AMPICILLIN RESISTANCE AND BACTERIAL DIVERSITY IN COLON CONTENT FROM GREY SEALS (*Halichoerus grypus*) AND HARBOUR SEALS (*Phoca vitulina*) AT THE COAST OF NORTHERN NORWAY

Thesis in Microbiology
for the degree Master of Pharmacy,
University of Tromsø, Norway
by
Vibeke Fam Kristiansen
2007

ACKNOWLEDGEMENTS

This work was conducted at the *Molecular Microbiology Laboratory*, Department of Pharmacology and Institute of Pharmacy and at the *Gut Microbiology Laboratory*, Department of Arctic Biology and Institute of Medical Biology, University of Tromsø. The study was supported by the Roald Amundsen Centre for Arctic Research (University of Tromsø) and the Norwegian Research Council.

The thesis supervisors were Professor Kaare M. Nielsen and Trine Glad, Department of Pharmacology and Institute of Pharmacy and Professor Monica A Sundset, Department of Arctic Biology and Institute of Medical Biology, University of Tromsø. Thanks to Kaare for encouragement and constructive guidance throughout the writing process. Thanks to Trine for giving me great insight into different molecular techniques, and for your advice and support during this year. A great thanks goes to Monica for your enthusiasm and for your helpful input during the writing of my thesis.

Thanks to my "lab partner" Pål B. for good company during this year and I also want to thank all the others at the microbiology lab.

A great thanks to Professor Karl-Arne Stokkan at the Department of Arctic Biology, University of Tromsø, for providing colon content collected from grey seals and harbour seals outside Ringvassøy in April-May 2006.

I would also like to thank Lill Hege Henriksen and the other personnel at the DNA Sequencing Laboratory at the University of Tromsø.

Finally; thanks to my amazing family and thanks to my dear Federico Pacitti for your love and for always being there for me, you mean everything to me. What should I have done without you?

Tromsø, May 2007
Vibeke Fam Kristiansen

ABSTRACT

Very little is known on the gut microbial ecology in seals. Environmental populations of bacteria, like those found in the gut of wildlife presumably unexposed to human antibiotic use, may be a reservoir of clinically important resistance genes. The purpose of this study was to characterize the bacterial diversity in the colon of grey seals (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*) at the cost of northern Norway by comparative sequence analysis of cloned 16S rRNA genes, and to determine the prevalence and diversity of bla_{TEM} genes. Colon contents was collected from one male harbour seal and one female grey seal (pregnant) outside Ringvassøy (69.91ºN, 19.02ºE) in April-May 2006. No aerobic ampicillin resistant isolates were detected in the colon content from neither the harbour seal nor the grey seal. However, bla_{TEM} alleles were detected in total DNA from tree of the eight colon samples of the grey seal, but no amplifications of the bla_{TEM} genes were obtained in total-DNA from the two colon samples of the harbour seal. This indicates that the prevalence of bla_{TEM} genes in the colon content of the harbour seal and the grey seal was low. A total of 153 assembled 16S rRNA gene sequences (~1,5 kb) were analyzed from the colon of the two seal species. From the harbour seal, 77 16S rRNA gene sequences were analyzed, identifying representatives associated with *Firmicutes* (all belonging to *Clostridiales* 49.4%), *Bacteroidetes* (all belonging to *Bacteroidales* 49.4%) and *Fusobacteria* (all belonging to *Fusobacteriales* 1.3%). From the grey seal, 76 16S rRNA gene sequences were obtained, including representatives from two bacterial phyla: *Firmicutes* (most of these were *Clostridiales* 72.4%) and *Bacteroidetes* (all belonging to *Bacteroidales*, 23.7%). The bacterial population in the colon of harbour seal and grey seal included species considered to be a part of the normal flora in e.g. humans and chickens. Only one clone from the harbour seal library showed >97% sequence similarity to their nearest database entries (BLAST). For the grey seal library about half of the clones showed <97% sequence similarity to their nearest database entries (BLAST). This indicates that several of the 16S rDNA sequences obtained from the seal colon represents novel bacterial species not yet isolated or characterized.

TABLE OF CONTENTS

1. INTRODUCTION

The aim of this study was (1) to contribute to a greater understanding of the distribution of antibiotic resistance genes (*Bla*$_{TEM}$-genes) in environments presumably unexposed to human antibiotic use, i.e. in seals for this thesis; and (2) to contribute to an increased understanding of the colon microbial ecosystem in harbour and grey seals.

1.1. Beta-lactam antibiotics

Different groups of antibiotics exist; substances within a group having similar mechanism of action. Beta-lactam antibiotics is one main group, and they all have in common a beta-lactam core structure which is essential for antimicrobial activity. All beta-lactam compounds consist of a four-membered beta-lactam ring containing three carbon atoms and one nitrogen atom (Mascaretti, 2003). The structure of their second ring allows these compounds to be classified into penicillins, cephalosporins, carbapenems or monobactams (Liras, 2006). Penicillin was first discovered in 1928, by Alexander Fleming, and this marked the start of the antibiotic era (Fleming, 1929). Penicillins, the most important group of antibiotics, can be divided into sub-groups according to their antimicrobial activity and resistance to beta-lactamases (Goodman and Gilman, 2001). Ampicillin, chosen for this study, is a broad-spectrum penicillin, meaning that it is effective against both Gram-positive and Gram-negative bacilli, but it is also susceptible to beta-lactamases (Kucers *et al*., 1997).

Beta-lactams act by inhibiting the synthesis of peptidoglycan, the last phase of bacterial cell wall synthesis (Tipper *et al.,* 1965). Peptidoglycan is an essential constituent of the bacterial cell wall. The cell wall provides rigid mechanical stability to the bacterium and is essential for bacterial growth and development. Dipeptid D-alanyl-D-alanin is the target for the transpeptidase, an enzyme that catalyzes the last cross-reaction in the synthesis of peptidoglycan. Beta-lactams act as a structural analog of the dipeptid D-alanyl-D-alanin and thus irreversible inhibit the transpeptidase (Yocum *et al*., 1979). The loss of enzymatic activities through beta-lactam activity disrupts the integrity of the cell wall, leading to lethal cell wall defects (Fisher *et al.,* 2005). The members of the genus *Chlamydia* have no measurable peptidoglycan, but are also susceptible to beta-lactams as peptidoglycan-containing bacteria. This shows that the complete mechanism of the beta-lactams are not yet fully understood (Moulder, 1993).

1.2. Beta-lactamases

Beta-lactams constitute the largest family of antimicrobial agents and the most extensively used in current clinical practice, but the progressive emergence of acquired resistance has limited the empirical use of beta-lactams and their efficacy in certain situations (Marin, 2003). Resistance to antimicrobials may arise through the mutation of normal cellular genes, the acquisition of foreign resistance genes (via conjugation, transduction, transformation or transposition), or a combination of these two mechanisms (Harbottle, 2006). Chromosomal-based determinants account for less than 5 % of acquired antibiotic resistance (Nwosu, 2001).

Resistance against beta-lactam antibiotics arise via alteration of the target site (e.g. Penicillin binding proteins), reduction of drug permeation across the bacterial membrane (e.g. efflux pumps) and production of beta-lactamase enzymes (Majiduddin *et al.,* 2002). The presence of the beta-lactamase enzymes is the most common cause of bacterial resistance to beta-lactam antimicrobial agents (Livermore, 1995, Majiduddin *et al.,* 2002). The beta-lactamase enzymes inactivate beta-lactam antibiotics by hydrolyzing the peptide bond of the characteristic four-membered beta-lactam ring rendering the antibiotic ineffective (Majiduddin *et al.,* 2002). Alteration of penicillin binding proteins are the most important resistance mechanism among Gram-positive bacteria, but beta-lactamases are most prevalent in Gram-negative species (Livermore, 1998, Philippon *et al.*, 2002, Jacoby *et al.*, 2005).

1.2.1. Extended-spectrum beta-lactamases

The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against beta-lactamase-mediated resistance to antibiotics, since they were effective against most beta-lactamase-producing organisms (Paterson *et al.* 2005). However, beta-lactamases which had the ability to confer resistance to the extended-spectrum cephalosporins were soon discovered (Knothe *et al.*, 1983). These new beta-lactamases were coined extended-spectrum beta-lactamases (ESBLs). ESBLs are a rapidly evolving group of beta-lactamases which can confer bacterial resistance against the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by beta-lactamase inhibitors such as clavulanic acid (Paterson *et al.,* 2005). Most ESBLs are derivatives of TEM or SHV enzymes (Jacoby *et al*., 1991, Bush *et al*., 1995) and they are most often found in *Escherichia coli* and *Klebsiella pneumoniae* (Bradford, 2001).

