I-complex plasmids - a story about incompatibility and host adaptation

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I-complex plasmids – a story about incompatibility and host adaptation

I-complex plasmiden – een verhaal over incompatibiliteit en adaptatie aan de host

(met een samenvatting in het Nederlands)

Proefschrift

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Ten doktorat dedykuję Rodzicom.

I dedicate this PhD thesis to my parents.

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Chapter 1

General introduction

Introduction

Before the discovery of antimicrobials, bacterial infections could only be treated with natural compounds such as honey,[1] chemicals such as mercury with high toxicity for the patient,[2] or more questionable practices such as leeching or bloodletting. The burden of bacterial infections was high, with high mortality and uncontrollable outbreaks of bacterial diseases, resulting in epidemics like the plague of Athens or the *Bla*ck Death.[3, 4] The discovery of penicillin by Alexander Fleming was a true revolution in human healthcare.[5] Back then, Fleming was already aware of the possibility of selection and spread of antimicrobial resistance. Therefore, in 1945 he was concerned about misuse of antimicrobials.[6]

"the public will demand [the drug and] … then will begin an era … of abuses. The microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and perhaps from there to others until they reach someone who gets a septicaemia or a pneumonia which penicillin cannot save. In such a case the thoughtless person playing with penicillin treatment is morally responsible for the death of the man who finally succumbs to infection with the penicillin-resistant organism. I hope the evil can be averted."

Penicillin was successful in controlling bacterial infections during World War II. Shortly after, just how Alexander Fleming predicted, resistant strains emerged and infections caused by penicillin resistant bacteria again became a serious clinical problem. The introduction of new antimicrobials like tetracycline and erythromycin were promising to treat infections successfully. Unfortunately, shortly after introduction of most new antimicrobials, there were reports of pathogens carrying an acquired resistance mechanism (Figure 1).

Figure 1 Timeline representing introduction of new antimicrobials and appearance of bacteria carrying resistance to these antimicrobials. Figure adapted from Ventola,2015.[7]

In the 1940s, the first experiences were reported that antimicrobials had a beneficial effect on animal growth.[8] In the 1960s, antimicrobial growth promoters (AGP's) were introduced more widely in chicken, cattle and pig production.[9] Soon it was noticed that this large scale application of antimicrobials increased the risk of resistance.[10] Sweden was the first country to ban AGP's while at the European Union level, the first ban was established on the 1st of July 1999. This ban included bacitracin, avoparcin, spiramycin, tylosin and virginiamycin, all belonging to drug classes which are also used in human medicine. In 2006, the European Union established a total ban on the use of antimicrobials as growth promoters. Only in 2017 the US Food and Drug Administration (FDA) established a ban for use of AGP's in the USA. Some of these products were re-licensed as therapeutics on prescription only and are still in use. Unfortunately, in 2018, 45 countries worldwide still use AGP's in livestock production,[11] contributing to an increased prevalence of AGP's resistant bacteria in animals and potentially in humans. However, this data is based on legislation implemented on a national level. Therefore, the data about actual use of AGP's remains a unknown. The most important driver for the global increase of antimicrobial resistance (AMR) is use of antimicrobials. To control antimicrobial resistance, misuse (e.g. overuse and inappropriate prescription of antimicrobials in animals and humans) should be prevented.

Antimicrobial resistance

Resistance mechanisms

Bacteria can develop resistance to antimicrobials in many different ways.[12] All of the described mechanisms can be encoded on the chromosome or plasmid. Plasmid-mediated resistance can spread both horizontally and vertically, whereas chromosome encoded resistance spreads only vertically.

The first resistance mechanism relies on the production of antimicrobial-inactivating enzymes like aminoglycoside modifying enzymes and β-lactamases such as penicillinases, (extended-spectrum)-β-lactamases and carbapenemases.[13] The second mechanism involves efflux pumps which, when overexpressed, remove the antimicrobial from the cytoplasm in the bacterial cell and thereby causing resistance. Besides non-specific multidrug efflux pumps affecting a wide range of antimicrobials, antimicrobial-specific pumps were described for tetracyclines, macrolides and chloramphenicol.[14] The third mechanism replaces the molecular target of the antimicrobial or creates an alternative pathway to bypass a molecule that is the target for the antimicrobial, resulting for instance in resistance to glycopeptides or trimethoprim.[15, 16] The last mechanism changes the target of antimicrobials, such as the bacterial gyrase or topoisomerase IV leading to quinolone resistance, the RNA Polymerase β subunit leading to rifampicin resistance or the dihydrofolate reductase gene leading to trimethoprim resistance.[15, 17, 18]

(Extended spectrum) β-lactamases

One of the most often reported class of antimicrobial resistance genes are β-lactamases. β-lactamases can hydrolyze β-lactam antimicrobials, like penicillins, cephalosporins, cephamycins, oxyiminocephalosporins and monobactams at a low rate,[19] by cleaving the amide bond in the β-lactam ring. Extended spectrum β-lactamases (ESBLs) are β-lactamases that can hydrolyze first-, second-, third-, and fourth generation cephalosporins,

penicillins and aztreonam, and are inhibited by β-lactamase inhibitors, like clavulanic acid.[20] There are multiple different families of ESBLs (Table 1), with *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ being the most common ones.[21] AmpC-type β-lactamases have a slightly different phenotype than ESBLs. They do not hydrolyze fourth generation cephalosporins and are resistant to cefoxitin and β-lactamase inhibitors. They are chromosomally located in *Enterobacteriaceae* such as *Citrobacter*, *Serratia* and *Enterobacter* and are frequently found on plasmids in *E. coli* and *Salmonella* . The most common genes encoding plasmid-mediated AmpC-type β-lactamases are CMY, ACC and DHA.[22]

Carbapenemases

Carbapenemases represent the most versatile family of β-lactamases and they belong to three molecular β-lactamases classes (Table 1). Class A contains serine carbapenemases, which includes the IMI, SME, and KPC enzymes (Table 1). They can all hydrolyze a broad variety of β-lactams, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanate and tazobactam.[23] Class B consists of metallo-β-lactamases which can hydrolyze cephalosporins and penicillins but not aztreonam.[23] They are resistant to commercially available β-lactamase inhibitors but susceptible to inhibition by metal ion chelators.[23] The most common enzymes representing this class are IMP and VIM (Table 1). Class D comprises of serine-carbapenemases and is the most diverse and the least understood class of the β-lactamases. The first enzymes identified had activity restricted to penicillins, while the OXA group contains enzymes active against cephalosporins and carbapenems and with widely differing sensitivities to inhibitors.[20]

Table 1 Classification of bacterial β-lactamases. This table was obtained from Bush and Jacoby, 2010. [24]

			Inhibited by			
Bush-Jacoby group (2009)	Molecular class (subclass)	Distinctive substrate(s)	CA or TZB	EDTA	Defining characteristic(s)	Representative enzyme(s)
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50

Table 1 continued

Bush-Jacoby group (2009)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
			CA or TZB	EDTA		
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
	B (B3)					L1,CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

Spread of AMR

Bacteria can spread resistance to antimicrobials in two ways: vertically or horizontally.[25] Vertical spread is associated with DNA replication when traits are transferred to the next generation through cell division, like the above described first and second mechanism. Two examples of mechanisms which spread resistance vertically are chromosomal mutation changes of the antimicrobial target like gyrase, topoisomerase IV, RNA Polymerase β or dihydrofolate reductase or overexpression of the efflux pumps, which represent mechanisms three and four in the above description.[15, 17, 18] Horizontal spread is driven by horizontal gene transfer (HGT) of mobile genetic elements such as insertion sequences, transposons and plasmids through processes like conjugation, transduction or transformation. Examples of horizontally spread resistance mechanisms are antimicrobial-inactivating enzymes such as β-lactamases, replacement of the molecular target of the antimicrobial or creating a bypass, such as β-lactam resistance among *Streptococcus pneumoniae*. This type of resistance can be obtained by the recombination of native penicillin binding protein (PBP) genes or acquisition of the *mecA* gene, encoding PBP2a, having a very low affinity for all β-lactams.[12]

Insertion sequence (IS) and transposons (Tn)

Insertion sequences (IS) and transposons (Tn) are DNA segments that can move themselves with associated resistance genes to new locations in the same or different DNA molecules within a single cell.[26] Typical IS-elements are between 0.7 and 2.5 kb in length with associated resistance genes.[27] Insertion sequences are grouped into families based on the nature of the enzymes catalysing their movement - transposases.[26] One example of a successful IS is IS*Ecp1*, which is associated with $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$ resistance genes clusters, $bla_{\text{CMY-2}}$, $bla_{\text{OXA-181}}$, *qnrB* and *rmtC*.[26, 28]

IS can create a composite transposon, which is a region bound by two copies of the same or related IS, that can mobilize resistance genes.[26] Unit transposons, in contrast to the composite transposons are bound by inverted repeats (IR) and not by a pair of IS. They include a transposase gene and an internal "passenger" gene, which may be an antimicrobial resistance gene.[26]

Plasmids

Plasmids are extrachromosomal pieces of DNA, mostly circular, that can vary in size from less than a kilobase to several megabases.[26] They are self-replicative and some of them encode conjugation or mobilization machinery, allowing plasmids to spread horizontally between bacterial cells. Additionally, plasmids can carry accessory genes encoding for antimicrobial resistance or other niche adaptive properties like metabolic functions, salt or heat tolerance.[29, 30]

A review summarizing the knowledge about plasmid mediated antimicrobial resistance (pAMR) in the family of *Enterobacteriaceae* was published.[31] The manuscript pointed out that some resistance genes occur widespread across plasmid groups, like $bla_{\text{CTX-M-9}}$ or $bla_{\text{SHV-12}}$,[32-36] while others are reported rarely and seem to be associated with a single plasmid group, like $bla_{\text{CTX-M-32}}$ on IncN plasmids.[35, 37] On the other hand, some plasmid

groups carry a wide variety of resistance genes, like IncI plasmids.[32, 35, 37-41], while others encode only a small variety of resistance genes, like IncK plasmids.[35, 42, 43]

Plasmid typing

The first plasmid typing scheme was based on incompatibility reactivities tested via conjugation.[44, 45] Later, replicon typing (Inc/rep typing) was established. It was based on Southern hybridization of 19 different plasmid probes with the tested plasmid.[46]Currently there are many plasmids known in bacteria that are identified by different methodologies, such as PBRT, PAR-T or MOB-typing, depending on the bacterial species involved. PBRT was designed mainly for plasmids associated with *Enterobacteriaceae* whereas MOB-typing can be used for *Enterobacteriaceae* and *Gammaproteobacteria* plasmids.[47]

Replication based incompatibility was used to create a method to distinguish plasmid groups in *Enterobacteriaceae*, called PCR-Based Replicon Typing (PBRT).[48] It is based on a set of primers that target plasmid group specific regions (such as *rep* genes, iterons, RNAI). This method classifies plasmids into "Inc" groups. Another plasmid typing tool is called plasmid partition gene typing (PAR-T).[49] It is based on a multiplex PCR targeting the plasmid partitioning system. PAR-T classifies plasmids into the same "Inc" groups determined by PBRT. PAR-T has slightly lower discriminatory power then PBRT, for example it doesn't recognize different variants of IncF plasmids. However, overall PAR-T and PBRT have very high correlation.

A third way of plasmid typing takes the mob genes into account, which encode for relaxases.[50] There are 6 groups of relaxases, and typing plasmids based on these genes shows a high correlation with PBRT typing, meaning that each Inc type predominantly contains a single MOB subfamily.

Plasmid incompatibility

Incompatibility is an inability of two plasmids to be stably maintained in one cell. It is caused by the presence of identical or closely related shared determinants between two plasmids.[51] This phenomenon can originate from partition or replication mechanisms.

Partitioning is a system ensuring proper segregation of plasmids or chromosomes to daughter cells after bacterial division (Figure 2). As a result of the partitioning process, plasmids are distributed equally to opposite ends of the nucleoid. Partition-based incompatibility can be caused by each of the partitioning mechanism components: ParA, ParB or parS. Excess of ParA was shown to interfere with the formation and/or the stability of the partition complex.[52] Overexpression of ParA results into a dose-dependent increase of incompatibility, up to a level where the plasmid lost is equal to a system with no partitioning at all. The strength of the incompatibility mediated by ParB is variable, and dependent on the level of protein expression. High levels of ParB lead to excessive plasmids polymerization, which lowers the number of plasmid units able to segregate.[53] Mild overproduction of ParB (5-fold) causes the activation of the ParB recombination activity, which results in high plasmid multimerization. This leads to a decreased number of segregation units (Figure 2), but it does not affect the copy number and thereby leads to missegregation.[54,55] Medium copy number plasmid incompatibility, mediated by parS, can be explained by random positioning of plasmids. Presence of an identical parS site on a

different plasmids leads to a distribution of plasmids along the cell length regardless of the plasmid type.[56] parS mediated incompatibility of low copy plasmids has a very strong effect. It can be explained by a different replication timing of plasmids.[56] It means that late replicating plasmids (close to the cell division) will not be properly segregated.

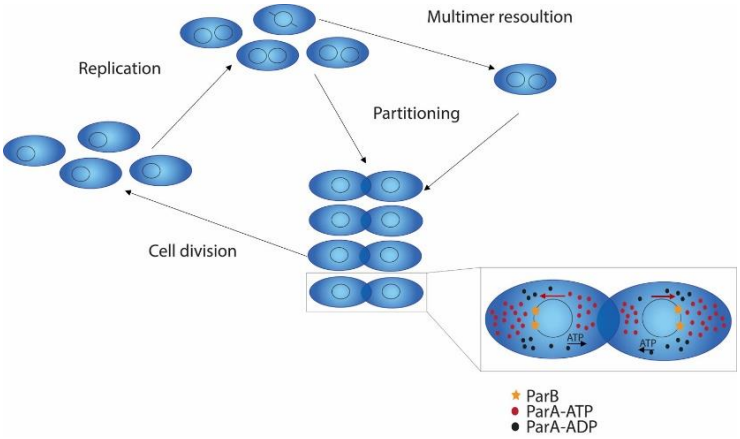

Figure 2 Plasmid partitioning. After replication, plasmids can be directly segregated by partitioning or used for plasmids forming multimers. In case the plasmid is used to form multimers, they first have to be converted back to monomers. Conversion back to monomers has to be completed first. Partitioning is mediated by the ParB protein, which is attached to the parS site. Plasmids are pulled to the opposite sites by depolymerization of the ParA protein. Figure adapted from Pinto et al., 2012. [57]

Plasmid replication-based incompatibility can be caused by two mechanisms: iterons or antisense RNA. Iterons are ~ 20 bp repeated DNA sequences that bind their cognate Rep proteins.[58] A stem-loop structure, antisense RNA, is complementary to the RNA molecules that play an essential role in the replication initiation. This binding has a different effect for different plasmid groups. For ColE1 plasmids it inhibits the formation of the RNA primer.[59-61] In the pT181 plasmid family it results in premature termination of the messenger RNA for the Rep protein.[62] For I-complex, IncF, IncL and IncM plasmids it inhibits translation of the rep mRNA.[62-71] Excessive amounts of iteron sequences or antisense RNA, originating from two different plasmids, are the basis of replication-mediated incompatibility. It leads to the destabilization of one or both plasmid replication control mechanisms and eventually plasmid loss.

I-complex plasmids

IncI-complex plasmids are low copy-number, narrow-host-range, conjugative plasmids, which size varies in size from 50 to 250 kb. The I-complex plasmid group consists of IncI1α, IncI1γ, IncI2, IncB/O, IncK and IncZ plasmids. Although these plasmids are closely related, not everything is known about their incompatibility and phylogeny. Additionally, members of I-complex are associated with the epidemic spread of resistance genes: IncK1 with $bla_{\text{CTX-M-14}}$, IncK2 with $bla_{\text{CMY-2}}$ and IncI1 with $bla_{\text{CTX-M-1}}$. Therefore, the I-complex plasmids are the main focus of this thesis.

The classification of I-complex plasmids is based on the serological and morphological similarities of its pili.[72, 73] I-complex plasmids, in contrast to most conjugative plasmids found in *Enterobacteriaceae*, produce two types of pili: a thicker, short rigid pilus that is essential for plasmid transfer in liquid or on solid agar, and a long, flexible pilus that is only required for plasmid transfer in liquid.[73-75]

Tight control of replication is crucial for all plasmids. For I-complex plasmids it is mediated by small RNA structure in the negative circuit This RNA structure, called RNAI is also the determinant of I-complex plasmid incompatibility.[76] A high degree of similarity between RNAI structures of I-complex plasmid group members (Figure 3) suggests close phylogenetic relatedness of these plasmids.

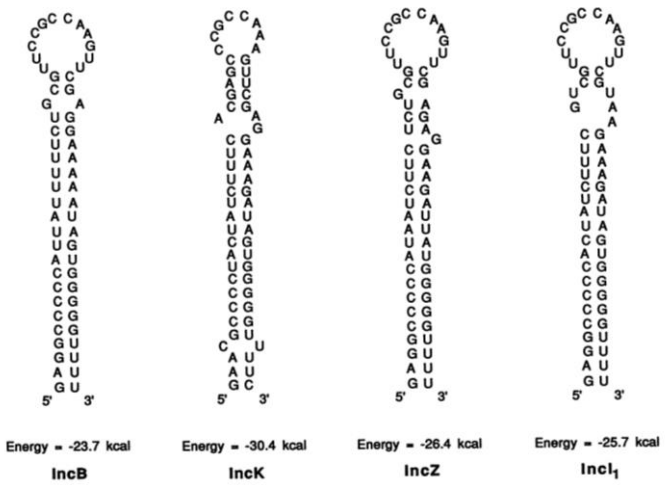

Figure 3 Comparison of RNAI structures of IncB/O, IncK, IncZ and IncI1 plasmids. This figure was obtained from Praszkier et al., 1991.[76]

Plasmid- and bacteria-host adaptations

A plasmid invading a new bacterial host faces many challenges due to differences in the environment. In order to survive and persist in a new host the plasmid has to adapt to the host by lowering its cost for the host or increasing benefits.

Plasmid-bacteria adaptations

Toxin-antitoxin system

A bacterial toxin-antitoxin system (TA system), encoded on a plasmid, ensures its stable inheritance. TA systems include a toxin inhibiting cell growth by interfering with the crucial processes. Every TA system additionally encodes an antitoxin which neutralizes the toxin. Four major types of TA systems have been described based on the antitoxin's nature and the way it inhibits the toxin. Type I and III are RNA molecules, whereas in

other classes, the antitoxin is a protein.[77] The most common plasmid encoded TA systems are CcdA/B, Hok/Sok, RelB/E and HigB/A.[78]

Plasmid fitness cost

Plasmids carrying antimicrobial or heavy metal resistance genes, virulence or catabolism related genes, can be beneficial for the bacterial host which would ensure successful persistence in selective environments. Some plasmids do not carry any obvious beneficial genes. Nonetheless, they are able to successfully persist in the host when the following three parameters are balanced: the fitness cost of the plasmid, the conjugation rate and plasmid loss rate.[79]

Sources of plasmid fitness cost

Plasmids pose a fitness cost, which is defined as a burden to a bacterial host.[80] This burden occurs in plasmid-bearing strains under conditions that do not select for plasmid-encoded genes and manifests itself in reduced growth rate, weakened competitiveness and increased phage sensitivity due to pilus formation during conjugation.[81] Therefore, in a mixed bacterial population, plasmid-free bacteria will out-compete the population containing the plasmid. There are multiple sources of plasmid fitness cost (Figure 4). Plasmid fitness cost initiates at the entry to the cell which triggers an SOS response, which can result in a delayed cell division, phage induction or mobilization of genomic islands.[82, 83] Further, plasmid replication results in a depletion of essential cellular components like RNA polymerase, tRNA and amino acids.[84] Plasmid conjugation is another costly process associated with high ATP demand.[80]

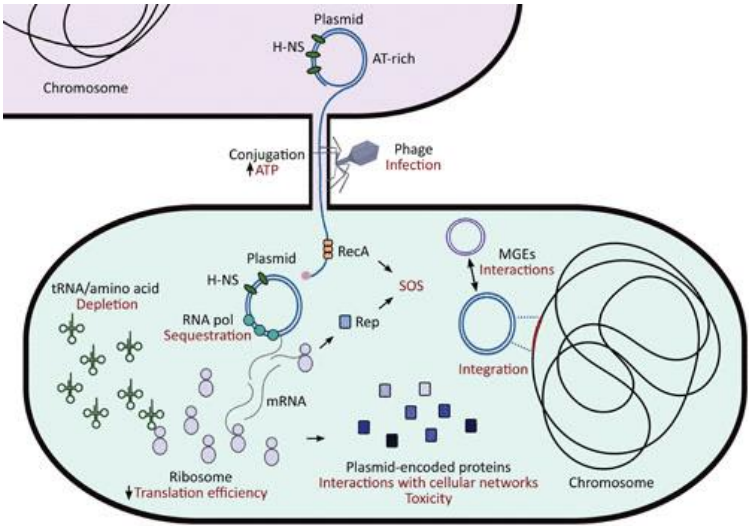

Figure 4 Fitness cost of the plasmid for the bacterial cell. This figure was obtained from San Millan and MacLean, 2017[80]

Ways to decrease plasmid fitness cost

High stability of plasmids in bacterial populations, despite their fitness cost, is counter intuitive and is referred to as the plasmid paradox. Plasmids can co-evolve together with their host which can greatly reduce the initial cost of the plasmid.[85] Moreover, bacterial hosts can induce changes in the plasmid that lower its fitness cost.[86] These changes can be mutations or deletions in the gene or region of which expression is very costly. Conjugation is a process with a high energy cost and some plasmids downregulate their conjugation rates in order to reduce fitness cost to the host. Examples are described where reduction or abolishment of plasmid conjugation was detected through spontaneous deletion of the conjugation region, or host induced changes in pilus production, presumably resulting in a fitness advantage within the population.[87, 88] Finally, reduction in plasmid fitness cost can also be achieved by silencing antimicrobial resistance genes, mediated by host proteins. For example, silencing of a resistance gene by the host H-NS proteins.[89] Expression of the pAMR upon plasmid arrival in the cell poses a high fitness cost which is later lowered due to the adaptation.

Plasmid adaptations to the environment

Plasmids can carry genes that allow the host bacteria to survive in a harsh environment of which resistance to antimicrobials is probably the best known and most broadly studied example. However, plasmids were also reported to encode other features that adapt them and/or their host to the environment. One example is an IncH plasmid for which the optimal conjugation temperature is 22-30 ^{o}C, which makes it well adapted to the transmission in aquatic environments compared to the IncF plasmid, which has optimal conjugation temperatures at 37-42 ^{o}C, which is the temperature of the mammals in which they are often present.[90, 91] A second example is a plasmid from faecal slurry which mediates salt tolerance in its *E. coli* host through overexpression of potassium transport system Kdp, which is an advantage for this bacterial host in the human gut.[29] Another example is a plasmid mediated ParDE toxin-antitoxin system providing a heat tolerance to *E. coli* often found in poultry, which have a higher body temperature than mammals.[30]

Aims and outline of the thesis

Because plasmids carrying antimicrobial resistance are a global problem, it is the aim of the thesis to understand interactions between plasmids, their physiology and phylogenetic relatedness, which will provide knowledge that is necessary to understand spread of plasmids and the antimicrobial resistance genes they carry. Understanding the pathways which allow plasmids to adapt to the bacterial host cell as well as animal host species will help to define treatment targets that could be targeted with interventions. Eventually that may lead to decrease or even elimination of plasmids carrying antimicrobial resistance.

Chapter 2 summarizes the knowledge about plasmids in the group of *Enterobacteriaceae* and the resistance determinants they carry. Additionally, it provides background knowledge about each plasmid group. Based on knowledge from Chapter 2, the I-complex plasmid group was chosen as a main focus of this thesis. The I-complex plasmid group is very complex and there are many knowledge gaps concerning (in)compatibility and phylogeny of the members of this group.

Chapter 3 gives an insight into the IncK plasmid group and compatibility reactivities of the members of this group. This chapter concludes that the IncK plasmid group can be subdivided into two separate lineages named IncK1 and IncK2.

Chapter 4 provides a general overview about compatibility and phylogeny of I-complex plasmids and re-examines the incompatibility relationship between IncB/O and IncZ plasmids. Hence filling the knowledge gap about I-complex plasmids mentioned above. The conclusion of this chapter is that IncB/O and IncZ plasmids could be considered one group named IncB/O/Z. An additional point made in Chapter 4 concerns differences between phenotypic and sequence-based typing of incompatibility groups.

Chapter 5 elaborates more in detail about the basis of incompatibility of IncK1 and IncK2 plasmids described in Chapter 3. The influence of point mutations in the RNA structure on the compatibility of IncK1 and IncK2 plasmids are assessed.

Chapter 6 looks at the host adaptation of IncK plasmids described in Chapters 3 and 4. It shows that the high conjugation rate of IncK2 plasmids possibly led to its adaptation to the chicken host.

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Chapter 2

Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*

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Abstract

Bacterial antimicrobial resistance (AMR) is constantly evolving and horizontal gene transfer through plasmids plays a major role. The identification of plasmid characteristics and their association with different bacterial hosts provides crucial knowledge that is essential to understand the contribution of plasmids to the transmission of AMR determinants. Molecular identification of plasmid and strain genotypes elicits a distinction between spread of AMR genes by plasmids and dissemination of these genes by spread of bacterial clones. For this reason several methods are used to type the plasmids, e.g. PCR-based replicon typing (PBRT) or relaxase typing. Currently, there are 28 known plasmid types in *Enterobacteriaceae* distinguished by PBRT. Frequently reported plasmids [IncF, IncI, IncA/C, IncL (previously designated IncL/M), IncN and IncH] are the ones that bear the greatest variety of resistance genes. The purpose of this review is to provide an overview of all known AMR-related plasmid families in *Enterobacteriaceae*, the resistance genes they carry and their geographical distribution.

Introduction

Even before the widespread therapeutic use of antibiotics, bacteria with penicillinase activity were discovered that could actively destroy penicillin in order to survive in penicillin-containing environments.[1] In the first reports on the spread of genetic material between bacterial cells, fertility factors were noted, which were not only capable of spreading antimicrobial resistance (AMR) but also of curing auxotrophic mutations through R-factors.[2–4] Later, it was recognized that these factors, designated plasmids, were autonomous DNA molecules capable of self-transmission between cells, and that they were also capable of mobilizing part of the chromosome through a process termed high-frequency recombination (Hfr).[5] The acquisition of novel genes by plasmids through mobile genetic elements such as transposons or insertion sequences, and their ability to replicate in a wide range of hosts, made them perfect vectors for the spread of AMR. Therefore, the identification of plasmid characteristics and behaviour in different bacterial hosts provides fundamental knowledge regarding the transmission of AMR. Molecular identification of plasmid and strain genotypes can distinguish whether the spread of AMR genes is driven by epidemic plasmids to different hosts or by clonal spread of bacterial organisms harbouring these plasmids with AMR genes.

In her review, Carattoli[6] focused mainly on resistance genes carried by 'epidemic plasmid types', which are defined as plasmids that have been detected in different countries, in bacteria of different origins and sources. The purpose of this review is to describe the characteristics of all currently known AMR-related plasmid families in *Enterobacteriaceae*, the resistance genes they carry and their geographical distribution.

Plasmid typing

The first plasmid typing scheme was developed by Datta and Hedges in 1971.[7,8] Transfer frequencies of plasmids belonging to different groups and their stable coexistence in bacterial cells were determined. Five incompatibility groups were defined based on conjugation experiments: W (based on a reference strain received from Tsutomu Watanabe, who discovered the phenomenon of incompatibility),[4] F (fi+), I (produce I-type pili), N and P. Later, this scheme was updated and 23 plasmid incompatibility groups were recognized: B, C, D, E, FI, FII, FIII, FIV, H, Iα, I2, Iγ, Iδ, Iζ, J, K, M, N, P, T, V, W and X.[9] Some additional annotations were made: plasmids incompatible with both IncA and IncC were designated IncA/C. Those previously named IncL were renamed IncM and former IncS were renamed IncH.[9]

Nowadays, the most frequently used plasmid typing scheme is called Inc/rep typing. The classification by Inc/rep typing is mostly consistent with the conjugation-based scheme. The first replicon typing method was based on Southern hybridization with 19 different replicons,[10] which were screened for their ability to express incompatibility towards the parental plasmids or miniplasmids used in their construction. Whenever possible, loci involved in plasmid copy number control were chosen rather than partition loci as these are present in all plasmids. Inc types have been independently identified in three different genera. Currently, there are 28 Inc types in *Enterobacteriaceae*, 14 in *Pseudomonas* and approximately 18 in *Staphylococcus*.[11]

Subsequently, PCR-based replicon typing (PBRT) was developed by Carattoli *et al.*[12] This scheme is based on a set of primers targeting different regions (such as *rep* genes, iterons, RNAI) specific for each plasmid group. Targets for identification of additional plasmid groups were added to the typing method by Garcia-Fernandez *et al.*[13] and Villa *et al.*[14] The method was adapted by Boot *et al.*[15] with the aim to speed up the procedure and to make it more sensitive using real-time PCR, which may increase sensitivity of detection of low-copy plasmid replicons.

Bousquet *et al.*[16] proposed a scheme which may be used in addition to PBRT. Different partition systems located on multidrug resistance (MDR) plasmids were identified which led to the design of a multiplex PCR method called plasmid partition gene typing (PAR-T). This method can be used for the classification of plasmids in *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella enterica*.

An alternative scheme for plasmid typing takes into account the differences in *mob* genes encoding for relaxases, which are important relaxosome components in both conjugative and mobilizable plasmids.[17] All known plasmid relaxases were divided into six groups and each family is specific in the details of its DNA-processing mechanism.[17,18] This relaxase or MOB classification does not detect IncR plasmids, as these do not contain a relaxase gene.[19] There is a high correlation with the PBRT scheme, which means that plasmids of each Inc type have relaxases of a single MOB subfamily (Figure 1). Therefore, high abundance of specific MOB families such as MOB_F and MOB_P correlates with abundant PBRT types such as the IncF complex, the IncI1 complex and the ColE-like plasmids. Some exceptions were explained by plasmid co-integration and secondary deletions.[20]

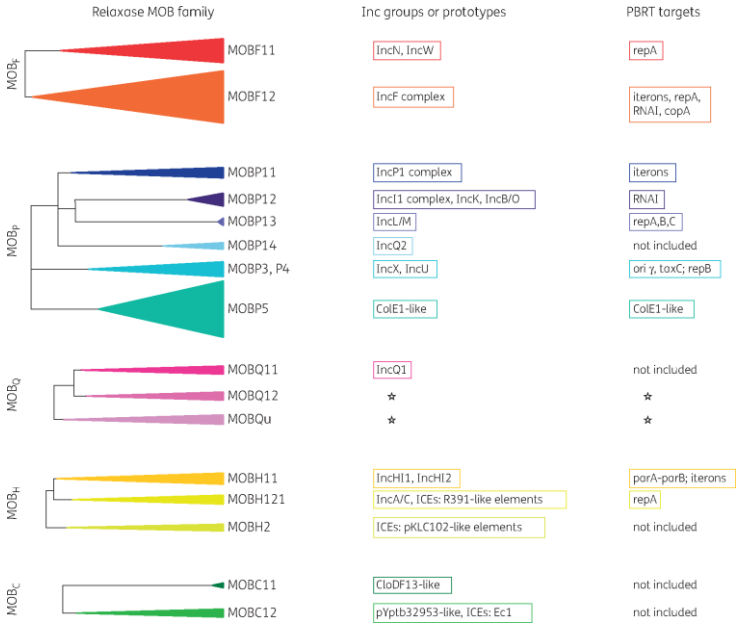

Figure 1 Inc/REP family distribution of gammaproteobacterial plasmids according to relaxase type. Adapted from Alvarado et al.[20] This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Another relaxase screening method, also called degenerate primer MOB typing (DPMT), was developed by Alvarado *et al.*[20] This scheme allows both typing of known plasmid groups and detection of plasmids not previously assigned to any Inc type.

Another typing scheme aimed at *mob* genes encoding for relaxases was developed by Compain *et al.*[21] and is called plasmid relaxase gene typing (PRaseT). This protocol distinguishes five relaxase clades arbitrarily designated HIα, HIβ, HIγ, HIδ and HIε among IncHI1 and IncHI2 plasmids. It also identifies IncX1–4 and ColE plasmids, which were initially untypeable with the PBRT method. In contrast to most other methods, PRaseT excludes a relatively large number of plasmid types such as IncFIV, IncFVI, IncFVII, IncY, IncR, IncI2, IncT, IncFIII-VII, IncJ and IncQ3 although these last three can also not be detected using the PBRT scheme.

