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Antimicrobial resistance and bacterial diversity in Arctic environments

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Paper I

Brusetti, L., T. Glad, S. Borin, P. Myren, A. Rizzi, P. J. Johnsen, P. Carter, D. Daffonchio and K. M. Nielsen (2008) Low prevalence of *bla*$_{TEM}$ genes in Arctic environments and agricultural soil and rhizosphere. Microbial Ecology in Health and Disease, 20: 27 – 36

Paper II

Glad T., P. Bernhardsen, K. M. Nielsen, L. Brusetti , M. Andersen, J. Aars and M. A. Sundset (2010) Bacterial diversity in faeces from polar bear (*Ursus maritimus*) in Arctic Svalbard. BMC Microbiology, 10:10

Paper III

Glad T., V. F. Kristiansen, K. M. Nielsen, L. Brusetti, A.-D. Wright and M. A. Sundset (2010) Ecological characterisation of the colonic microbiota in Arctic and sub-Arctic seals. Microbial Ecology, 60: 320-30

Paper IV

Glad, T., P. Barboza, A. Kohn, R. I. Mackie, A.-D. Wright, L. Brusetti, S. D. Mathiesen and M. A. Sundset. Effect of usnic acid, a natural antibiotic in lichens, on rumen microbial ecology and resistance in reindeer. In manuscript.

Introduction

Antimicrobials, use and resistance

An antibiotic is a substance produced by or derived from certain fungi, bacteria, and other organisms, that can kill or inhibit the growth of other microorganisms (Levy 1992). They are widely used in the prevention and treatment of infectious diseases. The term antibiotic is used to refer to a drug that cures infections caused by bacteria, while an antimicrobial agent is a general term that refers to a group of drugs that includes antibiotics, antifungals, antiprotozoals, and antivirals. The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928 when he observed that a common mold (*Penicillum*) produced a substance that lysed colonies of *Staphylococcus* spp. The first major development after the introduction of penicillin was ampicillin, which offered a broader spectrum of activity than either of the original penicillins. In the following decades, many new antibiotics with novel properties were discovered, including streptomycin, chloramphenicol, and tetracycline. Modification of already known antibiotics have led to several derivatives having different antimicrobial activities, pharmacokinetic properties, and resistance characteristics as compared to the older drugs (Levy 1992).

A major problem in treatment of infectious diseases is the emergence of drug resistant bacteria. Antibiotic resistance is a property of microorganisms being able to survive exposure to antibiotic to which they were once sensitive. In clinical terms resistance is linked to failure of therapy. The basic quantitative measure of *in vitro* activity of antibiotics is the minimum inhibitory concentration (MIC). The MIC is the lowest concentration of an antibiotic that results in inhibition of visual growth under standard conditions (Rapp 1999). Antibiotic resistance widespread today seems to have been rare in the pre-antibiotic era. Hughes and Datta (1983) found little antibiotic resistance when screening *Enterobacteriaceae* strains collected in various parts of the world from 1917 to 1954. When a new antibiotic is introduced, many bacteria are initially susceptible, but resistance development is often observed after a short time. For instance, pathogenic strains of *Staphylococcus aureus* were susceptible to penicillin when it was widely introduced in 1944. By 1946, about 6% of *S. aureus* were resistant to penicillin, and by 1948, more than 50% were resistant (Barber and Rozwadowska-Dowzenko 1948). However, the first β-lactamase (R-factor) giving resistance to penicillin was reported already in 1940 (Abraham and Chain 1940). There is a great diversity among bacteria, and they do not share all of the same biochemical and physiological

pathways. Therefore, not all antibiotics are active against all bacteria and they are intrinsically resistant to one or more antibiotics. Intrinsic resistance refers to resistant microorganisms without any chromosomal mutation or the acquisition of plasmid carrying resistance factors. Inherent features of the bacterial cell prevent antimicrobial action, and these properties are typically species characteristics. An example is *Pseudomonas aeruginosa*, a Gram negative soil organism, which has an outer membrane with porins that hydrophobic antibiotics can not penetrate. The Gram positive *Mycobacteria* produce an unusual bilayer outside the peptidoglycan layer that function as an efficient barrier (Fig. 1) (Nikaido 1994).

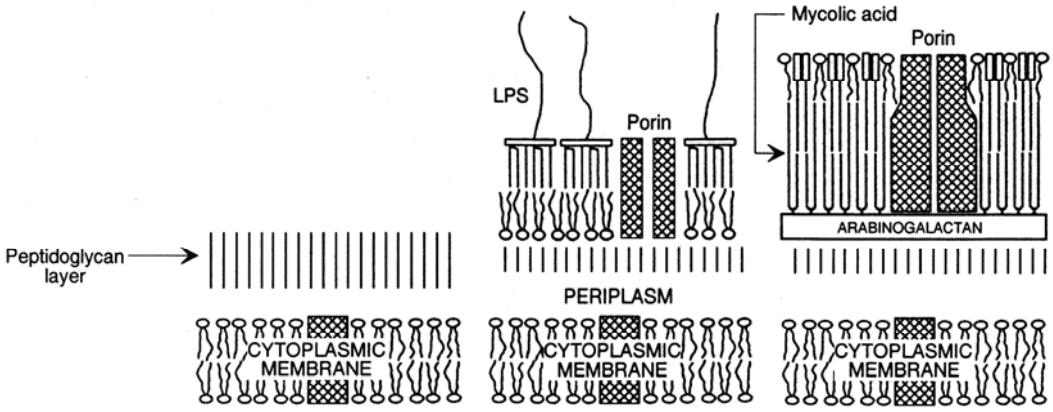

Fig. 1. Cell envelopes of bacteria. (**Left**) Most of the Gram-positive bacteria are covered by a porous peptidoglycan layer, which does not exclude most antimicrobial agents. (**Middle**) Gram-negative bacteria are surrounded by the outer membrane, which functions as an efficient barrier against many antibiotics. (**Right**). Mycobacteria produce an unusual bilayer, which functions as an exceptionally efficient barrier. From Nikaido (1994), reprinted with permission.

In contrast to intrinsic resistance, acquired resistance emerges through mutation of existing DNA or acquisition of new DNA by horizontal gene transfer (Thomas and Nielsen 2005). Horizontal gene transfer (HGT) is a process in which genetic material from an organism is transferred into a cell that is not its offspring. In bacteria, this can be done by transformation, conjugation, or transduction. Natural transformation is the stable uptake, integration, and functional expression of extracellular DNA from the environment into a recipient cell. Conjugative transfer is mediated by cell-to cell junctions and a pore through

which DNA can pass, although the nature of these structures remain elusive (Thomas and Nielsen 2005). Transduction is the bacteriophage-mediated transfer of both chromosomal and extra-chromosomal DNA from on bacterium to the other (Maloy *et al.* 1994). HGT is an important factor in evolution, enabling bacteria to acquire new characteristics. Chromosomal DNA acquired by HGT, that confers a selective advantage to the host, or mobile genetic elements that encode their own transfer and maintenance functions, have the potential to spread within a bacterial population (Thomas and Nielsen 2005; Babic *et al.* 2011). Environments where antibiotics are applied may be hotspots for HGT events, for instance in hospitals or animal husbandry (Fig. 2) (Schjørring and Krogfelt 2011).

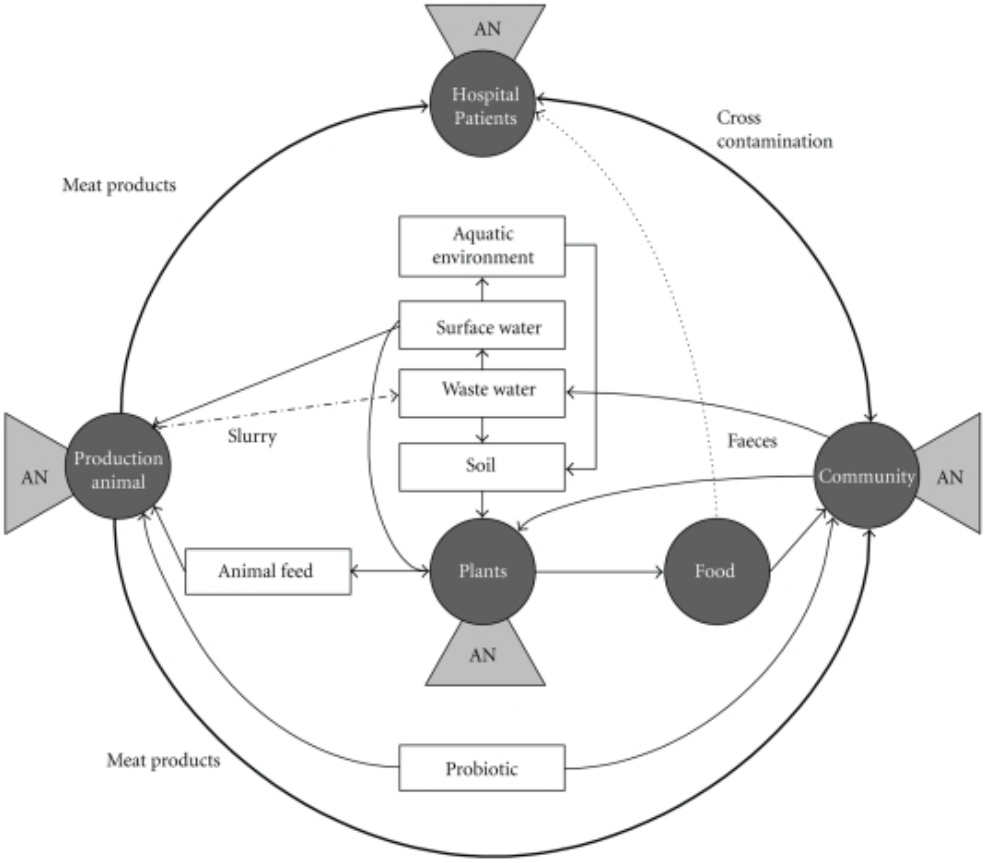

Fig. 2. Schematic presentation of the complexity of selection / development of antibiotic resistant bacteria in different known reservoirs. The possible routes of transmission throughout the environment of these resistant bacteria are suggested. The reservoirs where antibiotics are applied are also suggested as hotspots for horizontal gene transfer. AN: antibiotic treatment/pest control. From Schjørring and Krogfelt (2011), reprinted with permission.

Antibiotic use and overuse has been linked to the development of antibiotic resistance, by creating a selective environment for the resistant bacteria. Further, the use of antibiotic in one environment can lead to increased level of resistance in another, e.g. the antibiotic use in animal husbandry has an impact on the emergence of antibiotic resistance in human commensal bacteria. Microorganisms move easily between ecosystems: from humans and animals to soil and water and vice versa. Resistance genes by organisms in one ecosystem can easily be transferred among organisms in various ecosystems (Nwosu 2001; Smith *et al.* 2002; Schjørring and Krogfelt 2011), as demonstrated in Fig. 2.

