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MECHANISMS INVOLVED IN THE EXPRESSION OF THE PLASMID-ENCODED AmpC β-LACTAMASE GENE bla_{CMY-2} IN E. coli

By
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A Dissertation

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ABSTRACT

*Enterobacteriaceae* that express plasmid-encoded AmpC β-lactamases can be resistant to third generation cephalosporins and β-lactam/β-lactamase inhibitor combinations. Infections by these bacteria are associated with increased patient morbidity and mortality. Little is known about how plasmid-encoded AmpC genes are regulated or expressed. The experiments described in this dissertation sought to determine mechanisms that influence the transcription of *bla*<sub>CMY-2</sub>; the most common plasmid-encoded AmpC β-lactamase gene found in *E. coli* worldwide. Four clinical piperacillin/tazobactam-susceptible *E. coli* parent strains carrying *bla*<sub>CMY-2</sub> and twelve piperacillin/tazobactam-resistant mutants selected from the parent strains were the focus of study. It was hypothesized that sequence changes upstream of *bla*<sub>CMY-2</sub> were driving increased *bla*<sub>CMY-2</sub> expression and enabling resistance to piperacillin/tazobactam in the mutants. It was also hypothesized that transcription factors binding to sequence upstream of *bla*<sub>CMY-2</sub> promoter regions were influencing expression. Three important findings were made testing these hypotheses. 1) It was found that only 33% of the piperacillin/tazobactam-resistant mutants were overexpressing *bla*<sub>CMY-2</sub>. No upstream sequence changes were found in *bla*<sub>CMY-2</sub> overexpressing mutant strains. For all overexpression mutants, changes in *bla*<sub>CMY-2</sub> transcript level were associated with increased copy number of their *bla*<sub>CMY-2</sub> encoded plasmid. Two mutants with *bla*<sub>CMY-2</sub> on a 100 kb IncI1 plasmid had point mutations in the *inc* antisense RNA gene that controls IncI1 copy number. 2) Examination of upstream sequence for two parent strains identified a novel divergent tandem *bla*<sub>CMY-2</sub> arrangement flanking an IS5 insertion element. Strains with this feature had 2-fold higher *bla*<sub>CMY-2</sub> expression than a single arrangement strain. 3) The transcriptional activator Rob was identified binding sequence upstream of the distal *bla*<sub>CMY-2</sub> promoter sequence within the insertion element ISEcp1. Additional studies indicated Rob was activated by β-lactam exposure and therefore may enhance *bla*<sub>CMY-2</sub> expression when β-lactams are present. Further work will be needed to determine how gene copy number and Rob play a role in β-lactam resistance among clinical *E. coli* strains carrying *bla*<sub>CMY-2</sub>.
ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to Dr. Nancy Hanson. The funding and resources for this thesis work would never have existed without her hard work and dedication. The success of this work is also a direct result of her guidance and her boundless enthusiasm and desire to see good science.

I would also like to thank the members of my thesis committee, Dr. Richard Goering, Dr. Joseph Knezetic, Dr. Phil Lister, and Dr. Patrick Swanson for their dedication to guiding the progress and quality of this thesis work and my graduate education. Their advice and input were extremely valuable, and each provided materials and supplies, that without which, many of the experiments in this dissertation could not have been performed.

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I am also indebted to Dr. Ken Thomson, and Ellen Smith Moland for sharing their protocols, equipment, laboratory space, and their expertise with mutant selection and antibiotic susceptibility testing.

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<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>× g</td>
<td>Times gravitational force</td>
</tr>
<tr>
<td>6XHis-Rob</td>
<td>Rob protein expressed with six N-terminal histidines</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration curve</td>
</tr>
<tr>
<td>BAP</td>
<td>Blood agar plate, trypticase soy agar with 5% Sheep’s blood</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CSB</td>
<td>Cell suspension buffer</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DR</td>
<td>Nucleotide direct repeats</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-spectrum beta-lactamase</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>GTE</td>
<td>Glucose Tris-EDTA buffer</td>
</tr>
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## I. Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HTH</td>
<td>Helix-turn-helix</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IIR</td>
<td>Insertion sequence imperfect inverted repeat</td>
</tr>
<tr>
<td>IR</td>
<td>Insertion sequence inverted repeat</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence element</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani bacterial growth medium</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MHA</td>
<td>Mueller Hinton agar</td>
</tr>
<tr>
<td>MHB</td>
<td>Mueller Hinton broth</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin-sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>NAM</td>
<td>N-acetylmuramic acid</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at 600 nm light wavelength</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin binding protein</td>
</tr>
<tr>
<td>pET-rob</td>
<td>pET 100 plasmid with rob insert</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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## I. Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription - polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Shine Dalgarno sequence, or standard deviation</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>$T_m$</td>
<td>DNA melting temperature</td>
</tr>
<tr>
<td>Topt</td>
<td>Optimum probe hybridization temperature</td>
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II. Introduction and Review of the Literature

A. Introduction

This dissertation work tested the hypothesis that increased expression of the plasmid-encoded AmpC β-lactamase gene \( bla_{CMY-2} \) was associated with decreased piperacillin/tazobactam susceptibility in \( E. coli \) possessing \( bla_{CMY-2} \). Experiments were conducted to evaluate \( bla_{CMY-2} \) gene copy number, upstream DNA sequence, and cytoplasmic proteins binding to \( bla_{CMY-2} \) promoter regions. Three previously unrecognized mechanisms were identified that impacted the expression of \( bla_{CMY-2} \). One of these mechanisms included changes in plasmid copy number for a 100 kb IncI1 plasmid that encoded \( bla_{CMY-2} \) and directly affected \( bla_{CMY-2} \) expression. Second was the recognition of a novel tandem \( bla_{CMY-2} \) arrangement associated with insertion element IS5 that was associated with a 2-fold increase in \( bla_{CMY-2} \) transcription level. Third was the identification of the transcription factor Rob binding to sequence near a \( bla_{CMY-2} \) promoter region located within the insertion element ISEcp1. Evidence for the activation of Rob by β-lactam antibiotics was also observed, and this potentially makes the association with Rob a meaningful one for \( bla_{CMY-2} \) and other β-lactamase genes promoted by ISEcp1.

The following literature review contains a broad account of the subjects involved in these three discoveries. Section B is a review of β-lactam antibiotics. There is emphasis on the β-lactam drugs used for susceptibility testing in this study with particular emphasis on piperacillin/tazobactam which was used as a selection agent for \( bla_{CMY-2} \) overexpression mutants. Section C features a comprehensive review of β-lactam resistance mechanisms with special attention given to CMY-2 and other AmpC
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β-lactamases. Section D covers plasmids and plasmid replication with a focus on the replication of IncI1 plasmids. Section E is a brief review of insertion sequence elements IS\textit{Ecp1} and IS5. And lastly, section F reviews the transcription factor Rob and the role it plays in gene expression.

B. β-lactam Antibiotics

B. 1. Overview of β-lactam Antibiotics and their Activity

The β-lactams were one of the first classes of antibacterial chemotherapeutic agents to be mass produced, and they remain the most widely used class of antibiotics in the world today. Their development is one of the greatest achievements in the history of medicine. Since their introduction, β-lactams have expanded from basic fungal derivatives to semi-synthetic and synthetic agents. As of April 2013, at least 34 beta-lactam compounds had been approved for human use by the U.S. FDA (1). As an antibiotic class, they have characteristically been stable and effective at killing susceptible bacteria while remaining relatively non-toxic to humans and animals. These favorable properties are what have perpetuated their use. The basic structure of all β-lactam drugs is the β-lactam ring nucleus (Figure 1a). Lactam refers to the cyclic amide structure, and beta indicates the nitrogen is attached to the beta carbon. The nitrogen and all carbons except the carbonyl carbon (which is necessary for drug activity) can form bonds with additional atoms. These additional bonds define the class of the β-lactam drug. There are four major classes of β-lactams based on the ring structure connected to the β-lactam ring: 1) The addition of a saturated pentacyclic ring creates a penam (Figure 1b); 2) an unsaturated pentacyclic ring yields a penem (Figure 1c); 3) a
hexacyclic ring addition forms a cephem (Figure 1d); and 4) if no cyclic additions are made to the β-lactam, the molecule is classified as a monobactam. The properties of the drugs within each of these β-lactam divisions are discussed in the sections below.

**Figure 1.** Structural Nuclei of β-Lactam Classes

![Structural Nuclei of β-Lactam Classes](image)

The atom position numbering of these molecules begins with the atom adjacent to the β position carbon of the lactam molecule. This position is typically occupied by a sulfur atom for most penams and cephems, and a carbon for the penems. The position numbering continues clockwise back to the β-carbon, then counter clockwise around the β-lactam ring. Monobactams follow the conventional, International Union of Pure and Applied Chemistry (IUPAC) positioning scheme with the nitrogen atom as position 1, the carbonyl carbon as 2, and the α- and β-carbons at 3, and 4 respectively.

β-lactams are bactericidal drugs due to their ability to interfere with proteins that build and maintain the bacterial cell wall. The degree of interference that occurs, and the
specific proteins that are impaired depend on the drug being used, the concentration of
drug, and the properties of the bacterial cell that is being treated. Proteins that have been
recognized to bind β-lactam drugs are referred to as penicillin binding proteins (PBPs).
The PBPs of each bacterial species are given numerical designation (i.e. PBP1, PBP2, etc.) in descending molecular weight order. The numbers have no bearing on function or composition of the protein, and PBPs with identical numerical designations in different species may be entirely different proteins (e.g. PBP2 in *Escherichia coli* and in *Bacillus subtilis*) (2). In general however the higher molecular PBPs (e.g. *E. coli* PBP1, PBP2) tend to be bi-functional transglycosylase/ transpeptidases and lower molecular weight PBPs (*E. coli* PBP4, PBP5, PBP6) are usually monofunctional carboxypeptidases.

Since *E. coli* is the focus of this dissertation, the function of *E. coli* PBPs will be used as an example of PBP function and β-lactam interference. The cell wall of *E. coli*, like many other bacteria, is composed of strands of alternating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) sugars linked by β-(1,4)-glycosidic bonds (Figure 2) (3). The bonds between these units are catalyzed by PBP1A and PBP1B, and the strands of NAM-NAG heterodimers are referred to as glycan (4). In *E. coli*, the glycan strands average between 20 to 40 heterodimers in length depending on growth conditions (3). Each NAM unit has an amino acid side chain attached to it, primarily consisting of L-Ala–D-iGlu–m-A2pm–D-Ala–D-Ala pentapeptide as it extends from the NAM unit, with dibasic meso-diaminopimelic acid (m-A2pm) in the third position (5). Approximately 60% of amino acid side chains are cross-bridged to a side chain of a parallel glycan strand by a peptide bond (6). The cross-bridges provide rigidity for cell shape and osmotic stability, yet still allows for some flexibility (3, 7). The cross-bridges
are formed by transpeptidases (e.g. PBP2, PBP3) which first cleave the bond between a 4th and 5th D-alanine (Figure 2a). This cleavage provides the energy needed to form a peptide bond between the 4th position D-alanine and the D-center of m-A2pm of a parallel glycan strand (8, 9). A small percentage of cross-bridges are also formed between L-center of m-A2pm of one peptide chain and the D-center of m-A2pm of another peptide chain (6). DD-carboxypeptidases (e.g. PBP4, PBP5, PBP6) also cleave the bond between the 4th and 5th D-alanines (Figure 2) (10). They function to cleave the remaining 5th D-alanine of a cross-bridged side chain, or uncross-bridged side chains (Figure 2a, 2b). This activity is thought to limit excessive cross-bridge formation. In some cases the DD-carboxypeptidase (e.g. PBP 4) may also catalyze cross-bridge formation (11).

The *E. coli* PBPs mentioned above (PBP1A- PBP6) all have DD-peptidase activity with a conserved Ser-X-X-Lys active site (where X is any amino acid) (3, 10). For both carboxypeptidases and transpeptidases, the active site serine hydroxyl group oxygen interacts with the carbonyl carbon of a terminal D-Ala in the NAM peptide side chain (Figure 3a, 3b) and an acylenzyme intermediate is formed (Figure 3c). For carboxypeptidases under normal conditions, the acylenzyme bond is broken when the oxygen of a water molecule takes an electron from the carbonyl carbon attached to the enzyme, forming a carboxyl group, and cleaving the D-Ala–D-Ala peptide bond (Figure 3d) (10). With transpeptidases, the acylenzyme complex is broken when the ε-NH2-group of the m-A2pm residue accepts an electron from the adjacent D-Ala carbonyl carbon releasing the enzyme and forming the cross-link (3).
**Figure 2.** Actions of Transpeptidases and Carboxypeptidases in *E. coli* Peptidoglycan Peptide Cross-Bridge Formation

Transpeptidases (TP) cleave the bond between the 4\(^{th}\) and 5\(^{th}\) D-alanine of one N-acetylmuramic acid (NAM) pentapeptide side chain. This provides energy for the transpeptidase to catalyze the formation of a cross-bridge between the #4 D-alanine and the meso-diaminopimelic acid of an adjacent side chain.

Carboxypeptidases (CP) also cleave the bond between the 4\(^{th}\) and 5\(^{th}\) D-alanine between cross-bridged (a) and uncross-bridged (b) side chains. Cleavage of uncross-bridged side chains prevents excessive transpeptidase cross-linking and maintains cell shape.
β-lactam drugs that are able to interfere with PBPs do so because the carbonyl group of the β-lactam has similar structure to the carbonyl group of the D-Ala, D-Ala PBP target, and they fit into the active site of the PBP. When such an interaction occurs, the oxygen of the PBP serine hydroxyl group binds with the carbonyl carbon of the β-lactam ring. The ring opens up irreversibly and a stable ester bond is formed (Figure 4) (2). Unlike the acyl enzyme intermediates, the PBP-β-lactam bond cannot be easily disrupted by subsequent nucleophile attack from water or another amino group. The loss of DD-carboxypeptidase and/or transpeptidase function due to the β-lactam binding inhibits the formation of peptidoglycan cross-bridges, inhibits cell division, and leads to instability of the cell wall (12). In some cases, particularly with gram positive organisms, β-lactams trigger expression of autolysins that lead to cell destruction (13).
Figure 3. Molecular Diagram of DD-peptidase Hydrolysis of a D-Ala, D-Ala Peptide Bond

(a) The hydroxyl group of a serine molecule in the active site of the penicillin binding protein (PBP, i.e. carboxypeptidases and transpeptidases) attacks the carbonyl carbon of the terminal D-alanine and forms an acyl intermediate (b). Other residues in the enzyme active site form hydrogen bonds with a water molecule and position it to attack the carbonyl carbon of the terminal D-alanine (c). Formation of a bond between the water oxygen and the carbonyl carbon (d) breaks the peptide bond between the 4th and 5th D-alanine and deacylates the acylenzyme intermediate.
Figure 4. Penicillin Binding Protein Interaction with a β-Lactam Molecule (Penam)

(a) The β-lactam molecule has structural similarities to the D-alanine target that the PBP usually interacts with (see figure 3a) and they fit into the active site of the PBP enzyme. A key difference in the reaction with the β-lactam is that when the serine hydroxyl group attacks the carbonyl carbon, the bond with the nitrogen immediately breaks to relieve stress on the azetidinone structure (b). Due to steric hindrance and stability of the new bond, there is no subsequent hydrolysis attack on the carbonyl carbon. Instead, the PBP remains bound to the β-lactam molecule and is inactivated.
B. 2. **Penicillins**

The mold genus *Penicillium* was established in 1809 by Johann Heinrich Friedrich Link, but the therapeutic properties of this fungal group were already known to the ancient Egyptians and Bhutanese (14). In 1929 Alexander Fleming was the first to publish studies of the antibacterial properties of *Penicillium notatum* on cultures of *Staphylococcus aureus* and *Streptococcus pyogenes* (15). Ernst Boris Chain and Howard Florey later successfully treated mice with *Penicillium* extracts after experimentally infecting them with *S. aureus*, *S. pyogenes*, and *Clostridium septique* (*septicum*) and published their findings in August 1940 (16). The first treatment of a patient with *Penicillium* extracts occurred six months later in Oxford, England.

B. 2. a. **Benzylpenicillins.**

The β-lactam agents present in the *Penicillium* extracts of these early experiments have come to be known as penicillins. The penicillins are penams with the saturated pentacyclic ring being a thiazolidine. A side chain at C-6 distinguishes the different penicillin drugs. For example, a benzyl group at C-6 yields penicillin G (Figure 5) a phenoxyethylmethyl group at this location defines penicillin V. These two penicillins are produced naturally by *Penicillium* species and are referred to as benzylpenicillins. The benzylpenicillins, like most penicillins, are not stable in stomach acids and need to be administered parentally. They can distribute to most tissues and serous fluids, and can achieve therapeutic concentrations in the CNS and bone. Benzylpenicillins are primarily active against Gram-positive organisms with *E. coli* and other *Enterobacteriaceae* sustaining inherent resistance. More than 80% of circulating *S. aureus* strains are now
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resistant to benzylpenicillins [Minimal inhibitory concentration (MIC) ≥ 2 µg/ml], however benzylpenicillins still remains a viable treatment option for most meningococcal, and *Streptococcus pyogenes* infections (14, 17, 18).

**Figure 5.** Penicillin G

![Penicillin G](image)

B. 2. b. **Group M (Modified) Penicillins**

The emergence of penicillin resistance among *S. aureus* in the 1940s and 50s led to the development of the Group M or modified penicillins. Members of this group are semisynthetic and feature chemical modification to the penicillin G structure. One example is methicillin (Figure 6), which contains two methoxy groups at position 2’ and 6’ of the benzene ring. Another example is cloxacillin (Figure 7) which has an isoxzolyl group between the penam and the benzene ring, and a chlorine atom attached to the benzene. These additions increase the steric hindrance around the β-lactam carbonyl carbon atom protecting it from hydrolysis by narrow spectrum penicillinas. However, these additions also reduce the antibacterial activity of the molecule. Cloxacillin is also notable drug in this group because of its inhibitory effect against AmpC β-lactamases and can be used in phenotypic detection schemes for these enzymes (19, 20).

Today, the group M penicillins have limited use against *S. aureus*. Data from the National Nosocomial Infection Surveillance program in 2004 indicated that 44-48% of *S.*
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_aureus_ isolated from hospitalized patients in the U.S. are resistant to methicillin (21). This resistance is largely due to acquisition of a gene for an altered penicillin binding protein (PBP 2a) (see section I. C. 1. b). Many of these methicillin-resistant _S. aureus_ (MRSA) strains harbor additional resistance mechanisms against aminoglycosides, fluoroquinolones and macrolides making them difficult to treat.

B. 2. c. _α_ -Aminopenicillins (Group A Penicillins)

The need to treat Gram-negative bacterial infections led to the development of semisynthetic _α_ -aminopenicillins (Group A penicillins) in the 1960s. Two prominent members of this group are ampicillin, and amoxicillin (Figure 8) which are structured similar to penicillin G, but with an amino group between the penam and the benzyl group. The charged amino group enables the drug to penetrate the outer membrane of Gram-negative organisms and protect the β-lactam carbonyl carbon from hydrolysis by penicillinases. Another novelty of the _α_-aminopenicillins is that they can be taken orally. Bioavailability after oral administration ranges from 32-53% for ampicillin and 60-89% for amoxicillin (14). Unfortunately the _α_-aminopenicillins are not active against bacteria
with inducible AmpC β-lactamase expression (e.g. Enterobacter spp, Citrobacter spp, Pseudomonas aeruginosa) (14). The spread of plasmid-encoded TEM β-lactamases among E. coli, H. influenza and Proteus spp. during the 1970s also limited the use of Group A penicillins for treating infections of these species (22, 23).

**Figure 8.** Amoxicillin

B. 2. d. α-Carboxypenicillins and α-Sulfopenicillins

In the late 1960s the α-carboxypenicillin and α-sulfopenicillins were also developed to expand the spectrum of activity of penicillin against Gram-negative organisms. Instead of an amino group attached to the number 6 carbon, these drugs feature a carboxyl group (α-carboxypenicillins, e.g. carbenicillin, ticarcillin) or a sulfonic acid group (α-sulfopenicillins, e.g. sulbenicillin) attached at this location.

B. 2. e. 6-α-Penicillins

The development of 6-α-penicillins was first explored in the early 1970s in an attempt to create a better D-Ala-D-Ala analog from an α-aminopenicillin base (14). In the late 1980s temocillin (Figure 9) was introduced and featured a methoxy group attached to...
the number 6 carbon of the penam ring of ticarcillin. Temocillin showed improved \textit{in vitro} activity over ticarcillin against ampicillin-resistant \textit{Enterobacteriaceae} but it had little or no activity against Gram-positive species, anaerobes, or \textit{Pseudomonas} (24). This narrow spectrum led temocillin to fall out of favor with many healthcare providers, but its remarkable resilience against common extended-spectrum β-lactamases (ESBLs) (e.g. TEM, SHV, CTX-M) and AmpC β-lactamas has led to recent calls to revive its use, particularly for multidrug-resistant \textit{Acinetobacter baumannii} (25). It is thought that temocillin can help avoid over-reliance on carbapenems and selection of carbapenem-resistance. Additionally, temocillin appears to have less potential for selection of \textit{Clostridium difficile}-associated colitis (26).