Within other plasmid groups, different lineages can be identified by RFLP. This method, first introduced by Kiko *et al.*[22] in 1979, relies on digestion of plasmid DNA with restriction enzymes and comparison of obtained profiles.

An additional tool called plasmid multilocus sequence typing (pMLST) was developed to further differentiate plasmids within incompatibility groups. pMLST schemes were developed for IncA/C, IncI and IncN plasmids to increase the discriminatory power in the characterization of plasmids and to confirm epidemiological and evolutionary relatedness.[23–25] The IncHI2 subtyping is done by double locus sequence typing (DLST), as it includes only two targets.[26] The subtyping of F-plasmids is increasingly difficult due

to their potential multireplicon status. The replicon sequence typing scheme (RST) was developed for this purpose, leading to the FAB formula of a plasmid.[14] The FIA replicon is typed based on the sequence encoding the iterons and the replication protein RepA, for which 20 different alleles currently have been reported. The FIB replicon is typed based on the sequence of the *repB* gene for which 69 alleles were reported. The FII replicon is determined by the sequence of the *copA* gene for which 105 alleles were reported. Some additional species-specific FII replicons were also described including 5 alleles of *repA3* for *Salmonella* spp., 12 sequence variants for the region upstream of *repA* in *Klebsiella* spp. and 6 variants for the region downstream of *repA* in *Yersinia* spp., respectively referred to as FIIS, FIIK and FIIY. Finally, the FII replicon can also be replaced by the non-functional FIC for which five variants have currently been reported. Based on that, the FAB formula was created to type IncF plasmids.[14] Unfortunately, comparison of IncF plasmids with a defined FAB formula to those without is impossible and only general conclusions can be drawn.

One of the big challenges for plasmid replicon typing is multireplicon plasmids. The best known multireplicon plasmid is the earlier-mentioned IncF which can carry an FII, FIA and/or FIB replicon. Additionally, some plasmids can cointegrate, creating another type of multireplicon plasmid.[27–31] These pose a difficulty for typing and further understanding of plasmid and antimicrobial resistance epidemiology, as additional tests are required to distinguish between multiple plasmids present in the cell and a cointegrate.

The first plasmid incompatibility groups were defined and confirmed by conjugation. Nowadays, with more plasmid sequences publically available, it has become easier to study the genetic relationship between plasmids. We believe that in order to define new incompatibility (sub)groups it is necessary to confirm the data obtained through sequencing with conjugation-based incompatibility tests. For that reason, readers should be cautious when interpreting data from papers, not to mistake new replicon types for new plasmid incompatibility groups, without confirmation of the results by conjugation experiments.

Additionally, given the increasing availability of whole genome sequence data, the challenge is to trace back the typing schemes mentioned above to plasmid DNA sequences. This transition has recently been addressed by Orlek *et al.*,[32] who compared a curated dataset of publicly available plasmid sequences to replicon and MOB typing schemes.

Publication inclusion criteria

Publications chosen for this review were found on PubMed using the key words ‘resistance plasmid’ or ‘Inc plasmid’ as search criteria. Resistance determinants, described in the cited publications, were taken into account only if there was a clear linkage between plasmid Inc type and the resistance gene. The authors are aware of possible bias in the created database as many publications focus on ESBLs or carbapenemases. Additionally, the prevalence of plasmid types that are not included in the PBRT scheme may be underestimated. A description of plasmids associated with AMR is given below. A summary of prevalent plasmids is given in Table 1.

Table 1 Summary of plasmid features

Replicon type	Relaxase type	Size (kb)	Copy number	Transferability	Host range
IncF	MOB_F	45-200	low	conjugative	*Enterobacteriaceae*
IncI	MOBP	50-250	low	conjugative	narrow
IncK, IncB/O and IncZ	MOB_P	80-150	low	conjugative	narrow
IncA/C	MOB_H	18-230	low	conjugative	narrow
IncH	MOB_H	75-400	low	conjugative	wide host range (*Enterobacteriaceae*, several Gram negative organisms like *Aeromonas salmonicida, Vibrio anguillarum, and Yersinia ruckeri*)
IncP	MOB_P	70-275	low	conjugative	broad
IncL/M	MOB_P	50-80	low	conjugative	broad
IncN	MOB_F	30-70	low	conjugative	broad
Col	MOB_P	6-40	1-20	mobilizable	
IncX	MOB_P	30-50			narrow
IncR	not included	40-160		mobilizable	broad
IncW	MOB_F	up to 40	low	conjugative	broad
IncQ	MOB_Q	8-14	medium (4-12 copies/cell)	mobilizable	broad (including Alpha- Beta- Delta- and Gammaproteobacteria and Cyanobacteria)
IncT	MOB_H	~217	low	conjugative	narrow
IncU	MOB_P	29-60	low	conjugative	broad (Alpha-, Beta- and Gammaproteobacteria)

IncF plasmids

Plasmids belonging to the IncF group, or MOB_F according to relaxase typing,[19] are low-copy number, conjugative plasmids with size ranging from 45 to 200 kb. In the PBRT scheme, the target gene for these plasmids is the *repA* gene.[12] The host range is limited to the family of *Enterobacteriaceae*. In contrast to most other plasmid groups, IncF plasmids can encode several replicons; typical multireplicon IncF plasmids carry the FII replicon together with FIA and FIB. Additionally, it was shown that IncF plasmids with different F alleles are compatible.[14] In addition to this multireplicon status, IncF plasmids were reported to form cointegrates with IncI1, where the two replicon genes were separated by IS*100*, and an IncN replicon.[31,29]

IncF is the most frequently described plasmid type from human and animal sources (Figure 2) and it is mainly found in *E. coli*. The most frequently described resistance genes on IncF plasmids are ESBL genes, genes encoding carbapenemases, genes encoding aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance (PMQR) genes (Figure 3). As many research projects have focused on ESBLs, the collection of plasmids reported is likely to be biased towards those plasmids that encode ESBLs.

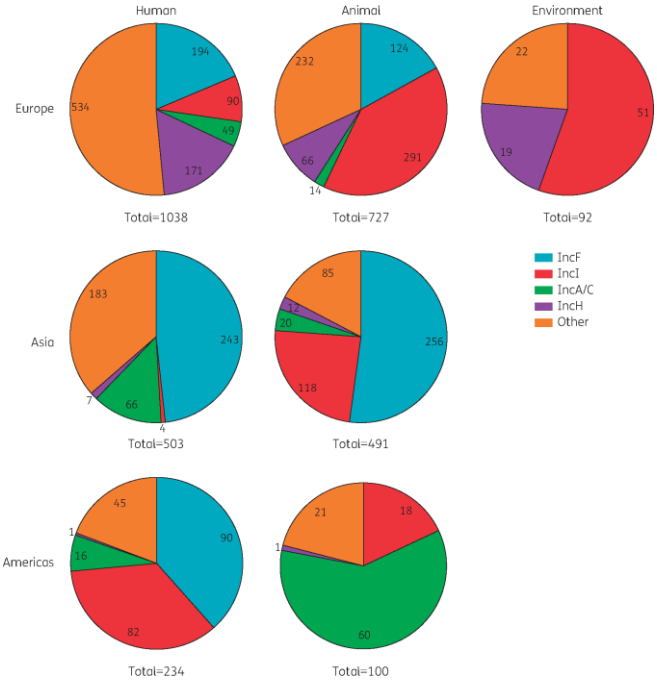

Figure 2 Distribution of different plasmid Inc types isolated from human, animal and environment across Europe, Asia and Americas (data from Table S1). Group 'other' includes: ColE, IncB/O, IncK, IncL/M, IncN, IncP, IncR, IncT, IncU, IncW, IncX, IncY and IncZ. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

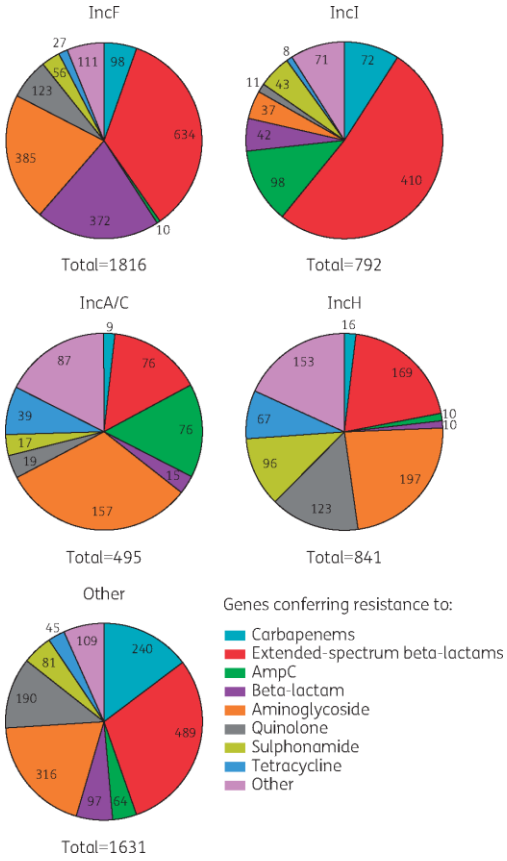

Figure 3 Distribution of genes encoding resistance to different antimicrobial classes carried by different plasmid Inc types (data from Table S1). Group 'other' includes genes encoding resistance to: trimethoprim, chloramphenicol, florfenicol, colistin, fosfomycin. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

The spread of *bla*$_{\text{CTX-M-15}}$ in human *E. coli* isolates is globally associated with IncFII plasmids in ST131 and ST405 clones.[33,34] The spread of *bla*$_{\text{CTX-M-14}}$ is associated with IncF plasmids in Korea and France,[35,36] while in Spain this gene is mainly located on IncK plasmids.[37,38] In Korea, dissemination of *bla*$_{\text{CTX-M-14}}$ was driven by horizontal transfer of the same IncF plasmid rather than clonal expansion of the host cell, since the same RST type of IncF plasmid was carried by *E. coli* strains of different sequence types.[35,] *bla*$_{\text{CTX-M-1}}$ located on an IncF plasmid was isolated only from animal sources.[39–43,] *bla*$_{\text{TEM-1}}$ on IncF is found only in *E. coli* strains mostly of human origin.[35,44–49] The spread of *bla*$_{\text{NDM}}$ and the *rmtB* gene (mostly reported in China) is also driven by IncF plasmids.[45,50,51]

No apparent correlations have been reported between any plasmid FAB formula and the resistance genes it encodes. However, trends in the prevalence of these plasmids have

been reported; F2: A−: B− is the predominant F plasmid type, F2: A1: B− was isolated only from humans and type F33: A−: B− seems to be disseminated mostly in China. Finally, both types F39: A−: B− and F2: A−: B− are found in combination with the N replicon as multireplicon plasmids.[51]

IncI plasmids

The I-complex plasmids contain incompatibility groups I, K, B and Z, which share morphological and serological similarities in their pili.[52] IncI, or MOB_P according to relaxase typing, is a group of low-copy-number, narrow-host-range, conjugative plasmids, which vary in size from 50 to 250 kb.[19] A typical feature for this plasmid group is the presence of a shufflon region at the 3′ end of the *pilV* gene which enables recombination between shufflon-specific sfx sites.[53] This recombination event selects one of seven different *pilV* genes, which is responsible for determination of the recipient specificity.[54] These plasmid rearrangements can cause possible difficulties during assembly of contigs obtained by WGS reads.[55] Incompatibility of IncI plasmids is expressed by a small, counter-transcript RNA, RNAI, which is also the target in the PBRT scheme. RNAI inhibits translation of RNA (RNAII) of the essential replication protein, RepA.[52]

Several variants exist within the IncI group: I1 (also named IncIα), I-γ and I2 (also named IncIδ). IncI1 and I-γ plasmids are very similar. However, there are some significant differences between their Inc RNA sequences. IncI-γ plasmid R621a lacks a stability region, which is conserved in IncI1 plasmids.[56] These plasmids also harbour different entry exclusion proteins ExcA that recognize different segments of their cognate TraY proteins thus allowing the transfer of IncI1 into recipient cells containing IncIγ and vice versa.[57]

Lv *et al.*[58] showed in a phylogenetic analysis of IncI2 plasmids that they are divided into three lineages. Additional phylogenetic analysis performed by Wong *et al.*[59] suggests that IncI2 plasmids can migrate between different bacterial species. Furthermore, they postulate cross-species migration with *E. coli* as a potential carrier.

The currently available PBRT scheme does not distinguish IncI-γ from IncI1. All IncI plasmids typed as IncI1 by PBRT should therefore be designated as IncI1-Iγ.[60] In the past, IncI-γ plasmids were typed using alignment with previously known sequences of reference plasmids: partially sequenced R621a from *E. coli*, and pSC138, isolated from the *S. enterica* serovar Choleraesuis.[61] Recently, Hiki *et al.*[62] proposed a PCR-RFLP method using CviAII enzyme, to differentiate between IncI1 and IncI-γ plasmids.

García-Fernández *et al.*[23] developed a pMLST scheme for IncI plasmids which is based on the allelic variation of five target genes: *repI, ard, trbA, sogS, pilL*. Currently there are 239 plasmid multilocus sequence types described (http://pubmlst.org/, last accessed 21 September 2017).

IncI2 plasmids can be distinguished from IncI1-Iγ by a set of PCR primers designed by Lv *et al.*[58] that target the *repA, rci, pilO, nikB* and *finO* genes.

IncI plasmids are described predominantly in Europe (Figure 2) in *E. coli* and *S. enterica* isolated from poultry sources. ESBL and plasmid-mediated (p)AmpC genes have been described on IncI plasmids, mostly located in *E. coli*, yet genes encoding for resistance to aminoglycosides, tetracyclines and quinolones are frequently found in *S. enterica* (Figure 3).[13,63–66] $bla_{\text{CTX-M-1}}$ is the most often identified gene on IncI plasmid ST7 and 3 (Table S1, available as Supplementary data at *JAC* Online) and has often been associated with *E. coli* ST10, 58, 117 and 131.[67,68] IncI plasmids carrying $bla_{\text{CTX-M-1}}$ have been identified all over Europe in *E. coli* from poultry. These isolates are considered a possible source of these plasmid/gene combinations in *E. coli* from human infections.[67] IncI plasmids belonging to clonal complex 5 (ST10 and 36) carry $bla_{\text{TEM-52}}$ and are frequently associated with *E. coli* ST10 in livestock.[67] IncI2 plasmids are found carrying $bla_{\text{CTX-M-55}}$ and $bla_{\text{KPC-3}}$.[58,69,70] Recently, IncI2 plasmids were described to be associated with the colistin resistance gene named *mcr-1* and its variants *mcr-1.3* and *mcr-1.5*.[71–73] It was reported in both human and animal sources in China, Japan, Denmark and Spain.[71,74–78] IncI-γ plasmids carry mostly the $bla_{\text{CMY-2}}$ gene.[61,62]

IncK, IncB/O and IncZ plasmids

IncK, IncB/O and IncZ plasmids, as they all belong to the I-plasmid complex, are discussed together.

According to relaxase typing, IncK and IncB/O belong to the MOB_{P} group.[19] The IncZ plasmid is not included in this typing scheme.[19] Their sizes vary between 80 and 150 kb. The presence of shufflons (widely distributed in the IncI plasmid family) was confirmed in IncK and IncZ plasmids, but not for IncB/O.[79,80] RNAI encodes antisense RNA for *repYZ* mRNA and is one of the elements responsible for plasmid incompatibility.

Recently it was shown that IncK plasmids can be divided into two compatible plasmid lineages, named IncK1 and IncK2.[81,82]

Originally, the IncB/O plasmid group was discovered and reported independently by two groups and termed IncB and IncO. However, Datta and Olarte[83] already mentioned that they may be synonymous.[84] Later, Bradley[228] referred to the IncO plasmid as IncB. In a review by Couturier,[10] IncB/O was mentioned as a plasmid group.

The fact that IncK, IncB/O and IncZ RNAI sequences, which are targets in the PBRT scheme, are very similar causes difficulties with typing. Carattoli *et al.*[12] already mentioned cross-reactivity between IncK and IncB/O replicons and aspecificity of PCR products based on primers included in the PBRT scheme was reported.[85] Considering these complications some researchers report IncK/B plasmid as a result of the inability to distinguish between IncK and IncB/O replicons.[86,87] In addition to IncK typing, as there is no pMLST scheme available, Dierikx *et al.*[88] made an RFLP scheme, using EcoRI and HindIII to differentiate plasmid variants. A recent paper by Moran *et al.*[89] showed that IncB/O-specific primers also detect the IncZ replicon.

The IncZ plasmid group was first discovered by Tschäpe and Tietze.[90] These plasmids could not be stably maintained together with an IncB plasmid. The authors suggested that it was caused by a 'dislodgement' phenomenon, which was defined as interactions leading

to the elimination of the resident plasmid or recombination between the two plasmids.[91] However, Praszkier *et al.*[52] later described that IncB/O and IncZ plasmids may be incompatible with each other.

IncK plasmids are mainly associated with the spread of *bla*$_{CMY-2}$ and *bla*$_{CTX-M-14}$ genes in Europe (especially in Spain and the UK) and are frequently found in *E. coli* from animal sources.[37,39,42,88,92–97] IncB/O plasmids are less prevalent, but carry a greater variety of resistance genes such as *bla*$_{CTX-M-1}$, *bla*$_{CMY-2}$, *bla*$_{ACC-4}$, *bla*$_{SCO-1}$, *bla*$_{TEM-1}$, *sul1*, *sul2*, *aad*, *strA*, *strB* and *aacA4*. (Table S1). IncZ plasmids have been reported to carry resistance to sulphonamides, ampicillin, tetracycline and chloramphenicol.[90]

IncA/C plasmids

IncA/C is a group of low-copy-number, conjugative, self-transferable plasmids with a size range of 40–230 kb, although smaller conjugative variants with sizes of 18–25 kb have also been reported.[98] According to relaxase typing, IncA/C belongs to MOB$_H$.[19] In the PBRT scheme *repA* is the target gene. IncA/C plasmids have a broad host range which include members of Beta-, Gamma- and Deltaproteobacteria.[99] The reference IncA/C plasmid, pRA1, was isolated from *Aeromonas liquefaciens* in 1971.[100]

Within this plasmid group, two variants have been identified: A/C$_1$ (corresponding to the IncA plasmid, with plasmid pRA1 as a reference) and A/C$_2$ (corresponding to the IncC plasmid). Although compatibility of IncA and IncC plasmids was confirmed,[101] later they were assigned to the same plasmid group called IncA/C.[102] IncA/C$_1$ and IncA/C$_2$ exhibit 26 SNPs in the *repA* gene.[103] Later analysis based on WGS data revealed that both plasmid groups carry regions that are unique to each backbone type.[104] Recently, two types were defined among the A/C$_2$ group, named type 1 and type 2, which diverged because of SNP accumulation and multiple insertions and deletions.[105] The two types differ in the *rhs* gene (named *rhs1* and *rhs2*) and an open reading frame between *traA* and *dsbC* (15 amino acid difference in the predicted protein). In addition, two short regions, i1 and i2, are present in type 2, but not type 1.[104]

A recent article by Hancock *et al.*[25] describes both a pMLST and a core genome pMLST (cgPMLST) for IncA/C plasmids. The pMLST includes four essential target genes for use with conventional PCR whereas the cgPMLST includes 28 conserved loci for a high resolution analysis of WGS data. Both schemes allow the distinction between type 1 and 2 A/C$_2$ plasmids.[25] Currently there are 12 pMLST sequence types for IncA/C and 37 cgpMLST available (http://pubmlst.org/, last accessed 6 March 2017). An important feature of the IncA/C$_2$ plasmid group is the presence of AMR islands named ARI-A and ARI-B. ARI-A is only found in type 1, whereas ARI-B is found in both type 1 and type 2 A/C$_2$ plasmids.[104] Both islands carry a great variety of genes, encoding factors responsible for resistance to many antimicrobial classes. Recently, Harmer and Hall[106] published a set of primers to distinguish IncA/C2 type 1 and type 2 plasmids, targeting *orf1832*/*orf1847*, *rhs1*/*rhs2* and insertions i1 and i2.

Walsh *et al.*[107] reported that bla$_{NDM-1}$-carrying IncA/C plasmids isolated from water sources have the highest transfer rate at 25 °C or 30 °C.

IncA/C plasmids are associated with MDR (Figures 2 and 3) and are spread worldwide. They are found in isolates from both human and animal sources and involved in the global spread of *bla*$_{CMY-2}$ (Table S1). IncA/C_2 are also found worldwide, in many sources and in different bacteria. IncA/C_2 can encode ESBLs (*bla*$_{TEM}$, *bla*$_{SHV,}$ but rarely *bla*$_{CTX-M}$), AmpC (*bla*$_{CMY}$, *bla*$_{DHA}$), carbapenemases (*bla*$_{OXA}$, *bla*$_{NDM}$, *bla*$_{IMP}$) and enzymes modifying groups of antibiotics: sulphonamides (*sul1*, *sul2*), aminoglycosides (*aphA1*, *aadA*, *aadB*, *strA*, *strB*, *aacC*), tetracyclines *tet*(A), chloramphenicol (*floR*, *catA1*) and trimethoprim (*dfrA*).[28,105,108–111]

IncH plasmids

IncHI is a group of low-copy-number plasmids with a wide host range, including the *Enterobacteriaceae* and several Gram-negative pathogens of fish such as *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Yersinia ruckeri*.[112] The size of both plasmid subgroups varies from 75 to 400 kb.

Members of the IncHI plasmid group (MOB_H according to relaxase typing[19]) were historically named IncH1, IncH2 and IncH3 (which contains only plasmid MIP233[113]). Members of each subgroup show a high level of DNA homology within the group, but not compared with the other two. Bradley *et al.*[114] introduced the new IncHII group based on incompatibility of these plasmids with other members of the IncH group, renaming existing plasmids to IncHI1, IncHI2 and IncHI3, respectively. The relationship between IncHI and IncHII groups is thought to be similar to that of IncFI with IncFII, which are related by antigenically similar pili.[115]

The IncHI plasmid group was divided into two groups due to incompatibility of some members with IncF plasmids. Accordingly, IncHI2 are compatible with IncF plasmids, but IncHI1, which possesses *repFIA*, are incompatible.[116] These distinctions were made based on DNA–DNA hybridization.[117]

To date, only four IncHII plasmids were reported.[118] Bradley *et al.*[114] found them in *K. pneumoniae* and these were reported to be unstable in the original host and *E. coli* K-12. Additionally, they observed that IncHII seems to be incompatible with IncD plasmids. Finally, it was concluded that plasmid loss is an example of dislodgement.

In the PBRT scheme the target site for typing IncHI1 plasmids is *parA-parB* and the iterons for IncHI2, while IncHI3 and IncHII are not included in this typing scheme. The first tool developed to characterize IncHI plasmids was RFLP. Seven different patterns were defined and a clear difference was made between plasmids isolated in 1993 and 1996.[119] As RFLP7 was predominant after 1996, it is possible that this group may have acquired genetic features which increased its fitness or its chance of survival. IncHI pMLST, developed by Phan *et al.*,[120] includes six loci: HCM1.043, HCM1.064, HCM1.099, HCM1.116, HCM1.178ac, and HCM1.259. In addition, two plasmid clusters were made based on the presence or absence of conserved regions (named A–E) and a transposon named Ins1056. Group 1 (containing ST1–4) consists of plasmids carrying region Ins1056, but most of them lack regions C, D and E. Group 2 (containing ST6–8) involves plasmids with regions C, D and E but without Ins1056. IncHI2 DLST includes

two loci: *smr0018* and *smr0199*.[26] Currently there are 14 sequence types for IncHI1 and 10 for IncHI2 available (http://pubmlst.org/, last accessed 3 June 2017).

Interestingly, the transfer rate of IncHI, but not IncHII, is temperature-dependent.[112,121] The efficiency is optimal at 22–30 °C.[122] A possible explanation was suggested by Alonso *et al.*,[123] who showed that the *trhRY* genes are required for conjugation. Temperature dependency suggests that these plasmids are potential vectors for the dissemination of genes among bacterial species in aqueous and soil environments.

IncHI1and IncHI2 plasmids have been isolated in Europe from both human and animal sources (Figures 2 and 3). Many of them are reported to be associated with multidrug resistance because, besides ESBL genes, they often carry genes encoding for resistance to sulphonamides, aminoglycosides, tetracyclines and streptomycin (Figures 2 and 3). IncHI1 plasmids are considered as a main carrier of a multiple resistant phenotype in *Salmonella* Typhi.[124] IncHI2 is occasionally reported as a multireplicon plasmid, also carrying a P replicon.[96,125] In addition to $bla_{\text{CTX-M-2}}$ carried by all IncHI2/IncP multireplicon plasmids, they can also encode $bla_{\text{TEM-1}}$.[41,96] Recently, an IncHI2 plasmid was reported to be associated with the novel colistin resistance genes *mcr-1* and *mcr-3*.[126–128] The IncHI3 plasmid was reported to carry $bla_{\text{NDM-1}}$ gene.[129]

IncHII plasmids were described to carry resistance to trimethoprim, streptomycin and spectinomycin, which were part of Tn*7*, and ampicillin, amikacin, chloramphenicol, gentamicin, kanamycin and tetracycline.[118]

IncP plasmids

IncP, MOB_P according to relaxase typing,[19] is a group of broad-host-range, low-copy-number plasmids ranging in size from 70 to 275 kb. The copy number is controlled by iterons which are also targeted in the PBRT scheme. IncP plasmids are classified in *Enterobacteriaceae* as IncP and in *Pseudomonas* spp. as IncP-1. Yakobson and Guiney[130] proposed to divide the IncP group into two subgroups named IncPα and IncPβ. Later, six subgroups of IncP plasmids were defined.[131] Classification of plasmids into subgroups is either based on the phylogeny of a single gene or multiple genes.[132] Within the *Enterobacteriaceae* family only plasmids from the IncPα and IncPβ groups have been reported.

IncP plasmids are often isolated from *Salmonella* Infantis from broilers in Japan. They were reported to carry genes conferring resistance to: (extended) spectrum β-lactams, sulphonamides, aminoglycosides and tetracyclines (Table S1).[87,94,109,133–138] IncP plasmids in human samples were mainly isolated from *E. coli* and *K. pneumoniae* (Table S1). Recently, an IncP plasmid was reported to be associated with the colistin resistance gene *mcr-1* and its variant *mcr-1.6*.[139,140] This plasmid co-harboured *dfrA1*, *tet*(A) and *sul1* resistance genes.

IncL/M plasmids

IncL/M, MOB_P according to relaxase typing, is a group of broad-host-range plasmids that range in size from 50 to 80 kb and have a low copy number.[19] In IncL/M, together with

IncI, IncK and IncB/O, replication is regulated by antisense RNA, which is also the determinant for plasmid incompatibility.[141] The target sites used in the PBRT scheme are *repA, repB* and *repC*.

Originally the plasmids IncL and IncM were separate groups. Richards and Datta[142] proposed placing IncL plasmids in the IncM group because repeated incompatibility experiments showed that IncL plasmids were incompatible with IncM plasmids. As a consequence, these groups were merged and named IncL/M. In contrast, Carattoli *et al.*[143] recently demonstrated that these plasmids should indeed be interpreted as separate incompatibility groups. This distinction was made by comparison of ExcA, TraY and TraX proteins (35%, 59% and 75% amino acid identity, respectively) and an update of the PBRT scheme has been proposed accordingly. Additionally, due to the differences in the RNAI sequence of IncM plasmids, two groups were defined and termed IncM1 and IncM2. Incompatibility tests did not confirm compatibility of IncM1 and IncM2 plasmids.

A 60 kb IncL plasmid, formerly designated IncL/M, is globally reported to be associated with $bla_{\text{OXA-48}}$, although this gene was also reported on IncF and IncP plasmids (Table S1). In hospitals, *K. pneumoniae* harbouring these IncL plasmids with $bla_{\text{OXA-48}}$ is considered to be a major cause of infections.[144] It was described that Tn*1999*, which carries $bla_{\text{OXA-48}}$, inserts in the *tir* gene which encodes for transfer inhibition protein.[145] That may be one of the causes of such a successful dissemination of this plasmid. IncL/M plasmids (typed before the report of Carattoli) can also carry $bla_{\text{CTX-M-1, -3, -14, -15}}$, $bla_{\text{TEM-1, -10, -52}}$, $bla_{\text{SHV-1}}$ and *armA* genes (Table S1).

IncN plasmids

IncN, MOB_{F} according to relaxase typing, is a group of broad-host-range plasmids, for which the copy number is controlled by iterons.[19] Their size ranges from 30 to 70 kb. The target site in the PBRT scheme for IncN is the *repA* gene. It was observed that plasmids belonging to the IncN group are often colocalized with IncF plasmids.[146] Yang *et al.*[147] reported a fusion of an IncN plasmid with an F33: A−: B− IncF plasmid (see the IncF section).

Recently, a new plasmid type, named IncN2, carrying a novel replicase gene (encoding Rep271) was described.[148] In contrast to IncN plasmids, IncN2 is not included in the PBRT scheme. A PCR to detect IncN2 was described by Netikul *et al.*[149]

Garcia-Fernandez *et al.*[24] developed the pMLST scheme for IncN plasmids. It involves three target genes: *repN*, *traJ* and *korA*. Currently, 16 different plasmid STs have been reported (http://pubmlst.org/; last accessed 6 March 2017).

IncN plasmids carry a great variety of resistance determinants against: extended-spectrum β-lactams, sulphonamides, quinolones, aminoglycosides, tetracyclines and streptomycin (Table S1). The $bla_{\text{CTX-M-1}}$ gene is often associated with IncN plasmid ST1. It is disseminated throughout Europe and isolated mainly from *E. coli* from animal sources. $bla_{\text{VIM-1}}$ was found in Spain, Greece and Italy. It was mainly isolated from human *K. pneumoniae* isolates. IncN plasmids often carry Tn*1721* encoding for *tetA* and *tetR* genes

and Tn*5393* carrying *strA* and *strB*.[150–153] Currently, the IncN2 plasmids have only been found in *Enterobacteriaceae* isolated from human samples from Thailand, Singapore and Australia.[149,154]

Colicinogenic plasmids

Colicins, which belong to the family of bacteriocins, are proteins produced by some strains of *E. coli* that are lethal for related *E. coli* strains. Colicins are encoded by genes predominantly located on plasmids.[155] One case of a chromosomally encoded colicin has been reported: colicin-like bacteriocin 28b produced by *Serratia marcescens*.[156] Two groups of colicins have so far been described based on cross-resistance: group A (TolA-dependent) containing the A, E1 to E9, K, L, N, S4, U and Y proteins. Group B is TonB-dependent and contains colicins B, D, H, Ia, Ib, M, 5 and 10.[155]

There are two groups of colicinogenic plasmids. The type I plasmids are small, mobilizable plasmids of 6–10 kb that contain approximately 20 copies per cell and mainly encode group A colicins. These plasmids have been frequently used for genetic engineering and biotechnology such as construction of vector pBR322.[157] The type II pCol plasmids are relatively large monocopy plasmids of about 40 kb that usually encode colicins of group B.

