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Behavior of *E. coli* ST131 regarding the acquisition of a bla_{OXA-48} encoding plasmid

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ABSTRACT

Background

Antimicrobial resistance has been a challenge ever since the discovery of antibiotics. The rising challenge of resistance has led to the increased use of last-resort antibiotics: carbapenems. It is predicted that a successful *E. coli* clone, ST131 is apt for the acquisition of carbapenem-resistance encoding plasmids. In this study, we aimed to understand how *E. coli* ST131 respond to acquisition of carbapenemase-encoding plasmids p50579417_3_OXA-48 and pK71-77-1-NDM, also comparing the response to other common *E. coli*: ST69, ST73 and ST95.

Method

Conjugation efficiency and plasmid persistence assays were conducted for clinical strains of ST131, ST69, ST73 and ST95. Conjugation efficiency, relative fitness and the plasmid persistence were measured and then analyzed with statistical analyses to investigate difference among *E. coli* ST131 clades and between ST131 the other *E. coli* clones.

Results

Among *E. coli* ST131 clades, clade A strains acquired p50579417_3_OXA-48 more efficiently than the other clades, but there was no difference in plasmid persistence when measured by serial transfers. However, plasmid persistence was higher for strains from clade A and B than C1 and C2 when measured directly from the overnight culture. ST131 strains acquired p50579417_3_OXA-48 less efficiently than the ST69 strain, but more efficiently than the ST73 strain, despite no observed fitness difference between strains. Furthermore, there was no difference in plasmid persistence among ST131 and non-ST131.

Conclusion

Our study suggests that *E. coli* ST131 clade C is less efficient in p50579417_3_OXA-48 acquisition and has lower plasmid persistence compared to *E. coli* ST131 clades A and B. Furthermore, although evidence from other studies suggest that *E. coli* ST131 is altogether fit to acquire and maintain carbapenemase genes and plasmids, our study does not observe any dominance for ST131´s acquisition and maintenance of p50579417_3_OXA-48 when compared to other common STs.

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ABBREVIATIONS

AMR	Antimicrobial resistance
CFU	Colony forming unit
CP	Carbapenemase-producing
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ESBL	Extended spectrum Beta-lactamase
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
FQ	Fluoroquinolones
FQR	Fluoroquinolone resistant
HGT	Horizontal gene transfer
KPC	*Klebsiella pneumoniae* carbapenemases
K. pneumoniae	*Klebsiella pneumoniae*
LB	Lysogeny Broth
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
mRNA	messenger RNA
NDM-1	New Dehli metallo-beta-lactamase 1
OXA-48	Oxacillinase 48
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
ST	Sequence type
TM	Tetrazolium maltose
TTC	Triphenyl tetrazolium chloride
UTI	Urinary tract infections
VIM	Verona-integron-encoded metallo-beta-lactamase

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1 INTRODUCTION

Antimicrobial drugs are one of the most important medicaments available, but the increasing resistance to these drugs is threatening their efficacy. Antimicrobial resistance (AMR) was already observed during the early days of antibiotics and has been considered a challenge ever since. So far, modification of existing compounds and the introduction of new antibiotic classes in the world of medicine have contributed to the avoidance of a crisis. Nonetheless, the rate of resistance has increased, yet the rate of antibiotic drug discovery has decreased extensively (1).

Escherichia coli is a bacterial species that can cause several types of infections, such as bacteremia and urinary tract infections (UTI). It is considered the leading cause of UTI, which are now among the most common bacterial infections in the world (2). *E. coli* is associated with the acquisition of various resistance genes, becoming multidrug-resistant (MDR), which leads to many problematic infections (3, 4).

Escherichia coli sequence type 131 (*E. coli* ST131) has become the dominant MDR *E. coli* lineage of UTI and bloodstream infections (5). This clone is further divided into 3 clades: A, B and C. Studies have suggested that acquisition of fluoroquinolone resistance has been one of the major drivers for the success of ST131 clade C (2). However, recent work has provided strong evidence that resistance to antimicrobials has not been the primary force of its success (2, 6). It has been proposed that the ability of ST131 clade C to offset the cost of acquiring plasmids is the cause of it being a dominant successful MDR lineage (5). Other common *E. coli* STs associated with UTI are ST69, ST73 and ST95 (7).

Plasmids are small, mostly circular, double stranded deoxyribonucleic acid (DNA) molecules found in bacteria apart from the host chromosome and may contain resistance genes. Plasmids containing resistance genes play an important role in the spread of antimicrobial resistance (8). The success of a plasmid and the resistance gene(s) it encodes is limited by several factors such as the fitness cost they impose, as well as their conjugation rate and stability (9). It is predicted that *E. coli* ST131 is apt for acquiring plasmids encoding carbapenem-resistance (3). Carbapenems are considered last resort antibiotics (10) and carbapenem resistance is of serious concern as it eliminates the usefulness of carbapenems in treatment of *E. coli* ST131 infections. Carbapenemases are enzymes that have the ability to confer carbapenem resistance, and the

most common carbapenemases described among *E. coli* worldwide are New Dehli-metallo beta-lactamase 1 (NDM-1) and oxacillinase 48 (OXA-48) (10). In this study, we mainly focus on the conjugation efficiency and persistence of OXA-48 and NDM-1 encoding plasmids in ST131.

1.1 Antimicrobial drugs

Since the discovery of penicillins by Alexander Flemming in 1928, antibiotics have emerged to become an important tool in medicine and have since shown to become beneficial in many ways (reviewed in (11)). Antibiotics have among other things extended life expectancies, decreased morbidity and mortality as well as prevented and treated infections. The first prescriptions for treating serious bacterial infections with antibiotics were in the 1940´s and penicillin showed to be effective in managing bacterial infections in soldiers. However, soon after, resistance to penicillin started to become a clinical problem. As penicillin resistance started to rise, new beta-lactam antibiotics were made (11). As resistance is spreading, there is a great need for a breakthrough in antibiotic discoveries as the introduction of new antibiotic compounds have decreased extensively since the 1980´s (11).

Antimicrobial agents can be classified in different ways, including spectrum of activity (broad or narrow spectrum), their chemical structure, their target sites and type of action (bactericidal or bacteriostatic) (12). Target sites are briefly explained in the section below.

1.1.1 Antibiotic target

Classification of antibiotics can be based on how they interfere with bacteria´s major processes (12). Four target sites for antibacterial action are mentioned below.

Cell wall synthesis: Beta-lactams and glycopeptides are two antibiotic classes that target cell wall synthesis. The cell wall is composed of peptidoglycan. The antibiotics interrupt processes in peptidoglycan synthesis, causing cell lysis (13).

Protein biosynthesis: Antibiotics that target protein biosynthesis include aminoglycosides, tetracycline, macrolides, and chloramphenicol. These antibiotics target essential parts of the bacterial ribosome, leading to the inhibition of protein synthesis (13).

DNA replication: Fluoroquinolones (FQ) are a class of antimicrobial drugs that target DNA replication by inhibiting the DNA gyrase, an enzyme that is essential for DNA replication (13).

Folic Acid: Folic acid is essential for the bacteria to grow. Two antibiotic classes that target the folic acid synthesis are sulfonamides and trimethoprim. Both inhibit enzymes that are essential in the folate biosynthetic pathway, leading to the inhibition of bacterial growth (14).

1.2 Mechanisms of antibiotic resistance

Bacteria can either be intrinsically resistant or the resistance can be acquired. Intrinsic resistance is the bacteria´s natural ability to withstand the antibiotic activity due to its structural or functional characteristics. An example of this is resistance due to the structural lack of an antibiotic target. Acquired resistance can be achieved through mutations or horizontal gene transfer (HGT). There are three main groups of acquired resistance mechanisms (15):

1. Poor penetration or antibiotic efflux causing decreased intracellular concentrations of the antibiotic.
2. Alteration of antibiotic target.
3. Modification or hydrolysis causing the inactivation of the antibiotic.

Once bacteria have acquired resistance, they continue to grow despite the presence of antibiotics, whilst sensitive bacteria will stop. This leads to the resistant mutants outnumbering sensitive bacteria, therefore spreading rapidly and causing the drug to be ineffective (1).

In the absence of antibiotic selective pressure, it has been hoped that bacteria would lose their resistance factors due to the strong physiological cost of maintaining resistance, leading the microbes to become sensitive towards antimicrobials (1) . There are four reasons as to why this resistance loss has not been widely observed (1):

1. The fitness cost is just not large enough, thus the resistance genes are kept in the population years after drug loss.
2. Neutralization of fitness cost due to mutations, or mechanisms leading to only activate resistance in presence of drugs.
3. As a result of the accumulation of mutations, the presence of the resistance gene might become essential for antimicrobial growth even in the absence of antibiotics. Such accumulation may be a result of long-term antibiotic selection for the resistance gene to be present.

4. In certain instances, antibiotic resistance mutations can lead to increased virulence that gives the mutant a fitness advantage when antibiotic selection is absent.

1.3 Beta-lactams' target and resistance

Beta-lactams are one of the most relevant class of antibiotics and are effective against *E. coli*, among other bacteria. These drugs are bactericidal meaning they cause cell death. Beta-lactams are classified into penicillins, cephalosporins, carbapenems and monobactams (16).

Antibiotic target: Beta-lactams target the Penicillin binding protein (PBP). Beta-lactam agents bind to the PBPs and inhibit the cross linking of glycan strands. This disrupts the peptidoglycan layer leading to bacterial lysis (13).

Antibiotic resistance: The most significant mechanism for antibiotic resistance towards beta-lactam agents is the acquisition of beta-lactamases. Beta-lactamases are enzymes that hydrolyze the beta-lactam ring, making it incapable of binding to PBPs (16).

Extended spectrum beta-lactamase-producing (ESBL) *Enterobacteriaceae*: ESBL-producing *Enterobacteriaceae* encode broad-spectrum beta-lactamases which cause the bacteria to become resistant to a large variety of penicillins and cephalosporins. Some ESBL-producing *Enterobacteriaceae* are insensitive to nearly all antibiotics regarding penicillin and cephalosporin classes. Treatment of ESBL-producing *Enterobacteriaceae* infections will then resort to increased carbapenem use (11).

1.4 E. coli

E. coli is a rod-shaped, Gram-negative bacterial species belonging to the *Enterobacteriaceae* family (17). *E. coli* is the leading cause of UTIs, which are now among the most common bacterial infections in the world (2).

Extraintestinal Pathogenic *E. coli* (ExPEC), is a versatile subset of *E. coli* strains that can cause UTIs and bacteremia, causing the ExPEC infections to be amongst the major causes of morbidity (4, 18). ExPEC strains are also associated with the acquisition of new and problematic resistance genes causing many ExPEC infections to be MDR (3, 4).

Due to the increased prevalence of ESBL-producing *Enterobacteriaceae*, treatment for serious bacterial infections have had to resort to last line antibiotics, such as the carbapenems. The increase in carbapenem use against *Enterobacteriaceae* has predictably led to the emergence of carbapenem resistant *E. coli* clones. Evidence suggests that a particular clone, *E. coli* ST131 is altogether fit to acquire and maintain carbapenemase genes and plasmids encoding them (3).

1.4.1 *E. coli* ST131 – clone of interest

E. coli ST131 has in recent years shown to be a globally dominant cause of human infections and is now a worldwide pandemic clone (18, 19). *E. coli* ST131 consists of clade A, clade B and clade C (20). Clade C is further divided into subclades C0, C1 and C2. Subclade C0 is composed of isolates susceptible to FQ. However, subclades C1 and C2 confer high-level resistance to FQ, and *E. coli* ST131 is now the predominant fluoroquinolone resistant (FQR) *E. coli* clone worldwide (20, 21). Subclade C2 is also associated with the ESBL gene $bla_{\text{CTX-M-15}}$ (20). The $bla_{\text{CTX-M-15}}$ gene has been found primarily on IncF plasmids in ST131. IncF plasmids spread rapidly among *Enterobacteriaceae*, and these plasmids containing $bla_{\text{CTX-M}}$ have been essential in the success of clade C2 (8, 22).

1.5 Plasmids

Apart from the host chromosome, plasmids can also be found in bacteria. These are small, frequently circular DNA molecules that among other genes, may contain resistance genes (8). Plasmids containing resistance genes play an important role in the spread of antimicrobial resistance through HGT (8).

Plasmids can be categorized into three groups: conjugative, mobilizable and non-mobilizable (23). Conjugative plasmids are self-transmissible and can transfer between species on their own. In the presence of conjugative plasmids or transposon, mobilizable plasmids are also able to transfer between species (24). Non-mobilizable plasmids, however, do not spread through conjugation (23, 25).

The host range of a plasmid is important for it to spread and its persistence can depend on the diversity of different host species enabling a plasmid to be maintained (24). The host range can be divided into 2 main groups, the narrow-host-range group, and the broad-host-range group (8). Broad-host range plasmids are easily transferable between various species, while the narrow-host range groups are restricted to certain species. (8) Plasmids belonging to

incompatibility groups A/C (IncA/C) and L/M (IncL/M) are examples of broad-host-range plasmid, while IncF is an example of a narrow-host-range group of plasmid (8). IncF resistance plasmids are able to acquire resistance genes and spread rapidly among *Enterobacteriaceae* (8). Both IncF and IncL/M plasmids can be found in *E. coli* ST131, the clone of interest in this thesis.

1.6 Plasmid persistence

Bacterial plasmids may encode a diversity of genes which can benefit the bacterial host, although, absolutely essential genes (required for cells to survive at normal conditions) are not generally encoded on plasmids. To be maintained in a bacterial host over a prolonged time period, a plasmid must either provide a selective advantage to the bacterial host, effectively ensure its proper segregation during cell division or be capable of efficient horizontal transmission (24).

1.6.1 Fitness cost

Despite encoding genes that may give the host cell an advantage in specific conditions, the plasmid may exert a fitness cost on the cell due to using the host machinery for gene expression and replication (26, 27). Cells carrying plasmids can therefore be outcompeted by cells not harboring plasmids when selection for plasmid-encoded traits is absent (26). However, mutations can compensate for the fitness cost, resulting in an extended plasmid persistence (27).

1.6.2 Plasmid inheritance

During cell division, an adequate amount of plasmid copies is important for plasmid inheritance. Plasmids can form multimers, resulting in reduced number of heritable units. As successful segregation requires multi-copy plasmids, the reduced number of units can decrease plasmid inheritance, and therefore also negatively affect the plasmid persistence (26). However, some plasmids may resolve formation of multimers through multimer resolutions system (24).

Low copy number plasmids can be ensured a stable maintenance through active partitioning during cell division. This mechanism ensures that both daughter cells inherit at least one plasmid. Another mechanism of plasmid stability is the post-segregational killing systems. Following unsuccessful segregation, these systems ensure growth arrest or cell death of plasmid-free cells. (24)

1.6.3 Plasmid transfer

As mentioned earlier, there are self-transmissible and mobilizable plasmids. These are able to transfer between species (24) and may lead to prolonged plasmid persistence through efficient conjugation (26). However, some plasmids are non-mobilizable and cannot be transferred by conjugation (25).

1.7 Horizontal gene transfer

Horizontal gene transfer allows genetic materials of bacteria to be exchanged between diverse species (28). HGT mechanisms include transformation, conjugation and transduction as shown in Figure 1. Transformation is the process where DNA is taken up from the environment and incorporated in the bacterial genome. Conjugation is the process in which plasmids are transferred directly between cells. Transduction is the process in which bacteriophages move genes from one cell to another (29).

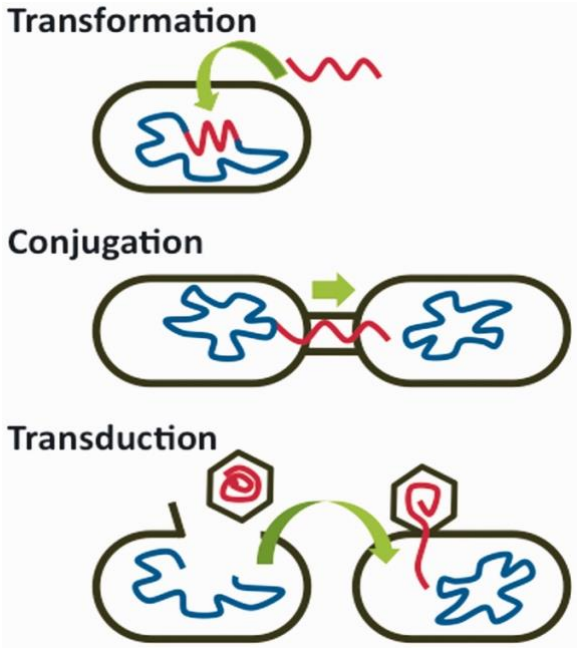


Figure 1 Mechanisms of horizontal gene transfer (29)

1.7.1 Conjugation

Conjugation is one of the mechanisms of HGT. In this mechanism, cell to cell contact is needed for DNA to be transferred from a donor to a recipient cell. Conjugative plasmids specify pili, which is required for the cell-to-cell contact. During conjugation, the two strands of DNA is unwound, where one strand is left on the donor cell and the other is transmitted to the recipient. The strands then serve as templates for the synthesis of DNA, and replicated DNA is recoverable from both donor and recipient cells (30).

1.8 Carbapenemases

Carbapenemases are enzymes that hydrolyze carbapenems, and the acquisition of these enzymes is the primary mechanism for carbapenem resistance in Gram-negative bacteria such as *E. coli* (31). Carbapenemases are now spreading, conferring resistance (32). Carbapenemase-producing (CP) *Enterobacteriaceae* are the most widespread antimicrobial resistant threat towards health services in the world (33). This is of serious concern, as the lack of other medications leaves carbapenems to often be the last line of effective therapy for the treatment of serious infections (34).

The genetic background of strains harboring the carbapenemase encoding gene may play a major role in its widespread. Incorporation of the genes in successful clones (*e.g., E. coli* ST131) may therefore be a significant factor in the emergence of the carbapenem resistance (35).

Carbapenemases in *Enterobacteriaceae* have been identified in 3 classes of beta-lactamases: A, B and D. Furthermore, uncommon chromosome encoded cephalosporinases produced by *Enterobacteriaceae* may hold an extended activity towards carbapenems and are considered as Ambler class C (34).

Class A beta-lactamases: There are a variety of class A carbapenemases observed, in which some are chromosome encoded and some are plasmid encoded. Regardless of where they are encoded, they all effectively hydrolyze carbapenems. In this group, *Klebsiella pneumoniae* carbapenemases (KPC) are the most common. KPC producers are usually MDR, especially towards beta-lactams and therefore limit treatment options for KPC infections (34).

Class B metallo-beta-lactamases: Unlike class A and D whose hydrolytic activities are serine-based, class B beta-lactamases require one or two zinc ions for its catalytic activity (31). This class of enzymes hydrolyzes all beta-lactams, except aztreonam. One of the most commonly identified enzymes in this group is the NDM-1 and has been identified worldwide (34).

Class D beta-lactamases: This class hydrolyzes narrow spectrum beta-lactams and weakly hydrolyze carbapenems, while they spare broad spectrum antibiotics. One of the most

frequently identified enzymes in this class is the OXA-48 and is increasingly found worldwide (31).

1.9 New Dehli metallo-beta-lactamase 1

New Delhi metallo-beta-lactamase 1 (NDM-1) is one of the most clinically significant carbapenemases, and alongside OXA-48 one of the most common carbapenemases among *E. coli* worldwide (10, 35). NDM-1 hydrolyzes beta-lactams including penicillins, cephalosporins and carbapenems, but spare monobactams such as aztreonam (35). The spread of NDM-1 causes concerns due to its resistance towards a wide range of antibiotics. Isolates producing NDM-1 has been found worldwide and been most frequently identified in *E. coli* and *K. pneumoniae* (34, 36). NDM-1 has been identified in *E. coli* ST131 as a source of community-acquired infection. Plasmids carrying $bla_{\text{NDM-1}}$ can also harbor a large quantity of other resistance genes such as carbapenemase and ESBL genes. The large quantity of resistance genes has rarely been observed in single isolates (34).