\textbf{Figure 9. Temocillin}

![Temocillin molecule](image)

\textbf{B. 2. f. Amidopenicillins}

Amidopenicillins were another group of penicillins developed in the early 1970s. These drugs feature an amidino group instead of a benzylamide group attached to the penam. One member of this group called amidinocillin (mecillinam) reached market but is no longer available in pharmaceutical preparations in the U.S. Mecillinam binds almost exclusively to PBP2 in \textit{E. coli} (27). Like the 6-α-penicillins, amidinocillin has no
activity against Gram-positives, anaerobes, or *Pseudomonas*. However, it has good activity against AmpC and ESBL producing *Enterobacteriaceae*, and is synergistic when used with other β-lactams and β-lactamase inhibitors (28-31). Because of this activity and its low incidence of side effects or *C. difficile*-associated disease, there has been some interest in the reintroducing mecillinam for treatment of infections by ESBL and AmpC producing *Enterobacteriaceae* (29, 30, 32-34).

B. 2. f. 1) N-acylpenicillins

The limited and diminishing spectrum of α-aminopenicillins in the 1970s led to the development of the N-acylpenicillins. The N-acylpenicillins are members of the α-aminopenicillins that feature an acyl group attached to the α-amino group. Any one of a variety of organic groups can be further attached to the acyl carbon, and the particular group that is attached, defines the subclassification of the N-acylpenicillin. There are four sub-groups based on molecular structure: carbamoyls, ureidopenicillins, acylureidopenicillins, and carbamoylureidopenicillins. Only the ureidopenicillins have made it into commercial production. The most prominent ureidopenicillin in clinical use today is piperacillin, which is part of the focus of this dissertation.

B. 2. f. 1) (a) Piperacillin

Piperacillin was introduced into clinical use during the early 1980s and remains one of the most active penicillins against Gram-negative bacteria available. It is also one of the few penicillins with legitimate activity against *Pseudomonas* species.
The piperacillin molecule features a 4-ethyl-2, 3-dioxo-piperazine R group attached to the N-acylpenicillin frame (Figure 10). The structure does not improve the outer membrane permeability over other α-aminopenicillins, but does slow down the rate of hydrolysis turnover by some β-lactamases (35-38). Piperacillin binds *E. coli* PBPs with some improved affinity over ampicillin, primarily binding PBP1, PBP2, and PBP3 and with less affinity to the smaller DD-carboxypeptidases (i.e. PBP4, PBP5, PBP6) (39).

![Figure 10. Piperacillin](Image)

Piperacillin is absorbed poorly through the gastrointestinal tract and must be administered parenterally. A 4 g intravenous (IV)dose (approximately 50mg/kg) can reach a peak serum level of 330 µg/ml in 20-30 minutes with an area under the plasma concentration curve (AUC) of 254.8 µg·ml/hr (40). The concentration of piperacillin in the CSF and bone average about 32% and 5% of the plasma concentration respectively (41, 42). Compared to the distribution of penicillin G, this is about equal for bone and 4-fold higher for cerebral spinal fluid (CSF). The plasma half-life of piperacillin is approximately one hour, with 22% binding to plasma proteins, and 50-65 % being
excreted unchanged in urine within 2 hours of administration (40). Patients with cystic fibrosis and renal insufficiency may achieve higher plasma concentrations due to slower rates of clearance (14, 43). In the human body, approximately 5% of piperacillin is metabolized by the liver into desethyl-piperacillin within two hours of administration, and this metabolite also exhibits some antibacterial activity (44, 45).

As noted above, piperacillin is notable for being the first penicillin to have legitimate activity against *Pseudomonas aeruginosa* and improved activity against *Klebsiella* spp (14, 46). Part of its activity against *P. aeruginosa* may be due its lower potential for β-lactamase induction (47). However, piperacillin on its own is ineffective against β-lactamase producing species of *Staphylococcus*, *Enterobacteriaceae* (e.g. AmpC producing *Citrobacter* spp. *Enterobacter* spp., *Serratia* spp.), *Haemophilus* spp., or *Neisseria* spp (46). These shortcomings were overcome to a large extent when piperacillin was formulated in combination with the β-lactamase inhibitor tazobactam in the mid-1980s. The combination remains a stalwart in the treatment of healthcare-associated infections. The activity and pharmacokinetics of piperacillin/tazobactam in combination are described further in Section I. B. 7. c.

B. 3. **Monobactams**

Monobactams are β-lactam compounds that feature a β-lactam ring without an adjoining pentacyclic or hexacyclic ring. Monobactam compounds are produced naturally by some bacteria in the genera *Acetobacter*, *Chromobacterium*, and *Pseudomonas*. Today, aztreonam is the only monobactam that is approved for clinical use in the U.S. It is a synthetic derivation of a monobactam (SQ-26180) produced by *Chromobacterium*
violaceum (14). The drug was first described in 1981 and was FDA approved in 1986 (48).

Monobactams are similar in structure to penicillins in that they all have an amide at position 3 of the β-lactam, but attached to the amide is a thiazolyl oxime group (similar to sulfonamides). Individual monobactams differ in the substitutions at positions 1 and 4 of the β-lactam ring and at the oxime side chain. All natural monobactams and aztreonam have a sulfate at position N1. Aztreonam (Figure 11) has additional methyl groups attached at position 4 that provides stability from β-lactamases, and a C(CH₃)₂COOH group attached to the oxime side chain which improves antipseudomonal activity (14).

Figure 11. Aztreonam

Azcronem can only be administered parenterally. A one gram IV delivery of aztreonam can achieve a 125 µg/ml peak concentration with a two hour half-life and an AUC of 191 µg·hr/ml (49). Sixty to sixty-eight percent of aztreonam is excreted in the urine unchanged, with urine concentrations reaching 25 times that of the plasma (49, 50). The drug is well distributed around the body with lung concentrations reaching roughly 100-200% of the serum concentration 2-4 hours after a 1 g dose (51). For patients with
cystic fibrosis (CF) lung concentrations are lower due to higher rates of excretion. This lowers achievable concentrations in these patients from 4 µg/ml to 2 µg/ml (52). To alleviate this excretion problem, inhaled aztreonam therapies have been developed for CF patients and have been reported to achieve improved lung function in CF patients with *P. aeruginosa* infection (53).

Aztreonam shows strong activity against *Haemophilus* spp., *Neisseria* spp. and non-β-lactamase producing *Enterobacteriaceae*. The drug preferentially binds to PBP3 of *E. coli* which is essential for septum formation in dividing cells (54). *E. coli* treated with aztreonam can typically be seen growing in long filaments without septation (55).

Aztreonam is notably resistant to hydrolysis by metallo-β-lactamases, and can act as an inhibitor of CTX-M-14 β-lactamases (56). Activity is variable against AmpC producing bacteria (57). Notable to this dissertation is the fact that the AmpC of *Citrobacter freundii* and its derivatives (e.g. CMY-2) can hydrolyze aztreonam (58). Aztreonam is typically ineffective against TEM and SHV class ESBL producing *Enterobacteriaceae* though actual rates of hydrolysis for some of these enzymes are very slow (59, 60). The drug has no activity against *Acinetobacter*, Gram-positive bacteria, or anaerobic bacteria (14, 61).

B. 4. Parenteral Cephalosporins

The cephalosporins are among the most diverse and heavily relied upon β-lactams used in clinical practice today. Their origins date back to 1945 when Giuseppe Brotzu of the University of Cagliari, Sardinia isolated the fungus *Cephalosporium acremonium* (now called *Acremonium crysogenum*) from a sewage outflow there (62-64). He had
observed that people who ate shellfish and swam in water near the outflow had a low incidence of *Salmonella* Typhi infections (63). He was able to demonstrate antibacterial properties of crude *C. acremonium* extracts against *S. Typhi* and other bacteria, but was unable to develop the agent any further (63, 64). A British medical officer stationed in Sardinia passed samples of the extracts and fungus on to Oxford University where researchers who had worked on penicillin were able to purify cephalosporin P1, cephalosporin N, and cephalosporin C (64). Cephalosporin C was of particular interest because despite having a lower antibacterial activity than penicillin against Gram-positive organisms, it did have activity against Gram-negative organisms. It was also stable in acidic solution to pH 2.5 indicating potential oral use, and it was stable against *Bacillus subtilis* penicillinases (65). The structure of cephalosporin C provided the basic framework for cephalothin (Figure 12) which includes an additional thiophene group, and was the first cephalosporin put into clinical use in 1964 (62).

Structurally, the cephalosporins are cephems that have a dihydrothiazine ring attached to the β-lactam nucleus. The structures of the individual cephalosporins differ in the R groups attached at the C3 position and the C7α carbon. The substitutions at C3 primarily affect the drug pharmacology, while substitutions at C7α can affect both microbiological activity and pharmacology (14, 66). The R groups of some drugs include complex structures such as tetrazole, thiadiazole, and dithiane rings. Typically the cephalosporins have been divided into generations (1st, 2nd, 3nd, 4th) based on chronological time of introduction, activity, and somewhat on product marketing (67). A stricter classification of activity and molecular structure divides the cephalosporins into seven structural groups that fall into four categories: limited spectrum (groups I and II),
extended-spectrum (Groups III, IV), narrow spectrum (Groups V, VI), and experimental cephalosporins (Group VII) (see Figure 13) (14). This scheme will be used to describe the parenteral cephalosporins below.

**Figure 12. Cephalothin**

![Cephalothin](image)

**B. 4. a. Limited Spectrum Cephalosporin Groups I and II**

The limited spectrum parenteral cephalosporins include those that were among the first to be introduced in the 1960s such as cephalothin and cefazolin. The category can be subdivided into Groups IA, IB, IIA, and IIB. Group IA are metabolized cephalosporins (e.g. cephalothin, cephapirin, cephacetrile) and carry an acetoxymethyl group at C3 that is cleaved during metabolism, resulting in a less active compound (68). The plasma half-life of these drugs are less than an hour but they have activity against most *Enterobacteriaceae* and penicillinase producing *S. aureus, S. pneumoniae, Haemophilus* spp., and *Neisseria* spp (14). The group IA drugs however are not active against enterococci, AmpC producing *Enterobacteriaceae, Pseudomonas, or Acinetobacter* (69). They are also prone to hydrolysis from ESBLs (70). Group IB cephalosporins are not metabolized and have a thiazole (e.g. cefazolin) or a pyridine ring (e.g. cephaloridine) structures attached through C3.
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Figure 13. Classification of Parenteral Cephalosporins

Limited Spectrum

- IA
  - Cephalothin
- IB
  - Cefazolin
  - Cephaloridine
- IIA
  - Cefamandole
  - Cefuroxime
- IIB
  - (Cephamycins)
  - Cefotaxim
  - Cefotetan

Extended Spectrum

- IIIA
  - Cefoperazone
  - Cefpiramide
- IIIB
  - Ceftriaxone
  - Cefotaxime
- IIC
  - Flomoxef
- IIID
  - (Carboxycephems)
  - Loracarbef
- IIIE
  - (Isocephem)
  - Cefepime
  - Cefpirome

Narrow Spectrum

- V
  - (Catechol Cepheps)
  - (Pyridal Cepheps)
- VI
  - Cefsuclidin
  - (C7-α-sulfoxypephems)
  - (C3-heteroarylthiocephems)

Experimental

- VII
  - (Sulfoxycephems)
  - Phosphocephems
  - Azacephem
They are also prone to hydrolysis from ESBLs (70). Group IB cephalosporins are not metabolized and have a thiazole (e.g. cefazolin) or a pyridine ring (e.g. cephaloridine) structures attached through C3. The lack of metabolic inactivation leads to improved half-lives of 1-3 hours and allows for better tissue penetration, but does not appreciably improve the \textit{in vitro} antibacterial activity over the Group IA cephalosporins (62, 71). Cephaloridine use was discontinued in the U.S. in the 1970s due to its association with acute renal toxicity (72).

The Group II cephalosporins include drugs considered to be second generation cephalosporins that were introduced in the 1970s. Members of this group each contain N-methylthiotetrazole group at C3. Group IIA drugs also feature benzyl or furan groups attached to C7α (e.g. cefamandole, cefuroxime). This group had better activity against \textit{Proteus} and \textit{Citrobacter} species than the Group I cephalosporins, and less susceptibility to TEM-1 and AmpC β-lactamases (73, 74). The Group IIB cephalosporins are the cephamycins which are defined by the presence of a methoxy group at C7α (e.g. cefotetan, cefoxitin) (Figure 14). The cephamycins have improved activity against \textit{Serratia marcescens}, indole positive \textit{Proteus} spp, TEM-1 producing \textit{E. coli}, and \textit{Klebsiella pneumoniae} (74-76). They also display some improved activity against anaerobes, but some have weakened or no activity against \textit{S. aureus} (e.g. cefotetan) (77, 78).

\textbf{Figure 14.} Cefoxitin
B. 4. b. **Extended-Spectrum Cephalosporin Groups III, IV**

The group III extended-spectrum cephalosporins can be subdivided into groups IIIA through IIIE. Groups IIIA, and IIIB contain drugs that are commonly used in clinical practice in the U.S. and are referred to as the 3rd generation cephalosporins. Some of the IIIA drugs possess an N-methylthiotetrazole group at C3 (e.g. cefotiam, cefoperazone, cefpiramide), and some feature an acyl heterocyclic group off the C7α position attached through a nitrogen and are referred to as N-acylcephalosporins (e.g. cefoperazone, cefpiramide, cefpimizole). The IIIA drugs were developed and introduced in the late 1970s and early 1980s. Cefoperazone and cefpiramide are notable for having effective antibacterial activity against *P. aeruginosa*. This activity is largely due to improved stability against AmpC beta-lactamases (79, 80). They also offered improved activity against *K. pneumoniae* but limited activity against *S. aureus* (79-82). Cefpiramide is also notable for attaining higher concentrations in bile than any other β-lactam (83).

The IIIB cephalosporins are distinguished by a 2-amino-5-thiazolyl group which enhances PBP binding activity, and a methoxyimino group attached through C7α that improves β-lactamase stability (14). Ceftazidime (Figure 15.) differs slightly in that it has an isobutyric residue attached through the C7α oxime. Because of the C7α oxime attachment, the IIIB cephalosporins are often referred to as oxyimino-cephalospoins. Members of Group IIIB primarily differ from one another by the functional groups attached at C3. The C3 R group may be simple acyl groups such as the acetoxymethyl group (i.e. cefotaxime, Figure 16), or heterocyclic groups as seen in ceftriaxone (Figure 17) or ceftazidime. The drugs generally have half-lives of 1 hour or more with ceftriaxone having a 6 hour half-life (14). The extended half-life of ceftriaxone is largely
due to its ability to bind plasma proteins (84). The Group IIIB cephalosporins are
excreted primarily (>70%) through the urine, with the exception being ceftriaxone which
is excreted approximately 50/50 in urine and bile (85, 86). The antibacterial spectrum of
the IIIB cephalosporins is notable for good activity against inducible AmpC producing
*Enterobacteriaceae* (e.g. *Enterobacter* spp., *Citrobacter* spp. and *S. marcescens*)
although they are not effective against strains that can produce AmpC at a constitutive
high level (87, 88). They are active against *Enterobacteriaceae* that produce early ESBLs
(TEM-1, SHV-1 CTX-M3) but not against more recent CTX-M ESBLs (CTX-M-14, -15)
(59, 89). The group IIIB drugs have excellent activity against *Haemophilus*

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**Figure 15. Ceftazidime**

![Ceftazidime](image)

**Figure 16. Cefotaxime**

![Cefotaxime](image)

**Figure 17. Ceftriaxone**

![Ceftriaxone](image)
influenzae and Neisseria spp., and ceftriaxone is often the drug of choice for prophylactic treatment of individuals exposed to meningococcal meningitis patients (17, 90-92).

Ceftazidime is notable for having strong activity against Pseudomonas (90). This activity is thought to stem from its inability to induce AmpC expression (93). Cefotaxime and ceftriaxone also have notable activity against penicillin-resistant S. pneumoniae (94, 95).

Group IIIC parenteral cephalosporins are oxa-1-cephems. Two drugs from this group were introduced into clinical practice, latamoxef (moxalactam) and flomoxef. These cephalosporins feature an oxazine ring joined to the β-lactam nucleus rather than dihydrothiazine. Moxalactam has three times more affinity for E. coli PBPs 3, 7, and 8 than benzylpenicillin and both drugs have low MICs for Enterobacteriaceae, including those producing ESBL and AmpC β-lactamases (96). They have moderate activity against anaerobes, but little or no activity against P. aeruginosa, Acinetobacter spp., Staphylococcus spp., or Enterococcus spp. (97-100). The drawback with these drugs is that they have been associated with interference of clotting factors and C. difficile associated disease (101-104). They are no longer available for clinical use in the U.S.

Group IIID cephalosporins are carbacephems which feature a cyclohexene attached to the β-lactam ring rather than a dihydrothiazine. The removal of the sulfur atom improves the molecular stability over dihydrothiazine analogs (105). A number of carbacephem molecules have been tested since 1970, but only one oral cephalosporin, Loracarbef, has been brought to market and is used clinically.

Group IIIE cephalosporins are the isocephem which feature presence of the sulfur, oxygen or nitrogen at position 2 of the cephem. None of these drugs have made it past the research and development stage (14).
The Group IV extended-spectrum cephalosporins are often referred to as fourth generation cephalosporins. Cefepime and cefpirome are only the two drugs in this group currently available for clinical use in the U.S. They were introduced in 1994 and 1995 respectively. Cefepime is the more frequently prescribed of the two drugs, and has come to be relied upon as one of the most active cephalosporins available for treating Gram-negative infections while having a spectrum that also covers many Gram-positive bacteria. Cefepime is currently recommended as empiric treatment for febrile neutropenia, severe community-acquired pneumonia, and hospital-acquired pneumonia (106-108).

Structurally, the Group IV cephalosporins contain an amalgam of functional groups seen in previous cephalosporin generations. For example, cefepime, and cefpirome each include the 2-amino-5-thiazolyl group common to the IIIB cephalosporins which enhances Gram-negative activity (Figure 18) (109). They also include a methoxyimino group as seen in the IIA cephalosporin cefuroxime which improves Gram-positive activity and stability against β-lactamases (110, 111). Both group IV drugs feature a C3 quaternary ammonium structure similar to ceftazidime which improves activity against *Pseudomonas* (109). The zwitter ionic properties created by the positive charge of the C3 quaternary ammonium group, and the negative charges of the C4 carboxyl group are a hallmark of the Group IV cephalosporins. These properties are thought to facilitate entry through the bacterial membrane (109, 111).

Cefepime does not interact readily with AmpC enzymes (112). As a result the drug has notable activity against *Enterobacteriaceae* that produce AmpC β-lactamases, including those which produce these enzymes on a constitutive high level (87, 113).
Cefepime also does not readily select for AmpC hyperproduction mutants as other cephalosporins can (93, 113).

However, group IV cephalosporins are ineffective against Enterobacteriaceae that produce ESBLs (56, 59, 114, 115). Both cefepime and cefpirome are active against methicillin-sensitive Staphylococcus aureus (MSSA) with cefpirome being slightly more active (115, 116). Cefepime is more active than cefpirome against Pseudomonas spp. but is less active then ceftazidime (116).

**Figure 18. Cefepime**

B. 4. c. **Narrow Spectrum Cephalosporins Groups V, VI**

The narrow spectrum cephalosporins are drugs which are designed for use against specific pathogens such as Pseudomonas or MRSA.

Group V cephalosporins, so called 5th generation cephalosporins by some, are catechol, and pyridone cephems featuring catechol or pyridone moieties at the C3 or C7α position of the molecule. Both functional groups mimic the structure of siderophores that bind soluble iron and are taken up by the TonB iron uptake system found in many bacteria (14). The structure thus creates an additional mechanism for entry into the
bacterial cell. Against clinical isolates, some catechol cephalosporins have shown activity similar to Group IIIB cephalosporins (117, 118). However the greatest potential of these drugs may be in the treatment of *Pseudomonas* spp with reduced drug permeability due to porin loss. Some formulations have shown exceptional activity against *P. aeruginosa*, including strains resistant to ceftazidime and imipenem (119, 120). However, most catechol cephalosporins tested have been found to be susceptible to β-lactamases (14). The activity of these drugs can also be reduced 4000-fold in *tonB*, and *cir, fiu* (catechol receptor) mutants (121). No clinical trials of these drugs have been done, and today they still remain experimental.

Group VI cephalosporins include the C7-α-sulfo cephalosporins, and C3-heteroarylthio cephalosporins. Cefsulodin is the only C7-α-sulfo cephalosporin that was introduced into clinical use. It was developed in the late 1970s and its activity is essentially specific for *Pseudomonas* with little activity against other Gram-negatives bacteria. Unfortunately its activity against *Pseudomonas* is not much better than other cephalosporins (122, 123). In the U.S. cefsulodin is not used therapeutically, and is primarily found as a component in cefsulodin-Irgasan-novobiocin (CIN) agar which is used to select for *Yersinia* spp in stool and food specimens (124).

