Molecular and biochemical characterisation of naturally occurring hyperexpressed and mutated extended spectrum AmpC β-lactamases in Norwegian clinical isolates of *Escherichia coli*

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CONTENTS

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ABBREVIATIONS

AM	Ampicillin
AT	Aztreonam
bp	base pairs
BURST	Based upon Related sequence type
CP buffer	Caption buffer
CS	Constant sequense
CT/CTL	Cefotaxim/clavulanic acid
ESBL	Extended spectrum β-lactamases
EtBr	Ethidium bromide
EXO	Exonuclease
FX	Cefoxitin
Hgt	Horisontal gene transfer
IEF	Isoelectric focusing
IP	Imipenem
IS	Insertion sequence
LB	Luria Bertani
MIC	Minimum Inhibitory Concentration
MLST	Multi locus sequence typing
MP	Meropenem
pl	isoeletic points
Pais	Pathogenicty islands
PBPs	Penicillin - binding - protein
PFGE	Pulsed - Field Gel electrophoresis
PM/PML	Cefepim/clavulanic acid
PP/Ptc	Piperacillin/tazobactam
SAP	Shrimp Alkaline Phosphatase
SLVs	singel locus variants
ST	Sequence type
TZ/TZL	Cefetazidim/clavulanic acid
XL	Amoxicillin/clavulanic acid

XM Cefpodoxim

SUMMARY

The prevalence of clinical *E. coli* isolates showing an AmpC phenotype with reduced susceptibility to cephalosporins is increasing worldwide. In *E. coli*, which contains the wild-type gene, hyperexpression is related to mutations in the promoter or in the attenuator regions. The most frequently described mutations change the promoter to resemble the *E. coli* consensus promoter or weaken the attenuator.

In this thesis, clinical *E. coli* isolates expressing reduced susceptibility to 3rd and 4th generation cephalosporins, isolated in Norway from 2003 through 2007, was characterised by sequencing their *ampC* gene control and gene coding region. 51 isolates were shown to have alterations in the *ampC* gene control region, while four isolates showed no alterations in the gene control region. Seven isolates were identified carrying an IS-element. Further studies of these by isoelectric focusing (IEF), real-time qRT-PCR (qRT-PCR), pulsed-field gel electrophoresis (PFGE), and multi locus sequence typing (MLST) revealed that they hyperexpressed AmpC enzymes and four of them were clonally related to each other. Further investigations of the four strains without promoter alterations characterising their gene coding region showed that all had unique mutations. IEF confirmed their production of AmpC enzymes, while qRT-PCR revealed that they did not hyperexpress these proteins. MLST studies identified two strains associated with the same STC complex. The cloning experiment did not give us any clear answers to why the four isolates without promoter mutations express reduced susceptibility to 3rd and 4th generation cephalosporins.

1. INTRODUCTION

Antimicrobial agents

Antimicrobial or antibacterial agents have derived their names from the term antibiotic. This term refers to natural metabolic products of fungi, actinomycetes and bacteria killing or inhibiting growth of microorganisms. Production of antibiotics is associated with natural soil microorganisms using the products in competition of space and nutrients with other microorganism. Sir Alexander Fleming was the first one to discover the bactericidal mechanism of penicillin in 1928, over 80 years ago. The observation of a mould inhibiting the growth of *Staphylococcus aureus* on a contaminated agar plate happened by chance. The mould was identified as a *Penicillum notatum* and Fleming gave the inhibitory substance the name penicillin [1]. Dr Howard W Florey and his colleagues in England were the first who managed to produce and purify the substance so it could be used for treatment purposes in 1941 [2]. From 1945 and up to 1980 numerous of new classes of antimicrobials were discovered with an increasing rate. In the 1980s and 1990s only already known classes were improved, and the search for new agents is still ongoing today. The process of finding new effective agents has an average time line of 10 years. Random screening of soil microorganisms was the technique used, but this method is more and more replaced by a rational design program [3].

Antimicrobial agents are either semi-synthetic or synthetic. Semi-synthetic agents are derived from natural products being chemically modified to enhance the efficacy. Synthetic agents like sulfonamides and quinolones are produced entirely from chemicals in laboratories [3].

Antibacterial agents can be classified in three different ways; by chemical structure, by target site, or according to whether they are bactericidal or bacteriostatic. Classification by the bactericidal or bacteriostatic activity can be vague, because some antibacterial agents have bactericidal effect against one bacterium, but only a bacteriostatic effect against the other. Characterisation by the chemical structure is useless alone because of the diversity among agents. Classification by target site is more convenient because it helps in understanding the molecular basis of antibacterial action. And the target sites differ from the hosts' mechanisms

to a greater or lesser extent so that the agents won't affect or be toxic to the patient infected. The five major mechanisms for actions are (i) inhibition of cell wall synthesis, (ii) inhibition of the cytoplamsmic membrane, (iii) inhibition of protein synthesis, (iv) inhibition of nucleic acid synthesis and (v) inhibition of folic acid synthesis [3].

Table 1.1. Classification of antimicrobials by target site

Target site	Antimicrobials
Inhibition of bacterial cell wall synthesis	Penicillins Carbapenems Cephalosporins Monobactams Glycopeptides Bacitracin
Inhibition of cytoplasmic membrane	Polymyxins
Inhibition of protein synthesis	Chloramphenicol Macrolides Lincosamides Steroid antibiotics Oxazolidinoses Aminoglycosides Tetracylines
Interference of nucleic acid synthesis	Quinolones Nitroimidazoles Rifampicin
Inhibition of folic acid synthesis	Sulphonamides Trimethoprim

Antimicrobial resistance

In 1941 when the first penicillin was taken into use in therapy the optimism that it could kill any bacterial infection was huge. This optimism regarding the new super-drug was soon shattered, as reports on resistant bacteria in hospitals arose shortly after the introduction. Resistance occurred mainly in the hospital acquired infections. Infections acquired in the community were unaffected. Studies during 1950 and 1960 reported that multidrug resistance could be transferred to susceptible recipient cells [4, 5]. As new antimicrobial agents were discovered, and taken into use, the bacteria responded by manifesting various forms of resistance. Over the years the use of antimicrobial agents increased and the resistance mechanisms utilised by pathogenic bacteria also increased in level and complexity [6]. Bacteria are still developing new enhanced mechanisms to survive the attack from our agents, while laboratories developing new ones decrease. This has become a global health problem as mechanisms for resistance have been reported for all known antimicrobials currently available for clinical use in human and veterinary medicine [5].

A bacterium's susceptibility and resistance to antibiotics can be defined in two ways; from a clinical point of view and from a microbial point of view. In a clinical point of view a bacterium is defined as resistant if it is not killed or inhibited by the maximum dose of a given antimicrobial agent. It's defined as susceptible if it responds to the therapy, and if the bacterium is not susceptible or resistant it's defined as intermediate resistant. This means that it might be removed if the concentration of the antibiotic is increased. In a microbial point of view a resistant bacterium possess resistance mechanisms expressed phenotypically or genotypically, whereas a susceptible bacterium lacks these properties [7].

Mechanisms of resistance

The main biochemical resistances mechanisms in bacteria are divided into four groups; (i) decreased import and increased export of the drug (mutated porins, up-regulated efflux pumps), (ii) structural alterations of the drug target (mutations, enzymatic modification), (iii)

hyperexpression or overproduction of the drug target, (iv) production of drug modifying or degrading enzymes (β-lactamase) [7, 8].

Resistance mechanisms in bacteria can either be an intrinsic property of the bacterium or acquired. Intrinsically resistant bacteria are naturally resistant where examples of properties are impermeability and lack of a susceptible target. This type of resistance has no additional genetic alterations and applies to the whole specie. Acquired resistance occurs by chromosomal mutations (deletions, point mutations, inversions, insertions etc.) or by acquisition of new DNA through horizontal gene transfer (HGT) [8].

Mutations

Chromosomal mutations occur randomly during the DNA replication at a very low frequency in all bacterial cells. The results of these mutations can be altered antibiotic targets, drug-inactivation, up- or down-regulation of efflux systems, and loss or inactivation of porins. Most of these mutations only affect one antimicrobial class but mutations in porins and efflux channels may contribute to resistance against several antibiotics [8, 9].

Acquisition of new DNA

Initially susceptible bacteria can acquire antibiotic resistance from genes encoding resistance through mechanisms of HGT like conjugation, transformation and transduction. Transfer can occur between strains of the same species and between species or genera. Mobilizable elements carrying these genes are plasmids, transposons, bacteriophages or chromosomal DNA fragments called pathogenicity islands (PAIs) [10].

Conjugation is the process when a donor bacteria transfer DNA, a self transmissible or mobilizable plasmid, to a recipient bacteria. This mechanism involves a separation of the mobile element into two strands where one of the strands move into the recipient, and both strands functions as templates replicating a complete double stranded molecule in each bacterium after the transfer. The recipient bacterium is called a transconjugant after it has received DNA from the donor. Other DNA in the cell can also be transferred along with the self-transmissible plasmid or DNA element. Gram-negative bacteria transfer plasmids through a pilus, an elongated proteinaceus structure, to adjacent bacteria. Gram-positive bacteria may produce sex pheromones making mating cells clump together to allow plasmid exchange [6, 10, 11].

Transformation involves the uptake of naked DNA, where DNA is released from the donor into the environment after cell lysis and taken up by a recipient cell, to become the transformant. If the new DNA is incorporated with its chromosomal DNA, a recombinant type of that bacterium will occur. Some bacteria are naturally transformable taking up environmental DNA by themselves while other bacteria need chemical or electrical treatment to become transformable. The uptake of DNA usually happens during a certain stage in the life cycle of bacteria that are naturally competent. 40 species are identified as naturally transformable today, including both Gram-negative (*Haemophilus influenza, Helicobacter pylori*) and Gram-positive bacteria (*Bacillus subtilis, Streptococcus pneumonia*). This mechanism is widely used in molecular genetics being the most convenient way to introduce new or altered DNA into a cell [6, 10, 12].

Transduction mechanism involves a bacteriophage transferring resistance genes from one bacterium to another. A transducing phage multiplies and accidently pack bacterial DNA into the phage head. Strains infected by the phage also receive the bacterial resistance genes. There are two types of transduction; generalised transduction transfers any region of DNA and specialised transduction transfers only DNA close to the attachment site of a lysogenic phage in the chromosome. This event rarely occurs because the incident of a phage packing host DNA by mistake is rare and transduced DNA must survive in the recipient cell to form a stable transductant [6, 10].

Resistance elements

Plasmids are extra-chromosomal genetic elements found in virtually all bacterial cells. They are self-replicating, transferable and mostly double-stranded covalently closed circular elements. Cells can contain several different plasmids, which can exist in multiple copies, differing in size, host range, transmission and their modes of replication. Even though plasmids are self-replicating they are dependent on both self-encoded and host-encoded factors in order to duplicate their genetic material. Plasmid copy number is defined by the number of copies of a certain plasmid in the cell directly after cell division. Some plasmids have a natural high copy number, while others have a lower copy number. Controlling

initiation of replication and partitioning is important so the plasmids are not lost or becomes a burden for the bacterial cell. Plasmids are classified into different incompatibility (Inc) groups according to their ability to coexist stably in the same cell. Those not able to coexist are the ones sharing common replication or partitioning systems, belonging to the same Inc-group. This results in an inability to control the copy number and plasmids may be cured from its host cell [13]. Plasmids are excellent cloning vectors as they are easy to purify, do not kill the host cell and can be made relatively small [10]. In a plasmid cloning vector, the inserted DNA will be replicated along with the vector, yielding many copies (clones) of the wanted DNA fragment [10, 13, 14].

Transposons are DNA elements able to move, transpose, from one place to another in the bacterial genome. Transposons exist in all organisms and the moving mechanism is called transposition. They have little or no target specificity facilitating insertion into any place in the genome. There are several different types of transposons, but generally they contain a transposase promoting transposition, inverted repeats in the ends, and short direct repeats of target DNA bracketing the transposons. Insertion sequence (IS) elements, composite transposons and non-composite transposons are all types of transposons able to carry around resistance genes from the chromosome to plasmids or the other way around. Conjugative transposons are another type containing genes for conjugative transfer from one bacterium to another. They are promiscuous transferring resistance genes between both Gram-negative and Gram-positive bacteria. Transposition is highly regulated and only occurs rarely [10, 15].

Integrons are genetic elements integrated in transposons found on groups of plasmids and in the bacterial chromosome. These gene capturing systems are evolved from site-specific recombination mechanisms, and a general integron encode a DNA integrase gene (*int*) and an adjacent recombination site (*att1*) [16]. Structurally they comprise two terminal invariable regions, constant sequences (CS), and a variable central section. In the variable site there's inserts of variable resistance genes integrated as gene cassettes. The gene cassettes are integrated in the attachment site (attI) of the integron which can contain many cassettes at once [14]. They can promote resistance to a wide variety of antibiotics (multiresistance). Integrons are unable to move by themselves, but their gene cassettes can be mobilized to other integrons or to secondary sites in the bacterial genome [16]. However integrons are often found as part of transposons and/or plasmids and can thus be mobilised. Integrons are mainly

found in gram-negatives like *Enterobacteriaceae* and plays an important part in the spread of antibiotic resistance [17].

β-lactam antibiotics

β-lactam antibiotics are one of the most important groups of antimicrobial agents administered worldwide, covering as much as 50% of all prescribed drugs. This is because they are highly effective and the side-effects are minimal. The β-lactams belongs to the group of antimicrobial agents inhibiting the cell wall synthesis. They have a bactericidal effect on the microorganisms and are classified into several groups; penicillins, cephalosporins, cephamycins, carbapenems, monobactams, and the β-lactamase inhibitors. The different groups consist of both natural and synthetic compounds varying in their chemical structure with the β-lactam ring as the common property. Each group have different ring structures and acyl side chains attached to the β-lactam ring [3, 7, 18].

Mechanism of action

Cell wall synthesis in bacteria is dependent on the penicillin-binding-protein (PBPs) enzymes transpeptidases, carboxypeptidases and transglycosylases. These enzymes complete the final stages of cross-linking the peptidoglycan polymers in the wall. β-lactams interfere during this stage directly inhibiting one or several of the enzymes. They act with the serine hydroxyl group of PBPs, replacing the D-ala-D-ala segment of the amino acid side chain of peptidoglycan, resulting in an irreversibly inhibition of the PBPs. Inhibition leads to bacteriolysis caused by autolysins activation of the cells autolytic system. This happens when precursors of the cell wall accumulate and the wall is unable to resist osmotic pressure.

Mechanisms of resistance

B-lactams efficiency is dependent on the targets accessibility, the degree of resistance to enzymatic inactivation by β-lactamases, and the ability of β-lactam to inhibit the target PBPs. Combinations or alterations of these parameters may result in resistance. The clinical important mechanisms of resistance are; enzymatic inactivation, alteration of the target site and inaccessibility to the target site [15, 19, 20].

Resistance by enzymatic inactivation

Antibiotic-inactivation enzymes, like β-lactamses, are the most important single cause of resistance to β-lactams. Over 700 unique enzymes have been identified and they are either chromosomally or plasmid mediated. These enzymes make biologically inactive products of the antibiotic by efficient hydrolysis of the amide bond in the β-lactam ring [19, 20].

Resistance by alteration of the target site

Resistance caused by alterations in PBPs can occur by acquisition of a resistant PBP, increased target PBP number and reduced affinity of the target PBP. PBPs with reduced affinity are an important mechanism of resistance to β-lactams when β-lactamases are absent [19, 20].

Resistance by alteration in access to the target site

B-lactams diffuse through porin channels in the outer membrane to get access to their target; the PBPs. Mutations in the porin genes of Gram-negative bacteria confer a decrease in permeability in the outer membrane. Synergistic effects can occur with the expression of β-lactamases or an active efflux, making the bacteria even more resistant [19, 20].

Cephalosporins

Cephalosporin was first identified in 1948 by Giuseppe Brotzu [21] and isolated from a fungus called *Cephalosporium.* Newton and Abraham isolated and purified the substance at Oxford University in 1955, and named it cephalosporin C. Cephalosporin C was acting very stable against penicillin β-lactamase and its *in vivo* activity and lack of toxicity was demonstrated by Florey in 1955 [21]. Numerous of cephalosporins have been developed since this year, and all commercially available cephalosporins are semi-synthetic [21]. Cephalosporins have the same mechanism of action and similar resistance mechanisms as penicillins.

A classification scheme divides the cephalosporins into generations according to their spectrum of antimicrobial activity and their historical development. Each newer generation

has significantly greater Gram-negative antimicrobial properties than the preceding generations:

(i) Moderate spectrum 1st generation (e.g. cefazolin, cephalexin and cephaloridine); are effective alternatives for treating staphylococcal and streptococcal infections.

(ii) Gram-negative spectrum 2nd generation (e.g. cefuroxime, cefcaclor and cefoxitin); have a greater Gram-negative spectrum of activity, useful for treating *E. coli*, *Klebsiella* and *Proteus* infections.

(iii) Broad spectrum 3rd generation (e.g. cefpodoxime, cefotaxime, ceftazidime and ceftriaxone); are not as effective against Gram-positive microorganisms as the first generation, but active against most members of the *Enterobacteriaceae*.

(iv) Extended spectrum 4th generation (e.g. cefepime, cefpirome and cefluprenam); display an increased stability against enzymatic degradation by β-lactamases (AmpC β-lactamases in particular), and an enhanced ability to penetrate the porins in the outer membrane of Gram-negative bacteria. They have the ability to cross the blood brain barrier and are thus also effective in meningitis.

Adapted from Naseer 2008 [7].

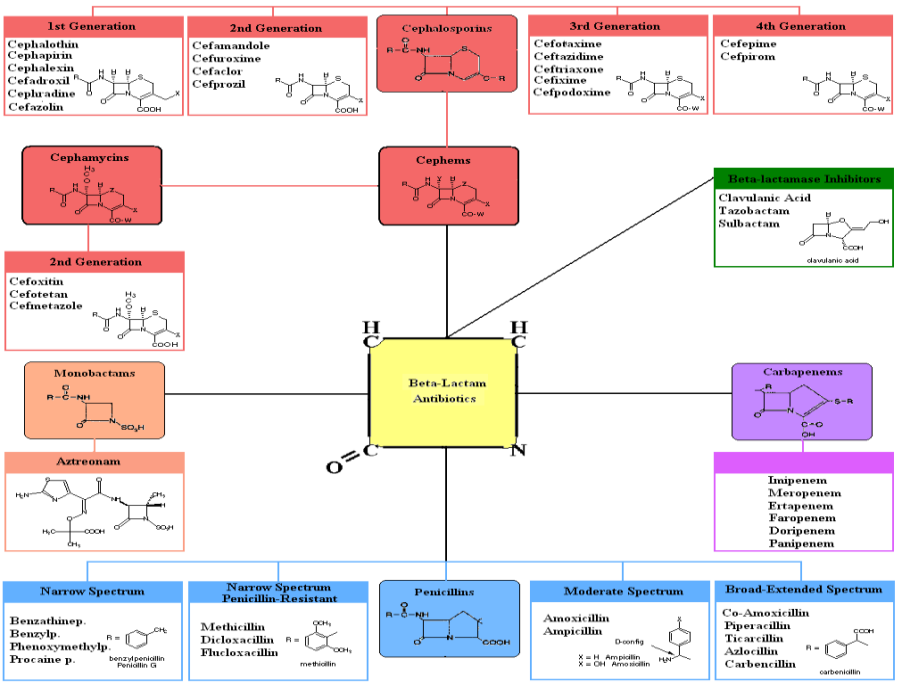

Figure 1.1. Schematic presentation of β-lactam antibiotics; Penicillins, Cephems (Cephalosporins, Cephamycins), Carbapenems, Monobactams and β-lactamase inhibitors, and subsequent sub-classes. Printed with permission from M. Umaer Naseer.

β-lactamases

The most important β-lactam resistance mechanism in Gram-negative bacteria is the production of β-lactamase enzymes. These enzymes are encoded on both chromosomes and plasmids, and possess different substrate profiles, inhibition profiles, molecular mass, isoelectric points, amino acid sequences and molecular structure [7]. β-lactamases that are chromosomally encoded can be expressed from a constitutive promoter giving high- or low level expression (e.g. *ampC* gene in *E. coli*), or they can be expressed from an inducible promoter (e.g. *ampC* gene in *Enterobacter*) [19, 22].

The first β-lactam hydrolysing enzymes were discovered in the 1940s in microorganisms like *E. coli*, *S. aureus* and *B. cereus*. This happened before penicillins were taken into use clinically and the term selective pressure did not exist. Today penicillins, cephalosporins, monobactams and carbapenems can all be hydrolysed by multiple members of the β-lactamase family [23]. During the last 40 years new β-lactams have been developed to fight resistance, but these have selected for more effective β-lactamases as extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC enzymes and carbapenems-hydrolysing β-lactamases (carbapenemases) [23-25].

Several classification schemes have been proposed for the classification of β-lactamases over the years. The two most frequently used are Amblers structural classes and Bush, Jacoby and Medeiros functional groups. Amblers classes A, B, C and D are based on the enzymes primary sequence similarity and catalytic mechanism. Class A, C and D share the same mechanism of action utilising an active-site serine, while class B require divalent metal cations (Zn++) to catalyse β-lactam hydrolysis. Bush, Jacoby and Medeiros divide the enzymes into four groups based on their substrate and inhibitor profiles. Group 1 are the cephalosporinases not inhibited by clavulanic acid or EDTA, group 2 are the penicillinases, cephalosporinases and broad-spectrum β-lactamases which to some extent are inhibited by β-lactamase inhibitors, group 3 are the metallo-β-lactamases, hydrolysing penicillins, cephalosporins and carbapenems, resistant to clavulanic acid, and group 4 are the penicillinases not inhibited by clavulanic acid [22, 26].

Table 1.2. Classification schemes for bacterial β-lactamases

Ambler classification scheme	
Class A	Serine Based Penicillinases
Class B	Zinc based metallo-β-lactamases
Class C	Serine Based Cephalosporinases
Class D	Serine based oxacillinases
Bush-Jacoby-Medeiros Scheme	
Group 1	Cephalosporinases resistant to clavulanic acid
Group 2	Penicillinases, cephalosporinases, and broad-spectrum β-lactamases susceptible to clavulanic acid
2a	Penicillinases
2b	Borad-spectrum penicillinases
2be	ESBLs
2br	Inhibitor resistant
2c	Carbenicillin hydrolysing
2d	Oxacillin hydrolysing
2e	Cephalosporinases
2f	Carbapenemases
Group 3	Metallo-β-lactamases resistant to clavulanic acid
Group 4	Penicillinases resistant to clavulanic acid

β-lactamases catalyse the irreversible hydrolysis of the amide (C-N) bond in β-lactams, with the use of a water molecule, leading to ineffective antimicrobials. Class A serine β-lactamase general mechanism of action involves activation of Lys73 or Glu66 followed by a nucleophilic attack by Ser70 on the carbonyl carbon of the β-lactam ring. Class C and D β-lactamases have more or less the same mechanism even though the activation of Ser70 is unknown. The mechanism behind hydrolysis remains unclear for the class B β-lactamases [7, 24].

