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Diagnostic of carbapenemase genes and their transmission through horizontal gene transfer

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Abbreviations and acronyms

A. baumannii	*Acinetobacter baumannii*	**ESBL**	extended-spectrum beta-lactamase
ANOVA	analysis of variance	**ESKAPE**	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species
bla	beta-lactamase	***et al.***	*et alii*
BLAST	Basic Local Alignment Search Tool	**EUCAST**	European Committee on Antimicrobial Susceptibility Testing
BHQ	Black Hole Quencher	**fw**	forward
bp	base pair	**FAM**	Carboxyfluorescein
BRIG	BLAST Ring Image Generator	**g**	gram
cDNA	complementary DNA	**GES**	Guiana extended-spectrum
C. freundii	*Citrobacter freundii*	**GIM**	German imipenemase
CFU	colony-forming units	**gyr**	gyrase
CIM	carbapenem-inactivation method	**h**	hour
cm^2	square centimeter	**HGT**	horizontal gene transfer
CO_2	carbon dioxide	**ICE**	integrative and conjugative elements
C. portucalensis	*Citrobacter portucalensis*	**i.e.**	*it est*
Ct	cycle threshold	**IMI**	imipenem-hydrolyzing beta-lactamase
CY	cyanine	**IMP**	imipenemase
DNA	desoxyribonucleic acid	**Inc**	incompatibility
E. cloacae	*Enterobacter cloacae*	**IS**	insertion sequence
E. coli	*Escherichia coli*	**JOE**	Dichloro-dimethoxy-fluorescein
e.g.	*exempli gratia*	***K. aerogenes***	*Klebsiella aerogenes*
E. hormaechei	*Enterobacter hormaechei*	**kb**	kilobase

KPC	*Klebsiella pneumoniae* carbapenemase	**PCR**	polymerase chain reaction
K. pneumoniae	*Klebsiella pneumoniae*	**pH**	potential hydrogen
LB	Luria Bertani	**qPCR**	quantitative real-time polymerase chain reaction
M	molar	**pol**	polymerase
MALDI TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry	**rv**	reverse
MDR	multidrug-resistant	**RAPD**	random amplification of polymorphic DNA
MF	McFarland	**RNA**	ribonucleic acid
mg	milligram	**rpm**	revolutions per minute
MHT	Modified Hodge test	**rRNA gene**	ribosomal RNA gene
MIC	minimum inhibitory concentration	**RT-qPCR**	reverse transcription quantitative polymerase chain reaction
min	minute	**s**	second
ml	milliliter	***S. aureus***	*Staphylococcus aureus*
MM	master mix	**SDS**	sodium dodecyl sulfate
mm	millimeter	***S. marcescens***	*Serratia marcescens*
mRNA	messenger RNA	**SME**	*Serratia marcescens* enzyme
NaCl	sodium chloride	**sp.**	species
NCBI	National Center for Biotechnology Information	**T4SS**	type IV secretion system
NDM	New-Delhi metallo-beta-lactamase	**T_m**	melting temperature
nm	nanometer	**VIM**	Verona integron-encoded metallo-beta-lactamase
NRL	National Reference Laboratory	**U**	unit
oriT	origin of transfer	**UV**	ultraviolet
OXA	oxacillinase	**WGS**	whole-genome sequencing
P	probe	**w/v**	weight per volume
P. aeruginosa	*Pseudomonas aeruginosa*	**µ**	micro
PBP	penicillin-binding protein	**°C**	degree Celsius

1 Introduction

1.1 The global burden of antimicrobial resistance

Infections with multidrug-resistant (MDR) bacteria are reported worldwide and represent an increasing threat to public health (Prestinaci *et al.* 2015). The excessive use and partly misuse of antibiotics, not only in public health but also in livestock and agriculture, are fostering this problem since bacteria can spread among humans, animals, and the environment (Michael *et al.* 2014; Prestinaci *et al.* 2015; Rousham *et al.* 2018; Shallcross and Davies 2014; Sun *et al.* 2020; Woolhouse *et al.* 2015). The development of antimicrobial resistance is a naturally occurring process, yet the selective pressure on bacteria under antibiotic treatment is accelerating mutations and the acquisition of new antimicrobial resistances (Abel zur Wiesch *et al.* 2011; Hay *et al.* 2018). Eradicating susceptible bacteria under constant antibiotic intake, by reducing the competition for resources, provides a growth advantage for bacteria harboring antimicrobial resistance genes. The appearance of resistant bacteria might be a rapid process in some circumstances, however, the eradication of them is more challenging (Levy and Marshall 2004). Resistant bacteria can emerge by mutations altering the antibiotic target structure that lower the inhibiting effect of the drugs (Aldred *et al.* 2014). Moreover, bacteria have the potential to share genetic material through horizontal gene transfer (HGT), which facilitates the spread of resistance genes, commonly located on mobile genetic elements (Read and Woods 2014). Since plasmids usually encode multiple resistance determinants, HGT represents a fast mechanism for the development of multidrug resistance (Bennett 2008).

MDR bacteria can cause severe infections, resulting in long hospital stays, since limited treatment options remain (World Health Organization 2020). Resistance to last-resort drugs, like carbapenems and colistin, are reported more frequently, thus infections are often linked to high mortality (Hay *et al.* 2018; Osei Sekyere *et al.* 2016). The number of deaths due to infections with MDR bacteria is predicted to increase over the next years (United Nations meeting on antimicrobial resistance 2016). Besides worse clinical outcomes, infections with MDR bacteria also represent an economic challenge, due to prolonged hospital stays resulting in higher medical costs (Dadgostar 2019; Zhen *et al.* 2018). A large variety of healthcare-associated infections is caused by ESKAPE pathogens, i.e., *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* species (Santajit and Indrawattana 2016). Especially the Gram-negative bacteria are of great concern since the interspecies transfer of mobile genetic elements is common and they consequently accumulate resistance mechanisms against different antibiotic classes. To counteract this problem, improvement of infection control in hospitals is implemented and antibiotic use is controlled closely (Coates *et al.* 2011).

1.2 Antibiotics – mechanism of action and bacterial evasion strategies

The discovery of penicillin by Alexander Fleming was an important event in human medicine for treating bacterial infections, hence leading to a drastic reduction of mortality caused by infections (Fleming 1929; Hutchings *et al.* 2019). Between 1950 and 1970, a large variety of different antibiotic classes were discovered, which prevent or decelerate bacterial growth by either interfering with the cell wall synthesis, impeding protein or nucleic acid synthesis, or by affecting metabolic pathways (see Figure 1, left part; Aminov 2010; van Hoek *et al.* 2011).

The bacterial cell wall and plasma membrane are essential for stabilization and protection against cell lysis due to osmotic pressure (Dörr *et al.* 2019). The maintenance of this complex structure is an active process in bacterial cells (Zeng and Lin 2013). Peptidoglycan represents the main component of the cell wall; therefore, antibiotics targeting different steps of the highly conserved peptidoglycan synthesis pathway are leading to destabilization of the bacterial cell and consequently cause cell death (Dörr *et al.* 2019; Elshamy and Aboshanab 2020; Kong *et al.* 2010; Zeng and Lin 2013). Beta-lactam antibiotics, such as penicillin, cefotaxime, or meropenem, are directed at the penicillin-binding proteins (PBP), which catalyze the cross-linking reaction of the peptidoglycan strands (Kapoor *et al.* 2017; O'Rourke *et al.* 2020). Another antibiotic class, the lipopeptides with polymyxin B as an example, is disrupting the cell by interacting with the lipopolysaccharides of the bacterial membrane (Peach *et al.* 2013; Hutchings *et al.* 2019).

Antibiotics can interfere with the nucleic acid synthesis that is crucial for bacterial replication, repair mechanism, transcription, and recombination (Yi and Lü 2019). As a variety of different protein complexes are involved in these essential processes, DNA replication is a favorable target for antibiotics, resulting in growth inhibition or cell death (Trojanowski *et al.* 2018; Windgassen *et al.* 2017). Antimicrobial compounds can affect various enzymes, such as DNA helicase, DNA-dependent RNA-polymerases, and topoisomerases, for instance, by preventing the unwinding of DNA, by priming and synthesizing a new DNA strand during replication, and by controlling DNA supercoils, respectively (Yi and Lü 2019). (Fluoro-)quinolones, such as nalidixic acid or ciprofloxacin, are targeting the topoisomerase II, also named DNA gyrase, and the topoisomerase IV, which are essential for unwinding and coiling of the bacterial genome, therefore blocking DNA replication (Aldred *et al.* 2014; Fàbrega *et al.* 2009; O'Rourke *et al.* 2020). Rifampicin, belonging to the ansamycins, is interacting with the DNA-dependent RNA polymerase, preventing transcription of genes into mRNA (Hutchings *et al.* 2019; Peach *et al.* 2013).

Translation of mRNA into a functional protein is another fundamental process for the viability of bacterial cells. Antibiotics, such as tetracyclines, aminoglycosides, or macrolides were developed targeting either the small subunit (30S) or the large subunit (50S) of the ribosome, impeding the initiation, elongation, or termination step in generating polypeptides (Arenz and Wilson 2016; Hutchings *et al.* 2019). Tetracyclines, as an example, prevent the binding of tRNAs to the ribosome (Lin *et al.* 2018).

The last option to suppress bacterial growth concerns metabolic pathways. Sulfonamides interfere with an enzyme of the folate biosynthesis pathway, inhibiting thymidine production, which is essential for various metabolic processes (Sköld 2000; van Hoek *et al.* 2011).

As antibiotics target several bacterial structures and pathways, microorganisms evolved various strategies to pass over susceptibility (see Figure 1, right part). MDR bacteria frequently accumulate different resistance mechanisms to withstand the toxic effect by diminishing the levels of incorporated antimicrobial substances, modifying or degrading the antibiotics, or altering and protecting the bacterial target sites (Munita and Arias 2016; Reygaert 2018). Depending on the bacterial species some resistances occur naturally, especially concerning changes in antibiotic absorption and release, as described for *P. aeruginosa* and *Escherichia coli* among others (Lomovskaya and Lewis 1992; Nikaido 1994). Spontaneous mutations furthermore contribute to intrinsic resistance (Martinez and Baquero 2000). Lowering the intracellular concentration can be accomplished by overexpression of chromosomally encoded non-specialized multi-component efflux pumps such as AcrAB/TolC in *E. coli* (Aldred *et al.* 2014; Peterson and Kaur 2018). Porins, channels in the outer membrane, can be modified or their expression can be down-regulated, which in turn prevents or lowers the uptake of antimicrobial agents (Goudarzi and Navidinia 2019; Peterson and Kaur 2018). A deletion mutation in the porin chromosomally encoded gene *oprD* in *P. aeruginosa*, for instance, reduces susceptibility to carbapenems (Kos *et al.* 2016).

Another major way for antibiotic resistance is the production of enzymes degrading or altering the antibiotic's structure. Depending on the enzyme variant, beta-lactamases hydrolyze the beta-lactam ring of penicillins, cephalosporins, or carbapenems, preventing interactions with the PBP (Fernandes *et al.* 2013; Goudarzi and Navidinia 2019; Levy and Marshall 2004; Zeng and Lin 2013). Aac(6')-Ib-cr, an aminoglycoside acetyltransferase modifies fluoroquinolones resulting in reduced toxic potency (Aldred *et al.* 2014; Goudarzi and Navidinia 2019).

Target alteration is another strategy to counteract the antibiotic effect. For instance, in PBPs structural changes by point mutations can occur, decreasing the affinity of beta-lactams to interact with the PBPs (Goudarzi and Navidinia 2019; Peterson and Kaur 2018; Zeng and Lin 2013). Since fluoroquinolones are targeting topoisomerases, changes in GyrA and ParC, subunits of gyrase and topoisomerase in Gram-negative bacteria, are likely, lowering interactions events, which is widely distributed in quinolone-resistant organisms (Aldred *et al.* 2014). *Qnr* resistance genes can protect topoisomerases and hinder quinolones to reach their target (Aldred *et al.* 2014; Goudarzi and Navidinia 2019).

Another strategy is the overproduction of the antibiotic target, which in turn reduces the inhibiting effect (van Hoek *et al.* 2011).

Many of the above-mentioned antibiotic resistance mechanisms are encoded on mobile genetic elements. Efflux pumps, such as OqxA/B, Qnr proteins, as well as Aac(6')-Ib-cr, contributing to quinolone resistance, have been found on plasmids (Hansen *et al.* 2004; Martínez-Martínez *et al.* 1998; Robicsek *et al.* 2006). Moreover, a large variety of beta-lactamases are transferable, including carbapenemases, promoting the distribution and acquisition of antibiotic resistance.

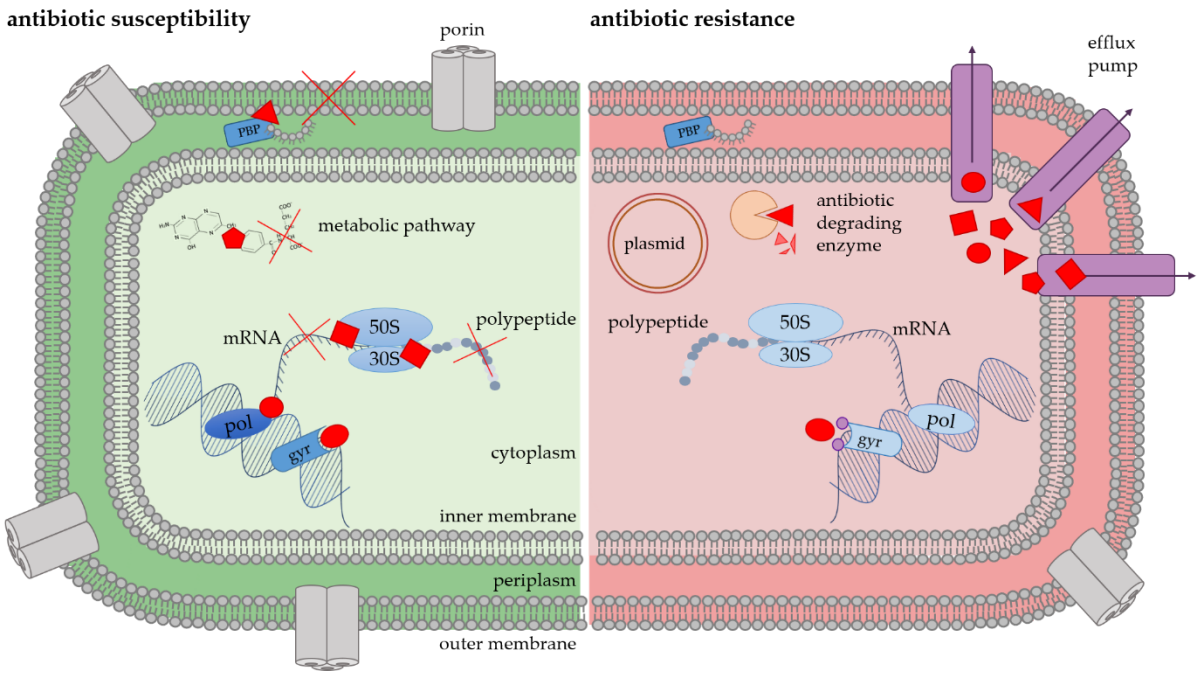

Figure 1: Mechanisms of different antibiotic classes to inhibit bacterial cell growth and bacterial strategies evolved to overcome antibiotic susceptibility.
The left part of the figure shows antibiotic classes that are targeting different bacterial enzymes involved in various processes, resulting in cell growth arrest or cell death. The transcription of bacterial DNA can be inhibited by (fluoro-)quinolones with nalidixic acid and ciprofloxacin as examples or by ansamycins with rifampicin as a representative. Antibiotics interfering with the DNA synthesis are illustrated as red circles and are targeting the topoisomerases (gyr) or the DNA-dependent RNA-polymerases (pol), needed for the unwinding of DNA and the generation of messenger RNA (mRNA), respectively. Another target of antibiotics represents the translation of mRNA to polypeptides and subsequently to proteins. Tetracyclines or aminoglycosides (e.g., gentamicin) are disturbing the 30S ribosomal subunit, whereas macrolides (e.g., erythromycin) inhibit the 50S subunit of the ribosome. Antibiotics impeding protein synthesis are symbolized with red rectangles. Metabolic pathways can be negatively influenced by inhibiting essential enzymes necessary for energy recovery. Sulfonamides with sulfamethoxazole as an example (red hexagon) inhibit the dihydropteroate synthase in the folic acid pathway. The last mode of action is targeting the cell wall synthesis, e.g., through inhibition of the penicillin-binding proteins (PBP) by beta-lactam antibiotics (e.g., penicillin, cefotaxime, or meropenem; red triangles), leading to destabilization of the bacterial cell.
The right part of the figure: Different strategies counteracting the toxic effect evolved, due to mutations or acquired antibiotic resistance genes (plasmid). To reduce the intracellular abundance of antimicrobial substances, efflux pumps can be overexpressed or porins can be down-regulated. Furthermore, alterations in enzyme structures, which often occur in topoisomerases (gyr), lower the binding affinity of antibiotics. Bacterial enzymes can be protected by proteins (purple circles at gyr) to hamper the binding of antibiotics. Another option is bacterial enzymes that can degrade antimicrobial substances. Source: own illustration based on descriptions in the main text (see Section 1.2).

1.3 Resistance to beta-lactam antibiotics

Beta-lactam antibiotics are widely used to treat infections caused by Gram-negative bacteria (Bush and Bradford 2016; Wilke *et al.* 2005). Since an increasing number of resistance mechanisms against penicillin and cephalosporins have emerged, carbapenems represent the last-line antibiotics for severe infections comprising a broad spectrum of activity (Greenhalgh and Edwards 1997; Joly-Guillou *et al.* 2010; Rhomberg and Jones 2003). Yet, carbapenem resistance can be caused by high expression levels of extended-spectrum beta-lactamases or cephalosporinases *ampC* if porin loss and/ or an increase in efflux pumps appear simultaneously (Elshamy and Aboshanab 2020). This kind of resistance can be found frequently in the non-fermenter *P. aeruginosa* (Quale *et al.* 2006). The more worrisome mechanism, however, is through carbapenemases, enzymes that can hydrolyze almost all beta-lactam antibiotics as well as carbapenems (Queenan and Bush 2007).

1.3.1 Attributes of beta-lactamases and their classification

Carbapenemase genes were described in the early 1990s to be plasmid-encoded, which enables their dissemination (Paton *et al.* 1993; Scaife *et al.* 1995; Watanabe *et al.* 1991). Over the last years, a large variety of carbapenemases have evolved that can be classified into Bush-Jacoby-Medeiros groups depending on their spectrum of activity (Bush *et al.* 1995) or Ambler classes based on amino acid similarities (see Table 1; Ambler 1980). The Ambler classification differentiates beta-lactamases by the enzymes' active site into serine- (Ambler class A, C, and D) and metallo-beta-lactamases (Ambler class B), harboring a serine residue and a zinc ion in the catalytic center, respectively, to mediate the cleavage of the beta-lactam ring (Ghuysen 1991; Jacoby and Munoz-Price 2005; Wang *et al.* 1999).

Different beta-lactamases with various spectrums of activity are classified into Ambler class A. Depending on the variant TEM- and SHV-type beta-lactamases can have narrow- (e.g., SHV-1; Matthew *et al.* 1979), broad- (e.g., TEM-1; Jacoby and Medeiros 1991) or extended-spectrum activity (e.g., TEM-3, SHV-2; Drawz and Bonomo 2010; Jacoby and Medeiros 1991; Sougakoff *et al.* 1988). Carbapenem inactivation ability was described for the *Klebsiella pneumoniae* carbapenemase (KPC), imipenem-hydrolyzing beta-lactamase (IMI), *Serratia marcescens* enzyme (SME), and the Guiana extended-spectrum (GES) beta-lactamases. Yet, not all GES enzymes can degrade carbapenems but comprise broad-spectrum activity against beta-lactamases such as GES-1 (Poirel *et al.* 2000). Initially, class B metallo-beta-lactamases were found chromosomally encoded, e.g., in *Stenotrophomonas maltophila* and *Aeromonas* sp. (Franco *et al.* 2010; Sánchez 2015). Nowadays, class B beta-lactamases are commonly plasmid-encoded and are all able to inactivate carbapenems, such as the German imipenemase (GIM), the imipenemase (IMP), the New-Delhi metallo-beta-lactamase (NDM), and the Verona integron-encoded metallo-beta-lactamase (VIM; Castanheira *et al.* 2004; Lauretti *et al.* 1999; Riccio *et al.* 2000; Watanabe *et al.* 1991; Yong *et al.* 2009). Class C mostly consists of cephalosporinases, however, for a few variants such as ACT-28 and CMY-10 carbapenem-hydrolyzing activity was described (Jousset *et al.* 2019; Kim *et al.* 2006). *AmpC* resistance genes are often

chromosomally encoded by *P. aeruginosa* but also in Enterobacterales, leading to low-level resistance without clinical relevance (Lindberg *et al.* 1985). Yet, the expression of these resistance genes can be induced under antibiotic treatment, resulting in non-susceptible bacteria (Jacobs *et al.* 1997; Lindberg *et al.* 1985; Santajit and Indrawattana 2016). Oxacillinases encompass numerous variants having narrow-, broad-, and extended-spectrum or carbapenem-hydrolyzing activity (Smith *et al.* 2019). Four important plasmid-encoded OXA groups can hydrolyze carbapenems: OXA-48-like, OXA-58-like, OXA-40/24-like, and OXA-23-like. Some OXA enzymes are also chromosomally encoded such as *bla*$_{\text{OXA-51}}$ in *Acinetobacter* species (Turton *et al.* 2006). For each group a large variety of carbapenemase variants have evolved, sometimes differing by only one amino acid exchange, such as OXA-163, belonging to the OXA-48-like group (Poirel *et al.* 2011). These mutations can lead to alterations in their enzymatic activity (Oueslati *et al.* 2015).

Table 1: Ambler classification of beta-lactamases based on their amino acid constitution.
The table was adapted from Bush *et al.* (1995), Diene and Rolain (2014), and Toussaint and Gallagher (2015).

Ambler class	Type	Example
A	Narrow-spectrum beta-lactamases	SHV-1
	Broad-spectrum beta-lactamases	TEM-1, TEM-2
	Extended-spectrum beta-lactamases	SHV-2, CTX-M-15, PER-1, VEB-1
	Serine carbapenemases	KPC-1, IMI-1, SME-1, GES-5
B	Metallo-beta-lactamases	VIM-1, IMP-1, NDM-1, GIM-1
C	Cephalosporinases	ACT-1, CMY-2, FOX-1, MIR-1
D	Oxacillinases	OXA-48-like, OXA-23-like, OXA-58-like, OXA-40/24-like

There are few treatment strategies left for infections with carbapenem-resistant Gram-negative bacteria, using either combination therapies with or without carbapenems or monotherapy with amikacin, colistin, tigecycline, or fosfomycin, dependent on the resistance pattern (Navarro-San Francisco *et al.* 2013; Tzouvelekis *et al.* 2014). However, antibiotics such as colistin are accompanied by adverse events, and reports of plasmid-encoded *mcr* resistance genes increased (Akajagbor *et al.* 2013; Carattoli *et al.* 2017; Huang *et al.* 2017; Wang *et al.* 2018). Furthermore, some inhibitors such as avibactam, sulbactam, tazobactam, or clavulanic acid can affect the serine residue in the active center of carbapenemases but do not affect metallo-beta-lactamases (English *et al.* 1978; Fisher *et al.* 1980; Lahiri *et al.* 2014; Pfaller *et al.* 2017). There are a few exceptions such as KPC-2, a class A carbapenemase, and the oxacillinase OXA-48, which are not affected by these inhibitors (Abboud *et al.* 2016; Drawz and Bonomo 2010; Papp-Wallace *et al.* 2010; Poirel *et al.* 2012). Coproduction of serine- and metallo-beta-lactamases in Gram-negative bacteria is described more frequently, resulting in the ineffectiveness of carbapenemase inhibitors (An *et al.* 2017; Wasfi *et al.* 2021; Wei *et al.* 2011; Xie *et al.* 2017).

1.3.2 Diagnostics of carbapenem-resistant bacteria

Different test strategies have been implemented in clinical microbiology laboratories, comprising several phenotypic and genotypic methods that vary in performance, costs, and time to results. In conventional microbiological diagnostics, primary phenotypic identification of carbapenem-resistant bacteria is common. Selective chromogenic agar, such as chromID® ESBL or chromID® CARBA agar have been developed to isolate carbapenemase-producing *Enterobacteriaceae*, showing sensitivities of 92.4 % (Vrioni *et al.* 2012) and 89.8 % to 92.4 % respectively (Simner *et al.* 2015; Vrioni *et al.* 2012). Yet, further diagnostics are necessary to allow differentiation between extended-spectrum beta-lactamases (ESBL) and carbapenemases by using the chromID® ESBL agar (Hrabak *et al.* 2014). Antimicrobial susceptibility of isolates growing on selective agar can be determined for instance by VITEK automated system (Codjoe and Donkor 2017), disk diffusion method (Vading *et al.* 2011), microdilution (Rotilie *et al.* 1975), or gradient strips containing meropenem or imipenem (Steward *et al.* 2003).

Some limitations were reported for phenotypic detection of isolates harboring carbapenemases with low enzymatic activity, such as OXA-48. OXA-48-producers can be overseen when screening with chromID® ESBL agar unless an ESBL gene is coexpressed (Bakthavatchalam *et al.* 2016; Carrër *et al.* 2010). Moreover, OXA-48 positive bacteria may have minimum inhibitory concentrations (MIC) below the clinical breakpoints of the EUCAST guidelines, thus showing sensitivity to meropenem (breakpoints: MIC ≤ 2 μg/ml; ∅ ≥ 22 mm). A study concerning phenotypic screening of OXA-48-like producers revealed 4.9 % undetected carbapenemase-positive bacteria since the MIC was below the threshold (Hopkins *et al.* 2019). Furthermore, issues in the detection of KPC carbapenemases were reported since MICs vary from susceptible to resistant. Depending on co-occurring resistance mechanisms, such as porin loss or increased copy numbers of the resistance gene, the MIC can be elevated under antibiotic treatment (Kitchel *et al.* 2010).

To investigate carbapenem hydrolyzing activity, the carbapenem inactivation method (CIM; van der Zwaluw *et al.* 2015), the Rapidec Carba-NP test (Poirel *et al.* 2015), or the Modified Hodge test (MHT) are available. Studies on carbapenemase detection by CIM reported sensitivities ranging from 78.0 % to 100 % (Aguirre-Quiñonero *et al.* 2017; Aktaş *et al.* 2017; Jing *et al.* 2018), leading to false-negative results for GES-producing *Enterobacteriaceae* (Aguirre-Quiñonero *et al.* 2017). For MHT false-positive results were reported in carbapenem-resistant *Enterobacteriaceae* harboring an ESBL or *ampC* resistance gene in combination with porin loss (Carvalhaes *et al.* 2010; Seah *et al.* 2011) and limitations were noticed concerning different carbapenemase variants in the non-fermenter *A. baumannii* (Bonnin *et al.* 2012a).

However, these methods are giving only limited detail on the present carbapenemase variant. Depending on the prevalence of carbapenemase-producers and the throughput of samples in the diagnostic microbiology laboratory, different detection methods might be favorable. For smaller laboratories in regions with fewer carbapenemase-harboring bacteria, rapid detection tests such as the immunochromatographic tests RESIST-4 O.K.N.V. and NG test Carba 5 might be suitable. RESIST-4 showed high sensitivity and specificity of 99.2 % and 100 %, respectively,

in detecting KPC, NDM, OXA-48-like, and VIM carbapenemases from Enterobacterales (Greissl *et al.* 2019). Some other studies revealed limitations in the detection of NDM (Kolenda *et al.* 2018; Saleh *et al.* 2018). The NG test Carba 5 designed for detecting NDM, KPC, VIM, IMP, and OXA-48-like carbapenemases in Enterobacterales and *Pseudomonas* sp. had a good performance with sensitivities and specificities of 97.3 % and 99.8 %, respectively (Han *et al.* 2020a; Hopkins *et al.* 2018; Jenkins *et al.* 2020). Yet, some IMP variants led to false-negative results (Hopkins *et al.* 2018). A few PCR assays based on hydrolysis probes or melt curve analysis are available to detect the most common carbapenemase genes *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{OXA-48}$-like, showing sensitivities and specificities close to 100 % (Hofko *et al.* 2014; Monteiro *et al.* 2012; Sadek *et al.* 2020; van der Zee *et al.* 2014; Yoshioka *et al.* 2021). Certain assays detect rare variants, such as GES and OXA-23-like carbapenemases, found in the non-fermenters *P. aeruginosa* and *A. baumannii* (Hofko *et al.* 2014; Monteiro *et al.* 2012; Yoshioka *et al.* 2021).

PCR-based assays are always biased methods since only selected resistance determinants are covered, which can lead to false-negative results. All mentioned approaches showed limitations in detecting carbapenemase-producers, that need to be considered before use. Usually, culture represents the initial screening method and further characterization of the isolates is done by phenotypic or genotypic methods, as described above. However, only one representative isolate, growing on the agar plate, is used for subsequent diagnostics, which can lead to false-negative results if the carbapenemase-producing isolate was not the selected one. Moreover, carbapenemase-producers that are not able to grow on the used culture medium would be missed. To counteract these issues, some PCR assays have been developed to detect carbapenemase genes from primary clinical samples (Huang *et al.* 2015; Lowman *et al.* 2014; McEwan *et al.* 2013; Monteiro *et al.* 2012).

Besides patient screening, monitoring of the hospital environment is crucial to impede the distribution of carbapenemases and to prevent nosocomial infections. Reservoirs of carbapenemase-harboring bacteria were often identified in aquatic clinical surroundings that represent a potential risk for outbreaks, as was reported, e.g., in France, Australia, and Switzerland for OXA-48-, IMP- and VIM-producers respectively (Catho *et al.* 2021; Jolivet *et al.* 2021; Leung *et al.* 2013).

1.3.3 Worldwide distribution of carbapenemases

Carbapenemase genes have spread drastically all over the world in the last decades. The widely distributed carbapenemases, also designated as the "big five", are KPC, NDM, IMP, OXA-48, and VIM (Bonnin *et al.* 2020b; Queenan and Bush 2007). Interestingly there are geographic accumulations of certain enzymes, such as NDM in South Asia, KPC in the US, South America, and China, VIM in Australia, and OXA-48 in Europe (Glasner *et al.* 2013; Han *et al.* 2020b; Munoz-Price *et al.* 2013; Poirel *et al.* 2012). KPC is also commonly found in European countries, being endemic in Greece and Italy (Cuzon *et al.* 2008; Fontana *et al.* 2010; Giani *et al.* 2012; Pournaras *et al.* 2009; Richter *et al.* 2012).

IMP carbapenemases are frequently identified in Southern Europe and Asia (Nordmann *et al.* 2011; Shigemoto *et al.* 2012; Watanabe *et al.* 1991). Besides geographic reliance, some carbapenemase variants are typical for certain bacterial species. For instance, *bla*$_{VIM-1}$ is frequently found in Enterobacterales, whereas *bla*$_{VIM-2}$ is mostly prevalent in *Pseudomonas* sp. (Kazmierczak *et al.* 2016; Matsumura *et al.* 2017; Papagiannitsis *et al.* 2015). The oxacillinases OXA-23-like, OXA-58-like, and OXA-40/24-like are commonly linked to *Acinetobacter* sp. (Afzal-Shah *et al.* 2001; Bertini *et al.* 2006; Bogaerts *et al.* 2006; Bou *et al.* 2000; Coelho *et al.* 2006; Donald *et al.* 2000). Yet, some OXA-23 and OXA-58 carbapenemase were sporadically detected in Enterobacterales (Bonnin *et al.* 2020a; La *et al.* 2014).

The National Reference Laboratory (NRL) in Germany is analyzing the carbapenemase situation every year in *Enterobacteriaceae* and the non-fermenters *A. baumannii* and *P. aeruginosa*. Based on this data there was a steady increase in the detection of carbapenemase-producers in *Enterobacteriaceae* from 2013 to 2019, whereas the frequency in the non-fermenters was fluctuating (see Figure 2 and Figure 3; Kaase 2014; Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020). The most abundant carbapenemases in *Enterobacteriaceae* comprise KPC, NDM, OXA-48-like, and VIM. The diversity of carbapenemase variants was raising over the years and more isolates were detected harboring two carbapenemases simultaneously. Other carbapenemases less frequently occurring, are IMI, GES, IMP, or GIM, however, their detection throughout the years accumulated.

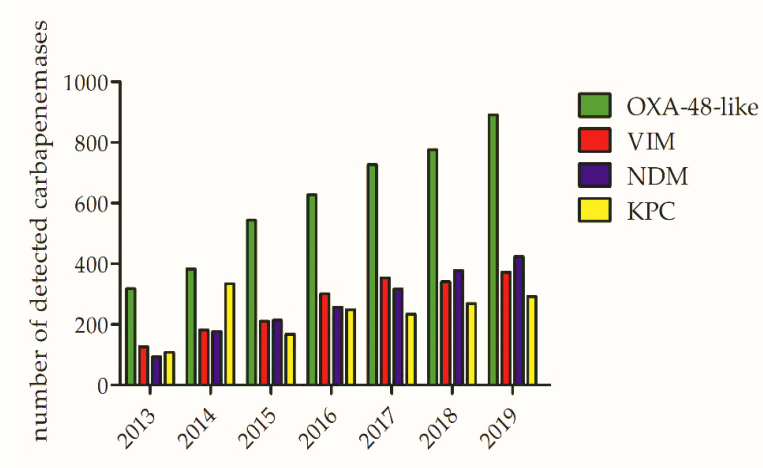

Figure 2: Carbapenemases detected in *Enterobacteriaceae* in Germany from 2013 to 2019 by the National Reference Laboratory.
Starting in 2013, there is a clear rise in carbapenemase detection with OXA-48-like being the most abundant enzyme over the years. Depending on the year the abundance of VIM, KPC, and NDM variants varied. The figure was created based on the data provided by the NRL (Kaase 2014; Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020).

The highest percentage of carbapenemase-producers in phenotypic resistant isolates was found in *A. baumannii* (> 90 %), with OXA-23 being the most prevalent enzyme, followed by OXA-72 and OXA-58. Furthermore, detection of NDM carbapenemases increased over the years (see Figure 3A). In contrast, around 24 % of the *P. aeruginosa* isolates were positive for carbapenemases, mostly VIM and IMP. GIM, NDM, and GES were detected more often in the years 2016 to 2019 (see Figure 3B; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020).

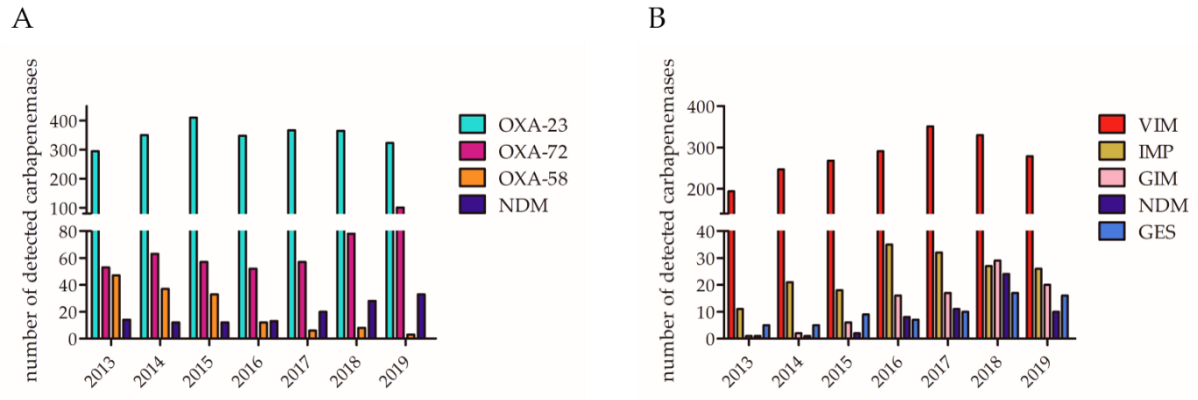

Figure 3: Carbapenemases detected in the non-fermenters *A. baumannii* (A) and *P. aeruginosa* (B) in Germany from 2013 to 2019 by the National Reference Laboratory.
Minor changes in the carbapenemase detection in the non-fermenters are present over the years. The most common carbapenemases in *A. baumannii* represent OXA-23 and OXA-72, whereas VIM is the predominant enzyme in *P. aeruginosa*. The figure was created based on the data provided by the NRL (Kaase 2014; Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020).

1.3.4 Mobile genetic elements

Mobile genetic elements, such as plasmids, transposons, or insertion sequences, integrated into the genome are commonly associated with resistance genes (Frost *et al.* 2005; Johnson and Grossman 2015). These integrated conjugative elements (ICE) encompass genes that allow excision from the chromosome and movement of DNA by one of the three major ways of HGT (see Section 1.4; Thomas and Nielsen 2005). Carbapenemase genes have been linked to several mobile genetic elements, elucidating their effective spread throughout the bacterial community (Johnning *et al.* 2018; Ludden *et al.* 2017). For instance, IMP, NDM, GES, and VIM carbapenemases are found in the direct surrounding of Tn3-like elements in *P. aeruginosa*, *Enterobacter cloacae*, and *E. coli* (Botelho *et al.* 2020; Botelho *et al.* 2018). KPC was identified next to IS*Kpn6* and IS26 in *P. aeruginosa* and NDM was found to be flanked by the IS91 family in *P. aeruginosa* and IS26 in *E. coli* (Botelho *et al.* 2020). OXA carbapenemases such as OXA-48 are often linked with Tn1999 and OXA-23 with IS*Aba1* (Partridge *et al.* 2018).
These transposons and insertion sequences can also be present on plasmids, which are circular DNA molecules that are independent of the host chromosome, hence replicate individually (Peterson and Kaur 2018). Plasmids consist of a backbone, containing genes essential for plasmid stability and maintenance as well as plasmid mobilization genes (Guynet *et al.* 2011; Lili *et al.* 2007; Macartney *et al.* 1997; Min *et al.* 1988). Furthermore, a variable region is present, in which accessory genes such as resistance genes accumulate. Depending on the genetic content in the backbone, plasmids can be classified as conjugative, mobilizable, and non-mobilizable plasmids. All genes needed for successful bacterial conjugation are present on conjugative plasmids. Mobilizable plasmids do not harbor those genes at all or just in parts, however, they can hijack the mating bridge of a conjugative plasmid or use the missing genes of another mobile genetic element to be transferred (Dionisio *et al.* 2019).

Plasmids are classified into incompatibility (Inc) groups based on the inability of plasmids to stably co-exist in a bacterial cell (Datta and Hedges 1971; Novick *et al.* 1976). Plasmids of the same Inc-type are usually closely related, thus using the same replication mechanism, which leads to failure in segregation and stable inheritance when two plasmids of the same Inc-type are present in one cell (Datta and Hedges 1971; Novick *et al.* 1976). Up to date, a variety of Inc-types was identified in Enterobacterales: IncA/C, IncB/O, IncF, IncH, IncK, IncL/M, IncN, IncP, IncQ, IncT, IncX, and IncY (Carattoli 2009; Couturier *et al.* 1988). Depending on the plasmids´ ability to spread between different taxonomic distantly or closely related bacterial species, plasmids comprise broad (e.g., IncN, IncW, IncP, or IncA/C) or narrow (e.g., IncF, IncH, or IncI) host ranges (Boyd *et al.* 1996; Fernández-Alarcón *et al.* 2011; Llosa *et al.* 1991; Mierzejewska *et al.* 2007; Pukall *et al.* 1996; Rozwandowicz *et al.* 2018). Carbapenemase genes were found on different Inc-type plasmids (Cai *et al.* 2008; Carattoli *et al.* 2006; Tato *et al.* 2007), for instance, *bla*$_{\text{NDM-1}}$ was detected on IncA/C, IncF, and IncL/M plasmids (Bonnin *et al.* 2012b; Carattoli *et al.* 2015; Hancock *et al.* 2017). The presence of resistance genes on broad host range plasmids enables interspecies transfer and explains their successful dissemination, as was shown for OXA-23 and OXA-58 found in *Acinetobacter* sp. and in Enterobacterales, respectively (Bonnin *et al.* 2020a; La *et al.* 2014). Usually, resistance genes against different antibiotic classes accumulate on plasmids, making the development of MDR bacteria more likely when transferred to bacteria already having a resistance gene-containing plasmid (Chen *et al.* 2020; Johnning *et al.* 2018).