The spread of ESBLs are one of the most serious threats to the usage of beta-lactam antibiotics (Jones *et al.*, 1998, Bradford, 2001, Shah *et al.*, 2004, Jacoby *et al.*, 2005).

1.2.2. *bla*$_{TEM}$ genes

There are different types of resistance genes coding for beta-lactamases, among them are the commonly occurring and clinically important *bla*$_{TEM}$ genes (Jacoby *et al.*, 2005, Livermoore, 1998). The classification system for the beta-lactamases that is most accepted today is the Ambler molecular classification that divides beta-lactamases into four major classes, designated A to D. All the beta-lactamases in the TEM family belongs to class A, which is the largest serine beta-lactamase class (Fisher *et al.*, 2005).

The TEM enzyme was first reported in 1965 from an *E. coli* isolate from a patient in Greece, named Temoneira (hence the designation TEM) (Datta *et al.*, 1965). Within a few years after its first isolation, the TEM-1 beta-lactamase spread worldwide and is now found in many different species of members of the family *Enterobacteriaceae*, and the species *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. TEM-1 is the most commonly encountered beta-lactamase in Gram-negative bacteria (Bradford, 2001). As of May 2007, the *bla*$_{TEM}$ group consist of about 150 different alleles, all encoding different amino acid polymorphisms that extend their substrate range (http://www.lahey.org/Studies/).

1.2.3. *Bla*$_{TEM}$-genes in natural environments

Not many studies have been done with regards to the distribution of *bla*$_{TEM}$-genes in the environment. Lorenzo *et al.* (unpublished) found a low prevalence of *bla*$_{TEM}$-genes when resistance genes in arctic and agricultural soil and rhizosphere were examined. A study by Gilliver *et al.* (1999) found that antibiotic resistance was very prevalent in populations of wild rodents living in northwest England. Overall, 90% of the isolates were ampicillin resistant, as a result of beta-lactamase expression in more than half the cases. Österblad *et al.* (1999) tested the faeces of moose, deer and vole in Finland and they found an almost complete absence of resistance in enterobacteria. None of the isolates were resistant towards ampicillin. The resistance found was to cefuroxime, and only one strain contained a TEM-type enzyme. These results disagree with those from the study of enterobacteria from english rodents, so it might be that the wild rodents are more exposed to antibiotics than the wild animals in Finland (Österblad *et al.*, 1999).

Cultivation will be used in this project to determine the prevalence of ampicillin resistant bacteria in the colon content of the two seal species. PCR will be used to determine

the prevalence and diversity of *bla*$_{TEM}$ genes in the total DNA extracted from the colon content of the two seal species.

1.3. Seals

Seals live mostly in the water, but they go on land to give birth in early spring and when they moult each summer. Depending on species, the pup is nursed for a period of a few days to several weeks. After breeding, the seals go to sea and fatten up and then hault out again to moult for a period of few weeks. During this period the seals fast and live off their body reserves. This results in dramatic seasonal changes in body fat. Seals show a high degree of fatness during winter, but lose weight during the moult in summer and stay lean until the autumn. They are carnivours animals, eating fish, squid and invertebrates (Blix, 2005). Seals have a typical carnivorous single stomach with a small intestine, rudimentary caecum and short colon (Olsen *et al.*, 1996). The length of the small intestine differs greatly among different species (from 5-25 times body length) and the reason for this is presently unknown (Mårtensson *et al.*, 1998).

The suborder of seals (*Pinnipedia*) is divided into three families: true seal (*Phocidae*), eared seals (*Otaridae*) and walrus (*Odobenidae*). No species of the eared seal family exist in Norwegian or adjacent waters. There is only one species in the walrus family that is arctic and it exists around the whole Nordkalotten. The true seals are divided into 3 subfamilies based on differences in teeth and skeleton-anatomy: *Phocinae*, *Monachinae* and *Cystophorinae*. Grey seals *(Halichoerus grypus)* and harbour seals *(Phoca vitulina)* that are included in this study, both belong to the *Phocinae* subfamily (Haug *et al.*, 1998).

1.3.1. Grey seals

The grey seals exist of both sides of the North-Atlantic. On the east side, they are distributed from Biscaya in the south to the Kola-coast in the north, while on the Norwegian coast they exist from Sør-Trøndelag to Finnmark. The male seals can be up to 230 cm long and 310 kg; the female seals being considerable smaller. The male grey seals are sexually mature at an age of 6-7 years; the female grey seals when they are 5 years old. Sexually mature females give birth to a pup each year in September-December. After 2-3 weeks, the pup is abandoned by the mother who goes off to breed with the males. The grey seals eat mainly fish naturally occurring at the coast line (Haug *et al.*, 1998).

1.3.2. Harbour seals

The harbour seals exist in northern parts of the Pacific Ocean and the Atlantic Ocean, along the whole Norwegian coast, on Kola and on the west side of Svalbard. They are relatively stationary through the year. The male seals can be up to 155 cm long and 100 kg; the female seals being a bit smaller. The harbour seals are sexually mature at an age of 4 years and sexually mature females gets a pup every year in June-July (Haug *et al.*, 1998). Harbour seals swim the day they are born and dive at age 2-3 days (Blix, 2005). The pups are nursed for a period of about 3 weeks. The harbour seals eat mainly fish; herring (*Clupea harengus*), Norway pout (*Trisopterus esmarkii)*, sand eels (*Ammodyt*es *tobianus)*, saithe (*Pollachius virens*) and Norway redfish (*Sebastes viviparus*)**.** They also eat flying squid (*Ommastrephes sagittatus*) and shellfish. The choice of food varies considerable between the seasons (Haug *et al.*, 1998).

1.3.3. Microbial diversity in seal

I am not aware of any studies done that have investigated neither the prevalence of bla_{TEM} genes nor the diversity of the microbial flora in colon content of seals. Previously studies done on seals have focused on analyzes of pathogenetic bacteria (isolated from various sites of stranded seals that were examined for bacterial infections) and their antimicrobial resistance patterns (Lockwood *et al.*, 2003, Johnson *et al.*, 1998, Thornton *et al.* 1995).

Until the 1980s, the determination of microbial community structure and the identification of microorganisms in environmental samples depended on culture-based studies, known to be biased as only a small part of a microbial community is accessed (Amann *et al.*, 1995). PCR (Polymerase Chain Reaction) and DNA sequence analysis are two major technological developments that have revolutionized microbial ecology and have permitted culture-independent characterization of microbial communities. In this thesis, these two molecular techniques will be used in the characterization of the bacterial diversity in colon content from grey seals and harbour seals in the Norwegian Sea.

2. OBJECTIVES

The objectives of this project were (1) to determine the total *colony forming units* (cfu) and the ampicillin resistance cfu in colon content from grey seals and harbour seals by cultivation, and if ampicillin resistant isolates were found, determine the proportion of beta-lactamase producers by the nitrocefin test; (2) to determine the prevalence and diversity of bla_{TEM} genes in the total DNA extracted from the colon content of both seal species, by PCR, agarose gel electrophoresis and DNA sequencing; (3) to characterize the bacterial diversity in the colon of grey seals and harbour seals by comparative sequence analysis of cloned 16S ribosomal RNA (rRNA) genes amplified from total DNA extracted from the colon content of both seal species, and to visualize the phylogentic relationships among the 16S rRNA sequences by constructing a phylogenetic tree based on bioinformatic analysis of the two bacterial 16S rRNA gene libraries.

3. MATERIALS AND METHODS

3.1 Collection of samples from seals

Colon contents was collected from one harbour seal and one grey seal outside Ringvassøy (69.91ºN, 19.02ºE) in April-May 2006 by Prof. Karl-Arne Stokkan, Department of Artic Biology, University of Tromsø (Table 1). The samples were collected immediately after the animals were killed, kept on ice during transport to the laboratory and within 24 hours transferred to -20°C.

Table 1. Data on the harbour seal (*Poca vitulina*) and the grey seal (*Halichoerus grypus*) harvested outside Ringvassøy, Troms County.

Species	Sex	Date collection	Comments
Harbour seal (n=1)	male	28.04.2006	Small individual
Grey seal (n=1)	female	19.05.2006	Pregnant

3.2 Determination of cfu

The colon content samples were taken from the freezer and cut in two pieces with a sterile scalpel. One half was used for cfu determination, and the other half was put back into the freezer for later isolation of total DNA.

One gram of colon content (fresh weight) was transferred to a test tube containing 9 mL 0,9 % NaCl and steam sterilized glass-pearls. The suspension was vortexed for 1 minute. Series of 10-fold dilutions were made to ensure that the plates had 15-300 colonies each. Three parallel dilutions were made of each sample. 100 µL of each dilution was spread onto three Chocolate agar plates (GC-Agar Base Medium, Oxoid CM367B, water, 5% defibrinated horseblood, 25 % Glucose solution, Vitox, Oxoid SR 090 H) with and without 50 µg/mL ampicillin. The dilution and plating was done on the bench in the laboratory using aseptic working technique. All cfu counts are given as cfu/g fresh weight.

