From the Division of Clinical Bacteriology,
Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

Incidence, emergence, persistence and mechanisms of antimicrobial resistance

in clinical isolates and normal microbiota

Sonja Löfmark

Karolinska Institutet

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ABSTRACT

The increasing resistance rate to antimicrobial agents is of global concern and needs to be readily addressed. Options for successful treatments of infectious diseases are becoming limited due to the spread of resistant strains and resistance genes in the clinic and in the community. The normal human microbiota, and especially the intestinal flora, is an important reservoir for the presence and transmission of resistance determinants. The *Bacteroides fragilis* group and *Propionibacterium acnes* are anaerobic opportunistic pathogens and members of the normal microbiota. Clinical isolates of these species are commonly resistant to a variety of antibiotics. Knowledge of the mechanisms behind the resistance is important for our understanding of its emergence, spread and persistence. It has also implications for the choice of antimicrobial therapy and the design of new drugs. In the present thesis, data on different aspects of antimicrobial resistance are presented.

Bacteroides species are resistant to a number of antimicrobial agents. The resistance mechanisms against metronidazole, an important drug for the treatment of anaerobic infections, are still not sufficiently elucidated. We could demonstrate that *nim* genes are present in a sub-population of European clinical *Bacteroides* spp. strains and are significantly associated with reduced susceptibility to metronidazole. These strains could be induced to form a highly resistant sub-population. We detected a new variant of the *nim* gene as well as a variety of upstream regulatory elements.

Patients with *acne vulgaris* are often heavily treated with antimicrobial agents creating a force for resistance development, which may also complicate the treatment of other infections. In the present study a number of known mechanisms behind erythromycin, clindamycin and tetracycline resistance in *P. acnes* isolates were characterised, but possibly newly evolved mechanisms were also involved. Different PFGE genotypes were shown to be distributed throughout Europe.

Antimicrobial agents do not only affect the pathogens targeted but have also an impact on the normal microbiota. A 7-day administration of clindamycin was shown to cause long-term disturbance in the faecal bacterial flora of healthy volunteers. This perturbation was recorded by analysing total faecal DNA and *Bacteroides* isolates from consecutively collected faecal samples. Molecular analysis of the faecal DNA revealed a shift in the *Bacteroides* community and enrichment of *erm* genes for up to two years following clindamycin exposure. Comparing these shifts with those in an unexposed control group where only minor changes in the

bacterial community were recorded further strengthens the dramatic impact of the antimicrobial administration. The negative influence the clindamycin exposure had on the *Bacteroides* community was also demonstrated among the cultured isolates. An immediate decrease in species and clonal diversity was noted and there was a strong selection of resistant strains and resistance determinants, especially the clindamycin specific *erm* genes, lasting for up to two years. The long-term stabilisation of *in vivo* acquired *erm* genes was shown to be due to a restoration of bacterial fitness, confirmed by an *in vitro* competition assay of isogenic isolates collected 21 days and 18 months after initiated administration. The initial biological cost of acquiring an *erm*-gene was further confirmed in an *in vitro* acquired transconjugant. In conclusion, short-term antibiotic administration may cause decreased clonal diversity and persistent enrichment of resistant clones in the normal intestinal microbiota, creating a reservoir of resistance, further adding to the global burden of resistance.

LIST OF PUBLICATIONS

I. **Löfmark S**, Fang H, Hedberg M, Edlund C. Inducible Metronidazole resistance and *nim* genes in clinical *Bacteroides fragilis* group isolates. Antimicrobial Agents and Chemotherapy, 2005, vol 49, s1253-1256

II. Oprica C, **Löfmark S**, Lund B, Edlund C, Emtestam L, Nord CE. Genetic basis of resistance in *Propionibacterium acnes* strains isolated from diverse types of infections in different European countries. Anaerobe, 2005, vol 11, s137-143

III. **Löfmark S**, Jernberg C, Jansson JK, Edlund C. Clindamycin-induced enrichment and long-term persistence of resistant *Bacteroides* spp. and resistance genes. Journal of Antimicrobial Chemotherapy, 2006, vol 58 s1160-1167.

IV. Jernberg C, **Löfmark S**, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. The International Society for Microbial Ecology Journal. Accepted.

V. **Löfmark S**, Jernberg C, Billström H, Andersson DI, Edlund C. Long-term persistence of resistant strains in the human intestinal microflora due to restored fitness. International Journal of Antimicrobial Agents. Submitted.

CONTENTS

LIST OF ABBREVIATIONS

ATCC	American type culture collection
CCUG	Culture collection University of Göteborg
CFU	Colony forming unit
CLSI	Clinical and laboratory standards institute
CT	Conjugative transposons
DGGE	Denaturing gradient gel electrophoresis
ERM	Erythromycin ribosomal methylase
ESBL	Extended spectrum beta-lactamases
EUCAST	European committee on antimicrobial susceptibility testing
IR	Inverted repeat
IS	Insertion sequence
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
MLS	Macrolide-lincosamide-streptogramin B
MLST	Multi locus sequence typing
MRSA	Methicillin resistant *Staphylococcus aureus*
NCCLS	National committee for clinical laboratory standards
NIM	Nitroimidazole resistance determinant
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
REP-PCR	Repetitive sequence based PCR
RFLP	Restriction fragment length polymorphism
TET	Tetracycline resistance determinant
T-RFLP	Terminal restriction fragment length polymorphism
VRE	Vancomycin resistant enterococci

INTRODUCTION

Antibiotics and antibiotic resistance

The advent of antimicrobial agents into clinical practice in the 1940's can be considered as one of the most notable therapeutic developments in the history of medicine. Successful treatment of previously mortal infections became a reality with the discovery of these potent drugs. By the 1960's the infectious diseases were considered to be under control or even eliminated. This optimism did not last long due to the entrance of new, re-emerging and antibiotic resistant infections. Still today infectious diseases are the second largest cause of mortality in the world accounting for more than 13 million deaths annually (Cohen, 2000).

Microbes introducing a serious confrontation are derived from both hospital settings, such as MRSA, VRE, and ESBL producing gram-negative rods, and from the community including multi-drug resistant (MDR) pneumococci, community acquired MRSA, MDR tubercolosis, DR malaria and HIV. Adding to the challenge in the field of infectious diseases are new environments that are favouring opportunistic bacteria as a consequence of the advances in clinical medicine. Another factor is the reduced therapeutic options due to the increasing antibiotic resistance. For anaerobic and several other bacterial infections the alternatives for treatment are already limited. The concept of a pre- and post-resistance era has been introduced since the antimicrobial resistance is changing the way the society is facing infectious diseases. This potential post-antibiotic era introduces an urgent health problem with increased morbidity, prolonged hospital stay, leading to greater direct and indirect costs for the individual and the society.

The misuse and overuse of antimicrobial agents causes spread and selection of resistant strains during and after treatment. Control of correct distribution and prescription of antibiotics is required, foremost in developing countries where other health issues overshadow the threat of antibiotic resistance. Comprehensive surveillance of resistance is also lacking in a global perspective and the overall picture is incomplete even though new reports are being published. Another factor contributing to the resistance problem is the vast amounts of antibiotics or analogous compounds used in agriculture that along with residues of antibiotics also found in the environment over long times create a more constant pressure selecting for resistance (Levy, 2002). In this aspect the normal flora is acting as a key player due to the abundance of species present and passing through which have an opportunity to interact with one another. There is a constant sharing of genetic material and the human microbiota has been

proposed as an important reservoir for spread of resistance genes. Many bacteria are resistant not only to one but to several groups of antibiotics and the emergence of MDR organisms can be attributed to the quantity of antibiotic used in clinical medicine and in animals for dietary consumption. The widely accepted belief that resistance is lost in the absence of antimicrobial pressure is today debated. Bacteria are genetically flexible and are fast to adapt and the complex phenomena of bacterial fitness deal with the ability of the organisms to compensate for the imposed cost of the acquired resistance mechanisms (Andersson, 2003).

The future brings challenges to the field of infectious medicine with the increased selective pressure, increased disease transmission and the fact that there have only been few new innovative discoveries in the last 30 years in the market of antibiotics. Research is urgently needed regarding identification of new compounds, new molecular targets, blocking of resistance mechanisms, non-antimicrobial based therapies such as vaccines and bacteriophages as well as exploring the field of functional genomics of antimicrobial producing organisms. Diagnostics have to be improved and standardized and take advantage of the evolving molecular techniques (Woodford and Sundsfjord, 2005). The possibilities of probiotics could also be further investigated. Other actions include improved rational use, reduced global selective pressure and education in different areas such as hospital hygiene and infection control measures. Shorter courses of antibiotic and cycling of the drugs are currently undergoing interventions. Much is being done and research is constantly bringing the field forward, yet more and faster action is needed (Andersson, 2003; Levy, 2002; Williams and Heymann, 1998).

Antibiotic agents- mechanisms of action and resistance

The term antibiotic was introduced already in the late 1800's and includes today compounds naturally produced by microorganisms, or synthetically and semi synthetically manufactured compounds that possess antibacterial activity not toxic to the host. Biochemical and structural differences of human eukaryotic cells and infectious organism make the action of the chemotherapeutic agents selectively toxic. Sulfonamide was the first antibiotic introduced in 1935 and for up to 30 years, new classes were discovered and developed with different spectrum and activity, the foremost example being penicillin. Antibiotics can be bactericidal, bacteriostatic or bacteriolytic and often show selectivity for gram-positive or gram-negative bacteria, depending on the differences in composition of the bacterial wall. Other factors also contribute to the activity of the antibiotics, such as preferred growing conditions and other physical properties of the bacteria (Bryskier, 2005).

Table 1 Some important families of antimicrobial compounds and their targets.

Mechanism of action	**Groups of antibiotics**
cell wall synthesis	beta-lactam agents, glycopeptides
cell membrane	polymyxin
protein synthesis	aminoglycosides, macrolides, lincosamides, streptogramins, oxozolidinones, tetracyclines, chloramphenicol
DNA/RNA	quinolones, metronidazole, rifampicin
Antimetabolite/folic acid metabolism	sulphonamide/trimetoprim

Bacteria are flexible and fast at changing appearance including modifying structures and targets. This is the basis of the evolution of antibiotic resistance, the bacteria's response to the amounts of antibiotics they are confronted with in our efforts to eradicate them. Resistance can be mediated by a number of different mechanisms, listed in table 2, that are either due to mutational events or uptake of genes. The genes encoding resistance can be transferred vertically in generations or horizontally by three major procedures; conjugation, transduction and transformation, of which conjugation is considered to be the clinically most important (Salyers and Amabile-Cuevas, 1997). Some bacteria are also intrinsically resistant to several groups of antibiotics, depending on the structure and physical properties of the bacteria. Resistance mechanisms to some of the antimicrobial groups of specific relevance to this thesis are described in detail below.

Table 2 Major mechanisms of resistance.

Mechanisms	**Groups of antibiotics**
Efflux pumps or altered permeability	tetracylines, quinolones, aminoglycosides, beta-lactam agents
Enzymatic inactivation of antibiotic	beta-lactam agents
Modification or protection of target	rifampicin, macrolides, beta-lactam agents
Alternative target	trimetoprim, sulphonamides
No activation of antibiotic	metronidazole

Metronidazole

Metronidazole, a nitroimidazole, is a pro-drug that needs to be activated by intracellular reduction in order to be effective against its target, the DNA molecule. It is active against a variety of anaerobic protozoa and anaerobic bacteria. Several mechanisms have been described conferring resistance to the nitroimidazoles. These include i) prevention of entry into the cell or active efflux, ii) reduced drug activation iii) alteration of DNA repair mechanisms, iv) target modification or loss, and v) inactivation of the drug (Land and Johnson, 1999).

Resistance genes conferring resistance to nitroimidazole (*nim* genes), have been isolated in a number of different genera of both gram-positive and gram-negative anaerobic bacteria, including *Bacteroides* (Reysset, 1996; Theron et al., 2004). The genes are usually found on low copy plasmids but have also been located to the chromosome and have been shown to be transferable by a conjugative process (Breuil et al., 1989; Reysset, 1996). So far seven members have been found, *nim*A-G (Gal and Brazier, 2004; Lofmark et al., 2005; Reysset, 1996). These genes are proposed to encode a reductase converting nitroimidazole to a non toxic derivative, thereby circumventing the toxic effect causing breakage of the DNA (Land and Johnson, 1999). Specific regulatory elements, insertion sequences, (IS) have been correlated to the *nim* genes and can regulate the expression of the genes by promoters carried on these mobile elements. Four of the *nim* genes have been described to be associated with different IS elements, which may also be coupled with other resistance genes (Reysset, 1996).

Clindamycin and erythromycin

Clindamycin, a lincosamide, and erythromycin, a macrolide, are chemically distinct compounds that both have a common target binding site on the ribosome and act by preventing peptide elongation. Resistance can therefore in some instances be conferred by the same mechanisms including i) mutation of the ribosomal RNA or ii) ribosomal proteins, iii) methylation of the ribosome, iv) extrusions of the antibiotic by efflux systems or v) enzymatic inactivation. The two latter have not been found in anaerobes to date. Mutations are seen in the conserved region V of the 23S rRNA moiety of the ribosomal 50S subunit and leads to impaired binding of the antibiotic to the ribosomal target. At least three specific point mutations have been observed, numbered according to corresponding positions in *E. coli,* and include A2058G, A2059G and G2057A transitions. The mutations have been found in species with one or two copies of the 23S rRNA, and can be heterozygous at these loci. Ribosomal protein L4 and/or L2 mutants have been identified and these can also be selected for in the presence of the antibiotic (Roberts, 2004).

Up to 40 *erm* genes (erythromycin ribosome methylases) have been described so far, and close to 30 of these in anaerobic species (Leclercq, 2002; Roberts, 2004). A single specific adenine in the 23S region (A2058) is methylated by these enzymes and thereby inhibits the binding of the antibiotic to the ribosome, thereby limiting its action. This is the most widely spread mechanism for resistance to MLS (macrolides, lincosamides and streptogramins) antibiotics. The *erm* genes can be inducibly or constitutively expressed. Erythromycin is a good inducer, and clindamycin can in some instances act as one (Roberts, 2004). Varying resistant phenotypes can arise from the inducible expression. The *erm* genes are divided into 21 classes (Leclercq, 2002) whereof four major classes, A, B, C and F, are detected in pathogenic bacteria. The host specificity is not clear since the different methylases are continuously found in new genera. The *erm*(F) is common in bacteroides, and other genes including *erm*(G) and (B) have been detected in this genus (Roberts, 2004). In *Propionibacterium* spp. the *erm*(X) has been described, but it is still a less common mechanism behind MLS resistance in this genus (Ross et al., 2002).