1.4 Horizontal gene transfer

The evolution of bacteria is driven by changes in the genetic content either through mutations or HGT whereby new genes potentially beneficial to the bacteria´s survival can be acquired, such as virulence, antimicrobial resistance, or metabolic genes (Heuer and Smalla 2007; Juan *et al.* 2015; Lerminiaux and Cameron 2019). Mutations and newly inserted genes can also be detrimental to bacterial fitness and are therefore eradicated from bacterial populations over time (Thomas and Nielsen 2005). There are three major ways, for bacterial intra- or interspecies substitution of DNA through HGT: transduction, natural transformation, and conjugation (Aminov 2011; see Figure 4).

Transduction is mediated by bacteriophages during the infection and replication process (see Figure 4A). The genetic material of the host can be packaged in the head of the newly assembled bacteriophages. After lysis of the host, the virus can spread the genes to other bacterial cells throughout their infection process (Heuer and Smalla 2007; Kleiner *et al.* 2020).

Transformation, the uptake of exogenous DNA (see Figure 4B), presupposes a competence state of the bacterial cell to enable the entrance of free DNA, which is a complex and tightly regulated process and activated under certain environmental conditions (Blokesch 2016; Peterson and Kaur 2018; Seitz and Blokesch 2013). The third process, conjugation, occurs by direct contact between two bacteria via a mating bridge through which genetic material can be transferred (see Figure 4C). Successful distribution of genes by HGT is restricted by various

factors, e.g., the host range of phages, the type of transferred genetic material, the imposed fitness costs for the new host, and the evasion of degradation in the recipient (Peterson and Kaur 2018).

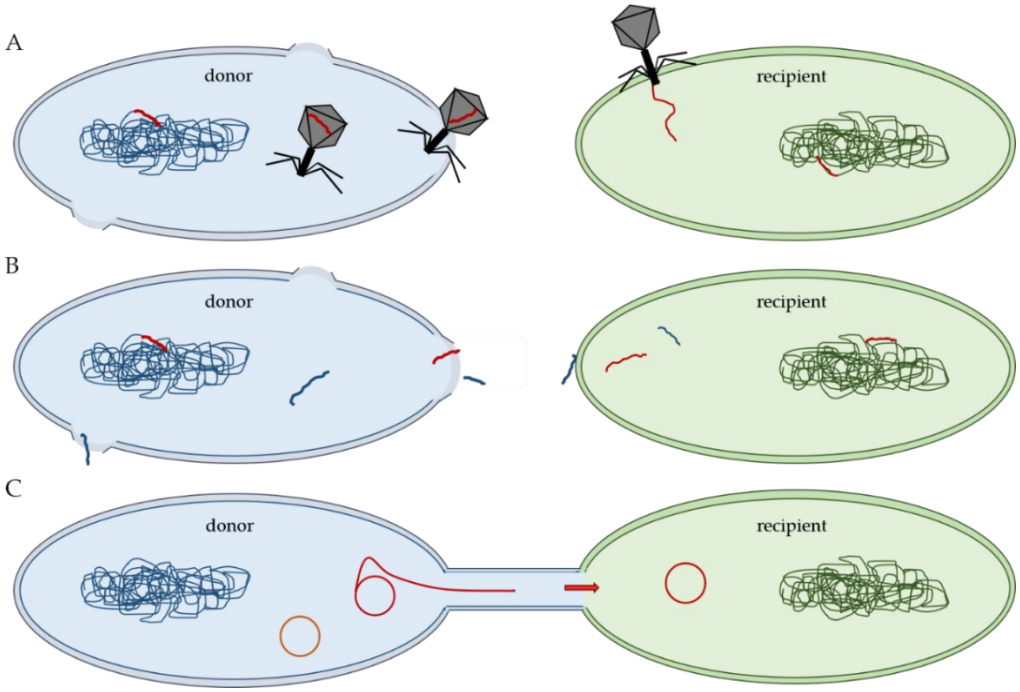

Figure 4: Exchange of genetic material through horizontal gene transfer (HGT)
The three main possibilities of HGT are transduction, transformation, and conjugation. A: Transduction is mediated by bacteriophages that infect and replicate within bacterial cells. During this replication process, the genetic material of the bacteria (donor) can be included in the phage particle, e.g., antimicrobial resistance genes (red wavy line) and transferred to another bacterial cell (recipient) with subsequent integration into the recipient chromosome. B: Released DNA of dead bacterial cells can be gathered up by competent recipient cells when certain environmental stimuli are present. C: Bacterial conjugation is an active process that occurs by direct contact between two bacteria. The donor builds up a mating bridge through which a copy of genetic material e.g., a plasmid, is transferred to the recipient (red and orange circle). The figure has been adapted from Furuya and Lowy (2006), and Vernikos and Medini (2014).

1.4.1 Bacterial conjugation

Since the discovery that bacteria can share plasmids via direct cell-to-cell contact by Joshua Lederberg and Edward Tatum in 1946, the process of F-plasmid conjugation has been studied extensively (Lederberg and Tatum 1946). A range of proteins have been described to be involved in the formation of the type IV secretion system (T4SS), yet the function of all proteins is not fully understood. Depending on the subtype of the T4SS it can be involved in the uptake of free DNA (transformation) and in transporting virulence factors from pathogenic bacteria to host cells (Alvarez-Martinez and Christie 2009; Hamilton *et al.* 2005; Hofreuter *et al.* 2003; Weiss *et al.* 1993). Conjugation-related genes can differ between the plasmid Inc-types, resulting in altered structures of the pilus, ranging from thick and rigid to thin and flexible (Bradley 1980; Bradley 1983). The pilus structure is facilitating conjugation either in solid biofilm formations or in aquatic environments, respectively (Bradley 1984; Bradley *et al.* 1980).

Self-transmissible plasmids encode all genes necessary for their transfer, namely *tra* (F-like plasmid), *vir* (Ti plasmid), or *trb*/*trh* (P-type plasmid) relative to the plasmid type with equivalent functions (Alt-Mörbe *et al.* 1996; Berger and Christie 1994; Frost *et al.* 1994). The T4SS is composed of 10 to 20 proteins and assembles within the membrane of the donor cell, creating the pore for transferring the single-stranded DNA (ssDNA; Koraimann and Wagner 2014). A key component of conjugation represents the relaxosome, an enzyme complex consisting of at least three proteins, TraI, TraY, and TraM, and chromosomally encoded integration host factors (Inamoto *et al.* 1994; Nelson *et al.* 1995). The bifunctional enzyme TraI catalyzes the unwinding of the plasmid and the nicking reaction at the oriT site, resulting in the cleavage of one plasmid strand (Datta *et al.* 2003; Ilangovan *et al.* 2017). TraY and TraM are accessory proteins of the relaxosome, promoting this reaction (Inamoto *et al.* 1994; Nelson *et al.* 1995; Ragonese *et al.* 2007). TraI persists covalently attached to the 5'-end of the nicked strand and is transferred to the recipient cell where the re-circulation of the plasmid is induced (Dostál *et al.* 2011).

TraC and TraD represent ATPases fueling the assembly of the T4SS and the transfer of mobile genetic elements (Gruber *et al.* 2016; Schandel *et al.* 1992). TraD is also known as the coupling protein that interacts with TraM of the relaxosome, necessary for the specific transfer of the conjugative plasmid (Disqué-Kochem and Dreiseikelmann 1997; Lu *et al.* 2008; Sastre *et al.* 1998). TraA represents pilin subunits; when acetylated by TraX they form the channel structure as a polypeptide and are anchored in the inner membrane via TraQ (Lawley *et al.* 2003; Lu *et al.* 2002; Majdalani and Ippen-Ihler 1996; Maneewannakul *et al.* 1993). TraL, present in the inner membrane, regulates pili production (Frost *et al.* 1994; Lawley *et al.* 2003). The proteins TraK, TraV, and TraB assemble to a complex and are the core proteins of the T4SS, which are incorporated in the outer membrane via the lipoprotein TraV. TraK persists in the periplasm and TraB in the inner membrane (Bragagnolo *et al.* 2020; Harris *et al.* 2001).

The conjugation process is tightly regulated by plasmid- and host-encoded factors such as FinO/P, AcrA as well as Tra proteins. *Tra* genes are under the control of three promotors; P_M controls the expression of *traM*, P_J regulates *traJ*, and P_Y operon is responsible for the vast majority of *tra* genes (Ippen-Ihler *et al.* 1972; Wong *et al.* 2012). *TraJ* expression is stimulated by host factors, such as the cyclic AMP receptor protein, depending on cellular conditions (Starcic *et al.* 2003). TraJ was identified as the main driver of *tra* gene expression, additionally, TraY and the chromosomal factor AcrA represent positive regulators of P_Y. TraY furthermore induces the expression of *traM* (Nelson *et al.* 1993; Silverman *et al.* 1991; Strohmaier *et al.* 1998). The presence of TraJ in the bacterial cell is adjusted on the mRNA level by the FinO/FinP regulation system. Interaction of the anti-sense RNA, *finP*, with *traJ* leads to degradation of the mRNA-duplex. The stability of the *finP* RNA is thereby affected by the plasmid-encoded gene *finO* (see Figure 5; Finnegan and Willetts 1971; Jerome *et al.* 1999; Timmis *et al.* 1978).

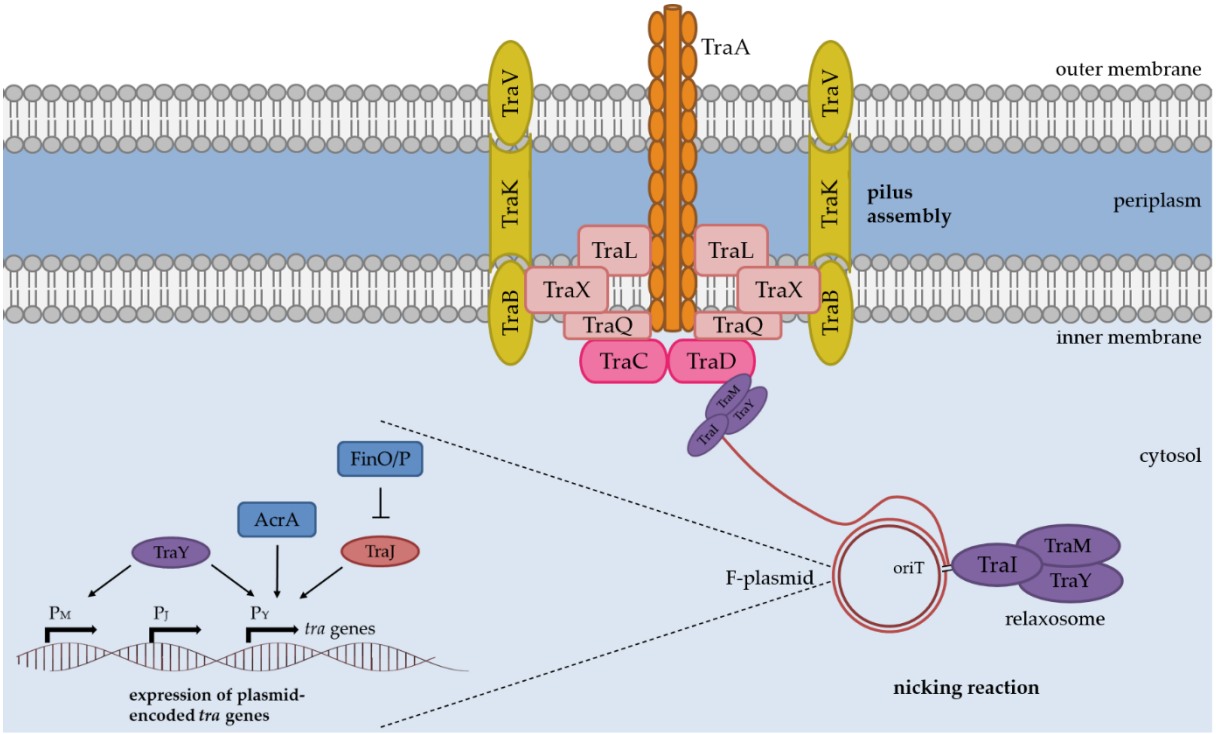

Figure 5: Gene regulation and assembly of the type IV secretion system (T4SS) to facilitate conjugation of F-plasmids.
Tra genes are under the control of three promoters P_M, P_J, and P_Y regulated by the host factor AcrA as well as by plasmid-encoded genes, i.e., *finO/P*, *traY*, and *traJ*. The key protein complex represents the relaxosome, composed of TraI, TraM, and TraY, whereby TraI induces the DNA strand break at the oriT. When the relaxosome is binding to the free 5'-end of the nicked strand, translocation of the strand to the T4SS occurs through the interaction of TraM with TraD. TraD and TraC proteins provide ATPase function to fuel the pilus assembly and the transfer process of the plasmid strand. The main components of the pilus are the pilin monomers (TraA) that built up a polypeptide forming a channel-like structure through the membranes of the host, which is supported by TraL, TraX, and TraQ. The core protein complex of the T4SS is represented by TraB, TraK, and TraV that are spanning through the outer membrane, periplasm, and inner membrane. The figure has been adapted from Mary *et al.* (2018), Sgro *et al.* (2019), Wong *et al.* (2012), and is based on the description in Section 1.4.1.

For successful conjugation direct contact between bacteria is essential. Bacterial cells communicate with each other via quorum sensing, which is involved in the transcriptional regulation of different processes, such as biofilm formation, virulence, and recognition of antibiotics (Nealson *et al.* 1970; Parsek and Greenberg 2005; Zhao *et al.* 2020; Zhu *et al.* 2020). Quorum sensing is considered to have a regulating impact on conjugation, thus contributing to the transfer of antimicrobial resistance genes (Singh and Meijer 2014; Zhang *et al.* 2018). Sublethal concentrations of antibiotics can have an elevating effect on plasmid transfer, the underlying mechanism, however, still needs to be discovered (Jutkina *et al.* 2018; Ohlsen *et al.* 2003; Shun-Mei *et al.* 2018).

1.4.2 SOS response in conjugational transfer

Diverse environmental factors can induce stress reactions in bacterial cells characterized by changes in the gene expression profile and often cell cycle arrest when DNA damage occurs (Takashima *et al.* 2020; Witkin 1976). The bacterial SOS response is triggered by the presence of ssDNA in the cell and consequently, various DNA repair mechanisms are induced to remedy the lesion (Sassanfar and Roberts 1990). There are two main regulators of the SOS response; LexA, a transcriptional inhibitor that controls the expression of repair genes, and the activator protein, RecA (Gudas and Pardee 1975). Without DNA damage, LexA is attached to the SOS box, a specific region in the promotor sequence, controlling more than 30 genes involved in different repair mechanisms (Courcelle *et al.* 2001; Fernández de Henestrosa *et al.* 2000). DNA damage activates RecA, which assembles at the ssDNA and in turn interacts in its' activated form with LexA resulting in self-cleavage of the transcriptional regulator into two domains and consequently inducing DNA repair gene expression (see Figure 6; Freitag and McEntee 1989; Joo *et al.* 2006; Little 1983; Little and Mount 1982; Luo *et al.* 2001; Zhang *et al.* 2010).

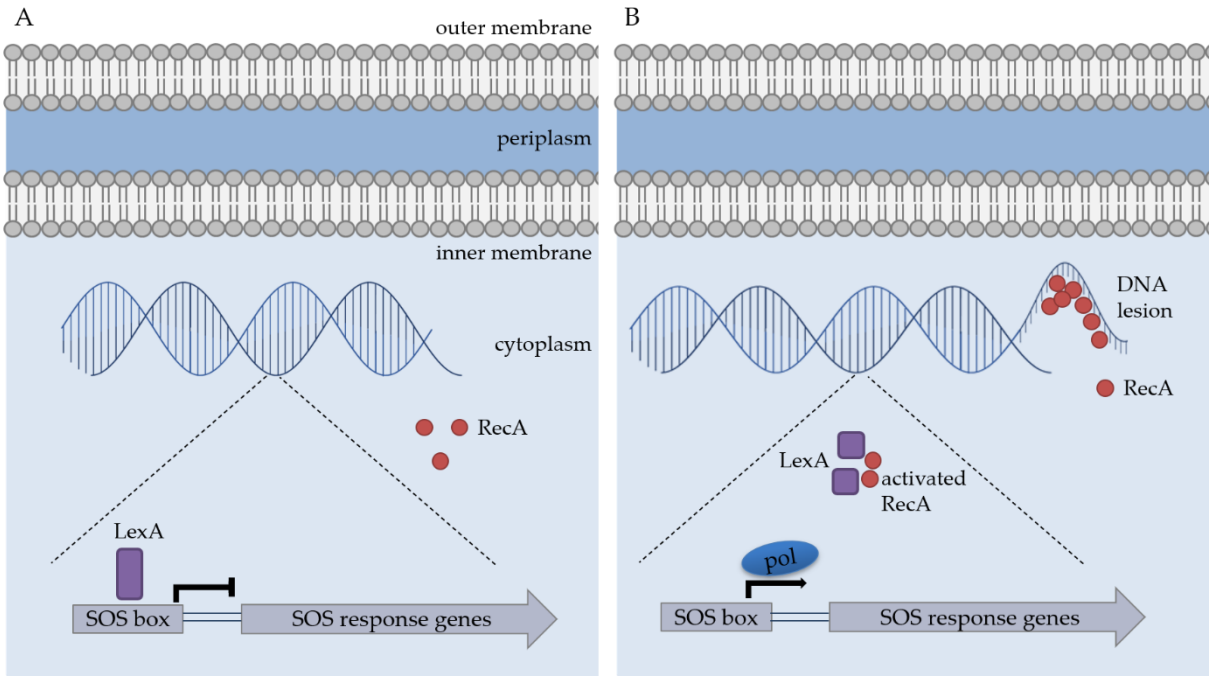

Figure 6: Regulation of the bacterial SOS response.
The bacterial stress response is usually inhibited by the transcriptional regulator, LexA. The occurrence of DNA damage and consequently the presence of single-stranded DNA (ssDNA) activates RecA, which accumulates at the DNA lesion. Activated RecA thereafter interacts with LexA, inducing auto-cleavage of the transcriptional regulator, which enables the RNA-polymerase (pol) to transcribe SOS response-related genes involved in various DNA repair mechanisms. The illustration is based on Maslowska *et al.* (2019), Qin *et al.* (2015), and the description of the main text in Section 1.4.2.

SOS genes are progressively expressed depending on the extent of DNA damage (Courcelle *et al.* 2001; Culyba *et al.* 2018). The order of transcription is relative to the binding affinity of the transcriptional regulator LexA to the SOS box upstream of the DNA repair genes (Courcelle *et al.* 2001; Culyba *et al.* 2018; Zhang *et al.* 2010). Various polymerases are described to contribute to mutagenesis during SOS response, facilitating the occurrence of `beneficial' mutations to the stressor (Cirz and Romesberg 2007; Jarosz *et al.* 2007; Napolitano *et al.* 2000). The error-prone polymerases, UmuCD and DinB, for instance, are specialized for abasic lesions and double-strand break repair, leading to frameshift or point mutations (Ponder *et al.* 2005; Reuven *et al.* 1998; Tang *et al.* 1999). The key genes *lexA* and *recA* are additionally controlled by an SOS box, enabling the down-regulation of the cellular stress response upon DNA damage repair and the reinduction after newly occurring DNA lesions (Friedman *et al.* 2005; Shimoni *et al.* 2009; Walker 1984).

Since the SOS response is elicited by DNA damage, antibiotics impeding DNA synthesis can provoke this stress pathway. Studies have shown that ciprofloxacin can induce the expression of the plasmid-encoded fluoroquinolone resistance gene *qnrB* in an SOS-dependent manner since an SOS box was found upstream of this resistance gene (Da Re *et al.* 2009; Wang *et al.* 2009). Yet, *qnrA* and *qnrS* expression were not related to this stress response (Da Re *et al.* 2009; Wang *et al.* 2009). It has been observed that integrases are under the control of LexA, connecting the SOS pathway to induction of HGT (Guerin *et al.* 2009). Since antimicrobial resistance genes are often linked to integrative elements, SOS response subsequently stimulates their distribution (Maslowska *et al.* 2019).

Activation of the SOS response during conjugation needs to be suppressed in the recipient cell to enable the successful transfer of plasmids. Since the single-stranded plasmid will trigger RecA accumulation and subsequently LexA cleavage, plasmids encode *psiB* to inhibit SOS response by interacting with free RecA (Althorpe *et al.* 1999; Bagdasarian *et al.* 1986; Jones *et al.* 1992; Petrova *et al.* 2009). However, induction of the SOS response was observed during conjugation processes in various studies, contributing to evolution by mutagenesis, movement of transposons, or reorganization of the recipient's chromosome (Baharoglu *et al.* 2010; Matic *et al.* 1995; Matic *et al.* 2000). Researchers suspect that the induction of the SOS response in the recipient cell might be dependent on the host range of the plasmid. On narrow host range plasmids suppressor genes of the SOS response are usually encoded such as *psiB*, whereas broad host range plasmids are inducing the stress response to adapt to the new host by genetic rearrangements (Baharoglu *et al.* 2010).

1.5 Research questions of the thesis

The thesis aimed to evaluate the performance of a newly developed automatized multiplex qPCR for detecting eight common carbapenemase genes (Probst *et al.* 2021a) in the context of the local carbapenemase epidemiology in Enterobacterales. It was validated whether the qPCR directly performed on primary patient specimens would outperform conventional culture-based diagnostics in terms of time and sensitivity.

The hospital environment was outlined as a potential source for nosocomial infections and possible outbreaks with carbapenemase-producing bacteria. Therefore, the value of the qPCR as an initial screening method for carbapenemase genes in wastewater samples was also assessed.

As the widely distributed *bla*$_{\text{VIM-1}}$ carbapenemase genes are usually plasmid-encoded, another objective of this thesis was to provide a better understanding of their transmission through HGT within Enterobacterales. Antibiotic treatment of patients exerts selection pressure on the bacterial community and is under suspicion to trigger HGT (Henderson-Begg *et al.* 2006; Wang *et al.* 2005). However, the factors facilitating plasmid transfer are poorly understood and it remains unclear whether antibiotics with a certain mode of action provoke conjugation (Bethke *et al.* 2020). Thus, the thesis intended to assess the influence of antibiotic treatment on conjugation and the genetic background contributing to efficient transmission.

Discovering bacterial species or special plasmid types that can spread carbapenemase genes efficiently under certain selective pressure might be used as diagnostic markers and may help to prevent the dissemination of resistance genes (Buckner *et al.* 2018; Lopatkin *et al.* 2017; Williams and Hergenrother 2008).

2 Materials and Methods

The following subsections 2.1 to 2.7 summarize all materials, devices, and software required for performing and analyzing the experiments.

2.1 Consumables

Table 2: List of consumables used for the experimental setups of this thesis and associated distributing companies.

Consumable	Company
BD MAX™ PCR Microfluidic PCR Cartridge	BD GmbH, Heidelberg, Germany
BeadBeater® zirconia-beads, 0.1 mm	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
ChromID® CARBA SMART agar plates	bioMérieux Deutschland GmbH, Nürtingen, Germany
ChromID® ESBL Agar	bioMérieux Deutschland GmbH, Nürtingen, Germany
Columbia Agar 5 % Sheep Blood	BD GmbH, Heidelberg, Germany
Conical Tubes, 0.3 ml	BD GmbH, Heidelberg, Germany
Micro TUBE AFA Fiber Pre-Slit Snap-Cap 6x16 mm	Covaris, Inc., Woburn, MA, USA
MIC test strip ciprofloxacin	bestbion dx GmbH, Köln, Germany
MIC test strip nalidixic acid	bestbion dx GmbH, Köln, Germany
Mueller Hinton E Agar	bioMérieux Deutschland GmbH, Nürtingen, Germany
Nunc™ 96-Well Polypropylene Storage Microplates	Fisher Scientific GmbH, Schwerte, Germany

2.2 Chemicals and reagents

Table 3: List of essential chemicals and reagents used in this thesis and associated distributing companies.

Reagent	Company
3-Aminocoumarin	Sigma-Aldrich Chemie, Steinheim, Germany
5-Azacytidine	Sigma-Aldrich Chemie, Steinheim, Germany
Acetic Acid, 100 % p.a.	Carl Roth, Karlsruhe, Germany
Ammonium acetate	Fisher Scientific GmbH, Schwerte, Germany
Aqua ad iniectabilia	B. Braun Melsungen, Melsungen, Germany
Cefotaxim 2g	Dr. Friedrich Eberth Arzneimittel GmbH, Ursensollen, Germany
Ceftazidim 2g	Dr. Friedrich Eberth Arzneimittel GmbH, Ursensollen, Germany
Ceftriaxon 2g	Fresenius Kabi Deutschland GmbH, Bad Homburg vor der Höhe, Germany

Reagent	Company
Chelex® 100 sodium form	Sigma-Aldrich Chemie, Steinheim, Germany
Ciprofloxacin Kabi Infusionslösung 100 mg/ 50 ml	Fresenius Kabi Deutschland GmbH, Bad Homburg, Deutschland
EREMFAT® i.v. 300 mg, active ingredient: rifampicin	Riemser Pharma GmbH, Greifswald, Germany
ESBL ChromoSelect Agar Base	Sigma-Aldrich Chemie, Steinheim, Germany
Ethanol, ≥ 99.8 % (GC)	Sigma-Aldrich Chemie, Steinheim, Germany
Ethidium bromide solution 1 %	Applichem GmbH, Darmstadt, Germany
LB-medium powder	NeoFroxx, Einhausen, Germany
Lysozyme, 100,000 units/mg	SERVA Electrophoresis GmbH, Heidelberg, Germany
Mitomycin C from *Streptomyces caespitosus*	Sigma-Aldrich Chemie, Steinheim, Germany
Nalidixic acid sodium salt	Sigma-Aldrich Chemie, Steinheim, Germany
Nuclease-free water	Qiagen GmbH, Hilden, Germany
PhiX control v3	Illumina, San Diego, CA, USA
Proteinase K	Merck KGaA, Darmstadt, Germany
QX DNA Alignment Marker 15 bp/ 5 kb	Qiagen GmbH, Hilden, Germany
QX DNA Dilution Buffer	Qiagen GmbH, Hilden, Germany
QX Size Marker 100 bp – 2.5 kb	Qiagen GmbH, Hilden, Germany
Sodium azide	Sigma-Aldrich Chemie, Steinheim, Germany
Sodium chloride	Carl Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	AppliChem GmbH, Darmstadt, Germany
TRIzol	Life Technologies by Thermo Fisher Scientific, Waltham, MA, USA

2.3 Kits

Table 4: List of kits required for the thesis and associated distributing companies.

Kit	Company
2x qPCRBIO SyGreen Mix Hi-ROX	PCR Biosystems, London, UK
BD MAX™ ExK™ DNA-2 Kit (4 snap configuration)	BD GmbH, Heidelberg, Germany
Direct-zol™ RNA Miniprep Plus Kit	Zymo Research Europe, Freiburg im Breisgau, Germany
DNeasy Blood and Tissue Kit	Qiagen GmbH, Hilden, Germany
Genomic DNA Clean & Concentrator-10	Zymo Research Europe, Freiburg im Breisgau, Germany

Kit	Company
Luna® Universal One-Step RT-qPCR Kit	New England Biolabs GmbH, Frankfurt am Main, Germany
MiSeq Reagent Kit v3 (600 cycle)	Illumina, San Diego, CA, USA
MiSeq Reagent Kit v3 (150 cycle)	Illumina, San Diego, CA, USA
MyTaq™ HS Mix	Bioline, London, UK
Nextera DNA Flex Library Prep Kit	Illumina, San Diego, CA, USA
Ovation® Complete Prokaryotic RNA-Seq DR Multiplex Systems 1-8 and 9-16 Kit	Tecan Genomics, AC Leek, the Netherlands
primaQUANT 5x qPCR-Probe-MasterMix	Steinbrenner GmbH, Wiesenbach, Germany
Q5® High-Fidelity 2X Master Mix	New England Biolabs GmbH, Frankfurt am Main, Germany
QIAxcel DNA Screening Kit	Qiagen GmbH, Hilden, Germany
Quant-iT™ PicoGreen™ dsDNA Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific, Waltham, MA, USA
RNA Clean & Concentrator Kit	Zymo Research Europe, Freiburg im Breisgau, Germany

2.4 Buffers and Solutions

Table 5: List of buffer and solution formulations used throughout the thesis.

Buffer/ Solution	Formulation
5-Azacytindine	2 mg/ml in 50 % acetic acid
3-Aminocoumarin	4 mg/ml in 50 % acetic acid
Ammonium acetate	3 M ammonium acetate in distilled water
Chelex	10 % (w/v) Chelex in *Aqua ad iniectabilia*
ESBL antibiotics	3 mg/ml Ceftazidim, 3 mg/ml Cefotaxim, 2 mg/ml Ceftriaxon in 10 ml *Aqua ad iniectabilia*
ESBL ChromoSelect Agar Base + ESBl and sodium azide (NaN_3)	4 % (w/v) chromID ESBL agar, autoclave, add 100 µg/ml NaN_3, 3 mg/ml Ceftazidim, 3 mg/ml Cefotaxim and 2 mg/ml Ceftriaxon
Luria-Bertani (LB)-medium	2 % (w/v) LB-powder in distilled water, autoclave
Lysozyme	10 mg/ml in *Aqua ad iniectabilia* or 40 mg/ml in *Aqua ad iniectabilia*
Mitomycin C	500 µg/ml in *Aqua ad iniectabilia*
Proteinase K	20 mg/ml in *Aqua ad iniectabilia* or 10 mg/ml in *Aqua ad iniectabilia*
Skim milk medium	10 % (w/v) skim milk in distilled water, autoclave
Sodium azide solution	50 mg/ml in *Aqua ad iniectabilia*
Sodium chloride solution	0.9 % (w/v), autoclave
Sodium dodecyl sulfate	10 % (w/v), sterile filtered

2.5 Devices

Table 6: List of devices used for performing the experiments in this thesis and associated distributing companies.

Device	Company
Bacterial Incubator Shaker	Infors, Bottmingen, Switzerland
BD MAX™ System	BD GmbH, Heidelberg, Germany
Centrifuge Heraeus Fresco 17	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge Heraeus Multifuge 3SR+	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge concentrator	Concentrator plus, Eppendorf AG, Hamburg, Germany
Covaris S-series Sonication System	Covaris, Inc., Woburn, MA, USA
MALDI-TOF MS	Bruker Daltonics, Göttingen, Germany
McFarland densitometer	DensiCHEK Plus Instrument, BioMérieux, Mary l'Etoile, France
Mini-Bead-Beater	BioSpec Products, Bartlesville, OK, USA
MIseq instrument (2×300 bp)	Illumina, San Diego, CA, USA
NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific, Waltham, MA, USA
QIAxcel Advanced System	Qiagen GmbH, Hilden, Germany
Real-Time PCR System	StepOnePlus, Thermo Fisher Scientific, Waltham, MA, USA
Thermoblock	TSC ThermoShaker, Biometra by Analytik Hena, Göttingen, Germany
Thermocycler, Primus 96 advanced gradient	VWR International, Radnor, PA, USA
UV Stratalinker® 1800	Stratagene California, La Jolla, CA, USA
VICTOR® Nivo™	PerkinElmer, Waltham, MA, USA
VITEK®2 automated system	bioMérieux Deutschland GmbH, Nürtingen, Germany

2.6 Software

Table 7: List of software used for running and analyzing the experiments, creating figures, and performing statistical analysis and associated manufacturer.

Software	Manufacturer
ABRicate	Available at: https://git.lumc.nl/bvhhornung/antibiotic-resistancepipeline/tree/master/tools/abricate
Ballgown	V3.13, available at: https://github.com/alyssafrazee/ballgown
BD MAX™ System software	V5.14 A, BD GmbH, Heidelberg, Germany
BRIG: BLAST Ring Image Generator	V0.95, available at: http://brig.sourceforge.net/

Software	Manufacturer
Burrows-Wheeler Aligner (bwa)	V0.7.17, available at: https://github.com/lh3/bwa
cell growth quantifier software	CG Quant 7.4, aquila biolabs GmbH, Baesweiler, Germany
DESeq2	V3.13, available at: https://bioconductor.org/packages/release/bioc/html/DESeq2.html
FastTree	V2.1.8, available at: http://www.microbesonline.org/fasttree/
FigTree	V1.4.2, available at: http://tree.bio.ed.ac.uk/software/figtree/
GffCompare	V0.12.6, available at: https://ccb.jhu.edu/software/stringtie/gffcompare.shtml
GraphPad Prism	V6.07, GraphPad Software, San Diego, CA, USA, www.graphpad.com
Inkscape	V0.92.4, Inkscape Project, 2020. Inkscape, available at: https://inkscape.org
Mauve	Version snapshot_2015-02-13, available at: http://darlinglab.org/mauve/mauve.html
NanoDrop software	V3.8.1, Thermo Fisher Scientific, Waltham, MA, USA
OligoAnalyzer™ Tool	V3.1, Integrated DNA Technologies, Inc., Coralville, Iowa, USA
PrimerQuest™ Tool	Primer3 software (version 2.2.3), Integrated DNA Technologies, Inc., Coralville, Iowa, USA
Prokka: rapid prokaryotic genome annotation	V1.14.6, available at: https://github.com/tseemann/prokka
qPCR software	StepOne Software v2.1, Thermo Fisher Scientific, Waltham, MA, USA
R	V4.1.0, available at: https://www.R-project.org/
Roary: Pan Genome Pipeline	V3.1.3, available at: https://sanger-pathogens.github.io/Roary/
Sickle: trimming tool for FASTQ files	V1.33, available at: https://github.com/najoshi/sickle
SPAdes	V3.10.0, available at: http://cab.spbu.ru/software/spades/
StringTie	V2.1.4, available at: https://ccb.jhu.edu/software/stringtie/

2.7 Oligonucleotides

Primers used for qPCR were obtained from Eurofins Genomics (Ebersberg, Germany) and hydrolysis probes from eurogentec (Seraing, Belgium). The oligonucleotides were resuspended in nuclease-free water to a final concentration of 100 pmol/µl and dilutions of 10 pmol/µl were prepared. Primers and probes were stored at - 20 °C until usage. Primers designed for this thesis were generated using the PrimerQuest™ and OligoAnalyzer™ Tool as described by Probst *et al.* (2021a).

Table 8: **List of oligonucleotides used for amplification of various genes throughout this thesis.**
The table lists sequences, melting temperature (T_m), and the reference. fw: forward primer, rv: reverse primer, P: hydrolysis probe.

Gene	Sequence (5′ to 3′)	T_m [°C]	Reference
*bla*NDM	fw: GCCACACCAGTGACAATATC rv: GTGCTCAGTGTCGGCAT P: FAM-ACTTGGCCTTGCTGTCCTTGATCA-BHQ1	62.3 62.5 68.8	Probst *et al.* 2021a
*bla*KPC	fw: CAGCTCATTCAAGGGCTT rv: CGTCATGCCTGTTGTCAG P: JOE-CACACCCATCCGTTACGGCAA-BHQ1	60.5 61.0 67.4	Probst *et al.* 2021a
*bla*VIM	fw: TCCAATGGTCTCATTGTCC rv: CATGAAAGTGCGTGGAGA P: Texas Red®-ATGAGTTGCTTTTGATTGATACAG CKTGG-BHQ2	60.0 60.3 67.2 - 68.4	Probst *et al.* 2021a
*bla*IMP	fw1: GGTGGAATAGAGTGGCTTAATTCTC rv1: GCCAAACCACTACGTTATCTKGAG P1: CY®5-CCCACGTATGCRTCTGAATTAACAAA TGARCTTCT-BHQ2 fw2: GGAATAGAGTGGCTTAATTCTC rv2: GCCAAACCACTACGTTATCTK P2: CY®5-ATGCRTCTGAATTAACAAATGARCTT CT-BHQ2	63.4 64.2 - 65.5 69.5 - 71.4 59.0 61.6 - 61.9 64.0 - 66.7	Probst *et al.* 2021a
*bla*OXA-23-like	fw: TAAATGGAAGGGCGAGAA rv: ACCTGCTGTCCAATTTCAG P: FAM-CCATGAAGCTTTCTGCAGTCCCAGTC-BHQ1	59.0 60.8 69.0	Probst *et al.* 2021a
*bla*OXA-40/24-like	fw: TGACTTTAGGTGAGGCAATG rv: GTTATGTGCAAGGTCATCGG P: JOE-TGCAAGACGGACTGGCCTAGAGCTAAT-BHQ1	60.6 61.5 70.6	Probst *et al.* 2021a

Gene	Sequence (5′ to 3′)	T_m [°C]	Reference
bla$_{OXA-58}$-like	fw: ATTGGCACGTCGTATTGG rv: CCCCTCTGCGCTCTACATA P: Texas Red®-AGTGAATTGCAACGTATTGGTTA TGGCA-BHQ2	60.7 63.2 68.1	Probst *et al.* 2021a
bla$_{OXA-48}$-like	fw: AGGGCGTAGTTGTGCTC rv: GTGTTCATCCTTAACCACGC P: CY®5-TCTTAAACGGGCGAACCAAGCAT-BHQ2	61.9 61.7 67.5	Probst *et al.* 2021a
16S rRNA gene	fw: TCCTACGGGAGGCAGCAGT rv: GGACTACCAGGGTATCTAATCCTGTT P: Cy5.5-CGTATTACCGCGGCTGCTGGCAC-BBQ	59.4 58.1 69.9	Nadkarni *et al.* 2002
traI	fw: GACGAGCAAGGCAAGACCG rv: CGGAAGCTAACCAGCAGGTGATA	63.8 64.8	Shun-Mei *et al.* 2018
traM	fw: TTCAGGGTTACTGGCTTCAC rv: CCGCGGACGCTGGTTAATTG	60.6 64.5	Dmowski *et al.* 2018
traK	fw: GCTGCGGTTGCGTTCCTG rv: ACTTGACCTTCCCCGTTTCC	64.8 62.9	Shun-Mei *et al.* 2018
traL	fw: GGGAGCCTGACTAATACAAC rv: ATGGAACGGGAATGCATCAC	58.3 61.6	Dmowski *et al.* 2018
virB4	fw: CTGTACCTGACGGTGCTTTATC rv: TCAGCGACGTTGAGCATTAC	61.3 61.3	This thesis
virB5	fw: CGTCCGTAACTGGTGATGTT rv: GTGGAAATCTGCTCGGTTAGT	60.8 60.9	This thesis
recA	fw: ACACCGGCGAGCAGGCACTGGAAA rv: ACGTGCCGCAAGGCCCATGTGA	72.6 71.8	Liu *et al.* 2019a
lexA	fw: GAAGAGGAAGAAGGGTTGC rv: CAATACGTGCGACAACGA	61.3 61.6	This thesis
rpoD	fw: CGTGGTAAGGAGCAAGGCTATC rv: TCACCTGAATGCCCATGTCG	63.2 63.2	Shun-Mei *et al.* 2018
nusG	fw: GTCCGTTCGCAGACTTTAAC rv: GCTTTCTCAACCTGACTGAAG	59.7 59.6	Kjeldsen *et al.* 2015

Gene	Sequence (5′ to 3′)	T_m [°C]	Reference
umuC	fw: ACGCAGACATGAGC rv: TACGCACACCTGTCAGA	53.2 58.5	Pourahmad Jaktaji and Pasand 2016
umuD	fw: ATCTTCGCTACTGATGGTAATG rv: TGTTCAGTGTGGCTTTCC	58.8 58.5	This thesis
dinB	fw: ACGTGCTCCTGAGTAGTT rv: AAGTGTGATCTGGTGATTCTG	61.5 61.4	This thesis
208	ACGGCCGACC	49.9	Pellegrino *et al.* 2006
272	AGCGGGCCAA	48.9	Pellegrino *et al.* 2006
rop-1	fw: CAACATGGCGGGATTCATA rv: GTATGCTGTTCTGGAGTTCTT	60.0 60.0	This thesis
rop-2	fw: AACTGGACGCACTCAATG rv: GCGTGTCTGTATGCTGTT	59.9 59.9	This thesis
rop-3	fw: GCAAGCCGTACTCAACAT rv: CGAGTTCGTGCAGTTTCT	60.0 59.8	This thesis

2.8 Surveillance of carbapenem-resistant bacteria in patients' samples

Rectal swabs and bacterial isolates were obtained from the microbiological diagnostics of the Department of Infectious Diseases, Medical Microbiology, Heidelberg University Hospital, Heidelberg, Germany in 2019. These samples were used to validate the developed multiplex qPCR for carbapenemase detection in the context of the current epidemiology (Probst *et al.* 2021b) and regarding the performance directly on primary patients' specimens (Probst *et al.* 2022; see Figure 7).