The key question addressed in this dissertation was the prevalence of, and the directionality of emergence and dissemination of resistance. Does antibiotic resistance facilitated by HGT emerge first in anthropogenic environments and then spread to pristine environments? Or is it a naturally occurring trait in pristine environments that can transfer into clinically relevant microbes?

Antibiotic resistance in soil

Antibiotic resistance genes have been detected in a variety of soil and sediment environments (Andersen and Sandaa 1994; D'Costa *et al.* 2006; Allen *et al.* 2008; Demanèche *et al.* 2008; Chronáková *et al.* 2010; Donato *et al.* 2010; Yang *et al.* 2010). To see if there are lower numbers of antibiotic resistance genes from environments without anthropogenic influence compared to environments heavily affected by agriculture/urban activity, Pei *et al.* (2006) studied the quantity of resistance genes in river sediments from pristine locations to agriculture and urban sites. They found that the kinds and quantities of resistance genes detected at the pristine site were consistently lower than at the agricultural/urban sites. In contrast to this, Yang and colleagues (2010) investigated the prevalence of antibiotic resistance genes in soil from several locations. They found that there was not a significant difference between the pristine and agricultural sites. A study on ampicillin resistant bacteria in agricultural and prairie soil showed a higher prevalence of resistant isolates in the prairie

soil (54.4%-69.6%) than the agricultural soil (0.4%-8%), indicating that bacterial communities not disturbed by agricultural practices might present a higher degree of antibiotic resistance, either intrinsic or acquired (Demanèche *et al.* 2008).

An explanation for the high level of antibiotic resistance in pristine soils is that the bacterial community is more exposed to soil microorganism producing antibiotics (Hansen *et al.* 2001; Anukool *et al.* 2004). The extent of antibiotic production in nature by soil organisms has been difficult to measure due to low levels of nutrients limiting growth and production. Lately, reports have been published, confirming that antibiotics are produced in soil at sufficiently high concentrations to inhibit growth in the surroundings of the producers (Hansen *et al.* 2001; Anukool *et al.* 2004). This may lead to development of antibiotic resistance mechanisms as a protection against the antibiotic-producing strains. Antibiotic resistance genes have been discovered in soil environments with minimal human induced selective antibiotic pressure. Allen and colleagues (2008) found a great variety of β-lactamase genes in remote Alaskan soil. These genes were distantly related to bla_{TEM} genes detected in clinical settings. Resistance genes have also been detected in marine sediments. Tetracycline-resistant gram-negative bacteria were found in four different marine sediments in Scandinavia. There were few resistant gene classes in unpolluted sediment and several determinant classes in polluted sediment (Andersen and Sandaa 1994). Resistance, including amoxicillin resistance genes, was also observed in pristine environments like miocene sediment excavated from a colliery (Chronáková *et al.* 2010).

There has been an increasing level of antibiotic resistance genes in soil during the last decades. Knapp *et al.* (2010) investigated the abundance of 18 resistance genes in several soils in the Netherlands from 1940 to 2008. They found a significantly increase in the abundance of resistance genes from all classes of antibiotics tested, including bla_{TEM} alleles giving resistance to ampicillin. The increase was notable for tetracycline resistance genes which were >15 times more abundant now than in the 1970s. The increase of antibiotic resistance is linked to the increasing amounts of antibiotics used in human medicine and agriculture. Agricultural use of antibiotic has shown to contribute to the spread of antibiotic resistance genes (Nwosu 2001). For instance was it demonstrated that manure and manure with antibiotic added to soil increases the level of antibiotic resistance in the soil bacteria (Binh *et al.* 2007; Heuer and Smalla 2007). In another study, Demanèche and colleagues investigated the presence of resistant bacterial isolates in agricultural and prairie soil and also

detected several *bla*$_{TEM}$ genes in both environments. The *bla*$_{TEM}$ alleles detected in the soils differed from alleles isolated in clinics (Fig. 3).

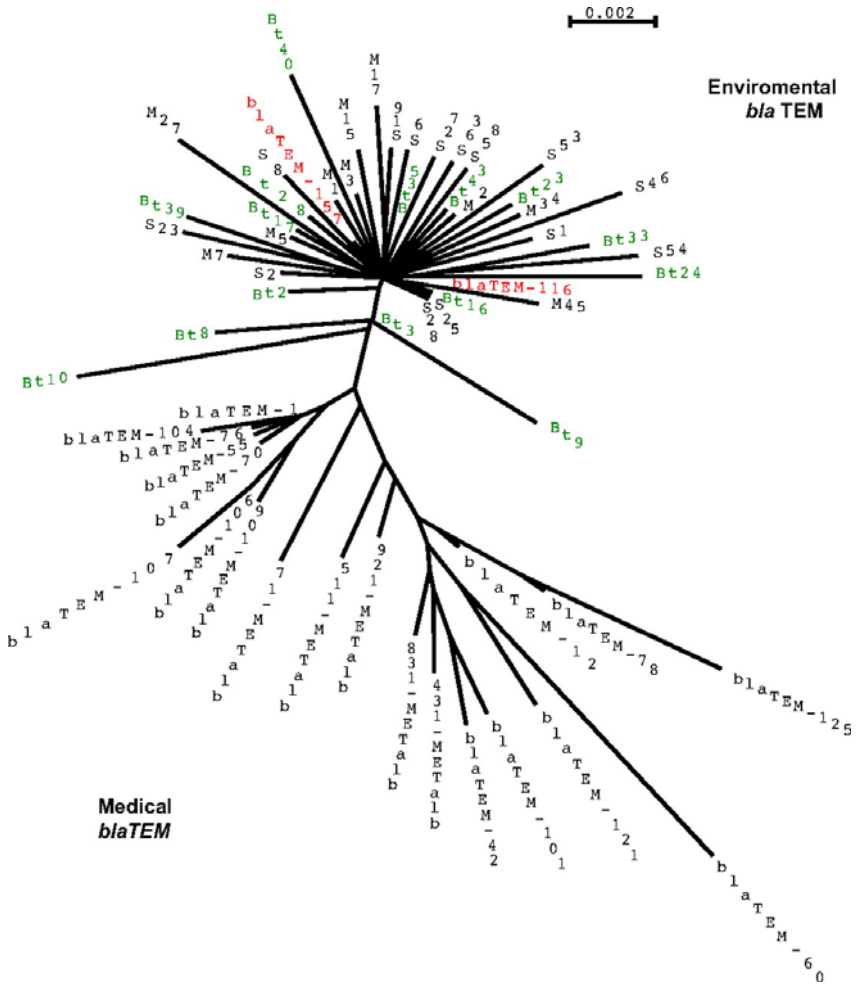

Fig. 3. Phylogenic relationship of *bla*$_{TEM}$ sequences isolated from medical origin and amplified from a transgenic corn field (Bt), a traditional corn field (*M*), and a prairie soil (*S*). Green shading identifies *bla*$_{TEM}$ sequences from the transgenic corn field. Black shading identifies *bla*$_{TEM}$ sequences from other origins. Red shading identifies the two *bla*$_{TEM}$ medical sequences that might originate from the environment. From Demanèche *et al*. (2008), reprinted with permission.

D'Costa and colleagues performed a survey on spore-forming bacteria isolated from urban, agricultural and forest soils, screening 481 isolates against 21 antibiotics (D'Costa *et al.* 2006). Every strain in the library was found to be multi-drug resistant. The antibiotics used for

screening included natural products, semi-synthetic derivatives, and completely synthetic compounds. They included agents introduced several decades ago and newly released products. The result from this study is most likely an underestimate because the cultivable bacteria represent only a fraction of the bacteria in soil. Metagenomic analysis of DNA extracted from savannah and orchard soils has shown the presence of antibiotic resistance genes, including determinants encoding resistance towards β-lactams, aminoglycosides and tetracycline (Riesenfeld *et al.* 2004; Donato *et al.* 2010).

Taken together, the above studies demonstrate that some soils represent an important environmental resistome. Further enumeration of antibiotic-resistant bacteria and antibiotic resistance genes in various soil environments will be helpful in understanding the baseline and movement of antibiotic resistance between different ecosystems in response to environmental pressure (Yang 2010). Little is known about the occurrence of ampicillin resistance in Arctic soil environments. In this dissertation, we have examined the prevalence of bla_{TEM} resistance genes in Arctic soils and sediments (paper I).

Antibiotic resistance in the gastrointestinal tract

Humans. The human gastrointestinal microbiota constitutes a reservoir of antibiotic resistance genes (Walker *et al.* 2001; Saenz *et al.* 2004; Baumgartner *et al.* 2007; Ehlers *et al.* 2009; Shahid *et al.* 2009; Sommer *et al.* 2009; Bailey *et al.* 2011). Ampicillin resistance genes (bla_{TEM}) have also been detected in the human microbiota (Ehlers *et al.* 2009; Sommer *et al.* 2009; Bailey *et al.* 2011). Sommer and colleagues (2009) studied the resistance reservoir in the oral and the gut microbiota of healthy humans. They found a great variety of resistance genes, and most of the genes identified using culture-independent sampling were distantly related to antibiotic resistance genes so far detected in pathogenic isolates. On the other hand, most of the genes from cultivable isolates were closely related to resistance genes in pathogenic isolates, indicating an evolutionarily close relationship to the resistance genes harbored by clinical pathogens. In the commensal microbiota, they identified a $bla_{\mathrm{TEM\text{-}1}}$ gene variant that has been reported in pathogenic strains of *Escherichia coli, Salmonella enterica,*

Klebsiella pneumonia, Haemophilus parainfluenzae, Serratia marcescens, Pseudomonas aeruginosa, and *Neisseria meningitidis* isolated around the globe (Sommer *et al.* 2009).

