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Characterization of conjugative plasmids in the gut microbiota from a preterm twin pair

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Sammendrag

Den økende spredningen av antibiotikaresistensgener er på vei til å bli et av verdens største utfordringer innen menneskers helse. Den humane tarmen har en meget høy tetthet av bakterier, noe som legger til rette for stor forekomst av genoverføring blant mikrobene som lever der. I denne avhandlingen ble tarmbakterier isolert fra et 20 dager gammelt prematurt tvillingpar som ikke hadde mottatt antibiotika. Isolatene ble undersøkt for antibiotikaresistens (AR), ved hjelp av antibiotika lappediffusjonstest, og konjugative plasmider av gruppene IncF IA, IncF IB, og IncI1, ved hjelp av kvantitativ PCR. Isolatene som inneholdt plasmider ble videre karakterisert ved hjelp av Illumina MiSeq helgenomsekvensering. Resultatene viste et høyt antall AR-gener hos begge spedbarn. Konjugative plasmider ble funnet hos 58% av de testede isolatene, og var av typen IncF IB og IncI1. IncI1 plasmidet ble vist å være overførbart sammen med et høyt antall AR-gener, via konjugasjonseksperimenter. I tillegg til dette ble én stamme funnet å kunne produsere en type colicin som hadde kapasitet til å inhibere vekst av enkelte kommensale tarmbakterielle stammer. Konklusjonen fra denne avhandlingen er at et stort resistom er tilstede i tarmen til spedbarn fra en veldig tidlig alder, selv uten antibiotisk selektiv påvirkning. Konjugative plasmider som var bærere av disse AR-genene var svært overførbare i konjugasjonsforsøk. Videre studier av konjugative plasmider er viktig for å utvide vår kunnskap om spredning av AR-gener i mikrobielle samfunn.

Abstract

The increasing spread of antibiotic resistance genes is becoming one of the most challenging issues for human health worldwide. The human gut holds a very high density of bacteria, which allows high rates of gene transmissions among the microbes living there. In this thesis, bacterial strains were isolated from the gut of a 20 days old preterm infant twin pair that had never received antibiotics. The isolates were screened for antibiotic resistance (AR) by antibiotic susceptibility test, and conjugative plasmids of the IncF IA, IncF IB and IncI1 group by quantitative PCR. The plasmid-containing isolates were further characterized by whole genome sequencing using Illumina MiSeq. The results revealed a high number of AR genes in both infants. Conjugative plasmids were present in 58% of the tested isolates, and where of IncF IB and IncI1. The IncI1 plasmid was shown to be transmissible, together with a high number of AR genes, through conjugation experiments. In addition to this, one strain was found to produce a colicin, which was able to inhibit the growth of some commensal gut bacterial strains. The conclusion from this study is that a large resistome is present in the gut of infants from a very early age, even without any antibiotic selective pressure. Conjugative plasmids carrying these AR genes were highly transmissible in conjugation experiments. Further studies of conjugative plasmids are important in order to expand our knowledge about the spread and persistence of AR genes in microbial communities.

Abbreviations and definitions

HGT – Horizontal Gene Transfer

ARG – Antibiotic Resistance Genes

MGE – Mobile genetic elements

Resistome – Collection of all genes that confer resistance to antibiotics

NEC – Neonatal necrotizing enterocolitis

DNA – Deoxyribonucleic acid

kb – kilobase pairs

Col V – Colicin V

MIC – Minimum Inhibitory Concentration

dNTP – deoxyribonucleotide triphosphate

ddNTP – dideoxyribonucleotide triphosphate

MH – Mueller Hinton

LB – Luria Bertani

TAE – Tris-Acetate EDTA

PCR – Polymerase Chain Reaction

NCBI – National Center for Biotechnology Information

BLAST – Basic Local Alignment Search Tool

RAST – Rapid Annotation using Subsystem Technology

MLST – Multi Locus Sequence Typing

pMLST – plasmid Multi Locus Sequence Typing

E.coli – Escherichia coli

E.faecalis – Enterococcus faecalis

S.flexneri – Shigella flexneri

S.epidermidis – Staphylococcus epidermidis

CFU – Colony Forming Units

ESBL – Extended Spectrum Beta Lactamase

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1. Introduction

Antibiotic resistance poses a worldwide threat to human health (Bennett 2008; Fischbach & Walsh 2009). The increasing resistance is limiting treatment options for bacterial infections (von Wintersdorff et al. 2016). HGT is a main contributor to the spread of ARGs (Lopatkin et al. 2016). The human gut contains a high number of bacteria, including many Gram-negatives. These commensal bacteria harbor numerous ARGs, which compromise the human gut associated resistome (a collection of all genes that confer resistance to antibiotics) (Sommer et al. 2009). These genes can be exchanged among the commensal microbiota, as well as to potential pathogens (Moore et al. 2013). The preterm gut microbiota has been shown to have an increased amount of bacteria belonging to the *Enterobacteriaceae* family (Barrett et al. 2013), which are hosts of different conjugative plasmids. Resistance genes associated with MGEs like conjugative plasmids deserve special attention due to their increased potential for dissemination in bacterial communities.

1.1 Gut microbiota

The gut microbiota plays an important role in our body as it helps to develop barrier and immune functions, break down certain nutrients and produce important vitamins (Lozupone et al. 2012). The colonization of the gut at birth is dependent on different factors like gestational age, mode of delivery, sanitation, diet, and antibiotic treatment (Adlerberth & Wold 2009). Vaginally-born term infants are initially colonized by microbial communities resembling maternal vaginal microbiota, including *Lactobacillus, Bifidobacterium* and *Prevotella* spp. Those delivered by caesarean section harbor communities that more closely resemble the skin microbiota. These include *Staphylococcus*, *Corynebacterium* and *Propionibacterium* spp. (Dominguez-Bello et al. 2010). The gut microbiota is dynamic in the first years of life, before stabilizing towards an adult microbiota by the end of the first 3-5 years of life (Rodriguez et al. 2015). In healthy adults, the gut microbiota consists of vastly diverse “species-level” phylotypes (Lozupone et al. 2012). Most of these phylotypes are bacteria belonging to just a few phyla, normally Bacteroidetes and Firmicutes (Eckburg et al. 2005).

1.1.1 Preterm infant gut microbiota

Preterm infants are infants born <33 weeks gestational age (Gibson et al. 2015). The microbiota of preterm infants is distinct from that of term infants (Moles et al. 2013). Their gut microbiota is less stable, more vulnerable to external influence, and has more limited diversity (Barrett et al. 2013). The gut colonization pattern has also been described as delayed, as compared to term infants (Moles et al. 2013).

Their early gut microbiota composition resembles bacterial communities of hospital surfaces (Gibson et al. 2015). *Proteobacteria* and other potential pathogens, such as members of the *Enterobacteriaceae* and *Enterococcaeceae* family, have been shown to be dominant in the preterm infant gut (Rodriguez et al. 2015). While levels of characteristic commensal families such as *Bacteroidaceae, Lactobacillaceae,* and *Bifidobacteriaceae* are decreased, compared to infants delivered at term (Barrett et al. 2013).

Preterm birth has also been associated with gut diseases like severe inflammatory intestinal disorder and NEC, likely due to delayed acquisition of beneficial species (Wang et al. 2009). In preterm infants, gut functions are not yet completed. Structural and immunological immaturities gives an increased risk for bacteria to translocate to systemic organs and tissues, and cause serious infections (Westerbeek et al. 2006). Antibiotics are therefore routinely given at a greater dose (Berrington et al. 2013).

1.1.2 Effect of antibiotic exposure on the gut microbiota

Antibiotic therapy can cause alterations to the gut microbiota, including reduced diversity and disruption of microbial development in infants (Rodriguez et al. 2015). Antibiotic treatment in preterm infants has been found to increase the abundance of opportunistic pathogens such as *Enterobacteriaceae* while lowering the abundance of bacteria linked to beneficial microbiota like *Bifidobacteriaceae, Bacillaceae,* and *Lactobacillaceae* (Westerbeek et al. 2006). Antibiotic therapy has also been shown to select for survival of resistant members of the microbial community and for members capable of acquiring ARGs. These populations can include opportunistic pathogens, as well as act as reservoirs for ARGs for transfer from commensal bacteria to pathogens (Gibson 2015). The increasing resistome is compromising successful treatment of serious infections (Alekshun & Levy 2007).

1.2 Antibiotic groups and mode of action

Classification of antibiotics is based on the chemical structure, and is characterized by a typical molecular core structure, which is responsible for drug activity. The antibacterial activity is due to inhibition of biochemical pathways that are involved in the biosynthesis of essential components of the bacterial cell (Aarestrup 2006).

Table 1 Examples of antibiotic groups, their mode of action and mechanisms for resistance against them (Aarestrup 2006).

	Antibiotic groups	**Mode of action**	**Mechanisms for resistance**
Inhibition of cell wall synthesis	Beta-lactams (penicillins and chephalosporins)	Bind to the transpeptidase penicillin binding protein (PBP), and blocks transpeptidation of peptidoglycan strands	Enzymatic inactivation of the antibiotic by cleavage of the drug or addition of a chemical group
Inhibition of protein synthesis	Chloramphenicols, macrolids, aminoglycosides, tetracyclins	Bind to the 50S or 30S subunit of the ribosome and lead to non-functional protein synthesis	Active efflux pumps to reduce the concentration of the antibiotic in the cytoplasm, Enzymatic inactivation
Inhibition of nucleic acid synthesis	Quinolones, coumarins, rifamycins	Interact with topoisomerase II (gyrase), topoisomerase IV, or RNA polymerase, and inhibit their function.	Structural modification of target making the antibiotic unable to bind to it
Inhibition of other metabolic processes	Sulfonamides, diaminopyrimidines (trimethoprim)	Block folic acid synthesis	Use of alternative metabolic pathways

Bacteria can be resistant to antibiotics by either primary (intrinsic) resistance, or acquired resistance. Primary resistance is when the bacteria are resistant to a specific antibiotic agent or class due to the lack of target for that specific antibiotic, or inaccessibility of that antibiotic in certain bacteria. Acquired resistance is due to acquisition of ARGs or mutational modification of chromosomal target genes. Antibiotic resistance in bacteria is caused by various mechanisms, often used in combination (Aarestrup 2006).

1.3 Horizontal gene transfer

Plasmids, integrons, and ARGs can be spread vertically from mother to daughter cell during cell division. They can also be spread through HGT between bacteria of the same or different species, through processes like conjugation, transduction or transformation (Aarestrup 2006).