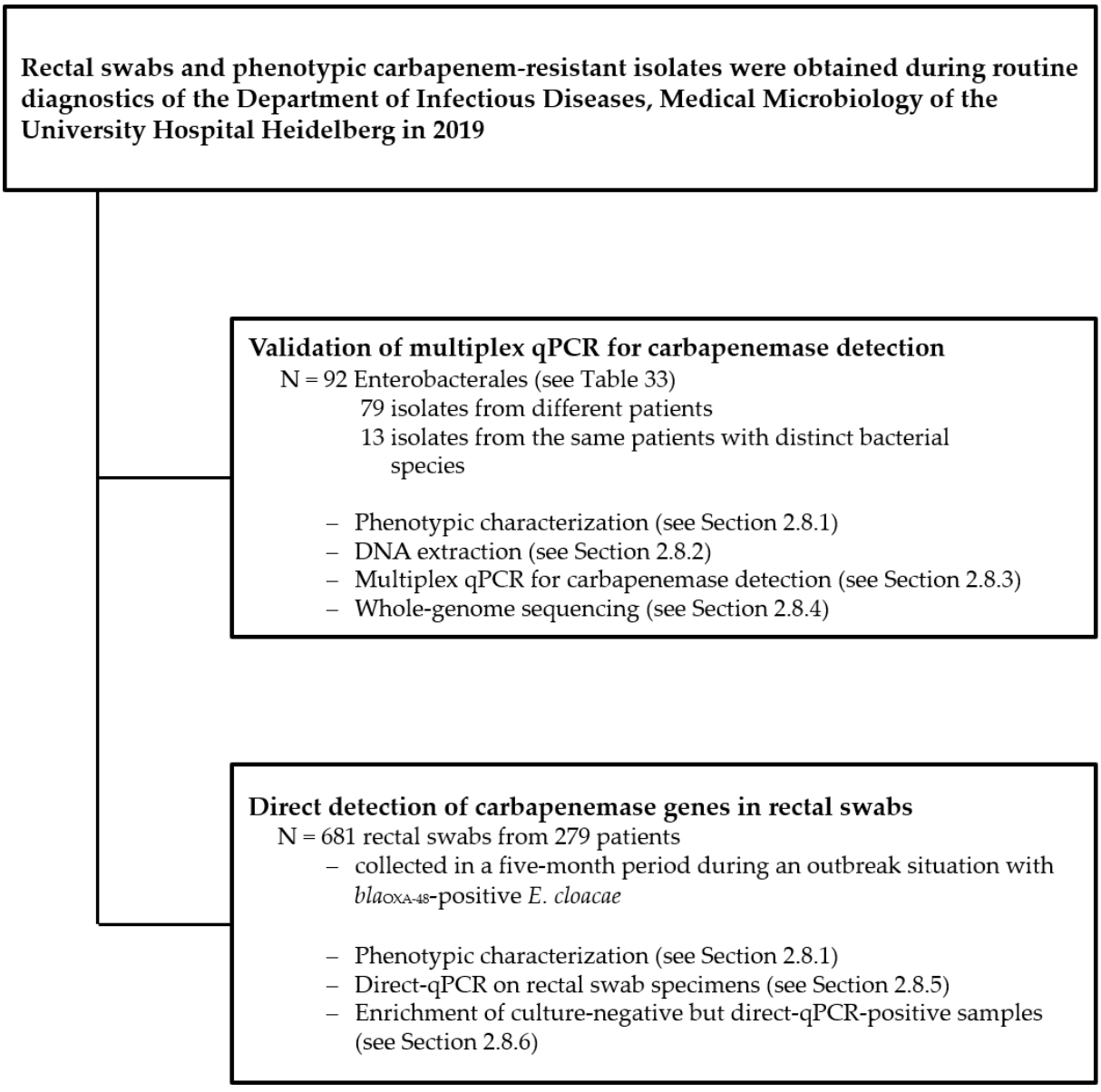

Figure 7: Overview of sample numbers and experimental procedure for carbapenemase screening in patients' samples by multiplex qPCR.

2.8.1 Phenotypic identification of carbapenem-resistant Gram-negative bacteria

Patients' specimens were plated on chromID® ESBL agar and antimicrobial resistance to meropenem and imipenem was determined with VITEK®2 automated system, which was evaluated considering the EUCAST guidelines of the respective year. MALDI-TOF MS was used to determine the bacterial species. The full phenotypic screening procedure was performed by staff of the microbiological diagnostics of the Department of Infectious Diseases, Medical Microbiology, Heidelberg University Hospital, Heidelberg, Germany. For long-term storage at - 20 °C skim-milk stocks were prepared, and isolates were regrown on Columbia Agar with 5 % sheep blood at 37 °C and 5 % CO_2.

2.8.2 DNA extraction for genotypic characterization

For genetic characterization of the phenotypic carbapenem-resistant isolates, DNA was extracted using the DNeasy Blood and Tissue Kit. A full loop (10 µl inoculation loop) of an overnight culture was resuspended in 180 µl of Buffer ATL. Then, 20 µl of Proteinase K (10 mg/ml) were added and the samples were incubated at 56 °C for 30 min. The samples were vortexed for 15 s and 200 µl of Buffer AL were pipetted to the samples. Before centrifugation at 6,000 x g for 1 min, the samples were transferred to the DNeasy Mini spin column. A washing step followed by adding 500 µl AW1 Buffer to the membrane and centrifugation at 6,000 x g for 1 min. After the second washing step with 500 µl AW2 Buffer and centrifugation at 20,000 x g for 3 min, the DNeasy Mini spin column was placed in a new nuclease-free 1.5 ml microcentrifuge tube. To elute the DNA, 200 µl of nuclease-free water were added directly on the membrane. After incubation for 1 min at room temperature, the samples were centrifuged at 6,000 x g for 1 min. DNA quality and quantity were determined by NanoDrop 1000 Spectrophotometer and Quant-iT™ PicoGreen™ dsDNA Assay Kit, according to the manufacturer's protocol, using the VICTOR® Nivo™ to determine fluorescence (excitation filter 480/30 nm, emission filter: 530/30 nm). The DNA was used for quantitative multiplex PCR (qPCR) and whole-genome sequencing (WGS) to identify carbapenemase-harboring bacteria. DNA was extracted for routine molecular characterization by Suzan Leccese, Selina Hassel, Nicole Henny, or Delal Sahin (Department of Infectious Diseases, Medical Microbiology and Hygiene at Heidelberg University Hospital in Heidelberg, Germany).

2.8.3 Carbapenemase gene detection by multiplex real-time PCR

A multiplex qPCR based on hydrolysis probes was used for identifying eight common carbapenemase genes: *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{OXA-23}$-like, *bla*$_{OXA-58}$-like, *bla*$_{OXA-40/24}$-like, and *bla*$_{OXA-48}$-like (Probst *et al.* 2021a). The two master mixes (MM1 and MM2) were prepared as described in Table 9 and Table 10. In MM1, primers and probes for amplifying the *16S rRNA* gene used as a positive control were added (Nadkarni *et al.* 2002).

Table 9: Composition of master mix 1 (MM1) for detecting *bla*NDM, *bla*KPC, *bla*VIM, *bla*IMP, and the *16S rRNA* gene as a positive control.

MM1	Final concentration	Volume for one reaction
Forward primer (IMP-fw1, IMP-fw2, 16S-fw)	0.3 µM	0.0375 µl
Reverse primer (IMP-rv1, IMP-rv2, 16S-rv)	0.3 µM	0.0375 µl
Probe (IMP-P1, IMP-P2)	0.2 µM	0.025 µl
Probe 16S-P	0.25 µM	0.03125 µl
Forward primer (NDM-fw, KPC-fw, VIM-fw)	0.4 µM	0.05 µl
Reverse primer (NDM-rv, KPC-rv, VIM-rv)	0.4 µM	0.05 µl
Probe (NDM-P, KPC-P, VIM-P)	0.15 µM	0.01875 µl
PrimaQUANT 5x qPCR-Probe-MasterMix	1x	2.5 µl
PCR-grade water		5.3375 µl
DNA		4 µl
Final volume		12.5 µl

Table 10: Composition of master mix 2 (MM2) for detecting *bla*OXA-23-like, *bla*OXA-58-like, *bla*OXA-40/24-like, and *bla*OXA-48-like.

MM2	Final concentration	Volume for one reaction
Forward primer (OXA-23-fw, OXA-40/24-fw, OXA-58-fw, OXA-48-fw)	0.4 µM	0.05 µl
Reverse primer (OXA-23-rv, OXA-40/24-rv, OXA-58-rv, OXA-48-rv)	0.4 µM	0.05 µl
Probe (OXA-23-P, OXA-40/24-P)	0.25 µM	0.03125 µl
Probe (OXA-58-P, OXA-48-P)	0.15 µM	0.01875 µl
PrimaQUANT 5x qPCR-Probe-MasterMix	1x	2.5 µl
PCR-grade water		5.5 µl
DNA		4 µl
Final volume		12.5 µl

Then, 10 µl of MM1 and MM2 were transferred to the BD MAX™ PCR cartridge and the PCR was run with the program described in Table 11 on the BD MAX™ system, using the PCR-only mode.

Table 11: PCR program for carbapenemase detection on the BD MAX™ system, using the PCR-only mode.

PCR step	Temperature	Time	Cycles
Initial denaturation	98 °C	3 min	1
Denaturation	98 °C	5 s	3
Annealing/Elongation	57 °C	47.8 s	
Denaturation	98 °C	5 s	37
Annealing/Elongation	61 °C	43 s	

2.8.4 Analyzing the bacterial resistome by whole-genome sequencing

To determine the full bacterial resistance pattern, carbapenem-resistant Gram-negative bacteria were analyzed by WGS. Library preparation was done with the Nextera DNA Flex Library Prep Kit performed by Suzan Leccese, Selina Hassel, Nicole Henny, or Delal Sahin. The WGS was conducted at the Department of Infectious Diseases, Medical Microbiology and Hygiene in Heidelberg, Germany, using the MIseq instrument (2 x 300 bp). Data analysis was performed by Dr. Sébastien Boutin (Department of Infectious Diseases, Medical Microbiology and Hygiene at Heidelberg University Hospital in Heidelberg, Germany). The quality control of raw sequencing reads was done by Sickle (parameters, q > 30; l > 45; Joshi and Fass 2011). The assembly was performed with SPAdes (Bankevich *et al.* 2012) and contigs < 1000 bp and < 10 x coverage were eliminated to exclude contaminations and sequencing errors in the draft genome. Prokka (Seemann 2014) and the NCBI Prokaryotic Genome Annotation Pipeline were used for annotation. The calculation of the core genome was done with Roary (core genome identity 100 %; Page *et al.* 2015). To determine the resistance pattern of the isolates, the databases ResFinder 3.0, ARG-ANNOT, and CARD-NCBI-BARRGD using ABRicate were chosen (Carattoli *et al.* 2014; Eichel *et al.* 2020; Zankari *et al.* 2012).

2.8.5 Carbapenemase detection directly from rectal swab specimens

During an outbreak situation with *bla*$_{\text{OXA-48}}$-positive *E. cloacae*, rectal swabs from the patients on the affected wards were collected (Probst *et al.* 2022). The cultural screening was conducted by the microbiological diagnostics of the Department of Infectious Diseases, Medical Microbiology, Heidelberg University Hospital, Heidelberg, Germany, as described in Section 2.8.1. To accelerate the time to results carbapenemase detection was performed directly on rectal swab specimens, using the multiplex qPCR (direct-qPCR; Probst *et al.* 2021a). The direct-qPCR was carried out on the BD MAX™ system, which combines DNA extraction and PCR in a fully automated procedure. The ExK™ DNA-2 Kit was used for DNA isolation by running the BD MAX™ ExK™ DNA-2 protocol (type 3: liquid MM with primers and probes). MM1 and MM2 were prepared two times concentrated for the direct-qPCR and the protocol described above was used (see Table 9 and Table 10). The extraction strips were equipped according to the manufacturer's protocol, and at position 3 50 µl of nuclease-free water were added for diluting the elution buffer. Finally, 100 µl of the liquid Aimes Collection and transport medium were transferred into the ExK DNA-2 sample buffer tube and vortexed for a few seconds. PCR was performed with the protocol described above (see Table 11).

2.8.6 Enrichment of carbapenemase-producers from rectal swab specimens

An additional enrichment step was performed for cultural negative rectal swabs that showed a positive result by direct-qPCR. Hereby, 5 ml LB-medium were inoculated with 100 µl rectal swab specimen and incubated overnight at 37 °C and 150 rpm. The bacterial suspension was then replated on chromID® ESBL agar and the PCR procedure using the ExK™ DNA-2 Kit was repeated for growing bacteria by adding one colony to the sample buffer tube as described in Section 2.8.5.

2.9 Surveillance of environmental contamination with carbapenemase-producers

Up to 40 ml of wastewater samples were collected at the Heidelberg University Hospital from different departments. The first collection was used to validate the performance of the qPCR when compared to cultural screening. With the second collection the wastewater treatment was optimized before using qPCR (Probst *et al.* 2022; see Figure 8).

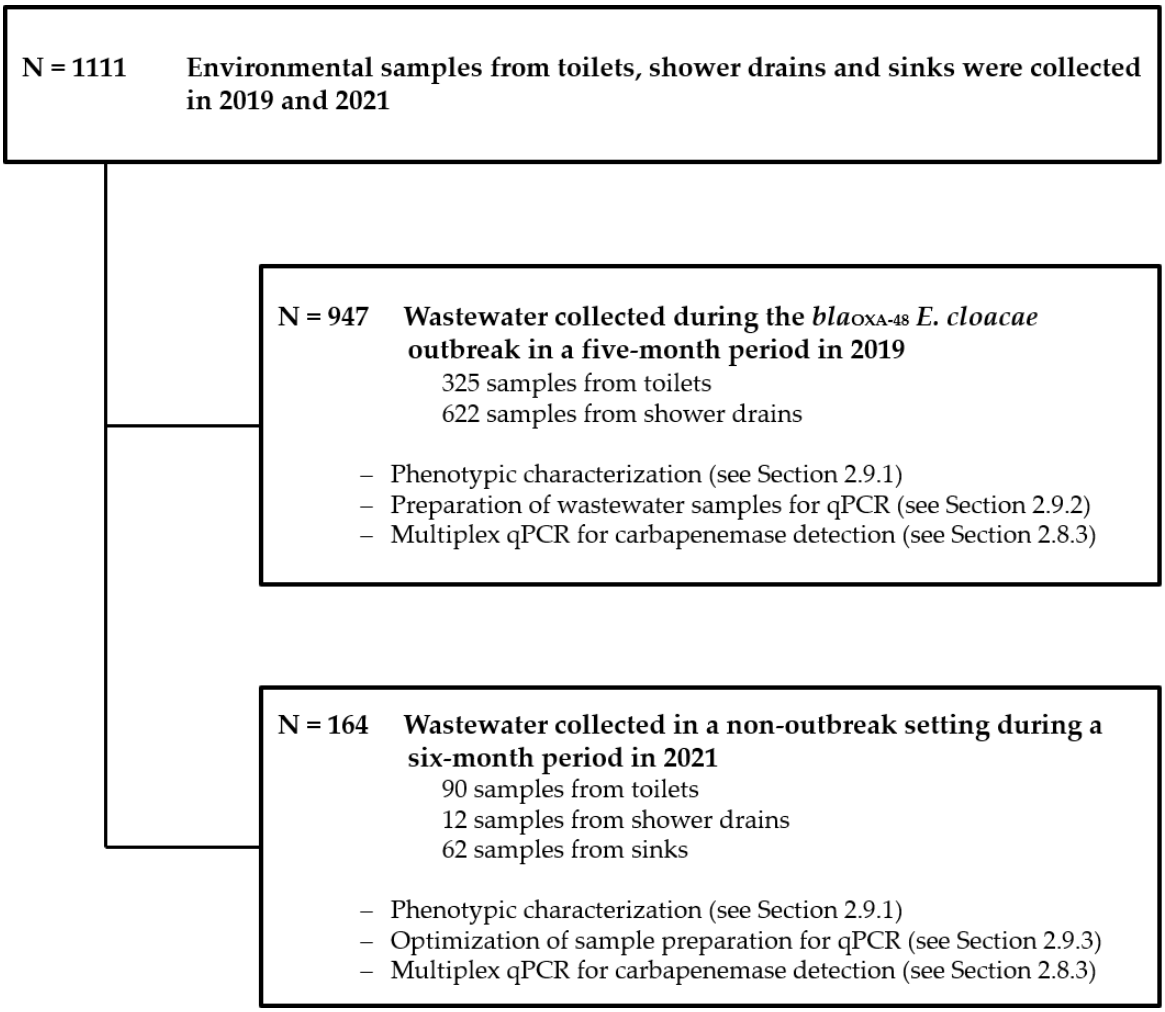

Figure 8: Overview of environmental samples and experimental procedure to detect carbapenemase genes in the hospital environment by multiplex qPCR.

2.9.1 Phenotypic screening of wastewater samples

The phenotypic screening of wastewater samples was performed by Delal Sahin, Annkathrin Wohlfahrt, or Michelle Mußler (Department of Infectious Diseases, Medical Microbiology and Hygiene at Heidelberg University Hospital in Heidelberg, Germany), using 10 µl of water to inoculate ChromID® CARBA SMART agar plates. The samples were incubated overnight at 37 °C with 5 % CO_2. The bacterial species was determined by using MALDI TOF MS (Probst *et al.* 2022).

2.9.2 Treatment of wastewater samples during the outbreak setting

For detecting carbapenemase genes by qPCR, the wastewater was concentrated by centrifugation of 5 ml at 4,300 rpm for 10 min and subsequently resuspending the pellet in 250 µl nuclease-free water. The samples were then incubated at 95 °C for 5 min to lyse the bacterial cells and centrifuged at 13,300 rpm for 5 min before using the supernatant in the qPCR (Probst *et al.* 2022). The qPCR procedure was performed as described in Section 2.8.3.

2.9.3 Optimization of the environmental screening

For the first experimental procedure of water sample treatment optimization was conducted, thus the qPCR was performed in parallel directly on untreated specimens, after overnight culture, and after centrifugation (see Section 2.9.2). The direct-qPCR was performed by adding 4 µl of wastewater to MM1 (see Table 9) and MM2 (see Table 10) using the procedure described in Section 2.8.3. For the overnight cultures, 5 ml of LB-medium were inoculated with 100 µl water sample and incubated at 37 °C and 150 rpm. After centrifugation at 4,300 rpm for 10 min, the pellet was resuspended in 250 µl nuclease-free water and silica-beads were added. Subsequently, a bead-beating step for 15 s using the Mini-Bead-Beater followed for mechanical disruption of the bacterial cells. All samples were incubated at 95 °C for 5 min and centrifuged at 13,300 rpm for 5 min before their use in the qPCR. The protocol for the multiplex qPCR can be found in Section 2.8.3.

2.10 Transfer of carbapenemase-encoding plasmids

Carbapenemase-positive bacteria used as donor strains were selected from the routine diagnostics of the Department of Infectious Disease, Medical Microbiology of the Heidelberg University Hospital between 2016 and 2019 (see Table 31). The isolates were tested regarding their conjugative ability without and with exposure to sublethal antibiotic concentrations. Additionally, different genetic mechanisms, putatively responsible for high transmission efficiencies were assessed (see Figure 9).

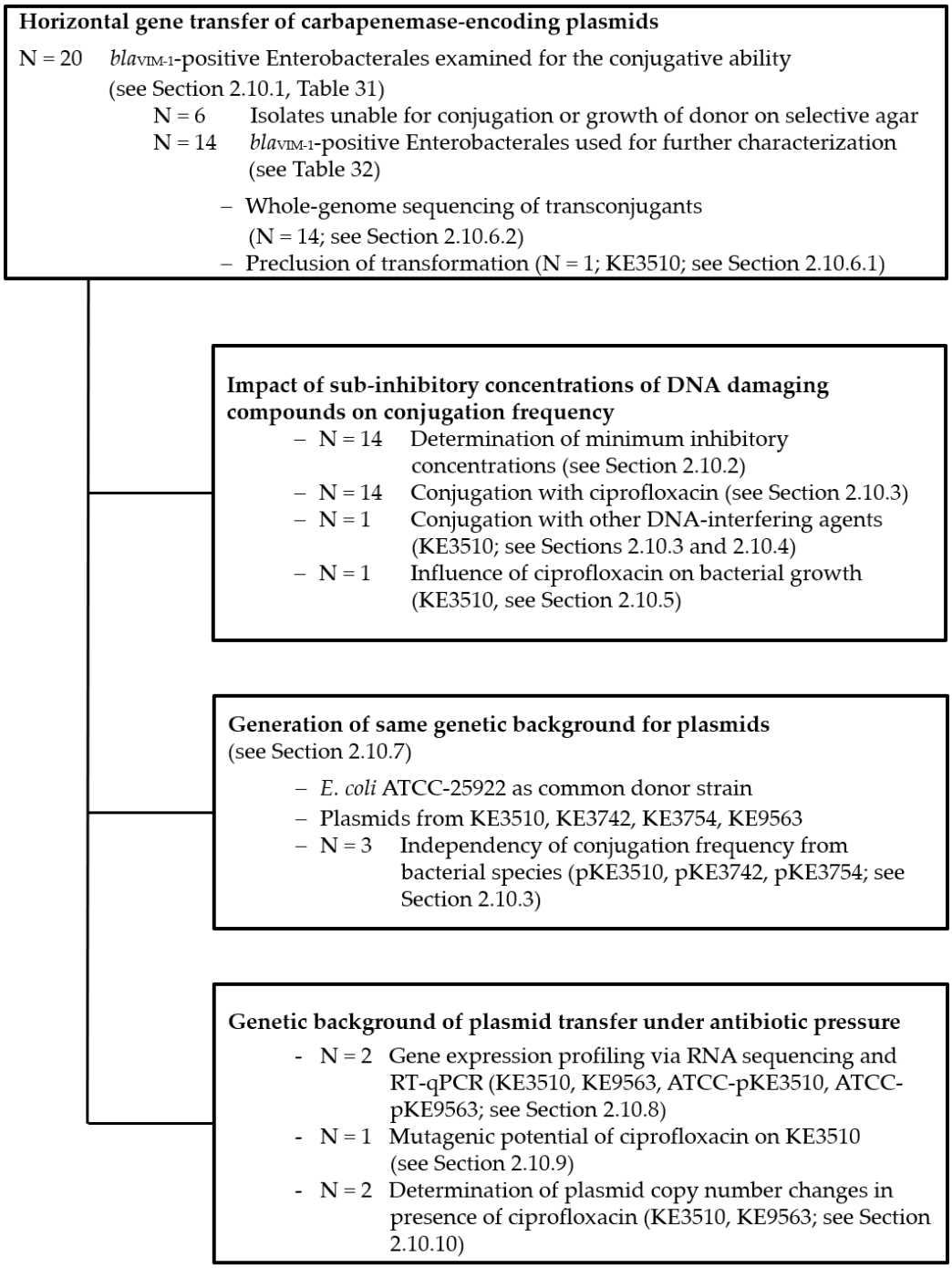

Figure 9: Overview of bacterial isolates and the experimental procedure to assess transmission of carbapenemase-encoding plasmids, the influence of antibiotic pressure, and the genetic background of plasmids with high conjugation frequencies.

2.10.1 Examination of conjugative ability

In vitro liquid mating experiments were carried out with the sodium azide resistant *E. coli* J53 as recipient to analyze whether a carbapenemase-positive isolate can transfer its plasmid (Göttig *et al.* 2015). Different isolates harboring *bla*$_{VIM-1}$-encoding plasmids were tested for their conjugative ability (see Table 31). Therefore, pre-cultures of donor and recipient, respectively, were prepared in 25 ml LB-medium and incubated at 37 °C and 150 rpm for 4 h. The bacterial cultures were adjusted to MF 3.0 ± 0.05 using the McFarland densitometer and 1 ml of donor and recipient were combined and mixed by vortexing for 5 s. After incubation of the bacterial suspension at 37 °C with 5 % CO_2 for 2 h, the conjugation process was interrupted by vortexing for 10 s. Then, 100 µl of different dilutions (1:2, 1:10, and 1:100), prepared in LB-medium, were plated on ChromoSelect Agar plates containing ESBL antibiotics and sodium azide and incubated at 37 °C with 5 % CO_2 for 48 h. Additionally, negative controls containing only the donor and the recipient strain (MF 3.0 ± 0.05), respectively, were added. The plates were evaluated for growth of the recipient, indicated by red colonies and countability of the transconjugants. Due to variability in growth and conjugation frequency, the experiments were performed in triplicates.

2.10.2 Determination of minimum inhibitory concentrations

To identify the influence of different antibiotics on the transmission of carbapenemase-encoding plasmids, MICs of several antimicrobial substances were determined by either gradient test strips or broth microdilution.

Gradient test strips were used to analyze the susceptibility to ciprofloxacin and nalidixic acid. Therefore, a bacterial solution of MF 0.5 ± 0.05 was prepared in 0.9 % NaCl, using the McFarland densitometer. The gradient test strips were transferred to a Mueller Hinton Agar plate, previously inoculated with the bacterial solution. After incubation overnight at 37 °C with 5 % CO_2, the zone of inhibition was evaluated (see Table 32).

Broth microdilution was used to determine antimicrobial resistance to mitomycin C, ethidium bromide, 5-azacytidine, and 3-aminocoumarin, which interfere with the bacterial DNA synthesis. The broth microdilution was performed in a 96-well plate, using 200 µl of bacterial suspension in LB-medium (MF 0.5 ± 0.05). Serial 1:10 dilutions of the antimicrobial substances were added with concentrations ranging from 0.1 µg/ml to 100 µg/ml. The broth microdilution was performed in triplicates for each antimicrobial compound. Additionally, a negative control containing only LB-medium and a positive control without antimicrobial compounds were added. After incubation at 37 °C with 5 % CO_2 overnight, the bacterial growth was evaluated according to the turbidity. The concentration that inhibited bacterial growth was used as the MIC.

2.10.3 Influence of antimicrobial compounds on conjugation frequency

To analyze the effect of different antimicrobial compounds on the transfer of carbapenemase-encoding plasmids, conjugation experiments were performed as described in Section 2.10.1 with the following modifications: After merging donor and recipient, different concentrations of antibiotics or DNA-interfering compounds (ciprofloxacin, nalidixic acid, 3-aminocoumarin, mitomycin C, ethidium bromide, 5-azacytidine) were added at concentrations below the MIC, at the MIC or higher than the MIC (1/32 MIC, 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, 2x MIC). Colonies were counted and the conjugation efficiency was calculated by dividing the numbers of transconjugants by the number of donors (MF 3.0 equals approximately 10^9 CFU/ml). Triplicates of the conjugation experiments were carried out in three independent experiments performed on different days.

2.10.4 Impact of UV-B on conjugation frequency

Transfer efficiency under UV-B exposure was investigated using the donor strain *Citrobacter amalonaticus* KE3510 and the recipient *E. coli* J53. After the pre-culture (see Section 2.10.1), 1 ml of MF 3.0 ± 0.05 donor and recipient, respectively, were merged in a Petri dish. UV-B exposure was carried out using the UV Stratalinker 1800 for 0.2 min (200 μJ/cm²), 0.5 min (500 μJ/cm²), 0.7 min (700 μJ/cm²), and 1 min (1 x 10^3 μJ/cm²), respectively, without the lid of the Petri dish. After 2 h of conjugation at 37 °C and 5 % CO_2 100 μl of the bacterial suspension was plated on selective agar and the number of transconjugants was evaluated after 48 h.

2.10.5 Influence of ciprofloxacin on bacterial growth

To evaluate the effect of sub-inhibitory ciprofloxacin concentrations on the growth of *C. amalonaticus* KE3510, growth curves were recorded with the CG Quant. Experiments were performed in three independent replicates by inoculating 10 ml LB-medium with 100 μl MF 0.5 ± 0.05 bacterial suspension prepared in LB-medium. Different antibiotic concentrations were added to the culture: 1/32 MIC, 1/16 MIC, 1/8 MIC, 1/4 MIC, and 1/2 MIC, respectively. The bacterial growth was recorded for 20 h at 37 °C, 5 % CO_2 and 200 rpm, with automatic quantification once an hour.

2.10.6 Confirmation of plasmid transfer via conjugation

2.10.6.1 Preclusion of bacterial transformation

To ensure that the transconjugants resulted from bacterial conjugation, transformation experiments were performed with inactivated bacteria and extracted DNA, respectively. The donor cells were lysed in the Mini-Bead-Beater for 15 s with an additional incubation at 95 °C for 10 min. Furthermore, the transformation was tested with isolated donor DNA that was added to the recipient *E. coli* J53. The remaining procedure was performed as described in Section 2.10.1.

2.10.6.2 Analysis of the transferred plasmid content

The transfer success of the *bla*$_{VIM-1}$-encoding plasmids to the recipient *E. coli* J53 was confirmed by WGS. Therefore, the transconjugants were lysed by resuspending three to four colonies in 170 µl TE-buffer (pH 8.0) and adding 15 µl lysozyme (40 mg/ml). The samples were then incubated at 37 °C and 800 rpm for 30 min. After adding 15 µl Proteinase K (10 mg/ml) an additional incubation step followed at 56 °C and 800 rpm for one hour. DNA was extracted with the Genomic DNA Clean & Concentrator-10 Kit. First, 600 µl of ChIP DNA Binding Buffer were added to the lysed cells and the mixture was transferred to the Zymo-Spin™ IC-XL Columns. After centrifugation at 12,000 rpm for 30 s, two washing steps followed by adding 200 µl of DNA Wash Buffer to the columns and centrifuging at 12,000 rpm for 1 min. To elute the DNA, 80 µl of nuclease-free water were pipetted directly on the membrane. The samples were incubated at 56 °C for 3 min before the DNA was eluted by centrifugation at 12,000 rpm for 30 s. The quality and quantity of the DNA was determined by NanoDrop 1000 Spectrophotometer.

Library preparation was performed as described in Section 2.8.4 by Suzan Leccese and Selina Hassel. To examine which plasmids were transferred to the recipient, the genetic content of the transconjugants and the recipient *E. coli* J53 were aligned, using Mauve (Darling *et al.* 2004) with the default settings. The alignment was manually screened for non-matching genes, which represented the plasmid content. These genes were annotated using Prokka (Seemann 2014). Additionally, Inc-type, insertion sequences and the present resistance genes were determined with the databases PlasmidFinder (Carattoli *et al.* 2014), ISFinder (Siguier *et al.* 2006), and ARG-ANNOT (Gupta *et al.* 2014), using ABRicate. For genes that were not annotated by Prokka and ABRicate, the identification was performed manually with UniProt BLAST (UniProt 2021). To assess similarities between the transferred *bla*$_{VIM-1}$ plasmid contents of the tested isolates, a core genome was calculated using Roary (Page *et al.* 2015). Genes present in the core needed to be abundant in all transconjugants (100 % identity). A multi-FASTA alignment of the plasmid content was created, using PRANK by default (Löytynoja 2014). Based on the alignment, a maximum-likelihood phylogenetic tree was generated with FastTree (Price *et al.* 2010). The tree illustration was optimized using FigTree by rooting the tree to the midpoint. According to the similarities of the plasmid Inc-types, the transferred plasmid content was illustrated with BRIG (Alikhan *et al.* 2011).

2.10.7 Generation of identical genetic background for different plasmids

The same genetic background for various plasmids was needed for some experimental setups. Therefore, the laboratory strain *E. coli* ATCC-25922 was used to avoid the impact of the different genomic content of the varying donor species on the conjugation frequencies. Additionally, issues in primer matching for gene expression profiling and determination of plasmid copy numbers were eradicated.

2.10.7.1 Transfer of *bla*$_{VIM-1}$-encoding plasmids to *E. coli* ATCC-25922

To transfer the *bla*$_{VIM-1}$-encoding plasmids to the rifampicin-resistant *E. coli* ATCC-25922, conjugation experiments were carried out. Rifampicin-resistant *E. coli* was generated by serial passaging, i.e., by increasing rifampicin concentrations to a final amount of 512 mg/l. By WGS an amino acid exchange (Q513L) in the *rpoB* gene could be found, confirming the rifampicin-resistant phenotype (Wu and Hilliker 2017; Zhou *et al.* 2013).

The conjugation procedure was performed as described in Section 2.10.1 with the following changes: After 2 h of conjugation, 1 mg/ml rifampicin was added to the bacterial culture and an additional incubation for 2 h ensued. Subsequently, 100 µl of the bacterial culture were transferred to a chromID® ESBL agar plate and incubated overnight at 37 °C and 5 % CO_2. The transconjugants were analyzed by random amplification of polymorphic DNA (RAPD, see Section 2.10.7.2) and qPCR (see Section 2.8.3) for transfer success.

2.10.7.2 Random amplification of polymorphic DNA

RAPD was performed to distinguish between *E. coli* ATCC-25922 and *E. coli* J53 to ensure that the conjugation to the recipient was successful. DNA was extracted using the chelex-based method. That means a loop of bacteria was transferred into 100 µl lysozyme (10 mg/ml) and incubated for 45 min at 37 °C and 450 rpm. After adding 1 µl of proteinase K (20 mg/ml) and 1 µl of 10 % SDS, samples were incubated for 30 min at 37 °C and 450 rpm. Then, 100 µl of 10 % chelex were added and samples were mixed gently. After incubation for 30 min at 56 °C and 450 rpm, the samples were vortexed for 10 s followed by an additional incubation step at 95 °C for 10 min. The samples were centrifuged for 5 min at 13,300 rpm and the supernatant was transferred into a new nuclease-free 1.5 ml microcentrifuge tube (Martin-Platero *et al.* 2010). To remove protein and carbohydrate contaminants, ethanol precipitation was performed, adding 0.3 M ammonium acetate and 175 µl of ethanol absolute to 45 µl of DNA. After inverting the tubes, an incubation at - 80 °C for 1 h followed. The samples were centrifuged for 20 min at 13,300 rpm and 4 °C to pelletize the DNA and the supernatant was discarded. Two washing steps with 300 µl ethanol (70 %, v/v) and centrifugation (13,300 rpm, 20 min, and 4 °C) followed. To completely remove the ethanol, the samples were dried in a centrifuge concentrator at 60 °C for 5 min and resuspended in 70 µl pre-warmed nuclease-free water (56 °C). DNA quality and quantity control were performed with the NanoDrop 1000 Spectrophotometer.

The master mix described in Table 12 was prepared for the PCR, using primers 208 and 272 (see Table 8). RAPD was performed by Michelle Mußler and Annkathrin Wohlfahrt.

Table 12: Composition of the master mix for RAPD.

Component	25 µl reaction	Final Concentration
MyTaq™ HS Mix	12 µl	1 x
Forward primer (25 µM)	0.5 µl	0.5 µM
Reverse primer (25 µM)	0.5 µl	0.5 µM
Template DNA	variable	10 ng
Nuclease-free water	to 25 µl	

The PCR was run in a thermocycler with the program shown in Table 13.

Table 13: PCR-program used for RAPD.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	94 °C	5 min	1
Denaturation	94 °C	45 s	
Annealing	30 °C	45 s	30
Extension	72 °C	30 s	

Illustration of the DNA fragments was performed on the QIAxcel Advanced System. Therefore, a 1:3 dilution of the PCR product with QX DNA Dilution Buffer was prepared to a final volume of 15 µl.

2.10.8 Gene expression profiling

To better understand whether the expression of conjugation-related genes or genes involved in SOS response have an impact on the conjugation frequency, quantitative reverse transcription PCR (RT-qPCR) and RNA sequencing were performed. RNA extraction and clean-up, described in Sections 2.10.8.1 and 2.10.8.2, was performed for all following gene expression experiments. Bacterial pre-cultures of *C. amalonaticus* KE3510, *E. cloacae* KE9563, *E. coli* ATCC-pKE3510, and *E. coli* ATCC-pKE9563, respectively, were prepared in 25 ml LB-medium and incubated at 37 °C and 150 rpm for 4 h. Subsequently, the bacterial suspension was adjusted to MF 3.0 ± 0.05 with the McFarland densitometer in LB-medium, and 2 ml of non-treated and ciprofloxacin-treated cultures (1/4 MIC and 1/2 MIC) were incubated at 37 °C with 5 % CO_2 for 4 h. Cells were harvested by centrifugation of 2 ml liquid culture at 13,300 rpm for 5 min. The supernatant was discarded.

2.10.8.1 Bacterial RNA extraction

Bacterial RNA was extracted with the Direct-zol™ RNA Miniprep Plus Kit. Silica beads and 700 µl of TriZol were transferred to the bacterial pellet, which was resuspended by vortexing for 30 s. After 3 min of incubation, the bacterial cells were lysed in the Mini-Bead-Beater for 30 s. Then, 700 µl of absolute ethanol were added and the samples were mixed thoroughly by inverting the tubes. To bind the RNA to the Zymo-Spin™ IIICG Column, 700 µl of the sample were added and a centrifugation step at 16,000 x g for 30 s followed. Before adding the prepared DNase I mix (5 µl DNaseI and 75 µl DNA Digestion Buffer per sample), the column was washed with 400 µl RNA Wash Buffer (centrifugation at 16,000 x g for 30 s). After 15 min of incubation with the DNase I mix at room temperature two washing steps with 400 µl Direct-zol™ RNA PreWash were performed. The columns were centrifuged each time at 16,000 x g for 30 s and the flow-through was discarded. The last washing step was performed by adding 700 µl of RNA wash buffer to the column with a centrifugation step at 16,000 x g for 2 min. To dry the Zymo-Spin™ IIICG Column an additional centrifugation step was done (17,000 x g, 1 min). After transferring the columns to a new RNase-free tube, the RNA was eluted by adding 50 µl of DNase/RNase-free water to the column and incubating the samples at room temperature for 3 min. The elution was done by centrifugation at 16,000 x g for 30 s. RNA quality and quantity were determined by NanoDrop 1000 Spectrophotometer.

2.10.8.2 RNA clean-up and concentrator

To remove genomic DNA a second DNase I treatment and RNA clean-up was performed using the RNA Clean & Concentrator Kit. A maximum of 20 µg RNA was used per reaction and 5 µl DNase I and DNA Digestion Buffer, respectively, were added. DNase/RNase-free water was added to a final volume of 50 µl and the mixture was incubated for 15 min at room temperature. Then, 100 µl of RNA binding buffer were transferred to the sample and mixed by vortexing. Subsequently, 200 µl of absolute ethanol were added. After mixing, the samples were transferred to the Zymo Spin IC Column and centrifuged at 16,000 x g for 30 s. Three washing steps followed by adding 400 µl RNA Prep Buffer, 700 µl RNA Wash Buffer, and 400 µl RNA Wash Buffer, respectively. The centrifugation of the samples was performed at 16,000 x g for 30 s. The last centrifugation step was performed at 16,000 x g for 2 min. After transferring the column in an RNase-free tube 15 µl DNase/RNase-free water were pipetted directly on the membrane and samples were centrifuged at 16,000 x g for 30 s. The concentration and quality of the RNA was measured with the NanoDrop 1000 Spectrophotometer. RNA was used for gene expression profiling if the quality was at least 1.8 based on the ratios A260/280 and A260/230.

To exclude DNA contaminations, a no-RT control was performed, without adding the Luna WarmStart® RT Enzyme Mix (20 x) to the reaction (see Section 2.10.8.3). Primers for amplifying the *16S rRNA* gene were used for the no-RT control. The RNA samples with cycle threshold (Ct)-values ≥ 33.0 for the *16S rRNA* gene in the no-RT control were used for gene expression profiling.

2.10.8.3 Expression of conjugation-related genes

Expression of conjugation-related genes was analyzed for the isolates *C. amalonaticus* KE3510 and *E. cloacae* KE9563. Non-treated isolates were compared to isolates after 4 h of 1/4 MIC and 1/2 MIC ciprofloxacin exposure, respectively. The cDNA synthesis and the amplification of the desired genes were combined, using the Luna® Universal One-Step RT-qPCR Kit. The experiments were performed in triplicates in two independent experiments. The genes *rpoD*, *traI*, *traM*, *traK*, *traL*, *virB4* (*traC*), and *virB5* (*traQ*, see Table 8) were targeted by PCR using the protocol in Table 14.

Table 14: Composition of the master mix for the RT-qPCR with the Luna® Universal One-Step RT-qPCR Kit.

Component	20 µl reaction	Final Concentration
Luna Universal One-Step Reaction Mix (2x)	10 µl	1 x
Luna WarmStart® RT Enzyme Mix (20x)	1 µl	1 x
Forward primer (10 µM)	0.8 µl	0.4 µM
Reverse primer (10 µM)	0.8 µl	0.4 µM
Template RNA	variable	250 ng
Nuclease-free water	to 20 µl	

The times and temperatures listed in Table 15 were used to analyze the gene expression on the StepOnePlus™ system. A melt curve was included to ensure specific amplification of the target genes.

Table 15: Program used for analyzing the gene expression profile with the Luna® Universal One-Step RT-qPCR Kit.

