



Potentially pathogenic bacteria isolated from diverse habitats in Spitsbergen, Svalbard

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Abstract

The Arctic ecosystem, a reservoir of genetic microbial diversity, represents a virtually unlimited source of microorganisms that could interact with human beings. Despite continuous exploration of Arctic habitats and description of their microbial communities, bacterial phenotypes commonly associated with pathogenicity, such as hemolytic activity, have rarely been reported. In this study, samples of snow, fresh and marine water, soil, and sediment from several habitats in the Arctic archipelago of Svalbard were collected during Summer, 2017. Bacterial isolates were obtained after incubation on oligotrophic media at different temperatures and their hemolytic potential was assessed on sheep blood agar plates. Partial (α) or true (β) hemolysis was observed in 32 out of 78 bacterial species. Genes expressing cytolytic compounds, such as hemolysins, likely increase the general fitness of the producing microorganisms and confer a competitive advantage over the availability of nutrients in natural habitats. In environmental species, the nutrient-acquisition function of these compounds presumably precedes their function as toxins for mammalian erythrocytes. However, in the light of global warming, the presence of hemolytic bacteria in Arctic environments highlights the possible risks associated with these microorganisms in the event of habitat melting/destruction, ecosystem transition, and re-colonization.

Keywords Arctic · Svalbard · Hemolysins · Climate change · Pathogens · Virulence

Introduction

Even though cold environments have long been regarded as extreme due to low temperatures, low water and nutrient availability and high levels of UV radiation, they are actively inhabited by a variety of microorganisms (Maccario et al. 2015). Ecosystems such as tundra, permafrost, and glaciers are regarded as biomes, harboring species from the

Bacteria, *Archaea*, and *Eukarya* domains (Wagner 2008; Kirby et al. 2012) and constitute major pools of genomic diversity (Edwards 2015). The Arctic, spanning more than 7 million km² across regions of Alaska, Canada, Northern Europe, Greenland, Iceland, and Russia (Kirby et al. 2012), has great potential for research focused on the diversity of microbial communities as well as their interactions with the environment. However, this ecosystem is highly sensitive to perturbations and environmental changes (Davidson et al. 2011). Due to the Arctic's low adaptative capacity (Gitay et al. 2002), the consequences of environmental disturbances are usually amplified (Doney et al. 2012; Kirby et al. 2012). As a result of climate change, the annual mean temperature in the Arctic has increased twice as much as in other parts of the world (Davidson et al. 2011; Kirby et al. 2012; Mateos-Rivera et al. 2016) altering a variety of habitats and raising concerns about diversity loss and the possible (re)emergence of diseases (Kurane 2010; Davidson et al. 2011; Reich et al. 2012; Altizer et al. 2013; Wang et al. 2016).

Stressful conditions brought about by a changing climate can heavily influence competition within bacterial communities and might even play a key role in the

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expression of virulence genes (Mekalanos 1992; Livermore 2003; D'Amico et al. 2006). Bacterial species can produce extracellular proteins, such as enterotoxins, coagulases and cytolytins, aimed at reducing the competition in their environment or at inflicting damage in neighboring cells to gain access to their nutrients (Tomita and Kamio 1997; Madigan et al. 2012; Harwood et al. 2018). Among these toxins, hemolysins are of special interest. Since their activity is more easily observed in mammalian erythrocytes, they are frequently referred to as hemolysins, even though red blood cells are rarely their only target *in vivo* (Tomita and Kamio 1997). Secreted by a variety of organisms, hemolysins are responsible for damaging cellular membranes, causing cell lysis, destroying neighboring tissues, and are considered a major virulence determinant in infection models of laboratory animals (Bhakdi et al. 1994; Bayley 1997; Tomita and Kamio 1997; Madigan et al. 2012).

Bacteria can exhibit three different types of hemolytic activity (Buxton 2005): Beta (β) hemolysis, when the toxin causes the complete lysis of the red blood cells. Often referred to as true lysis, it manifests as a clear, transparent area in the blood agar cultures; alpha (α) hemolysis, when lysis does not occur but the hemoglobin of the red blood cells is reduced to methemoglobin and a brown/green colored area can be observed in blood agar cultures; gamma (γ) hemolysis, or non-hemolysis, when no damage to the cells is caused and no change in the agar plate is observed.