Beta-lactamases are located in the periplasmic space between the outer and cytoplamsmic membranes of Gram-negative bacteria [19].

Chromosomal β-lactamases are encoded and found in all *Enterobacteriaceae,* except from *Salmonella*. *E. coli*, *Klebsiella, Proteus* and *Shigella spp* produce low basal levels of the intrinsic AmpC β-lactamase and are susceptible to ampicillin and other β-lactams. Examples of bacteria producing small amounts inducible AmpC β-lactamases are *Enterobacter spp, Citrobacter freundii, Serratia, Morganella morganii, Providencia spp* and *Pseudomonas aeruginosa*. These bacteria are intrinsic resistant to ampicillin, first generation

cephalosporins and are not inhibited by β-lactamase inhibitors, but only when the expression is high enough.

Plasmid-mediated β-lactamases were identified in Gram-negative bacteria in the early 1960s, and the TEM β-lactamases were the first ones[22]. During the 1970s plasmid encoded β-lactamases like TEM-1, TEM-2, SHV-1 (broad-spectrum) and OXA-1 (oxacillin-hydrolysing) had spread to *Enterobacteriaceae, P. aeruginosa, Haemophilus influenza* and *Neisseria gonorrhoeae*. It is believed that all plasmid-mediated β-lactamases has their origins from the chromosome. Chromosomal *ampC* genes of *Enterobacter*, *C. freundii*, *Serratia* and *P. aeruginosa* have moved onto plasmids which have been recovered from clinical isolates of *E. coli* and *Klebsiella pneumonia*. The source organisms are unknown for many types, and genes encoding these enzymes are often located on transposons [19, 22, 24]. SHV-2 was the first enzyme to hydrolyse newer β-lactams with an increased spectrum of activity (ESBL) and today numerous of TEM, SHV and OXA-derived ESBLs are identified. Resistance to β-lactamase inhibitor/β-lactam drug combinations, penicillins, cephamycins, first, second, and third generations cephalosporins, and monobactams is seen in these strains. Susceptibility only to cefepime and imipenem is retained [19].

Class C β-lactamases – the AmpC cephalosporinases

The first description of an AmpC β-lactamase came in 1940 and in 1965 scientists began to study the genetics behind these enzymes and their resistance [27]. This enzyme was identified in an *E. coli* isolate and had the ability to hydrolyse penicillin. In 1981 the whole sequence of the *ampC* gene in *E. coli* was obtained, which differed from the penicillinases sequence, but still with a serine at its active site [27]. Since the first AmpC enzyme was discovered, numerous of new variants have been identified [27]. Producers of the chromosomal AmpC β-lactamases are Gram-negative bacteria, where the majority is found in the *Enterobacteriaceae* family, including *Citrobacter*, *Salmonella, Serratia, Enterobacter* and *Shigella*. But these enzymes are also observed in strains like *Pseudomonas, Aeromonas* and *Ochrobactrum* [28]. AmpC β-lactamases can also be found encoded on plasmids [20].

The AmpC cephalosporinases are classified into Amblers structural class C β-lactamases and into Bush, Jacoby and Medeiros functional group 1 [26].

Physically the AmpC enzymes location is in the periplasmic space of the bacteria. AmpC enzymes have a molecular mass between 34 and 40 kDa and isoelectric points usually above 8 [27].

The enzymatic properties of the AmpC β-lactamases confer resistance to a variety of β-lactams. These enzymes have the ability to hydrolyse penicillins, cephalosporins, cephamycins (cefoxitin, cefotetan), oxyiminocephalosporins (ceftazidime, cefotaxime, ceftriaxone) and monobactams (aztreonam) to a little extent. They are not inhibited by commercially available β-lactamase inhibitors. They are usually susceptible to carbapenems and fourth-generation cephalosporins (cefepime, cefpirome). Bacterial clinical isolates with mutated AmpC enzymes mediating reduced susceptibility to cefepime have been reported. Carbapenem resistance in an AmpC producing strain can be achieved by porin mutations or the loss of porins in the outer membrane. Inhibitors like clavulanic acid, sulbactam and tazobactam have little effect towards these enzymes (some are inhibited by tazobactam and sulbactam). They are poorly inhibited by *p*-chloromercuribenzoate and not at all by EDTA. Good inhibitors are substances like cloxacillin, oxacillin and aztreonam, and boronic acid reversibly inhibiting AmpC [20, 24, 27, 28].

The expression of AmpC β-lactamase is usually low but can be inducible by a complex mechanism linked to the *ampR* gene. Overexpression is linked to mutations in AmpD and AmpR leading to hyperinducibility or constitutive hyperproduction of the enzyme. Mutations in AmpG can lead to constitutive low-level expression. Additional features to AmpC regulation is found in different bacteria. Examples are *E. coli* and *Shigella* both lacking AmpR which confer noninducible AmpC production regulated by promoter and attenuator mechanisms instead. The amount of AmpC enzymes are important to the resistance, as well as the rate substrates are delivered to the enzyme [27].

Porin channels and efflux pumps plays an important role for the resistance spectrum. The concentration of a β-lactam in the periplasmic space is dependent on the outer membranes permeability. A decrease in porin channels or an increase of efflux pumps will lower the amount of β-lactams in the periplasmic space leading to higher enzyme efficiency. Molecules with both positive and negative charges (cefepime and cefpirome) penetrate the outer bacterial membrane faster than molecules with a net positive charge (cefotaxime, ceftriaxone) [27, 29].

Plasmid-mediated AmpC β-lactamases have been found in isolates worldwide, and are capable to compromise the use of β-lactams. CMY-1 was the first enzyme discovered in a *K. pneumoniae* isolate in South Korea 1989[30]. Plasmid encoded AmpC enzymes are divided into families according to differences in their amino acid sequences. There are 43 variants of CMY, 7 variants of FOX, 4 variants of ACC, LAT and MIR, 3 variants of ACT and MOX, and 2 variants of DHA [27]. Plasmid determined enzymes can be very closely related to certain chromosomal AmpC β-lactamases, and amino acid sequence similarity can be 100% for some groups. They confer resistance to penicillins, oxyimino-β-cephalosporins, cephamycins, and variably to aztreonam. They are susceptible to cefepime, cefpirome, and carbapenems, and inhibited by aztreonam, cloxacillin and boronic acid. Their molecular size range from 38 to 42 kDa, and the size of their plasmids vary between 7 to 180 kb. Bacteria carrying plasmid-mediated AmpC enzymes are often multiresistant encoding resistance against antibiotics like aminoglycosides, chloramphenicol, sulfonamide and tetracycline. Plasmid-borne *ampC* genes are usually not inducible because of the absence of *ampR* regulatory gene. Genetic elements involved in the mobilization of the *ampC* genes onto plasmids are transposable elements (transposons) and integron like elements. The IS-element IS*Ecp1* is involved in the transposition and mobilization of chromosomal *bla* genes onto plasmids (CMY family), and insertion sequence common region (IS*CR1*) is associated with gene mobilization into complex class 1 integrons. Plasmid-borne *ampC* genes are not associated with characteristic gene cassettes [20, 27, 31].

Extended-spectrum cephalosporinases with enhanced hydrolysis activity against oxyimino-β-lactam substrates are described for both chromosomal and plasmid-mediated AmpC enzymes. Alterations in the amino acid sequence like insertions, deletions and substitutions occur in the Ω-loop or near the R2 loop. Ω-loop mutations make the enzyme more accessible for substrates with bulky R1 side chains. An R2 loop mutation makes this binding site wider. The opposite effect can occur on both mutation sites. Structural gene mutations are often followed by promoter mutations increasing the level of expression of the mutant gene [27, 32].

Clinical relevance

Gram-negative bacteria that are able to develop hyperexpression of AmpC β-lactamases are clinically important. Several studies have confirmed that patients treated with broad-spectrum cephalosporins developed bacterial strains showing a decrease in their susceptibility. This development of resistance upon therapy is a great concern [27].

Escherichia

The genus *Escherichia* is a small group of Gram-negative rods classified under the family of *Enterobacteriaceae*. *Escherichia* comprise a total number of six species including *Escherichia albertii*, *E. blattae*, *E. fergusonii*, *E. hermannii*, *E. vulneris* and *E. coli.* They are widely distributed in the intestines of humans and animals. *Escherichia* are facultative anaerobic, non-sporulating, motile with peritrichous flagella or non-motile. They ferment D-glucose and other sugars, catalase positive, oxidase negative, reduce nitrate to nitrite, and have a GC content between 39 and 59% in their DNA [33].

Escherichia coli

E. coli is the one most often isolated from human specimens of all the six species. It is almost a permanent constituent of the bowel flora of healthy individuals, and may also colonize the lower end of urethra and vagina. This is the most important medical specie, functioning as

an opportunistic pathogen for both humans and animals. *E. coli* is capable to cause extraintestinal and intestinal infections in both healthy and immunocompromised individuals. Pathogenic clones of *E. coli* can cause urinary tract infections (UTI), diarrheal diseases, neonatal meningitis and septicaemia, and is one of the most versatile of all bacterial pathogens. The main route for transmission is contact and ingestion (faecal-oral), and presence of *E. coli* in water or food implies faecal contamination. Contamination is usually associated with pathogenic strains. The bacterium possesses O (somatic), H (flagellar), K (capsular) and F (fimbrial) antigens which can be used to characterise pathogenic strains by serotyping [3, 33].

Clinical significance

E. coli is one of the most common causes of hospital acquired infections, and accounts for more infections than any other single species overall. One million deaths per year are caused by pathogenic *E. coli* [34].

Aims of study

1. Characterise Norwegian clinical *E. coli* isolates for genetic alterations in the chromosomal *ampC* gene that can lead to increased resistance to cephalosporins by an increase of the mRNA level.
    a. Mutations in the *ampC* gene control region
    b. Mutations in the *ampC* gene coding region
2. Characterise the functional properties of mutations in the *ampC* gene coding regions that can change the catalytic efficacy of the AmpC enzymes against extended spectrum cephalosporins.

2. MATERIALS

Strain collection

A total of 55 clinical *E. coli* isolates with a chromosomal AmpC phenotype where chosen for this study. All strains where collected from 12 diagnostic Norwegian clinical microbiology laboratories over a time period from 2003 through 2007. Prior to this study each laboratory performed antimicrobial susceptibility testing in agreement with the Norwegian Working Group on Antibiotics (NWGA) guidelines and the recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST). The Norwegian Reference Centre for Detection of Antimicrobial Resistance (K-res) received and tested the strains further to establish a final resistant profile with a panel of β-lactam Etests. AmpC multiplex PCR was carried out to exclude presence of plasmids and acquired *ampC* genes.

In this study the main group of isolates that was characterised had an AmpC phenotype with reduced susceptibility to third generation cephalosporins, without clavulanic acid synergy. Three of the chosen isolates for this study did not fulfil the AmpC phenotype selection criteria, expressing intermediary susceptibility to fourth generation cephalosporins, with clavulanic acid synergy. Etest selection criteria for the chosen AmpC phenotype isolates where cefotaxim and ceftazidim (3^{rd} generation) MIC values >4. The Etest selection criteria for the three isolate not expressing the AmpC phenotype where cefepime (4^{th} generation) MIC values ≥1. The information on clinical isolates is given in Table 2.1 and for the complete Etest see APPENDIX 1.

Table 2.1. Data from clinical and sensitive isolates and control strains

				MIC Etest (mg/L)						
Ref. no	**Recieved from**	**Year of isolation**	**Material**	**CT**	**TZ**	**FX**	**PX**	**PM/PML***	**Boronic acid test**	**AmpC multiplexPCR**
K2-68	Ullevål	2003	Nasal secretion	4	16	64	96	<0.25	Pos	Neg
K4-30	Ahus	2003	Urine	6	16	256	256	0.5	Pos	Neg
K4-37	Ullevål	2003	Blood	4	12	96	64	0.38	Pos	Neg
K8-02	Vestfold	2003	Pus	16	12	>256	256	0.25	Pos	Neg
K9-38	UNN	2004	Operationwound	6	8	64	64	<0.25	Pos	Neg
K9-66	Stavanger	2004	Urine	6	16	256	192	<0.25	Pos	Neg
K14-27	Vestfold	2004	Urine	6	24	256	128	0.38	Pos	Neg
K14-35	Vestfold	2004	Matter	32	64	>256	>256	1.5	Pos	Neg
K15-08	Molde	2004	Blood	3	16	128	64	0.38	Pos	Neg
K22-31	Telelab	2005	Urine	4	24	192	128	<0.25	Pos	Neg
K25-19	Vestfold	2005	Urine	6	16	256	>254	<0.25	Pos	Neg
K25-65	Haukeland	2005	Blood	12	12	128	>256	3	Pos	Neg
K29-48	Haukeland	2005	Urine	4	6	128	48	<0.25/0.094	Pos	Neg
K29-77	Haukeland	2006	Bloodculture	4	6	256	96	<0.25/0.125	Pos	Neg
K29-80	Haukeland	2006	Urine	4	8	256	64	<0.25/0.094	Pos	Neg
K30-01	Haukeland	2006	Urine	4	48	128	256	<0.25/0.19	Pos	Neg
K30-18	Vestfold	2006	Urine	4	12	64	128	<0.25/0.064	Pos	Neg
K30-39	Telelab	2006	Urine	8	24	> 256	256	0.25/0.19	Pos	Neg
K30-44	Haukeland	2006	Urine	3	6	192	48	3/4	Pos	Neg
K30-45	Østfold	2006	Bloodculture	4	8	192	64	<0.25/0.125	Pos	Neg
K33-01	Østfold	2006	Urine	4	12	128	64	<0.25/0.125	Pos	Neg
K33-03	Haukeland	2006	Urine	>256	24	32	> 256	>16/<0.064	Pos	Neg
K33-24	Østfold	2006	Urine	3	2	256	8	1.5/0.38	Pos	Neg
K33-54	Østfold	2006	Urine	4	12	256	128	0.25/0.094	Pos	Neg
K34-08	Haukeland	2006	Urine	4	4	192	64	<0.25/0.094	Pos	Neg
K34-10	Vestfold	2006	Pus	4	8	128	64	<0.25/0.125	Pos	Neg
K34-32	Østfold	2006	Urine	4	8	> 256	96	<0.25/0.094	Pos	Neg
K34-43	UNN	2006	Urine	8	24	256	128	0.38/0.38	Pos	Neg
K34-61	Telelab	2006	Unknown	6	32	256	96	<0.25/0.125	Pos	Neg
K34-69	Vestfold	2006	Abscess	4	6	96	64	<0.25/0.094	Pos	Neg
K34-76	UNN	2006	Urine	6	8	64	64	0.75/0.5	Pos	Neg
K36-13	Ullevål	2006	BAL	6	>32	192	256	0.38/0.125	Pos	Neg
K36-22	Sørlandet	2006	Urine	4	6	128	64	<0.25/0.094	Pos	Neg
K36-30	Sørlandet	2006	Urine	4	8	32	64	<0.25/0.094	Pos	Neg
K36-50	Østfold	2007	Urine	4	16	48	96	<0.25/0.094	Pos	Neg
K41-19	UNN	2007	Urine	4	16	64	64	<0.25/0.19	Pos	Neg
K41-22	Haukeland	2007	Blood	6	16	96	96	<0.25/0.125	Pos	Neg
K41-34	Haukeland	2007	Blood	4	6	192	64	<0.25/0.125	Pos	Neg
K41-39	Haukeland	2007	Urine	4	6	256	64	<0.25/0.125	Pos	Neg
K41-52	Haukeland	2007	Urine	4	8	96	64	<0.25/0.125	Pos	Neg
K41-53	Ålesund	2007	Blood	4	16	256	96	0.25/0.125	Pos	Neg
K41-64	Vestfold	2007	Abscess	48	128	96	> 256	0.5/0.38	Pos	Neg
K41-71	Sørlandet	2007	Urine	4	4	> 256	48	0.38/0.19	Pos	Neg
K44-58	UNN	2007	Urine	4	6	192	64	<0.25/<0.064	Pos	Neg
K44-60	Haukeland	2007	Urine	2	3	96	6	1/0.19	Pos	Neg
K44-77	Haukeland	2007	Urine	4	16	96	128	<0.25/<0.064	Pos	Neg
K45-79	Vestfold	2007	Urine	4	8	128	64	<0.25/<0.064	Pos	Neg
K46-03	Sørlandet	2007	Urine	4	16	96	64	<0.25/0.094	Pos	Neg
K46-23	Ullevål	2007	Urine	32	48	96	> 256	0.38/0.25	Pos	Neg
K46-34	Haugesund	2007	Urine	6	12	64	96	<0.25/0.125	Pos	Neg
K46-40	UNN	2007	Urine	8	8	> 256	256	<0.25/<0.064	Pos	Neg
K46-46	Levanger	2007	Blood	24	128	> 256	> 256	0.38/0.38	Pos	Neg
K46-48	Haukeland	2007	Operationwound	4	8	128	48	<0.25/<0.094	Pos	Neg
K46-52	UNN	2007	Blood	4	12	64	96	<0.25/0.125	Pos	Neg
K46-73	UNN	2007	Urine	8	48	> 256	256	<0.25/0.094	Pos	Neg
ATCC 25922	Control	Unknown	Unknown	≤1	≤1	≤4	≤0,25	ND	ND	ND

*test results withot clavulanate acid were tested by diskdiffusion

Table 2.2. Bacterial growth media used in this study

Medium	Content
Brain Heart Infusion Broth (BHI)	Oxoid Brain Heart Infusion, dH_2O
Mueller Hinton agar	Difco Mueller Hinton medium, dH_2O
Green agar with 100 µg/L Ampicillin	
Green agar	
Deep freeze culture broth	Oxoid Brain Heart Infusion, glycerol (Merck), dH_2O
LB broth	
LB agar	
LB agar with 50 µg/L Kanamycin	
LB agar with 100 µg/L Ampicillin	
Saline	0.9% NaCl
SOC-medium	
Psi-medium	5 g yeastextract, 20 g trypthon, 5 g $MgSO_4$ per L medium

Table 2.3. Chemicals and reagents used in this study

Method	Chemicals	Contents
Electrophoresis of DNA	SeaKem® LE Agarose	LONZA
	Gel loading buffer 6x	Promega
	Ethidium Bromide	Sigma, Germany
	1 Kb plus DNA ladder	New England Biolabs, Beverly, USA
	0.5 x TBE buffer with pH 8.0	Tris-Base, borate, EDTA
Etest	Ampicillin, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Ampoxicillin/clavulanic acid, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Piperacillin, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Piperacillin/tazobactam, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Cefoxitin, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Cefuroxime, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Cefpodoxime, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Cefotaxime, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Cefotaxime/clavulanic acid, Etest strips with range 0.25-16/0.016-1 µg/ml	AB Biodisk, Solna, Sweden
	Ceftazidime/clavulanic acid, Etest strip with range 0.5-32/0.064-4 µg/ml	AB Biodisk, Solna, Sweden
	Cefepime/clavulanic acid, Etest strips with range 0.25-16/0.064-4 µl/ml	AB Biodisk, Solna, Sweden
	Aztreonam, Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
	Meropenem, Etest strips with range 0.002-32 µg/ml	AB Biodisk, Solna, Sweden
	Ceftazidime , Etest strips with range 0.016-256 µg/ml	AB Biodisk, Solna, Sweden
PFGE	Low range PFGE Marker	New England Biolabs, Beverly, USA
	PIV-buffer	10 mM Tris-HCl pH 7.6, 1 M NaCl
	Basis-buffer	100 mM EDTA, 6 mM Tris-HCl pH 7.6, 1 M NaCl, 0.5% Brij 58
	TE-buffer	10 mM Tris-HCl pH 8.0, 0.1 mM EDTA
	10 x TBE buffer, pH 8.0	108 g Tris-base, 55 g boric acid, 9.3 g EDTA, dissolve in ddH_2O to 1000 ml
	Lysis-buffer (for 5 plugs)	10 ml basic buffer, 0.02 g deoxycholate, 0.05 g N-laurosylsarcosine, 0.01 g lysozyme (1 mg/ml), 1 µl Rnase ONE (10U/µl)

	Proteinase K Stock Solution	100 mg Proteinase K, 10 ml 50 mM Tris-Cl pH 8.0, 10 mM $CaCl_2$
	ESP-buffer	0.5 M EDTA pH 9-9.5, 1% Na-laurylsarcosine
	2% LMP agarose	10 ml PIV buffer, 0.2 g LMP agarose
IEF	10 mM Tris-HCL pH 7.0	
	Ampholine PAGplate polyacrylamide gel, pH 3.5-9.5	GE Healthcare, Oslo, Norway
	Nitrocefin solution (0.5 g/L)	Tris-HCL (10mM, pH 7.95), DMSO
	Kerosene	
	Cathode solutions	1 M NaOH
	Anode solutions	1 M H_3PO_4
	IEF Protein Standards pI 4.45-9.6	Bio-Rad Laboratories, Hemel Hempstead, UK
Cloning	IPTG	
	X-gal	
	Cybersafe	Invitrogen
Competent cells	TFB1	30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2x2H_2O$, 50 mM $MnCl_2x4H_2O$, 15% Glycerol
	TFB2	10 mM MOPS, 75 mM $CaCl_2xH_2O$, 10 mM RbCl, 15% Glycerol

Table 2.4. Kits and enzymes used in this study

Method	Reagents, kits and enzymes	Producer
Isolation of DNA	MagAttract DNA Mini M48 kit (192)	
PCR	ReddyMix x 2	
	RedTaq	
	JumpStart RED Taq	
Purification of PCR products	E.Z.N.A Cycle Pure Kit	
	GE Healthcare illustra™ GFX™ PCR and Gel Band Purification Kit	
	ExoSAP-IT	
DNA sequencing	BigDye®Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
Isolation of RNA	RNAprotect® Bacteria Reagent	Qiagen
	RNeasy® Mini Kit	Qiagen
cDNA synthesis	High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
qRT-PCR	qRT Master Mix Plus	Eurogentec
PFGE	*XbaI*	Promega, Madison, USA
	Buffer 2 (10 x)	
	BSA	
Isolation of plasmid	E.Z.N.A Plasmid Midiprep Kit	
	E.Z.N.A Plasmid Miniprep Kit	
Cloning	pGEM®-T Easy Vector Systems	
	pBK-CMV Phagemid Vector Cloning Kit	
	2 x Rapid Ligation Buffer, T4 DNA Ligase	
	T4 DNA Ligase (3 Weiss units/µl)	
	EcoRI Buffer (NEB)	
	EcoRI	
	NotI	
	100 x BSA Buffer	
	10 x T4 Ligase Buffer	
	T4 DNA Ligase enzyme (400 000 U/mL)	

Table 2.5. Oligonucleotide primers used for PCR and direct sequencing

PCR	*Primer*	Sequence 5'-3'	References
AmpC whole region	*ampC F*	TTCCTGATGATCGTTCTGCC	Mammeri *et al.*, 2008
	ampC R	AAAAGCGGAGAAAAGGTCCG	Mammeri *et al.*, 2008
AmpC whole region	*ampC intF*	GGTTCGGTCAGCAAAACATT	This study
	ampC intR	GCAAGTCGCTTGAGGATTTC	This study
16S rDNA	*16S F*	AGAGTTTGATCMTGGCTCAG	Weisburg *et al*., 1991
	16S R	ACGGHTACCTTGTTACGACTT	Weisburg *et al*., 1991
MLST	*adk F*	ATTCTGCTTGGCGCTCCGGG	Achtman *et al*., 2006
	adk R	CCGTCAACTTTCGCGTATTT	Achtman *et al*., 2006
	fumC F	TCACAGGTCGCCAGCGCTTC	Achtman *et al*., 2006
	fumC R	GTACGCAGCGAAAAAGATTC	Achtman *et al*., 2006
	gyrB F	TCGGCGACACGGATGACGGC	Achtman *et al*., 2006
	gyrB R	ATCAGGCCTTCACGCGCATC	Achtman *et al*., 2006
	icd F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	Achtman *et al*., 2006
	icd R	GGACGCAGCAGGATCTGTT	Achtman *et al*., 2006
	mdh F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	Achtman *et al*., 2006
	mdh R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	Achtman *et al*., 2006
	purA F	CGCGCTGATGAAAGAGATGA	Achtman *et al*., 2006
	purA R	CATACGGTAAGCCACGCAGA	Achtman *et al*., 2006
	recA F	CGCATTCGCTTTACCCTGACC	Achtman *et al*., 2006
	recAR	TGTTCGAAATCTACGGACCGGA	Achtman *et al*., 2006
Cloning ampC gene	*EcoRI F*	ATATGAATTCTTTTGTATGGAAACCAGACC	Mammeri *et al*., 2008
	NotI R	ATATGCGGCCGCAAAAGCGGAGAAAAGGTCCG	Mammeri *et al*., 2008
pGEM-T easy vector	*pGEM F*	CAGGGTTTTCCCAGTCAC	Promega
	pGEM R	GCGGATAACAATTTCACAC	Promega
pBK-CMV phagemid vector	*T3 F*	AATTAACCCTCACTAAAGGG	Stratagene
	T7 R	GTAATACGACTCACTATAGGGC	Stratagene

3. METHODS

Preparation of stock cultures (B-culture)

A stock culture of bacteria is stored in -70°C (deep freeze storage) and prepared from a freeze broth containing an osmotic protector such as glycerol or DMSO.