Cycle Step	Temperature	Time	Cycles
Reverse Transcription	55 °C	10 min	1
Initial Denaturation	95 °C	1 min	1
Denaturation	95 °C	10 s	40
Extension	60 °C	30 s	
Melt Curve	60 °C - 95 °C	various	1

Gene expression was normalized to the housekeeping gene *rpoD* and the non-treated control, using the ΔΔCt method. Ct-values of the conjugation-related genes were subtracted from the Ct-value of the housekeeping gene for the non-treated and the antibiotic-treated samples, respectively, leading to the ΔCt-value. Then, the ΔCt-value of the treated sample was subtracted from the ΔCt-value of the non-treated sample for each gene, resulting in the ΔΔCt-value. Fold change was determined using the following equation: $2^{-\Delta\Delta Ct}$. For graphic representation, $\log_2$(fold change) was used. Statistics were performed with the ΔCt-values (see Section 2.11).

2.10.8.4 Expression of SOS response-related genes

The expression of SOS response-related genes was analyzed using the *E. coli* ATCC-25922, harboring the *bla*$_{VIM-1}$-encoding plasmids from *C. amalonaticus* KE3510 (ATCC-pKE3510) and *E. cloacae* KE9563 (ATCC-pKE9563), respectively. The isolates were treated with 1/4 MIC and 1/2 MIC ciprofloxacin for 4 h (see Section 2.10.3). RNA was extracted subsequently following the protocols described in Sections 2.10.8.1 and 2.10.8.2. Bacterial cDNA was prepared using the RevertAid First Strand cDNA Synthesis Kit. In total, 200 ng RNA were used for cDNA synthesis. To exclude DNA contamination, a no RT-control was used, adding nuclease-free water instead of the RevertAid M-MulV RT. For one reaction the amounts of reagents listed in Table 16 were used and the cDNA synthesis was performed in the thermocycler using the program described in Table 17.

Table 16: Composition of the master mix for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit.

Component	20 µl reaction	Final Concentration
Random Hexamer Primer (100 µM)	1 µl	
5x Reaction Buffer	4 µl	1 x
RiboLock RNase Inhibitor (20 U/µl)	1 µl	20 U
dNTP-Mix (10 mM)	2 µl	
RevertAid M-MulV RT (200 U/µl)	1 µl	200 U
Total RNA	variable	200 ng
Nuclease-free water	to 20 µl	

Table 17: Program used for generating cDNA with the RevertAid First Strand cDNA Synthesis Kit.

Cycle Step	Temperature	Time	Cycles
Step 1	25 °C	5 min	1
Step 2	42 °C	60 min	1
Step 3	70 °C	5 min	1

A 1:4 dilution of the cDNA was used for gene expression analysis using the StepOnePlus™ system. The PCR program, including a melt curve for specific amplification control, can be found in Table 19. Primers for amplifying the housekeeping gene *rpoD*, and the SOS response-related genes *recA*, *lexA*, *umuC*, *umuD*, and *dinB* were used (see Table 8 and Table 18). The gene expression was calculated with the ΔΔCt method (see Section 2.10.8.3). Experiments were performed as triplicates in two independent experiments.

Table 18: Composition of the master mix for amplification of SOS response-related genes.

Component	20 µl reaction	Final Concentration
2x qPCRBIO SyGreen Mix Hi-ROX	10 µl	1x
Forward primer (100 µM)	0.08 µl	0.4 µM
Reverse primer (100 µM)	0.08 µl	0.4 µM
Template cDNA	variable	0.25 ng/µl
Nuclease-free water	to 20 µl	

Table 19: Program used to analyze SOS response-related gene expression.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	20 s	1
Denaturation	95 °C	3 s	40
Extension	60 °C	30 s	
Melt Curve	60°C - 95°C	various	1

2.10.8.5 RNA Sequencing

Gene expression analysis by RNA sequencing was done for *C. amalonaticus* KE3510 and *E. cloacae* KE9563 4 h post-treatment with 1/4 MIC of ciprofloxacin and a non-treated control. The experimental procedure for RNA extraction and clean-up was described in Sections 2.10.8.1 and 2.10.8.3. Transcriptomics was performed on the MiSeq instrument at the Department of Infectious Diseases, Medical Microbiology, Heidelberg University Hospital, Heidelberg, Germany. Libraries were prepared with the Ovation® Complete Prokaryotic RNA-Seq DR Multiplex Systems 1-8 and 9-16 Kit, using 500 ng of RNA. The experimental procedure was performed according to the manufacturer's protocol. Analysis of the raw sequencing data was done by Dr. Sébastien Boutin.

First, an index of the reference genome was created for mapping the transcripts of *C. amalonaticus* KE3510 and *E. cloacae* KE9563, respectively, to the reference genome using the Burrows-Wheeler Aligner (Li and Durbin 2009). After that all annotations were assembled and compared to the original reference, using StringTie (Pertea *et al.* 2015) and GffCompare (Pertea 2020). Subsequently, Ballgown (Frazee *et al.* 2015) and DESeq2 (Love *et al.* 2014) were used to compare the diverse expression between control and the ciprofloxacin-treated samples. Differential analysis and data visualization were done with the R software (R-Core-Team 2020).

2.10.9 Mutagenesis of *C. amalonaticus* KE3510 due to ciprofloxacin treatment

To evaluate whether ciprofloxacin in sublethal concentrations causes mutations, a pre-culture of *C. amalonaticus* KE3510 was performed in 25 ml LB-medium at 37 °C and 150 rpm for 4 h. The bacterial suspension was subsequently adjusted to MF 3.0 ± 0.05, and 0.0625 µg/ml ciprofloxacin (1/4 MIC) were added to a final volume of 2 ml. Additionally, a control of KE3510 without antibiotic treatment was included. After an additional 4 h of incubation, cells were harvested and cell lysis, as well as DNA extraction, was performed as described in Sections 2.10.1 and 2.8.2. Library preparation, WGS, and analysis of the raw sequencing data was done as described in Section 2.8.4. A core genome was calculated using Roary (95 % identity), to determine genes that are present or absent in the control and the mutant, respectively.

Validation of gene deletion in the mutant was performed by PCR. Therefore, the DNA used for WGS was chosen as a template for amplification of the regulatory gene *rop* with the master mix described in Table 20.

Table 20: Composition of the master mix for amplification of the regulatory gene *rop*.

Component	25 µl reaction	Final Concentration
Q5® High-Fidelity 2X Master Mix Mix	12.5 µl	1 x
Forward primer (100 µM)	0.075 µl	0.3 µM
Reverse primer (100 µM)	0.075 µl	0.3 µM
Template DNA	variable	10^5 copies
Nuclease-free water	to 25 µl	

The PCR was run in the thermocycler using the protocol described in Table 21.

Table 21: Program used to analyze the presence of the regulatory gene *rop*.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	
Annealing and Extension	60 °C, 55 °C[a]	30 s	40
Final Extension	72 °C	2 min	

[a] PCR was tested with different annealing and extension temperatures, using either 60 °C or 55 °C.

DNA amplicons generated by PCR were visualized using the QIAxcel Advanced System with the procedure described in Section 2.10.7.2.

2.10.10 Determination of plasmid copy numbers

To analyze whether the number of plasmid copies is changing under treatment with sub-inhibitory concentrations of ciprofloxacin, qPCR was performed. Pre-cultures of the donor strains *C. amalonaticus* KE3510, *E. coli* ATCC-pKE3510, and ATCC-pKE9563 were prepared (see Section 2.10.8). The cultures were adjusted to MF 3.0 ± 0.05 using the McFarland densitometer and incubated for 4 h under ciprofloxacin exposure (1/4 MIC and 1/2 MIC). Subsequently, the cells were harvested by centrifugation for 5 min at 13,300 rpm, and the DNA was extracted with the chelex-based method, as described in Section 2.10.7.2. DNA quality and quantity were determined by NanoDrop 1000 Spectrophotometer and Quant-iT™ PicoGreen™ dsDNA Assay Kit. Genomic copy numbers were calculated with the average genome size of *C. amalonaticus* and *E. coli* ATCC-25922, respectively, and the samples were adjusted to 10^5 copies/µl. Primers amplifying the chromosomal *rpoD* gene and the plasmid-encoded *bla*$_{VIM-1}$ gene were used to determine plasmid copy numbers, using the 2x qPCRBIO SyGreen Mix Hi-ROX (see Table 22).

Table 22: Composition of the master mix for amplification of chromosomal and plasmid-encoded genes.

Component	20 µl reaction	Final Concentration
2x qPCRBIO SyGreen Mix Hi-ROX	10 µl	1x
Forward primer (10 µM)	0.4 µl	0.2 µM
Reverse primer (10 µM)	0.4 µl	0.2 µM
Template DNA	variable	10^5 copies
Nuclease-free water	to 20 µl	

Amplification was done on the StepOnePlus™ system with the protocol described in Table 23.

Table 23: Program used for amplification of the chromosomal *rpoD* and the plasmid-encoded *bla*$_{VIM-1}$ gene.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	20 s	1
Denaturation	95 °C	3 s	40
Extension	60 °C	30 s	
Melt Curve	60°C - 95°C	various	1

For comparing the plasmid numbers of the different isolates, ratios of the Ct-values of the plasmid-encoded gene to the chromosomal gene were created. The experiments were performed in three independent experiments as triplicates.

2.11 Statistics

Statistical analysis of the data in this thesis was done with GraphPad Prism. The experiments were performed in biological replicates, whereby the exact number of repetitions is mentioned in the Materials and Methods section and each figure caption. One-way analysis of variance (ANOVA) was performed to determine the significance of conjugation frequencies and gene expression between the control and the antibiotic-treated samples. The Dunnett test was done as a follow-up test to ANOVA to compare the mean of all treated samples with the mean of the control. Statistically significant results were labeled as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Calculation of confidence intervals was done with the Clopper-Pearson interval. To analyze the magnitude of relation, Pearson's Product-Moment Correlation was used.

2.12 Ethics

The ethical committee was consulted regarding the use of patient samples for quality improvement in infection prevention and control through active surveillance of multidrug-resistant organisms at the Heidelberg University Hospital (S474/2018).

3 Results

Parts of the following results have been published previously (Probst *et al.* 2022; Probst *et al.* 2021b); a complete attribution can be found in Section 8.

3.1 Surveillance of carbapenem-resistant bacteria

3.1.1 Local epidemiology of carbapenem-resistant Enterobacterales

During a routine microbiological diagnostics procedure, phenotypic carbapenem-resistant Enterobacterales were collected at the Heidelberg University Hospital in 2019. Antimicrobial susceptibility testing by VITEK®2 revealed 92 phenotypic carbapenem-resistant bacteria. Carbapenemase detection was performed by qPCR and compared to WGS for validation. In this collection, 80.4 % of the isolates (74/92) were tested positive for a carbapenemase gene by qPCR. WGS confirmed the presence of these genes in all samples, but initially, six discordant results appeared. Isolates harboring *bla*$_{\text{KPC-2}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{VIM-1}}$ were considered as negatives during the first run of analysis. In two additional isolates, harboring two carbapenemase genes simultaneously, *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{KPC-2}}$, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{KPC-2}}$, respectively, just one gene was identified initially. After lowering the coverage threshold and reanalyzing the raw sequencing data, six carbapenemase genes were found by WGS (see Table 24 and Table 33).

Table 24: Comparing the performance of qPCR and WGS for carbapenemase detection in Enterobacterales. This table has been originally published as part of Table 1 by Probst *et al.* (2021b).

		WGS	
		negative	positive
qPCR	negative	18	0
	positive	6 (0)[a]	68 (74)[a]

[a] After reanalyzing the raw sequencing data

The most common carbapenemase gene was *bla*$_{\text{OXA-48}}$ (40.5 %), followed by *bla*$_{\text{VIM-1}}$ (21.6 %), *bla*$_{\text{KPC-2}}$ (12.2 %), and *bla*$_{\text{NDM-5}}$ (9.4 %). *Bla*$_{\text{NDM-1}}$ (2.7 %), *bla*$_{\text{OXA-244}}$ (2.7 %), *bla*$_{\text{KPC-3}}$ (1.4 %), and *bla*$_{\text{OXA-232}}$ (1.4 %) genes were less abundant. Isolates positive for two different carbapenemase genes were detected occasionally (8.1 %). The predominant species harboring carbapenemase genes was *E. cloacae* (n = 30), followed by *K. pneumoniae* (n = 17) and *E. coli* (n = 15). Carbapenemase genes were less frequently detected in *Citrobacter freundii* (n = 7), *Klebsiella oxytoca* (n = 3), and *S. marcescens* (n = 2, see Figure 10).

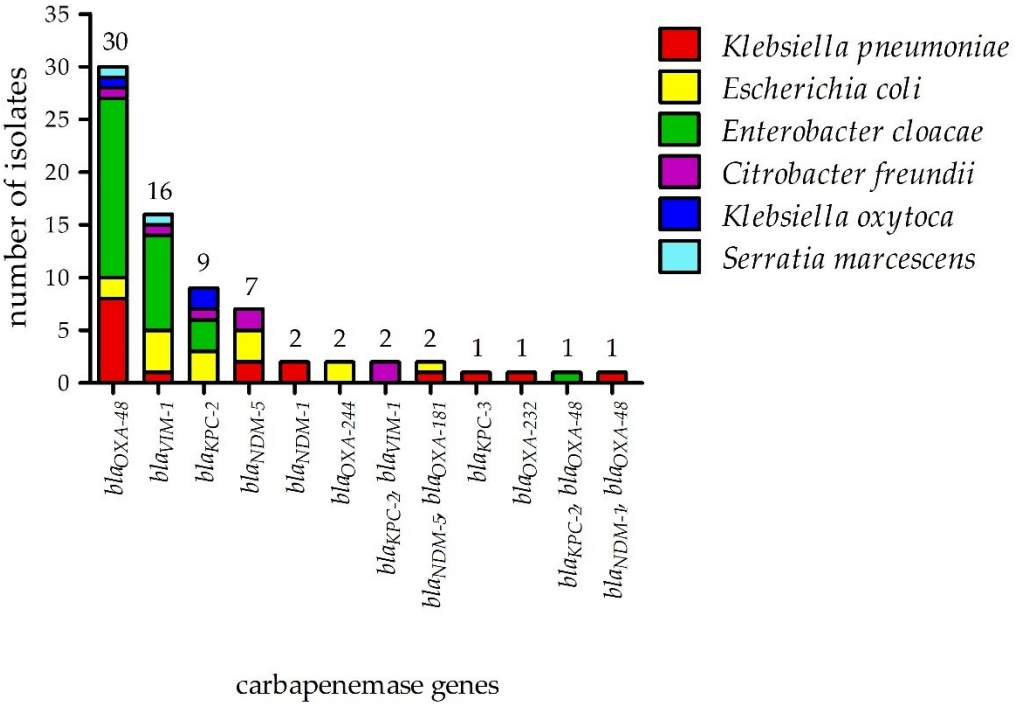

Figure 10: Carbapenemase genes identified by WGS in phenotypic carbapenem-resistant Enterobacterales. *E. cloacae* (n = 30), *K. pneumoniae* (n = 17), *E. coli* (n = 15), *C. freundii* (n = 7), *K. oxytoca* (n = 3), *S. marcescens* (n = 2). This figure has been originally published as Figure 1 by Probst *et al.* (2021b).

In 18 isolates the two methods revealed no carbapenemase gene; thus, they were designated as negatives. Using WGS different *ESBL* and *AmpC* resistance genes as well as multidrug efflux pumps and porins were identified in these isolates, which may explain the carbapenem resistance phenotype (see Table 25).

Table 25: ESBL, AmpC, efflux pumps, and porins identified in carbapenemase-negative Enterobacterales that showed phenotypic resistance to meropenem and imipenem.

Isolate	Species	ESBL	AmpC	Efflux pump	Porin
KE9249	*E. cloacae*	-	ACT-55	-	-
KE9347	*E. cloacae*	-	ACT-15	-	-
KE9591	*E. cloacae*	PDC-138	-	MexA-N, MexP, MexQ, MexV, MexW, MexX, MexY, OprJ, OprM, OprN	OpmB, OpmH, OpmD, OpmE
KE9576	*E. coli*	TEM-1	EC-18, AmpH	AcrA, AcrB, AcrD, AcrE, AcrF, AcrS, TolC, EmrA, EmrB, EmrR	-
KE9599	*E. coli*	-	AmpC	-	-
KE9623	*E. coli*	SHV-12	EC-18, AmpC1, AmpH	AcrA, AcrB, AcrD, AcrE, AcrF, AcrS, TolC, EmrA, EmrB, EmrK, EmrR, EmrY	-
KE9633	*K. aerogenes*	CTX-M-14-b, CTX-M-15	-	KpnE, KpnF, KpnG, AcrA	OmpK37

Isolate	Species	ESBL	AmpC	Efflux pump	Porin
KE9068	*C. freundii*	TEM-1, SCO-1	CMY-48	-	-
KE8986	*E. cloacae*	-	MIR-17	AcrA	-
KE9344	*E. cloacae*	TEM-1	ACT-35	-	-
KE9475	*E. cloacae*	-	ACT-15	-	-
KE9083	*E. coli*	CTX-M-27	EC-5, AmpH	AcrA, EmrE, AcrB, AcrD, AcrE, AcrF, AcrS, TolC, EmrA, EmrB, EmrK, EmrR, EmrY	-
KE9425	*K. aerogenes*	-	AmpC	-	-
KE9614	*K. aerogenes*	CTX-M-15	CMY-48	-	-
KE9309	*K. pneumoniae*	SHV-187, CTX-M-15	-	KpnE, KpnF, KpnG, AcrA	OmpK37
KE9095	*K. pneumoniae*	OXA-1, SHV-106, CTX-M-15	-	KpnE, KpnF, KpnG, AcrA	OmpK37
KE9171	*K. pneumoniae*	OXA-1, SHV-187, TEM-1, CTX-M-15	-	KpnE, KpnF, KpnG, AcrA	OmpK37
KE9039	*S. marcescens*	SST-1	-	-	-

3.1.2 Accelerating the detection of colonized patients during an outbreak situation

Since qPCR delivered similar performance to WGS in detecting carbapenemase genes, it was investigated whether the implementation of qPCR can optimize outbreak investigations by accelerating the necessary diagnostics. During a nosocomial outbreak with *bla*$_{\text{OXA-48}}$ positive *E. cloacae* in 2019, patients on the affected wards were screened weekly for colonization with carbapenemase-positive bacteria. The rectal swabs were analyzed in the microbiological diagnostics by culture screening on selective chromID® ESBL agar and antimicrobial susceptibility testing. To shorten the time to results, qPCR for carbapenemase detection was performed directly on rectal swab specimens and the results were compared to culture-based diagnostics for validation. In total, 681 rectal swabs were analyzed. Cultural screening and qPCR led to carbapenemase-positive results in 28 cases (4.1 %). *Bla*$_{\text{OXA-48}}$-like was found in 25 rectal swabs and only in three patients' specimens, *bla*$_{\text{KPC}}$ was detected. The direct-qPCR did not lead to false-negative results. In ten cases the qPCR seemed to be more sensitive than culture-based diagnostics since Ct-values were between 14.7 and 32.9 for detecting *bla*$_{\text{OXA-48}}$-like carbapenemase genes. Additional enrichment culture and replating on selective chromID® ESBL agar plates revealed growth of *bla*$_{\text{OXA-48}}$-like harboring Enterobacterales in five cases (see Table 26). The other five samples remained negative, even after enrichment. Yet, *bla*$_{\text{OXA-48}}$-positive Enterobacterales have been identified by cultural screening in preceding and following patients' samples, indicating that the direct-qPCR results are true positives. Although the genotypic confirmation by PCR was not always performed, phenotypic resistance to the carbapenem antibiotics meropenem and imipenem was confirmed for these isolates.

Table 26: Comparison of direct-qPCR and culture screening for detecting carbapenemase-positive bacteria in rectal swab specimens during an outbreak setting.
This table has been originally published as Table 1 by Probst *et al.* (2022).

	qPCR and culture comparison				Test performance		Predictive values	
	True-positive (n)	True-negative (n)	False-positive (n)	False-negative (n)	Sensitivity (95 % CI)[b]	Specificity (95 % CI)[b]	Positive predictive value (95% CI)[b]	Negative predictive value (95% CI)[b]
qPCR on rectal swabs	28	643	10[a]	0	100 % (87.7 % - 100 %)	98.5 % (97.2 % - 99.3%)	73.7 % (60.2 % - 83.8 %)	100 %

[a] An additional overnight enrichment revealed bacterial growth on chromID® ESBL plates with *bla*$_{\text{OXA-48}}$-like positive isolates.

[b] "exact" Clopper-Pearson confidence intervals for binomial distributions.

3.1.3 Identification of contaminants in the hospital environment as potential sources for carbapenemase transmission

Besides patient screening, screening the hospital environment is crucial to ensure they do not become sources of nosocomial infections with MDR Gram-negative bacteria. To identify potential sources in hospitals, wastewater samples from toilets and shower drains of the affected wards were collected during the outbreak period with *bla*$_{\text{OXA-48}}$-positive *E. cloacae*. Since the direct-qPCR on rectal swabs showed good performance results for detecting the presence of *bla*$_{\text{OXA-48}}$, qPCR was also used to directly test wastewater samples. In total, 947 environmental samples were screened for *bla*$_{\text{OXA-48}}$. In 42 cases OXA-type carbapenemase was detected by qPCR but not by cultural screening. Furthermore, 33 samples were positive by culture but remained negative by qPCR. Hence, the qPCR showed a sensitivity of 62.1 % and specificity of 95.1 %. Positive and negative predictive values were 56.3 % and 96.1 %, respectively (see Table 27).

Table 27: Comparison of direct-qPCR and culture screening for detecting carbapenemase-positive bacteria in environmental samples during an outbreak setting.
This table has been originally published as Table 2 by Probst *et al.* (2022).

	qPCR and culture comparison				Test performance		Predictive values	
	True-positive (n)	True-negative (n)	False-positive (n)	False-negative (n)	Sensitivity (95 % CI)[a]	Specificity (95 % CI)[a]	Positive predictive value (95 % CI)[a]	Negative predictive value (95 % CI)[a]
qPCR on wastewater	54	818	42	33	62.1 % (51.0 % - 72.3 %)	95.1 % (93.5 % - 96.5 %)	56.3 % (47.8 % - 64.3 %)	96.1 % (95.0 % - 97.0 %)

[a] "exact" Clopper-Pearson confidence intervals for binomial distributions.

As the qPCR used in this study was designed to detect the eight most common carbapenemase genes (Probst *et al.* 2021a), the assay displayed the abundance of several carbapenemase variants in the environment, besides *bla*$_{OXA-48}$-like. *Bla*$_{OXA}$ carbapenemases, particularly *bla*$_{OXA-48}$-like, were predominantly found in the shower drains. Additionally, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{IMP}$ were detected. In water samples of the toilet basins, fewer carbapenemase genes were found. The most abundant gene was *bla*$_{VIM}$, followed by *bla*$_{OXA-58}$-like, *bla*$_{NDM}$, *bla*$_{OXA-48}$-like, and *bla*$_{IMP}$. During the five-month study period, the qPCR revealed the presence of various carbapenemase genes in wastewater from shower drains and toilet basins in almost all patient rooms (see Figure 11).

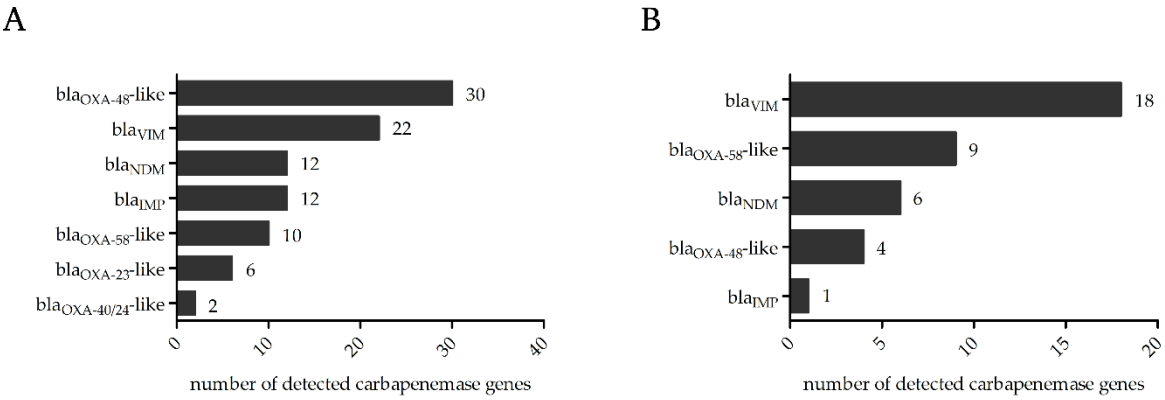

Figure 11: Non-duplicated carbapenemase genes detected in hospital environmental samples by direct-qPCR. A: carbapenemases found in wastewater samples from shower drains. B: carbapenemase genes found in wastewater samples from toilet basins. This figure has been originally published as Figure 1 by Probst *et al.* (2022).

3.1.4 Optimization of direct environmental screening for carbapenemase genes

Since the qPCR on environmental samples during the outbreak setting was not reliable in carbapenemase detection, the sample preparation was modified. In addition to the concentrated water samples, untreated wastewater, and overnight cultures in LB-medium were tested. For this purpose, environmental samples were collected monthly during a half-year period from toilet basins, shower, and sink drains. In total, 164 samples were analyzed, of which 104 samples were negative with all tested methods and for 60 samples either growth on selective agar or a positive qPCR result was observed (see Table 28).

In 50.0 % of the samples, the cultural screening was positive, but the qPCR remained negative (30/60). However, *P. aeruginosa* was growing in 93.3 % on the selective plates (28/30). As the chromID® CARBA SMART agar is optimized for detecting OXA-48, NDM-1, and KPC carbapenemases in *Enterobacteriaceae*, it might be that the non-fermenters harbor other mechanisms leading to carbapenem resistance. In four samples only (6.7 %), concordant results occurred between the culture-based screening and the qPCR with differently pre-treated samples. The other 26 samples were positive by qPCR but remained negative with the cultural screening (43.3 %). Comparing the three different sample preparations, the detection of carbapenemases directly from the untreated water sample and the concentrated water was

successful in just 3 and 4 samples, respectively, thus suggesting that the overnight culture might be the better choice.

Table 28: Comparison of different sample preparations before using qPCR to cultural screening on selective chromID® CARBA SMART agar for detecting carbapenemase-positive bacteria in environmental samples.

chromID® CARBA SMART	Origin and number of samples	qPCR on untreated wastewater sample	qPCR on concentrated wastewater sample	qPCR on the liquid overnight culture of wastewater sample
P. aeruginosa	shower (n = 1) sink (n = 26) toilet (n = 1)	negative	negative	negative
K. pneumoniae	sink (n = 1)	negative	negative	negative
E. cloacae	toilet (n = 1)	negative	negative	negative
P. aeruginosa	toilet (n = 1)	*bla*VIM	*bla*VIM	negative
E. cloacae	toilet (n = 2)	negative	negative	*bla*NDM, *bla*OXA-48-like
E. cloacae, *C. freundii*	toilet (n = 1)	negative	negative	*bla*NDM
negative	toilet (n = 6)	negative	negative	*bla*VIM
negative	toilet (n = 6)	negative	negative	*bla*NDM
negative	toilet (n = 1)	negative	*bla*NDM	*bla*NDM
negative	toilet (n = 2)	negative	negative	*bla*NDM, *bla*OXA-48-like
negative	toilet (n = 1)	negative	negative	*bla*NDM, *bla*VIM
negative	toilet (n = 1)	negative	negative	*bla*VIM, *bla*OXA-48-like
negative	toilet (n = 1)	negative	negative	*bla*NDM, *bla*KPC
negative	toilet (n = 1)	negative	negative	*bla*NDM, *bla*KPC, *bla*VIM
negative	toilet (n = 1)	negative	negative	*bla*NDM, *bla*VIM, *bla*IMP
negative	sink (n = 1)	negative	negative	*bla*OXA-58-like
negative	toilet (n = 1)	negative	negative	*bla*KPC, *bla*OXA-58-like
negative	toilet (n = 1)	negative	negative	*bla*OXA-23-like, *bla*OXA-58-like
negative	toilet (n = 1)	*bla*NDM	negative	*bla*NDM, *bla*KPC, *bla*VIM
negative	sink (n = 1)	negative	*bla*OXA-23-like	negative
negative	sink (n = 1)	*bla*NDM, *bla*KPC, *bla*IMP, *bla*OXA-23-like, *bla*OXA-58-like	*bla*OXA-23-like, *bla*IMP	negative
negative	shower (n = 11) sink (n = 32) toilet (n = 61)	negative	negative	negative

3.2 Transmission of carbapenemase-encoding plasmids via conjugation

Carbapenemase genes are mostly plasmid-encoded, which facilitates their dissemination via HGT (Maiden 1998). Especially in environments with high bacterial cell densities and the presence of numerous species, conjugation events are likely (Ogilvie *et al.* 2012). It is commonly assumed that antibiotic treatment can foster HGT (Zhang *et al.* 2013). Yet, the mechanism behind this effect remains unknown. To identify highly transmissible plasmids and conditions impacting the plasmid transfer, *in vitro* liquid mating experiments were carried out with various *bla*$_{VIM-1}$-positive clinical isolates, used as donors. The sodium azide resistant laboratory strain *E. coli* J53 was used as the recipient. In the following sections donor strains are named as "KE" followed by a number identifying the individual bacterial isolate. Transconjugants were designated with either "J53-pKE" or "ATCC-pKE" depending on the used recipient strain. Plasmids derived from the donors are indicated as "pKE".

3.2.1 Impact of plasmid Inc-types and bacterial species on the transfer efficiency

A large variety of plasmid types found in numerous bacterial species have evolved over the last decades, which can be classified into different Inc-types (Shintani *et al.* 2015). Conjugation experiments were performed with 14 Enterobacterales donor strains comprising *C. amalonaticus*, *C. freundii*, *Citrobacter portucalensis*, *Enterobacter hormaechei*, *E. cloacae*, *E. coli*, *Klebsiella aerogenes*, *K. pneumoniae*, and *S. marcescens*. All isolates harbor a *bla*$_{VIM-1}$ carbapenemase gene and different plasmids types, such as IncN2, IncN1, IncHI2, IncR, IncL/M, IncFII, IncFIB, IncB/O/K/Z, IncA/C, and Col-like plasmids (see Figure 12). Most of the isolates carry multiple plasmids with up to six different plasmid types as a maximum, which was detected for the two *E. hormaechei* KE3436 and KE9474.

To identify the transferred plasmid content, transconjugants were analyzed by WGS using the genome of the recipient *E. coli* J53 as a reference. Interestingly, mostly just one plasmid was transferred to *E. coli* J53, except for *C. portucalensis* KE4494 and *K. aerogenes* KE4495, where two plasmids were shared simultaneously (see Figure 12, blue squares). Multiple donor strains isolated from one patient, transferred the same plasmids even though different plasmid types were present (see Table 31 and Figure 12).

The isolates *C. amalonaticus* KE3510, *E. hormaechei* KE3754, and *E. coli* KE3742, gained from patient 2, were all positive for the IncN2-type plasmids. *E. hormaechei* KE3754 additionally harbored an IncR-type plasmid, whereas in *E. coli* KE3742 two further plasmids were detected (IncFIB-type and IncFII-type), which were not transferred. Another pair of IncN1-type plasmids were found in the isolates *E. coli* KE4472 and *C. freundii* KE4473 from patient 3. *E. coli* KE4472 further contained an IncB/O/K/Z plasmid, and *C. freundii* KE4473 harbored two types of IncFIB plasmids, but only the IncN1-type plasmids were shared with the recipient. In clinical samples from patient 4, *C. portucalensis* KE4494 and *K. aerogenes* KE4495 were found to have both an IncA/C and an IncFII(pECLA)-type plasmid, which were conjugated simultaneously. Additionally, both isolates harbor an individual plasmid, Col440I, and IncFII(Yp), respectively. The last plasmid pair present in *S. marcescens* KE9538 and *E. cloacae*

KE9563, isolated from patient 8, represents an IncL/M plasmid, which is the only one present in these isolates. The remaining donor strains were gained from different patients. The transfer of just individual plasmids might suggest that a few of the present plasmids were not self-transmissible, preventing the transfer to the recipient.

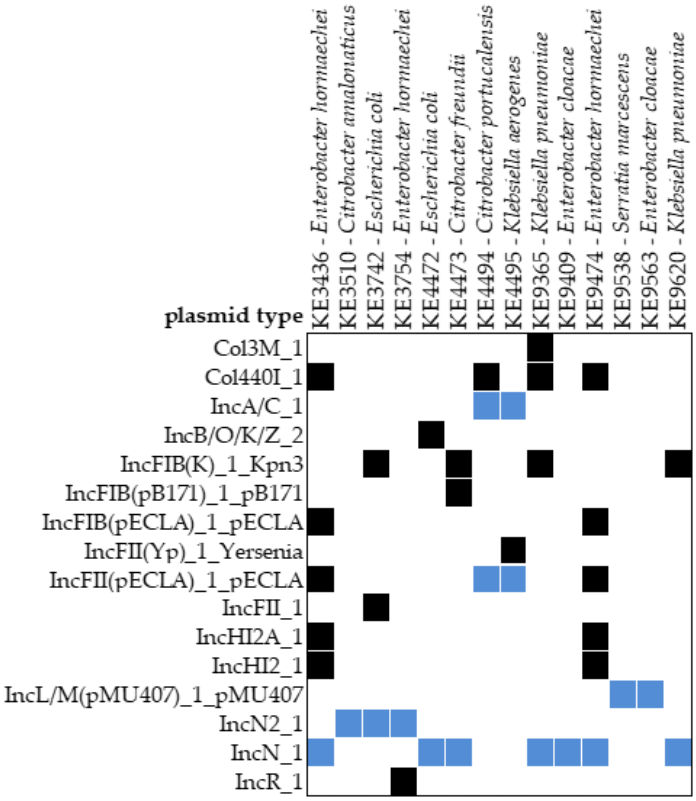

Figure 12: Plasmid Inc-types identified by WGS in the *bla*VIM-1-positive isolates used as donors for conjugation experiments.
Colored squares (black and blue) represent all plasmid types present in the respective donor strains. The blue squares indicate plasmids that were transferred to the recipient *E. coli* J53.

As the Inc-type was identified by WGS, conjugation efficiencies for different plasmid types could be examined. The liquid mating experiments revealed a conjugation frequency approximately 7 to 13 times higher for IncN2-type plasmids than for IncN1-, IncA/C-, IncFII-, and IncL/M-type plasmids (see Figure 13), suggesting that the IncN2 plasmids found in three isolates of patient 2 were highly conjugative plasmids.

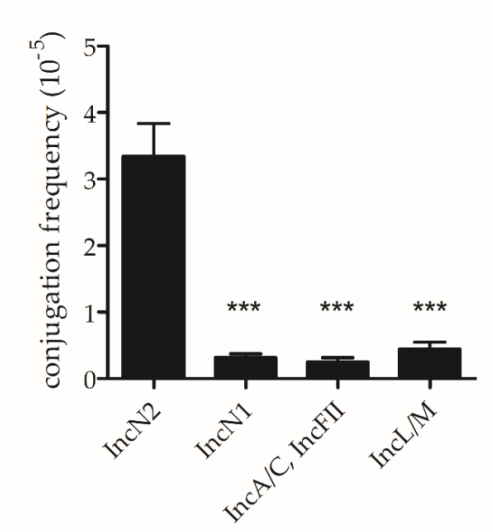

Figure 13: Conjugation frequencies of different plasmid Inc-types summarized from all conjugation experiments carried out without antibiotic exposure.
The following donor strains were used: IncN2 (n = 3): *C. amalonaticus* KE3510, *E. hormaechei* KE3754, and *E. coli* KE3742; IncN1 (n = 7): *E. coli* KE3742, *E. coli* KE4472, *C. freundii* KE4473, *K. pneumoniae* KE9620, *E. hormaechei* KE3436, *E. hormaechei* KE9474, *E. cloacae* KE9409, and *K. pneumoniae* KE9365; IncA/C, IncFII (n = 2): *C. portucalensis* KE4494 and *K. aerogenes* KE4495; IncL/M (n = 2): *E. cloacae* KE9563 and *S. marcescens* KE9538. Conjugation experiments for each plasmid were performed as triplicates in three independent experiments. * indicates statistical significance from a one-way ANOVA for unpaired data.

Furthermore, the impact of the transferred plasmid content size was assessed (see Figure 15 to Figure 20), showing a significant correlation between smaller plasmids and higher conjugation frequencies (Pearson's product-moment correlation: $R^2 = -0.5985776$, p-value = 0.02372).
Besides the plasmid type, the bacterial species of the donor also seemed to have an impact on the conjugation efficiencies, although the plasmid content was comparable (see Figure 14). Even with the same plasmid type, differences in transfer efficiencies of various donor strains could be observed, indicating that not only the plasmid Inc-type but also the donor and recipient species influence the efficiency of the transfer. *C. amalonaticus* (IncN2-type) showed the highest conjugation frequency, suggesting that this species represents an efficient donor. However, other *Citrobacter* sp. harboring different plasmid types (IncN1 and IncA/C, IncFII) showed a significantly lower conjugation frequency. Based on the tested isolates, *Klebsiella* sp. seemed to be an inefficient donor for plasmid transfer to *E. coli* recipient strains.

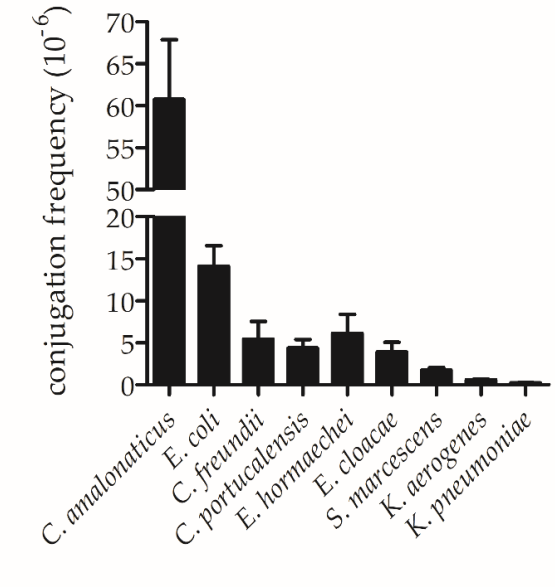

Figure 14: Conjugation frequencies of different donor species summarized from all conjugation experiments carried out without antibiotic exposure.
C. amalonaticus (n = 1), *E. coli* (n = 3), *C. freundii* (n = 1), *C. portucalensis* (n = 1), *E. hormaechei* (n = 2), *E. cloacae* (n = 2), *S. marcescens* (n = 1), *K. aerogenes* (n = 1), *K. pneumoniae* (n = 2). Conjugation experiments for each isolate were performed as triplicates in three independent experiments.

3.2.2 Analysis of the transferred plasmid content

The carbapenemase-encoding plasmids were most likely transferred by conjugation since transformation experiments with the inactivated donor *C. amalonaticus* KE3510 and pure DNA, respectively, did not lead to the growth of *E. coli* J53 transconjugants on the selective plate.
To assess genetic similarities of the transferred plasmid content, a core genome was calculated and illustrated by a mid-rooted Neighbor-Joining phylogenetic tree (see Figure 30 and Figure 31). As expected, most of the different plasmid Inc-types grouped together. The two IncL/M-type plasmids derived from *S. marcescens* KE9538 and *E. cloacae* KE9563 cluster together with most of the IncN1-type plasmids. Yet, the IncN1-type pKE9365 showed large variations when compared to the other IncN1-types and seemed to have more similarities with the IncA/C, IncFII-type plasmids. Based on the phylogenetic trees the plasmids were separated into different groups and illustrated with BRIG to depict differences in the transferred genes (see Figure 15 to Figure 20). All conjugated plasmids contained *tra* or *virB* genes, which are essential

for initiating the conjugation process and the plasmid transfer. Additionally, a gene for replication *repA* or *repE* was present, which is necessary for regulating the plasmid copy number and various antibiotic resistance genes could be annotated. Different transposons and insertion sequence families were present on the plasmids, which can modulate the genetic content, by interrupting or deleting genes, as well as by influencing their expression (Muñoz-López and García-Pérez 2010; Siguier *et al.* 2014).

The IncN2-positive strains *C. amalonaticus* KE3510, *E. coli* KE3742, and *E. hormaechei* KE3754, were all isolated from the same patient. *C. amalonaticus* KE3510 was the first isolate and approximately one month later *E. coli* KE3742 and *E. hormaechei* KE3754 were gained (see Table 31). All isolates harbored plasmid homologs, indicating *in vivo* interspecies transfer (see Figure 15). Only the pKE3742 content, illustrated by a pink circle in Figure 15 showed a gap of approximately 7 kbp, annotated as Tn3 family transposon, the transposase gene *tnpR*, and the receptor encoding gene *tcp* (Abe *et al.* 1999). The Tn3 family transposon missing in one of the three plasmids might indicate an incomplete plasmid transfer.