ColE1 plasmids, MOB_P according to relaxase typing,[19] are regulated by an antisense RNA, which by binding to a pre-primer RNA alters its secondary structure and prevents its subsequent processing to form a primer for the initiation of DNA synthesis.[158] Additionally, Davison[159] confirmed that incompatibility of ColE plasmids is expressed by loop II′ of RNA I which interacts with both the loop I and loop II regions of RNA II. A single mutation in this region can give rise to two different ColE1 plasmids, with independent copy numbers, replication and resistance level.[160]

Although ColE plasmids have been found to carry different AMR genes, they are predominantly associated with spread of *qnrS1* and *qnrB19* genes.[13,63,161–163] They are most often found in *S. enterica* strains isolated from human samples. Surprisingly, *qnrB* genes carried by ColE plasmids were found frequently (27%) in a remote Amazonas population which had no previous exposure to therapeutic antibiotics.[164] Additionally, β-lactamases $bla_{CTX\text{-}M\text{-}17}$, $bla_{CMY\text{-}31}$ and $bla_{CMY\text{-}36}$ carried by ColE1-like plasmid were described.[165,166] Moreover, Herrera-Leon *et al.*[161] reported that ColE plasmids, in addition to *qnrS1*, also frequently harboured *sul2*, *strA/B* and *tetA* genes. ColE plasmids were reported to carry novel colistin resistance genes *mcr-4* and *mcr-5*.[167,168] ColE1 plasmids bearing different AMR genes can further coexist in the same bacterial host, providing multiresistant phenotypes.[169]

IncX

IncX, MOB_P according to relaxase typing, is a group of narrow-host-range plasmids.[19] IncX plasmids have six known subtypes (X1–X6) and their size ranges from 30 to ~50 kb. PBRT includes primers that recognize only IncX1 and 2, for which the target site is ori γ, meaning that the prevalence of IncX3–6 may be underestimated. Johnson *et al.*[170] designed a set of primers targeting the *taxC* gene, which allows the differentiation of

plasmids belonging to groups X1–4. Recently, based on differences in the *taxC* gene, novel subgroups X5 and IncX6 were identified.[171] Acquisition of IncX plasmids has caused phage type conversion in *Salmonella* Enteritidis.[172] This is interesting from an epidemiological perspective, since phage typing was traditionally used in epidemiological studies on *Salmonella*.[173,174] IncX plasmids were shown to be able to form cointegrants with pSLT (*Salmonella* serotype-specific plasmid-carrying virulence genes) which resulted in a broadening of the host range of the new plasmid.[175]

IncX plasmids were present in *Salmonella* strains which were isolated before antibiotics were commonly used.[176] Nowadays, IncX plasmids are isolated mainly from both *Salmonella* and *E. coli* from human and animal sources (Table S1). These plasmids encode primarily AMR determinants against extended-spectrum β-lactams and quinolones. In addition, tetracycline and trimethoprim resistance determinants can be carried by IncX plasmids (Table S1). Genes encoding carbapenemases (mainly bla_{KPC} and bla_{NDM}) are reported on IncX plasmids.[177–179] Recently, an IncX4 plasmid was reported to be associated with the colistin resistance genes *mcr-1* and *mcr-2*.[75,126,180]

Rarely detected plasmids

A number of plasmids are not often reported in literature but, as most of them have a broad host range and can carry multiple AMR genes, these are involved in the continuous spread of resistance genes.

IncR

The IncR plasmids range in size from 40 to 160 kb and are not included in the MOB typing as these plasmids do not contain a relaxase gene.[19] Sequencing results indicate that these plasmids do not possess conjugational transfer genes.[181] However, because of their broad host range, Bielak *et al.*[182] postulated that IncR plasmids are mobilizable. IncR plasmids can form multireplicon cointegrates with IncA/C or IncN plasmids.[27,28] The first IncR plasmid was detected from a *Salmonella* Montevideo isolate and conferred a multidrug-resistance phenotype, including resistance to aminoglycosides, chloramphenicol and tetracycline.[13] In addition, IncR plasmids have been reported to carry genes conferring resistance to antibiotics belonging to many classes including: β-lactams, sulphonamides, quinolones, aminoglycosides, tetracyclines, chloramphenicol and trimethoprim (Table S1).

IncW

IncW, MOB_F according to relaxase typing,[19] is a group of low-copy-number, broad-host-range plasmids with sizes up to 40 kb. Host species are Alpha-, Beta-, Gamma-, Deltaproteobacteria and Bacteroidetes.[183] IncW plasmids are considered the smallest conjugative plasmids. The IncW plasmid R388 was shown to be essential for mobilization of the plasmids RSF1010 (IncQ) and ColE1.[184] In the PBRT scheme the *repA* gene is the target site.

IncW plasmids were found in many bacterial sources in the 1980s.[183] Although primers recognizing these plasmids are included in the PBRT, IncW plasmids are currently rarely detected.

The reference IncW plasmid (pSa) was shown to carry genes conferring resistance to chloramphenicol, tetracyclines, sulphonamides, gentamicin and trimethoprim.[185] Later, plasmids carrying a subset of these genes were reported.[183,186] IncW plasmids were also shown to encode the carbapenemase *bla*$_{KPC-2}$ and metallo-β-lactamase *bla*$_{VIM-1}$ genes.[187,188]

IncQ

The IncQ group can be subdivided into two major groups, of which IncQ1 belongs to the MOB$_Q$ group, while IncQ2 belongs to the MOB$_P$ group, according to relaxase typing. These groups are not detected by PBRT.[19] IncQ is a group of mobilizable plasmids with a medium-range copy number (4–12 copies/cell) and a size range from 8 to 14 kb. These plasmids have a broad host range including Alpha-, Beta-, Delta- and Gammaproteobacteria and Cyanobacteria. It was proposed that its broad host range is a result of the presence of genes required for plasmid replication.[189] The IncQ reference plasmid RSF1010 encodes RepA, -B and -C proteins,[190] but it also requires host-encoded single-strand binding proteins, DNA gyrase and the γ subunit of the DNA polymerase III.[191]

IncQ plasmid incompatibility is expressed through direct repeats at *oriV*, which was confirmed in both RSF1010 and R1162.[192,193] Becker and Mayer[229] reported that introduction of additional direct repeats into the origin of replication of R1162 resulted in a decreased copy number. This suggests that the lack of a partitioning or stability system leads to a high copy number, which prevents plasmid loss. Additionally, it was proven that members of subclasses of the IncQ family are compatible with each other due to evolution of their iteron sequences.[194]

Rawlings and Tietze[189] suggested dividing the IncQ family into two groups based on their Rep protein similarities. Plasmids RSF1010, pIE1107, pIE1115, pIE1130 and pDN1 form the first group (IncQ1), and pTF-FC2 and pTC-F14 make up the second (IncQ2).[195] Further subgroups were defined according to their iteron sequence variability and incompatibility. Plasmids which are incompatible with RSF1010 were designated IncQ-1α. Plasmids that were incompatible with pIE1107, pIE1115 or pDN1 were designated IncQ-1β. IncQ-1γ was assigned to plasmids which are incompatible with pIE1130. Furthermore, IncQ2 was subdivided into IncQ-2α for pTF-FC2 and IncQ-2β for pTC-F14.[189]

Additionally, after *in silico* alignment of *repA, repB* and *repC* genes from known IncQ plasmids,[195] Loftie-Eaton and Rawlings[195] proposed two new subclasses named IncQ3 and IncQ4, of which the latter consists of only one member, pPNAP08.[196]

IncQ plasmids were reported to carry *bla*$_{CMY-4}$, *bla*$_{GES-1}$ and the *sul2-strA-strB* gene cluster.[197–199]

IncT

Rts1, a reference plasmid for the IncT group and MOB$_H$ according to relaxase typing,[19] is a low-copy-number, narrow-host-range, 217 kb plasmid originally isolated from *Proteus vulgaris*.[200] The target site in the PBRT scheme is the *repA* gene. Most plasmids belonging

to this group exhibit thermosensitive replication and conjugation, which are stable at 37 °C and 25 °C, respectively, but inhibited at 42 °C and 37 °C.[201] Interestingly, R394 isolated from *Proteus rettgeri* did not show this thermosensitive phenotype for stability and conjugation.[202] This phenomenon was later explained by the fact that R394 is a cointegrate plasmid containing IncT and IncN replicons.[30]

Early isolated IncT plasmids were carrying kanamycin- (Rst1) or sulphonamide resistance genes (R485). Recently, a *Proteus mirabilis*-carrying $bla_{\text{CTX-M-2}}$ on an IncT plasmid was reported in Japan.[203–205] IncT plasmids were also reported to carry $bla_{\text{OXA-181}}$.[206]

IncU

IncU (MOB_P according to relaxase typing[19]) is a group of broad-host-range plasmids isolated from Alpha-, Beta- and Gammaproteobacteria.[207] These plasmids are low-copy-number with sizes ranging between 29 and 60 kb.[208] The first IncU plasmids were isolated from *Aeromonas salmonicida*.[209] The stabilization module of RA3 encodes seven homologues to IncP plasmid products and its conjugative transfer region is highly similar to the one from the PromA plasmid,[210] which is a novel family of broad-host-range plasmids, which seem to have no phenotypical effect on the host (cryptic plasmids).[211]

Plasmid Rms149 has been classified as a novel IncG plasmid; however, later studies showed that this plasmid is a member of the IncU group.[212,213]

IncU plasmids were reported to carry resistance to: trimethoprim, chloramphenicol, ampicillin, tetracyclines, sulphonamides, kanamycin and streptomycin.[208,214]

IncD

The first IncD plasmid was mentioned by Datta.[9] Later, Coetzee *et al.*[215] reaffirmed existence of this group performing compatibility experiments. However, the group is not included in the PBRT scheme.

IncD plasmids belong to the IncF-like group of plasmids based on classification by genetic relatedness and pilus structure.[216] Plasmids examined by Coetzee *et al.*[215] were conjugative and can be transferred between members of the *Enterobacteriaceae* family. Transfer between other families has not been determined. They can carry resistance determinants to ampicillin and kanamycin. Unfortunately, no plasmid of this incompatibility group was fully sequenced and there are no reports revealing the functional biology or prevalence of these plasmids.

IncJ

In 1972 Coetzee *et al.*[202] described a new plasmid called R391 and assigned it to the new IncJ incompatibility group. Later it was discovered that R391 rather belongs to the group of integrative and conjugative elements (ICEs). These elements are integrated in the chromosome, but after excision they circularize, replicate autonomously and are self-transmissible via conjugation.[217]

Recent work of Carraro *et al.*[218] shows that stability of R391 family members depends on replication starting at *oriT* by TraI. Additionally, these plasmids encode the toxin–antitoxin system *hipAB*, although this is not highly conserved. These results suggest that ICEs are more similar to plasmids than was previously thought but, as these are not actually plasmids, they are not detected by the PBRT scheme.

IncY

IncY is a group of prophages which replicate as autonomous plasmids. Their size range is approximately 90–100 kb and they are low-copy-number plasmids.[219] Although they contain iterons close to the *repA* gene which is also the target in the PBRT scheme, their involvement in the incompatibility reaction was not confirmed.[220]

IncY were confirmed to confer resistance to ampicillin and carry the bla_{SHV-2} gene.[219,221] Additionally they are reported to be associated within a cell with IncF and/or IncI plasmids or IncHI2.[96,134,222–227]

Untypeable plasmids

Many authors report plasmids which they designate as 'untypeable'. There are reports of untypeable plasmids with variable sizes (20–260 kb) that carry genes encoding for different antimicrobial classes: β-lactams including cephalosporins and carbapenems, sulphonamides, quinolones and aminoglycosides (Table S2).

Although the PBRT scheme is widely used, it is recognized that it cannot detect all known plasmid types. The false negative results that were described for IncL/M have been solved by a new set of primers that was described for subdividing these plasmids. For other plasmids such as IncX3–4 and the IncFIII-VII replicons, currently no PCR methods have been described that can be used in addition to the PBRT scheme. Another consideration is the continuous rearrangement and mutations that plasmids undergo, which may also occur in the regions that are used for plasmid typing. This may result in novel untypeable plasmids evolving from currently well-studied plasmid types.

Conclusions and future perspective

Since the first incompatibility experiments performed by Couturier *et al.*[10] in 1988, a lot has changed. Different typing methodologies are used in literature, which hampers a comparison of results from these studies. Nowadays, the PBRT scheme is the most commonly used technique for plasmid typing of *Enterobacteriaceae*, as it facilitates rapid identification of the dominant replicon types. Its use has led to a more unified way of plasmid identification, which in turn has resulted in a large expansion of our knowledge of plasmid epidemiology. The commercially available PBRT kit is kept up to date by periodic inclusion of newly described targets for plasmid identification. The main disadvantage, however, is that it can only detect plasmids included in the scheme, and that some plasmids harbour more than one replication machinery. Typing plasmids according to the relaxase gene has a higher discriminatory power, but it misses plasmids which do not contain a relaxase gene.

Carattoli[6] has provided an extensive overview of plasmids and their associated resistance genes. The work presented here provides an update about all known resistance plasmids in *Enterobacteriaceae*.

A great variety of plasmids can be found in human, animal and environmental isolates. The most abundant plasmids, often referred to as epidemic plasmids, are IncF, IncI, IncA/C and IncH. There are differences in prevalence of certain plasmids from different sources and on different continents. Animals in Europe are mainly colonized by *E.coli*-carrying IncI plasmids, while in Asian animals the dominant plasmid is IncF and in animals from North and South America the dominant plasmid type is IncA/C. From human sources, IncF is the most abundant plasmid isolated in Asia and North and South America, while in Europe isolates are more diverse, including IncI and IncH. Next to IncF, IncA/C also seems to be abundant among humans in North and South America.

ESBLs are the most frequently described enzymes conferring resistance to antimicrobials encoded on plasmids. Enzymes hydrolysing aminoglycosides and genes encoding for resistance to quinolones and sulphonamides are often co-transferred through transposons located on a plasmid. We also show that various plasmids seem to be associated to a different range of antibiotic resistance gene classes, e.g. IncF carry a wide variety of gene classes, while IncI plasmids are mainly associated with ESBLs. Some plasmids even have a strong correlation with specific genes, like IncL/M with $bla_{\text{OXA-48}}$, or IncK plasmids with $bla_{\text{CMY-2}}$ or $bla_{\text{CTX-M-14}}$. However, the exact nature of these specific relationships is still not fully understood.

Given the fact that the number of studies performed on all continents varies and certain resistance determinants are studied more intensively, the data presented in this article will inevitably be slightly biased. Therefore, the observed differences should be interpreted with care. Most papers describe data from Europe. Additionally, most of them focus on β-lactamases, which means that the prevalence of other antibiotic classes may be underestimated. Furthermore, most plasmids are typed using the PBRT scheme, which means that the prevalence of the plasmids not included in that scheme can be underestimated.

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Transparency declarations
None to declare.

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presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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Chapter 3

Plasmids of distinct IncK lineages show compatible phenotypes.

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Abstract

bla$_{CMY-2}$ and *bla*$_{CTX-M-14}$ are among the most prevalent AmpC and ESBL β-lactamase genes associated with IncK plasmids. In this study a set of 37 IncK plasmids was subjected to whole genome sequencing and phylogenetic analysis was performed. As an outgroup, IncB/O plasmid sequences were added to the analysis. Phylogenetic trees based on IncK plasmids' accessory genomes showed distinct clusters. One cluster included IncK plasmids encoding *bla*$_{CTX-M-14}$ and isolated from *Escherichia coli* from various sources. A second cluster included IncK plasmids associated with *bla*$_{CMY-2}$ isolated from *E. coli* only from poultry. IncB/O plasmids included in this analysis, instead of clustering together, form two separate clusters that position in between IncK1 and 2 plasmids. Incompatibility experiments, performed using representative plasmids from both clusters, show that these two plasmids can be present together in one cell and therefore are compatible. Additionally, stability tests showed that these plasmids can be stable in one cell without selective pressure for at least 72h. In conclusion, the IncK plasmid group consists of two compatible plasmid lineages which are which are referred to here as IncK1 and IncK2.

Introduction

Antimicrobial resistance is an increasing problem worldwide for human and veterinary medicine and which is often spread by the exchange of plasmids encoding resistance genes on plasmids and other mobile genetic elements. In the Gram-negative *Enterobacteriaceae*, highly related plasmids are often found to be incompatible. Plasmid incompatibility is defined as an inability of two plasmids to be stably maintained in one cell without selection. Incompatibility can be caused by replication or partitioning-related mechanisms.[1] Although not much is known about incompatibility determinants in the IncK plasmid group, they were extensively studied in the closely related IncB/O group. Siemering *et al.* proved that the RNAI structure is responsible for incompatibility in the IncB/O group and that the nucleotides in the loop and upper stem are crucial for the structure formation and its function.[2]

Resistance to cephalosporins can be encoded by ESBL and AmpC β-lactamases. $bla_{\text{CMY-2}}$ is the most prevalent AmpC gene and $bla_{\text{CTX-M-14}}$ is one of the most prevalent ESBL genes.[3] Both enzymes encoded by these genes are often described to be encoded on IncK plasmids isolated worldwide. *Escherichia coli* encoding ESBL or AmpC genes on IncK plasmids are found predominantly in poultry but they have also been reported in other species including humans, calves and pigs.[4-8] IncK plasmids, together with IncB/O, IncZ and IncI, belong to the I-complex of plasmids and their sequences are highly related with frequent recombination between these groups.[9] For that reason, studying the complete plasmid sequences of IncK, together with other members of the I-complex, is helpful in understanding the successful spread of IncK plasmids.

When studying a large set of IncK plasmid sequences from various sources from the Netherlands, De Been *et al.* created a SNP based phylogenetic tree of IncK plasmids which showed that two separate lineages of IncK plasmids were present, one cluster contained plasmids carrying $bla_{\text{CMY-2}}$ and the other $bla_{\text{CTX-M-14}}$.[10] The purpose of this study is to further investigate the possible existence of multiple lineages of IncK plasmids and determine the incompatibility relationship within and between the IncK lineages and IncB/O plasmids, representing other plasmid from the I-complex. IncB/O plasmid was chosen as the most closely related plasmid to the IncK group. Additionally, *in silico* analysis of IncK and IncB/O plasmids was performed to establish phylogenetic relationship between these closely related plasmids.

Methods

Strain collection

E. coli isolates carrying $bla_{\text{CMY-2}}$ or $bla_{\text{CTX-M-14}}$, obtained from various epidemiological studies, were screened for IncK plasmids using previously described primers.[11] Plasmids in *E. coli* were selected for further analysis based on the resistance gene it encoded, the animal or human source and variety of studies to assure utmost diversity, minimizing a potential sampling bias.

Table 1 E. coli strains containing plasmids sequenced in the study

Plasmid name	Replicon	Beta-lactamase	Source	Origin	Accession number	Reference
546	IncK	CTX-M-1, TEM-1B	cattle	Netherlands	ERR1551804	this study
762-6	IncK	CTX-M-14	dog	Netherlands	ERR1551800	[12]
527-1	IncK	CTX-M-14	cattle	Netherlands	ERR1551796	this study
713-4	IncK	CTX-M-14	dog	Netherlands	ERR1551798	[12]
0291	IncK	CTX-M-14	cattle	Netherlands	ERR1607720	this study
0380	IncK	CTX-M-14	cattle	Netherlands	ERR1607721	this study
0590	IncK	CTX-M-14	cattle	Netherlands	ERR1607723	this study
1-42	IncK	CTX-M-14	human	Netherlands	ERR1607727	this study
NRS1	IncK	CTX-M-14	human	Netherlands	ERR1607726	this study
0405	IncK	CTX-M-14	cattle	Netherlands	ERR1607722	this study
047	IncK	CTX-M-14	cattle	Netherlands	ERR1551803	this study
WF11	IncK	CTX-M-14	redshank	Netherlands	ERR1607718	[13]
0058	IncK	CTX-M-14	cattle	Netherlands	ERR1607719	this study
707-3	IncK	CTX-M-14	dog	Netherlands	ERR1551797	[12]
754-7	IncK	CTX-M-14	dog	Netherlands	ERR1551799	[12]
NRS7	IncK	CTX-M-14	human	Netherlands	ERR1607724	this study
NRS9	IncK	CTX-M-14	human	Netherlands	ERR1607725	this study
0286	IncB/O	CTX-M-1	cattle	Netherlands	ERR1551805	this study
118-2	IncK	CMY-2	cattle	Netherlands	ERR1551795	this study
UMC606	IncK	CMY-2	human	Netherlands	ERR1551802	this study
UMC328	IncK	CMY-2	human	Netherlands	ERR1551801	this study
T1.09	IncK	CMY-2	poultry	Netherlands	ERR1607717	this study
T237	IncK	CMY-2	poultry	Netherlands	ERR1607704	this study
38-52-2	IncK, Col(MG828)	CMY-2	poultry	Netherlands	ERR1607714	this study
T498	IncK	CMY-2	poultry	Netherlands	ERR1607711	this study
T506	IncK	CMY-2	poultry	Netherlands	ERR1607712	this study

Table 1 continued

Plasmid name	Replicon	Beta-lactamase	Source	Origin	Accession number	Reference
39-62-1	IncK	CMY-2	poultry	Netherlands	ERR1607715	this study
T494	IncK	CMY-2	poultry	Netherlands	ERR1607710	this study
5-3	IncK	CMY-2	poultry	Netherlands	ERR1607716	this study
T10.2	IncK	CMY-2	poultry	Netherlands	ERR1607702	this study
T26A	IncK	CMY-2	poultry	Netherlands	ERR1607713	this study
T394	IncK	CMY-2	poultry	Netherlands	ERR1607705	this study
T.1.1	IncK	CMY-2	poultry	Netherlands	ERR1607701	this study
T491	IncK	CMY-2	poultry	Netherlands	ERR1607709	this study
T235	IncK	CMY-2	poultry	Netherlands	ERR1607703	this study
T425	IncK	CMY-2	poultry	Netherlands	ERR1607706	this study
T442	IncK	CMY-2	poultry	Netherlands	ERR1607707	this study
T476	IncK	CMY-2	poultry	Netherlands	ERR1607708	this study

Plasmid transformation and conjugation

IncK plasmids were isolated from the *E. coli* strains using Wizard Plus SV kit (Promega) according to the manufacturer's instructions and electroporated to *E. coli* DH10B ElectroMAX (Thermo Fisher Scientific). Transformation was performed according to the manufacturer's instructions. Selection of transformants was performed on Luria-Bertani agar plates containing 2 μg/ml cefotaxime. Additionally, plasmids were conjugated into *E. coli* W3110 and MG1655. Briefly, the overnight culture of *E. coli* DH10, carrying IncK plasmid, and *E. coli* W3110 or MG1655 were renewed in fresh LB medium (Tritium Microbiologie) and grown to OD600 = 0.5, then mixed together in 1:1 ratio and grown for 1.5 h at 37 °C. Exconjugants were recovered on LB agar supplemented with appropriate antibiotics. Five colonies were recovered from every plate and subjected to the PCR reactions targeting the IncK replicon and resistance gene encoded on the plasmid to confirm the conjugation.

Whole genome sequencing (WGS)

E. coli DH10 and W3110 isolates containing the plasmids were sequenced with Illumina Miseq sequencing using 2x250 bp reads and 300 bp insert size. Assembly was performed with SPAdes using default settings.[14] Chromosomal contigs were removed by mapping these against the DH10 and W3110 genome sequences using BLAST.[15] Remaining plasmid contigs were annotated with Prokka.[16] Antimicrobial resistance genes were determined using the Resfinder tool.[17] Core and pan genome determination and whole plasmid-based phylogeny were performed with Roary using the non-paralog splitting method.[18] Toxin-antitoxin systems were predicted using TA finder based on toxin-antitoxin database (TADB).[19] Virulence genes were predicted with VirulenceFinder 2.0.[20]

Plasmid contigs were submitted to the European nucleotide archive (http://www.ebi.ac.uk/ena) with accession numbers listed in Figure 1.

Phylogenetic tree analysis

A phylogenetic tree was constructed from both newly sequenced and sequences downloaded from ncbi using FastTree.[21] Additional IncK and IncB/O plasmid sequences were obtained from Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/resources/downloads/plasmids/), Genbank and the European nucleotide archive. The phylogenetic tree was visualized using ITOL.[22]

IncK-specific PCR

Due to accumulation of SNPs in the target region of previously designed primers for IncK plasmids, some of the typing results were inconclusive.[11] Therefore, a new set of primers was designed (Table 2). New (K/B fw-new and K rv-new) and previously reported primers were mixed in a 1:1 ratio to a final concentration of 2.5 pmol/µl each to improve detection of IncK plasmids. PCR was performed using GoTaq Green Master Mix (Promega) with initial denaturation of 2 minutes at 95 °C, followed by 25 cycles of 1 minute at 94 °C, 30 seconds at 63 °C, and 1 minute at 72 °C, and finally 5 minutes at 72 °C.

Table 2 Primers used in the study

Primer name	Primer sequence	Target	Reference
K/B fw	5' gcggtccggaaagccagaaaac 3'	RNAI	[11]
K rv	5' tctttcacgagcccgccaaa 3'		[11]
K/B fw new	5' aggatccgggaagtcagaaaac 3'	RNAI	this study
K rv new	5' tctttcacgatcccgccaaa 3'		this study
K1 fw	5' atcgtcaggatccgggaagtc 3'	RNAI and *repY* gene	this study
K1 rv	5' gagcgattgtgccgtgtatt 3'		this study
K2 fw	5' atgctcgcggtccggaaagcc 3'	RNAI and *repY* gene	this study
K2 rv	5' gtgccgtgcgttaatgcactgcaa 3'		this study
CMY-F	5' atgatgaaaaaatcgttgctgc 3'	bla_{CMY} gene	[23]
CMY-R	5' gcttttcaagaatgcgccagg 3'		[23]
TEM F	5' gcggaacccctatttg 3'	bla_{TEM} gene	[24]
TEM R	5' accaatgcttaatcagtgag 3'		[24]
CTX-M-9-1F	5' tggtgacaaagagagtgcaacg 3'	$bla_{CTX\text{-}M\text{-}group\text{-}9}$ genes	[25]
CTX-M-9-4R	5' tcacagcccttcggcgat 3'		[25]
CTX-M-14/17 fw	5' ctattttacccagccgcagc 3'	$bla_{CTX\text{-}M\text{-}14}$ gene	[26]
CTX-M-14/17 rv	5' gttatggagccacggttgat 3'		[26]
ssb fw	5' tggtgctgttcggcaagctc 3'	*ssb* gene	this study
ssb rv	5' ccttacgtccacggccttttc 3'		this study
ISEcp1A	5' gcaggtctttttctgctcc 3'	IS*Ecp1* transposase	[27]
ISEcp1B	5' tttccgcagcaccgtttgc 3'		[27]

Two pairs of primers, targeting the RNAI and part of the *repY* gene (Table 1), were designed to discriminate between the IncK1 and IncK2 plasmid lineages. PCR reactions were performed using GoTaq Green Master Mix (Promega) as described above with the annealing temperature of 55 °C.

Plasmid incompatibility

Conjugation between *E. coli* W3110 and MG1655, carrying either an IncK or IncB/O plasmid, was performed to determine incompatibility relationship between different IncK plasmids and between IncK and IncB/O plasmids. To determine the incompatibility of members of the IncK2 lineage, in which all plasmids carry the bla_{CMY-2} gene, an additional set of primers was used, targeting the *ssb* gene and IS*Ecp1* (Table 1), which allowed distinction between the two selected IncK2 plasmids. Compatibility of IncK plasmids was tested within a group (IncK1 or IncK2) as well as between the groups. As a positive control, compatibility of IncK plasmids was tested against IncB/O plasmids.

Plasmid stability

Stability of two IncK plasmids residing in one host was determined (adapted from Jafar *et al.* [28]). 50 colonies carrying both IncK1 and IncK2 plasmids were subsequently plated on LB plate or LB plate supplemented with appropriate antibiotics. Replating all colonies was repeated three times, every 24 hours. Presence of both plasmids was confirmed by PCR, amplifying the IncK replicons and the resistance genes. Stability was determined as a percentage of colonies carrying both plasmids in absence of the selection pressure compared to those plated on the selective plate.

Results

Phylogenetic trees analysis

Analysis of the phylogenetic tree based on presence/absence of accessory genes of IncK and IncB/O plasmids (Figure 1) revealed presence of two major clusters containing IncK plasmids. The first cluster, designated IncK1, contains plasmids carrying $bla_{CTX-M-14}$. IncK1 shows high similarity to the previously described pCT.[29] The second cluster, IncK2, includes IncK plasmids carrying bla_{CMY} genes. All IncK2 plasmids were associated with *E. coli* isolates from poultry sources. In contrast, the IncK1 plasmids were associated withisolates from various sources, being human, cattle, dog and redshank. IncB/O plasmids included in this analysis, instead of clustering together, form two separate clusters that position in between IncK1 and 2 plasmids.

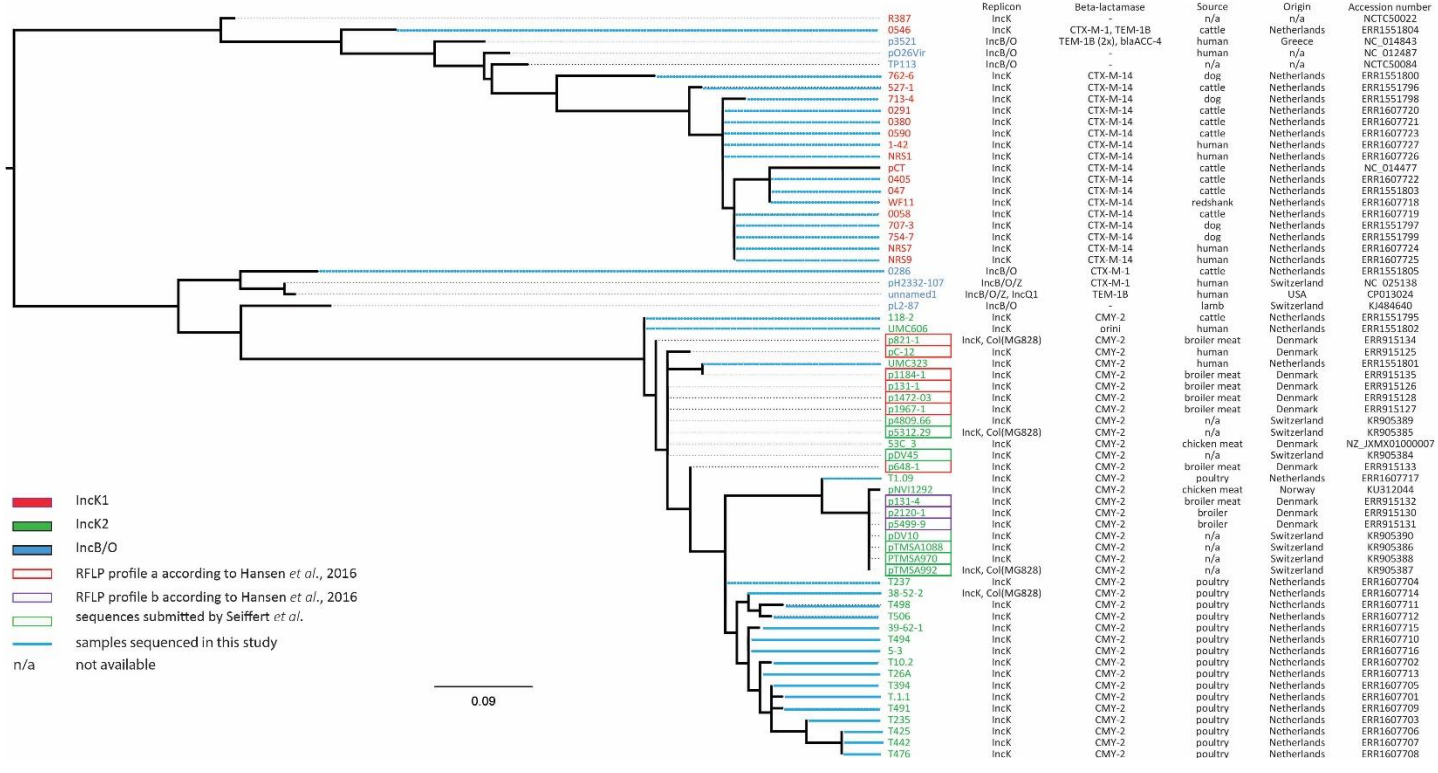

Figure 1 Phylogenetic tree of IncK and IncB/O plasmids, based on accessory genome

Plasmid characteristics

Plasmid sizes ranged from 79,176 to 168,100 bp, with an average GC content of 52.6% and 104 coding sequences. The core genome of all IncK plasmids include transfer and partition systems and the shufflon recombinase with the *pilV* gene. None of the sequenced plasmids contain known virulence genes. As a reference, one closed plasmid sequence for each group was chosen for a more detailed comparison: IncK1 – 713 and IncK2 – pTMSA1088. The replication initiation protein gene (*repA*) of IncK1 plasmid 713 shares 100% homology with the same gene of the previously described IncK1 plasmid – pCT, whereas IncK2 plasmid pTMASA1088 *repA* gene has no detectable homology with *repA* of the pCT plasmid. The *rep* gene that is the most similar to the one from pTMSA1088 is the *rep* gene from IncZ plasmid pCERC10 (98.73% identity). Overall, IncK1 and IncK2 plasmids show high levels of homology (Figure 2a). The pilus encoding region of pCT (10324 bp) shows 99.97% identity over 99% of the length of the region with IncK1 plasmid 713 and 82.85% identity over 74% of the length of the region with IncK2 plasmid pTMSA1088. The site-specific recombination system (shufflon) gene *rci* of the pCT plasmid shares 100% identity with IncK1 plasmid 713 and 93.40% identity over 99% of the gene length with IncK2 plasmid pTMSA1088. Another important plasmid feature is entry exclusion mediated by *excA* and *traY*. IncK1 plasmid 713 shares 100% similarity with *excA* and *traY* genes of pCT plasmid. IncK2 plasmid pTMSA1088 shares 97% similarity over 99% of *excA* and 96.21% similarity over 99% of the *traY* gene of the pCT plasmid. The resistance region of almost all IncK1 plasmids, excluding R387 and 0546, was located between the *traK* and *traL* genes, similar to the reference pCT plasmid. $bla_{\text{CTX-M-14}}$, carried by IncK1 plasmids, was associated with the IS*Ecp1* upstream and IS*903* downstream. In contrast, $bla_{\text{CMY-2,}}$ carried by IncK2 plasmids, lack IS*903*, except for one $bla_{\text{CMY-2}}$ and the $bla_{\text{CTX-M-1}}$ carrying plasmid, which both lacked IS*Ecp1* as well as IS*903*. IncK1 and IncK2 vary in presence or absence of several genes (Figure 2b) which may be caused by the presence of multiple insertion sequences.