Tetracycline

Tetracycline acts by blocking the protein synthesis. Resistance mechanisms involve i) efflux pumps actively removing the antibiotic from the cell, ii) ribosomal protection and iii) alteration of the antibiotic molecule and there are also other genes found with unknown functions to date (Roberts, 2005). In *P. acnes* a single G-C transition in the 16S rRNA subunit has been shown to confer clinical resistance (Ross et al., 1998). The presence of different *tet* genes are according to available analysis different to some extent between gram-positive and gram-negative, aerobic and anaerobic bacteria. Some genera also harbour more than one type of *tet* gene. All the three resistance mechanisms have been found on mobile elements such as plasmids, transposons and integrons. The type of element these genes are associated with is argued to influence their ability to spread to new genera. Resistance due to acquisition of the *tet*(Q) gene, conferring resistance by ribosomal protection, is commonly seen in *Bacteroides* spp. The *tet*(Q) gene is most often carried on conjugative transposons together with the *erm*(F) resistance gene (Roberts, 2005).

Beta-lactam agents

There are three major resistance mechanisms against the group of beta-lactam agents; i) enzymatic hydrolysis of the drug by beta-lactamases, ii) introduction of penicillin binding proteins as alternative targets and iii) altered outer membrane permeability leading to limited access of the antibiotic. The most common mechanism of resistance is the production of beta-lactamases encoded on either the chromosome or plasmids, which can

spread between different bacterial species (Hedberg and Nord, 1996). Beta-lactamase producing organisms have a double clinical role in infections since they can also protect other susceptible bacteria by releasing the enzymes into the environment. The beta-lactamases are common in anaerobes and are specifically well studied within the *Bacteroides fragilis* spp group (Fang, 2001). The cephalosporinases are frequently observed among this group of bacteria and include the cefoxition hydrolyzing enzymes CepA and the less often detected CfxA (Avelar et al., 2003; Hecht, 2004; Paula et al., 2004).

Acquisition, transmission and persistence of resistance

The emergence and spread of resistant bacteria and resistance genes depend on different factors but the major pressure is antibiotic usage. The capacity of the resistant bacteria to transmit, whether between individuals in community, from contaminated water sites or between animals and humans through the food chain, are determined by different components. These include the ability to colonise, the genetic basis of resistance (i.e. mutation rates and horizontal transfer of genes) as well as the relative fitness of resistant and susceptible strains. The stability of the resistant strains depend also on the rate of formation of resistant mutants and the rate and extent of the genetic compensation of the cost (Bjorkman and Andersson, 2000). In the clinical setting exogenous cross transmission, introduction of medical devices, medical disruption of the gastric barrier and decreased colonisation resistance create opportunities for colonisation by resistant nosocomial pathogens. Hospital hygiene and infection control measures are therefore crucial for prevention of the transmission of resistant bacteria (Agvald-Ohman C, 2006; Stiefel and Donskey, 2004).

The normal flora of especially the intestine is a perfect place for efficient transmission of resistance genes. This wet and warm environment with nutrients in abundance comprises high numbers of species and cells acting as targets for resistance mechanisms and also constitutes a large gene pool of resistance determinants. The intestinal tract is also a reservoir of nosocomial pathogens including *Enterococcus* spp, *Clostridium* spp, *Escherichia coli, Bacteroides* spp and *Candida* (Salyers et al., 2004; Stiefel and Donskey, 2004). There is a selective pressure each time an antibiotic is administered and prolonged exposure of sub-inhibitory concentrations favours resistance development. Other ecosystems, such as the skin flora are also influenced by the antibiotic pressure and contribute to the exchange of resistance genes. The use of antibiotics in agriculture has increasingly been acknowledged to contribute to the global burden of antibiotic resistance with transient and colonising resistant bacteria originating from ingested foods (Salyers et al., 2004).

Transfer of resistance genes within the human intestine occurs to a high extent, as shown by several studies but the picture is far from complete (Lester et al., 2006; Salyers et al., 2004; Scott, 2002; Shoemaker et al., 2001). An initial selection of resistance genes occurs in the commensal flora and may then be transferred to pathogens, which was first described in 1959 between a commensal *E. coli* and the food pathogen *Shigella dysenteriae* (Andremont, 2003). Lester et al (Lester et al., 2006) showed that vancomycin resistance determinant *vanA* can be transferred *in vivo* between strains in the gut of healthy volunteers. The source was an *E. faecium* isolate of animal origin and the recipient a human gut isolate of the same species. The spread of genes from gram-positive to gram-negative bacteria is comprehensive and the genes are readily expressed, while the reverse direction is generally not true (Courvalin, 2005). Plasmid mediated conjugation between *Enterococcus faecalis* and *E. coli* has been demonstrated in an animal model (Doucet-Populaire et al., 1992). The majority of these studies are on minor populations of the human microbiota and more information is needed on anaerobes, including the *Bacteroides* spp as well as the less characterised gram-positive anaerobic species that comprises a substantial part of the flora (Salyers et al., 2004). A number of gram-positive resistance genes have appeared in *Bacteroides* but there seem to be barriers against movement of certain types of elements *in vivo* (Shoemaker et al., 2001). These barriers lie in the transfer process itself and in the replication of exogenous DNA. The process of conjugation was earlier thought to occur only between closely related species and what was then called a broad host range is now re-defined (Salyers and Amabile-Cuevas, 1997).

The experiments performed in order to study this gene flux are generally performed *in vitro* and this brings the question of how to interpret the data. Evidence is needed for whether events shown under laboratory conditions also can occur in nature. Animal models give better predictions but they still differ from humans and especially germfree animals comprise a different environment where the bacteria under study will dominate the flora. Retrospective studies elucidating the transfer of DNA sequences between different species and genera are another approach for gathering evidence of gene movement (Salyers and Amabile-Cuevas, 1997; Shoemaker et al., 2001). In a study by Shoemaker et al (Shoemaker et al., 2001), data is presented on a recent event of gene transfer. Since 1970 the incidence of *Bacteroides* strains carrying the *erm* genes had increased, indicating a horizontal transfer. The *erm*(F) had previously been found but the *erm*(B) and (G), of gram-positive origin, were detected more recently.

Stabilisation of resistant bacterial clones

Resistance can be stably maintained even in the absence of antibiotic pressure and resistance genes are found in individuals with no history of antibiotic consumption (Salyers and Amabile-Cuevas, 1997). Compensatory events, genetic linkage and no-cost resistance are other forces behind maintenance and stability of resistant strains and resistance determinants but also environmental contamination of low concentrations of antibiotic compounds favours persistence. Generally it is believed that the acquisition of resistance mechanisms to antibiotics is related to a cost for the bacteria or a loss in fitness, as measured by reduced ability to grow and compete, and a decrease in virulence, transmission and clearance. Estimating the cost of resistance can be performed retrospectively, prospectively or experimentally (Bjorkman and Andersson, 2000). An experimental setup can be pair-wise competitions between antibiotic susceptible and resistant bacteria with genotypic relationship and calculating the relative rates of growth and ability to compete. The cost of resistance has been reported both for resistance conferred by mutations in the chromosome as well as for acquirement of extra chromosomal elements. Most studies on fitness and cost of resistance have been focusing on chromosomal mutations. When resistance genes on acquired elements have been analysed, it has mainly been performed by using cloning vectors and/or lab strains *in vitro*. Data are still scarce concerning natural host-plasmid interactions as well as from *in vivo* studies, even though these kind of studies have been performed (Bjorkman and Andersson, 2000; Enne et al., 2004; Gustafsson et al., 2003).

Bacteria tend to evolve fast and adapt to the challenges presented. This compensation of the resistance cost is due to compensatory events within the chromosome or in the mobile element the resistance determinant is carried on. Second site compensatory mutations can occur after a period of host adaptation while true reversions are not so common (Bjorkman and Andersson, 2000). Plasmids have also been demonstrated to improve host fitness (Bouma and Lenski, 1988). Once antibiotic resistance has been established on mobile elements it is hard to eliminate, and this has been shown in several studies (Enne et al., 2004; Johnsen et al., 2002; Salyers and Amabile-Cuevas, 1997). This is also further emphasised by the long-term persistence of resistant clones following antibiotic administration demonstrated in a few studies, including the work presented in this thesis (Jernberg et al., 2006; Lofmark et al., 2006; Sjolund et al., 2005; Sjolund et al., 2003).

Methods to study genetic relationships

There are a number of different methods for typing of bacterial relationship below species level. The pulsed-field gel electrophoresis

(PFGE) has been considered the gold standard among the genotyping methods. It is still an important tool for analysing spread of resistant clones, for example in the case of out-breaks at hospital wards. The basis of the method is the cleavage of the intact, agarose embedded genome with a specific restriction enzyme. DNA fragments of varying sizes can then be separated on an agarose gel. The method has some drawbacks including the subjective interpretation of banding pattern and the fact that it is hard to compare results between different labs. PFGE is also time consuming compared to some of the other methods available. Another method to study clonality is repetitive sequence based PCR (rep-PCR) that uses different outward oriented primers to create specific segments of DNA for each species and down to clonal level. The banding pattern is analysed on an agarose gel. The rep-PCR is shown to be close to as discriminative as the golden standard PFGE and also faster and easier to perform (Olive and Bean, 1999). Different primers can be used binding to distinct sequences including repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences and BOX elements. The BOX sequences are mosaic repetitive elements used for differentiation of bacterial relationship. Rep-PCR is becoming the most widely used method for DNA typing (Olive and Bean, 1999) and have been used for differentiation of *Bacteroides* species strains (Moraes et al., 2000). To follow regional changes between laboratories or hospitals there is a need for direct sequence based methods, where the actual genetic code is compared. These methods have limitations as well, including the methodology as well as the choice of sequence to base the differentiation on (Olive and Bean, 1999).

Mobile elements

The increase in resistance among bacterial pathogens, as well as among the normal flora, is much due to the ease of which a variety of genetic elements carrying resistance genes can transfer between even distantly related species. The stability of these mobile elements and their rapid adaptation to the host is also an important factor. There are a number of different mobile elements characterised, and the categorisation of them differs (Mahillon and Chandler, 1998; Salyers and Amabile-Cuevas, 1997; Summers, 2006). Conjugation has been shown to be a method for transfer showing the broadest host range in the laboratory, but transformation and transduction should not be ruled out as important contributors to the spread *in vivo*. There are two types of conjugal elements commonly associated with a broad host range; the plasmids and the conjugative transposons (CT) (Salyers and Amabile-Cuevas, 1997). These and some of the other mobile elements are described below.

Plasmids
Plasmids responsible for the spread of resistance genes are often large circular independently replicating structures. There are conjugative or self-transmissible plasmids and mobilisable plasmids. The self-transmissible plasmids carry genes needed for conjugation, but can also co-transfer other elements carrying resistance genes, for example the smaller non-conjugative mobilisable plasmids. The latter carries genes allowing it to use the provided conjugal apparatus (Salyers and Amabile-Cuevas, 1997; Summers, 2006). DNA transferred to another cell by a conjugative plasmid can integrate and recombine with the chromosome of the recipient or remain associated with the plasmid. Virulence factors and resistance genes are examples of sequences that plasmids frequently carry, often in association with transposons and integrons (Summers, 2006).

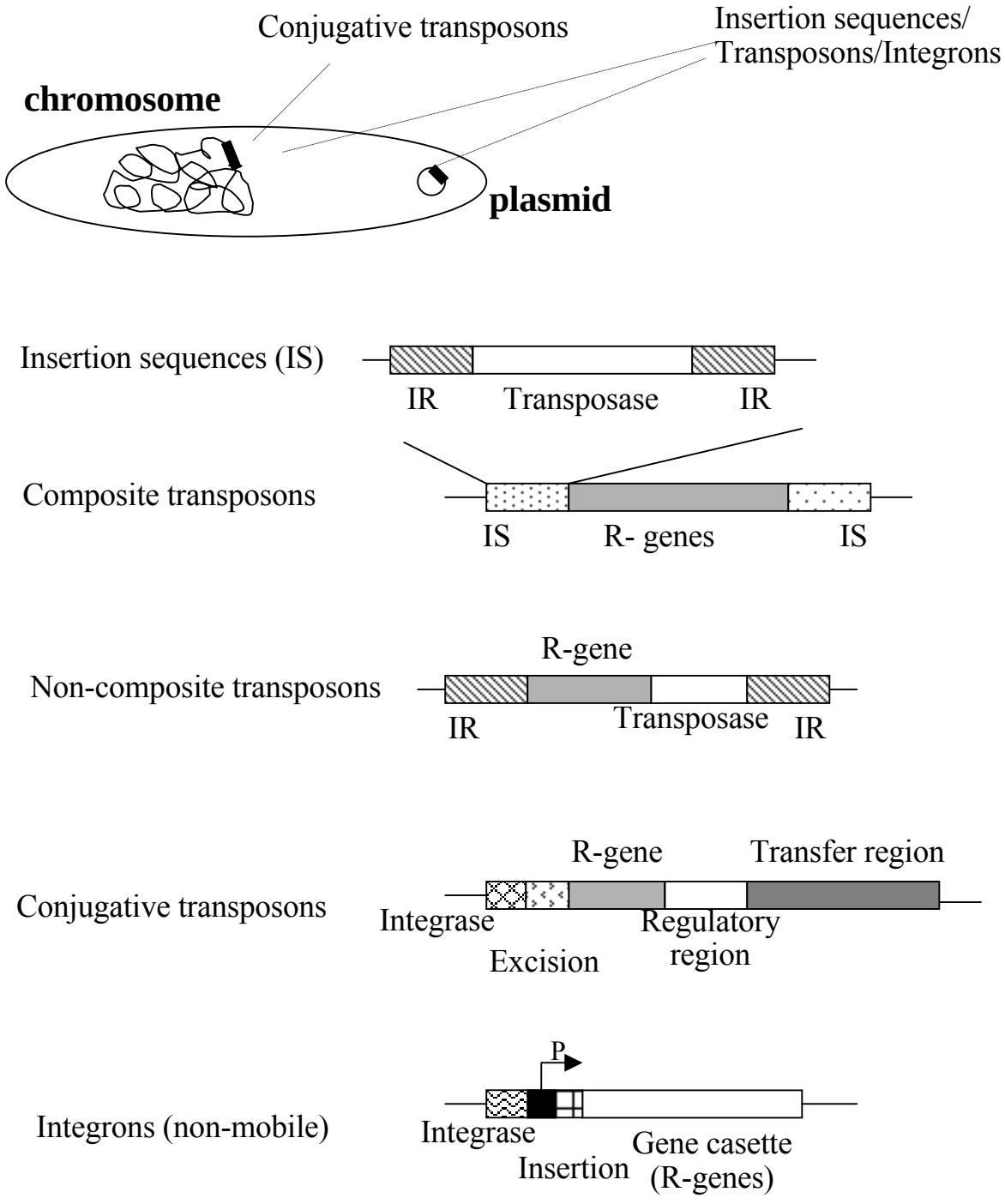
Conjugative transposons
The conjugative transposons (CTs) differs from transposons (described below) in that they take an intermediate circular form, transfer by conjugation and do not create target site duplication when they integrate (Salyers and Amabile-Cuevas, 1997). The CTs range from 18 to 150 kb and normally integrate to chromosome, but can in some cases be found extra chromosomally. The genes needed for conjugal transfer are carried by the element, as well as an integrase mediating the integration. The CTs often contain genes not only for antibiotic resistance but also genes encoding for heavy metal resistance such as mercury, which helps stabilise the mobile elements as well as the resistance genes in the population. The incidence of the CT elements in commensals have not been much studied, but they are responsible for spread of various resistance genes in the *Bacteroides* group and other bacteria inhabiting or passing through the gastro-intestinal tract. This extensive movement can be attributed to their broad host range and they are found in both gram-positive and gram-negative strains (Scott, 2002). The importance of CTs and their responsibility for transfer within *Bacteroides* group is described in a study by Shomaker et al (Shoemaker et al., 2001). The presence of the CTs is greater than its own transfer since they can co-transfer different elements and can provide mating apparatus for plasmids, or by forming a co-integrate with the plasmid and thereby make it mobile (Salyers and Amabile-Cuevas, 1997; Scott, 2002; Whittle et al., 2002). As more bacterial genomes are sequenced, the real extent of gene transfer between related and unrelated species will be revealed.