The distinction between α and β hemolysis is of relevance in some areas, such as the assessment of the safety of probiotics (Jeon et al. 2017), but it is not generally made in other settings because both types of hemolysis are usually considered a virulence determinant and one of the many phenotypes studied to determine the clinical relevance of the species and/or strains (Bayley 1997; Ramachandran 2013). For this reason, in this study, isolates with either α -hemolytic or β -hemolytic activity are referred to as hemolytic.

The expression of hemolysins has infrequently been assessed in bacterial isolates from environmental samples (Bevivino et al. 2002; González-Rodríguez et al. 2007; Albarral et al. 2016) and much less is known about the hemolytic activity of Arctic species. As warming global temperatures are favoring the geographic expansion of pathogens and increasing the risk of human exposure to infectious disease (Revich et al. 2012; IPCC 2014), it is increasingly important to study the presence of potential pathogens in Arctic environments and the expression of virulent phenotypes.

For this purpose, bacteria from several habitats in Spitzbergen, the biggest island of the Svalbard archipelago (Norway) in the High Arctic were isolated and taxonomically classified. We tested whether or not the isolates displayed hemolytic activity in an effort to highlight the possible

implications of their presence in the context of public health, habitat alteration, and destruction caused by climate change.

Materials and methods

Sampling sites

Samples of snow, glacier forefield soil, sediment, glacier meltwater, pond, and marine water were collected in triplicate from various sites in the High Arctic island of Spitzbergen, Svalbard (Norwegian territory) during summer of 2017 (Fig. 1; Table 1). During the sampling process, sterile nitrile gloves were used and sampling was carried out facing the wind. For the samples of snow and soil, 5 cm of the surface were discarded and a disinfected shovel or sterile spoon was used to store the sample in sterile plastic bags (Whirl-Pak bags; Nasco). Water and sediment samples were collected using sterile collection bottles and a Niskin sampler. All samples were frozen at $-20\text{ }^{\circ}\text{C}$ until processing in the lab.

Cultivation and isolation of bacteria

Samples were thawed overnight at $4\text{ }^{\circ}\text{C}$ prior to culture. All samples were plated on oligotrophic media R2A (Oxoid) in duplicate. Serial dilutions were used for all sample types and the plates were incubated at 5, 17, and $37\text{ }^{\circ}\text{C}$ for as long as 4 months, in sterile plastic bags to conserve the humidity of the agar media. Negative control plates were prepared with sterile water frozen prior to culturing and thawed following the same procedure as the samples. Two control plates were streaked for each testing temperate and incubated in the same conditions and for the same duration as the samples. Liquid and enrichment cultures were not used.

Morphologically different colonies were selected as soon as they became visible on the plates, and transferred successively to new R2A plates to obtain pure cultures. Axenic cultures were confirmed by Gram staining and DNA sequencing as described below.

16S rRNA gene sequencing and phylogenetic characterization

Genomic DNA was extracted from the isolates using a bacterial DNA extraction kit (OMEGA BIO-TEK) and the 16S rRNA gene was amplified via PCR using universal primers 8F (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3') and 1541R (5'-AAG-GAG-GTG-ATC-CAG-CCG-CA-3') (Tam et al. 2015) and ready-to-use PCR reaction mix (MangoMix, Bionline). Two negative controls using nuclease-free water were prepared for each DNA extraction as well as PCR reaction. The PCR conditions were initial denaturation at $94\text{ }^{\circ}\text{C}$ for 2 min, followed by 30 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for