Other mammals. Antibiotic resistant *E. coli* have been isolated from faecal samples from domestic and wild animals, with domestic species carrying bacteria with resistance to the largest number of agents (Sayah *et al.* 2005). In pigs, resistance levels are known to be high. Lapierre *et al*. (2008) found that 83% of isolates from pigs were resistant to at least one antimicrobial agent. Resistance in one environment can influence the level of resistance in another. Bacterial isolates from wild animals that lived in close proximity to food animal agriculture were more likely to carry resistance to antimicrobials (Kozak *et al.* 2009). Possibly the wild animals were exposed to resistant *E. coli* isolates (and their genes) from livestock, or to antibiotics through contact with animal feed. Tetracycline resistance was the most common resistance in the wild animals. In the same study, 85% of the *E. coli* isolated from pigs was resistant to one or more antibiotics, and the most prominent type of resistance was towards tetracycline as was the case with the wild animals. Tetracycline is often the first-line antimicrobial in disease prevention and growth promotion in food animals. In rural England, 90% of bacterial isolates from mice and voles were resistant to β-lactam antibiotics, with more than 50% showing β-lactamase activity (Gilliver *et al.* 1999). In contrast to these results, faecal bacterial isolates from moose, deer and voles in Finland had almost no resistance (Osterblad *et al.* 2001). Finland is not as densely populated as England, and the environmental impact from agriculture is less. Thus, the wild animals in the finish study might be less influenced by human activities than the wild rodents in England.

Also in ruminants, antibiotic resistance has been observed. Sundset and colleagues (2008) isolated a reindeer rumen bacterium, *Eubacterium rangiferina*, able to tolerate and grow in the presence of the natural antibiotic usnic acid. Tetracycline (TetW) genes have been detected in whole rumen contents from free-ranging Svalbard reindeer and Norwegian reindeer fed a commercially produced pellet feed (M.A. Sundset, unpublished data). In healthy lactating dairy cows, ampicillin resistance was the dominant resistance type with 21% of *E. coli* isolated resistant to this substance (Houser *et al.* 2008).

Despite an increased knowledge of the level of antibiotic resistance in the gastrointestinal tract of wild and domesticated animals and other environments outside clinical settings, little is known about antibiotic resistant microorganisms in the gastrointestinal tract of wild animals in the Arctic. Polar bears on Svalbard and seals in the

Greenland Sea are mammals which are likely to not to have been influenced by human activities, and thereby interesting subjects for an investigation of the prevalence of resistance determinants in their gastrointestinal tracts (paper II and III).

Antibiotics

Betalactam antibiotics / Ampicillin

β-Lactam antibiotics belong to a broad class of antibiotics that includes penicillin derivatives, carbapenemes, monobactams, and cephalosporins. They are the most widely used group of antibiotics available. In 2009, the sales of veterinary antimicrobial agent approved for therapeutic use in animals in Norway amounted to 6,137 kg of active substance. The penicillins accounted for 47% of this, and the proportion has increased from 24% in 1995. No antibiotics have been used as growth promoters in animals in Norway since 1997 (NORM/NORM-VET 2009). Total sales in 2009 of antimicrobial agents for therapeutic use in farmed fish were 1,313 kg of active substance and no β-lactam antibiotics were sold. This is a decrease of 98% from 1987, while the production of farmed fish increased from 55,100 tons to 940,000 tons in the same time period. The significant decrease has been mainly attributed to the introduction of vaccines against bacterial infections (NORM/NORM-VET 2009). The human consume of antibiotics for systemic use were 19.4 DDD (defined daily doses) / 1000 inhabitants/day with penicillins accounting for 43%. The use of ampicillin has been stable over the last years, with 0.10 DDD / 1000 inhabitants / day.

The β-lactam substances all contain a β-lactam ring necessary for their antimicrobial activity (Fig. 4). The β-lactam antibiotics are bactericidal. They act by inhibiting cell wall synthesis. β-Lactams act by binding to penicillin-binding proteins (PBPs) and thereby inhibiting the peptidoglycan synthesis; specifically, the transpeptidation reaction that cross-links the peptide side chain of the polysaccharide peptidoglycan backbone. They also stimulate other endogenous enzymes that weaken the peptidoglycan layer (autolysins). The destruction of peptidoglycan leads to bacterial lysis, although the exact mechanism of killing is unsolved (Timbury *et al.* 2002).

Ampicillin is a β-lactam antibiotic that has been used since 1961 (Fig. 4). In addition to being active against most Gram-positive bacteria like the penicillins, ampicillin is also active against some Gram-negative bacteria like *Haemophilus influenzae* and *E. coli*.

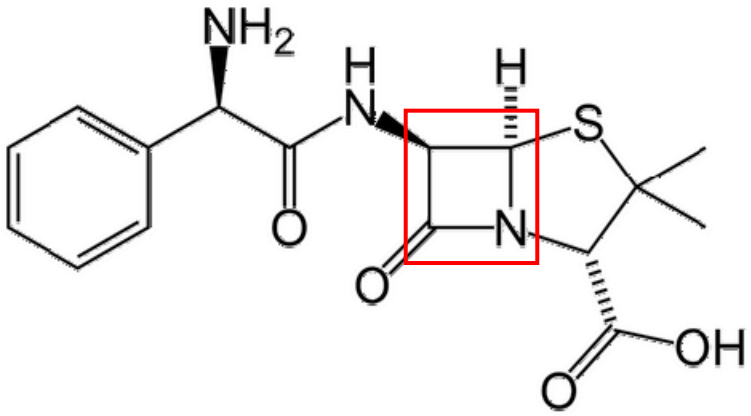

Fig 4. Chemical structure of ampicillin. The β-lactam ring is indicated in red. From www.freebase.com, reprinted with permission.

There are three main mechanisms of antibiotics resistance, and the most widespread is the production of enzymes that degrade or modify the antibiotic. The other mechanisms are alteration of target site and altered permeability or forced efflux (Wilke *et al.* 2005).

The most common cause of bacterial resistance to β-lactam antimicrobial agents is the production of β-lactamases (Livermore 1995), a family of enzymes that hydrolyzes the β-lactam ring, thereby inactivating the antibiotic molecule prior to binding with PBPs. More than 890 unique protein sequences for β-lactamases have been recorded (Bush *et al.* 1995). A variety of transferable genes encoding β-lactamases (*bla*) have been described in clinical environments including $bla_{\text{CTX-M}}$, bla_{GES}, bla_{HER}, bla_{OXA}, bla_{OXY}, bla_{SED}, bla_{SHV}, bla_{SPM}, bla_{VEB}, bla_{VIM}, and ampC alleles (Jacoby 2006). Among the most common *bla* genes is the $bla_{\text{TEM-1}}$ gene, a representative of the bla_{TEM} group that now consists of more than 180 different alleles, all encoding different amino acid polymorphisms that extend their substrate

range (http://www.lahey.org/Studies/temtable.asp). The TEM-1 enzyme is, for instance, responsible for most of the ampicillin resistance seen in *E. coli* isolates (Sanders and Sanders 1992).

The first TEM β-lactamases were discovered in an *E. coli* strain isolated in 1963 (Datta and Kontomichalou 1965). In the past decades, an increasing prevalence of "extendedspectrum" β-lactamases (ESBLs), which inactivate a wider spectrum of β-lactam antibiotics, has been encountered (Jacoby and Medeiros 1991). Most ESBLs are produced by mutations in the $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-2}}$ genes. The newer variants of the bla_{TEM} alleles have only been found in clinical isolates and are likely emerging as a result of random point mutations and strong directional selection. Specific ampicillin resistance-encoding bla_{TEM} alleles are also present in various bacterial cloning vectors such as the pUC series, and have been inserted in some transgenic plant cultivars including commercially used maize lines (e.g. event Bt176). In response to the development of β-lactamases, specific inhibitors have been developed to conserve the activity and extend the spectrum of any accompanying β-lactam drug against β-lactamase–producing microorganisms.

β-Lactam antibiotics constitute the largest group of antibiotics used in human and veterinary medicine and β-lactamases are the most frequent cause of resistance towards these agents. The bla_{TEM} enzymes are widespread in clinical and environmental settings and thus we expected these enzymes also to be prevalent in Arctic environments expected not to be influenced by human activities. We therefore sampled a range of Arctic environments and determined the prevalence of the bla_{TEM} gene.

Usnic acid

The increase in antibiotic resistance and failure of treatment has stimulated a search for new agents, both developing synthetic agents and looking for natural substances. Usnic acid is a secondary component produced by several lichens, including *Cladonia*, *Cetraria* and *Usnea*, with an antimicrobial activity protecting the lichen against bacterial infections (Ingólfsdóttir 2002). Lichens are a symbiotic consortium of fungi and photosynthetic green algae or cyanobacteria (Cocchietto *et al.* 2002). A wide variety of phenolic secondary compounds are synthesized within their fungal component and typically deposited onto the

outer surface of hyphae rather than inside compartments of the cells (Romagni *et al.* 2000). Usnic acid is a protective metabolite functioning against UV radiation and as an anti-herbivore. It was first described by the German scientist Knop in 1844 (Knop 1844). It is a yellow cortical pigment and occurs in two enantiomeric forms, (+) and (-), depending on the projection of the angular methyl group at the chiral 9b position (Fig. 5). The two enantiomers have different biological activities (Romagni *et al.* 2000). In addition, two other natural isomers, (+) and (-) isousnic acids also occur in lichens. Both enantiomers are active against Gram positive bacteria and mycobacteria. *In vitro* studies have shown the antimicrobial activity of usnic acid against clinical isolates of *Enterococcus faecalis, Enterococcus faecium,* and *Staphylococcus aureus* (Ingólfsdóttir 2002). Usnic acid is highly lipophilic, and can behave as a membrane uncoupler (Backor *et al.* 1997). It can pass through biological membranes and dissipate the proton gradient, disrupting the tight coupling between electron transport and adenosine triphosphate (ATP) synthesis. This forms the basis of its antimicrobial activity. In addition to the antibacterial activity, usnic acid has also antiviral, antiprotozoal, antimycotic, antiproliferative, and anti-inflammatory activities (Ingólfsdóttir 2002).

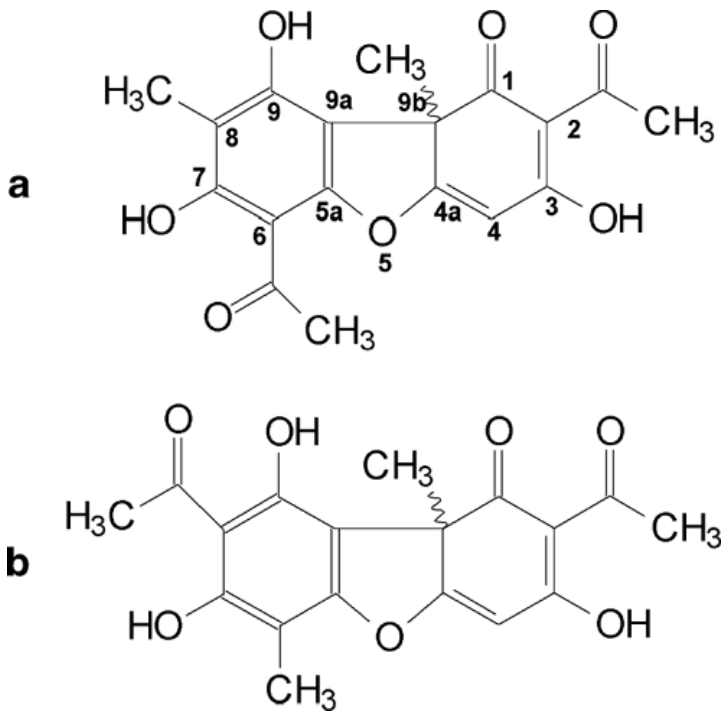

Fig. 5. Chemical structure of usnic (a) and isousnic (b) acid. From Guo *et al*. (2008), reprinted with permission.