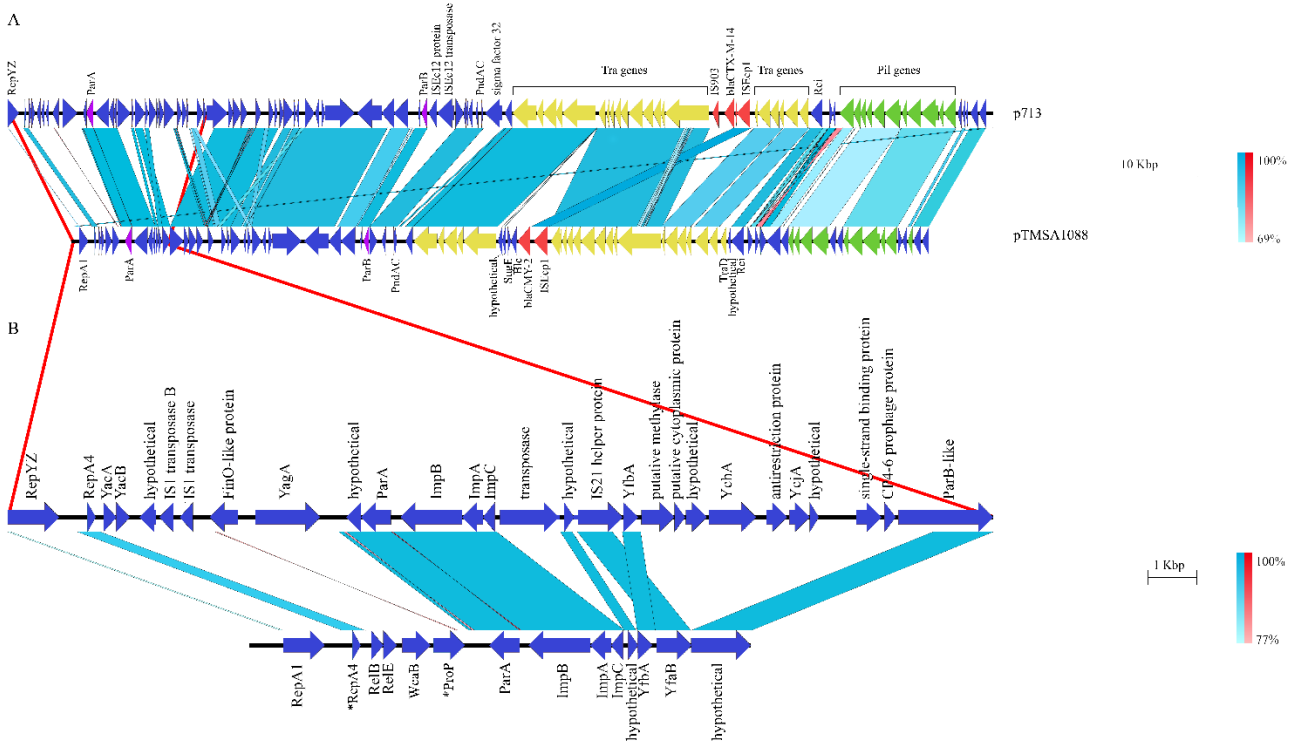

*Figure 2 Comparison of the IncK1 and IncK2 plasmid sequences (2a) with an additional focus on the highest variable region in the plasmid scaffolds (2b). * partial gene sequence.*

Plasmid incompatibility and stability

Due to the accumulation of SNPs in the target region of IncK1 plasmids, some typing results were difficult to reproduce using previously designed primers.[11] Therefore, a new set of primers was designed (Table 2). Additionally, two pairs of primers, targeting RNAI and part of the *repY* gene, were designed to discriminate between the IncK1 and IncK2 plasmid lineages. Selected plasmids were subjected to incompatibility tests, which were performed via conjugation of two *E. coli* strains carrying different IncK or IncB/O plasmids. Testing exconjugants revealed that IncK1 and IncK2 plasmids were compatible (Table 3). In contrast, plasmids belonging to the same group, either IncK1 or IncK2 were incompatible. Compatibility of IncK1 and IncK2 was checked with IncB/O plasmids to confirm that neither of the lineages were mistyped IncB/O plasmids. Additionally, stability of the plasmids from both lineages in one host was checked. Stability was determined as a percentage of colonies carrying both plasmids in absence of selective pressure compared to those plated on selective agar. After 72 hours, 98% of the IncK1 plasmids were still present using selective agar and 100% using nonselective agar. The IncK2 plasmid showed 100% stability on both selective and non-selective agar.

Table 3 Incompatibility test results

Mating pairs		
Plasmid 1 (plasmid ID)	Plasmid 2 (plasmid ID)	Incompatibility results
IncK1 (p754)	IncK2 (p118)	compatible
IncK1 (p0291)	IncK2 (p118)	compatible
IncK1 (pWF11)	IncK1 (p527)	incompatible
IncK2 (p39_62_1)	IncK2 (pT1.09)	incompatible
IncK1 (p754)	IncK1 (p0546)	incompatible
IncK2 (pT10.2)	IncK1 (p0546)	compatible
IncK1 (p754)	IncB/O (p0289)	compatible
IncK2 (p118)	IncB/O (p0289)	compatible
IncK1 (p0291)	IncB/O (p0289)	compatible

Discussion

IncK plasmids, together with IncI, IncB/O and IncZ plasmids belong to the I-complex plasmid group. Until now, IncK was considered as a single incompatibility group. In the present study we sequenced a collection of IncK plasmids isolated from different sources in the Netherlands.

To examine the genetic basis of IncK, we performed phylogenetic analysis of the accessory genomes of selected IncK plasmids. The analysis showed existence of two separate lineages named IncK1 and IncK2. The IncK1 plasmids examined here, predominantly encode $bla_{\text{CTX-M-14}}$ and were isolated from *E. coli* from human and various animal sources. IncK2 plasmids encode mainly $bla_{\text{CMY-2}}$ and were isolated predominantly from poultry. This suggests that IncK2 plasmids are specific to *E. coli* from poultry as a host. It is possible that IncK2 plasmids encode genes, that are not present on IncK1 plasmids, that provide an advantage in poultry. Additionally, Hansen *et al* (7) presented

two IncK plasmid RFLP types (SalI-digested), which form two distinct clusters, both within the IncK2 lineage (Figure 1). More in depth research is needed to examine the basis of the apparent host specificity of IncK2 plasmids.

To examine the relationship between these plasmid groups we performed incompatibility testing, using conjugation. We were able to obtain heteroplasmid strains, that carried both IncK1 and IncK2 plasmids. The obtained results demonstrate that IncK1 and IncK2 plasmids from a single lineage are incompatible, but between lineages these plasmids are compatible.
Stability tests showed that combinations of IncK1 and IncK2 plasmids can be stably maintained in one cell for at least 72 h without selection. Therefore, we suggest to divide the IncK plasmid group into two separate subgroups, designated IncK1 and IncK2 in the present study. Obtained results confirm previous observations of Seiffert *et al*, who submitted sequences of seven IncK2 plasmids to Genbank (Figure 1).

Phylogenetic analysis based on plasmids' accessory genomes showed two distinct clusters of IncB/O plasmids. These results open up a possibility of existence of two separate IncB/O subgroups. Additional experiments to confirm the compatibility of these plasmids need to be performed to confirm this hypothesis.

Plasmid-based replicon typing (PBRT) is a very useful tool, allowing easy identification of plasmids' replicon group.[11] However, high similarity of IncK and IncB/O RNAI sequences, which are targets in the PBRT classification scheme, causes difficulties with identification, which was shown in this study as well as in a previous study.[30] Inconsistencies in the PBRT outcomes for IncK and IncB/O plasmids mean that prevalence of these plasmid groups can be underestimated, because some of the published results may be based on misclassifications. These inaccuracies cause difficulties in understanding the epidemiology of IncK and IncB/O plasmid groups – their sources, resistance genes carried and possible spreading routes. All the above highlights the need to improve the identification method for these plasmids. Additional research is needed to fully understand the genetic relationship and compatibility between members of the I-complex plasmid group, which will provide knowledge to better understand these plasmids' epidemiology.

A shortcoming of this study is the fact that the phylogenetic analysis could be influenced by the geographical bias of the origin of the plasmids included in this study. These findings should therefore be confirmed using an extended collection of plasmids from a more diverse geographical background.

This study showed the existence of two compatible IncK plasmid lineages, designated IncK1 and IncK2. A similar phylogenetic distinction was shown for IncB/O plasmids, but experimental confirmation is needed to determine if the IncB/O plasmid group also consists of two lineages. Moreover, this study highlights the need to improve the identification method for the IncK and IncB/O plasmid groups.

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Chapter 4

Incompatibility and phylogenetic relationship of I-complex plasmids.

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Abstract
Plasmid incompatibility is the inability of two plasmids to be stably maintained in one cell, resulting in loss of one of the plasmids in daughter cells. Dislodgement is a phenotypically distinct form of incompatibility, described as an imperfect reproduction, manifesting in rapid exclusion of a resident plasmid after superinfection. The relationship between plasmids of the phenotypic incompatibility groups IncB/O and IncZ is unclear. Their inability to co-exist was initially referred to as dislodgement while other research reached the conclusion that IncB/O and IncZ plasmids are incompatible. In this manuscript we re-evaluated the relationship between IncB/O and IncZ plasmids to settle these conflicting conclusions. We performed dislodgement testing of R16Δ (IncB/O) and pSFE-059 (IncZ) plasmids by electroporation in a bacterial cell and checked their stability. Stability tests of the obtained plasmid pair showed that the IncB/O plasmid was exclusively and almost completely lost from the heteroplasmid *Escherichia coli* population. Other IncB/O – IncZ pairs could not form a heteroplasmid population, using conjugation or electroporation. Our data supports the previous suggestion that IncB/O and IncZ plasmids may be considered phenotypically incompatible.

Key words
Plasmid, dislodgement, incompatibility IncB/O, IncZ

Introduction

The I-complex plasmid group, designated due to the morphological and serological properties of their pili,[1, 2] consists of phenotypically incompatible plasmids including IncI1α and IncI1γ, IncI2, IncK, IncB/O and IncZ.

The first widely adopted plasmid typing scheme was based on phenotypically screened incompatibility, which is defined as an inability of plasmids to be stably maintained in a single cell.[3, 4] Incompatibility of I-complex plasmids is caused by a small stem-loop structure called RNAI.[5] Interaction of RNAI with *repB* (or *repY*, which is an equivalent of *repB* in I-type plasmids) is required for translation of replication initiation protein RepA.[6] Presence of two almost identical RNAI structures coming from two different plasmids leads to copy number destabilization and plasmid loss. Replication-based incompatibility results in a roughly even spread of loss of the plasmids upon superinfection, regardless of which plasmid was resident and which plasmid was coming into the recipient cell. The phenomenon can easily be confused with partition-based incompatibility and its phenotypically distinct form – dislodgement.[7] Partition-based incompatibility describes the phenomenon where superinfection leads to the stable maintenance of a specific plasmid type over another, regardless of whether this plasmid was the resident or the invading plasmid.[8] Plasmid dislodgement refers to the phenomenon where, upon superinfection, the resident plasmid is always lost and (nearly) all recipient cells will only contain the invading plasmid. Plasmid dislodgement was described for several plasmid groups including IncI, IncH and IncT.[9-11] For some plasmid groups dislodgement was later disproven. It was shown that the IncT plasmid R394, which showed dislodgement with IncN plasmids, was in fact a co-integrate of these two replicons.[12] Another plasmid feature that can be confused with plasmid incompatibility is entry exclusion. It is a process where transfer of plasmid between bacterial cells is inhibited due to identical or closely related exclusion proteins, often encoded on related plasmids.[13]

Tschäpe and Tietze first described the IncZ plasmid group and reported an unusual relationship of IncB (R16) and IncZ (pIE545) plasmids.[14] In a heteroplasmid bacterial population carrying both IncB/O and IncZ plasmids, the resident one was rapidly and exclusively lost. This observation was designated plasmid dislodgement. To test the incompatibility relationships, they used wild-type plasmids. Later, Praszkier *at al.* cloned minireplicon versions of IncB (pMU707) and IncZ (pIE545) into pBR322 and introduced these into IncB or IncZ minireplicon containing cells.[15] They obtained the same results of loss of the resident plasmid, but this was interpreted as incompatibility of IncB and IncZ plasmids, followed by the suggestion that these groups are effectively the same group of plasmids.

After the introduction of PCR-Based Replicon Typing (PBRT) and the introduction of a reliable and commercially available kit, this molecular method has become a universal standard to identify plasmids in *Enterobacteriaceae*.[16] The grouping in this scheme has also become the basis for *in silico* replicon typing in whole-genome sequencing (WGS) data. Nonetheless, discrimination between replicon types based on phenotypical data is still useful for more in-depth studies of plasmids.[17, 18] In this study we describe the DNA sequence of a plasmid R16Δ, derivative of the archetype IncB/O plasmid R16,[19] using both short and long read sequencing techniques. We compared the RNAI sequences of R16Δ to other plasmids that represent either IncB/O or IncZ. We tested incompatibility

of IncB/O and IncZ plasmids using conjugation and electroporation. Additionally, we have performed stability testing between IncB/O and IncZ plasmids to determine their ability to stably persist in bacterial cells together.

Materials and Methods

Plasmid and bacterial stains used

The strain carrying R16Δ was obtained from the Belgian Nuclear Research Centre.[19] All other plasmids described in this study were recovered from human and animal samples that were collected and analysed in the Dutch national AMR monitoring program as reported in MARAN-report (www.wur.nl/en/Research-Results/Projects-and-programmes/MARAN-Antibiotic-usage.htm) and the EU-SAFEFOODERA project: The Role of Commensal Microflora in the transmission of ESBLs (ref. 08176) (Table 1). *E. coli* OT-ESBL-0589 was isolated from cattle in 2011, *E. coli* SFE-059 was isolated from a human urine sample in 2009 and *E. coli* SFE-199 was isolated from poultry meat in 2009. As a recipient in conjugation experiments, we used *E. coli* MG1655-YFP.[20]

Table 1 Plasmids or plasmid sequences used in this study

Plasmid name	Incompatibility group	Reference	Accession number
R16Δ	IncB/O	this manuscript	PRJEB30795
R16	IncB/O	[19]	MK758104
SFE-059	IncZ	this manuscript	MN335639
SFE-199	IncZ	this manuscript	MN335638
OT-ESBL-0589	IncZ	this manuscript	MN335640
pIE545	IncZ	[15]	M93064.1 (only replication region)
pMU707	IncB/O	[15]	M93062.1 (only replication region)
p3521	IncB/O	[21]	GU256641.1

Sequencing

Illumina sequencing was performed on a Miseq platform using 2x250 bp reads and a 300 bp insert size. Long-read sequencing libraries were prepared according to the manufacturer's protocols using sequencing kit SQK-LSK108 and native barcoding kit EXP-NBD103 (Oxford Nanopore Technologies). Sequencing was performed on the MinION MK1B with flowcell type R9.4 (Oxford Nanopore Technologies). The hybrid assembly was performed using Unicycler v0.4.6 with default settings.[22] Annotation was performed using prokka.[23] The resistance region of plasmid R16Δ was analysed using Galileo AMR (ARC BIO).[24] Toxin-antitoxin systems were predicted using TA finder based on TADB.[25] Virulence genes were predicted using VirulenceFinder 2.0.[26] RNAI structure prediction was performed using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) with default settings. Levenshtein distance of IncB/O and IncZ RNAI sequences was calculated using the online tool "Levenshtein Distance" (https://planetcalc.com/1721/).

Phylogenetic analysis

Phylogenetic trees were constructed from both newly sequenced plasmids and downloaded sequences. Alignment was performed with MAFFT.[27] For the RNAI-based phylogenetic tree we used Q-INS-I strategy and the gap opening penalty was set to 1.00. For the *repA*-based phylogenetic tree, default settings were used. Phylogenetic trees were built using IQ-TREE with the default settings.[28] Phylogenetic trees were visualised using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Dislodgement testing

Electroporation

Plasmid R16Δ was isolated using the Wizard Plus SV Miniprep kit (Promega) and transferred to *E. coli* DH10B Electro MAX cells (Thermo Fisher Scientific) by electroporation. *E. coli* DH10B Electro MAX were transformed using: 2.0 kV, 200 **Ω**, 25 μF conditions with plasmid R16Δ miniprep DNA and transformants were selected on LB plates supplemented with 3 μg/ml tetracycline (Sigma-Aldrich). Electrocompetent *E. coli* cells carrying IncB/O or IncZ plasmids were prepared from a liquid culture (OD_{600} = 0.5) in LB with appropriate antibiotics. Cultures were spun down for 10 min with 6000 rpm at 4 °C. Pellets were washed twice with ice-cold water, resuspended in ice-cold 10% glycerol and frozen at -80 ºC. *E. coli* DH10B with R16Δ (DH10B-R16Δ) was transformed with plasmid pSFE-059 (IncZ) and transformants were selected on LB plates supplemented with 3 μg/ml tetracycline and 2 μg/ml cefotaxime (Sigma-Aldrich). The obtained strain was designated DH10B-R16Δ/pSFE-059. The presence of both replicons in the cell after electroporation was confirmed with replicon-specific PCRs using primers listed in Table 2. Additionally, we performed the same experiment with the reverse order of plasmids, namely plasmid pSFE-059 was transferred first to *E. coli* DH10B and later plasmid R16Δ was added as a superinfecting plasmid. The obtained strain was designated DH10B-pSFE-059/R16Δ. These strains were used for stability experiments.

Table 2 PCR primers used in the study

Primer name	Primer sequence	Incompatibility
R16 fv	5’ cacaatcgctccgccataag 3’	B/O
R16 rv	5’ gcaggcagtttcttcagacg 3’	B/O
IncZ fv	5’ accgtcaggtaaagaacccg 3’	Z
IncZ rv	5’ ccgcgacattatcatgcgtt 3’	Z
3521 fv	5’ cgcttccggagatactcagc 3’	Z
3521 rv	5’ ccagagattcaaccctgtgc 3’	Z

Liquid conjugation

The overnight culture of *E. coli* DH10B, carrying an IncB/O or IncZ plasmid, and *E. coli* MG1655-YFP were renewed in fresh LB broth (Tritium Microbiologie) and grown to OD_{600} = 0.5, then mixed in 1:1 ratio and grown for 16h at 37 °C. Transconjugants were recovered on LB plates supplemented with appropriate antibiotics. Five colonies were recovered from each plate and subjected to PCR reactions using primers targeting the IncB/O or IncZ replicons (Table 2). Positive controls were included in each experiment, in which either pSFE-059 or pR3521 were conjugated into an empty *E. coli* MG1655-YFP. All experiments were performed in triplicate. As an additional control, compatibility of IncB/O and IncZ plasmid pairs was examined using both electroporation and

conjugation as described. Plasmids pSFE059 and pR3521 were used as resident plasmids and plasmids pSFE199, OT-ESBL-0589, pSFE059 and pR3521 were used as an incoming plasmid (Table 3).

Table 3 Pairs of plasmids used for the dislodgement and incompatibility testing.

donor / recipient	pSFE-059	pR3521
pSFE-059	np	x
pOT-ESBL-0589	np	x
pSFE-199	x	x
pR3521	x	np

np = experiment was not performed, x = experiment was performed but no transconjugants or transformants were obtained.

Stability

Stability of IncB/O and IncZ plasmids was examined in LB broth without antibiotic selection. *E. coli* DH10B-R16Δ/pSFE-059 and DH10B-pSFE-059/R16Δ were each grown overnight in LB broth without antibiotics and plated on non-selective LB plates. After overnight incubation, 100 colonies were replicated onto LB plates supplemented with 3 µg/ml tetracycline, 2 µg/ml cefotaxime or combinations of both antibiotics. 10 µl of the overnight culture was used to inoculate 10 ml of LB broth and grown overnight again. Culturing was repeated once more resulting in 72h of growth. Plating was repeated after each sampling point. Stability tests were performed in triplicate. Ten colonies from each sampling time point that grew on double selection plates were tested with PCR for presence of IncB/O and IncZ plasmids.

Results

Sequence analysis

All plasmids in this study were sequenced using both Illumina and Nanopore sequencing platforms followed by hybrid assembly of the data. Overall, IncB/O and IncZ plasmids share a high degree of homology (Figure 1). Besides plasmid R16Δ, which lost some conjugal transfer genes, the conjugation region of IncB/O and IncZ plasmids are highly similar. The region encoding genes responsible for the pili formation seems to be more diverse. IncZ plasmids share only about 80% homology compared to the 11,727 bp pili region of p3521, 87% homology to the same regions of pSFE-059 and pOT-ESBL-0589, and 75% for pSFE-199. The entry exclusion system inhibits the transfer of closely related plasmids and in I-complex plasmids it is mediated by two proteins ExcA and TraY.[29] The *excA* gene of plasmid p3521 shares 93% identity with plasmids pSFE-199 and pSFE-059 and 96% with plasmid OT-ESBL-0589. The *traY* gene of plasmid p3521 shares 89% identity with plasmids pSFE-199 and pSFE-059 and 98% with plasmid OT-ESBL-0589. The gene encoding the RepA protein does not share homology between IncB/O and IncZ plasmids, which is in line with previous reports.[15]

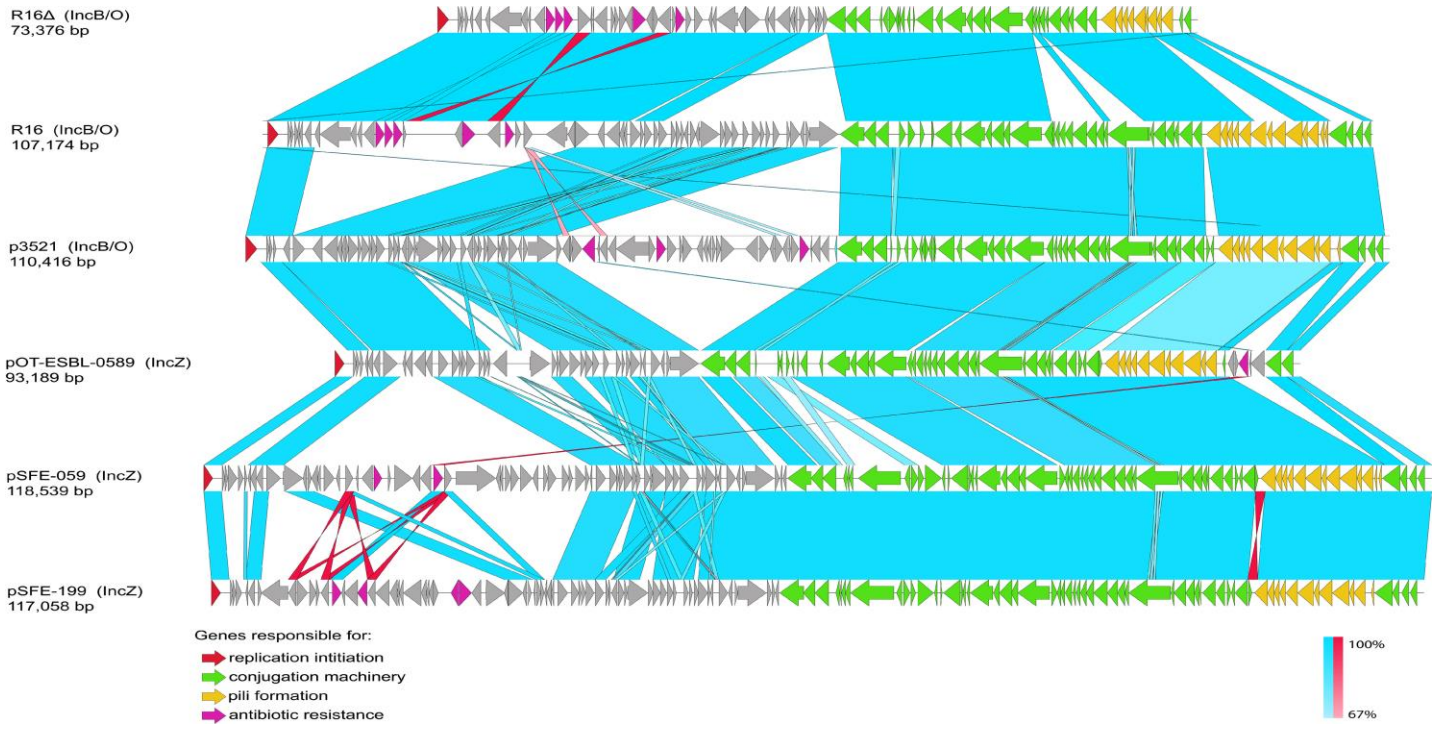

Figure 1 Comparison of the four plasmids sequenced in this study with previously sequenced IncB/O plasmid p3521. These are linearized representations of the plasmid sequences. Open reading frames are indicated by coloured arrows to match the gene's functions as indicated at the bottom of the figure. Regions of homology are connected by blue boxes, inversions are indicated by red boxes.

R16Δ

R16Δ is a 73,376 bp IncB/O non-conjugative derivative of plasmid R16. R16Δ shares 94% identity with the replication region of pMU707 (2167 bp), the reference IncB/O plasmid. It contains RelE/ParE and PndAC family plasmid stabilization systems. The resistance region of plasmid R16Δ is identical to the previously published plasmid R16.[30]

Although plasmid R16 was previously described as capable of conjugative transfer,[14] all three conjugation attempts of R16Δ in the present study failed. We compared the conjugation and pili encoding regions of R16Δ with R16. This analysis showed that the transfer region of plasmid R16Δ was incomplete (Figure 2). Some essential genes for conjugation were absent, among which *traC, pilO, pilQ, sogL, traM, traO, traQ*.[31-33]

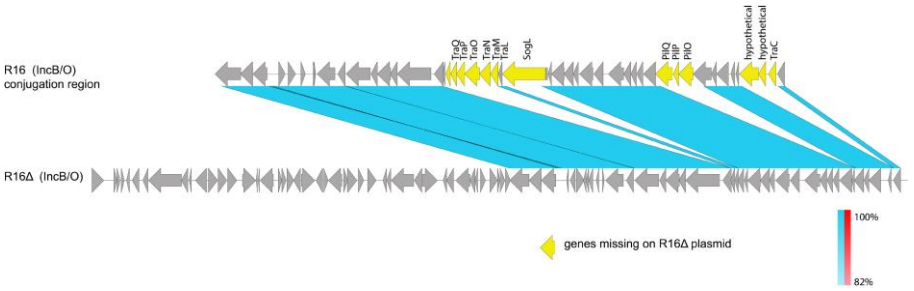

Figure 2 Comparison of the conjugation region of R16 plasmid (top) with whole sequence of plasmid R16Δ (bottom). Yellow arrows indicate genes missing on plasmid R16Δ.

IncZ plasmids pSFE-059, pSFE-199, pOT-ESBL-0589

The three IncZ plasmids that were sequenced contained regions of high similarity but the region that is known to contain accessory functions differed significantly. As a result of these differences, the sizes varied where pOT-ESBL-0589 was 93.189 bp, pSFE-059 was 118.539 bp and pSFE-199 was 117.058 bp. A comparison of the replication regions of these plasmids to the reference pIE545 show that these have 98%, 100% and 98,37% sequence identity respectively. All three of the plasmids contained the RelBE or ParDE toxin antitoxin systems which act in plasmid stabilization.

In plasmid pOT-ESBL-0589, the ESBL gene *bla*$_{\text{CTX-M-1}}$ is associated with IS*Ecp1* which is likely responsible for it's insertion, identical to the region described in the IncI1 plasmid pCTX1261 (HF549090).[34] Of note is that the region has not inserted in the region that is usually associated with accessory functions but within the conjugation region.

In pSFE-199 and pSFE-059 incorporation of various integrative elements into the accessory regions of the plasmids have led to mosaic regions in which multiple antimicrobial resistance genes are present (Figure 3). In pSFE-199 the tetracycline resistance gene *tetA* and its regulator *tetR* were incorporated by insertion of Tn*1721*. A partial copy of Tn*21* is present, which is interrupted by insertions of IS*26* and a partial copy of IS*Ecp1*. This region is similar in sequence and structure to an integration previously described in IncN and IncI plasmids[35] encodes for the ESBL gene *bla*$_{\text{CTX-M-1}}$ and the macrolide resistance gene *mphA*. In the accessory region of pSFE-059, an aminoglycoside resistance gene *aadA22* is part of a class 1 integron as previously

described in *Salmonella* Chailey.[36] The ESBL gene $bla_{CTX\text{-}M\text{-}32}$ is flanked by a copy of IS*Ecp1* which was interrupted by an insertion of IS*Kpn26* and *orf477*, as previously described in *E. coli* isolated in Portugal.[37] This complete cassette is flanked by two copies of IS*26.*

Plasmids pSFE-059 and pSFE-199 carry a type V secretion system protein involved in secretion to the cell surface.[38]

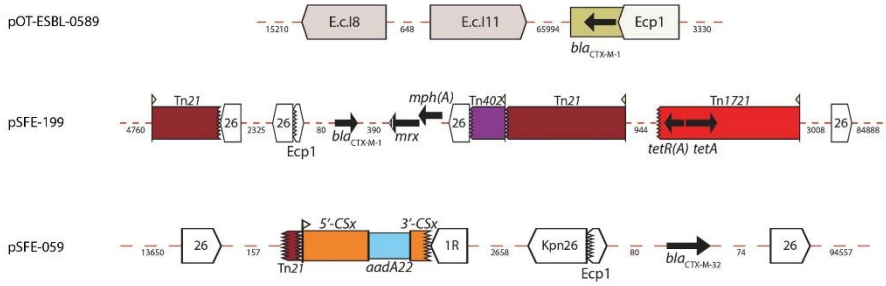

Figure 3 Resistance region of plasmids pOT-ESBL-0589, pSFE-199 and pSFE-059. Copies of previously described mobile elements are shown as blocks, partial mobile elements show jagged lines at the edges. Complete resistance genes are depicted as arrows in the orientation of transcription. Inverted and direct repeats are shown at the edge of a mobile element by stemloop structures.

Comparison of the incompatibility and replication regions

An alignment of the DNA sequence of the RNAI structure of available sequences of IncB/O and IncZ plasmids was performed (Figure 4). The nucleotide sequence and the resulting stem loop structure of the RNAI molecule after transcription differ between plasmids from different groups while they are strongly conserved within plasmid groups, which is in line with previous findings.[15, 18, 30, 39] There is 83% identity between the IncB/O and IncZ RNAI DNA sequence, and they have a Levenshtein distance of 9 mutations/indels.[40]

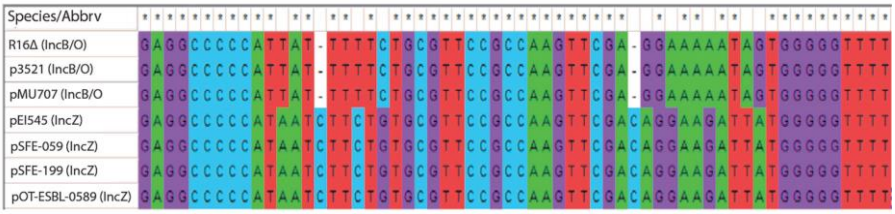

Figure 4 Comparison of RNAI sequences of IncB/O and IncZ plasmids. Asterisks on top of the sequence indicate conserved nucleotides.