The C3-heteroarylthio cephalosporins are anti-MRSA cephalosporins. Many of the drugs in this group are similar to group III cephalosporins in that they have a 2-amino-5-thiazolyl group and a methoxyamino group attached at C7α, and they have cyclic groups attached to C3 through a thioether group. The key difference with many of the C3-heteroarylthio cephalosporins is that at the C3 position they also have an additional thioether that attaches a saturated aminoethyl group. This extension improves
the affinity to PBP2a, while the addition of an ammonia group on the side chain (or formulation as a sodium salt) provides enough positive charge that avoids excessive serum protein binding (125). One of the more effective drugs in this group that is under development has been RWJ-54428 which has in vitro activity against MRSA, as well as glycopeptide-intermediate *S. aureus* (GISA), vancomycin-resistant *Enterococcus faecalis* and *E. faecium* (VRE), and ampicillin resistant-VRE (126, 127). In general, its activity against other Gram-positive pathogens is effective, but it has essentially no activity against Gram-negative organisms (126).

B. 4. d. Experimental Cephalosporins, Group VII

A number of attempts have been made to create cephalosporins with changes at position 1, such as attaching an oxygen to the sulfur (sulfoxyccephem), or changing the sulfur to a phosphorus (phosphocephem) or nitrogen (nitrocephem) (14). Drugs with these modifications remain experimental.

B. 5. Oral Cephalosporins

The oral cephalosporins can be divided into Group I limited spectrum, Group II extended-spectrum, and Group III broad spectrum cephalosporins.

The Group I oral cephalosporins are α-aminocephalosporins which are an emulation of the α-aminopenicillins. The group can be further subdivided into groups IA and IB. The Group IA drugs have aliphatic attachments at the C3 position. A prominent member of this group is cephalaxin (Figure 19) which was the first oral cephalosporin introduced in 1967. The Group IB oral cephalosporins feature polar additions at C3 such
as an oxygen (cefoxadine) or a chlorine (cefaclor, loracarbef). These polar additions improve activity against Gram-negative bacteria (14).

Figure 19. Cephalexin

As of 2010, cephalexin was cited as the most commonly used cephalosporin in the United States with 21.9 million prescriptions per year, and it was the second most commonly used β-lactam antibiotic (second to amoxicillin, 51.1 million prescriptions per year) (128). The popularity of cephalexin stems from its inexpensiveness ($0.50-$0.80 per 250 mg cap) and lack of significant side effects rather than its spectrum of activity (129). The spectrum of activity is actually somewhat ambiguous since the Clinical Laboratory Standards Institute has not established cephalexin breakpoints against any bacteria, and its susceptibility range for Enterobacteriaceae is based on using cephalothin as a surrogate (130). There has been some question about the accuracy of this approach especially when using some automated analyzers (131). None the less, cephalexin remains a recommended empiric therapy for urinary tract infections, prophylaxis for dermatologic surgery patients, treatment of staphylococcal cystic fibrosis infections, and as empiric treatment for non-purulent cellulitis (132-137). And despite having weak or no
activity against viradans group streptococci, cephalexin is also recommended as prophylaxis for patients at risk for endocarditis following oral surgery (138, 139). Cephalexin is active against *E. coli*, *Klebsiella* spp., MSSA, *S. saprophyticus*, and beta and gamma hemolytic streptococci, but has only weak activity against *Proteus*, and no activity against enterococci, *Pseudomonas*, or *Enterobacteriaceae* with inducible AmpC β-lactamases (140, 141). Cefaclor has slightly better activity than cephalexin against *Staphylococcus* and Gram-negatives, but is still inactive against many of the same species as cephalexin (140, 141).

The Group II extended-spectrum oral cephalosporins were attempts to make oral versions of the parenteral Group II cephalosporins. Cefuroxime axetil is the only member of this group to be brought to market in the U.S. in 1988. It contains an acetoxysethyl group attached to the C4 position that allows for gastric intestinal absorption (142). The group is then rapidly cleaved off by esterases in the intestine to release the active drug (143). Cefuroxime shows activity against Gram-negatives, and *Staphylococcus* similar to that of cefaclor, but has notably improved activity against α-hemolytic streptococci (14, 144).

Group III broad spectrum oral cephalosporins are all structurally similar to IIIB parenteral cephalosporins in that they feature a 2-amino-5-thiazolyl group attached through the C7α position. Some of the drugs in this group also have a methoxyimino group attached through this position (e.g. cefpodoxime, cefetamet). These methoxyimino oral cephalosporins are designed as prodrug formulations with acyl groups attached by an ester linkage to the C4 carboxyl group. Other Group III drugs have a larger imino acid group (e.g. cefixime) or a hydroxime group (cefdinir) attached through the C7α rather
than a methoxyimino group. They also feature a vinyl group attached through C3. These configurations allow for gastric buffering capacity and enable gastrointestinal absorption without requiring a prodrug attachment (145).

The Group III drugs have improved activity over Group I and II oral cephalosporins for Gram-negative bacteria, including species and strains that the earlier groups have little or no activity against, such as ampicillin-resistant *H. influenza*, AmpC producing *Enterobacteriaceae*, and *Proteus* (146-148). However, these improvements come with a general decrease in activity against Gram-positive bacteria. Three of the group III oral cephalosporins: cefixime, ceftabutin, and cefetamet, are notable for their lack of activity against MSSA (MIC50 concentrations between 32->64 µg/ml) (149). Some authors subclassify these drugs as Group IIB oral cephalosporins based on this lack of activity (14). Cefdinir and Cefditoren maintain activity against Gram-positives that is comparable to the Group I and II drugs (14).

**B. 6. Carbapenems**

The carbapenems are the most heavily relied upon β-lactam antibiotic for treatment of infections caused by *P. aeruginosa*, and ESBL and AmpC producing *Enterobacteriaceae*. For many, they are the last remaining broad-spectrum β-lactams in the antibiotic armamentarium. The first identified carbapenem, thienamycin, was isolated in the mid-1970s from culture filtrates of *Streptomyces cattleya* (150). Thienamycin and other early carbapenems displayed excellent antibacterial activity against both Gram-positive and Gram-negative organisms including *Pseudomonas* (151). However, the early carbapenems were found to be rapidly hydrolyzed by renal peptidases, in
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particularly, dihydropeptidase I (DHP-I) (152). Later carbapenems were semisynthetic
derivatives designed to be stable against DHP-I, and to be less neurotoxic to patients (14).

The carbapenems are structurally distinguished by a dihydropyrrole ring attached
to the β-lactam (Figure 20, 21, 22). In general the carbapenems are molecularly small and
behave as zwitter ions. These characteristics allow them to penetrate bacterial membranes
more efficiently through porins and gain access to PBPs (153). Attachments at the C1
position, such as a methyl group (e.g. doripenem; meropenem, Figure 21; ertapenem,
Figure 22) or an alkoxy group (e.g. Ro-40-3485) improve stability against DHP-I
hydrolysis but also reduces antibacterial activity (14, 154). Groups attached through a
thiol at the C2 position are also necessary for protection from DHP-I hydrolysis and
affect antibacterial activity. Drugs with cyclic groups in this position (doripenem,
ertapenem, meropenem,) have better activity against Gram-positive organisms, and the
attachments here offset any loss of activity due to attachments at the C1 position (14).
When basic functional groups (carbonyl, or amino group) are part of the C2 attachments,
there is improved activity against *Pseudomonas* (14).

![Figure 20. Imipenem](image1)

![Figure 21. Meropenem](image2)
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The key to the activity of the carbapenems is that they exhibit both PBP binding bactericidal activity, and a β-lactamase inhibition effect (155-157). The hydroxyl group off the β position of the C6 carbon is responsible for this inhibitory property (158). For serine β-lactamases, the inhibition occurs after the β-lactam ring forms an acyl-enzyme intermediate with the serine active site. Instead of being immediately hydrolyzed, the molecule rotates slightly supported by hydrogen bonds between the β-C6 hydroxyl and adjacent residues (e.g. Asn132 and Gln166 associated water for TEM-1) (159). The positioning prevents an immediate nucleophile attack on the β-lactam oxyanion thus prolonging the acyl-enzyme configuration and slowing the rate of drug inactivation (159).

Imipenem was the first carbapenem FDA approved for clinical use in 1985. Because it lacks a C1 attachment, it is required to be coadministered with cilastatin, a dihydropeptidase inhibitor. A one gram dose of imipenem can achieve a plasma concentration of 58.9 µg/ml with a half-life of about 30 minutes and AUC of 85.7 µg·hr/ml (160, 161). Approximately 13% of the drug may be bound to plasma proteins, and 70% is removed in the urine unchanged (160, 161). Meropenem was the second carbapenem approved by the FDA in 2005. It is a C1 β-methyl carbapenem that does not
need cilastatin co-administration, and has pharmacokinetics similar to imipenem but with half-life extended closer to one hour (162, 163). Ertapenem was approved in 2006 and is a C1-β methyl carbapenem with an extensive C2 chain. The structure yields weaker antibacterial activity than imipenem or meropenem, and is more reliant on available porins for entry into Gram-negative bacteria, but has improved pharmacokinetic stability (14). A one gram 30 minute infusion of ertapenem yields plasma concentrations of 191 µg/ml with a half-life more than 4 hours and an AUC of 688 µg·hr/ml (164, 165). Approximately 92% of ertapenem is protein bound at peak concentrations, and 45% is excreted in the urine (166).

The instability of carbapenems under laboratory conditions is notable and must be considered when evaluating pharmacokinetics, or bacterial susceptibility tests. Drug suspensions are generally unstable at room temperature and should be stored at -70 °C if not used immediately (167, 168). Imipenem can be inactivated in media containing thioglycolate or with pH under 4.0, or exceeding 8.0 (169, 170).

The activity of carbapenems against Gram-positive organisms including anaerobes is quite potent. Three exceptions to this are MRSA, methicillin-resistant *Staphylococcus epidermidis*, and *Enterococcus faecium* (14, 171). In addition, ertapenem is not active against *Nocardia* spp (14, 172).

Carbapenems have remarkably strong activity against most Gram-negative bacteria. ESBL and AmpC producing *Enterobacteriaceae* are generally susceptible even though carbapenems can induce AmpC production (173, 174). The carbapenems have high binding affinity for PBP-2 of *E. coli*, with weaker affinity for PBP 1a and 1b (175-177). Ertapenem additionally binds PBP3 with strong affinity (177). The inactivation of
PBP2 by carbapenems yields *E. coli* cells that are short and rounded rather than rod shaped (176, 178, 179).

Notable Gram-negative bacteria that are not susceptible to carbapenems include *Elizabethkingia* spp. (formerly *Chryseobacterium*), and *Stenotrophomonas maltophilia*, which have intrinsic metallo-β-lactamases (180, 181). *P. aeruginosa* strains are generally susceptible but strains deficient in production of the porin OprD can be resistant (182-184).

Perhaps one of the most troubling developments in infectious disease over the last decade has been the spread of mobile non-intrinsic carbapenemases among Gram-negative bacteria. Resistance among *Enterobacteriaceae* in the United States has been driven primarily by *Klebsiella pneumonia* carbapenemase (KPC), while in Europe and Asia, mobile metallo-β-lactamases, such as NDM-1, play a larger role (185). Among *A. baumannii*, OXA β-lactamase producing strains have been the main source of resistance (186). These enzymes are discussed in more detail in Section I. C. 3.

**B. 7. β-lactamase Inhibitors**

β-lactamase inhibitors are β-lactam compounds that can bind with β-lactamase enzymes and inhibit their activity. Three inhibitors have been approved for clinical use in the United States. They are clavulanic acid, sulbactam, and tazobactam. All three have structures and bodily distribution comparable to penicillins (14). The clavulanic acid molecule is somewhat unique in that it is an oxazolidine penam (Figure 23). At the time of its discovery it was the first such structure of this kind to be found in nature (187). Sulbactam and tazobactam have sulfone thiazolidine rings attached to the β-lactam and
are derived semisynthetically (Figure 24, 25). The oxazolidine and sulfone structures play an important role in the inhibition activity of these molecules in that they can be protonated after the β-lactam ring has formed an acylenzyme complex with the β-lactamase active site. The protonation opens up the five-membered ring and leaves the inhibitor molecule susceptible for further degradation through nucleophilic attack by water or amino acids adjacent to the β-lactamase active site (157). The nucleophilic attacks lead to β-elimination of the inhibitor fragments which form linkages to the attacking amino acids impairing further activity of the enzyme (157). For clavulanic acid, water can protonate the molecule, whereas sulfone thiazolidine inhibitors require amino acids from the β-lactamase to donate the protons (188). This translates to a higher reactivity for clavulanic acid but lower stability. The extent of the reaction between the inhibitor and β-lactamase, or the ability for any reaction to occur between the two at all, depends on the particular inhibitor β-lactamase combination. Some of these relationships and the activity of the individual inhibitors are described below.
B. 7.a. **Clavulanic Acid**

Clavulanic acid is derived from *Streptomyces clavuligerus* and it was the first \( \beta \)-lactamase inhibitor to be introduced into clinical practice; first in the United Kingdom in 1981, and later in the United States in 1984. Clavulanic acid is typically found in 125 mg doses combined with 250 mg amoxicillin and sold as Augmentin\textsuperscript{®}, or in combination with ticarcillin as Timentin\textsuperscript{®}.

Orally administered clavulanic acid has 70% bioavailability, and a 125 mg dose can reach a peak plasma concentration of 5.5 µg/ml, with a 47 minute half-life, and AUC of 6.1 µg·hr/ml (189). It is excreted equally (50/50) through urine and bile (14). When co-administered with amoxicillin, clavulanic acid can achieve slightly higher plasma levels than when administered alone (7.7 µg/ml) but amoxicillin levels are essentially unchanged (16.2 µg/ml alone versus 15.9 µg/ml combined) (189).

The antibacterial spectrum of amoxicillin/clavulanic acid is notable for its activity against penicillinase producing *S. aureus*, *H. influenzae*, *Moraxella catarrhalis*, *Bacteroides* spp. and *N. gonorrhoeae* (190). It is also active against *Enterobacteriaceae* producing TEM-1 and SHV-1 \( \beta \)-lactamases (190). It has limited or no activity against bacteria with inducible chromosomal and plasmid AmpC \( \beta \)-lactamases, and clavulanic acid can actually induce AmpC \( \beta \)-lactamase expression, reducing the activity of its coadministered \( \beta \)-lactam (190-193). This induction may be due to the ability of clavulanic acid to bind PBPs, which it does more strongly than the other \( \beta \)-lactamase inhibitors (194). Interestingly, clavulanic acid in combination with piperacillin does have
activity against chromosomal AmpC producing Enterobacteriaceae and Pseudomonas, but this pairing has not been marketed (195).

B. 7. b. Sulbactam

Since 1987, sulbactam has been available in a 2:1 ampicillin:sulbactam formulation sold as Unasyn®. Sulbactam is not absorbed well orally and must be administered parentally (14). An orally administered ampicillin/sulbactam diester compound called sultamicillin is available but only in Europe.

For sulbactam to be effective, it typically must be used at higher concentrations than clavulanic acid (1 g verses 0.125 g). A 30 minute IV infusion of 2 g ampicillin with 1 g sulbactam can achieve plasma concentrations of 99.8 µg/ml ampicillin and 52.2 µg/ml sulbactam in an adult with normal kidney function (196). Half-life is approximately one hour for each drug with an AUC varying by age from 118-182 µg·hr/ml for ampicillin, and 68-110 µg·hr/ml for sulbactam, with levels higher in older adults (196). About 38% of sulbactam is protein bound and 60% is excreted via the kidneys (196).

Compared to ampicillin/clavulanic acid (4:1), ampicillin/sulbactam (2:1) has improved activity against Enterobacteriaceae producing chromosomally encoded AmpC β-lactamases (Citrobacter, Enterobacter, Morganella) (197). However, it has limited activity against E. coli expressing plasmid-encoded AmpC β-lactamases (198). Additionally, sulbactam is less effective than clavulanic acid or tazobactam in inhibiting early TEM, SHV, and OXA type β-lactamases in nitrocefin hydrolysis protection assays, and in vitro susceptibility studies (195, 199).
Despite these shortcomings, ampicillin/sulbactam remains among the first-line empiric drugs recommended for lower respiratory tract infections. This is primarily because of its continued efficacy against \textit{S. pneumoniae} and β-lactamase producing \textit{Haemophilus influenzae} \cite{107}. It is also interesting to note that sulbactam as a lone drug, demonstrates \textit{in vitro} antibacterial activity against multidrug-resistant \textit{Acinetobacter baumannii} (MIC$_{50}$ 2 µg/ml, MIC$_{90}$ 32 µg/ml) and may be useful as part of combination therapy for treating serious infections by resistant \textit{Acinetobacter} strains \cite{200, 201}.

\textbf{B. 7. c. Tazobactam, and Piperacillin/Tazobactam}

Tazobactam was first introduced in 1993 as an 8:1 piperacillin:tazobactam formulation called Zosyn$^\text{®}$. Structurally, tazobactam is similar to sulbactam in that it has a sulfone thiazolidine ring attached to the β-lactam ring, but differs in that it has a triazole moiety attached to the C3 carbon rather than a pair of methyl groups (Figure 25).

The concentration needed for tazobactam to be effective (~500 mg) is lower than sulbactam, but greater than clavulanic acid. Similar to clavulanic acid, tazobactam demonstrates increased plasma levels when co-administered with a β-lactam drug. A 500 mg tazobactam dose can yield a plasma concentration of 24.3 µg/ml without piperacillin, and 27.2 µg/ml when co-administered \cite{202}. The increase appears to be due to reduced renal excretion, whereas clavulanic acid elevation appears to be due to increased absorption \cite{189, 202}. Piperacillin plasma levels also appear higher when co-administered (213 µg/ml versus 223 µg/ml), though half-lives of both drugs (each one hour) appear unaffected by co-administration \cite{202, 203}. Co-administration also appears to improve tazobactam penetration into chemically induced blister fluid from 6.4 to 11.3
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μg/ml (202). Approximately 42.5% and 60.0% of piperacillin and tazobactam respectively is excreted in the urine unchanged, and 0.7% and 0.2 % respectively is excreted in the bile within 12 hours (204).

A 4 g piperacillin, 0.5 g tazobactam IV infusion over 30 minutes yields skin concentrations of both drugs about equal to that of plasma concentration (202, 205). Ninety minutes after infusion, muscle concentrations of both drugs are about 20-30% of plasma concentration, and fatty tissue concentrations are approximately 10% of plasma concentration (205, 206). Concentrations in pneumonic lung interstitial fluid compared to serum concentrations are about 53% -56.8% for piperacillin, and 66.7 – 91.3% for tazobactam, making piperacillin/tazobactam an effective treatment of pneumonia (206, 207). In bone tissue, piperacillin and tazobactam each achieve about 20% of their plasma concentration (208).

The above pharmacokinetic findings are based on 30 minute infusions of piperacillin/tazobactam. Recently the use of four hour piperacillin/tazobactam infusions has been gaining support (209). The extended infusions are mathematically projected to increase the length of time that free drug remains above MIC, though actual pharmacokinetic studies have not been done (210, 211). Cohort studies indicate that this approach improves patient outcomes and is cost efficient (212, 213). In the future this may be the way piperacillin/tazobactam will be administered. The effect these prolonged infusions may have on the development of piperacillin/tazobactam resistant mutants is still unknown.

Clinically, the broad spectrum antibacterial activity of piperacillin combined with the strong β-lactamase inhibition activity of tazobactam, make piperacillin/tazobactam
the most potent β-lactam/β-lactamase inhibitor combination commercially available. In many cases because of its activity against β-lactamase producing bacteria, it is reserved for patients with healthcare-associated infections where such bacteria are more common. Current Infectious Disease Society of America (IDSA) recommendations include the use of piperacillin/tazobactam as a first line treatment for healthcare-associated pneumonia, surgical site infections, and neutropenic fever (106, 202, 203, 214).

Piperacillin/tazobactam is active against methicillin-sensitive *S. aureus*, Vancomycin-sensitive *Enterococcus faecalis*, and penicillin-resistant *S. pneumonia* (215, 216). It also has broad activity against anaerobic bacteria, including *C. difficile*, and is not a risk factor for *C. difficile* associated disease (217-222).

Susceptibility surveillance data from North America indicate that most *E. coli* (62 - 97 %) are susceptible to piperacillin/tazobactam (Table 1), as are the majority of non-ESBL producing *Klebsiella* spp. (87 - 96%), however, ESBL producing *Klebsiella* spp. are mostly insusceptible (28 – 51 % susceptible) (223-228). Most inducible AmpC producing Gram-negative bacteria remain susceptible to piperacillin/tazobactam, including *C. freundii* (60 – 90.5%) *Enterobacter* spp (54 - 83%), and *P. aeruginosa* (88-90%) (224-226, 229). Most *Acinetobacter* spp. remain susceptible to piperacillin/tazobactam as well (59 – 70%), but this may vary by location and patient population (224-226, 230).