The first recorded use of usnic acid is as an antimicrobial in traditional Chinese medicine, under the name Song Lo. It has also been used to treat pulmonary tuberculosis, fever, wounds, athlete's foot and other dermal lesions. Further, it has been used in perfumery, cosmetics, sunscreen, toothpaste, shampoo and deodorants (Guo *et al.* 2008). Usnic acid has also been used as diet supplement for weight loss as it increases metabolic activity and reduces the sense of hunger. The problem with the supplement was the toxic property of usnic acid. There have been at least 21 reports of liver injury or failure, including one death, attributed to weight loss dietary supplement containing usnic acid (Guo *et al.* 2008). In addition to being toxic to humans, it is toxic to most animals. It was reported that intake of the lichen *Xanthoparmelia chlorochroa* caused the death of 400-500 elk (*Cervus canadensis*) in Wyoming (Roach *et al.* 2006). It has also been demonstrated that usnic acid is toxic to sheep at doses of 485 - 647 mg usnic acid / kg / day resulting in muscle damage and even death (Dailey *et al.* 2008). The mammals known to consume usnic acid containing lichens in large quantities are reindeer, musk deer and muskoxen (Green 1987; Ihl and Klein 2001; Sundset *et al.* 2010).

Very little is known about what kind of mechanisms reindeer employ to cope with usnic acid. It has been indicated previously that the reindeer rumen microbiota can utilize usnic acid as a source of energy (Palo 1993). Recent studies by Sundset and colleagues (2010) showed no trace of usnic acid in fresh rumen contents collected from reindeer eating usnic acid containing lichen. This indicates that usnic acid is rapidly degraded and detoxified by rumen microbes. The mechanism by which this is achieved is still unknown, but this finding suggests that reindeer harbour rumen microbes that are resistant to secondary metabolite and even capable of metabolizing them (Sundset *et al.* 2010). In this thesis, we investigated a potential effect of usnic acid on the reindeer rumen microbiota. In order to identify possible resistant bacteria we screened reindeer rumen isolates for the ability to grow in presence of usnic acid. The results are presented in paper IV.

Arctic environments

The main focus of this thesis is antimicrobial resistance in Arctic environments (Fig. 6). We have investigated the prevalence of bla_{TEM} alleles in soils/sediments and the gastrointestinal tract of seals and polar bears. These environments were selected because they have little or no

anthropogenic influence. Another Arctic animal, the reindeer, has the ability to tolerate and utilize usnic acid, a lichen substance that is toxic to most animals. We studied a potential effect of the natural antibiotic usnic acid on the rumen microbial flora of reindeer, and screened for usnic acid resistant bacterial isolates in the rumen. The following sections give a short introduction to the Arctic environments examined.

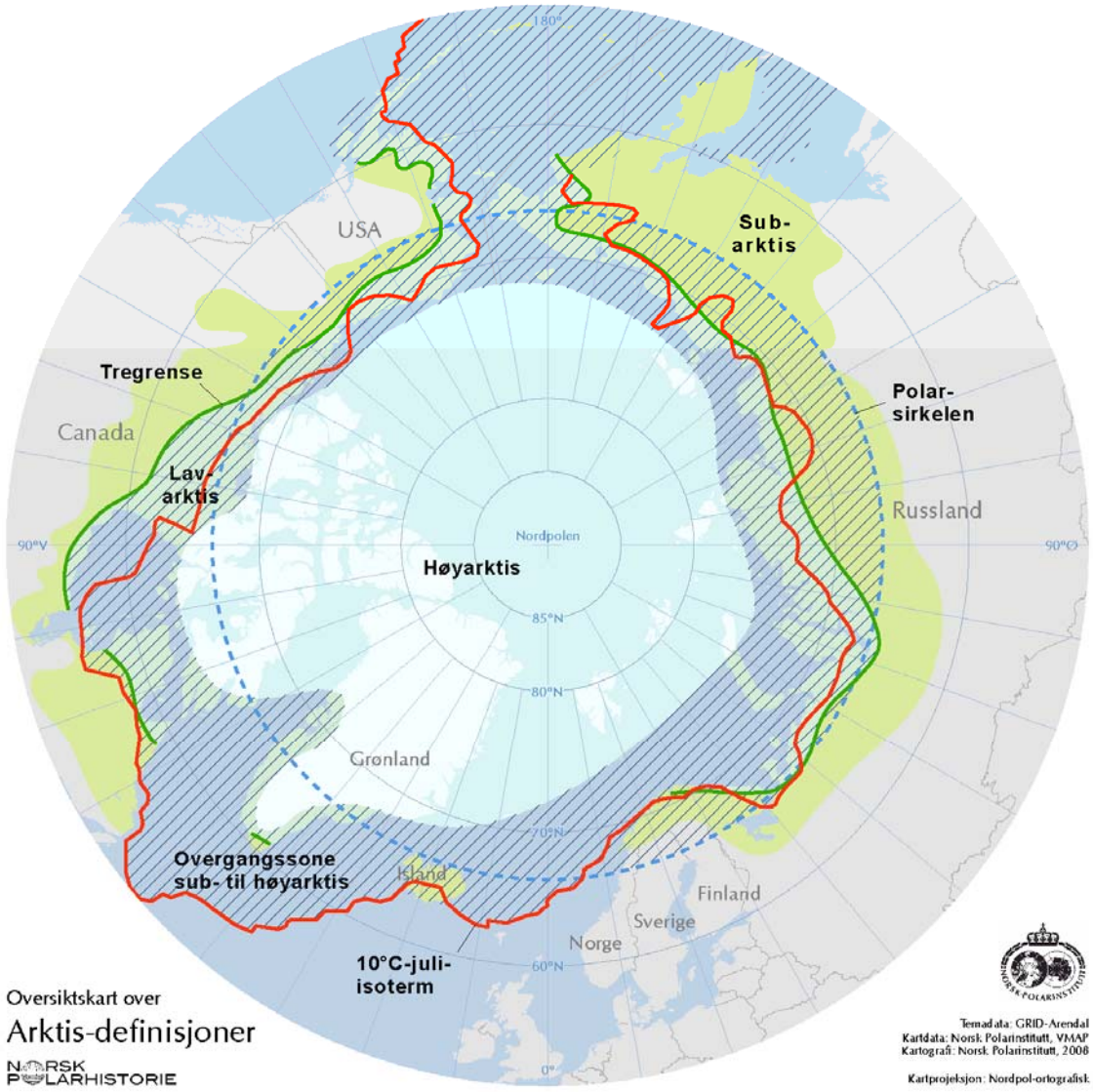

Fig. 6. The Arctic region. The red line points out the border where the average temperature for the warmest month (normally July) is below 10°C, the green line shows the northernmost tree line, and the blue dotted line indicates the Arctic Circle. From Norsk Polarhistorie / Norsk Polarinstitutt (Norwegian Polar Institute) 2008, reprinted with permission.

The Arctic is a region located at the northern-most part of the earth (Fig. 6). The region was named after Arctos, which is Greek for bear, and the prominent constellation *Ursa Major* (great bear) points to the north pole star Arcturus. There is no general agreement on the geographical limits of the region. The area can be defined as north of the Arctic Circle (66° 33'N), the approximate limit of the midnight sun and the polar night. Alternatively, it can be defined as the region where the average temperature for the warmest month (normally July) is below 10°C; the northernmost tree line roughly follows the isotherm at the boundary of this region. In the Arctic this isotherm encircles the Arctic Ocean and includes Greenland, Svalbard, parts of Iceland and most of the northern coast and all off lying island of Russia, Canada and Alaska (Fig 6) (Blix 2005).

Soils and sediments

Soil constitutes the major habitat of terrestrial microorganisms, and it is a complex and dynamic microhabitat. Microorganisms in soil are responsible for degradation of organic compounds and mineral cycling. Important plant polymers such as cellulose and lignin are degraded exclusively by soil microbes. Since carbon and nitrogen are the chief macro elements essential for all organisms, the soil microbes play an important role in cycling of these elements in the biosphere. Sediments are fragments of inorganic or organic material that has been deposited as a layer of solids by wind, water, or ice. When glaciers retreat, new terrestrial habitats are exposed, and microorganisms present have a key role in soil development, nutrient cycling and facilitating plant colonization (Schütte *et al.* 2010). Bacteria comprise the bulk of the biomass and chemical activity in sediments. They are well suited to their role as sediment chemists, as they are the right size and have the required metabolic versatility to oxidize the organic carbon in a variety of different ways (Nealson 1997).

Higher number of bacteria occurs in the organically rich surface layers than in the underlying mineral soils. Particularly high numbers of microbes occur in association with plant roots. Microbial diversity in soil ecosystems exceeds, by far, that of eukaryotic organisms. One gram of soil may harbour up to 10^{10} microorganisms. We also find high numbers of bacteria in arctic soil and sediments, ranging from 10^8 to 10^9 per gram. In

permafrost sediments, 10^7-10^9 cells per gram sediment were found, and in subglacial environments, 10^6-10^9 cells per gram has been detected (Sharp *et al.* 1999).

The bacterial diversity in soils and sediments in Arctic environments is high, despite the extreme environmental conditions. However, we have limited insight to the soil and sediment microbial diversity as a large proportion of the bacterial taxa are unlike those previously described. Schütte *et al.* (2010) found that more than 70% of 16S rRNA sequences in samples from the foreland of Midtre Lovénbreen at Svalbard could not be classified into a genus. Torsvik *et al.* (1996) calculated the presence of about 4,000 different bacterial genomes per gram pristine soil whereas 7,000 genomes in marine sediments, by taking the average genome size of soil bacterial isolate as a unit.

Polar bear

Polar bears (*Ursus maritimus*) are major predators in the Arctic marine ecosystem. They have a circumpolar distribution and are closely associated with sea ice, which they use as substrate for both hunting and movement (Mauritzen 2002). In winter and spring they are commonly found in the shore fast ice with deep snow drift, the floe edge, and areas of moving ice with a large degree of ice cover (Blix 2005).