Two phylogenetic trees, based on the *repA* gene and RNAI regions, were constructed independently in order to examine the relatedness of plasmids from the I-complex based on these regions. The *repA*-based phylogenetic tree shows two main clusters. One is formed by IncK1, IncB/O and IncI plasmids and the other from IncK2 and IncZ plasmids

(Figure 5). Representative IncI2 plasmid (R721) is phylogenetically further apart from IncB/O and other IncI plasmids. The RNAI-based phylogenetic tree shows a different relationship between these plasmid groups (Figure 6). IncK1 and IncK2 plasmids are phylogenetically distant from IncB/O, IncZ and IncI plasmids. We were unable to identify homologs of RNAI on the archetype IncI2 plasmid R721.

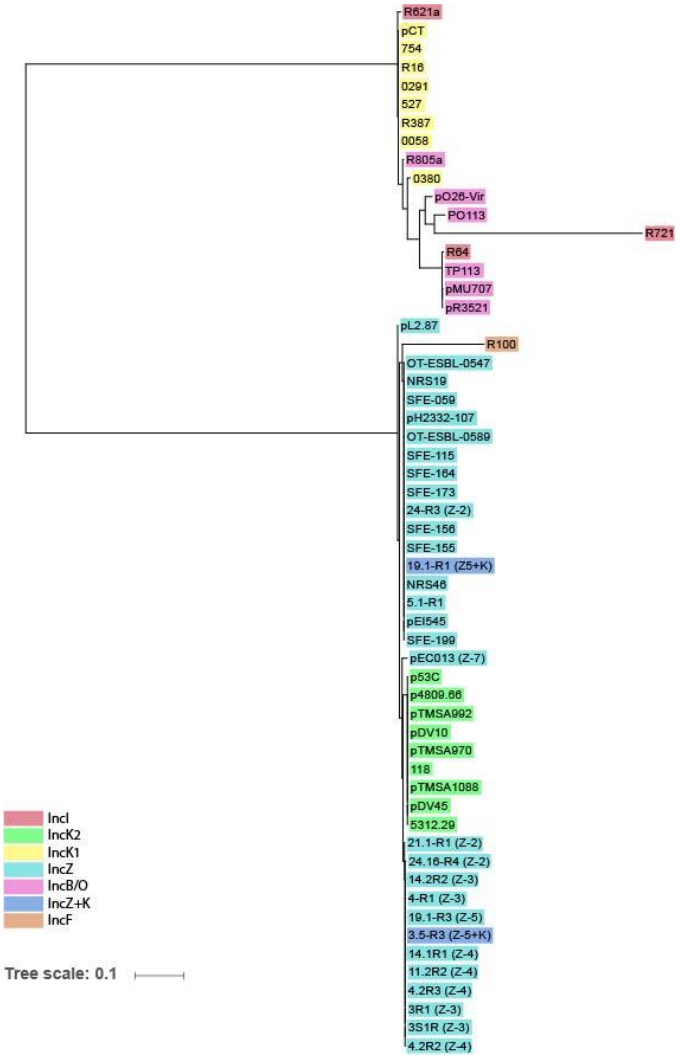

Figure 5 RepA-based phylogenetic tree of I-complex plasmids. For visualization purposes, branches intersected by // are displayed as 10% of the actual length. Plasmid names are coloured based on the incompatibility group in which they were organised in the original studies, based phenotypic incompatibility typing, PBRT or in silico PBRT. See supplementary table (S1) for accession numbers.

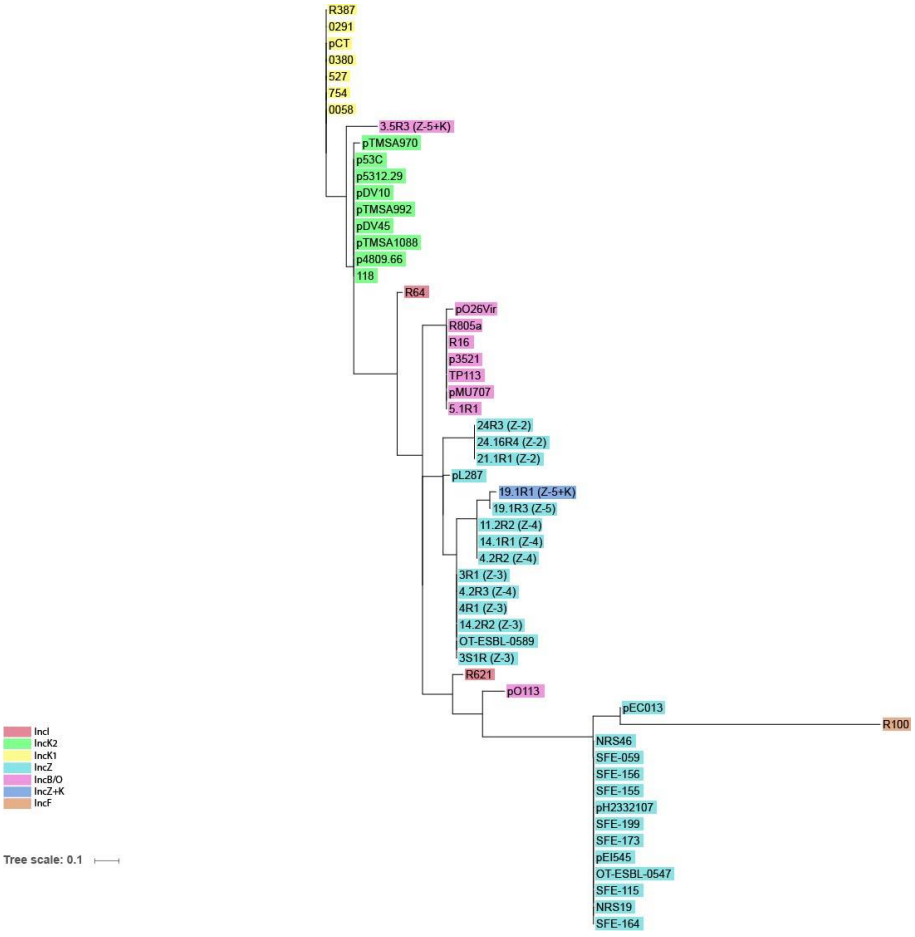

Figure 6 RNAI-based phylogenetic tree of I-complex plasmids. Plasmid names are coloured based on the incompatibility group in which they were organised in the original studies, based phenotypic incompatibility typing, PBRT or in silico PBRT. See supplementary table (S1) for accession numbers.

Testing for incompatibility and dislodgement

To determine the relationship of the IncZ plasmids with IncB/O plasmids, we performed a series of electroporation and conjugation experiments with *E. coli* strains carrying one IncB/O or IncZ plasmid (Table 3). R16Δ and pSFE-059 were chosen to represent IncB/O and IncZ in an experiment where both plasmids were introduced into a single cell by two independent rounds of electroporation, resulting in cells containing both plasmids when cultured on double selective agar plates. When both plasmids were in the same bacterial cell, we observed a rapid loss of the plasmid R16Δ when cultured in non-selective media, while plasmid pSFE-059 remained 100% stable in *E. coli* cells, as determined by subculturing individual colonies on single selective or double selective agar plates (Figure 7). This occurred regardless of the order in which the plasmids entered the bacterial cell.

Part of the population, which was growing on double selection plates, contained both IncB/O and IncZ plasmids, which means that for at least three consecutive rounds of O/N culture, plasmid R16Δ was not lost completely. Presence of both plasmids in the cell was confirmed by PCR in ten colonies. *E. coli* DH10B containing either plasmid R16Δ or pSFE-059 were tested separately in order to confirm their stability and both were stable for three consecutive rounds of O/N culture without selection (data not shown).

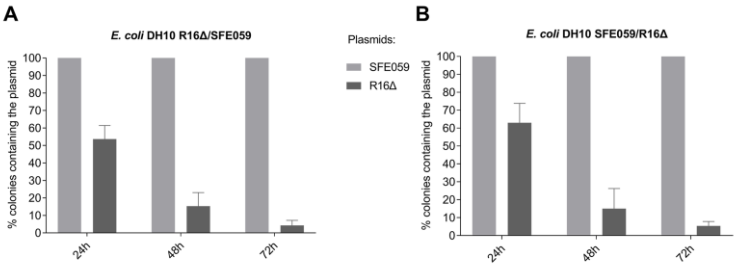

Figure 7 Stability of single IncZ (pSFE-059) and IncB/O (R16Δ) and combination of both plasmids in E. coli DH10B R16Δ/SFE059 and E. coli SFE059/R16Δ grown without antibiotic pressure. Stability experiments were performed in triplicate. Bars represent standard deviation.

Two additional IncZ and one IncB/O plasmids were chosen to test the compatibility of these plasmid groups via conjugation (Table 3). All plasmids were used in donor and recipient cells in triplicate. None of the tested plasmid pairs resulted in a heteroplasmid population, which was confirmed by PCRs targeting both plasmids. Compatibility of the same plasmid combinations was additionally tested via electroporation. All tested plasmids were used both as a resident or superinfecting plasmids. Heteroplasmid populations were also not obtained via electroporation, which was confirmed by PCRs targeting both plasmids.

To demonstrate the ability of tested plasmids (Table 3) to conjugate to the recipient cells, control conjugation experiments were performed for all tested plasmids (data not shown). All tested plasmids can be successfully conjugated to *E. coli* MG1655-YFP. The possibility to electroporate tested plasmids to the recipient cells was examined by control electroporation for all tested plasmids (data not shown). All tested plasmids were successfully electroporated into *E. coli* DH10.

Discussion

In this study we sequenced the archetype IncB/O plasmid R16Δ and three IncZ plasmids – pSFE-059, pSFE-199, pOT-ESBL-0589 using Illumina and Nanopore sequencing techniques. Having the full plasmid sequence, rather than a collection of contigs, is essential for detailed plasmid sequence analysis.[41]

Plasmid R16 was originally reported to be conjugative, but our experiments, supported by the DNA sequence analysis, showed that the plasmid we were working with lost the ability to conjugate, probably due to several deletions in the tra- and pil-regions. These genes are responsible for pilin maturation, DNA processing and entry exclusion.[42] These deletions

may have occurred after long-term storage, but we could not locate any other source for this specific plasmid. As such, this R16 derived plasmid is referred to here as R16Δ. Recently, a sequence of wild type R16 plasmid, obtained from a stock culture from the 1950s, which was still conjugative, was published.[30] This plasmid is substantially bigger in size than R16Δ sequenced in this study. Besides parts of conjugation and pilus formation genes, R16Δ lacks a region (20 942 bp) containing a nickase, plasmid SOS inhibition proteins A and B and hypothetical proteins.

In this study we used three IncZ plasmids together with the R16Δ plasmid, which was originally used to describe the phenomenon of dislodgement.[14] Dislodgement, which can be mistaken with incompatibility, was defined as a rapid exclusion of a resident plasmid after superinfection.[7] Two previous studies examined dislodgement between IncB/O and IncZ plasmids: Tschäpe and Tietze used only wild-type plasmids, while Praszkier *et al.* used multicopy pBR322-derivatives of IncB/O and IncZ plasmids with minireplicon versions.[14, 15] Similar results were obtained, but these were interpreted differently, resulting in different conclusions. The first group reported dislodgement of the plasmids, while the latter designated IncB/O and IncZ plasmids as incompatible.

In this study we created a bacterial strain carrying IncB/O plasmid R16Δ and IncZ plasmid pSFE-059. Both plasmids were completely stable in a single plasmid population after three culturing rounds. However, plasmid R16Δ was highly unstable and almost completely lost from a heteroplasmid population created using electroporation, whereas plasmid pSFE-059 was 100% stable after three rounds of consecutive batch cultures, in the absence of selective pressure. One of the reasons only plasmid R16Δ was lost can be its inability to conjugate. Plasmid pSFE-059, even if lost from the cell, can be re-introduced to plasmid-free cells via conjugation. Therefore, we did not only observe a rapid loss of the resident plasmid from the heteroplasmid population, which would indicate dislodgement, as it was suggested in previous studies, but specifically of the IncB/O plasmid that was tested. Compatibility of five other IncB/O and IncZ plasmid pairs was tested (Table 3). We were unable to create a bacterial strain carrying both plasmids from any of these pairs using conjugation or electroporation. Plasmid pSFE-059, which was present in one cell together with R16Δ, was not detected in one cell with any other IncB/O plasmid. These results suggests that the plasmid pair of pSFE-059 - R16Δ was exceptional and does not represent the relationship between IncB/O and IncZ plasmids. Therefore, our results do not support the hypothesis that the relationship between IncB/O and IncZ plasmids should be considered as dislodgement. Overall, the presented results demonstrate that IncB/O and IncZ plasmids are incompatible.

In this study we created phylogenetic trees of I-complex plasmids: based on the *repA* gene and the RNAI region. As shown in Figure 4, the stem loop structure of the RNAI molecule differs between plasmids from different groups, while they are strongly conserved within plasmid groups. This is in line with previous findings.[15, 18, 39, 43] According to our knowledge this is a first attempt to summarize and confirm the work that was previously published on I-complex plasmids on a larger sample set. The *repA*-based phylogenetic tree shows a clear clustering of plasmids: the first clusters contains IncK1, IncB/O and IncI plasmids and the second cluster contains IncK2 and IncZ plasmids. Recently Zhang *et al.* also published a *repA*-based phylogenetic tree of I-complex plasmids.[39] Even though both trees were constructed on plasmid sets with a minimal overlap, the clustering of plasmids is the same as described in this study. The RNAI-based tree shows completely different

clustering as compared to the *repA*-based tree. IncK1 and IncK2 plasmids are phylogenetically distant from IncB/O, IncZ and IncI plasmids. Phylogenetic group discrepancies between RNAI-based and other conserved plasmid genes-based analysis were shown for plasmid R471.[44] According to the core genome-, ExcA-, TraX- and TraY-based phylogenetic trees this plasmid belongs to the IncL plasmids group but RNAI-based analysis position it within IncM1 cluster. Seven types of amplicons of IncZ plasmids were published previously.[18, 45] Six out of seven types were included in the phylogenetic analysis presented in this study. Although here we used only the RNAI sequence instead of the whole amplicon, similar clustering was seen as previously described except for plasmid 4.2-R3, which was typed as Z-4, but clustered here together with Z-3 plasmids. In the light of previous findings, showing that IncZ plasmids may have formed by a recombination of IncFII and IncI1α,[46] we decided to include IncFII plasmid R100 in our analysis. Plasmid R100 clusters closely with IncZ plasmids, which shows that the findings of this study are in line with previous reports.[45] Presented results confirm on a larger sample set that the evolution of the plasmid replication initiation protein (RepA) and incompatibility determinant (RNAI), which are both part of the replication system, can be independent of each other.

This manuscript combines classical incompatibility testing with modern sequence and phylogenetic analysis. It highlights that sequence-based plasmid types do not necessarily correspond with plasmid incompatibility groups designated using phenotypic methods. Both methods provide valuable information, which can be used for different purposes. Phenotypic incompatibility provides an insight about the possibilities of plasmid transmission while sequence-based plasmid types allow to associate plasmids with certain hosts or regions. We believe that both typing methods are valuable and they both should be used in typing newly discovered or redefined plasmid groups. A good illustration are recent studies in which compatibility of the IncL/M and IncA/C plasmid groups were revised, based on phenotypic incompatibility experiments.[44, 47] This resulted in splitting the groups into IncL, IncM and IncA with IncC respectively.

In conclusion, the presented results suggest that the IncB/O and IncZ plasmids are incompatible and, based on phenotypic tests, could therefore be considered one plasmid group. This is in contrast to the different lineages that have been observed using sequence-based techniques. Additional research, including more plasmids of the different lineages, should be performed to confirm our findings.

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Chapter 5

Understanding the genetic basis of the incompatibility of IncK1 and IncK2 plasmids.

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Abstract

Incompatibility is the inability of two plasmids to be stably maintained in one cell. It is caused by the presence of identical or closely related shared determinants between two plasmids originating from partition or replication mechanisms. For I-complex plasmids, replication- based incompatibility is caused by the small antisense RNA stem-loop structure called RNAI. The RNAI structure of two compatible IncK plasmid groups, IncK1 and IncK2, differs only by five nucleotides. In this study we constructed minireplicons containing the replication region of IncK1 or IncK2 plasmids coupled with a kanamycin resistance marker. Using minireplicons excludes involvement of the incompatibility structures other than RNAI. Additionally, we performed single nucleotide mutagenesis targeting the five nucleotides that differ between the IncK1 and IncK2 RNAI sequences. The obtained results show that a single nucleotide change in the RNAI structure can change compatibility of plasmids. Only nucleotides on top of the RNAI stem loop structure have an effect on minireplicon incompatibility with wildtype plasmids while mutations in the stem of the RNAI structure had no significant effect on incompatibility. Understanding the molecular basis of incompatibility is relevant for future in silico predictions of plasmid incompatibility.

Importance

Antimicrobial resistance is a global health threat that is predicted to increase over the next decades. The spread of antimicrobial resistance genes between bacteria through horizontal gene transfer is partially responsible for this. In Gram negative organisms, this is mediated mostly through conjugative plasmids. Plasmid epidemiology is studied nowadays through molecular diagnostics although previously, a phenotypic scheme was used based on the inability of related plasmids to be maintained together, referred to as incompatibility. The molecular basis of incompatibility is not completely understood yet but more knowledge on this topic can contribute to designing interventions that help combat plasmid mediated antimicrobial resistance.

Introduction

Antimicrobial resistance is a health threat that is spreading globally and threatens human and veterinary medicine. One of the factors that has facilitated this spread more easily is the transfer of mobile genetic elements between bacteria, for which plasmids are mostly responsible in Gram negative bacteria such as *Escherichia coli*.[1]

Incompatibility is the inability of two plasmids to be stably maintained in one cell. This phenotypic trait was long used as a plasmid typing tool for epidemiological studies but this has now mostly been replaced by molecular diagnostics.[2] Incompatibility is caused by the presence of identical or closely related shared determinants between two plasmids, originating from partition or replication mechanisms.[3]

Partition-based incompatibility can be caused by each of the partitioning mechanism components: ParA, ParB or *parS*. Excess of ParA was shown to interfere with the formation and/or the stability of the partitioning complex, provoking a dose-dependent increase of incompatibility, up to a maximum loss rate corresponding to a random inheritance.[4] The strength of the incompatibility mediated by ParB is variable and dependent on the level of protein overexpression where high levels lead to excessive plasmid sequestration through their *parS* sites, which lowers the number of units able to segregate.[5] Mild overproduction of ParB (5-fold) causes the activation of the ParB recombination activity, leading to high plasmid multimerization, a decreased number of segregation units and missegregation.[6,7] Incompatiblity due to identical copies of *parS* affect plasmids due differences in timing of the replication, affecting low copy number plasmids most severely.

Replication of plasmids can be regulated by one of the two mechanisms: iterons or antisense RNA molecules of replication initiation proteins, both of which can cause incompatibility. Iterons are repeated DNA sequences of approximately 20 bp that bind their cognate *repA* which prevents its translation.[8] Antisense RNA molecules form a stem-loop structure complementary to the RNA molecules of the replication initiation proteins leading to different effects for different plasmid groups. For ColE1 plasmids it leads to the inhibition of formation of the RNA primer.[9-11] In pT181 family plasmids, it results in premature termination of the messenger RNA for the Rep protein.[12] For IncF, IncL, IncM and I-complex plasmids, it inhibits translation of the *rep* mRNA.[12-18] Replication-mediated incompatibility is based on the presence of excessive amounts of iteron sequences or antisense RNA, originating from two different plasmids, which leads to the destabilization of one or both plasmids.

Involvement of the antisense RNA (RNAI structure) in replication control, and therefore incompatibility, was extensively studied for IncB/O plasmids.[19] In order to test the involvement of the replication control region, and not the partitioning region in incompatibility, a set of minireplicons was constructed. A minireplicon is a derivative subunit containing the replication region of a wild type plasmid ligated with a gene encoding a selection marker.[20] The role of nucleotides in the loop domain and upper stem of the RNAI structure in the interaction with RNAII was studied in detail.[19] Using systematic mutagenesis of nucleotides in the RNAI structure, it was concluded that three nucleotides on the top of the loop (C37, C38 and C39) are crucial for the kissing interaction with RNAII. Mutations at other positions only had significant effects if the mutation caused a base mismatch and therefore altered the structure. Additionally, few nucleotides

in the upper stem also had substantial effect of the RNAI-RNAII interactions. These results provide insights into the basis of incompatibility of IncB/O plasmids, but may also be helpful in understanding incompatibility of plasmids closely related to IncB/O, like IncK and IncZ.

Two compatible lineages of IncK plasmids were described in literature.[21, 22] Five SNPs were described that differ in the RNAI sequence of IncK1 and IncK2 plasmids, which may contribute to the compatibility of these plasmids. In this paper we examined the exact role of these five SNPs in the RNAI structure for the compatibility of IncK1 and IncK2 plasmids.

Materials and methods

Plasmids and vectors used

Plasmid pT.1.09 described in this study was recovered from a poultry sample, plasmid p754 was recovered from a dog sample (Table 1).[21] Vector pMW2 is a pBR322-derivative carrying the kanamycin resistance gene *aph(3')-III*.[28]

Table 1 Plasmids used and constructed in this study

Plasmid name	Resistance gene	Source	Reference
p754	$bla_{CTX\text{-}M\text{-}14}$	dog	[21]
pT.1.09	$bla_{CMY\text{-}2}$	poultry	[21]
pIncK1 mini	*aph(3')-III*	-	this study
pIncK2 mini	*aph(3')-III*	-	this study
pIncK1 mini RNAI_delA2	*aph(3')-III*	-	this study
pIncK1 mini RNAI_T10C	*aph(3')-III*	-	this study
pIncK1mini RNAI_G25T	*aph(3')-III*	-	this study
pIncK1 mini RNAI_G41C	*aph(3')-III*	-	this study
pIncK1 mini RNAI_G41C	*aph(3')-III*	-	this study
pIncK2 mini RNAI_insA2	*aph(3')-III*	-	this study
pIncK2 mini RNAI_G3C	*aph(3')-III*	-	this study
pIncK2 mini RNAI_C9T	*aph(3')-III*	-	this study
pIncK2 mini RNAI_T24G	*aph(3')-III*	-	this study
pIncK2 mini RNAI_C40G	*aph(3')-III*	-	this study

Minireplicon construction

Minireplicons were constructed by amplifying the replication region using the rep754 mini fw and rv primers for the IncK1 plasmid and repT1.09 mini fw and rv primers for IncK2 (Table 2, Supplementary Figure S1). The PCR reaction was performed with an annealing temperature of 57 ºC. The PCR product was purified using the Gene Clean Turbo Kit (MpBio), digested with BamHI and KpnI for IncK1 and SacI and KpnI (Thermo Fisher Scientific) for IncK2. The digested replication region was ligated into vector MW2, using T4 Ligase (Thermo Fisher Scientific). The ligated product was electroporated into *E. coli*

DH10 Electro MAX competent cells (Thermo Fisher Scientific). *E. coli* DH10 Electro MAX were transformed using: 2.0 kV, 200 Ω, 25 μF conditions, using 5 μL minireplicon plasmid DNA. Cells were recovered in 1 ml SOC medium (Invitrogen) for 30 min. Transformants were grown on agar plates containing 25 μg/ml of kanamycin (Sigma). The presence of the minireplicon was confirmed by PCR using IncK replicon targeting primers (K1 fw and rv and K2 fw and rv). All created minireplicons are listed in Table 1.

Single nucleotide mutagenesis was performed with the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific) according to the manufacturers protocol, using primers with the designated mutation (Table 2). PCR was performed with the annealing temperature of 62 °C using Phusion HSII Taq polymerase. The ligation product was electroporated into *E. coli* DH10 Electro MAX. Transformants were plated on agar plates containing 25 μg/ml of kanamycin. The presence of the minireplicon was confirmed with PCR using primers targeting the replication region (Table 2). The presence of the mutation was confirmed by Sanger sequencing.

The RNAI structure of the wt and mutated RNAI genes was predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

Table 2 Primers used in this study. "Mini" in the primer name means that the primer targets the minireplicon. Each primer name additionally depicts the mutation that is introduced in the RNAI structure, using this primer.

Primer name	Primer sequence	Reverse primer used	Reference
rep754 mini fw	5' catggtaccggcctgcagttctgacagac 3'		This study
rep754 mini rv	5' atgtgatcataggcacggtgctgcgtttg 3'		This study
repT1.09 mini fw	5' cagggtaccactgagccagataccagtt 3'		This study
repT1.09 mini rv	5' caggagctctacgagcgtgtactgaggac 3'		This study
K1 fw	5' atcgtcaggatccgggaagtc 3'		[21]
K1 rv	5' gagcgattgtgccgtgtatt 3'		[21]
K2 fw	5' atgctcgcggtccggaaagcc 3'		[21]
K2 rv	5' gtgccgtgcgttaatgcactgcaa 3'		[21]
Phu-754-R1	5' gggataagtatatatgaaaccgtaccagag 3'		This study
Phu-754-R2	5' tagtaggggcgttcacagaatacgggataa 3'		This study
Phu-T1.09-R1	5' gggataagtatatatgaaaccgtgtcagag 3'		This study
Phu-T1.09-R2	5' tagtgggggcctcacagaatacgggataag 3'		This study
Kan fw	5' atgatgctatggctggaagg 3'		This study
Kan rv	5' cgcagaaggcaatgtcatac 3'		This study
CTX-M-14 fw	5' ctattttacccagccgcagc 3'		[29]
CTX-M-14 rv	5' gttatggagccacggttgat 3'		[29]
CMY fw	5' atgatgaaaaaatcgttgctgc 3'		[30]
CMY rv	5' gcttttcaagaatgcgccagg 3'		[30]

Table 2 continued

Primer name	Primer sequence	Reverse primer used	Reference
Phu-754-A2-Del-Fw	5’ gtattctgtgacgcccctactatctttcacg 3’	Phu-754-R1	This study
Phu-754-C4G-Fw	5’ gtattctgtgaaggcccctactatctttcacg 3’	Phu-754-R1	This study
Phu-754-T10C-Fw2	5’ gtattctgtgaacgccccc actatctttcacg 3’	Phu-754-R1	This study
Phu-754-G25T-Fw	5’ tctttcacgatcccgccaaagttcgaggaaagat 3’	Phu-754-R2	This study
Phu-754-G41C-Fw	5’ tctttcacgagcccgccaaagttcgacgaaagat 3’	Phu-754-R2	This study
Phu-T1.09-A2-Ins-Fw	5’ gtattctgtgaaggcccccactatctttcacg 3’	Phu-T1.09-R1	This study
Phu-T1.09-G3C-Fw	5’ gtattctgtgacgcccccactatctttcacg 3’	Phu-T1.09-R1	This study
Phu-T1.09-C9T-Fw	5’ gtattctgtgaggcccctactatctttcacg 3’	Phu-T1.09-R1	This study
Phu-T1.09-T24G-Fw	5’ tctttcacgagcccgccaaagttcgacgaaagat 3’	Phu-T1.09-R2	This study
Phu-T1.09-C40G-Fw	5’tctttcacgatcccgccaaagttcgaggaaagat 3’	Phu-T1.09-R2	This study

Stability

The stability of minireplicons was determined in triplicate by overnight culturing in LB broth without selection. Serial dilutions of the culture were plated on LB agar plates without selection and incubated overnight at 37 ºC. 40 colonies were picked and PCR was performed to amplify the resistance gene. Stability was determined as a percentage of colonies containing the minireplicon. All results were statistically analysed using the Mann-Withney U test.

Incompatibility testing

IncK minireplicons are non-conjugative, therefore electroporation was chosen as a method to deliver plasmids into the bacterial cell. IncK1 or IncK2 plasmids were electroporated into *E. coli* DH10B according to the manufacturer protocol (see above). *E. coli* cells carrying IncK1 or IncK2 wt plasmid were made electrocompetent from 250ml liquid culture of OD_{600} 0.5 in lysogeny broth (LB) supplemented with 25 µg/ml kanamycin (Sigma-Aldrich) for the minireplicon and 2 µg/ml cefotaxime (Sigma-Aldrich) for the IncK wt plasmid. Cultures were spun down for 10 min at 3560 x g at 4 °C. Pellets were washed twice with 250 and 125 ml ice-cold water, washed with 10 ml ice-cold 10% glycerol and finally resuspended in 0.5 ml ice-cold 10% glycerol and frozen at -80 ºC. For electroporation, minireplicons were isolated using the Wizard Plus SV Miniprep kit (Promega) and transformed as described above. Transformants were subsequently selected on LB plates supplemented with 25 µg/ml kanamycin and 2 µg/ml cefotaxime.

To test the incompatibility of IncK plasmid and the minireplicon combinations, the heteroplasmid population was grown over night and plated on the non-selective plates. For detailed description see the "stability" paragraph.

Results

RNAI structures comparison

The RNAI structures of wt IncK1 and IncK2 plasmids, which consist of 61 or 60 nucleotides respectively, mainly differ in the top loop region. The top loop of the IncK2 RNAI structure is substantially bigger (12 nucleotides) compared to the one from IncK1 (8 nucleotides). The RNAI sequences of the wt IncK1 and IncK2 plasmids differ by 5 nucleotides of which one is located in the loop of the molecule.

RNAI structures of the wt plasmids as well as the mutated structures were predicted (Figure 1). All these nucleotide positions that are different between IncK1 and IncK2 plasmid were subjected to the mutagenesis. Every mutated nucleotide was substituted with a corresponding nucleotide from the opposite plasmid group. Most of the introduced mutations did not result in the change of the shape of the RNAI structure. Only mutation G25T for IncK1 and T24G for IncK2 resulted in a conformational change where the IncK1 mutant resembles IncK2 and vice versa. (Figure 1).

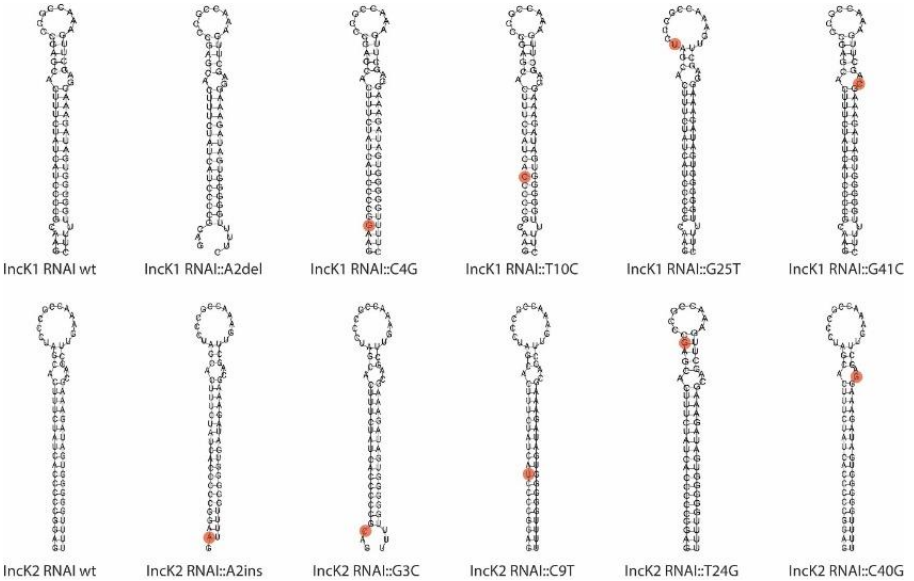

Figure 1 RNA structure prediction of all mutated RNAI variants. Red circles point out the different mutation sites of the various minireplicons that were tested.

Minireplicon stability

We examined the effect of point mutations in the RNAI structure on the compatibility of IncK plasmids. To determine the effect of the RNAI structure only and exclude involvement of any other plasmid structures, we designed minireplicons that contain the

replication region of the IncK plasmid, and a kanamycin resistance cassette, which were ligated in a MW2 vector.