In 1999 CLSI guidelines recommended that *E. coli* and *K. pneumonia* strains identified as producing ESBLs be automatically reported as resistant to all penicillins including piperacillin/tazobactam (231). However, as discussed above, piperacillin/tazobactam remains active against many of these ESBL producing strains.
This activity does not appear to be offset by increased inoculum, and use of piperacillin/tazobactam to treat patients infected with these strains has been associated with successful outcomes (232-235). As a result, CLSI repealed the drug restriction recommendation in 2010, and currently the CLSI does not recommend restricting active drugs from use against ESBL producing *Enterobacteriaceae* (236). Furthermore, use of piperacillin/tazobactam appears not to select for ESBL producing *Enterobacteriaceae* in cohort studies (236-240).

In cohort studies of patient populations where ESBLs are prevalent, the use of piperacillin/tazobactam did not select for increased piperacillin/tazobactam resistance in *K. pneumoniae* (237, 240). However in other less defined strain populations, piperacillin/tazobactam has been found to select for its own resistance in clinical *E. coli* and *Klebsiella* strains (241, 242). It remains unknown what mechanisms led to resistance in these studies. It is possible that overproduction of AmpC β-lactamases played a role, as observed in the piperacillin/tazobactam resistant mutants generated in the experiments for this dissertation.
Table 1. *Enterobacteriaceae* Piperacillin/Tazobactam Susceptibility Data from North American Studies

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C. Bacterial Resistance to β-Lactam Drugs

Bacterial resistance to β-lactam drugs is neither an unusual or unexpected event in nature. The “simple” single cell design of bacteria puts them in immediate contact with their environment, and each species must quickly respond and adapt to life-threatening environmental changes in order to survive. Through millennia of evolution, bacteria have acquired or developed enzymes and response mechanisms to deal with environmental threats in their particular niche. The general plasticity of bacterial genomes allows for DNA to be acquired, lost, or undergo some level of mutation that enables their evolution to continue. The relative short generation time of most bacteria ensures that strains with beneficial genomic changes can be expanded quickly.

β-lactam molecules are an environmental threat that bacteria have evolved means to protect themselves from. Typically bacteria employ three different strategies to combat them: 1) alterations to penicillin binding protein targets, 2) alterations to cell permeability, and 3) β-lactamases. The review below will expand on each of these three mechanisms with a focus on how they apply to *E. coli* where feasible.

C. 1. Altered β-lactam Target

As mentioned in Section B.1., penicillin binding proteins (PBPs) are the affected targets of β-lactam antibiotics. Bacteria that can produce low β-lactam affinity PBPs can become resistant to the killing effects of β-lactams. There are four basic ways low affinity PBP-mediated resistance can be acquired: 1) overproduction of a low affinity PBP, 2) acquisition of a foreign low affinity PBP, 3) a recombination of a susceptible PBP gene with foreign low affinity gene fragments, and 4) point mutations within a PBP gene
sequence that lower β-lactam affinity. These four types of alterations are primarily seen in Gram-positive bacteria since altered PBP composition often leads to changes in peptidoglycan shape, and Gram-positive bacteria with their external cell wall have capacity to accommodate these changes.

C. 1. a) Overproduction of Low-affinity PBPs

Overexpression of natural low affinity PBPs is a mechanism by which bacteria shift to overproduce a carboxypeptidase or transpeptidase that is less compatible to β-lactam binding. The peptidase often has less efficient activity, which hampers growth, but also prevents excessive cell wall cross-linking. Examples include PBP4 of S. aureus, and PBP5 of Enterococcus faecium (246, 247). S. aureus PBP4 has carboxypeptidase and transpeptidase activity, and actually has the ability to hydrolyze the acyl-enzyme intermediate when bound to a β-lactam, which classifies it as β-lactamase (248). E. faecium PBP5 only has transpeptidase activity (249). The low affinity PBP overproduction in these examples seems to come from changes at the level of transcription, and can enable resistance to penicillins, cephalosporins, and carbapenems (246, 250, 251). The excessive production of these low affinity PBPs come at a cost of cell stability for Enterococcus and an increase in vancomycin susceptibility for S. aureus (252, 253).

C. 1. b. Acquisition of a Foreign Low-affinity PBP

Acquisition of a foreign low affinity PBP has most infamously been demonstrated by S. aureus strains that have acquired the meca gene for PBP2A. Production of this PBP
enables resistance to methicillin and other β-lactams. The origins of the *mecA* gene are uncertain though an 87.8% homologous PBP gene exists in *Staphylococcus sciuri* (254). The *mecA* gene travels as part of a SCC*mec* cassette, of which several types have been identified (255). There is evidence that SCC*mec* can be acquired and lost by strains, but the mechanism for its transfer is uncertain (256). Several horizontal gene transfer mechanisms have been considered (transformation, transduction, plasmids etc.) but the precise means of transfer between two given strains may vary by circumstance and environmental conditions, and may be limited to receptive strain types (257-260).

C. 1. c.  Recombination with Foreign Low-affinity PBP Gene Fragments

Sometimes transfer and recombination of foreign gene fragments rather than an entire gene cassette may be all that is necessary to acquire a lower affinity PBP. The recombination of low-affinity PBP gene fragments as a mechanism for β-lactam resistance has been best documented in strains of *Streptococcus pneumonia*, *Neisseria gonorrhoeae*, and *N. meningitidis*. Each of these species is known to be competent for transformation (261-263). In *S. pneumoniae*, the foreign low affinity PBP DNA is believed to be acquired from commensal oral streptococci *S. oralis* and *S. mitis*, with several portions recombined within the homologous PBP genes forming a mosaic (264, 265). Most resistant clinical isolates harbor three mosaic genes encoding, PBP1a, PBP2b, and PBP2x (266). The recombination of foreign PBP DNA appears to be a rare event, and most of the worldwide penicillin resistance in *S. pneumoniae* has been manifested by the circulation of a limited number of successful clones (267). In *N. gonorrhoeae*, and *N. meningitidis*, mosaic recombinations of the PBP2 *penA* gene have been found with
sequence blocks from commensal *Neisseria flavescens*, *Neisseria cinerea* or *Neisseria perflava* (268-270). For *N. gonorrhoeae* these alterations remove oral cephalosporins from the treatment options. Likewise, for *N. meningitidis*, these recombination events, though rare, potentially create fatal empiric meningitis treatment failures.

C. 1. d. **PBP Gene Point Mutations that Lower β-lactam Affinity**

Lastly, point mutations can occur in natural PBP genes to lower β-lactam affinity without importation of foreign DNA. One of the more notable instances of this has been reported in the already low affinity PBP5 of *E. faecium*. The most active mutations reported are those which change the active site position 466 to a serine, position 485 to an alanine, and position 629 to a valine (271-273). The mutations can increase ampicillin MIC 10-fold, or penicillin MIC 100-fold (272, 273). Today 73–86% of *E. faecium* isolated in the Unites States are resistant to ampicillin (245, 274). Rare *S. aureus* pbpB (PBP2) mutations have been reported and associated with increased β-lactam resistance, but at a level far lower than strains expressing PBP2a (275, 276). Point mutations have also been reported among *H. influenza* encoding PBP3A which causes non-β-lactamase mediated β-lactam resistance, a rare but troubling event for laboratories that only measure *H. influenza* β-lactam susceptibility by a rapid β-lactamase test (277).

C. 2. **Altered Cell Permeability**

Cell permeability mediated β-lactam resistance is essentially exclusive to Gram-negative bacteria since these organisms have their cell wall and PBPs located behind an outer membrane. The protective outer membrane is asymmetric, with the inner side
composed primarily of phospholipids and the outer side featuring lipopolysaccharides (LPS). The LPS units, like phospholipids, are amphiphilic with a lipid A anchor in the membrane, and a hydrophilic core of oligosaccharides at the surface. In many cases, an extension of oligosaccharide units (called the O antigen in *E. coli*) extends from the LPS core. Phosphate groups attached to the core give the LPS molecule an overall negative charge that repels hydrophobic antibiotics, bile salts, detergents, proteases, lipases, and lysozyme; and yet the lipid strands of lipid A prevent penetration of hydrophilic molecules from diffusing through the membrane too (278). To cross this barrier and reach their targets, most β-lactam antibiotics must gain entry through outer membrane porins, and avoid expulsion via efflux pumps. The composition and the number of porins and efflux pumps present in the outer membrane can impact the antibacterial activity of a β-lactam drug. The relationship of β-lactam drugs with the porins and efflux pumps of *E. coli* is described further below.

C. 2. a. Porins

OmpC and OmpF are considered the two major porins identified with β-lactam passage through the outer membrane of *E. coli* (279, 280). Both are structurally related to one another and have homologues in other *Enterobacteriaceae* [e.g. OmpK35 (OmpF) and OmpK36 (OmpC) in *Klebsiella pneumonia*]. Each porin is a β-barrel composed of 16 amphiphilic anti-parallel beta strands with eight connecting loops (L1 - L8) between different β-strands (Figure 26B) (281, 282). In the membrane, the porins form trimers joined by hydrophobic interactions and the interlinkage of loop L2 (Figure 26A) (281-283). For each porin monomer, the peptide loop L3 folds into the central pore forming a
“constriction zone” with an internal diameter of 2.5 Å (OmpC) to 6.0 Å (OmpF) (281, 284, 285). At the constriction zone, the L3 loop which is acidic (negatively charged), faces the inner wall of the barrel which is basic (positively charged) and together they form a transverse electric field that can restrict molecules on the basis of charge, yet allow passage of polar and zwitterionic molecules such as water and some β-lactams (286-288).

**Figure 26.** OmpF Porin

OmpF tripartite porin top view (A) and OmpF monomer lateral view (B) proposed by Nikaido (285). Green Bands represents β-barrel amino acid chains. Blue chain represents loop 2 which interlocks the monomers together. Gold chain represents loop 3 which forms the constriction zone and creates a negatively charged side of the porin channel.

The amount of porins in the outer membranes is not static for *E. coli*, and quantities of OmpF and OmpC naturally change under different environmental conditions. In general, the wider and less selective OmpF porin is downregulated in hypertonic and oxidative stress conditions while the more selective OmpC porin can be
simultaneously upregulated to keep a fairly constant number of porins (289, 290). The response systems that govern these changes include the EnvZ-OmpR two component system which downregulates \textit{ompF} and upregulates \textit{ompC} in hypertonic conditions (291). The transcription factors SoxS, MarA, and Rob, can upregulate the expression of MicF, an antisense RNA that interferes with translation of the \textit{ompF} transcript under oxidative stress and weak acid condition (290, 292).

Porin loss in β-lactam resistant \textit{Enterobacteriaceae} clinical isolates has not been studied in large scale, so the degree to which porin alterations impact β-lactam susceptibility in the clinical setting is largely unknown (293). Studies that have examined porin loss in clinical isolates have described them in clonal or sequential strains where the loss of porin expression is seen after continued antibiotic exposure (294-297). The cause for the loss of porin expression is unknown, or not explored in many cases (297, 298). In others, amber mutations and insertion element disruptions of the porin gene have been found (294-296). The loss of both OmpF and OmpC has been associated with greater β-lactam MICs than observed with loss of just one of the porins (294-296). However, in controlled experimental conditions, OmpC and OmpF porin loss alone seems to have minimal influence on β-lactam susceptibility in \textit{E. coli} (59, 299-301). In these conditions high production of an effective β-lactamase was more strongly correlated with resistance to broad spectrum β-lactams (59, 299-301).

For some β-lactam drugs, OmpF and OmpC may not be the primary route of entry to the periplasm. For example, piperacillin is relatively unaffected by the loss of OmpF and OmpC in \textit{E. coli}, and conduction studies have shown that piperacillin interacts very weakly or not at all with OmpF (288, 301). This may be due to both the large size and
negative charge of piperacillin, whereas OmpF and OmpC prefer small divalent molecules. The somewhat hydrophobic nature of piperacillin may enable it to diffuse through the outer membrane or pass through other porins that are more receptive to its chemical composition.

Recent studies have found that porin channel modification rather than porin loss may be an overlooked means by which Enterobacteriaceae can achieve β-lactam resistance. For example, changes to amino acids in the constriction zone of OmpC can affect the transverse electric charge across the zone and decrease β-lactam permeability in E. coli and Enterobacter aerogenes (287, 302). The ability to retain a porin this way and yet still achieve clinical resistance can have important pathogenicity implications since the complete loss of OmpC has been associated with vulnerability to phagocyte killing, reduced survival alkaline conditions, and reduced virulence in a mouse model.(303-305)

C. 2. b. Efflux Pumps

Even if antibiotics are able to breach the Gram-negative outer membrane, the drugs can still be expelled from the periplasmic space or cytoplasm via efflux pumps. In the genome of E. coli there are at least 37 genes encoding for efflux pumps (306). Twenty of these genes have been shown to be associated with changes in antibiotic susceptibility (306). The pumps can be categorized by sequence into five classes of membrane transporters: 1) ATP binding cassette (ABC), 2) major facilitator superfamily (MFS), 3) multidrug and toxic compound exporters (MATE), 4) resistance-nodulation-division (RND) and, 5) small multidrug resistance (SMR). Of these, the RND type are the only exporters with evidence of β-lactam efflux, with pumps AcrAB-TolC and AcrEF-TolC
II. Introduction and Review of the Literature

having the strongest association with changes in β-lactam susceptibility (307). However, the impact that efflux pumps have on β-lactam resistance in clinical *E. coli* strains appears ancillary to other resistance mechanisms.

The AcrAB-TolC pump is the most well studied efflux pump in *E. coli* and it is linked to the efflux of a wide range of chemicals and toxins (308). The pump is composed of three components: AcrA, AcrB, and TolC; and it is a homologue of the *Pseudomonas aeruginosa* MexAB-OprM pump (308, 309). For each pump, three TolC proteins join to form a single 12 banded β-barrel channel which traverses the outer membrane and extends a long barrel of α-helices down into the periplasm (Figure 27) (309, 310). AcrB is a 1049 amino acid protein that forms a homotrimer which connects to TolC in the periplasm via disulfide crosslinks (311). Each AcrB monomer has a TolC docking domain, a periplasmic pore domain with a vestibule for collection of molecules to be expelled from the periplasm, and a transmembrane domain that imbeds the pump into the cytoplasmic membrane and facilitates proton movement to the cytoplasm (312, 313). AcrA is a thin sickle shaped protein that surrounds the AcrB-TolC junction in the periplasm and is essential for the function of the AcrAB pump (314-316). One AcrA monomer is believed to support each AcrB monomer (317). It is speculated that the AcrA has a hinge like ability that allows for the pump motion and possibly assists with the opening of the TolC channel (314).
Genes *acrAB* and *tolC* can be upregulated in *Enterobacteriaceae* by the MarA, SoxS, and Rob transcription factors in response to high salts, acids, and oxidative stress (318-321). The negative regulator AcrR also releases from its binding site upstream of *acrAB* under the same conditions (322). Constitutive *acrAB* expression can be seen when mutations occur in the negative regulation network, such as an *acrR* deletion, *marR* insertion, and SoxR nonsense mutations (323-325). In laboratory studies of *E. coli*, multiple regulator mutations appear necessary to produce enough efflux pumps to achieve a 4-fold change in MIC to ampicillin or naladixic acid (326). In studies of clinical isolates, only single *acrAB* disregulation mutations have been reported, and resistance to
a given antibiotic was typically only present when strains possessed an additional pertinent resistance mechanism (e.g. a β-lactamase or an altered quinolone target) (327-332). Also, in these disregulated and resistant strains, the average levels of *acrAB* expression are only slightly higher (2-4 fold) than susceptible strains (324, 328, 333-336). This may indicate that only small changes in *acrAB* expression can be tolerated by clinical strains, and that the AcrAB-TolC efflux pump may only play a small role in the resistance seen.

With respect to piperacillin and piperacillin/tazobactam susceptibility, changes in *acrAB* expression and regulation appear to have little impact, and kinetic studies by Lim et al. found AcrAB-TolC efflux velocity of piperacillin to be among the slowest of all β-lactam drugs tested (330, 337, 338).

C. 3. **β-lactamases**

While porins and efflux pumps are general defense mechanisms to environmental stressors and toxins, β-lactamases are a specific defense against β-lactam molecules. Currently nothing more strongly correlates with Gram-negative β-lactam resistance than the production of β-lactamases (299, 339). Many of the β-lactamases (Groups A, C, and D) are serine hydrolases, which are believed to have originated from the DD-peptidases (i.e. transpeptidases, carboxypeptidases, see section I. B. 1.) that cleave the terminal D-Ala-D-Ala of the N-acetylmuramic acid pentapeptide side chain. β-lactamases feature a smaller enzymatic active site than their DD-peptidase counterparts (340). The site does not easily accommodate peptidoglycan D-Ala-D-Ala targets, but readily accepts the cyclic amide of the β-lactam molecule (340). The production of β-lactams and
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β-lactamases seems to have co-evolved in early Gram-negative bacterial species (e.g. Elizabethkingia, Chromobacterium) that can produce both molecules (341). Since β-lactams in these species are produced in times of stationary phase starvation, it is thought that they are a means of competition, and the surviving members producing β-lactamases can also breakdown the β-lactams to use the molecules as nutrient (341). The dissemination of β-lactamase genes into non-β-lactam producing bacteria likely occurred through genetic exchange mechanisms (e.g. transformation, transduction, plasmid conjugation). The acquisition became maintained by some species because of the selective advantage it provided for life in β-lactam rich environments. With the dawn of the penicillin era, β-lactam-rich environments became more plentiful, and the spread of β-lactamases accelerated. The acquisition and development of new and modified β-lactamase enzymes has continued to meet the challenge of each newly introduced β-lactam drug.

Currently more than 900 β-lactamases have been reported to genetic databases held at the Lahey Clinic and Pasteur Institute (342, 343). The nomenclature of β-lactamases is usually limited to a three letter designation that may arbitrarily represent the drug activity, the locations from which the enzyme was first isolated, or the name of a patient or researcher (344). Numbers following the letter designation refer to the chronology in which different gene variations were reported and recorded (345). These variations typically represent novel amino acid sequences, though some older designations represent only nucleotide differences (e.g. TEM-10 and TEM-23) (346). The current nomenclature for β-lactamase genes uses the notation _bla_ followed in subscript by the three letter enzyme family identifier, and a hyphenated numeric designation (345).
Two classifications schemes have been devised to group the different β-lactamases enzymes. One scheme devised by Bush, Jacoby, and Medeiros, organizes the enzymes based on phenotypic hydrolytic activity (347). This scheme is hampered by the fact that closely related enzymes can fall into different groups based on small mutations that alter activity, or conversely, divergent enzymes can be grouped together based on similar β-lactamase activity (348). A more robust scheme devised by Ambler is based on amino acid sequence and divides the β-lactamases into four groups (A-D) (349). The Ambler classification will be used to describe β-lactamases in the brief review below.

C. 3. a. **Class A β-lactamases**

The class A β-lactamases are defined by three conserved motifs in their active site located at residues Ser-X-X-Lys73, residues Ser-Asp-Asn132, and Lys/Arg-Ser/Thr-Gly-Ala237 (350). The serine at residue 70 of the active site classifies these enzymes as serine dependent β-lactamases. Two notable and well distributed Class A enzymes are TEM-1 and SHV-1. Both phenotypically behave as penicillinases, with little or no activity against cephalosporins, however later amino acid variations of these enzymes (e.g. TEM-3, SHV-2) conferred activity against cephalosporins and aztreonam (351, 352). The ability of these enzymes to hydrolyze oxyimino cephalosporins by at least 10% of the rate they do benzylpenicillin, and yet be inhibited by clavulanic acid defined the variants as extended-spectrum β-lactamases (ESBLs) (348). Certain TEM and SHV mutations have also confirmed resistance to β-lactamase inhibitors, however these mutations also generally disable ESBL activity (350, 353, 354).
Another major group of class A β-lactamases with ESBL activity is the CTX-M family of enzymes. This group of β-lactamases is derived from *Kluyvera* spp. and was first isolated in Japan in 1986 (355). In the 2000s, the CTX-M type ESBLs emerged as the most predominant ESBL found in healthcare and community *Enterobacteriaceae* isolates around the world (356, 357). Unlike most TEM and SHV ESBLs, CTX-M hydrolyze group IIIB parenteral cephalosporins cefotaxime and ceftriaxone better than ceftazidime (358, 359). They also exhibit greater inhibition from tazobactam than clavulanic acid (358, 359). The enzymes CTX-M-14 and CTX-M-15 have been the most prevalent variants of the CTX-M group (356, 357). Interestingly, and of relevance to this dissertation, the genes for both enzymes are typically found downstream of an IS*Ecp1* element which provides promoter sequences and may provide a means of transposition (360, 361). The arrangement likely provides a competitive advantage over strains possessing other ESBL arrangements. *E. coli* sequence type strain ST-131 has been associated with carriage of an IS*Ecp1-bla*$_{CTX-M-15}$ arrangement. ST-131 strains with this arrangement are considered part of a pandemic responsible for the spread of *bla*$_{CTX-M-15}$ worldwide. The strain type has also been credited with allowing ESBL producing *E. coli* infections to occur in the general public, not just healthcare-associated infections (362-365).