Polar bears were heavily hunted during the first half of the 20th century all over the Arctic. Through the 1950s and 1960s, there was an increase in the recorded number of polar bears killed. The polar bears were completely protected in the Soviet Union from 1956, and in 1973, the international "Agreement on the Conservation of Polar Bears" was signed (Prestrud and Stirling 1994). The world population of polar bears is currently believed to be about 20,000-25,000 animals that can be divided into 19 subpopulations throughout the circumpolar Arctic (Aars *et al.* 2005). The Barents Sea subpopulation is one of these, inhabiting the geographic regions of Svalbard, the Barents Sea and Franz Josef Land. The size of this subpopulation is estimated to be approximately 2650 individuals (Aars *et al.* 2009).

Polar bears are long-lived, late maturing carnivores that have relatively low rates of reproduction and natural mortality. They have a monogastric digestive system with a simple and relatively short intestine typical of a carnivorous animal, and with the caecum completely lacking (Larsen *et al.* 2004). Polar bears are mostly carnivorous and feed mainly on seals,

although white whale and narwhal carcasses, birds, bird eggs and carrion can be important food items during times of the year when seals are less available (Smith 1980; Gjertz and Lydersen 1986; Lowry *et al.* 1987; Smith and Sjare 1990; Rugh and Shelden 1993; Stempniewicz 1993; Donaldson *et al.* 1995). When feeding on seal blubber, they have a digestive efficiency of more than 90% (Blix 2005). In Svalbard, polar bear predation on reindeer on land has also been observed (Derocher *et al.* 2000).

Seal

The hooded seal (*Cystophora cristata*), the harbour seal (*Phoca vitulina*), and the grey seal (*Halichoerus grypus*) belong to the family *Phocidae*, which is one of three main groups within the suborder *Pinnipedia* (Fig. 7). Pinnipeds are fine-footed mammals which have derived from a common bearlike ancestor, diverging 25 to 27 million years ago into the presently known families. Members of the family *Phocidae* are called earless or true seals. They have a thick layer of fat which contributes to the streamlining of the body and reduces the costs of swimming, and provides insulation against the ice-cold arctic water as well as storage for periods of fasting during breeding and moult. They use their hind-flippers when swimming and have short front flippers which they use when moving on land (Blix 2005).

Seals have a typical carnivorous gastrointestinal tract, consisting of a single stomach, a small intestine, a rudimentary caecum and a short simple colon (Olsen *et al.* 1996; Mortensson *et al.* 1998). The length of the small intestine differs greatly among different species (five to 25 times body length), but the reason for this is unknown (Mortensson *et al.* 1998). The digestive tract of mammals harbours a complex microbial ecosystem with representatives from *Bacteria*, *Archaea* and *Eucarya*, playing a key role in the nutrition and health of the animal (Woese *et al.* 1990).

Fig. 7. Hooded, harbour and grey seal. The photo of hooded seals is by Erling S. Nordøy, and the other two are by Havforskningsinstituttet (Institute of Marine Research). All photos are printed with permission.

Hooded seal

The hooded seal (Fig. 7) is abundant in western and central regions of the Arctic and sub-Arctic North Atlantic. They are named for the inflatable nasal sac occurring in sexually mature males. When inflated, it is a hood that covers the front of the face and the top of the head. Adult males measure about 2.6 meters and typically weigh between 200-360 kg, while mature females measure 2.2 meters and weigh between 150-250 kg. One of three major breeding sites is the Greenland Sea near Jan Mayen, where they gather together in late March for breeding and then reappear in the drift ice to moult in July. Between pupping and moulting, they make long individual trips away from the ice edge, mostly to open water. Sometimes, they visit coastal areas; the Faeroe Islands, Norway or Iceland (Folkow *et al.* 1996). The diet of hooded seals consists of a variety of fish and invertebrates, including Greenland halibut, Atlantic argentine, redfish, herring, polar cod, squid, and crustaceans (euphausiids, amphipods) (Haug *et al.* 2000; Potelov *et al.* 2000; Tucker *et al.* 2009).

Harbour seal

The harbour seal, also known as common seal, is one of the most common seal species in the world (Fig. 7). They are the most wide ranging of the pinnipeds, and exist in northern parts of

the Pacific Ocean and the Atlantic Ocean, along the Norwegian coast, on Kola and the west side of Svalbard (Bigg 1981; Henriksen *et al.* 1997). In North Norway, the largest concentration is in Vesterålen, Nordland, where Haug and colleagues (1998) estimated an abundance of approximately 1000 seals in 1998. The harbour seal is more resident than e.g. the hooded seal. Harbour seals can be up to 2 meters long and weight between 50-170 kg, males being slightly larger than females. The diet composition of harbour seals may reflect differences in the prey species assemblages encountered in various habitats. In northern Norway, they eat mainly fish like saithe, cod, herring, and sculpin (Berg *et al.* 2002). The Norwegian coast is utilised for harvesting and farming marine resources. The harbour seals being a fish feeder foraging in coastal water may interact with local fisheries and fish farms, thereby being exposed to human activities (Henriksen and Moen 1997; Bjorge *et al.* 2002).

Grey seal

Grey seals (Fig. 7) are found only in the North Atlantic with three main groups located in the Northeast Atlantic, the Northwest Atlantic and the Baltic Sea. The Northeast Atlantic population is centred around the British Isles, ranging from Iceland, eastward along the coast of France, and north to Norway and the Kola peninsula (Bonner 1981). The systematic name, *Halichoerus grypus*, means the hooked-nose sea pig. The males grow to an average of 2.2 meters in length and weigh about 220 kg, females reaching an average of 1.8 meters and an average weight of 150 kg. The population along the Norwegian coast consists of 5,800 to 6,600 individuals with the largest abundance in mid Norway (Nilssen and Haug 2007). As is the case with harbour seals, grey seals have a resident nature and are exposed to human activities through interaction with local fisheries and fish farms. Their diet is dominated by fish like cod, saithe, sand eel, herring and catfish (Hammond *et al.* 1994; Mikkelsen *et al.* 2002). Regional variations in diet may reflect variations and abundance and availability of potential prey. Adults feed on larger prey than younger seals (Mikkelsen *et al.* 2002).

Reindeer

Reindeer (*Rangifer tarandus*) are highly adaptable ruminants that developed 15 million years ago (Randi *et al.* 1998; Mathiesen *et al.* 2005). They are widely distributed and numerous in the circumpolar north counting (Eurasia, North America, the Arctic islands of Scandinavia, Finland, the western region of Russia and in West Siberia) counting approximately five million individuals (Banfield 1961). The reindeer belong to the *cervidae* family and include seven subspecies living in diverse habitats (Banfield 1961). Some subspecies migrate between summer and winter ranges like most of the Eurasian tundra or mountain reindeer (*Rangifer tarandus tarandus*), wild tundra caribou (*Rangifer tarandus groenlandicus*) and Alaskan caribou (*Rangifer tarandus granti*), while others, such as the reindeer on Svalbard (*Rangifer tarandus platyrhynchus*), live on the tundra all year round.

Reindeer are grazing herbivores adapted to a seasonal variation in available nutrients in their forage. The summer diet consists of green plants and leaves with high nutritive value. The winter diet, especially on the Scandinavian mainland, is dominated by lichens and some wintergreen plants (Mathiesen *et al.* 2005). Lichens are an important source of carbohydrates but low in protein and minerals, and the reindeer are unique because of the ability to digest and utilize these (Nieminen and Heiskari 1989; Aagnes *et al.* 1995; Storeheier *et al.* 2002). Lichens play a significant role in nature almost everywhere they occur and form the dominant vegetation on approximately 8% of the Earth's terrestrial surface, fundamentally influencing the growth and development of other plants and animals sharing the same environment (Nash 1996; Brodo *et al.* 2001). The ruminant reindeer have a compartmentalised stomach-system where symbiotic rumen microorganisms take part in degradation of plant cell wall carbohydrates (Mathiesen *et al.* 2005).

Microbial diversity

We find bacteria almost everywhere, in soil, sediments, gut, rumen, and sea; the main diversity of life is microbial. Even in habitats that seem incompatible with life, like Arctic ice, the Dead Sea and hot springs, microbes grow. Microbial diversity is the degree of variation of microbes within a given ecosystem. In some ecosystems, like the human gut, we have a greater knowledge about the microbiota. In others, like the gut of wild Arctic mammals, we know less. In microbial ecology, diversity can be determined using phenotypic or genotypic approaches.

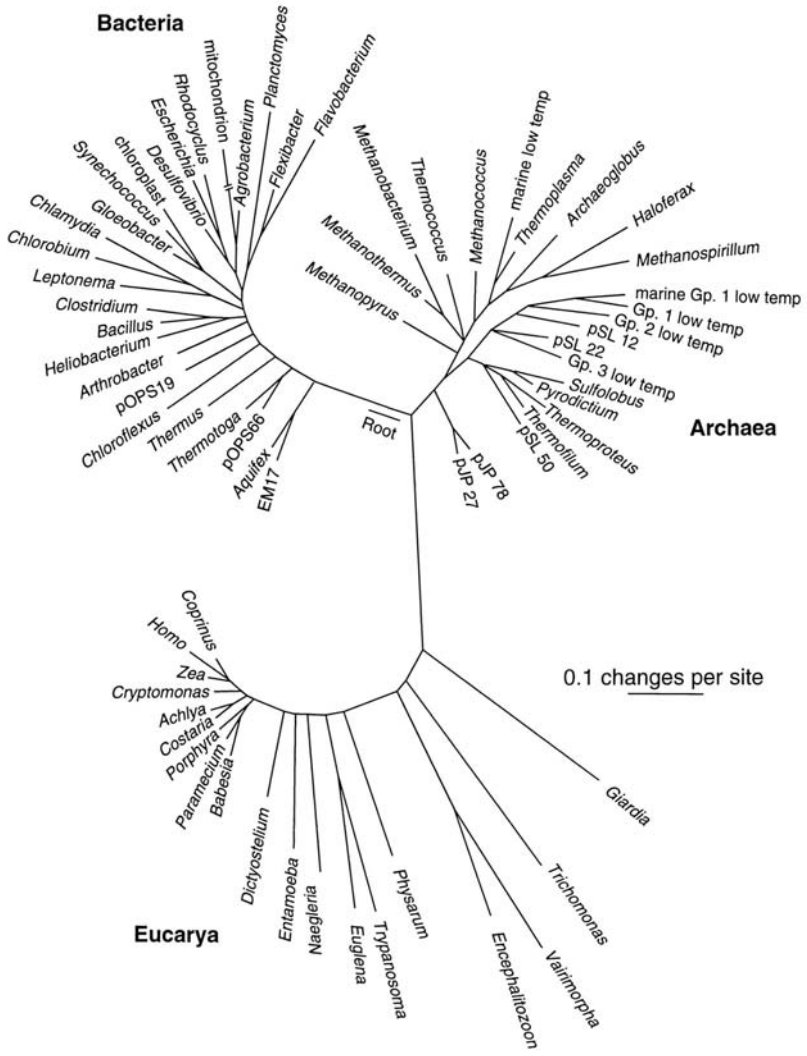

Fig. 8. Universal phylogenetic tree based on small-subunit rRNA sequences. Sixty-four rRNA sequences representative of all known phylogenetic domains were aligned, and a tree was produced using FASTDNAML. That tree was modified, resulting in the composite one shown, by trimming lineages and adjusting branch points to incorporate results of other analyses. The scale bar corresponds to 0.1 changes per nucleotide. From Pace (1997), reprinted with permission.