The stability of the minireplicons was examined in triplicate after 24 hours of culture without antibiotic selection and was defined as the percentage of colonies that contained the minireplicon at the end of experiment. The IncK1 wt minireplicon had a higher stability compared to the IncK2 wt minireplicon (p=0.034). All IncK1 mutated minireplicons have a higher stability than the corresponding IncK2 mutated minireplicons ($p \leq 0.05$). For IncK1 plasmids there were no statistically significant differences between the wt minireplicon and mutated variants (Figure 2). For IncK2 minireplicons, mutations A2ins, T24T and C40G resulted in a statistically significant increase in stability of the minireplicon compared to wt (p=0.037 for A2ins and C40G and p=0.034 for T24T). On the other hand, mutation C9T caused statistically significant decrease in stability of the minireplicon compared to the IncK2 wt (p=0.037).

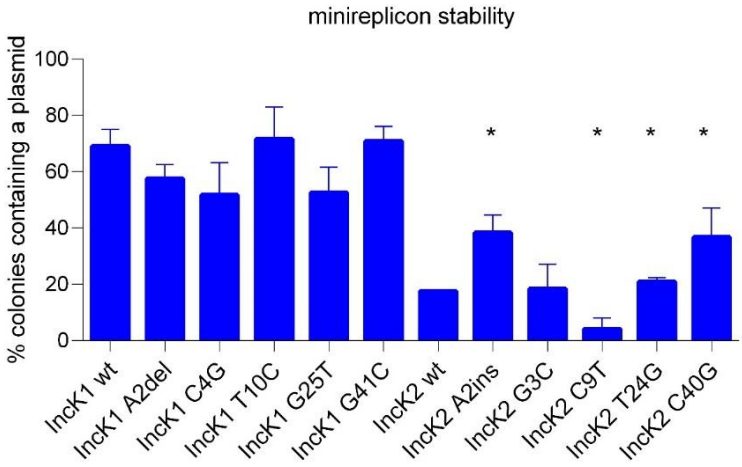

Figure 2 Stability of wt and mutant minireplicons. Asterisks indicate the mutant minireplicons for which stability was significantly different compared to the minireplicons containing the wt RNAI($p \leq 0.05$)

Minireplicon incompatibility

Incompatibility of all mutated minireplicons was checked in triplicate against the parental and opposite wt minireplicons IncK plasmids. Surprisingly, we obtained colonies carrying the IncK1 wt plasmid and the IncK1 wt minireplicon after plating the transformants on double selective agar plates. We were not able to create cells carrying both the IncK2 wt plasmid and InK2 wt minireplicon. According to the expectations, we obtained heteroplasmid clones carrying the IncK1 wt plasmid and the IncK2 minireplicons and vice versa. To further examine the unexpected results of the IncK1 wt plasmid and the IncK1 minireplicons, we performed overnight incompatibility tests to assess if these plasmids could be stably present together in one cell without antibiotic selection. None of the 40 tested colonies contained both the IncK1 wt plasmid and IncK1 minireplicon after 24h culture, which demonstrates that these plasmids are incompatible (Figure 3).

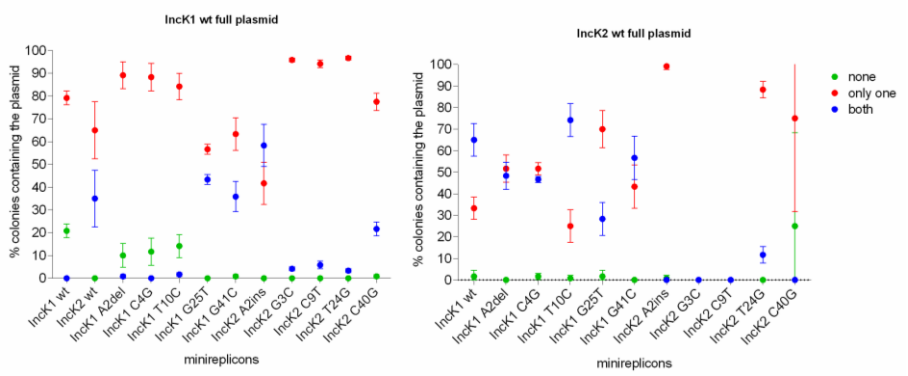

Figure 3 Compatibility of wt and mutated minireplicons tested in triplicate against parental and non-parental IncK plasmids. "X" means that the heteroplasmid strain was not obtained.

All heteroplasmid strains were created by electroporating a mutated minireplicon with its parental (exact same replications system) or non-parental (similar replication system) IncK wt full plasmid into one cell. The incompatibility of all obtained heteroplasmid cells are shown in Figure 3. We determined the percentage of cells carrying either both a minireplicon and IncK wt plasmid, only one of these or none. All minireplicons were compatible with their non-parental IncK plasmid. In case a cell does only contain one plasmid we do not differentiate between the minireplicon or IncK wt plasmid in Figure 3. In most cases the minireplicon was lost from the heteroplasmid strain. However, in case of the IncK2 minireplicon C40G together with the IncK2 wt plasmid the results deviated between replicates, where either the minireplicon or both minireplicon and the wt plasmid were lost. This is represented by the large standard deviation in Figure 3. Besides, mutations G25T and G41C have a critical effect on compatibility of the IncK1 minireplicon with the IncK1 parental plasmid. Mutations A2del and T10C also cause some degree of compatibility with the IncK1 parental plasmid. For the IncK2 minireplicons, only mutation T24G changes the compatibility with its parental plasmid.

Based on our incompatibility experiments, we compared the stability of single minireplicons to the heteroplasmid strains containing both the minireplicon and their (non)parental IncK plasmids (Figure 4). All minireplicons that were co-existing with their parental plasmid had a statistically significant lower stability in comparison with cells containing only the minireplicon (Figure 4). The only exception is mutation G25T on the IncK1 minireplicon, where there was no statistical difference in stability between the heteroplasmid or single plasmid strains. This is also the only mutation that restored full compatibility of the minireplicon and its parental plasmid. Surprisingly, the same mutation did not restore full compatibility of the IncK2 minireplicon and the IncK2 wt plasmid. However, both mutation G25T on the IncK1 minireplicon and T24G for the IncK2 minireplicon caused reduced compatibility with their non-parental plasmid. Moreover, stability of the IncK2 minireplicon was improved in a heteroplasmid strain containing an IncK1 wt plasmid.

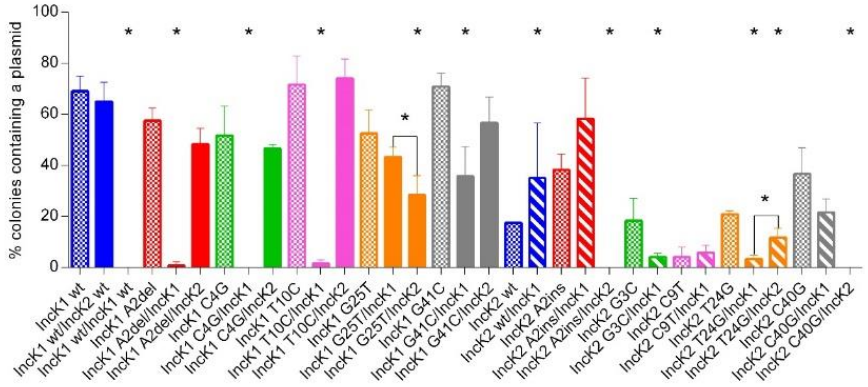

*Figure 4 Comparison of single minireplicons stability with its stability in the heteroplasmid strain (co-existing with parental or non-parental plasmids). Sample names under the graph show minireplicons used before the "/" and IncK wt plasmid used after "/". Dotted bars represent stability of the minireplicon alone, full bars represent stability of minireplicons with the IncK1 wt plasmid and striped bars represent the stability of minireplicons with the IncK2 wt plasmid. * Means that the minireplicon stability in the heteroplasmid sample is statistically significantly different from the stability of a single minireplicon.*

Discussion

In this manuscript we examined the molecular basis of incompatibility of IncK plasmids by determining the effect of point mutations in the RNAI structure on incompatibility. We created minireplicons carrying the replication region of either the IncK1 or IncK2 plasmid, in which we subsequently introduced point mutations in the RNAI structure.

The minireplicons constructed in this study consisted of the replication region of IncK1 or IncK2 plasmids and a kanamycin resistance gene. The copy number of the plasmid is regulated by the replication region. IncK plasmids were shown to have a copy number of 1-2 per cell.[23] Lack of the partitioning system on the low-copy plasmid leads to random segregation during cell division and therefore rapid plasmid loss. This could explain the instability of the minireplicons created in this study. High instability of minireplicons was previously reported for IncL/M plasmids.[24]

The results obtained in this study showed that mutations in the loop and upper stem of the RNAI structure have a critical effect on the compatibility of IncK plasmids. Similar findings were previously demonstrated for IncB plasmids.[19] Additionally, different mutations cause a different degree of incompatibility for IncK1 and IncK2 plasmids. For IncK1 minireplicons, mutations G25T and G41C have a critical effect on their compatibility with the IncK1 parental plasmid. However, mutations A2del and T10C have only a small influence on the compatibility. These findings are in line with previous research which showed that different mutations in the RNAI structure can cause a different degree of repA inhibition.[19] However, for IncK2 minireplicons, only mutation T24G changed compatibility towards its parental plasmid. Inability to detect heteroplasmid

clones carrying IncK2 minireplicons may be due to its very low stability and not due to incompatibility caused by binding of the RNAI to the *repA* mRNA.

Incompatibility of two plasmids is caused by the combined effect of replication and partitioning system interference. The fact that the minireplicons constructed in this study lack the partitioning system may be the reason why the observed incompatibility was weaker than expected. The obtained results indicate that replication-based incompatibility may not be a straight-forward system based on plasmids being present or not in one cell. It may be similar to partitioning-based incompatibility of IncF plasmids, where both strong and weak incompatibility was reported.[25]

In recent years, compatibility of many plasmids was re-examined.[21, 22, 26, 27] The most used and well established method to determine compatibility of two plasmids is conjugation of both into one cell, followed by selective plating. However, the present study has indicated the added value of examining plasmid incompatibility over time. This greatly affected the interpretation of results.

This manuscript demonstrates the molecular basis of incompatibility of IncK1 and IncK2 plasmids. It shows that a single mutation in the RNAI structure of the IncK1 plasmid can change its compatibility. The same mutation does not fully change compatibility of the IncK2 plasmid which suggests that it is mediated by more than 1 mutation. Further research in this field, including more plasmid types, can possibly lead to a design of a sequence-based tool predicting plasmid compatibility.

Funding

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We would like to thank Arjen Timmerman and Heleen Zweerus for the help with stability experiments.

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Supplementary

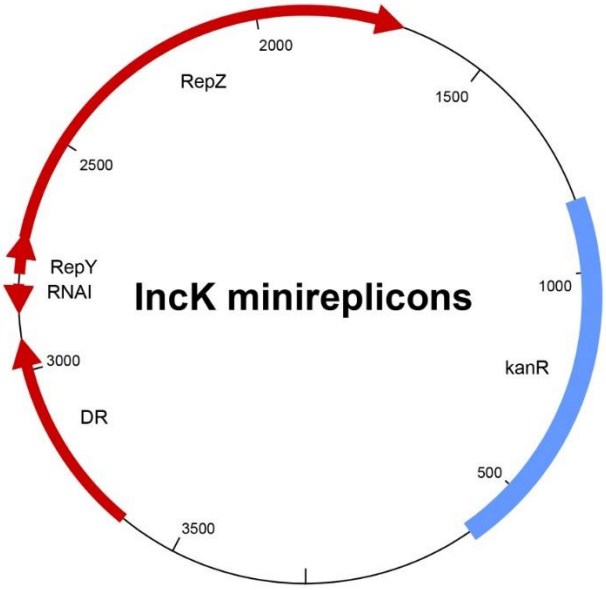

Figure S1 Map of the IncK minireplicons. Red arrows indicate the replication region of the wt IncK plasmids, the blue box indicates the selective marker – a kanamycin resistance gene. The size of the IncK1 minireplicon is 3789 bp and IncK2 3922 bp.

Chapter 6

Successful Host Adaptation of IncK2 Plasmids

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Abstract

The IncK plasmid group can be divided into two separate lineages named IncK1 and IncK2. IncK2 is found predominantly in poultry while IncK1 was reported in various mammals, including animals and humans. The physiological basis of this distinction is not known. In this manuscript we examined fitness cost of IncK1 and IncK2 plasmids at 37 ºC and 42 ºC, which resembles mammalian and chicken body temperatures, respectively. We analyzed conjugation frequency, plasmid copy number and plasmid fitness cost in direct competition. Additionally, we measured levels of σ-32 in *Escherichia coli* carrying either wild type or conjugation-deficient IncK plasmids. The results show that IncK2 plasmids have a higher conjugation frequency and lower copy number at 42 ºC compared to IncK1. While the overall fitness cost to the host bacterium of IncK2 plasmids was higher than that of IncK1, it was not affected by the temperature while the fitness cost of IncK1 was shown to increase at 42 ºC compared to 37 ºC. These differences correlate with an increased expression of σ-32, a regulator of heat-shock protein expression, in *E. coli* with IncK2 compared to cells containing IncK1. This effect was not seen in cells containing conjugation deficient plasmids. Therefore, it is hypothesized that the assembly of the functional T4S may lead to these increased levels of σ–32. Increased activation of CpxR at 42 ºC may explain why IncK2 plasmids, and not IncK1, are predominantly found in chicken isolates.

Keywords

plasmid, IncK2, conjugation, sigma-32, chicken

Introduction

Antimicrobial resistance is a global health threat and was responsible for an estimated 33110 infection-related deaths in European Union in 2015.[1] As antimicrobial resistance (AMR) is often encoded on plasmids, it is crucial to understand the dynamics of plasmid spread. One of the determinants influencing plasmid spread is plasmid fitness cost, which is defined as a burden on the bacterial host, manifesting in reduced growth rate and weakened competitiveness of plasmid-bearing strains under conditions that do not select for plasmid-encoded genes.[2] Plasmid fitness cost can derive from many processes. Entrance into the cell triggers an SOS response which may delay cell division.[3] Moreover, replication causes depletion of essential cellular components like RNA polymerase, tRNA and amino acids.[4] A recent review of San Millan and Maclean describes these mechanisms in greater detail.[5]

Carrying a plasmid may not only be a burden, but also provide a certain evolutionary advantage.[6-8] It was shown that plasmids entering a new host, where they initially pose a fitness cost, after short- term evolution, become advantageous.[9] This phenomenon counteracts efforts to lower AMR levels as it seems that plasmids can persist in their host even without selective pressure.

The fact that plasmids can show a high stability in bacterial populations in the absence of apparent selective pressure is counter intuitive to the fitness burden that they impose on the bacterial host cell, this is also referred to as the plasmid paradox.[5] With the advancement of studies on plasmid fitness cost it became clear that plasmids evolved strategies to neutralize their fitness cost. Based on many plasmid fitness cost studies described in literature, Harrison and Brockhurst (2012) pointed out that simultaneous evolution of plasmid and its host (coevolution) is the most important factor in reducing fitness cost.[10] The phenomenon of coevolution partly resolves the plasmid paradox. Another way to neutralize plasmid fitness cost is compensatory evolution. Plasmids posing a high fitness cost, can minimize it through evolutionary changes induced by the bacterial host.[11] One example is mutations in the CheY protein of *Pseudomonas moraviensis*, which were reported to decrease plasmid fitness cost.[12] Another example reported to explain a decrease in plasmid fitness cost is an IS-mediated deletion of a 25 kb plasmid fragment containing three resistance determinants, a restriction anti-restriction system, and the main conjugation machinery.[13] Moreover, it was shown for ColE plasmids that compensatory evolution enables coexistence of multiple copies of a plasmid.[14] Another strategy is gene silencing, which prevents expression of xenogeneic DNA, which in turn can be harmful to the bacterial cell or cause severe fitness consequences.[15] Expression of these genes can be switched on in certain environmental conditions. Gene silencing can be mediated by H-NS or plasmid partitioning proteins. H-NS proteins act as a master regulator, which can affect transcription of up to 60 genes in *Escherichia coli*.[16] Silencing of only resistance genes was also reported.[17] Silencing of genes due to the partition proteins was reported for F and P1 plasmids.[18, 19] Compensatory mutations to reduce fitness cost can result in converging evolution of plasmids that were once closely related and can result in adaptation to specific niches.

It was shown that IncK plasmids can be divided into two separate lineages named IncK1 and IncK2.[20, 21] IncK2 is found predominantly in poultry sources while IncK1 was reported in various animal and human sources. A possible explanation could be adaptation of IncK2 plasmids to poultry specific characteristics like e.g., a slightly higher body temperature

compared to other animals and humans.

Environmental temperature is known to have major effects on bacterial evolution, which is also influenced by the body temperature of a colonized host.[22] Most research focuses on temperatures lower than 37 ºC, mimicking environmental or food storage conditions.[23, 24] There is only limited data available on plasmid fitness cost in elevated temperatures. The body temperature of chickens is 42 ºC vs. 37–39 ºC of mammals, depending on the animal species involved. The higher chicken body temperature was demonstrated to induce a heat-shock response for optimal fitness of *Salmonella* residing in chicken ceca.[25] A heat-shock response can further influence plasmid fitness cost by increasing conjugation frequency or biofilm formation.[26-28] Zahrl *et al.* (2006, 2007) showed that assembly of T4S triggers activation of the extracytoplasmic stress, which is sensed by the two-component system CpxRA.[29, 30] That leads to increased levels of σ-32, which in turn is responsible for the heat-shock response. These findings suggest that elevated temperatures may play an important role in plasmid adaptation to the animal host.

In recent years several methods to measure plasmid fitness cost were developed. The most widely used in vitro experiments focus on bacterial growth and direct competition between plasmid-bearing and plasmid-free strains.[31-33] Furthermore, a mouse model was used to assess plasmid fitness cost *in vivo*.[33] Fitness cost can also be assessed indirectly by measuring the conjugation rate or the rate of biofilm formation.[26]

The goal of this research was to examine the fitness cost of IncK1 and IncK2 plasmids on its bacterial host. To achieve that, growth rates, conjugation frequency, direct competition and plasmid copy numbers were determined at 37 ºC and 42 ºC. Additionally, we determined levels of σ-32 in *E. coli* with and without the presence of IncK plasmids.

Materials and methods

Plasmids and bacterial strains

In this study, we used IncK1 plasmids p754 and p527, isolated from *E. coli* obtained from dog and cattle, respectively. The IncK2 plasmids pT.1.09 and pT.10.2, isolated from *E. coli* obtained from poultry. The IncK1 plasmids used in this study carry $bla_{\text{CTX-M-14}}$, while the IncK2 plasmids carry $bla_{\text{CMY-2}}$. The *E. coli* MG1655 strain, used as recipient for conjugation experiments, encodes resistance to chloramphenicol. All experiments were performed at 37 ºC and 42 ºC. These temperatures were chosen to resemble the body temperatures of mammals and chickens, respectively.

Conjugation rate

Conjugation was performed as previously described.[20] Briefly, liquid cultures of donor and recipient cells at OD600 0.5 were mixed in 1:1 ratio and incubated for 18 h at 37 ºC or 42 ºC. Donor and transconjugant cells were recovered on LB plates or LB plates supplemented with 2 mg/L cefotaxime and 25 mg/L chloramphenicol, respectively. All experiments were performed in triplicate. Conjugation frequency was calculated as the number of transconjugants per donor cell. Obtained data was analyzed using the Mann-Whitney test with $p > 0.05$ considered statistically significant.

traY gene mutagenesis

To obtain a non-conjugative IncK plasmid for competition experiments, a kanamycin resistance gene was knocked-in to the *traY* gene using the RedET system (Gene Bridges). Primers used for the mutagenesis are listed in Supplementary Table S1. Mutagenesis was performed according to the kit protocol. Insertion of the resistance cassette in the *traY* gene was confirmed by PCR using primers "K1 traY fw" and "K1 traY rv" for IncK1 plasmids and "K2 traY fw" and "K2 traY rv" for IncK2 (Supplementary Table S1).

Stability and competition

The stability of the mutant plasmids, as well as the gene insertions, were measured over the course of five consecutive days without selection. 50 µl of bacterial suspension with a density 0.5 McFarland was incubated in 5 mL LB broth and grown at 37 ºC or 42 ºC. Cultures were renewed daily in fresh LB in 1:1000 ratio after overnight incubation for 5 days. Samples of the overnight cultures were plated on LB plates and after overnight incubation 100 separate colonies were replicated on LB plates with 2 mg/L cefotaxime or 15 mg/L kanamycin.

Competition experiments between *E. coli* DH10B and *E. coli* DH10B IncK1-traY::kan, or *E. coli* DH10B and *E. coli* DH10B IncK2-traY::kan, were performed in triplicate. The procedure to test the fitness cost was the same as the stability tests, but using multiple strains in competition. Fitness cost was calculated as previously described.[34] Briefly, the CI (competition index) was calculated as the ratio of the mean cfu for three independent competition experiments between the resistant and susceptible strains at a given time point (t_1) divided by the same ratio at t_0. The selection coefficient, *s*, was calculated as the slope of the linear regression model: $s = \ln(CI)/\ln(d)$, where *d* is the dilution factor. The selection coefficient estimates the difference between the relative fitnesses of the two competitors over the entire competition experiment. The relative fitness (*w*) was calculated with the formula $w = 1 - s$. Obtained data were analyzed using the Mann-Whitney test.

Plasmid copy number

To determine the plasmid copy number, three independent DNA extractions using QIAamp DNA Midi Kit (Qiagen)+ were performed for each strain, and qPCRs (BioRad), targeting the IncK replicon and *uidA* gene, were carried out in triplicate for each extraction. Plasmid copy number per chromosome was calculated using the formula described by San Millan *et al.* (2015)[35] $c_n = [(1 + E_c)^{Ctc}/(1 + E_p)^{Ctp}] \times (S_c/S_p)$, where c_n is the plasmid copy number per chromosome, S_c and S_p are the sizes of the chromosomal and plasmid amplicons (in bp), respectively, E_c and E_p are the efficiencies of the chromosomal and plasmid qPCRs (relative to 1), respectively, and Ctc and Ctp are the threshold cycles of the chromosomal and plasmid reactions, respectively. Plasmid copy number was determined at 37 ºC and 42 ºC using K/B fv and K rv primers for IncK2, K/B fv new and K rv new primers for IncK1 and uidA fw and uidA rv for the chromosomal target (Supplementary Table S1). Obtained data were analyzed using the Mann-Whitney test.

Sigma-32 levels analysis

σ-32 levels were analyzed in whole-cell lysates corresponding to 0.3 OD_{600}. 4 mL of the culture was centrifuged at 3500 *g* for 1 min. The pellets were resuspended in 100 µL of

the protease inhibitor mixture (cOmplete^{TM}, Mini Protease Inhibitor Cocktail, Roche). 15 uL of the samples were loaded on 12% polyacrylamide gels. Immunological detection of σ-32 was performed using a monoclonal anti-σ-32 antibody (Neoclone), diluted 1:2000 in PBS buffer with 0.5% gelatin, and 0.1% Triton x-100 (Sigma Aldrich). As secondary antibodies, HRP conjugated goat anti-mouse IgG antibodies (Sigma Aldrich) diluted 1:8000 in PBS buffer with 0.5% gelatin and 0.1% Triton x-100 were used. The chemiluminescent detection was performed using the Clarity ECL system (Bio Rad). As a positive control for the specificity of the antibodies, a strain with mutated σ-32 was used.[36] *rpoH-lacZ* gene fusion results in higher molecular weight of the protein, which enables correct identification with western blot. σ-32 protein levels were standardized to the *E. coli* DH10 sample at 37 ºC or 42 ºC The relative quantity of σ-32 is an average of three independent experiments. Obtained data were analyzed using the Mann-Whitney test.

Results

Conjugation rate

Conjugation rate experiments in liquid broth matings were performed at 37 ºC and 42 ºC to assess differences in the spread potential of IncK1 and IncK2 plasmids. At 37 ºC there was no difference in conjugation rate between IncK1 and IncK2 plasmids (Figure 1). At 42 ºC, the rate of both plasmid types significantly decreased compared to 37 ºC. However, the rate of IncK1 was decreased much further than that of the IncK2 plasmids, compared to 37 ºC. At 42 ºC there is a significant difference in conjugation rate between IncK1 and IncK2 plasmids (p = 0.0039) (Figure 1).

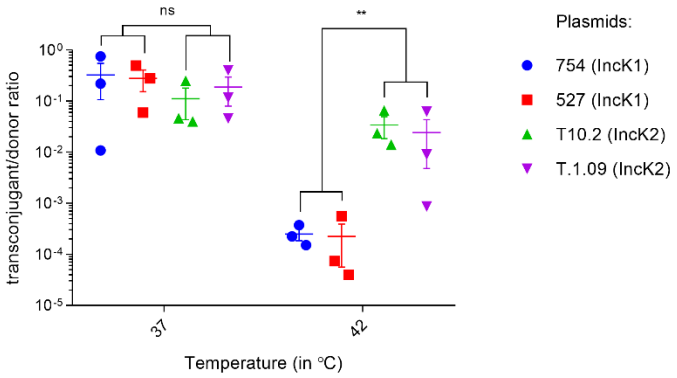

*Figure 1 Triplicate measures of conjugation rates of IncK plasmids at 37 and 42°C. Bars depict the mean and standard deviation. ** depict statistical significance (*p = 0.0039*).*

Plasmid copy number

The plasmid copy number of IncK1 and IncK2 plasmids in *E. coli* DH10 were assessed at 37 ºC and 42 ºC. For both of the IncK1 and IncK2 plasmids that were tested here, the plasmid copy number at 37 ºC was consistently just under 1 copy per chromosome, reflecting a bias in the DNA isolation method that was used.[37] Assuming that this bias is equal for all isolates, all plasmids, besides T.109, showed a significant difference in copy number at 37 ºC and 42 ºC. The difference in copy number between IncK1 and IncK2

plasmids at 42 °C was statistically significant (p = 0.0039), while there was no difference at 37 °C (Figure 2). Both IncK1 and IncK2 plasmids showed a statistically significant increase in copy number at 42 °C compared to 37 °C (p = 0.0039 and p = 0.0250, respectively).

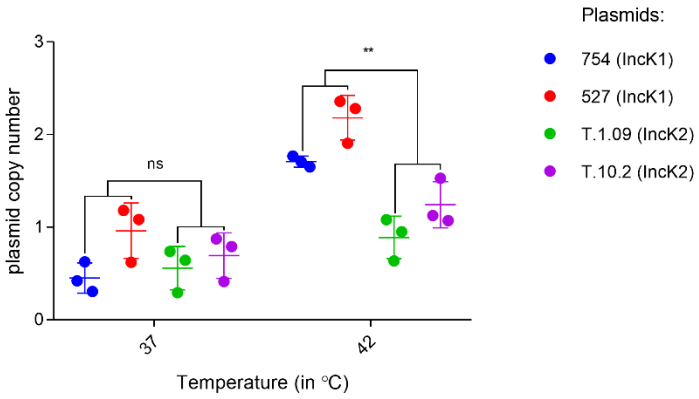

*Figure 2 IncK1 and IncK2 plasmid copy numbers at 37 and 42 °C. All the measurements were performed in triplicate. Bars depict the mean and standard deviation. ** depict statistical significance (p = 0.0039).*

Fitness cost

To make IncK plasmids non-transmissible, knock-in mutants were created by inserting a kanamycin resistance cassette into the *traY* gene.[38] Conjugation experiments were performed to confirm the inability of conjugative transfer of IncK plasmids (data not shown). Stability of the IncK plasmid with the insertion in the *traY* gene was confirmed during the competition experiment. The *E. coli* DH10B IncK-traY::kan stably maintained these plasmids throughout the duration of the experiment.

At both 37 °C and 42 °C IncK1 plasmids had a lower fitness cost compared to IncK2 plasmids (T.1.09) (p = 0.0201) (Figure 3A). Due to a large standard deviation at 42 °C, plasmid T.10.2 was excluded from the statistical analysis. Differences between 37 °C and 42 °C were significant for IncK1 plasmids (p = 0.0495), but not for IncK2 (Figure 3B).

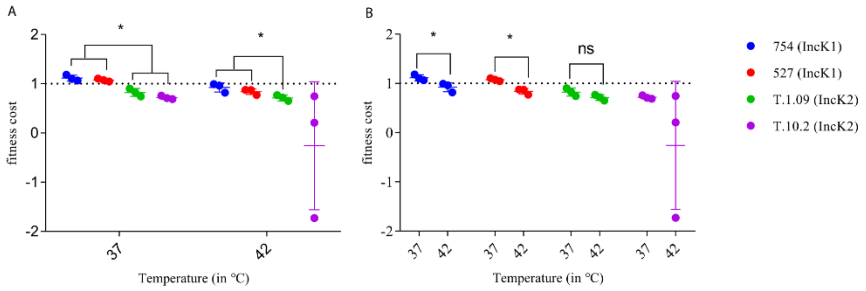

*Figure 3 Fitness cost of IncK1 and IncK2 plasmids measured at 37 and 42 °C. All the measurements were performed in triplicate. Bars depict the mean and standard deviation. (A) Comparison of plasmid fitness cost by temperature. * depict statistical significance (p = 0.0201). (B) Comparison of plasmid fitness cost by plasmid type. * depict statistical significance (p = 0.0495).*

Sigma-32 levels

The levels of σ-32 in lysates of *E. coli* cell suspensions at OD 0.3, with or without IncK1 or IncK2 plasmids were measured. In our experiment, a statistically significant difference ($p = 0.037$) in σ-32 upshift between 37 ºC and 42 ºC between IncK1 and IncK2 plasmid carrying *E. coli* was observed (Figure 4). The difference in σ-32 upshift between 37 ºC and 42 ºC was not observed when testing non- transmissible IncK plasmids (IncK-traY::kan).

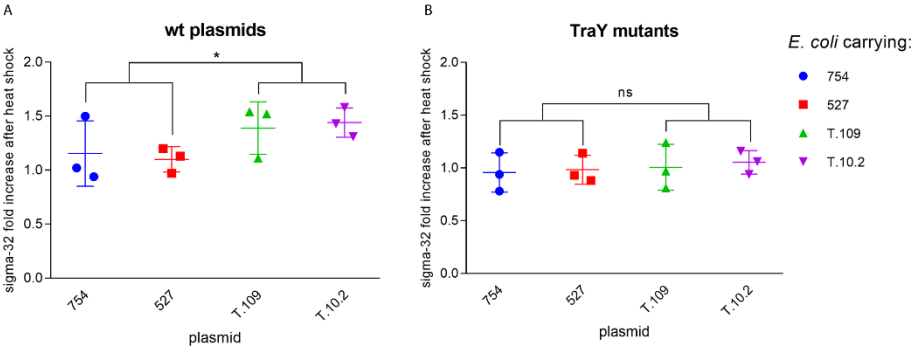

*Figure 4 (A) σ-32 fold increase between 37 and 42 °C in Escherichia coli carrying wt plasmids. * depict statistical significance (p = 0.037). (B) σ-32 fold increase between 37 and 42 °C in E. coli carrying plasmids with mutated TraY; Bars depict the mean and standard deviation.*

Discussion

While most attention in previous studies has focused on plasmid behavior at temperatures below mammalian body temperature, it is known that elevated temperatures can lead to a higher conjugation rate, biofilm formation, and a higher plasmid copy number per cell.[26-28, 39-41]

In the present study we have examined various attributes of IncK1 and IncK2 at both 37 °C and 42 °C to resemble the mammalian and chicken body temperature, as these are the respective niches of IncK1 and IncK2.[20, 21] These attributes included several well studied properties including the conjugation frequency, plasmid copy number and plasmid fitness cost of plasmids from the IncK group, as well as the levels of expression of σ-32 to assess the extra-cytoplasmic stress level induced by the plasmid conjugation. All of these experiments were performed with the IncK1 plasmids p754 and p527 isolated from dog and cattle, respectively, and the IncK2 plasmids pT.1.09 and pT.10.2 isolated from poultry. Conjugative plasmids pose a difficulty during direct competition as it is impossible to differentiate between cells that originally had a plasmid and the ones that received it via conjugation during the experiment. One of the solutions can be to introduce mutations in the conjugation pathway of a plasmid. It was shown that mutation of the *traY* gene of IncK plasmids effectively prevents conjugation.[42]

Measurement of the plasmid copy number at 37 °C resulted in a consistent copy number just below 1 copy per chromosome for all four plasmids that were tested here. As all cells were grown on selective media before the experiment, an actual copy number below 1 copy per chromosome is likely caused by a bias in the DNA isolation method.[37] Nonetheless, this bias is presumed equal for all plasmids that were tested and differences in the relative abundance of plasmids can still be compared between the samples. At 37 °C there are no differences in plasmid copy number between IncK1 and IncK2 plasmids but at 42 °C IncK1 plasmids have statistically significant higher copy numbers compared to IncK2 plasmids.