Insertion sequence elements, transposons and integrons
There is a sliding definition between IS and transposons and there are functional similarities between different types of mobile elements (Mahillon and Chandler, 1998; Summers, 2006). The IS elements,

sometimes defined as small transposons, are the simplest mobile element that carry no genetic information other than what is required for its movement and are generally less than 2.5 kb in size. They comprise a heterogeneous class of mobile elements with over 500 variants identified. The IS elements are thought to be involved in plasticity of prokaryote genomes and can control the expression of genes. They can be found both on the chromosome and on plasmids and in multiple copies. Non-composite transposons are analogous with IS elements, but differs in that they carry genetic markers, such as resistance genes, in addition to the transposase. When two IS elements surround or are integrated on both sides of a gene or a gene array they make a functional composite transposon entity. The IS elements are often flanked by inverted or sometimes direct repeats, recognised by an enzyme, the transposase. The transposase is needed for the transposition of the element and catalyses breakage and reunion of DNA. The promoter of the IS that can control the expression of other gene carried with the transposons is found in the inverted repeat (IR) domain (Mahillon and Chandler, 1998). The presence of an IS element upstream of a functional gene has been assigned a role in the expression of several resistance genes in *Bacteroides* species, including those for metronidazole, erythromycin/clindamycin, cefoxitin and carbapenems (Soki et al., 2006; Whittle et al., 2002).

Integrons on the other hand are short, non-mobile element often located on transposons and specialised in creating gene clusters. They provide an integration site for incoming gene cassettes that can contain over 5 different resistance genes. In addition to the integrase, the integrons carry a promoter for expression of the operon created by the integrase action (Salyers and Amabile-Cuevas, 1997; Summers, 2006).

Figure 1 Schematic overview of elements involved in the transfer and regulation of resistance genes. The elements are located on the chromosome or on plasmids. The inverted repeats (IR) often contain a promoter (P) for regulation of resistance (R) genes. The conjugative transposons carry genes coding for conjugal transfer and can form intermediate circular forms.



The human normal bacterial microflora

From the moment we are born, microbes will start to colonise all surfaces of the human body exposed to the environment, such as the skin and the mucous membranes lining the mouth and oral cavity, upper respiratory tract, the gastro-intestinal tract and parts of the female genital tract. These microorganisms include numerous bacteria and are collectively called the normal microbiota, normal microflora or normal flora. They co-exist with their host throughout life but our knowledge of this rich and complex community is still sparse and far from complete. This becomes more evident with the introduction of molecular methods that can be used to study different aspects of the flora. The establishment of these techniques has increased our insight into the diversity of species present, and also the role of the microbiota in human health and disease. The concept of 'normal flora' is discussed below; what are the functions and how can we study this diverse and variable microbiota.

The role and composition of the normal flora

The microorganisms colonising our bodies has been estimated to weigh 1 to 2 kg, with bacteria dominating and represented by up to 800 different species (Backhed et al., 2005; Dethlefsen et al., 2006; McFarland, 2000). These microbes have also been referred to as an extra organ carrying out essential physiological processes. The normal microbiota acts as a barrier against invading pathogens by forming a protective layer or biofilm, producing substances acting as bacteriocins (Hooper and Gordon, 2001). There is a finely tuned balance on going with the host from birth that depends on life style, health status, diet, age and gender but also on the host genotype. Studies have also demonstrated a discrepancy between countries (Mueller et al., 2006), but still more research is needed on what these differences mean. Different body sites presenting different habitats also display varying assembly of microorganisms, but the majority of the bacteria are found in the distal colon. It has been established that anaerobic bacteria are predominating in this environment and *Bacteroides* spp. are among the most commonly recovered genera. There is also a broad range of functions in which the bacteria are involved including our immune system, vitamin production and degradation of unabsorbed carbohydrates (Guarner and Malagelada, 2003). Sequencing of the complete *Bacteroides thetaiotaomicron* genome revealed the highest representation of enzymes involved in the break down of plant polysaccharides, of any bacteria sequenced (Xu et al., 2003). The products of this metabolism are essential fatty acids forming an important dietary component increasingly recognised to have potentially significant role in protecting against large bowel cancer.

The composition of the normal flora is not a static state, but fluctuates over time, as shown by research performed on both animals and humans. Little is still known of the complex community of the normal flora due to the difficulty in finding representative samples and measurements, as described below. Ongoing research is trying to elucidate its composition and function in human health and disease. The definition of what is a normal state in regard to the composition of the flora is still under re-evaluation. The perturbation of the collaboration between microbes and host has been associated with health conditions such as asthma and allergies. The diversity among the flora is important for the variety of functions its members are involved in (Hooper and Gordon, 2001).

Normal flora of the skin

The microbes inhabiting the skin have to endure harsh conditions but exhibit an unexpectedly stable pattern over time. The composition differs with the environment of the area of the skin. Factors influencing the constitution include temperature, moisture, pH, oxygen, UV radiation, concentration of skin lipids, and other microbes. The major members present are various genera of bacteria and yeasts. Gram-positive bacteria are favoured because of their ability to endure physical stress. Both anaerobic and aerobic bacteria are found including *Staphylococcus* spp, *Micrococcus* spp, *Corynebacterium* spp, *Propionibacterium* spp and *Acinetobacter* spp (Bojar and Holland, 2004).

Normal flora of the gastrointestinal tract

The intestine is the main habitat for the human flora and it is estimated using culturing methods that bacteria comprise 60% of faecal mass with levels of 10^{11} to 10^{12} cfu/gram faeces. The density in the stomach is sparser with less than 100 org/ml gastric juice, and the upper smaller bowel harbours around 10^{5} cfu/ml, increasing closer to the colon (Dethlefsen et al., 2006). The colonic bacteria are also more diverse with much of the bacterial world represented. Anaerobes outnumber the aerobes with 100:1 to 1000:1 and *B. fragilis* group dominates the cultivable species. Within this group *B. thetaiotaomicron* is the most prevalent (Duerden, 1980). Other gram-negative anaerobes include *Prevotella* spp and *Fusobcterium* spp. Eubacteria has been shown to represent a large proportion along with clostridia and anaerobic cocci as well as non-spore forming gram-positive anaerobic rods with low pathogenicity. Among the facultative anaerobes, *E. coli* and various streptococci and enterococci can be mentioned (Jouisimies-Somer et al., 2002). There is a discrepancy between culture methods and molecular based approaches. But it is still clear that our knowledge of the composition of the flora is far from complete. According to molecular methods, only 20-40 % of the gut flora has been cultured (Eckburg et al., 2005)

Analysis of the normal microbiota

A number of different methods can be used for analysis of this complex microbiota. Traditionally, microbiological analyses have been used but the expansion of molecular approaches introduced, and the discovery of today uncultivable microorganisms has influenced this field of research into taking a new direction. These methods are well suited for analysing heterogenic environmental niches, such as the normal microbial flora and especially the abundant flora found in the intestines (Dethlefsen et al., 2006). The routine for analysis of the skin flora remains based on phenotypic traits including sugar fermentation and extracellular enzymes (Bojar and Holland, 2004).

The conventional microbiological methods used are necessary for further analysis and characterization of isolates, including susceptibility testing and phenotypical expression. Our knowledge on cultures and cells of the microorganisms dates back to the 18th century and is still valuable in understanding the world of bacteria. The drawback with these traditionally used microbiological assays include the time consuming laboratory work load and high cost of the media used. A limit is also that they will seldom reveal any information below species level.

The numbers of molecular techniques deal with different aspects of detection, identification and quantification. Many methods used to try to resolve the phylogenetic diversity are based on universally conserved sequences of the ribosomal RNA/DNA, or other species-specific genes. Molecular cloning, oligonucleotide hybridisation, flow cytometry, real time PCR and microarrays are employed for detection and quantification. Sequencing is mainly used for identification. Denaturation grade gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) are examples of techniques that can be applied for profiling of complex microbial communities in directly extracted DNA, thereby bypassing the necessity for cultivation. These approaches can be used with species specific or universal reverse primer during PCR amplification of 16S rRNA genes. The advantage of T-RFLP is its ability to reveal a fingerprint at a given moment of the whole community and to track changes over time. This makes it ideal for comparing and monitoring spatial and temporal changes over time according to treatments or other external influences (Dethlefsen et al., 2006).

When considering the variety of different methods the aspect of bias has to be considered. Some methods have limited sensitivity and rare phylotypes are missed, as for example with broad range PCR based methods (Dethlefsen et al., 2006). There is also the disadvantage that live and dead

cells are not distinguished with these approaches. The sampling procedure and extraction methods are also important for the outcome. There is always the question of what is a representative sample? The intestinal flora is a well-studied niche, most often represented by faecal samples. The faeces is used as an indicator of what can be found among the mucosal microflora and is convenient and non invasive. The composition between the two environments differs somewhat but when disturbed by for example an antibiotic it should be reflected in faeces (Jernberg et al., 2005). According to a study by Eckburg et al analysing multiple colonic mucosal sites and faeces of healthy volunteers (Eckburg et al., 2005), the greatest variation was explained by faecal-mucosa divergence, after accounting for the difference in composition of bacteria seen between subjects. The bacterial community of the mucosa is more intricate to analyse, as a biopsy is needed and more difficult to obtain. Most often the wall of the intestine is washed and then the sample is taken. This means that the actual composition may not be what is seen in the analysis of the sample. The approach to studying the flora should be reflected against what is the effort and cost, and the actual goal of the analysis.

Effect of antibiotic administration on the normal flora

Antimicrobial agents can influence the normal flora in different ways and cause different symptoms and states of disease. The density or the number of bacteria and the diversity of species and specific clones can be affected as well as resistance levels and accumulation of resistance genes. Selection of resistant microorganisms is a common consequence of insufficient absorption of the drug. The impact of administered antibiotics depends on factors such as the spectrum of the agent, the dose, route of administration, drug concentrations in the different body departments as well as action of the antibiotic and its inactivation *in vivo* (Sullivan et al., 2001). Disturbance of the intestinal flora has been shown to lead to gastritis and diarrhoea (Finegold, 1970; Levy, 2000). Antibiotic with broad spectra, e.g. clindamycin tend to be disturbing to the flora, and can cause pseudomembranous colitis with diarrhoea due to *Clostridium difficile* overgrowth (Sullivan et al., 2001). Overgrowth of VRE has been shown in patients treated with antianaerobic drugs (Lautenbach et al., 2003; Stiefel et al., 2004). Some antibiotics can disturb the metabolism and absorption of vitamins and others may alter the susceptibility to infections (Levy, 2000). Other aspects include the selection of resistant strains and co-selection of resistance genes.

Table 3 Impact of selected groups of antibiotics on the normal intestinal microflora based on culture methods.

Agents	*Anaerobes*	*Enterococci*	*Enterobact-eriaceae*	*Overgrowth of resistant*	
				Enterobact-eriaceae	*C.difficile*
penicillin V					
cephalosporins (high conc in GI tract)	↓	↑	↓↓	+	+
cephalosporins (others)		↑	↓	+	+
tetracyclines			↓	+	
vancomycin	↓	↓	↓		
metronidazole					
clindamycin	↓↓	↑		+	++

Modified from (Edlund, 1993; Sullivan et al., 2001)

The effects different antibiotics perform on the intestinal bacteria have been investigated in a number of studies while effects on the flora of the skin and other compartments are less elucidated (Sullivan et al., 2001). In a study by (Vowels et al., 1996) analysing the effect of topical erythromycin on the aerobic bacteria on the skin, a static density of the total aerobic flora was demonstrated. There was at the same time an increase in the level of erythromycin resistant coagulase negative staphylococci. The increased resistance lasted 6 weeks before returning to base levels. Resistance to clindamycin was also co-selected. Many studies have recorded short-term quantitative changes to the bacteria comprising the human normal microbiota (Sullivan et al., 2001). Qualitative changes such as the alterations in resistance levels are also commonly recorded. What is lacking is information on the long-term effects of antibiotic administration. There is also a need for deeper analysis of what happens with the diversity at species and clonal level and what impact this could have. Our normal flora has often been said to be our allies and we should encourage the usage of antimicrobials with a minimal effect on the normal microbiota. Probiotics prepared in different formulas can be helpful in preventing disturbances of the gastro-intestinal flora (Sullivan et al., 2001; Sullivan and Nord, 2005).

Anaerobic bacteria

Anaerobic bacteria can be defined as those either able to grow in the absence of oxygen, or those that require the absence of oxygen to grow. They are found as endogenous human flora colonising many different

parts of the body, and can be involved in various infections with severe morbidity and high mortality. Besides humans, anaerobic bacteria are common in animal gut and soil sediments. Anaerobic bacteria are often slow growing and fastidious. It is a heterogeneous group of organisms requiring different conditions and media for growth. Identification of anaerobes in clinical specimens or other samples collected involve direct examination using gram stain and biochemical tests, but also gas liquid chromatography, DNA probe screening techniques or direct nucleotide sequencing. Blood agar plates, often containing additional supplements and reducing agents are often used for isolation of anaerobes. The pure cultures can be identified further with colony morphology and different biochemical tests, including rapid identification tests detecting bacterial enzymes. Susceptibility testing can be performed by broth- and agar-dilution techniques. However the agar diffusion method using antibiotic discs is not the method of choice for anaerobic organisms (Jenkins, 2001; Jouisimies-Somer et al., 2002).