1.10 Oxacillinase 48

OXA-48 is a class D beta-lactamase and is one of the most common carbapenemases among *E. coli* worldwide (10). Even though class D primarily has been found in *Acinetobacter* species, OXA-48 has only been found in *Enterobacteriaceae*, and was first described in *K. pneumoniae* (37, 38). OXA-48 hydrolyzes penicillins and carbapenems leading to its resistance towards these antibiotics, but spare the extended-spectrum cephalosporins (38).

The gene encoding OXA-48, $bla_{\text{OXA-48}}$, is found in a self-transferable, epidemic IncL/M plasmid with broad host range. This plasmid has a high efficiency of transfer and conjugates at a high rate among the *Enterobacteriaceae* species (35, 39). The high transfer efficiency of this plasmid is proposed to be the cause of the OXA-48´s successful spread (35). This plasmid does not carry any other resistance determinants, but 80% of isolates being positive for OXA-48 are reported to coproduce ESBLs. In addition, there has been reported coproduction of OXA-48, NDM-1 and Verona-integron-encoded metallo-beta-lactamase (VIM). These coproductions cause the isolates to be resistant towards all beta-lactams available (39).

2 AIM AND HYPOTHESIS

It is hypothesized that clade C ST131 is pre-adapted for the acquisition of carbapenemase encoding plasmids, with little or no cost from a bacterial perspective. The aim of this study is to understand how *E. coli* ST131 responds to acquisition of carbapenemase-encoding plasmids (p50579417_3_OXA-48 and pK71-77-1-NDM) and compare them to other common STs such as ST69, ST73 and ST95, so that predictions about future successful *E. coli* ST131 plasmid combinations can be made. We therefore focus to investigate differences in conjugation efficiency and plasmid persistence among ST131 clades, and between ST131 and non-ST131 strains.

3 MATERIALS AND METHOD

3.1 Materials

3.1.1 Strains

E. coli is the target organism in this thesis. The main strains in study included 14 ST131 strains, one ST69 strain, one ST95 strain and two ST73 strains. The study was expanded to three mutants, deriving from one of the ST73 strains, conferring ciprofloxacin resistance. Other strains with less significant roles were also used, as shown in

Table 1.

Table 1 List of all strains used throughout the thesis.

Strain	Description	Usage	Source
MP15-01	Clinical *E. coli* ST131 clade A isolates	To study plasmid behavior	(40)
MP15-03			
MP23-07			
MP15-04	Clinical *E. coli* ST131 clade B isolates		
MP15-05			
MP15-06			
MP15-07	Clinical *E. coli* ST131 clade C1 isolates		
MP15-08			
MP15-09			
MP15-10	Clinical *E. coli* ST131 clade C2 isolates		
MP15-11			
MP15-12			
MP15-13			
MP15-14			
MP23-25	Clinical *E. coli* ST69 isolate		
MP23-27	Clinical *E. coli* ST73 isolate		
MP23-30	Clinical *E. coli* ST95 isolate		
MP04-02	Clinical *E. coli* ST73 isolate		(41) (42)
MP23-49	MP04-02 derivative gyrAS83L		(43, 44)

MP23-50	MP04-02 derivative gyrAS83L parCE84K		(43, 44)
MP23-51	MP04-02 derivative gyrAS83L parCE84K gyrAD87G		(43, 44)
ATCC 29522	Reference *E. coli* strain	Quality control in e-test	(44)
MP08-01	Laboratory *E. coli* strain MG1655	Negative control for $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ in PCR test	(45)
MP14-23	08-01 derivative Δ*malF*	To generate strains 23 60 and 23_61	(45)
MP10-31	Laboratory *E. coli* strain DH5α carrying p50579417_3_OXA-48	To generate strains 23_60	Unpublished
MP09-76	Laboratory *E. coli* strain DH5α carrying pK71-77-1-NDM	To generate strains 23 61	Unpublished
MP08-04	Clinical *E. coli* ST410 K71-77 isolate carrying pK71-77-1-NDM and another plasmid	Positive control for $bla_{\text{NDM-1}}$ in PCR test	(46)
MP08-05	Clinical *E. coli* ST405 50579417 isolate carrying p50579417_3_OXA-48 and 4 other plasmids	Positive control for $bla_{\text{OXA-48}}$ in PCR test	(47)
MP23-60	14_23 transconjugant carrying p50579417_3_OXA-48	Donors in conjugation assays with NORM and EcoSens clinical isolates and derivatives	This work
MP23-61	14_23 transconjugant carrying pK71-77-1-NDM		

3.1.2 Growth media and solutions

Different growth media, both in liquid and solid form were used to cultivate bacteria. These included selective and non-selective, as well as rich and minimal media.

3.1.2.1 Lysogeny broth (LB) and LB agar

Lysogeny broth (LB) is a rich, non-selective medium containing the essential nutrients for most bacteria to grow without applying selective pressure. When antibiotic selection is required, LB can be supplemented with the desired antimicrobial drug.

LB was prepared by adding 20 g LB broth (BD Difco™, Miller) to 800 mL distilled water. The mix was then autoclaved at 121°C and stored in room temperature.

LB agar (solid version of LB) was prepared similarly, adding 12 g select agar (Sigma Aldrich) to the mix. After autoclaving, the mix was cooled down to approximately 50°C, before being poured into sterile petri-dishes, dried overnight and stored at 4°C.
If the medium was supplemented with ampicillin, the antibiotic was added after cooling down the autoclaved mix. Final concentration of ampicillin used in this thesis was 100 mg/L.

3.1.2.2 M9 minimal medium

M9 minimal medium used in this thesis was a selective medium using either maltose or lactose as a nutrient for the cultivation of bacteria. Growth of bacteria would depend on if the strains coded for the complete maltose or lactose utilization operon.

Prior to M9 minimal medium preparation, solutions (Table 2) required for the medium were made by mixing, autoclaving at 121°C and cooling down to room temperature. M9 minimal media was then prepared by mixing all solutions (Table 3), before being poured into sterile petri-dishes, dried overnight and stored at 4°C.

If supplemented with ampicillin, the volume of sterile H_2O was reduced to 21.2 mL, adding 0.8 mL of ampicillin with a final concentration of 100 mg/L in the mix.

Table 2 List of chemicals used to make solutions for M9 minimal media preparation.

Chemical	**Manufacturer**	**Amount (g)**	**dH_2O volume (mL)**	**Volume created (mL)**
Select agar	Sigma-Aldrich	12	to 600	600
5xM9 salts: **Sodium phosphate dibasic(Na_2HPO_4)**	Sigma-Aldrich	33.88	to 1000	1000
Potassium phosphate monobasic (KH_2PO_4)	AnalaR NORMAPUR,	15.0		
Sodium Chloride (NaCl)	VWR BDH CHEMICALS	2.5		
Ammonium chloride ≥ 99.5% (NH_4Cl)	Sigma-Aldrich	5.0		
Magnesium sulphate ($MgSO_4$)	VWR, BDH CHEMICALS	9.62	to 80	80
Calcium chloride dihydrate ($CaCl_2$ * $2H_2O$)	Sigma-Aldrich	1.176	to 40	40
Maltose monohydrate ≤ 99%	Sigma-Aldrich	20	to 100	100
Lactose monohydrate	Merck	4	to 20	20

Table 3 General recipe for preparation of 800 mL M9 minimal media

Chemical	**Volume for 800 mL**
Select agar 2%	600 mL
5xM9 salts	160 mL
1M $MgSO_4$	1.6 mL
200 mM $CaCl_2$	0.4 mL
20% Maltose or 20% lactose	16 mL
Sterile H_2O	22 mL

3.1.2.3 Tetrazolium maltose (TM) media

Tetrazolium maltose (TM) medium is a non-selective rich medium. The medium was supplemented with maltose as carbon source and Triphenyl tetrazolium chloride (TTC) as an indicator (making it a differential medium). Bacteria that were able to utilize maltose would appear as white colonies, unlike bacteria that could not utilize maltose which would appear as red colonies. This made it possible to distinguish between strains encoding the complete maltose operon.

All chemicals (Table 4), except 20% maltose and TTC (5%) were mixed and autoclaved at 121°C. The mix was then cooled down to approximately 50°C, where 20% maltose and TTC (5%) were added. The solution was then poured in petri-dishes, dried overnight and stored at 4°C.

Table 4 General recipe used to make 800 mL of TM media

Chemical	Manufacturer	Amount for 800 mL
Bacteriological peptone	Oxoid	8 g
Yeast extract	Fluka	0.8 g
Sodium Chloride (NaCl)	AnalaR NORMAPUR, VWR BDH CHEMICALS	4 g
Select agar	Sigma-aldrich	12 g
dH2O		Up to 760 mL
20% maltose	Sigma-Aldrich	40 mL
TTC (5%)	Sigma-Aldrich	800 µL

3.1.2.4 80% glycerol

To make 80% glycerol, 6.4 mL of dH_2O was added to 73.6 mL of 87% glycerol (VWR, BDH CHEMICALS). The solution was then autoclaved at 121°C, cooled down and stored in room temperature.

3.1.2.5 0.9% NaCL

To make 0.9% NaCl (saline), 3.6 g NaCl (AnalaR NORMAPUR, VWR BDH CHEMICALS) was added to 400 mL of dH_2O. The solution was then autoclaved at 121°C, cooled down and stored in room temperature.

3.1.3 Equipment

Several equipment was used throughout the project, with the most frequently used listed in (Table 5)

Table 5 List of frequently used equipment.

Equpment/Materials	Brand	Usage
Incubator	Termaks	Incubate solid cultures for antimicrobial growth
Incubator shaker	Multitron, infors	Incubate and shake liquid cultures (in tubes) for antimicrobial growth
Centrifuge	VWR Microstar 17	Conjugation assay to concentrate cells.
McFarland Densitometer	DEN-1, Biosan	Obtain density of 0.5 McFarland in E-test
Thermo cycler	BIO RAD T100	PCR
96 deep well plates	VWR	Conjugation and plasmid persistence assay

3.2 Methods

The main focus of this study was to investigate differences in conjugation efficiency and plasmid persistence. Conjugation and plasmid persistence assays were therefore performed, in addition to PCR and phenotype characterization to confirm transconjugants. Other minor methods such as making overnight cultures, freeze stocks and plating are also described in this section.

3.2.1 Making overnight cultures

3.2.1.1 In culture tubes

2 mL of LB broth was added to a culture tube. A 1 µL inoculating loop was then used to either touch an isolated colony from a plate or from a freeze stock and was then added into the culture tube. The culture tube was then placed in an incubator shaker at 37°C and 225 rpm. There was

always a control tube containing pure LB broth alongside the overnight cultures to ensure the media was free from contamination.

In some instances, 2 µL ampicillin 100 mg/mL were added obtaining a final ampicillin concentration of 100 mg/L in the culture. In these cases, the ampicillin was supplemented and vortexed right before the bacterial strain was added.

3.2.1.2 In 96 deep well plates

In a 2 mL 96-well plate, 1 mL of LB broth was added in each well. The antimicrobials were then inoculated as described in section 3.2.1.1, but added in a chess pattern (Figure 2). The 96-well plate was placed in an incubator at 37°C and 700 rpm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB
B	LB	S1	LB	S2	LB	S3	LB	S4	LB	S4	LB	LB
C	LB	LB	S5	LB	S5	LB	S6	LB	S6	LB	S7	LB
D	LB	S8	LB	S9	LB	S10	LB	S11	LB	S12	LB	LB
E	LB	LB	S13	LB	S14	LB	S15	LB	S16	LB	S17	LB
F	LB	S18	LB	S19	LB	S20	LB	S21	LB	S22	LB	LB
G	LB	LB	S23	LB	S24	LB	S25	LB	S26	LB	S27	LB
H	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB

Figure 2. Illustration of bacterial cultivation in a 96 well plate following a chess pattern. S represents strains, and all white cells represent wells where strains could be cultivated. All other wells were supplemented with LB in order to check for contamination.

3.2.2 Bacterial cultivation on solid media

3.2.2.1 Streaking

On a solid media plate, bacteria were streaked using three 1 µL inoculating loops. The first loop was used to obtain the bacteria by either touching an isolated colony or taking from a freeze stock. The loop was then placed on the media and used to streak back and forth horizontally on one third of the plate. The plate was turned 90° and a new loop was used to touch the previous streak, then streaked back and forth horizontally on another area of the plate. The plate was turned 90°and a third loop was used to repeat the process (Figure 3). The plates were the incubated at 37°C overnight (rich media) or 48 hours (minimal media).

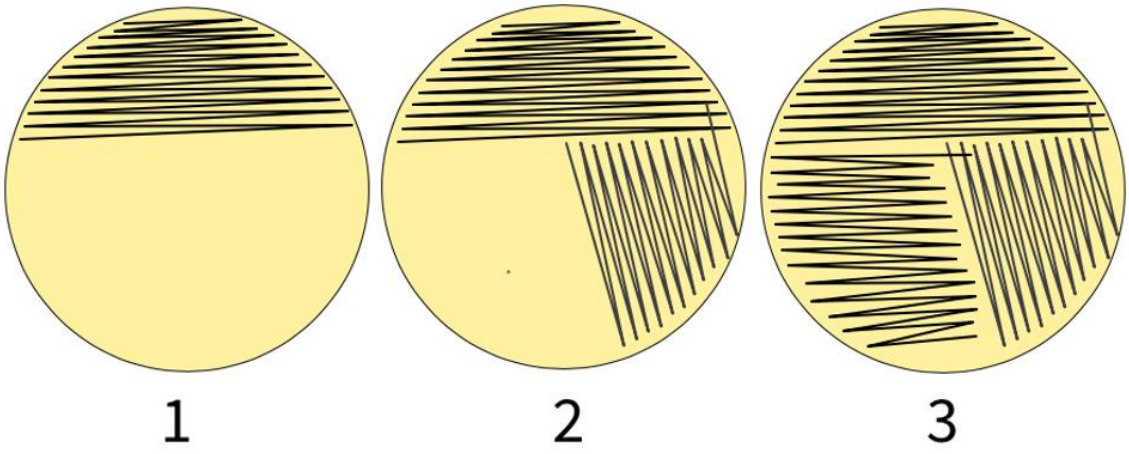


Figure 3 An illustration of how bacteria were streaked on plates.

3.2.2.2 Plating

Glass beads were used to spread liquid culture on a solid media. 10-20 glass beads were poured on a solid media plate. The wanted amount of liquid culture was then pipetted on the plate. The plate was shaken back and forth so that the beads would move vertically across the plate. The plate was then turned approximately 40° and shaken again. This was repeated several times so that the beads would distribute the liquid inoculum throughout the whole area of the plate. To remove the beads, the plate was turned around so that the beads would fall into the lid and then removed.

3.2.3 Polymerase chain reaction (PCR) and gel electrophoresis

Polymerase chain reaction (PCR) and gel electrophoresis were techniques used in this thesis to analyze DNA sequences in strains, making it possible to detect presence of a gene or distinguish strains based on primers and amplicon size. In this thesis, PCR was mainly conducted to confirm presence of p50579417_3_OXA-48, pK71-77-1-NDM, and to distinguish between donor and recipient strains. Other PCRs were conducted to confirm presence of *lacZ* and absence of other plasmids (IncFIA, IncB/O/K/z, IncFIB_2, IncX4 and IncFII).

3.2.3.1 PCR amplifying the replicon region of p50579417_3_OXA-48 and pK71-77-1-NDM:

Primers IncL/M and IncA/C_2 (forward and reverse) (Appendix 3) were used in a PCR to amplify replicon region of p50579417_3_OXA-48 and pK71-77-1-NDM respectively, thereby confirming the presence of the plasmids. Gel electrophoresis was conducted for analyzing and

visualizing results. For the presence of *repA*, the PCR product would show a band at an amplicon size of 790 bp for p50579417_3_OXA-48 and 466 bp for pK71-77-1-NDM.

3.2.3.2 PCR amplifying the chromosomal region between *rpoS* and *ygbN*

Primers *rpoS-F* and *ygbN*-R (Appendix 3) were used to distinguish *E. coli* MG1655 from other strains. Using these primers, *E. coli* MG1655 would show a band at an amplicon size of 2.2 bp, while other strains (excluding the ST69 strain) used in this thesis would show a band at an amplicon size of approximately 4 bp.

3.2.3.3 Procedure

Prior to PCR preparation, materials needed for the PCR mix (Table 6) were prepared. Templates were required for positive control, negative control, and test strains. They were made by taking a 1 µL inoculating loop to pick a single colony from each of the grown strains and thereafter added to 50 µL of sterile H_2O in a PCR tube. Primers were diluted by mixing primer and 1x TAE at a 1:10 ratio, giving the primer a final concentration of 1 µM in the PCR mix. The PCR mix was prepared by adding all materials (Table 6) except the templates in an Eppendorf tube. A volume of 19.2 µL of PCR mix was then transferred to PCR tubes, where 0.8 µL of template was added. The process was done on ice and with gloves to delay and prevent the occurrence of unwanted enzymatic activity.

The PCR was run using the reactions shown in Table 7. Agarose gel 0.8% was made by mixing agarose (Lonza, Seakem ®) with 1X TAE. The mix was heated in the microwave. After cooling it down, 3 µL ethidium bromide 1 mg/mL was added. The solution was then poured on the form to make the gel. PCR samples were then made by mixing 1X loading dye and PCR product to a final volume of 12 µL. The PCR samples and 12 µL smart ladder was pipetted into separate wells in the gel, and gel electrophoresis was run at 100 V for 45 minutes.

Table 6 General recipe to make 20 µL of PCR mix.

Material	**Volume (µL)**
dH_2O	5.2
dreamTAQ (Thermo Scientific)	10
Primer forward (10 µM)	2
Primer reverse (10 µM)	2
Template	0.8

*Table 7 Standard PCR reaction conditions used throughout the thesis. * These conditions were used for the PCR amplification of chromosomal region between rpoS and ygbN.*

Initial denaturation	94 °	3 min	
Denaturation	92 °	20 sec	
Annealing	58° *56°	20 sec	30 cycles
Extension	72°	1 min 10 sec *5 min	
Final extension	72°	5 min	
Storing	4°	infinity	

3.2.4 Phenotype test

3.2.4.1 Distinguish strains

By using TM plates, a phenotypic test could be conducted to test if the strains were able to use maltose as carbon source. To do so, they were streaked on TM media and then incubated at 37°C overnight (16-18 hours).

3.2.4.2 Checking growth conditions

Different strains were streaked on M9 minimal media + maltose and LB agar plates to check respectively the ability to grow with maltose as sole carbon source and for any possible contamination. This was to ensure the use of right conditions for further experiments throughout the project.

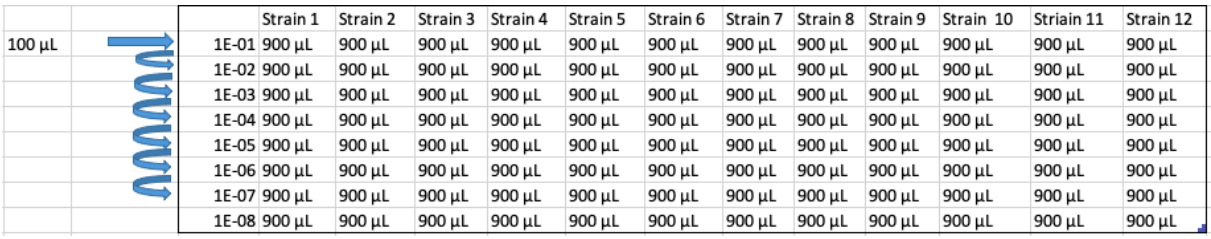
The strains were streaked on either LB agar or M9 minimal media plates and then respectively incubated overnight (16 -18 hours) or for 48-72 hours depending on growth response.