A few class A β-lactamases are also known to have carbapenemase activity. These include GES, IMI, SME, and KPC enzymes. Each has at least 30% amino acid identity to one another (366). The GES enzymes behave as ESBLs, but have some carbapenemase activity when Asn or Ser substitutions occur at Gly170 (367). GES carbapenemases are particularly concerning when produced by bacteria with innate low
permeability to β-lactams such as *P. aeruginosa* or *A. baumannii* (367, 368). The *bla*<sub>GES</sub> genes have been found located on transmissible plasmids encoded within class I integrons (368, 369). The prevalence of these enzymes is very low, though a number of variants (at least 22) have been found distributed around the world (342). IMI β-lactamases have also only been reported rarely. Currently they have only been found in *Enterobacter* species (370, 371). Similarly, SME enzymes have been found exclusively in strains of *Serratia marcescens*. Three variants of SME have been described to date, and their occurrence has been sporadic (372). The carbapenemase activity of both IMI and SME is strongest against imipenem, though the activity for each can be inhibited by clavulanic acid (371, 373).

KPC enzymes are the newest of the class A carbapenemases, and in the last 10 years they have emerged as the most prevalent of them all. In some New York healthcare facilities, up to one-third of *Klebsiella pneumoniae* isolates have been found to possess *bla*KPC (374). At least 12 KPC variants (KPC-2 to KPC-13) have been reported to date with KPC-2 and KPC-3 being the most prevalent (342, 375). The *bla*KPC genes have typically been found located on self-transferable plasmids within a Tn<sub>4401</sub> transposon (376). Plasmid and transposon mobility may account for the rapid spread of *bla*KPC among *K. pneumoniae* strains and movement into other species such *A. baumannii*, *E. coli*, and *P. aeruginosa*, among others (377-379).

The production of KPC can decrease susceptibility to essentially all β-lactam drugs (380, 381). In addition, KPC producing *K. pneumoniae* strains frequently have reduced permeability through loss of ompK35, and demonstrate resistance to other classes of antibiotics (380-382). Treatment of KPC producing bacterial infections is still a
challenging and evolving practice. Formal clinical trials for treating infections by KPC producing organisms have yet to be done, and available treatment data has only come from case reports and case series. To this point, combination therapies using β-lactamase inhibitors, polymyxins, or aminoglycosides in combination with a carbapenem have shown the most success (383). New drugs such as tigecycline have had success too, but appear to work best when used in combination with another drug (383).

C. 3. b. Class B β-Lactamases

The class B β-lactamases are considered metallo-β-lactamases because of their requirement of zinc ions to catalyze their β-lactamase reactions. These enzymes are not members of the Ser-X-X-Lys aminotransferase superfamily like class A, C, and D β-lactamases, or PBPs. Rather, they belong to the HAHAD family of metalloproteases which are not related to cell wall construction enzymes at all, and are derived from a variety of enzymes such as, arylsulphatases, glyoxalases, and polyketide cyclases (384). The metallo-β-lactamases also react with β-lactams differently. Their amino acids do not directly interact with the β-lactam molecule, and they do not form an acylenzyme intermediate. Instead, their zinc ion positions and charges a water molecule to carry out the nucleophile attack on the beta-lactam ring, and the water immediately forms a hydroxyl group on the carbonyl carbon inactivating the β-lactam (350).

The hydrolytic activity of metallo-β-lactamases can enable resistance to all β-lactams except monobactams, though for many enzymes the extent of β-lactam resistance is dependent on the permeability of the host species or strain (385). The enzymes are not inhibited by β-lactamase inhibitors, but can be inhibited by metal
chelators such as ethylenediaminetetraacetic acid (EDTA) which can be used in β-lactamase identification schemes.

The class B β-lactamases can be divided into three subgroups (B1-B3) with B1 and B3 requiring two zinc atoms, and B2 requiring just one (386). The B2 and B3 enzymes are primarily chromosomally encoded β-lactamases that are inherent to the species they are found in. The B3 enzymes include GOB-1 β-lactamase from *Elizabethkingia menigosepticum* (genus formerly *Chyseobacterium*), FEZ-1 from *Legionella gormannii*, and L1 from *Stenotrophomonas maltophilia*, which is actually encoded on a conserved plasmid (350, 387). Subclass B2 includes CphA from *Aeromonas hydrophila* and Sfh-I from *Serratia fonticola*. Subgroup B1 includes enzymes BeII from *Bacillus cereus*, CcrA of *Bacteroides fragilis*, B-1 from *E. menigosepticum*, and IND-1 from *Elizabethkingia indologenes* (350). This subgroup also includes mobile metallo-β-lactamases IMP, VIM, and NDM-1 which are arguably the most clinically relevant Group B1 enzymes. IMP and VIM both have more than 30 variants, with worldwide distribution, and they are found primarily among *P. aeruginosa* and *A. baumannii* (386).

In a very short period of time NDM-1 has become one of the most prevalent carbapenemases in Europe and Asia (388). Unlike other metallo β-lactamases, NDM-1 does not appear to rely on host impermeability to achieve carbapenem resistance, and it is found primarily in *Enterobacteriaceae* (*K. pneumoniae*, and *E. coli*) (389). Another troubling aspect is that NDM-1 producing strains typically carry resistance mechanisms to aztreonam, fluoroquinolones, macrolides, and aminoglycosides; conferring extreme drug resistance (390).
Treatment of metallo-\(\beta\)-lactamase producing *Enterobacteriaceae* infections has yet to be fully studied. Much like KPC associated infections, most success has been seen with combination therapy utilizing aminoglycosides, colistin, and tigecycline with or without carbapenems (386). Some limitations to this are encountered with the *Enterobacteriaceae* tribe Proteae (Proteus, Morganella, Providencia spp) for which tigecycline and colistin are ineffective (386). Therefore, the spread of metallo-\(\beta\)-lactamases into these species has been garnered with great concern.

C. 3. c. **Class C (AmpC) \(\beta\)-Lactamases**

The Class C \(\beta\)-lactamases are also known as AmpC \(\beta\)-lactamases. They are cephalosporinases that are not clinically inhibited by clavulanate or sulfone \(\beta\)-lactamase inhibitors such as tazobactam, but they only poorly hydrolyze Group IV cephalosporins, and they are inhibited by cloxacillin and oxacillin (57). AmpC \(\beta\)-lactamases can enable resistance against penicillins, cephalosporins (including Group IIB and III cephalosporins), monobactams, \(\beta\)-lactamase inhibitor combinations and even carbapenems in some bacteria with reduced permeability (300, 391). The AmpC \(\beta\)-lactamases are believed to be the first \(\beta\)-lactamases to have evolved in nature and are considered the progenitors of all other serine \(\beta\)-lactamases (i.e. class A and D enzymes) (392). They likely derived from DD-peptidases, and may in fact still contribute to cell wall construction in some way (393). It is speculated that AmpCs evolved as a mechanism to defend against naturally occurring cephalosporins since the enzymes are more suited to binding and hydrolyzing cephalosporins than penicillins (57, 394).
AmpC genes are naturally encoded in the genome of at least 49 species of Proteobacteria (57). Many more have been postulated through analysis of genetic databases, however, due to their close relation with DD-peptidases, the production of functional enzyme needs to be determined phenotypically (57). Within the Enterobacteriaceae family, notable AmpC producers include Citrobacter spp., Enterobacter spp. Escherichia spp., Morganella morganii, Serratia marcescens and Shigella spp.

For Citrobacter spp., Enterobacter spp. M. morganii, and S. marcescens, production of the AmpC enzyme can be induced by the actions of some β-lactams, whereas in Escherichia spp and Shigella spp. production is not inducible. The induction mechanism is based on the accumulation of peptidoglycan 1,6-anhydro-NAM-oligopeptide degradation products which bind and conformationally alter the bla<sub>ampC</sub> transcription repressor AmpR to become a transcriptional activator, and upregulate the transcription of bla<sub>ampC</sub> (57). The chain of events that lead to excess 1,6-anhydro-NAM-oligopeptide begins with the β-lactams binding to PBPs. Inhibition of different PBPs both low and high molecular weight may play a roll. Loss of low molecular weight PBPs may lead to excessive cross-bridges, which may trigger enhanced cell wall recycling, leading to more peptidoglycan breakdown products (10). Loss of high molecular weight PBPs leads to the loss of peptide cross-bridges between parallel peptidoglycan strands. The absence of cross-bridges leaves the peptidoglycan strands more vulnerable to cleavage by transglycosylases (395). Slt transglycosylase appears to be particularly important in this cleavage, and cuts the peptidoglycan strands into N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid-oligopeptide disaccharide units (396). The disaccharides are
transported from the periplasm to the cytoplasm by the AmpG muropeptide MFS transporter, and are cleaved into the monosaccharides N-acetylglucosamine, and 1,6-anhydro-NAM-oligopeptide by NagZ (397, 398). The latter of these monosaccharides can then bind AmpR, modifying its activity at the \( \text{bla}_{\text{ampC}} \) promoter to upregulate \( \text{bla}_{\text{ampC}} \) expression. The AmpD N-acetyl-anhydromuranmyl-L-alanine amidase serves as a negative control for this system by removing the peptides from 1,6-anhydro-NAM-oligopeptide which makes the molecule incapable of converting AmpR into its transcription activator configuration (399). Mutations in \( \text{ampD} \) are the most common genetic alterations that lead to constitutive \( \text{bla}_{\text{ampC}} \) overexpression in bacteria with an inducible \( \text{bla}_{\text{ampC}} \) systems (57, 400). \( \text{ampR} \) mutations can also lead to constitutive overexpression but are less common (57).

For species such as \textit{E. coli} which lack an \( \text{ampR} \) gene, the expression of \( \text{bla}_{\text{ampC}} \) remains low and is not inducible. Strains have evolved with mutations or insertions in the \( \text{bla}_{\text{ampC}} \) promoter region that lead to higher constitutive level of \( \text{bla}_{\text{ampC}} \) expression (401-403). In clinical \textit{E. coli} isolates, \( \text{bla}_{\text{ampC}} \) promoter changes have historically been the most common mechanism for AmpC mediated resistance, however in the last decade, plasmid-encoded AmpC genes have become increasing more common means of AmpC mediated resistance in \textit{E. coli} (404, 405).

C. 3. c. 1) \textbf{Plasmid-encoded AmpC β-lactamases}

With the proliferation of TEM and SHV β-lactamases in the 1980s, clinicians became more reliant on group IIB and group III cephalosporins, and beta-lactamase inhibitors to treat \textit{Enterobacteriaceae} infections. It is thought that this created the
selection pressure for AmpC β-lactamase genes to move on to plasmids and disseminate (198). In 1990 the first verified plasmid-encoded AmpC β-lactamase MIR-1 was reported (406). By July 2012 the Lahey Clinic β-lactamase Classification Database had listed at least 136 recognized plasmid-encoded AmpC genes placed in 9 different designations [ACC (5 genotypes), ACT (16), CFE (1), CMY (92), DHA (8), FOX (10), LAT (1), MIR (5), and MOX(8)] (342). As with other β-lactamases, the three letter designations for these enzymes are somewhat arbitrary and may refer to the location where the enzyme was first isolated (e.g. MIR, Miriam Hospital) or the β-lactam drug it was found to have particular activity against (e.g. CMY, cephemycins). While the genes may be encoded on plasmids that carry additional resistance genes, they have not been found in resistance gene cassettes, and they remain largely associated with independent insertion elements (57). Some plasmid-encoded AmpC genes like ACT-1, DHA-1, DHA-2, and CMY-13 have an ampR gene comigrated onto the plasmid, and they are inducible (407-410). The others appear to rely on enhanced constitutive expression gained from promoters located on the neighboring insertion elements (57, 87, 411).

A troubling aspect that persists with plasmid-encoded AmpC β-lactamases is that there are no established CLSI guidelines for detecting them in the clinical setting. As a result they go largely undetected. Infections by Enterobacteriaceae that produce plasmid-encoded AmpC β-lactamases are typically treated based on the phenotypic characteristics of the isolate, and not their enzymatic potential. Failure to recognize a plasmid-encoded AmpC infection and properly treat them has been associated with poor patient outcomes (412). The results of the piperacillin/ tazobactam mutation studies of this dissertation (Section III. A.) demonstrates how blaCMY-2 producing E. coli that are susceptible to
piperacillin/tazobactam, can spawn resistant mutants upon exposure to piperacillin/tazobactam. Today there are both phenotypic and molecular based detection methods that have been developed to identify plasmid-encoded AmpCs in Enterobacteriaceae (413, 414). These methods will likely become essential for healthcare facilities that strive to tailor appropriate therapies for their patients.

C. 3. c. 1) (a) **CMY Enzymes and CMY-2**

The CMY enzymes are the most diverse and the most common of the plasmid-encoded AmpC enzymes. Genetically, the CMY group includes three different families which probably should be subdivided in the future. CMY-1,-8, -9, -10, -11, and -19 are related to chromosomal \textit{bla}_{ampC} of \textit{Aeromonas sobria}, whereas the remainder of CMY enzymes are related to the \textit{bla}_{ampC} of \textit{Citrobacter freundii} (See dendrogram Figure 28) (198, 415). CMY-1 was first characterized in 1989 and CMY-2 was reported seven years later. By contrast, more than 60 new CMY genotypes have been recognized in the last five years (2008-2012) (342). It is uncertain if the expansion of CMY enzymes represents further evolution or dissemination of CMY genes. Most likely it is sequencing efforts on the part of researchers that are driving the expansion. Some of the more recently described \textit{Citrobacter} origin CMY enzymes fall into a subgroup with several amino acid differences from the \textit{bla}_{CMY-2} family of enzymes. Many of the GenBank submissions of this group appear to represent sequencing of the \textit{Citrobacter freundii} chromosomal \textit{ampC}, and they do not demonstrate evidence of plasmid location. Perhaps this group should also not be classified as true CMY β-lactamases? Two exceptions may
be CMY-64 and -75 which were found in *E. coli* and *Klebsiella pneumonia* isolates respectively and thus appear to be mobilized AmpCs of *Citrobacter* origin.
Figure 28. $bla_{CMY}$ Phylogenetic Tree

Dendrogram created with Clustal W from Lahey Database designated CMY family sequences available in GenBank.
Even with the recent increase in CMY diversity, CMY-2 has remained the most frequently encountered CMY enzyme worldwide (243, 405, 416-419). It has been isolated from Enterobacteriaceae on 6 continents, and is often associated with E. coli, Salmonella, or Klebsiella; with E. coli being the most frequent of the three. CMY-2 has been associated with Enterobacteriaceae isolated from poultry, livestock, and patients with livestock exposures (420-423). The association with livestock is thought to be related to the use of cephalosporins as growth enhancers on feedlots; a practice which as of January 2012 has been banned in the United States (424-427).

In studies that have looked at the genetic relations of blaCMY-2 carrying E. coli, the dissemination of blaCMY-2 has not been linked to any particular PFGE strain or plasmid profile (404, 416, 428-430). Among the plasmid types encoding blaCMY-2, incompatibility types IncI1 and IncA/C have been the most common (404, 431, 432). They have been found with about equal frequency in some studies, and in some locations a shift may be occurring where blaCMY-2 encoded on IncI1 plasmids is being found more frequently in recent isolates (404, 432).

blaCMY-2 is typically found with the insertion sequence element ISEcp1 in its upstream region. It is thought by some that ISEcp1 may mobilize blaCMY-2, transposing them together as a unit (see Section I. E. 1.) (432). However the common pairing of blaCMY-2 with the upstream ISEcp1 is more likely due to the strong promoter sequence, provided by the 3’ end of ISEcp1.

Three other CMY genes found with notable frequency are CMY-4, -7, -16. These enzymes differ from CMY-2 by single nucleotide/amino acid substitutions. They have similar activity as CMY-2 but appear less active against ceftazidime, cefepime, and
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carbapenems (198, 433). Like CMY-2, the genes for all three enzymes are typically preceded by a full or augmented ISecp1 element (434-438). Interestingly, these three CMY enzymes are frequently found in Enterobacteriaceae carrying additional β-lactamases (439-441). Notably, \textit{bla}_\text{CMY-4} and \textit{bla}_\text{CMY-16} have been identified in strains producing \textit{bla}_\text{NDM-1} and their isolation frequency may continue to expand with the expansion of \textit{bla}_\text{NDM-1} carrying isolates (389, 441).

C. 3. d. Class D β-lactamases

Class-D beta-lactamases consist almost entirely of one group, the OXA β-lactamases. Groups LCR and NPS are two rarely encountered class D members, each with only one enzyme in their group. Like β-lactamases classes A and C, the group D enzymes have a serine active site (Ser-X-X-Lys73). However, these enzymes also contain unique Tyr/Phe-Gly-Asn146, and Trp-X-X-Gly235 motifs not found in the other β-lactamase enzymes (442).

To date, more than 250 OXA variants have been designated (342). There is great variability in this group and some members have no more than 30% amino acid identity with one another (443). The first members of this group were found to hydrolyze oxacillin faster than benzylpenicillin and this property earned the group its namesake as OXA oxacillinases (443). Early OXA enzymes (OXA-1, OXA-2) defy inhibition from β-lactamase inhibitors, though they can be inhibited by 100 mM sodium chloride. OXA-1 also has the ability to hydrolyze Group IV cephalosporins (444). Early OXA enzymes do not hydrolyze Group III cephalosporins but that is of little consequence when they are expressed by ESBL producing strains (445, 446). For example, many of the recently
emerged ST131 *E. coli* strains express both OXA-1 and CTX-M-15 (447, 448). The combination creates a formidable β-lactam resistance profile that severely limits β-lactam therapeutic options.

OXA variants have also emerged that are themselves ESBLs. For example, an Asp substitution at Gly150 in OXA-2, yields extended-spectrum activity (OXA-15) (443). Carbapenemase activity is seen in members of the OXA-23, OXA-40, OXA-48, OXA-58 and OXA-143 families (443). For the most part, these enzymes have been almost exclusively found in *Acinetobacter baumannii*. The notable exception to this is OXA-48 which is found in *Klebsiella pneumoniae* and *E. coli* (449). Interestingly, it has recently been proposed that many of the OXA enzymes are not found in *Enterobacteriaceae* because they may interfere with peptidoglycan assembly (450). The extent of OXA incompatibility remains to be determined, but even with species barriers, the OXA Group D β-lactamases remain important β-lactam resistance adjunct to species and strains already possessing other resistance mechanisms.

**D. Plasmids and Plasmid-mediated Antibiotic Resistance**

The term “plasmid” was coined by Joshua Lederberg in the early 1950s, and has come to refer to extrachromosomal DNA elements which under normal growth conditions, are not essential for the host organism and can be lost or gained without consequence; but under a given environmental condition, they can provide the host with a selective survival advantage (451-453). Since this definition was made, there have been instances where essential genes have been found encoded on plasmids as conserved parts of a bacterial genome (e.g. *Borrellia burgdorferi*) (454). To some researchers, plasmids
are regarded as simple organisms (431, 455). While not all agree with that assessment, plasmids are uniformly understood to serve as vehicles by which bacteria can obtain or deliver genetic code. They play an important role in bacterial evolution and genetic exchange, and can probably best be described as mobile pools of extrachromosomal DNA shared by populations of bacteria (456).

Among *Enterobacteriaceae*, plasmids are typically double stranded closed circular supercoiled DNA elements 50 to 200 kb in size, capable of autonomous replication with mechanisms encoded in their framework to govern their replication and copy number (431). The plasmids often carry genes for toxin-antitoxin addiction systems, or bacteriocins that create their own selective environment by killing daughter cells and neighboring cells that do not carry the plasmid (457, 458).

As discussed in the above sections of this dissertation, plasmids frequently encode genes for antibiotic resistance traits that provide survival advantages to their hosts. Resistance genes play an important role in the discovery of plasmid biology in the late 1950s and early 1960s (459). In the past decade, the growing problem of multidrug-resistance phenotypes among *Enterobacteriaceae* has almost exclusively been linked to plasmid-encoded resistance mechanisms (460-462).

There are different theories as to why some antibiotic resistance traits are sustained on plasmids rather than transferred to the chromosome. For one, the ability to keep non-essential DNA outside the chromosome allows the bacteria to replicate its essential chromosomal genes faster than if additional genes were added into the chromosome. Second, it provides the host cell the option of losing the plasmid if selective antibiotic pressure is not kept and it becomes more beneficial to expel the plasmid.
Thirdly, as described in the data of this dissertation, an extrachromosomal plasmid can increase in copy number, and thus increase expression of plasmid-encoded resistance genes, enabling enhanced antibiotic resistance.

D. 1. Plasmid Classification and Incompatibility

Schemes to classify plasmids initially started with phenotypic traits that were imparted by genes contained on the plasmid. Among Enterobacteriaceae, plasmid traits included “fertility” (F) plasmids, colicin associated (Col) plasmids, and antibiotic resistance (R) plasmids. Later it was found that strains that carried these plasmids also produced serologically distinct pili (conjugation or sex pili), and this led to a classification scheme based on pili serotype (463). Serotyping in this era was cumbersome since it required the production of specific antibodies, and use of an electron microscope to visualize antibody agglutination to the pili. By the late 1960s it was recognized that strains possessing a given plasmid type could not accept plasmids of the same serotype (464). The use of incompatibility as a classification scheme was then adopted over serology because of its relative simplicity, however, this method could be confounded by extraneous factors that mimicked incompatibility and prevented plasmid transfer (455). Further research into plasmid biology during the 1970s and 80s found that incompatibility was a function of replication control mechanisms. DNA sequencing of the plasmid replicon regions (rep typing) was found to be a more robust way to distinguish plasmid types, and this is the agreed method of plasmid typing today (455). There are at least 39 incompatibility “Inc” groups from IncA/C to IncZ with several
subgroups within some groups (e.g. IncFI – IncFVII). Multiplex PCR schemes now exist that can readily identify plasmids of these known replication types (465-467).