The method that was used to measure the burden on the fitness cost of the host bacterium was previously described by Santos-Lopez and uses direct competition between bacteria with and without a plasmid.[34] At 37 °C, both of the IncK1 plasmids have a small fitness advantage for the *E. coli* while IncK2 plasmids present a burden. Although these differences are minor, there is a significant effect which may lead to selection of IncK1 over IncK2 *in vivo* at this temperature. At 42 °C, the IncK1 plasmid also has a lower fitness cost compared to the IncK2 plasmid. Looking at each plasmid separately, IncK1 plasmids have a significantly higher fitness cost at 42 °C compared to 37 °C while for IncK2 plasmids this difference is not significant.

In this manuscript we measured fitness cost of IncK plasmids in an indirect as well as a direct manner. Conjugation rate and copy number are examples of the plasmid's characteristics that indirectly influence the fitness cost the plasmid causes to the host. By performing competition experiments we demonstrate in a direct way the burden of carrying an IncK plasmid for the *E. coli* host. We believe that measuring multiple fitness cost related parameters will allow for a better understanding of plasmid fitness cost.

Additionally, to determine a potential stress response we measured levels of σ-32 in *E. coli* carrying either IncK1 or IncK2 plasmids. The IncK2 plasmids causes a higher upshift of σ-32 levels, compared to the IncK1 plasmids. This effect is no longer observed when the *traY* gene is disabled by creating a knock-in mutant, which inactivates the assembly of the conjugation machinery. These results are in line with the model proposed by Zahrl *et al.* (2006) which links conjugation to a heat shock response, which was manifested by elevated levels of σ-32.[29] In their model, the assembly of a functional TS4-system generated a stress signal which is sensed by CpxRA envelope stress signaling system, which subsequently leads to a transcriptional induction of both extra-cytoplasmic stress genes as well as the *rpoH* gene (encoding σ-32).

The model proposed by Zahrl *et al.* (2006) demonstrates that a lower conjugation rate of both IncK1 and IncK2 plasmids at 42 °C compared to 37 °C was associated with

cytoplasmic stress.[29]
This suggests that cytoplasmic stress caused by the high level of σ-32 at 42 °C decreased the conjugation rate. Upregulation of σ-32 in IncK1-carrying cells leads to the cytoplasmic stress, which was proven to decrease plasmid conjugation. These findings explain lower conjugation level of IncK1 plasmid compared to IncK2 at 42 °C.

The role of the two-component system CpxRA in bacterial virulence was previously discussed in literature, with conflicting results. Some groups reported the role of CpxRA in pathogenesis through modulation of expression of virulence factors and regulators.[43-47] Others showed that activation of CpxRA inhibits the virulence.[48, 49] Vogt *et al.* (2010) reported that depending on the level of induction of Cpx response, it can promote or inhibit the assembly of *E. coli* bundle- forming pilus.[50] More recent research has shown that CpxR plays a crucial role in regulation of genes important for colonization of *Salmonella* and avian pathogenic *E. coli* (APEC).[51, 52]
The results presented in this study add environmental context to the plasmid induced bacterial stress model described by Zahrl *et al.* (2006) (Figure 5).[29] Bacteria carrying an IncK2 plasmid that enters the chicken body, have a higher conjugation rate compared to an IncK1 plasmid, which results in higher expression and assembly of T4S. Using conjugation deficient mutants confirmed the role of increased conjugation rate of IncK2 plasmid in the presented pathway. Assembly of T4S leads to extracytoplasmic stress, which is sensed by CpxRA. Activation of the Cpx response leads to increased levels of σ-32. Increased levels of CpxRA were reported to have a role in colonization of *Salmonella* and *E. coli* APEC.[51, 52] All of the above data leads to the possible explanation why IncK2, and not IncK1 plasmids, are predominantly found in the chicken isolates by promoting a fitness advantage to the host bacterium at relatively high temperatures.

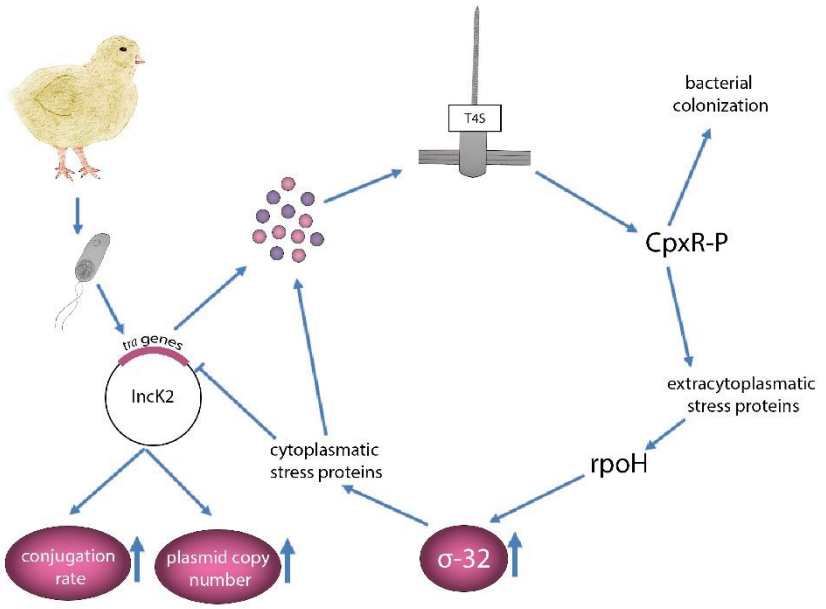

Figure 5 Schematic presentation of the environmental context to the plasmid induced bacterial stress model adapted from Zahrl et al. (2006), which explains the high

*prevalence of IncK2 (and not IncK1) in poultry isolates. E. coli carrying an IncK2 plasmid that enters the chicken gut expresses the tra proteins. Assembly of a T4S induces extracytoplasmic stress sensed by CpxR. CpxR, which was associated with increased bacterial colonization, induces the σ-32 response encoded by rpoH gene, resulting in a further upregulation of cytoplasmic stress proteins. *Arrow pointing up means less decrease in conjugation rate of IncK2 plasmid compared to IncK1 at 42°C.*

Obtained results show how chicken's body temperature influences plasmid fitness cost in *E. coli* host. Increased temperature leads to higher conjugation rate of IncK2 plasmids, which by inducing stress response, activates CpxRA which was proven to be involved in bacterial colonization. Fitness cost of IncK2 plasmid, in contrast to IncK1 plasmid, is not changed with higher temperature. Moreover, IncK1 plasmid copy number is abnormally high at the chicken body temperature, which may lead to the instability. These data shed a light on IncK2, and not IncK1, plasmid's success in invading chicken and gives a possible target to eliminate these plasmids from chicken isolates.

Data availability statement

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

Ethics statement

Faecal samples were taken either after excretion by the animal or by rectal swabs. All sampling falls within the guidelines of the Dutch Animals Act (https://zoek.officielebekendmakingen. nl/stb-2011-345.html) and the Animal Welfare Body Utrecht (http://www.ivd-utrecht.nl/en/), meaning no additional ethics approval was required.

Author contribution

MB, JW, BG-Z, DM, and JH designed the study, while MR and MB designed the laboratory experiments that were performed by MR. LM-G analyzed the data statistically. MR wrote the original draft. MB, LM-G, JW, BG-Z, DM, and JH critically reviewed the manuscript.

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Supplementary

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.02384/full#supplementary-material

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Chapter 7

General discussion

Antimicrobial resistance (AMR)

Antimicrobial resistance is a global problem. The World Health Organization (WHO) published a list of bacteria for which new antibiotics are urgently needed (ESKAPE list), which includes carbapenem-resistant *Acinetobacter baumannii, Pseudomonas aeruginosa* and carbapenem-resistant and ESBL-producing *Enterobacteriaceae* as critical targets.[1] Moreover, resistant bacteria from either humans, animals or the environment can spread from one domain to another (Figure 1). Travel and global trade of food and livestock are driving factors of the spread of AMR between countries. Therefore, a "One Health" collaboration between WHO, the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) was established in order to join efforts in combating AMR. All three organizations adopted a resolution on the containment of antimicrobial resistance in their general assemblies in 2015, followed by a resolution at the UN general assembly in September 2016.

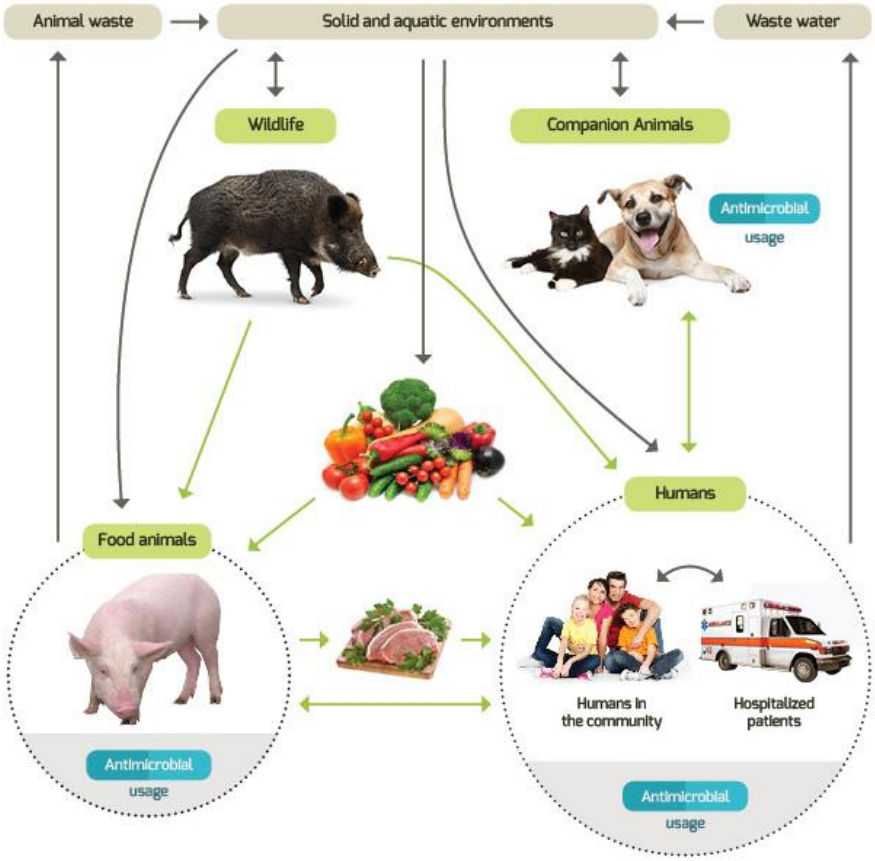

Figure 1 Routes of AMR spread. Figure obtained from EFFORT project. [2]

Mechanisms of AMR

AMR can be acquired through mutations in the chromosomal DNA, which can only spread vertically in bacterial clones by cell division. Examples are resistance to rifampicin, streptomycin, fluoroquinolones and oxazolidinones that arise due to the mutations in target genes: DNA polymerase, *rrs* or *rspL* genes, and DNA topoisomerase II and IV, respectively.[3] Another example of a vertically spreading resistance determinant is the chromosomally encoded SCC*mec* cassette of *Staphylococcus aureus*.[4]

Bacterial cells can also become resistant to antimicrobials via Horizontal Gene Transfer (HGT). HGT is mediated by three distinct processes. First of all by transformation, which is a process of DNA uptake. Second of all by transduction, which is DNA transfer mediated by phages and lastly conjugation, a process of plasmid exchange between two bacterial cells.[5] Conjugation of plasmids is the most important mechanism for the spread of AMR in *Enterobacteriaceae*.

Scope of the thesis

Plasmids are important carriers of antimicrobial resistance genes in *Enterobacteriaceae*. To understand their evolutionary success and high persistence, more knowledge about interactions between plasmids and their host, their physiology and genetic relatedness is necessary. Therefore, the aim of the thesis was to examine the genetic and physiological basis for the global spread of a highly successful group of epidemic plasmids, the IncI-complex.

Chapter 2 provides background knowledge about each plasmid group, reviews the knowledge about plasmids in *Enterobacteriaceae* and the resistance determinants they carry.

Chapter 3 describes IncK plasmids and compatibility patterns of the members of this group. This chapter concludes that the IncK plasmid group can be subdivided into two separate lineages designated IncK1 and IncK2.

Chapter 4 describes the phylogenetic relationship between plasmids belonging to the I-complex. It revises the incompatibility relationship between IncB/O and IncZ plasmids. The conclusion of this chapter is that IncB/O and IncZ plasmids are incompatible and it is suggested that these groups could be merged.

Chapter 5 elaborates about the molecular basis of incompatibility of IncK1 and IncK2 plasmids described in **Chapter 3**. It showed that one mutation in the loop of the RNAI structure of IncK1 plasmid can change its compatibility pattern. Mutations at other positions had a milder effect on compatibility.

Chapter 6 studies the host adaptation of IncK plasmids described in **Chapters 3 and 4.** It shows that the high conjugation rate of IncK2 plasmids initiates heat-shock response which mediates its adaptation to the chicken's high body temperature.

Plasmids

Plasmids spreading through conjugation are the main driver of the dissemination of antimicrobial resistance. However, spread is restricted by certain mechanisms that prohibit plasmids from spreading randomly through populations. One of the plasmids' features is their incompatibility.

Incompatibility

Incompatibility is the inability of two plasmids to be stably maintained in one cell. It is caused by the presence of identical or closely related, shared determinants between two plasmids.[6] It can be caused by plasmid partitioning or replication mechanisms. Partitioning is a system ensuring proper segregation of plasmids, after replication, to the daughter cells. Partition-based incompatibility can be caused by each of the partitioning mechanism components: ParA, ParB or *parS*.[6] Inappropriate plasmid segregation to the daughter cells leads to plasmid loss. Replication-based incompatibility of I-complex plasmids, IncF and IncL and IncM, is mediated by a small stem loop structure – RNAI, which inhibits translation of the *rep* mRNA.[7-16]

Chapter 5 discusses the role of point mutations in the structure of the RNAI molecule on the compatibility of IncK1 and IncK2 plasmids, building on work which was previously performed for IncB/O plasmids.[17] Expanding our knowledge about the molecular basis of plasmid incompatibility may be a milestone to create an *in silico* plasmid compatibility prediction tool. Such a tool would essentially be a database of known incompatibility determinants and its published genetic variants with the annotation of the effect on incompatibility. **Chapter 5** and other previously published manuscripts provide the knowledge that is necessary to create such a tool. They provide information about genetic variation of the incompatibility region and effect of these mutations of the incompatibility. Therefore, the starting point of creating such a database would be extracting all these available data. A next step would be to create a machine learning algorithm which would predict, based on current knowledge, if a plasmid of interest would be compatible or not with members of its own subgroup. Every probable "compatible" outcome should be further confirmed experimentally and added to the model in order to further improve the predictions.

Phenotype vs genotype

Plasmid incompatibility was historically tested via assessing the conjugation and stability of both plasmids in one cell. Later, a new method testing plasmid incompatibility based on DNA hybridization of the plasmid with a set of probes was developed. Unfortunately, both methods are time consuming and pose certain difficulties. The conjugation-based method requires selection markers, like antimicrobial resistance genes, on both tested plasmids. Furthermore, the conjugation-based method can only be used for conjugative plasmids, which is a big limitation of this method. Additionally, some other plasmid encoded features, which have nothing to do with incompatibility, can compromise the experiment. One of these features is entry exclusion, which is a barrier to the physical transfer of DNA between cells carrying isogenic or closely related entry exclusion systems.[18] The hybridization method is based on a set of probes which solved the problems of plasmids being non-conjugative or not having appropriate selective markers.[19]

However, the hybridization method does not allow testing for partitioning-based incompatibility. In order to test for partitioning-based incompatibility, both tested plasmids need to be present in the same cell and undergo a full cycle of replication and cell division. All of the above creates a need for a better solution.

Plasmid typing

Plasmids found in *Enterobacteriaceae* can be categorized into groups, using typing tools, based on their distinct features. One of the plasmid typing tools is called plasmid partition gene typing (PAR-T) and it is based on a multiplex PCR targeting the plasmid partitioning system.[20] An important relaxosome component, the relaxase encoded by the *mob* gene, is a target for another plasmid typing scheme called MOB-typing, dividing all known relaxases into six groups.[21, 22] A third typing scheme is Inc/rep typing. Inc/rep typing is the most frequently used plasmid typing scheme in *Enterobacteriaceae*.[23] It is based on a set of primers targeting genetic regions specific for each plasmid group (such as *rep* genes, iterons, RNAI) and is referred to as PCR-Based Replicon Typing (PBRT).[23] Inc/rep typing classifies plasmids into Inc groups and currently, there are 40 known plasmid types in *Enterobacteriaceae* (**Chapter 2**, Fang *et al*, 2018).[24] In *Enterobacteriaceae*, there is a high correlation between MOB and Inc/rep schemes, which means that plasmids of related replicon types have relaxases of a single MOB (sub) family.

Plasmid nomenclature

In the era of whole genome sequencing (WGS), more complete plasmid sequences are available, which allows comparison of these sequences. Determination of plasmid incompatibility groups is very important for epidemiological studies and proper plasmid mediated antimicrobial resistance surveillance. Additionally, it may lead to a discovery of interesting geographical associations like endemic IncP plasmid pSI54/04 in poultry from Hungary.[25] Further research showed a high prevalence of this plasmid in Austria and Poland, which may indicate a common source.[26]

With WGS being widely used for diagnostics, surveillance and epidemiological studies, it is crucial to establish harmonised plasmid nomenclature and properly annotated databases. **Chapter 4** discusses differences between plasmid groups determined with WGS and using classical incompatibility testing. It highlights that sequence-based plasmid types do not necessarily correspond with plasmid incompatibility groups designated using phenotypic methods. Therefore, it creates confusion when new plasmid groups are defined based on sequence differences in the conserved parts of the plasmid, but the name is still preceded by "Inc". One example is the IncX plasmid groups.[27, 28] Another example is previously proposed scheme introducing Z-2 till Z-6 amplicon types.[29] In my opinion this practice is confusing and should be avoided. For the new plasmid groups designated by sequence similarities, I propose the addition of "type" and number to the general plasmid group name for example "IncZ type 1". New names containing "Inc" should be created only when compatibility with other group members was confirmed. The main advantage of the scheme proposed here is the incorporation of sequence-based plasmid typing into existing Inc-typing based on incompatibility tests.

Plasmid mediated AMR (pAMR)

Plasmid mediated spread of AMR (pAMR) is a huge problem worldwide and its negative consequences for human health are well documented.[30] **Chapter 2** summarizes the knowledge about plasmids and pAMR in *Enterobacteriaceae* together with their hosts. It also highlights the crucial role of plasmids and their characteristics on the spread of AMR. Some plasmids are "generalists" – found in many counties in the world and isolated from various sources, others seem to be region or host specific. As an example, IncF plasmids are spread all over the world and can be isolated from various sources and bacterial species, while IncK2 plasmids are less often isolated and found predominantly in poultry. On the other hand, the main plasmid type isolated from *Enterobacteriaceae* in European poultry is IncI1, whereas in Asia, in poultry IncF is more abundant. These differences emerge from each plasmid's features, like its host range or fitness effects, as well as local evolution within the host and/or in the specific region of the world. Only detailed studies of particular plasmid – host/environment interactions will allow to understand the basis of this relationship.

Chapter 2 points out dependencies of plasmid epidemiology that can sometimes be explained by antimicrobial use (AMU) within a region. A recent example is the transferable colistin resistance gene *mcr-1*, discovered in 2015 in China.[31] Since then, *mcr-1* and other transferable colistin resistance genes were retrospectively discovered all around the world. Authors of the first manuscript describing *mcr-1* suggested that the high prevalence of this gene was caused by the use of colistin as a growth promotor in China. Besides, selection pressure by inappropriate use of polymyxins, HGT and host adaptation of a successful and epidemic plasmid type, IncI2 encoding *mcr-1* have attributed to its occurrence. Therefore, this gene can be found in animal and human reservoirs around the world. These findings led to the decision to ban the use of colistin as a feed additive in China, which became effective on April 30, 2017.[32] **Chapter 2** additionally shows that the prevalence of some plasmid-resistance gene combinations cannot be explained by AMU. It means that at some point these plasmids were selected for and because of their adaptations they managed to persist in many hosts all over the world. One example is I-complex plasmids with IncK1 plasmid encoding $bla_{\text{CTX-M-14}}$, IncK2 plasmid encoding $bla_{\text{CMY-2}}$, and IncI plasmid encoding $bla_{\text{CTX-M-1}}$ (**Chapters 2 and 3**).

Information about plasmid and resistance gene associations will allow studying source attribution and risk assessment for pAMR spread.[33, 34] Drivers of pAMR persistence and spread, other than antimicrobial resistance, are discussed below in the chapter "Drivers of the spread of pAMR". All above examples show that plasmids are the determining agents of the spread of resistance genes. Therefore, more attention should be paid to monitoring plasmids and not resistance genes only.

In 2005, a review similar to **Chapter 2** was published, laying the groundwork for a pAMR database.[35] Unfortunately, an update was not publicly available until the publication of **Chapter 2** in 2017. Although several plasmid databases have been created, they only focus on closed plasmid sequences.[36, 37] The drawback of these databases is the fact that most plasmids published nowadays are sequenced only with the Illumina platform, which does not always result in a full plasmid sequence. Therefore, such a database is incomplete and

would be a sub-optimal source for epidemiological studies. As such, one of the conclusions arising from **Chapter 2** is the urgent need to create a curated database containing the sequences of all AMR-carrying plasmids. Such a database will not only allow quick and easy access to all published plasmid types with their resistance genes, but can also be used to trace the spread of pAMR. Ideally, such a database should be managed by a consortium of several research institutes based on reports sent by individual researchers. A team from one institute should be responsible for the curation of the database. Other institutes can be involved in data preparation and quality control. Funding for the database would be provided by large publicly funded organizations such as FDA, CDC, ECDC and EFSA. Such a database would have a misbalance in the content based on the fact that in many regions in the world proper surveillance is not in place and/or WGS is not widely available. These gaps would have to be filled by the introduction of the surveillance programmes.

I-complex plasmids

The main focus of this thesis is the I-complex plasmids which are divided into four different incompatibility groups, I1, I2, K, B/O and Z, each with serologically and morphologically similar pili.[38] These low-copy-number plasmids have a size varying between 50 and 250kb, are conjugative and have a narrow-host-range.[39]

The I-complex plasmids are often found in *E. coli* and *S. enterica* strains isolated from poultry sources within Europe. (**Chapter 2**) The I-complex plasmids have been associated with a broad range of resistance genes which are often introduced into a specific region with other accessory functions and introduced via small transposable elements. The majority of the resistance encoding for ESBL and plasmid-mediated (p)AmpC genes were found on plasmids isolated from *E. coli* and *S. enterica*. (**Chapter 2**)

Each plasmid from the I-complex carries an unique set of resistance genes. Colistin resistance gene *mcr-1* and variant *mcr-1.3* and *mcr-1.5* are associated with IncI2 plasmids and have been isolated in different countries from both animal and human sources.[31, 40-44] The $bla_{\text{CMY-2}}$ gene is predominantly carried by plasmids from the IncI1-γ group.[45, 46] However, the spread of both $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-14}}$ in Europe (in particular in Spain and the UK) is mainly associated with plasmids from the IncK group isolated from animal sources.[47-55] Although less prevalent, IncB/O plasmids carry a wide range of resistance genes including $bla_{\text{CTX-M-1}}$, $bla_{\text{CMY-2}}$, $bla_{\text{ACC-4}}$, $bla_{\text{SCO-1}}$, $bla_{\text{TEM-1}}$, *sul1*, *sul2*, *aad*, *strA*, *strB* and *aacA4*.[46, 56-62] Genes encoding for resistance to ampicillin, chloramphenicol, sulphonamides and tetracycline have been found on plasmids belonging to the IncZ group.[63]

I-complex plasmid phylogeny

Studying plasmid phylogeny is very important for understanding plasmid relatedness and evolution which can be crucial during outbreaks. **Chapter 4** describes a phylogenetic analysis of I-complex plasmids based on both the *repA* gene and the RNAI region. Both *repA* and RNAI are conserved within a plasmid group. However, a phylogenetic tree of I-complex plasmids based on *repA* and RNAI shows different clustering of the members of plasmid groups. The *repA*-based phylogenetic tree shows a clear clustering of plasmids: the first cluster contains IncK1, IncB/O and IncI plasmids and the second cluster contains

IncK2 and IncZ plasmids. According to the RNAI-based tree, IncB/O, IncZ and IncI plasmids are phylogenetically distant from IncK1 and IncK2 plasmids. There are other examples of different plasmid clustering when different plasmid-backbone-genes were chosen to construct the phylogenetic tree.

Phylogenetic group discrepancies between RNAI-based, and other conserved plasmid genes-based analysis were shown for plasmid R471.[64] According to the core genome-, ExcA-, TraX- and TraY-based phylogenetic trees, this plasmid belongs to the IncL plasmid group, but RNAI-based analysis positions it within IncM1 cluster. These differences arise from separate evolution of discussed genes. A good solution for plasmid phylogenetic analysis can be the use of whole plasmid sequencing instead of single genes. Using whole plasmid multi locus sequence typing, similarly like it is already used for bacterial genomes, would provide a much higher discrepancy power and would exclude bias caused by separate evolution of plasmid genes. However, in order to study phylogeny of plasmid incompatibility, only its determinant should be used for the analysis. For example, RNAI for I-complex plasmids.

Drivers of pAMR spread

Antimicrobial use is one of the main drivers of dissemination of plasmids harbouring AMR-genes. However other factors like heavy metals can select for plasmids encoding its resistance in the absence of antimicrobials. Another crucial factor influencing plasmid spread and survival without selection pressure is its fitness cost.

Additional resistance carried - heavy metals

Metals are normally present in the soil and bacteria require them for growth. However, heavy metals can be toxic to bacteria, animals and plants, sometimes even at low concentrations. Therefore, over time, bacteria developed many resistance mechanisms to overcome toxic metal concentrations.[65]

Certain metals are commonly used in human medicine instead of antimicrobials. Copper and silver are used in various types of coatings for example: urinary catheters, protective respiratory face masks and Cu-impregnated socks.[66] Cu/Ag ionizers can be used for controlling *Legionella* in drinking water distribution systems in hospitals to mitigate nosocomial infections.[67] In animal production the metals are used as feed additives and it was proven that these animals can shed high concentrations of the metals.[68] However, the European Union is taking actions to limit animal exposure to heavy metals by restricting the use of zinc oxide in piglets.[69]

All examples described above show that bacteria in the environment, animals and humans are frequently exposed to heavy metals. Several studies demonstrated that presence of antimicrobial resistance genes can be highly correlated with the presence of heavy metals in the environment[70-73] which can be even stronger than the correlation between antimicrobial resistance genes and the presence of antimicrobials in the environment.[70]

A report on the co-existence of antimicrobial resistance genes and biocide or heavy metal resistance genes [74] showed that many of those genes are associated with antimicrobial resistance genes. One example is the *qacEΔ* gene, mediating tolerance to quaternary

ammonium compounds, that is correlated, amongst others, with: sulphonamide, streptomycin and tetracycline resistance genes, co-located on class-1 integrons. Another example is the cadmium resistance gene (*cadD*), which is correlated with *mph(C)*, *msr(A)* and *aph(3')-IIIa* conferring resistance to macrolides and aminoglycosides. The presented data shows that plasmids can encode numerous co-selecting genes that are beneficial for the bacteria in certain environments and increase their persistence.

Chapter 2 focuses on antimicrobial resistance genes, since these are considered to be the most recognisable plasmid selection factor. The evidence described above indicates that other factors can also select for the spread of plasmids. A database of antibacterial biocide and metal resistance genes was created together with a web-based tool to predict the resistance.[74] I believe that such a database should be included in a routine plasmid and bacteria analysis pipeline, together with an AMR-gene database like ResFinder, in order to better understand the drivers of plasmid spread and persistence.

Plasmid fitness cost and host specificity

Besides the presence of selective agents, fitness cost and adaptation to the host are key factors determining a success of plasmid spread. Plasmid fitness cost is defined as a burden to the bacterial host posed by a plasmid. It can be expressed by reduced growth rate or weakened competitiveness of plasmid-bearing strains under non-selective conditions.[75] However, plasmids can be beneficial to the host by providing fitness advantage. Such a benefit provided by a plasmid can be a driver of plasmid's spread. A high persistence of plasmids in absence of antimicrobial pressure, even though the plasmid fitness cost can be (relatively) high, is counter intuitive. Plasmids can adapt towards a certain niche or host by lowering its fitness cost through mutations or by carrying genes encoding for beneficial traits. For example, plasmids isolated from *Polaromonas* spp. from the Arctic and Antarctic Glaciers carry genes encoding proteins involved in [Fe-S] cluster assembly.[76] This cluster was never reported on plasmids before and may help to restore proteins that were damaged by UV-radiation. Another example comes from the bovine rumen plasmidome, where rumen-enriched functions like polyketide synthase, glycosyl transferase and hydrolase are encoded.[77] Another example IncHI plasmid pEQ1 encoding a *fos* operon, which is involved in the metabolism of scFOSs and therefore provides an advantage in the horse with carbohydrate-rich diet.[78] This plasmid-encoded feature increases bacterial adaptation to an animal host. **Chapter 3** highlights the importance of the correlation of certain plasmids with their animal or human host. IncK2 plasmids were found predominantly in poultry isolates, whereas IncK1 plasmids are reported in various sources. Additionally, **Chapter 6** showed that an IncK2 plasmid has a higher fitness cost compared to IncK1 at 42 °C. One of the traits that can reduce plasmid fitness cost is decreasing the conjugation rate. However, IncK2 plasmids have a substantially higher conjugation rate at 42 °C compared to IncK1 plasmids. All of the obtained results were pointing out that the IncK2 plasmid is encoding a non-obvious beneficial trait which allows it to persist in the bacterial cell, regardless of its high fitness cost. Further investigation showed that a higher conjugation rate of the IncK2 plasmid was inducing a stress response in the host bacteria. This led to the higher expression of σ-32, which is responsible for the heat shock response. All these results indicate adaptation to the chicken body temperature, which is higher than in other animals. In conclusion, the presented

evidence demonstrates that other features, like metabolic pathways, protective functions and certain physiological advantages can be selective for plasmids in a niche, the same way as antibiotic resistance is selective in the presence of antimicrobials.

The obtained results point out an interesting fact: high plasmid fitness cost and its persistence is a paradox. Therefore, it is worthwhile, while examining plasmids with a high fitness cost, to look for the adaptive traits that can be encoded on the plasmid. Understanding these adaptive traits, that stabilise plasmids in the population even without selective pressure, can point out future targets to destabilize plasmids during antimicrobial therapies.

All plasmids have to balance low fitness cost, allowing quick and easy spread to different hosts and niches, with high fitness cost and higher host adaptation. Using networks (described later) to model plasmid fitness cost in each host, may possibly provide knowledge about genes that are present only in certain animal/human host. Such an information may serve to predict the likelihood of plasmid spread.

Possibilities of containing pAMR

AMR surveillance

Although surveillance as such does not contain pAMR, knowledge of the trends in prevalence patterns of antimicrobial resistance genes carried by plasmid groups will help to understand how AMR spreads. Surveillance of AMR is the first step to achieve that.