Since anaerobic bacteria require special sampling and handling they are easily overlooked in the clinical setting. Many laboratories are often not equipped to culture and handle the samples. Anaerobes are often involved in mixed infections making it a complex and time consuming task to elucidate the role of the different agents. This knowledge has increased among clinicians and microbiologist overall, but there is a tendency to carry out fewer cultures and minimize analysis. This is partly because of the time and economic factors, but also the availability of effective antibacterial agents for empirical therapy, as well as other treatment options including surgery and drainage (Jenkins, 2001). One problem with reduced testing is that, on top of the misdiagnosis and mistreatment, we are missing information on development of resistance when the bacteria are not properly identified. There should therefore be an awareness of usual antimicrobial susceptibility patterns of the organisms. Species that should be considered for susceptibility testing include the *B. fragilis* group, other anaerobic gram-negative bacilli such as *Prevotella* and *Clostridium* species as well as other bacteria known to be especially virulent (Finegold, 1997; Hecht, 2006; Rosenblatt, 1997).

Bacteroides fragilis group

Bacteria belonging to the *Bacteroides fragilis* group are gram-negative rods, phenotypically characterised by bile resistance. The *Bacteroides* genus has undergone changes under the past years and includes the *Bacteroides fragilis* group and other *Bacteroides* species. The *B. fragilis* group currently includes 10 species: *B. fragilis, B. thetaiotaomicron, B. ovatus, B. distasonis, B. vulgatus, B. uniformis, B. caccae, B. eggerthi, B. stercoris* and *B. merdae.* Three new members have been proposed

B. nordii, B. salyersae and *B. goldsteinii* (Song et al., 2005; Song et al., 2004). With the introduction of molecular techniques, the taxonomy will continue to be re-evaluated, not only in this group of bacteria. Some of the species within the group display similar biochemical properties and are difficult to differentiate. The clinically most important are the *B. fragilis* species although only representing a minor part of the normal microbiota where *B. thetaiotaomicron* is the most commonly found species (Duerden, 1980; Hedberg and Nord, 2003; Jenkins, 2001; Lofmark et al., 2006).

Table 4. Relative frequency of specific species within the *B. fragilis* group in clinical specimens and normal microbiota

Bacteroides	**Percentage**	
	Clinical samples	**Normal microbiota**
B. fragilis	65	7
B. thetaiotaomicron	16	39
B. distasonis	2	13
B. vulgatus	8	8
B. ovatus	4	13
B. uniformis	1	19
B. caccae	<1	1

(Hedberg and Nord, 2003; Lofmark et al., 2006)

Propionibacterium acnes

The *Propionibacterium acnes* species belong together with *P. avidum, P. granulosum, P. propionicum* and *P. lymphophilum* to the *Propionibacterium* genus. They are gram-positive non motile, non spore forming rods found on the skin as part of the normal flora, but can also be recovered from other sites. *Propionibacterium acnes* is together with *P. granulosum* mainly found on sebum rich areas, such as the head, chest and back. The *Propionibacterium* genus is not strictly anaerobic, or lipophilic but are optimally cultured under anaerobic conditions with the supplement of Tween 80. *Propionibacterium acnes* is also a key factor in development of acne, and is involved in other serious infections increasingly recognised as an opportunistic pathogen (Bojar and Holland, 2004).

Anaerobic bacteria in infections

Infections involving anaerobes are often mixed together with facultative anaerobes. They can be severe and life threatening. The source is in most cases the normal endogenous flora, which can be the cause of skin and soft tissue infections as well as intra abdominal, wound and female genital tract infections. Postoperative infections following abdominal surgery impose serious problems where the enteric bacteria can escape the colon.

Characteristically, anaerobic infections are marked by necrosis and suppuration. The most common genera isolated are the gram-negative rods including *Bacteroides, Prevotella, Porphymonas,* and *Fusobacterium* species. Gram-positive bacteria include *Peptostreptococcus*, *Clostridium* and *Actinomyces* specis. Bacteroides is one of the most important groups due to its production of several virulence factors and also beta-lactamases (Jenkins, 2001; Jouisimies-Somer et al., 2002). *Propionibacterium acnes* has been ascribed a causative role in a variety of serious infections and is involved in the process of *acne vulgaris*, a chronic inflammatory disease common among young persons. It is often persistent and associated with an impact on their life situation even though it is not life threatening. The management of the disease renders a high cost to the society (Bojar and Holland, 2004). The role of non-cultivable species in diverse anaerobic infections is still not established, but the collection of data is ongoing.

The anaerobes are generally of low virulence, but there are virulence factors that can contribute to the establishment and progress of the disease. These include the ability to adhere to or invade epithelial surfaces, production of enzymes, toxins or other pathogenicity factors and the presence of cell surface constituents such as lipopolysaccharide (endotoxin) and/or capsular polysaccharides. The polysaccharide capsule of *Bacteroides* species is of advantage in adherence, abscess formation and it also prevents phagocytosis (Pumbwe et al., 2006). *Propionibacterium acnes* are very hardy and can survive and persist in human tissues, explaining the long-term inflammation in acne lesions. The anaerobic bacteria originating from our normal flora are opportunistic and can take advantage of these virulence factors and display pathogenic behavior in compromised patients (Aldridge, 1995).

The treatment of anaerobic infections includes antibiotic therapy, in combination or as single regimens, but also surgery. Agents with anti-anaerobic activity against the most clinical important anaerobes are the nitromidazoles (metronidazole), penicillins often combined with a beta-lactamase inhibitor, cephalosporins, carbapenems, chloramphenicol, and certain newer quinolones. Clindamycin were previously considered the gold standard but the emergence of resistant strains has limited its activity and it is no longer considered first line therapy for the bacteroides group or other anaerobic infections. It can still be used for mixed infections (Vedantam and Hecht, 2003). For *P. acnes* the drugs of choice depends on the severity of the disease. In mild acne topical antibiotics are used, including tetracyclines, clindamycin and erythromycin. In moderate to severe *acne vulgaris* oral treatment with erythromycin or tetracycline is recommended. There is not a complete elimination of bacteria with the usage of antibiotics and a combination therapy with non-antibiotic drugs

may be the best approach. In the case of *P. acnes* associated with severe infection, benzylpenicillin is recommended (Bojar and Holland, 2004).

Antimicrobial resistance in anaerobic bacteria

The resistance among the anaerobes is prevalent and generally increasing, some are resistant to close to all of the most commonly used agents. This can lead to adverse outcomes, which is why it is important to follow resistance development regularly as the susceptibility pattern changes with usage. The use of broad-spectrum empirical antibiotics may not be effective, at the same time as it may promote resistance. The role of resistant organisms might also be underestimated, as they are difficult to isolate in mixed infections and often efficiently removed with surgery (Hecht, 2004).

Antibiotic resistance in the Bacteroides fragilis group

The increasing resistance within the *B. fragilis* group is a growing clinical problem, as the therapeutical options are becoming limited. The levels of resistance are known to vary between species and among surveys, institutions and countries. The reported resistance rates are high against several antibiotics including clindamycin with up to 69% resistance, cefotetan (up to 85%), cefoxitin (up to 21%), and piperacillin (up to 52%). Resistance to tetracycline and ampicillin is nearly universal, even among the normal flora, thus these agents are not considered effective for empirical treatment. Resistance against agents commonly used for empiric therapy such as beta-lactams/beta-lactamases inhibitors, carbapenems and metronidazole is increasing, but is still at low-level (< 5%) (Aldridge et al., 2001; Hedberg and Nord, 2003).

Metronidazole was introduced in 1960 and by 1978 the first resistant *Bacteroides* strain was described (Ingham et al., 1978). The levels of resistance to this agent have remained low, but treatment failures attributed to resistance have been reported (Rotimi et al., 1999). Low-level resistant strains may be overlooked, as the breakpoint set by CLSI is high at 32mg/L. Another factor contributing to underestimation of the true resistance rates is the practice in some busy laboratories to identify obligate anaerobes by susceptibility to metronidazole. Growth around the disks is presumed to be facultative anaerobes with naturally reduced susceptibility, and not investigated further (Brazier et al., 1999). In a survey performed by Ulger et al (Ulger (Toprak), 2004) there was a high-level of resistance recorded to eight different antibiotics for both clinical *B. fragilis* isolates as well as *B. thetaiotaomicron* isolated from healthy volunteers. Virtually all genetics behind different antibiotic resistances have been found on transmissible elements, which are major features in

Bacteroides species. This further emphasises the importance of action against resistance development among anaerobes (Whittle et al., 2002).

Antibiotic resistance in P. acnes

There has been an increased prevalence in resistance among *P. acnes* isolated from acne patients, to clindamycin, erythromycin and tetracycline. According to a European study 50% of patients diagnosed with acne were colonised with clindamycin- and erythromycin resistant strains while the level of resistance against tetracycline was 20%. Resistance has also emerged in *P. acnes* isolated from systemic infections, but great variations according to geographic location are seen. Long-term treatments with different antibiotics are common and the likelihood of colonisation by resistant *P. acnes* strains increases with treatment duration. Antibiotic resistance among strains is one cause of treatment failures. There are also reports of high incidences of carriage of resistant strains among close contacts (Nord and Oprica, 2006).

AIMS OF THE THESIS

General aims

The overall aims of this thesis were to study different aspects of antibiotic resistance in clinical isolates and the normal flora using an array of microbiological and molecular tools. The focus was on emergence and persistence of resistance among the opportunistic anaerobic bacteria *Bacteroides fragilis* group and *Propionibacterium acnes*. The strains were collected both from clinical samples and *Bacteroides* species of the normal flora of healthy volunteers exposed to an antibiotic were also analysed. We wanted to investigate the long-term impact of antimicrobial administration on normal faecal flora. The goal was also to try to explain and characterise the mechanisms behind the resistances, the genetic relationship and the cost associated with the carriage of the determinants.

Specific aims

To examine the incidence and role of specific genes conferring metronidazole resistance (*nim* genes) and associated upstream regulatory elements, so called insertion sequences (IS) among clinical *Bacteroides* isolates of European origin.

To examine the susceptibility to induction of metronidazole resistance among clinical *Bacteroides* isolates positive or negative for the *nim* genes.

To characterise resistance mechanisms in clinical *P. acnes* isolates resistant to clindamycin and erythromycin or tetracycline.

To investigate the genetic diversity of resistant *P. acnes* strains from different infections and geographic origin.

To follow the long-term influence of a one-week clindamycin administration to healthy volunteers on the members of *Bacteroides* group species from consecutively collected faecal samples. Focus was on selection and development of resistance and resistance mechanisms as well as variations in the species diversity compared to a control group not exposed to any antibiotic.

To trace changes in the composition of the normal microbiota over time following a short-term course of clindamycin. This included analysis of the total bacterial, as well as *Bacteroides* specific, fingerprints in faecal DNA.

To analyse the total accumulation of resistance genes over time in faecal DNA as well as clonal distribution and stabilisation of resistant clones among the bacteroides isolates in healthy volunteers.

To determine the comparative fitness over time of resistant *Bacteroides* clones after *in vivo* acquirement of resistance genes, selected for and stabilised for 18 months after clindamycin exposure. A transconjugant with an *in vitro* acquired resistance gene was also investigated regarding fitness.

MATERIAL AND METHODS

Below the material and methods used in the different studies of this thesis are generally described. For more detailed descriptions see respective papers I-V.

Sampling and microbiological assays

Collection and culture of samples

In the five studies presented in the present thesis, anaerobic bacteria belonging to *Bacteroides* group and *Propionibacterium acnes* species, isolated from various sources were analysed. Both clinical isolates from infection sites and isolates from healthy volunteers were collected and cultured under anaerobic conditions (**Paper I-V**).

Clinical Bacteroides fragilis isolates

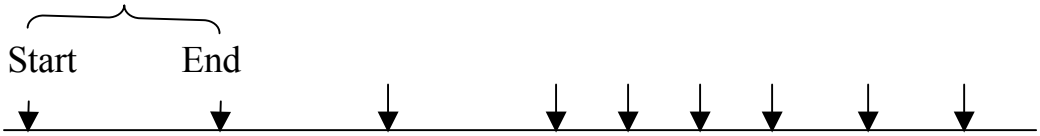
A total of 1502 clinical *B. fragilis* group isolates, originating from a previous surveillance study of antimicrobial susceptibility including 19 European countries, were analysed. Typing to species level had previously been performed by biochemical tests. *B. fragilis* spp. dominated and abdominal infections and wounds were the main sources. The strains were collected during a 2-year period (1999-2001). Susceptibility testing to seven different antimicrobials had also been performed (Hedberg and Nord, 2003) (**Paper I**).

Clinical Propionibacterium acnes isolates

A total number of 36 clindamycin and erythromycin resistant strains and 27 tetracycline resistant clinical *P. acnes* strains were cultured and analysed. Susceptibility testing was performed as recommended by Clinical and Laboratory Standards Institute (CLSI), former NCCLS. The isolates were collected between 1996 and 2002 from different geographical areas in Europe. The strains derived both from acne patients and from various other infections and had previously been identified by gram staining and gas-chromatographic analysis as well as by biochemical tests using Rapid Ana (Remel Inc., Lenexa, USA) (**Paper II**).

Faecal Bacteroides spp isolates from healthy volunteers
Faecal samples were collected from eight healthy volunteers over a two-year period. Four subjects had received clindamycin (4 x 150 mg) for 7 days, and four subjects were included as a control group and did not receive any antibiotic. The samples were collected at pre exposure and at eight different time points over the following 24 months and for the control group at corresponding intervals.

Clindamycin exposure



Bacteroides isolates were morphologically identified to genus level and presumed bacteroides colony-forming units (cfu) were enumerated and, if possible, 20 colonies of *Bacteroides* spp. were picked from each sample. Biochemical tests including fermentation of different sugars were performed for species typing (Morgan et al., 1976) (**Paper III-V**).

Antibiotic susceptibility testing

Susceptibility to the different antibiotics were determined by the agar dilution method, as recommended by CLSI, and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (**Papers I-V**).

Susceptibility to the following antibiotics was analysed in the thesis and the valid breakpoints according to CLSI and EUCAST, respectively, are listed below:

B. fragilis group	(CLSI)	
Metronidazole	≥32 mg/L	paper I, previously performed
Clindamycin	≥8 mg/L	paper III
Tetracycline	≥16 mg/L	paper III
Ampicillin	≥2 mg/L	paper III
P. acnes	(EUCAST)	
Clindamycin	≥0.25 mg/L	paper II
Erythromcyin	≥0.5 mg/L	paper II
Tetracycline	≥2 mg/L	paper II

Induction of metronidazole resistance

A total of 17 bacteroides strains, including two reference strains, were tested for induction of metronidazole resistance using a broth micro dilution technique. The strains were susceptible or expressed reduced susceptibility to metronidazole (MIC 0.0064-8 mg/L), and 12 were positive and 5 were negative for *nim* genes. Bacterial colonies were suspended in PBS and adjusted to McFarland 0.5 (~1.5 x 10^5 cfu/ml). Five µl of the suspension from each strain was added to a row of micro titer wells with increasing concentrations of metronidazole in the range 0-256 mg/L, in a total volume of 200µl broth per well and incubated at 37°C (**Paper I**).