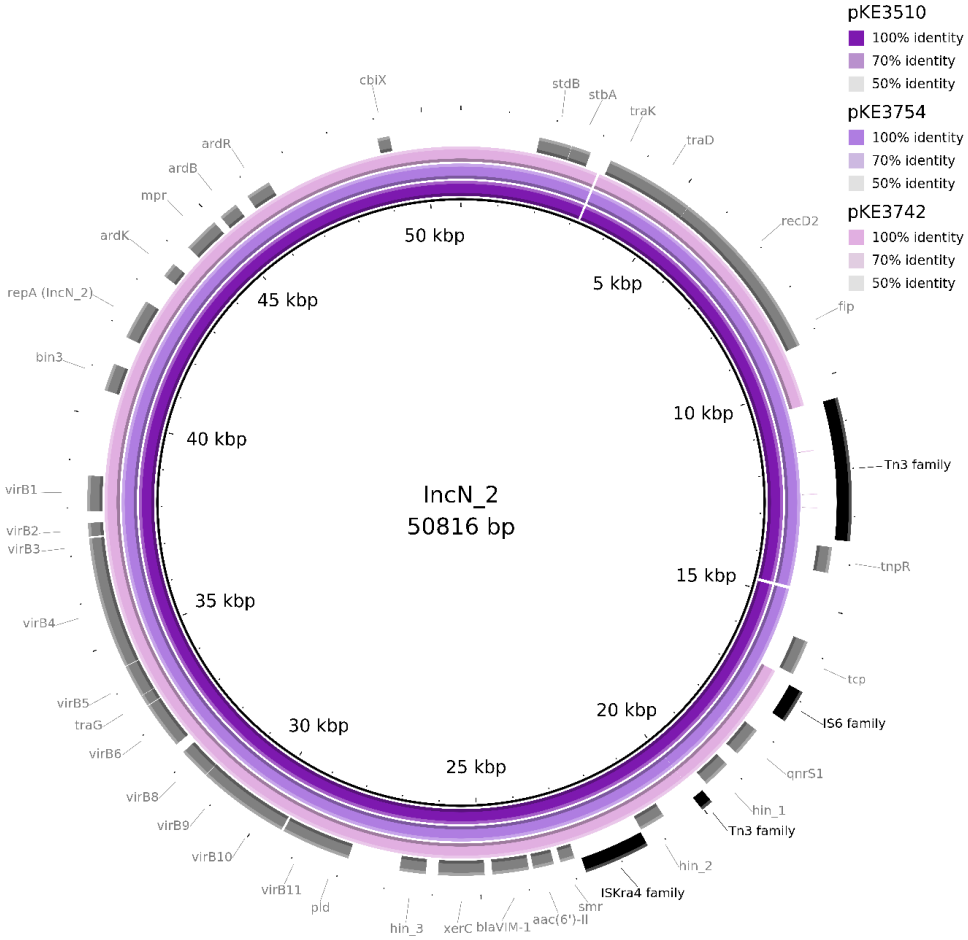

Figure 15: IncN2-type plasmid content transferred to *E. coli* J53 from *C. amalonaticus* KE3510, *E. coli* KE3742, and *E. hormaechei* KE3754, respectively.

The genetic content of the transconjugants J53-pKE3754 (pink) and J53-pKE3742 (light purple) was mapped to J53-pKE3510 (dark purple) and used as the reference. Annotated genes are figured as arc-shaped grey bars and insertion sequences, as well as transposons, are illustrated as arc-shaped black bars.

The bacterial strains *C. freundii* KE4473 and *E. coli* KE4472 were isolated from patient 3 on the same day (see Table 31). They contained similar plasmid content with some gene deletions found in pKE4472, comprising a transposon of the Tn3 family and the genes *hin* and *rhsD*, the former encoding a site-specific recombinase (Henderson *et al.* 1999) and the latter a protein linked with the type VI secretion system (Koskiniemi *et al.* 2013). Moreover, pKE4473 contained the gene *ytbA* whose function is currently unknown. The donor *C. freundii* KE4473 harbors a second carbapenemase gene, *bla*$_{KPC-2}$ (see Figure 32), which could not be found in the transferred genetic content. This indicates that *bla*$_{VIM-1}$ and *bla*$_{KPC-2}$ carbapenemase genes were located on different plasmid types, however, only the IncN1-type plasmid harboring *bla*$_{VIM-1}$ was transferred (see Figure 16).

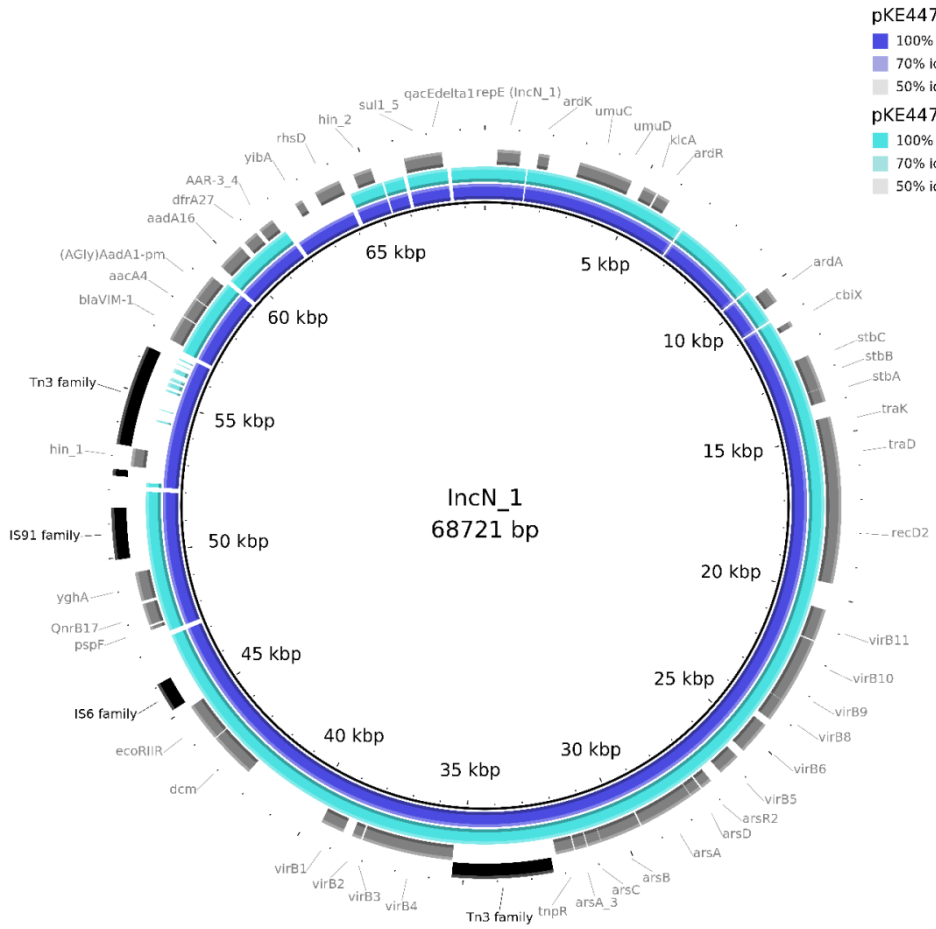

Figure 16: IncN1-type plasmid content transferred to *E. coli* J53 from *C. freundii* KE4473 and *E. coli* KE4472, respectively.
The genetic content of the transconjugants J53-pKE4472 (light blue) was mapped to J53-pKE4473 (dark blue) and used as the reference. Annotated genes are figured as arc-shaped grey bars and insertion sequences, as well as transposons, are illustrated as arc-shaped black bars.

The IncN1-type plasmids pKE3436, pKE9474, pKE9409, and pKE9620 were clustering together in the phylogenetic tree (see Figure 31), thus showing similarities in the genetic content (see Figure 17). The plasmid contents of pKE3436, pKE9474, and pKE9409 share the same genes, even though they were all isolated from different patients (see Table 31). These plasmids originate from *Enterobacter* spp., hence, suggesting that this plasmid is widely distributed in this genus. The pKE9620 content displays more variations, e.g., regarding deletions of the transposons Tn21-like element and Tn3-like element as well as of the IS6-family. These variations could be due to genus disparity since the plasmid pKE9620 was derived from a *K. pneumoniae* donor strain.

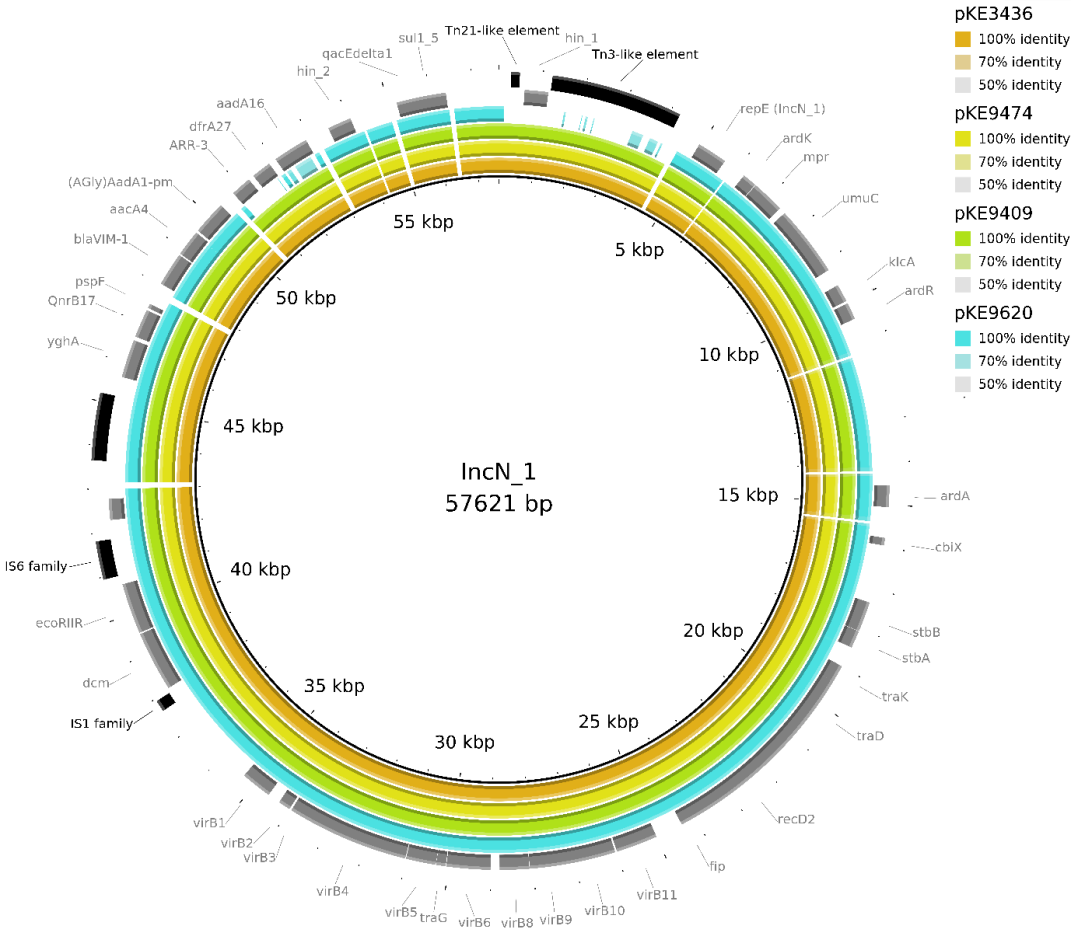

Figure 17: IncN1-type plasmid content transferred to *E. coli* J53 from *E. hormaechei* KE3436, *E. hormaechei* KE9474, *E. cloacae* KE9409, and *K. pneumoniae* KE9620 respectively.
The genetic content of the transconjugants J53-pKE9474 (yellow), J53-pKE9409 (green), and J53-pKE9620 (cyan) were mapped to J53-pKE3436 (orange) and used as the reference. Annotated genes are figured as arc-shaped grey bars and insertion sequences, as well as transposons, are illustrated as arc-shaped black bars.

The IncN1-type plasmid pKE9365 showed large variations in the genetic content and was more comparable to the IncA/C, IncFII plasmids (see Figure 18, 30, and 31). Although this plasmid was found in *K. pneumoniae* it does not show high similarity with the other IncN1-plasmid pKE9620 also derived from *Klebsiella* sp.

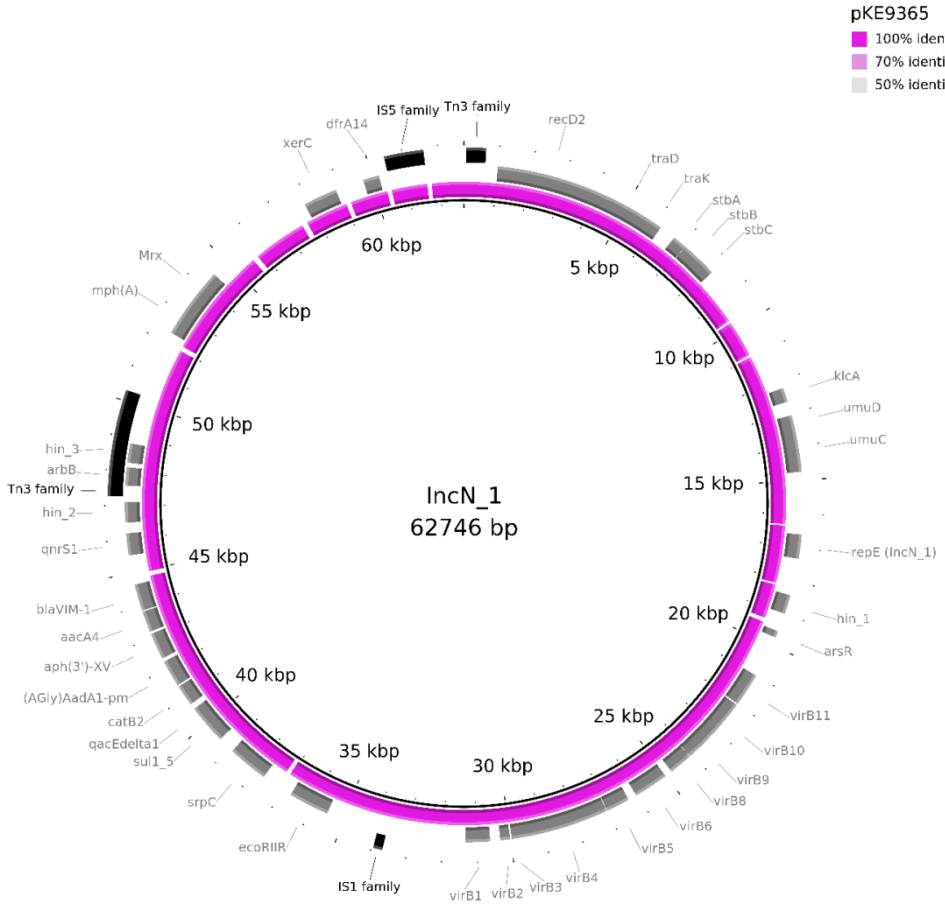

Figure 18: IncN1-type plasmid content transferred to *E. coli* J53 from *K. pneumoniae* KE9365.
Annotated genes are figured as arc-shaped grey bars, and insertion sequences, as well as transposons, are illustrated as arc-shaped black bars.

The donors *C. portucalensis* KE4494 and *K. aerogenes* KE4495 originated from the same patient (see Table 31) and were the only isolates that transferred two plasmid types IncA/C and IncFII simultaneously. The genetic content of both plasmids could not be separated after sequencing; thus, Figure 19 illustrates a combination of the genes encoded by the two plasmids. The plasmid content pKE4494 showed some deletions when compared to pKE4495, regarding the insertion sequences of the IS3 family and the genes *smc* and *hin1*, which are involved in the structural maintenance of the chromosome (Harvey *et al.* 2002) and the site-specific inversion of DNA (Henderson *et al.* 1999), respectively.

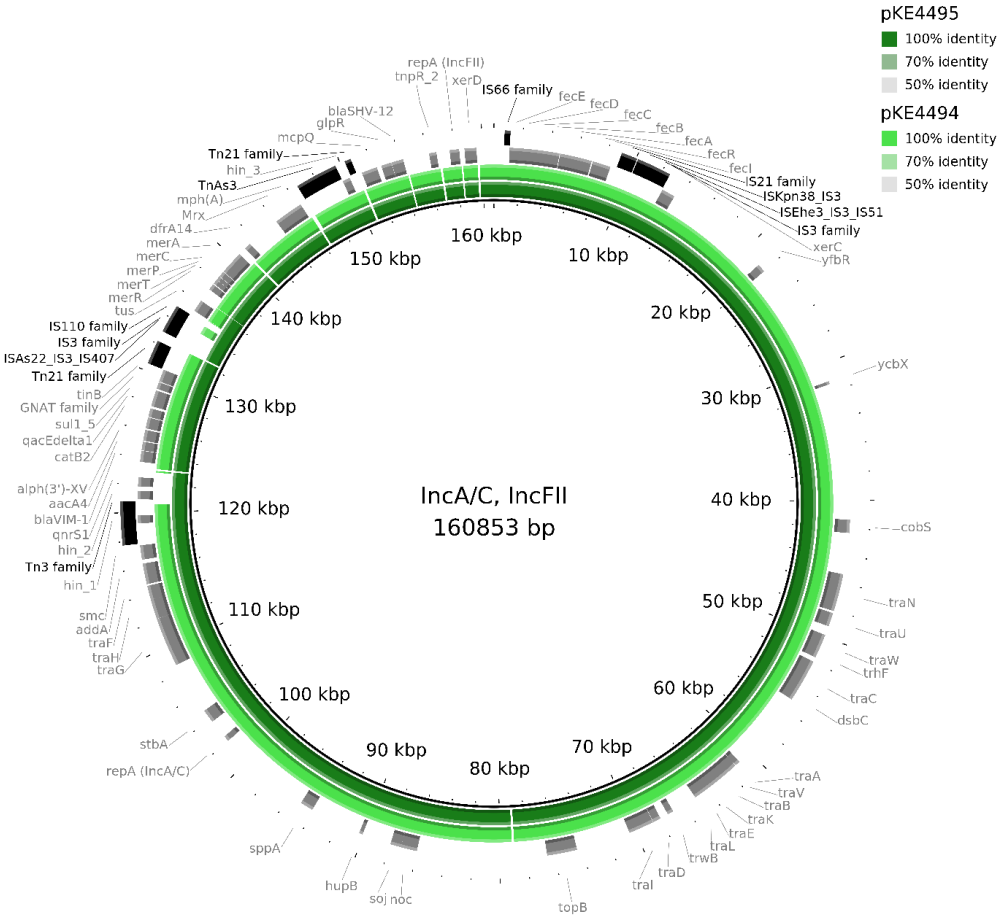

Figure 19: IncA/C- and IncFII-type plasmid content transferred to *E. coli* J53 from *K. aerogenes* KE4495 and *C. portucalensis* KE4494, respectively.

The genetic content of the transconjugants J53-pKE4494 (light green) was mapped to J53-pKE4495 (dark green) and used as the reference. Annotated genes are figured as arc-shaped grey bars and insertion sequences, as well as transposons, are illustrated as arc-shaped black bars.

E. cloacae KE9563 and *S. marcescens* KE9538 originated from patient 8 (see Table 31). The IncL/M-type plasmids showed a high degree of homology with an additional segment found in the pKE9563, containing the *yibA* gene, which is involved in the bacterial stress response, e.g., to antibiotics (see Figure 20; Cui *et al.* 2018; Reyes *et al.* 2011).

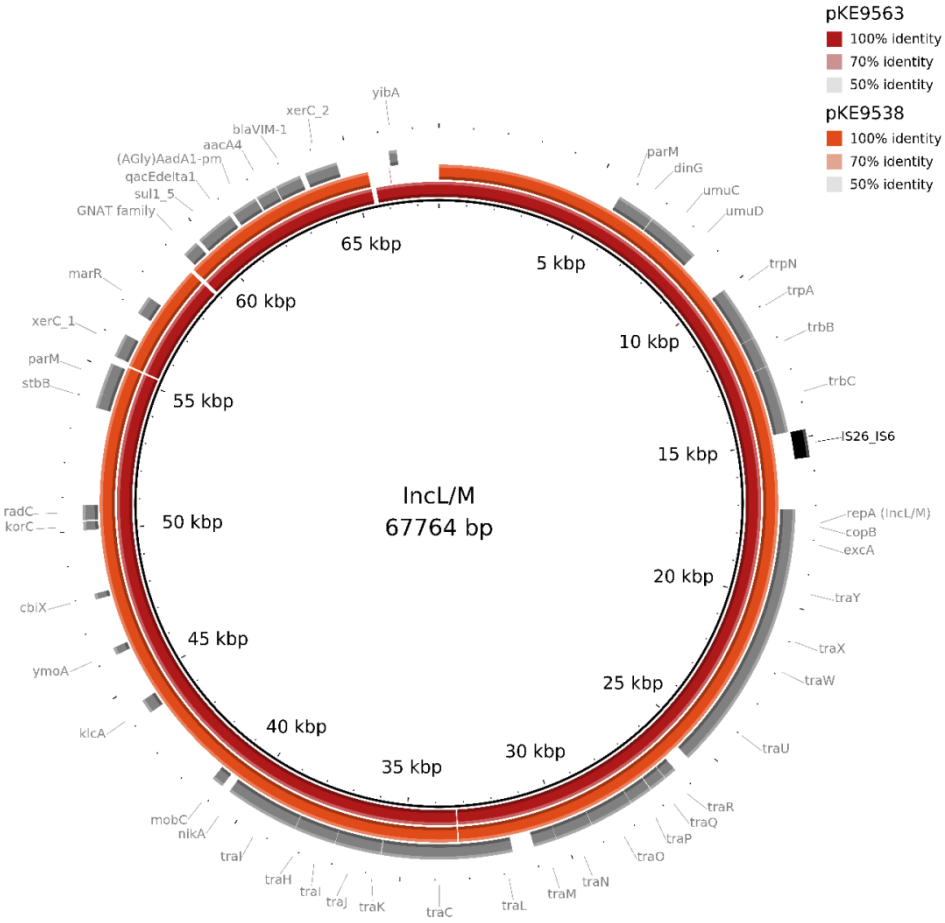

Figure 20: IncL/M-type plasmid content transferred to *E. coli* J53 from *E. cloacae* KE9563 and *S. marcescens* KE9538, respectively.
The genetic content of the transconjugants J53-pKE9538 (light red) was mapped to J53-pKE9563 (dark red) and used as the reference. Annotated genes are figured as arc-shaped grey bars and insertion sequences, as well as transposons, are illustrated as arc-shaped black bars.

3.2.3 Impact of ciprofloxacin on the transfer of various carbapenemase-encoding plasmids

Preliminary *in vitro* liquid mating experiments were performed by Xiaojie Li with the donors *C. amalonaticus* KE3510, *E. coli* KE3742, and *E. hormaechei* KE3754, harboring a *bla*$_{VIM-1}$ plasmid, and the recipient strain *E. coli* J53. Under exposure to sub-inhibitory concentrations of the fluoroquinolone antibiotics ciprofloxacin, levofloxacin, and moxifloxacin, conjugation frequency was increased compared to the control (Li and Nurjadi 2019).

Since this effect was present only for the *bla*$_{VIM-1}$-encoding IncN2 plasmid, the aim was to understand whether this event was common for *bla*$_{VIM-1}$-encoding plasmids. Therefore, the 14 isolates, whose successful conjugation was confirmed by WGS, were screened with a quarter and half of their ciprofloxacin MIC for increased plasmid transfer to the recipient *E. coli* J53 (see Figure 21).

Elevation of conjugation frequency was observed for *C. amalonaticus* KE3510, *E. coli* KE3742, and *E. hormaechei* KE3754, hence the results observed by Xiaojie Li could be reproduced for the three IncN2-positive isolates. Additionally, *E. coli* KE4472 showed a significantly higher conjugation frequency under 1/4 MIC ciprofloxacin exposure (see Figure 21A-D). The other tested isolates led to similar results in the treated samples compared to the non-treated control or even to a decrease in conjugation efficiency under ciprofloxacin treatment (see Figure 21E-N). The results indicate that the fluoroquinolone antibiotic has not a common impact on the transmission of *bla*$_{VIM-1}$ plasmids.

Since the effect of increased conjugation frequency was seen under ciprofloxacin exposure, it is likely that genes conferring resistance to fluoroquinolone antibiotics play an important role in this effect. Quinolone resistance genes *qnrA*/*B*/*S* and *oqxA*/*B*, encoding efflux pumps (Rodríguez-Martínez *et al.* 2013), could be found in almost all donor strains, except for *E. cloacae* KE9563 and *S. marcescens* KE9538 (see Figure 32). However, only the isolates from patient 2, *C. amalonaticus* KE3510, *E. hormaechei* KE3754, and *E. coli* KE3742, as well as *E. coli* KE4472 showed the rise in transfer efficiency.

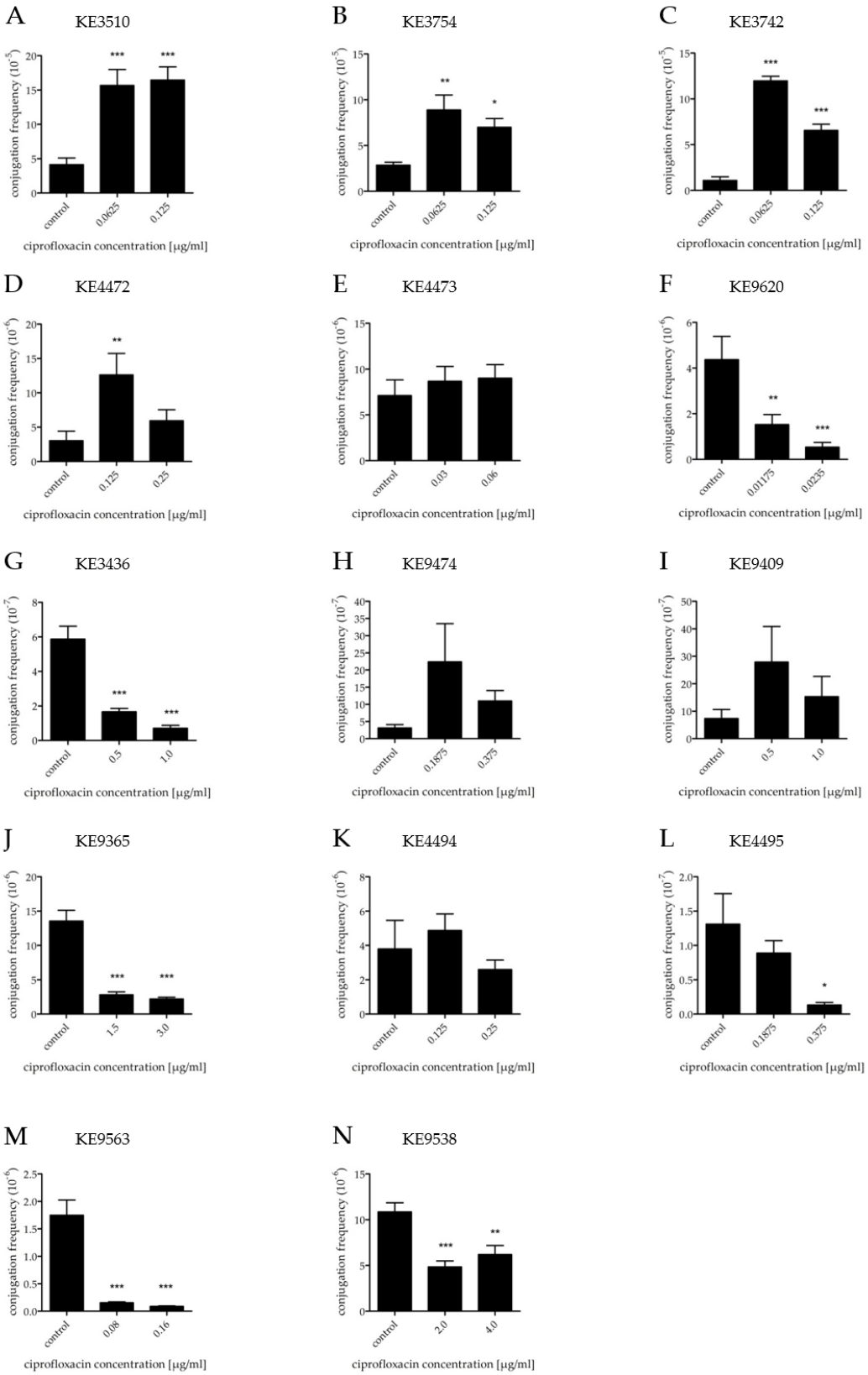

Figure 21: Screening of *bla*VIM-1-positive isolates for increased conjugation frequency with 1/4 MIC and 1/2 MIC of ciprofloxacin.

A: *C. amalonaticus* KE3510, B: *E. hormaechei* KE3754, C: *E. coli* KE3742, D: *E. coli* KE4472, E: *C. freundii* KE4473, F: *K. pneumoniae* KE9620, G: *E. hormaechei* KE3436, H: *E. hormaechei* KE9474, I: *E. cloacae* KE9409, J: *K. pneumoniae* KE9365, K: *C. portucalensis* KE4494, L: *K. aerogenes* KE4495, M: *E. cloacae* KE9563, N: *S. marcescens* KE9538. Results are summarized from three independent experiments performed as triplicates. * indicates statistical significance from a one-way ANOVA for unpaired data.

3.2.4 Transfer-induced impact of ciprofloxacin is independent of the antibiotic-mediated selection

Since the fluoroquinolone antibiotic ciprofloxacin is interfering with the DNA synthesis, leading to growth arrest and subsequently may cause cell death; various concentrations of ciprofloxacin below the MIC were tested to exclude an artifact due to growth inhibition. The lowest tested concentration (0.0078 µg/ml), which did not influence the growth of *C. amalonaticus* KE3510 (see Figure 22B) already showed a significantly increased conjugation frequency when compared to the control (see Figure 22A). All other concentrations up to the MIC showed the same effect, even though the growth was inhibited with rising antibiotic concentration. The highest tested concentration (0.5 µg/ml), which represents two times the MIC, led to similar transfer efficiency compared to the control without antibiotic treatment, even though the growth was markedly inhibited. As the increased conjugation frequency was also detected for 1/32 MIC and 1/16 MIC without growth inhibition, it can be assumed that it is not an artifact due to the ciprofloxacin-mediated selection pressure.

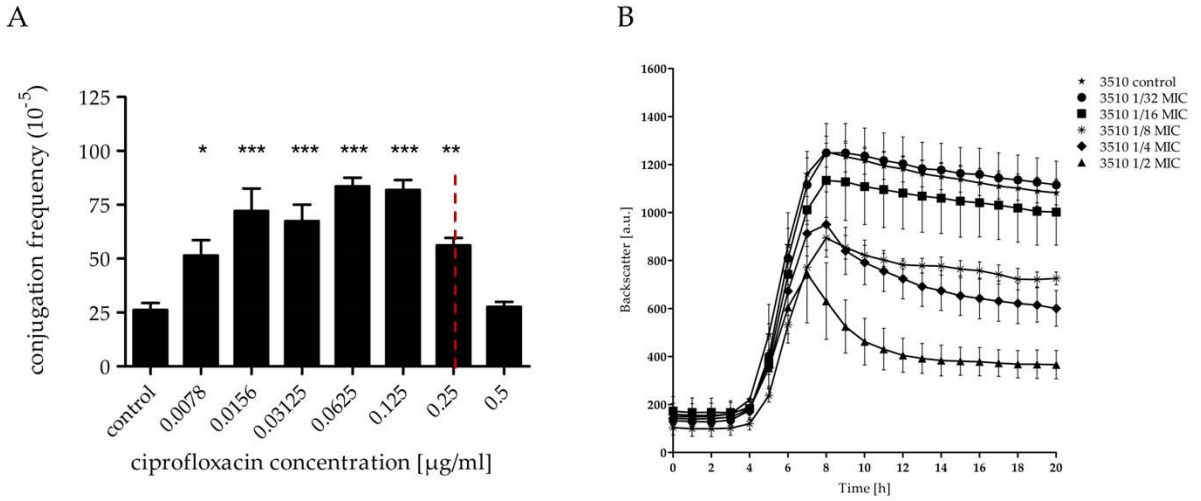

Figure 22: Influence of different ciprofloxacin concentrations on transfer efficiency and bacterial growth of *C. amalonaticus* KE3510.

A: The following titrations of ciprofloxacin were used for the investigation of conjugation frequency and growth inhibition: 1/32 MIC, 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, and 2xMIC. The red dotted vertical line indicates the MIC. Results for conjugation are summarized from three independent experiments performed as triplicates. B: The growth curve was performed as triplicates in independent experiments with the above-mentioned ciprofloxacin concentrations for 20 h. * indicates statistical significance from a one-way ANOVA for unpaired data.

3.2.5 Increased conjugation frequency is a plasmid-specific event

To determine whether the effect of increased conjugation frequency with sub-inhibitory concentrations of ciprofloxacin is independent of the bacterial species, two other isolates from the same patient (*E. coli* KE3742 and *E. hormaechei* KE3754) harboring plasmid homologs to pKE3510 were analyzed (see Figure 23). The conjugation experiments of the two strains with *E. coli* J53 as recipient revealed similar results as *C. amalonaticus* KE3510, showing an approximately ten-times higher conjugation frequency when treated with low ciprofloxacin concentrations.

Additionally, the three plasmids were transferred to a rifampicin-resistant *E. coli* ATCC-25922 to generate plasmid carriers with the same genetic background and the conjugation to *E. coli* J53 was repeated. The effect was less strongly marked for ATCC-pKE3510, ATCC-pKE3742, and ATCC-pKE3754, respectively, ranging from two-times to four-times higher conjugation frequency under sub-inhibitory ciprofloxacin concentrations. ATCC-pKE3742 and ATCC-pKE3754 showed a significantly higher increase under 1/4 MIC ciprofloxacin, whereas for ATCC-pKE3510 the tendency of elevated conjugation is visible. The success of the plasmid transfer from *E. coli* ATCC-25922 harboring the *bla*$_{\text{VIM-1}}$ plasmid to *E. coli* J53 was approved by RAPD (see Figure 34).

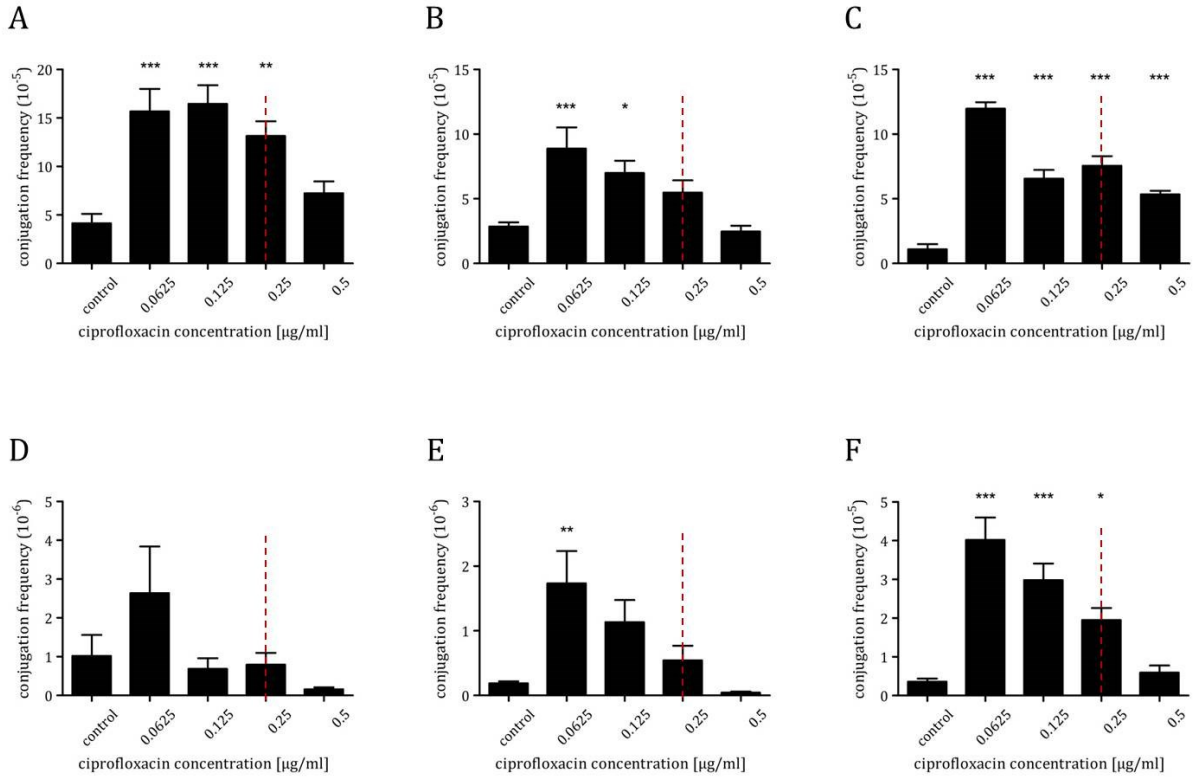

Figure 23: Sub-inhibitory concentrations of ciprofloxacin increase the conjugation frequency of pKE3510, pKE3754, and pKE3742, independent of the used donor strain.

A: *C. amalonaticus* KE3510, B: *E. coli* KE3742, C: *E. hormaechei* KE3754, D: *E. coli* ATCC-pKE3510, E: *E. coli* ATCC-pKE3742, F: *E. coli* ATCC-pKE3754. The red dotted vertical line indicates the MIC. Results are summarized from three independent experiments performed as triplicates. * indicates statistical significance from a one-way ANOVA for unpaired data.

3.2.6 DNA-interfering substances elevate the conjugation frequency

Different antibiotic classes were tested in preliminary experiments by Xiaojie Li for their influence on conjugation efficiency; just the fluoroquinolone antibiotics showed a reinforcing effect on the conjugation frequency with sub-inhibitory concentrations (Li and Nurjadi 2019). Here, other substances, differently affecting the DNA synthesis, were tested for their impact on plasmid transfer of *C. amalonaticus* KE3510 to *E. coli* J53. Aminocoumarin inhibits the subunit B of the DNA gyrase and quinolones in contrast interfere with subunit A (Alt *et al.* 2011; Hooper *et al.* 1987). The antitumor drug mitomycin C covalently attaches to the DNA strands, acting as a cross-linker (Paz *et al.* 2012; Wei *et al.* 2001). Azacytidine inhibits DNA methylation (Christman 2002) and ethidium bromide intercalates in the DNA (Sigmon and Larcom 1996). Only nalidixic acid, which was the first quinolone antibiotic discovered (Takahashi *et al.* 2003), showed a significantly higher conjugation with half of the MIC. For the other substances, a tendency of increased conjugation could be observed. UV-B induces different types of DNA damage (Sinha and Häder 2002), which significantly reduced the conjugation compared to the control, even with the shortest possible exposure time (see Figure 24).