1.3.1 Conjugation

Bacterial conjugation is a process where DNA, generally in the form of conjugative plasmids, is transferred from a donor to a recipient cell, through close contact between the cells (Huddleston 2014). Conjugative plasmids, capable of transferring through conjugation, contain at least 25 transfer (*tra*) genes encoding expression of pili, synthesis and transfer of DNA during mating, and interference with the ability of the plasmid to serve as recipient (Holmes 1996). In order for conjugation to happen, a mating-pair formation (mpf), usually in the form of pili, needs to be established between the donor and the recipient cell. A single strand copy of the donor DNA is transferred from the donor to the recipient cell. In the recipient cell, the transferred DNA is recircularized, before a complementary strand is synthesized. This process results in a transconjugant containing a copy of the conjugative plasmid (De La Cruz et al. 2010). The process of conjugation is shown in figure 1.

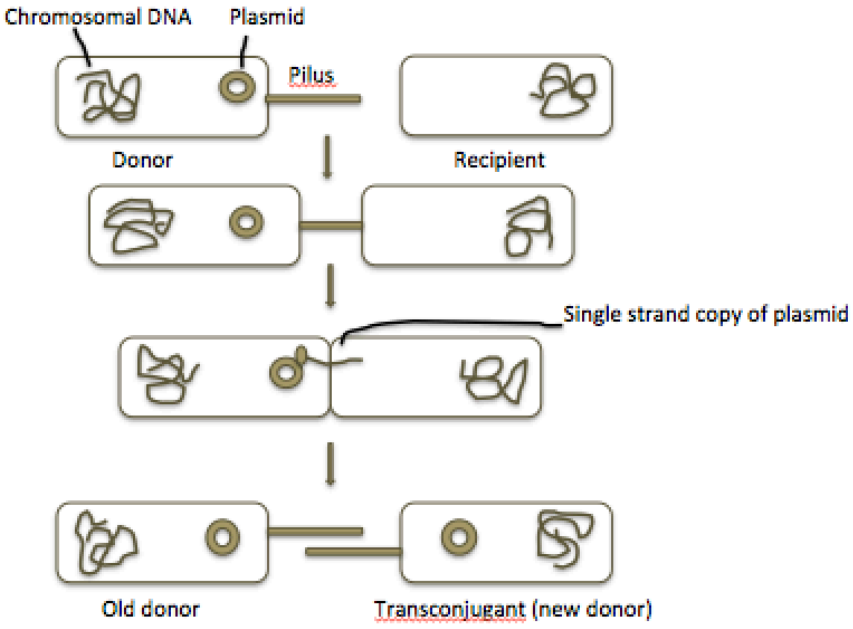

Figure 1 The conjugation process. The donor bacterium, containing a plasmid, is connecting its pilus to a receptor on the plasmid-free recipient bacterium. The pilus is pulling the two cells closer together. A single strand copy of the plasmid is synthesized and is transported through the pilus to the recipient strain, where it forms a copy of the donor plasmid (figure made for this thesis).

1.3.2 Transduction

Bacteriophages can transfer genes from one bacterial cell to another during an infection of bacteria. This way of HGT is called transduction. In order to replicate, bacteriophages inject their DNA into a host bacterium, where it can produce new phage particles. Inside the host cell, the phage-borne genes is expressed, their DNA is replicated and packaged into new phage particles. The new phage particles are then released by lysis of the bacterial cell (lytic cycle). However, phages can also integrate into the host cell chromosome as a "prophage", and stay dormant for longer periods (lysogenic cycle). External conditions, like low nutrient level or UV-irradiation can activate the prophage to go into the lytic cycle. During the phage assembly, resistance plasmids or other genes from the host cell may accidentally be packed into the phage heads. This DNA may integrate into other bacterial cells during infection of new host cells (Aarestrup 2006).

1.3.3 Transformation

Transformation is transfer of free DNA into competent recipient cells. The free DNA in the environment is often a result of cell death or active excretion (Norman et al. 2009). This process of transformation is an active uptake initiated by the recipient cell, were the extracellular DNA is heritable integrated. Unlike conjugation and transduction, which rely on extrachromosomal genetic elements, transformation is part of the normal physiology of the competent cell. Competent cells express a set of proteins dedicated to the uptake and homologous recombination of the free DNA (Johnsborg et al. 2007).

1.4 Plasmids

In bacteria, ARGs are often found in extra chromosomal, double stranded genetic elements called plasmids. They vary in size from a few to more than several hundred kb, and in copy number from one to several hundred per cell (Waters 1999). They are typically composed of "household" regions coding for functions responsible for plasmid replication, stability, and transfer. In addition they often harbor one or more elements that are advantageous for the survival of the host, for example antimicrobial resistance or virulence genes (Carattoli et al. 2005). These additional genes are often found within transposable elements, which increases their potential for dissemination in the microbial community (Couturier et al. 1988).

If a plasmid fails to get included in a daughter cell during bacterial cell division, the lineage becomes plasmid-free or "cured". Since a plasmid imposes a metabolic burden for the host, the lineages without the plasmid will be favored (Million-Weaver & Camps 2014). If a strain is cured, the genes of the lost plasmid can become extinct over time, unless a selective pressure is restored, or the genes have been successfully transmitted onto the host chromosome or another plasmid. Plasmids have several mechanisms that promote plasmid stability to prevent curing (Zielenkiewicz & Ceglowski 2001). One mechanism is active partitioning. High copy number plasmids can normally rely on adequate distribution by chance, based on random diffusion. However, low copy number conjugative plasmids have to rely on active mechanisms to ensure transfer to the daughter cell during cell division. This usually includes a nucleoprotein complex (the segrosome), encoded by the genes *par*A and *par*B. The complex ensures that the plasmids are actively moved into position during the cell division. Another mechanism that promotes plasmid stability is plasmid addiction systems. This is systems that ensures plasmid presence by killing the host cells that does not contain the plasmid after segregation, so-called post-segregational killing (PSK). An example of addiction system is the ccdAB toxin-antitoxin (TA) system of the F-plasmid (Norman et al. 2009).

1.4.1 Conjugative plasmids

Conjugative plasmids can be self-transmissible, if they contain a self-sufficient conjugative transfer system. This is a *tra*-gene complex, which contains all components of the transfer apparatus. Conjugative plasmids are often found in low copy numbers (<10 copies/cell), which is minimizing the metabolic burden on the host, and thus gives a selective advantage (Thomas 2001).

The conjugation process of conjugative plasmids requires close contact between the donor and the recipient cell (Aarestrup 2006). In Gram-negative bacteria, mpf is established by a plasmid-encoded pilus, assembled by a type IV secretion system (T4SS). The pilus binds to a specific outer membrane protein (a *ompA* gene product) on the recipient cell. A DNA processing enzyme, relaxase, introduces a strand-specific nick within a sequence called the origin of transfer, *oriT* in the donor DNA. The relaxase, bound to the 5´ end of the nicked DNA, mediate the transfer of a single strand of the DNA from the donor to the recipient, through the pilus. In the recipient cell, the

enzyme then recognizes the 3´end of the *oriT,* and recircularizes the single strand by reversing the nicking process. A new complementary strand is synthesized in both the donor (to replace the transferred strand), and in the recipient cell (Derbyshire & Gray 2014).

Conjugative plasmids can be classified based on incompatibility (Inc) groups. Plasmids with the same replication controls belong to the same Inc group and are "incompatible". This means they cannot be in the same cell at the same time. Plasmids with different replication controls are compatible, and can be found together in one cell (Novick 1987). This system is based on introducing a plasmid of unknown Inc group into a strain carrying a plasmid of a known Inc group, through HGT, for example conjugation. If the host plasmid is eliminated the incoming plasmid is assigned to the same Inc group (Carattoli et al. 2005).

IncF

IncF plasmids are low copy number plasmids, often >100 kb in size, limited to a host range of the *Enterobacteriaceae* family (Villa et al. 2010). They contribute to the fitness of the bacterial host by virulence and antibiotic resistance genes. Virulence traits like bacteriocins, siderophores, cytotoxins, and adhesion factors, often found in species like *Salmonella enterica*, *Shigella spp*. and enterohaemorhagic *Escherichia coli* (EHEC), enteropathogenic *E.coli* (EPEC), and enteroinvasive *E.coli* (EIEC), are encoded by IncF plasmids, designated in these species as virulence plasmids (Johnson & Nolan 2010). IncF plasmids have especially been associated to the spread of ESBLs, quinolone, and aminoglycoside resistance (Villa et al. 2010). IncF plasmids is one of the most frequently encountered plasmid types, and was found in >50% of *E.coli* from feces of healthy, antibiotic-free humans in the USA (Johnson, T. J. et al. 2007). Their addiction systems contribute to the promotion of plasmid spread, and their virulence and resistance genes gives them a selective advantage (Villa et al. 2010).

The IncF plasmid group was first defined based on the production of type F pili susceptible to phage Ff (Carattoli 2009). All the plasmids in the IncF group carry the same conjugative transfer systems (Tra). They require two complexes for conjugative transfer; the Mpf, and the DNA transfer and replication system (Dtr), which combines DNA processing functions and factors for transport of DNA from the donor to the

recipient cell. The conjugative transfer system of IncF plasmids (TraF) produces characteristic flexible pili, which allow conjugation in liquid media. The F transfer region contains 35 genes and has a rather complex organization (Thomas 2001).

IncI

IncI plasmids are, like the IncF plasmids, limited to the *Enterobacteriaceae* family. The IncI group was first defined based on that the plasmids were producing type I thin conjugative pili susceptible to phage Ifl (Carattoli 2009). The IncI group is further divided into two classes based on replicon typing and conjugation apparatus. One class includes IncI1, B and K groups, and the other includes IncI2 plasmids (Thomas 2001). The IncI plasmids have a complex transfer region that specifies the formation of a thin, flexible pilus and a thick and rigid pilus essential for DNA transfer. IncI1 plasmids have a total of 49 genes that encode transfer in liquid and on solid media (Thomas 2001).

IncI1 plasmids are noted as being in possession of different ARGs. They have especially been associated with the spread of several ESBL genes, detected in both animal and human fecal samples (Carattoli 2009). They are also characterized by the presence of a cluster encoding the type IV pili, contributing to adhesion and invasion of shiga-toxigenic *E.coli* (Kim & Komano 1997). The IncI1 plasmids has been identified more frequent in pathogenic than commensal strains (Johnson, Timothy J. et al. 2007).

1.4.2 Mobilizable plasmids

Mobilizable plasmids are often less than 30 kb in size (Smillie et al. 2010). They usually carry a mobilization region (*mob*), encoding specific relaxsome components, and the origin of transfer (*oriT*). They do not however carry the rest of the machinery needed for self-transfer (Francia et al. 2004). Yet, mobilizable plasmids have the ability to be transferred through conjugation if a self-transmissible plasmid is also present in the donor cell.