3.2.5 Conjugation assays

3.2.5.1 Construction of new donors

Overnight cultures were made for the donor strain (supplemented with ampicillin 100 mg/mL) and recipient strains in culture tubes. In a new culture tube, 50 µL of donor culture and 50 µL of recipient culture was added to 900 µL LB broth for conjugation. The tubes were incubated at 37° C for 24 hours without shaking. A dilution series was made for each conjugation mix on a 96 deep well plate (see Figure 4) where 100 µL of conjugation mix was added to 900 µL

saline in the first row (dilution=10^{-1}), thereby taking 100 µL of the mix in the first row and adding to 900 µL saline in the second row (10^{-2}). This was repeated until dilution 10^{-6} was obtained. A volume of 100 µL of dilutions 10^{-1}, 10^{-3}, 10^{-5} were then plated on M9 minimal media with lactose and ampicillin. The plates were then put in the incubator at 37° C for 48 hours. Figure 4 Illustration of a serial dilutionThe transconjugants were then purified and PCR tested.



		Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9	Strain 10	Striain 11	Strain 12
100 µL	1E-01	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-02	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-03	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-04	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-05	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-06	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-07	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
	1E-08	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL

Figure 4 Illustration of a serial dilution on a 96 well plate.

3.2.5.2 Measuring conjugation efficiency

Three biological replicates of conjugation assays were conducted using newly constructed donor from section 3.2.5.1.

An overnight culture of the donor strain was made in a culture tube with ampicillin while overnight cultures of recipient strains were made in 96-well plates. Ampicillin was removed from the donor culture by centrifuging the culture at 1200 x g for 10 minutes and resuspending the bacterial pellet in 2 mL fresh LB. This was done twice to remove any remaining ampicillin. Donor and recipient cultures were mixed at a 1:100 ratio, giving a final volume of 550 µL. The culture mixes were then centrifuged at 1200 x g for 10 minutes and resuspended in 200 mL LB. From the culture mix, 100 µL was added to a 0.05 µM nitrocellulose membrane filter (VMWP) placed on top of an LB agar plate. The plates were incubated at 37° for 24 hours. From the same culture mix, 50 µL was added to 450 µL saline in a 96-well plate. A serial dilution from 10^{-1} to 10^{-7} was made (see Figure 4). To estimate initial donor and recipient colony forming units (CFUs), 100 µL of dilutions 10^{-4} and 10^{-5} was plated on LB agar +ampicillin plates, while 100 µL of dilution 10^{-6} and 200 µL of dilution 10^{-7} was plated on TM plates. The plates were incubated for 16-18 hours at 37° C. After 24 hours, conjugation was halted, and cells were resuspended by adding each filter to a 50 mL falcon tube containing 5 mL phosphate-buffered saline (PBS). The tubes were then vortexed for 1 minute each. To estimate final CFUs for each

mating suspension, a dilution series on 96 deep well plate was made. A volume of 100 µL of dilutions 10^{-4} and 10^{-5} was plated on LB agar +ampicillin plates, while 100 µL of dilution 10^{-6} and 200 µL of dilution 10^{-7} was plated on TM plates. A volume of 100 µL from all the dilutions and the undiluted PBS cultures were plated on M9 minimal media with maltose to get the CFUs for the transconjugants. The plates were incubated at 37°C for 48 hours.

The transconjugants were purified and confirmed by phenotypic characterization and PCR. Freeze stocks were then made from the confirmed transconjugants. **Error! Reference source not found.**

3.2.6 Purification of colonies

Transconjugants were streaked on M9 minimal media plates containing ampicillin. After cultivation, the colonies were streaked on new plates. For purification of colonies, the colonies were streaked two times.

3.2.7 Freeze stocks

Overnight cultures were made in culture tube from purified colonies. Ampicillin was added to the overnight cultures if the strain was ampicillin resistant. From the overnight cultures, 750 mL culture was added into cryovials containing 250 mL 80% glycerol whilst pipetting up and down to obtain a homogenous solution. The cryovials were then stored in a -80° C.

3.2.8 Conjugation efficiency

The conjugation efficiency for each strain replicate was calculated as shown in Equation 1.

Equation 1 Calculation of conjugation efficiency

$$Conjugation\ efficiency = log_{10}(\frac{\frac{CFU}{mL} transconjugants}{\frac{CFU}{mL} recipients})$$

where CFU/mL for transconjugants and recipients were calculated by taking the average CFU/mL obtained from the plated dilutions.

3.2.9 Relative Fitness

Fitness was measured for each strain replicate by using Equation 2:

Equation 2 Calculation of relative fitness

$$Relative\ fitness = \frac{log_2\left(\frac{Rfinal}{Rinital}\right)}{log_2\left(\frac{Dfinal}{Dinitial}\right)}$$

where R is the average CFU/ml for the plated dilutions of recipient strains, and D is the average CFU/mL for the plated dilutions of donor strains.

3.2.10 Plasmid persistence

The transconjugants obtained from the conjugation experiment (Appendix 5) were used to test plasmid persistence over time. Unless otherwise stated, three transconjugants were tested per strain, such that each transconjugant served as a biological replicate. **Error! Reference source not found.**

3.2.10.1 Procedure

Overnight cultures were made in 96 well plates supplemented with ampicillin. 10 µL of the overnight culture was transferred to 990 µL fresh LB broth in a new 96-well plate using a chess pattern. The 96 well plate was incubated at 37 °C and 700 rpm for 24 hours. The transfer was repeated for three days. A dilution series of 10^{-1} to 10^{-6} was made on a 96 deep well plate. Different dilutions with different volumes were plated on LB+ampicillin plates depending on dilutions giving CFUs between 30-300. A volume of 100 µL of dilution factor 10^{-6} was plated on LB agar. The plates were then put in an incubator at 37 °C for 24 hours. This was repeated for each transfer. From the overnight cultures, a phenotype test was conducted by plating 100 µL of the dilution 10^{-6} on a TM plate.

Plasmid persistence was calculated twice, one based directly from the overnight cultures and the other from the serial transfers.

Plasmid persistence based on overnight cultures were calculated as shown in Equation 3.

Equation 3 Calculation of plasmid persistence based on overnight cultures

$$Plasmid\ persistence = \frac{\frac{CFU}{mL}\ of\ P}{\frac{CFU}{mL}of\ T}$$

where P is plasmid carrying cell and T is total number of cells.

Plasmid persistence from serial transfers were calculated by performing a linear regression where the independent variable (x-axis) = timepoint and the dependent variable (y-axis) = log10

of plasmid persistence (Equation 3) for each timepoint. The graph then identified an equation for the trendline (Equation 4).

Equation 4 Equation obtained from linear regression.

$$y = ax + b$$

where plasmid persistence for serial transfers were calculated to be the slope (a).

3.2.11 Antimicrobial gradient strip testing

The purpose of the antimicrobial gradient strip test was to determine if the strains had different levels of resistance to ampicillin. The test was also conducted for a sensitive strain, ATCC 25922, used as quality control. Ampicillin strips with a known concentration gradient was used. When the strip was placed on the agar surface, ampicillin would immediately transfer into the agar. Antimicrobial growth would then depend on the sensitivity to the different concentrations of ampicillin. Minimum inhibitory concentration (MIC) values were read as the lowest concentration on the strip leading to growth inhibition and could be seen where the growth ellipse intersected with the strip.

3.2.11.1 Procedure

The strains were cultivated on LB agar plates supplemented with ampicillin. This was necessary as the method required freshly grown colonies (24-48 hours). Colonies were thereby suspended in saline to obtain a density of 0.5 McFarland. The inoculum was streaked on a Mueller Hinton II agar plate (produced in the University Hospital of Northern Norway) for confluency with a sterile cotton swab and the help of a rotator. Ampicillin strips 0.016-256 mg/L (Liofilchem) were then placed on the agar plate with the scale facing up. Using sterile tweezers, the strip was tapped gently from the lowest to highest concentration to prevent bubbles under the strip. The plates were then placed in an incubator at 37°C for 18 hours (+/- 20 minutes). The MIC values were then analyzed.

3.2.12 Bioinformatics

Different web tools were used to analyze the assembled genomes of the clinical strains (Table 1).

3.2.12.1 Multilocus sequence type (MLST)

MLST 2.0 is a web tool designed to identify the sequence types of different bacterial species (48). Assembled genomes were uploaded and analyzed using *E. coli* scheme one.

3.2.12.2 *fumC fimH* (CH) type

CHtyper is a web tool designed to identify *fumC and fimH* in *E. coli* (49). Assembled genomes were uploaded and analyzed with 85% identity threshold.

3.2.12.3 Serotype

SerotypeFinder is a web tool used to classify and characterize *E. coli* based on the O:H serotype (50). Assembled genomes were uploaded and analyzed using 85% identity threshold and a minimum length of 60%.

3.2.12.4 ResFinder

ResFinder is web tool used to identify acquired antimicrobial resistance genes and chromosomal mutations in different species (51, 52). Assembled genomes were uploaded and analyzed for both chromosomal mutations and acquired AMR genes with 85% identity threshold and 60% minimum length.

3.2.12.5 PlasmidFinder

The PlasmidFinder is a web tool designed to identify and classify plasmids from *Enterobacteriaceae* species. The web tool also assigns plasmid into different Inc groups (53). Assembled genomes were uploaded and analyzed with a threshold of 85% identity and 60% minimum length.

3.2.13 Statistical Analysis

Statistical analyses were performed using IBM SPSS statistics (version 27). In general, statistical tests were chosen with guidance from Laerd Statistics (54)

4 RESULTS

The aim of this thesis is to elucidate the impact of carbapenemase encoding plasmids on *E. coli* ST131. Therefore, we studied two plasmids: IncL/M p50579417_3_OXA-48 and IncA/C pK71-77-1-NDM, which respectively encode $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$. We used a total of 14 *E. coli* clinical strains belonging to ST131 (three clade A, three clade B, three clade C1 and five clade C2). As a reference, we included three clinical strains each belonging to other dominant STs: ST69, ST73 and ST95.

We confirmed the sequence type of the strains by analyzing their genomes with bioinformatical tools (Appendix 1). Furthermore, all ST131 clade A strains had CHtype 40-41 and serotype H5:O16, while all clade B strains had CHtype 40-22 and serotype H4:O25 and clade C strains had CHtype 40-30 and serotype H4:O25. ResFinder results showed that none of the strains encoded beta-lactamases (Appendix 2). Ampicillin sensitivity for the strains was also confirmed by antibiotic susceptibility test in another thesis (unpublished). Results from PlasmidFinder showed that none of the strains harbored IncA/C nor IncL/M plasmids (Appendix 2).

4.1 Donor construction

To be able to transfer p50579417_3_OXA-48 and pK71-77-1-NDM into the different clinical strains, we required counter selectable donors. Therefore, we constructed them as MG1655 Δ*malF* derivatives which were unable to grow on medium containing only maltose as carbon source. DH5a strains (Δ*lacZ)*, MP10-31 and MP09-76 harboring p50579417_3_OXA-48 and pK71-77-1-NDM were used to transfer the plasmids into MG1655 Δ*malF,* generating transconjugants MP23-60 and MP23-61 respectively. Prior to the conjugation step, PCR amplification confirmed the presence of pK71-77-1-NDM in MP09-76 and p50579417_3_OXA-48 in MP10-31, also confirming the absence of the other plasmids tested (mentioned in section 3.2.3). After conjugation, PCR confirmed the presence of p50579417_3_OXA-48 in MP23-60 and pK71-77-1-NDM in MP23-61, as well as *lacZ* in both strains. Furthermore, the transconjugants appeared as red colonies on the TM plates, which confirmed the lack of *malF*. These transconjugants were later used as donors for measuring conjugation efficiency for the clinical strains.

4.2 Phenotypic test for growth and contamination

After the donor construction, phenotypic tests to check growth and contamination were conducted for the recipient clinical strains. Results showed no contamination, and all strains were able to grow on minimal media with maltose. However, the strain belonging to ST95 grew slower, requiring an incubation time of 72 hours in 37°C.

4.3 Methodological considerations

Numerous methods were attempted for the conjugation assay throughout the thesis. The different methods included differences in donor and recipient ratio, liquid and filter matings, different culture volumes and different ampicillin concentrations on minimal media with maltose. Conjugation with donor MP23-61 did not result in transconjugants with most strains using these methods. Therefore, pK71-77-1-NDM was excluded, making p50579417_3_OXA-48 the focus of this thesis.

4.3.1 CFUs obtained from conjugation

Three biological replicates for filter conjugation of p50579417_3_OXA-48 in clinical strains were performed. Transconjugants were obtained for most strain replicates, although some strains had issues with CFUs obtained from the different dilutions.

For strains MP15-01 and MP15-03, both ST131 clade A strains, results showed that a 10-fold increase in dilution did not result in a 10-fold decrease of CFUs (Table 8). The pattern was observed for all three biological replicates, therefore two additional replicates were conducted for these strains. The method for conjugation was altered to which the PBS containing conjugation filters were vortexed for five minutes, as opposed to one minute. Results from the modified method showed the same pattern as for the first three replicates. As the CFUs did not correspond to the 10-fold dilutions, the conjugation efficiency was calculated based on the CFUs from the two lowest and the two highest dilutions, each pair giving a different conjugation efficiency.

For strain MP15-12, an ST131 clade C2 clinical strain, all three replicates showed CFUs that did not correspond to the 10-fold dilutions (see Table 8). Two additional biological replicates were performed but showed the same pattern. We did not obtain countable transconjugants for two of the replicates, therefore they were excluded from the conjugation efficiency measurement.

For strain MP15-13, another ST131 clade C2 clinical strain, the results for initial donor CFUs did not show any colonies on any of the biological replicates. No transconjugants were obtained for this strain, and it was therefore excluded from the thesis.

Strain MP23-27, an ST73 clinical strain, showed poor emergence of transconjugants. Therefore, a total of four replicates were conducted for this strain, where only two resulted in countable CFUs. However, one of these replicates showed CFU=1 when incubated for 72 hours. This replicate was excluded from the study, leaving only one replicate for MP23-27. The strain was excluded altogether from measuring conjugation efficiency and plasmid persistence as it was not seen fit to base the measurement on only one replicate.

For strain MP23-30, a variety of dilutions were plated for each replicate. For each replicate, the CFU/mL also varied. We conducted six replicates for this strain. Only four of the replicates resulted in a detectable number of transconjugants. For one replicate, we obtained CFU=233 for dilution 10^{-5}, but for the rest of the replicates transconjugants were only obtained on the lowest dilutions plated, but CFU < 2. Due to the limitations of dilutions plated for each replicate, we were not able to determine whether CFUs were consistent with the 10-fold dilution. The low number of transconjugants therefore contributes to lower confidence when using the CFUs in analysis for conjugation efficiency.

Table 8 Example of CFUs pattern obtained from 100 µL dilutions of different strains that deviated from expected 10-fold decrease.

Strain	Dilution					
	10^{0}	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
MP15-01	526	52	39	25	19	2
MP15-03	1141	309	242	251	135	19
MP15-12	*TNTC	319	0	Not plated	Not plated	Not plated

* Too numerous to count.

4.3.2 Plasmid persistence assays

Three biological plasmid persistence assays were conducted, each using a transconjugant obtained from each of three biological strain replicates from the conjugation assays. Prior to the plasmid persistence assays, the transconjugants were confirmed by PCR and phenotype

characterization. Results from the phenotype characterization were not clear, as it was difficult to determine if colonies were red or white. They were therefore further confirmed by PCR amplification of the chromosomal region between *rpoS* and *ygbN*.

PCR could not confirm presence of p50579417_3_OXA-48 in two MP15-12 (ST131 clade C2) replicates and one MP04-02 (ST73) replicate. Furthermore, PCR results showed that the bacterial host for one MP15-07 (ST131 clade C1) replicate was MG1655 Δ*malF*. These replicates were therefore excluded from the plasmid persistence assays, where strain MP15-12 was excluded altogether. All strains excluded from conjugation and plasmid persistence assays are shown in Appendix 4.

From the overnight cultures of the plasmid persistence assays, new phenotypic characterization on TM plates further confirmed the transconjugants.

4.4 Determining differences among *E. coli* ST131 subclades

Statistical analysis was conducted to determine if there were any differences in conjugation efficiency and plasmid persistence among ST131 clades.

4.4.1 Conjugation efficiency

All transconjugants with detectable CFUs, unless otherwise stated in section 4.3.1 were used in the analysis of conjugation efficiency. As explained in section 4.3.1, we calculated the conjugation efficiency of clade A in two different ways, thus we conducted two analyses. Two one-way ANOVAs were conducted to determine whether the conjugation efficiency differed among the four different *E. coli* ST131 clades: A (n = 3), B (n = 3), C1 (n = 3) and C2 (n = 4).

The first analysis was conducted using the conjugation efficiency based on the CFUs of the highest dilutions of clade A. Boxplots showed no outliers and a Shapiro-Wilk test verified that data was normally distributed ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed homogeneity of variances ($p = 0.109$). One-way ANOVA analysis ($F(3,9) = 12.58$, $p = 0.001$) showed statistically significant difference among clades (Figure 5). A Tukey HSD post hoc showed statistically significant differences between clade A and all the others, but no statistically significant differences ($p > 0.05$) between clades B, C1 and C2.

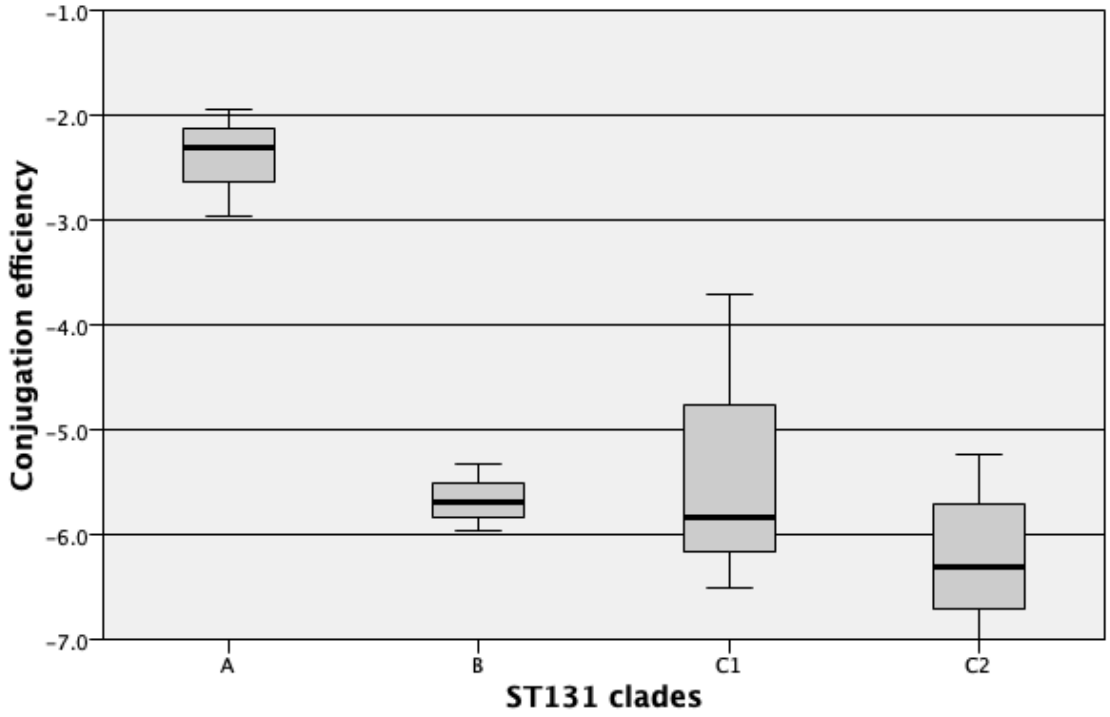


Figure 5. Distribution of conjugation efficiency of ST131 clades, where conjugation efficiency for clade A is based on the CFUs for the highest dilutions. The x-axis represents the different ST131 clades and the y-axis represents log10 (transconjugants/recipients). Each value in a boxplot represents the average log10 of the conjugation efficiency of each strain. For clade A n = 3, B n = 3, C1 n = 3 and C2 n = 4. The conjugation efficiency increased from clade C2 (M = -6.21, SD = 0.73), to clade B (M = -5.66, SD = 0.32), to clade C1 (M = -5.35, SD = 1.46) to clade A (M = -2.40, SD = 0.52), in that order. One-way ANOVA with Tukey HSD post hoc revealed that conjugation efficiency increased significantly from clade C2 to A (3.80, 95% CI (1.76 to 5.85), p = 0.001), C1 to A (2.94, 95% CI (0.76 to 5.13), p = 0.010) and B to A (3.26, 95% CI (1.07 to 5.44), p = 0.005).