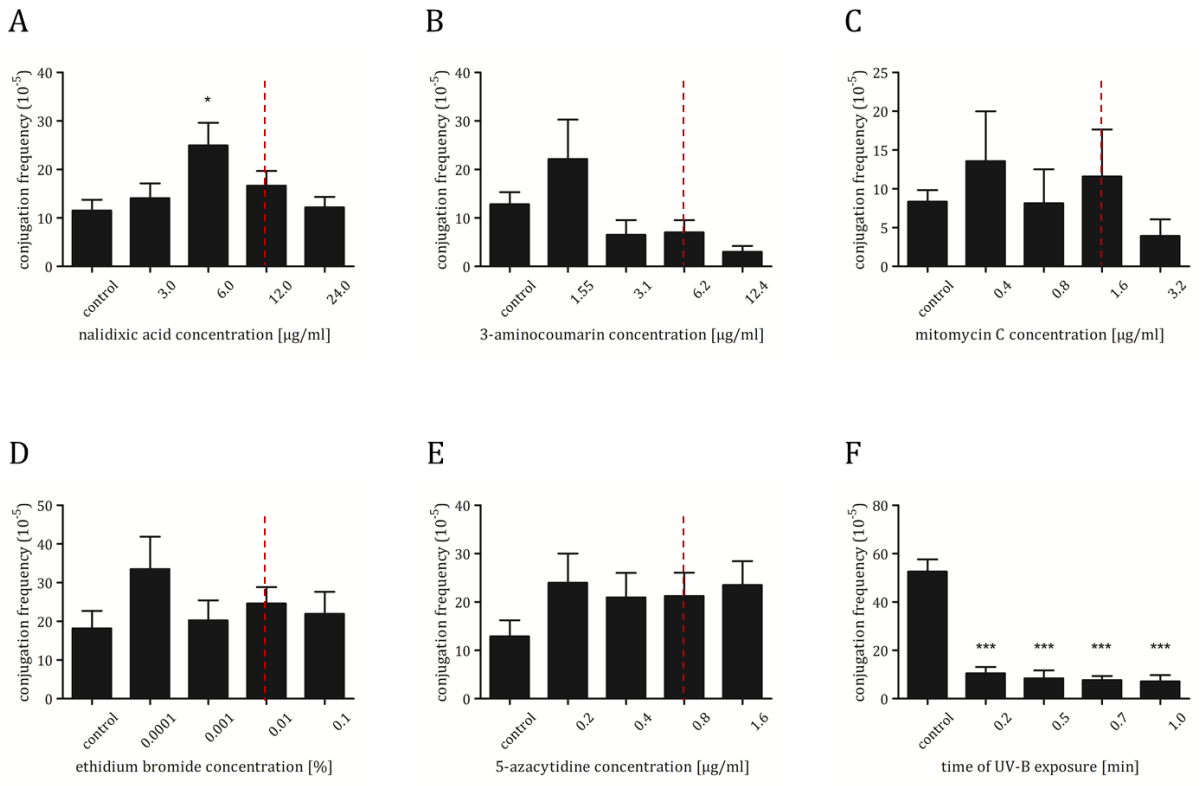

Figure 24: Influence of sub-inhibitory concentrations of DNA-interfering substances with a different mode of action on the conjugation frequency of *C. amalonaticus* KE3510 to *E. coli* J53.
A: Nalidixic acid, B: 3-Aminocoumarin, C: Mitomycin C, D: Ethidium bromide, E: 5-Azacytidine, F: UV-B light. The red dotted vertical line indicates the MIC. Results are summarized from three independent experiments performed as triplicates. * indicates statistical significance from a one-way ANOVA for unpaired data.

3.3 Mechanism of increased conjugation frequency

Different mechanisms were already considered to have an impact on the conjugation frequency, like overexpression of the conjugation-related genes or the induction of the SOS response and its mutagenic potential (Beaber *et al.* 2004; Liu *et al.* 2019a; Shun-Mei *et al.* 2018). Furthermore, higher transfer rates were associated with elevated plasmid copy numbers in the donor cell (Dimitriu *et al.* 2020; Gulyás *et al.* 2019). To further investigate the increased conjugation frequency of the IncN2 plasmid derived from *C. amalonaticus* KE3510 these considerations were addressed in the following sections.

3.3.1 Influence of conjugation and SOS response-related gene expression on the transfer efficiency

Gene expression profiling was performed by RNA sequencing to provide a comprehensive picture of up- and down-regulated genes after 4 h of treatment with 1/4 MIC ciprofloxacin compared to the non-treated control. The time of 4 h post-ciprofloxacin exposure has been chosen since preliminary RNA sequencing data generated by Xiaojie Li after 2 h of treatment did not lead to a clear result (Li and Nurjadi 2019).

Two isolates were chosen for gene expression profiling: *C. amalonaticus* KE3510, which showed the effect of increased conjugation with sublethal concatenations of ciprofloxacin and *E. cloacae* KE9563, where the antibiotic did not seem to affect the transfer (see Figure 21). Only a small number of genes could be found to be significantly up- or down-regulated in the treated sample compared to the control in both isolates (see Figure 25).

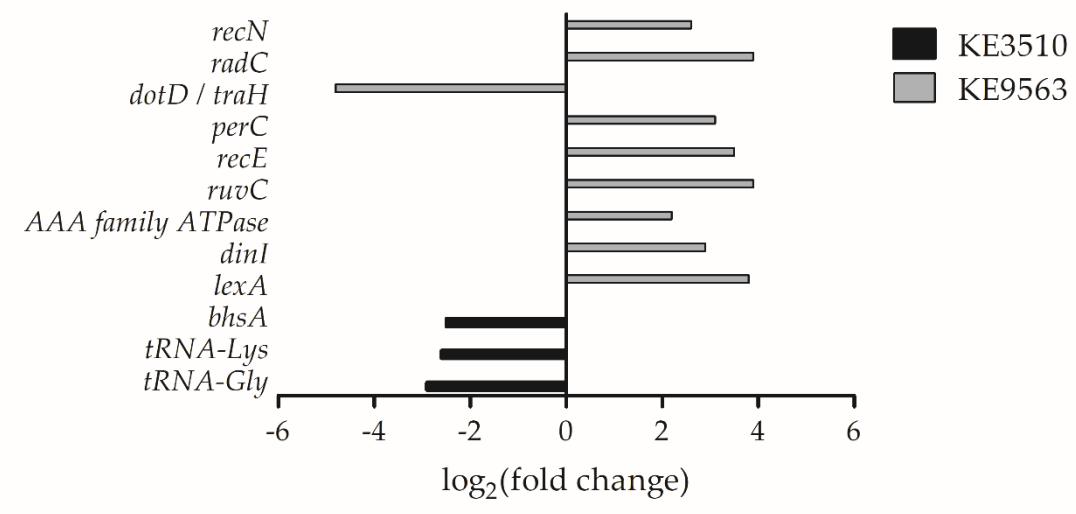

Figure 25: Gene expression profiling of *E. cloacae* KE9563 and *C. amalonaticus* KE3510 after 4 h of 1/4 MIC ciprofloxacin treatment.

Bars represent $\log_2$ (fold change) of genes significantly differentially regulated compared to the control. Three repetitions of RNA sequencing were carried out for both isolates. Black bars represent genes of KE3510, and grey bars depict KE9563-originated genes.

Most of the up-regulated genes found in *E. cloacae* KE9563 are involved in the cellular response to DNA damage. Proteins responsible for various repair processes and SOS response-related proteins showed a 4- to 16-fold increase compared to the untreated isolate. LexA and RecA are key proteins of the SOS response, negatively regulating and inducing this cellular process, respectively (Brent and Ptashne 1981). DinI was up-regulated, which represents an inhibitor of active RecA, and was therefore involved in the negative regulation of this pathway (Yasuda *et al.* 2001). *RecE*, *radC*, *ruvC*, and *recN* encode proteins contributing to various DNA repair mechanisms. The exodeoxyribonuclease VIII RecE initiates double-strand repair (Muyrers *et al.* 2000) and is linked to the recombination pathway (Kolodner *et al.* 1994), in which *radC* is also involved (Attaiech *et al.* 2008). RuvC proteins are endonucleases that cleave Holliday structures and impact DNA repair (Iwasaki *et al.* 1991). The DNA repair protein recN is part of the SOS response-mediated double-strand break repair mechanism (Chan *et al.* 1994; Uranga *et al.* 2017). In addition to the SOS response and DNA repair genes, conjugation-related genes of *E. cloacae* KE9563 were affected by ciprofloxacin. The gene *traH*, which is involved in the pilus assembly (Arutyunov *et al.* 2010), was found to be 32-fold decreased compared to the control. An AAA family ATPase, comprising the enzymes VirB4 and VirB11, was up-regulated under antibiotic treatment. These ATPases are joining the assembled pilus complex and provide the required energy for pilus biogenesis (Guglielmini *et al.* 2014). Furthermore, the expression of a transcriptional regulator influencing the type III secretion system was induced, which is involved in the control of niche adaption (Mellies *et al.* 2017).

Gene expression profiling by RNA sequencing revealed only three genes for *C. amalonaticus* KE3510, which are up to 8-fold lower expressed under ciprofloxacin exposure compared to the non-treated control. Two genes encode tRNAs and the third gene *bhsA* contributes to stress resistance (Hwang *et al.* 2020) and is involved in stress-related biofilm formation (Zhang *et al.* 2007).

Since few genes were found to be significantly down-regulated, gene expression of the individual experiments for *C. amalonaticus* KE3510 was evaluated. Various genes involved in the conjugative process, DNA replication, DNA repair mechanism, and antimicrobial resistance genes were considered (see Table 29). However, there was a large batch effect of the three repetitions, indicated by the cluster dendrogram and high standard deviations, thus gene expression is ambiguous (see Figure 26 and Table 29).

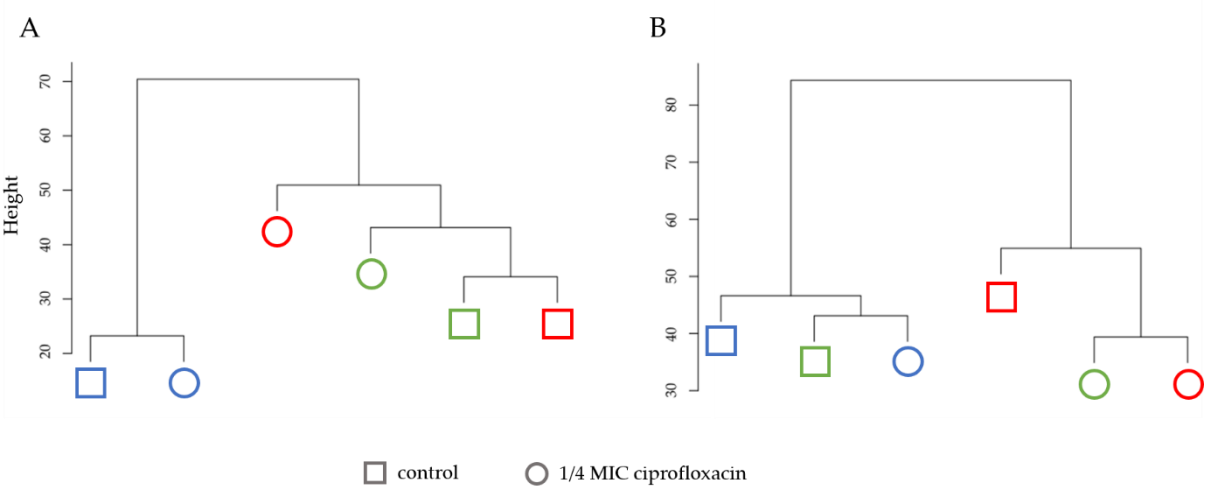

Figure 26: Cluster dendrogram illustrating the batch effect of RNA sequencing repetitions.
Dendrogram based on the log2 transform RNA sequencing gene counts normalized concerning the library size. A: Cluster dendrogram of *C. amalonaticus* KE3510; B: Cluster dendrogram of *E. cloacae* KE9563. Rectangles represent the untreated control, whereas circles illustrate samples treated with 1/4 MIC ciprofloxacin. Each repetition is represented by an individual color.

Table 29: Regulation of genes involved in conjugation, SOS response and general stress response, contributing to antibiotic resistance, DNA repair, and DNA replication. Gene expression was investigated for *C. amalonaticus* KE3510 by RNA sequencing in three independent experiments after 4 h of treatment with 1/4 MIC ciprofloxacin. Gene regulation is shown as mean $\log_2$ fold change (FC), normalized to the control without antibiotic treatment. The standard deviations show variations of the three replicates.

Description	Genes	Mean $\log_2$(FC)	Standard deviation	Protein function
Conjugation-related genes	*pilQ*	0.4	1.2	PilQ: Type IV pilus biogenesis and competence protein (Komano *et al.* 2000)
	virB6	0.4	0.7	VirB6, VirB8: Type IV secretion system proteins, which are involved in the membrane translocase component (Bhatty *et al.* 2013)
	virB8	0.9	0.4	
	virB9	0.2	0.1	VirB9: putative channel protein of the type IV secretion system (Christie and Vogel 2000)
	virB11	0.3	0.6	VirB11: ATPase of the type IV secretion system (Fronzes *et al.* 2009)
	virB4	-0.2	0.7	VirB4: ATPase of the type IV secretion system (Guglielmini *et al.* 2013)
SOS response-related genes	*lexA*	0.2	0.8	LexA: repressor of the SOS response-dependent gene expression (Gudas and Pardee 1975)
	recA	0.5	1.2	RecA: recombinase activated by ssDNA; positive regulator of the SOS response (Gudas and Pardee 1975)
	umuC	1.4	2.9	UmuC, UmuD: mutagenesis-specific DNA polymerase V (Cafarelli *et al.* 2013; Reuven *et al.* 1998)
	umuD	0.9	0.4	
	dinF	0.4	0.9	DinF: DNA damage-inducible protein protects against oxidative stress (Rodríguez-Beltrán *et al.* 2012)
	dinB	0.3	1.0	DinB: DNA polymerase IV involved in translesion repair (Cafarelli *et al.* 2013; Wagner *et al.* 1999)
	yedK	-0.6	1.4	YedK: putative SOS response-associated protein, involved in error-prone repair (Aravind *et al.* 2013; Mohni *et al.* 2019)
	dinI	-0.6	0.6	DinI: DNA damage-inducible protein, stabilizes the RecA-ssDNA-complex or prevents the binding of RecA to ssDNA depending on the intracellular concentration (Lusetti *et al.* 2004)
	recX	-0.3	0.2	RecX: regulatory protein, inhibitor of RecA (Cox 2007; Drees *et al.* 2004)
	polB	0.8	0.3	Pol B: DNA polymerase II, inducible by SOS response, necessary for DNA repair (Hughes *et al.* 1991)

General stress response genes	*uspA*	-0.7	1.4	
	uspB	-0.8	1.5	
	uspC	-0.6	0.7	Usp proteins: universal stress proteins, induced by various outside influences, e.g., starvation, oxidative stress, or heat shock (Diez *et al.* 2000; Nachin *et al.* 2005; Vollmer and Bark 2018)
	uspE	-0.2	0.9	
	uspF	-1.4	1.6	
	bssS	-0.9	1.2	BssS: stress-related regulation of biofilm formation (Domka *et al.* 2006)
Antibiotic resistance genes	*acrA*	0.2	0.5	
	acrE	0.1	0.7	AcrA, AcrB, AcrE, AcrF: multidrug efflux transporter (Hayashi *et al.* 2016; Kawamura-Sato *et al.* 1999; Zgurskaya and Nikaido 1999)
	acrF	0.6	1.0	
	mdfA	0.6	1.6	MdfA: putative membrane protein involved in multidrug efflux (Edgar and Bibi 1997)
	mdtA	0.5	0.9	
	mdtC	0.2	0.8	MdtA, MdtC, MdtK, MdtH, EmrD: multidrug-resistance proteins involved in multidrug efflux (Nagakubo *et al.* 2002; Yin *et al.* 2006)
	mdtH	0.5	0.7	
	emrD	2.2	2.6	
	oqxB17	0.8	0.8	OqxB17: multidrug efflux transporter permease subunit, conferring fluoroquinolone resistance (Surleac *et al.* 2020)
	ompL	0.2	0.8	OmpL: outer membrane proteins (UniProt 2021)
	ampC	0.2	0.4	AmpC: class C beta-lactamase (Henderson *et al.* 1997)
	marA	-1.5	1.9	
	marR	-0.6	0.6	MarA, MarR: multiple antibiotic resistance proteins confer ciprofloxacin and tetracycline resistance (Beggs *et al.* 2020; Jair *et al.* 1995; Pourahmad Jaktaji and Ebadi 2013)
	mdtB	-1.2	2.5	MdtB, MdtM: multidrug resistance proteins involved in multidrug efflux (Holdsworth and Law 2013; Kim and Nikaido 2012)
	mdtM	-0.7	0.6	
	ompD	-1.2	1.3	
	ompW	-1.3	2.2	OmpD, OmpW, OmpX: outer membrane porin proteins contributing to antibiotic resistance, adhesion, and biofilm formation (Hong *et al.* 2006; Hu *et al.* 2011; Li *et al.* 2018; Vogt and Schulz 1999)
	ompX	-0.6	1.5	

DNA repair genes	*radA*	0.5	0.7	RadA: belongs to the RecA/RadA/Rad51 protein superfamily and is involved in conjugational recombination and DNA repair (Zhou *et al.* 2006)
	recN	0.8	0.3	RecN: involved in dsDNA repair and activates RecA (Uranga *et al.* 2017)
	recO	0.4	0.6	RecO: involved in DNA repair and the RecF pathway (Umezu *et al.* 1993)
	recC	0.2	0.7	
	recB	0.1	0.3	RecB, RecC, RecD: subunits of the RecBCD protein complex that repairs double-strand breaks (Dillingham and Kowalczykowski 2008)
	recD	0.6	0.9	
	recJ	0.3	0.5	RecJ: single-stranded DNA-specific exonuclease involved in homologous recombination and mismatch repair (Han *et al.* 2006)
DNA replication-related genes	*uvrD*	0.2	0.3	UvrD: DNA helicase II that takes part in mismatch repair (Ordabayev *et al.* 2019)
	holA	0.8	1.1	HolA, HolE: subunit delta and theta of DNA polymerase III (Dong *et al.* 1993; Slater *et al.* 1994)
	holE	0.1	0.3	
	parC	0.3	0.2	ParC, parE: subunit A and subunit B of DNA topoisomerase IV, involved in unwinding of DNA and site-specific recombination (Kato *et al.* 1990; Lavasani and Hiasa 2001; Zechiedrich *et al.* 1997)
	parE	-0.5	0.6	
	topA	0.3	0.6	TopA: DNA topoisomerase I, involved in unwinding of DNA (Roca 1995)
	dnaB	-0.1	0.2	DnaB: Replicative DNA helicase, necessary for initiation and elongation step of DNA replication (Marszalek and Kaguni 1994)
	topB	-0.1	0.2	TopB: DNA topoisomerase III, relaxes supercoiled DNA (Broccoli *et al.* 2000)
	gyrA	-0.3	1.5	gyrA, gyrB: DNA gyrase subunits that initiate DNA supercoiling (Gellert *et al.* 1976)
	gyrB	-0.2	1.3	

Various studies revealed the impact of conjugation-related gene overexpression on enhanced conjugation frequency (Dmowski *et al.* 2018; Møller *et al.* 2017; Poidevin *et al.* 2018; Shun-Mei *et al.* 2018). Since the RNA sequencing did not provide clear insights into the elevated transfer efficiency of *C. amalonaticus* KE3510 under ciprofloxacin treatment, the expression of important conjugation-related genes was examined by RT-qPCR. *C. amalonaticus* KE3510 and *E. cloacae* KE9563 were treated for 4 h with 1/4 MIC and 1/2 MIC ciprofloxacin, respectively. For normalization, the housekeeping gene *rpoD* was used. The genes *traI* and *traM* are essential for the initiation of the conjugation; *traI* encodes a helicase that is responsible for the nicking reaction at the oriT site of the plasmid (Lu *et al.* 2008), and TraM represent accessory proteins of the relaxosome complex (Lu *et al.* 2008). The genes *virB5*, *traL*, and *traK* are important for the pilus assembly and extension (Bragagnolo *et al.* 2020; Yeo *et al.* 2003). The ATPase VirB4 fuels the process of T4SS assembly and protein transport (Peña *et al.* 2012). However, there were no significant changes in gene expression observed in the ciprofloxacin-treated isolates compared to the control (see Figure 27). Comparable to the RNA sequencing, the three repetitions showed partly strong variations in gene expression, leading to high standard deviations, especially for the genes *virB5* and *traL*, impeding a clear direction of regulation. Yet, most of the considered genes were down-regulated in *C. amalonaticus* KE3510 and up-regulated in *E. cloacae* KE9563, especially under treatment with 1/2 MIC ciprofloxacin, which seemed to be contradictory to the observed effect of elevated conjugation (see Figure 21A, M). Gene expression analysis was performed for two plasmid Inc-types that might differ in *tra* and *virB* gene sequences. Therefore, primer mismatching resulting in variations of amplification efficiencies might bias the gene expression profiling.

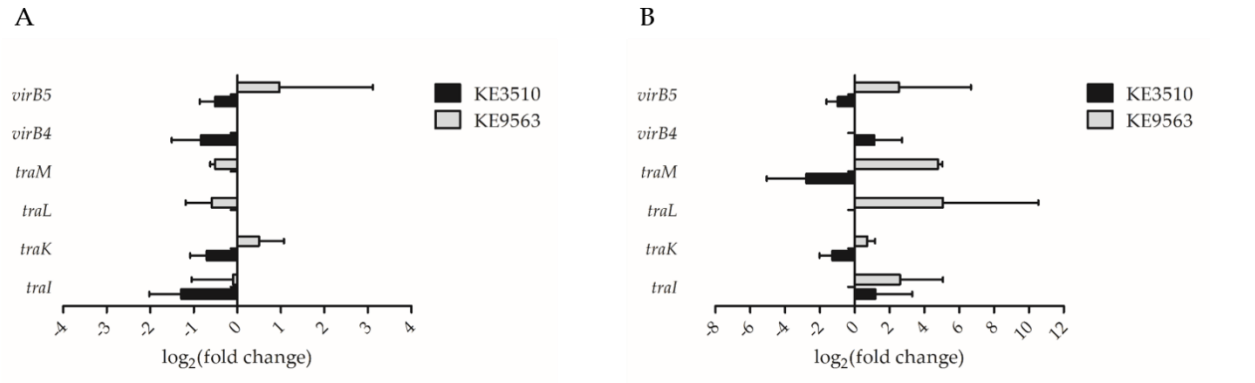

Figure 27: Gene expression of conjugation-related genes in *C. amalonaticus* KE3510 and *E. cloacae* KE9563 after 4 h treatment with ciprofloxacin.

A: Gene expression under 1/4 MIC ciprofloxacin. B: Gene expression under 1/2 MIC ciprofloxacin. Gene expression experiments were performed as triplicates in two independent experiments. Normalization was performed using the ΔΔCt method and *rpoD* as a housekeeping gene. Statistical significance was calculated for ΔCt-values, using a one-way ANOVA with Dunnett's post hoc test. No significantly regulated gene expression was observable.

It was already shown that fluoroquinolone antibiotics can trigger the SOS response, due to their interference in DNA synthesis causing DNA lesions (Dörr *et al.* 2009; Lewin *et al.* 1989; Piddock and Wise 1987). To investigate the role of SOS response in the conjugative transfer of the plasmids pKE3510 and pKE9563, the expression of SOS response-related genes was assessed under ciprofloxacin treatment. Since the two plasmids were derived from different bacterial species, *E. coli* ATCC-25922 harboring the *bla*$_{VIM-1}$ plasmid of *C. amalonaticus* KE3510 and *E. cloacae* KE9563, respectively, was used for analyzing the SOS response-related gene expression to avoid artifacts caused by different efficiencies in primer matching. The gene expression was analyzed after 4 h of treatment with 1/4 MIC and 1/2 MIC ciprofloxacin, respectively, compared to the control without treatment (see Figure 28).

The key genes *lexA* and *recA* were up-regulated in both isolates, ATCC-pKE9563, and ATCC-pKE3510, under 1/4 MIC and 1/2 MIC ciprofloxacin treatment, approving the RNA sequencing results for SOS response induction in *E. cloacae* KE9563. Additionally, the genes *umuC* and *umuD*, encoding subunits of DNA polymerase IV (Tang *et al.* 2000), lately expressed in the SOS response, were up-regulated. However, *umuC* was found to be lower expressed in ATCC-pKE3510 treated with 1/4 MIC ciprofloxacin and in ATCC-pKE9563 treated with 1/2 MIC ciprofloxacin. The down-regulation of *umuC* is contradictory to the RNA sequencing data, where *umuC* was up-regulated in two of the three repetitions under 1/4 MIC ciprofloxacin exposure. Similar results in up- and down-regulation were observed for the gene *dinB*, encoding the DNA polymerase IV necessary for SOS-induced error-prone repair (Tang *et al.* 2000). Statistical analysis revealed that there was no significant difference between the untreated control and the ciprofloxacin-treated isolates. There was no clear distinction in the expression of SOS response-related genes that might explain the variations in conjugation frequencies between the plasmids pKE3510 and pKE9563.

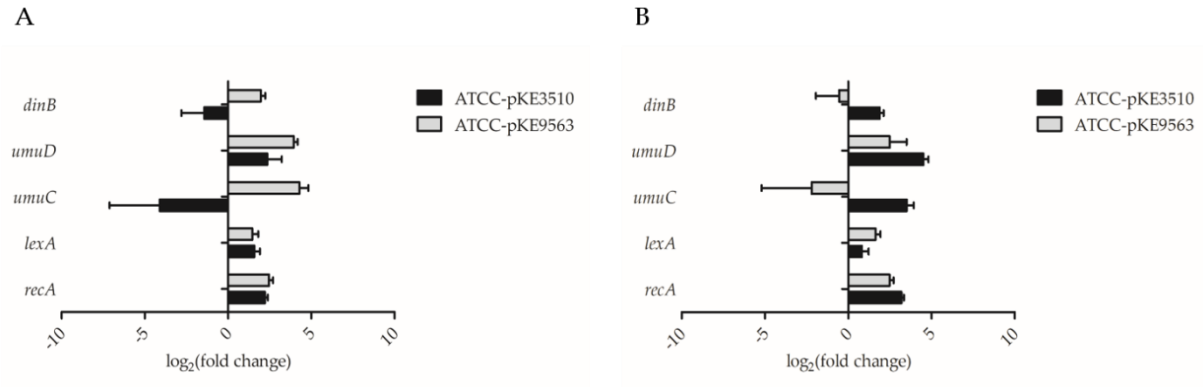

Figure 28: Gene expression of SOS response-related genes in *C. amalonaticus* KE3510 and *E. cloacae* KE9563 after 4 h treatment with ciprofloxacin.

A: Gene expression under 1/4 MIC ciprofloxacin. B: Gene expression under 1/2 MIC ciprofloxacin. Gene expression experiments were performed as triplicates in two independent experiments. Normalization was performed using the ΔΔCt method and *rpoD* as a housekeeping gene. Statistical significance was calculated for ΔCt-values, using a one-way ANOVA with Dunnett's post hoc test. No significantly regulated gene expression was observable.

3.3.2 Mutagenesis of donor strains due to ciprofloxacin treatment

Since SOS response-related genes were expressed under ciprofloxacin treatment, mutagenesis due to error-prone DNA polymerases UmuC/D and DinB is likely. To investigate changes in the genetic content of *C. amalonaticus* KE3510 under 1/4 MIC ciprofloxacin exposure (mutant) WGS was performed. Comparison of the genome to the untreated control revealed some gene deletions in the mutant (see Table 30).

Table 30: Changes in the genetic content of *C. amalonaticus* KE3510 due to 4 h of 1/4 MIC ciprofloxacin treatment revealed by WGS.
A core genome was calculated by Roary using 95 % identity. "-" indicates the absence of the gene, "+" represents the presence of the gene.

Annotation	Control KE3510	Mutant KE3510 ciprofloxacin treated
Regulatory protein Rop	+	-
Plasmid mobilization protein MobA	+	-
Uncharacterized protein (n = 8)	+	-
Uncharacterized protein (n = 3)	-	+

In total 10 genes were not detected in the mutant, two of which could be annotated. The other genes were designated as uncharacterized proteins with functions so far unknown. The regulatory protein Rop was of special interest since it controls the copy number of the bacterial plasmid (Cesareni *et al.* 1982; Som and Tomizawa 1983). A loss of this gene, negatively regulating the plasmid replication, indicates a high copy number in the mutant and might be an explanation for increased conjugation frequencies. Yet, the validation by PCR failed to detect the *rop* gene in the control strain, thus the gene deletion could not be confirmed.
The plasmid mobilization gene *mobA* was absent in the mutant, which also impacts the replication of the plasmid and subsequently the copy number (Frey *et al.* 1992). Deletion of the *mob* gene might therefore lead to a higher copy number.

3.3.3 Influence of plasmid copy number on the mechanism of increased conjugation frequency

The potential deletion of the *rop* gene in the ciprofloxacin-treated *C. amalonaticus* KE3510, which is involved in replication control of the plasmid, might have led to an increased copy number in the mutant compared to the untreated control. Thus, the plasmid copy numbers of the two isolates were determined by qPCR, amplifying the plasmid-encoded gene *bla*$_{VIM-1}$ and the chromosomal housekeeping gene *rpoD*. However, no changes in the number of plasmids relative to the chromosome could be observed (see Figure 29A).

To determine whether the IncN2-type plasmid, present in *C. amalonaticus* KE3510 is a high copy number plasmid compared to the IncL/M-type of *E. cloacae* KE9563, the plasmid numbers were estimated by the coverage of the contigs containing the plasmid-associated *repA* gene. WGS data revealed a higher coverage for KE3510 than for KE9563, indicating a higher plasmid copy number in *C. amalonaticus* (see Figure 29C), which might cause elevated transfer rates. Copy numbers of IncN2-type and IncL/M-type plasmids were validated by qPCR, amplifying the genes *bla*$_{VIM-1}$ and *rpoD*. To avoid bias in *rpoD* amplification due to different species of the donors, the plasmids were transferred to the rifampicin-resistant *E. coli* ATCC-25922. Similar results could be observed compared to WGS, since a slightly lower ratio was seen for ATCC-pKE3510, suggesting a higher number of plasmid copies (see Figure 29B, black bars). The impact of ciprofloxacin on the plasmid copy numbers of *E. coli* ATCC-pKE3510 and ATCC-pKE9563 was determined after 4 h of 1/4 MIC and 1/2 MIC ciprofloxacin exposure. There was no remarkable difference between control and ciprofloxacin-treated isolates detected.

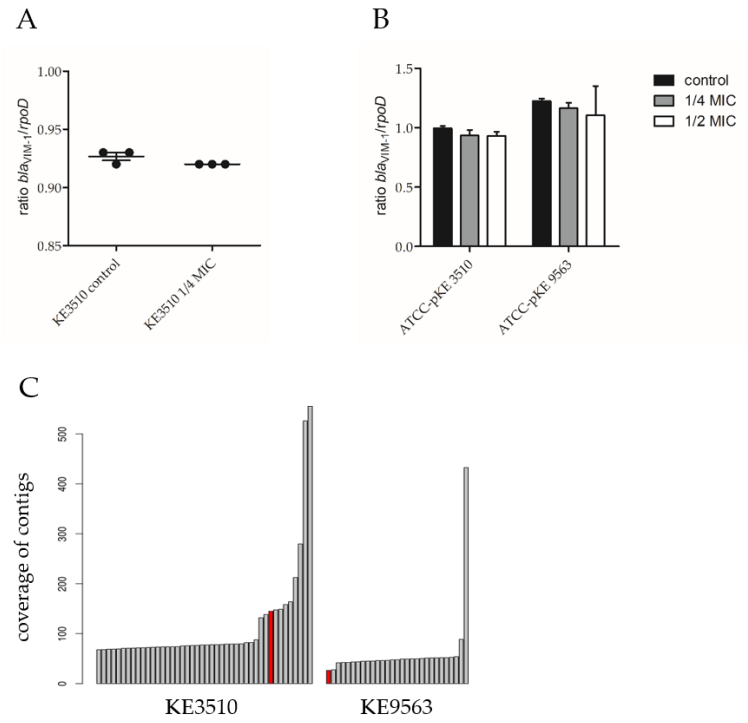

Figure 29: Effect of ciprofloxacin on the plasmid copy number of pKE3510 and pKE9563.
A: Evaluation of plasmid copy numbers in the control *C. amalonaticus* KE3510 and the mutant treated with ciprofloxacin. The experiment was carried out in technical triplicates, amplifying the plasmid-encoded *bla*$_{VIM-1}$ and the chromosomal *rpoD* gene. B: Influence of ciprofloxacin on alteration of the plasmid copy number in *E. coli* ATCC-pKE3510 and ATCC-pKE9563. Amplification of the chromosomal *rpoD* housekeeping gene and *bla*$_{VIM-1}$ plasmid-encoded gene were used for evaluation of the plasmid amounts relative to the chromosome. Experiments were performed as biological triplicates in two independent experiments. No statistically significant changes could be observed, using a one-way ANOVA for unpaired data. C: Determination of plasmid copy number in *C. amalonaticus* KE3510 and *E. cloacae* KE9563 by evaluating the coverage of the different contig. The red bar indicates the plasmid-associated contig, containing the *repA* gene.

4 Discussion

4.1 Surveillance of carbapenem-resistant bacteria

4.1.1 Local epidemiology of carbapenemase-producing Gram-negative bacteria

Detection of carbapenem-resistant bacteria is the first essential step to counteract the increasing problem of resistance gene distribution. Hence, the carbapenemase epidemiology of Gram-negative bacteria, showing antimicrobial resistance to meropenem and imipenem, was evaluated at the Heidelberg University Hospital in 2019. OXA-48 was with 40.5 % the most prevalent carbapenemase in Enterobacterales, followed by VIM-1 (21.6 %), KPC-2 (12.2 %), and NDM-5 (9.5 %, see Figure 10). The local epidemiology was comparable to data of the German NRL from the years 2014 to 2019, with *bla*$_{\text{OXA-48}}$ being the predominant carbapenemase in all years. Moreover, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{KPC-2}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{KPC-3}}$, *bla*$_{\text{OXA-181}}$, and *bla*$_{\text{NDM-5}}$ were frequently detected (Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020; see Figure 2). The bacterial species, *K. pneumoniae* and *E. coli*, harboring carbapenemases were overrepresented in the analysis done by the NRL compared to the local study (Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020). In 2019, *E. cloacae* was at a maximum of 38.8 % in Germany (Pfennigwerth 2020) in the other years this species was less often detected (Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019). On the contrary, *E. cloacae* was the most abundant species (40.5 %), mainly harboring *bla*$_{\text{OXA-48}}$ in the collection of the Heidelberg University Hospital (see Figure 10). Compared to the data of the NRL, local surveillance revealed a higher prevalence of carbapenemase-producers (80.4 %) in phenotypic resistant Enterobacterales. However, this could be an overestimation due to an outbreak with *bla*$_{\text{OXA-48}}$-positive *E. cloacae* that year (Nurjadi *et al.* 2021a). Carbapenemase genes were identified in 38.2 % to 47.1 % of the isolates sent to the NRL between 2014 and 2019 (Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020).

Studies on carbapenemase-harboring Enterobacterales in other countries showed a similar prevalence of resistance genes. In Austria, OXA-48 was the most abundant enzyme, followed by KPC, from 2011 to 2016 (Segagni Lusignani *et al.* 2020b). Surveillance performed in Spain between 2014 and 2016 revealed either OXA-48 or KPC as the most prevalent carbapenemases, depending on the year (López-González *et al.* 2019). In contrast to the local study, *K. pneumoniae* was the most common carbapenemase-producer in Austria and Spain. The *E. cloacae* abundance remained below 15 % in both studies (Segagni Lusignani *et al.* 2020a; Segagni Lusignani *et al.* 2020b). In Italy, KPC was predominant in *K. pneumoniae* (Bartolini *et al.* 2017). In the study from Heidelberg University Hospital, *K. pneumoniae* isolates were mostly OXA-48 producers. Worldwide, OXA-48, KPC, NDM, VIM, and IMP variants were reported as the "big five" carbapenemases in

Enterobacteriaceae (Nordmann *et al.* 2011; van Duin and Doi 2017; Walsh 2010). These carbapenemases and most of their variants can be detected with the qPCR assay used in this study (Probst *et al.* 2021a). However, in some geographic regions, less frequent carbapenemase types, such as GES, GIM and IMI can appear in *Enterobacteriaceae* (Kaase 2015; Kaase and Pfennigwerth 2016; Pfennigwerth 2017; Pfennigwerth 2018; Pfennigwerth 2019; Pfennigwerth 2020). As these genes are not included in the multiplex qPCR panel, the genes are not detected, and their prevalence may be underestimated. Hence, the surveillance of carbapenemase genes by WGS is essential to react to changes in prevalence and consequently adapt the qPCR to the local epidemiology. However, rare carbapenemases were not detected by WGS throughout the surveillance study in 2019 (see Figure 10 and Table 33).

In 18 phenotypic resistant Enterobacterales, no carbapenemase gene was identified by WGS and qPCR. Thus, phenotypic carbapenem resistance may have been mediated by mechanisms other than carbapenemases. Narrow-/broad- and/or extended-spectrum beta-lactamases like CTX-M, SHV, and TEM as well as AmpC resistance genes, *bla*$_{ACT}$, *bla*$_{CMY}$, *bla*$_{MIR}$, *bla*$_{EC}$, *bla*$_{ampH}$, and *bla*$_{ampC}$ were found in the non-carbapenemase-producers by WGS. Additionally, the isolates harbor different multidrug efflux pumps and porins, like MexA-N, OprJ/M/N, AcrA-F, TolC, EmrA, or KpnE-G (see Table 25). A few studies on beta-lactam resistance due to changes in membrane permeability and increased efflux in combination with increased ESBL and/or AmpC expression have been published. In *P. aeruginosa* for instance, overexpression of the MexAB-OprM efflux system and the loss of the outer membrane OprD porin was linked to carbapenem resistance (Codjoe and Donkor 2017; Köhler *et al.* 1999; Li *et al.* 1994; Pai *et al.* 2001). The porins OmpK35/36 in *K. pneumoniae* and OmpF/C in *E. coli* were indicated to play a role in beta-lactam resistance as well as higher expression of the efflux pumps KpnF/A in combination with ESBL and AmpC (Maurya *et al.* 2019; Nordmann *et al.* 2012). Overexpression of *acrA*, a component of an efflux pump, was reported to contribute to imipenem resistance in *Enterobacter aerogenes* (Bornet *et al.* 2003). In *E. coli* the most important efflux pumps are AcrAB-TolC, EmrAB-TolC, and MdtM that, when overexpressed, can lead to carbapenem resistance (Chetri *et al.* 2019).

Differentiation between the mechanism leading to carbapenem resistance is important since infection control and prevention measures are adopted correspondingly. Carbapenemase genes are usually located on mobile genetic elements that can be disseminated within the bacterial community and are thus more critical.

4.1.2 Accelerating the detection of carbapenemase genes in patients' specimens

In an outbreak setting, timely identification of patients infected or colonized with carbapenemase-producers is essential for earlier treatment of the patients and for initiating dissemination prevention strategies. Thus, screening directly on primary samples was evaluated throughout the *bla*$_{\text{OXA-48}}$ *E. cloacae* outbreak in 2019 compared to the conventional culture. The direct-qPCR was performed on the automatized BD MAX™ system, leading to results within 3 h (Probst *et al.* 2021a). Compared to the cultural screening, the qPCR showed high sensitivity and specificity of 100 % and 98.5 %, respectively, and was in 10 cases even more sensitive than the culture, indicated by a clear amplification curve (see Table 26; Probst *et al.* 2022). According to the patient documentation, the detection of *bla*$_{\text{OXA-48}}$-like in those samples was comprehensible since carbapenemase-producers were found in previous or subsequent rectal swabs and clinical samples, respectively. Finding more carbapenemase genes by direct-qPCR on primary patients' specimens compared to culture is concurrent with other commercialized assays like the Check-Direct CPE (Huang *et al.* 2015; Lau *et al.* 2015; Nijhuis *et al.* 2013), NucliSENS EasyQ KPC assay (Hindiyeh *et al.* 2008; McEwan *et al.* 2013), and XPert CARBA-R assay (Jin *et al.* 2020; Tenover *et al.* 2013). Since the cultural screening of carbapenemase-producers was performed on chromID® ESBL agar plates, the limitations in detecting *bla*$_{\text{OXA-48}}$-like carbapenemases mentioned above need to be considered. Low enzymatic activity, as well as low-level expression of the carbapenemase genes, might lead to a susceptible phenotype (Carrër *et al.* 2010). Additionally, the bacterial load in antibiotic pre-treated patients' specimens might be reduced or bacterial growth may be inhibited, thus, the culture remained negative. Heteroresistance can be present in the population, comprising susceptible and resistant bacteria. However, the resistant phenotype is often unstable and might only be induced under antibiotic exposure (Pereira *et al.* 2021). The time needed for identification is the main drawback of culture-based diagnostics since 24 h to 48 h are needed to determine carbapenemase-producers in patients' samples, leading to a delay in optimal treatment and timely initiation of hygienic measures.