The samples were incubated at 37°C for 72 hours. Colonies on the Chocolate agar plates with and without 50 µg/mL ampicillin were counted. Some colonies were picked, purified and stored in a solution containing 3% Brain Heart Broth (Fluka) and 16% glycerol (Merck) at
-70ºC.

3.3 PCR

3.3.1 DNA extraction

3.3.1.1 Environmental samples

A sub-sample (180-220 mg) of colon content was transferred to a test tube. DNA was extracted by using a QlAmp® DNA Stool Mini Kit from Qiagen, following the instructions in the "Protocol for Isolation of DNA from Stool For Pathogen Detection". The DNA was stored frozen at -20°C.

3.3.1.2 Single bacterial colonies

Template DNA was made by dissolving one pure colony in a test tube containing 100 µL deionised distilled sterilized H_2O. The samples were heated at 100°C for 10 minutes, and then spun down at 9400 g for 5 minutes. The supernatants were transferred to a new test tube (Glad *et al.*, 2001).

3.3.2 Quantification of DNA

The concentration of DNA obtained by using the Stool Mini Kit, was measured using a Nano Drop® ND-1000 Spectrophotometer. The wavelength used to estimate the nucleic acid content in the samples was 260 nm; the wavelength of light maximally absorbed by nucleic acids. The ratio of absorbance at 260 and 280 nm (260/280) was used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of contaminants.

3.3.3 PCR amplification

A 16S rRNA control PCR was set up to make sure that the DNA could be amplified. For the PCR, 1 µl template was added to 24 µl PCR mix containing 12,5 µl HotStar Mastermix (HotStarTaq Master Mix, Qiagen), 10 µl dH_2O (Qiagen), 0,75 µl 0,3 mM forward primer, 27 F (Sigma) and 0,75 µl 0,3 mM reverse primer, 1494 R (Sigma) (Table 2).

The 16S rRNA control PCR was run on a Peltier Thermal Cycler-200 (MJ Research) programmed as follows: an initial temperature at 94 °C for 15 min to activate the HotStar Taq DNA Polymerase, followed by 5 cycles of denaturation at 94 °C for 4 min, annealing at 50 °C for 45 sec, and polymerization at 72 °C for 1 min, followed by 30 cycles of denaturation at 92 °C for 45 sec, annealing at 55 °C for 45 sec, and polymerization at 72 °C for 1 min. The polymerization was completed by an additional 7 min of incubation at 72 °C.

To detect *bla*$_{TEM}$-genes, 4 µl template was added to 56 µl PCR reaction mix containing 30 µl HotStar Mastermix (HotStarTaq Master Mix, Qiagen), 23,6 µl dH_2O (Qiagen), 1,2 µl *Bla*$_{TEM}$ forward primer (Sigma), 1,2 µl *Bla*$_{TEM}$ reverse primer (Sigma) (Table 2). The primers used for *bla*$_{TEM}$-detection have been reported to bind to all TEM-alleles (Brusetti, 2004, Poirel *et al*., 1999).

Table 2. PCR primers employed in this study

Primer	Sequence [a]
Bla$_{TEM}$ F	CAT TCC CGT GTC GCC CTT ATT CC
Bla$_{TEM}$ R	GGC ACC TAT CTC AGC GAT CTG TCT A
16S rRNA 27 F	AGA GTT TGA TCC TGG CTC AG
16S rRNA 1494 R	CTA CGG CTA CCT TGT TAC GA
M13 F	GTA AAA CGA CGG CCA G
M13 R	CAG GAA ACA GCT ATG AC
338Bact	ACT CCT ACG GGA GGC AGC

[a] Sequences given in 5' to 3' direction.

The amplifications were carried out with a Peltier Thermal Cycler-200 (MJ Research) programmed as follows: an initial temperature at 95 °C for 15 min, followed by 33 cycles of denaturation at 95 °C for 1 min, annealing at 61 °C for 1 min, and polymerization at 72°C for 1 min. The polymerization was completed by an additional 10 min of incubation at 72 °C. The positive controls used were *E. coli* with TEM-3, TEM-6, TEM-9 and TEM-10 (provided by A. Sundsfjord, the National Competence Center for Antibiotic Resistance Detection, University Hospital of North-Norway, Tromsø). Water and *E. coli* with SHV 2 (provided by A. Sundsfjord) were used as a negative control.

For the 16S rRNA clone library PCR, 2 µl template was added to 48 µl PCR reaction mix containing 25 µl HotStar Mastermix (HotStarTaq Master Mix Kit, Qiagen), 20 µl dH_2O (Qiagen), 1,5 µl 0,3 mM primer 27 F and 1,5 µl 0,3 mM primer 1494 R (Table 2). The 16S rRNA genes were amplified using universal primers (Weisburg *et al*., 1991, Urzi *et al*., 2001) that should amplify most bacterial DNA. The PCR for the 16S rRNA clone library was performed using a Peltier Thermal Cycler-200 (MJ Research) programmed as follows: an initial temperature at 94 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and polymerization at 72 °C for 2 min. The polymerization was completed by an additional 3 min of incubation at 72 °C.

3.3.4 Agarose gel electrophoresis

The PCR product was analysed by gel electrophoresis using a 1 % agarose gel. The gel was prepared by boiling 1,0 g agarose powder (Cambrex) with 100 ml of TBE buffer (0,01 M Tris-borate, 0,02 M EDTA, pH 8,0) in a microwave oven and placing the solution on a casting tray containing 4 µl 10 mg/ml Ethidium Bromide (Gibco BRL and Sigma). When set, the gel was placed in the electrophoresis chamber filled with TBE buffer. Each PCR reaction (15 µl) was mixed with 3 µl loading buffer (40 % sucrose + bromophenol blue 0,25 % w/v) and loaded onto the gel. 5 µl of 1 Kb Plus DNA Ladder (Invitrogen) was used as a size marker. A BioRad Power Pac 200 or 300 was used for running the gel at 90 V for approximately 1 hour. The gel was visualized and images documented using a GelDoc 2000 Transilluminator and the program Quantity One GelDoc (BioRad).

3.4 DNA sequencing

3.4.1 Cloning and transformation

First, different samples from the same seal were pooled after DNA extraction and PCR amplification, then the cloning reaction and the transformation was performed according to the instruction manual of the TOPO TA Cloning® Kit for Sequencing (Invitrogen). PCR products were cloned into the vector pCR4-TOPO (Invitrogen) and transformed into chemically competent *E. coli* TOP10 (Invitrogen). Clones were plated onto LB-Agar (Fluka) containing ampicillin (Roche and Calbiochem) 100 µg/mL and X-gal 40 µg/mL. As a transformation control the pUC19 plasmid (Invitrogen) was included to check the transformation efficiency of the competent cells. The samples were incubated over night at 37°C. Single positive clones were picked, and streaked out on LB (Fluka) plates containing 50 µg/mL ampicillin (Roche and Calbiochem). Purified colonies were inoculated into 0,85 mL of LBB (Invitrogen) containing 100 µg/mL ampicillin. The culture was grown over night in a shaking incubator at 37°C. 0,15 mL of sterile glycerol (Merck) was added to each culture the next day and then stored at -80°C.

3.4.2 Plasmid extraction

Purified colonies were cultured overnight in 3 mL Luria Broth Base (Invitrogen) medium containing 100 µg/mL ampicillin. The culture was grown overnight in a shaking incubator at 37°C. The bacterial cells were harvested by centrifugation at > 6800 g in a table-top microcentrifuge for 3 minutes at room temperature. For plasmid isolation the QIAprep Spin Miniprep Kit from Qiagen was used. The instructions according to the "Protocol for plasmid DNA purification using the QIAprep spin miniprep kit and a microsentrifuge" were followed. The plasmids were analyzed for inserts by agarose gel electrophoresis as described in section 3.3.4. 5 µl of each sample was mixed with 1 µl loading buffer (40 % sucrose + bromophenol blue 0,25 % w/v) and loaded onto the gel.