1.4.3 Non-mobilizable plasmids

Some plasmids are called non-mobilizable because they are neither conjugative nor mobilizable. Non-mobilizable plasmids lack the relaxase protein, and thus do not have the ability to be self-transmissible (Smillie et al. 2010). They can however spread by natural transformation, by transduction, or from mother to daughter cells during

replication (Smillie et al. 2010). Non-mobilizable plasmids are often very large, and can be >300 kb in size. Some have suggested that these large plasmids in a way could serve as a secondary chromosome for the bacterial host (Harrison et al. 2010).

1.4.4 Accessory elements

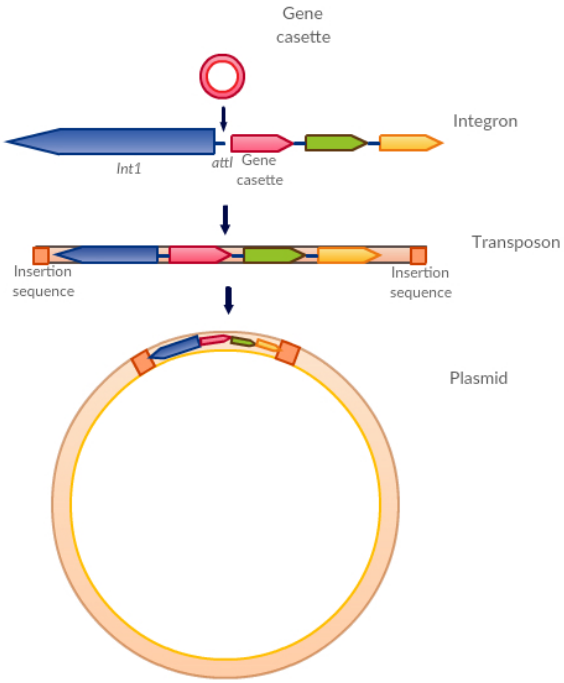

Figure 2 Mobile genetic elements. Gene cassettes containing ARGs can be found inside an integron. An integron can be found within a transposon, which again can be found within a conjugative plasmid (figure made for this thesis).

Integrons

Integrons are genetic elements consisting of an *Int*-gene (figure 2). The gene encodes a site-specific recombination enzyme, called integrase, and a corresponding recombination site, *attI* (Recchia & Hall 1995). At the *attI*-site, gene cassettes containing several DNA elements (for example ARGs) can be inserted (Hall & Collis 1995). A gene cassette exists as a small, non-replicating double stranded circular DNA molecule as an intermediate in the process of moving from one integron to another or in the re-assortment of gene cassettes within a particular integron (Bennett 2008). Several gene cassettes can be inserted in one integron, and up to 8 gene cassettes have been found in the same integron. There are approximately around 100 different gene

cassettes containing ARGs known today, contributing to multidrug resistance (Gogarten 2009). Integrons are not mobile itself, but are often found together with a transposon or in close association with conjugative plasmids (Bennett 1999).
Most resistance integrons belong to a structure known as class 1 integron (*Int1*). These elements consist of two terminal constant sequences and a highly variable central section. The *attI* site and the promoter from which gene cassettes are expressed are located near the 5´end. The 3´end accommodates part of a gene, *qacEΔ1*, that confers resistance to quaternary ammonium compounds, followed by a *sul*-gene, that confers resistance to sulfonamides (Bennett 2008).

Transposons

Transposons are MGEs capable of transporting itself from one DNA site to another through recombination. They carry both DNA sequences that function as recombination sites and genes encoding proteins that participate in recombination (Liebert et al. 1999). The recombinases responsible for transposition are usually called transposases or integrases (Kostriken et al. 1981). In addition to genes encoding their own transposase, the transposon may carry genes that provide a function useful to its host cell (e.g antibiotic resistance genes) (Watson 2014). Transposons have the ability to either move from one site to another within a DNA molecule or move from one DNA molecule to another, for example from one plasmid to another, or from one plasmid to a bacterial chromosome (Bennett 2008). An example of a transposon in relation to other MGEs is shown in figure 2.

Bacteriocins

Bacteriocins are proteinaceous antimicrobial molecules produced by various bacteria, to kill closely related strains. In the last decade bacteriocins have been shown great interest due to their potential as both a next-generation antibiotic and a food preservative (Perez et al. 2014). A bacteriocin can help the producer cell to outcompete other bacterial strains, for example when there is a lack of nutrients (Alvarez-Sieiro et al. 2016).

Colicin is a bacteriocin produced by strains of *E.coli*, that harbor a colicinogenic plasmid, pCol. It is a protein that can kill closely related strains of *E.coli*. They have a narrow target range due to the presence of specific receptors on the surface of the

sensitive strains, to which the colicin binds (Cascales et al. 2007). Colicins are named by an alphabet letter according to what receptor it binds on the sensitive strain. If several colicins can bind to the same receptor they are named by the alphabet letter of the receptor, followed by a number (e.g Colicin E: E1 to E9) (Cascales et al. 2007).

Colicin V is a peptide that kills sensitive cells by gaining access to their inner membrane from the periplasmic face and disrupting their membrane potential. ColV synthesis, export, and immunity require four plasmid-borne genes (*cva*A, *cva*B, *cvaC*, and *cvi*) (Gérard et al. 2005). *cva*C is the gene coding for the functional peptide, and synthesizes a 103-amino-acid primary translation product with a conserved double glycine leader peptide at its N terminus (Havarstein et al. 1994). An ABC-exporter composed of three proteins (CvaA, CvaB, ans TolC) is needed for secretion of the peptide. The *cvi*-gene codes for an immunity protein, which protect the cell from being killed by its own ColV production. All these genes need to be present and functional in order for the cell to produce the ColV (Gérard et al. 2005).

1.5 Microbiology methods

1.5.1 Antibiotic susceptibility

Antibiotic susceptibility can be evaluated by determining the MIC of a given antibiotic, which is the lowest antibiotic concentration that completely inhibits growth of the bacterial isolate. A strain can be defined resistant, intermediate, or susceptible based on clinical breakpoints (Aarestrup 2006). The European Committee of Antibiotic Susceptibility Testing (EUCAST) has standardized methods and breakpoints for antibiotic susceptibility testing with Kirby-Bauer antibiotic disk diffusion test (EUCAST 2013). The principle for this method is to place an antibiotic-impregnated disk on the surface of agar inoculated with the test bacterium. The antibiotic will diffuse out in the agar, producing an antibiotic concentration gradient were there will be high concentration near the disk. After incubation, a clear zone around the antibiotic disk is seen if the bacterium is susceptible to the antibiotic. The wider the zone, the more susceptible the bacterium is. If the bacterium manages to grow up to the disk it is considered resistant. The zone width is also dependent on the antibiotics initial concentration, its solubility, and its diffusion rate through agar. The zone width can therefore not directly be compared between different antibiotics (Willey et al. 2009).

1.6 Sequencing

1.6.1 Sanger sequencing

Sanger sequencing is a first-generation sequencing method, known as chain-termination sequencing or dideoxy sequencing. It was developed in 1977, by Frederick Sanger and his colleagues. The method is based on synthesis of DNA strands that are complementary to a template DNA strand (GATC-Biotech 2017). A DNA template, primers, DNA polymerase, dNTPs, and ddNTPs are used in the reaction to synthesize complementary DNA fragments. The ddNTPs are labeled with a fluorescent dye, distinct for each base. The DNA polymerase is adding dNTPs to the 3´-end of new growing DNA fragments. When a ddNTP is added, the DNA elongation is terminated. This random incorporation of dNTPs and ddNTPs produces fragments of different sizes with the labeled ddNTP at the end of each fragment (Sanger et al. 1977). The DNA fragments can be separated by size using gel electrophoresis. A laser is then used to excite the fluorescent dyes, and the emitted fluorescence from each base can be read by a detector and determines the nucleotide sequence of the original DNA template (GATC-Biotech 2017).

1.6.2 Next generation sequencing

Sequencing technologies have rapidly improved over the last decades. Next generation sequencing, or high-throughput sequencing, is a technology that can sequence millions of DNA sequences at the same time, at reduced costs and time (Jay & Hanlee 2008). Next generation sequencing methods can be used to sequence whole genomes. This is done by so-called shotgun sequencing, where the genome is randomly fragmented and amplified before sequencing. Several next generation platforms have been developed. The Illumina platform is one of them.

Using Illumina sequencing method, the isolated and purified DNA needs to be processed into a library suitable for sequencing. The DNA library preparation includes random fragmentation of the high-molecular weight DNA into smaller fragments, followed by ligation of adapter sequences at the ends of each fragment. The adapters are specific sequences that make the fragments compatible to the further steps of the sequencing process. They also contain unique barcodes for discrimination of the different samples after sequencing. After the adapter ligation, the libraries are pre-amplified and denaturated before loading to a flow cell. The Illumina platform uses

bridge amplification for colony generation on the flow cell. The flow cell is covered with several oligonucleotides (primers), complementary to the adapter sequences of the DNA fragments that are being sequenced. When the fragments bind to these primers, a DNA polymerase can synthesize a complementary fragment DNA strand. The double stranded DNA is then denaturated, and the initial library strand is removed. The copied fragments attached to the flow cell binds at the opposite end to an adjacent complementary primer on the flow cell surface. The hybridized primer is extended by a polymerase and forms a double bridge between the two primers. The bridge is then denaturated, resulting in two copies of covalently bound single stranded templates. This bridge amplification cycle is repeated until multiple bridges have been synthesized. Finally, the revers strands are cleaved and off, leaving only the forward strands to be sequenced (Buermans & Den Dunnen 2014).

A sequencing primer hybridizes to the adapters on the DNA fragments, and fluorescently labeled dNTPs with a blocked 3´OH-group binds complementary to the adapter sequences. The fluorescence of each nucleotide is detected. Because of the colony formation, this fluorescent signal is strong enough to be detected with a camera. After the detection, the fluorescent color is removed, and a new fluorescent-labeled dNTP is incorporated. The signals from all the detections are finally put together to form the DNA sequence (Illumina 2010).

1.7 Aim of the study

The aim of this thesis was to address the diversity of conjugative plasmids in the gut microbiota of a preterm infant twin pair, and their role in the spread and persistence of multidrug resistance. To achieve the main aim, we had the following sub-goals:

- Identification and characterization of strains collected from stool samples of a preterm twin pair
- Investigation of the strains for AR pattern
- Whole genome sequencing of the strains
- Screening for conjugative plasmids
- Testing of plasmid stability over time
- Experimental testing of various traits and virulence factors found in the genomes of the strains
- Testing of conjugative transfer ability

The methods used to achieve the aim and sub-goals includes Sanger sequencing, qPCR, Illumina shotgun sequencing, generation time experiments, and conjugation experiments.