Analysis of resistance mechanisms

DNA extraction

DNA extraction of bacterial isolates was performed by boiling the bacteria in order to destroy the cell wall. When centrifuging, the debris will collect in the bottom of the tube and DNA is found in the supernatant (**Paper I-V**). For the repetitive PCR the whole suspension was used as template, without boiling. To extract DNA from faecal samples a DNA spin kit for soil (Q-BIOgene, USA) was employed (**paper III-V**).

PCR/Real time PCR

Conventional and/or real time PCR were performed in order to screen for resistance genes and mutational events and upstream regulatory elements (Table 5). Different program profiles were employed for the different target genes depending on the length of the expected product and the primers designed; the protocols are described in the specific papers (**Paper I-V**). The real time PCR method was also used to quantitatively determine the relative increase of *erm* resistance genes in total DNA from faecal samples collected over time. Calculations of relative increase were made with the 16S rRNA gene as a reference gene for normalisation of the levels of the resistance genes in each sample. The level of specific gene increase was calculated as the fold increase compared to levels at day 0, prior to clindamycin administration (**Paper IV**).

Restriction Fragment Length Polymorphism

The method was used for determination of known *nim* gene types (A-E) using a single enzyme (Hsp92II) that was chosen to sufficiently cleave the PCR product of the gene, instead of using two enzymes (TacI and HpaII) that have previously been used for discrimination of the different *nim* types (Stubbs et al., 2000). The difference in banding patterns was also anticipated to reveal any new *nim* types (**Paper I**).

Sequencing

Conserved regions of 23S and 16S rDNA genes in *P. acnes* (**Paper II**) were amplified by PCR and sequenced in order to find mutations known to confer resistance to MLS and tetracycline, respectively. The metronidazole resistance conferring *nim* genes in *Bacteroides* were examined by sequencing to confirm the results of the RFLP (**Paper I**). Alignments were performed in clustalW/EMBL database (**Paper I and II**).

Efflux pump mediated resistance

To test for efflux pump activity in strains not positive for the genes or mutations screened for in the respective paper, reserpine, an agent blocking all efflux pumps, was added to the agar media (**Paper II and III**).

Table 5 Resistance genes, mutational events and upstream regulatory elements screened for.

Gene/mutation	**Resistance to**	**Method**	**Paper**
nim genes and IS elements	metronidazole	PCR, RFLP, sequencing	(I)
*erm*F/G/B/X	MLS	RT PCR, sequencing	(II-V)
tet(Q)	tetracycline	RT PCR, sequencing	(III)
*cep*A	beta-lactams	RT PCR, sequencing	(III)
*cfx*A	beta-lactams	RT PCR, sequencing	(III)
23S rRNA specific mutations	MLS	PCR, sequencing	(II)
16S rRNA specific mutations	tetracycline	PCR, sequencing	(II)
Efflux mediated resistance	MLS	reserpine; efflux inhibitor	(II and III)

Genotyping and genetic diversity

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was used to determine clonal relationship of clinical isolates of *P. acnes* from different sources and with different resistance patterns. Restriction enzyme *spe*I was used. The specific protocol was developed in the present study. Dendrograms were created for analysis of clustering of the banding patterns of the cleaved genomes (**Paper II**).

Repetitive sequence based PCR

Rep-PCR was used for discrimination of the *Bacteroides* isolates using three different sets of primers (REP, ERIC and BOX). They have previously been shown to give separate banding patterns enabling differentiation of species, and down to clonal level for the bacteroides group. Any extra inserted mobile elements could possibly be detected with a genotyping method like this one, revealing different sized fractions of the analysed genome (**Paper III-V**).

T-RFLP

The genetic diversity and any shift in the composition of the bacteroides group and of the total bacterial community within faecal samples was analysed over time using terminal restriction fragment length polymorphism (T-RFLP). For amplification of *Bacteroides* spp and universal eubacterial 16S rDNA specific reverse primers were used and the forward primer was in both cases fD1-FAM, fluorescently labelled at the 5'end. Terminal restriction of the PCR products was performed using the restriction enzyme *Hae*III. Analysis of the different fragments would give fingerprints over time disclosing any changes caused by the impact of antibiotic, compared to control group. The relative abundance of the fluorescent TRFs was calculated and the statistical significance of clustering patterns was determined (**Paper IV**).

Conjugation and competition

Induction of rifampicin resistance

A *B. thetaiotaomicron* isolate deriving from an originally clindamycin susceptible, *erm* negative clone, collected from a healthy volunteer pre-administration as described in paper III, was used as a recipient in the *in vitro* conjugation experiments. Spontaneous mutations conferring rifampicin resistance was induced as a genetic marker in the recipient strain, by culturing the bacteria in broth containing high concentrations of rifampicin (20000 mg/L) (**Paper V**).

Conjugation by filter mating

In vitro transfer of the *erm*(G) gene was performed by conjugation using a filter mating method. A rifampicin resistant, clindamycin susceptible, *B. thetaiotaomicron* strain (see above) was used as the recipient. An *in vivo* isolated clindamycin resistant isolate of a different species (*B. vulgatus*) and positive for the *erm* gene was used as the donor. The obtained transconjugant, resistant to rifampicin and clindamycin, and positive in PCR for the *erm*(G) gene, was collected for further analysis of fitness (**Paper V**).

Competition assay

Bacterial fitness and the cost of acquiring a resistance gene was assessed over time using an *in vitro* competition assay. Pairwise competition was performed for both clones A and B with the isogenic susceptible parent strains and the two *erm* positive, clindamycin resistant *in vivo* isolates (**Paper III**). For subject A, the *in vitro* obtained transconjugant was also used in a competition experiment. The experiments were performed separately for all resistant isolates collected at day 7, after 21 days and after 18 months after initiation of clindamycin administration (**Paper V**).

Statistical methods

Statistical methods included descriptive statistics (**paper I-III**) and calculation of odds ratio (OR) (**Paper I** and **II**). Analysis of T-RFLP data included the multivariate statistical analyses methods correspondence analysis and principal components analysis. One-way ANOVA was used for statistical significance of clustering patterns between subjects (**paper IV**).

RESULTS

Genetic basis of resistance in European clinical isolates

Antibiotic susceptibility and resistance mechanisms

Among the 1502 *Bacteroides* species strains analysed, a total of 30 strains (2%) were positive for *nim* genes, of which two expressed high-level resistance (≥ 64 mg/L) to metronidazole. The *nim*A genotype was the most common, even though all known *nim* types (A-E) could be detected and a new *nim* (F) was identified. As reported previously (Hedberg and Nord, 2003), resistance to metronidazole was low and only six strains were considered resistant according to breakpoints of ≥ 32 mg/L, while twelve strains expressing low-level resistance (8-16 mg/L) were found. Among the six resistant strains, four had an MIC of 64 mg/L and were negative for *nim*. Two distinct populations could be recognised when looking at the distribution of *nim* positive and *nim* negative strains, among the strains investigated. A significant relative risk of metronidazole resistance in *nim* positive strains was revealed (odds ratio of 26 [95% confidence interval 4.6 to 149]). The risk of having reduced susceptibility (MIC ≥8 mg/L) was even higher for *nim* positive strains (odds ratio of 53 [95% confidence interval 19 to 147]). The most common species among the *nim* positive strains was *B. fragilis*; this is also in accordance with the collection from which they were isolated, where *B. fragilis* constituted the majority of the clinical isolates. No correlation was noted between metronidazole resistance, *nim* type and species, even though the two high-level resistant strains belonged to *B. fragilis* species (**Paper I**).

IS elements could only be observed in *nim* positive strains and not in the *nim* negative clinical isolates or type strains analysed, but no clear correlation was seen between species or MICs and the presence of IS element. Seven *nim* positive strains lacked the IS elements looked for, where of one was highly resistant to metronidazole. The IS elements were shown by PCR to be located directly upstream of the *nim* gene in the majority of the isolates harbouring the sequence (**Paper I**).

Reversible high-level resistance was induced in 11 of 12 *nim* positive *Bacteroides* spp. strains tested for susceptibility to induction. The strains were grown for 3 passages in sub inhibitory concentrations of metronidazole using a broth microdilution technique. Nine of the induced strains also harboured an IS element. Irreversible resistance was triggered in two of the strains. The phenomenon of induction of resistance could not be detected in strains negative for *nim* genes, and neither for the strain carrying the *nim*F genotype (**Paper I**).

Among the 36 clindamycin and erythromycin resistant *P. acnes* isolates examined, 56% harboured any of the specific mutations in the peptidyl transferase region of domain V in 23S rRNA explaining the resistance. The *erm*(X) gene could not be detected in any of the isolates. The group I genotype, an A2058G transition, was the most common and found in 39% of all strains. This mutation was generally associated with high-level resistance to erythromycin and varying levels to clindamycin. The majority of the strains (79%) with the group I genotype belonged to one cluster according to the PFGE analysis. Presence of mutations was most common among the 22 strains from the Swedish acne patients, and also among this group the group I genotype was the most frequent. None of the ten susceptible clinical strains, or the two reference strains included contained the specific mutations or the resistance gene. All the resistance conferring mutations screened for could be detected among the resistant isolates. There were five main genetic lineages overall among the 36 erythromycin/clindamycin resistant strains as determined by PFGE (**Paper II**).

The majority of the 27 tetracycline resistant strains were isolated from Swedish patients diagnosed with acne, constituting 70% of the isolates. Twelve of these strains expressed high-level resistance to tetracycline, which was shown to be associated with a G to C transition in 16S rRNA. All the strains harbouring the mutation clustered together according to the PFGE analysis and were isolated from Swedish patients diagnosed with acne. All remaining strains showed low or intermediate resistance to tetracycline with MIC values within the range of 2-8 mg/L and harboured wild type alleles. None of the 27 tetracycline resistant isolates showed reduced resistance when exposed to an efflux pump inhibitor. The two reference strains and ten clinical isolates included for the efflux assay were all susceptible and revealed no mutations, or reduced MIC in the presence of the efflux pump inhibitor. Seven PFGE clusters were identified among the tetracycline resistant strains and beside the cluster of the highly resistant strains harbouring the mutation, one cluster could be seen with genetically related strains with varying resistance levels that were mainly from Swedish patients diagnosed with acne (**Paper II**).

Impact of antibiotic administration on the normal microbiota

Emergence of resistance and resistance mechanisms

Resistance levels of clindamycin, tetracycline and ampicillin in healthy volunteers were recorded in intestinal *Bacteroides* spp. over a two-year period. After analysis of the two groups included in the study, one receiving clindamycin for 7 days and the other not exposed to any

antibiotic, we could detect a clear increase and establishment of clindamycin resistance among the bacteroides isolates as a direct response to the introduced antibiotic pressure. Screening of the collected isolates for a number of specific resistance genes revealed a distinct difference for the exposed and non-exposed group regarding presence and persistence of resistance determinants over time, especially to clindamycin. A selection and establishment of isolates carrying either *erm*(F) and *erm*(G) genes following clindamycin administration was apparent among the exposed subjects (**Paper III**). This selection of *erm* genes type F and G in total DNA extracted from faecal samples was also established by real time PCR (**Paper IV**). The increase of *erm*(B) in total DNA analysed with real time PCR was low, which was also indicated by the absence of this gene in the analysed *Bacteroides* isolates (**Paper III** and **IV**). The calculated fold increase of the analysed *erm* genes varied to some extent between the subjects in the exposed group, but were still at a significantly higher level compared to the control group where the presence of *erm* genes only fluctuated around the base line over time. In two of the exposed subjects the resistance genes were still 1000- fold increased after 24 months compared to pre-administration (**Paper IV**).

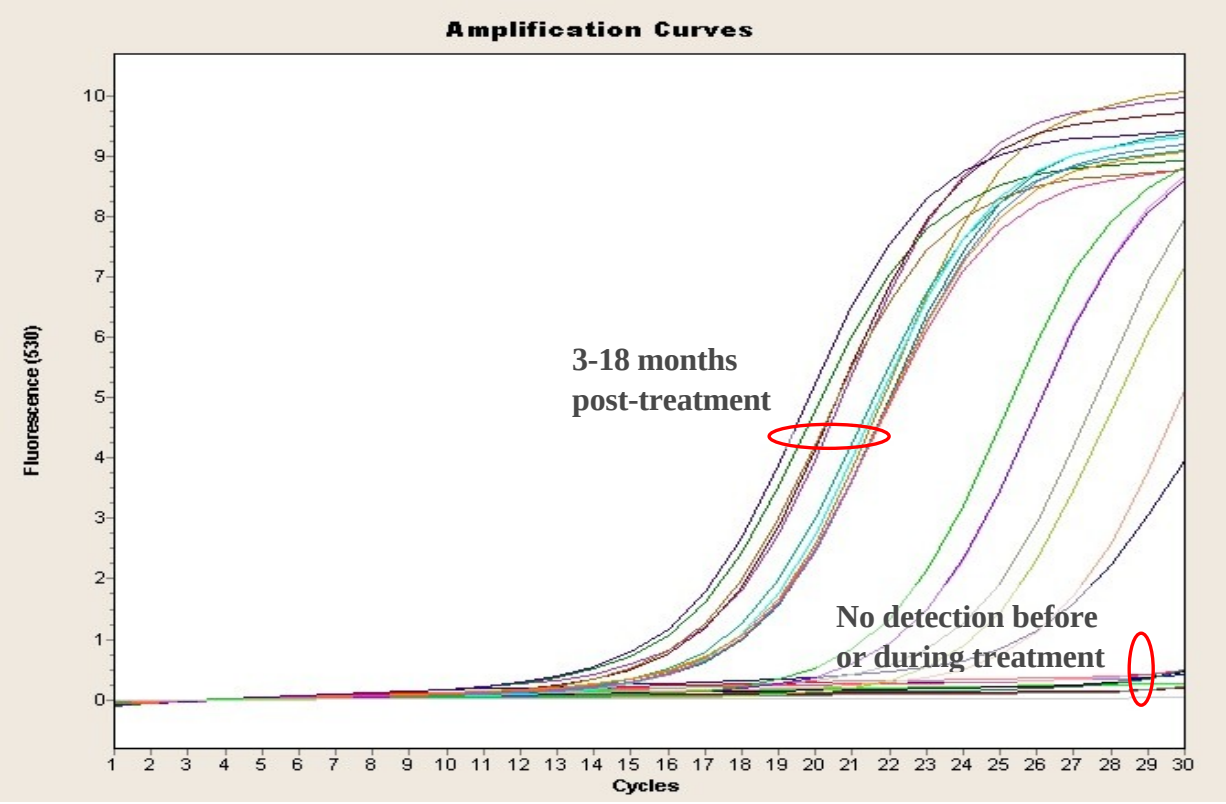
Already on the last day of clindamycin administration the average resistance levels had increased from 9% to 95% and this elevated resistance to clindamycin could still be detected after two years, mainly due to selection of a few single dominating resistant strains. The levels of resistance varied somewhat between the subjects, but 100% resistance was recorded in all subjects for at least one time point during the study period. High prevalence of resistance could be observed directly after exposure and for up to nine months in three subjects. This was in contrast to the control group where the clindamycin resistance levels were below 1% during the whole study period (**Paper III)**. These results are confirmed by the analysis of *erm* genes from the total faecal DNA (**Paper IV**). The clindamycin resistant isolates negative for the specific *erm* genes (B, F, G) were screened with universal primers but failed to reveal the presence of other *erm* genes. A weak efflux pump according to the reserpine assay might explain the low-level clindamycin resistance in one strain stabilised over time. The carriage of *erm* genes was correlated to the resistance levels, and was generally associated with high-level resistance (≥64 mg/L). None of the clindamycin resistance determinants screened for were found in the control group (**Paper III**).