3.4.3 Cycle sequencing reaction mix

The concentration of extracted plasmid DNA was determined spectrophotometrically with a Nano Drop® ND-1000 Spectrophotometer (260 nm). Between 150-300 ng of the template DNA was used in each cycle sequencing reaction. Template was added to a mix containing 2.0 µL BigDye v3.1 (Applied Biosystems), 3,0 µL 5 x Sequencing Buffer (Applied

Biosystems) and 3,2 µL 1 µM primer. Water was added to get a total volume of 20 µL. 3 different primers were used, M13F (Invitrogen), M13R (Invitrogen) and Bact338 (OPERON Biotechnologies), added to separate sequencing reactions (Table 2). Reactions were carried out with a Peltier Thermal Cycler-200 (MJ Research) programmed as follows: an initial denaturation at 96° C for 3 minutes, followed by 26 cycles of denaturation at 96°C for 15 sec, annealing at 50°C for 15 sec and polymerization at 60°C for 4 min. The sequencing was performed on a 2130xl Genetic Analyzer and performed by personnel at the DNA Sequencing laboratory at the University of Tromsø, who also precipitated the sequencing reactions. The resulting sequences were assembled using SeqManTMII v.5.05 (DNASTAR Inc.).

3.4.4 DNA sequence analysis

3.4.4.1 Phylogenetic analysis

The sequences were initially compared with sequences obtained from the Ribosomal Database Project II Release 9.24 and then subsequently through BLAST (Basic Local Alignment Search Tool) (Altschul *et al.,* 1997) at the National Center for Biotechnology Information (NCBI) web site. The 16S rRNA gene sequences were automatically aligned (multiple sequence alignment) by CLUSTAL W in the software package BioEdit v. 7.0.5.3 to give a uniform length. The phylogenetic analyses were performed using the neighbor-joining method (Saito *et al.,* 1987) with a Kimura-2 correction in the software MEGA v.3.1. Statistical significance of branching was verified by bootstrapping (Felsenstein, 1985) involving construction and analysis of 1000 trees from the data set in the software MEGA.

3.4.4.2. Diversity analysis

The clones generated from the seal colons were assigned to operational taxonomic units (OTU) based on a 97% sequence identity criterion. Standard diversity and richness indices, including the Shannon Index (Shannon *et al.,* 1949) and the Chao1 Index (Chao, 1984), were calculated using the FastGroupII web-based bioinformatics platform for analyses of 16S rRNA gene based libraries after trimming the 5' end without N in the first 10 base pairs and the 3' end with Bact (517-534) of 70% similarity (Yu *et al.,* 2006).

4. RESULTS

4.1 Determination of cfu

The results from the cultivation of colon aerobic bacteria, on Chocolate agar plates with and without ampicillin, are given in Table 3.

Table 3. Colony counts in frozen colon contents from the harbour seal and the grey seal in culture media with and without ampicillin.

Sample	Total counts cfu/g	Amp^r counts cfu/g	Amp^r fraction (%)
Harbour seal	1.6 (+/- 0,2) x 10^5	< 11.1	< 0.01
Grey seal	2.1 (+/- 0,1) x 10^4	< 11.1	< 0.05

4.2 PCR

4.2.1 Quantification of DNA

DNA was extracted from the colon contents of the harbour seal and the grey seal using the Mini Kit from Qiagen and ranged between 1.8-102.3 ng/µL in a total volume of 200 µL.

4.2.2 Amplification of the 16S rRNA gene

The 16S rRNA PCR showed that all the eight extracted DNA products (total-DNA extracted from the colon of the harbour seal and the grey seal, in addition to six bacterial isolates from the grey seal) were PCR-amplifiable. Fig. 1 shows a picture of the results from the 16S rRNA control PCR.

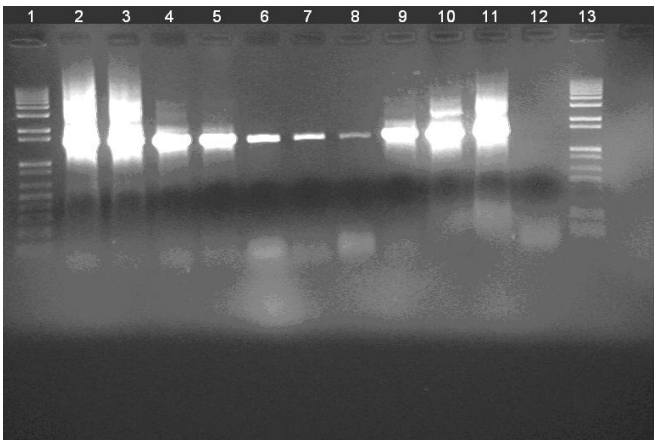


Fig. 1. The agarose gel showing the products from the 16S rRNA control PCR. Well 2 and 3 contains total-DNA extracted from the colon content of the harbour seal and the grey seal, respectively. Well 4-9 contains genomic DNA of six various single colon bacterial isolates from the grey seal. Well 10 and 11 contains positive controls; *E. coli* with TEM 9 and TEM 10, respectively. Water in well 12 was used as a negative control. Lane 1 and 13 contains the ladder (1 Kb Plus DNA Ladder, Invitrogen).

4.2.3 *Bla*$_{TEM}$ amplification

Total-DNA extracted from 11 environmental samples (2 colon samples from the harbour seal and 9 colon samples from the grey seal) were screened for the presence of *bla*$_{TEM}$-genes. Fig. 2 shows a picture of one of the agarose gels ran to detect *bla*$_{TEM}$-genes. Positive signals are observed in lane 5, 7 and 13, all belonging to the grey seal. No amplifications of the *bla*$_{TEM}$ genes were obtained in total-DNA from colon samples of the harbour seal.

Fig. 2. The agarose gel showing the products from the *bla*$_{TEM}$ PCR. Well 3 and 4 contains parallel PCR products from the harbour seal. Well 5-13 contains parallel PCR products from the grey seal. Water and *E. coli* with SHV 2 in well 1 and 20 were used as negative controls. Well 15-18 contains positive controls; *E. coli* with TEM 3, TEM 6, TEM 9 and TEM 10, respectively. Lane 2 and 19 contains the ladder (1 Kb Plus DNA Ladder, Invitrogen). Well 14 is empty.

Table 4. Colon bacterial diversity (% of clones) based on 16S rRNA clone libraries from the grey seal (*Halichoerus grypus*) and harbour seal (*Poca vitulina*)

Library (*n* = numbers of clones)	**Phylum (%)**		
	Firmicutes	**Bacteroidetes**	**Fusobacteria**
Harbour seal (CSH) (n = 77)	49.4	49.3	1.3
Grey seal (CSG) (n = 76)	76.3	23.7	0

4.3 DNA sequencing

A total of 459 16S rRNA gene sequences (~1.5 kb length) were assembled and analyzed from the colon of the two seal species. From the harbour seal, 77 16S rRNA gene sequences were analyzed identifying representatives associated with *Firmicutes* (all belonging to *Clostridiales* 49.4%), *Bacteroidetes* (all belonging to *Bacteroidales* 49.4%) and *Fusobacteria* (all belonging to *Fusobacteriales* 1.3%) (Table 4, Fig. 3). The coverage of the harbour seal library was 66% (Table 5). Of these only one clone showed >97% sequence similarity to their nearest database entries (BLAST). From the 77 clones in the harbour seal library, 39 distinct OTUs were identified.

From the grey seal, 76 16S rRNA gene sequences were obtained, including representatives from two bacterial phyla: *Firmicutes* (most of these were *Clostridiales* 72.4%)

and *Bacteroidetes* (all belonging to *Bacteroidales,* 23.7%) (Table 4, Fig. 3). The coverage of the grey seal library was 84%, and about half of the clones showed <97% sequence similarity to their nearest database entries (BLAST) (Table 5). From the 76 clones in the grey seal library, 27 distinct operational taxonomic units (OTUs) were identified. For the harbour seal library, 97 OTUs was predicted by the Chao1 nonparametric estimator approach, and 39 OTUs for the grey seal library (Table 5). The Shannon Index, a measure of diversity, was highest for the harbour seal library with 3.25, while the diversity value for the grey seal library was 2.91 (Table 5).

Table 5. Diversity indices of the bacterial clone library for the harbour seal and the grey seal calculated by the FastGroupII web-based bioinformatic platform (Yu *et al*. 2006)

Library	Valid sequences (n)	OTUs (n)[a]	Coverage (%)[b]	Chao1[c]	Shannon Index[d]	Novel strains (%)[e]
Harbour seal (CSH)	77	39	66	97	3.25	98.7
Grey seal (CSG)	76	27	84	39	2.91	51.3

[a] Operational taxonomic units based on the 97% sequence identity criterion; [b] The coverage of the clone library was calculated with the formula [1-n/N] as described by Good (1953) where *n* is the number of phylotypes represented by one clone and *N* is the total number of clones; [c] Chao1 is a nonparametric estimator of the minimum richness (i.e., number of ribotypes) in a sample. It is based on the number of rare ribotypes (singletons and doublets) and used to predict the total number of OTU present (the species richness); [d] The Shannon Index is a nonparametric diversity index that combines estimates of richness (total numbers of ribotypes) and evenness (relative abundance of each ribotype) indicating diversity. It takes into account the abundance of individual taxa and can be used as an overall indicator of the level of diversity in a sample; [e] Percentage of novel strains with <97% identity to any cultured bacterial sequence or clone sequences published in the public database.