2. Material and methods

2.1 Cohort description and strain isolation

The strains were collected from two healthy preterm twins that were admitted in the University and Polytechnic hospital La Fe in Valencia, Spain. The preterm infants were born at gestational age 30 by emergency caesarean section and the samples were collected 20 days after birth. The infants weighed 1410 g and 1630 g after birth. The infants were breast-fed and stayed at the hospital until sampling, even though they did not show any complications or sign of infections. No antibiotics were given until sampling. The collection and isolation of strains was done by Anuradha Ravi, a PhD student at Professor Knut Rudi´s lab.

Bacterial strain isolation from fecal samples.

MH agar (Sigma Aldrich, Spain) was used to plate 0.2 g of fecal sample diluted to up to 10^{-4} dilution from the corresponding twins. The plates were incubated at 37°C overnight. Individual colonies were picked at random out at 10^{-3} and 10^{-4} dilution plates and streaked onto fresh MH agar plates to get pure cultures. The isolated pure cultures were then stored with 35% glycerol at -80°C until further analysis.

Thirteen isolates from twin one and 61 isolates from twin two were revived from glycerol stock for experiments. 100 µL of the stock were cultured in 3 mL MH broth (Sigma Aldrich, Norway) and incubated aerobically at 37°C for 18-24 h. in a shaking incubator at 130 RPM. One µL of the cultured isolates were spread on MH-agar plates with an inoculation loop and incubated at 37°C for 18-24 h. One colony from each plate were spread on a new MH-agar plate and incubated at 37°C for 18-24 hours to make sure it was a pure culture.

2.2 Antibiotic susceptibility

The antibiotic susceptibility pattern of the isolates was determined with antibiotic disk diffusion on MH-agar (Thermo Fisher, USA) plates, following Eucast´s guidelines (Matuschek et al. 2014). The isolates were suspended in 0,85% saline to the density of 0.5 McFarland, corresponding to a cell number of approximately 1-2 $x10^8$ CFU/mL. A cotton swab was used to spread the suspension on MH agar, by swabbing in three directions. Antibiotic disks were put on the agar surface and the plates were incubated

at 35±1°C for 18-24 h. For each antibiotic disk the zone diameter was read to the nearest millimeter and compared to Eucast´s clinical breakpoint table for susceptibility. The antibiotics used in the initial antibiotic susceptibility testing were 5 µg of Trimethoprim, 10 µg of Gentamicin, 5 µg of Ciprofloxacin, 10 µg of Cefpodoxime, 25 µg Sulphamethoxazole/trimethoprim, and 30 µg of Amoxicillin-clavulanic acid. The antibiotic susceptibility cartridges were obtained from Oxoid, ThermoFisher Scientific, USA.

2.3 DNA extraction

DNA from the strains was isolated using the Mag Midi DNA extraction kit (LGC Genomics, UK). 200 µL of the isolates were mixed with 200 µL STAR buffer (Roche, USA) and acid washed glass beads (Sigma Aldrich, Norway, <106 µm; 0,25 g), and lysed in FastPrep96 (MP Biomedicals, USA) at 1800 rpm for 40 seconds, three times. The samples were cooled on ice between the runs. After the lysis the samples were centrifuged at 13000 rpm for 5 minutes, and 50 µL of the supernatant was transferred to a KingFisher plate (Thermo Scientific, USA). 50 µL lysis buffer and 5 µL Proteinase was added to degrade cellular proteins at 55°C for 10 minutes in the KingFisher Flex robot (Thermo Scientific, USA). The DNA extraction was done by the KingFisher Flex robot, using paramagnetic beads to bind negatively charged DNA. Several washing steps were preformed to remove salts and other impurities. Then the DNA was eluted in elution buffer and stored at -20°C until further use.

2.4 Qualitative and quantitative nucleic acid measurements

2.4.1 PCR

The primers and the specific annealing temperatures for each reaction are shown in table 2.

Qualitative PCR

Each reaction was carried out with a final concentration of 1x 5X HotFirePol Blend Master Mix Ready to Load (Solis Biodyne, Estonia), 0.2 µM forward primer, 0.2 µM revers primer (Solis Biodyne, Estonia), 7.5-150 ng/µL DNA template, and 18 µL nuclease free H_2O (Amresco, USA) per sample. Initial denaturation was at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec (table 2), elongation at 72°C for 45 sec. Then a final elongation was set at 72°C for 7 min, before cooling at 4°C ∞. All reactions were performed on a 2720 Thermal Cycler

(Applied Biosystems, USA). Nuclease free water was used as negative control in all reactions.

Quantitative PCR

Each reaction was carried out with a final concentration of 1x 5X HOT FIREPol® EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia), 0.2 µM forward primer, 0.2 µM revers primer (Solis BioDyne, Estonia), 1-50 ng/µL DNA template, and 14.2 µL nuclease free H_2O per sample. The reactions were performed in 96 well LightCycler qPCR plates (Roche, Germany). The reaction was performed in the LightCycler 480 (Roche, Germany). Initial denaturation was at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing for 1 min (see table 2 for specific temperatures). Then a final elongation was set at 72°C for 30 sec, where fluorescence was measured after each cycle. After each run, a high resolution melting (HRM) curve analysis was performed to verify the desired amplicon. Nuclease free water was used as negative control in all reactions.

Table 2 Primers used in this study.

Primer target	Primer sequence (5´-3´) FW-RV	Amplicon size (bp)	Annealing temperature (°C)	References
Bacterial DNA (16S rRNA)	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	450	60	(Nadkarni et al. 2002)
IncF IA plasmid	CCATGCTGGTTCTAGAGAAGGTG GTATATCCTTACTGGCTTCCGCAG	462	60	(Carattoli et al. 2005)
IncF IB plasmid	GGAGTTCTGACACACGATTTTCTG CTCCCGTCGCTTCAGGGCATT	702	60	(Carattoli et al. 2005)
IncI1 plasmid	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT	139	60	(Carattoli et al. 2005)
Integron 1	ACGAGCGCAAGGTTTCGGT GAAAGGTCTGGTCATACATG	565	53	(L'Abee-Lund & Sorum 2001)
E.coli (23S rRNA)	GGTAGAGCACTGTTTTGGCA TGTCTCCCGTGATAACTTTCTC	87	60	(Chern et al. 2011)

Abbreviations: FW, Forward; RV, Revers

2.4.2 Agarose gel electrophoresis

The size of the PCR products was determined using gel electrophoresis with a 1% agarose (Sigma Aldrich, Germany). This was prepared by dissolving agarose in 1x TAE

buffer. The electrophoresis ran at 90 V for 30-50 min. A 100 bp DNA ladder (Solis BioDyne, Estonia) was used as size marker for the DNA fragments. The fragments were visualized using The Molecular Imager® Gel Doc™ XR Imaging system with Quantity One 1-D analysis software v.4.6.7 (Bio-Rad, USA), using UV-light.

2.4.3 DNA quantification

DNA concentrations were measured on the Qubit™ fluorometer (Life Technologies, USA), by using the dsDNA (double-stranded DNA) High Sensitivity Assay Kit (Life Technologies, USA). The measurements were done following the kit protocol, mixing 198 µL of Working solution (Quant-iT™ reagent diluted 1:200 in Quant-iT™ buffer) with two µL of the DNA sample. Calibration of the instrument was performed before the measurements as recommended by manufacturer.

2.5 Sequencing

2.5.1 Sanger sequencing

DNA of the isolates were amplified using qualitative PCR (see 2.4.1 PCR), before they were purified with 0,8x AMPure® XP beads (Beckman Coulter, USA). The samples were mixed with 16S rRNA primers (see table 2), and sent to GATC BioTech, Norway to be sequenced.

2.5.2 Illumina whole genome sequencing

The DNA concentration of the samples were measured using the Qubit™ fluorometer (see 2.4.3 DNA quantification), and the samples were diluted to 0,2 ng/µL. The tagmentation and indexing of the samples were performed using the Nextera XT DNA sample preparation kit (Illumina, USA). The samples were purified using 0,6x AMPure® XP beads. The Illumina index PCR products were normalized and pooled, together with a PhiX control (Illumina, USA). The pooled samples were purified with 0,6X AMPure® XP beads.

The DNA concentration of the pooled samples was quantified using droplet digital PCR (Bio-Rad, USA). The amplicon library was loaded on to a flow cell, following the Illumina protocol for whole genome sequencing, before loading on the MiSeq® system (Illumina, USA).

2.6 Analytic approaches

2.6.1 Annotation

The annotation of the assembled genomes from the Illumina sequencing was performed using the RAST server ((Aziz et al. 2008) http://rast.nmpdr.org/). This is an online-based tool used for annotation of bacterial and archaeal genomes. The RAST was used with the recommended parameters and settings.

2.6.2 BLAST

The BLAST was used through NCBI´s web site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This is a tool providing access to biomedical and genomic information. The BLAST finds regions of similarity between biological sequences, and the program compares nucleotide or protein sequences to sequence databases to calculate the statistical significance. The Nucleotide BLAST was used with the recommended parameters and settings. The annotation was visualized using CLC Sequence Viewer 6 (Qiagen, USA).

2.6.3 PlasmidFinder

PlasmidFinder 1.3 was used to search for plasmids in the genome ((Carattoli et al. 2014) https://cge.cbs.dtu.dk/services/PlasmidFinder/). The PlasmidFinder was used with the recommended parameters and settings.

2.6.4 ResFinder

ResFinder 2.1 was used to search for ARGs in the genome ((Zankari et al. 2012) https://cge.cbs.dtu.dk/services/ResFinder/). The ResFinder was used with the recommended parameters and settings.

2.6.5 MLST

DTU`s MLST online tool was used to find the sequence type of the *E.coli* strains ((Larsen et al. 2012) https://cge.cbs.dtu.dk/services/MLST/). The MLST was used with the selected configuration: "*Escherichia coli* #1" and selected type of reads: "Assembled Genome/Contigs".

2.6.6 pMLST

DTU´s pMLST online tool was used to identify the plasmid types present in the genome. ((Carattoli et al. 2014) https://cge.cbs.dtu.dk/services/pMLST/). The pMLST was used with the recommended parameters and settings.

2.6.7 Bactibase

Bactibase is a database that contains calculated or predicted physiochemical properties of over 200 bacteriocins produced by bacteria. The database allows rapid prediction of identification, structure, and fuction of bacteriocins (http://bactibase.pfba-lab-tun.org/main.php). The bactibase was used to characterize the bacteriocin genes found, using the BLASTp tool with the recommended parameters and settings.