AMR surveillance programmes in the EU can be divided based on different types of resistance surveillance: in commensals and pathogens in the food chain - European Food Safety Authority (EFSA), in the general population for humans - European Centre for Disease Prevention and Control (ECDC) and ECDC coordinated network for hospital acquired infections - Healthcare-associated Infections Surveillance Network (HAI-Net)

A surveillance system financed by the veterinary pharmaceutical industry is called Centre Européen d'Etudes pour la Santé Animale (CEESA).[79] It conducts four AMR resistance surveillance and monitoring programmes across Europe. First of all The European Antimicrobial Susceptibility Surveillance in Animals (EASSA), which examines the antimicrobial susceptibility of zoonotic and commensal bacteria in healthy food animals; secondly VetPath, looking at major disease-causing bacterial pathogens in food-producing animals; thirdly ComPath, monitoring susceptibility of pathogens from diseased cats and dogs and lastly MycoPath, focusing on disease-causing mycoplasma in food-producing animals.[80]

So far, all the monitoring programmes in the EU are based on phenotypic information. Whole genome sequencing (WGS) has the advantage of revealing genetic background of antimicrobial resistance which can further help understand how the resistance spreads in bacterial communities. Multiple studies have proven that WGS mostly provides data that is in agreement with phenotypic results.[81-84] WGS is not adapted as a routine surveillance tool in the EU yet. However, in 2019, EFSA prepared a joint report together with ECDC about collection and analysis of whole genome sequencing data from food-borne

pathogens and other relevant microorganisms isolated from human, animal, food, feed and food/feed environmental samples.[85] The report proposed a gradual shift of the surveillance towards WGS. WGS is undoubtedly the tool with the highest discriminatory power available so far. However, complexity of the data analysis requires implementation of strict guidelines about sequencing procedure, including DNA isolation, WGS library preparation, WGS sequencing machine, data analysis and reporting the results in order to be able to compare the data from different countries. Additionally, the fact that implementation of WGS as a routine surveillance tool is still impossible in lower-income countries, decreases the value of WGS as a global surveillance tool. Additionally, recovering complete plasmid sequence from the short-read WGS is still a challenge. Only obtaining complete plasmid sequences will allow correct attribution of pAMR to a plasmid type. In 2014 WGS for AMR surveillance was introduced in the USA.[86] As an alternative, whole metagenome sequencing (WMS) has been proposed.[87] WMS allows to sequence all the bacterial genomes present in a given sample, which, in contrast to WGS, also provides information about bacteria that cannot be cultured. A recent publication demonstrated how WMS can be used for global monitoring of antimicrobial resistance from urban sewage.[88] The main advantage of global sewage surveillance is that samples representing healthy human populations can be easily obtained and analysed without ethical concerns. Unfortunately, analysis of the resistome of such pooled samples does not allow to distinguish between chromosome and plasmid-encoded resistance mechanisms. Moreover, it will not identify the bacterial species that encodes the AMR-genes found.

Surveillance of pAMR is not routinely performed alongside surveillance of AMR and pathogens yet. Advancement of WGS as a surveillance tool would allow to perform pAMR monitoring. Such a system would provide the necessary amount of information to understand global spread and prevalence of pAMR.

A recent study examined attributable sources of carriage of *E. coli* with a variety of β-lactam resistance genes.[89] Studies like this would greatly benefit from including information about the plasmid, on which the resistance gene is carried. WGS could provide the data about all plasmids found in an isolate and the resistance genes they carry. With complete plasmid sequences being available, multiple analysis like comparative genomics and phylogenetic analysis based on single nucleotide polymorphism (SNPs) can be performed.[90] These results can be further used for the epidemiology and source attribution analysis, which will greatly improve our knowledge about pAMR spread. Additionally, knowing the complete plasmid sequence together with all resistance genes carried by the particular plasmid, may influence the intervention strategies to reduce or possibly eliminate pAMR. For all plasmid-curing methods (discussed in detail below) it is important to know which plasmids are present in a niche and to obtain their sequence. Targeting only some of the plasmids can result in establishing further spread of the remaining plasmids. Finally, for a CRISPR-Cas based method, which could target resistance genes on the plasmid,[91] it is crucial to know which resistance gene is encoded on which plasmid, so all of the AMR plasmids can be eliminated. All above arguments show a great need to implement pAMR surveillance.

Reduction of antibiotic use

In 2015, the WHO adopted a Global Action Plan in which one of the objectives was to optimize the use of antimicrobial medicines in human and animal health. Additionally, it calls for the development and implementation of National Action Plans. While several countries have already made changes in policy, others are still drawing up their National Action Plans. One example is the Netherlands, where after government and the animal industry measures were taken by to reduce antimicrobial usage, a 64% reduction of total AMU was obtained.[92] Additionally, antimicrobial usage and resistance levels was continuously monitored.[93] One study concluded that reduction of antimicrobial use reduced total resistance levels in the pig and veal calf industry over a five year period (2009-2014). Further decrease in use of these drugs was projected to have even clearer impact. However, relationships in dairy cattle and poultry were weaker than expected. It was suggested that this is the result of co-selection of multi-resistant bacteria with combinations of plasmid and chromosomally encoded AMR-genes. Another Dutch study analysed antimicrobial resistance trends for individual antimicrobial classes in commensal *E. coli* from broilers, slaughter pigs and veal calves over a period of 18 years.[94] It showed that after the intervention on antimicrobial use, since 2009 there was a significant decrease in resistance trends in broilers and slaughter pigs for all examined antimicrobials.

Unfortunately, the problem of multi-resistant strains limits the outcome of reduced antimicrobial use. Many of the resistance genes are grouped in mobilizable cassettes which can be shared between strains. It means that reduction of use of one antimicrobial is not enough to eliminate such a resistance cassette. Additionally, many plasmids carry genes which encode for resistance to heavy metals, which is an additional selection marker, as mentioned above. European Union made a directive specifying maximum limits for heavy metals such as arsenic, lead, mercury and cadmium in certain feed materials as feed additives.[95] However, removing all of the heavy metal contamination from the environment is nearly impossible. Additionally, the fact that all of these resistance genes are encoded on mobile genetic elements creates a threat of acquiring new resistance genes and therefore changing the associations patterns that we know now.

Influence of farming strategy and meat production on containing pAMR

Farming structure and management can highly influence the outcome of antimicrobial use reduction intervention. Farming management mainly influences the spread of AMR-carrying bacteria. However, understanding how farming management influences spread of AMR-carrying bacteria can help containing pAMR spread.

Poultry and pig production is organized as a pyramidal structure. If there is transfer of resistance determinants from higher levels (parent, grand-parent animals), a top down approach is important to prevent transfer of resistance to the lower levels. Broilers have a very short life-cycle and there is no 'microbiological' contact between mother and offspring except through bacteria transferred with the egg. As farms have an all in-all out management, the exposure of animals to resistant bacteria from previous cycles is relatively low. However, in previous studies it was demonstrated that a Grand Parent stock (GPS) chicken can already be contaminated with $bla_{\text{CMY-2}}$[96] and spread of AMR between levels of the pyramid was shown previously.[97-99] For laying flocks the same holds, except

for a considerable longer lifecycle. In pigs, a comparable vertical system is used but until piglets are weaned, they are exposed to their mother's microbiome. In such a pyramidal structure, the origin of animals is much better controlled compared to e.g. European veal calves who are coming from many different dairy farms, bringing in bacteria from many countries. These young animals are also highly susceptible for infections, which results in relatively high AMU.[100] The differences in breeding system and farm management are reflected in the levels of resistance for these different groups of animals. It shows that not all resistance genes can be eliminated by improved hygiene of the stables and AMR in the parental animals should be addressed.

pAMR prevention

A strategy named competitive exclusion was proposed to protect newborn animals from colonization by bacteria carrying pAMR. Competitive exclusion is the protective effect of a natural intestinal bacterial flora in limiting colonization with certain bacterial pathogens.[101] Several studies proved that challenging young chicks with probiotics provides a short term decrease of colonization by *E. coli* and *S. enterica* and a reduction of ESBL-producing *E. coli* in the faeces of chickens.[102-106]

Chapter 6 showed that because IncK2 plasmids cause a fitness cost to their bacterial host, it can possibly be outcompeted by a plasmid-free bacterium. However, another study with a longer observation period showed that plasmid-carrying *E. coli* eventually spread to all tested animals.[107]

Strategies to eliminate pAMR

Conjugation inhibitors

Conjugation was previously targeted in order to stop pAMR spread using various chemical compounds targeting conjugation.[108] It is postulated that lowering, or even abolishing, conjugation will stop the spread of pAMR. Secondly, targeting one of the conjugation machinery components with specific antibodies demonstrated to protective effect against *S. aureus* and *E. faecium* in the mouse model.[109] Unfortunately, conjugation inhibitors tend to be replicon specific and none of the known substances can fully prevent conjugation. Therefore, conjugation inhibitors will probably not be the solution to the pAMR problem. On the other hand, antibodies directed against conjugation systems show very promising results and hopefully it will result in a future therapy. Despite its perceived success in animal trials, there are no products developed for use in practice. Possibly due to strict safety regulations that limit licencing of such products.

Chapter 6 points out that the high conjugation rate of the IncK2 plasmid was a driver of its adaptation to the chicken host. Inhibiting conjugation would remove the advantage of IncK2 plasmids and may lead to IncK2 plasmid eradication from chickens.

Plasmid curing – incompatibility

Understanding the basis of plasmid incompatibility and their classification to certain incompatibility groups is of crucial importance for the possibility of containing pAMR plasmids *in vivo*. Several published studies describe the curing of pAMR plasmids due to plasmid interference.[110, 111] The principal of the method is the introduction of the vector carrying the incompatibility determinant against a target plasmid. A recent paper described

successful *in vivo* elimination of plasmid using plasmid interference in mice with normal gut microbiota.[112]

Chapters 3 and **4** discuss incompatibility experiments performed on plasmids belonging to the IncK, IncB/O and IncZ plasmid groups. It allowed to determine that the IncK plasmid group should be divided into IncK1 and IncK2. It was also found that IncB/O and IncZ plasmids are incompatible and should be considered one group, named IncB/O-Z. Additionally **Chapter 5** showed that only a single mutation in the RNAI structure can change the incompatibility pattern of an IncK1 plasmid. Such sensitivity of the incompatibility system is a major obstacle in designing an incompatibility-based plasmid curing system. Plasmid curing is highly replicon specific and requires delivery of a modified vector. Additionally, small changes in the replication control machinery can provide "immunity" to the curing vector.

Understanding the basis of incompatibility of different plasmids can result in a design of a vector that can target multiple plasmids at the same time, which would make this method way more effective against pAMR. The main limitation of available solutions is the use of GMO strains, the necessity to use antimicrobials to select for incompatibility vectors and the possibility of development of resistance to the vector. Overcoming the problem of the need to use antimicrobial selection along with the change in regulations about the use of GMOs could make it a viable option. However, the use of GMOs as therapeutics is a very sensitive issue with a lot opposition based on perception of risks related to use in patients.

CRISPR-Cas based systems

CRISPR-Cas is a bacterial system that recognizes and degrades foreign DNA. The system is based on the Cas protein binding and cleaves specific CRISPR RNA (crRNA), originating from spacer DNA segments.[110] A CRISPR-Cas system can be engineered to target AMR plasmids. Many targets like: AMR genes, toxin-antitoxin system or plasmid backbone genes like replicase were chosen and as a result plasmid loss or antimicrobial re-sensitization was obtained.[91, 113, 114] Additionally, the CRISPR-Cas system can be used as anti-plasmid vaccination.[115] The biggest problem with the CRISPR-Cas approach is the delivery system that requires either conjugation, transformation, use of phagemids or bacteriophages. Additionally, most studies were performed *in vitro* with only limited *in vivo* attempts performed only on a skin colonization model.[115]

Antisense nucleotides

An interesting alternative to tackle the pAMR problem can be antisense oligonucleotides, which are gene expression inhibitors.[116] They can be targeted against every essential bacterial process, therefore developing resistance seems unlikely. The main challenges of antisense nucleotide therapy are finding an effective prokaryotic delivery system, lack of knowledge about both the exact RNA structure of the target and the region of RNA where the interference will have the best effect.[116]

Applicability of the known methods

Unfortunately, all described methods have disadvantages. More research is needed to improve the available methods and to make them future proof. Moreover, applicability of these methods has to be tested on hosts with a functional microbiome. None of the

presented methods was fully successful by itself. The main problems are lack of efficient delivery system and not satisfactory efficiency. In my opinion, a combination of different strategies may be more useful. Combining the challenge of newborn animals with probiotics followed by administration of anti-plasmid and anti-conjugation drugs may decrease the plasmid load enough that it would be eliminated. Because of the possible high cost of such an approach, besides humans, only animals higher up the pyramid should be treated like GPS in poultry. Additionally, using such a therapy in fast-growing broiler chickens creates an opportunity for rapid development of resistance. It remains questionable if EMA or EFSA will ever allow the licencing of drugs that interfere with basic prokaryotic genetic or cellular functions and for which a true safety assessment will be very complex to perform and conclude upon.

Global consequences of containing pAMR

Balanced intestinal microbiota play a crucial role in protecting humans from pathogens.[117] Containing bacteria with pAMR from the gut microbiome may result in a dysbiosis caused by the competition between plasmid-carrying and plasmid-free strains. Removal of the costly plasmid would shift the fitness cost/benefit ratio and further possible eradication of certain strains from the gut. As an example, eradication of *Helicobacter pylori* from the human gut was proven to cause dysbiosis.[118] Such a dysbiosis could make humans or animals more vulnerable to certain bacterial infections. Moreover, reduction or loss of some plasmid-carrying species, which will enable other (resistant) species to exploit empty niches.[119]

Microorganisms in the gut form genetic exchange communities (GEC). Both wide- and narrow host range plasmids in natural populations spread mainly towards their kin (clone-mates).[120] The bias of transfer towards the kin was 10-fold higher compared to non-kin. It was shown that lowered antibiotic pressure (resulting in sub-lethal antibiotic concentrations) leads to more rapid evolution of resistance, reduced cost of the resistance and what's most important – higher plasmid spread.[121] This may result in higher rate of organisms moving to a different niche or host.

So far, the only available solutions allow to eliminate conjugative plasmids or the one carrying certain resistance determinants. Although few alternatives for antimicrobials are available, like antimicrobial peptides and phage therapy, they are all predicted to select for clones that are naturally resistant to this treatment.

In my opinion containing pAMR requires multidisciplinary collaboration of microbiologists, epidemiologists, medical doctors and mathematicians in order to predict and fully understand the consequences of our interventions.

Conclusions and future perspective

The results presented in this thesis highlight the importance of plasmids in the spread of AMR. It especially underlines the importance of understanding plasmid compatibility and correlation with certain bacterial and/or animal hosts. Knowing and understanding these differences will help improving pAMR surveillance as a basis for control.

This thesis also points out the necessity to create a database of all sequenced plasmids. It would make global plasmid surveillance and tracking of resistance more feasible. An interesting approach to present and manage large amounts of plasmid sequence data is a network database.[122] It analyses plasmids in a gene-sharing network, which allows to determine genes important for adaptation to ecological or geographical environments. Using a network approach instead of classical phylogenetic analysis would provide higher discriminatory power to study plasmid relationships and eliminate difficulties caused by the separate evolution of plasmid genes. Hopefully, future plasmid databases will be arranged as networks.

Many aspects of plasmid phylogeny or adaptations to the host are not known. This thesis provides new insights into the phylogeny and compatibility of I-complex plasmids. It demonstrates that IncB/O and IncZ, for which compatibility was argued in the past, are incompatible. This thesis also shows that the IncK plasmid group should be divided into IncK1 and IncK2. These findings are of crucial importance for plasmid typing and epidemiology. Only by understanding plasmids and their relationships, we can try to understand the spread of AMR.

This thesis also raises the issue of plasmid nomenclature. Plasmid groups designated by incompatibility testing should not be confused with the groups based on sequence homology. There is an urgent need to apply changes in the plasmid nomenclature in order to avoid confusion about true (in)compatibility of plasmid groups. The solution presented in this thesis is a proposal and could be adjusted. Existing plasmid databases contain many sequences that are inadequately annotated and/or lack metadata. Creating a plasmid database as mentioned before, with appropriate annotation and metadata available, would help creating and sustaining appropriate plasmid nomenclature. Because creating a new database is very costly, an alternative solution can be more stringent curation of existing databases.

Additionally, this thesis elaborates on the basis of compatibility of IncK plasmids. It highlights that a single mutation in the replication control can change the compatibility of the plasmid. This knowledge is especially important to apply plasmid curing methods in order to eliminate pAMR. Additionally, the knowledge about the molecular basis of plasmid incompatibility can be a basis for the design of an incompatibility prediction tool. Such a machine learning-based prediction tool would allow quick and reliable plasmid compatibility prediction from WGS data instead of laborious conjugation-based compatibility testing.

Moreover, this thesis shows the basis of IncK2 plasmid adaptation to the chicken host. It highlights the necessity to study plasmids in the host environment context. Understanding plasmid adaptation to the host can provide new targets to eliminate pAMR. Creating plasmid databases would allow using machine learning algorithms to define bacterial host attribution and signatures of host adaptations.[123, 124]

Several methods to eliminate pAMR were developed, but only few were assessed *in vivo.* More research should be devoted to assess possibilities to eliminate pAMR *in vivo*. Creating combined therapy including probiotics treatment followed by anti-plasmid

treatment could be considered. Further reduction of AMU will greatly benefit the efforts to contain pAMR. However, because containing pAMR poses a great challenge, options for alternatives of antimicrobials should be explored, especially. Such an alternative antimicrobial should be used exclusively in human as last resort treatment.

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Appendix

Summary

Antimicrobial resistance (AMR) is a growing problem worldwide. Within the group of, *Enterobacteriaceae* AMR spreads mostly through mobile genetic elements like plasmids. Plasmids are extrachromosomal pieces of DNA, mostly circular, that can vary in size from less than a kilobase to several megabases. They are self-replicative and some of them encode conjugation or mobilization machinery, allowing plasmids to spread horizontally between bacterial cells. Additionally, plasmids can carry accessory genes encoding for antimicrobial resistance or other niche adaptive properties like metabolic functions and heavy metal tolerance.

An important plasmid feature is the inability of two similar plasmids to be stably maintained in one cell, which is called plasmid incompatibility. This phenomenon is regulated by partition or replication mechanisms. Replication-based incompatibility is caused by the RNAI structure. Based on incompatibility, plasmids are categorized into 40 "Inc" groups.

Chapter 2 provides an overview of all known AMR-related plasmid families in *Enterobacteriaceae*, the resistance genes they carry and their geographical distribution. The identification of plasmid characteristics and their association with different bacterial hosts provides crucial knowledge to understand the contribution of plasmids to the transmission of AMR determinants. Molecular identification of plasmid and strain genotypes elicits a distinction between spread of AMR genes by plasmids and dissemination of these genes by spread of bacterial clones.

The I-complex plasmid group, designated due to the morphological and serological properties of their pili, consists of phenotypically incompatible plasmids including IncI1α, IncI1γ, IncI2, IncK, IncB/O and IncZ. **Chapter 3** analyzed IncK plasmids: their animal hosts, phylogenetic relationships and incompatibility. $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-14}}$ are among the most prevalent resistance genes associated with IncK plasmids. Phylogenetic analysis based on IncK plasmids' accessory genomes showed distinct clusters. One cluster included IncK plasmids encoding $bla_{\text{CTX-M-14}}$ and isolated from *Escherichia coli* from various sources. A second cluster included IncK plasmids associated with $bla_{\text{CMY-2}}$ isolated from *E. coli* from poultry only. IncB/O plasmids included in this analysis, instead of clustering together, form two separate clusters that position in between IncK1 and K2 plasmids, which was further analysed in **Chapter 4**. Incompatibility experiments show that IncK plasmids from two distinct phylogenetic clusters can be present together in one cell and therefore are compatible. Thus, the IncK plasmid group consists of two compatible plasmid lineages which are referred to here as IncK1 and IncK2.

The relationship between plasmids of the phenotypic incompatibility groups IncB/O and IncZ is unclear. Their inability to co-exist was initially referred to as dislodgement, meaning that in a bacterial population carrying both IncB/O and IncZ plasmids, the resident one was rapidly and exclusively lost. In contrast, other research reached the conclusion that IncB/O and IncZ plasmids are incompatible. In **Chapter 4** we re-evaluated the relationship between IncB/O and IncZ plasmids to settle these conflicting conclusions. Our data supports the previous suggestion that IncB/O and IncZ plasmids may be considered phenotypically incompatible.

For I-complex plasmids, replication-based incompatibility is caused by the small antisense RNA stem-loop structure called RNAI. The RNAI structure of the two compatible IncK plasmid groups, IncK1 and IncK2, differs only by five nucleotides. In **Chapter 5** we constructed minireplicons. Minireplicons are plasmids derivatives which only contain their replication region and a selection marker. Minireplicons allow to study the influence of only the replication region on the compatibility of the plasmid. In our case the minireplicon containing the replication region of IncK1 or IncK2 plasmids coupled with a kanamycin resistance marker. Then, we performed single nucleotide mutagenesis targeting the five nucleotides that differ between the IncK1 and IncK2 RNAI sequences. The obtained results show that a single nucleotide change in the RNAI structure can change the compatibility of these plasmids.

The IncK plasmid group can be divided into two separate lineages named IncK1 and IncK2. IncK2 is found predominantly in poultry while IncK1 was reported in various mammals, including cattle, cats, dogs and humans. The physiological basis of this distinction is not known. In **Chapter 6** we examined fitness cost of IncK1 and IncK2 plasmids at 37 °C and 42 °C, which resembles mammalian and chicken body temperatures, respectively. We analyzed conjugation frequency, plasmid copy number and plasmid fitness cost in direct competition. Additionally, we measured levels of σ-32 in *E. coli* carrying either wildtype or conjugation-deficient IncK plasmids. The results show that IncK2 plasmids have a higher conjugation frequency and lower copy number at 42 °C compared to IncK1. While the overall fitness cost to the host bacterium of IncK2 plasmids was higher than that of IncK1, it was not affected by the temperature, while the fitness cost of IncK1 was shown to increase at 42 °C compared to 37 °C. These differences correlate with an increased expression of σ-32, a regulator of heat-shock protein expression, in *E. coli* with IncK2 compared to cells containing IncK1. This effect was not seen in cells containing conjugation deficient plasmids. Therefore, it is hypothesized that the assembly of the functional T4S may lead to these increased levels of σ-32. Increased activation of CpxR at 42°C may explain why IncK2 plasmids, and not IncK1, are predominantly found in chicken isolates.

The results presented in this thesis highlight the importance of plasmids in the spread of AMR. It underlines the importance of understanding plasmid compatibility and its association with certain bacterial and/or animal hosts. Knowing and understanding these differences will help improving plasmid mediated antimicrobial resistance (pAMR) surveillance as a basis for control. This thesis provides new insights into the phylogeny and compatibility of I-complex plasmids. It demonstrates that IncB/O and IncZ, for which compatibility was argued in the past, are incompatible. This thesis also shows that the IncK plasmid group should be divided into IncK1 and IncK2. These findings are of crucial importance for plasmid typing and epidemiology. Only by understanding plasmids and their relationships, we can try to understand the spread of AMR. Additionally, this thesis elaborates on the basis of compatibility of IncK plasmids. It highlights that a single mutation in the replication control can change the compatibility of the plasmid. Moreover, this thesis shows the basis of IncK2 plasmid's adaptation to the chicken host. It highlights the necessity to study plasmids in the host environment context.

Nederlandse samenvatting

Antimicrobiële resistentie (AMR) is wereldwijd een groeiend probleem. Binnen de groep van *Enterobacteriaceae* vindt verspreiding voornamelijk plaats via mobiele genetische elementen zoals plasmiden. Plasmiden zijn stukjes extrachromosomaal DNA, veelal circulair, die variëren in grote van minder dan een kilobase tot meerdere megabases. Ze zijn zelf replicerend en kunnen coderen voor de conjugatie of mobilisatie machinerie, waardoor plasmiden zich horizontaal kunnen spreiding tussen bacteriële cellen. Verder kunnen plasmiden extra genen bevatten die coderen voor antimicrobiële resistentie of andere adaptieve eigenschappen zoals metabole functies of tolerantie tegen zware metalen. Een belangrijke eigenschap van plasmiden is het onvermogen om samen met en vergelijkbaar plasmide stabiel in één cel in stand te blijven, dit heet incompatibiliteit. Dit fenomeen wordt gereguleerd door partitie of replicatie mechanismen. Incompatibiliteit gebaseerd op replicatie wordt veroorzaakt door een RNAI-structuur. Op basis van hun incompatibiliteit kunnen plasmiden in 40 verschillende "Inc" groepen worden ingedeeld.
Hoofdstuk 2 geeft een overzicht van alle bekende AMR gerelateerde plasmidefamilies in *Enterobacteriaceae*, de resistentiegenen die ze bevatten en hun geografische verspreiding. De identificatie van plasmide kenmerken en hun associatie met verschillende bacteriële gastheren geeft cruciale kennis over de bijdrage van plasmiden in de transmissie van AMR. Moleculaire identificatie van genotypen van plasmiden en stammen geeft onderscheid tussen verspreiding van AMR-genen veroorzaakt door plasmiden of door de verspreiding van bacteriën.
De I-complex plasmide groep, gekenmerkt door de morfologische en serologische eigenschappen van hun pili, bestaat uit fenotypisch incompatibele plasmiden waaronder IncI1α, IncI1γ, IncI2, IncK, IncB/O en IncZ. **Hoofdstuk 3** analyseert IncK plasmiden: hun dierlijke gastheren, fylogenetische relaties en incompatibiliteit. *Bla*$_{\text{CMY-2}}$ en *bla*$_{\text{CTX-M-14}}$ behoren tot de meest voorkomende resistentiegenen die geassocieerd zijn met IncK plasmiden. Fylogenetsiche analyse op basis van IncK genomen toonde verschillende clusters aan. Eén cluster omvatte IncK plasmiden coderende voor *bla*$_{\text{CTX-M-14}}$ geïsoleerd uit *Escherichia coli* uit verschillende bronnen. Een tweede cluster bevatte IncK plasmiden die geassocieerd waren met *bla*$_{\text{CMY-2}}$, en alleen geïsoleerd zijn uit *E. coli* afkomstig van pluimvee. IncB/O plasmiden clusteren in deze analyse niet samen, maar in plaats daarvan vormen ze twee afzonderlijke clusters, gepositioneerd tussen IncK1 en K2 plasmiden. Dit is verder onderzocht in **Hoofdstuk 4**. Incompatibiliteit experimenten tonen aan dat IncK plasmiden van twee verschillende fylogenetische clusters samen in één cel aanwezig kunnen zijn, en dus compatibel zijn. De groep van IncK plasmiden bestaat daarom uit twee compatibele plasmide lijnen die hier worden aangeduid als IncK1 en IncK2.
De relatie tussen plasmiden van de fenotypische incompatibiliteit groepen IncB/O en IncZ is onduidelijk. Het onvermogen om naast elkaar stabiel in één cel te bestaan werd aanvankelijk aangeduid als 'dislodgement', wat betekent dat in een bacterie populatie die zowel IncB/O- als IncZ-plasmiden bevat, het initiële plasmide snel verloren gaat. Ander onderzoek kwam daarentegen tot de conclusie dat IncB/O en IncZ plasmiden niet compatibel zijn. In **Hoofdstuk 4** hebben we het verband tussen IncB/O en IncZ plasmiden opnieuw geanalyseerd in relatie met deze tegenstrijdige conclusies. Onze gegevens ondersteunen de suggestie dat IncB/O en IncZ plasmiden als fenotypisch incompatibel kunnen worden beschouwd.
Voor plasmiden uit het I-complex wordt incompatibiliteit gebaseerd op replicatie veroorzaakt door een kleine complementaire RNA hairpin genaamd RNAI. De RNAI

structuur van de twee compatibele IncK plasmide groepen, IncK1 en IncK2, verschilt slechts vijf nucleotiden. In **Hoofdstuk 5** hebben we minireplicons geconstrueerd. Minireplicons zijn derivaten van plasmiden die alleen nog hun replicatie gebied en een selectiemerker bevatten. Met minireplicons kan de invloed van enkel het replicatiegebied op de compatibiliteit van het plasmide worden bestudeerd. In ons geval bevat het minireplicon het replicatie gebied van IncK1 of IncK2 plasmiden gekoppeld aan een kanamycine resistentie gen. Vervolgens hebben we puntmutaties aangebracht op de vijf nucleotiden die verschillen tussen de IncK1 en IncK2 RNAI sequenties. De verkregen resultaten tonen aan dat de verandering van één enkel nucleotide in de RNAI structuur de compatibiliteit van deze plasmiden kan veranderen.
De groep IncK plasmiden kan worden onderverdeeld in twee aparte lijnen, genaamd IncK1 en IncK2. IncK2 wordt voornamelijk aangetroffen bij pluimvee, terwijl IncK1 is geïsoleerd uit verschillende zoogdieren waaronder runderen, katten, honden en mensen. De fysiologie achter dit onderscheid is niet bekend. In **Hoofdstuk 6** onderzochten we de fitness kosten van IncK1 en IncK2 plasmiden bij 37 °C en 42 °C, die respectievelijk lijken op de lichaamstemperatuur van zoogdieren en kippen. We hebben de conjugatiefrequentie, het aantal kopieën van het plasmide en de fitness kosten in directe competitie geanalyseerd. Ook hebben we expressie niveaus van σ-32 gemeten in *E. coli* die ofwel wildtype ofwel conjugatie deficiënte IncK plasmiden bevatte. De resultaten laten zien dat IncK2 plasmiden in een hogere frequentie conjugeren en een lager aantal kopieën hebben bij 42 °C in vergelijking met IncK1.
Hoewel de totale fitness kosten voor de bacterie met IncK2 plasmiden hoger was in vergelijking tot IncK1, werd dit niet beïnvloed door de temperatuur, ook werd aangetoond dat de fitness kosten van IncK1 bij 42 °C stegen in ten opzichte van 37 °C. Deze verschillen correleren met een verhoogde expressie van σ-32, wat de expressie van hitteschok eiwitten reguleert, in *E. coli* die IncK2 plasmiden bevat in vergelijking met IncK1 plasmiden. Dit effect werd niet gezien in cellen die conjugatie deficiënte plasmiden bevatte. Daarom wordt verondersteld dat de vorming van functioneel T4S kan leiden tot deze verhoogde niveaus van σ-32. Verhoogde activatie van CpxR bij 42 °C kan verklaren waarom IncK2 plasmiden, en niet IncK1, voornamelijk worden aangetroffen in kippen.
De resultaten in dit proefschrift benadrukken het belang van plasmiden bij de verspreiding van AMR. Het legt de nadruk op de noodzaak van het begrijpen van01 plasmide compatibiliteit, en de associatie ervan met bepaalde bacteriële en/of dierlijke gastheren. Kennis en begrip van deze verschillen zal helpen bij het verbeteren van plasmide-gemedieerde antimicrobiële resistentie (pAMR) monitoring als basis voor controle. Dit proefschrift biedt nieuwe inzichten in de fylogenie en compatibiliteit van I-complex plasmiden. Het toont aan dat IncB/O en IncZ, die in het verleden als compatibel werden beschouwd, incompatibel zijn. Dit proefschrift laat ook zien dat de IncK plasmide groep moet worden verdeeld in IncK1 en IncK2. Deze bevindingen zijn van cruciaal belang voor de typering en epidemiologie van plasmiden. Alleen door een beter begrip van plasmiden en hun relaties kunnen we proberen de verspreiding van AMR te begrijpen. Daarnaast wijdt dit proefschrift uit over de basis van compatibiliteit van IncK plasmiden. Het benadrukt dat een enkele mutatie in de replicatie de compatibiliteit van het plasmide kan veranderen. Bovendien toont dit proefschrift de basis aan van de aanpassing van IncK2 plasmiden aan de kip als gastheer, en benadrukt het de noodzaak om plasmiden te bestuderen in de relatie met de omgeving van hun gastheer.

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About the author

Marta was born on 27th of April 1990 in Warsaw in Poland. In 2011 she completed a one-month internships at Warsaw University of Technology, working on L-lactide polymerization with a catlyst containing magnesium, calcium, zinc, tin and immobilized enzymes. The same year she finished another one-month internship in the Institute of Biochemistry and Biophysics Polish Academy of Sciences working on global phenotyping of lactic acid bacteria. She completed her bachelor at the University of Warsaw, Institute of Microbiology. Her thesis was entitled "Construction of a recombinant plasmid for mutagenesis of *Campylobacter jejuni Cj0046* gene" which she followed up with a master's program in the same institute. In 2013 she completed a three months internship at Utrecht University, Department Infectious Diseases & Immunology on determining if antibodies against flagellin can block TLR5 activation. In 2014 she obtained her master's degree with the thesis on „Profile of substrate proteins of Dsb system from *Campylobacter jejuni* and *Helicobacter pylori*". In 2014 Marta started her PhD at the Veterinary Faculty of Utrecht University. Her research on plasmids, phylogeny and host adaptation is the subject of this thesis.