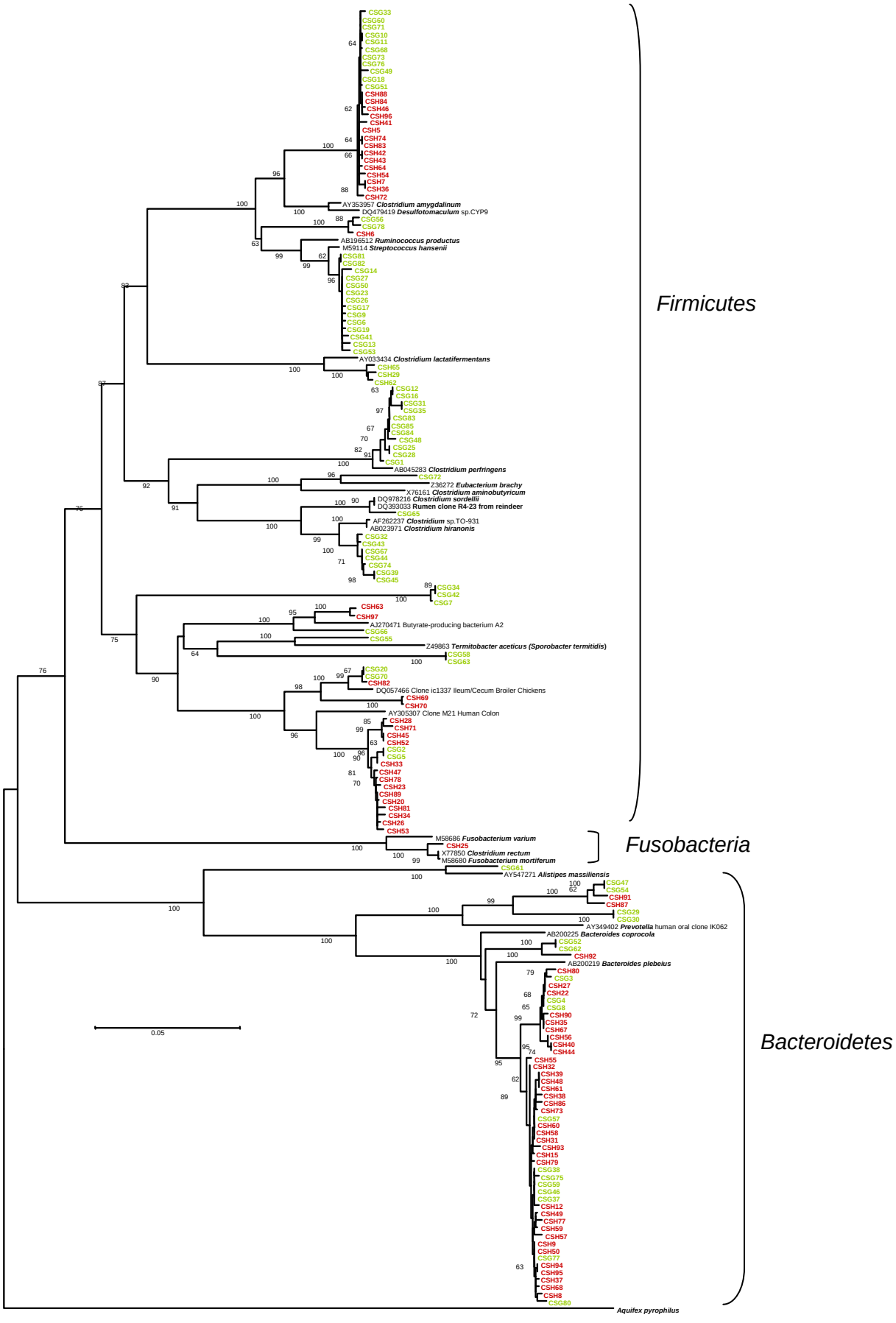
Fig. 3 shows the phylogenetic relationships among the 16S rRNA sequences from both seal species. A cluster of totally 46 clones, with representatives from both seals, were related to *Bacteroides plebeius* previously isolated from human faeces. The clones CSH 92, CSG 52 and 62 were 93-94% similar to *Bacteroides coprocola* previously isolated from human faeces. A cluster of 6 clones (CSG 29, 30, 47, 54 and CSH 87, 91) were 90-91% similar to *Prevotella,* which are members of the human oral flora. Only one clone (CSG 61) was closely related to *Alistipes massiliensis* previously isolated from human sample, with 95% sequence identity.

One clone (CSH 25) belonged to the phylum *Fusobacteria* and was closest related to *Clostridium rectum* with 97% sequence identity.

A cluster of 26 clones, represented from both seal species, were related to *Clostridium amygdalinum*. Three clones, CSG 56, CSG 78 and CSH 6, clustered together and were similar to only uncultured bacterial clones. A group of 14 clones, all belonging to the grey seal, was related to *Ruminococcus productus*. Three clones from the harbour seal formed a cluster with

a sequence identity value of 96% to *Clostridium lactatifermentans*, isolated from the caeca of a chicken. Only clones from the grey seal, in total 11 clones, were related to *Clostridium perfringens*. A single clone (CSG 72) was found with *Eubacterium brachy* as the closest relative, but at a sequence identity of only 92%. *Clostridium sordellii* was the closest relative to one single clone from the grey seal (CSG 65), with 98% sequence identity. A cluster of seven clones, all from the grey seal, were 97% similar to *Clostridium* sp. TO-931. A group of three clones from the grey seal (CSG 7, 34 and 42) had no culture representatives. CSH 63, 97 and CSG 66 were closest related to butyrate-producing bacterium A2-207 from the human gut, with sequence identity values from 94 to 96%. One clone (CSG 55) was most closely related to *Sporobacter termitidis*, isolated from the digestive tract of a wood-eating termite; however, the sequence identity was only 92%. Two clones from the grey seal (CSG 58 and 63) were similar to only uncultured bacterial clones. Bacterium ic1337, isolated from the ileum and cecum of broiler chickens, was the closest relative to five clones (CSH 69, 70, 82 and CSG 20, 70), with sequence identity values from 93 to 97%. A group of 16 clones, belonging to both seal species, were closest related to Butyrate-producing bacterium M21 from the human colon.

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences from the colon contents of one harbour seal (CSH, red text) and one grey seal (CSG, green text) (edited to a uniform length of 1341 bp) associated with *Firmicutes*, *Fusobacteria* and *Bacteroidetes.* The tree was constructed using the neighbor-joining method with the Kimura-2 parameter model for nucleotide change. Statistical significance of branching was verified by bootstrapping (Felsenstein 1985) involving construction and analysis of 1000 trees from the data set. Bootstrap values > 60% are shown near nodes. The *Aquifex pyrophilus* sequence was used as an outgroup for rooting the tree. The scale bar represents a 5% sequence divergence, and reference sequences were obtained from the GenBank Database.


CSG33
CSG60
CSG71
CSG10
CSG11
CSG68
CSG73
CSG76
CSG49
CSG18
CSG51
CSH88
CSH84
CSH46
CSH96
CSH41
CSH5
CSH74
CSH83
CSH42
CSH43
CSH64
CSH54
CSH7
CSH36
CSH72
AY353967 Clostridium amygdalinum
DQ479419 Desulfotomaculum sp.CYP9
CSG56
CSG78
CSH6
AB196512 Ruminococcus productus
M59114 Streptococcus hansenii
CSG81
CSG82
CSG14
CSG27
CSG50
CSG23
CSG26
CSG17
CSG9
CSG6
CSG19
CSG41
CSG13
CSG53
AY033434 Clostridium lactatifermentans
CSH65
CSH29
CSH62
CSG12
CSG16
CSG31
CSG35
CSG83
CSG85
CSG84
CSG48
CSG25
CSG28
CSG1
AB045283 Clostridium perfringens
CSG72
Z36272 Eubacterium brachy
X76161 Clostridium aminobutyricum
DQ978216 Clostridium sordellii
DQ393033 Rumen clone R4-23 from reindeer
CSG65
AF262237 Clostridium sp.TO-931
AB023971 Clostridium hiranonis
CSG32
CSG43
CSG67
CSG44
CSG74
CSG39
CSG45
CSG34
CSG42
CSG7
CSH63
CSH87
AJ270471 Butyrate-producing bacterium A2
CSG66
CSG55
Z49863 Termitobacter aceticus (Sporobacter termitidis)
CSG58
CSG63
CSG20
CSG70
CSH82
DQ057466 Clone ic1337 Ileum/Cecum Broiler Chickens
CSH69
CSH70
AY305307 Clone M21 Human Colon
CSH28
CSH71
CSH45
CSH52
CSG2
CSG5
CSH33
CSH47
CSH78
CSH23
CSH89
CSH20
CSH81
CSH34
CSH26
CSH53
M58686 Fusobacterium varium
CSH25
X77850 Clostridium rectum
M58680 Fusobacterium mortiferum
CSG61
AY547271 Alistipes massiliensis
CSG47
CSG54
CSH91
CSH87
CSG29
CSG30
AY349402 Prevotella human oral clone IK062
AB200225 Bacteroides coprocola
CSG52
CSG62
CSH92
AB200219 Bacteroides plebeius
CSH80
CSG3
CSH27
CSH22
CSG4
CSG8
CSH90
CSH35
CSH67
CSH56
CSH40
CSH44
CSH55
CSH32
CSH39
CSH48
CSH61
CSH38
CSH86
CSH73
CSG57
CSH60
CSH58
CSH31
CSH93
CSH15
CSH79
CSG38
CSG75
CSG59
CSG46
CSG37
CSH12
CSH49
CSH77
CSH59
CSH57
CSH9
CSH50
CSG77
CSH94
CSH95
CSH37
CSH68
CSH8
CSG80
Aquifex pyrophilus
0.05
Firmicutes
Fusobacteria
Bacteroidetes