2.7 Plasmid stability

The generation time for the strains were determined by adding $1,5x10^8$ CFU/mL of bacteria in MH-broth and incubating at 130 rpm in 37°C. McFarland densitometer (BioSan, Latvia) was used to calculate the doubling time of the strains. The number of cells was detected at time 0 and every 10 minutes, until the cells reached the stationary phase, by reading the optical density (OD) with a McFarland densiometer. The generation time for the subject strains were calculated. This was used for the plasmid stability experiments where the stability of the plasmid was investigated. $1,5x10^8$ CFU/mL of the strains were incubated in MH-broth in 37°C at 130 rpm for 102 generations. The generation time experiment showed that the two *E.coli* strains grew for three generations before reaching stationary phase after 180 minutes of incubation under these conditions. After every 180 minutes of incubation the samples were transferred to new tubes with MH-broth to a concentration of $1,5x10^8$ CFU/mL cells and incubated for another 180 minutes. This was repeated until the strains had grown for 102 generations. DNA was extracted from every 180 minute and was screened for IncF IB using qPCR and IncF IB primers. Bacteria from the first and the last generation were also spread on MH agar, both with and without antibiotics (300 µg/mL sulfamethoxazole) to compare the number of colonies.

2.8 Bacteriocin-testing

To test if the isolates were producing colicin, an ammonium sulfate precipitation was done to precipitate the proteins and test it against a colicin sensitive strain (indicator strain *E.coli* LMG 2700).

2.8.1 Ammonium sulfate precipitation

100 µL of the isolates were cultured in 50 mL of LB broth (Sigma Aldrich, Norway) and incubated at 130 rpm in 37°C for 18-24 h. Then the cultures were centrifuged at 5000 G for 15 minutes at 4°C. The supernatant were transferred to new flasks and ammonium sulfate was added to a saturation of 50 %. The online tool "Ammonium Sulfate Calculator" from EnCor Biotechnology Inc. was used to calculate the amount of ammonium sulfate to add at 4°C (Inc. 2017). (The tool takes in account the molecular weight of ammonium sulfate and its solubility in water at different temperatures, and calculates at which concentration the wanted saturation is reached. The calculation also takes in consideration the increase in volume when the ammonium sulfate is added). The flasks were left at 4°C for 18-24 h before centrifuged at 25 000 rpm at 4°C for 45 minutes. The supernatant was removed and the pellet (precipitate) was resuspended in 500 µL 1xPBS. The precipitate was heat treated at 70°C for 5 minutes to kill all living cells.

2.8.2 Soft agar overlay method

The indicator was grown in LB broth and incubated at 37°C for 18-24 h. Forty µL of the indicator strain was mixed with 5 mL of 0,7 % LB agar (Sigma Aldrich, Norway) and poured on the surface of a LB agar dish. The dish was left to dry for 5 minutes before adding 2 µL of the bacteriocin precipitate from the isolates on the agar surface, and then incubated at 37°C for 18-24 h. A clear inhibition zone where the precipitate was added could be seen in the agar if the isolate had produced a substance that could kill the indicator strain. To check if this substance was of proteinaceous nature (bacteriocin), 2 µL of proteinase K was added next to 2 µL of the precipitate. If the proteinase K inhibited the bacteriocin ("half moon" inhibition zone) the isolate was most likely producing a bacteriocin.

2.9 Conjugation

Selected isolates were used for conjugation studies. The recipient (*E. coli* DH5α-Rif^{R}) and the donor strains were streaked out on MH agar containing 32 mg/L rifampicin (Oxoid, ThermoFisher Scientific, USA) to make sure the recipient grew and the donor did not grow here. The antibiotics used to select for the transconjugants were 25 µg Sulphamethoxazole, 25 µg Streptomycin, 30 µg Tetracyclin, 30 µg Chloramphenicol, 5 µg Trimethoprim, 5 µg Cefotaxime, and 2 µg Ampicillin (obtained from Oxoid, ThermoFisher Scientific, USA) and 100 µg Streptomycin, 10 µg Ampicillin, 5 µg Trimethoprim, and 240 µg Sulfamethoxazole (obtained from Rosco Diagnostica, Denmark).

2.9.1 Conjugation in broth

Conjugation experiments were performed at the Norwegian Veterinary Institute in Oslo, guided by Marianne Sunde. Donor and recipient strains were cultured in 4 mL LB broth and incubated at 37°C for 18-24 h. 500 µL of the recipient and 10 µL of the donor was mixed in 4 mL LB broth for mating and incubated at 37°C for 4 h. The mating mix, together with a 1:10 and 1:100 dilution of the mating mix in 0,85% NaCl, was streaked out on MH agar containing 32 mg/L rifampicin by swabbing in three directions with a cotton stick. Disks with the relevant antibiotics (antibiotics that the donor is resistant to) were put on the agar surface and the plates were incubated at 37°C for 18-24 h. Growth on MH agar with rifampicin and colonies inside the inhibition zones was most likely to be a positive conjugation. The strains were mated for another 24 h before the mating mix and a 1:10 and 1:100 dilution of the mating mix in 0,85% NaCl was streaked out on new MH agar containing 32 mg/L rifampicin with relevant antibiotic disks, to increase the chance of successful conjugation.

Colonies inside the inhibition zones, the potential trans-conjugants, were selected and streaked out on blood agar and lactose agar (blue agar) to inspect the colony morphology and their ability to ferment lactose. The purpose of this was to ensure that the potential transconjugants were DH5α-Rif^{R}, and not donor strains with mutation being able to grow on the plates containing rifampicin. The recipient/transconjugant strains have smaller colonies than the donor strains. They are also lactose non-fermenting, unlike the donor strains which are able to ferment lactose. The trans-conjugants were also tested for resistance against the relevant antibiotics by disk

diffusion test to see which resistance genes that had been transferred. The antibiotic disks used to select for transconjugants were both from Oxoid, ThermoFisher Scientific, USA and Rosco Diagnostica, Denmark. The antibiotic disks used for the antibiotic susceptibility testing of the transconjugants were all from Oxoid, ThermoFisher Scientific, USA.

2.9.2 Conjugation on agar

Donor and recipient strains were streaked out on blood agar (Norwegian Veterinary Institute) and incubated at 37°C for 18-24 h. One inoculation loop (1 µL) of the recipient and one inoculation loop of the donor was streaked out together on a blood agar dish and incubated at 37°C for 4 h. One 1 µL inoculation loop of the mixed strains was dissolved in 1,2 mL NaCl (0,85 %) and streaked out on MH agar containing 32 mg/L rifampicin with a cotton stick, by swabbing in three directions. Relevant antibiotic disks were put on the agar and incubated at 37°C for 18-24 h. The strains were mated for another 24 h before the mixture was streaked out on new MH agar containing 32 mg/L rifampicin with relevant antibiotic disks, to increase the chance of successful conjugation. The trans-conjugants were selected as described in “Conjugation in broth”.

3. Results

3.1 Identification of strains

The isolates were identified using qPCR with *E.coli* specific primers. The isolates showing negative results were identified further with Sanger sequencing. Fifty isolates out of 74 (67,6%) were identified as *E.coli*, while 22 out of the 74 isolates (29,7%) were identified as *Enterococcus faecalis*. In addition, *Shigella flexneri* and *Staphylococcus epidermidis* were identified. The identification of each isolate is shown in appendix A.

3.2 Antibiotic susceptibility testing

The isolates were grouped based on 13 different antibiotic resistance patterns. The *E.coli* strain 1 and *E.coli* strain 2 did not show resistance towards any of the tested antibiotics. Both of these strains originated from twin 2. The strains with pattern V, also originated from twin 2. All the other groups with different resistance patters contained strains originating from both twins. The antibiotic resistance patterns are shown in table 3.

Table 3 Antibiotic resistance patterns of 74 isolates from the gut microbiota of a preterm infant twin pair.

Pattern	Strain	ID	W	CN	CIP	CPD	SXT	AMC
I	1-2	*E.coli*	S	S	S	S	S	S
II	3	*E.coli*	S	S	S	R	S	S
III	4-17	*E.coli/E.faecalis*	S	I	S	R	S	S
IV	18-25	*E.coli/E.faecalis/S.flexneri*	S	R	S	R	S	S
V	26-28	*E.coli*	R	S	S	S	R	S
VI	29-34	*E.faecalis*	S	R	S	R	S	S
VII	35-36	*E.coli/E.faecalis*	S	R	I	R	S	S
VIII	37	*S.epidermidis*	S	R	R	R	S	S
IX	38-53	*E.coli*	R	S	S	R	R	S
X	54	*E.coli*	R	S	I	R	R	S
XI	55	*E.coli*	R	I	S	R	R	R
XII	56-72	*E.coli*	R	S	S	R	R	R
XIII	73	*E.coli*	R	I	S	R	R	R
XIV	74	*E.coli*	R	R	S	R	R	I

Abbreviations: W, trimethoprim; CN, gentamicin; CIP, ciprofloxacin; CPD, cefpodoxime; SXT, sulfamethoxazole trimenthoprim; AMC, amoxycillin clavulanic acid; S, susceptible; I, intermediate: R, resistant

The most commonly occurring resistance was towards cefpodoxime antibiotic, which was present in 93,2% of the tested isolates. Resistance towards trimethoprim and sulfamethoxazole/trimethoprim was always found together, and present in *E.coli*

strains. Fifty percent of the isolates were multi-drug resistant (resistant to >3 antibiotics), where all except one isolate (strain 37, *S.epidermidis*) were *E.coli*. The relationship and overlap of the antibiotic resistance patterns of the isolated strains are shown in figure 3.

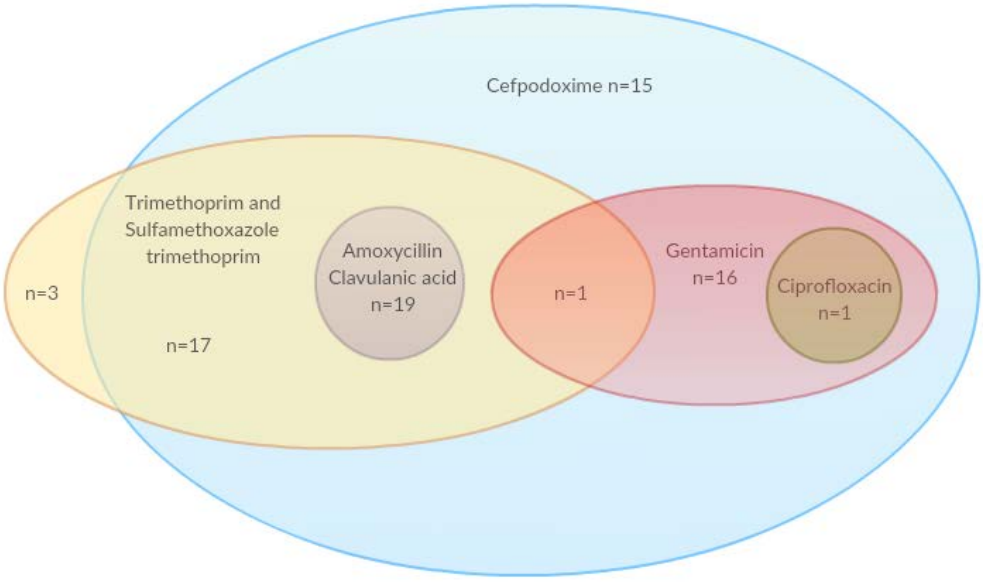

Figure 3 Antibiotic resistance pattern of 74 isolates from the gut microbiota of a preterm infant twin pair. "n" is the number of isolates. Resistance for trimethoprim and sulfamethoxazole trimethoprim was always co-occurring, and is being represented together with the color yellow.