Procedure:

1. 8-10 overnight colonies was taken from an overnight incubated plate and inoculated in 1 ml freeze broth.
2. The broth was homogenized on a vortex mixer and stored at -70°C.
3. Control plates were inoculated and incubated at 37°C overnight to check for contamination.
4. If the control plate was contaminated the strain was purified by re-incubating it on a new plate before storage.

(Freezing of bacterial strains, version 1.4? Methodology, General Bacteriology section, Department of Microbiology, University Hospital of Northern Norway)

Rubidiumchloride (RbCl) Competent cells

In this method the cell walls of the bacteria were treated with rubidium chloride to make them competent.

Procedure:

1. 1% of an overnight LB broth culture was inoculated in 100 ml Psi-broth, and incubated at 37°C until OD600 showed 0.4, approximately two hours after incubation.
2. The suspension was incubated on ice for 15 minutes and then centrifuged for five minutes at 4500 rpm.

3. The supernatant was discarded and the pellet resuspended in 40 ml cold TFB1, and incubated on ice for five minutes.
4. A new round of centrifugation was performed at 4500 rpm for five new minutes.
5. The supernatant was discarded and the pellet resuspended in 3 ml cold TFB2.
6. The suspension was then dispersed 100 µl in 30 Eppendorftubes and immediately frozen on dry ice.
7. The tubes with competent cells were stored at -80°C.

(Personal communication.)

Isolation of DNA from bacterial cells

Two different methods were performed isolating DNA from the bacterial strains; rapid template isolation and the use of the robot GenoM^{TM}-48.

Rapid template isolation of DNA for PCR

Rapid template isolation allows fast and easy preparation of PCR-amplifiable DNA. A simple boiling step makes the bacterial cell lysate, and DNA becomes available for the PCR amplification process.

Procedure:

1. 8-10 bacterial colonies were collected and dissolved in 1 ml TE-buffer in an Eppendorftube.
2. After centrifugation for 5 minutes at 5000 rpm, the supernatant was discarded.
3. The pellet was resuspended in 100µl TE-buffer.
4. The sample was boiled for 10 minutes at 100°C, and spun for 5 minutes at 5000 rpm.
5. The Eppendorftube was placed on ice, and supernatant was transferred to a new clean tube and stored at -20°C.

(Extraction of DNA from bacteria, version 1.0, Methods for DNA work, Department of Medical Microbiology, University of Tromsø)

Isolation of DNA using the robot GenoM^{TM}-48 Ver 2.0 (Geno Vision, USA)

The GenoM^{TM}-48 robot uses the technology of GenoPrep^{TM} products and performs all steps of the DNA isolation procedure, including lysis, washing steps and elution. This isolation of DNA relies upon the binding of DNA to the GenoPrep^{TM} Beads. These are magnetic and DNA has a tendency to bind to the silica (glass) surface of the magnetic beads in the presence of a chaotropic salt solution (sodium iodide, guanidinium thiocyanate or guanidinium hydrochloride).

Procedure:

1. An inoculum of 0.5 McFarland was prepared in 0.9% NaCl.
2. The sample tubes were placed on the robot.
3. The robot performed all steps of isolation, including sample lysis, binding to the GenoPrep^{TM} beads, washing steps and the elution.
4. When finished the screw caps were replaced and samples were stored at 2 to 8°C.

(Mag Attract DNA Mini M48 kit (192))

Amplification of DNA

Polymerase Chain Reaction (PCR)

The PCR is used for enzymatic amplification of a specific or defined DNA fragment *in vitro*. The reaction requires a DNA template, complementary oligonucleotide primers, dNTPs and heatstable DNA polymerase. The PCR thermocycler is a heating block that rapidly change between temperatures and can be programmed to do this in different cycles. PCR amplification of DNA involves three stages with alternating temperatures. In the first stage, at 94°C, DNA is completely denatured to single-stranded DNA. The second stage, at 40-60°C, involves annealing to the target DNA by primers complementary to the 3' ends of opposite strands of the DNA. In the third stage, at 72°C, DNA polymerase extends each primer, duplicating the target sequence. These three stages are repeated 30 to 40 times yielding new duplicates every cycle, resulting in many copies of the wanted DNA fragment, the PCR product.

Procedure:

1. The mastermix contents were thawed on ice before use, and the PCR mastermix was made on a separate bench to avoid contamination. See table 3.1 for a general PCR reaction recipe.
2. The PCR mastermix and template were transferred to special PCR tubes and spun down with a mini centrifuge.
3. The tubes were placed on the PCR machine and the PCR program, with the right cycling conditions pre-installed, was started. See table 3.2 for a general PCR cycling program.

Table 3.1. Mastermix contents for a general PCR

Mastermix contents	Quantity
Reddy Mix x2	25 µl
ddH2O	19 µl
Primer Forward (50 pmol/µl)	1 µl
Primer Reverse (50 pmol/µl)	1 µl
Template	4 µl
Total volume	50 µl

Table 3.2. General PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	94/95°C	2-5 min	
Denaturation	94/95°C	30-60 sec	x 30
Annealing	*)	30-90 sec	x 30
Elongation	72°C	90-120 sec	x 30
Terminal elongation	72°C	5 min	
Hold	4°C	∞	

*) Primer melting temperature

(Principle for PCR version 2.0, Department of microbiology and virology, IMB, University of Tromsø.)

PCR-based detection of ampC β-lactamase gene control and gene coding regions

For specific mastermix contents and PCR cycling program parameters used in this PCR-reaction see Table 3.3 and Table 3.4.

Table 3.3. Mastermix contents for ampC whole region PCR

Mastermix contents	Quantity
Reddy Mix x 2	25 µl
ddH_2O	19 µl
Primer Forward (50 pmol/µl) *)	1 µl
Primer Reverse (50 pmol/µl) *)	1 µl
Template	4 µl
Total volume	50 µl

*) See Table 2.5.

Table 3.4. *ampC* gene control and coding region PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	94°C	3 min	
Denaturation	94°C	30 sec	x 30
Annealing	55°C	30 sec	x 30
Elongation	72°C	90 sec	x 30
Terminal elongation	72°C	5 min	
Hold	4°C	∞	

PCR-based detection of ampC β-lactamase gene coding region

For specific mastermix contents and PCR cycling program parameters used in this PCR-reaction see Table 3.5 and Table 3.6.

Table 3.5. Mastermix contents for AmpC Cloning PCR

Mastermix contents	Quantity
Reddymix x 2	25 µl
ddH_2O	19 µl
Primer forward (50 pmol/µl) *)	1 µl
Primer reverse (50 pmol/µl) *)	1 µl
Template	4 µl
Total volume	50 µl

*) See Table 3.5.

Table 3.6. *bla*$_{ampC}$ gene coding region PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	94°C	3 min	
Denaturation	94°C	30 sec	x 30
Annealing	56°C	30 sec	x 30
Elongation	72°C	90 sec	x 30
Terminal elongation	72°C	5 min	
Hold	4°C	∞	

16S rDNA PCR

The identification of different species in bacteria can be established with both phenotypic and genotypic methods. Phenotypic methods are often not completely reliable because all strains within a given specie not necessarily are positive for a common property. And it's proven that the same strain can possess biochemical variability. Genotypic methods are therefore developed based on analysis of PCR products derived from selected target DNA sequences for species and genus identification. 16S ribosomal DNA (rDNA) sequence determination is

often used as an alternative or supplement to traditional identification techniques. The rRNA genes are highly conserved and present in all bacteria containing some sequence areas with sequence specific variations that can be used in taxonomic characterisation. Universal primers recognising 16S rDNA from most bacteria are used for amplification and sequencing.

For specific mastermix contents and PCR cycling program parameters used in this PCR-reaction see Table 3.7 and Table 3.8.

Table 3.7. Mastermix contents for 16S rDNA PCR

Mastermix contents	Quantity
JumpStart RED Taq	12.5 µl
ddH_2O	9.6 µl
16S rDNA F (50 pmol/µl) *)	1 µl
16S rDNA R (50 pmol/µl) *)	1 µl
Template	2.5 µl
Total volume	25 µl

*) See Table 3.5.

Table 3.8. 16S rDNA PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min	
Denaturation	95°C	30 sec	x 30
Annealing	55°C	30 sec	x 30
Elongation	72°C	60 sec	x 30
Terminal elongation	72°C	7 min	
Hold	4°C	∞	

The PCR products were pre-sequenced, sequenced and detected automatic with ABI PRISM^{TM} 3100 Genetic Analyser (PE Biosystems) (see below). The obtained sequences were compared to 16S rDNA sequences available in GenBank-, EMBL- and DJB-databases by the BLASTN program on NCBIs homepage: http://www.ncbi.nlm.nih.gov/BLAST/.

(Identifying genotype, version 1.1 Methodology, General Bacteriology section, Department of Microbiology, University Hospital of Northern Norway)

Multi locus sequence typing (MLST) PCR

Multi-locus sequence typing (MLST) is a typing technique utilising multiple housekeeping genes loci in characterisation of isolates from particular bacterial species. This method takes advantage of the variation present in the nucleotide sequences of these gene fragments. All the unique sequences for a given locus are assigned a novel allelic number. The combination of the multiple loci numbers defines a particular strains sequence type (ST). Relationships among the isolates are identified by comparing their allelic profiles. Isolates closely related have identical STs, or STs that differ at a few loci, while unrelated isolates have unrelated STs. Materials required for the ST determination are available in laboratories all over the world and the MLST data are easily exchanged. This technique can be highly automated and can be able to screen hundreds and thousands of samples in a short matter of time. MLST schemes are available for several prokaryotic and eukaryotic pathogens [35, 36].

Procedure:

1. The E. coli MLST uses internal fragments of the following seven housekeeping genes:
    - *adk* (adenylate kinase)
    - *fumC* (fumarate hydratase)
    - *gyrB* (DNA gyrase)
    - *icd* (isocitrate/isopropylmalate dehydrogenase)
    - *mdh* (malate dehydrogenase)
    - *purA* (adenylosuccinat dehydrogenase)
    - *recA* (ATP/GTP binding motif)

2. The PCR amplification properties and the product length of the different genes:

Gene	Product length	Annealing temperature
adk	583 bp	54°C
fumC	806 bp	54°C
gyrB	911 bp	60°C
icd	878 bp	54°C
mdh	932 bp	60°C
purA	816 bp	54°C
recA	780 bp	58°C

3. All gene fragments with the same annealing temperature were run in the same PCR cycle reaction. For mastermix contents and PCR cycling program parameters see table 3.9 and table 3.10, respectively.
4. The PCR products were pre-sequenced, sequenced and detected automatic with ABI PRISM^{TM} 3100 Genetic Analyser (PE Biosystems) (see below). The sequences were blasted on MLSTs homepage (http://mlst.ucc.ie/mlst/dbs/Ecoli/dbs/Ecoli) to obtain the ST types of the bacterial isolates.

Table 3.9. Mastermix contents for MLST PCR

Mastermix contents	**Quantity**
RedTaq	12.5 µl
ddH_2O	9.6 µl
Primer Forward (50 pmol/µl) *)	0.2 µl
Primer Reverse (50 pmol/µl) *)	0.2 µl
Template	2.5 µl
Total volume	25 µl

*) See Table 3.5.

Table 3.10. MLST PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	
Denaturation	95°C	60 sec	x 30
Annealing	54-60°C*)	60 sec	x 30
Elongation	72°C	120 sec	x 30
Terminal elongation	72°C	5 min	
Hold	4°C	∞	

*) Primer melting temperature

MLST was performed according to the scheme developed by Achtman *et al* [34] (http://web.mpiib-berlin.mpg.de). Sequence alignments were performed using BioEdit sequence Alignment Editor.

BURST algorithm analysis (eBURST)

The BURST algorithm is a simplified model for bacterial evolution trying to identify groups of related genotypes that are mutually exclusive based on MLST data. First the algorithm attempts to identify the founding (ancestral) genotype for each group, designated sequence type (ST). The founder is the ST with the greatest number of single locus variants (SLVs). In the next step genotype variants are predicted based on the way the clones have emerged and diversified from the founding genotype. Related genotypes are then clustered, centred on the predicted founder, and this cluster is referred to as a clonal complex (STC).

The allelic profiles were obtained by BURST (Based Upon Related Sequence Types) analysis online (http://eburst.mlst.net/).

Agarose gel electrophoresis of DNA

The most common way to separate DNA molecules according to size is electrophoresis in agarose or polyacrylamide gels. Electrophoresis in horizontal agarose gels stained with ethidium bromide (EtBr) is the most widely used method. EtBr binds to the DNA between the stacked base-pairs (bp) and is exhibiting a strong orange-red fluorescence when illuminated with ultraviolet light. Molecules larger than 200 bp are separated by agarose gels, and molecules smaller than this should be separated by polyacrylamide gels. Agarose is a linear polymer extracted from seaweed, with a basic structure consisting of D-galactose and 3,6-anhydro-L-galactosidase. Agarose melts at 100°C and becomes solid again when the temperature decrease to under 50°C. The gel matrix solidified density is determined by the concentration of the agarose. DNA is negatively charged at a neutral pH value and will migrate toward the anode when an electric field is applied across the gel. Several parameters determine the migration rate; DNA molecular size, agarose concentration, composition of the electrophoresis buffer, DNA conformation, direction of the electrical field, and the presence of intercalating dyes.

Procedure:

1. A 1% agarose gel was made by adding agarose to 0.5 x TBE buffer.
2. The agarose solution was boiled until all the agarose was dissolved in a microwave oven.
3. After 5 minutes of cooling 0.5 µl 10 mg/ml EtBr was added per 10 ml gel.
4. An appropriate comb was placed in a sealed mould, and agarose was poured into the mould.
5. The gel was allowed to cool for at least 20 minutes before the seal and the comb was removed. The gel was then placed in an electrophoresis chamber filled with 0.5 x TBE buffer.
6. The PCR products was mixed with 1 µl 6 x loading buffer/5 µl PCR product and applied to a well. 5 µl of marker (Kb+) was loaded into the wells flanking the samples.
7. The gel was run at 110 V for 50 minutes prior to photographing in a UV transilluminator at 260 nm.

(Gel electrophoresis, version 1.2, DNA methods, Department of medical microbiology, University of Tromsø)

Automatic DNA sequencing

Pre-sequencing: Purification of PCR-products from excess components.

A PCR-reaction use primers, dNTPs and DNA polymerase to produce multiple copies of a specific DNA sequence. These components are able to influence the following sequencing reaction because they exist in large quantities in the PCR product mix. Several methods are available removing these compounds. In this study three different methods were used, two different purification kits and purification by the use of ExoSAP.

Purification kits:

Purification kits use spin-column technology with selective binding properties of uniquely designed silica-membranes. The caption buffer (CP-buffer) effectively binds single or double stranded PCR-products as small as 50 bp, and removes 99.5% of the primers smaller than 40 bp. Nucleic acid adsorb to the silica-gel membrane while contaminants pass through during centrifugation. The ethanol containing Wash Buffer efficiently washes away impurities. After the washing of the products are finished, DNA is eluted in elution buffer or TE-buffer.

Purification of PCR product by E.Z.N.A Cycle Pure Kit

Procedure:

1. The whole PCR-product volume was transferred to a clean 1.5 ml microfuge tube, and 6 volumes of CP-buffer were added.
2. The sample was applied to a HiBind® DNA spin-column with a 2 ml collection tube and centrifuged at 13 000 rpm for 1 minute.
3. Flow-through liquid was discarded, and the column was washed by adding 750 µl Wash Buffer and centrifuged at 13 000 rpm for 1 minute.
4. Flow-through was discarded and the washing step repeated.
5. The liquid was discarded and the empty column was centrifuged for 1 minute at 13 000 rpm to dry the column matrix. (Critical for good DNA yields.)
6. The HiBind® column was placed into a clean 1.5 ml microfuge tube.
7. PCR-products were eluted by adding 30 µl TE-buffer directly onto the column matrix and centrifuged for 1 minute at 13 000 rpm.
8. Concentration and purity of the DNA was measured using NanoDrop.

Purification of PCR product by GE Healthcare illustra™ GFX™ PCR DNA and Gel Band Purification Kit

Procedure:

1. A volume of up to 100 µl was transferred to a clean 1.5 ml eppendorftube, and 500 µl Capture Buffer type 2 was added to the sample and mixed thoroughly.
2. The Capture Buffer type 2-sample mix was loaded onto a GFX® MicroSpin column assembled with a collection tube.
3. The column was spun for 30 seconds at 13 000 x g, and flow-through liquid was discarded.
4. 500 µl Wash Buffer type 1 was added to the column, and spun for 30 seconds at 13 000 x g to wash the column.

5. Flow-through liquid was discarded and the column transferred to a clean 1.5 ml eppendorftube.
6. 10-50 µl Elution Buffer type 4 was added directly onto the centre of the membrane in the column, and the sample was incubated for 1 minute at room temperature.
7. The sample was spun at 13 000 x g for 1 minute to recover the purified DNA.
8. Concentration and purity of the DNA was measured using NanoDrop.

Purification of PCR product by the use of ExoSAP

This purification method removes the excess primers, dNTPs and DNA polymerase with the use of two hydrolytic enzymes, Shrimp Alkaline Phosphatase (SAP) and ExonucleaseI (Exo). SAP removes the remaining dNTPs and Exo removes the remaining primers. Both of the enzymes inactivates at 80°C for 15 minutes.

Procedure:

1. 1 µl ExoSAP-IT was added to 50 µl PCR product held on ice. New clean caps were placed on the PCR tubes.
2. The samples were spun down and placed in the PCR-machine. The following PCR cycling program was used for the purification reaction:

37°C	1 min
37°C	60 min
85°C	15 min
4°C	∞

3. The purified PCR-products were stored at -20°C.

(Purification of PCR products by the use of ExoSAP, Version 1.0 Methodology, General Bacteriology section, Department of Microbiology, University Hospital of Northern Norway)

Taq-terminator reaction

The sequence reaction is carried out in both directions with both forward and reverse primers. Each of these reactions is performed by a single primed PCR using purified DNA as template. To ensure that the correct sequence is obtained all DNA sequencing reactions are performed bi-directionally. The sequencing reaction is based on DNA synthesis in the presence of 3'-dye labelled dideoxynucleotide triphosphate (ddNTPs, dye terminators) in addition to the standard deoxynucleotide triphosphates. The site of polymerisation is located at the 3' OH group where dideoxynucleotides can be incorporated into a growing chain lacking this group and because of that terminates the DNA synthesis. In the automated fluorescent sequencing, fluorescent dye labels are incorporated into DNA extension products using 3'dye labelled terminators. During the sequencing reaction fragments with various lengths will be produced, each corresponding to the point at which a ddNTP was incorporated. After precipitating the Taq-terminator amplicon, the samples are denatured and loaded onto the ABI PRISM® 3100 Genetic Analyser that separate, detect, and analyse the fluorescent labelled DNA fragments using capillary electrophoresis. Molecules from the samples are injected into thin, fused-silica capillaries and the DNA fragments migrate towards the other end of the capillaries, with the shorter fragments moving faster than the longer fragments. As the fragments enter the detection cell, a laser beam detects the last incorporated base in each fragment. Since the four different ddNTPs are labelled with different dyes which emit light at different wavelength when excited by an argon ion laser, the laser is able to identify the A, C, G and T order and thereby the complete sequence of the DNA. DNA sequencing results were analyzed using the computer programs EditSeq and SeqMan (DNA Star from Lasergene).