5. DISCUSSION

5.1. Aerobic bacterial counts

Aerobic total counts determined by cultivation of frozen colon contents were 1.6 (+/- 0.2) x 10^5 cfu/g in the harbour seal and 2.1 (+/- 0.1) x 10^4 cfu/g in the grey seal (Table 3). The counts for the grey seal may be a bit higher, because it was difficult to resolve if one or several colonies gave rise to single colony forming units. If in doubt, it was counted as one cfu.

No colonies were observed on the Chocolate agar plates with ampicillin, indicating that there are no ampicillin resistance among cultivable bacteria in frozen colon contents (one gram each) from the harbour seal and the grey seal used in this study. Larger samples of the colon content may have allowed detection of ampicillin resistant colonies. The detection limit was calculated to be less than 11.1 cfu/g colon content. The disadvantage of this method was that only the culturable ampicillin resistant bacteria in the colon content are recovered, still this method enables the identification of the bacterial host.

Culture methods are generally laborious and time consuming identifying bacteria from their phenotypic patterns, which varies with the expression of their genes and is influenced by the environment. Culture-based studies are also known to be biased, as only a small percentage of the bacteria visualised by direct count procedures (microscope) can be cultured (Amann *et al.*, 1995). The reason for this inability to culture the majority of the bacteria, include the selectivity of the media that are used, the stress imposed by the cultivation procedures, the necessity of strictly anoxic conditions as most of the bacteria inhabiting the gastrointestinal tract are obligate anaerobes, and also the interactions between the organisms that can limit their ability to grow (Zoetendal *et al.*, 2003). Furthermore, as the seal colon with its contents had been frozen following collection, some of the bacteria present are also likely to be dead. The circumvention of these limitations in studying the colon bacterial populations requires the application of culture-independent approaches based on PCR and DNA sequence analysis that are two major technological developments permitting culture-independent characterization of microbial communities. Both molecular techniques were applied in this thesis to characterize both the presence of resistance genes and the bacterial diversity in colon content from grey seals and harbour seals. However, although sequencing of cloned 16S rRNA amplicons gives significant information about the identity of the uncultured bacteria, quantification of the data will not be accurate since also PCR and cloning steps are also not without bias (von Wintzingrode *et al.*, 1997, Leser *et al.*, 2002). Positive controls known to

give a product in PCR reactions were used in all the PCR experiments performed in this study to distinguish between errors in preparation of the reaction mix and sample-related problems (Fig. 1, Fig. 2). If the positive control yields a product but the sample of interest does not, it could be that inhibitory material is present in the PCR or that very low levels of template DNA are present. Also false-positive reactions due to contamination of the PCR with DNA fragments from a source other than the added template could be a problem in PCR. This is especially likely to occur when universal primers are used, like in this case. Therefore, a negative control (water), to which no template DNA was added, was used in every PCR experiment to check for contaminating DNA (Röling and Head, 2005).

5.2 Presence of *bla*$_{\text{TEM}}$ genes

Very few studies have been conducted regarding the distribution of *bla*$_{\text{TEM}}$-genes in the environment (Lorenzo *et al.*, unpublished, Gilliver *et al.*, 1999, Österblad *et al.*, 1999). The search for *bla*$_{\text{TEM}}$ genes in the colon bacterial population in our seals was performed by *bla*$_{\text{TEM}}$ specific PCR of total-DNA extracted from colon content of both species. Three samples, all from the grey seal, yielded bands of the expected size on the agarose gel (Fig. 2), indicating that ampicillin resistant bacteria in the colon content existed in this seal, probably in low proportions, even though no growth on the plates with ampicillin was observed (Table 3). Hybridized with *bla*$_{\text{TEM}}$ probes could possibly have given a higher number of PCR products positive for *bla*$_{\text{TEM}}$-genes, but this method was not applied due to limited time. Moreover, there could be undetected *bla*$_{\text{TEM}}$-genes in the colon content of the harbour seal and the grey seal, because only a small part of the colon content was examined for the presence of these resistance genes. It should be noted that the detection limit of *bla*$_{\text{TEM}}$-genes was not determined in this study.

In a study done by Matthew and Hedges (1976), TEM-1 and TEM-2 beta-lactamases were found in a number of different species belonging to the *Enterobacteriaceae* family. Bradford (2001) also states that the TEM-1 beta-lactamase is found in many different species of *Enterobacteriaceae*. *Bla*$_{\text{TEM}}$ genes was detected in total DNA from colon content from the grey seal (Fig. 2), but there were no representatives of the family *Enterobacteriaceae* among the 153 clones analyzed from the harbour seal and the grey seal colon (Table 4, Fig. 3). However, diversity studies done on colon content of hooded seals (*Cystophora cristata*) in the Greenland sea, showed clones that were similar to *Escherichia coli*, belonging to the *Enterobacteriaceae* family (Glad *et al*., 2007). This could mean that if a larger number of

clones were screened, species belonging to the family *Enterobacteriaceae* might be detected. Whether the PCR products by the bla_{TEM} specific primers in the total DNA extracted from the colon content of the grey seal belongs to species in the *Enterobacteriaceae* family, remains to be determined.

5.3 Bacterial phylogeny

The phylogenetic diversity of the colon bacterial community in harbour and grey seals determined by 16S rRNA gene sequence analysis (Fig. 3), indicated that they harbour bacteria similar to species previously isolated from the gastrointestinal tract of chickens and humans (Kitahara *et al.* 2005, van der Wielen *et al.*, 2002, Barcenilla *et al*., 2000, Bjerrum *et al*., 2006, Louis *et al*., 2004). The majority of the sequences belonged to the *Bacteriodetes* (49.4% for the harbour seal; 23.7% for the grey seal library) and *Firmicutes* (49.4% for the harbour seals; 76.3% for the grey seal library) phyla (Fig. 3; Table 4). This is consistent with findings in human intestinal tract (Wang *et al*., 2005). DNA was extracted from 9 environmental samples from the colon of the grey seal, and only two from the harbour seal. For the 16S rRNA clone library PCR, only 2 µl template was added to each PCR reaction mix, then again only 4 µL (pooled) PCR product have been used in each TOPO cloning reaction. In each step DNA is lost, so there is no guarantee that the less abundant sequences have been covered, but hopefully the dominant ones have been detected. However, different samples from the same seal were pooled after DNA extraction and PCR amplification because the bacterial composition could vary within the colon. This was done to ensure maximum sampling diversity.

The colon samples used in this study were collected in April-May, when the seals' food intake is supposed to be normal. The composition and the density of the colon microflora is expected to be relatively representative, since the samples were not taken during moulting, when very little feeding occurs. For instance in reindeer that are ruminants, the microorganisms of the rumen starts to die off at an alarming rate when met with episodes of starvation. Mathiesen *et al.* (1984) found that the bacteria in the reindeer rumen were reduced to only 0.3 % of the original population after 3 days of starvation. Also later studies have shown that this starvation condition has a vast impact on the rumen microflora (Aagnes *et al.*, 1995). We could expect that something similar could happen in the colon of the seals during moulting. The effects of changes in diet may also influence the colonic bacterial population in

seals, as their choice of food may vary considerable between seasons (e.g. Haug *et al.,* 1998; Gjertz *et al.,* 2000).