3.3 Screening of MGEs

All isolates were screened for IncF IA, IncF IB and, IncI1 plasmids using qPCR. None of the isolates contained an IncF IA plasmid, but 42 out of 74 isolates (56,8%) contained an IncF IB plasmid. The IncI1 plasmid was present in 41 out of the 74 isolates (55,4%). All of the IncF IB and IncI1 positive isolates were *E.coli* strains, and were found in both twins (appendix A).

The isolates were also screened for *Int1*, where 39 out of 74 isolates (52,7%) were positive. All the *Int1* positive isolates contained the IncF IB and the IncI1 plasmids as well. The relationship and overlap of the isolates containing IncF IB, IncI1 and *Int1* are shown in figure 4.

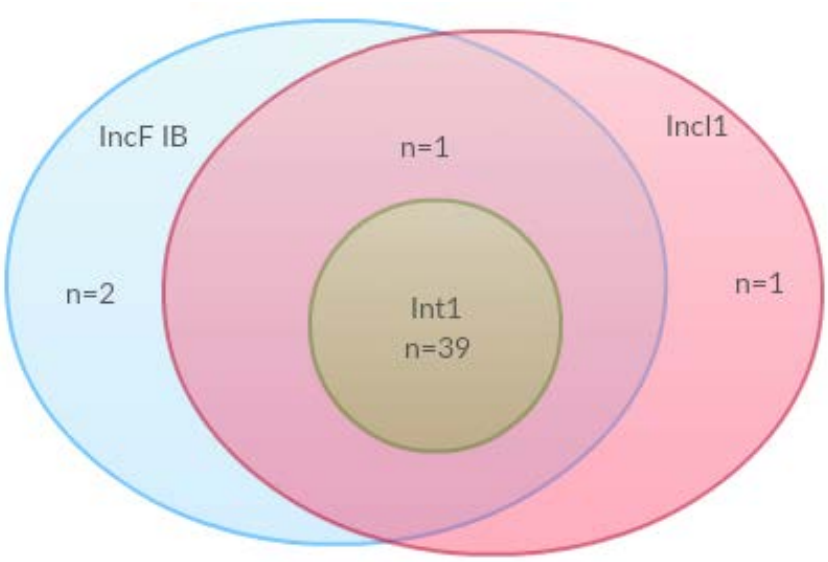

Figure 4 Venn diagram of the IncF IB, IncI1, and *Int1* containing strains isolated from the gut microbiota of a preterm infant twin pair. "n" is the number of isolates.

3.4 Whole genome sequencing

3.4.1 Selection of strains

Seventeen isolates were chosen for whole genome sequencing. The selected isolates were chosen to get a variety of the following criteria; isolates from both twins, isolates that were plasmids positive and isolates that were plasmid negative, both *E.coli* and *E. faecalis* strains, and isolates with different antibiotic resistance pattern. The identification and a summary of information about the selected isolates are shown in table 4.

Table 4 Strains chosen for whole genome Illumina sequencing, isolated from the gut microbiota of a preterm infant twin pair.

Strain	Twin	Resistance pattern	ID	IncF IB	IncI1	Int1
1	2	I	E.coli	Pos	Neg	Pos
3	2	II	E.coli	Pos	Pos	Pos
6	1	III	E.faecalis	Neg	Neg	Neg
7	2	III	E.coli	Neg	Neg	Neg
18	2	IV	E.faecalis	Neg	Neg	Neg
26	2	V	E.coli	Pos	Pos	Pos
32	2	VI	E.faecalis	Neg	Neg	Neg
35	1	VII	E.faecalis	Neg	Neg	Neg
36	2	VII	E.coli	Neg	Neg	Neg
39	2	IX	E.coli	Pos	Pos	Pos
40	2	IX	E.coli	Pos	Pos	Pos
46	1	IX	E.coli	Pos	Pos	Pos
56	1	XII	E.coli	Pos	Pos	Pos
62	2	XII	E.coli	Pos	Pos	Pos
68	2	XII	E.coli	Pos	Pos	Pos
73	2	XIII	E.coli	Pos	Pos	Pos
74	2	XIV	E.coli	Pos	Pos	Pos

The quality filtering of the reads, merging and *de* novo assembly of the reads into contigs was performed by Anuradha Ravi.

3.4.2 Identification of plasmids

The plasmids present in the genomes of the isolates were identified using PlasmidFinder. The plasmids identified are shown in table 5.

Table 5 Identification of plasmids in strains isolated from the gut microbiota of a preterm infant twin pair, using PlasmidFinder.

Strain	Plasmid	Identity (%)	Query cover (%)	Accession no.
1	IncFIB	96,63	100	AP001918
	IncFII	98,03	100	AY458016
3, 26, 35, 39, 40, 46, 56, 62, 68, 73, 74	IncFIB	98,39	100	AP001918
	IncFII	96,95	99,62	AY458016
	IncI1	99,3	100	AP005147

The PlasmidFinder also identified some other plasmids like Col156, p0111, and ColRNAI. However, these were placed on very small contigs and did not contain any ARGs.

3.4.3 BLAST-search of plasmids

A NCBI BLAST-search of the contigs containing plasmid genes showed that 10 of the 11 *E.coli* strains, showed identity towards "*Salmonella enterica* subsp. enterica serovar Kentucky plasmid pCS0010A", with accession number CP002090. One *E.coli* strain (strain 1), on the other hand, showed identity towards a plasmid named "*Escherichia coli* plasmid pEC16I_2", with accession number AP009379. The plasmids Genbank number, query cover, identity and most important genes are shown in appendix B.

For all the *E.coli* strains, except strain 1, conjugative plasmid genes were present in two different contigs. One contig contained plasmid replication genes (*rep*A) and the other contained transfer genes needed for conjugative transfer (*tra*-genes). A NCBI BLAST-search of the contig with the replication genes showed identity to an IncF plasmid, while the contig with the transfer genes showed identity to an IncI plasmid.

3.4.4 Identification of resistance genes

The resistance genes present in the genome of the isolates were identified using ResFinder. The patterns of resistance genes identified in the strains are shown in table 6.

Table 6 Antibiotic resistance genes found in strains isolated from the gut microbiota of a preterm infant twin pair, using using "ResFinder".

		Resistance gene pattern and strain numbers					
		A	**B**	**C**	**D**	**E**	**F**
Phenotype	**Gene**	1	3	6	7,18,32,36	26,74	35,39,40,46,56,62,68,73
Sulfonamide	*sul*1	x					
	*sul*3		x			x	x
Aminoglycoside	*str*A		x			x	x
	*str*B		x			x	x
	*aad*A1	x	x			x	x
	*aad*A2		x			x	x
	aph(3`)-la					x	x
Phenicol	*cat*A1	x					
	*cml*A1		x	x		x	x
Beta-lactam	*bla*SHV-12		x				x
	*bla*TEM-1B					x	x
Tetracyclin	*tet*(B)		x			x	x
	tet(M)			x	x		
Macrolide	*lsa*(A)			x	x		
	mph(A)					x	x
Trimethoprim	*dfr*A14					x	x

3.4.5 MLST identification

From the MLST of the *E.*coli strains containing a conjugative plasmid, strain 1 was found to be MLST-type ST-59 (100% identity). The other 10 strains (strain 3, 26, 39, 40, 46, 56, 62, 68, 73 and 74) were found to be MLST-type ST-345 (100% identity).

3.4.6 pMLST identification

The sequence type of the IncF and IncI plasmids were identified using pMLST search tool. The sequence type of the plasmids is shown in table 7.

Table 7 pMLST-type of plasmids in strains isolated from the gut microbiota of a preterm infant twin pair.

Strain	Plasmid	Sequence type	Identity (%)
1	IncF	F4:A-:B10	100
	IncI	-	0
3, 26, 35, 39, 40, 46, 56, 62, 68, 73, 74	IncF	F24:A-:B1	100
	IncI	ST-3	100

3.5 Plasmid stability

Two *E.coli* strains (strain 1 and 74) were chosen for plasmid stability experiments. A contamination was discovered in strain 1, and therefore the results from this were not considered.

From the last generation of strain 74, 55 cfu were growing on the agar with the antibiotic, while 87 cfu were growing on the agar without antibiotics. All the colonies from the last generation, growing on the agar without antibiotics, were screened for IncF IB using heat-boil lysis of the cells and qualitative PCR with IncF IB primers. All the colonies were positive for the IncF IB plasmid.

The IncF IB plasmid was persistent throughout all 102 generations. The CT-values from the screening of the IncF IB plasmid, together with the 16S rRNA, in strain 74 every third generation for 102 generations are shown in figure 5.

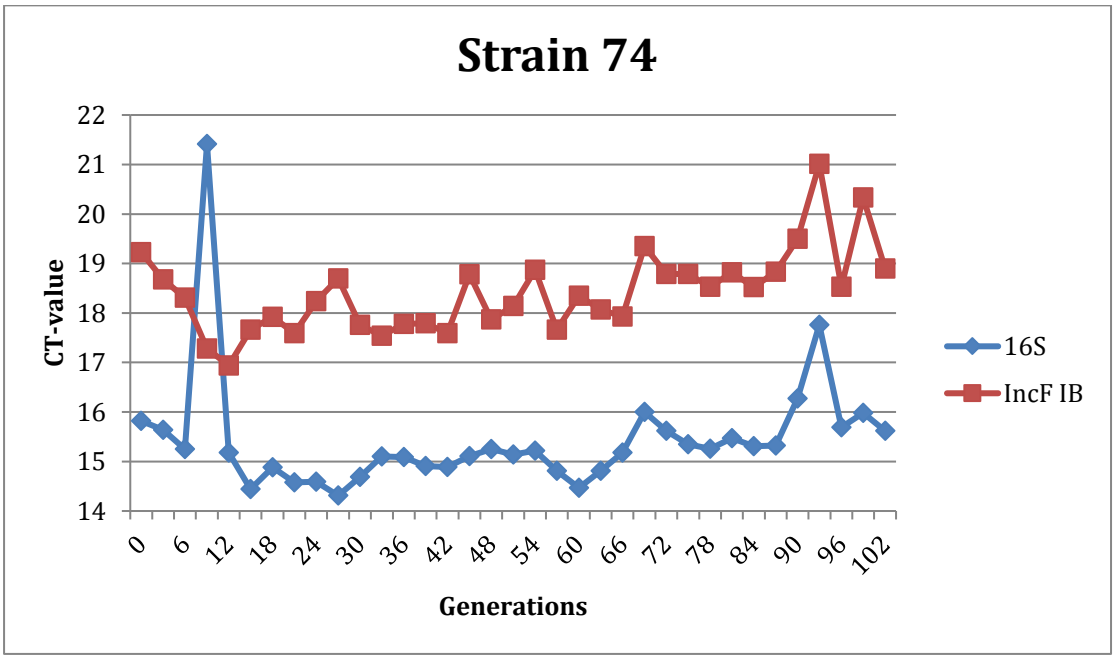

Figure 5 CT-values of 16S rRNA and IncF IB every 3rd generation for 102 generations, in *E.coli* strain 74, isolated from the gut microbiota of a preterm infant.

