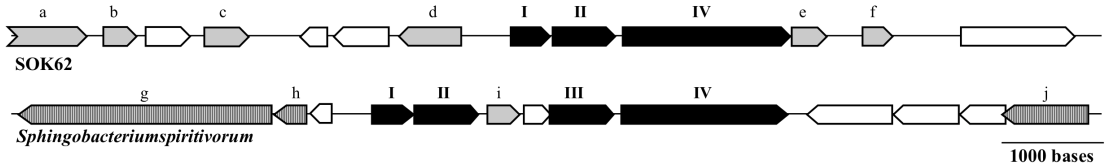

Fig. 4. Genetic organization of the *mer*-operon in *Flavobacterium* isolate SOK62 and *Sphingobacterium spiritvorum*. Black reading frames indicate genes encoding proteins assumed to be involved in mercury resistance (I – ArsR family transcriptional regulator, II – Mercuric transport protein, III – Mercurial lyase (MerB), IV – Mercury (II) reductase (MerA)); grey/stribed reading frames indicate genes encoding proteins involved in conjugation or transposition (g – Protein found in conjugative transposon, h – TraF, j – Protein found in conjugative transposon); grey reading frames indicate genes encoding other functions (a – Multicopperoxidase, b – Ferrodoxin, c – Universal stress protein, d - High affinity Fe2+/Pb2+ permease, e – Heavy metal transporting ATPase, f – Protein with DNA binding domain excisionase, i – Copper-translocating P family ATPase) , while white reading frames indicate genes encoding hypothetical or conserved proteins.

Table 1. Distribution of *merA* and plasmids in the mercury resistant bacterial arctic isolates.

Phylum	Subgroup	Isolate[1]	*merA*[2]	Plasmid[3]	Plasmid pattern[4]
Proteobacteria	Alpha I	SOK5, SOK19, SOK19y, 8D1	+	+	III
	Alpha II	30D12, 8D12b	-	-	
	Beta I	SOK35, SOK17a,SOK17b, SOK18t, SOK48, SOK51, SOK57	-	-	
	Beta II	SOK15	+	+	II
	Beta III	8D36	-	-	
	Gamma I	SOK32, SOK33, SOK43, SOK52	+	-	
		SOK61, SOK67, SOK71	-	+	V
		SOK70	+	+	V
		SOK80	+	+	IV
	Gamma II	8D32, 8D45, 8D48b, 8D48s	-	+	IV
		SOK41, SOK44, SOK50, SOK54, SOK59 SOK65, SOK68, SOK73, SOK75	+	+	IV
		SOK84, SOK85, SOK89	+	-	
		SOK19w, SOK84s, SOK90, SOD12, 8D21 8D26, 8D41, 8D55s, 8D55t, 8D56, 8D64b	-	-	
	Gamma III	SOK13	+	-	
Bacteroidetes	Flavo I	SOK18b	-	-	
	Flavo II	SOK62	+	-	
		SOK29, SOK79	-		
	Sphingo	SOK70s, 8D64s	-	-	
Actinobacteria	Actino I	8D5s	+	-	
		8D5b, 8D7, 8D8, 8D10, 8D11, 8D12, 8D13	-	-	
	Actino II	68F56	-	-	
Firmicutes	Bacilli I	SOK1b	+	+	I
	Bacilli II	SOK27	-	-	
	Bacilli III	SOK38	-	-	
	Bacilli IV	BD41	-	-	

[1] "SOK" indicates that the isolate originated from freshwater; "8", "30" and "68" that the isolate was from snow and "BD" that is was from brine

[2] "+" indicates that *merA* was detected by PCR or whole genome sequencing (SOK62), while "–" indicates that *merA* PCR was performed but with negative result

[3] "+" indicates that plasmid(s) were isolated, while "–" that it plasmid isolation was attempted but with negative result.