Phenotypic diversity is related to the expression of genes under a given set of conditions. As most of the bacteria, especially in environmental samples, do not grow under laboratory conditions, only a minor part of the phenotypic diversity in a population can be observed. For example in soil, the population of colony forming bacteria is not representative for the total community as only 0.1-1% of the bacteria is cultivable (Torsvik *et al.* 1996). Genetic diversity measures the genetic composition in microbial communities independent of environmental conditions. Over three decades of molecular-phylogenetic studies, researchers have compiled an increasingly robust map of evolutionary diversification showing that the main diversity of life is microbial, distributed among the domains, *Bacteria*, *Archaea*, and *Eucarya*, suggested by Woese (1990) (Fig 8). The application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional requirement for cultivation has resulted in the discovery of many unexpected evolutionary lineages; members of some of these lineages are only distantly related to known organisms.

Taxonomic classification

Classification of life has for a long time been based on their phenotype, starting with Aristotle classifying all life as being plants or animals. Carolus Linnaeus lived in the 18th Century and created the familiar hierarchical classification scheme of life: kingdom, family, class, order, family, genus and species, based on phenotype. In the mid-19th century, Ernst Haeckel introduced a third kingdom for microorganisms, in addition to the two for plants and animals. A challenge was to classify microbes with little distinct morphology or shape. A revolution in microbial classification came with the work of Carls R. Woese in the 1970s. He proposed that variations in the sequences of DNA encoding ribosomal RNA (rRNA) in different organisms would provide information regarding evolutionary relatedness. Woese studied evolutionary relationships among microorganisms and in the process, he and colleagues discovered a split in the "prokaryotes". He originally thought that these were primitive organisms and called them the *Archaea*. So, rather than kingdoms of life, Woese argued for the three domains *Eucarya*, *Archaea*, and *Bacteria* (Woese *et al.* 1990), with a common ancestor (Fig. 8).

Traditionally, biodiversity measures are based upon the species with binomial Liennaen names as the descriptive unit. The species concept is used in all branches of biology (de Queiroz 2005). Most biologists know the “the biological species definition” established by

Mayr in 1942: “species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated form other such groups” (Mayer 1942). Species diversity can be expressed as species richness, which is an index (single number) expressing the ratio between the number of species and the number of individuals in a collection. Many diversity indices, like the Shannon index (Shannon and Weaver 1949), take into account both species richness and species abundance.

The traditional species concept does not fit the bacterial world, and microbiologists lack a widely accepted theoretical species definition. One primary criterion for assigning bacteria to the same species is if their reciprocal, pair-wise DNA-DNA hybridization is ≥70%. In addition, all strains within a species must have a certain degree of phenotypic consistency, and species descriptions should be based on more than one type of strain (Achtman and Wagner 2008). Microbes with 16S rRNAs that are ≤98.7% identical are always members of different species, because such strong differences correlates with <70% DNA-DNA similarity. The opposite might not be true, and distinct species have been occasionally described with 16S rRNAs that are >98.7% identical. Even though not generally accepted, microbial ecologists typically assign 16S rRNA sequences that are >97% identical to the same species, and sequences with >93% identity are designated the same genus. The DNA-DNA hybridization concept, as most of the other concepts used today, includes methodological considerations.

Horizontal gene transfer (HGT) has an important role in the evolution of microbial genomes and has been introduced as a disruptive force that challenges the species concept (Doolittle and Papke 2006). HGT is recognized as a major cause of antibiotic resistance since the 1960’s, e.g. there is evidence that resistance has been transferred to *Staphylococcus aureus* on more than 20 occasions (Grundmann *et al.* 2006). Studies on *S. aureus* and *Streptococcus pneumoniae* has revealed that HGT has facilitated acquired antibiotic resistance (Harris *et al.* 2010; Croucher *et al.* 2011). Other types of genes, like mobile genetic elements, plasmids and prophages, have also been widely disseminated by HGT. Genomic islands are often associated with HGT and can introduce complete metabolic pathways and hence rapid environmental adaption. With the exception of rapid acquisition antibiotic resistance, little evidence exists for recent, real-time evolution, due to frequent and ongoing HGT. Most horizontally acquired sequence changes are eventually lost, especially if they reduce fitness, and most genes introduced is transient and lost over evolutionary time and thereby not a

reason for turning down the concept of microbial species (Achtman and Wagner 2008). On the other hand, Dagan and Martin (2007) argue that at least 65% of prokaryote gene families has been affected by HGT during the course of evolution.

There is a lack of knowledge about the prevalence of antimicrobial resistance genes in the gastrointestinal tract of Arctic mammals, and little is known about the bacteria inhabiting these environments. To increase knowledge of the diversity in the gastrointestinal tract of polar bears and Arctic and sub-Arctic seals, we have applied DNA sequencing and phylogenetic analysis to examine the microbiota in faeces and colon content from these animals.

Hypotheses and objectives of the study

Hypotheses

- Ampicillin resistance, encoded by *bla*$_{TEM}$ alleles, is prevalent in non-clinical bacterial populations in Arctic environments that have little or no human influence.

- Polar bear and seals harbour a distinct, diet-dependent gastrointestinal microbiota that is distinct from currently described microbial diversity.

- The natural antibiotic, usnic acid, has an effect on the reindeer rumen microbiota ecology, and the rumen harbour usnic acid resistant bacteria.

Objectives

- Determine the prevalence and allele diversity of *bla*$_{TEM}$ resistance genes in a range of environments, including soils and sediments (paper I), and the gastrointestinal tract of polar bear (paper II) and seals (paper III).

- Describe the diversity of the main microbiota of polar bear (paper II) and seals (paper III).

- Examine the effect of usnic acid on rumen bacterial diversity in reindeer, and screen for usnic acid resistant bacterial strains (paper IV).

Summary of papers

Paper I

The prevalence of *bla*$_{TEM}$ genes conferring ampicillin resistance (Amp^r) in different soils was determined to clarify the environmental distribution of resistance determinants of major clinical importance. Samples were collected from 14 sites in New Zealand, mainland Norway, Svalbard, and 2 soil microcosms made of compost purchased in Italy. The Amp^r bacteria represented 1.7-100% of the cultivable microflora with an average of 28%. Approximately 1200 Amp^r isolates were further analyzed. Although >50% of the resistant isolates were capable of β-lactam-ring (nitrocefin) degradation, none carried a PCR-detectable *bla*$_{TEM}$ gene. The proportion of *bla*$_{TEM}$ genes in the culturable Amp^r isolates was <0.07%. The overall *bla*$_{TEM}$ gene prevalence was determined by *bla*$_{TEM}$-specific PCR of DNA extracted directly from the environmental sample. DNA hybridization was performed on selected samples with a detection limit of ~11 *bla*$_{TEM}$ genes per PCR sample. Our analysis indicates that the prevalence of *bla*$_{TEM}$ carrying bacteria is <1 per 1000 to 100 000 bacteria in the samples analyzed. The study suggests that *bla*$_{TEM}$ genes are rare in soil environments, in contrast to their increasing prevalence in some clinical and commensal bacterial populations. The frequent observation of nitrocefin-degrading capacity among the sampled isolates suggests that other mechanisms conferring enzyme-mediated resistance to β-lactam antibiotics are widespread in Arctic and agricultural soil environments.

Paper II

Polar bears (*Ursus maritimus*) are major predators in the Arctic marine ecosystem, feeding mainly on seals, and living closely associated with sea ice. Little is known of their gut microbial ecology and the main purpose of this study was to investigate the microbial diversity in faeces of polar bears in Svalbard, Norway (74-81°N, 10-33°E). In addition, the level of *bla*$_{TEM}$ alleles, encoding ampicillin resistance (Amp^r), was determined. In total, ten samples were collected from ten individual bears, rectum swabs from five individuals in 2004 and faeces samples from five individuals in 2006. A 16S rRNA gene clone library was constructed, and all sequences obtained from 161 clones showed affiliation with the phylum

Firmicutes, with 160 sequences identified as *Clostridiales* and one sequence identified as unclassified *Firmicutes*. The majority of the sequences (70%) were affiliated with the genus *Clostridium*. Aerobic heterotrophic cell counts on chocolate agar ranged between 5.0×10^4 to 1.6×10^6 colony forming units (cfu)/ml for the rectum swabs and 4.0×10^3 to 1.0×10^5 cfu/g for the faeces samples. The proportion of Amp^r bacteria ranged from 0% to 44%. All of 144 randomly selected amp^r isolates tested positive for enzymatic β-lactamase activity. Three % of the amp^r isolates from the rectal samples yielded positive results when screened for the presence of bla_{TEM} genes by PCR. Bla_{TEM} alleles were also detected by PCR in two out of three total faecal DNA samples from polar bears. The bacterial diversity in faeces from polar bears in their natural environment in Svalbard is low compared to other animal species, with all obtained clones affiliating to *Firmicutes*. Furthermore, only low levels of bla_{TEM} alleles were detected in contrast to their increasing prevalence in some clinical and commensal bacterial populations.

Paper III

Dominant colonic bacteria in wild hooded (n=9), harbour (n=1) and grey (n=1) seals were identified using 16S rRNA gene clone libraries (313 clones), revealing 52.7% *Bacteroidetes*, 41.5% *Firmicutes*, 4.5% *Proteobacteria* and 1.0% *Fusobacteria*. Thirty (77%) of the 39 phylotypes identified were novel, showing <97% sequence similarity to their nearest cultivated relatives. Mean colonic bacterial cell density, determined by real-time PCR, was high (12.8 log10 cells/g wet wt) for the hooded seals, while the number of methanogenic *Archea* was low (4.0 log10 cells/g wet wt). The level of ampicillin (Amp^r) and tetracycline-resistant (Tet^r) isolates was investigated by cultivation. Aerobic Amp^r isolates were only detected in colon contents from four hooded seals, whereas aerobic Tet^r isolates were found in seven of the nine hooded seals. These data provide novel insight to the gut microbiota of Arctic and sub-Arctic seals living in the wild.