The second analysis was conducted using the conjugation efficiency based on the lowest dilutions of clade A. Boxplots showed no outliers and a Shapiro-Wilk test verified that data were normally distributed ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed homogeneity of variances ($p = 0.084$). One-way ANOVA analysis (F (3,9) = 2.31, $p = 0.144$) showed no statistically significant differences (Figure 6), also shown with Tukey´s post hoc ($p > 0.05$).

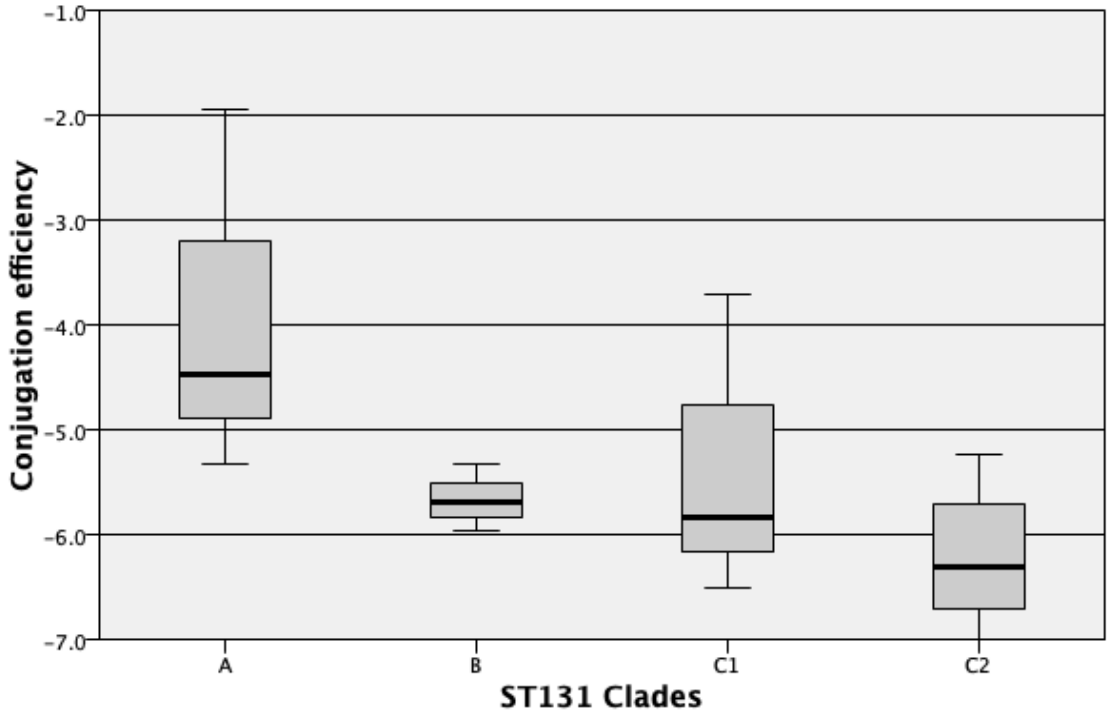


Figure 6. Distribution of conjugation efficiency of ST131 clades, where the conjugation efficiency for clade A is based on the lowest dilutions. The x-axis represents the different ST131 clades and the y-axis represents log10 (transconjugants/recipients). Each value in a boxplot represents the average log10 of the conjugation efficiency of each strain. For clade A n = 3, B n = 3, C1 n = 3 and C2 n = 4. The conjugation efficiency increased from clade C2 (M = -6.21, SD = 0.73), to clade B (M = -5.66, SD = 0.32), to clade C1 (M = -5.35, SD = 1.46) to clade A (M = -3.9, SD = 1.76), in that order. One-way ANOVA and Tukey HSD post hoc (p > 0.05) revealed no statistically significant difference in conjugation efficiency.

4.4.2 Relative fitness

The relative fitness of the different strains might affect the conjugation efficiency. We therefore measured it to understand if there were any differences in the growth rates of recipients.

A one-way ANOVA was conducted to determine whether the relative fitness differed between ST131 clades: clade A (n = 3), clade B (n = 3), clade C1 (n = 3) and clade C2 (n = 4). Boxplots showed no outliers and a Shapiro-Wilk test verified that data was normally distributed ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed homogeneity of variances ($p = 0.35$). The results (Figure 7) showed no statistically significant difference in relative fitness between the clades (F (3, 9) = 1.82, $p = 0.21$) along with Tukey´s post hoc ($p > 0.05$).

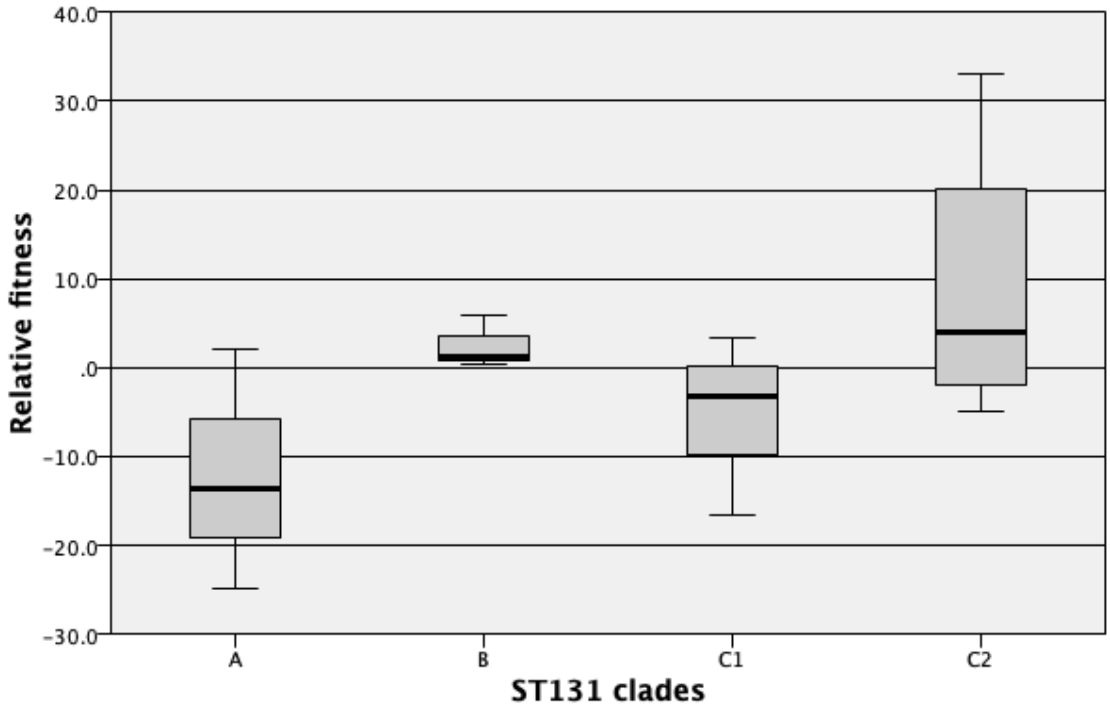


Figure 7. Distribution of the relative fitness of ST131 clades. The x-axis represents the different ST131 clades and the y-axis represents their fitness relatively to the donor strain MP23-60. Each value in a boxplot represents the average of the relative fitness of each strain. For clade A n = 3, B n = 3, C1 n = 3 and C2 n = 4. The fitness increased from clade A (M = -12.14, SD = 13.57) to clade C1 (M = -5.49, SD = 10.21) to clade B (M = 2.57, SD = 2.96) to clade C2 (M = 9.00, SD = 16.75). One-way ANOVA with Tukey HSD post hoc (p > 0.05) revealed no statistically significant difference in relative fitness.

4.4.3 Plasmid persistence

As mentioned in section 4.3.2 MP15-12 (ST131 clade C2) was excluded from the plasmid persistence assays, reducing the sample size of clade C2 to three strains.

4.4.3.1 Plasmid persistence based on overnight cultures

A one-way Anova was conducted to determine whether plasmid persistence was statistically significantly different between *E. coli* ST131 clades: A (n = 3), B (n = 3), C1 (n = 3) and C2 (n = 3). Boxplots showed no outliers and Shapiro-Wilk and Levene´s tests verified, respectively, that the data was normally distributed and the groups showed equal variances ($p > 0.05$). The results showed that there was a statistically significant difference (F (3,8) = 80.06, $p < 0.001$) in plasmid persistence between the clades (Figure 8). Tukey´s post hoc test shows that the increase from clades C1 and C2 to clades A and B were statistically significant, but showed no difference between clades A and B nor between C1 and C2.

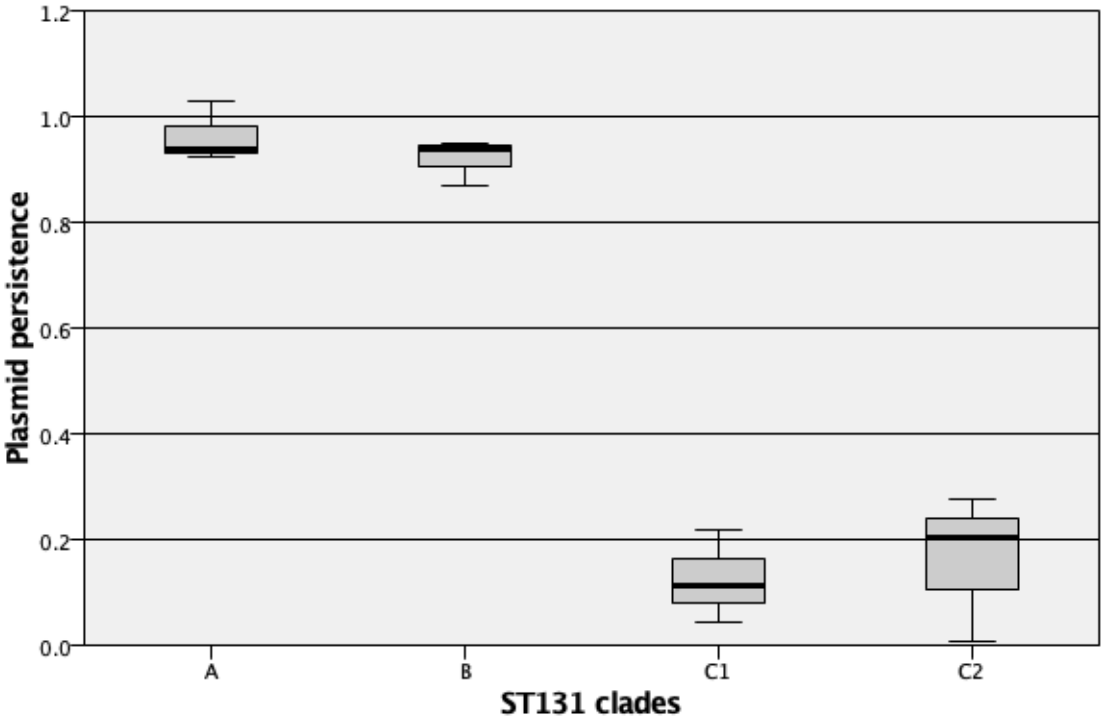


Figure 8. Distribution of plasmid persistence measured from the overnight culture for the different ST131 clades. The x-axis represents the different ST131 clades and the y-axis represents the frequency of plasmid-carrying cells in the population. Each value in a boxplot represents the average of the plasmid persistence of each strain. For all clades n = 3. Plasmid persistence increased from clade C1 (M = 0.12, SD = 0.09), to clade C2 (M = 0.16, SD = 0.14), to clade B (M = 0.92, SD = 0.04) to clade A (M = 0.96, SD = 0.06). One-way ANOVA with Tukey HSD post hoc revealed that plasmid persistence increased significantly from clade C1 to A (0.84 (95% CI, 0.61 to 1.07), p < 0.001), C2 to A (0.80 (95% CI, 0.57 to 1.03) p < 0.001), C1 to B (0.79 (95% CI, 0.56 to 1.02) p < 0.001) and C2 to B (0.76 (95% CI, 0.52 to 0.99) p < 0.001).

4.4.3.2 Plasmid persistence based on serial transfers

A one-way ANOVA was also performed to determine whether the plasmid persistence based on serial transfers differed between the four different *E. coli* ST131 clades: A, B, C1 and C2. Boxplots showed no outliers and Shapiro-Wilk test verified that the data was borderline not normally distributed for clade A ($p = 0.049$), but was normally distributed for the rest of the clades ($p > 0.05$). Assumption of equal variances was not met ($p = 0.010$), as assessed by Levene´s test of homogeneity of variances and results from welch ANOVA were interpreted. The results showed no statistically significant difference (Welch´s $F(3, 3.70) = 1.85$, $p = 0.287$, along with Games-Howell post hoc ($p > 0.05$)) between the clades (Figure 9).

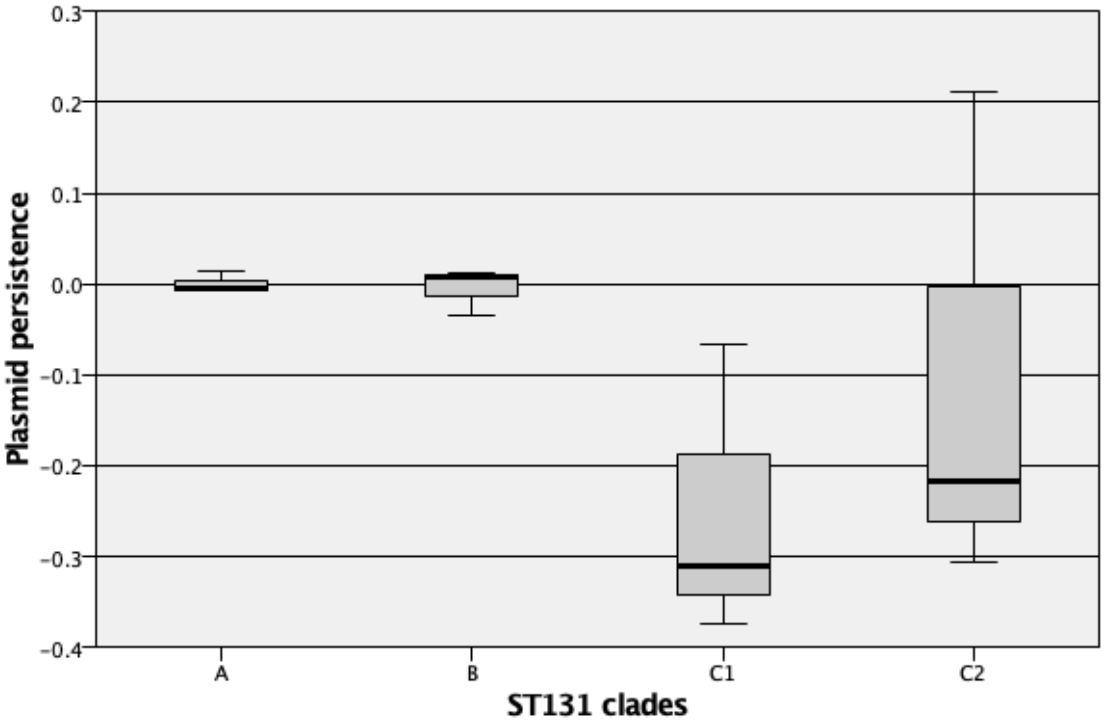


Figure 9. Distribution of plasmid persistence based on serial transfers for the different ST131 clades. The x-axis represents the different ST131 clades, and the y-axis represents the rate of plasmid persistence (slope obtained from the linear regression of the daily frequency of plasmid-carrying cells in the population). Each value in a boxplot represents the average of the plasmid persistence of each strain. For clades n = 3. Plasmid persistence increased from clade C1 (M = -0.25, SD = 0.16), to clade C2 (M = 0.10, SD = 0.28), to clade B (M = -0.01, SD = 0.03) to clade A (M = 0.00, SD = 0.11). Welch ANOVA with Games-Howell post hoc (p > 0.05) revealed no statistically significant difference in plasmid persistence.

4.5 Antibiotic susceptibility test

Antibiotic susceptibility test was conducted to determine whether the transconjugants (Appendix 5) had different levels of resistance to ampicillin, which could explain the low detection of transconjugants for clades C1 and C2 in media containing ampicillin 100 mg/L.

The quality control strain showed an MIC of 12 mg/L where the growth ellipse intersected MIC between 8-12 mg/L, as shown in Figure 10. Results showed that all strains were resistant, and had an MIC over 256 mg/L. However, all ST131 clade C2 strains and an ST131 clade C1 strain showed lower bacterial density in the area close to the ampicillin strips (Figure 11). The lower density of bacterial growth might indicate plasmid loss, also corresponding to the results obtained for the plasmid persistence.

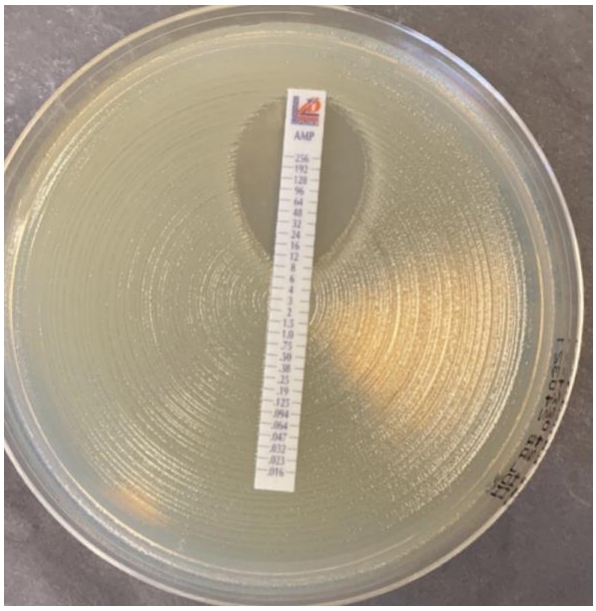


Figure 10 Antibiotic susceptibility test of a quality control, giving an MIC of 12 mg/L.