D. 1. a. **IncI1 Plasmids**

The IncI1 plasmids are also known as IncIκ, Col1B-P9, R64, and R144 plasmids. They were first reported in 1967 as plasmids associated with *E. coli* resistance to tetracycline and aminoglycosides (468-470). This resistance was determined to be associated with carriage of the *tetD* gene which is located on the Tn10 transposon, and whose product has since been proposed to upregulate multiple drug-resistance mechanisms similar to the way the transcription factors MarA, SoxS, and Rob do (471, 472). Most circulating IncI1 plasmids found today no longer carry *tetD*. (P. Kurpiel, personal observation, GenBank search.)

IncI1 plasmids have been found in *E. coli* on six continents, and in other *Enterobacteriaceae* species such as *Salmonella enterica*, and *Klebsiella pneumoniae* (431). *Enterobacteriaceae* carrying IncI1 plasmids have been particularly associated with resistance plasmids in livestock animals (473, 474). This association is likely due to the antibiotics being provided to the animals, and the resistance genes encoded on the IncI1 plasmids. A variety of resistance genes have been identified on IncI1 type plasmids. As mentioned in Section I. C. 3 c. 1 (a), they are one of the most common vehicles for CMY type AmpC β-lactamase genes (e.g. *bla*_{CMY-2}, *bla*_{CMY-7}). They are also seen as frequent carrier of CTX-M type ESBL genes (e.g. *bla*_{CTX-M-14}, *bla*_{CTX-M-15}), and VIM-1 metallo-β-lactamases (431). IncI1 plasmids have also been found to carry genes for ribosomal
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methylases (rmtB), macrolide phosphotransferase (mphA), and pentapeptide (qnr) quinolone resistance genes (431).

In addition to antibiotic resistance genes, IncI1 plasmids are known to carry a pilS gene which is considered a virulence gene. The gene product is a Type IV prepillin, or thin pillin, that is important for conjugal transfer of the plasmid (475-477). The thin pillin appears vital for the formation of biofilm, and E. coli adherence to epithelial cells and abiotic surfaces (478).

D. 2. Plasmid Replication

There are two general replication mechanisms for circular plasmids in Enterobacteriaceae: theta replication, and rolling-circle replication (454). Plasmids associated with antibiotic resistance in Enterobacteriaceae typically use theta replication during normal growth, but use rolling circle during conjugation (431). In either mechanism, replication begins at an origin of replication (ori region). The plasmid usually provides at least one protein for replication initiation, and relies on host cell DNA replication machinery (i.e. primases, polymerases, helicases, ligases, etc.) to carry out replication.

Since incessant plasmid replication would threaten the survival of the host bacterial cell, it needs to be carefully controlled. Replication and copy number of theta replicating plasmids is usually controlled by RNA based mechanisms. This may involve limiting production of DNA polymerase priming RNAs (e.g. ColE1 plasmids), or through antisense RNA molecules that block translation of plasmid specific initiation proteins (e.g. IncI1 plasmids). These control mechanisms are also responsible for the
incompatibility that is observed, by not allowing incoming plasmids with the same replication control systems to replicate upon entry into the cell and take up residence (454).

D. 2. a. IncI1 Replication

The copy number of IncI1 plasmids is usually limited to one or two copies per cell (479). This is maintained by the combination of a Col1B colicin addiction system which fosters survival of daughter cells that carry the plasmid, and a tightly controlled plasmid replication system (480). Replication of IncI1 plasmids is initiated by the protein RepZ, and copy number is maintained by controlling repZ expression at the level of translation through multiple mechanisms. The first level of control involves the inaccessibility of the repZ Shine-Dalgarno sequence (SD). The sequence is located on the stem of an RNA stem loop structure referred to as SL III (Figure 29A.) that inhibits ribosome binding (481). Secondly, even when the sequence is available, it is a weak SD sequence meeting only 14% of the E. coli SD consensus (482). To help initiate translation, the repZ transcript must form an mRNA pseudoknot just upstream of the SD sequence (481). This likely serves to halt a scanning ribosome at the translation initiation site (482). For the pseudoknot to form, four RNA nucleotides 80-83 nt upstream of the repZ start codon at the top of the upstream stem loop I (SL I) must bind with four complementary nucleotides that lie 3-6 nucleotides upstream of the repZ SD, and 16-19
nucleotides upstream of the start codon\(^1\) (479). As mentioned above, the repZ SD is located on the stem of a stem loop, and complementary nucleotides needed for the pseudoknot are also located on this stem. In order to make these nucleotides available, an upstream overlapping gene called repB (repY) must be translated (Figure 29b) (481, 482). The repB gene codes for a 29 amino acid polypeptide with no known function other than its translation assists repZ translation (482). Ten base pairs of the 3’ end of repB sequence overlap the 5’ end of repZ (Figure 30) (483). The repB SD is slightly better for translation initiation, meeting 29% E. coli SD consensus. When repB is translated, the ribosome translates through the SL III stem loop relaxing the RNA structure and making the pseudoknot nucleotides available for pseudoknot formation (482).

Yet even these controls are not enough to maintain low plasmid copy number. An interfering RNA mechanism is present which further controls copy number. The interfering RNA called Inc is approximately 70 nucleotides long (length may vary from 66 to 79 nucleotides) and it is counter-transcribed from the complementary DNA of the repZ mRNA upstream non-coding region, 92-170 nucleotides from the repZ start codon\(^1\) (see figure 30) (484). This is the region that forms the SL1 stem loop structure describe above. The inc and repZ transcription promoters both match about 75% the E. coli consensus and are expressed at approximately equal levels (485).

Incl1 repZ Inhibition as proposed by Praszker and Pittard (482). (a) repZ is transcribed as part of a polycistronic transcript that includes repB. The repZ Shine Delgarno sequence (SD) (green blocks) is obscured on a stem loop structure SL3. (b) The repB SD (yellow blocks) is available, and upon its translation SL 3 is straightened. (c) With the straightening of SL3 a pseudoknot forms by the bonding of complementary nucleotides (in orange) on SL1 and an area upstream of the repZ SD. This pseudoknot improves the translation of repZ. (d) This translation is inhibited by Inc antisense RNA (Light grey) which is complementary to SL1. The joining of Inc with SL1 relies on the initial contact between three complementary nucleotides at the top of the hairpin loops in a “kissing complex”. (e) Inc then joins SL1 in a double stranded “inhibition complex” which blocks ribosome access to the repB SD and thus prevents repZ translation.
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The nature of Inc interference was deduced by scientists at the University of Tokyo through mutation and RNA binding studies (486). They found that three of the four nucleotides (GGC) responsible for the pseudoknot formation located on the apex of the SLI loop are responsible for the initial interaction between Inc and the \textit{repZ} transcript \cite{487}. The initial joining of these complementary nucleotides forms what is called a “kissing complex” \cite{482}. The ability to form this complex is the critical step in IncI1 plasmid copy number of control \cite{487}. Mutations in the loop sequence can change the shape of the loop and affect the ability for the kissing complex to form. This in turn can lead to an increase plasmid copy number \cite{479, 484}. Once the kissing complex has formed, the remaining complementary nucleotides of Inc and SL1 can bind to form an inhibition complex \cite{482}. The complex blocks the translation of \textit{repB}. Without \textit{repB} translation, the pseudoknot cannot form, and \textit{repZ} cannot be translated.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig30}
\caption{\textit{inc repB}, \textit{repZ} Gene Alignment}
\end{figure}

Dotted line indicates transcription direction. Block Arrows indicate gene coding direction.
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E. Insertion Sequence Elements

Bacterial insertion sequence (IS) elements have been defined as small (0.75 to 2.5 kb) phenotypically cryptic segments of DNA which only encode transposase genes that govern their own transposition (488). The IS elements contain specific inverted repeat (IR) end sequences on the left (5´) and right (3´) ends of the element (in relation to the transposase coding direction). The ends define the element, and are recognized by the element’s transposase for binding and excision (489). The IR sequences are often imperfect inverted repeats (IIR) and not exact inverted copies of one another. However, flanking the IR sequences there can be true direct repeats (DR) formed through DNA gap repair of the staggered double strand cuts made in the target DNA sequence by the IS element transposase during insertion. The DR are typically 2-14 nucleotides in length (489). There are varying degrees of target sequence specificity for different transposases. Some IS elements will only insert into specific sequences (e.g. IS91) (490). While others seem to have only minor target sequence requirements, or a preference for GT or AT rich sequences (489).

The identification of IS elements came in the early 1970s through the study of *E. coli* mutants that had lost function of the *gal* operon. Some of the mutants had polar mutations affecting only part of the operon, and they could revert back to wild type seemingly undoing a deletion mutation (491, 492). The IS element found disrupting the *gal* operon later became known as IS1 (493). As studies of these elements progressed, it was found that IS elements could not only interrupt gene expression, but also provide promoters to downstream genes, or move genes in a composite transposition (494, 495).
The initial nomenclature for IS elements began with assigned numbers (488). Later nomenclature used initials to describe the organism and genetic location of the IS element (496). For example, for the IS element IS\textit{Ecp1}, “Ec” refers to \textit{E. coli}, “p” refers to plasmid location. Today the website IS Finder (www-is.biotoul.fr) is a repository for new and established IS elements. There are currently 3,821 bacterial IS elements recognized from 713 different bacterial species (497, 498).

Transcriptional promoter sequences are often found within IS elements. They are usually located on the 3’ end of the element. The function of these promoters is not believed to just altruistically assist expression of downstream genes, but rather to help promote expression of the transposase gene when the IS element is in a circular intermediate form called a transpososome (489). Evidence for this comes from insertion elements like IS\textit{21}, IS\textit{30} that have a -35 sequence on their 3’ end and a -10 sequence on their 5’ end, and they only form a functional -35 and -10 promoter region when the two ends of the elements are joined end to end (499, 500). Thus these promoters facilitate expression of the transposase at a time when it is most needed (i.e. to break out of the transpososome and insert into a larger DNA molecule). When both the intact -35 and -10 sequences are located on the 3’ end of the element, there can be benefits for the host bacterial cell if promotion of the gene immediately downstream provides a competitive advantage, such as an antibiotic resistance gene. This is discussed more with IS\textit{Ecp1} in the section below.
II. Introduction and Review of the Literature

E. 1. Insertion Sequence ISEcp1

ISEcp1 (also known as ISEcp1B) is a 1656 bp insertion sequence that is member of the IS1380 family (489). It has no known species of origin and it has no apparent sequence target, but may prefer 5 bp AT rich sequences such as TATGA, TATCA, TACAT, TATAC, and TTCAT (361). ISEcp1 was first reported in 1999 as an element located on a plasmid upstream of a blacMY-4 gene in a clinical isolate of E. coli from England (501). It has since been reported from around the world as an immediate upstream element to at least 16 different kinds of CTX-M class ESBL genes, 7 different kinds of plasmid-encoded AmpC genes of the CMY-2 family, and two aminoglycoside resistance genes (Table 2) (437, 501-519). As mentioned in the β-lactamase section (I. C. 3.), large scale surveillance studies of ESBL genes have found blactx-M-14, and blactx-M-15 promoted by ISEcp1 to be the dominant ESBL types of E. coli in Europe, China, and Canada (508, 520, 521). Likewise, ISEcp1 promoted blacmy-2 is the most common plasmid-encoded AmpC β-lactamase arrangement found in E. coli worldwide (522-524). The reports of ISEcp1 in association with antibiotic resistance genes come from at least 17 different countries, involving 7 different species of bacteria (Table 2). In these studies, a wide variety of pulsed field patterns, plasmid types, and gene arrangements have been seen, indicating that the distribution of ISEcp1 is not due to the distribution of a common clone, or a common plasmid (504, 506, 511, 513, 515, 516, 525).
## Table 2. Publications Detailing IS*Ecp1* Upstream of Antibiotic Resistance Genes

<table>
<thead>
<tr>
<th>Publication Reference</th>
<th>Country</th>
<th>Bacteria Species</th>
<th>Type of Resistance Gene</th>
<th>Gene</th>
<th>Number of Isolates Reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>(502) China</td>
<td>Enterococcus casseliflavus</td>
<td>adenyllytransferase</td>
<td>APH(2&quot;)-LE</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(503) Japan</td>
<td>P. mirabilis</td>
<td>16s rRNA Methyltransferase</td>
<td>RmtC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(504) France</td>
<td>K. pneumoniae, P. mirabilis</td>
<td>AmpC</td>
<td>ACC1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>(504) Germany</td>
<td>Klebsiella pneumoniae</td>
<td>AmpC</td>
<td>ACC1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(505) Poland</td>
<td>Proteus mirabilis</td>
<td>AmpC</td>
<td>CMY-4, CMY-12, CMY-14, CMY-15</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>(506) Taiwan</td>
<td>E. coli</td>
<td>AmpC</td>
<td>CMY-2</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>(504) Tunisia</td>
<td>K. pneumoniae, S. enterica</td>
<td>AmpC</td>
<td>CMY-2, CMY-4, CMY-7</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>(437, 501) United Kingdom</td>
<td>E. coli</td>
<td>AmpC</td>
<td>CTX-M3, CTX-M15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>(512) India</td>
<td>E. aerogenes, E. coli, K. pneumoniae</td>
<td>ESBL</td>
<td>CTX-M15</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>(513) Portugal</td>
<td>E. coli</td>
<td>ESBL</td>
<td>CTX-M14, CTX-M15, CTX-M32</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(514) South Korea</td>
<td>E. coli</td>
<td>ESBL</td>
<td>CTX-M12</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>(515) Spain</td>
<td>E. coli</td>
<td>ESBL</td>
<td>CTX-M3, CTX-M14, CTX-M27, CTX-M40, CTX-M55</td>
<td>351</td>
<td></td>
</tr>
<tr>
<td>(517) Turkey</td>
<td>E. coli</td>
<td>ESBL</td>
<td>CTX-M15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>(518, 519) Vietnam</td>
<td>E. coli, Klebsiella pneumoniae</td>
<td>ESBL</td>
<td>CTX-M-like, CTX-M17</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>
ISEcp1 is frequently credited for the mobilization of the β-lactamase genes associated with it. Experiments by Lartigue et al. have demonstrated that ISEcp1 can transpose downstream β-lactamase genes through a “one-ended” transposition process (361). By this mechanism ISEcp1 transposase (TnpA) is less specific about the sequences it recognizes as right IIR, and when ISEcp1 attempts to excise itself from a genetic environment, TnpA may cut at sites further downstream of the actual right IIR and mobilize genes on its 3′ side. The left and right IIR sequences of ISEcp1 only have 32% consensus with one another, so the TnpA transposase of ISEcp1 may not be specific about its endonuclease excision site. In the one-ended transposition studies by Lartigue et al., the longest segments transposed by this method included 3,808 bp downstream of ISEcp1 which would be sufficient to include most β-lactamase genes (361).

However, even if a single ISEcp1 element can mobilize downstream genes by itself, it does not completely explain its wide distribution as an upstream element of prevalent β-lactamases. In many gene arrangements the ISEcp1 tnpA transposase gene has been interrupted and is not functional (504, 509). What does remain in essentially all arrangements are the proximal (3′–most) -35 and -10 promoter sequences located within ISEcp1 -104 to -66 bp upstream from the 3′ end. In reports describing ISEcp1 upstream of β-lactamases, the ISEcp1 3′ end is usually positioned -145 to -40 bp upstream of the translation start codon of the β-lactamase gene (510, 525, 526). The promoter sequence in this arrangement has been shown to be necessary to achieve effective expression of downstream blaCMY and blaCTX-M β-lactamase genes in E. coli hosts (360, 411, 527). So, while the antibiotic resistance genes downstream of ISEcp1 may vary, the presence and positioning of the promoters remain remarkably consistent, and the survival advantage
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this arrangement provides may be the true reason why it is frequently encountered upstream of the most common plasmid-encoded AmpC and ESBL genes.

E. 2. Insertion Sequence IS5

IS5 was once considered a member of the IS4 family but is now considered the lead member of its own insertion element family (497). It is a 1,195 bp element primarily associated with *E. coli*. There are 11 copies of IS5 in *E. coli* K-12 MG1655 and up to 18 copies in *E. coli* W3110 (528). However there are a number of sequenced *E. coli* strains that contain no IS5 at all, particularly O157: H7 strains, so the element is not as ubiquitous among the *E. coli* as once thought (528). IS5 has been found to have nucleotide target sequence YTAR. Some recognized targets include CTAG, CTAA, CTAA, or CAAG (497). The ability of IS5 to transpose with high frequency is somewhat notorious, and it has been found to interfere with genetic experiments in *E. coli* K-12 (529, 530) In fact, its discovery in 1974 was made through one such accident (530).

IS5 has a GATC methylation site within the promoter region of its transposase gene that is believed to affect its transposition ability. The arrangement is similar to that of IS10 which also has a methylation site in its promoter region (531). For IS10, it has been found that methylation at the promoter decreases transposase gene transcription 12-fold, and decreases the rate of transposition 1000-fold (531). If such a control mechanism existed for IS5, it could account for the high IS5 transposition frequency observed when *E. coli* strains are maintained in relatively stagnant cultures; a time when DNA methylation activity is low (529).
IS5 is also notable for its ability to recombine with itself. These recombination events have been shown to manifest tandem gene amplification of antibiotic resistance associated genes located between two IS5 elements (532, 533). Recombination events with IS5 are believed to be quite frequent and have made the presence flanking direct repeats somewhat rare for IS5 (497). The nature of these recombinations has not been widely studied. There is a putative chi recombination site 5´-CATGGTGG-3´ located within IS5 at nucleotides 176-183 which may play a role in recombination events centered on the element (P. Kurpiel unpublished observation).

There has been some evidence that IS5 can upregulate expression of downstream genes. In fact, IS5 insertion at the bglA promoter is a common way for E. coli to gain function of its cryptic bgl operon. However, the mechanism of promoter activation is not completely understood. Schnetz and Rak found that insertion of IS5 upstream or downstream of the of the bglA promoter improved bglA expression 60-fold (534). They also found that a fragment containing only the first 25 bp of the IS5 5´ end and the last 32 bp of the 3´end had almost the same level of upregulation as the whole element, so long as the full IS5 transposase gene was also present somewhere in the cell (534). This led them to conclude that the transposase somehow acts on these ends to improve expression of the downstream gene, but evidence to support this theory has not been substantiated.

**F. The Rob Protein**

The Rob protein (also known as CbpB, RobA) is a 289 amino acid, 33 kDa, helix-turn-helix (HTH) DNA binding protein that is a member AcrA/ Xyls family of transcription activators. Rob is closely related (~50% amino acid identity) to the
transcription factors MarA and SoxS (535, 536). It is also closely related to TetD (60% amino acid identity), which is a transcription factor encoded on the Tn10 transposon (472, 535). All four proteins share a conserved (~100 residue) N-terminal domain present in all ArcA/Xyls family members (536). Rob differs in that it has an extended C-terminal end (~200 residues) that is structurally similar to the galactose binding portion of the E. coli galactose-1-phosphate uridyltransferase enzyme, GalT (537). Interestingly, Rob also has a negative influence on the expression of glaT (538).

Rob binds the same promoter sequences as MarA and SoxS with different degrees of affinity, and with different levels of transcriptional activation/repression transcription (536, 537, 539, 540). The binding regions for these proteins are approximately 20 bp in length and referred to as “marboxes” (541). They have a consensus sequence of 5’-AYNGCACNNWNNRYYAAYN-3’ (where N = any base; R = purine A/G; W = weak bonding A/T; Y = pyrimidine C/T) (541, 542). The marboxes may be aligned on the non-coding strand, essentially in the reverse direction of the promoter and the downstream gene coding direction (Figure 31). This reverse alignment is the most frequent marbox configuration and is referred to as a class I marbox (539, 541). The class I marboxes are usually positioned 15 to 27 bp upstream of the -35 promoter sequence. The Class II marbox on the other hand aligns with the gene coding direction and in most cases actually overlaps the -35 sequence extending 18 to 19 bp upstream of the -10 sequence (539, 541). Evidence indicates that all three proteins exert influence at the Class I marbox sites by interacting with the RNA polymerase alpha subunit, perhaps stabilizing it (540). For class II binding sites the presence of the alpha subunit is not necessary for an influence to be exerted (540). Both SoxS and MarA appear to bind with the RNA
polymerase holoenzyme prior binding the marbox ("prerecruitment"), whereas Rob appears to bind DNA first and the RNA polymerase joins later (543). MarA, SoxS, and Rob can all bend DNA to some extent upon binding, and this too may also improve transcription initiation at some promoters (537, 540).