Procedure:

1. The ABI PRISM^{TM} BigDye® Terminator v3.1 Cycle Sequencing Kit was used for the cycle sequencing. The reaction for each template contained the following reagents (with A, C, G and T in one tube):

BigDye 3.1	1 µl
Template (30-300 ng)	3-6 µl
BigDye sequencing buffer (5x)	2.5 µl

Primer (2 pmol/µl)	1.6 µl
ddH_2O	up to 20 µl
Total volume	20 µl

2. The reactions were mixed in PCR tubes held on ice and placed in the PCR machine. The following PCR cycling parameters was used for the sequencing reaction:

    96°C for 1 minute

    25 cycles:

        96°C for 10 seconds

        50°C for 5 seconds

        60°C for 4 minutes

    4°C until the samples are removed from the machine.

Precipitation of the terminator reaction and the DNA-sequencing (ABI PRISM® 3100 Genetic Analyzer, Applied Biosystems) were performed at the Sequencing Lab Unit, UiT. The nucleotide sequence is analyzed automatically and the result is presented as an electropherogram. The sequence is stored in the four-letter code for computer analysis. For further references, and detailed description of preparations of gels, running of samples, data collection software, instrument hardware and Macintosh maintenance; see BigDye Terminator v3.1 Cycle Sequencing Kit Protocol (2002): http://docs.appliedbiosystems.com/pebiodocs/04337035.pdf

Isolation of RNA from bacterial cells

When analysing RNA it is important that they represent the *in vivo* gene expression in a bacteria as truly as possible. A combination of two separate events is able to greatly affect the gene expression profile. The first is that the half-life of bacterial RNA is very short and they are enzymatically degraded within few minutes. This results in loss or reduction of many transcripts. The second event involves a higher expression of specific genes induced by the

handling and processing of the bacterial cells before lysis. RNA should be stabilized *in vivo*, since most changes in the gene expression profile occur during or directly after the harvesting of bacterial cells. This ensures reliable gene expression analysis. RNAprotect® Bacteria Reagent stabilizes RNA before bacterial cells are lysed. Released RNA can then be purified with either RNeasy Mini Kit or RNeasy Midi Kit.

Enzymatic lysis of bacteria by Qiagen RNAprotect® Bacteria Reagent

RNAprotect® Bacteria Reagent stabilizes the RNA before bacterial cells are lysed. This ensures that the cells gene expression profile is not disturbed and allows efficient disruption. The reagent prevents both RNA transcripts degradation and induction of genes. Two volumes of RNAprotect Bacteria Reagent are added directly to one volume of bacterial culture providing immediate stabilization of RNA. The bacterial cells are then lysed to release RNA.

Procedure:

1. 2-3 single colonies were inoculated in 5 ml LB-broth overnight at 37°C in ambient air with agitation (150) rpm.
2. 100 µl of the overnight culture was transferred into 30 ml LB-broth in Erlenmeyer bottles and incubated at 37°C with agitation (200 rpm) until $O.D_{600nm}$ reached 0.5-0.7.
3. 1 ml bacterial culture was transferred to an Eppendorftube, and 500 µl RNA protect was added. The tube was immediately vortexed for 5 seconds and incubated at room temperature for 5 minutes.
4. The tube was centrifuged for 10 minutes at 10 000 rpm (5000 x g) in an Eppendorf fuge.
5. The supernatant was decanted and residual supernatant was removed by gently dabbing the inverted tube once on a paper towel.
6. Bacteria pellet was kept on dry ice until the next step was started.
7. 200 µl TE-buffer containing lysozym (1 mg/ml) was added and the tube vortexed for 10 seconds.
8. The suspension was incubated for 10 minutes at room temperature and mixed every 2 minutes by vortexing.

9. 700 µl RLT-buffer containing 28 µl dtt per 700 µl RLT-buffer was added and vortexed vigorously.
10. 500 µl 96% EtOH was added and mixed by pipetting or shaken vigorously.

(RNAprotect® Bacteria Reagent Handbook Protocol 1)

RNA extraction and purification by RNeasy® Mini Kit

The released RNA is purified using RNeasy Mini Kit. The method combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Up to 100 µg RNA longer than 200 bp are allowed to bind to the silica-membrane with a specialized high-salt buffer system. The total RNA binds to the membrane and contaminants are efficiently washed away. RNAs shorter than 200 nucleotides are selectively excluded which provides an enrichment of mRNA. The resulting high-quality RNA is suitable for use in several downstream applications as quantitative real-time RT-PCR and northern, dot, and slot blotting.

Procedure:

1. 700 µl lysate was added to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15-20 seconds at 8000 x g (≥10 000 rpm).
2. Flow-through was discarded and step 1 repeated.
3. 350 µl RW1-buffer was added, the tube centrifuged 15-20 seconds at 8000 x g and the flow-through was discarded.
4. 80 µl incubation mix was added to the spin column and incubated for 15 minutes at room temperature.
5. 350 µl RW1-buffer was added and incubated for another 5 minutes at room temperature and centrifuged for 15-20 seconds at 8000 x g.
6. The spin column was placed in a new 2 ml collection tube, 500 µl RPE-buffer was added and the tube centrifuged for 15-20 seconds at 8000 x g to wash the column matrix.
7. 500 µl RPE-buffer was added and the tube centrifuged for 2 minutes at 8000 x g.

8. The spin column was placed in a new 1.5 ml Eppendorftube. 30 µl RNase free water was added to elute the RNA by centrifugation for 1 minute at 8000 x g.
9. The tube was kept on ice and the sample was divided in pre-marked Eppendorftubes and quickly frozen at -70°C for storage.
10. 3-5 µl was kept to measure RNA concentration and purity by using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

(RNAprotect® Bacteria Reagent Handbook Protocol 7)

Measurement of DNA and RNA concentration with NanoDrop ND-1000 Spectrophotometer, Thermo Technologies

The NanoDrop ND-1000 spectrophotometer use a patented sample retention system holding 1 µl of sample not needing traditional containment devices such as cuvettes and capillaries. The sample is held in place between two optical surfaces using fiber optic technology and surface tension defining the pathlength in a vertical orientation. During each measurement cycle sample is assessed at both a 1 mm and 0.2 mm path providing extensive dynamic range (2 ng/µl to 3700 ng/µl for dsDNA). This eliminates the need to perform dilutions or to make assumptions regarding sample concentration. Interference caused by incident light and transmitting light passing through containment walls of traditional cuvettes, microcell cuvettes and capillaries are removed with the direct coupling to the optics. Total measurement cycle time, including preparation and removal of the sample, is approximately 30 seconds.

Procedure:

1. 1-2 µl of ddH_2O was loaded onto the lower optical surface. The lever arm was closed and tapped a few times to bathe the upper optical surface. The lever arm was then lifted and both optical surfaces were wiped off with a Kimwipe.
2. The NanoDrop software was opened and selected for the nucleic acid module.

3. The spectrophotometer was initialized by placing 1 µl ddH_2O onto the lower optical surface, lowering the lever arm and then select "initialize" in the NanoDrop software. After initialization was completed (10 sec) both optical surfaces were cleaned with a Kimwipe.
4. A blank measurement was performed by loading 1 µl ddH_2O or buffer selecting "blank". When the blank was completed both optical surfaces were cleaned.
5. The nucleic acid sample was measured by loading 1 µl and selecting "measure". Once the measurement was completed both of the optical surfaces were cleaned.
6. After all the samples had been measured the optical surfaces were washed as described in step 1.

(Current Protocols in Molecular Biology Volume 1, Quantitation of DNA and RNA with Absorption and Fluorescence Spectroscopy, A.3D.9, Supplement 76: http://www.nanodrop.com/Science.aspx?Cat=Protocols)

cDNA synthesis of RNA

This PCR cycle reaction kit allows the reverse transcription of total RNA into single stranded cDNA suitable for quantitative PCR applications like real-time RT-PCR.
Procedure:

1. The RNA was diluted to a final concentration of 40 ng/µl with RNase free water.
2. The cDNA synthesis was performed with one sample per RNA extract, and each reaction had a total volume of 20 µl. 10 µl cDNA Mastermix + 10 µl RNA (40 ng/µl).
3. To check for DNA contamination in the qRT-PCR one sample per RNA extract had water instead of the reverse transcriptase in the cDNA Mastermix.

<u>cDNA Mastermix for 1 sample of 20 µl:</u>

10x RT buffer	2.0 µl
25x dNTP mix	0.8 µl

10x RT random primers	2.0 µl
Reverse transcriptase (ddH_2O)	1.0 µl
RNase inhibitor	1.0 µl
Nuclease free H_2O	3.2 µl
Total volume	10.0 µl

4. The reverse transcription PCR program:

Step	Temperature	Time
1	25°C	10 min
Step 2	37°C	120 min
Step 3	85°C	5 sec
Step 4	4°C	Max 1 hour, freeze at -20°C immediately

5. The samples were frozen at -20°C immediately.

(High-Capacity cDNA Reverse Transcription Kits Protocol, Applied Biosystems 2006)

Quantitative Real Time PCR (qRT-PCR)

This strategy for relative quantitation of gene expression allows quantifying differences in the expression level of a specific gene between different samples (isolates). Output data is expressed as a fold-change or a fold-difference of expression levels. RT-PCR gives the ability to monitor the progress of the PCR as it occurs. Data is collected during the reaction process instead of at the end of the reaction. Reactions are characterized by the point in time during cycling when amplification of target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic target, the sooner a significant increase in fluorescence is observed. There are two types of chemistries available to detect PCR products using Sequence Detection Systems (SDS) instruments; TaqMan® chemistry (“fluorogenic 5’ nuclease chemistry”) and SYBR®

Green I dye chemistry. In this study the TaqMan chemistry using a fluorogenic probe, enabling the detection of a specific PCR product as it accumulates during PCR cycles, was used.

The stepwise process starts with construction of an oligonucleotide probe containing a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. If the target sequence is present, the probe anneals downstream form one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. This cleavage of the probe, separates the reporter dye from the quencher dye, increasing reporter dye signals, and removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in fluorescence intensity proportional to the amount of amplicon produced.

Procedure:

1. The 96-well plate was set up with each cDNA synthesis sample as a duplicate. This generated a total of 8 qRT-PCR samples per cDNA sample, 4 samples for the gene of interest and 4 samples for the normalization gene. A mastermix control for each gene was also put up where the cDNA was replaced with water.
2. The cDNA samples were diluted 1:10, and each of the qRT-PCR reactions had a total volume of 25 µl consisting of 20 µl mastermix and 5µl diluted cDNA.

   qRT-PCR mastermix 60 x primer/probe assay for 20 samples:

60 x primer/probe stock	8.5 µl
Nuclease free water	141.5 µl
2 x Taqman Universal mastermix	250 µl
Total volume	400 µl

3. After the samples were loaded on the plate, it was sealed and put into the real time machine.

Chromosomal analysis by Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel electrophoresis (PFGE) is a technique for resolving large chromosomal DNA fragments. Macrorestricted linear bacterial DNA fragments from 100 bp to 10 Mbp can be separated. The method basically involves electrophoresis in agarose where two electrical fields are applied alternately at different angles for defined time periods. Activation of the first electrical fields causes the coiled molecules to be stretched in the horizontal plane and start moving through the gel. Interruption of this field and application of the second field, force the molecules to move in the new direction. Since there is length dependent relaxation behaviour when a long-chain linear molecule undergoes conformational changes in an electrical field, the smaller molecule, the quicker it realigns itself with the new field and is able to continue to move through the gel. Larger molecules take longer time to realign. In this way, smaller molecules draw ahead of larger molecules draw ahead of larger molecules and separate according to size.

PFGE involves the embedding of bacteria in agarose plugs to protect the chromosomes from fragmentation due to mechanical damage. The cells are lysed by treatment of the plugs with cell-lysis enzymes such as lysozym and lysostaphin. Extensive washing removes contaminants. The remaining genome is then digested *in situ* with restriction enzymes that have few recognition sites resulting in a relatively small number of restriction fragments (10-30). The separation of restriction fragments is affected by agarose concentration, buffer concentration, buffer temperature, initial and final switch times, voltage, total electrophoresis run time and field angle.

Preparation of PFGE plugs

Procedure:

1. One single colony was inoculated in 5 ml BHI-medium and incubated over night at 37°C with agitation (250 rpm).

2. 50 µl of the culture was transferred to 5 ml BHI and incubated for another 4 hours at 37°C, shaking (250 rpm). The bacterial cells should be in exponential growth when harvested.
3. The culture was transferred to a 15 ml Falcon tube and centrifuged at 3500 rpm for 10 minutes.
4. The supernatant was discarded and the cell pellet was resuspended in 1 ml cold PIV-buffer.
5. 495 µl of the suspension was transferred to a new Eppendorftube. 5 µl of lysozym (100 mg/ml) was added and the suspension was mixed well by vortexing and equilibrated at 50°C on a water bath.
6. 500 µl of 2 % LMP-agarose equilibrated at 50°C was added, and the bacteria/agarose solution was mixed properly.
7. The mixture was then cast into a plug mould.
8. The plug was incubated at 4°C for 15 minutes to solidify, and transferred to a T-tube containing 2 ml lysisbuffer.
9. The plug was then incubated for another 2 hours at 37°C for 2 hours with careful slow shaking.
10. The lysisbuffer was removed and the plug washed with 1 ml ddH_2O for 15 minutes.
11. The plug was incubated in 1 ml of ESP-buffer containing 50 µg/ml proteinase K-solution overnight at 50°C.
12. The plug was washed in 1 ml TE-buffer for 30 minutes at room temperature. Repeated twice.
13. The plug was transferred to a new tube containing 1 ml TE-buffer (with 50 µM thiurea) and stored at 4°C for further use.

Xba1 digestion and electrophoresis

Procedure:

1. A thin slice of the plug, approximately 1 mm, was cut off and transferred to an Eppendorftube.
2. (The plug was washed 4 x 30 minutes in TE-buffer to remove thiurea.)

3. The following restriction enzyme mix was made:

   XbaI mixture for 12 plugs: 125 µl x 12 = 1500 µl

   Buffer 2 (10x): 12.5 µl x 12 = 150 µl

   20 U XbaI: 20 U x 12 = 240 U → 240:20 U/µl = 12 µl

   BSA (100x): 15 µl

   ddH_2O: 1323 µl

4. 125 µl of the restriction enzyme mix was added to each plug before incubation at 37°C overnight.
5. The plug was then washed with TE-buffer for 4 x 30 minutes.
6. 1 % agarose was dissolved in 100 ml 0.5 x TBE-buffer by boiling, and equilibrated at 50°C in a water bath.
7. The gel equipment was prepared. Plugs were placed onto the tip of the gel comb, and mounted directly into the gel by inserting the comb before pouring the agarose. The gel solidified for 30 minutes at room temperature.
8. The electrophoresis chamber was filled with 1.8-2 liters of TBE-buffer. Circulation and cooler was then turned on to let the buffer cool down to 12°C.
9. When the gel had solidified the comb was removed and the gel released from the mould. All excess agarose was carefully removed.
10. The support plate was pressed down in the frame in the electrophoresis chamber, and the following parameters was run in the program:

    Pulse time: 1-20 s

    Total run time: 21 h

    Voltage: 6.0 V/cm = 200 V

    Angle: 120°

    Buffer temperature: 12°C

    Gel running buffer: 0.5 x TBE

11. When the program was finished, the gel was stained with EtBr (500 ml H_2O + 50 µl EtBr) for 20 minutes, destained in ddH_2O for 40 minutes and photographed in a UV transilluminator at 260 nm.

(Methods UiT)

Isoelectric focusing (IEF)

Isoelectric focusing (IEF) is an electrophoretic method that separates proteins according to their isoelectric points (pI). This technique is dependent on the presence of a pH gradient. Under the influence of an electric field in a pH gradient the protein will migrate to the position where its net charge is zero. A protein with a positive net charge will migrate toward the cathode, becoming less positively charged the further it moves through the gradient until it reaches its pI. A protein with a negative net charge will migrate towards the anode and become less negatively charged until it reaches its pI. The focusing effect of this method is that proteins are concentrated at their pIs and the separation is precise with very small charge differences. If a protein diffuse away from its pI it immediately gains charge and migrates back to its isoelectric point. The degree of resolution in IEF is determined by the electric field strength performed at a high-voltage greater than 1 000 V.

Procedure:

1. One single colony was inoculated in 5 ml LB-broth and incubated over night at 37°C with agitation (200 rpm).
2. The culture was transferred to a 15 ml Falcon tube kept on ice and centrifuged at 4000 rpm for 10 minutes at 4°C.
3. The supernatant was discarded and the pellet was resolved in 1 ml 10 mM Tris-HCL pH 7.0 (buffer).
4. The sample was sonicated on ice 2 x 30 seconds by 25% amplitude, pulsing 1 second on/1 second off, and inspected visually for “clearing” of the solution.
5. The sample was centrifuged at 4000 rpm for 20 minutes at 4°C.

6. The supernatant was collected and kept on ice.
7. The Multiphore II electrophoresis unit was connected to the MultiTemp II thermostatic circulator and the temperature was set to 10°C.
8. The gel was cut into right size according to the sample numbers and the protecting films removed.
9. A few ml of kerosene was loaded onto the cooling plate and the gel was positioned on the plate making sure no air bobbles was trapped under it.
10. Electrode strips were soaked evenly in 2-3 ml anode (+) and cathode (-) electrode solutions and applied to the long edges of the gel.
11. Pre-run the gel for 15 minutes, with the right running conditions.
12. An applicator strip (IEF/SDS) was used to load 20 µl of the samples on the gel depending on the expected pI-value.
13. The program was started again and these were the running conditions used:

pH range	3.5-9.5
Voltage (V)	1500 V
Current (mA)	50 mA
Power (W)	30 W
Time (h)	1.5 h

14. After the program finished the gel was removed and placed in a tray.
15. Nitrocefin was prepared and added to the gel for staining of the proteins.

Nitrocefin solution:

Tris-HCL (10mM, pH 7.95)	19 ml
DMSO	1 ml
Nitrocefin (0.5 g/l)	0.010 g
Total volume	20 ml

16. The gel was visually inspected for red bands during staining. Pictures were taken with a camera when staining was satisfactory.

17. Marker bands were measured on the picture to plot the values in a pI-curve, measuring and reading their pI-values from the marker pI-curve.

(Protocol for isoelectric focusing of β-lactamases, Department of Microbiology, University Hospital of North Norway.)

Cloning

Cloning is a technique based on the isolation and multiplying of target DNA fragments, like genes, by PCR. The multiplied genes are ligated into an expression vector and then transformed into a new bacterium expressing only this DNA fragment. This method is a helpful tool testing the properties of a gene expressing resistance against antibiotics.

Isolation of plasmids with E.Z.N.A Plasmid Midiprep Kit

The Plasmid Midiprep kit combines the power of HiBind® technology with the time tested consistency of alkaline-SDS lysis of bacterial cells to deliver high quality DNA. The columns bind reversibly to DNA or RNA under certain optimal conditions allowing proteins and other contaminants to be removed. Omega Bio-Tek's midi-columns facilitate the binding, washing, and elution steps enabling multiple samples to be processed simultaneously. Yields vary according to plasmid copy number, but 50 ml overnight culture can typically produce 100-200 µg high copy plasmid DNA. Purified DNA is suitable for most downstream applications including automated fluorescent DNA sequencing, restriction endonuclease digestion, and other manipulations.

Procedure:

1. One single bacterial colony was inoculated in 30 ml LB medium (with 100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated over night at 37°C with an agitation of 250 rpm.

2. The suspension was centrifuged at 4000 x g for 10 minutes at room temperature and the medium was discarded.
3. Cell pellet was resuspended in 2.25 ml Solution I/RNase A.
4. Cell suspension was then transferred to a 15-30 ml centrifuge tube. 2.25 ml of solution II and 50 µl of OB Protease Mixture were added to the suspension and incubated at room temperature for 3-5 minutes.
5. 3.2 ml Solution III was added and the tube gently mixed until a white flocculent precipitate formed.
6. The suspension was centrifuged at 12 000 rpm for 10 minutes at 4°C to pellet the cellular debris and genomic DNA.
7. 1 ml Equilibration Buffer was added into a HiBind®DNA Midi column, pre-inserted in a 15 ml collection tube, and was incubated for 4 minutes at room temperature to equilibrate the membrane. The tube was then spun at 3000 rpm for 3 minutes.
8. The clear supernatant was carefully aspirated and 3.75 ml was added to a clean equilibrated HiBind®DNA Midi column assembled in a 15 ml collection tube.
9. The tube was centrifuged at 5250 rpm for 5 minutes.
10. Flow-through was discarded and step 8 and 9 was repeated until all the cleared lysate had been passed through the column.
11. 3.5 ml Buffer HB was added to the column and centrifuged at 5250 rpm for 5 minutes. Flow-through was discarded.
12. The column was washed by adding 3.5 ml of DNA Wash Buffer and spun at 5250 rpm for 5 minutes. Flow-through was discarded.
13. The column was then washed with 3.5 ml absolute ethanol and centrifuged at 5250 rpm for 5 minutes. Flow-through was discarded.
14. An additional centrifugation at 5250 rpm for 10 minutes was performed to dry the empty column matrix.
15. The column was placed in a new clean 15 ml centrifuge tube and 0.5 ml DNA Elution Buffer was added directly onto the column matrix.
16. After 2 minutes of incubation at room temperature the tubes was centrifuged at 5250 rpm for 5 minutes to elute the DNA.
17. Concentration and purity of the DNA was measured using NanoDrop.

Isolation of plasmids with E.Z.N.A Plasmid Miniprep Kit

The Plasmid Miniprep kit combines the power of HiBind® technology with the time tested consistency of alkaline-SDS lysis of bacterial cells to deliver high quality DNA. The columns bind reversibly to DNA or RNA under certain optimal conditions allowing proteins and other contaminants to be removed. HiBind® mini columns facilitate the binding, washing, and elution steps enabling multiple samples to be processed simultaneously. Yields vary according to plasmid copy number, but 1.5 ml overnight culture can typically produce 8-12 µg high copy plasmid DNA. Purified DNA is suitable for most downstream applications including automated fluorescent DNA sequencing, restriction endonuclease digestion, and other manipulations.

Procedure:

1. One single bacterial colony was inoculated in 5 ml LB medium (with 100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated over night at 37°C with an agitation of 250 rpm.
2. The suspension was centrifuged for 1 minute at 10 000 x g at room temperature and the medium was discarded.
3. Cell pellet was resuspended in 250 µl Solution I/RNase A.
4. 250 µl solution II was added and the tube gently mixed by inverting and rotating the tube several times to obtain a clear lysate.
5. 350 µl solution III was then added and mixed immediately by inverting the tube several times until a flocculent white precipitate formed.
6. The suspension was centrifuged at 13 000 x g for 10 minutes at room temperature in a mini centrifuge from.
7. The clear supernatant was carefully aspirated, and added into a clean HiBind® Miniprep Column assembled in a 2 ml collection tube.
8. To completely pass the lysate through the column the tube was centrifuged at 13 000 x g for 1 minute at room temperature.
9. Flow-through liquid was discarded and 500 µl Buffer HB was added to wash the column. The sample was centrifuged at 13 000 x g for 1 minute at room temperature.