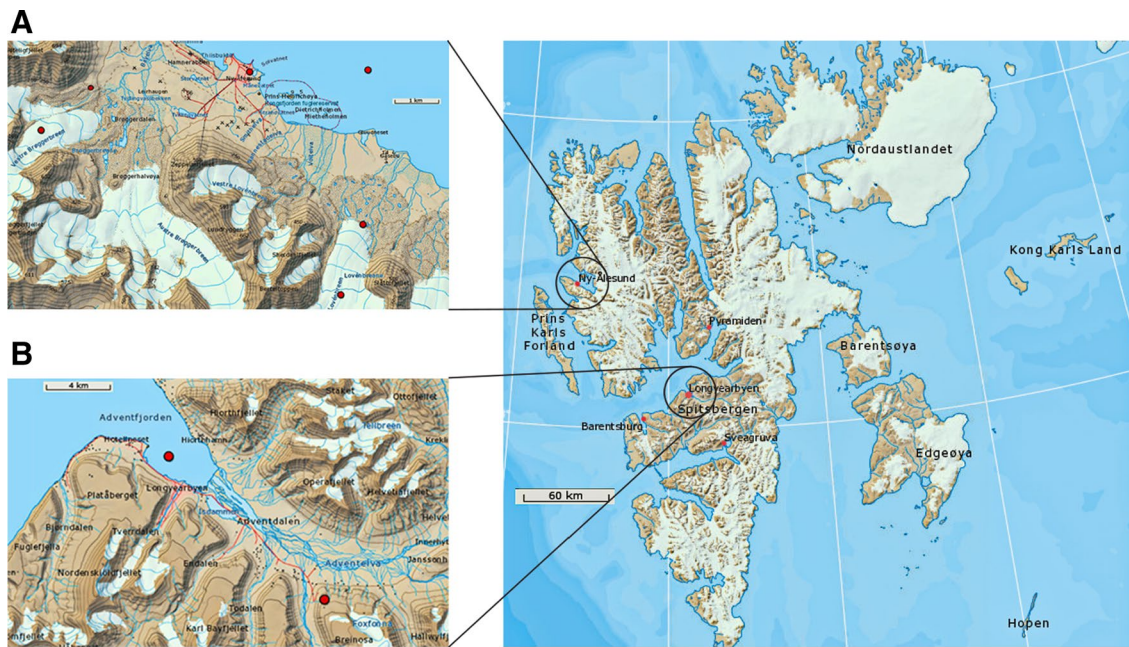


Fig. 1 Sampling sites in the Svalbard archipelago, Norway. The samples were taken from locations on the island of Spitsbergen: **a** sampling sites around the area of Ny-Ålesund and **b** sampling sites

around the area of Longyearbyen (Map adapted from TopoSvalbard, courtesy of the Norwegian Polar Institute, available at: <https://toposvalbard.polar.no/>)

Table 1 Sampling locations for this study and colony forming units (CFU) for all samples after incubation for 4 months

Sample collected	Site description	Site coordinates	CFU at 5 °C	CFU at 17 °C	CFU at 37 °C
Snow	Seasonal pond up the valley of Adventdalen	78° 09' 24" N 16° 01' 59" E	1×10^3	1×10^3	2×10^3
	Midtre Lovénbreen (ML) glacier	78° 53' 08" N 12° 02' 44" E	2×10^2	6×10^1	0
	Vestre Brøggerbreen (VB) glacier	78° 54' 42" N 11° 43' 42" E	0	0	0
Water	Meltwater from ML glacier	78° 53' 25" N 12° 03' 15" E	6×10^1	8×10^1	4×10^1
	Water from a pond near the town of Ny-Ålesund	78° 55' 34" N 11° 56' 21" E	2×10^3	6×10^3	0
	Seawater from Adventfjorden	78° 14' 27" N 15° 36' 59" E	3×10^3	9×10^3	1×10^4
Soil	Seawater from Kongsfjorden	78° 55' 33" N 12° 02' 29" E	1×10^2	2×10^2	3×10^1
	Forefield from ML glacier	78° 53' 54" N 12° 03' 59" E	2×10^5	1×10^8	5×10^3
Sediment	Forefield from VB glacier	78° 55' 20" N 11° 46' 38" E	1×10^5	8×10^7	1×10^4
	marine sediment from Adventfjorden	78° 14' 27" N 15° 36' 59" E	2×10^5	2×10^5	2×10^4
	Pond sediment near the town of Ny-Ålesund	78° 55' 34" N 11° 56' 21" E	9×10^4	2×10^8	2×10^5

Colony forming unit (CFU) counts given in CFU/ml for all samples except soil and sediment, which are given as CFU/g

20 s, annealing at 60 °C for 25 s, extension at 70 °C for 90 s, and a final extension step at 72 °C for 5 min.

Amplicons were visualized on 2% agarose gels and those reactions with the expected size (approx. 1500 bp) were purified (Monarch cleanup kit, New England Biolabs) and Sanger sequenced (Seqlab-Microsynth GmbH, Germany).

Using the BLASTN interface,¹ the DNA sequences were aligned and taxonomically assigned to characterized

reference taxa (16S ribosomal bacteria database). The 16S rDNA sequences obtained in this study were deposited in the NCBI GenBank nucleotide database. The accession numbers assigned are MH714605–MH714685 (Table S1).