Paper IV

The reindeer rumen microbiota have interacted with dietary lichens through millions of years of evolution, and recent data indicate that symbiotic bacteria in the reindeer rumen may have evolved mechanisms to resist and even detoxify secondary compounds such as usnic acid in lichens. Reindeer (n=3) were given *ad libitum* access to a concentrate diet supplemented with usnic acid for 17 days to examine the effect of this antibiotic phenolic compound on the rumen microbiota. Sampling was conducted before, during (days 9 and 17) and 8 days after the usnic acid supplementation. Denaturing gradient gel electrophoresis (DGGE) profiling and phylogenetic analysis of 16S rRNA gene sequences from the DGGE profiles were used to determine the effect of the usnic acid on bacterial diversity and to identify dominant phylotypes. Population densities of bacteria and methanogenic archaea associated with both the liquid and particle fraction of the rumen contents were estimated by real-time PCR. Furthermore, rumen bacterial isolates (n=65) were screened for resistance towards four different lichen acids (usnic, atranoric, fumarprotocetraric and lobaric acid). Two bacterial isolates, with 16S rRNA genes that had a 100% sequence identity to *Pseudobutyrivibrio ruminis* and 98% identity to *Butyrivibrio hungatei*, respectively, were found to be resistant to all four lichen acids, confirming that the reindeer rumen does host bacteria able to grow in the presence of these antibiotic secondary compounds. Representatives from four different phyla (*Verrucomicrobiota*, *Bacteriodetes*, *Proteopbacteria* and *Firmicutes*) were detected among the bacterial 16S rRNA gene sequences obtained from the DGGE gels. Bacterial numbers varied little between samples, ranging from 1.1×10^{11} to 9.8×10^{11} cells/g wet weight, while numbers of methanogenic archaea ranged from 1.5×10^{9} to 2.1×10^{10} cell numbers/g wet weight independent of the supplementation of usnic acid. Furthermore, the DGGE profiles did not reveal significant effects of the usnic acid treatment on rumen bacterial diversity, suggesting that lichen secondary compounds may be rapidly degraded by rumen microbes and consequently do not influence the dominant populations of rumen bacteria.

General discussion

Environmental resistance

Ampicillin is a semisynthetic β-lactam antibiotic that has been commercially used on a global scale since the 1960s (Palumbi 2001). The most common resistance mechanisms towards ampicillin is the production of β-lactamases encoded by bla_{TEM} alleles (Livermore 1995).

The key question addressed in this thesis work is the directionality of resistance emergence and dissemination. That is, is transferable antibiotic resistance emerging first in clinical and anthropogenic environments and then spreads to pristine environments, or is antibiotic resistance a naturally occurring trait in pristine environments that can transfer into clinically relevant microbes? Our tested hypothesis stated that there is a natural occurrence of bla_{TEM} alleles in microbial populations localised in environments with little human influence. These bla_{TEM} reservoirs could therefore contribute to resistance development in clinical settings.

The important feature of pristine environments is that they are not subjected to major anthropogenic influences. An environment can be described as the totality of circumstances surrounding and organism or group of organisms, especially, the combination of external physical conditions that affect and influence the growth, development, and survival of organisms (www.thefreedictionary.com). Owing to the movement of antibiotics, and antibiotic resistance genes in the environments, e.g. on wind and feathers, maybe it is unlikely that any environment can be considered truly pristine (Kallenborn *et al.* 2007). However, environments have a various degree of human influence, from hospitals with a strong antibiotic selection pressure to the Arctic considered to be one of the last outposts of wilderness with minimal human influence on the ecology of the antimicrobial resistance. It seems likely that e.g. soil contain antibiotic resistant bacteria, even in the absence of human selection pressure, because many soils contain low concentrations of compounds that select for resistance. For example, soil may be rich with microorganisms that produce β-lactam antibiotics, such as penicillin and cephalosporins (Martin and Liras 1989).

We found low levels of bla_{TEM} alleles in the environments examined: Arctic soils and sediments, and the gut of polar bear and Arctic and sub-Arctic seals. This was the case even in

agricultural soil from Tromsø and compost made of household waste, the environments expected to have the most human influence. Four amp^r isolates from polar bear rectal swabs carried *bla*$_{TEM}$ alleles. The isolates were identified as *E. coli* and the four alleles were identical and inserted in Tn3 transposons which are found in a wide variety of bacteria. Reports on antimicrobial resistance in Arctic environments are few, but resistance has been observed in Arctic birds. Eight percent of *E. coli* isolates were resistant and ampicillin resistance was one of the most frequent resistances observed (Sjölund *et al.* 2008). The mechanism of the ampicillin resistance was not examined. However, the birds in the region migrate from up to six different continents and thereby may have acquired drug-resistant bacteria during wintering or stop at lower latitudes. One *E. coli* isolate showed a resistance profile similar to a pattern common in clinical isolates supports the theory of introduction by migration and transfer of bacteria between birds (Sjölund *et al.* 2008). As described in the introduction, antibiotic resistant bacteria have been detected in a wide range of environments, including various soils, sediments, and animal gut. In remote soil in Alaska, antimicrobial resistance genes were detected through functional metagenomics. Allan and colleagues found diverse and ancient β-lactamases capable of conferring resistance to *E. coli* (Allen *et al.* 2008). However, none of the Alaskan soil β-lactamases were related to the *bla*$_{TEM}$ alleles investigated in our study. In the polar bear rectal swabs, we detected amp^r isolates with β-lactamase activity that were *bla*$_{TEM}$ PCR negative. A study on *bla* genes in agricultural and non-agricultural (prairie) soil in France showed that the non-agricultural soil had higher levels of amp^r bacteria (54.4% to 69.6%) than the agricultural soil (0.4% to 8.0%). The *bla*$_{TEM}$ alleles detected in the soil differed from alleles isolated in clinics. Two exceptions were *bla*$_{TEM}$ 116 and 157 which clustered together with the soil alleles, thus they may be of environmental origin (Fig. 3) (Demanèche *et al.* 2008).

It is well known that resistant bacteria with transferable antibiotic resistance are present in the gastrointestinal tract of farm animals. Also in the gut of wild animals, bacteria with transferable resistance have been found (Rolland *et al.* 1985; Gilliver *et al.* 1999; Osterblad *et al.* 2001; Sayah *et al.* 2005; Rwego *et al.* 2008; Sjölund *et al.* 2008; Kozak *et al.* 2009). It seems that the closer contact to human activities, the higher levels of resistance (Rolland *et al.* 1985; Rwego *et al.* 2008; Kozak *et al.* 2009). For instance, Kozak and colleagues (2009) found that bacteria isolated from wild small animals that lived in close proximity to food animal agriculture were more likely to carry resistance to antimicrobials than isolates from more natural environments. The same trend was observed between baboons

in their natural habitat with limited or no contact with humans and individuals living close to humans and daily contact with human waste (Rolland *et al.* 1985; Rwego *et al.* 2008). In rural England, 90% of bacterial isolates from mice and voles were resistant to β-lactam antibiotics, with more than 50% showing β-lactamase activity (Gilliver *et al.* 1999). In contrast to these results, faecal bacterial isolates from moose, deer and voles in Finland had almost no resistance (Osterblad *et al.* 2001). Finland is not as densely populated as England, and the load from agriculture is less. Thus, the animals in the finish study might be less influenced by human activities than the wild rodents in England. The trend that the closer contact with human activities increases the possibility of resistance is consistent with our findings of low levels of resistance in gut flora of polar bears and Arctic and sub-Arctic seals. Event though two of the seals in our study were captured close to the cost of northern Norway and we can not exclude an influence from human activities.

Even though we detected low levels of bla_{TEM} alleles in our study, some environments, like soil and gastrointestinal tract, are reservoirs of antibiotic resistant organisms and their associated genes, as describe above. A debate is going on whether these environmental reservoirs affect the pathogen microbiota. Most resistance genes in pathogen bacteria are acquired through horizontal gene transfer via mobile genetic elements as plasmids, and some of these plasmids have been found without antibiotic resistance elements before the antibiotics were introduced (Hughes and Datta 1983). During the large-scale antibiotic production era, more of these plasmids have become vectors of resistance genes. Despite the gaps in our knowledge, there are some indications of transfer of resistance genes from the environment to the clinic. As mentioned above, Demanèche and colleagues suggests that $bla_{\text{TEM-116}}$ and $bla_{\text{TEM-157}}$, which are found in clinical isolates, originate from the environment (Fig. 3) (Demanèche *et al.* 2008). Other scientists have found that the source of CTX-M, an extended-spectrum β-lactamase having a global negative impact on the treatment of infectious disease, is likely the environmental bacterial genus *Kluvyera*, particularly *K. ascorbata* and *K. georgiana* (Rodriguez *et al.* 2004). These examples link environmental resistance to the clinic and the strong anthropogenic selection pressure provide for a transmission and dissemination of resistance genes.

In conclusion, we found low prevalence of bla_{TEM} alleles in the soil, sediments and gastrointestinal tract of polar bear and seals, and this is in contrast to our hypothesis. However, we sampled only few environments and few animals were included. A screening on

a larger scale would give a better picture of the prevalence of resistance genes. We detected ampicillin resistant isolates showing β-lactamase activity (nitrocefin test). Thus, there might be other types of β-lactamases present, and functional metagenomic analysis of the samples could reveal the nature of these.

Bacterial diversity

In general, little is known about the microbiota of wild animals, and this is also the case in Arctic environments. The intestinal microbiota of Arctic mammals has rarely been examined for carriage of antimicrobial resistance genes, and little is known of these bacterial populations in general. We wanted to develop an understanding of the gastrointestinal microbiota of polar bear and seals. The polar bear microbiota consisted of members of the *Firmicutes* (Fig. 9) (Glad *et al.* 2010a). Another research group has looked for *Clostridia perfringens* isolates in faeces of polar bear on Svalbard, and they detected this species in 44% of the samples (Jores *et al.* 2008). Almost all clones in our study affiliated with the order Clostridiales, and the nearest relative (99.9% sequence identity) to the most abundant phylotype was *Clostridium perfringens*. In a study on captive polar bears, the faecal microbiota was found to be dominated by the facultative anaerobes *Enterobacteriaceae* and enterococci, and the *Clostridium* cluster I, that represents both *Proteobacteria* and *Firmicutes* (Schwab and Gänzle 2011). In Fig. 9, the findings in polar bear and seals are compared to human and pig intestinal microbiota. Most of the *Firmicutes* isolated from the human intestine were members of the *Clostridia* class (Eckburg *et al.* 2005).