10. Flow-through liquid was discarded and 700 µl DNA Wash Buffer was added, and the tube centrifuged at 13 000 x g for 1 minute to wash the column.
11. The empty column was centrifuged for 2 additional minutes at 13 000 x g to dry the column matrix.
12. The column was placed into a clean 1.5 ml eppendorftube and 50 µl DNA Elution Buffer was added directly onto the column matrix and centrifuged for 1 minute at 13 000 x g to elute DNA.
13. Concentration and purity of the DNA was measured using NanoDrop.

pGEM®-T Easy Vector Systems

The pGEM®-T Easy vector is a linearized high-copy number vector with T-overhangs at the insertion site improving the efficiency of ligation of PCR products. The vector contains numerous restriction sites within the multiple cloning regions.

Ligation

Procedure:

1. PCR was run on the desired gene inserts, controlled with gel electrophoresis and purified with a purification kit. DNA concentration and purity was measured using NanoDrop ND-1000.
2. The appropriate amount of PCR product (insert) to include in the ligation reaction was calculated using the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

$$\frac{50 \text{ ng vector} \times 1.1 \text{ kb insert}}{3.0 \text{ kb vector}} \times \frac{3}{1} = 55 \text{ ng insert}$$

3. The pGEM®-T Easy Vector tube was briefly centrifuged to collect the contents at the bottom of the tube.
4. The ligation reaction was set up as described below:

Ligation mix:	
2x Rapid Ligation Buffer, T4 DNA Ligase	5 µl
pGEM-T Easy Vector (50 ng)	1 µl
PCR product (55 ng)	X µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Nuclease free water (up to 10 µl)	X µl
Total volume	10 µl

5. The reaction was mixed by pipetting and incubated for 1 hour at room temperature.

Transformation

Procedure:

1. Two LB-plates with treated with 14 µl IPTG and 50 µl X-gal was prepared and equilibrated to room temperature.
2. 2 µl of the ligation mix was transferred to a new clean Eppendorftube held on ice.
3. A tube of frozen DH5α high efficiency competent cells was put on ice until just thawed (5-15 minutes). Cells were mixed gently by flicking the tube.
4. 50 µl of cells was carefully transferred into the tube with the ligation mix.
5. The tube was gently flicked to mix the content and incubated on ice for 20 min.
6. The cells were heat-shocked for 50 seconds in a water bath at exactly 42°C.
7. The tube was immediately returned to the ice for 2 minutes.
8. 950 µl SOC-medium with room temperature was added to the tube and the suspension was incubated for 1 hour at 37°C with slow shaking (150 rpm).

9. 100 µl transformation culture was plated onto the prepared LB-plates, and incubated at 37°C overnight (16-24 hours).

pBK-CMV Phagemid Vector Cloning Kit

The pBK-CMV phagemid vector is a cloning vector derived from a high-copy-number pUC-based plasmid. This vector allows expression in both eukaryotic and prokaryotic systems. Prokaryotic expression is driven by the lac promoter, which is repressed in the presence of X-gal and IPTG. Kanamycin resistant colonies containing vector with insert will be white and can express the inserted gene as a fusion protein.

Cutting of the pBK-CMV and the *ampC* gene with restriction enzymes *EcoR1* and *Not1*

Procedure:

1. The pBK-CMV plasmid and the desired insert were prepared for the ligation reaction by cutting their DNA with *EcoR1* and *Not1* restriction enzymes.
2. Gene cutting mastermix contents:

Purified PCR product (insert) and plasmid:	
Purified PCR product/plasmid (30 µg)	30/x µl
EcoR1 Buffer (NEB)	10 µl
ddH_2O	51/x µl
EcoR1 restriction enzyme	4 µl
Not1 restriction enzyme	4 µl
100 x BSA Buffer	1 µl

Total volume	100 µl

3. The tubes with the cutting mixes were incubated at 37°C for 2 hours.
4. After the cutting all samples were run in 1% agarose gel electrophoresis with Cybersafe as the fluorescence marker instead of EtBr to protect the DNA.
5. After the electrophoresis was done the cut fragments were purified.

Purification of the cut fragments with E.Z.N.A Gel Extraction Kit

The Gel Extraction kit uses the HiBind® technology to recover DNA bands in the size range 50 bp to 40 kb from all grades of agarose gel. The yield is more than 85% of the total product. DNA bands of interest is excised from the gel, dissolved in Binding Buffer, and applied to a HiBind® DNA spin column. The HiBind® matrix specifically, but reversibly, binds to DNA or RNA under certain optimal conditions so that proteins and other contaminants can be removed. DNA is eluted with Elution Buffer and ready for other applications after a rapid washing step. The product is then suitable for ligation, PCR, sequencing, restriction digestion, or various labelling reactions.

Procedure:

1. Agarose gel electrophoresis was performed to fractionate the DNA fragments.
2. After adequate separation of bands had occurred, the DNA fragment of interest was carefully excised using a wide, clean, sharp scalpel and UV-light.
3. The appropriate volume of the gel slice was determined by weighing it in a clean 1.5 ml Eppendorftube. An equal volume of Binding Buffer (XP2) was added, and the mix incubated at 55°C-60°C for 7 minutes or until the gel had completely melted. The tube was vortexed every 2-3 minutes.
4. A HiBind® DNA Mini Column was placed in a 2 ml collection tube.
5. 700 µl of the DNA/agarose solution was applied to the column and centrifuged at 10 000 x g for 1 minute at room temperature.
6. Liquid was discarded and the column was placed back in the collection tube.
7. 300 µl of Binding Buffer (XP2) was added to the column and centrifuged at 10 000 x g for 1 minute at room temperature. Flow-through was discarded.

8. 700 µl of SPW Wash Buffer was added to wash the column and centrifuged at 10 000 x g for 1 minute at room temperature.
9. Liquid was discarded and the empty column was centrifuged for 2 additional minutes at ≥ 13 000 x g to dry the column matrix.
10. The column was placed in a new clean Eppendorftube and 30 µl of Elution Buffer was added directly onto the column matrix and incubated for 1 minute at room temperature. To elute DNA the tube was centrifuged at ≥ 13 000 x g for 1 minute.
11. Concentration and purity of the DNA was measured using NanoDrop.

Ligation of *ampC* gene into pBK-CMV

Procedure:

1. The appropriate amount of PCR product (insert) to include in the ligation reaction was calculated using the following insert-to-vector molar ratio equation:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

$$\frac{\text{50 ng vector x 1.2 kb insert}}{\text{4.5 kb vector}} \times 3/1 = 40 \text{ ng insert}$$

2. To prepare the ligation reaction the following components were mixed in an Eppendorftube:

pBK-CMV (50 ng)	x µl
10 x T4 ligase buffer	2 µl
T4 DNA ligase enzyme (400 000 U/ml)	1 µl
Insert (40 ng)	x µl

ddH_2O (up to 20 µl)	x µl
Total volume	20 µl

3. The ligation reaction was mixed gently and incubated for 10 minutes at room temperature.

Transformation of the recombinant plasmid pBK-CMV into the recipient *E. coli* strain SNO3

Procedure:

1. E. coli SNO 3 was thawed on ice (5-15 minutes) and mixed gently by flicking the tube.
2. 20 µl of the ligation mix and 0.5 µl of "clean" pBK-CMV were added carefully to the tube with 100 µl E. coli SNO 3 cells.
3. The tube was gently flicked to mix the transformation suspension and incubated on ice for 20 minutes.
4. The cells were heat-shocked for 50 seconds in a water bath at exactly 42°C.
5. The tube was immediately returned to the ice for 2 minutes.
6. 500 µl room tempered SOC-medium was added to the tube, and the suspension was incubated for 1 hour at 37°C with slow shaking (150 rpm).
7. 100 µl transformation reaction culture was plated onto LB-kanamycin plates (50 µg/ml) and incubated at 37°C overnight (16-24 hours).

PCR amplification control of insert

The presence and size of the DNA insert in pBK-CMV may be determined and controlled by PCR amplification of the DNA from individual colonies.

Procedure:

1. Each of the 5 transformed single colonies picked were inoculated in an Eppendorftube containing 20 µl ddH_2O.
2. All samples were streaked onto LB-kanamycin patch plates (50 µg/ml) for future reference and incubated at 37°C overnight (16-24 hours).
3. The cell suspensions were then boiled at 100°C for 10 minutes to lysate the cells and release DNA.
4. The tubes were centrifuged at 13 000 rpm for 1 minute to form a pellet.
5. The tubes were immediately placed on ice and supernatant was transferred to new clean Eppendorftubes.
6. A PCR amplification reaction was prepared containing the following components:

Reddymix x 2	10 µl
ddH_2O	7.6 µl
Primer forward (2 pmol/µl)	0.2 µl*See table 3.5.
Primer reverse (2 pmol/µl)	0.2 µl*See table 3.5.
Templat	2.0 µl
Total volume	20 µl

7. The PCR was performed using the following cycling parameters:

1 cycle:

94°C for 4 minutes

50°C for 2 minutes

72°C for 2 minutes

30 cycles:

94°C for 30 seconds

54°C for 90 seconds

72°C for 1 minute

72°C for 5 minutes

4°C until the samples are removed from the machine.

8. The PCR-products was analyzed to determine insert sizes using standard 1% (w/v) agarose gel electrophoresis. The expected size of the PCR product should be 167 bp plus the size of the insert.

Sequencing of the plasmid insert

After the analyzing of the insert verified that the product had the right fragment size, the plasmid with insert was sequenced.

Procedure:

1. The reaction for each plasmid template contained the following reagents:

BigDye 3.1	1 µl
Template (100-300 ng dsDNA)	3-6 µl
BigDye sequencing buffer (5x)	2.5 µl
Primer (2 pmol/µl) *See table 3.5.	1.6 µl
ddH_2O	up to 20 µl
Total volume	20 µl

3. The following PCR cycling parameters was used for the sequencing reaction:

96°C for 5 minutes

30 cycles:

96°C for 10 seconds

50°C for 5 seconds

60°C for 4 minutes

4°C until the samples are removed from the machine.

Susceptibility testing by Etest

Etest is a quantitative method to confirm bacteria susceptibility against antibiotics. This test determines the MIC (Minimum Inhibitory Concentration) value of the actual strain, establishing the lowest concentration of an antibiotic inhibiting visible growth of bacteria. A thin plastic strip is covered with a continuous antimicrobial gradient of an antibiotic on one side and a number scale on the other. The antibiotic is immediately released into the agar when applied to an inoculated agar plate forming a stable continuous gradient of antibiotic concentrations underneath the strip. When the bacterial growth has become visible after incubation an ellipse formed inhibition zone is seen centred along the strip. The MIC value is red from the scale (µg/ml) where the ellipse edge intersects the strip. MIC measures are accurate and reproducible.

Procedure:

1. The E-test strips were taken out of the -20°C freezer and allowed to achieve room temperature before use (1 hour).
2. Colonies from an overnight green agar plate with 100 µg/ml ampicillin were selected and inoculated in a 0.9 % NaCl to adjust the suspension to match the 0.5 McFarland turbidity standards.
3. A sterile non-toxic swab was dipped into the bacterial suspension, and excess fluid was removed by pressing the swab against the inside wall of the test tube. The surface of a MH-agar plate was swabbed by the use of a rotator to ensure an even distribution of the inoculum.
4. The surface was allowed to dry for 15 minutes.
5. Two (small MH plate) to six (large MH plate) E-test plastic strips were applied onto the inoculated agar surface (Simlex C76 robot, vacuum pen, tweezers), ensuring that no air bobbles got trapped under the strip.
6. The plates were incubated at 37°C for 16-18 hours, before reading the MIC values at the point of intersection between the inhibition edge and the E-test strip.

MIC interpretive standards (µg/ml) for E. coli can be found in Table 3.11. The breakpoints are from AFAs breakpoints *Enterobacteriaceae* ISBN 978-82-92345-11-5 Sensitive (S) ≤ and Resistant (R) >, and from $MIC_{50/90}$ after Lorian (Ed.). Antibiotics in Lab. Medicine. 4th Ed. 1996, ISBN 0-683-05169-5[1].

Table 3.11 MIC interpretative standards (µg/ml)

Antimicrobial agent	**S ≤**	**R >**
Ampicillin	0,5	8
Amoxicillin/clavulanic acid	0,5	8
Piperacillin/tazobactam	8	16
Cefoxitin[1]	2	4
Cefuroxime	0,5	8
Cefpodoxime	1	2
Cefotaxime	1	2
Aztreoname	1	8
Meropeneme	2	8
Ceftazidime	1	8

(Microbiological Methods version 2.0, Determination of MIC-values by Etest, Department for microbiology and virology, IMB.)

4. RESULTS

Sequencing of the control and gene coding region of the *ampC* gene

Sequencing was performed to analyse the chromosomal *ampC* genes for mutations in the control and gene coding region. Detection of promoter mutations was based on the nucleotide sequence, while detection of mutations in the gene coding region was based on the nucleotide mutations leading to a change in the amino acid sequence. All cephalosporin resistant (n=55) and sensitive (n=10) isolates were sequenced. The sensitive isolates were sequenced as controls for natural variations in the amino acid sequence. *E. coli* K12 sequence functioned as the control strain for the nucleotide sequence alignment. A complete alignment of the promoter sequences can be found in APPENDIX 2.

51 (91%) of the resistant isolates had mutations in the promoter regions. The changes included C→T mutations at position -42 (n=30, 54.5%), G→A mutations at position -18 (n=30, 54.5%), TTGTCA→TTGACA changes in the -35 box (n=7, 12.7%) and TACAAT→TATAAT changes in the -10 box (n=4, 7.3%). Nine (16.4%) isolates had a single nucleotide insertion in the spacer regions between the -35 and -10 boxes, shifting their length of this region to the optimal 17 bp. These represents the most important and essential mutations for increased resistance against cephalosporins. Eighteen (32.7%) isolates had mutations in the attenuator region (+17 - +37). In addition there were variable substitution mutations and spacer mutations at other positions in the promoter of the isolates. Twentyeight (50.9%) of the isolates with the C→T mutation at position -42, also contained mutations at position -88 (C→T), -82 (A→G), -18 (G→A) and -1 (C→T). Seven (12.7%) of the isolates with promoter mutations had an IS-element, IS*911*, between the -35 box and -10 box interrupting the promoter region. All these isolates had additional G→A mutations at position -28 and C→T mutations at position -73 in the promoter region. These isolates were selected for further genotypic studies to analyse clonal relatedness. Five (9.1%) of the resistant isolates had no mutations at any positions in the promoter. Strain K15-8 had no alterations in the promoter, but is identified with a large insertion sequence at position -17/-18 recognized as the putative IS-element IS*Ec10* from *E. coli* CFT073 in a previous study [37].

Amino acid sequence analysis revealed 4 (7.2%) isolates with interesting properties for further studies that did not possess any alterations in the promoter or an IS*911* IS-element. One isolate, K41-64, showed no essential promoter mutations or special mutations in the gene coding region explaining its decreased susceptibility against cephalosporins. Three isolates, K33-24, K44-60 and K46-23, had no essential promoter mutations, but contained mutations in the gene coding region differing from the sensitive sequences that might be important to the increased resistance.

These four isolates were chosen for further studies by cloning (see below).

See Table 4.1 and 4.2 (NB: x = unknown aa due to uncomplete nucleotide sequence) for summarized results.

Table 4.1. Molecular features of the promoter

Strain	-42	-35 box	-18	Spacer insertions	-10 box	Attenuator[a]	IS*911*
K2-68	C -> T		G -> A				
K4-30	C -> T		G -> A				
K4-37	C -> T		G -> A				
K8-02				-14 T		Pos	
K9-38	C -> T		G -> A				
K9-66	C -> T		G -> A				
K14-27	C -> T		G -> A			Pos	
K14-35	C -> T		G -> A			Pos	
K15-08							
K22-31			G -> A				
K25-19	C -> T		G -> A				
K25-65				-17 C		Pos	
K29-48							Pos
K29-77						Pos	Pos
K29-80						Pos	Pos
K30-01	C -> T		G -> A				
K30-18	C -> T		G -> A				
K30-39				-14 T		Pos	
K30-44							Pos
K30-45	C -> T		G -> A				
K33-01	C -> T		G -> A				
K33-03		TTGTCA -> TTGACA					
K33-24							
K33-54		TTGTCA -> TTGACA			TACAAT -> TATAAT	Pos	
K34-08							Pos
K34-10				-14 T		Pos	
K34-32				-16 C, -15 G, -14 T		Pos	
K34-43	C -> T		G ->A				
K34-61	C -> T			-14 T		Pos	
K34-69	C -> T		G -> A			Pos	
K34-76	C -> T		G -> A				
K36-13	C -> T		G -> A				
K36-22	C -> T		G -> A				
K36-30	C -> T		G -> A				
K36-50	C -> T		G -> A				
K41-19	C -> T		G -> A			Pos	
K41-22			G -> A	-14 T			
K41-34		TTGTCA -> TTGACA		-16 C			
K41-39							Pos
K41-52	C -> T		G -> A				
K41-53	C -> T		G -> A			Pos	
K41-64							
K41-71	C -> T		G -> A				
K44-58		TTGTCA -> TTGACA			TACAAT -> TATAAT		
K44-60							
K44-77	C -> T		G -> A			Pos	
K45-79						Pos	Pos
K46-03	C -> T		G -> A				
K46-23							
K46-34	C -> T		G -> A			Pos	
K46-40		TTGTCA -> TTGACA			TACAAT -> TATAAT		
K46-46		TTGTCA -> TTGACA		-14 T		Pos	
K46-48	C -> T		G -> A				
K46-52	C -> T		G -> A				
K46-73		TTGTCA -> TTGACA			TACAAT -> TATAAT		

a Positive for attenuator means attenuator mutation

Table 4.2. Amino acid sequence muations of the *ampC* gene coding regions from resistant and sensitive *E. coli* clinical strains

Strain number	2	4	8	23	28	37	40	92	102	104	105	110	130	140	157	191	196	201	209	210	217	222	231	232	234	236	248	251	254	255	256	257	260	261	263	277	298	300	304	306	312	316	321	323	325	367	371	372	373	374	375	376	377
K2-68											A									P								R	M	N		R		D					G		H					A							
K4-30															T					P								R	M	N		R		D					G		H					A	X	X	S	X	L	X	X
K4-37															T											T	C	R	M	N		R		D					G		H					A							
K8-02		M							I		A			D			H	T	S														T				I									A						X	
K9-38											A									P								R	M	N		R		D					G		H					A							
K9-66											A									P								R	M	N		R		D					G		H					A							
K14-27											A									P								R	M	N		R		D					G		H					A						X	
K14-35											A									P		X						R	M	N		R		D					G		H					A		X				X	X
K15-08			T													K			S																		I					P				A							
K22-31											A									P								R	M	N		R		D					G		H					A							
K25-19															T					P								R	M	N		R		D					G		H					A							
K25-65			T													K			S													F			I		I					P				T					T		
K29-48			T				R									K			S																I		I					P				T					T		
K29-77			T				R									K			S																I		I					P				T		X		X	T		
K29-80			T				R									K			S																I		I					P				T					T		
K30-01											A									P								R	M	N		R		D					G		H					A							
K30-18											A									P								R	M	N		R		D					G		H					A							
K30-39			T													K			S													F			I		I					P				T					T		
K30-44			T				R									K			S																I		I					P				T					T		
K30-45											A									P								R	M	N		R		D					G		H					A							
K33-01											A									P			V					R	M	N		R		D					G		H					A		X					
K33-03			T													K			S													F					I					P				T					T		
K33-24		M									A							T	S														T				I						A			A							
K33-54					L				I		A			D				T	S														T				I								C	T							
K34-08			T				R									K			S																I		I					P				T					T		
K34-10			T													K			S													F			I		I					P				A							
K34-32			T				R									K			S																		I					P			H	T							
K34-43											A									P								R	M	N		R		D					G		H					A							
K34-61			T													K			S													F			I		I					P				T					T		
K34-69															T					P				I				R	M	N		R		D					G		H					A							
K34-76											A									P								R	M	N		R		D					G		H					A							
K36-13											A									P								R	M	N		R		D					G		H					A							
K36-22											A									P	L							R	M	N		R		D					G		H					A							
K36-30										X	A									P								R	M	N		R		D					G		H					A							
K36-50											A									P								R	M	N		R		D					G		H					A							
K41-19											A									P								R	M	N		R		D					G		H					A							
K41-22											A									P								R	M	N		R		D					G		H					A							
K41-34			T				R									K			S						V										I		I					P				T				X	T	X	
K41-39			T				R									K			S																I		I					P				T					T		
K41-52											A									P								R	M	N		R		D					G		H					A						X	
K41-53											A									P								R	M	N		R		D					G		H					A							
K41-64				K							A															T	C	R	M	N		R		D					G							T							
K41-71								E																		T	C	R	M	N		R		D					G		H					A							
K44-58			T													K			S																		I			X		P				A			S	T	L	Y	S
K44-60				K							A	S														T	C	R	M	N		R		D					G							T							
K44-72	L		T			D										K			S																		I					P				T					T		
K44-77											A									P								R	M	N		R		D					G		H					A							
K45-79			T													K			S																		I					P				A							
K46-03											A									P								R	M	N		R		D					G		H					A							
K46-23		M									A							T	S														T				I						A			A							
K46-34													T													T	C	R	M	N	H	R		D			I	V								A							
K46-40			T													K			S																		I					P				A							
K46-46			T													K			S																		I					P				A							
K46-48				K							A																			T						K	I							V		T							
K46-52											A									P								R	M	N		R		D					G		H					A							
K46-73			T													K			S																		I					P				A							
Sensitive isolates																																																					
K18-03			T				R									K			S																I		I					P				T		X		X	T	X	X
K27-39			T													K			S													F			I		I					P				T		X		X	T	X	
K27-41			T													K			S													F			I		I					P				T					T		
K27-42															T					P								R	M	N		R		D					G		H					A							
K27-43			T				R									K			S																		I					P			H	T						X	
K27-44			T													K			S																		I					P				A							
K27-45				K							A																C	R	M	N	H	R		D			I	V								A		X		X	X	X	
K27-46													T													T	C	R	M	N	H	R		D			I	V								A							
K27-47	L		T			D										K			S													F			I		I					P				T		X		X	T		

Amino acid positions in the gene coding sequence containing mutations

Further analysis of isolates with the IS-element IS911 and isolates without promoter mutations

Pulsed-Field Gel Electrophoresis (PFGE)

*Xba*I digested DNA from the seven isolates carrying IS-element IS*911*, in front of the gene coding region, was examined by PFGE to establish a potential clonal relationship among them. Analysis showed that six isolates had identical band patterns on the gel, indicating a clonal relationship between these strains. The last strain had a band pattern that didn't resemble the others indicating no clonal relationship to these strains (presented in Figure 4.1).