Hemolytic activity of the isolates

Isolates were inoculated on Columbia agar with sheep blood (Oxoid). The media consisted of peptone (23 g l^{-1}), starch (1 g l^{-1}), sodium chloride (5 g l^{-1}), and agar (10 g l^{-1}) with 5% sterile sheep blood added after autoclave sterilization.

¹ Available at: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Table 2 Types of hemolysis, by sample type, observed in the bacterial isolates

Sample type	Phyla	α (Partial) hemolysis	β (Complete) hemolysis	γ (Non) hemolysis
Snow	<i>Actinobacteria</i>			S20, S28, S34
	<i>Firmicutes</i>		S44, N83	N82
Water	<i>Actinobacteria</i>	An34, S10, S32, S60, N106	S23b, S58	S22, S31, S54, N84, N91
	<i>Firmicutes</i>			S5, N54, N85, N93
	<i>Proteobacteria</i>			N87, N92
Soil	<i>Actinobacteria</i>		N18, N28, N41, N42	N47
	<i>Bacteroidetes</i>			N72
	<i>Firmicutes</i>		N2, N34	N7, N9, N10a, N17, N57b, N81b
	<i>Proteobacteria</i>		N60, N71	N15, N30, N30b, N43, N78
Sediment	<i>Actinobacteria</i>	S26	S3, S8, S24	An21, An24, S16, S25, S72, S73, S81, S87
	<i>Bacteroidetes</i>		N36a	S65
	<i>Firmicutes</i>	An58, S27b, S70, N58	S7, S71, N23, N24, N39	S15, S77, S90, N36
	<i>Proteobacteria</i>	N61		S35, S67, S84, S88, N96

The bacteria were streaked across the plate to observe individual colonies' phenotypes and a section of the same plate without streak was inoculated via needle puncture to search for oxygen-labile hemolysins. The plates were incubated for 18 h at room temperature (approx. 25 °C).

An isolate was considered α -hemolytic when the colonies caused a green or brown discoloration in the surrounding medium, β -hemolytic when true lysis of the red blood cells resulted in a clear, transparent zone surrounding the colonies and gamma γ -hemolytic or non-hemolytic, when there was no reaction in the surrounding medium (Buxton 2005).

Results

Bacterial cultures and phylogenetic characterization

After 4 months of cultivation of samples from Spitzbergen in oligotrophic media (R2A) at different temperatures (5, 17, and 37 °C), colony forming units (CFU) were counted on all plates. Samples of snow had the lowest viable count (up to 2×10^3 CFU ml⁻¹) in comparison to samples of soil (up to 1×10^8 CFU g⁻¹) or sediment (up to 2×10^8 CFU g⁻¹), which had the highest (Table 1).

A total of 78 unique isolates were obtained and about 83% of these were from incubation temperatures of 17 °C or 37 °C (Table S1). Gram-positive genera accounted for 59/78 isolates. Nutrient-rich samples, i.e., sediment and soil, yielded the majority of isolates (55/78), followed by seawater (12/78), snow (6/78), melt water (4/78), and finally pond water (1/78).