**Figure 31.** Positioning of Class I and Class II Marboxes

Adapted From Martin *et al.* (523). Positioning of Marboxes for different genes under the influence of MarA, SoxS, and Rob with respect to the RNA polymerase -35 and -10 promoter sequences. Black arrows indicate the marbox sequence direction and N-terminal to C-terminal positioning of Rob when bound.
### Table 3. *E. coli* Genes under the Influence of Rob

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>acnA</em></td>
<td>Aconitase A</td>
</tr>
<tr>
<td><em>acrA, acrB</em></td>
<td>Component of AcrAB efflux pump</td>
</tr>
<tr>
<td><em>aldA</em></td>
<td>Aldehyde dehydrogenase, NAD-dependent;</td>
</tr>
<tr>
<td><em>fpr</em></td>
<td>Ferredoxin-NADP reductase. Reduces oxidized NADPH and ferredoxin</td>
</tr>
<tr>
<td><em>fumC</em></td>
<td>Fumarate hydratase. Needed when acetate instead of glucose is used as a carbon source</td>
</tr>
<tr>
<td><em>inaA</em></td>
<td>Unknown, Deletion mutants are susceptible to weak acids</td>
</tr>
<tr>
<td><em>mdaB</em></td>
<td>FMN-dependent NADH-quinone reductase</td>
</tr>
<tr>
<td><em>micF</em></td>
<td>Antisense RNA, blocks OmpF translation.</td>
</tr>
<tr>
<td><em>nfnB</em></td>
<td>Dihydropteridine reductase</td>
</tr>
<tr>
<td><em>nfo</em></td>
<td>Endonuclease IV with intrinsic 3'-5' exonuclease activity</td>
</tr>
<tr>
<td><em>nfsA</em></td>
<td>Nitroreductase A</td>
</tr>
<tr>
<td><em>pgi</em></td>
<td>Glucose-6-phosphate isomerase</td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td><em>ybjC</em></td>
<td>Membrane protein part of the NfsA operon</td>
</tr>
<tr>
<td><em>yhbW</em></td>
<td>Function unknown</td>
</tr>
<tr>
<td><em>yncE</em></td>
<td>Unknown, predicted member of the fur regulon</td>
</tr>
<tr>
<td><em>zwf</em></td>
<td>Glucose-6-phosphate dehydrogenase, needed for oxygen and nitrogen intermediates</td>
</tr>
</tbody>
</table>
There are approximately 17 genes with known function that are under the combined influence by MarA, SoxS, and Rob (Table 3) (318). By and large, the products of these genes have roles in maintaining cell function under oxidative or acid stress conditions, and expelling or limiting entry of toxins into the cell. MarA seems to be more responsive to acid stress. Its expression is negatively controlled by the repressor MarR, which is thought to release this control after direct interaction with acids like salicylate (544). SoxS on the other hand is more responsive to oxidative stress. SoxS is regulated by SoxR, which under non-oxidative stress conditions, binds to block transcription of soxS (545). When SoxR is oxidized, it changes shape and acts as an activator of soxS and improves its expression approximately 20-fold (545).

Our understanding of Rob and its role in activating genes in the MarA/SoxS/Rob regulon is still evolving. Rob was first reported in 1993 as a protein that bound DNA on the right side of the E. coli origin of replication (535). It thus earned its name as a “right-origin-binding” protein (535). The activity at the origin of replication remains speculative. Loss of rob does not appear to affect replication, but it may help curb replication in times of stress (535). Skarstad et al. estimated that there were 5,000 – 10,000 Rob monomers per E. coli cell, but interestingly about 75% of Rob precipitates out with the insoluble cell fraction, indicating that it may normally be bound up with insoluble components within the cell (535). In E. coli genomic cDNA arrays performed at the University of Wisconsin, Rob transcript levels have been shown to be remarkably consistent under a wide variety of treatment conditions (log phase, heat shock, cold shock, acid shock, ciprofloxacin treatment), with an estimated 1-2 transcript copies per cell (546). In stationary phase 3-4 copies of rob transcript were estimated to be present
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Kakeda et al. presented evidence that rob may be up-regulated in the sigma S response. They found that expression from the rob promoter increased during stationary phase and in glucose and phosphate starvation medium; whereas expression from the rob promoter in these conditions was reduced in a sigma S null mutant (548).

A short time after Rob was discovered, studies looked into its binding and regulatory characteristics with known MarA and SoxS regulated genes. Two of the first papers by Ariza et al and Jiar et al. studied the influence of Rob on inaA, fpr, fumC, micF, nfo, sodA, and zwf (536, 540). In these experiments, excess Rob was found to upregulate expression of these genes, but not to the same degree that SoxS could for genes inaA, sodA, or zwf. These early expression papers are perhaps flawed by the lack of understanding of the activation (e.g. oxidative stress, acid stress conditions) necessary to enable Rob to behave as a transcription factor (discussed below) and Rob may in fact have equal or greater influence on these genes.

Rob is known to affect antibiotic susceptibility of bacterial cells. Work by Ariza et al. with E. coli, and Lee et al. with Enterobacter cloacae, found that excess Rob was associated with reduced susceptibility to puromycin, tetracycline, chloramphenicol, and tobramycin (536, 549). One mechanism affecting this sensitivity may be the AcrAB efflux pump. Work by Rosenberg et al. demonstrated that Rob binds near the acrAB operon promoter and up-regulates acrAB expression, particularly after exposure to bile salts and decanoate (321). Interestingly, it appears that SoxS and MarA do not act at the acrAB promoter, at least not in the presence of decanoate (321). Rob also binds the micF promoter and up-regulates its expression (536, 540). MicF is an antisense RNA that downregulates expression of ompF, the gene for outer membrane porin OmpF. As
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described in Section I. C. 2. a, downregulation or loss of the OmpF porin can limit entry of antibiotics (e.g. β-lactams, fluoroquinolones) into the cell, and this is likely another mechanism by which Rob alters antibiotic susceptibility.

In 2000 Kwon et al. published X-Ray crystallography studies of Rob binding DNA at the \textit{micF} promoter (537). There are two DNA binding HTH structures at the N-terminal region of Rob, and they bind two areas within the marbox sequence through hydrogen and van der Waals bonding (537). The N-proximal HTH interacts with the DNA major groove at the conserved tetramer sequence GCAC. Kwon \textit{et al.} referred to this as the A-box (Figure 32). The C-proximal HTH of Rob forms a hydrogen bond between Arg 90 and the phosphate backbone of the coding DNA strand. For the \textit{micF} promoter this bond occurs with the thymine 14 nt from the 5´ end of the marbox. This is slightly upstream of a second marbox conserved region referred to by Kwon \textit{et al.} as the B-box. This is different from MarA binding at the \textit{micF} promoter, where the C-proximal HTH binds the DNA major groove just upstream of the B-box (537, 550). Sequences of the B-box are variable for different marboxes, indicating that this site is less specific than the A-box (539, 541). Indeed, Kwon \textit{et al.} inserted different nucleotides within the B-box area and this did not appear to affect binding of Rob (537). Similar substitutions carried out by Li and Demple found changes in the A box decrease Rob binding 100-fold, whereas changes in the B-box only decreased binding 2-4 fold (551).

As mentioned above, Rob is maintained in fairly abundant quantities within the \textit{E. coli} cell in a variety of conditions yet most of the protein remains bound up in the insoluble fraction of the cell under normal growth conditions. Early studies found that the deletion of \textit{rob} had essentially no effect on growth, except when stress was applied (321,
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535, 552). This indicated that Rob may have a post-translational activation mechanism. In 2002 Rosner et al explored this possibility using a \textit{rob+ ΔsoxS, ΔmarA} strain (553). They found that oxidative stress inducing agents 4,4′-dipyridyl, and 2,2′-dipyridyl led to increased expression of \textit{inaA}, but was diminished in a \textit{Δrob, ΔmarA, ΔsoxS} triple knockout. Overexpression of \textit{rob} under normal growth conditions did not seem to affect \textit{inaA} expression, only the combination of \textit{rob} expression and stress did. When \textit{rob} was put on a low copy number plasmid (pta108) without any \textit{rob} transcription initiation control mechanism, the combination of excess \textit{rob} and oxidative stress was deleterious to

\textbf{Figure 32.} Rob Protein Binding to the \textit{micF} promoter region

Illustration of the Rob protein interaction with A Box and B Box sequences within the marbox at the \textit{micF} promoter adapted from Kwon et al. (521). The N-terminal-most helix-turn-helix motif interacts with the major groove of the A box whereas the C-terminal-most helix-turn-helix interacts with the DNA backbone just upstream of the B-box.
the *E. coli* host (553). Interestingly, mutant strains that were able to survive had deletions or amber mutations that removed the C-terminal end of Rob. Without the C-terminal portion, Rob maintained basal level of expression, but *inaA* upregulation in response to 2,2′-dipyridyl, or 4,4′-dipyridyl was abolished (553).

Rosner *et al.* also conducted NMR studies that indicated 2,2′-dipyridyl molecules bound to the C-terminus of Rob. This led them to conclude that direct binding of toxins was a possible mechanism of activation (553). These binding studies were performed *in vitro* using excess 2,2′-dipyridyl. It seems unlikely that *E. coli* would produce Rob to specifically react with a manmade agent that is not abundant in nature. Also 2,2′-dipyridyl did not enhance the binding of *rob* to promoters in *in vitro* electrophoretic mobility studies (553). Rosenberg *et al.* evaluated fluorescent emission spectra of Rob after the addition of bile salts and decanoate, and reported spectral shifts that were indicative of direct binding (321). Salts with the strong binding tended to be associated with stronger expression of genes influenced by Rob. These results are intriguing, but only provide indirect evidence of a substrate binding activation mechanism for Rob.

Later work by Griffith *et al.* in 2009 proposed a more general Rob activation mechanism of “sequestration-dispersal” (554). This work built on earlier findings by Azam *et al.* that found that Rob could be visualized as intracellular clusters with immunofluorescent staining (555). What Griffith *et al.* did was create a chimera protein that had a Rob C-terminal end and a SoxS N-terminal end so that it could undergo Rob activation as seen by Rosner *et al.* but be detected by the SoxS antibodies they had on hand. They found that upon treatment with 4,4′-dipyridyl, the Rob clusters dispersed into
the cytoplasm (Figure 33). The dispersal coincided with up-regulation of the MarA/SoxS/Rob influenced gene *inaA* (554).

**Figure 33.** Dispersal Activation of Rob/SoxS Chimera in *E. coli* Following Treatment with Dipyridyl and Deconoate.

[Images of bacterial cells stained with Alexa 488 immunofluorescent staining showing dispersal activation of Rob/SoxS chimera protein in *E. coli* strain RA4468.]

From Griffith *et al.* 2009 (554). Alexa 488 immunofluorescent staining of Rob-SoxS chimera protein in *E. coli* strain RA4468. Cells are treated with 4,4′-dipyridyl and deconoate as labeled on the left. Times after treatment indicated above.

The feasibility of the sequestration-dispersal mechanism proposed by Griffith *et al.* was also supported by additional degradation studies that found MarA and SoxS proteins had half-lives of 1-3 minutes, and the half-life of Rob was greater than 20 hours. This indicated that Rob may indeed sit in clusters for long periods of time waiting for a dispersal stimulus to arrive. The long half-life of Rob depended on the presence of the C-terminal end. Removing just six amino acids from the end of Rob reduced its half-life to 10 minutes, and half-life became shorter with successive removals (554). Along with the decrease in half-life came commensurate reductions in *inaA* expression similar to
what was seen by Rosner et al. (553, 554). The removal of just six amino acids from the
Rob C-terminal end also eliminated its ability to form sequestration clusters (554).

So, ostensibly what Griffith et al. propose is that Rob lingers within the cell,
residing in insoluble clusters that are dependent on the presence of the C-terminal end to
form. While in clusters, Rob remains protected from proteases. The clusters dissociate by
an unknown mechanism upon the presence of an oxidative stress stimulus. The “active”
Rob once freed from the cluster, acts as a transcription factor, up-regulating genes whose
products ultimately reduce the cell permeability to toxins and reduce the effects of
oxidation. Rob then likely relinquishes its transcriptional activation duty to MarA or
SoxS. These factors are slower to respond due to their requirement to be transcribed and
translated post stimulus, but they have more specific binding properties than Rob, and
they are eliminated more readily by proteases once the stimulus is discontinued. The
question to be solved in the future is: what holds Rob in clusters, and what is the
mechanism for its release? By learning to inhibit the dispersal mechanism we may find a
way to add potency to antibiotics against E. coli and other Enterobacteriaceae.

G. Summary of the Literature Review

This literature review sought to review β-lactam antibiotics and β-lactam
resistance mechanisms to put into context the evolution of β-lactam drugs and resistance
mechanisms that have led to the current therapy challenges we face today. Some key
points to reiterate from Sections I. C. 3. a and C. 3. c. 1) (a) include the emergence of the
plasmid-encoded CMY-2 AmpC β-lactamase, and CTX-M-14 and CTX-M-15 ESBLs
whose genes are promoted by the insertion element ISEcp1. These β-lactamases are the
most prevalent in their respective classes, and they are driving resistance against third
generation cephalosporins. It was mentioned in section I. C. 3. c. that there are currently
no CLSI recommendations for the clinical detection of plasmid-encoded AmpC β-
lactamases like CMY-2. Also in this section it was noted that AmpC enzymes like
CMY-2 can enable resistance to penicillins such as piperacillin, and are not inhibited by
sulfone β-lactamase inhibitors such as tazobactam. It was stated that in studies of
piperacillin/tazobactam therapy, piperacillin/tazobactam resistant *E. coli* and *Klebsiella
pneumoniae* can emerge during therapy (Section I. B. 7. c.) We hypothesized that the
emergence of piperacillin/tazobactam resistance in *bla*<sub>CMY-2</sub> carrying *E. coli* was due to
overexpression of *bla*<sub>CMY-2</sub>. Experiments in this dissertation sought to evaluate *bla*<sub>CMY-2</sub>
carrying *E. coli* that were susceptible to piperacillin/tazobactam but spawned resistant
mutants upon exposure to piperacillin/tazobactam. One aim was to compare the parents
and mutants for sequence changes in their *bla*<sub>CMY-2</sub> promoter regions. Upon study of the
*bla*<sub>CMY-2</sub> promoter regions of two *E. coli* strains, a novel tandem *bla*<sub>CMY-2</sub> arrangement
was found. A review of IS5 in Section I. E. 2. provided background for the significance
of this finding. Another aim sought to identify transcription factors binding to *bla*<sub>CMY-2</sub>
 promoter regions. The transcription factor Rob was identified in these studies. A review
of Rob in Section I. F. discussed the properties of Rob. The last aim was to evaluate
*bla*<sub>CMY-2</sub> gene copy number in the mutants with increased *bla*<sub>CMY-2</sub> expression. It was
found that overexpression mutants had an increase in plasmid copy number equal to their
increase in *bla*<sub>CMY-2</sub> expression. For one set of mutants *bla*<sub>CMY-2</sub> was encoded on an IncI1
plasmid. The review on IncI1 replication control mechanisms in section I. D. 2. a.
II. Introduction and Review of the Literature

provided context to the IncI1 copy number control mechanisms, and the point mutations that were found in the antisense RNA gene *inc* of two mutants.
III. Experimental Methods

A. Bacterial Strains.

The strains used in this study are listed in Table 4. Briefly, CUMC-50 was obtained as part of a surveillance study for cefoxitin-resistant *E. coli* in the Omaha, Nebraska metropolitan area during 2003. *E. coli* strains CUMC-201, CUMC-214, CUMC-243 were all obtained as part of another community surveillance study for cefoxitin-resistant *E. coli* in the Omaha, Nebraska area between January 1, and June 30, of 2005 (556). *Klebsiella pneumoniae* strain KPVAMC50 was obtained as part of a 24 hospital national study for *K. pneumoniae* with intermediate or full resistance to cefoxitin or imipenem (557). Strains *E. coli* Misc-341 (Bil-1), *E. coli* Misc-345 (Lat-2) are older *bla*CMY-2 strains from the collections of the Creighton University Center for Research in Anti-infectives and Biotechnology (CRAB). *K. pneumoniae* Kleb-249 came from Rady Children’s Hospital in San Diego, California. *Proteus mirabilis* strain PM-IMP-27, and *Salmonella enterica* sv. Typhimurium strain Sal-100 were submitted to CRAB of as part of reference testing for antibiotic resistance mechanisms (439).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Source</th>
<th>Patient Demographic</th>
<th>Location</th>
<th>Date</th>
<th>How Obtained</th>
<th>Genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUMC-50</td>
<td><em>E. coli</em></td>
<td>Urine</td>
<td>Outpatient, 11y/o female</td>
<td>Omaha, NE</td>
<td>March 2003</td>
<td>Cefoxitin resistant <em>E. coli</em> study, Omaha</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(88)</td>
</tr>
<tr>
<td>CUMC-50M</td>
<td><em>E. coli</em></td>
<td>Etest Assay Mutant</td>
<td>Creighton University</td>
<td>Jan 2004</td>
<td>Etest assay of CUMC-50</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(88)</td>
<td></td>
</tr>
<tr>
<td>CUMC-50M2</td>
<td><em>E. coli</em></td>
<td>Agar Mutant Selection</td>
<td>Creighton University</td>
<td>Nov 2006</td>
<td>Mutant selection from CUMC-50</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(88)</td>
<td></td>
</tr>
<tr>
<td>CUMC-50M8</td>
<td><em>E. coli</em></td>
<td>Agar Mutant Selection</td>
<td>Creighton University</td>
<td>Nov 2006</td>
<td>Mutant selection from CUMC-50</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(88)</td>
<td></td>
</tr>
<tr>
<td>CUMC-201</td>
<td><em>E. coli</em></td>
<td>Urine</td>
<td>Hospital inpatient</td>
<td>Omaha, NE</td>
<td>Jan 1-June 30 2005</td>
<td>Cefoxitin resistant <em>E. coli</em> study, Omaha</td>
<td>Tandem <em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(88, 556, 558)</td>
</tr>
<tr>
<td>CUMC-214</td>
<td><em>E. coli</em></td>
<td>Urine</td>
<td>Outpatient</td>
<td>Omaha, NE</td>
<td>Jan 1-June 30 2005</td>
<td>Cefoxitin resistant <em>E. coli</em> study, Omaha</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(556)</td>
</tr>
<tr>
<td>CUMC-214M1</td>
<td><em>E. coli</em></td>
<td>Agar Mutant Selection</td>
<td>Creighton University</td>
<td>Dec 2006</td>
<td>Mutant selection from CUMC-214</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>CUMC-243</td>
<td><em>E. coli</em></td>
<td>Urine</td>
<td>Nursing home patient</td>
<td>Omaha, NE</td>
<td>Jan 1-June 30 2005</td>
<td>Cefoxitin resistant <em>E. coli</em> study, Omaha</td>
<td>Tandem <em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(556, 558)</td>
</tr>
<tr>
<td>MISC-341</td>
<td><em>E. coli</em></td>
<td>Pakistan</td>
<td>1989</td>
<td></td>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; (BIL-1)</td>
<td>(559)</td>
</tr>
<tr>
<td>MISC-345</td>
<td><em>E. coli</em></td>
<td>Athens, Greece</td>
<td>Oct 1994</td>
<td></td>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; (LAT- 2)</td>
<td>(560)</td>
</tr>
<tr>
<td>Kleb-249</td>
<td><em>K. pneumoniae</em></td>
<td>Rady Children’s Hospital San Diego, CA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; (87)</td>
<td></td>
</tr>
<tr>
<td>KPVAMC50</td>
<td><em>K. pneumoniae</em></td>
<td>USA</td>
<td>1996-2000</td>
<td></td>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>(557)</td>
</tr>
<tr>
<td>PM-IMP-27</td>
<td><em>Proteus mirabilis</em></td>
<td>Wound</td>
<td>Outpatient</td>
<td>Council Bluffs, IA</td>
<td>2010</td>
<td>Creighton CRAB reference testing</td>
<td><em>bla</em>&lt;sub&gt;IMP-27&lt;/sub&gt;</td>
<td>(561)</td>
</tr>
<tr>
<td>Sal-100</td>
<td><em>Salmonella enterica sv. Typhimurium</em></td>
<td>Stool</td>
<td>14 month old w/ travel history</td>
<td>Australia</td>
<td>March 2000</td>
<td>Creighton CRAB reference testing</td>
<td><em>bla</em>&lt;sub&gt;CMY-7, bla*&lt;sub&gt;SHV-9, bla*&lt;sub&gt;OXA-30</td>
<td>(439)</td>
</tr>
</tbody>
</table>
B. Agar MICs

Agar dilution minimal inhibitory concentration (agar MIC) methods were used to determine antibiotic susceptibility levels of the bacterial strains in this dissertation study. The method involves spotting bacterial cell suspensions on Mueller Hinton agar (MHA) plates containing measured concentrations of antibiotic. Different concentrations of antibiotic are used, and the lowest concentration at which the bacterial strain no longer grows is interpreted as the minimal inhibitory concentration of that drug for that strain.