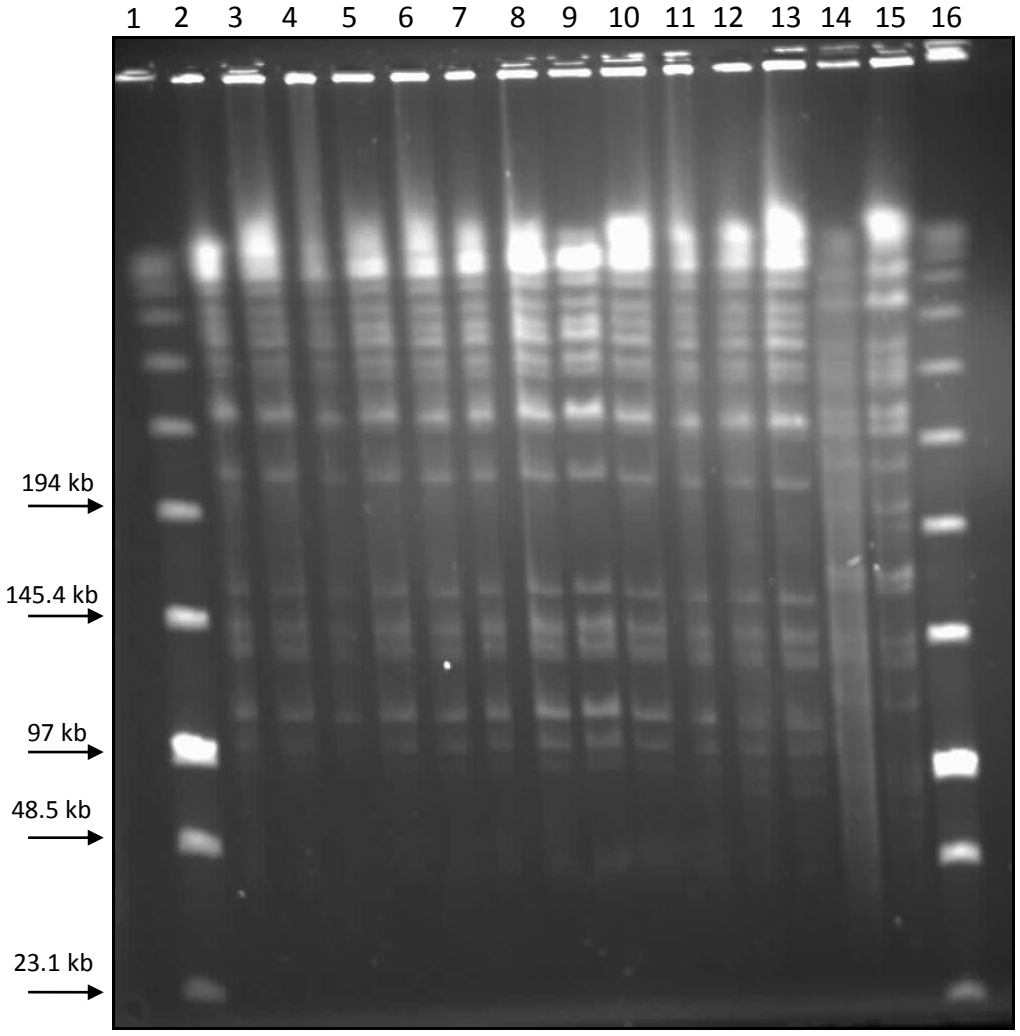

Figure 4.1. PFGE of *XbaI* digested DNA from isolates carrying IS-element IS*911*. Lane 1: Low range PFGE marker, lane 2 and 3: K29-48, lane 4 and 5: K29-77, lane 6 and 7: K29-80, lane 8 and 9: K30-44, lane 10 and 11: K34-08, lane 12 and 13: K41-39, lane 14 and 15: K45-79, lane 16: Low range PFGE marker.

Multi Locus Sequence Typing (MLST)

To confirm that the six isolates with IS*911* were clonally related to each other, and that the last isolate is not, they were typed by MLST. The four isolates without promoter mutations that were selected for cloning of the *ampC* gene were also included in this genotypic analysis to identify their founders and to examine if there are any similarities among them. A total of 11 strains were typed.

MLST analysis identified five different STs for these 11 strains; ST131, ST550, ST38, ST1291 and ST410 (presented in Figure 4.2). The six isolates that were clonally related by PFGE all belonged to ST131, confirming their clonal relatedness. The IS*911* strain not related to these strains by PFGE belonged to ST550. Two of the four isolates without mutation alterations belonged to ST38. The last two strains belonged to ST410 and ST1291. See Table 4.2 for results.

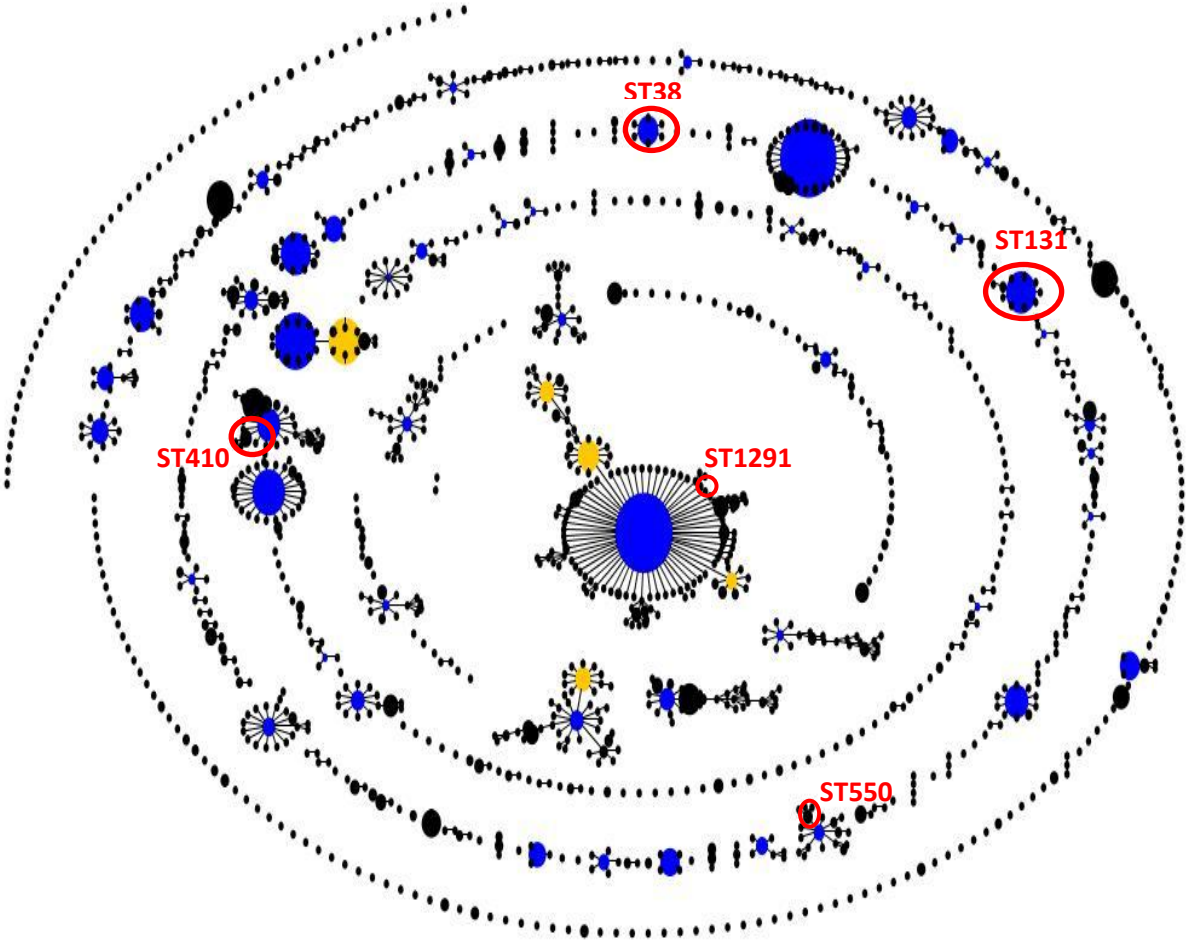

Figure 4.2. eBURST picture of the different STs obtained from the MLST sequencing. ST types are marked with red ellipses.

Isoelectric Focusing (IEF)

IEF was performed to establish whether the 11 selected isolates K29-48, K29-77, K29-80, K30-44, K33-24, K34-08, K41-39, K41-64, K44-60, K45-79, K46-23 with an AmpC phenotype expressed β-lactamases. The isoelectric points (pIs) were determined by comparison with reference β-lactamases *bla*$_{TEM-1}$ (pI 5.4) and *bla*$_{SHV-1}$ (pI 7.6), and naturally coloured IEF Protein Standards pI 4.45-9.6.

IEF analysis revealed that all isolates expressed β-lactamases, with a pI value between 9.1 and 9.2 corresponding to the alkaline profile of AmpC β-lactamases (Figure 4.3 and Table 4.3). Control strains A2-6 and A2-7 expressing bla$_{TEM-1}$ and bla$_{SHV-1}$, respectively, had bands at pI 5.4 and 7.6 exclusively.

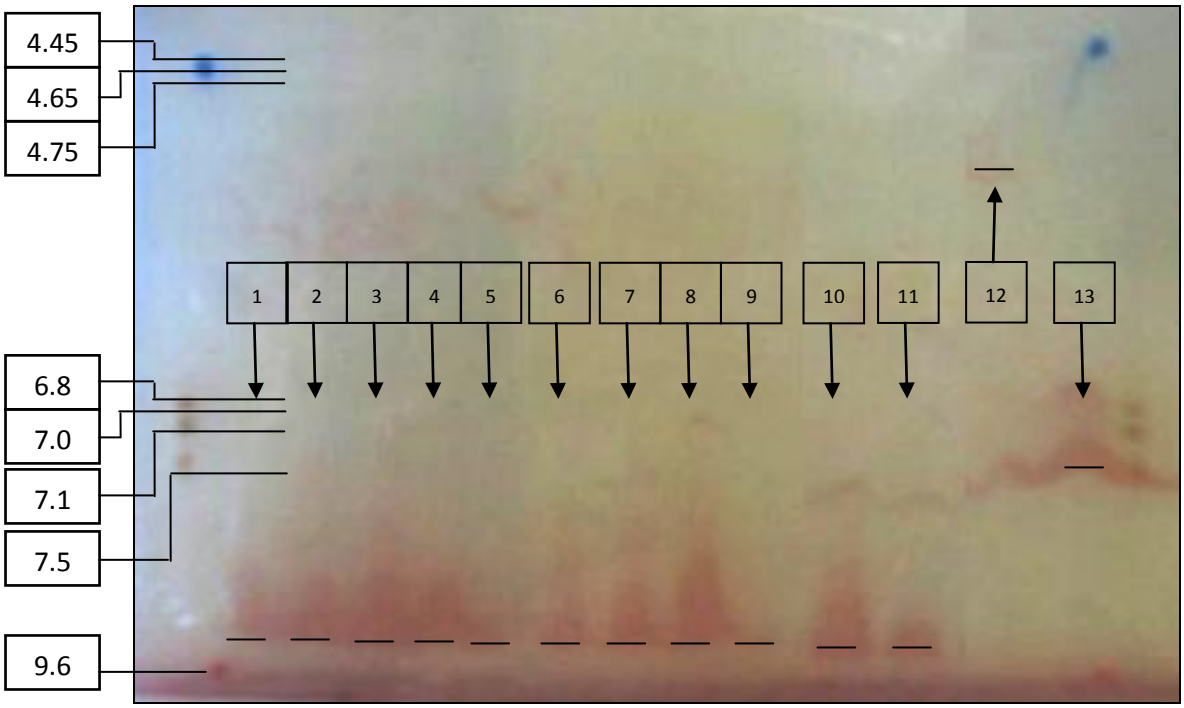

Figure 4.3. Modified picture of the nitrocefin-stained gel with red β-lactamase bands. IEF protein standards pI 4.45-9.6, lane 1: K29-48, lane 2: K29-77, lane 3: K29-80, lane 4: K30-44, lane 5: K33-24, lane 6: K34-08, lane 7: K41-39, lane 8: K41-64, lane 9: K44-60, lane 10: K45-79, lane 11: K46-23, lane 12: A2-6, lane 13: A2-7.

Real-time qRT-PCR

Real-time qRT-PCR was performed to analyse the expression level of chromosomal *ampC* genes in the four isolates without alterations within the promoter and one of the isolates with IS*911*. The *ampC* target gene was normalized against *gapA* (glyceraldehyde 3-phosphate dehydrogenase). *E. coli* ATCC 25922 mean normalized expression level of mRNA was set as 1.0, and used to calibrate the mean normalized expression levels of *ampC* mRNA from the isolates as fold differences.

The four isolates without alterations in the promoter region had no increase in expression of the *ampC* gene, and most of them showed a slightly lower expression, compared with the control strain ATCC 25922. The one isolate with the IS-element IS*911* showed a 60-fold increase of expression compared to the control (Table 4.3).

Table 4.3. Summary of results: MLST, qRT-PCR and IEF

	MLST			
Strain	**ST**	**STC**	**qRT-PCR expression**	**pI by IEF**
IS*911* isolates				
K29-48	131	None	ND	9.1
K29-77	131	None	ND	9.1
K29-80	131	None	ND	9.1
K30-44	131	None	57.3	9.1
K34-08	131	None	ND	9.1
K41-39	131	None	ND	9.1
K45-79	550	ST14	ND	9.1
Neg promoter mutation strains				
K33-24	38	ST38	0.83	9.1
K41-64	410	ST23	0.66	9.1
K44-60	1291	None	0.31	9.1
K46-23	38	ST38	0.67	9.2

Functional characterization of *ampC* gene coding regions

The functional properties of the mutated amino acid sequence of the gene coding regions in the four strains, K33-24, K41-64, K44-60 and K46-23, lacking promoter alterations, were investigated by cloning. K33-24 and K46-23 had amino acid mutations at positions 4 (M), 201 (T) and 321 (A) differing from *E. coli* K12 and the sensitive isolates. It should be noted that one strain had the same alteration at position 4, and two strains had the same changes at position 201. K41-64 did not have any unique mutations differing from *E. coli* K12 or the sensitive isolates. K44-60 had one mutation in position 110 (S) as the only isolate containing this alteration. For the complete amino acid sequence alignment see APPENDIX 3.

The gene coding regions were amplified without their own promoter, cloned into the plasmid pBK-CMV and the recombinant plasmids were transformed into the expression strain *E. coli* SNO3 [38]. *E. coli* ATCC 25922 (A-81) was functioning as the initially sensitive control strain.

All clones were examined by antimicrobial susceptibility testing by the following Etest strips; ampicillin (AM), amoxicillin/clavulanic acid (XL), piperacillin (PP), piperacillin/tazobactam (Ptc), cefoxitin (FX), cefuroxim (XM), cefpodoxim (PX), cefotaxim (CT), cefotaxim/clavulanic acid (CT/CTL), ceftazidim/clavulanic acid (TZ/TZL), cefepime/clavulanic acid (PM/PML), aztreonam (AT), meropenem (MP) and ceftazidim. See Table 4.4 for results.

Table 4.4. MIC (mg/L) values of β-lactams for recombinant clones with no promoter mutations

Strain	AM	XL	PP	Ptc	FX	XM	PX	CT	CT/CTL	TZ/TZL	PM/PML	AT	MP	TZ
pBKA-81	>256	64	6	6	64	32	48	2	2/>1	3.0/3.0	<0.25/<0.064	3	0.032	1.5
pBK33-24	>256	64	12	6	>256	48	>256	3	4/>1	6.0/4.0	<0.25/<0.064	6	0.032	3
pBK41-64	>256	96	24	6	24	16	96	3	6/>1	16.0/1.5	<0.25/<0.64	4	0.032	4
pBK44-60	>256	64	16	6	12	12	96	3	3/>1	6.0/>4	<0.25/<0.064	4	0.032	3
pBK46-23	>256	64	12	8	>256	96	128	3	4/>1	4.0/>4.0	<0.25/<0.064	6	0.032	3
pBK-CMV	2	3	1	1	4	3	0.5	0.047	<0.25/0.032	<0.5/0.19	<0.25/<0.064	0.064	0.023	0.125
SNO 3	2	2	1	1	4	3	0.5	ND	<0.25/0.047	<0.5/0.125	<0.25/<0.064	ND	0.032	0.125

5. DISCUSSION

AmpC β-lactamases (cephalosporinases) are a clinically important group of enzymes produced by many Gram-negative bacteria. These enzymes are encoded on the chromosome of *E. coli* and expressed on a constitutive low basal level in their cells. When these cephalosporinases are overexpressed in *E. coli* they confer resistance to penicillins and cephalosporins, but not to cefpirome and cefepime.

Detection of AmpC resistance determinants by sequencing

Two main mechanisms explaining the hyperexpression of the *ampC* gene in 51 (92.7%) of 55 clinical isolates were identified in this study. The first mechanism involves mutations in the control regions essential sites (n = 44, 79.9%) of the gene. The second mechanism revealed was the insertion of an IS-element IS*911* (n = 7, 12.7) in the control regions of the gene.

30 (54.5%) of the isolates with essential promoter alterations had a C→T mutation at position -42. Base substitution at this position is reported in many previous studies [37, 39-45] as the most essential alteration for hyperproduction of AmpC enzymes in *E. coli*. Strains possessing the -42 alteration usually have an additional G→A base substitution at the -18 site. In this study 29 (52.7%) of the strains had this mutation. Two strains were identified differing from this. One strain only had the substitution at position -18 while the other only had the one at position -42. The -42 mutation creates a new perfect TTGACA -35 box upstream of the normal box and in this way modifies the transcription initiation site. The -18 mutation creates a new -10 box with the optimal distance of 17 bp from the new -35 box. Both mutations can create a stronger promoter independent of each other. Studies have reported all three combinations of these mutations suggesting they are the most common and favoured promoter-region variations affecting *ampC* expression [41]. In this study this seems to be the trend as well.

A total of seven (12.7%) strains had alterations in their -35 box changing the sequence from TTGTCA to TTGACA, and four (7.3%) of these also had additional mutations in the -10 box (TACAAT → TATAAT). The -35 box and the -10 box play an important part in the level of gene transcription. It is proved that the closer these boxes are to the *E. coli* sigma 70 promoter

consensus sequences, the stronger the promoter will turn out to be. Both sequences are defined, and -35 has the consensus TTGACA whereas TATAAT is the consensus for -10. Several previous studies have confirmed the importance of these mutation sites in the promoter, indicating that the mutation in the -10 box are more rare compared to the -35 one, inducing moderate promoter strengths [39-41, 43-50].

Eight (14.5%) of the isolates had a single nucleotide insertion in the spacer region between the -35 box and the -10 box, resulting in an optimal 17 bp distance between them. Two of these strains also had the mutation TTGTCA→TTGACA in the -35 box, and two isolates had substitutions at position -42 and -18. The insertion mutation between the -35 and -10 boxes is another important property affecting the promoter strength. In the wild type *E. coli* the distance between these boxes are normally 16 bp, while the optimal distance for transcription is 17 bp. Mutations occurring in this spacer region changes the distance and creates a stronger promoter described in many previous studies [39, 40, 42, 44, 47, 50]. Previous studies imply that the insertion of a single nucleotide between these boxes in the *ampC* gene control region is enough to increase the expression and mediate resistance [41, 47-49].

A total of 18 (32.7%) isolates had various mutations in their attenuator region. Attenuator mutations only play a small part in increasing *ampC* promoter strength. All strains had attenuator mutations combined with other more essential mutations. See table 4.1 for details. Attenuator mutations are previously described in other studies [50-52].

Seven (12.7%) isolates, K29-48, K29-77, K29-80, K30-33, K34-08, K41-39, K45-79, did not have any essential promoter mutations known to up-regulate the transcription of the *ampC* gene, but had an IS*911* inserted between the -35 box and -10 box in the promoter region. It should be noted that three of the strains contained mutations in the attenuator site. Previous studies have described cases where the insertion of IS-elements in promoter regions can cause hyperproduction of the *ampC* gene by creating an alternative promoter. These reports include the IS-elements IS*2*, IS*Ec10*, IS*10* and IS*911* [37, 41, 53]. In a previous study the IS*911* created a stronger -35 box and a 17 bp spacer region to the -10 box [50]. This will generate a promoter sequence looking more like the *E. coli* consensus promoter sequence causing hyperproduction of AmpC enzymes. Hyperexpression of this chromosomal *ampC* gene control region was investigated in one of the strains, K30-44, by real-time qRT-PCR. The increase of expression compared to *E. coli* ATCC 25922 was almost 60-fold higher and

correlated well with findings in Canadian studies from 2005 [41] and 2007 [50]. These seven isolates were examined further to investigate a possible clonal relationship between them.

Five (9.1%) isolates had no mutations at all in their promoter regions. One of the strains, K15-8, has been characterised in a previous Norwegian study and was identified with the IS-element IS*Ec10* inserted in the promoter region, explaining the hyperproduction of AmpC enzymes [37]. The remaining four (7.1%) strains K33-24, K41-64, K44-60 and K46-23 had no obvious mutations in the gene control region to explain the observed resistance profiles against β-lactams.

The IEF results showed that the seven IS*911* strains K29-48, K29-77, K29-80, K30-44, K34-08, K41-39 and K45-79 and the four isolates K33-24, K41-64, K44-60 and K46-23 without any mutations in the promoter, produced AmpC enzymes with pI-values 9.1 and 9.2, as expected. This experiment verified that strains with an AmpC phenotype actually produced AmpC enzymes, though this experiment is not able to quantify the level of expression or the AmpC enzymes hydrolytic profiles.

Amino acid analysis of *ampC* gene coding regions

Amino acid sequences of the *ampC* gene coding regions of all resistant and sensitive strains were aligned against *E. coli* K12 amino acid *ampC* gene coding region. The purpose was to identify mutations in the gene coding sequences. We focused on the four strains K33-24, K41-64, K44-60 and K46-23, that lacked obvious promoter or attenuator mutations or insertion of IS-elements that could give hyperexpression of the *ampC* gene. Ten sensitive strains were used as a control for natural sequence variations occurring in the coding region.