The harbour seal library had the lowest coverage of the two libraries, with only 66% coverage, which means that we statistically have been able to cover only 66% of the bacterial diversity in the colon (Table 5). Covering a larger part of the diversity with this method would require the screening of a larger number of clones. To cut down the number of clones to be sequenced a restriction cutting of cloned PCR products or a method called D/TGGE (denaturering/thermal gradient gel electrophoresis) could be used to detect similar or identical rRNA sequences. DGGE detect sequence variation within cloned genes by determination of melting behaviour. Although the fingerprints obtained by DGGE with general bacterial primers are not informative on phylogenetic composition, bands can be excised from gels and sequenced to allow a more detailed phylogenetic analysis. Even though DGGE only yield one diagnostic band per clone, it could be problematic in complex environments, where DGGE can generate large numbers of bands, to obtain single bands, free from contamination with other rRNA gene fragments, which can be sequenced directly (Röling and Head, 2005).

Although PCR is the most sensitive technique to detect sequences that are present in very low concentrations in the environment, PCR are not without biases. To ensure a high diversity of rRNA PCR products for the cloning, we used as few PCR cycles as possible, 30 cycles, because reports in the literature have indicated that amplification through many cycles may decrease the observed diversity (Bonnet *et al*, 2002).

From the harbour seal, 77 16S rRNA gene sequences were analyzed and only one of these clones showed >97% sequence similarity to their nearest database entries (BLAST) (Table 5). Most of the 16S rRNA sequences obtained from the seal colon represented novel bacterial species not yet isolated or characterized, but some of these could also be possible chimeras. Chimeras are defined as rRNA gene fragments replicated from different templates, and thus representing a complete rRNA sequence that does not exist naturally in a living organism (Röling and Head, 2005). Chimera formation lead to an overestimation of diversity (Röling and Head, 2005), but can be diminished by increasing the elongation time and decreasing the number of cycles (Wang *et al.,* 1997). A number of computer programs that can identify possible chimeras have been developed, and these sequences should be rejected from further analysis.

Several methodological limitations may have influenced the outcome of our investigation. The cloning efficiency was quite low and in total 15 cloning reactions were carried out to get a representative amount of clones for sequencing. The transformation

efficiency for the positive control pUC19 was very variable, however, a bigger amount of clones was not necessarily obtained the times the transformation efficiency for the pUC19 was high. To improve the cloning efficiency, several factors influencing the cloning efficiency were changed. Instead of adding only 2 µL of the cloning reaction to the competent cells, according to the instructions, the total volume of the cloning reaction (6 µL) was added. It could also be that the competent cells had been exposed to heat and thawed, so several cloning kits with competent cells were tried. The time the TOPO cloning reaction are added to the competent cells, is also known to be a critical step. A new 16S rRNA clone library PCR was also carried out. LB-plates without ampicillin added, were included as a control, and *E. coli* cells were always growing very well on those. Other factors causing the low cloning efficiency might be that the insert was too large or that the concentration of the PCR products was too low.

5.4 Conclusions

In conclusion, this study has contributed to a greater understanding of the distribution of antibiotic resistance genes in environments presumably unexposed to human antibiotic use. No aerobic ampicillin resistant isolates were detected in the colon content from neither the harbour seal nor the grey seal. However, bla_{TEM} alleles were detected in total DNA from three of the eight colon samples of the grey seal, but no amplifications of the bla_{TEM} genes were obtained in total-DNA from the two colon samples of the harbour seal, indicating that the prevalence of bla_{TEM} genes in the colon content of the harbour seal and the grey seal was low. Further investigations are however needed to draw any conclusions. This thesis also presents data on novel bacterial diversity in the colon of two different seal species determined by comparative sequence analysis of 16S rRNA genes. A total of 153 16S rRNA gene sequences (~1,5 kb) were analyzed from the colon of the two seal species. From the harbour seal 77 16S rRNA gene sequences were analyzed, identifying representatives associated with *Firmicutes* (all belonging to *Clostridiales* 49.4%), *Bacteroidetes* (all belonging to *Bacteroidales* 49.4%) and *Fusobacteria* (all belonging to *Fusobacteriales* 1.3%). From the grey seal, 76 16S rRNA gene sequences were obtained, including representatives from two bacterial phyla: *Firmicutes* (most of these *Clostridiales* 72.4%) and *Bacteroidetes* (all belonging to *Bacteroidales*, 23.7%). The bacterial population in the colon of harbour seal and grey seal included species considered to be a part of the normal flora in e.g. humans and chickens (Kitahara *et al.* 2005, van der Wielen *et al.*, 2002, Barcenilla *et al*., 2000, Bjerrum *et al*., 2006, Louis *et al*., 2004).

Only one clone from the harbour seal library showed >97% sequence similarity to their nearest database entries (BLAST). For the grey seal library about half the clones showed <97% sequence similarity to their nearest database entries (BLAST). This indicates that several of the 16S rDNA sequences obtained from the seal colon represents novel bacterial species not yet isolated or characterized.

5.5 Further considerations

Resistance to antibiotics is an increasingly common problem and the origin(s) of the resistance and the selection mechanisms responsible for maintaining a high prevalence of resistance are still unknown. Several studies suggest that some antimicrobial resistance determinants originate from natural microbial populations not believed to encounter exposure to pharmaceutically-produced antibiotics (Mazel *et al.*, 1999, Seveno *et al.*, 2002). However, further investigation/studies regarding the distribution of antibiotic resistance genes remains very important for future attempts to manage resistance.

A vast majority of the microorganisms in the gastrointestinal tract of wild animals has still not been isolated and characterised (e.g. Tajima *et al*. 1999; Sundset *et al*. 2007). Identifying the bacteria in the gastrointestinal tract is the first important step in studying ecosystems, for later to determine the role and function of the different microbes in the gastrointestinal tract. Therefore, the generation of clone libraries from more animals and the deposition of novel sequences in the DNA databases remain very important.

6. REFERENCES

Aagnes T H, Sørmo W, Mathiesen S D. 1995. Ruminal microbial digestion in free-living, in captive-fed, and in starved reindeer (*Rangifer tarandus tarandus*) in winter. Appl Environ Microbiol **61**: 583-591.

Altschul S F, Madden T L, Schäffer A A, Zhang J, Chang Z, Miller W, Lipman D J 4. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25**: 3389-3402.

Amann R I, **Ludwig W**, **Schleifer K H**. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59**: 143-169.

Barcenilla A, **Pryde S E**, **Martin J C**, **Duncan S H**, **Stewart C S**, **Henderson C**, **Flint H J**. 2000. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl Environ Microbiol **66**:1654-1661.

Bjerrum L, **Engberg R M**, **Leser T D**, **Jensen B B**, **Finster K**, **Pedersen K**. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult Sci **85**: 1151-1164.

Blix, Arnoldus Schytte. 2005. Arctic Animals and their Adaptions to Life on the Edge. Trondheim: Tapir Academic Press.

Bonnet R, Suau A, Doré J, Gibson G R, Collins M D. 2002. Differences in rDNA libraries of faecal bacteria derived from 10- and 25-cycle PCRs. Int J Syst Evol Microbiol **52**: 757-763.

Bradford P A. 2001. Extended-spectrum ß-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev **14**: 933-951.

Brusetti L. 2004. Effects of transgenic Bt176 maize protected against *Ostrinia nubilalis* on the structure and diversity of bacterial communities in complex environments. A tesi di Dottorato di Ricerca, University of Milano.

Bush K, Jacoby G A, Medeiros A A. 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother **39:** 1211–1233.

Chao A. 1984. Non-parametric estimation of the number of classes in a population. Scand J Stat **11**: 783-791.

Datta N, **Kontomichalou P**. 1965. Penicillinase synthesis controlled by infectious R factors in *Enterobacteriaceae*. Nature **208**: 239-241.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783-791.

Fisher J F, Meroueh S O, Mobashery S. 2005. Bacterial resistance to β-lactam antibiotics: Compelling opportunism, compelling opportunity. Chem Rev **105**: 395-424.

Fleming A. 1929. On antibacterial action of cultures of a penicillium with special reference to their use in isolation of *B. influenzae*. British Journal Exp Path **10**: 226-235.

Gilliver M A, **Bennett M**, **Begon M**, **Hazel S M**, **Hart C A**. 1999. Antibiotic resistance found in wild rodents. Nature **401**: 233-234.

Gjertz I, Kovacs K M, Lydersen C, Wiig Ø. 2000. Movements and diving of adult ringed sels (*Phoca hispida*) in Svalbard. Polar Biology **23**: 651-655.

Glad T, Klingenberg C, Flægstad T, Ericson J U, Olsvik O. 2001. Rapid detection of the methicillin-resistance gene, mecA, in coagulase-negative *Staphylococci*. Scand J Infect Dis **33**: 502-506.

Glad T, Kristiansen V F, Sundset M A, Nordgård L, Nielsen K M. 2007. Bacterial diversity and antibiotic resistance in the colon of the hooded seal. Poster presented at the 2007 Conference on Gastrointestinal Function. Chicago, April 16-18th, 2007.