After all these considerations, these observations show that the detection of carbapenemase genes in primary samples might not only be a useful tool during outbreak settings, as already shown in other studies (Giani *et al.* 2012; Naas *et al.* 2013) but also during the screening of newly admitted or hospitalized patients with bacterial infections. Patients with an initially negative screening for carbapenemase-producers, becoming positive after 48 h of hospitalization, are counted as nosocomial infections (Jain *et al.* 2021). However, the resistant bacterial load might be below the detection limit of the selective agar in the initial patients' specimens. Antibiotic treatment during the hospitalization period may select for these pathogens (Bonhoeffer *et al.* 1997; Gullberg *et al.* 2011), leading to a cultural positive result in the next screening. Those patients, putatively negative at admission may already be identified as colonized with carbapenemase-producers by detecting the presence of resistance genes. Consequently, using the direct-qPCR as initial

screening prior to cultural diagnostics might prevent the overestimation of hospital-acquired infections due to the low sensitivity of the detection method.
Even though the direct-qPCR results obtained during the outbreak setting were promising, careful evaluation needs to be done before using as an initial screening method for carbapenemase-producers in a routine diagnostic procedure. Further studies in a non-outbreak period would be needed to determine the number of false-negatives by direct-qPCR and by culture (Lowman *et al.* 2014). Additionally, the local carbapenemase resistance situation should be closely monitored to react to changes in epidemiology and adapt the qPCR approach, accordingly.
Molecular-based diagnostics using multiplex qPCRs as a primary assay is already applied for detecting pathogens causing gastrointestinal infections directly from stool samples with good performances. For example, the Seegene Allplex™ Gastrointestinal assays, the Luminex xTAG Gastrointestinal Pathogen Panel, and the BD MAX™ Enteric panel showed sensitivities of 81 % to 100 %, 92 %, and 78 %, respectively, depending on the pathogen (Martín *et al.* 2018; Yoo *et al.* 2019; Zimmermann *et al.* 2020). The Seegene Allplex™ Gastrointestinal assay also showed higher detection rates than cultural screening (Amrud *et al.* 2018). Thus, adjusting the conventional microbiological diagnostics for carbapenem-resistant bacteria to a primary genotypic method may increase sensitivity and shorter time to results. Positive samples identified by qPCR could then be further differentiated by conventional culture, to determine bacterial species and susceptibility to other antibiotic classes. Using qPCR approaches to detect the full bacterial resistance genes has technical limitations in the number of targets to be included. Detecting resistance genes in patients' samples is a lot more extensive than pathogen determination since a large variety of genes are involved in resistance. Moreover, antibiotic resistance caused by mutations or overexpression of certain genes is difficult to detect by qPCR. Therefore, metagenomics would be needed for changing the diagnostics away from the present pathogen-specific resistance towards the present resistome in the patients' specimens. This might be beneficial to get an understanding of whether antibiotic treatment will select for certain bacteria harboring resistance genes, or whether transfer of plasmids from commensals to pathogens occur, however, needs to be validated first.

4.1.3 Implementing WGS in microbiology routine diagnostics

WGS is a powerful tool to get insights into the full gene repertoire of certain isolates and the microbial composition of patients' samples. So far, this approach is mostly used for surveillance purposes and is not yet standardized in clinical diagnostic laboratories, thus critical evaluation of the parameters during the analysis of raw sequencing data is crucial to ensure high reliability in resistance gene prediction. Quality control parameters are individually defined regarding the coverage of the assembly, quality of de novo assembly, and the eradication of possible DNA contaminants, which can impact the sensitivity (Ellington *et al.* 2017). Additionally, the

completeness of the database, used for annotation of the resistance genes, influences the results (Ruppé *et al.* 2020).

Throughout the surveillance study from 2019 on carbapenemase-producing Enterobacterales, the comparison of qPCR and WGS revealed limitations in detecting resistance genes by sequencing. Due to coverage issues, WGS showed a lower sensitivity since six genes were missed by the sequencing approach (see Table 24 and Table 33; Probst *et al.* 2021b).

For quality control, concerning misassemblies and DNA impurities, a 25x coverage, generally applied, was used for the full genome, and contigs below 10x coverage and 1000 bp length were eliminated, thus true signals for carbapenemase genes were invisible. The performance of resistance gene prediction might be influenced by low abundant resistance determinants due to plasmid loss during DNA isolation or low-copy number plasmids. This data indicates that the non-curated draft genome should be used to raise the accuracy in detecting carbapenemase genes in Enterobacterales (Probst *et al.* 2021b).

WGS showed an encouraging performance in resistance gene prediction with a concordance of 100 % to qPCR after reanalysis (see Table 24). Other studies on antimicrobial susceptibility detection of various pathogens by sequencing approaches revealed similar sensitivities of about 82.6 % to 99.1 % when compared to standard phenotypic methods (Bradley *et al.* 2015; Mason *et al.* 2018; McDermott *et al.* 2016; Ruppé *et al.* 2020; Zankari *et al.* 2017). Nonetheless false-positive and false-negative results need to be further reduced, since they can lead to inappropriate antibiotic therapy of patients, potentially resulting in the development of bacterial resistance (Su *et al.* 2019).

One big advantage of WGS is the comprehensive amount of information gained, which is useful for susceptibility determination, genetic relationship, species identification, tracking outbreaks and transmission chains, and for identifying new resistance mechanisms in phenotypic resistant isolates (Balloux *et al.* 2018; Hendriksen *et al.* 2019; Nurjadi *et al.* 2021b). Compared to other genotypic methods, WGS is an unbiased approach, not limited in the number of targets (Su *et al.* 2019). Performing WGS on primary samples brings an advantage for detecting slow-growing bacteria, such as *Mycobacterium tuberculosis* (Doyle *et al.* 2018), and fastidious bacteria that are difficult to culture. Yet, WGS requires a longer time to results, and the costs are too high for clinical routine diagnostics (Su *et al.* 2019). Furthermore, automatization of the process will be needed prior to use in diagnostics, and both easy to handle pipelines and data management needs to be assessed (Yee *et al.* 2020). To ensure high accuracy in antimicrobial susceptibility prediction, bioinformatics expertise or standardized parameters for analyzing raw sequencing data are needed. Resistance caused by overexpression of certain genes or point mutations might be difficult to detect by WGS, which is another drawback of using this approach in clinical diagnostics (Ellington *et al.* 2017; Ruppé *et al.* 2020).

4.1.4 The role of the hospital environment in carbapenemase outbreaks and transmission

The hospital environment has always been considered to play an important role as a reservoir for MDR bacteria and sources for transmission. Various places in hospitals were found to be contaminated with potentially pathogenic bacteria, including door handles, hospital equipment, piping systems, and different surfaces in bathrooms (Bhatta *et al.* 2018; Chaoui *et al.* 2019; Chemaly *et al.* 2014; Otokunefor *et al.* 2020; Rodrigues *et al.* 2020). Microbiome studies of the bacterial community in hospital drains revealed a high diversity, comprising opportunistic and clinically relevant bacteria, such as *Pseudomonas*, *Aeromonas*, *Enterobacter*, *Staphylococcus*, or *Sphingomonas* species, just to mention a few (Constantinides *et al.* 2020; Pirzadian *et al.* 2020). Some bacterial species can form biofilm facilitating their protection and persistence over long periods in the drains. Furthermore, biofilm can create perfect niches for clinical isolates, derived from infected patients (Chaoui *et al.* 2019; Chng *et al.* 2020; Pirzadian *et al.* 2020). Biofilms were pointed out to be hot spots for HGT due to high cell densities and complex species constitution, thus new MDR bacteria can evolve in the hospital environment (Abe *et al.* 2020; Ghigo 2001; Mairi *et al.* 2018; Ory *et al.* 2019). The presence of various antimicrobial compounds in wastewater is fostering this issue since selective pressure favors the abundance of resistant bacteria (Abe *et al.* 2020; Brown *et al.* 2006; Mairi *et al.* 2018; Ory *et al.* 2019; Verlicchi *et al.* 2012). Transmission of MDR bacteria between the hospital environment and patients is often linked to outbreak events as shown in various studies (Brooks *et al.* 2016; Chng *et al.* 2020; Decraene *et al.* 2018; Khan *et al.* 2018; Kizny Gordon *et al.* 2017; Oberauner *et al.* 2013; Wendel *et al.* 2016).

The *bla*$_{\text{OXA-48}}$-positive *E. cloacae* outbreak at the Heidelberg University Hospital was also connected to environmental reservoirs (Nurjadi *et al.* 2021a). To identify potential contaminations, wastewater of toilets and shower drains was analyzed weekly during the outbreak period by cultural methods. To accelerate the time to results, multiplex qPCR for carbapenemase detection was performed directly on the concentrated wastewater samples without cultural enrichment, which led to a sensitivity and specificity of 62.1 % and 95.1 %, respectively. A few more false-negative samples by qPCR were obtained compared to cultural screening (see Table 27), indicating that the concentration step before qPCR might not be the optimal solution, especially for samples with low bacterial load.

Therefore, different sample treatment options, prior qPCR, were tested in a non-outbreak setting with water samples from sinks, toilet basins and shower drains. Besides the concentration by centrifugation, the qPCR was directly performed on water samples and after enrichment in LB-medium. The overnight culture showed the best performance when compared to untreated and concentrated water samples (see Table 28). Using the untreated water was problematic since just a small volume of water (4 µl) was used for the qPCR, which the genes of interest might not be present in. To increase sensitivity another concentration method, using filters, for instance, could be considered (Jutkina *et al.* 2016). Other studies, performing an initial enrichment step for environmental samples, showed higher sensitivities by qPCR (Knoester *et al.* 2014; Kotsanas *et al.*

2013; Leung *et al.* 2013). Yet, the sampling technique and the sampling volume also need to be considered, since it was shown to have an impact on the performance of detection methods (Lerner *et al.* 2013; Perry *et al.* 2018). The enrichment in LB-medium revealed various carbapenemase-producers that were not detected by cultivation on selective agar. The selection pressure might be too high, suggesting that an initial enrichment culture might also be beneficial for cultural screening, albeit enrichment itself is biased since some bacteria might grow more efficiently and thus inhibiting the growth of low abundant or slowly growing species. Yet, resistant strains can also be counter-selected since the LB-medium represents a non-selective culture medium. Using culture-based methods *P. aeruginosa* could be found in numerous samples, however the qPCR remained negative (see Table 28). Since the chromID® CARBA SMART agar is optimized for detecting VIM, NDM, KPC, and OXA-48 in *Enterobacteriaceae*, other mechanisms regarding the membrane permeability might lead to carbapenem resistance and allow growth of *P. aeruginosa* on the selective medium.

Diverse carbapenemase genes were detected by qPCR in the hospital environment during the outbreak setting as well as in the non-outbreak collection (see Figure 11 and Table 28). Detection of OXA-23-like, OXA-40/24-like, OXA-58-like, and VIM suggests the presence of *A. baumannii* and *P. aeruginosa* since these carbapenemases can be frequently found in those species (see Figure 3; Leitner *et al.* 2015). The high detection rates of *bla*$_{\text{OXA-48}}$ carbapenemase genes during 2019 could be linked to the *E. cloacae* outbreak. Further optimization of the sampling procedure and the pre-treatment of the samples need to be performed prior to application as a routine screening method. In summary, the multiplex qPCR might be better suited for carbapenemase detection in patients' specimens than in wastewater samples due to the higher sensitivity. However, low densities of carbapenemase-producers are less likely to colonize in environmental niches or infect patients. As high loads of carbapenemase-producers could be detected in wastewater samples by qPCR, samples with dangerous levels contributing to possible outbreak events may be identified, as was shown for the *bla*$_{\text{OXA-48}}$-positive *E. cloacae* strain (Nurjadi *et al.* 2021a).

4.2 Transmission of plasmids through conjugative transfer

Conjugation frequency was determined in this thesis by the ratio of transconjugants to the initial number of donors. This calculation is taking no account of the expanding population of the donor, recipient, and transconjugants, respectively (Alderliesten *et al.* 2020). Yet, Prensky *et al.* (2021) discovered that there is a significantly lower growth rate due to an increased lag phase of newly emerging transconjugants, which might diminish the potential bias of population dynamics, however, this was not addressed in this thesis.

A large variety of studies have been performed on analyzing the impact of different parameters on the conjugation efficiency during *in vitro* liquid mating experiments. High variations could be observed by changing cell density, temperature, pH, nutrition, or mating time (Alderliesten *et al.* 2020; Fernandez-Astorga *et al.* 1992; Johnsen and Kroer 2007; Rochelle *et al.* 1989). In this study, it could be observed that alterations of the surface area, using Petri dishes instead of culture tubes, led to a ten-times higher transfer rate of pKE3510 derived from *C. amalonaticus* (see Figure 21A and Figure 24F). Besides the influence of environmental factors on conjugation frequencies, the focus was placed on different factors, such as relatedness of donor and recipient, the plasmid Inc-type, co-existing plasmids, as well as host- or plasmid-encoded genes that might have a regulatory impact on the transfer.

4.2.1 Effect of donor and recipient relatedness on plasmid transmission

Carbapenemase-positive isolates belonging to the families *Enterobacteriaceae* (*Citrobacter* sp., *Enterobacter* sp., *Escherichia* sp., and *Klebsiella* sp.) as well as *Yersiniaceae* (*Serratia* sp.) were used in this thesis to assess the transfer efficiency of *bla*$_{\text{VIM-1}}$-encoding plasmids to the recipient strain *E. coli* J53. The highest conjugative transfer was observed for the donor *C. amalonaticus*. Even *E. coli* donors showed lower conjugation rates, which is contradictive to previously published studies (Alderliesten *et al.* 2020; Dimitriu *et al.* 2019; see Figure 14).

Alderliesten *et al.* (2020) performed a meta-analysis in this context, revealing the importance of taxonomic relatedness in conjugation frequencies. For donor and recipient strains belonging to different classes, more than 700-fold lower transfer rates were observed compared to those belonging to the same species (Alderliesten *et al.* 2020). Dimitriu *et al.* (2019) pointed out that even between closely related *E. coli* isolates variations regarding conjugation efficiency can be observed. The restriction-modification system, a defense mechanism against foreign DNA, is under suspicion to limit conjugation. Closely related species share similar restriction enzymes, which might cause less suppression on transfer than in distantly related donors and recipients (Dimitriu *et al.* 2019; Roer *et al.* 2015).

Aside from *C. amalonaticus*, all tested donor strains, including other *Citrobacter* sp., showed lower transfer rates than *E. coli* (see Figure 14). *S. marcescens*, belonging to a different taxonomic family, was in line with *Klebsiella* sp., the most inefficient donor in this study, which is more consistent

with previously published results. The dependency of donor and recipient relatedness on plasmid transfer efficiency could be nicely observed for the IncN1 plasmid homologs pKE3436, pKE9474, and pKE9409 derived from *E. hormaechei* and *E. cloacae* (see Figure 17). Differences in conjugation frequency of approximately one order of magnitude could be detected (see Figure 21).
The elevated transfer rates of *C. amalonaticus* KE3510 might be biased and overbalanced by the high conjugative IncN2 plasmid present in this donor strain. Hence, besides the relatedness of donor and recipient, the plasmid Inc-type seemed to play an important role in transfer efficiency (see Section 4.2.2).

4.2.2 Impact of plasmid Inc-type on transfer efficiency

Throughout the thesis, various isolates harboring different Inc-type *bla*$_{VIM-1}$ plasmids were tested for their conjugative ability. *In vitro* liquid mating experiments revealed significantly higher transfer rates for IncN2-type plasmids than for IncN1, IncA/C, IncFII, and IncL/M plasmids, respectively (see Figure 13).
The correlation between Inc-type and transfer rates, however, differ between publications. Gama *et al.* (2017) published a study on several Inc-types showing variations in conjugation by eight orders of magnitude. IncF-type plasmids had the highest transfer rates in their study, followed by IncW, IncA/C, IncP-1, and IncN, whereas IncX-type and IncH-type had the lowest efficiency (Gama *et al.* 2017). Lower transfer rates of IncA/C compared to IncF plasmids were confirmed by another working group, showing an 85-times difference (Alderliesten *et al.* 2020). Bethke *et al.* (2020) examined transfer rates of different plasmid Inc-types conferring resistance to beta-lactam antibiotics. The highest efficiencies could be observed for IncN/F and IncI plasmids, whereas IncB/O/K/Z plasmids showed lower conjugation frequencies (Bethke *et al.* 2020).
The observed high conjugation efficiency of IncN2 plasmids in this thesis is in line with the study of Bethke *et al.* (2020), but inconsistent with the lower frequency detected by Gama *et al.* (2017). Yet, in both studies, the IncN-type was not further subdivided into IncN1 and IncN2 plasmids. Depending on the subclassification of IncN, efficiencies were quite different for *bla*$_{VIM-1}$ plasmid transfer (see Figure 21). However, all IncN2 plasmids, derived from a single patient, showed a high degree of genetic similarity (see Figure 15), which might bias the high conjugative ability, observed for this Inc-type. Contradictory results were obtained for *bla*$_{VIM-1}$ containing IncFII plasmids showing the lowest transfer rates in this thesis compared to the studies mentioned above. Yet, IncFII plasmids were conjugated simultaneously with IncA/C plasmids, which might have an impeding effect (see Section 4.2.3).
Gama *et al.* (2017) suggested that there is no correlation of plasmid Inc-type and conjugation efficiencies since plasmids belonging to similar Inc-types showed variations (Gama *et al.* 2017). Indeed, mating experiments resulted in huge differences between IncN subgroups, yet similar conjugation frequencies could be observed for IncN1-type plasmids (n = 7), pointing towards a

correlation between Inc-type and conjugation efficiencies for *bla*$_{VIM-1}$-encoding plasmids (see Figure 21).

Plasmid Inc-types described in Enterobacterales differ in size and copy number accordingly, which might contribute to transfer efficiencies. Small plasmids are usually present in high copy numbers, whereas large plasmids are less abundant with low copy numbers that spread less frequently (Rodríguez-Rubio *et al.* 2020; San Millan *et al.* 2014).

In two studies smaller plasmid sizes were linked to significantly higher transfer rates, which was attributed to shorter transfer times of small mobile genetic elements (Potron *et al.* 2014; Thomas and Nielsen 2005). Thomas and Nielsen (2005) proposed that large elements are often shared incomplete due to the collapse of the mating bridge during the conjugation process.

The tested *bla*$_{VIM-1}$ plasmids derived from Enterobacterales vary in size from 50.8 kb (IncN2) to around 80.4 kb (fusion plasmid content of IncA/C and IncFII; see Figure 15 to Figure 20). Correlation of the plasmid size to transfer frequencies revealed significantly higher transfer rates for small plasmids, such as pKE3510, pKE3742, and pKE3754 (see Figure 13), being in line with the published observations.

The copy number of the IncN2 plasmid pKE3510 and the IncL/M plasmid pKE9563 was estimated by WGS regarding the coverage of the copy number regulating gene *repA* (see Figure 29C), pointing towards more copies of pKE3510. Hence, the smaller size and the consequently higher copy numbers of the IncN2 plasmids might have favored the high transfer rates observed in this thesis.

4.2.3 Co-occurring plasmids manipulate conjugation frequency

Most of the *bla*$_{VIM-1}$-positive Enterobacterales included in this study harbor more than one plasmid except for *C. amalonaticus* KE3510 (IncN2), *E. cloacae* KE9409 (IncN1), *S. marcescens* KE9538 (IncL/M), and *E. cloacae* KE9563 (IncL/M, see Figure 12). Plasmids are commonly linked to a reduction in host fitness since energy is required for their maintenance, hence, bacteria harboring a single plasmid replicate faster than those with numerous plasmids (Gama *et al.* 2017; Dahlberg and Chao 2003; Haft *et al.* 2009; San Millan *et al.* 2014; Yates *et al.* 2006). However, isolates used in this thesis comprise up to six different plasmids (see Figure 12), indicating that carrying multiple plasmids might provide more advantages to environmental conditions and thus might lower metabolic costs (San Millan *et al.* 2014). Yet, harboring fewer plasmids does not seem to be beneficial for transfer efficiencies since lower frequencies were obtained for *E. cloacae* KE9409, *S. marcescens* KE9538, and *E. cloacae* KE9563, respectively, compared to isolates with more plasmids such as *E. hormaechei* KE9747 and *E. hormaechei* KE3436 (see Figure 12 and Figure 21).

Studies on co-existing plasmids in the donor cell revealed interactions between the mobile genetic elements, resulting in a reduced or reinforced conjugation efficiency, whereby inhibition events occurred more often (Gama *et al.* 2017).

The isolate *C. freundii* KE4473 harbors two carbapenemase genes, *bla*$_{VIM-1}$ and *bla*$_{KPC-2}$, yet only the *bla*$_{VIM-1}$ gene could be found in the transconjugant, indicating a negative effect of the IncN1 on the IncFIB plasmid, which probably encodes the *bla*$_{KPC}$ gene (see Figure 16 and Figure 32). This inhibition event could be observed for most donors with multiple plasmids since only one Inc-type was transferred except for *C. portucalensis* KE4494 and *K. aerogenes* KE4495 that transferred the IncA/C and the IncFII plasmid simultaneously.

It is worth mentioning that not only plasmids present in the donor strain influence the transfer efficiency but also plasmids already abundant in the recipient strains (Alderliesten *et al.* 2020; Benz *et al.* 2021). The conjugation can be facilitated if conjugative plasmids are present in donor and recipient cells, which could stabilize the mating pair formation (Dionisio *et al.* 2019; Gama *et al.* 2017). This aspect was not addressed in this thesis since the laboratory strain *E. coli* J53 that does not contain any plasmids was used as a recipient. However, it is critical for complex bacterial communities that occur in the gut or environmental reservoirs where a variety of potential recipient strains is present (Benz *et al.* 2021).

Regulatory genes, important for transmission efficiencies, can be chromosomally or plasmid-encoded and can influence the plasmid itself or co-occurring plasmids. These genes can be impacted by transposons and insertion sequences resulting in inactivation or overexpression of conjugation-related genes that in turn alter the plasmid transfer (see Section 4.2.4).

4.2.4 Impact of regulatory genes and transposable elements on plasmid transfer

Depending on the plasmid type, different regulatory genes impacting conjugation frequencies were determined. Self-regulatory genes such as *fin* were described on IncF, IncL, IncN, and IncX plasmids, drastically diminishing plasmid transfer (Dionisio *et al.* 2019; Gama *et al.* 2017; Gasson and Willetts 1975; Haft *et al.* 2009; Takahashi *et al.* 2011). Yet, mutations in this gene can have an elevating effect on conjugation (Dionisio *et al.* 2019; Gama *et al.* 2017; Gasson and Willetts 1975; Haft *et al.* 2009; Takahashi *et al.* 2011). Besides self-regulation, plasmids encode genes to limit the transfer of co-existing plasmids. *PifC* encoded by IncF plasmids and *osa* found on IncW plasmids were described to prevent the transfer of IncW and IncP plasmids, respectively, by interacting with the coupling protein, which is essential for the DNA transfer (Getino *et al.* 2017; Santini and Stanisich 1998; Tanimoto and Iino 1983). Moreover, differences in the plasmid-encoded *tra* genes, necessary for conjugation, were considered to contribute to a 40-times higher transfer efficiency of a *bla*$_{OXA-48}$-encoding IncL/M plasmid than an IncL/M plasmid containing a *bla*$_{NDM}$ carbapenemase gene (Potron *et al.* 2014).

Antimicrobial resistance genes are often linked to transposable elements, allowing their transition within the host chromosome and co-existing plasmids. In this context, Che *et al.* (2021) point out that the association of resistance genes to insertion sequences and transposons, which are often present on conjugative plasmids, is important for the successful distribution within the complex bacterial community. Transposable elements can further reduce metabolic costs by translocating beneficial genes of transiently present plasmids to the chromosome or to stably existing plasmids, thus contributing to the evolution of new MDR bacteria (Che *et al.* 2021).

Some plasmids were designated as "superspreaders" since they can be transferred highly efficiently. In those plasmids, insertion sequences or transposons were identified as the most probable reason for increased transfer rates by interrupting genes negatively regulating conjugation or increasing the expression of conjugation-related genes. In this context, different plasmid loci were described such as the *tir* gene, a possible inhibitor for conjugation (Tanimoto *et al.* 1985). Integration of the transposon tn1999 into *tir* was frequently observed in *bla*$_{\text{OXA-48}}$-encoding IncL/M plasmids, resulting in increased conjugation frequencies (Berger *et al.* 2013; Espedido *et al.* 2013; Potron *et al.* 2014). Insertion of an IS3 element into *finO*/*P* genes led to constitutive expression of *traJ*, the transcriptional activator of the conjugation machinery (Cheah and Skurray 1986; Yoshioka *et al.* 1987). Dmowski *et al.* (2018) demonstrated that inactivation of o*rf35* and *orf36*, potential regulators of the plasmid mobilization genes, facilitate plasmid transfer. Another study, performed by Yamaichi *et al.* (2015), identified a short region, designated as Hft (high-frequency transfer) locus upstream of the *tra* genes. Insertion of transposons into this region drastically increased plasmid transfer. This observation was confirmed by Poidevin *et al.* (2018), revealing *traA* overexpression as a consequence of Hft interruption, which in turn might lead to hyperpiliation enabling more cell-to-cell contacts and subsequently higher conjugation efficiencies. The operon *stbABC* was described on conjugative plasmids, being necessary for plasmid stability in the bacterial cell. Inactivation of the *stbA* gene by insertion of transposable elements led to higher plasmid instability likewise to increased conjugation frequencies (Guynet *et al.* 2011).

Various insertion sequences and transposons could be identified in the transferred *bla*$_{\text{VIM-1}}$ plasmid content, yet regulatory genes mentioned above were not present (see Figure 15 to Figure 20). However, the plasmids were not fully closed, and some genes could not be successfully annotated, suggesting that some of the hypothetical proteins might contribute to a regulatory mechanism.

The expression of *tra* genes of the highly conjugative IncN2 plasmid derived from *C. amalonaticus* KE3510 compared to an IncL/M plasmid present in *E. cloacae* KE9563, showing lower transfer rates, was investigated by RNA sequencing and RT-qPCR, which is part of the discussion Section 4.3.2.

4.3 The role of antibiotic treatment in plasmid transfer

Besides inhibiting microbial growth, antibiotics may influence bacterial metabolism, modulating different bacterial processes, such as biofilm formation, adhesion, or toxin secretion (Andersson and Hughes 2014; Fitzpatrick *et al.* 2002; Ishikawa and Horii 2005; Li *et al.* 2005). Selection pressure caused by sublethal antibiotic concentrations has been outlined in many studies to be the key driver in conjugation, especially when the plasmid brings beneficial features concerning metabolism, virulence, or resistance (Aminov 2011; Baharoglu *et al.* 2010; Hohnstock *et al.* 2000; Johnsen and Kroer 2007; Karkman *et al.* 2018; van Elsas and Bailey 2002). Treatment of patients with antibiotics often results in drug concentrations below the therapeutic level in the human gut and consequently in excrements and wastewater, since a few antimicrobial compounds are not fully absorbed (Chee-Sanford *et al.* 2009). The human gut, as well as the hospital environment, are ideal surroundings for horizontal gene transfer since high cell densities and a diverse bacterial community is present and thus are termed as "melting pots" for bacterial conjugation (Ronda *et al.* 2019; Kurokawa *et al.* 2007; Quigley 2013). Investigations of *in vivo* and *in vitro* conjugation frequencies revealed higher plasmid transfer rates in living organisms, highlighting the clinical relevance for patients under antibiotic treatment (Feld *et al.* 2008; Göttig *et al.* 2015).

However, in the literature, it is still contentious whether antibiotics have a direct promoting effect on plasmid transfer or rather act as a selective driver on the bacterial population, involving donors, recipients, and transconjugants, or if both assumptions play a role (Lopatkin *et al.* 2016a). In the following section, this issue regarding the transfer of *bla*$_{\text{VIM-1}}$-encoding plasmids under antibiotic treatment is discussed. Additionally, the impact of different substances inhibiting DNA replication and potential mechanism leading to increased transfer rates under selective pressure are addressed (see Sections 4.3.2 and 4.3.3).

4.3.1 DNA-interfering substances enhance *bla*$_{\text{VIM-1}}$ plasmid transfer

Preliminary experiments regarding the influence of different antibiotic classes on the plasmid transfer rates of *C. amalonaticus* KE3510 disclosed an elevating effect exclusively under treatment with fluoroquinolone antibiotics (Li and Nurjadi 2019).

Likewise, the facilitating effect of antibiotic classes on conjugation differs between bacterial strains and plasmid types, as suggested by the following publications. Ohlsen *et al.* (2003) showed an approximately three-fold increase in conjugation events between *S. aureus* strains in presence of sublethal gentamicin concentrations, whereas no impact by tetracycline, ciprofloxacin, or erythromycin could be observed. Elevated plasmid transfer in presence of gentamicin could be confirmed by Jutkina *et al.* (2018), using a mixture of sewage-derived donors and *E. coli* as recipient. Sulfamethoxazole could induce transfer rates, whereas other tested antibiotics such as ciprofloxacin, cefotaxime, erythromycin, and trimethoprim, did not impact the conjugation (Jutkina *et al.* 2018). On the contrary, erythromycin led to a 31-times higher transfer efficiency of

an IncFIB/FII/Col156 plasmid from *E. coli*, which remained unaffected by norfloxacin, chloramphenicol, and kanamycin (Bethke *et al.* 2020). The inducing effect, determined for the *bla*$_{VIM-1}$-harboring *C. amalonaticus* KE3510, by fluoroquinolones was also described in a study by Shun-Mei *et al.* (2018) on IncP plasmid transfer between *E. coli* and *P. aeruginosa*. Likewise, ciprofloxacin could elevate the transfer of *bla*$_{CTX-M}$-containing IncI1 and IncFII plasmids, as was the case for cefotaxime and ampicillin (Liu *et al.* 2019a).

Throughout this study it was investigated whether ciprofloxacin facilitating conjugation is widely spread in Enterobacterales, harboring a *bla*$_{VIM-1}$ plasmid. Besides *C. amalonaticus* KE3510, the two isolates *E. coli* KE3742 and *E. hormaechei* KE3754 harboring IncN2 plasmid homologs and *E. coli* KE4472 (IncN1) showed enhanced transfer rates in the presence of the antibiotic (see Figure 21). The results indicate that the increase in conjugation efficiencies triggered by fluoroquinolones is not common for carbapenemase-encoding plasmids, since it could be observed just for two individual plasmids (14.3 %). These observations are supported by Ohlsen *et al.* (2003) and Bethke *et al.* (2020), where just a small proportion of the screened *S. aureus* and clinical *E. coli* isolates showed increased transfer rates under treatment with gentamicin, chloramphenicol, and erythromycin, respectively.

It was suggested that antibiotics trigger the transfer of plasmids, if the corresponding resistance gene is present, which could be approved by Jutkina *et al.* (2016) and Scornec *et al.* (2017) regarding the conjugation of tetracycline resistance genes in the presence of tetracycline. Additionally, Scornec *et al.* (2017) observed higher transfer rates under macrolide, lincosamide, and streptogramin treatment, which are all affecting the bacterial ribosome (Scornec *et al.* 2017).

Most of the *bla*$_{VIM-1}$ plasmids included in this study carry the quinolone resistance genes *qnrS* or *qnrB* (see Figure 32), however, elevated transfer rates could only be seen for a small proportion. The conjugation frequency of the plasmids pKE4495 and pKE9409, for instance, did not increase, even though quinolone resistance genes were present (see Figure 21 and Figure 32).

To ensure that ciprofloxacin has a direct impact on the increased conjugation of the IncN2 plasmid, the antibiotic's inhibiting effect on bacterial growth and the independence of the donor species was assessed. The growth rate of *C. amalonaticus* KE3510 was not reduced at 1/32 MIC and 1/16 MIC, respectively, and increased transfer rates could be observed compared to the control (see Figure 22), indicating that the higher IncN2 plasmid transfer is independent of the antibiotic effect. Lopatkin *et al.* (2016b) proposed that selection caused by antimicrobial agents is negligible within the first hours of conjugation, aligning with the study from Prensky *et al.* (2021), which suggests a prolonged lag-phase of newly emerging transconjugants. Thus, it is unlikely that the higher conjugation frequencies obtained for *C. amalonaticus* KE3510 under ciprofloxacin treatment are caused by selection since *in vitro* liquid mating experiments were carried out only for 2 h.

A similar increase in conjugation frequency was observed for the three IncN2-type plasmids (pKE3510, pKE3742, and pKE3754) derived from different bacterial species (*C. amalonaticus*, *E. hormaechei,* and *E. coli*), demonstrating that the higher transfer is a plasmid-specific effect,

independent of the donor species. This observation was confirmed by using the laboratory strain *E. coli* ATCC-25922 as a donor for pKE3510, pKE3742, and pKE3754, showing a similar increase in conjugation even though it was less strongly marked for pKE3510 (see Figure 23).
The observation of just fluoroquinolones promoting the transfer of *bla*$_{VIM-1}$ plasmids resulted in the hypothesis that DNA-damaging agents generally increase conjugation frequency. All tested substances boosted plasmid transfer from *C. amalonaticus* KE3510 to *E. coli* J53, which was, however, only significant for the quinolone antibiotic nalidixic acid (see Figure 24). Of note, nalidixic acid can prevent plasmid transfer of IncF and IncR plasmids, whereas IncN plasmids are not inhibited (Burman 1977). This investigation suggests an Inc-type dependency, which is in line with the elevated transfer of IncN2 plasmids in this thesis.
Studies on the bacterial transcriptome in presence of 5-azacytidine, mitomycin C, and aminocoumarins revealed changes especially in genes involved in DNA repair mechanisms under the control of the SOS response (Barbé *et al.* 1986; Dapa *et al.* 2017; Keller *et al.* 2001; Lal *et al.* 1988; Militello *et al.* 2016), pointing to a potential mechanism for elevated plasmid transfer.
Additionally, the impact of UV irradiation was tested, which significantly reduces transfer events of the *bla*$_{VIM-1}$ plasmid (see Figure 24). Depending on the energy intensity, however, exposure to UV can also induce plasmid transfer as noticed by Guo *et al.* (2015). Yet, excessive DNA damage might have been caused in *C. amalonaticus* KE3510, hence conjugation was inhibited.
In summary, the conjugation enhancing effect of ciprofloxacin seemed to be independent of the bacterial species and thus is likely to be caused by the plasmid Inc-type. However, the Inc-type alone cannot be used as an explanation since the homologs pKE4472 and pKE4473 (IncN1) did not show similar transfer rates (see Figure 16). Based on these observations, the genetic content is of high importance, regarding transposons or insertion sequences that might impact the expression of conjugation-related *tra* genes (see Sections 4.2.4 and 4.3.2). Furthermore, the SOS response is suspected of contributing to the elevating transfer effect, as only DNA-damaging agents increased the frequency (see Sections 4.3.2 and 4.3.3).

4.3.2 Altered gene expression under antibiotic treatment enhances plasmid transfer

The gene expression profiles of two Enterobacterales were investigated after 4 h of ciprofloxacin exposure that showed different responses to the antibiotic. Genes involved in the conjugation process and the SOS response of *C. amalonaticus* KE3510 and *E. cloacae* KE9563 were of particular interest. However, RNA sequencing did not result in distinct gene expression between the control and antibiotic-exposed donors due to high variations between the replicates (see Figure 25, Figure 26, and Table 29). Targeting several *tra* genes, essential for the T4SS and the conjugation process, by RT-qPCR led to slightly differentially regulated and rather down-regulated genes in presence of 1/4 MIC and 1/2 MIC ciprofloxacin, respectively (see Figure 27).

Up-regulation of *tra* genes was linked to increased transfer rates in a few publications, yet the underlying mechanism remains mostly unknown. Shun-Mei *et al.* (2018) discovered a higher expression of several *tra* genes after 8 h of antibiotic exposure correlating with elevated plasmid transfer. Another study, examining conjugation rates of IncI1 plasmid harboring the ESBL gene *bla*$_{\text{CTX-M-1}}$, showed up-regulation of various plasmid mobility genes (*traF, traI, traJ, traK, traN, traO, traW*) after less than 1 h, which was consistent with higher conjugation (Møller *et al.* 2017). Liu *et al.* (2019b) investigated that cefotaxime exposure induced expression of *traF* depending on the up-regulation of the *bla*$_{\text{CTX-M-1}}$ resistance gene and on the chromosomally encoded *rfaH* gene, a transcriptional regulator activating several operons including the pilus formation.

The expression of *traI*, *traM*, *traK*, *traL*, *virB5*, and *virB4* of an IncN2 (pKE3510) and an IncL/M (pKE9563) plasmid was assessed via RT-qPCR. These genes are involved in the relaxosome, the pilus assembly, and in providing energy for the process. The RT-qPCR, as well as the RNA sequencing, were performed after 4 h of ciprofloxacin exposure. Compared to the other studies, 4 h might be the wrong point in time to see a distinct change in transcription since *tra* gene expression was proposed to provide major fitness costs of conjugative plasmids to the bacterial cells and is thus tightly controlled (Dahlberg and Chao 2003; Kohler *et al.* 2018). The increased plasmid transfer of *C. amalonaticus* KE3510 could be caused by the high expression of another *tra* gene, which was not targeted by RT-qPCR.

Since just DNA-damaging agents induced the plasmid transfer of *C. amalonaticus* KE3510, the contribution of SOS response is likely. Up-regulation of the DNA repair genes, *dinB*, *umuC*, *umuD*, and the main regulators, *lexA* and *recA*, could be determined by RT-qPCR under 1/4 and 1/2 MIC ciprofloxacin, respectively, for both isolates, *E. coli* ATCC-pKE3510 and *E. coli* ATCC-pKE9563 (see Figure 28). RNA sequencing revealed elevated expression of several genes in *E. cloacae* KE9563, involved in the bacterial stress response to DNA damage (see Figure 25). As *E. cloacae* KE9563 did not harbor resistance genes to fluoroquinolones, it might be an explanation for the strong induction of the SOS pathway. *C. amalonaticus* KE3510 comprises the *qnrS1* gene, facilitating resistance to fluoroquinolones (see Figure 32). Thus, the expression of genes related to the DNA repair mechanism might be less markedly influenced (see Table 29). These results suggest that the level of SOS response induction depending on the caused DNA damage might be critical for plasmid conjugation efficiency.

However, the impact of SOS response on higher conjugation frequencies was excluded by Shun-Mei *et al.* (2018), Møller *et al.* (2017), and Liu *et al.* (2019a), since the expression was either not consistent with the observed elevated plasmid transfer or there was no differential regulation of the repair genes under antibiotic treatment. On the other hand, studies revealed the contribution of the SOS pathway to increased conjugation of an ICE SXT (Beaber *et al.* 2004) or an ESBL-encoding plasmid (Crane *et al.* 2018).

The antibiotic novobiocin, belonging to the aminocoumarins, can prevent induction of the SOS response by targeting *recA* gene expression, whereas ciprofloxacin promotes expression of the

SOS operon (Schröder *et al.* 2014; Schröder *et al.* 2012). Plasmid transfer of *C. amalonaticus* KE3510 was strongly elevated under ciprofloxacin exposure but was less strongly pronounced under aminocoumarin treatment (see Figure 21 and Figure 24), suggesting that the SOS response plays a role in elevating transfer. However, the gene expression in presence of other DNA-damaging agents was not assessed for *C. amalonaticus* KE3510 in this thesis.

In summary, the expression profiling of *tra* genes did not contribute to the disclosure of increased conjugation frequency of the IncN2-type plasmid derived from *C. amalonaticus* KE3510 exposed to sublethal concentrations of fluoroquinolone antibiotics. Under consideration of the mentioned limitations, these data indicate that 4 h post-ciprofloxacin exposure might be the wrong point in time to detect changes between the treated sample and the control.

Based on the observed correlation of DNA-interfering compounds with increased conjugation for *C. amalonaticus* KE3510, the involvement of SOS response and its mutagenic potential was still in focus, even though the related genes were not significantly up-regulated, which is further discussed in the next section.

4.3.3 Mutagenic effect of DNA-interfering antibiotics

Antibiotics exert mutagenic potential on bacteria, resulting in favorable and detrimental genomic changes (Sniegowski *et al.* 2000; Taddei *et al.* 1997). Besides the extensively studied SOS-dependent mutations induced by fluoroquinolones (Long *et al.* 2016; McKenzie *et al.* 2000; Migliorini *et al.* 2019; Song *et al.* 2016), other antibiotics, such as the beta-lactam ceftazidime, enhanced genetic variability of *P. aeruginosa* and *E. coli* (Blázquez *et al.* 2006; Pérez-Capilla *et al.* 2005). Likewise, streptomycin and rifampicin facilitated mutations in various bacterial species (Gustafsson *et al.* 2003; Ren *et al.* 1999).

Ciprofloxacin exposure induced up-regulation of the SOS-dependent error-prone polymerase genes *umuC/D* and *dinB* in *C. amalonaticus* KE3510 (see Figure 28) that can bypass DNA lesions or lead to point mutations, respectively (McKenzie *et al.* 2001; Wagner *et al.* 1999). Hence, the genomes of *C. amalonaticus* KE3510 isolates with 1/4 MIC ciprofloxacin treatment and without antibiotic exposure were examined by WGS, revealing changes in the genomes (see Table 30). Some deletions could be observed in the treated *C. amalonaticus* isolate with two interesting gene candidates: *rop* and *mobA*. Both genes are related to the regulation of plasmid replication and consequently the plasmid copy number (Cesareni *et al.* 1982; Frey *et al.* 1992; Som and Tomizawa 1983).