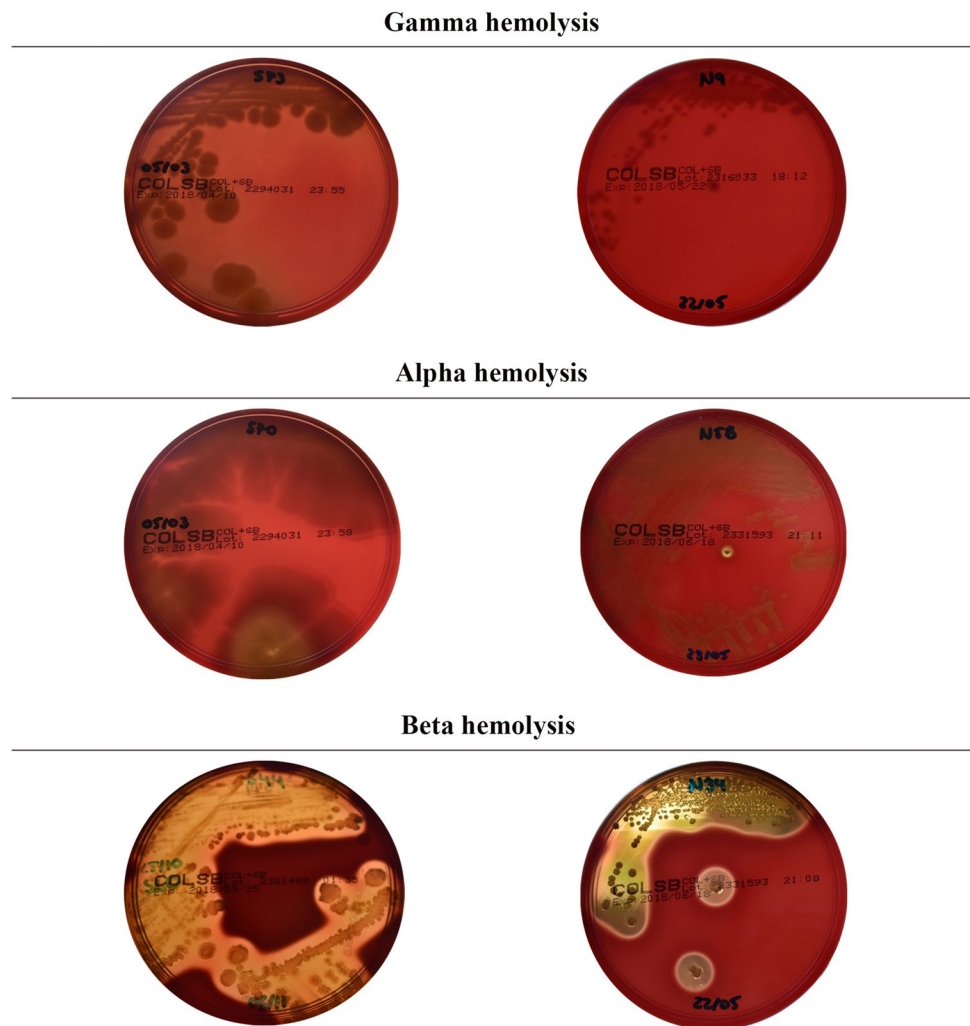
The 16S rRNA gene sequencing revealed that the 78 unique isolates (Table S1) belong to the phyla *Actinobacteria* (32 isolates), *Firmicutes* (28), *Proteobacteria* (15), and *Bacteroidetes* (3), representing a total of 37 different genera. The most abundant genera within each phylum were: 5 *Arthrobacter* spp. (*Actinobacteria*), 12 *Bacillus* spp. (*Firmicutes*) and 7 *Pseudomonas* spp. (*Proteobacteria*). In contrast, the three *Bacteroidetes* species belong to three different genera, i.e., *Pedobacter*, *Flavobacterium*, and *Algoriphagus* (Table S1).

When compared to their closest neighbors in the GenBank database, three isolates had 16S rDNA sequence identities lower than 97% (Table S1) possibly indicating that they correspond to new species: An21 (944nt, 93% identity to *Propionocyclava tarda*, GenBank accession number NR_112669.1), N24 (945nt, 83% identity to *Bacillus clausii*, GenBank accession number NR_026140.1), and N54 (838nt, 94% identity to *Psychrobacillus psychrodurans*, GenBank accession number NR_025409.1).

Hemolytic activity of the isolates

Results of the incubation of pure cultures on blood agar are shown in Table 2, while examples of the observed phenotypes are shown in Fig. 2. After incubation, 32/78 isolates were hemolytic, representing the aforementioned four phyla and out of which 28/32 corresponded to Gram-positive genera. Nutrient-rich samples, i.e., soil and sediment, harbored more hemolytic bacteria (23/32) compared to other sample types such as snow (2/32) or water (7/32). Gamma (non-hemolytic) isolates, 46/78, were distributed among all four phyla and all sample types (Table 2).

Fig. 2 Examples of hemolytic phenotypes observed in Arctic isolates inoculated on Columbia agar supplemented with sheep blood and incubated at room temperature (25 °C) for 18 h. Isolates shown are S73/N9 for γ -hemolysis, S70/N58 for α -hemolysis, and S44/N34 for β -hemolysis



Discussion

To gain insight into the presence and diversity of environmental bacteria that display potential pathogenic phenotypes, samples from the Arctic archipelago of Svalbard were cultured. Altogether, 78 genetically diverse isolates of 37 different genera were obtained and their hemolytic activity was assessed.