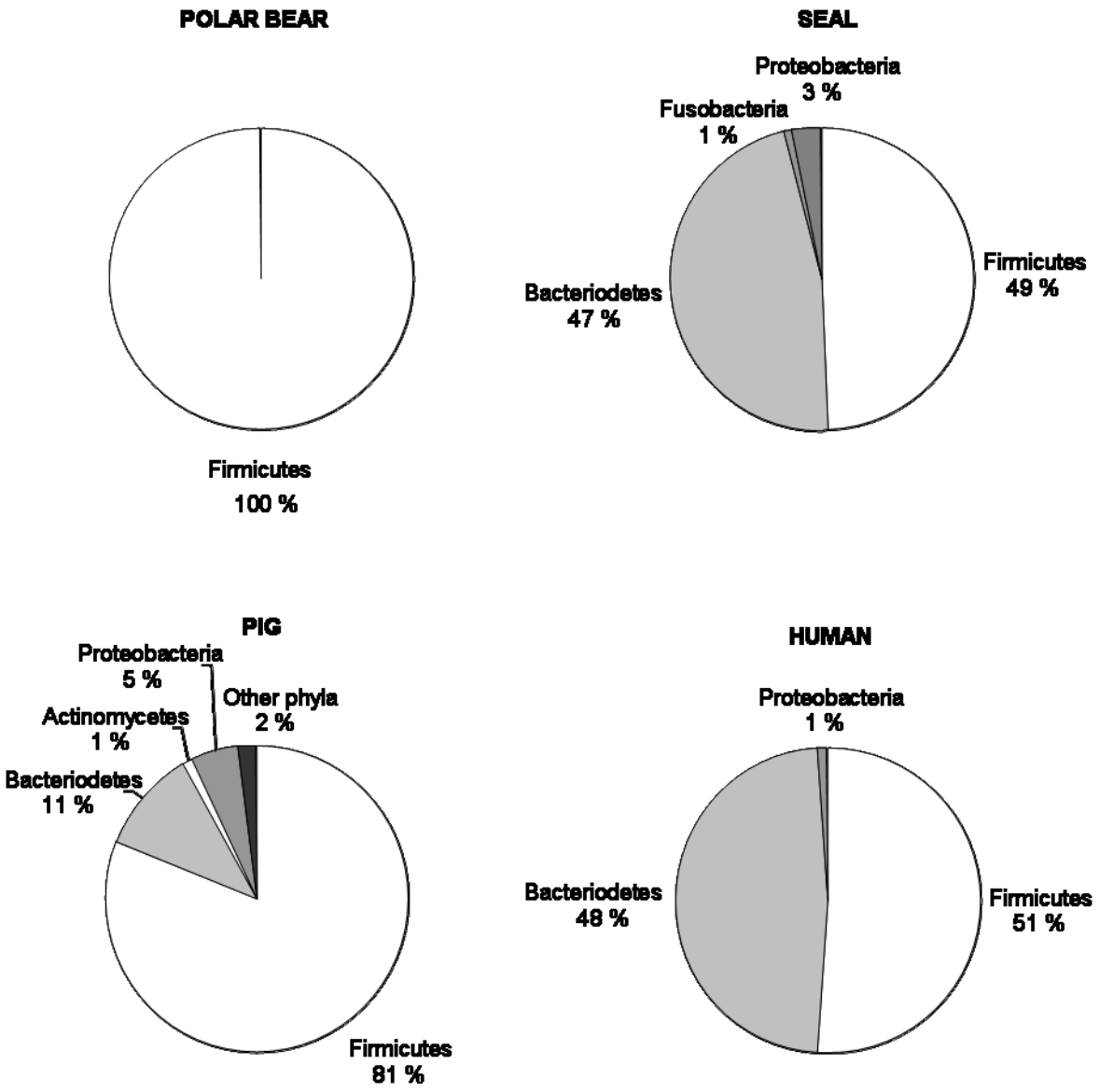

Fig. 9. Pie charts showing the distribution of sequence or phylotype affiliation in the faeces of polar bear (Glad *et al.* 2010a), colon content of seals (average % of clone sequence affiliation from three species of seal) (Glad *et al.* 2010b), ileum, cecum and colon content of pigs (Leser *et al.* 2002), and multiple colonic mucosal sites and faeces of humans (Eckburg *et al.* 2005).

The colon content from seals demonstrated a more diverse microbiota. The grey seal colon content was dominated by bacteria from the phylum *Firmicutes* (76%), the harbour seal of bacteria from both *Firmicutes* (50%) and *Bacteriodetes* (49%), and the harbour seals had a dominance of *Bacteriodetes* (68%). In Fig 9, the mean values of the percentage sequence affiliation for the three seal species are presented for comparative purposes. As discussed in

the paper, the limitations of this study is the small number of seals included (Glad *et al.* 2010b). Our result are consistent with findings by Ley *et al*. (2008) that carnivore have a lower microbial diversity in the gastrointestinal tract than omnivore and herbivores. Ley and colleagues state that both phylogeny and diet influence the composition of the intestinal microbiota.

When we compare the intestinal diversity data from polar bear with other bears there are some divergence. In the wild grizzly bear microbiota, both representative from the *Firmicutes* and *Proteobacteria* dominated. In captive grizzly bears, the *Enterobacteriaceae*, belonging to the *Proteobacteria* dominated. Even though placed in the order *Carnivora*, the grizzly bears living inland feed on plant material and only occasionally catch prey (Schwab *et al.* 2009). As members of *Proteobacteria* are well known to exist widely in the intestine of animals, we could have expected to find them in our study on polar bear as well. In giant pandas, also belonging to the order *Carnivora*, 13 phylotypes have been identified, and this low number is consistent with our results in polar bear. The phyla detected were the *Proteobacteria* (62%) and *Firmicutes* (38%) (Wei *et al.* 2007). The diet of the pandas is strictly vegetative, consisting of bamboo. However, they have a short (four times their own body length) and simple gastrointestinal tract that is characteristic of the carnivores. Most other herbivores have a more complex intestinal tract 10 to 22 times their own body length (Wei *et al.* 2007). Thus, it seems like the shared anatomy of the gut system of bears have a stronger influence on the composition of the intestinal microbiota than diet.

Our hypothesis was that polar bear and seals harbour a distinct diet-depending gastrointestinal microbiota. The phylogenetic analysis of the microbiota of faeces from polar bear does not support the hypothesis, as we detected only members of the phyla *Firmicutes*, order *Clostridiales*, which are prevalent also in the human gastrointestinal tract. Only 6% of the sequences were novel, showing <97% similarity to sequences representing the nearest cultivated relative. The low diversity can be linked to the small numbers of clones analysed, and a high throughput sequencing strategy would have revealed a higher diversity. Further, it seems like the anatomy of the gastrointestinal tract of bears has a stronger influence on the microbiota composition than diet. The seal colon content microbiota displayed a greater diversity then of the polar bear, the average distribution on a phylum level was similar to the one found in humans, even though there were differences between the seal species (Fig. 9).

81% of the sequences were novel, thus the seal microbiota were distinct from currently described microbial diversity.

Effect of usnic acid

Reindeer herding is affected by the global climate change as this influence the availability and quality of feed (Turunen *et al.* 2009). Current global climate models predict deeper snow cover, increasing rainfall, warm thawing and freezing cycles and a higher risk of ice layer formation on the soil and within the snow during the winter (Turunen *et al.* 2009). Developing appropriate methodologies for assessing the adaptive capacity, the vulnerability and the resilience of social-ecological systems to global changes remains a challenge (Tyler *et al.* 2007). Access to different pasture habitats and the availability of different forage plants can greatly affect the growth rate and survival of reindeer. The predicted increase in UV-B radiation is also likely to increase concentration of phenolic compounds such as tannins in plants and usnic acid in lichens eaten by reindeer (Nybakken and Julkunen-Tiitto 2006; Turunen *et al.* 2009).

A large and diverse microbiota such as the reindeer rumen may harbour microbes that can tolerate and detoxify plant secondary metabolites (Sundset *et al.* 2007; Sundset *et al.* 2008; Sundset *et al.* 2009a; 2009b). Our hypothesis was that usnic acid has an effect on the rumen microbial ecology. Reindeer were fed usnic acid supplemented feed and the rumen microbiota was investigated by DGGE profiling, revealing no effect after 9 and 17 days of treatment. This might be due to a rapid degradation of the usnic acid in the rumen by bacteria. Sundset and colleagues (2010) did not detect usnic acid in the rumen short time after the reindeer had been eating usnic acid containing feed, which supports this theory. The presence of usnic acid resistant isolates in the reindeer rumen, as demonstrated in this study and by Sundset *et al*. (2008), indicates that the rumen microorganism in these animals have adapted mechanisms to deal with this antibiotic and toxic compound. Genome sequencing and knock out studies of bacteria capable of growing in presence of usnic acid can reveal the mechanisms involved in the detoxification process that allow the utilization of lichens as a diet for reindeer.

General conclusions

This dissertation has sought to explore antibiotic resistance and bacterial diversity in Arctic environments, and the key question was the directionality of resistance emergence and dissemination. Our tested hypothesis stated that there is a natural occurrence of bla_{TEM} alleles, encoding resistance to ampicillin, in microbial populations localised in environments with little human influence. However, we found low levels of bla_{TEM} alleles in the environments examined. We know little about the microbes that constitutes the microbial environment in the gastrointestinal tract of wild Arctic animals. Studying the bacterial diversity in faeces of polar bear on Svalbard revealed an uncomplicated microbiota consisting of members of the phyla *Firmicutes*, order *Clostridiales*, with few 16S rRNA sequences being novel. The seal colon content microbiota displayed a greater diversity and was more distinct with 81% of the sequences being novel. The circumpolar reindeer has the ability to tolerate and probably also utilize usnic acid, a natural antibiotic found in lichen eaten by reindeer. We studied the potential effect of the usnic acid on the rumen microbiota of reindeer and the presence of usnic acid resistant isolates. No effect was detected by the methods used, which might be due to a rapid degradation of the usnic acid in the rumen by bacteria following intake. Rumen isolates that were able to grow in the presence of usnic acid were detected.

We conclude that even though low levels of bla_{TEM} alleles were detected in the Arctic environments examined in this thesis work, some environments (including soil and the gastrointestinal tract) have shown to be reservoirs of antibiotic resistant organisms and their associated genes. There is little knowledge about whether these environmental reservoirs affect the pathogen microbiota, but there have been some indications of transfer of resistance genes from the environment to the clinic. Antibiotic treatment is our primary method of treating infectious diseases, and more studies of the environmental resistance reservoirs are important to our future ability to fight infections.

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Paper I

Paper II

Paper III

Paper IV

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