Moderate to high-levels of tetracycline resistance among the *Bacteroides* spp. was observed in both groups during the investigation period. Levels of 77% and 47% resistant isolates were recorded in the exposed and the

control group, respectively. Thus, tetracycline resistance was present both with and without coincident clindamycin resistance, although tetracycline resistance increased with the enrichment of clindamycin resistance, as compared to the control group where the clindamycin resistance was low during the entire study period. Resistance determinant *tet*(Q) was detected in both the exposed and the control group over time and phenotypic tetracycline resistance was associated with *tet*(Q) genes in 99% of isolates from the exposed group and in 59% of isolates from the control group. The level of isolates carrying *tet*(Q) rose directly after the clindamycin exposure and was still high after two years compared to the control group, having more stable levels over time. Over all a co-selection of *tet*(Q) and *erm*(F) genes was detected, the association being more pronounced in two of the exposed subjects (**Paper III**).

Resistance to ampicillin was high in both groups, at close to 100% at all time points. The beta-lactamases *cepA* and *cfxA* were detected at low-levels in the bacteroides isolates. The *cepA* gene was found in both groups, exclusively in *B. fragilis* isolates while the *cfxA* gene was only observed in the exposed group, in high-level ampicillin resistant *B. ovatus* and *B. vulgatus* isolates, mainly originating from one subject (**Paper III**).

Figure 2 Selection of *erm*(F) in faecal samples collected over a two year period from one subject exposed to clindamycin, in a real time PCR analysis.



Changes in the composition of the bacterial community

In the T-RFLP analysis with universal primers, reflecting the total bacterial community, a statistically significant shift in the composition of the flora could be detected after clindamycin exposure. This was recorded for all four subjects, while the *Bacteroides* communities were altered in three of the four subjects as an effect of clindamycin administration, as assessed by T-RFLP analysis with bacteroides specific primers. The samples from the control group analysed by the bacteroides specific primers clustered more over time, and the differences between the samples were not as large as in the exposed group. This was also in accordance to the results from the analysis of these samples with the universal primers. The samples from the control group clustered more tightly together over time and showed a higher similarity than the samples from the exposed group that were more scattered, especially directly after clindamycin exposure (**Paper IV**).

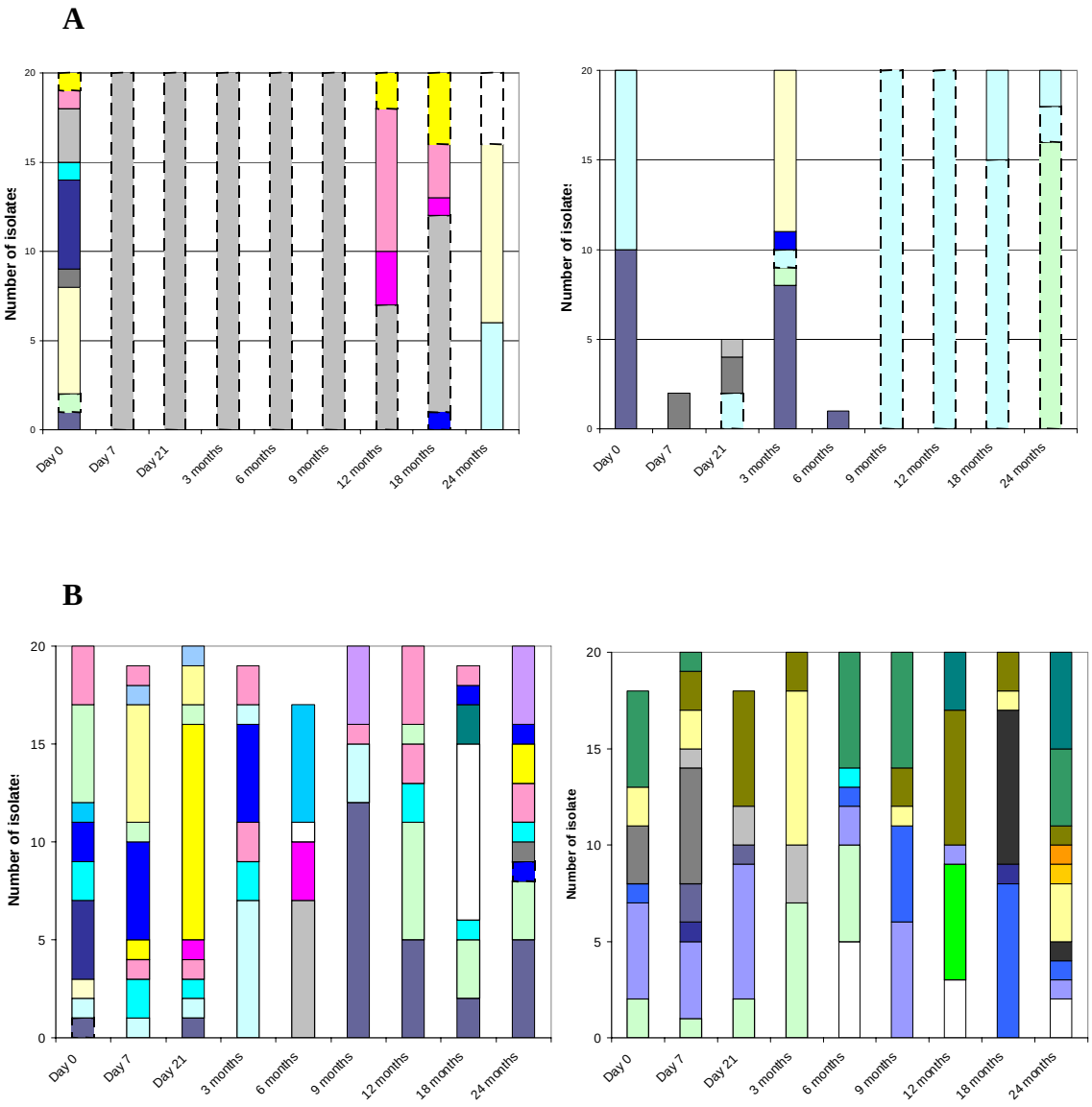
The variation of specific *Bacteroides* species was clearly reduced after clindamycin administration as assessed by biochemical tests and genotypic identification by rep-PCR. In the control group a higher diversity during the entire investigation period could be detected, reflecting a normal fluctuation of the bacteroides community in the human intestine (**Paper III**). The overall number of isolates detected was also reduced by the clindamycin exposure as the antibiotic eradicated components of the bacteroides community in the normal flora. The most prevalent species overall in both groups was *B. thetaiotaomicron,* although the composition differed between the two groups during the study period. After clindamycin exposure, clindamycin resistant strains of *B. thetaiotaomicron* dominated the bacteroides flora up to 24 months. While these strains comprised a minority of the isolates pre-exposure, they completely dominated the cultivable flora after 7 days, and still after two years they were found at an increased level. In contrast, these alterations over time could not be detected in the control group (**Paper III** and **IV**).

Selection and stabilisation of resistant clones

Specific clones were selected for and stably maintained for up to two years as a response to clindamycin administration, analysed by rep-PCR (**Paper IV**). In three of the exposed individuals, clindamycin resistant genotypes could be detected up to at least 18 months post-administration. From another exposed subject, a single *erm* negative, low-level clindamycin resistant strain (MIC 8 mg/L), came to dominate the bacteroides community in all but one of the subsequent samples. This *B. thetaiotaomicron* clone constituted 5% of the analysed colonies day 0. In the control group some specific clones remained over the study period,

but never dominated the flora. In general there were more transient clones detected in this group. From two of the exposed subjects one clone each was identified by rep-PCR, that was susceptible to clindamycin at day 0 but gained resistance conferred by the uptake of *erm* genes, and persisted over the entire study period (**Paper IV**). These two clones were further analysed regarding their persistence and domination in the population (**Paper V**). A regain of the initially reduced fitness, caused by *in vivo* acquisition of *erm* genes, was observed for the two resistant, *erm* positive strains. This was shown by challenging them against their susceptible parental counterparts in *in vitro* comparative fitness experiments. A compensation could be detected for both clones when analysed 14 days after discontinuation of administration and remained stable still after 18 months. The suppression of *Bacteroides* spp cfu under the detection limit observed directly after clindamycin administration in one of the subjects (**Paper III**) might be attributed to an initial loss in fitness. In one subject this initial loss of fitness could be recorded in the *in vitro* pairwise competition assay of the isolate collected at the last day of administration. This was confirmed by analysis of an *erm*(G) positive transconjugant with reduced ability to grow and compete *in vitro* (**Paper V**). Two weeks following the last day of clindamycin exposure, neither of the two *in vivo* isolated clones showed any disadvantages in growth rate and were both detected from the faecal samples again (**Paper IV**).

Figure 3 Distribution of specific *Bacteroides* species clones identified by rep-PCR (**Paper III**). A) Selection of clindamycin resistant clones (dashed bars) after 7-days clindamycin exposure in two healthy subjects and B) normal variation of susceptible clones in two non-exposed subjects. Each colour represents a unique clone. The dominating *B. thetaiotaomicron* clones from subjects (A) were further analysed in fitness experiments (**Paper V**).



DISCUSSION

The spread and long-term persistence of resistant bacteria and resistance determinants is of great concern on a global level. Antimicrobial resistance among pathogens limits the options for successful treatment of infectious diseases and increases the risk for complications and fatal outcome for patients with severe infections. The consequences of a resistance gene pool is most evident in the hospital setting, especially in the ICU and other wards with high antibiotic pressure and severely ill patients. Nevertheless, increasing resistance rates are badly complicating the treatment of community-acquired infections, and the role of the normal microbiota as a reservoir of resistance cannot be neglected in this aspect. The impact the exposure of antimicrobial agents can have on the normal flora in a short time span has previously been documented, but the present results revealing a long-term enrichment and persistence of resistant clones and resistance genes for up to two years after one week antibiotic exposure were unexpected. These resistant strains also took over and dominated among the isolated *Bacteroides* over time. The findings that sub-inhibitory concentrations of metronidazole can induce high-level resistance in *Bacteroides* spp. strains further emphasize the importance of addressing the threat of antibiotic resistance and the consequences it imposes on human health.

The major force behind the development of resistance is the consumption of antibiotics. Global action is needed since no nation can protect itself with the situation we have today of worldwide travel and trade. In 2001 WHO launched the Global Strategy for Containment of Antimicrobial Resistance that recognises the global problem of resistant pathogens (http://www.who.int/mediacentre/factsheets/fs194/en/). In Europe the European Antimicrobial Surveillance system (EARSS) and European Surveillance of Antimicrobial Consumption (ESAC) monitors the progress and development of antimicrobial resistance and usage of antibiotic agents. The prevalence of resistance varies substantially between different geographic regions, mainly related to the higher consumption in southern and eastern Europe than in northern Europe. The Swedish Strategic Programme for the Rational use of Antimicrobial Agents and Surveillance of Resistance (STRAMA) was founded in order to create a national overview of the surveillance projects and activities in Sweden, to promote prudent antimicrobial use and to initiate other actions needed to be taken to minimize further development and spread of resistance.

Exposure of antibiotics often results in ecological disturbances of the normal microbiota that can be both qualitative, as with the selection of

resistant strains, but also quantitative resulting in a decreased colonisation resistance. *Bacteroides fragilis* group members and *Propionibacterium acnes* are opportunistic pathogens that can take advantage of the situation when the immune system is impaired or the protective physical barrier is breached. Essentially all infections caused by anaerobic bacteria originate from the endogenous flora. Anaerobes are overlooked in the clinical settings and the resistance development neglected because of the shortcomings in collection and transport procedures, their slow growing nature and general susceptibility. The anaerobic infections are often cleared with surgery and drainage, but antibiotics still play a major role in successful treatment (Giamarellou and Kanellakopoulou, 1997). Antibiotic resistance in anaerobes is increasing and surveillance of both emergence and persistence of resistant strains as well as associated resistance mechanisms is therefore vital.

In this thesis we demonstrated that *nim* genes are present in approximately 2% of European clinical *Bacteroides* group spp. isolates and that the presence of these genes is significantly associated with a reduced susceptibility to metronidazole. The *nim* genes are the most widely studied mechanism behind metronidazole resistance, although other mechanisms also contribute to resistance. Metronidazole is considered to be the cornerstone of antimicrobial agents used for treatment of anaerobic organisms and even though the levels of resistance are still low, they are increasing and more studies are urgently required on the exact nature behind resistance. A new variant of the *nim* gene was detected in one strain that did not confer clinical resistance to metronidazole, which could possibly be related to the lack of upstream IS regulatory elements. The fact that *nim* negative strains were isolated expressing high-level resistance indicates the importance of other additional mechanisms of resistance.

The findings in our study revealed that sub-inhibitory levels of metronidazole could easily induce both reversible and irreversible resistance in *nim*-positive strains *in vitro*, which will likely be of clinical importance. These findings are supported by Gal *et al* (Gal and Brazier, 2004) and by another study showing that the phenomenon of induction also occurs in *nim* negative strains (Schaumann et al., 2005). The IS elements associated with the *nim* genes in paper I were likely to be involved in the induction of resistance by upregulating the expression of the genes. Two strains were irreversibly triggered to high-level resistance and some other additional yet unknown mechanism was probably responsible for the increased resistance, possibly a mutation. In the two strains susceptible to induction but negative for IS element, other unknown upstream regulators might have been present. Notably, metronidazole exposure has also been shown to enhance pathogenicity in both susceptible

and resistant *Bacteroides* strains (Diniz et al., 2003; Diniz et al., 2000) and to select for global changes in the regulation of the physiology of the resistant strain possibly protecting it and preventing metronidazole activation (Diniz et al., 2004).