In accordance to what has been reported before in Arctic environments (Amato et al. 2007), more isolates were obtained from nutrient-rich samples like soil or sediment (55/78), which are known to have notably higher numbers of viable cells compared to other sample types such as snow or seawater (Amato et al. 2007; Hansen et al. 2007; Kirby et al. 2012).

The isolates showed growth at temperatures well above those they endure in the environments from which they were obtained. In fact, 65/78 isolates were obtained from incubation temperatures of 17 or 37 °C, an indication of their psychrotolerant and mesophilic nature and

confirming that psychrophilic and psychrotolerant microorganisms inhabit the same environments but the latter are more abundant (Moyer and Morita 2007; De Maayer et al. 2014). Sequencing of the 16S rDNA gene revealed that the isolates belonged to four phyla usually found in Arctic environments, namely *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Amato et al. 2007; Maccario et al. 2014; Wang et al. 2016).

Bacterial species isolated from polar habitats which display hemolytic phenotypes are virtually undocumented. In this study, however, we observed an important number of bacteria displaying hemolytic activity (32/78 isolates). Gram-positive species are the most common producers of cytolytic toxins (Masignani et al. 2006; Ramachandran 2013), which we confirmed by finding 28 Gram-positive hemolytic species vs. only four Gram-negative ones (Table S1).

Some of the closest neighbors of the isolates we obtained have been found to produce a variety of cytolytic and cytotoxic compounds, among others, pore-forming compounds

such hemolysin I, II, and III, hemolysin BL as well as other non-specific cytolytic compounds such as surfactin. This is the case for isolates S7, S44, S70, S71, N34, closely related to species of the *Bacillus* genus: *B. cereus* (Ramarao and Sanchis 2013), *B. methylotrophicus* (Harwood et al. 2018), *B. wiedmannii* (Miller et al. 2016), *B. licheniformis* (Harwood et al. 2018) and *B. amyloliquefaciens* (Phelps and McKillip 2002). Under stressful conditions, *Bacillus* spp. can release compounds that cause the lysis of the majority of the population to release the nutrients and resources needed for sporulating cells (Harwood et al. 2018). Other members of the *Firmicutes*, i.e. *Carnobacterium* spp., and *Enterococcus* spp. have also been reported as hemolytic (Vancanneyt et al. 2002; De Vos et al. 2009), confirming our observations for isolates An58 and S27b.

Actinobacteria species are generally regarded as an abundant source of metabolites such as antibiotics, antioxidants, and hemolytic as well as anti-hemolytic compounds (Goodfellow et al. 2009; Suthindhiran and Kannabiran 2009; Pang et al. 2016). We observed members of the genera *Micromonospora*, *Streptomyces*, and *Oerskovia* (isolates S3, S10, N18, N28, and N41) that exhibited hemolytic activity, supporting previous reports of species in these genera that might be considered opportunistic pathogens (McNeil and Brown 1994; Pang et al. 2016; Mohammadipanah and Momenilandi 2018; Takahashi and Nakashima 2018).

Furthermore, we found isolates that display hemolytic activity for which the genus and/or closely related species had been previously described as non-hemolytic. For instance, Funke et al. (1997) reported that *Brevibacteria* are non-hemolytic (Funke et al. 1997). However, isolate S24 (*Brevibacterium* spp.) was beta-hemolytic in our assays. Similarly, *Bacillus clausii* isolates had been previously reported as non-hemolytic (Funke et al. 1997; Jeon et al. 2017), whereas in our assays, *Bacillus* spp. isolates N23 and N24 possess beta-hemolytic activity.

Isolates S8, S23b, S26, and S60, which were identified as *Microbacterium* spp., displayed hemolytic activity. Even though reference to hemolytic phenotypes is rare, members of the *Microbacterium* genus are increasingly isolated from clinical samples and are considered opportunistic human pathogens (Goodfellow et al. 2009).

Finally, to the best of our knowledge, the closest neighbors to isolates An34, S32, S58, N2, N36a, N39, N42, N60, N61, N71, N83, and N106, which are, respectively, *Leifsonia kafniensis*, *Tessaracoccus flavescens*, *Salinibacterium amurskyense*, *Exiguobacterium undae*, *Pedobacter nyackensis*, *Carnobacterium funditum*, *Streptomyces fulvissimus*, *Pseudomonas helmanticensis*, *Psychrobacter nivimaris*, *Pseudomonas lurida*, *Bacillus idriensis*, and *Salinibacterium xinjiangense*, had not been previously assessed for hemolytic activity but some reports exist of these species or other members of their genera with

pathogenic phenotypes (Ostroff et al. 1990; Ko et al. 2006; Ülbegi-mohyla et al. 2009; Leisner et al. 2012; Tayabali et al. 2015; Chen et al. 2017; Milivojevic et al. 2018).