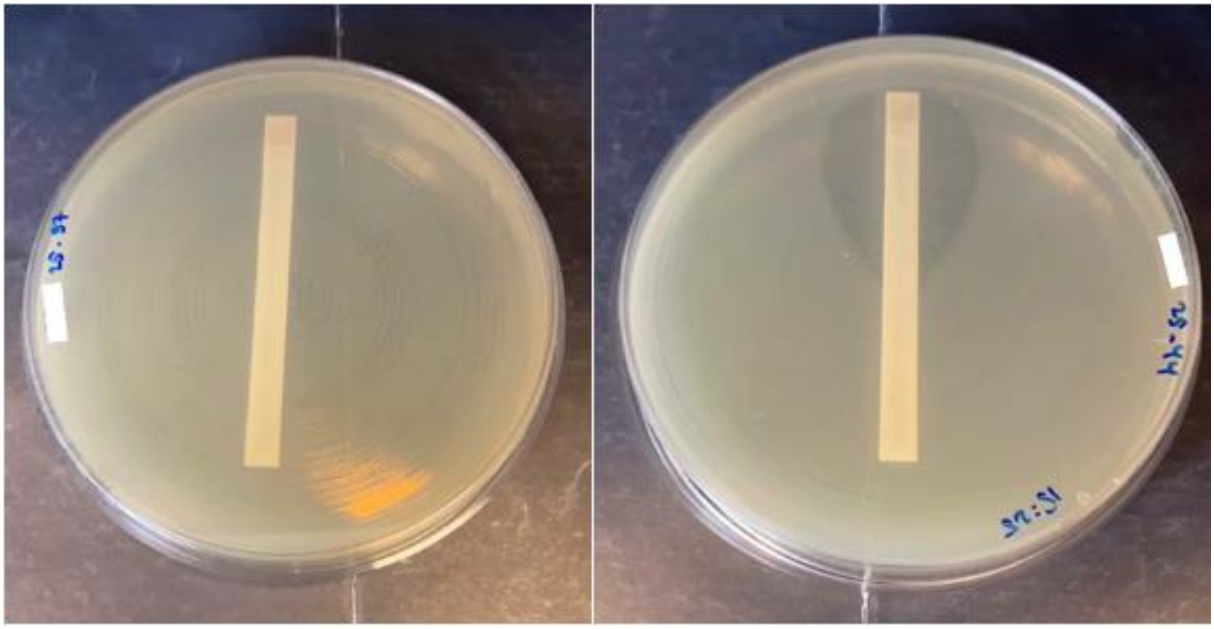


Figure 11. Antibiotic susceptibility test of two randomly selected strains, both giving an MIC above 256 mg/L. The picture on the left shows full density of growth, while the picture on the right shows an example of a strain giving less dense growth around the ampicillin strip.

4.6 Impact of ciprofloxacin resistance

Results above show a statistically significant difference in response of p50579417_3_OXA-48 acquisition between clade C strains and clade A and B strains. Since it is known that clade C is associated with FQ resistance, we wanted to determine whether this would impact the response shown by clade C. We therefore investigated the differences in conjugation efficiency and

plasmid persistence between a ciprofloxacin-sensitive ST73 strain and its three derivatives carrying one, two or three of the mutations required for ciprofloxacin resistance (55) (Table 1). The strains analyzed were MP04-02 without mutations, MP23-49 with a *gyrA*S83L mutation, MP23-50 with *gyrA*S83L and *parC*E84K mutations and, MP23-51 with *gyrA*S83L, *parC*E84K and *gyrA*D87G mutations.

4.6.1 Conjugation efficiency

A one-way ANOVA was conducted to determine whether the conjugation efficiency of ST73 was different from its mutants. Difference between the strains were analyzed based on their number of mutations: zero (n = 3), one (n = 3), two (n = 3) and three (n = 3). Boxplots showed no outliers although Shapiro-Wilk test showed that data was not normally distributed for strains with one mutation (p = 0.045) but normally distributed for the rest of the strains (p = 0.05). The assumption of equal variances was met, as assessed by Levene´s test of homogeneity of variances (p = 0.674). The analysis was continued by performing a one-way ANOVA followed by Dunnett's post hoc. The results of the one-way ANOVA (F (3,8) = 0.276, p = 0.841) and Dunnett´s post hoc (p > 0.05) showed no statistically significant difference between ST73 and its mutants (Figure 12).

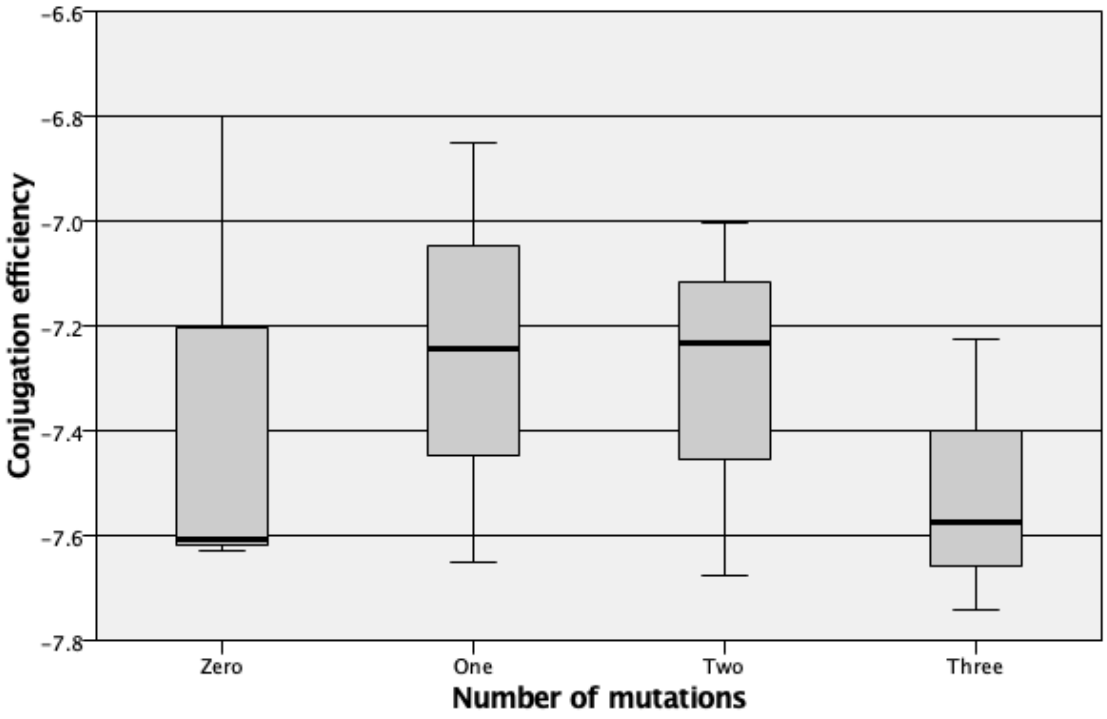


Figure 12. Distribution of conjugation efficiency based on the number mutations in each strain. The x-axis represents the different ST73 derivatives and the y-axis represents log10 (transconjugants/recipients). Each value in a boxplot represents the log10 of conjugation efficiency for each strain replicate with the corresponding number

of mutations. For all strains n = 3. Conjugation efficiency increased from three mutations (M = -7.51, SD = 0.0.26), to zero mutations (M = -7.34, SD = 0.47), to two mutations (M = -7.30, SD = 0.34) to one mutation (M = -7.25, SD = 0.40), in that order. One-way ANOVA with Dunnett´s posts hoc showed no statistically significant difference (F (3,8) = 0.276, p = 0.841) between the strains.

Moreover, a one-way ANOVA was conducted to determine whether the relative fitness differed between ST73 and its mutants. Boxplots showed no outliers and a Shapiro-Wilk test verified that data was normally distributed ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed equal variances ($p = 0.289$). Results showed that there was no statistically significant difference (F (3,8) = 0.755, $p = 0.550$, Dunnett´s post hoc ($p > 0.05$)) of relative fitness between ST73 and the strains conferring ciprofloxacin resistance (Figure 13).

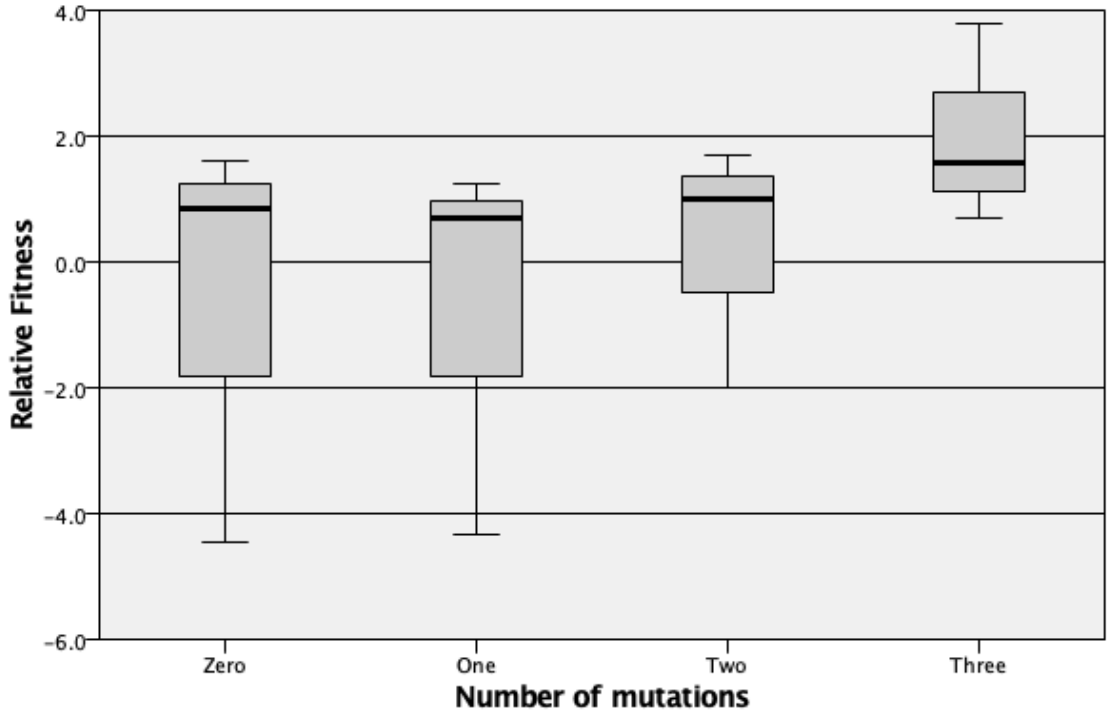


Figure 13. Distribution of relative fitness based on number of mutations in each strain. The x-axis represents the different ST73 derivatives and the y-axis represents their fitness relatively to the donor strain MP23-60. Each value in a boxplot represents the relative fitness for each strain replicate with the corresponding number of mutations. For all strains n = 3. Relative fitness increased from one mutation (M = -0.79, SD = 3.08) to no mutations (M = -0.67, SD = 3.31) to two mutations (M = 0.24, SD = 1.97) to three mutations (M = 2.03, SD = 1.60). One-way ANOVA with Dunnett´s post hoc showed no statistically significant difference (F (3,8) = 0.755, p = 0.550) between the strains.

4.6.2 Plasmid persistence

As mentioned in section 4.3.2, a replicate of MP04-02 (ST73) was excluded from the plasmid persistence assay, therefore reducing the sample size of MP04-02 to two strains.

4.6.2.1 Plasmid persistence of overnight cultures

A one-way ANOVA was conducted to determine whether the plasmid persistence in the sensitive ST73 was different from its mutant derivatives. Boxplot showed no outliers and a Shapiro-Wilk test showed that data was not normally distributed for 23-49 ($p = 0.031$) but was normally distributed for the other strains ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed equal variances ($p = 0.12$). The results show no statistical difference ($F(3,7) = 2.25$, $p = 0.170$, with Tukey´s post hoc ($p > 0.05$)) between ST73 and the mutants (Figure 14).

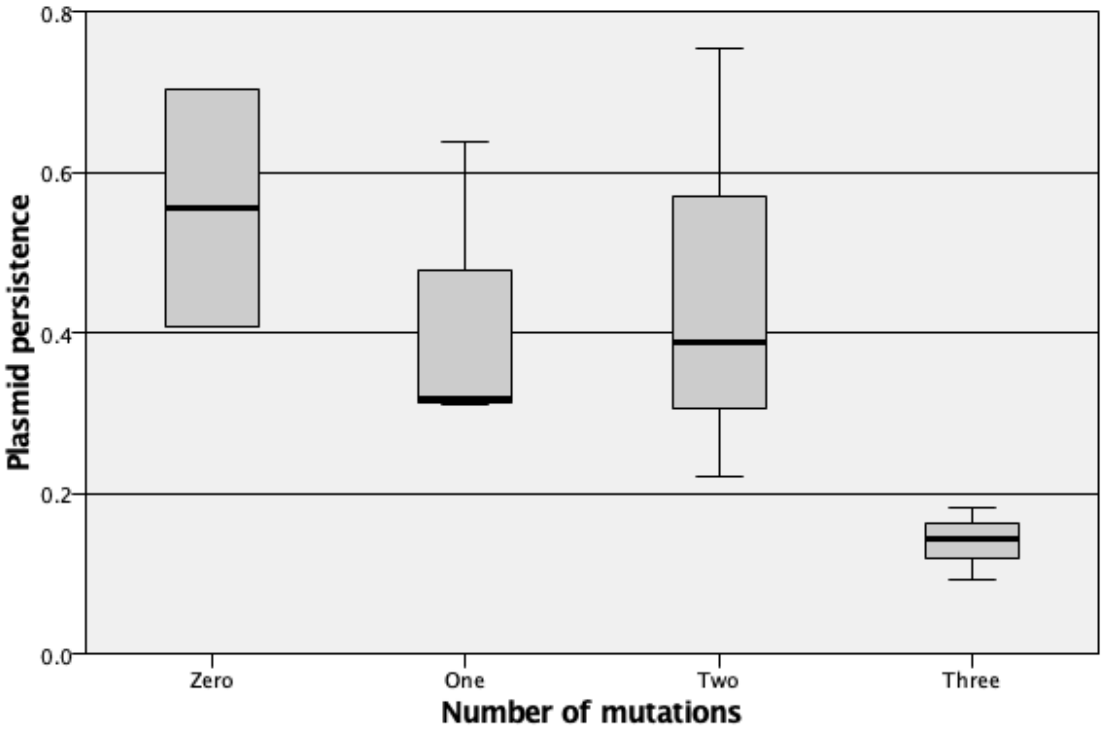


Figure 14. Distribution of plasmid persistence from overnight cultures based on number of mutations in each strain. The x-axis represents the different ST73 derivatives and the y-axis represents the frequency of plasmid-carrying cells in the population. Each value in a boxplot represents the plasmid persistence for each strain replicate with the corresponding number of mutations. For zero n = 2, one n = 3, two n = 3 and three = 3. The plasmid persistence increased from three mutations (M = 0.14, SD = 0.04), to one mutation (M = 0.42, SD = 0.19) to two mutations (M = 0.45, SD = 0.27) to no mutations (M = 0.56, SD = 0.21). One-way ANOVA with Dunnett´s post hoc showed no statistically significant difference (F (3,7) = 2.25, p = 0.170) between the strains.

4.6.2.2 Plasmid persistence from serial transfers

As Levene´s test for homogeneity of all strains showed heterogeneity ($p < 0.05$), multiple t-tests with Bonferroni corrections were run to determine difference between ST73 and its mutants conferring ciprofloxacin resistance (Figure 21).

Independent sample t-tests were performed between the *gyrA*S83L mutant MP23-49 and the triple (*gyrA*S83L, *parC*E84K and *gyrA*D87G) mutant MP23-51 (all n = 3) against the parental strain (n = 2). Boxplots showed no outliers for any of the strains. Shapiro-Wilk tests showed that data were normally distributed (p > 0.5). Homogeneity of variances was verified for each comparison by Levene´s test of homogeneity of variances (p > 0.05).

Results from the t-test (0.16 (95% CI, 0.07 to 0.24), *t*(3.00) = 5.91, p = 0.03) showed that there was a statistical significant difference in the plasmid persistence between the parental strain and the *gyrA*S83L mutant MP23-49. However, there was no statistical significant differences between the parental strain and the triple (*gyrA*S83L, *parC*E84K and *gyrA*D87G) mutant MP23-51 from the parental strain (t-test, 0.00 (95% CI, -0.73 to 0.73), *t*(3.00)= 0.01, p =1.00).

Shapiro-Wilk tests showed that data were not normally distributed for the double mutants (p = 0.030). Mann-Whitney U test was performed between the double (*gyrA*S83L and *parC*E84K) mutant MP23-50 (n = 3) against the parental strain (n = 2). Boxplots showed no outliers for any of the strains. Distribution of plasmid persistence were not similar as assessed by visual inspection. Results show no statistically significant difference between the double mutant MP23-50 (Mean rank = 2.0) and the parental strain MP04-02 (Mean rank = 2.0) (*U* = 0.00, z = -1.73, p = 0.6).

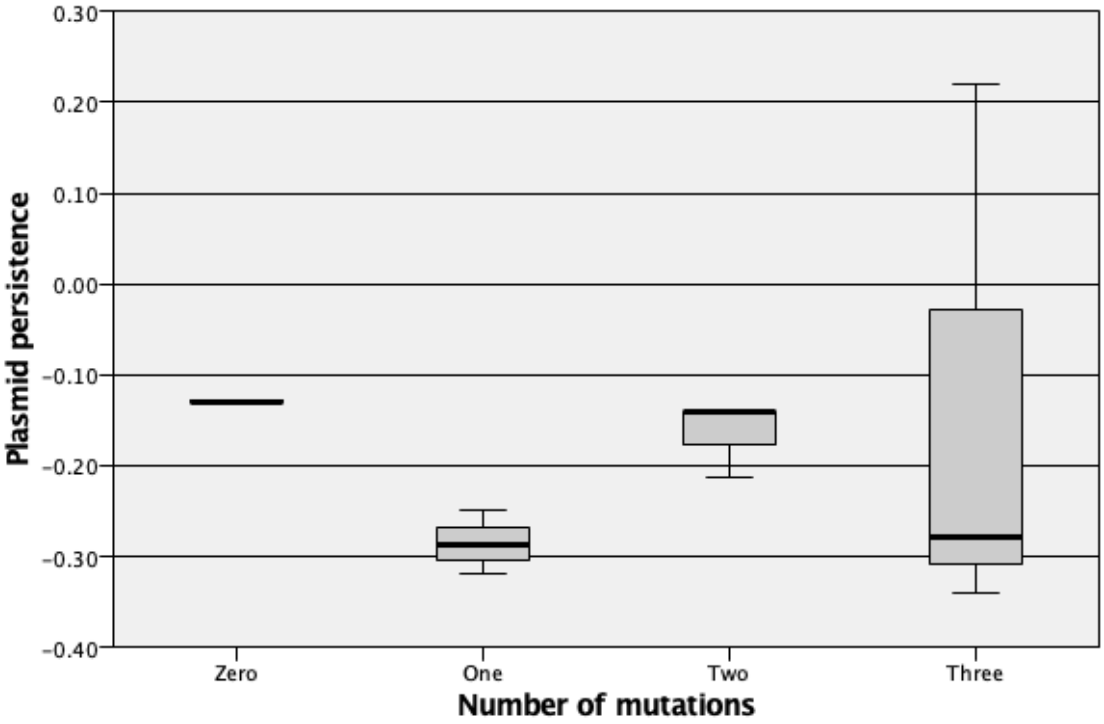


Figure 15. Distribution of plasmid persistence from serial transfers based on number of mutations in each strain. The x-axis represents the different ST73 derivatives and the y-axis represents the rate of plasmid persistence (slope obtained from the linear regression of the daily frequency of plasmid-carrying cells in the population). Each value in a boxplot represents the plasmid persistence for each strain replicate with the corresponding number of mutations. For zero n=2, one n=3, two n=3 and three=3. Plasmid persistence increased from one mutation (M= -0.29, SD= 0.04), to two mutations (M= -0.16, SD= 0.04), to three mutations (M= -0.13, SD= 0.31) to no mutation ((M= -0.13, SD=0.00). Multiple t-tests show statistically significant difference between zero and one mutations (0.16 (95% CI, 0.07 to 0.24), t(3.00)= 5.91, p =0.03), but no statistically significant difference between zero mutations and the other number of mutations.