Patients with *acne vulgaris* are often heavily treated with antibiotics creating a large selective pressure for development of resistant strains. There is a risk for inter-person spread of the enriched resistant strains, which has been described as being common between close contacts, but there is also a spread of these strains in the general population (Nord and Oprica, 2006). In our study we found several unique pulsotypes of erythromycin and clindamycin resistant *P. acnes,* collected from different infections throughout Europe. The most common mechanism for MLS resistance was an A-G transition in the 23S rRNA, which is in accordance with previous findings (Ross et al., 2001). Strains harboring this mutation were primarily isolated from Swedish patients diagnosed with acne. The type III mutation conferring low-level resistance to erythromycin was more sparsely detected which might be attributed to a cost to the bacteria resulting in reduced fitness, not balanced by the relatively low-level of resistance it is associated with. No strains were positive for the *erm*(X) gene, which has also previously been shown to be a less common mechanisms for resistance in *P. acnes* (Ross et al., 2002).

The genetic variation among the tetracycline resistant *P. acnes* isolates in the study was less diverse. Forty-four percent of these isolates had the specific point mutation in the 16S rRNA, whereof all were isolated from Swedish acne patients. Unlike a previous study (Ross et al., 2001) all these strains expressed high-level of resistance to tetracycline. The strains negative for the mutation showed greater variability and had lower MIC value than the isolates harbouring the mutation. In a test with an efflux pump inhibitor, the MICs of all strains were unaffected, indicating that resistance was not mediated by enhanced efflux from the bacterial cell. An alternative explanation is that reserpine was unable to inhibit the relevant pump-protein. Erythromycin and clindamycin are the most commonly used topical agents for treatment of mild to moderate acne while topical tetracycline is less effective. For oral treatment erythromycin and tetracyclines are commonly used, while clindamycin is not frequently recommended due to possible side effects. Clindamycin is mainly excreted in the bile with high concentrations in faeces leading to ecologically negative effects that can lead to overgrowth of *C. difficile* and antibiotic associated diarrhoea. In the present thesis we demonstrated major disturbances in the intestinal flora of healthy volunteers after clindamycin exposure, leading to decreased clonal diversity and persistent enrichment of resistant clones.

A few studies have shown that a restriction of the antibiotic pressure may successfully lead to decreased resistance levels in the community (Austin et al., 1999; Seppala et al., 1997) It seems that what is determining the possibility of reversing resistance levels in individuals and society is the ease at which compensatory events can occur in the resistant population, counteracting the biological costs conferred by the acquired resistance. Co-selection of resistance by the use of unrelated antibacterial groups due to multi-drug resistance is also a factor that renders a reverse to a susceptible population more difficult. Some previous reports have described the long-term effect and stabilisation of resistant strains and resistance genes following exposure of different antimicrobial compounds (Sjolund et al., 2005; Sjolund et al., 2003). The present findings of specific *Bacteroides* clones acquiring resistance determinants *in vivo* and persisting for up to 24 months due to restored fitness is of importance for further understanding and explaining stabilisation of resistance in spite of the abolished antibiotic pressure. The molecular mechanisms behind restored fitness for these strains remain to be elucidated. The fact that the main force behind a potential reversibility of resistance has theoretically been the fitness cost of resistance emphasizes the importance of these kinds of studies. The data we present are of significance as the initially decreased fitness of the bacteria after the acquirement of *erm* genes in the human intestine was also confirmed by analysing an *in vitro* obtained transconjugant.

In the present thesis a selection and long-term stabilisation of resistant strains and resistance genes in the faecal samples were recorded, as a response to clindamycin exposure. This was demonstrated both by the analysis of total faecal DNA and by studying cultured *Bacteroides* spp. isolates. We also observed the stabilisation of clones over time with reduced susceptibility to clindamycin, but negative for the *erm* genes. This might be explained by other mechanisms of resistance or by the fact that the universal primers used did not bind to all possible variants of the gene. A weak efflux pump according to the reserpine assay might explain the low-level clindamycin resistance in one strain stabilised over time. The increase of *erm*(B) was low, which was also indicated by the absence of this gene in the analysed *Bacteroides* isolates. The *erm*(B) is not commonly found among *Bacteroides* species but appears to originate from gram-positive species, primarily streptococci and enterococci (Shoemaker et al., 2001). Mainly clindamycin specific resistance genes were found in our study, but there was also a co-selection of tetracycline resistance genes. This simultaneous carriage of other resistance determinants has great impact on their selection and dissemination, as the presence of CTs that may carry multiple resistance determinants are tightly connected with the *Bacteroides* spp. In the presence of tetracycline these mobile elements

have shown induced transfer frequencies, up to 10000 fold. The *erm*(F) is known to often be connected with the *tet*(Q) and is commonly found in bacteroides isolates, as we also showed in our work. The beta-lactamases *cepA* and *cfxA* were detected at low levels. The *cepA* was exclusively found in *B. fragilis* isolates while the *cfxA* gene was only observed in the exposed group, in high-level ampicillin resistant *B. ovatus* and *B. vulgatus* isolates. Resistance to beta-lactams and cefoxitin is increasing worldwide and the resistances have in some cases shown to be associated with *cfxA*, and more commonly with the *cepA* (Avelar et al., 2003; Hecht, 2004; Paula et al., 2004).

Antibiotic administration can disturb the balance of the endogenous flora and not only select for resistant strains and resistance genes but also cause a shift and a decrease in the diversity within the bacterial community. Especially the anaerobes have been shown to be affected by clindamycin, recorded as a substantial decrease in numbers of bacteria (Sullivan et al., 2001). In the present work we demonstrated a shift in the total intestinal bacterial population as assessed by T-RFLP, as well as a more long-term disturbance of the *Bacteroides* community in response to clindamycin exposure. The total bacterial community was observed to roughly stabilise and return to a pre-administration composition after approximately three months. The analysis of DNA samples from a control group revealed minor and more time dependent variations, reflecting what could be considered to be a normal state. The ingestion of food and other external factors affect the composition of the flora, the population dynamic in such a complex environment is not static but fluctuates somewhat over time. When these variations are compared to the large shift observed in the exposed group the dramatic impact of the antibiotic is even further strengthened.

The insight of the nature and status of resistance mechanisms, how they emerge and persist in the clinical settings as well as in the normal microbiota is of importance for our understanding and solving of the global antimicrobial resistance problem we are confronting. This knowledge is critical for the choice of antimicrobials and the design of new antimicrobial agents, as well as in prevention of dissemination of resistant strains in clinical medicine

GENERAL SUMMARY

In the present thesis we present important findings of prevalence of resistance mechanisms, induction of resistant subpopulations in clinical isolates as well as the selection and stabilisation of resistant strains and resistance genes in the normal microbiota after exposure to an antimicrobial agent. The increasing levels of antibiotic resistance among not only clinically important pathogens but also in bacteria constituting the normal human microbiota and other complex ecosystems, is of global concern. The administration of an antibiotic does not only affect the organism towards which it is targeted but also disturbs the normal flora in different aspects. The anaerobic *Bacteroides fragilis* spp and *Propionibacterium acnes* spp are two members of the normal flora but are also opportunists found to be involved in severe infections. The resistance among clinically isolated anaerobes is increasing. We have investigated different aspects of resistance and mechanisms of resistance in these two bacterial species. We also followed changes over time in the total faecal bacterial community following antibiotic administration, as well as the impact on genetic diversity.

The major findings in this thesis are:

- Presence of *nim* genes significantly increases the risk for having reduced susceptibility to metronidazole. A new variant of the *nim* gene was detected in one strain but did not confer clinical resistance to metronidazole, which could possibly be related to the lack of upstream IS regulatory element. The fact that *nim* negative strains were isolated expressing high-level resistance indicates the importance of other additional mechanisms of resistance.

- Sub-inhibitory concentrations of metronidazole can induce reversible high-level metronidazole resistance in clinical *Bacteroides* isolates harbouring certain *nim* and IS variants. The findings of strains that were irreversibly triggered to high-level resistance indicate additional mechanisms, possibly a mutation in the regulatory regions.

- Erythromycin/clindamycin and tetracycline resistant clinical *Propionibacterium acnes* isolates from different geographic origins displayed well-known mechanisms of resistance but other possibly new mechanisms seem to have appeared. The majority of the resistant isolates harboured a specific mutation in the ribosome, which is the binding site of the antibiotic molecule; the 23S subunit

for erythromycin and clindamycin and the 16S for tetracycline. Other mutations in adjacent positions were also detected in the erythromycin and clindamycin resistant isolates. No active efflux pumps, looked for by a reserpine assay were detected among the tetracycline resistant strains.

- Different PFGE genotypes were distributed throughout Europe among the erythromycin/clindamycin and tetracycline resistant clinical *Propionibacterium acnes* isolates from various infections. The majority of the erythromycin and clindamycin resistant strains that harboured type I mutation belonged to one cluster. All tetracycline resistant isolates with the mutation were from Swedish patients diagnosed with acne and were all clustered together in the PFGE analysis.

- A 7-day clindamycin administration selected for a long-term persistence of resistant *B. thetaiotaomicron* clones seen already at the last day of administration. In the control group only one strain was found with low-level resistance to clindamycin. The natural variation of species and clones over time seen in the control group was readily reduced following antibiotic exposure.

- The gain of an *erm* gene *in vitro* severely reduced fitness of the bacteria. The *in vivo* acquisition of the *erm* genes also resulted in a loss of fitness as shown by the low competition rates seen for the isolate collected at the last day of clindamycin administration in a pairwise competition assay. An initial reduction in number of detectable isolates after clindamycin administration was also detected. The persistence of two resistant *Bacteroides* clones in the intestine, with acquired *erm* genes, was due to restored fitness determined by the competition assay.

- A selection of *erm* resistance genes among the collected bacteroides isolates, as well as in the total DNA extracted from the collected faecal samples occurred as a response to a short-term clindamycin administration. In specific isolates analysed there was a co-selection of a tetracycline resistance determinant. The resistance genes persisted among the faecal flora for up to two years. In the control group the *erm* genes could not be detected.

- A long-term disturbance of the *Bacteroides* community in the total faecal DNA was noted as consequence of the 7-day clindamycin administration, while for the total bacterial community normalisation back to pre-exposure levels was seen after three

months. When the results were compared to a control group the dramatic impact of the antibiotic was even further strengthened.

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REFERENCES

Agvald-Ohman C, L. B., Hjelmqvist H, Hedin G, Struwe J, Edlund C., 2006, ICU stay promotes enrichment and dissemination of multiresistant coagulase-negative staphylococcal strains., *Scand J Infect Dis.* **38**(6-7)**:**441-447.

Aldridge, K. E., 1995, The occurrence, virulence, and antimicrobial resistance of anaerobes in polymicrobial infections, *Am J Surg* **169**(5A Suppl)**:**2S-7S.

Aldridge, K. E., Ashcraft, D., Cambre, K., Pierson, C. L., Jenkins, S. G., and Rosenblatt, J. E., 2001, Multicenter survey of the changing in vitro antimicrobial susceptibilities of clinical isolates of Bacteroides fragilis group, Prevotella, Fusobacterium, Porphyromonas, and Peptostreptococcus species, *Antimicrob Agents Chemother* **45**(4)**:**1238-43.

Andersson, D. I., 2003, Persistence of antibiotic resistant bacteria, *Curr Opin Microbiol* **6**(5)**:**452-6.

Andremont, A., 2003, Commensal flora may play key role in spreading antibiotic resistance, *Asm News* **69**(12)**:**601-607.

Austin, D. J., Kristinsson, K. G., and Anderson, R. M., 1999, The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance, *Proc Natl Acad Sci U S A* **96**(3)**:**1152-6.

Avelar, K. E., Otsuki, K., Vicente, A. C., Vieira, J. M., de Paula, G. R., Domingues, R. M., and Ferreira, M. C., 2003, Presence of the cfxA gene in Bacteroides distasonis, *Res Microbiol* **154**(5)**:**369-74.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I., 2005, Host-bacterial mutualism in the human intestine, *Science* **307**(5717)**:**1915-20.

Bjorkman, J., and Andersson, D. I., 2000, The cost of antibiotic resistance from a bacterial perspective, *Drug Resist Updat* **3**(4)**:**237-245.

Bojar, R. A., and Holland, K. T., 2004, Acne and Propionibacterium acnes, *Clin Dermatol* **22**(5)**:**375-9.

Bouma, J. E., and Lenski, R. E., 1988, Evolution of a bacteria/plasmid association, *Nature* **335**(6188)**:**351-2.

Brazier, J. S., Stubbs, S. L., and Duerden, B. I., 1999, Metronidazole resistance among clinical isolates belonging to the Bacteroides fragilis group: time to be concerned?, *J Antimicrob Chemother* **44**(4)**:**580-1.

Breuil, J., Dublanchet, A., Truffaut, N., and Sebald, M., 1989, Transferable 5-nitroimidazole resistance in the Bacteroides fragilis group, *Plasmid* **21**(2)**:**151-4.

Bryskier, A., 2005, Historical review of antibacterial chemotherapy, in: *Antimicrobial agents; antibacterial and antifungals* (A. Bryskier, ed.), ASM press, Washington DC, pp. 1-12.

Cohen, M. L., 2000, Changing patterns of infectious disease, *Nature* **406**(6797)**:**762-7.

Courvalin, P., 2005, Antimicrobial Drug Resistance: "Prediction Is Very Difficult, Especially about the Future", *Emerg Infect Dis* **11**(12)**:**1503-1506.

Dethlefsen, L., Eckburg, P. B., Bik, E. M., and Relman, D. A., 2006, Assembly of the human intestinal microbiota, *Trends Ecol Evol* **21**(9)**:**517-23.