The strains K33-24 and 44-60 are two of the four cefepime selected strains. These isolates had mutations in the gene coding sequences differing from K12 and the sensitive isolates which can be important for the resistance (see Table 4.2). These were selected for further sequence analysis (see below).

Strain K41-64 is one of the AmpC selected strains. This isolate has no exceptional mutations compared to K12 or in the gene coding sequence that can explain the decreased susceptibility (see Table 4.2). This strain was selected for further sequence analysis (see below).

Strain K46-23 is one of the AmpC selected strains. This isolate has mutations in the gene coding sequence differing from K12 and the sensitive isolates which can be important for the resistance (see Table 4.2). This strain was selected for further sequence analysis (see below).

These 4 strains were selected for further studies, to examine if the alterations in their gene coding region enhance their ability to hydrolyse broad-spectrum and extended-spectrum cephalosporins (see below).

Mutations in the *ampC* coding sequence can modify the enzymes structure and in that way broaden their substrate profiles. Previous studies performed by Mammeri *et al* have found several mechanism of resistance producing cephalosporinases with broadened substrate activity, expanded-spectrum AmpC (ESAC) β-lactamases. These mechanisms involved amino acid insertions [54, 55] and substitutions [56] of the *ampC* gene coding sequence altering their enzymes structure and resistance profiles.

The expression of the chromosomal *ampC* genes in the strains without promoter mutations or insertions was investigated by real-time qRT-PCR. And the results revealed no increased expression compared with *E. coli* ATCC 25922. These results did not unveil any surprises knowing only isolates with control region mutations hyperexpress *ampC*. This confirms that other factors are involved contributing to these strains resistance profiles against cephalosporins.

MLST analysis results showed that two of the isolates without promoter mutations, K33-24 and K46-23, was identified as ST38 belonging to ST complex 38. One strain, K41-64, was identified relating to ST complex 23 as ST410. And the strain K44-60 was identified relating to ST complex 10 as ST1291. STC38 related strains are globally distributed uropathogenic and enteroaggregative *E. coli* isolates from Brazil, Germany, India and Nigeria. Japan recently identified ST38 strains in CTX-M producing isolates [57], and a Norwegian study recently reported CMY-2 producing multidrug resistant isolates related to STC 38 [58].

Functional characterisation of the *ampC* gene coding regions

This study of the functional characterisation of the *ampC* gene coding sequence was interesting, because a recent study on *E. coli* isolates conferring reduced susceptibility to all cephalosporins, including cefepime and cefpirome, were identified with a novel resistance

mechanism, involving structural modifications in designated regions of the cephalosporinase sequence [45]. The functional properties of the *ampC* gene in the four strains K33-24, K41-64, K44-60 and K46-23 were investigated to establish whether mutations in the amino acid sequences reduce their susceptibility to cephalosporins or broaden their substrate activity. The cloned *ampC* gene coding region from *E. coli* ATCC 25922 (A-81) functioned as the susceptible control. The empty pBK-CMV vector transformed into SNO3 and SNO3 without any plasmid functioned as controls without any expressed AmpC enzymes. The four selected strains gene coding regions were cloned into the high copy vector pBK-CMV followed by expression in *E. coli* SNO3 [38]. The MIC values for selected β-lactam antibiotics were determined with a panel of Etests to establish the resistance profiles for all clones and controls. From this point the selected strains will be discussed separately based on their initial selection criteria for this study. K33-24 and K44-60 were chosen based on their decreased susceptibility to cefepime without clavulanic acid synergy, not fulfilling the AmpC selection criteria. K41-64 and K46-23 were chosen based on their AmpC phenotype profile showing decreased susceptibility to cefotaxime and ceftazidime without clavulanic acid synergy.

E. coli ATCC 25922 is susceptible to cephalosporins. When its gene was cloned into a high copy vector expressing and producing AmpC enzymes on a high level in SNO3, this strain had decreased susceptibility towards penicillins and cephalosporin antibiotics. In Table 4.3 where the MIC values are listed we see that SNO3 pBKA-81 is resistant to penicillins and cephalosporins, but were still susceptible to meropenem (MP).

Both pBK-CMV and SNO3 are completely susceptible towards all the β-lactam panels in this test, confirming they do not have any AmpC production in their cells.

Comparing pBK33-24 and pBK44-60 to pBKA-81 there is not a significant difference for any of the β-lactams tested. pBK44-60 has more or less identical MIC values compared to pBKA-81. pBK33-24 is expressing somewhat higher MIC values for cefoxitin and cefpodoxime compared to pBKA-81 and pBK44-60. Since these strains were cefepime selected one could expect an increased cefepime/clavulanic acid MIC compared to pBKA-81, but this was not observed. This suggests that the elevated resistance for cefepime in these isolates could be due to a porin mutation leading to a decrease in permeability in the outer membrane, rather than the mutations in the *ampC* gene coding regions.

pBK41-64 and pBK46-23 did not either express any higher MIC values compared to pBKA-81. pBK41-64 has almost identical values as the control for most of the β-lactams. pK46-23 is expressing higher MIC values for cefoxitin and cefpodoxime, like pBK33-24, compared to pBKA-81 and pBK41-64. We also expected to see an increase for the cefepime/clavulanic acid MICs in these strains as well, but there were no change in the catalytic efficacy in these either. When it comes to these two strains we have no good answers to why they have a typical AmpC phenotype resistance profile, because this study suggest that the gene coding mutations have nothing to say for the catalytic efficacy. A possible explanation for these strains can be that they possess a plasmid-mediated *ampC* gene that the multiplex PCR are unable to detect, like the ACC-1 β-lactamase. We cannot exclude that these enzymes have higher catalytic efficiencies against typical substrates for AmpC enzymes, but that such subtle differences are masked by the high expression of the genes from a high copy vector.

Genotypic studies of isolates with the IS-element IS*911*

PFGE analysis revealed that six of the seven IS*911* isolates were clonally related to each other. Further epidemiological data for these seven strains were collected. The six isolates K29-48, K29-77, K29-80, K30-44, K34-08 and K41-39 were all collected from patients in different institutions from the same area in Bergen. The seventh isolate K45-79 was collected from a patient in a hospital in Vestfold. Further patient data revealed that three, K29-77, K29-80, K30-44, of the six related strains were isolated from the same patient with one month between each isolation. This patient's first isolate K29-77 was isolated in the hospital in January 2006, the second isolate K29-80 was isolated in the nursing home in February 2006, and the third isolate K30-44 was isolated in a new nursing home in March 2006. The three other isolates were collected from different patients in the same nursing home. The first strain K29-48 was isolated in December 2005, the second strain K34-08 was isolated in August 2006, and the third strain K41-39 was isolated in March 2007. The most likely link between these clonal strains is a medical centre both nursing home use. The clonal outbreak of this *E. coli* clone probably started in December 2005 when the first strain was isolated in this medical centre. This is, to our knowledge, the first clonal outbreak of an *E. coli* with the IS*911* reported in Norway.

The clonal relationship between the four strains, carrying IS*911* in their promoter region, was confirmed with MLST typing identifying all isolates as ST131. The isolate from Vestfold was identified as ST550 belonging to ST complex 14.

Previous studies of MLST sequence types in clinical *E. coli* have identified that strains of ST131 are globally disseminated presenting successful, often highly virulent and resistant clone lineages. ST131 strains are associated with the global dissemination of CTX-M-15 type ESBL [15, 59], extended spectrum cephalosporin-resistance and plasmid-mediated quinolone resistance in the UK [60, 61], ciprofloxacin-resistance in eight European countries [62, 63], and with CMY-2 producing isolates in Norway [58]. They have also been isolated from stool samples of healthy people [64] and of cats and dogs within households [65]. One recent report describes the transmission of an ST131 *E. coli* between a father and daughter causing serious illness [66].

Concluding remarks

Results from the characterising of the gene control regions confirmed that the most usual mechanisms of resistance in these strains are mutations in the important and already known mutation sites in this region. These mutations increase the level of mRNAs and hence AmpC enzymes in the cells making them more resistant. Those isolates not having promoter mutations did not hyperexpress AmpC on mRNA level, suggesting other mechanisms responsible for their resistance.

The characterising of the gene coding regions gave us two different explanations. The cloned cefepime selected *ampC* genes had no change in the β-lactam MICs compared to the sensitive clone suggesting porin mutations making the cell more impermeable as a mechanism for their resistance against cefepime. The cloned AmpC selected *ampC* genes had no change either in the β-lactam MICs compared to the sensitive clone. This result leaves us with no reason for the resistance. These isolates should be studied further to reveal their mechanism of resistance.

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APPENDIX 1. Complete Etest for all clinical and sensitive strains

Strain number	Ampicillin	Amoxy/clav	Piperacillin	Pip/tazo	Cefoxitin	Cefuroxim	Cefpodoxim	Cefotaxim	Cefotax/clav	Ceftazidime	Cefta/clav	Cefepim/clav	Aztreonam	Imipenem	Meropenem	Cefox/boronic
K2-68	256	24	192	12	64	ND	96	4	4/>1	16	16/>4	<0,25	3	0.25	0.016	ND
K4-30	>256	48	24	24	256	ND	256	6	8/>1	16	24/>4	0,5	4	0.5	0.023	ND
K4-37	>256	32	64	24	96	ND	64	4	4/>1	12	16/>4	0,38	6	0.25	0.023	ND
K8-02	>256	24	32	24	>256	ND	256	16	16/>1	12	12/>4	0,25	16	0.19	0.023	ND
K9-38	>256	32	24	16	64	ND	64	6	6/>1	8	8/>4	<0,25	4	0.25	0.016	ND
K9-66	>256	48	12	12	256	ND	192	6	12/>1	16	16/>4	<0,25	4	0.25	0.023	ND
K14-27	>256	32	32	24	256	ND	128	6	12/>1	24	32/>4	0,38	6	0.25	0.016	ND
K14-35	>256	96	96	64	>256	ND	>256	32	>16/>1	64	32/>4	1,5	16	0.75	0.032	ND
K15-08	>256	32	32	32	128	ND	64	3	4/0.5	16	24/>4	0,38	12	0.25	0.023	ND
K22-31	>256	24	16	6	192	ND	128	4	4/>1	24	16/>4	<0,25	6	0.19	0.016	ND
K25-19	>256	48	24	8	256	ND	>256	6	8/>1	16	16/>4	<0,25	6	0.25	0.023	ND
K25-65	>256	48	6	4	128	ND	>256	12	16/>1	12	12/>4	3	6	0.19	0.023	ND
K29-48	> 256	64	32	6	128	ND	48	4	2 / > 1	6	6 / > 4	< 0,25 / 0,094	4	0,38	0,032	ND
K29-77	> 256	32	32	8	256	ND	96	4	3 / > 1	6	6 / > 4	< 0,25 / 0,125	8	0,38	0,023	Pos
K29-80	> 256	32	32	8	256	ND	64	4	3 / > 1	8	6 / > 4	< 0,25 / 0,094	6	0,25	0,023	Pos
K30-01	> 256	48	24	12	128	ND	256	4	6 / > 1	48	> 32 / > 4	< 0,25 / 0,19	4	0,19	0,016	Pos
K30-18	> 256	32	12	4	64	ND	128	4	3 / > 1	12	12 / > 4	< 0,25 / 0,064	12	0,25	0,023	Pos
K30-39	> 256	96	48	24	> 256	ND	256	8	4 / > 1	24	24 / > 4	0,25 / 0,19	16	0,75	0,094	Pos
K30-44	> 256	24	32	6	192	ND	48	3	2 / >1	6	3 / 4	3 / 4	6	0,19	0,023	Pos
K30-45	> 256	32	16	6	192	ND	64	4	3 / > 1	8	8 / >4	< 0,25 / 0,125	8	0,19	0,023	Pos
K33-01	> 256	32	> 256	12	128	ND	64	4	4 / > 1	12	8 / > 4	< 0,25 / 0,125	8	0,25	0,016	Pos
K33-03	> 256	24	> 256	2	32	ND	> 256	> 256	> 16 / 0,5	24	24 / 0,5	> 16 fant / < 0,064	48	0,25	0,023	Pos
K33-24	> 256	16	> 256	12	256	ND	8	3	1,5 / > 1	2	1,5 / 0,5	1,5 / 0,38	1,5	0,19	0,023	Pos
K33-54	> 256	32	> 256	24	256	ND	128	4	3 / > 1	12	8 / > 4	0,25 / 0,094	8	0,25	0,032	Pos
K34-08	> 256	64	48	12	192	48	64	4	3 / > 1	ND	4 / 4	< 0,25 / 0,094	6	0,38	0,032	Pos
K34-10	> 256	48	16	12	128	64	64	4	3 / 1	ND	8 / 2	< 0,25 / 0,125	6	0,38	0,023	Pos
K34-32	> 256	48	32	24	> 256	128	96	4	4 / > 1	ND	8 / 2	< 0,25 / 0,094	8	0,38	0,094	Pos
K34-43	> 256	48	32	12	256	128	128	8	8 / > 1	ND	24 / > 4	0,38 / 0,38	8	0,38	0,19	Pos
K34-61	> 256	128	32	24	256	96	96	6	6 / > 1	ND	32 / > 4	< 0,25 / 0,125	16	1	0,19	Pos
K34-69	> 256	32	> 256	> 256	96	256	64	4	4 / > 1	ND	6 / 2	< 0,25 / 0,094	8	0,25	0,032	Pos
K34-76	> 256	32	24	8	64	32	64	6	4 / > 1	ND	8 / > 4	0,75 / 0,5	2	0,19	0,094	Pos
K36-13	> 256	48	48	24	192	128	256	6	8 / > 1	ND	> 32 / > 4	0,38 / 0,125	12	0,25	0,047	Pos
K36-22	> 256	48	24	6	128	> 256	64	4	3 / > 1	ND	6 / 4	< 0,25 / 0,094	12	0,19	0,047	Pos
K36-30	> 256	48	12	6	32	64	64	4	3 / > 1	ND	8 / > 4	< 0,25 / 0,094	6	0,19	0,047	Pos
K36-50	> 256	48	24	8	48	64	96	4	3 / > 1	16	ND	< 0,25 / 0,094	8	0,25	0,032	Pos
K41-19	> 256	32	24	8	64	48	64	4	4 / > 1	ND	16 / 2	< 0,25 / 0,19	6	0,25	0,047	Pos
K41-22	> 256	32	24	12	96	96	96	6	3 / > 1	ND	16 / 4	< 0,25 / 0,125	12	0,25	0,023	Pos
K41-34	> 256	32	96	12	192	> 256	64	4	2 / > 1	ND	6 / 2	< 0,25 / 0,125	16	0,25	0,047	Pos
K41-39	> 256	32	64	8	256	64	64	4	3 / > 1	ND	6 / 2	< 0,25 / 0,125	6	0,25	0,032	Pos
K41-52	> 256	32	24	8	96	96	64	4	2 / > 1	8	6 / 1,5	< 0,25 / 0,125	4	ND	0,023	Pos
K41-53	> 256	24	16	8	256	256	96	4	2 / > 1	16	12 / 4	0,25 / 0,125	8	ND	0,012	Pos
K41-64	> 256	32	128	16	96	> 256	> 256	48	> 16 / > 1	128	> 32 / > 4	0,5 / 0,38	12	ND	0,023	Pos
K41-71	> 256	48	> 256	12	> 256	48	48	4	2 / > 1	4	2 / 1,5	0,38 / 0,19	6	ND	0,094	Pos
K44-58	> 256	24	32	24	192	96	64	4	4 / > 1	6	4 / 0,5	< 0,25 / < 0,064	8	ND	0,012	Pos
K44-60	> 256	8	96	8	96	64	6	2	1 / 0,25	3	2 / 0,25	1 / 0,19	0,75	ND	0,023	Pos
K44-72	48	8	8	8	256	> 256	12	2	1,5 / > 1	2	1 / 0,19	1 / 0,094	1,5	ND	0,016	Pos
K44-77	> 256	24	32	12	96	128	128	4	6 / > 1	16	12 / 2	< 0,25 / < 0,064	8	ND	0,023	Pos
K45-79	> 256	24	32	24	128	96	64	4	4 / > 1	8	6 / >4	< 0,25 / < 0,064	4	ND	0,016	Pos
K46-03	> 256	32	> 256	8	96	64	64	4	4 / > 1	16	8 / > 4	< 0,25 / 0,094	6	ND	0,023	Pos
K46-23	> 256	24	48	1,5	96	> 256	> 256	32	> 16 / > 1	48	> 32 / > 4	0,38 / 0,25	8	ND	0,032	Pos
K46-34	> 256	32	96	24	64	96	96	6	8 / > 1	12	8 / > 4	< 0,25 / 0,125	12	ND	0,023	Pos
K46-40	> 256	16	48	12	> 256	256	256	8	8 / > 1	8	6 / 4	< 0,25 / < 0,064	12	ND	0,023	Pos
K46-46	> 256	48	256	64	> 256	> 256	> 256	24	> 16 / > 1	128	> 32 / > 4	0,38 / 0,38	128	ND	0,25	Pos
K46-48	> 256	32	16	4	128	48	48	4	3 / > 1	8	4 / 1,5	< 0,25 / < 0,094	6	ND	0,047	Pos
K46-52	> 256	32	48	8	64	48	96	4	3 / > 1	12	8 / 4	< 0,25 / 0,125	4	ND	0,047	Pos
K46-73	> 256	16	256	48	> 256	> 256	256	8	16 / > 1	48		< 0,25 / 0,094	16	ND	0,023	Pos
Sensitive stammer!																
K18-03	>256	16	>256	3	4	ND	64	12	16/0.094	1	0.75/0.19	3/>0.064	2	0.19	0.023	ND
K27-39	≥32	16	ND	≤4	8	ND	1	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-41	≥32	4	ND	≤4	≤4	ND	≤0.25	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-42	≥32	4	ND	≤4	8	ND	0.5	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-43	4	≤2	ND	≤4	≤4	ND	0.5	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-44	8	4	ND	≤4	8	ND	2	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-45	4	4	ND	≤4	≤4	ND	≤0.25	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-46	8	4	ND	≤4	8	ND	1	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
K27-47	8	≤2	ND	≤4	≤4	ND	0.5	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND
ATCC 25922	4	≤2	ND	≤4	≤4	ND	≤0.25	≤1	ND	≤1	ND	ND	≤1	ND	≤0.25	ND

APPENDIX 2. Nucleotide alignment of *ampC* promoter sequences.