Good I J. 1953. The population frequencies of species ans the estimation of population parameters. Biometrika **40**: 237-264.

Goodman & Gilman's. 2001. Pharmacological principles of therapeutics, 10th edition, McGraw-Hill, New York.

Harbottle H, **Thakur S**, **Zhao S**, **White D G**. 2006. Genetics of antimicrobial resistance. Anim Biotechnol **17**: 111-124.

Haug T, and Norges forskningsråd. 1998. Sjøpattedyr : om hval og sel i norske farvann. Oslo: Universitetsforl.

Jacoby G A, Medeiros A A. 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother **35:** 1697–1704.

Jacoby G A, **Munoz-Price L S**. 2005. The new beta-lactamases. N Engl J Med **352**: 380-391.

Johnson S P, Nolan S, Gulland F M. 1998. Antimicrobial susceptibility of bacteria isolated from pinnipeds stranded in central and northern California. J Zoo Wildl Med **29**: 288-294.

Jones R N, Pfaller M A. 1998. Bacterial resistance: A worldwide problem. Diagn Microbiol Infect Dis **31**: 379-388.

Kitahara M, **Sakamoto M**, **Ike M**, **Sakata S**, **Benno Y**. 2005. *Bacteroides plebeius* sp. nov. and *Bacteroides coprocola* sp. nov., isolated from human faeces. Int J Syst Evol Microbiol **55**: 2143-2147.

Knothe H, **Shah P**, **Krcmery V**, **Antal M**, **Mitsuhashi S**. 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection **11**: 315-317.

Kucers A, Crowe S M, Grayson M L, Hey J F. 1997. The use of antibiotics. A clinical review of antibacterial and antiviral drugs. 5th Edition, Butterworth-Heinemann, Oxford, United Kingdom.

Leser T D, Amenuvor J Z, Jensen T K, Lindecrona R H, Boye M, Moller K. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol. 68: 673-690.

Liras P, **Martin J F**. 2006. Gene clusters for beta-lactam antibiotics and control of their expression: why have clusters evolved, and from where did they originate? International Microbiology **9**: 9-19.

Livermore D. 1995. β-Lactamases in Laboratory and Clinical Resistance. Clin Microbiol Rev **8**: 557-584.

Livermore D. 1998. β-Lactamase-mediated resistance and opportunities for its control J Antimicrob Chemother Suppl. D **41**: 25-41.

Lockwood S K, Chovan J L, Gaydos J K. 2003. Aerobic bacterial isolations from harbor seals (*Phoca vitulina*) stranded in Washington: 1992-2003. J Zoo Wildl Med **37**: 281-291.

Louis P, **Duncan S H**, **McCrae S I**, **Millar J**, **Jackson M S**, **Flint H J**. 2004. Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. J Bacteriol **186**: 2099-2106.

Majiduddin F K, Materon I C, Palzkill T G. 2002. Molecular analysis of beta-lactamase structure and function. Int J Med Microbiol **292**: 127-137.

Marin M, **Gudiol F**. 2003. beta-Lactam antibiotics. Enfermedades Infecciosas Microbiologia Clinica **21**: 42-55.

Mascaretti OA. 2003. Bacteria versus antibacterial agents, an integrated approach. Washington DC: American Society for Microbiology Press.

Mathiesen S D, Rognmo A, Blix A S. 1984. A test of the usefulness of a commercially available mill "waste product" as feed for starving reindeer. Rangifer **4:** 28-34.

Matthew M, **Hedges R W**. 1976. Analytical isoelectric focusing of R factor-determined beta-lactamases: correlation with plasmid compatibility. J Bacteriol **125**: 713-718.

Mazel D, Davies J. 1999. Antibiotic resistance in microbes. Cell. Mol. Life Sci. **56**:742-754.

Moulder J W. 1993. Why is Chlamydia sensitive to penicillin in the absence of peptidoglycan? Infect Agents Dis **2**: 87-99.

Mårtensson P-E, Nordøy E S, Messelt E B, Blix A S. 1998. Gut length, food transit time and diving habit in phocid seals. Polar Biology **20**: 213-217.

Nwosu V C. 2001. Antibiotic resistance with particular reference to soil microorganisms. Res Microbiol **152**: 421-430.

Olsen M A, Nilssen K T, Mathiesen S D. 1996. Gross anatomy of the gastrointestinal system of harp seals (*Phoca groenlandica*). J. Zool., Lond **238:** 581-589.

Österblad M, **Norrdahl K**, **Korpimaki E**, **Huovinen P**. 1999. Antibiotic resistance. How wild are wild mammals? Nature **401**: 233-234.

Paterson D L, Bonomo R A. 2005. Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev **18**: 657-686.

Philippon A, Arlet G, Jacoby G A. 2002. Plasmid-determined AmpC-type ß-lactamases. Antimicrob Agents Chemother **46**: 1-11.

Poirel L, Karim A, Mercat A, Le Thomas I, Vahaboglu H, Richard C, Nordmann P. 1999. Extended spectrum β-lactamase-producing strain of *Acinetobacter baumanni* isolated from a patient in France. J Antimicrob Chemother **43**: 157-166.

Röling W F M, Head I M. 2005. Prokaryotic systematics: PCR and sequence analysis of amplified 16S rRNA genes. In: Osborn AM, Smith CJ (EDS). Molecular microbial ecology. Taylor Francis, NY, pp. 25-63.

Saito N, Nei M. 1987. The neighbour-joining method: a new method for constructing phylogenetic trees. Mol Biol Evol **4**: 406-425.

Seveno N, Smalla K, Van Elsas J D, Collard J-C, Karagouni A, Kallifidas D, Wellington E M H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. Rev. Med. Microbiol. **13**:1-13.

Shah A A, Hasan F, Ahmed S, Hameed A. 2004. Characteristics, epidemiology and clinical importance of emerging strains of Gram-negative bacilli producing extended-spectrum β-lactamases. Res Microbiol **155:** 409-421.

Shannon C E, Weaver W. 1949. The mathematical theory of communication. 1 University of Illinois Press, Urbana.

Sundset M A, Præsteng K E, Cann I K O, Mathiesen S D, Mackie R I. 2007. Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. Microbial Ecology. DOI 10.1007/s00248-007-9254-x.

Tajima K, Aminov R I, Nagamine T, Ogata K, Nakamura M, Matsui H, Benno Y. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol **29**: 159-169.

Thornton S M, Nolan S, Gulland F M. 1995. Bacterial isolates from California sea lions (*Zalophus californianus*), harbor seals (*Phoca vitulina*), and northern elephant seals (*Mirounga angustirostris*) admitted to a rehabilitation center along the central California coast, 1994-1995. J Zoo Wildl Med **29**: 171-176.

Tipper D J, **Strominger J L**. 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci USA **54**: 1133-1141.

Urzi C, Brusetti L, Salamone P, Sorlini C, Stackebrandt E, Daffonchio D. 2001. Biodiversity of *Geodermatophilaceae* isolated from altered stones and monuments in the Mediterranean basin. Environ Microbiol **3**: 471-479.

van der Wielen P W, **Rovers G M**, **Scheepens J M**, **Biesterveld S**. 2002. *Clostridium lactatifermentans* sp. nov., a lactate-fermenting anaerobe isolated from the caeca of a chicken. Int J Syst Evol Microbiol **52**:921-925.

von Wintzingerode F, Gobel U B, Stackebrandt E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev **21**: 213-229.

Wang G C Y, Wang Y. 1997. Frequency of formation of chimeric molecules is a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. Appl Environ Microbiol **63**: 4645-4650.

Wang M, Ahrne S, Jeppsson B, Molin G. 2005. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol Ecol **54**: 219-231.

Weisburg W G, Barns S M, Pelletier D A, Lane D J. 1991. 16 S Ribosomal DNA amplification for phylogenetic study. J Bacteriol **173**: 697-703.

Yocum R R, Waxman D J, Rasmussen J R, Strominger J L. 1979. Mechanism of penicillin action: Penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases. Proc Natl Acad Sci USA. **76**: 2730-2734.

Yu Y, Breitbart M, McNairnie P, Rohwer F. 2006. FastGroupII: A web-based bioinformatics platform for analyses of large 16S rDNA libraries. BMC Bioinformatics **7**: 57-65.

Zoetendal E G, Koike S, Mackie R I. 2003. A critical view on molecular microbial ecology of the gastrointestinal tract. In: ´t Mannetje L, Ramirez-Aviles L, Sandoval-Castro C, Ku-Vera JC (EDS). Matching herbivore nutrition to ecosystems biodiversity. IV International symposium on the nutrition of herbivores. pp. 59-78.