Diniz, C. G., Arantes, R. M., Cara, D. C., Lima, F. L., Nicoli, J. R., Carvalho, M. A., and Farias, L. M., 2003, Enhanced pathogenicity

of susceptible strains of the Bacteroides fragilis group subjected to low doses of metronidazole, *Microbes Infect* **5**(1)**:**19-26.
Diniz, C. G., Cara, D. C., Nicoli, J. R., Farias, L. D., and De Carvalho, M. A., 2000, Effect of metronidazole on the pathogenicity of resistant Bacteroides strains in gnotobiotic mice, *Antimicrob Agents Chemother* **44**(9)**:**2419-23.
Diniz, C. G., Farias, L. M., Carvalho, M. A., Rocha, E. R., and Jeffrey Smith, C., 2004, Differential gene expression in a Bacteroides fragilis metronidazole-resistant mutant, *J Antimicrob Chemother*.
Doucet-Populaire, F., Trieu-Cuot, P., Andremont, A., and Courvalin, P., 1992, Conjugal transfer of plasmid DNA from Enterococcus faecalis to Escherichia coli in digestive tracts of gnotobiotic mice, *Antimicrob Agents Chemother* **36**(2)**:**502-4.
Duerden, B. I., 1980, The isolation and identification of Bacteroides spp. from the normal human faecal flora, *J Med Microbiol* **13**(1)**:**69-78.
Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A., 2005, Diversity of the human intestinal microbial flora, *Science* **308**(5728)**:**1635-8.
Edlund, C., Nord CE., 1993, Ecological impact of antimicrobial agents on human intestinal microflora, *Alpe Adria Microbiology Journal* **3:**137-164.
Enne, V. I., Bennett, P. M., Livermore, D. M., and Hall, L. M., 2004, Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure, *J Antimicrob Chemother* **53**(6)**:**958-63.
Fang, H., 2001, ß-Lactam and metronidazole resistance in the Bacteroides fragilis group, Stockholm,, pp. 64 ;.
Finegold, S. M., 1970, Interaction of antimicrobial therapy and intestinal flora, *Am J Clin Nutr* **23**(11)**:**1466-71.
Finegold, S. M., 1997, Perspective on susceptibility testing of anaerobic bacteria, *Clin Infect Dis* **25 Suppl 2:**S251-3.
Gal, M., and Brazier, J. S., 2004, Metronidazole resistance in Bacteroides spp. carrying nim genes and the selection of slow-growing metronidazole-resistant mutants, *J Antimicrob Chemother*.
Giamarellou, H., and Kanellakopoulou, K., 1997, Bacteriologic and Therapeutic Considerations in Intra-abdominal Surgical Infections, *Anaerobe* **3**(2-3)**:**207-12.
Guarner, F., and Malagelada, J. R., 2003, Gut flora in health and disease, *Lancet* **361**(9356)**:**512-9.
Gustafsson, I., Cars, O., and Andersson, D. I., 2003, Fitness of antibiotic resistant Staphylococcus epidermidis assessed by competition on the skin of human volunteers, *J Antimicrob Chemother* **52**(2)**:**258-63.
Hecht, D. W., 2004, Prevalence of antibiotic resistance in anaerobic bacteria: worrisome developments, *Clin Infect Dis* **39**(1)**:**92-7.
Hecht, D. W., 2006, Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing, *Anaerobe* **12**(3)**:**115-21.
Hedberg, M., and Nord, C. E., 1996, Beta-lactam resistance in anaerobic bacteria: a review, *J Chemother* **8**(1)**:**3-16.
Hedberg, M., and Nord, C. E., 2003, Antimicrobial susceptibility of Bacteroides fragilis group isolates in Europe, *Clin Microbiol Infect* **9**(6)**:**475-88.
Hooper, L. V., and Gordon, J. I., 2001, Commensal host-bacterial relationships in the gut, *Science* **292**(5519)**:**1115-8.
Ingham, H. R., Eaton, S., Venables, C. W., and Adams, P. C., 1978, Bacteroides fragilis resistant to metronidazole after long-term therapy, *Lancet* **1**(8057)**:**214.
Jenkins, S., 2001, Infections due to anaerobic bacteria and the role of antimicrobial susceptibility testing, *Reviews in medical microbiology* **1:**1-12.

Jernberg, C., Löfmark, S., Edlund, C., and Jansson, J. K., 2006, Long term ecological impact of antibiotic administration on the human intestinal flora, *manuscript*.
Jernberg, C., Sullivan, A., Edlund, C., and Jansson, J. K., 2005, Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism, *Appl Environ Microbiol* **71**(1)**:**501-6.
Johnsen, P. J., Simonsen, G. S., Olsvik, O., Midtvedt, T., and Sundsfjord, A., 2002, Stability, persistence, and evolution of plasmid-encoded VanA glycopeptide resistance in enterococci in the absence of antibiotic selection in vitro and in gnotobiotic mice, *Microb Drug Resist* **8**(3)**:**161-70.
Jouisimies-Somer, H., Summanen, P., Citron, D., Baron, E., Wexler, H., and Fingeold, S., 2002, Wadsworth-KTL Anerobic Bacteriology Manual, Star publishing company, Belmont, CA.
Land, K. M., and Johnson, P. J., 1999, Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa, *Drug Resist Updat* **2**(5)**:**289-294.
Lautenbach, E., LaRosa, L. A., Marr, A. M., Nachamkin, I., Bilker, W. B., and Fishman, N. O., 2003, Changes in the prevalence of vancomycin-resistant enterococci in response to antimicrobial formulary interventions: impact of progressive restrictions on use of vancomycin and third-generation cephalosporins, *Clin Infect Dis* **36**(4)**:**440-6.
Leclercq, R., 2002, Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications, *Clin Infect Dis* **34**(4)**:**482-92.
Lester, C. H., Frimodt-Moller, N., Sorensen, T. L., Monnet, D. L., and Hammerum, A. M., 2006, In vivo transfer of the vanA resistance gene from an Enterococcus faecium isolate of animal origin to an E. faecium isolate of human origin in the intestines of human volunteers, *Antimicrob Agents Chemother* **50**(2)**:**596-9.
Levy, J., 2000, The effects of antibiotic use on gastrointestinal function, *Am J Gastroenterol* **95**(1 Suppl)**:**S8-10.
Levy, S. B., 2002, The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance, *J Antimicrob Chemother* **49**(1)**:**25-30.
Lofmark, S., Fang, H., Hedberg, M., and Edlund, C., 2005, Inducible metronidazole resistance and nim genes in clinical Bacteroides fragilis group isolates, *Antimicrob Agents Chemother* **49**(3)**:**1253-6.
Lofmark, S., Jernberg, C., Jansson, J. K., and Edlund, C., 2006, Clindamycin-induced enrichment and long-term persistence of resistant Bacteroides spp. and resistance genes, *J Antimicrob Chemother*.
Mahillon, J., and Chandler, M., 1998, Insertion sequences, *Microbiol Mol Biol Rev* **62**(3)**:**725-74.
McFarland, L. V., 2000, Normal flora: diversity and functions, *Microbial Ecology in Health and Disease* **12:**193-207.
Moraes, S. R., Goncalves, R. B., Mouton, C., Seldin, L., Ferreira, M. C., and Domingues, R. M., 2000, Use of rep-PCR to define genetic relatedness among Bacteroides fragilis strains, *J Med Microbiol* **49**(3)**:**279-84.
Morgan, J. R., Liu, P. Y., and Smith, J. A., 1976, Semi-microtechnique for the biochemical characterization of anaerobic bacteria, *J Clin Microbiol* **4**(4)**:**315-8.
Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., Cresci, A., Silvi, S., Orpianesi, C., Verdenelli, M. C., Clavel, T., Koebnick, C., Zunft, H. J., Dore, J., and Blaut, M., 2006, Differences in fecal microbiota in different European study

populations in relation to age, gender, and country: a cross-sectional study, *Appl Environ Microbiol* **72**(2)**:**1027-33.
Nord, C. E., and Oprica, C., 2006, Antibiotic resistance in Propionibacterium acnes. Microbiological and clinical aspects, *Anaerobe* **12**(5-6)**:**207-10.
Olive, D. M., and Bean, P., 1999, Principles and applications of methods for DNA-based typing of microbial organisms, *J Clin Microbiol* **37**(6)**:**1661-9.
Paula, G. R., Falcao, L. S., Antunes, E. N., Avelar, K. E., Reis, F. N., Maluhy, M. A., Ferreira, M. C., and Domingues, R. M., 2004, Determinants of resistance in Bacteroides fragilis strains according to recent Brazilian profiles of antimicrobial susceptibility, *Int J Antimicrob Agents* **24**(1)**:**53-8.
Pumbwe, L., Skilbeck, C. A., and Wexler, H. M., 2006, The Bacteroides fragilis cell envelope: quarterback, linebacker, coach-or all three?, *Anaerobe* **12**(5-6)**:**211-20.
Reysset, G., 1996, Genetics of 5-nitroimidazole resistance in Bacteroides species, *Anaerobe* **2**(2)**:**59-69.
Roberts, M. C., 2004, Resistance to macrolide, lincosamide, streptogramin, ketolide, and oxazolidinone antibiotics, *Mol Biotechnol* **28**(1)**:**47-62.
Roberts, M. C., 2005, Update on acquired tetracycline resistance genes, *FEMS Microbiol Lett* **245**(2)**:**195-203.
Rosenblatt, J. E., 1997, Can we afford to do anaerobic cultures and identification? A positive point of view, *Clin Infect Dis* **25 Suppl 2:**S127-31.
Ross, J. I., Eady, E. A., Carnegie, E., and Cove, J. H., 2002, Detection of transposon Tn5432-mediated macrolide-lincosamide-streptogramin B (MLSB) resistance in cutaneous propionibacteria from six European cities, *J Antimicrob Chemother* **49**(1)**:**165-8.
Ross, J. I., Eady, E. A., Cove, J. H., and Cunliffe, W. J., 1998, 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium, *Antimicrob Agents Chemother* **42**(7)**:**1702-5.
Ross, J. I., Snelling, A. M., Eady, E. A., Cove, J. H., Cunliffe, W. J., Leyden, J. J., Collignon, P., Dreno, B., Reynaud, A., Fluhr, J., and Oshima, S., 2001, Phenotypic and genotypic characterization of antibiotic-resistant Propionibacterium acnes isolated from acne patients attending dermatology clinics in Europe, the U.S.A., Japan and Australia, *Br J Dermatol* **144**(2)**:**339-46.
Rotimi, V. O., Khoursheed, M., Brazier, J. S., Jamal, W. Y., and Khodakhast, F. B., 1999, Bacteroides species highly resistant to metronidazole: an emerging clinical problem?, *Clin Microbiol Infect* **5**(3)**:**166-169.
Salyers, A. A., and Amabile-Cuevas, C. F., 1997, Why are antibiotic resistance genes so resistant to elimination?, *Antimicrob Agents Chemother* **41**(11)**:**2321-5.
Salyers, A. A., Gupta, A., and Wang, Y., 2004, Human intestinal bacteria as reservoirs for antibiotic resistance genes, *Trends Microbiol* **12**(9)**:**412-6.
Schaumann, R., Petzold, S., Fille, M., and Rodloff, A. C., 2005, Inducible metronidazole resistance in nim-positive and nim-negative bacteroides fragilis group strains after several passages metronidazole containing columbia agar plates, *Infection* **33**(5-6)**:**368-72.
Scott, K. P., 2002, The role of conjugative transposons in spreading antibiotic resistance between bacteria that inhabit the gastrointestinal tract, *Cell Mol Life Sci* **59**(12)**:**2071-82.
Seppala, H., Klaukka, T., Vuopio-Varkila, J., Muotiala, A., Helenius, H., Lager, K., and Huovinen, P., 1997, The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in

group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance, *N Engl J Med* **337**(7)**:**441-6.
Shoemaker, N. B., Vlamakis, H., Hayes, K., and Salyers, A. A., 2001, Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon, *Appl Environ Microbiol* **67**(2)**:**561-8.
Sjolund, M., Tano, E., Blaser, M. J., Andersson, D. I., and Engstrand, L., 2005, Persistence of resistant Staphylococcus epidermidis after single course of clarithromycin, *Emerg Infect Dis* **11**(9)**:**1389-93.
Sjolund, M., Wreiber, K., Andersson, D. I., Blaser, M. J., and Engstrand, L., 2003, Long-term persistence of resistant Enterococcus species after antibiotics to eradicate Helicobacter pylori, *Ann Intern Med* **139**(6)**:**483-7.
Soki, J., Gal, M., Brazier, J. S., Rotimi, V. O., Urban, E., Nagy, E., and Duerden, B. I., 2006, Molecular investigation of genetic elements contributing to metronidazole resistance in Bacteroides strains, *J Antimicrob Chemother* **57**(2)**:**212-20.
Song, Y., Liu, C., Lee, J., Bolanos, M., Vaisanen, M. L., and Finegold, S. M., 2005, "Bacteroides goldsteinii sp. nov." isolated from clinical specimens of human intestinal origin, *J Clin Microbiol* **43**(9)**:**4522-7.
Song, Y. L., Liu, C. X., McTeague, M., and Finegold, S. M., 2004, "Bacteroides nordii" sp. nov. and "Bacteroides salyersae" sp. nov. isolated from clinical specimens of human intestinal origin, *J Clin Microbiol* **42**(12)**:**5565-70.
Stiefel, U., and Donskey, C. J., 2004, The Role of the Intestinal Tract As a Source for Transmission of Nosocomial Pathogens, *Curr Infect Dis Rep* **6**(6)**:**420-425.
Stiefel, U., Pultz, N. J., Helfand, M. S., and Donskey, C. J., 2004, Increased susceptibility to vancomycin-resistant Enterococcus intestinal colonization persists after completion of anti-anaerobic antibiotic treatment in mice, *Infect Control Hosp Epidemiol* **25**(5)**:**373-9.
Stubbs, S. L., Brazier, J. S., Talbot, P. R., and Duerden, B. I., 2000, PCR-restriction fragment length polymorphism analysis for identification of Bacteroides spp. and characterization of nitroimidazole resistance genes, *J Clin Microbiol* **38**(9)**:**3209-13.
Sullivan, A., Edlund, C., and Nord, C. E., 2001, Effect of antimicrobial agents on the ecological balance of human microflora, *Lancet Infect Dis* **1**(2)**:**101-14.
Sullivan, A., and Nord, C. E., 2005, Probiotics and gastrointestinal diseases, *J Intern Med* **257**(1)**:**78-92.
Summers, A. O., 2006, Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem, *Anim Biotechnol* **17**(2)**:**125-35.
Theron, M. M., Janse Van Rensburg, M. N., and Chalkley, L. J., 2004, Nitroimidazole resistance genes (nimB) in anaerobic Gram-positive cocci (previously Peptostreptococcus spp.), *J Antimicrob Chemother*.
Ulger (Toprak), N., Celik, C., Cakici, O., Soyletir, 2004, Antimicrobial susceptibilities of Bacteroides fragilis and Bacteroides thetaiotaomicron strains isolated from clinical specimens and human intestinal microbiota, *Anaerobe* **10**(5)**:**255-259.
Vedantam, G., and Hecht, D. W., 2003, Antibiotics and anaerobes of gut origin, *Curr Opin Microbiol* **6**(5)**:**457-61.
Whittle, G., Shoemaker, N. B., and Salyers, A. A., 2002, The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes, *Cell Mol Life Sci* **59**(12)**:**2044-54.
Williams, R. J., and Heymann, D. L., 1998, Containment of antibiotic resistance, *Science* **279**(5354)**:**1153-4.

Woodford, N., and Sundsfjord, A., 2005, Molecular detection of antibiotic resistance: when and where?, *J Antimicrob Chemother* **56**(2)**:**259-61.
Vowels, B. R., Feingold, D. S., Sloughfy, C., Foglia, A. N., Konnikov, N., Ordoukhanian, E., Starkey, P., and Leyden, J. J., 1996, Effects of topical erythromycin on ecology of aerobic cutaneous bacterial flora, *Antimicrob Agents Chemother* **40**(11)**:**2598-604.
Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., Hooper, L. V., and Gordon, J. I., 2003, A genomic view of the human-Bacteroides thetaiotaomicron symbiosis, *Science* **299**(5615)**:**2074-6.