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                  10                  20                  30                  40                  50                  60                  70                  80
1 T G C A C G A T C T G A A A A T C C A C G T A C C T G C G G G C A A A T G G G T T T T C T A C G G T C T G G C T G C T A T C C T G A C A G T T G T C A C G C T G A T T G G E.coli K-12
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K2-68
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K4-30
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K4-37
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K8-2
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K9-38
1 . . . . . . . . . . . . . G . . T . . . . . G . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K9-66
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K14-27
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K14-35
1 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - N N . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K15-8
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K22-31
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K25-19
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K25-65
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K29-48
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K29-77
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K29-80
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K30-1
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K30-18
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K30-39
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K30-44
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K30-45
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K33-1
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K33-24
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . A . . . . . . . . K33-3
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . K33-54
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K34-10
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K34-32
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K34-43
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K34-61
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K34-69
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K34-76
1 . . . . . . . . . . . . . . - . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K34-8
1 . . . . . . . . . . . . . G . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K36-13
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K36-22
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K36-30
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K36-50
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K41-19
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K41-22
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . A . . . . . . . . K41-34
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K41-39
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K41-52
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K41-53
1 . . . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K41-64
1 . . . . . . . . . . . . . - . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K41-71
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . K44-58
1 . . . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K44-60
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K44-72
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K44-77
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . K45-79
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K46-03
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . K46-23
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K46-34
1 . . . . . . . . . . . . . . - . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . K46-40
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . K46-46
1 . . . . . . . C . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K46-48
1 . . . . . . . . . . . . . . . . T . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . K46-52
1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . K46-73
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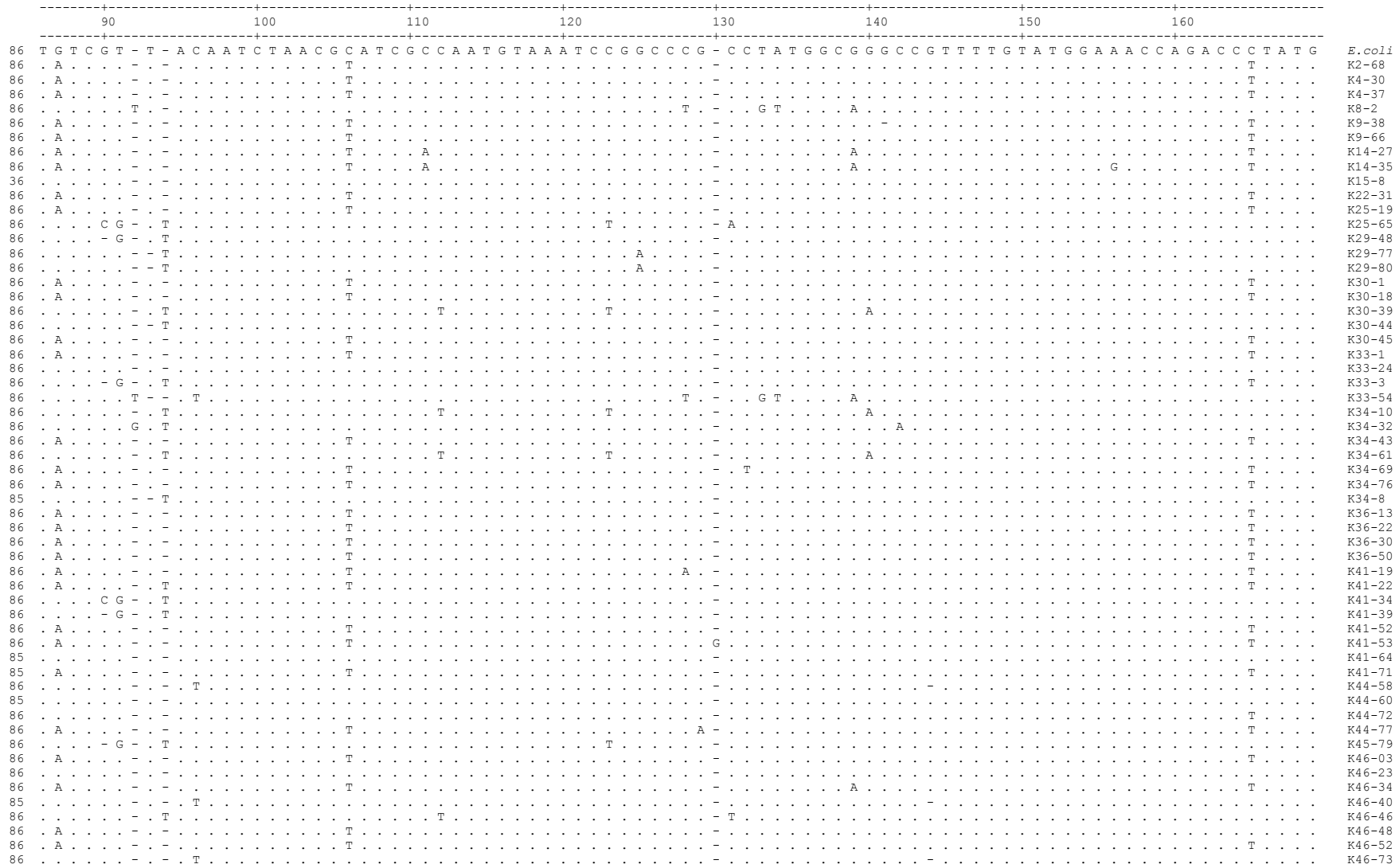
90
100
110
120
130
140
150
160
E.coli K-12
K2-68
K4-30
K4-37
K8-2
K9-38
K9-66
K14-27
K14-35
K15-8
K22-31
K25-19
K25-65
K29-48
K29-77
K29-80
K30-1
K30-18
K30-39
K30-44
K30-45
K33-1
K33-24
K33-3
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K34-61
K34-69
K34-76
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K41-39
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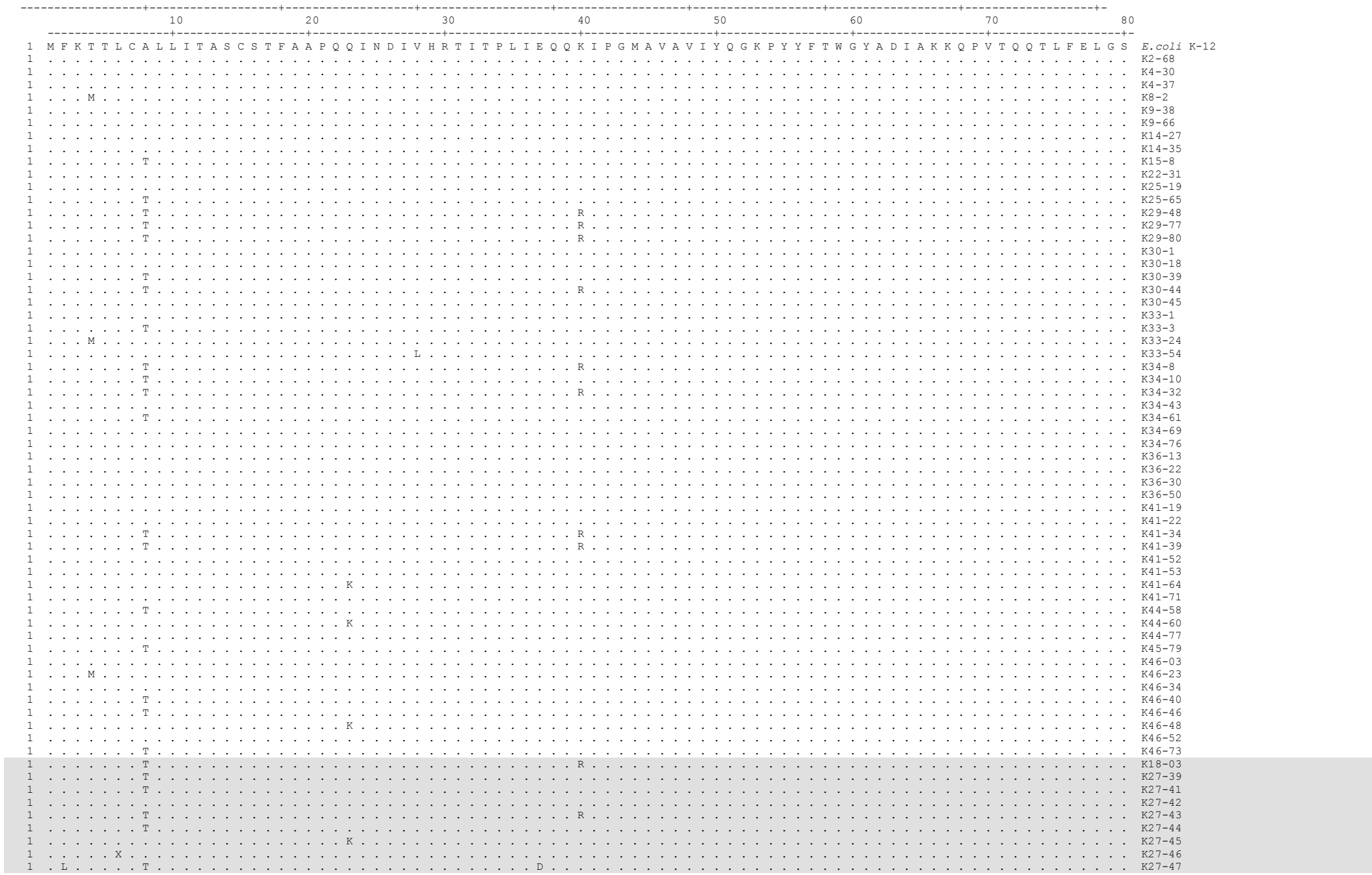
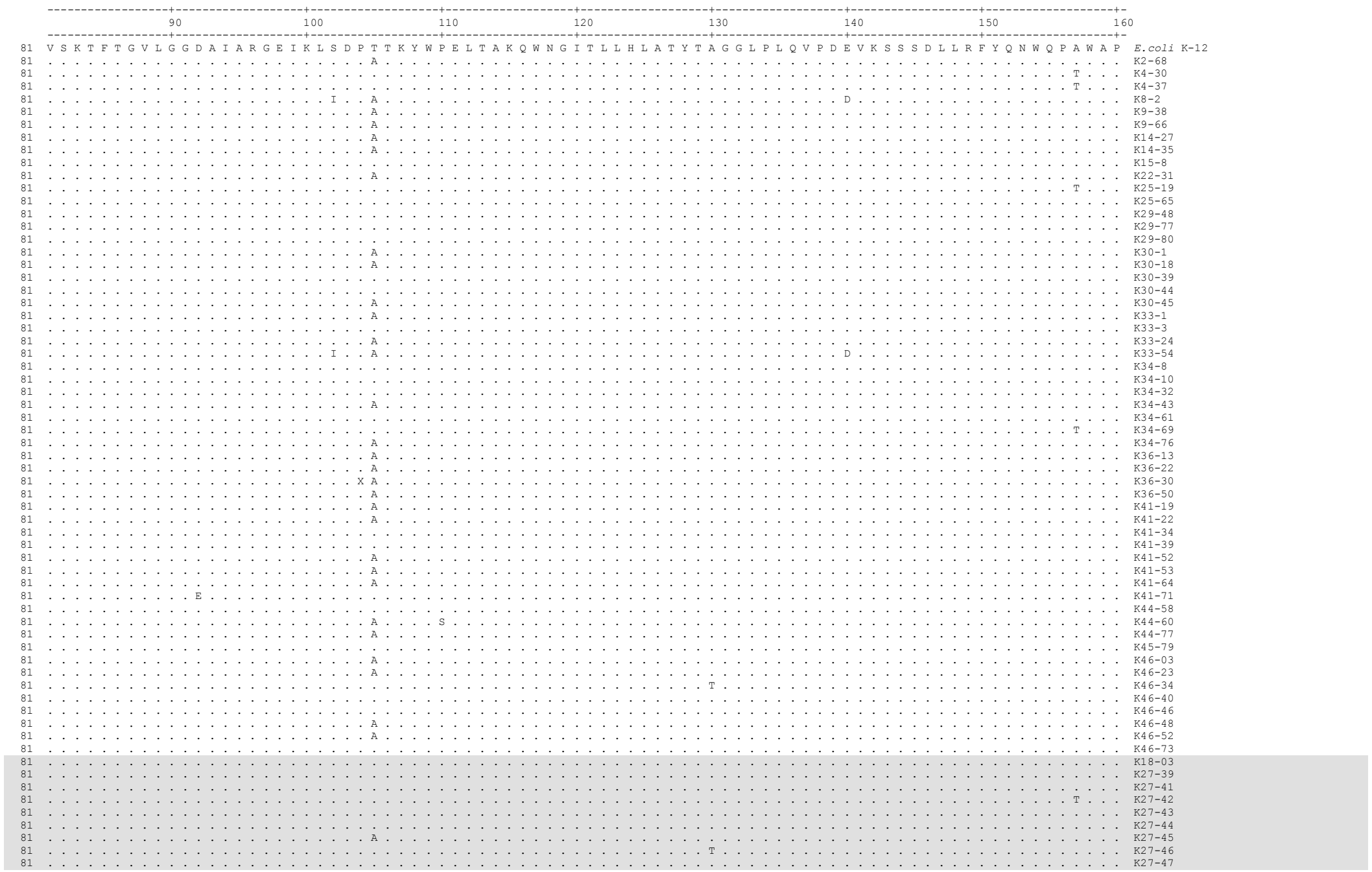
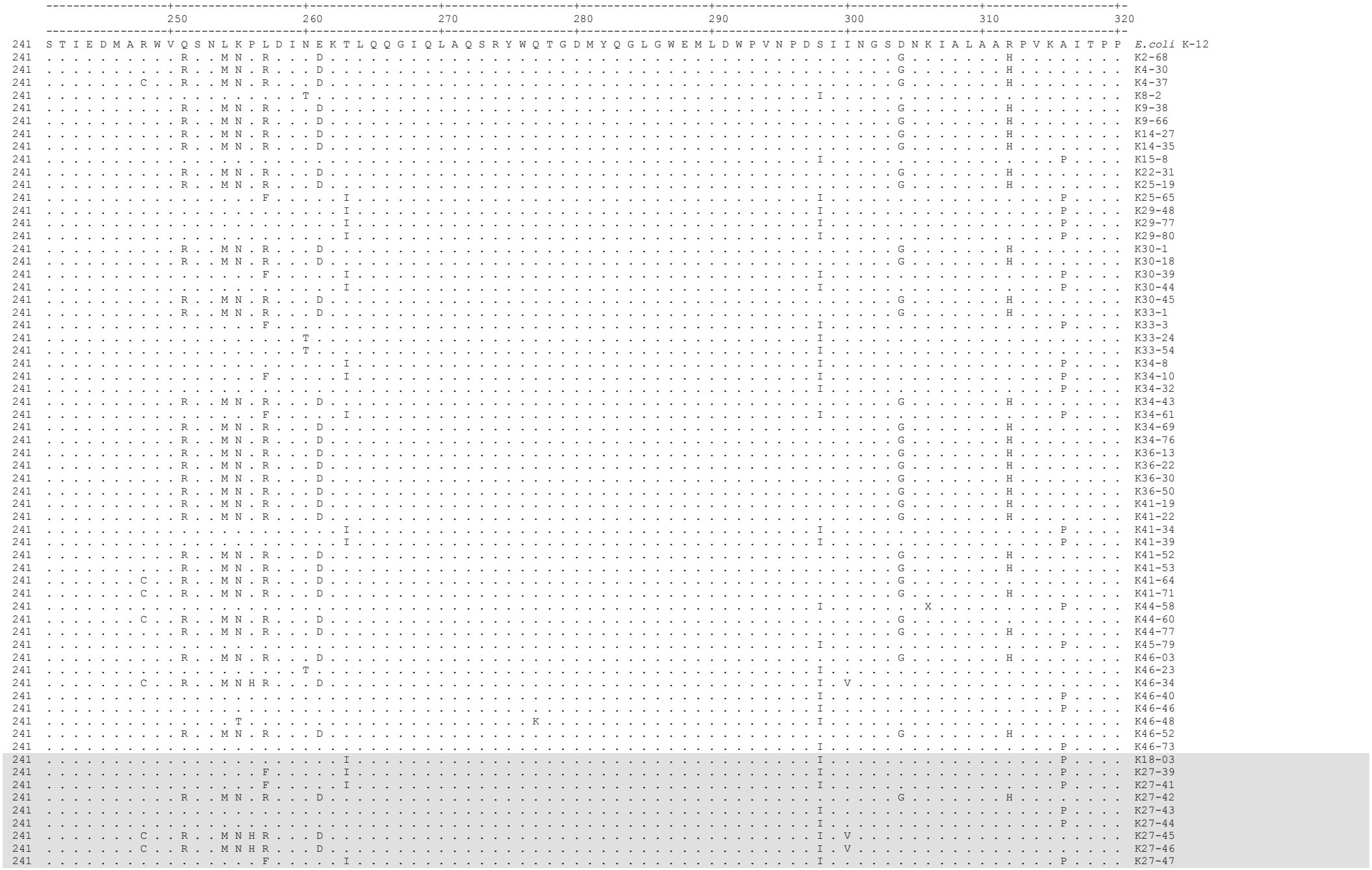
K41-53
K41-64
K41-71
K44-58
K44-60
K44-72
K44-77
K45-79
K46-03
K46-23
K46-34
K46-40
K46-46
K46-48
K46-52
K46-73

APPENDIX 3: Amino acid alignment of the complete *ampC* gene coding region for *E. coli* K-12, cephalosporin resistant and sensitive (grey) strains. Amino acids that differ from E. coli K-12 are shown.




90 100 110 120 130 140 150 160
81 V S K T F T G V L G G D A I A R G E I K L S D P T T K Y W P E L T A K Q W N G I T L L H L A T Y T A G G L P L Q V P D E V K S S S D L L R F Y Q N W Q P A W A P E.coli K-12
81 . . . . . A K2-68
81 . . . . . T K4-30
81 . . . . . T K4-37
81 . . . . . I . . A . . . . . D K8-2
81 . . . . . A K9-38
81 . . . . . A K9-66
81 . . . . . A K14-27
81 . . . . . A K14-35
81 . . . . . K15-8
81 . . . . . A K22-31
81 . . . . . T K25-19
81 . . . . . K25-65
81 . . . . . K29-48
81 . . . . . K29-77
81 . . . . . K29-80
81 . . . . . A K30-1
81 . . . . . A K30-18
81 . . . . . K30-39
81 . . . . . K30-44
81 . . . . . A K30-45
81 . . . . . A K33-1
81 . . . . . K33-3
81 . . . . . A K33-24
81 . . . . . I . . A . . . . . D K33-54
81 . . . . . K34-8
81 . . . . . K34-10
81 . . . . . K34-32
81 . . . . . A K34-43
81 . . . . . K34-61
81 . . . . . T K34-69
81 . . . . . A K34-76
81 . . . . . A K36-13
81 . . . . . A K36-22
81 . . . . . X A K36-30
81 . . . . . A K36-50
81 . . . . . A K41-19
81 . . . . . A K41-22
81 . . . . . K41-34
81 . . . . . K41-39
81 . . . . . A K41-52
81 . . . . . A K41-53
81 . . . . . A K41-64
81 . . . . . E K41-71
81 . . . . . K44-58
81 . . . . . A . . . . S K44-60
81 . . . . . A K44-77
81 . . . . . K45-79
81 . . . . . A K46-03
81 . . . . . A K46-23
81 . . . . . T K46-34
81 . . . . . K46-40
81 . . . . . K46-46
81 . . . . . A K46-48
81 . . . . . A K46-52
81 . . . . . K46-73
81 . . . . . K18-03
81 . . . . . K27-39
81 . . . . . K27-41
81 . . . . . T K27-42
81 . . . . . K27-43
81 . . . . . K27-44
81 . . . . . A K27-45
81 . . . . . T K27-46
81 . . . . . K27-47


170 180 190 200 210 220 230 240
161 G T Q R L Y A N S S I G L F G A L A V K P S G L S F E Q A M Q T R V F Q P L K L N H T W I N V P P A E E K N Y A W G Y R E G K A V H V S P G A L D A E A Y G V K E.coli K-12
161 . . . . . P K2-68
161 . . . . . P K4-30
161 . . . . . T K4-37
161 . . . . . H . . . . T . . . . . S K8-2
161 . . . . . P K9-38
161 . . . . . P K9-66
161 . . . . . P K14-27
161 . . . . . P . . . . . X K14-35
161 . . . . . K . . . . . S K15-8
161 . . . . . P K22-31
161 . . . . . P K25-19
161 . . . . . K . . . . . S K25-65
161 . . . . . K . . . . . S K29-48
161 . . . . . K . . . . . S K29-77
161 . . . . . K . . . . . S K29-80
161 . . . . . P K30-1
161 . . . . . P K30-18
161 . . . . . K . . . . . S K30-39
161 . . . . . K . . . . . S K30-44
161 . . . . . P K30-45
161 . . . . . P . . . . . V K33-1
161 . . . . . K . . . . . S K33-3
161 . . . . . T . . . . . S K33-24
161 . . . . . T . . . . . S K33-54
161 . . . . . K . . . . . S K34-8
161 . . . . . K . . . . . S K34-10
161 . . . . . K . . . . . S K34-32
161 . . . . . P K34-43
161 . . . . . K . . . . . S K34-61
161 . . . . . P . . . . . I K34-69
161 . . . . . P K34-76
161 . . . . . P K36-13
161 . . . . . P . . . . . L K36-22
161 . . . . . P K36-30
161 . . . . . P K36-50
161 . . . . . P K41-19
161 . . . . . P K41-22
161 . . . . . K . . . . . S . . . . . V K41-34
161 . . . . . K . . . . . S K41-39
161 . . . . . P K41-52
161 . . . . . P K41-53
161 . . . . . T K41-64
161 . . . . . T K41-71
161 . . . . . K . . . . . S K44-58
161 . . . . . T K44-60
161 . . . . . P K44-77
161 . . . . . K . . . . . S K45-79
161 . . . . . P K46-03
161 . . . . . T . . . . . S K46-23
161 . . . . . T K46-34
161 . . . . . K . . . . . S K46-40
161 . . . . . K . . . . . S K46-46
161 . . . . . K46-48
161 . . . . . P K46-52
161 . . . . . K . . . . . S K46-73
161 . . . . . K . . . . . S K18-03
161 . . . . . K . . . . . S K27-39
161 . . . . . K . . . . . S K27-41
161 . . . . . P K27-42
161 . . . . . K . . . . . S K27-43
161 . . . . . K . . . . . S K27-44
161 . . . . . K27-45
161 . . . . . T K27-46
161 . . . . . K . . . . . S K27-47


250 260 270 280 290 300 310 320
S T I E D M A R W V Q S N L K P L D I N E K T L Q Q G I Q L A Q S R Y W Q T G D M Y Q G L G W E M L D W P V N P D S I I N G S D N K I A L A A R P V K A I T P P
E.coli K-12
K2-68
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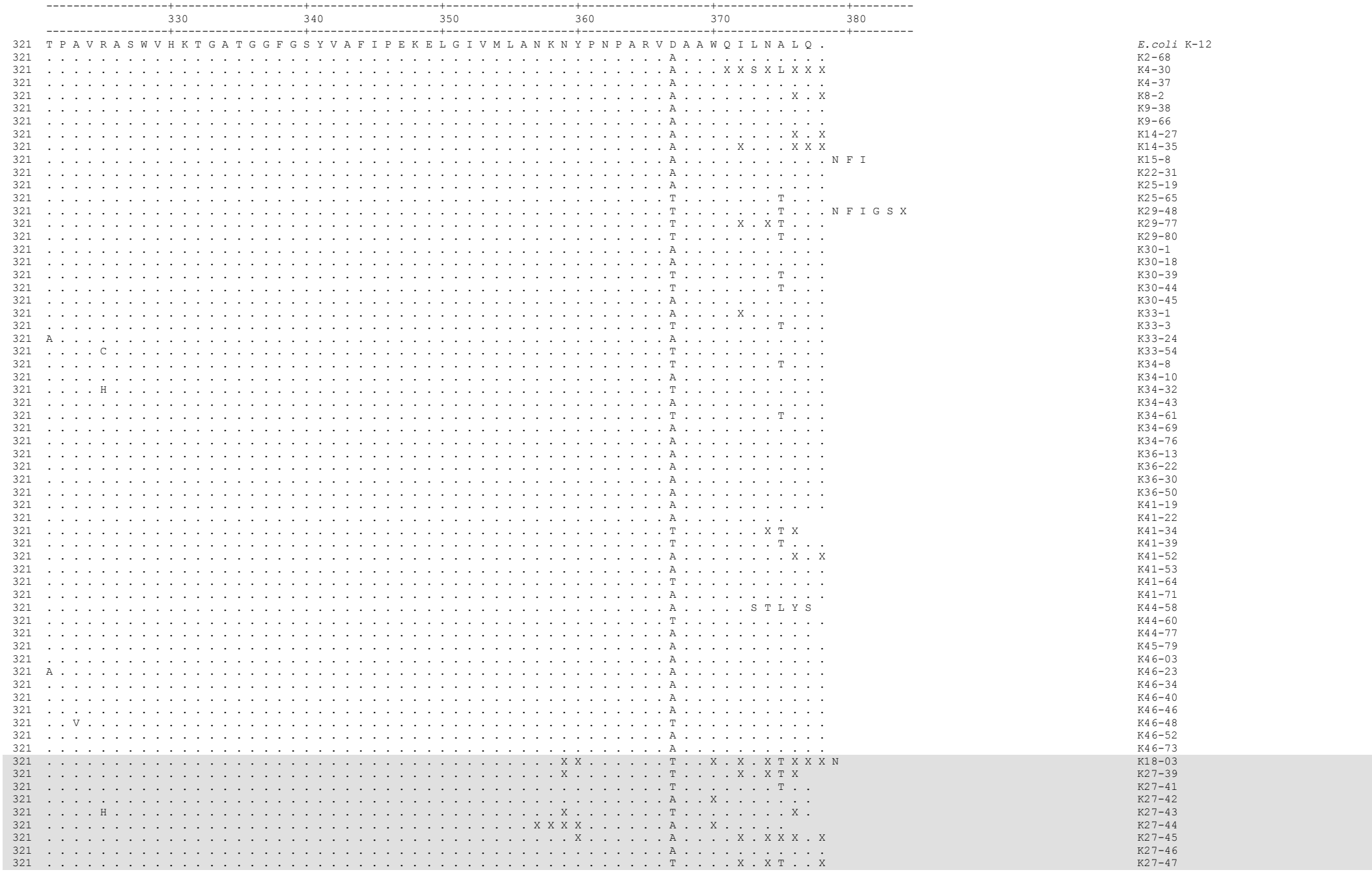
K27-46
K27-47


330
340
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380
321 T P A V R A S W V H K T G A T G G F G S Y V A F I P E K E L G I V M L A N K N Y P N P A R V D A A W Q I L N A L Q .
E.coli K-12
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