When cytotoxins from environmental species are seen occurring across many sample types, and bacterial genera, as it's shown here, it can be inferred that the production of toxins constitutes part of the many mechanisms devised to survive in severe environmental conditions. In fact, the expression of this phenotype is aimed at reducing surrounding microbial competition or at obtaining scarce nutrients (mainly iron contained in neighboring cells or tissues) and increasing the competitiveness and survival rates of the species (Harding et al. 2011; Harwood et al. 2018). Importantly, while the production of hemolysins is often considered a virulence determinant when assessing the relevance of pathogens in clinical settings (Bhakdi et al. 1994), it has been noted that in environmental species, the nutrient acquisition and competition functions presumably precede their virulent/toxic functions in potential hosts (Falkow 2004). Microbial communities evolve under the influence of external abiotic factors as well as the selective pressure of the surrounding microorganisms. New genotypes and phenotypes are the result of the mutually exerted selection and in turn, they shape the functional properties of the community as a whole (Vayssier-Taussat et al. 2014).

Interestingly, the adaptations allowing survival in cold environments could also allow colonization of human-associated environments (D'Amico et al. 2006). Since several virulence properties of bacterial species, such as hemolytic toxins, are expressed while transitioning between environments, e.g. snow to melt water to sediment, and constitute a survival strategy (Mekalanos 1992), their expression could be used as an indicator of pathogenicity. In fact, the pathogenic potential of an organism is clearly related to the toxins it produces and hemolysins represent one the most potent and versatile tool with which invading microbes damage the host cells (Bhakdi et al. 1994). Since hemolytic activity is rarely regarded as a housekeeping function (Mekalanos 1992; Masignani et al. 2006), the different habitats of Svalbard may be considered as potentially harboring pathogenic bacterial species.

Due to its remote location, geological and biological history, the Svalbard archipelago in the High Arctic is a prime natural system for studying the effects of environmental disturbances brought about by climate change (Isaksen et al. 2007; Park et al. 2011). Warming temperatures in polar areas will likely favor the expansion of forest ecosystems and agricultural activities which will be followed by expanding human settlements and consequently, human-associated pathogens (Gitay et al. 2002; Revich et al. 2012). Altered climatic conditions directly affect disease patterns (Epstein 2001) and research addressing the sensitivity of endemic

microorganisms and their adaptation mechanisms is becoming of greater importance (Kirby et al. 2012; Reich et al. 2012; Boetius et al. 2015).

It is important to consider the risks associated with potentially virulent environmental bacteria and the possibility of their dispersal to new habitats (Harding et al. 2011) through the destruction/disappearance of natural barriers (melting glaciers, thawing permafrost) acting as ecological filters (Park et al. 2011; Underwood 2014) as well as increased animal migrations (Allen et al. 2010) and a marked increase in human activities near the poles (Vayssier-Taussat et al. 2014; Edwards 2015).

The results of this study represent, to the best of our knowledge, the first observations of hemolytic phenotypes in culturable bacteria from Arctic environmental samples. The expression of hemolysins is likely an adaptation mechanism used to survive the stressful conditions of the polar habitats. Moreover, the presence of this potentially virulent phenotype in natural environments provides valuable information about the evolution of clinically relevant species and opportunistic pathogens. Finally, our research contributes further knowledge useful to predict and prepare responses to the countless consequences and global alterations brought about by a drastically changing climate.

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Author contributions DMA carried out the sampling, performed the experiments and wrote the manuscript with input from all co-authors. FB and DW supervised the project and contributed to the interpretation of the results and valuable discussion.

Compliance with ethical standards

Conflict of interest FB is Managing Director of Dr. Brill+Partner GmbH. DMA was employed by Dr. Brill+Partner GmbH as part of a research network (European Union's Horizon 2020 research and innovation programme, Marie Skłodowska-Curie Grant agreement No 675546) and declares no conflict of interest. DW declares no competing interests.

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