[4] Plasmid pattern grouping based on gel migration of the plasmids (Fig. 3)

Table 2. Types of *merA* genes and similarity to sequences reported in the literature.

merA type	Isolate	Sub-Group	Size of *merA* sequence(bp)	BLAST results			
				Similarity (%)		Protein accession no.	Most closely related MerA
				DNA[1]	AA[2]		
1	SOK5, SOK19, SOK 19y	Alpha I	725-743	99	100	CBI60722	*Sordaria macrospora*
2	SOK15	Beta II	245	100	100	BAA20337	*Pseudomonas* sp.K-62 pMR26
3	SOK32, SOK33, SOK43, SOK52, SOK80	Gamma I	1140-1154	99	99	YP_001202176	*P.fluorescens* SBW25 pQBR103 Tn*5042*
3	SOK41, SOK44, SOK50, SOK54, SOK59, SOK65, SOK68, SOK73, SOK75, SOK84, SOK85, SOK89	Gamma II	185-1156	99-100	99-100	YP_001202176	*P.fluorescens* SBW25 pQBR103 Tn*5042*
3	SOK13	Gamma III	680	100	100	YP_001202176	*P.fluorescens* SBW25 pQBR103 *Tn5042*
4	SOK70	Gamma I	1032	100	100	CAA67451	*Pseudomonas* sp Tn*4051*
5	SOK62	Flavo II	1659	-	82	ZP_03969449.1	*Sphingobacterium spiritivorum* ATCC 33300
6	8D5s	Actino I	1095	80	92	ZP_05707785	*Corynebacterium genitalium* ATCC 33030
7	SOK1b	Bacilli	411	68	79	YP_003429000	*Bacillus pseudomfirmus* OF4

[1] blastN
[2] blastX

Table S1. Primers used for amplifying *merA*.

Primer	Sequence	Target (MerA)	Length	Annealing Temp (°C)	Reference
MerA2-f	CCA TCG GCG GCA CYT GCG TYA A	Low GC	1249 bp	61	(Barkay et al, 2010)
MerA2-r	CGC YGC RAG CTT YAA YCY YTC RRC CAT YGT	Low GC	1249 bp	61	(Barkay et al, 2010)
MerA3-f	CGT SAA CGT SGG STG CGT GCC STC CAA G	High GC	1246 bp	64	(Barkay et al, 2010)
MerA3-r	CGA GCY TKA RSS CYT CGG MCA KSG TCA	High GC	1246 bp	64	(Barkay et al, 2010)
Bact-Fw436	GGT GGC ACT TGT GTT AAT G	*L. blandensis*	1295 bp	59	Rasmussen et al, 2008
Bact-Rev	CTT GCA CAA CAA CTC AAT TTA G	*L. blandensis*	1295 bp	59	Rasmussen et al, 2008
A1s-n-F	TCC GCA AGT NGC VAC BGT NGG	*Proteobacteria*	1329-1638	62	Chatziefthimiou et al, 2007
MerA5-R	CGC YGC RAG CTT YAA YCY YTC RRC CAT YGT	*Proteobacteria*	1329-1638	62	Chatziefthimiou et al, 2007
Act-Fw	CSG AVT TGG TST ACG TCG C	*Actinobacteria*	397 bp	62	Oregaard et al, 2007
Act-Rv	ATG AGG TAS GGG	*Actinobacteria*	397 bp	62	Oregaard et al, 2007
Fir-Fw	GTT TAT GTW GCW GCY TAT GAA GG	*Firmicutes*	455 bp	64	Oregaard et al, 2007
Fir-Rev$_{1892}$	CCT GCA CAR CAA GAT AAT TTB GA	*Firmicutes*	455 bp	64	Oregaard et al, 2007
Al-Fw	TCC AAG GCG MTG ATC CGC GC	*α Proteobacteria*	~ 800 bp	63	Oregaard et al, 2007
Al-Rv	TAG GCG GCC ATG TAG ACG AAC TGG TC	*α Proteobacteria*	~ 800 bp	63	Oregaard et al, 2007
A7s-n-F	CGA TCC GCA AGT GGC IAC BGT	*γ/β Proteobacteria*	288 bp	60	Schaefer et al, 2007
55-n-R	ACC ATC GTC AGR TAR GGR AAV A	*γ/β Proteobacteria*	288 bp	60	Schaefer et al, 2007
UmerA-F	CTG GTT GTG AAG AAC AT	*Firmicutes*	1556 (1792)	50	Stapleton et al, 2004
UmerA-R	TCC TTC TGC CAT TGT TA	*Firmicutes*	1556 (1792)	50	Stapleton et al, 2004
SFmerA-f	CGC AGC AGG AGA GTG CTT TA	*S. fridigimarina*	1629 bp	54	This study
SFmerA-r	TAT CCA TAC AAG GGA TGA GCT GCC	*S. fridigimarina*	1629 bp	54	This study

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