3.6 Colicin production

Using CLC and NCBI BLAST-search engines, colicin genes were found in 11 *E.coli* strains (strain 1, 3, 26, 39, 40, 46, 56, 62, 68, 72, and 74). The genes in one of these strains (strain 1) were annotated as “Colicin protein”. The NCBI BLAST search showed identity to colicin E1 genes.

The genes in the remaining 10 strains were annotated to colicin V genes, including *cva*A, *cva*B, *cva*C, *cvi*, and *tol*C. The *cva*A, *cva*B, and *cva*C genes were located in the same operon, whereas *cvi*, and *tol*C were located different places in the genome.
Proteins from the 11 strains were precipitated and tested for activity against a colicin-sensitive strain. Only one strain (strain 1) was able to inhibit growth of the colicin-sensitive strain. To test if the inhibition was due to a protein such as a bacteriocin, proteinase K was added next to the protein precipitate on the agar surface, and a "half moon" inhibition zone was formed. This means that the proteinase inhibited the precipitate and proved it was a possible colicin.

The precipitated colicin protein from strain 1 was further tested on the rest of the other non-producing strains (n=10). However, none of these were inhibited by the colicin. In addition, the colicin was tested on 31 Norwegian isolates to see if the colicin-producers could inhibit the growth of these commensal bacteria. The Norwegian isolates were from early and late colonizers of the infant gut. Three of the Norwegian isolates were inhibited by the colicin from strain 1. All the inhibited isolates were early colonizers. The colicin produced by strain 1 was identified by Bactibase as Colicin E1, with a 43% identity and a 48% query cover.

3.7 Conjugative transfer

Six isolates containing conjugative plasmids and antibiotic resistance genes were selected for conjugation experiments. The transmission potential of the conjugative plasmid containing strains was determined.
Strain 1 did not give any transconjugants, indicating the presence of a non-conjugative plasmid. The rest of the strains showed transconjugants. The transmission of IncF IB, IncI1 and Int1 in the transconjugants was detected using qualitative PCR. The conjugation results of the strains are shown in table 8.

Table 8 Antibiotic resistance and MGEs profile in donor-, recipient-, and transconjugant strains.

	Strain	TE	C	S	W	RL	Amp	CTX	Rif	IncF IB	IncI1	Int1
Recipient[2]	***E.coli*-Rif R**	S	S	S	S	S	S	S	R	Neg	Neg	Neg
Donor[1]	**26**	R	R	R	R	R	R		S	Pos	Pos	Pos
	39	R	R	R	R	R	R	R	S	Pos	Pos	Pos
	40	R	R	R	R	R	R	R	S	Pos	Pos	Pos
	46	R	R	R	R	R	R	R	S	Pos	Pos	Pos
	73	R	R	R	R	R	R	R	S	Pos	Pos	Pos
Transconj. Broth mating	**26**	S	R	R	R	R	R		R	Neg	Pos	Pos
		S	S	R	S	S	R		R	Neg	Pos	Neg
	39	S	R	R	R	R	R	S	R	Neg	Pos	Neg
		S	R	R	R	R	R	S	R	Neg	Pos	Pos
	40	S	R	R	R	R	R	R	R	Neg	Pos	Pos
		S	S	R	S	S	R	R	R	Neg	Pos	Neg
	46	S	R	R	R	R	R	R	R	Neg	Pos	Pos
		S	R	R	R	R	R	R	R	Neg	Pos	Pos
		S	R	R	R	R	R	R	R	Neg	Pos	Pos
		S	R	R	R	R	R	R	R	Neg	Pos	Pos
	73	S	R	R	R	R	R	R	R	Neg	Pos	Pos
		S	R	R	R	R	R	R	R	Neg	Pos	Pos
		S	R	R	R	R	R	R	R	Neg	Pos	Pos
Transconj. Agar mating	**39**	S	S	R	S	S	R	R	R	Neg	Pos	Neg
	40	S	S	R	S	S	R	R	R	Neg	Pos	Neg
	46	S	S	R	S	S	R	R	R	Neg	Pos	Pos
	73	S	S	S	S	S	R	R	R	Neg	Pos	Neg
		S	S	S	S	S	R	R	R	Neg	Pos	Neg

1 The donor strains were *E.coli* isolated from the gut microbiota of a preterm infant twin pair
2 The recipient strain were *E.coli* DH5α-Rif^{R}
Abbreviations: TE, tetracyclin; C, chloramphenicol; S, streptomycin; W, trimethoprim; RL, sulfamethoxazole; Amp, ampicillin; CTX, Cefotaxime; Rif, Rifampicin; S, susceptible; R, resistant.

Antibiotics used to select for transconjugants were chosen based on the ARGs found using the "ResFinder" tool. The same antibiotics were used to check the antibiotic susceptibility pattern of the transconjugants. Cefotaxime antibiotic was not tested or used for strain 26, as no resistance gene for this antibiotic was found in this strain.

4. Discussion

4.1 Antibiotic resistance patterns

The antibiotic susceptibility screening revealed a high occurrence of resistance in strains from the preterm infant gut microbiota. This was surprising as the samples were taken only short time after birth and the twins had not been given any antibiotics prior to sampling. This suggests that a rather large resistome is present in the gut of infants immediately after birth, even without any antibiotic selective pressure. The results support what others have found in studies done on ARGs in the gut microbiota of healthy infants (Rose et al. 2017; Zhang et al. 2011). Earlier studies done on transmission of mothers gut microbiota to the infants have shown that maternal bacteria and ARGs can be transferred from the mother to their child even before birth (de Vries et al. 2011; Gosalbes et al. 2016). Infants can also acquire ARGs from the hospital environment upon delivery (Zhang et al. 2011). In this study, both the twins carried strains with the same plasmid type and the same ARGs. This suggest that there is a cross resistance between infants in the hospital. It is therefore possible that preterm infants may have a greater risk of acquiring ARGs, as they often spend a longer time period at the hospital after birth, compared to infants born at term (Whyte 2010). Earlier studies have found microbes living in the hospital environment to resemble those in the gut of premature infants (Brooks et al. 2014). The large number of ARGs discovered in the preterm twins is of major clinical importance (Sommer & Dantas 2011). In the event of an infection, pathogenic bacteria may acquire the ARGs from the commensal microbiota (Karami et al. 2007; Shoemaker et al. 2001). This will limit treatment options and reduce the chances of overcoming the infection.

Resistance to cefpodoxime occurred most frequently among the included isolates. However, it should be taken in consideration that approximately one third of the isolates were *E.faecalis*, which are intrinsic resistant to cephalosporins. Nevertheless, resistance to cefpodoxime was present in almost all the *E.coli* isolates tested as well.

Resistance to sulfamethoxazole and trimethoprim were also widely distributed among the *E.coli* strains. Resistance towards these two antibiotics is often found together (Skold 2001). Sulfonamide resistance genes have almost exclusively been found on class 1 integrons, which are commonly located on conjugative plasmids (Hammerum

et al. 2006; Rådström et al. 1991; Sunde et al. 2015; Wu et al. 2010). This is probably one of the reasons for the wide distribution of these resistance genes.

4.2 Conjugative plasmids in the gut microbiota

The gut microbiota contains many members of the *Enterobacteriaceae* family, known to harbor conjugative plasmids carrying various ARGs (Carattoli 2009). Resistance genes encoding resistance to major antibiotic classes like β-lactams, aminoglycosides, tetracyclines, chloramphenicols, and quinolones have been found on these plasmids (Yang et al. 2015). In this study, isolates from preterm infant fecal samples were screened for IncFIA, IncF IB and IncI1 conjugative plasmids, as these are the most frequently encountered plasmid types (Carattoli 2009; Villa et al. 2010). The results revealed that a large percentage of the tested isolates contained either IncF IB or IncI1, or both conjugative plasmids. The plasmids were present in strains from both twins. The large abundance of these conjugative plasmids found in the gut microbiota of healthy infants is of major clinical importance as the plasmids contribute to the spread of ARGs (Huddleston 2014).

The IncI1 plasmid was found to be of pMLST-type ST-3. These plasmids, together with IncF plasmids, have been shown to play a key role in the dissemination of β-lactam resistance genes (Zurfluh et al. 2014). Beta-lactam resistance may be due to production of ESBLs, which are enzymes that catalyze the hydrolysis of the β-lactam ring in these antibiotics, mediating resistance to extended-spectrum cephalosporins (Picozzi et al. 2013). The persistence and spread of ESBL producing bacteria is a big threat to human health as they give resistance to a big group of antibiotics like penicillins, cephalosporins and other related β-lactam compounds (Matagne et al. 1998). It is possible that some of the *E.coli* strains isolated in this study are ESBL producers, considering that many contained an IncI1 plasmid of ST-3 together with β-lactam resistance genes. However, more extensive screening of the isolates needs to be done in order to determine whether they really are ESBL producers.

4.3 Conjugative transfer

Most of the selected ARGs were transferred from the donors to the recipient strain during the conjugation experiment. The high frequency of isolates containing self-transferable resistance plasmids was a bit unexpected and disturbing, as the mating

process only lasted for 4-24 hours. It was expected that the genes present on the IncF IB and IncI1 plasmids would be transferred. However, even though the IncI1 plasmid had been transferred to all the transconjugants, there was no consistency in which ARGs had been transferred as well. The integron was only transferred to some of the transconjugants, mostly by broth mating, even though the IncI1 plasmid was transferred to all transconjugants in both liquid and on solid media. It is therefore likely that the integron was placed on a different MGE that has not been tested yet. In addition to this, there was a higher frequency of transfer of ARGs and integron during the broth mating, compared to the agar mating. It could be that another MGE that transfers more easily in liquid media was present in the donor strains and was not detected from the sequencing results. In future work it would be interesting to further sequence the recipient and the transconjugant strains to investigate what element is being transferred together with the integron, besides the IncI1 plasmid.