Studies performed on mutating or deleting these regulatory genes resulted in higher plasmid copy numbers, as was shown for *rop* (Lin-Chao *et al.* 1992; Moser *et al.* 1984) and *copA* (Dimitriu *et al.* 2020). Higher copy numbers, however, can increase the fitness costs for the host, which is consequently lowering the plasmid transfer (Haugan *et al.* 1995). In contrast, Dimitriu *et al.* (2020) correlated the rise of plasmid replication with elevated conjugation frequencies, suggesting that

the expanded presence of plasmid mobility genes might lead to the higher activity of the transfer machinery. This assumption is supported by Yang *et al.* (2021), showing that mutations in the *pcnR* gene increased copy numbers and conjugation frequencies of an *mcr-1* encoding IncI plasmid despite higher fitness costs for the host.

The discovery of *rop* and *mobA* deletions strongly indicates that ciprofloxacin treatment may prevent or decelerate cell division of *C. amalonaticus* KE3510 (see Figure 22 B), yet the replication process of the plasmid may still be functional, resulting in high plasmid copy numbers under antibiotic treatment. However, analyzing the plasmid copy numbers of *C. amalonaticus* KE3510 by qPCR, targeting the chromosomal gene *rpoD* and the plasmid-encoded $bla_{\text{VIM-1}}$ did not support this hypothesis (see Figure 29). Since the confirmation of *rop* deletion by PCR failed, the absence of this gene in the mutant may also be explained by using short-read sequencing. Long-read sequencing could be beneficial to ensure the gene has not been lost between two contigs.

Even though different potential mechanisms were assessed in this thesis, such as *tra* gene expression, SOS response involvement, plasmid copy number, and mutagenesis, the causes of elevated transfer rates of IncN2 plasmids could not be fully uncovered with the performed experiments.

5 Outlook

Direct-qPCR for carbapenemase identification in primary patients' samples showed promising results during the outbreak situation with *bla*$_{\text{OXA-48}}$-positive *E. cloacae*. Nonetheless, confirmatory studies during a routine screening of newly admitted patients should be carried out to investigate the false-negative rates of direct-qPCR and culture-based diagnostics. Further monitoring of carbapenemase variants by WGS would be essential to timely adapt the qPCR to potential changes in local epidemiology. Screening of the hospital environment by multiplex qPCR revealed reservoirs of various carbapenemase-producers that pose risk for new outbreak situations. The performance of the qPCR is not yet adequate. However, the initial enrichment of bacteria in a nutritious medium was beneficial, which might also be worth considering before cultural screening with selective agar to achieve better sensitivities.

Conjugation experiments revealed that not all plasmids are transferred with the same efficiency. High transfer rates of *bla*$_{\text{VIM-1}}$-encoding plasmids could mainly be correlated to the plasmid type since IncN2 plasmids showed significantly higher conjugation frequencies compared to other Inc-types within Enterobacterales. Co-existing plasmids as well as taxonomic relatedness of donor and recipient seemed to play minor roles in transfer efficiency. To further validate the dependency of high conjugation frequencies on the plasmid type, more *in vitro* liquid mating experiments should be carried out with IncN2 plasmids compared to other plasmid types.

Sub-inhibitory concentrations of antibiotics did not seem to have a common elevating impact on carbapenemase plasmid transfer. Gene expression profiling regarding *tra* genes after 4 h and 2 h of ciprofloxacin treatment did not lead to clear-cut results, suggesting that this may not be a suitable point in time to see an up-regulation. Since the conjugation process is tightly controlled, other times should be considered, to determine whether overexpression of *tra* genes contributed to elevated plasmid conjugation. As only DNA-damaging agents could provoke an increase in conjugation efficiencies of *C. amalonaticus* KE3510 harboring the IncN2-type plasmid, the involvement of SOS response was likely, yet SOS response-related genes were not significantly up-regulated. Further experiments using SOS response inhibitors such as zinc pyrithione (Crane *et al.* 2018) would be beneficial to assess whether this stress response has an impact on conjugation.

Altogether, the results presented in this thesis indicate that the ability of bacteria to transfer plasmids highly efficiently is predominantly dependent on the plasmid type. The increased conjugation frequencies under antibiotic treatment are, however, not common for *bla*$_{\text{VIM-1}}$-encoding plasmids. Since neither altered expression of *tra* genes, nor a clear contribution of SOS response, nor mutagenesis resulting in higher plasmid copy numbers could be determined as the main mechanism for increased plasmid transfer, more investigation is required to see whether general genetic markers exist to determine highly conjugative plasmids. Hence, it would be helpful to close the plasmids and get a clear picture of all abundant genes by a sequencing approach using longer reads.

6 Summary

Carbapenemase-producing bacteria are distributed worldwide in healthcare facilities with continuously increasing numbers and are drastically limiting treatment options for severe bacterial infections. Hygienic measures and infection control are essential to counteract their distribution. Hence, sensitive, and reliable detection methods are needed to identify patients, colonized with carbapenem-resistant bacteria, and environmental contaminations, which are often involved in nosocomial infections and outbreaks. One of the major aims of this thesis was to evaluate a newly developed multiplex qPCR as a primary method for carbapenemase detection directly from rectal swab specimens and environmental samples.

The qPCR showed excellent performances for the current local carbapenemase epidemiology and revealed the value of initial genotypic detection when compared to phenotypic methods. Besides the temporal advantages to initiate dissemination preventions strategies, the selection of growing isolates for further characterization by cultural screening is avoided. For wastewater samples, the direct-qPCR was less accurate, yet environmental reservoirs with critical levels of carbapenemase-producers could be identified, which may serve as potential sources for hospital-acquired infections and outbreaks.

Carbapenemase genes are usually found on plasmids that facilitate their transmission within the bacterial community through horizontal gene transfer. Selection pressure under antibiotic therapy is supposed to contribute to the transmission of resistance genes. Therefore, the investigation of transferability of *bla*$_{\text{VIM-1}}$-encoding plasmids between Enterobacterales and the impact of antibiotic therapy on conjugation was the second aim of this dissertation.

The efficient transfer could be mainly attributed to the plasmid Inc-type for the tested Enterobacterales. An IncN2-type plasmid and its two homologs emerged as highly transmissible plasmids in this thesis. Furthermore, regulatory genes and transposons or insertion sequences, present on the plasmid play an important role in transmission, even though a certain gene could not be pointed out. Minor contributions could be ascribed to co-existing plasmids that usually have an impeding effect and to the negative correlation of donor and recipient relatedness to conjugative transfer.

Sublethal concentrations of the fluoroquinolone antibiotic ciprofloxacin were able to induce conjugation of the IncN2-type plasmids and an IncN1-type plasmid, indicating that this effect is not widely distributed among the tested *bla*$_{\text{VIM-1}}$-encoding plasmids (14.3 %). Different mechanisms were assessed that potentially contribute to elevated IncN2 plasmid transfer of *C. amalonaticus* KE3510. Overexpression of conjugation-related *tra* genes could not be confirmed at the tested time. Since other DNA-interfering substances were able to induce the IncN2 plasmid transfer, the contribution of the DNA damage-inducible SOS response was likely. Expression of SOS response-related genes was slightly increased, suggesting a mutagenic effect of ciprofloxacin in an SOS-dependent manner. Deletions of two genes, regulating the plasmid copy number, were observed in the mutant. An increase of plasmid copy numbers under ciprofloxacin treatment,

however, could not be confirmed for *C. amalonaticus* KE3510, therefore, the exact mechanism of how antibiotics contribute to higher plasmid transfer rates could not be uncovered.

Altogether, data presented in this dissertation point out that highly efficient transfer is not widely distributed among carbapenemase-positive bacteria and only few plasmids can be positively influenced by antibiotic therapy. A small number of bacteria harboring highly transmissible plasmids, however, is sufficient to successfully distribute resistance genes throughout the bacterial community and contribute to the development of new multidrug-resistant bacteria.

7 Zusammenfassung

Weltweit nimmt die Zahl an Carbapenemase-produzierenden Bakterien in Gesundheitseinrichtungen zu. Für schwere Infektionen mit diesen Bakterien verbleiben nur noch wenige Therapieoptionen, weshalb Infektionskontrollen und Hygienemaßnahmen essenziell sind, um der weiteren Verbreitung entgegenzuwirken. Effiziente und sensitive Screening-Methoden sind daher wichtig, um eine frühe Identifizierung von Patienten, die mit Carbapenem-resistenten Bakterien besiedelt sind, zu gewährleisten. Zudem spielen auch Kontaminationen in der Krankenhausumgebung bei nosokomialen Infektionen sowie bei Ausbrüchen eine große Rolle und sollten daher vom Screening nicht ausgeschlossen werden.

Ein Hauptziel dieser Dissertation war es, eine neu entwickelte multiplex qPCR als primäre genotypische Methode zur Carbapenemase-Detektion aus Rektalabstrichen sowie aus Umgebungsproben im Vergleich zum kulturellen Verfahren zu validieren.

Die qPCR hatte eine hohe Sensitivität für die lokale Carbapenemase-Resistenzsituation und erwies sich als zuverlässige initiale Screening-Methode mit Vorteilen gegenüber dem kulturellen Verfahren. Zusätzlich zur Zeitersparnis bei der Isolation kolonisierter Patienten, entfällt die Selektion und Auswahl individueller Bakterienkolonien zur weiteren Charakterisierung, da die gesamte Komposition der Probe betrachtet wird.

Im Vergleich zur Detektion von Carbapenemasen in Rektalabstrichen, wies die qPCR bei den Umgebungsproben eine geringere analytische Empfindlichkeit auf. Daher können nur Umgebungen mit einem hohen Besiedelungsgrad zuverlässig identifiziert werden, die jedoch auch ein höheres Risiko der Verbreitung von Carbapenemase-positiven Bakterien mit sich bringen.

Carbapenemase-Gene sind meist auf Plasmiden zu finden, was die Übertragung zwischen Bakterien durch horizontalen Gentransfer erleichtert. Selektionsdruck, der während der Antibiotikatherapie auf Bakterien entsteht, steht im Verdacht den Transfer von Resistenzgenen zu fördern. Ein weiteres Ziel dieser Dissertation war es daher, die Übertragungsmöglichkeiten von *bla*$_{\text{VIM-1}}$-Plasmiden innerhalb der Enterobacterales zu ermitteln und den Einfluss von Antibiotika auf die Konjugation zu beurteilen.

Homologe des Plasmid-IncN2-Typs wiesen eine hohe Übertragungseffizienz auf, weshalb der Plasmid-Typ eine große Rolle zu spielen scheint. Dennoch haben auch regulatorische Gene sowie Transposons und Insertionssequenzen einen nicht zu vernachlässigenden Einfluss, wie zahlreiche Publikationen aufzeigen. Konjugation wird außerdem durch koexistierende Plasmide meist negativ beeinflusst sowie durch den Verwandtschaftsgrad von Donor und Rezipient.

Geringe Konzentrationen des Fluorochinolon-Antibiotikums Ciprofloxacin erhöhten die Übertragungsfrequenz lediglich bei den IncN2-Plasmiden sowie bei einem IncN1-Plasmid, weshalb dieser Effekt bei den getesteten *bla*$_{\text{VIM-1}}$-Plasmiden nicht weit verbreitet war (14,3 %). Um mehr über die genetischen Hintergründe des induzierten IncN2-Plasmidtransfers von *C. amalonaticus* KE3510 herauszufinden, wurden verschiedene potenzielle Mechanismen

überprüft. Eine Überexpression der für die Konjugation verantwortlichen *tra*-Gene konnte zum getesteten Zeitpunkt nicht bestätigt werden. Da auch andere DNA-interagierende Substanzen zu einer erhöhten Konjugation führten, ist ein Einfluss der durch DNA-Schäden induzierbaren SOS-Antwort naheliegend. An dieser stressinduzierten Reaktion beteiligte Gene waren geringfügig hochreguliert, weshalb eine SOS-abhängige Mutagenese von *C. amalonaticus* KE3510 untersucht wurde. Es konnten Deletionen zweier Gene festgestellt werden, die zur Regulation der Plasmidkopienzahl benötigt werden. Ein gesteigerte Konjugationsrate aufgrund einer erhöhten Anzahl an Plasmiden konnte experimentell allerdings nicht bestätigt werden.

Zusammenfassend konnte gezeigt werden, dass nur ein geringer Anteil an Carbapenemase-positiven Bakterien ihr Plasmid hoch effizient übertragen kann, was in Einzelfällen durch Antibiotikatherapie noch gefördert wird. Dies ist von klinischer Relevanz, da auch eine geringe Anzahl an Bakterien mit hoch-übertragbaren Plasmiden ausreichend ist, um Resistenzgene erfolgreich zu verbreiten und zur Entstehung neuer multiresistenter Bakterien beizutragen.

8 Personal publications

Parts of this dissertation have already been published in the following articles:

1. Probst, K., Nurjadi, D., Heeg, K., Frede, A.-M., Dalpke, A. H. and Boutin, S. (2021). Molecular Detection of Carbapenemases in Enterobacterales: A Comparison of Real-Time Multiplex PCR and Whole-Genome Sequencing. Antibiotics 10 (6), 726, doi: 10.3390/antibiotics10060726.

2. Probst, K., Boutin, S., Späth, I., Scherrer, M., Henny, N., Sahin, D., Heininger, A., Heeg, K. and Nurjadi, D. (2022). Direct-PCR from rectal swabs and environmental reservoirs: A fast and efficient alternative to detect *bla*$_{\text{OXA-48}}$ carbapenemase genes in an *Enterobacter cloacae* outbreak setting. Environ Res, 111808, doi: 10.1016/j.envres.2021.111808.

Publication 1 is based on the results in Chapter 3.1.1 and the discussion parts 4.1.1 and 4.1.3 of this dissertation. The experimental data incorporated in this publication was generated, and interpreted by the doctoral candidate, with exception of the whole-genome sequencing of the bacterial isolates and WGS data analysis, which was performed by Selina Hassel, Suzan Leccese, Nicole Henny, Delal Sahin, and Dr. Sébastien Boutin, respectively. The original manuscript was written by the doctoral candidate.

Publication 2 consists of results from Sections 3.1.2 and 3.1.3 and the discussion parts 4.1.2 and 4.1.4. Culture-based diagnostics of the rectal swabs and the environmental samples were performed by the staff of the routine diagnostics, Delal Sahin and Nicole Henny. The qPCR for carbapenemase detection, the interpretation of the data, and the writing of the original manuscript were performed by the doctoral candidate.

Further publications:

3. Probst, K., Boutin, S., Bandilla, M., Heeg, K. and Dalpke, A. H. (2021). Fast and automated detection of common carbapenemase genes using multiplex real-time PCR on the BD MAX system. J Microbiol Methods 185, 106224, doi: 10.1016/j.mimet.2021.106224.

4. Kocer, K., Boutin, S., Probst, K., Heeg, K. and Nurjadi, D. (2020). Whole-genome sequencing disproves two suspected transmission events of *bla*$_{\text{NDM}}$ between *Pseudomonas aeruginosa* and Enterobacterales in hospitalized patients. J Hosp Infec 106 (2), 372-375, doi: 10.1016/j.jhin.2020.07.006.

5. Nurjadi, D., Scherrer, M., Frank, U., Mutters, N. T., Heininger, A., Späth, I., Eichel, V. M., Jabs, J., Probst, K., Müller-Tidow, C., Brandt, J., Heeg, K. and Boutin, S. (2021a). Genomic Investigation and Successful Containment of an Intermittent Common Source Outbreak of OXA-48-Producing *Enterobacter cloacae* Related to Hospital Shower Drains. Microbiol Spectr, e0138021, doi: 10.1128/Spectrum.01380-21.

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Appendix

Table 31: Bacterial isolates used in this thesis for conjugation experiments and gene expression profiling.

Isolate number	Carbapenemase gene	Species	Origin	Date of isolation
J53	-	*E. coli* J53	laboratory strain, sodium azide resistant, available at: https://www.atcc.org/products/baa-2731	-
ATCC-25922	-	*E. coli* ATCC-25922	laboratory strain	-
KE3436	*bla*$_{VIM-1}$	*E. hormaechei*	isolate patient 1	2016/10/06
KE3510	*bla*$_{VIM-1}$	*C. amalonaticus*	isolate patient 2	2016/10/14
KE3742	*bla*$_{VIM-1}$	*E. coli*	isolate patient 2	2016/11/15
KE3754	*bla*$_{VIM-1}$	*E. hormaechei*	isolate patient 2	2016/11/15
KE4472	*bla*$_{VIM-1}$	*E. coli*	isolate patient 3	2017/04/25
KE4473	*bla*$_{VIM-1}$, *bla*$_{KPC-2}$	*C. freundii*	isolate patient 3	2017/04/25
KE4494	*bla*$_{VIM-1}$	*C. portucalensis*	isolate patient 4	2017/04/28
KE4495	*bla*$_{VIM-1}$	*K. aerogenes*	isolate patient 4	2017/04/28
KE9365	*bla*$_{VIM-1}$	*K. pneumoniae*	isolate patient 5	2019/05/16
KE9409	*bla*$_{VIM-1}$	*E. cloacae*	isolate patient 6	2019/06/07
KE9474	*bla*$_{VIM-1}$	*E. hormaechei*	isolate patient 7	2019/08/03
KE9538	*bla*$_{VIM-1}$	*S. marcescens*	isolate patient 8	2019/09/28
KE9563	*bla*$_{VIM-1}$	*E. cloacae*	isolate patient 8	2019/10/14
KE9620	*bla*$_{VIM-1}$	*K. pneumoniae*	isolate patient 9	2019/12/03
KE9414	*bla*$_{VIM-1}$	*E. cloacae*	isolate patient 10	2019/06/14
KE9462	*bla*$_{VIM-1}$	*E. cloacae*	isolate patient 11	2019/07/12
KE9511	*bla*$_{VIM-1}$, *bla*$_{KPC-2}$	*C. freundii*	isolate patient 13	2019/09/03
KE9548	*bla*$_{VIM-1}$	*E. cloacae*	isolate patient 12	2019/10/06
KE9549	*bla*$_{VIM-1}$	*E. coli*	isolate patient 12	2019/10/06
KE9559	*bla*$_{VIM-1}$	*C. freundii*	isolate patient 14	2019/10/14

Table 32: Determination of the ciprofloxacin MIC for isolates harboring a carbapenemase-encoding plasmid.

Sample ID	Carbapenemase gene	Species	MIC ciprofloxacin	Dilution used for conjugation
KE3436	*bla*$_{VIM-1}$	*E. hormaechei*	8.0 µg/ml	1:2
KE3510	*bla*$_{VIM-1}$	*C. amalonaticus*	0.25 µg/ml	1:100
KE3742	*bla*$_{VIM-1}$	*E. coli*	0.25 µg/ml	1:10
KE3754	*bla*$_{VIM-1}$	*E. hormaechei*	0.25 µg/ml	1:100
KE4472	*bla*$_{VIM-1}$	*E. coli*	0.5 µg/ml	1:2
KE4473	*bla*$_{VIM-1}$, *bla*$_{KPC-2}$	*C. freundii*	0.5 µg/ml	1:10
KE4494	*bla*$_{VIM-1}$	*C. portucalensis*	0.047 µg/ml	1:10
KE4495	*bla*$_{VIM-1}$	*K. aerogenes*	2.0 µg/ml	1:2
KE9365	*bla*$_{VIM-1}$	*K. pneumoniae*	0.75 µg/ml	1:2
KE9409	*bla*$_{VIM-1}$	*E. cloacae*	2.0 µg/ml	1:10
KE9474	*bla*$_{VIM-1}$	*E. hormaechei*	6.0 µg/ml	1:2
KE9538	*bla*$_{VIM-1}$	*S. marcescens*	0.32 µg/ml	1:2
KE9563	*bla*$_{VIM-1}$	*E. cloacae*	0.12 µg/ml	1:100
KE9620	*bla*$_{VIM-1}$	*K. pneumoniae*	0.75 µg/ml	undiluted

Table 33: Phenotypic carbapenem-resistant Enterobacterales collected in 2019, analyzed by qPCR and WGS. Quality control parameters for WGS: coverage and N50. This table has been originally published as part of Table 1 by Probst *et al.* (2021b).

Sample ID	Species	qPCR	WGS	WGS Reanalyzed	Coverage	N50
KE9539	*E. coli*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		48	535,993
KE9246	*E. coli*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		53	135,761
KE9526	*E. cloacae*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		52	363,822
KE9478	*E. cloacae*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		96	363,822
BK31926	*E. coli*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		29	120,862
KE9621	*K. pneumoniae*	*bla*$_{KPC}$	*bla*$_{KPC-3}$		35	386,401
KE9498	*C. freundii*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		31	200,582
KE9038	*K. oxytoca*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		50	285,607
KE9326	*K. oxytoca*	*bla*$_{KPC}$	negative	*bla*$_{KPC-2}$	42	109,274
KE9511	*C. freundii*	*bla*$_{KPC}$, *bla*$_{VIM}$	*bla*$_{KPC-2}$, *bla*$_{VIM-1}$		30	198,406
KE9378	*C. freundii*	*bla*$_{KPC}$, *bla*$_{VIM}$	*bla*$_{KPC-2}$	*bla*$_{KPC-2}$, *bla*$_{VIM-1}$	39	201,178
KE9132	*E. cloacae*	*bla*$_{KPC}$	*bla*$_{KPC-2}$		49	363,822
KE9520	*K. pneumoniae*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		53	186,575
KE9434	*K. pneumoniae*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		34	292,061
KE9521	*E. coli*	*bla*$_{NDM}$, *bla*$_{OXA-48}$-like	*bla*$_{NDM-5}$, *bla*$_{OXA-181}$		61	106,471
KE9395	*E. coli*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		54	94,083
KE9433	*E. coli*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		36	214,212
KE9636	*K. pneumoniae*	*bla*$_{NDM}$, *bla*$_{OXA-48}$-like	*bla*$_{NDM-1}$, *bla*$_{OXA-48}$		27	383,090
KE9616	*C. freundii*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		50	186,958
KE9522	*E. coli*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		103	269,697
KE9593	*K. pneumoniae*	*bla*$_{NDM}$, *bla*$_{OXA-48}$-like	*bla*$_{NDM-5}$, *bla*$_{OXA-181}$		38	296,725
D3014	*C. freundii*	*bla*$_{NDM}$	*bla*$_{NDM-5}$		36	186,959
KE9449	*K. pneumoniae*	*bla*$_{NDM}$	negative	*bla*$_{NDM-1}$	25	220,843
KE9500	*K. pneumoniae*	*bla*$_{NDM}$	*bla*$_{NDM-1}$		33	536,321
KE9382	*E. cloacae*	*bla*$_{OXA-48}$-like	*bla*$_{OXA-48}$		27	374,725
KE9492	*K. pneumoniae*	*bla*$_{OXA-48}$-like	*bla*$_{OXA-232}$		30	242,997
KE9629	*E. coli*	*bla*$_{OXA-48}$-like	*bla*$_{OXA-244}$		33	238,467
KE9025	*E. cloacae*	*bla*$_{OXA-48}$-like	*bla*$_{OXA-48}$		49	272,750
KE9469	*E. cloacae*	*bla*$_{OXA-48}$-like	*bla*$_{OXA-48}$		76	374,315
KE9472	*E. cloacae*	*bla*$_{OXA-48}$-like	*bla*$_{OXA-48}$		98	382,653

Sample ID	Species	qPCR	WGS	WGS Reanalyzed	Coverage	N50
KE9424	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		36	184,292
KE9499	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		66	486,681
KE9400	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		45	208,351
KE9468	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		80	383,026
KE9638	*E. coli*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-244}}$		37	156,925
KE9493	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like, *bla*$_{\text{KPC}}$	*bla*$_{\text{OXA-48}}$	*bla*$_{\text{KPC-2}}$, *bla*$_{\text{OXA-48}}$	44	530,933
KE9456	*K. oxytoca*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		28	223,596
KE9443	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		27	225,118
KE9354	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		66	486,663
BK32270	*E. coli*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		35	117,967
KE9626	*E. coli*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		53	196,578
KE9208	*S. marcescens*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		58	2,797,497
D2902	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		64	302,960
KE9541	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		47	427,613
KE9554	*C. freundii*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		39	165,554
KE9338	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		47	374,725
KE9355	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		109	486,681
KE9328	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		43	274,145
KE9510	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		31	491,022
D3070	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		44	372,768
KE9428	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		36	486,663
KE9527	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	negative	*bla*$_{\text{OXA-48}}$	27	339,153
D3018	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		25	473,650
D3082	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		62	486,663
KE9637	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		36	876,600
D3081	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		85	383,026
EX1012	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		39	223,327
D3078	*E. cloacae*	*bla*$_{\text{OXA-48}}$-like	*bla*$_{\text{OXA-48}}$		54	486,828
KE9366	*E. coli*	*bla*$_{\text{VIM}}$	*bla*$_{\text{VIM-1}}$		38	215,473
KE9563	*E. cloacae*	*bla*$_{\text{VIM}}$	*bla*$_{\text{VIM-1}}$		35	377,920
KE9409	*E. cloacae*	*bla*$_{\text{VIM}}$	*bla*$_{\text{VIM-1}}$		46	486,118
KE9414	*E. cloacae*	*bla*$_{\text{VIM}}$	*bla*$_{\text{VIM-1}}$		38	161,463

Sample ID	Species	qPCR	WGS	WGS Reanalyzed	Coverage	N50
KE9365	*K. pneumoniae*	*bla*VIM	*bla*VIM-1		32	232,474
KE9538	*S. marcescens*	*bla*VIM	*bla*VIM-1		40	1,130,420
KE9585	*E. cloacae*	*bla*VIM	*bla*VIM-1		25	287,090
KE9559	*C. freundii*	*bla*VIM	*bla*VIM-1		41	163,976
KE9549	*E. coli*	*bla*VIM	*bla*VIM-1		47	279,067
KE9548	*E. cloacae*	*bla*VIM	*bla*VIM-1		46	230,814
KE9579	*E. coli*	*bla*VIM	*bla*VIM-1		39	112,495
KE9474	*E. cloacae*	*bla*VIM	*bla*VIM-1		38	290,132
KE9462	*E. cloacae*	*bla*VIM	*bla*VIM-1		33	502,528
KE9560	*E. cloacae*	*bla*VIM	*bla*VIM-1		40	290,117
KE9575	*E. cloacae*	*bla*VIM	*bla*VIM-1		38	389,538
KE9536	*E. coli*	*bla*VIM	negative	*bla*VIM-1	44	377,920
D2923	*E. cloacae*	negative	negative		58	203,439
KE9347	*E. cloacae*	negative	negative		79	439,426
KE9591	*E. cloacae*	negative	negative		47	279,225
KE9576	*E. coli*	negative	negative		27	228,481
KE9599	*E. coli*	negative	negative		37	281,932
KE9623	*E. coli*	negative	negative		50	93,960
KE9633	*K. aerogenes*	negative	negative		40	495,847
KE9068	*C. freundii*	negative	negative		46	176,242
KE8986	*E. cloacae*	negative	negative		47	230,847
KE9344	*E. cloacae*	negative	negative		48	235,301
KE9475	*E. cloacae*	negative	negative		40	208,042
KE9083	*E. coli*	negative	negative		57	208,544
KE9425	*K. aerogenes*	negative	negative		48	902,223
KE9614	*K. aerogenes*	negative	negative		62	429,809
D3017	*K. pneumoniae*	negative	negative		27	232,937
KE9095	*K. pneumoniae*	negative	negative		66	237,389
KE9171	*K. pneumoniae*	negative	negative		54	481,561
KE9039	*S. marcescens*	negative	negative		40	1,228,444

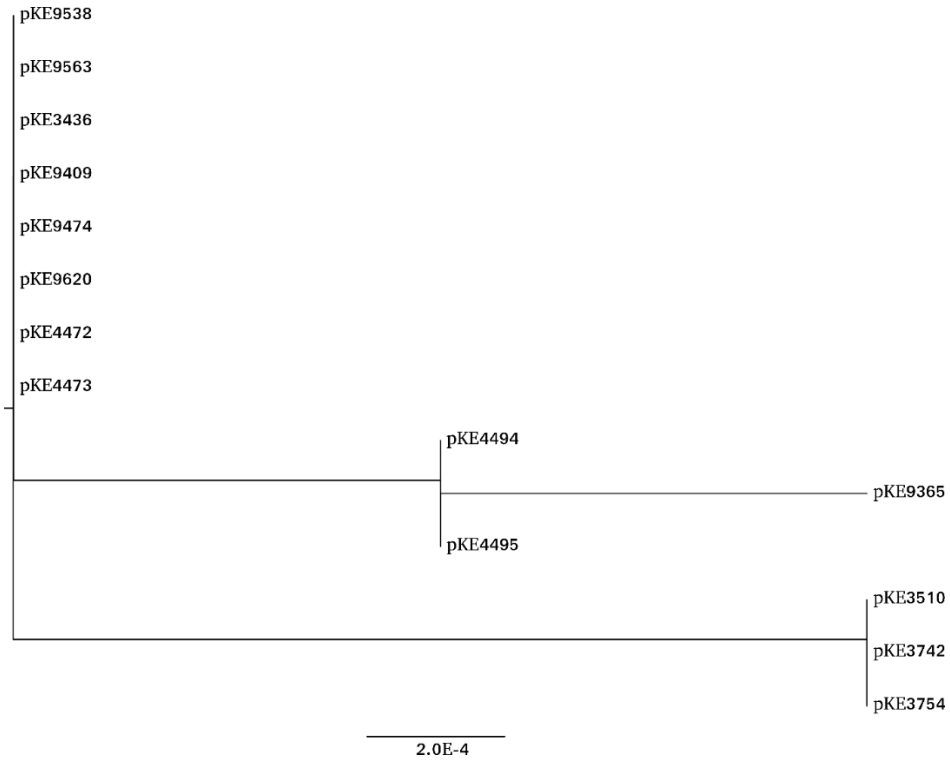

Figure 30: Neighbor-Joining mid-rooted phylogenetic tree of various Inc-type plasmids derived from Enterobacterales.

The tree is based on the core genome calculated by Roary of the plasmid contents from IncN2 (pKE3510, pKE3742, pKE3754), IncN1 (pKE9365, pKE3436, pKE9474, pKE4473, pKE4472, pKE9409, pKE9620), IncL/M (pKE9538, pKE9563) and IncA/C, IncFII (pKE4494, pKE4495).

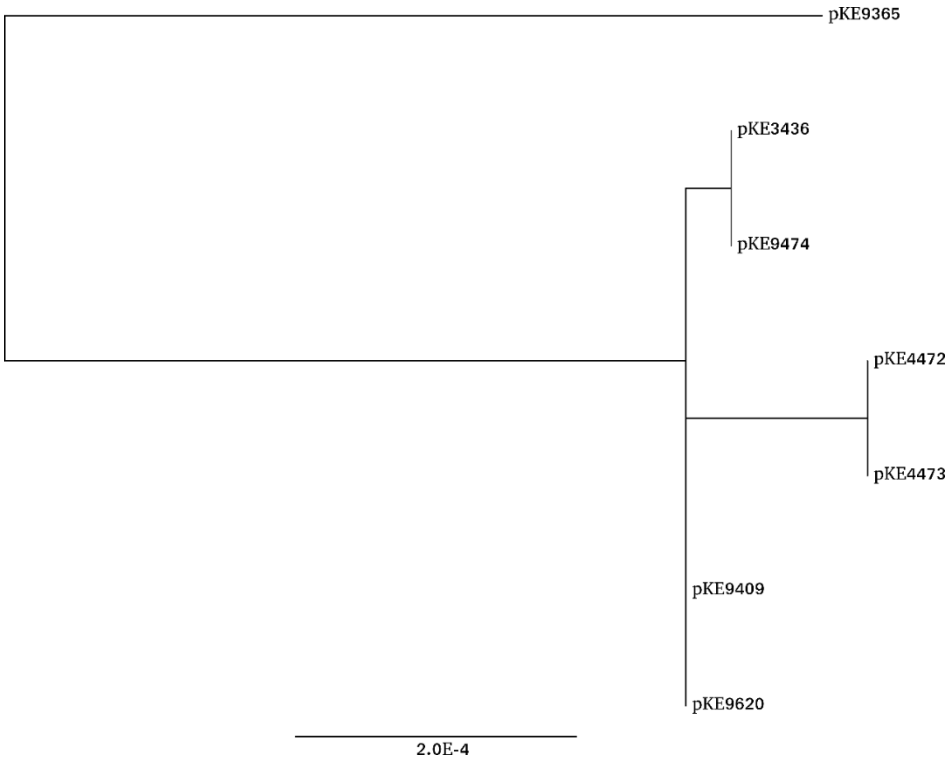

Figure 31: Neighbor-Joining mid-rooted phylogenetic tree of IncN1-type plasmid content.
The tree is based on the core genome calculated by Roary of the plasmid contents from pKE9365, pKE3436 pKE9474, pKE4472, pKE4473, pKE9409, and pKE9620.

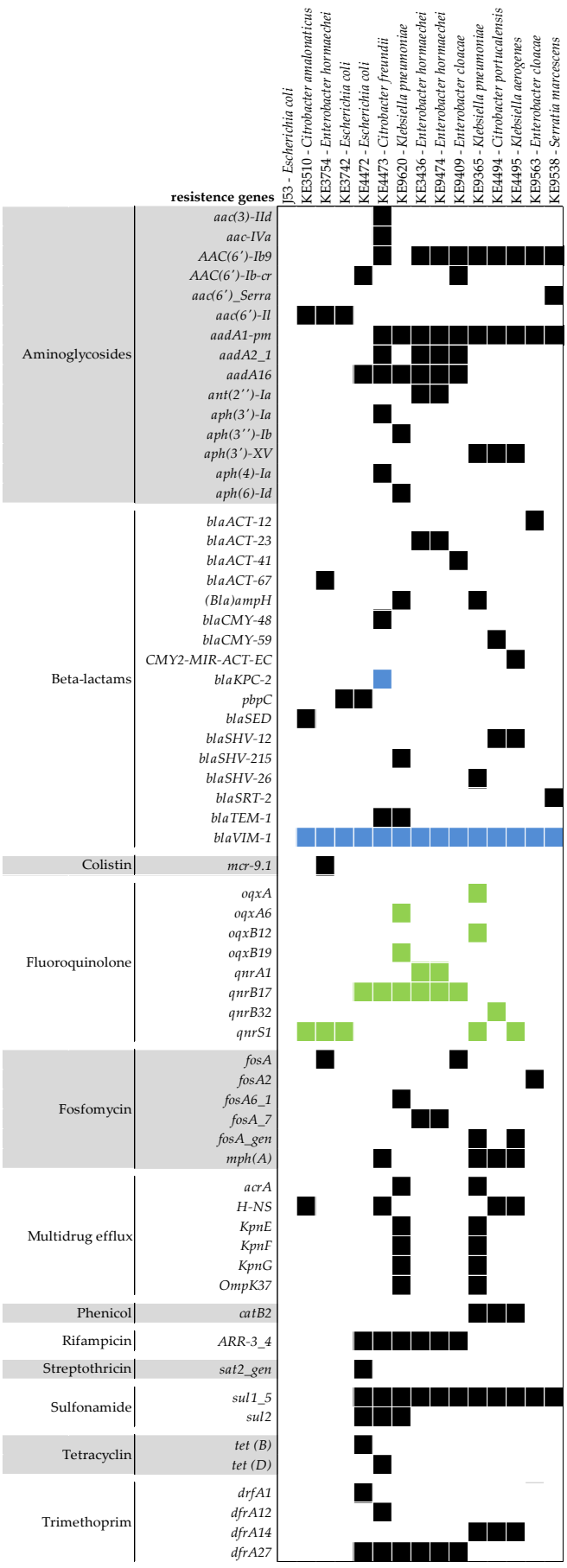

Figure 32: Resistance genes of *bla*VIM-1 harboring donor strains used for conjugation experiments.

Different resistance patterns were determined comprising antimicrobial resistance genes against aminoglycosides, beta-lactams, colistin, fluoroquinolones, fosfomycin, phenicol, rifampicin, streptothricin, sulfonamides, tetracyclines, and trimethoprim, as well as genes involved in multidrug efflux. Blue squares: carbapenem-resistance genes, green squares: fluoroquinolone resistance genes, black squares: resistance genes against other antibiotic classes.

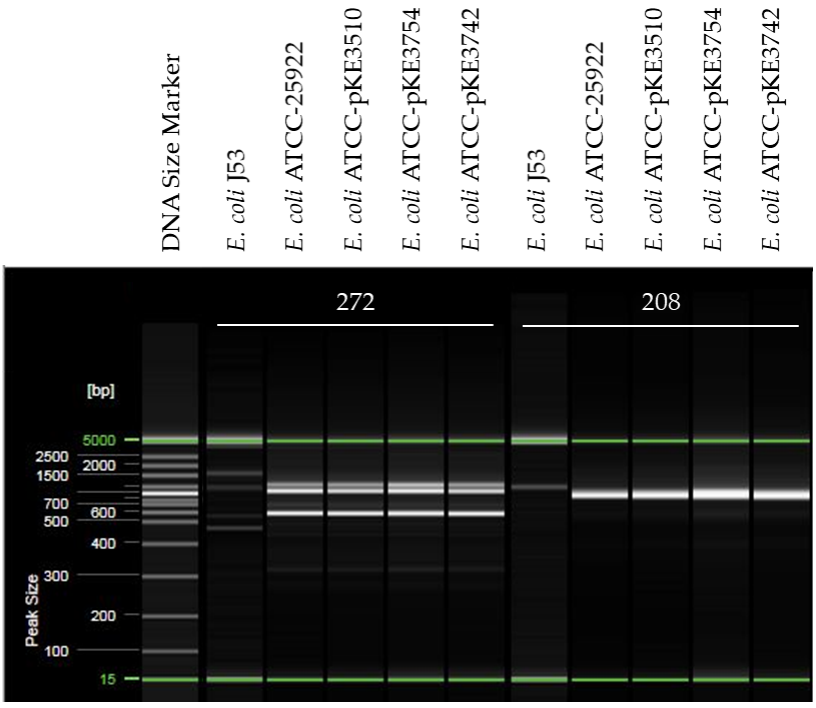

Figure 33: Verification of successful plasmid transfer from the donor strains *C. amalonaticus* KE3510, *E. hormaechei* KE3754, and *E. coli* KE3742, respectively, to the recipient strain *E. coli* ATCC-25922.

RAPD was performed for the transconjugants as well as for the recipient *E. coli* ATCC-25922, using the primer 272 and 208, visualized with the QIAxcel Advanced System. For both primers, all ATCC-strains revealed bands with the same size, which are different from the other recipient strain *E. coli* J53 also used for conjugation experiments.

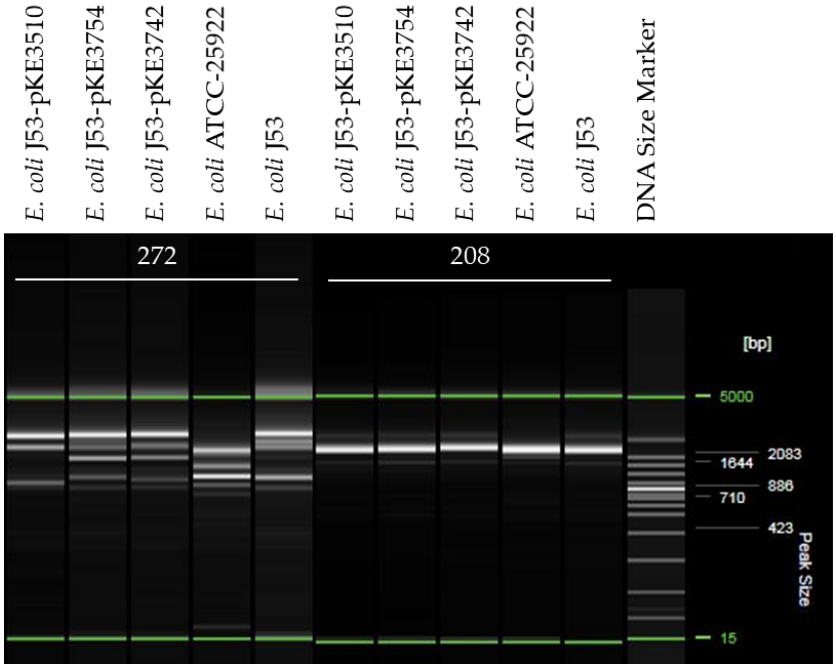

Figure 34: Verification of successful plasmid transfer from the donor strains *E. coli* ATCC-pKE3510, ATCC-pKE3754, and ATCC-pKE3742, respectively, to the recipient strain *E. coli* J53.

RAPD was performed for the transconjugants as well as for the strains *E. coli* ATCC-25922 and *E. coli* J53, using the primer 272 and 208, visualized with the QIAxcel Advanced System. For both primers, all J53-strains revealed similar bands which were different from the donor strain *E. coli* ATCC-25922 and were consistent with the recipient strain *E. coli* J53.

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