These strains were ampicillin resistant (MIC > 256 mg/L) but all (except for one replicate) showed lower bacterial density in the area close to the ampicillin strips as reported for most clade C ST131 strains. The lower density of bacterial growth is in agreement with the high plasmid loss observed.

4.7 Determining difference between ST131 and other STs.

We wanted to understand how *E. coli* ST131 strains responded to p50579417_3_OXA-48 acquisition compared to other common STs. Statistical analyses was therefore conducted to determine if there were any differences in conjugation efficiency, fitness, and plasmid persistence. To determine whether it was possible to merge ST69, ST73 and ST95 into one group, analyses were performed to check if there were significant differences between them.

In addition to the clinical strains that were the focus of this thesis, we also included another ST73 strain, MP04-02, mentioned above in the section concerning ciprofloxacin resistant

mutants. Since we had to exclude MP23-27, the other ST73 strain, we were able to use the data retrieved from MP04-02 to represent ST73.

4.7.1 Conjugation efficiency

A one-way ANOVA was conducted to determine whether the conjugation efficiency differed between clinical isolates ST69 (n = 3), ST73 (n = 3) and ST95 (n = 4). Boxplots showed no outliers, but according to a Shapiro-Wilk test the data was not normally distributed for ST73 ($p = 0.045$), but normally distributed for the other STs ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed homogeneity of variances ($p = 0.105$). One-way ANOVA results (F (3,7) = 16.82, $p < 0.002$) showed that there was a statistic significance in conjugation efficiency between the groups. Tukey HSD post hoc showed that the conjugation efficiency of ST73 differed from those of the other STs (Figure 16). However, there was no statistically significant difference between ST69 and ST95. Due to the statistically significant difference between ST73 and the two other sequence STs, the groups were not merged, and each sequence type was therefore analyzed individually towards ST131.

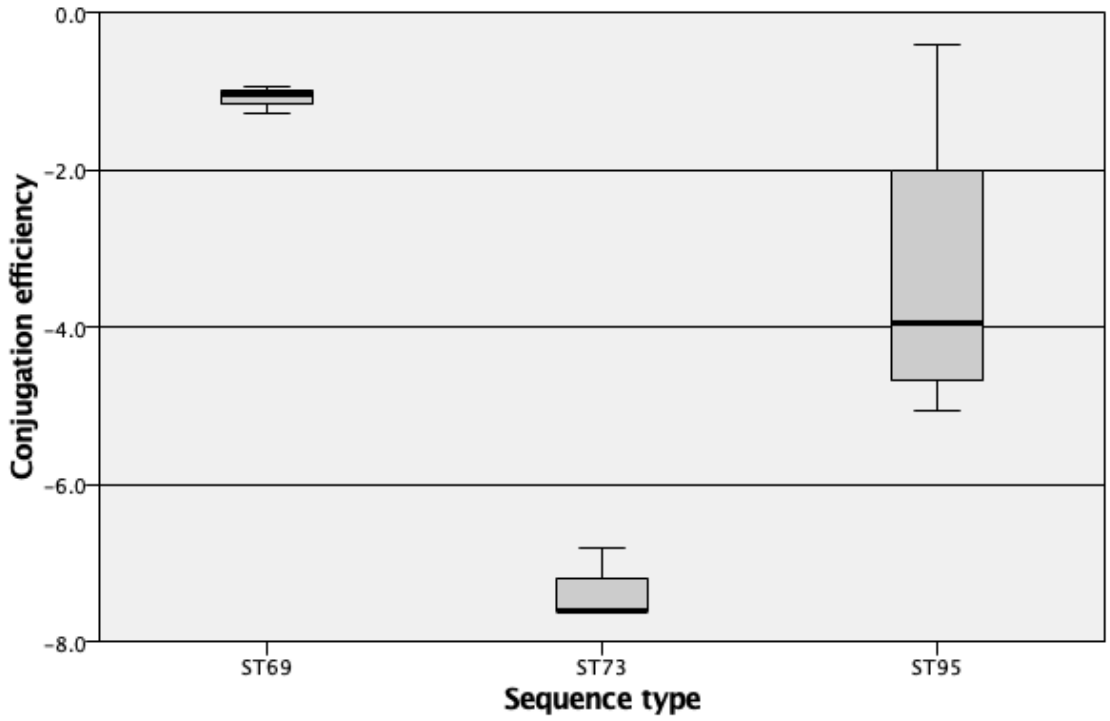


Figure 16. Distribution of conjugation efficiency for the different non-ST131 strains. Each value in a boxplot represents log10 of the conjugation efficiency of each strain replicate. The x-axis represents the different non-ST131 strains and the y-axis represents log10 (transconjugants/recipients). For ST69 n = 3, ST73 n = 3, and ST95 n = 4. Conjugation efficiency increased from ST73 (M = -7.34, SD = 0.47), to ST95 (M = -3.34, SD = 2.04), to ST69 (M = -1.08, SD = 0.18) in that order. One-way ANOVA and Tukey HSD post hoc revealed that conjugation efficiency increased significantly from ST73 to ST95 (4.00, 95% CI (0.93 to 7.07)), (p = 0.015) and ST73 to ST69 (6.26, 95% CI (2.98 to 9.58), (p = 0.002).

As explained in section 4.3.1, we calculated the conjugation efficiency of ST131 clade A in two different ways, thus we conducted two analyses for determining conjugation efficiency between ST131 and the other STs.

The first analysis was done based on the conjugation efficiency from the CFUs of the highest dilutions of clade A. Levene´s test of homogeneity showed inequal variances ($p < 0.05$), and multiple t-tests with Bonferroni corrections were therefore performed. In all cases, boxplots showed no outliers and Shapiro-Wilk tests verified that data were normally distributed ($p > 0.05$). For the comparison between ST131 ($n = 13$) and ST69 ($n = 3$), a Welch t-test was conducted due to heterogeneity of variances, as assessed by Levene´s test of homogeneity of variances ($p = 0.026$). The result showed a statistically significant increase (3.92 (95% CI, -4.96 to -2.88), $t(13.04)= -8.15$, $p < 0.001$) from ST131 to ST69. For the two remaining comparisons, homogeneity of variances was verified by Levene´s test of homogeneity of variances ($p > 0.05$) and independent sample t-tests were run. The results showed a statistically significant increase (2.34 (95% CI, 0.17 to 4.50), $t(14.00)= 2.31$, $p = 0.036$) from ST73 to ST131, but no significant difference between ST131 and ST95 (-1.66 (95% CI, -3.82 to 0.49), $t(15.00) = -1.65$, $p = 0.36$). Results are shown in Figure 17.

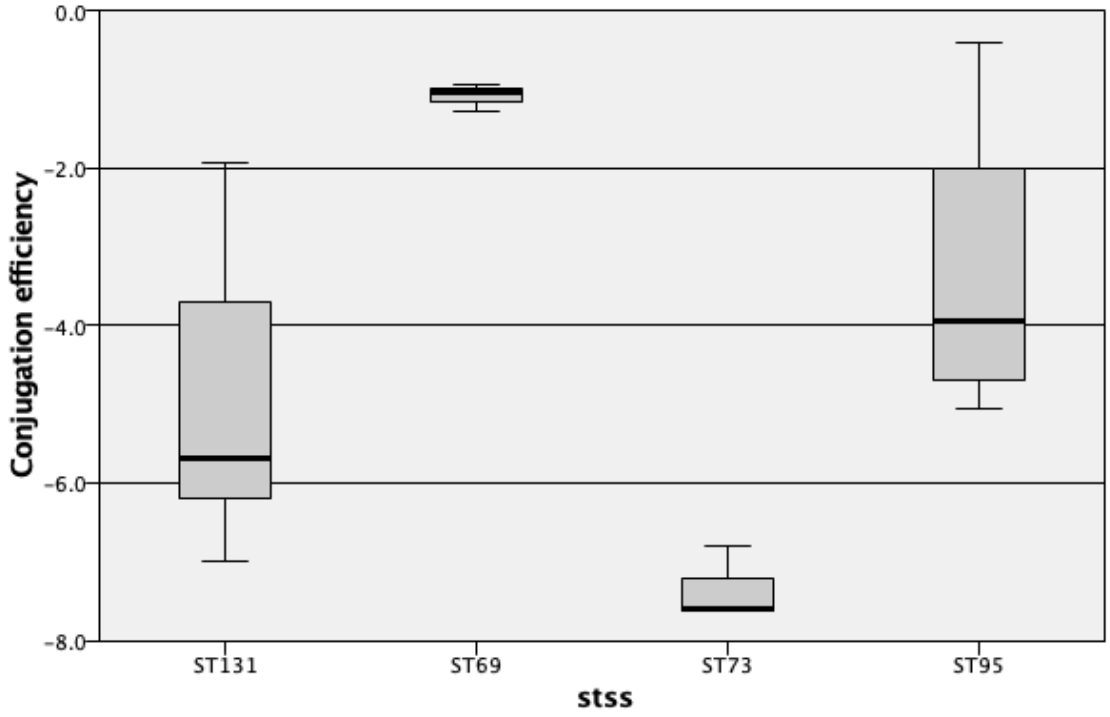


Figure 17. Distribution of conjugation efficiency among STs, where conjugation efficiency of ST131 is based on the highest dilutions of clade A. The x-axis represents the different STs and the y-axis represents log10 (transconjugants/recipients). For ST131 strains, each value in a boxplot represents average log10 of the conjugation efficiency of each strain. For the non-ST131 strains, each value in a boxplot represents the average log10 of the conjugation efficiency of each strain replicate. For ST131 n = 13, ST69 n = 3, ST73 n = 3, and ST95 n = 4. Conjugation efficiency increased from ST73 (M = -7.34, SD = 0.47), to ST131 (M = -5.00, SD = 1.70) to ST95 (M = -3-34, SD =2.04), to ST69 (M = -1.08, SD = 0.18) in that order. Multiple t-tests revealed that increase from ST131 to ST69 (3.92 (95% CI, -4.96 to -2.88), t*(13.04) = -8.15, p < 0.001) and decrease from ST131 to ST73 (2.34 (95% CI, 0.17 to 4.50),* t*(14.00)= 2.31, p = 0.036) were statistically significant.*

The second analysis was done based on the conjugation efficiency of CFUs from the lowest dilutions of clade A. Levene´s test of homogeneity of variances showed equal variances (p = 0.198), and a one-way Anova with Dunnett´s post hoc was therefore conducted with ST131 as control group. A Shapiro-Wilk test showed that data was not normally distributed for ST73 (p = 0.045) but normally distributed for the other STs (p > 0.05). Boxplots showed two outliers for ST131. A one-way ANOVA was conducted despite outliers on ST131 and because data for ST73 were borderline normal. The conjugation efficiency was statistically significantly different between the STs (F (3, 19) = 13.32, p < 0.001). Dunnett´s post hoc with ST131 as control showed a statistically significant increase from ST131 to ST69 and ST95 (Figure 18).

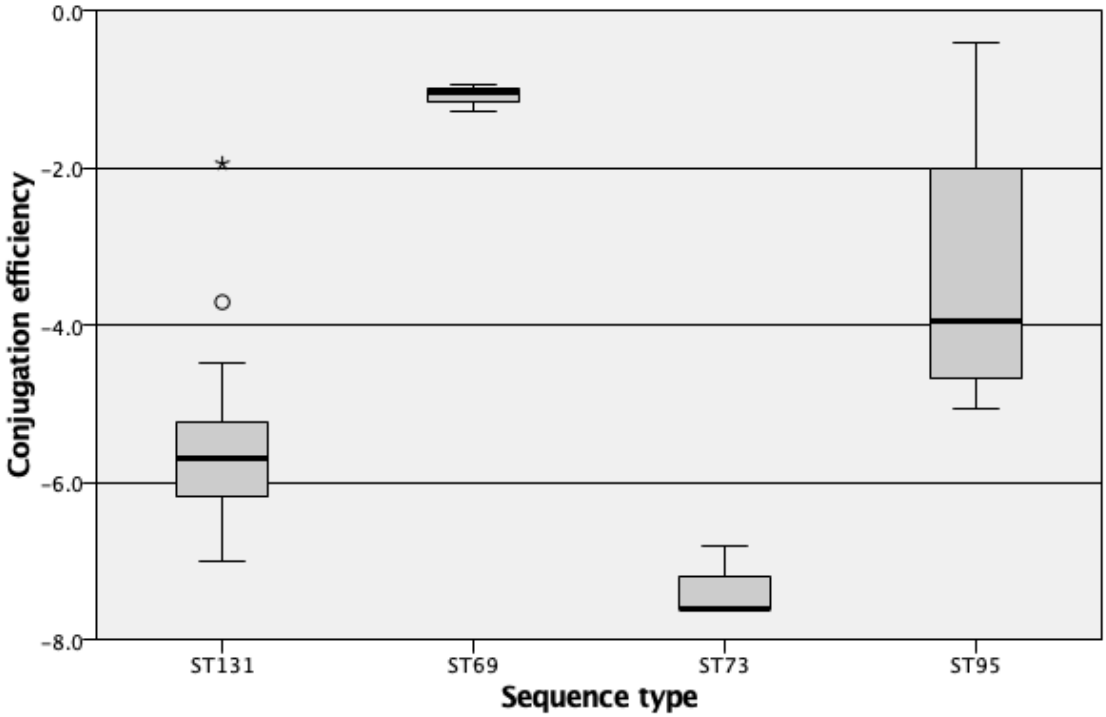


Figure 18. Distribution of conjugation efficiency among STs, where conjugation efficiency of ST131 is based on the lowest dilutions of clade A. The x-axis represents the different STs and the y-axis represents log10 (transconjugants/recipients). For ST131 strains, each value in a boxplot represents average log10 of the conjugation efficiency of each strain. For the non-ST131 strains, each value in a boxplot represents the average log10 of the conjugation efficiency of each strain replicate. Asterix (*) represent an outlier that is 3 boxlengths from the edge of the box, while a circle represents an outlier that is 1.5 boxlengths from the edge. For ST131 n =13, ST69 n = 3, ST73 n = 3, and ST95 n = 4. Conjugation efficiency increased from ST73 (M= -7.34, SD= 0.47) to ST131 (M= -5.35, SD=1.34), to ST95 (M = -3.34, SD = 2.04), to ST69 (M = -1.08, SD = 0.18). One-way ANOVA and Dunnett´s post hoc revealed that conjugation efficiency increased significantly from ST131 to ST69 (4.27, 95% CI (2.02 to 6.51)), (p < 0.001) and from ST131 to ST95 (2.011, 95% CI (0.003 to 4.01)), (p = 0.05), with the last increase being borderline significant.

4.8 Fitness

A one-way ANOVA was conducted to determine whether the relative fitness differed between clinical isolates ST69 (n = 3), ST73 (n = 3) and ST95 (n = 4). Boxplots showed no outliers and a Shapiro-Wilk test showed that data was not normally distributed for ST73 (p = 0.03) but was normally distributed for the other strains (p > 0.05). Furthermore, Levene´s test of homogeneity of variances showed homogeneity of variances (p = 0.064). The results (F (2, 7) = 2.48, p = 0.154) with Tukey´s post hoc showed no statistically significant difference of relative fitness between the sequence types (Figure 19). These sequence types were therefore merged into one group, non-ST131, for further analysis against ST131.

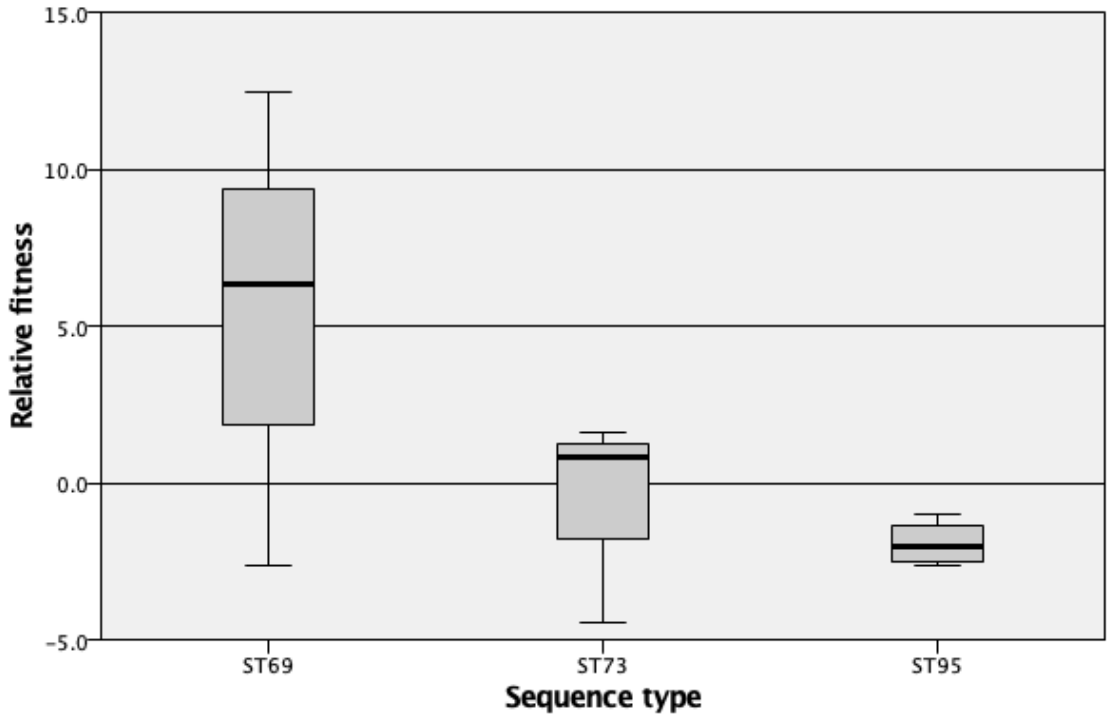


Figure 19. Distribution of relative fitness for non-ST131 strains. The x-axis represents the different non-ST131 strains and the y-axis represents their fitness relatively to the donor strain MP23-60. Each value in a boxplot represents the relative fitness of each strain replicate. For ST69 n = 3, ST73 n = 3 and ST95 n = 4. The relative fitness increased from ST95 (M = -1.90, SD = 0.73) to ST73 (M = -0.66, SD = 3.31) to ST69 (M = 5.39, SD = 7.61). One-way ANOVA and Tukey HSD post hoc (p > 0.05) revealed no statistically significant difference in relative fitness.

Boxplots showed outliers on both groups, and Shapiro-Wilk´s test showed that data was not normally distributed for non-ST131 (p = 0.028), but normally distributed for ST131 (p = 0.159). Distribution of relative fitness were not similar as assessed by visual inspection. A Mann-Whitney U test was performed to determine if there was a difference in relative fitness between ST131 (n = 13) and non-ST131 (n = 10). Results showed no statistically significant difference between ST131 and non-ST131, U = 66.00, z = 0.062, p =1.00 (Figure 20).