From the BLAST-search of the plasmids it was found that the IncF IB plasmid was carrying the replication genes, while the IncI1 plasmid was carrying the transfer genes. It is therefore hypothesized that these plasmids could be sharing the genes, and that the IncF IB plasmid would transfer along with the IncI1 plasmid during conjugation. However, the IncF IB plasmid did not transfer to any of the recipients and it is therefore likely that this particular plasmid is a non-mobilizable plasmid. The IncF IB plasmid has generally been found to be conjugative (Carattoli 2009). It is possible that the IncF IB plasmid studied in this thesis for some reason has lost its transfer abilities due to mutation. Since the plasmid only showed on average 65% identity towards the "*Salmonella enterica* subsp. enterica serovar Kentucky plasmid pCS0010A" identified with the BLAST-search, it is likely that the low identity match is caused by the lack of the transfer genes that this plasmid normally possess.

4.4 Plasmid stability

The results of the IncF IB screening of strain 74 for over 100 generations showed that the IncF IB plasmid in this strain was very stable. Even though the CT-value was varying a bit between the generations, the plasmid was still present in the strain after growing for 102 generations, without any antibiotic selective pressure.

From the last generation, there was 58,2% more colonies growing on the agar without the antibiotic than on the agar with the antibiotic. Here one could expect that the increase in CFU could be due to growth of bacteria that had lost the plasmid because

of the lack of antibiotic pressure. Yet, all the colonies growing on the agar without the antibiotic still contained the IncF IB plasmid. The reason for the stability of the plasmid could be due to survival genes like addiction and persistence systems. Even though the IncF IB plasmid was found to be relatively stable, there are however some weaknesses in this experiment that should be taken in consideration. In order to increase the reliability of the results, the experiment should have been performed using parallels. This way, any experimental errors would more easily be detected and the result would gain more trust. For example is it likely that the results from sample 1 could have been trusted despite the contamination, if there had been included parallels that were not contaminated. In future studies of plasmid stability, the number of samples and parallels of each sample should therefore be increased to get more trustworthy results.

4.5 Colicin production

The isolate of MLST-type ST-59 was the only strain producing a colicin. Even though the colicin genes of this strain showed identity towards colicin E1, it cannot be said for certain that it is a colicin E1 before the protein has been characterized further with methods like mass spectrometry. However, it is very likely that what was being produced was a bacteriocin of type colicin based on the genes found and its ability to inhibit growth of a colicin-sensitive strain.

Even though colicin genes were found in the other tested isolates as well, they were not able to produce the colicin. This may be due to lack of transporters that prevents them from transporting the colicin out of the cell, or mutations that lead to a non-functional colicin (Cascales et al. 2007). Their genes did however protect them from being inhibited by the colicin-producing strain because of their *cvi* –immunity gene (Cascales et al. 2007).

The colicin-producing strain was able to inhibit the growth of three Norwegian isolates taken from the commensal gut microbiota of infants. This suggests that this strain has the capacity to outcompete other bacteria in the gut. Further studies need to be done in order to determine whether or not this is beneficial for the host. If the colicin-producers inhibit the growth of commensal strains and alters the gut microbiota, the colicin-producer is considered virulent to the host (Saarela et al. 2000). However, if the colicin-producers kill closely related virulent bacteria or pathogens, this may be favorable to the gut microbiota of the host (Corr et al. 2007; Rea et al. 2011). More research on this

field may offer possibilities of using bacteriocins as an alternative to antibiotics in the future (Rea et al. 2011).

4.6 Future work

The study of the spread and persistence of ARGs in microbial communities is important in order to develop new methods for overcoming the worldwide problem of antibiotic resistant bacteria. For future work it would be of interest to sequence the recipient and transconjugant strains from the conjugation experiment. This way it is possible to find out what MGE beside the IncI1, was transferring with the integron from the donor strains to the recipient. It would also be very interesting to investigate the rate of transmission of these MGEs in the gut (*in vivo*).

In addition, it would be interesting to investigate whether the β-lactam resistant isolates are ESBL producers, as ESBL producing bacteria is an increasing problem all over the world (Picozzi et al. 2013).

The colicin produced by one isolate in this study should be further characterized in order to know what type of colicin it is. In the future it would be interesting to study the benefit potential of this colicin in the gut microbiota.

Finally, it would be of interest to repeat the plasmid stability experiment at a bigger scale, for both the IncF IB and IncI1 plasmids. This would include increased number of tested isolates, increased number of parallels of each isolate, and also test the stability over a longer time period.

5. Conclusion

A high percentage of multi-drug resistant bacterial isolates were detected in fecal samples from a Spanish preterm infant twin pair. These infants were not given antibiotics prior to sampling. Most of these isolates were *E.coli,* containing IncF IB and IncI1 plasmids, in addition to Integron 1. Conjugation experiments proved the IncI1 plasmid transmissible in all the isolates tested, along with several ARGs. The IncF IB plasmid was not transmissible in any of the tested isolates, and hence was not conjugative as first expected. The integron was only transmissible in some of the tested isolates, which indicates that the integron may be placed on a different MGE that was not detected in this study. It would be of interest to further investigate what mobile elements are transferring along with the integron, besides the IncI1 plasmid.

The IncF IB plasmid was found stable for over 100 generations in the *E.coli* strain. However, due to the lack of parallels the results from the stability experiment cannot be verified. For future work, it is suggested to increase the number of strains and include several parallels for each strain when determining the plasmid stability. It would also be of interest to investigate the stability of the IncI1 plasmid.

One *E.coli* strain was found to produce a colicin, which were able to inhibit growth of gut-commensal strains. For further work it would be interesting to investigate whether or not the colicin-producing strain is beneficial for the gut microbiota of the host.

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Appendix

Appendix A: Strain information

Table A Twin number, strain identification, antibiotic susceptibility pattern and MGEs information of all strains.

Strain	Twin	ID	W	CN	CIP	CPD	STX	AMC	IncF IB	IncI1	Int1
1	2	E.coli	S	S	S	S	S	S	Pos	Neg	Neg
2	2	E.coli	S	S	S	S	S	S	Neg	Neg	Neg
3	2	E.coli	S	S	S	R	S	S	Pos	Pos	Pos
4	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
5	1	E.coli	S	I	S	R	S	S	Pos	Neg	Neg
6	1	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
7	2	E.coli	S	I	S	R	S	S	Neg	Neg	Neg
8	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
9	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
10	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
11	2	E.coli	S	I	S	R	S	S	Neg	Neg	Neg
12	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
13	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
14	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
15	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
16	2	E.faecalis	S	I	S	R	S	S	Neg	Neg	Neg
17	2	E.coli	S	I	S	R	S	S	Pos	Pos	Pos
18	1	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
19	2	S.flexneri	S	R	S	R	S	S	Neg	Neg	Neg
20	2	E.coli	S	R	S	R	S	S	Neg	Neg	Neg
21	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
22	2	E.coli	S	R	S	R	S	S	Neg	Neg	Neg
23	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
24	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
25	1	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
26	2	E.coli	R	S	S	S	R	S	Pos	Pos	Pos
27	2	E.coli	R	S	S	S	R	S	Pos	Pos	Pos
28	2	E.coli	R	S	S	S	R	S	Pos	Pos	Pos
29	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
30	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
31	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
32	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
33	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
34	2	E.faecalis	S	R	S	R	S	S	Neg	Neg	Neg
35	1	E.faecalis	S	R	I	R	S	S	Neg	Neg	Neg
36	2	E.coli	S	R	I	R	S	S	Neg	Neg	Neg
37	2	S.epidermidis	S	R	R	R	S	S	Neg	Neg	Neg
38	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
39	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
40	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
41	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
42	1	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
43	1	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
44	1	E.coli	R	S	S	R	R	S	Pos	Pos	Pos

45	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
46	1	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
47	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
48	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
49	1	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
50	1	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
51	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
52	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
53	2	E.coli	R	S	S	R	R	S	Pos	Pos	Pos
54	1	E.coli	R	S	I	R	R	S	Pos	Pos	Pos
55	2	E.coli	R	I	S	R	R	R	Pos	Pos	Pos
56	1	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
57	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
58	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
59	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
60	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
61	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
62	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
63	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
64	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
65	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
66	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
67	2	E.coli	R	S	S	R	R	R	Neg	Pos	Neg
68	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
69	2	E.coli	R	S	S	R	R	R	Neg	Neg	Neg
70	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
71	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
72	2	E.coli	R	S	S	R	R	R	Pos	Pos	Pos
73	2	E.coli	R	I	S	R	R	R	Pos	Pos	Pos
74	2	E.coli	R	R	S	R	R	I	Pos	Pos	Pos

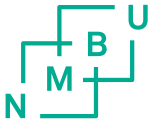
*W, trimethoprim; CN, gentamicin; CIP, ciprofloxacin; CPD, cefpodoxime; SXT, sulfamethoxazole trimenthoprim; AMC, amoxycillin clavulanic acid; S, sensitive; I, intermediate: R, resistant

Appendix B: BLAST-search of plasmid and integron genes

Table B BLAST-search of contigs containing plasmid and integron genes.

Strain	Gene	Plasmid name	Genbank	Query cover (%)	Identity (%)
1	IncF IB	Escherichia coli SE15 plasmid pECSF1 DNA	AP009379	98,6	99,9
	Transfer	Escherichia coli strain MVAST0167 plasmid pMVAST0167_1	CP014493	97,8	99,6
	Int1	Escherichia coli plasmid pEC16I_2, complete sequence	KU997026	100,0	100,0
3	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid	CP002090	52,4	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	100,0	100,0
	Int1	Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	AY509004	96,6	99,7
26	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky	CP002090	60,8	100,0
	Transfer	Escherichia coli plasmid pC49-108, complete sequence	KJ484638	56,5	100,0
	Int1	Salmonella enterica serovar Schwarzengrund	CP001125	95,3	100,0
39	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	65,2	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	83,5	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12	HQ419283	92,0	100,0
40	IncF IB	Salmonella enterica serovar Kentucky plasmid pCS0010A	CP002090	66,5	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	100,0	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12	HQ419283	95,4	99,9
46	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	99,8	100,0
	Transfer	Escherichia coli plasmid pC59-112, complete sequence	KJ484637	83,4	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12 genomic sequence	HQ419283	95,3	99,9
56	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	65,2	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	59,5	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12	HQ419283	95,9	99,9
62	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	65,2	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	93,0	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12 genomic sequence	HQ419283	95,9	99,9
68	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	70,3	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	95,2	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12 genomic sequence	HQ419283	95,9	99,9
73	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	65,2	100,0

	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	59,5	100,0
	Int1	Klebsiella pneumoniae strain S8 clone fosmid 4D12	HQ419283	95,0	100,0
74	IncF IB	Salmonella enterica subsp. enterica serovar Kentucky plasmid pCS0010A	CP002090	36,5	100,0
	Transfer	Escherichia coli FAP1 plasmid unnamed 2 sequence	CP009580	59,5	100,0
	Int1	Escherichia coli partial pEC279 plasmid class I integron	FM242709	99,7	100,0



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