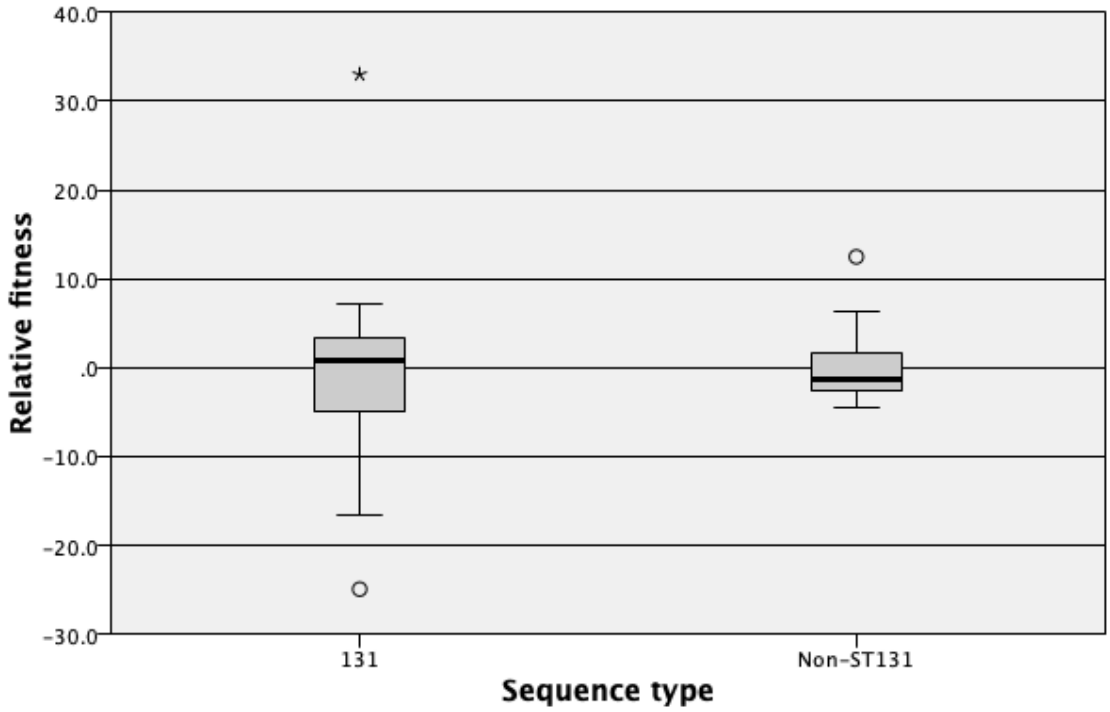


Figure 20. Distribution of relative fitness for ST131 and non-ST131. The x-axis represents the ST and the y-axis represents their fitness relatively to the donor strain MP23-60. For ST131, each value in a boxplot represents the average relative fitness of each strain. For non-ST131, each value in a boxplot represents the relative fitness for each strain replicate. ST131 (n = 13) and non-ST131 (n = 10). Asterix (*) represent an outlier that is 3 boxlengths from the edge of the box, while a circle represents an outlier that is 1.5 boxlengths from the edge. Mann-Whitney U test showed no statistically significant difference in mean ranks for ST131 (11.92) and nonST131 (12.10).

4.9 Plasmid persistence

As mentioned in section 4.3.2 an MP04-02 (ST73) replicate was excluded from the plasmid persistence assays, reducing the sample size of ST73 to two strains.

4.9.1 Based on overnight cultures

A one-way ANOVA was conducted to determine whether the plasmid persistence was statistically significantly different between ST69 (n = 3), ST73 (n = 2) and ST95 (n = 3). Boxplots showed no outliers and a Shapiro-Wilk test showed that data was normally distributed ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed equal variances ($p = 0.06$). The results (F (2,5) = 3.4, $p = 0.117$) with Tukey´s post hoc ($p > 0.05$) show no statistically significant difference (Figure 21). The STs can therefore be merged into one group, non-ST131, when determining difference between ST131 and non-ST131.

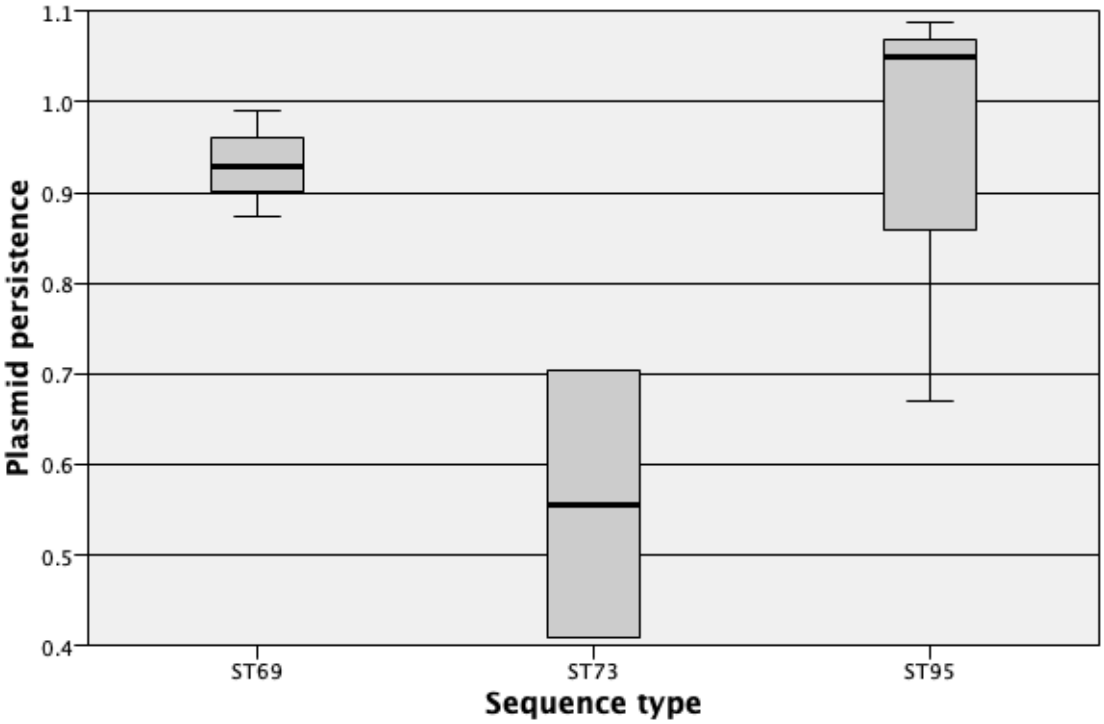


Figure 21. Distribution of plasmid persistence measured from overnight cultures for the different non-ST131 STs. The x-axis represents the different non-ST131 and the y-axis represents the frequency of plasmid-carrying cells in the population. Each value in a boxplot represents the plasmid persistence of each strain replicate. For ST69 n = 3, ST73 n = 2 and ST95 n =3. The plasmid persistence increased from ST73 (M=0.56, SD= 0.21), to ST69 (M= 0.93, SD=0.06), to ST95 (M=0.94, SD= 0.23). One-way ANOVA and Tukey HSD post hoc (p>0.05) revealed no statistically significant difference in relative fitness.

Shapiro-Wilk tests showed that data were not normally distributed for ST131 (p = 0.008), but normally distributed for non-ST131 (p = 0.439). Mann-Whitney U test was performed between ST131 (n = 12) and non-ST131 (n = 8). Boxplots showed no outliers in for any of the strains. Distribution of plasmid persistence were not similar as assessed by visual inspection. Results show no statistically significant difference between ST131 and non-ST131 (U = 28.00, z = -1.54, p = 0.14) (Figure 22).

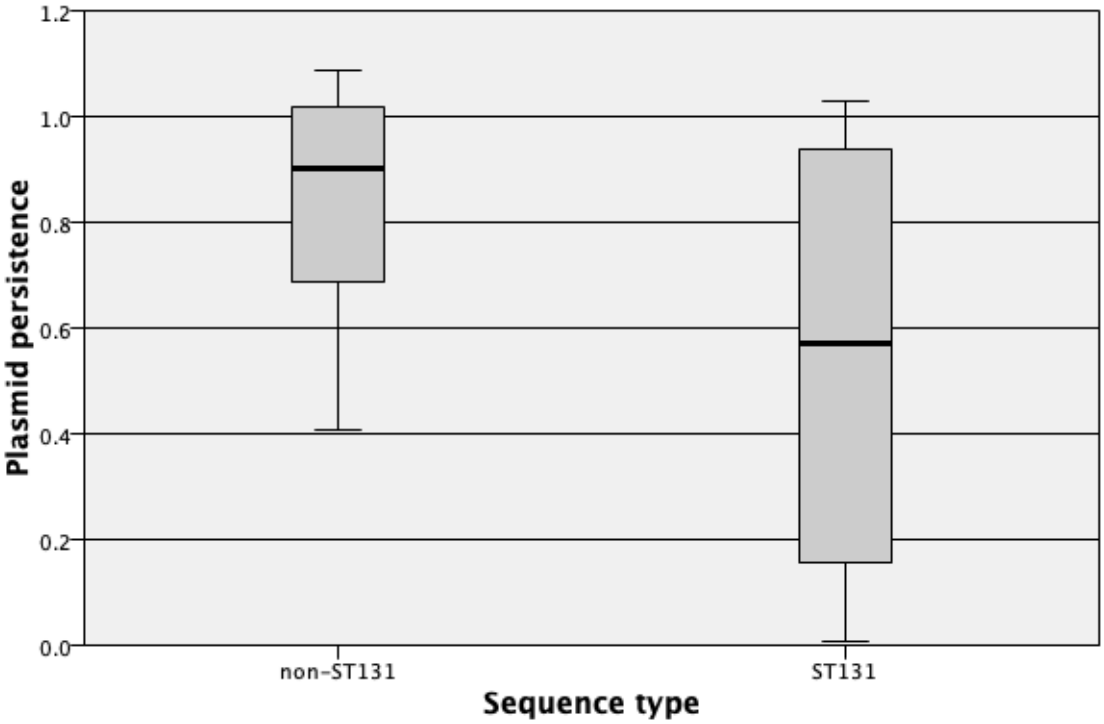


Figure 22. Distribution of plasmid persistence measured from overnight cultures for ST131 and non-ST131. The x-axis represents the different non-ST131 and the y-axis represents the frequency of plasmid-carrying cells in the population. For ST131, each value in a boxplot represents the average plasmid persistence of each strain. For non-ST131, each value in a boxplot represents the plasmid persistence for each strain replicate. ST131 (n = 12) and non-ST131 (n = 8). Mann-Whitney U test showed no statistically significant difference in mean ranks for ST131 (8.83) and non-ST131 (13.00) (U = 28.00, z = -1.54, p= 0.14).

4.9.2 Measured from serial transfers

A one-way ANOVA was conducted to determine whether the plasmid persistence differed between clinical isolates ST69 (n = 3), ST73 (n = 2) and ST95 (n = 3). Boxplots showed no outliers and a Shapiro-Wilk test verified that data was normally distributed ($p > 0.05$). Furthermore, Levene´s test of homogeneity of variances showed equal variances ($p = 0.078$). Results showed that there were statistically significant differences of plasmid persistence between the sequence types (F (2,5) = 42.16, $p < 0.001$). Multiple comparisons by Tukey´s post hoc showed statistically significant increase from ST73 to ST69 and ST95 (Figure 23). These sequence types were therefore not merged into one group and were analyzed separately against ST131.

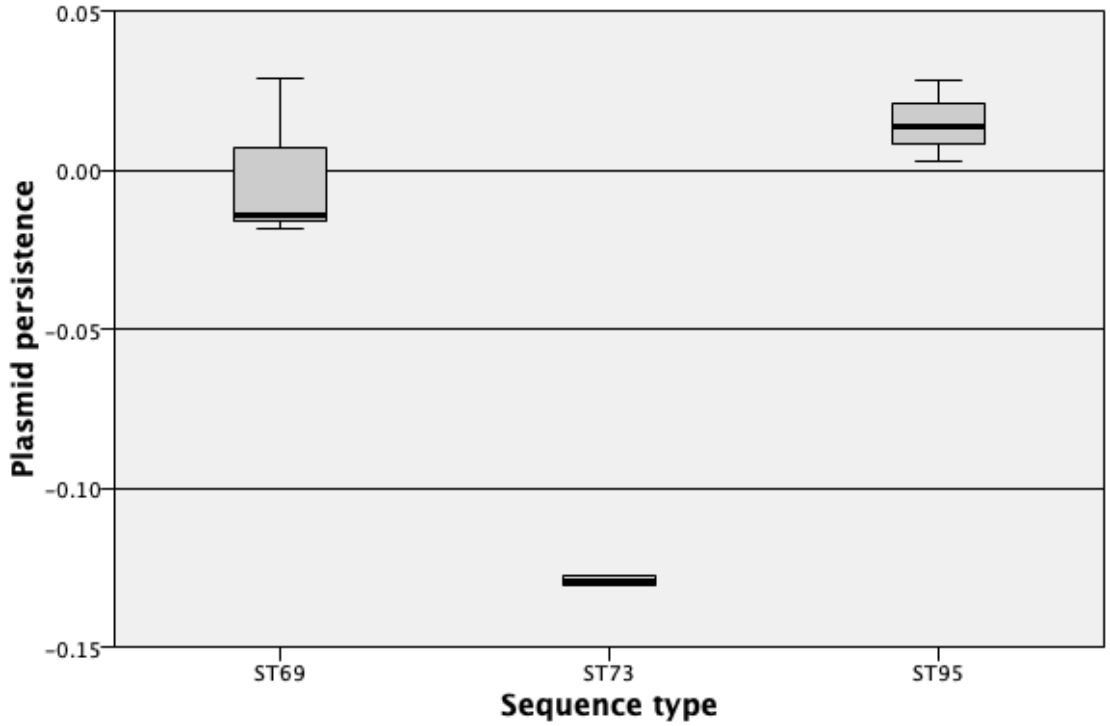


Figure 23. Distribution of plasmid persistence measured from overnight cultures for non-ST131 strains. The x-axis represents the different non-ST131 and the y-axis represents the rate of plasmid persistence (slope obtained from the linear regression of the daily frequency of plasmid-carrying cells in the population). Each value in a boxplot represents the plasmid persistence of each strain replicate. Plasmid persistence increased from ST73 (M = -0.13, SD = 0.002), to ST69 (M = -0.001), SD = 0.03) to ST95 (M = 0.015, SD=0.01). One-way Anova with Tukey´s post hoc showed statistically significant increase from ST73 to ST69 (0.13, 95% CI (0.07 to 0.18), (p=0.001) and ST73 to ST95 (0.14, 95% CI (-0.20 to -0.09), (p < 0.001).

A welch t-test was conducted for ST131 (n = 12) and ST69 (n = 3) due to heterogeneity of variances as assessed by Levene´s test of homogeneity of variances (p = 0.042). Boxplots showed no outliers and a Shapiro-Wilk test verified that data was normally distributed (p > 0.05). Results from the t-test (-0.09 (95% CI, -0.20 to 0.02), $t(12.50) = -1.70$, p = 0.35) (Figure 24) showed that there was no statistical significant difference between the plasmid persistence in ST131 and ST69.

An independent sample t-test was run for ST131 (n = 12) and ST73 (n = 2). Boxplots showed no outliers and a Shapiro-Wilk test verified that data was normally distributed (p > 0.05), although no data was obtained for ST73. Equal variance was assessed by Levene´s test of homogeneity of variances (p = 0.057). Results from the t-test (0.04 (95% CI,-0.24 to 0.32) (Figure 24), $t(12.00) = 0.31$, p = 1.00) showed that there was no statistical significant difference in the conjugation efficiency between the ST131 and ST73.

A welch t-test was conducted for ST131 (n = 12) and ST95 (n = 3) due to heterogeneity of variances as assessed by Levene´s test of homogeneity of variances (p = 0.029). Boxplot showed no outliers and a Shapiro-Wilk test showed that data was normally distributed (p > 0.05). Results from the t-test (-0.10 (95% CI,-0.22 to 0.01), *t*(11.44) = -2.07, p = 0.31) (Figure 24) showed that there was no statistical significant difference between the plasmid persistence of ST131 and ST95.

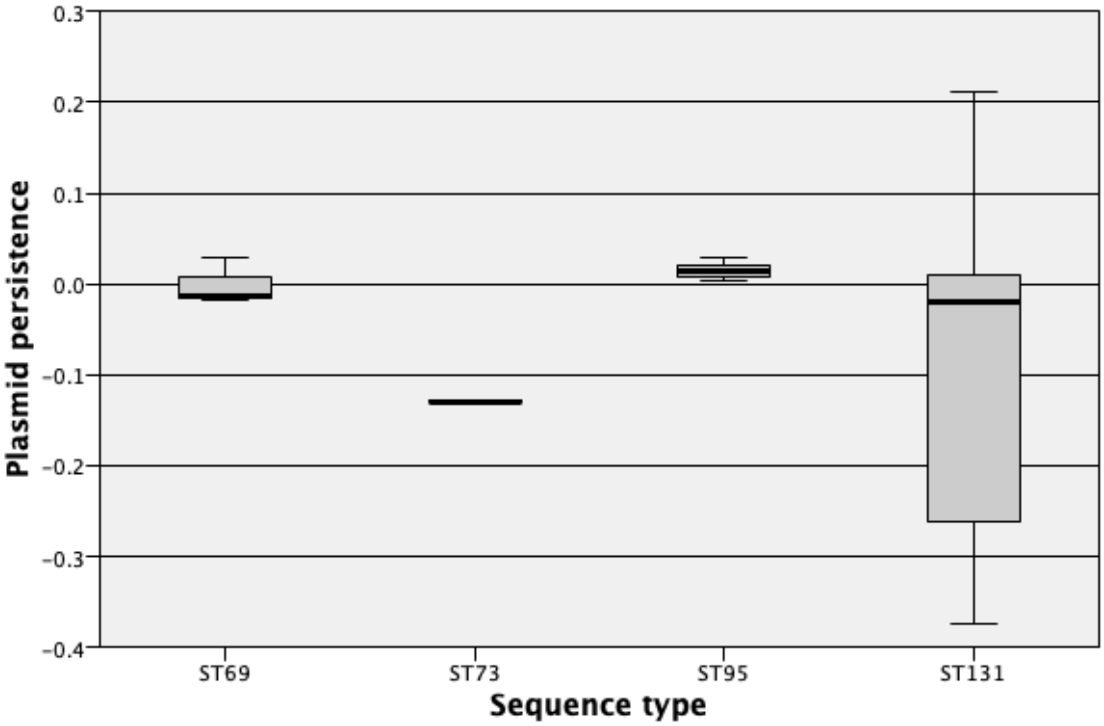


Figure 24. Distribution of plasmid persistence based on serial transfers for the different STs. The x-axis represents the different STs and the y-axis represents the rate of plasmid persistence (slope obtained from the linear regression of the daily frequency of plasmid-carrying cells in the population). For ST131, each value in a boxplot represents the average plasmid persistence of each strain. For non-ST131, each value in a boxplot represents plasmid persistence in each strain replicate. For ST131 n = 12, ST69 n = 3, ST73 n = 2, ST95 n = 3. Plasmid persistence increased from ST73 (M = -0.13, SD = 0.002), to ST131 (M = -0.09, SD = 0.17), to ST69 (M = -0.001), SD = 0.03) to ST95 (M = 0.015, SD = 0.01). Multiple t-tests show no statistically significant differences between ST131 and the other STs (p > 0.05).

5 DISCUSSION

The aim of this study is to understand how *E. coli* ST131 responds to the acquisition carbapenemase-encoding plasmids (p50579417_3_OXA-48 and pK71-77-1-NDM) and compare them to other common STs such as ST69, ST73 and ST95. We therefore investigated differences in conjugation efficiency and plasmid persistence among ST131 clades, and between ST131 and non-ST131.

5.1 General discussion

Among *E. coli* ST131, clade A strains acquired p50579417_3_OXA-48 more efficiently than the other clades, but there was no difference in plasmid persistence when measured by serial transfers. However, plasmid persistence was higher for strains from clade A and B than C1 and C2 when measured directly from the overnight culture. Although all ST131 transconjugants were resistant to ampicillin, we generally observed a lower bacterial density around the ampicillin strip for strains of clade C, more frequently for C2. This result support lower plasmid stability in the clade.

It has been proposed that the ability of ST131 clade C to offset the cost of acquiring MDR plasmids is the cause of it being a dominant drug-resistant successful lineage (5). However, the data obtained in this thesis suggest that clade A strains acquire p50579417_3_OXA-48 more efficiently and that this is not due to differences in the relative fitness amongst strains of the different clades. Furthermore, the data suggest that the plasmid is less stable in strains of clade C than clades A and B, and therefore indicating that it may not be able to maintain the plasmid to the same extent.

A study from Ellaby et al. (56) for the emergence of diversity in CP *E. coli* in England showed that the OXA-48 producers were most often found in clade A. Furthermore, the study revealed that OXA-48 found in clade A was associated with IncL/M replicons. The findings from the study, combined with the results from this thesis indicate that clade A might be more prone to acquire and maintain plasmid-encoded OXA-48, compared to the other clades.

Other studies have also shown the widespread of FQR clade C and it has been hypothesized to be the major driver of clade C's success as a dominant lineage (2). Our results suggest that ciprofloxacin resistance had no impact on clade C strains' fitness and p50579417_3_OXA-48

acquisition. However, this resistance may affect plasmid stability. Therefore, using an ST73 strain, we tested how these mutations affected plasmid stability. Our results show that the *gyrA*S83L mutation alone decreased plasmid persistence, but two or three mutations altogether no longer affected plasmid maintenance. Therefore, ciprofloxacin resistance does not explain the lower plasmid persistence observed in clade C.

ST131 strains acquired p50579417_3_OXA-48 less efficiently than the ST69 strain, but more efficiently than the ST73 strain, despite no observed fitness difference between strains. Other studies have also observed ST73 to have had lower plasmid acquisition (57). Furthermore, there was no difference in plasmid persistence among ST131 and non-ST131. As observed for ST131 clade C2, two ST73 transconjugants showed lower bacterial density around the ampicillin strip. However, the third transconjugant was resistant with high density growth surrounding the ampicillin strip, although the presence for p50579417_3_OXA-48 was not confirmed in this transconjugant. This would indicate that the *bla*$_{\text{OXA-48}}$ gene could be incorporated in the chromosome. This could also further support the suggestion that lower density of bacterial growth could be explained by plasmid loss.

E. coli ST131 has in recent years shown to be a globally dominant cause of human infections and is now a worldwide pandemic clone (18, 19). It is predicted that *E. coli* ST131 is apt for the acquisition of carbapenem-resistance encoding plasmids (3). As for *bla*$_{\text{OXA-48}}$, our study does not observe any dominance for ST131's acquisition and maintenance of p50579417_3_OXA-48 when compared to other common STs. Studies have also shown that clade C is the most dominant lineage of ST131 (22). As mentioned above, we see that clade C show lower plasmid acquisition and maintenance, which might explain why ST131 does not differ in the success of harboring *bla*$_{\text{OXA-48}}$ compared to other common STs.

5.2 Troubleshooting

We experienced some issues throughout the thesis, mainly concerning the CFUs obtained from the conjugation assays.

Two of the clade A strains, MP15-01 and MP15-03 showed CFUs that were not consistent across dilutions (Table 8). Due to the pattern seen in all 5 biological replicates, we speculate if the cause for the pattern might have been cell clumping. The risk of cell clumping would

decrease with higher dilution, as the number of cells would decrease. This might also explain why the highest dilution (10^{-5}) show a 10-fold decrease from the previous dilution (10^{-4}).

Due to the inconsistency issue, we wanted to understand if the choice of dilution for the conjugation efficiency of MP15-01 and MP-03 would impact the main results. The conjugation efficiency was therefore estimated twice: one calculation based on the CFUs from the two highest dilutions and another from the two lowest dilutions. The results from the highest dilutions showed that clade A strains acquired p50579417_3_OXA-48 more efficiently than the other clades, and that ST131 strains differed from ST69 and ST73 strains. However, results from the lowest dilutions show no difference among clades, while ST131 strains differed from ST69 and ST95 strains. The difference in outcomes between the highest and lowest dilutions show that the choice of dilutions from the clade A strains had an impact on the main findings. Although the hypothesis for cell clumping was neither tested nor confirmed, the discussion of main results was mainly based on the conjugation efficiency from CFUs of the highest dilutions. This is due to the thought of decreased risk of cell clumping in higher dilutions.

Conjugation with a clade C2 strain, MP15-13, as a recipient did not obtain any transconjugants. Results from the initial plating for donor and recipient CFUs from all three biological replicates showed no donor colonies. It can be speculated that MP15-13 may be a phage or toxin producing strain, causing the cell lysis of the donor cells, which in turn prevents conjugation to occur. Pilot tests for phage and toxins have been conducted for the strains, but due to time restrictions this was not further investigated in this thesis.

5.3 Limitations of this study

The main limitation of this study was the conjugation assay. We conducted several methods to achieve detectable CFUs for both p50579417_3_OXA-48 and pK71-77-1-NDM but had to exclude pK71-77-1-NDM as we did not achieve detectable transconjugants for most strains.
We continued with the conjugation assay for p50579417_3_OXA-48, although the method resulted in low number of transconjugants for several strains. The considered countable range for CFUs is 25-200 or 30-300 (58), reducing the risk of CFUs being skewed. As we obtained CFUs outside the limits for several strains, we consider the calculations as the estimated CFUs. Overall, the low number of transconjugants obtained contributed to a lower confidence in conjugation efficiency analysis.

To avoid this limitation, we could have attempted using another donor in the conjugation assay, which might have contributed to an increase in number of transconjugants obtained.

6 CONCLUSION AND FUTURE ASPECTS

In this study, we aimed to understand how *E. coli* ST131 responded to acquisition of carbapenemase-encoding plasmids (p50579417_3_OXA-48 and pK71-77-1-NDM) and compare them to other common STs such as ST69, ST73 and ST95. We therefore investigated differences in conjugation efficiency and plasmid persistence among ST131 clades, and between ST131 and non-ST131.

E. coli ST131 has in recent years shown to be a globally dominant cause of human infections and is now a worldwide pandemic clone (18, 19) and studies have shown that clade C is the most dominant lineage of ST131. Our study suggests that *E. coli* ST131 clade C is less efficient in p50579417_3_OXA-48 acquisition and has lower plasmid persistence compared to *E. coli* ST131 clades A and B. Furthermore, although evidence from other studies suggest that *E. coli* ST131 is altogether fit to acquire and maintain carbapenemase genes and plasmids (3), our study does not observe any dominance for ST131´s acquisition and maintenance of p50579417_3_OXA-48 when compared to other common STs.

Further research for *E. coli* ST131´s response to acquisition of $bla_{\text{OXA-48}}$ encoding plasmids is essential to better understand the difference in response among clades. In elucidating a possible reason for ST131 clade C´s difference in behavior from the other clades, our study was limited to the possible impact of FQR. We therefore further suggest research investigating other factors that might possibly affect the acquisition and persistence of $bla_{\text{OXA-48}}$ encoding plasmids in ST131, such as restriction modification systems. Furthermore, the study of $bla_{\text{NDM-1}}$ encoded plasmids was excluded in this thesis, and further research for *E. coli* ST131´s behavior regarding acquisition of $bla_{\text{NDM-1}}$ encoding plasmids is yet to be conducted. Clarification of factors affecting spread of carbapenemase-encoding plasmids in *E. coli* ST13 is essential to able to predict future successful *E. coli* ST131 plasmid combinations. We also hypothesized the production of phages in one of our ST131 strains, and further research of phage production in *E. coli* ST131 may contribute to the understanding for its success as clone.

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8 SUPPLEMENTARY DATA

Appendix 1 Results from MLST, CHtyper and SerotypeFinder for clinical strains.

Strains	MLST	CHtype	Serotype
MP15-01	131	40-41	H5:O16
MP15-03	131	40-41	H5:O16
MP15-04	131	40-22	H4:O25
MP15-05	131	40-22	H4:O25
MP15-06	131	40-22	H4:O25
MP15-07	131	40-30	H4:O25
MP15-08	131	40-30	H4:O25
MP15-09	131	40-30	H4:O25
MP15-10	131	40-30	H4:O25
MP15-11	131	40-30	H4:O25
MP15-12	131	40-30	H4:O25
MP15-13	131	40-30	H4:O25
MP15-14	131	40-30	H4:O25
MP23-07	131	40-41	H5:O16
MP23-25	69	35-27	H18 O:17/44/77
MP23-27	73	24-30	H1:O6
MP23-30	95	38-30	H7:O1

Appendix 2 Results from Plasmidfinder and ResFinder for clinical strains.

Strains	Plasmids	Resistence
MP15-01	Col-like, IncFIB, IncFII	Tetracycline
MP15-03	Col-like, IncFIB, IncFII	Folate pathway antagonist, macrolide, tetracycline
MP15-04	3 Col-like, IncFIB, IncFII	Tetracycline
MP15-05	Col-like, IncFIB, IncFII	Tetracycline
MP15-06	Col-like, IncFIB, IncFII	Tetracycline
MP15-07	4 Col-like, IncFIA, IncFIB, IncFII, IncX4	Fluoroquinolone
MP15-08	Col-like, IncFIA, IncFIB, IncFII	Fluoroquinolone, tetracycline
MP15-09	2 Col-like, IncFIA, 2 IncFIB, IncFII	Folate pathway antagonist, macrolide, tetracycline, fluoroquinolone
MP15-10	Col-like, IncFIA, IncFIB, IncFII	Fluoroquinolone, tetracycline
MP15-11	2 Col-like, IncFIA, IncFIB, IncFII	Fluoroquinolone, tetracycline
MP15-12	Col-like, IncFIA, IncFIB, IncFII	Folate pathway antagonist, macrolide, tetracycline, fluoroquinolone
MP15-13	2 Col-like, IncFIA, IncFIB	Folate pathway antagonist, macrolide, tetracycline, fluoroquinolone
MP15-14	Col-like, IncFIA, 2IncFIB, IncFII	Folate pathway antagonist, macrolide, tetracycline, fluoroquinolone
MP23-07	3 Col-like, IncFIA, IncFIB, IncFII, IncQ	Folate pathway antagonist
MP23-25	Col-like	None
MP23-27	No plasmid	None
MP23-30	Col-like	None

Appendix 3 Primers used in this thesis.

Primer (reference)	Sequence 5´to 3´ (ref)	Usage	Reference
IncL/M forward	GGGTGAAAAATATCTCCAGTGGCGA	Confirm presence of p50579417_3_OXA-48	Adapted from (59, 60), 11 nucleotides (nt) different
IncL/M reverse	CTGCAGGGGCGATTCTTTAGG		(59, 60)
IncA/C_2 forward	GAGAACCAAAGACAAAGACCTGGA	Confirm presence of pK71-77-1-NDM	(59, 60)
IncA/C_2 reverse	ACGACAAACCTGGATTGCTTCCTT		Adapted from (59, 60), 11 nt different
IncFIA forward	CCATGCTGGTTCTAGAGAAGGTG	Confirm absence of plasmid IncFIA on MP09_76	(59, 60)
IncFIA reverse	GTATATCCTTACTGGCTTCCGCAG		(59, 60)
IncB/O/K/z forward	TCAGCGTTCCGGCATCTTCAC	Confirm absence of plasmid IncB/O/K/z on MP10-31	Adapted from (59, 60), 6 nt different
IncB/O/K/z reverse	ACGATCCGGAAAGTCAGAAAAC		Adapted from (59, 60), 3 nt different

IncFIB_2 forward	ATCCAAATTGACGTCAGGCC	Confirm absence of plasmid IncFIB_2 on MP10-31	Unpublished
IncFIB_2 reverse	GCAGAATATTGACGCCAGCA		Unpublished
IncX4 forward	AGCAAACAGGGAAAGGAGAAGACT	Confirm absence of plasmid IncX4 on 10_31	(61)
IncX4 reverse	TACCCCAAATCGTAACCTG		(61)
IncFII forward	CTGATCGTTTAAGGAATTTT	Confirm absence of plasmid IncFII on 10_31	(62)
IncFII reverse	CACACCATCCTGCGCTTA		(62)
rpoS forward	AGTCAGAATACGCTGAAAGTTCATG	Distinguish between MG1655 and clinical strains	Unpublished
ygbN reverse	GGTTGCCAAAATATCGCCACTC		Unpublished
lacZ forward	GTTTAAACAGTGAGCGCAACGCAATTAATG	Confirm presence of *lacZ*	Unpublished
lacZ reverse	GTTTAAACTACCATTCGCCATTCAGGCTGCG	Confirm presence of *lacZ*	Unpublished

Appendix 4 List of excluded strains from conjugation efficiency and plasmid persistence

Strains	Replicate	Excluded from	Reason
MP15-07	1	Plasmid persistence assay	Transconjugant not confirmed by phenotype characterization, nor by PCR amplification of *rpoS-ygbN region*
MP15-12	All	Plasmid persistence assay	Presence of p50579417_3_OXA-48 confirmed for one replicate only.
MP15-13	All	Conjugation efficiency and plasmid persistence	No transconjugants obtained.
MP23-27	All	Conjugation efficiency and plasmid persistence	Only obtained transconjugants for one replicate.
MP04-02	1	Plasmid persistence assay	PCR did not confirm presence of p50579417_3_OXA-48

Appendix 5 New strains obtained from conjugation assay.

Parental strain	**Transconjugant strains**	**Notes**
MP15-01	MP25_01-MP25_03	ST 131 clade A
MP15-03	MP25_04-MP25_06	ST 131 clade A
MP15-04	MP25_07-MP25_09	ST 131 clade B
MP15-05	MP25_10-MP25_12	ST 131 clade B
MP15-06	MP25_13-MP25_15	ST 131 clade B
MP15-07	MP25_16-MP25_17, MP25_59	ST 131 clade C1
MP15-08	MP25_18-MP25_20	ST 131 clade C1
MP15-09	MP25_21-MP25_22, MP25_60	ST 131 clade C1
MP15-10	MP25_23-MP25_25	ST 131 clade C2
MP15-11	MP25_26-MP25_28	ST 131 clade C2
MP15-12	MP25_29	ST 131 clade C2
MP15-14	MP25_30-MP25_32	ST 131 clade C2
MP23-07	MP25_33-MP25_35	ST 131 clade A
MP23-25	MP25_36-MP25_38	ST69
MP23-27	MP25_39-MP25_40	ST73
MP23-30	MP25_41-MP25_42, MP25_61	ST95
MP04-02	MP25_43-MP25_45	ST73 K56-02
MP23-49	MP25_46-MP25_48	ST73 K56-02 *gyrA*S83L
MP23-50	MP25_49-MP25_51	ST73 K56-02 *gyrA*S83L *parC*E84K
MP23-51	MP25_52-MP25_54	ST73 K56-02 *gyrA*S83L *parC*E84K *gyrA*D87G

Appendix 6 Calculated results for conjugation efficiency, relative persistence.

Strains	Conjugation efficiency log10 -highest dilutions (SD)	Conjugation efficiency log10 -lowest dilutions (SD)	Relative fitness (SD)	Plasmid persistence (overnight) (SD)	Plasmid persistence (serial transfers) (SD)
MP15-01	-2.96, (0.13)	-5.32, (0.38)	2.04 , (7.37)	0.93, (0.03)	-0.01, (0.03)
MP15-03	-2.3,(0.26)	-4.47, (0.37)	-13.54,(23.30)	0.94, (0.04)	0.01, (0.02)
MP15-04	-5.33, (0.32)	-5.33, (0.32)	0.46, (0.09)	0.94, (0.07)	0.01, (0.02)
MP15-05	-5.96, (0.22)	-5.96, (0.22)	5.96, (6.81)	0.87, (0.08)	0.01, (0.02)
MP15-06	-5.69, (0.36)	-5.69, (0.36)	1.30, (2.70)	0.95, (0.48)	-0.03, (0.08)
MP15-07	-3.70, (0.95)	-3.70, (0.95)	3.36, (8.99)	0.05, (0.06)	-0.07, (0.26)
MP15-08	-5.83, (0.29)	-5.83, (0.29)	-16.66, (27.06)	0.22, (0.15)	-0.37, (0.19)
MP15-09	-6.51, (0.60)	-6.51, (0.60)	-3.16, (30.11)	0.11, (0.12)	-0.31, (0.11)
MP15-10	-6.42, (0.29)	-6.42, (0.29)	0.86, (0.17)	0.28, (0.04)	-0.22, (0.08)
MP15-11	-6.99, (0.05)	-6.99, (0.05)	7.09, (4.80)	0.01, (0.00)	0.21, (0.26)
MP15-12	-5.24, (1.12)	-5.24, (1.12)	-4.94, (7.44)	Excluded	Excluded
MP15-13	Excluded	Excluded	Excluded	Excluded	Excluded
MP15-14	-6.18 (0.49)	-6.18 (0.49)	33.02, (50.48)	0.20, (0.16)	-0.31, (0.08)
MP23-07	-1.94, (0.16)	-1.94, (0.16)	-24.92, (44.52)	1.03, (0.09)	-0.01, (0.04)
MP23-25 replicate one	-1.29	-1.29	-2.64	0.99	-0.01
MP23-25 replicate two	-0.93	-0.93	12.49	0.93	-0.02
MP23-25 replicate three	-1.03	-1.03	6.32	0.87	0.03
MP23-27 replicate one	Excluded	Excluded	Excluded	Excluded	Excluded
MP23-27 replicate one	Excluded	Excluded	Excluded	Excluded	Excluded
MP23-27 replicate one	Excluded	Excluded	Excluded	Excluded	Excluded
MP23-30 replicate one	-0.42	-0.42	-0.98	1.09	0.01

MP23-30 replicate two	-3.58	-3.58	-2.35	0.67	0.03
MP23-30 replicate three	-4.31	-4.31	-1.67	1.05	0.00
MP23-33 replicate four	-5.06	-5.06	-2.61	*Excluded	*Excluded
MP04-02 replicate one	-7.63	-7.63	-4.46	Excluded	Excluded
MP04-02 replicate two	-7.60	-7.60	0.85	0.70	-0.13
MP04-02 replicate three	-6.80	-6.80	1.62	0.41	-0.13
MP23-49 replicate one	-7.24	-7.24	-4.33	0.64	-0.25
MP23-49 replicate two	-7.65	-7.65	0.71	0.31	-0.32
MP23-49 replicate three	-6.85	-6.85	1.26	0.32	-0.29
MP23-50 replicate one	-7.23	-7.23	-1.99	0.75	-0.21
MP23-50 replicate two	-7.68	-7.68	1.01	0.22	-0.14
MP23-50 replicate three	-7.00	-7.00	1.71	0.39	-0.14
MP23-51 replicate one	-7.58	-7.58	3.81	0.09	0.22
MP23-51 replicate two	-7.74	-7.74	0.70	0.14	-0.34
MP23-51 replicate three	-7.22	-7.22	1.58	0.18	-0.28