The antibiotic plates were cast by mixing 9 ml of molten MHA (50°C) with 1 ml of antibiotic suspension in Mueller Hinton broth (MHB) or phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mm KH₂PO₄) for tazobactam. All antibiotics were initially suspended in 10 ml of PBS, except carbapenems which were suspended in sterile nanopure water. Suspensions were syringe filtered through a 0.22 µm Microcon® Luer-lock filter (Millipore, Billerica, MA) and serially diluted as needed in 3 ml of MHB except tazobactam which was kept at 100 X final concentration (400 µg/ml) and added to agar by pipetting in a 100 µl volume before mixing. The concentration of antibiotic for the initial suspension was calculated using Equation 1 which accounts for the manufacturer’s denoted drug potency. Controls for these studies included a no-antibiotic control plate to assure that reasonable growth was attained on the agar, and MIC control strains with established MICs to the antibiotic being tested. The control strains used were those recommended by Clinical Laboratory Standards Institute (CLSI) and included *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922; and *E. coli* ATCC 35218 (130). The strains to be tested were cultured from frozen stocks overnight at 37°C on trypticase soy agar with 5% sheep blood (blood agar plate, BAP) (Becton
Dickenson, Franklin Lakes, NJ; and Remmel, Lenexa, KS). A few to several colonies growing on the BAP were collected on a sterile cotton swab and used to inoculate 5 ml of MHB that was grown overnight (16-20 hours) at 37°C with 120 rpm rotation. It was important to inoculate the broth cultures with more than one colony in the event that mutations or plasmid loss had occurred in cells represented by one of the colonies. The following day, the overnight broth culture contained approximately $10^9$ CFU/ml of bacteria and 100 µl of the culture was transferred to a 9.9 ml tube of sterile PBS. This yielded a suspension of $10^7$ CFU/ml from which 650 µl was loaded into a representative well of a Steers Foltz plate replicator. The replicator deposited on to the MHA plate an approximate 10 µl volume of suspension that contained approximately $10^5$ CFU. Plates were spotted, and then incubated 16 to 20 hours at 37°C before being evaluated. In cases where growth was sparse at the inoculation spot, growth was considered positive if three or more colonies were present.

**Equation 1.** Determination of Drug Mass Required for Agar MIC Assays

$$\text{Amount of drug added to initial suspension in } \mu g = \frac{(\text{uppermost conc } \mu g/ml) \times (\text{ml diluent}) \times (\text{ml final agar volume})}{(\text{drug potency})}$$

Potency is given as a fraction by the manufacturer. The equation was also used for drug suspensions other than agar plates.
C. Etest® Assays for Antibiotic Susceptibility

Etest® assays (AB BioDisk, Solna, Sweden) were conducted as recommended by the manufacturer and the CLSI (130). Strains to be tested were grown overnight on BAP plates and several colonies from the culture were lifted with a sterile cotton swab and suspended in 5 ml of MHB to a 0.5 McFarland turbidity level. Turbidity was measured with a DU-6 spectrophotometer (Beckman-Coulter, Brea, CA) to assure that the OD$_{600}$ of the suspension was at or near 0.113 (0.5 McFarland). A fresh cotton swab was saturated in the cell suspension and wrung out by rolling the head against the sides of the tube. The swab was then used to inoculate the surface of a 90 mm petri plate containing 20 ml of MHA by streaking in three directions, turning the plate 60° before repeating. This method results in a confluent lawn of growth. After streaking, the plate was allowed to rest 20-30 minutes at room temperature so excess moisture could be absorbed by the agar. The AB BioDisk Etest® strips were stored at -20°C and allowed to warm up to room temperature for 30 minutes before using to limit condensation. The strips were lifted from their packaging with an AB BioDisk Etest® manual applicator and applied to the inoculated plate in a single motion. The plates were incubated at 37°C for 16 to 20 hours after which the MICs were interpreted and plates were photographed.

D. Mutant Selection by Antibiotic Agar Suspension

In an effort to select mutants emerging from *E. coli* strains exposed to piperacillin/tazobactam and meropenem, suspensions of bacterial cells were mixed with inhibitory/superinhibitory concentrations of these antibiotics in molten MHA. Colonies that were able to grow in the antibiotic agar were considered “putative mutants”.
Selected putative mutants were collected, cultured, and had antibiotic sensitivities measured by agar dilution. Putative mutants that had MICs 4-fold higher than their parent strain for the selection drug were considered mutants. Mutant frequencies were estimated using the number of colonies from corresponding cell viability plates as a denominator.

The parent strains used in the mutation assays were grown overnight on BAP plates, and colonies from the overnight culture were used to inoculate a sidearm flask containing 70 ml of MHB to an OD_{600} of 0.1. The MHB culture was grown in a 37°C incubator shaking at 120 rpm until an OD_{600} of 0.5 was achieved. When cells reached the desired optical density, the entire culture was poured into a 250 ml Nalgene® centrifuge bottle (Thermo Fisher, Waltham, MA) and cells were concentrated by centrifugation at 6000 rpm for 15 minutes at 4°C. The cell pellet was resuspended in 45 ml of sterile phosphate buffered saline (PBS) (Fisher Scientific Cat# BP2438-4, 0.137M sodium chloride, 0.0027M potassium chloride, and 0.0119M phosphate). This yielded an approximate 10^9 CFU/ml cell suspension. One ml of the suspension was serially diluted to 10^8 and 10^7 CFU/ml in PBS.

For cell viability plates, the three cell suspensions (10^9, 10^8, 10^7 CFU/ml) were serially diluted in PBS to 10^1 CFU/ml. One milliliter of the 10^1, 10^2, and 10^3 CFU/ml dilutions were plated by mixing with 9 ml of molten MHA. All platings were done in duplicate. See Figure 34 for a schematic of the mutation and cell viability studies.

For the plates containing antibiotic, one ml from each of the three suspensions (10^9, 10^8, 10^7 CFU/ml) were plated with 8 ml of molten MHA, and 1 ml of the appropriate antibiotic suspension in MHB. Piperacillin/tazobactam preparations also
Figure 34. Schematic for Mutant Selection by Piperacillin/Tazobactam Agar Suspension

Mix 8 ml agar, 1 ml antibiotic suspension and 1 ml of bacteria suspension.

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III. Experimental Methods

included 100 µl of 400 µg/ml tazobactam suspended in PBS. Final plate concentrations of 2X, 4X and 8X the parent strain MIC for piperacillin, and 1X, 2X, and 4X the parent MIC for meropenem studies were used. All antibiotic agar plating was done in duplicate.

The solidified plates for viability counts and mutant selection were incubated for 16 to 20 hours at 37°C before reading. If pinpoint colonies were present, the plates were allowed to sit on the bench at room temperature for an additional 20 hours and colonies were recounted. Only plates that had between 30 and 300 colonies were used to calculate the putative mutant frequency.

E. DNA Template Preparation, Primer Design, and End Point PCR

PCR assays were used to amplify DNA regions for sequencing, cloning, and as a means of genetic detection in various experiments. DNA templates for these assays were usually obtained by heat lysis preparations of bacterial cells. The preparations were made from overnight cultures grown in Luria Bertani (LB) broth. Cells from 1 ml of the overnight culture were concentrated by centrifugation at 2500 × g (5300 rpm in microfuge). The supernatant was removed and the cells were resuspended in 500 µl of sterile nanopure water and held at 100°C for 10 minutes. The lysed cell debris was concentrated by centrifugation at 15,000 × g (13,000 rpm microfuge) and the supernatant containing genomic DNA was collected and stored at -20°C until needed.

Primers used in PCR assays were synthesized by IDT Technologies (Coralville, IA). A few rules were used to guide primer design. Primer size was limited to 18-24 bp, with GC content between 44% and 57%. Primer melting temperature ($T_m$) (as calculated by IDT Oligo Analyzer 3.1) was kept between 46 – 60°C (562). The 3’ ends of the
primers were typically designed to end in GC, CG, CC, or GG; and the last nucleotide on the 5´end was usually a G or C. When possible, primers were sought that did not have all G and Cs in the last 5 nucleotides on the 3´end. Long runs of any single nucleotide were avoided and limited to at most 3 consecutive like nucleotides. Using IDT Oligo Analyzer 3.1, primers with hairpins exceeding 45°C $T_m$ were avoided; also homodimers or heterodimers that formed complexes with predicted Gibbs free energy values $\leq -9 \Delta G^\circ$ were similarly avoided.

When a PCR assay was performed to confirm or detect the presence of a genetic sequence, a standard Taq polymerase (Invitrogen, Carlsbad, CA, cat # 18038-240) was employed. The reactions were set up in 50 µl reactions as follows, with final concentrations denoted by [ ]:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.25 µl</td>
<td>Sterile nanopure water</td>
</tr>
<tr>
<td>5.00 µl</td>
<td>10 x PCR buffer (200 mM Tris-HCl pH 8.0, 500 mM KCl) [1×]</td>
</tr>
<tr>
<td>1.50 µl</td>
<td>50 mM MgCl₂ [1.5 mM]</td>
</tr>
<tr>
<td>1.00 µl</td>
<td>10 mM dNTP mix [0.2 mM]</td>
</tr>
<tr>
<td>1.00 µl</td>
<td>Forward primer 100 pmol/µl [2 pmol]</td>
</tr>
<tr>
<td>1.00 µl</td>
<td>Reverse primer 100 pmol/µl [2 pmol]</td>
</tr>
<tr>
<td>0.25 µl</td>
<td>Taq polymerase 5 units/µl [.025 units/µl]</td>
</tr>
<tr>
<td>2.00 µl</td>
<td>DNA template (~500 ng/µl) [20 ng/µl]</td>
</tr>
<tr>
<td>50.00 µl</td>
<td>^{-}</td>
</tr>
</tbody>
</table>

All items except the DNA template were added to the reaction tube inside a biological safety cabinet. Reaction parameters consisted of a 5 minute denaturation at 94°C, followed by 25 cycles of: 30 seconds at 94°C; 30 seconds at $T_m -2°C$ for annealing, where $T_m$ is the estimated melt temperature of the primer with the lowest
III. Experimental Methods

estimated melt temperature; and a 2 minute extension at 72°C. Annealing time was sometimes reduced to 15 seconds if mispriming was an issue. For expected products between 500 and 1000 bp, extension time was reduced to 1 minute. For products less than 500 bp, extension was set for 30 seconds.

In PCR reactions where sequence fidelity was critical, such as amplifications for sequencing or cloning, high fidelity PCR reactions were performed using an Invitrogen Platinum® Taq DNA polymerase High Fidelity (Invitrogen cat# 11304). The reactions were set up with the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile nanopure water</td>
<td>37.75 µl</td>
<td></td>
</tr>
<tr>
<td>10 x PCR buffer (600 mM Tris-SO₄ pH 8.9, 180 mM (NH₄)₂SO₄) [1×]</td>
<td>5.00 µl</td>
<td></td>
</tr>
<tr>
<td>50 mM MgSO₄ [2 mM]</td>
<td>2.00 µl</td>
<td></td>
</tr>
<tr>
<td>10 mM dNTP mix [0.2 mM]</td>
<td>1.00 µl</td>
<td></td>
</tr>
<tr>
<td>Forward primer 100 pmol/µl [2 pmol/µl]</td>
<td>1.00 µl</td>
<td></td>
</tr>
<tr>
<td>Reverse primer 100 pmol/µl [2 pmol/µl]</td>
<td>1.00 µl</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase 5 units/µl [0.025 units/µl]</td>
<td>0.25 µl</td>
<td></td>
</tr>
<tr>
<td>DNA template (~500 ng/µl) [20 ng/µl]</td>
<td>2.00 µl</td>
<td></td>
</tr>
<tr>
<td>DNA template (~500 ng/µl) [20 ng/µl]</td>
<td>50.00 µl</td>
<td></td>
</tr>
</tbody>
</table>

Reaction parameters consisted of a 5 minute denaturation at 94°C, followed by 25 cycles of: 30 seconds at 94°C, 15 seconds at \((T_m - 2°C)\), and 2 minutes at 68°C. A final 7 minute extension at 68°C was performed after the 25 cycles to ensure that all products were extended to completion and tailed with an extra adenosine nucleotide if TA cloning was to be carried out (see Section II. H. below). Primers used in sequencing PCR reactions are listed in Table 5 below.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>GenBank Accession</th>
<th>Nucleotide Binding Location</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMY-7 PE1</td>
<td>TGGAGAAAGAGGCTGTCAG</td>
<td>JF300163</td>
<td>1190-1172</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; upstream sequencing</td>
</tr>
<tr>
<td>CUMC-50 seq 1</td>
<td>GAAGGTCATCAAGAAGGTGC</td>
<td>NC_011077</td>
<td>8150-8170</td>
<td></td>
</tr>
<tr>
<td>CMY25F1</td>
<td>CAATGTGTGAGAAGCAGTC</td>
<td>JF300163</td>
<td>945-963</td>
<td></td>
</tr>
<tr>
<td>CMY-7 PE2</td>
<td>GGGTGATGGTGCGATTAACGATATCG</td>
<td></td>
<td>1250-1225</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; sequencing</td>
</tr>
<tr>
<td>CMY-2 int-F</td>
<td>GCACCTAGCCACCTATACG</td>
<td>JF300163</td>
<td>1519-1537</td>
<td></td>
</tr>
<tr>
<td>CMY-2 int-R</td>
<td>CTGGTCATTGCCTCTTCG</td>
<td></td>
<td>1725-1708</td>
<td></td>
</tr>
<tr>
<td>Citro-AmpC-3’Flank</td>
<td>GTTAAGTGTAGATGACAGCAGGG</td>
<td>NC_011077</td>
<td>10537-10516</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; sequencing, tandem <em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; IS5 sequencing</td>
</tr>
<tr>
<td>ISEcp1-2 201-F1</td>
<td>GATATGTAATCATGAAGGTGGTGC</td>
<td></td>
<td>1555-1533</td>
<td></td>
</tr>
<tr>
<td>ISS-Int-F1</td>
<td>CATGCTACGCTTCACTGC</td>
<td>HQ680722</td>
<td>1803-1821</td>
<td>Tandem <em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; IS5 sequencing</td>
</tr>
<tr>
<td>ISS-Int-R1</td>
<td>GAACTGTCGCTTATGATGTC</td>
<td></td>
<td>2484-2465</td>
<td></td>
</tr>
<tr>
<td>ISS Int F2</td>
<td>CATCATCAAGCGACAGTTC</td>
<td></td>
<td>2466-2485</td>
<td></td>
</tr>
<tr>
<td>rob Flank F</td>
<td>CTATTACACGAGCAATTAGTTC</td>
<td>NC_000913</td>
<td>4633526 - 4633504</td>
<td><em>E. coli</em> rob sequencing</td>
</tr>
<tr>
<td>rob Flank R</td>
<td>CTGCCTAATGTCTATTTCTC</td>
<td></td>
<td>4632339 - 4632359</td>
<td></td>
</tr>
</tbody>
</table>
All reactions were carried out using a Perkin Elmer 480 thermocycler (PerkinElmer, Waltham, MA). Positive controls were used whenever feasible, and negative controls consisted of a “hood water control” that used 2 µl of sterile nanopure water from the biological safety cabinet to assure that no contamination had occurred at that location. A bench negative control consisting of the bench water used to make the DNA template, or if the DNA template was prepared from a commercial DNA preparation kit, or gel extraction kit, then the buffer from the kit that suspended the final DNA product was used. When reactions were complete, 5 µl of the PCR product was mixed with 3 µl of loading dye (66 % glycerol, 33.3 % TE, 0.6% ficoll, 0.00625 % xylene cyanol, 0.00625% bromophenol blue) (TE is Tris-EDTA; 10 mM Tris-HCl, 1 mM EDTA). The PCR product-loading dye mixture was loaded into the well of an agarose gel (Certified Molecular biology Grade, Bio-Rad, Hercules, CA, Cat # 161-3100) buffered in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). When expected products were greater than 1000 bp in size, products were run on a 1% agarose gel; if a product was expected between 1000 and 500 bp, a 1.5% agarose gel was used; if the product was less than 500 bp, then a 2% agarose gel was used. Three microliters of 1 µg/µl 100 bp, or 1 kb ladder (Invitrogen, Cat# 15628-019, and 15615-0116) was loaded into lanes flanking PCR reaction products. Products were usually electrophoresed through the gel at 60 volts for 4.5 hours, then stained for 5 seconds in 10 µg/ml ethidium bromide solution, and destained in deionized water for 30-60 minutes with gentle agitation on a rotary shaker.
F. Genome Walking

Genome walking was performed to obtain upstream sequence of the $bla_{CMY-2}$ gene in strain CUMC-50. The procedure involves restricting the DNA with a blunt cutting restriction enzyme and ligating an adapter sequence to the blunt ends. The adapter contains sequence recognized by an adapter primer, which can be used along with a primer that is specific for the known DNA region to amplify unknown sequence between the two. A second nested PCR is performed using additional primers specific for the adapter and the known sequence to assure specificity. The nested PCR product can then be sequenced to determine the DNA sequence between the adapter and the known DNA region. Primers can then be designed for the newly identified DNA sequence and additional PCRs can be performed to “walk” further into the unknown region using the adapter ligated fragment library.

For CUMC-50 genome walking a GenomeWalker™ Universal Kit (Clonetech, Mountain View, CA) was used according to manufacturer directions. Since $bla_{CMY-2}$ was located on a large plasmid, DNA template was prepared using a BacMax™ DNA Purification Kit (Epicentre® Biotechnologies, Madison, WI). Digestions of the plasmid DNA, and a kit human DNA control were carried out with each of the kit restriction enzymes (DraI, EcoRV, PvuII, and Stu I) in overnight reactions. For plasmid DNA digestions the restriction reactions consisted of the following:
### III. Experimental Methods

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Plasmid DNA (~50 ng/µl)</td>
<td>~0.5 ng/µl</td>
</tr>
<tr>
<td>8</td>
<td>Restriction enzyme (10 U/µl)</td>
<td>0.1U/µl</td>
</tr>
<tr>
<td>10</td>
<td>Kit 10X restriction buffer</td>
<td>1×</td>
</tr>
<tr>
<td>57</td>
<td>Deionized water</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The digestion was carried out for 18 hours with a brief vortexing after the first 2 hours.

The following day, 5 µl of the digestion was separated by electrophoresis through a 0.8 % agarose gel for 20 hours at 35 volts to assure plasmid band was no longer visible. DNA fragments were purified by phenol chloroform extraction. For this, a 95 µl volume of phenol was added to the remaining restriction digest reaction. The mixture was briefly vortexed and separated by centrifugation for 1 minute at 15,000 × g (13,000 rpm bench top centrifuge). The aqueous phase was placed in a new microfuge tube, mixed with 95 µl of chloroform, and again separated by centrifugation for 1 minute at 15,000 × g. The aqueous phase was transferred to a sterile 1.5 ml microfuge tube and DNA was precipitated by mixing with 190 µl of 95% ethanol, 9.5 µl of 3 M sodium acetate and 20 µg of glycogen (5 µl of a 10 µM solution). The precipitated DNA was concentrated to a pellet by centrifugation at 15,000 × g. The pellet was washed with 100 µl of 75% ethanol and centrifuged at 15,000 × g for 5 min. The ethanol was removed and the pellet was air dried for 5 minutes before being suspended in 20 µl of TE.

The adapter sequence was ligated to the restriction fragments in ligation reactions consisting of the following components:
III. Experimental Methods

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 µl</td>
<td>Digested purified DNA</td>
</tr>
<tr>
<td>1.9 µl</td>
<td>GenomeWalker™ Adapter (25 µM) [6 µM]</td>
</tr>
<tr>
<td>1.6 µl</td>
<td>10X ligation buffer [2X]</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>T4 DNA Ligase (6 units/µl) [0.375 U/µl]</td>
</tr>
<tr>
<td>8.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

The reactions were incubated at 16°C overnight then stopped by incubating at 70°C for 5 minutes, after which 72 µl of TE was added to each tube.

The PCR reactions in which the ligated fragments were used as templates also included a positive control pre-constructed adaptor linked human DNA library and negative (water) controls. Human DNA control primers were provided in the kit. Primers used for bla<sub>CMY-2</sub> genome walk PCRs are listed in Table 6. The first PCR reactions were set up as follows:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 µl</td>
<td>Sterile nanopure water</td>
</tr>
<tr>
<td>5 µl</td>
<td>Kit 10X PCR Buffer [1 ×]</td>
</tr>
<tr>
<td>1 µl</td>
<td>10 mM dNTP mix [0.2 mM]</td>
</tr>
<tr>
<td>1 µl</td>
<td>Kit Adapter Primer 1 (10 µM) [0.2 µM]</td>
</tr>
<tr>
<td>1 µl</td>
<td>Gene specific primer (10 µM) [0.2 µM]</td>
</tr>
<tr>
<td>1 µl</td>
<td>Kit 50× hot start polymerase mix (Advantage 2) [1×]</td>
</tr>
<tr>
<td>1 µl</td>
<td>Template DNA (adapter ligated restricted DNA)</td>
</tr>
<tr>
<td>50 µl</td>
<td></td>
</tr>
</tbody>
</table>

All reactions were amplified with a PerkinElmer 480 thermocycler using a two-step touchdown PCR approach with the following settings: 7 cycles of: 94°C for 25 seconds and 72°C for 3 minutes; followed by 32 cycles of: 94°C 25 sec and 67°C for 3min. All cycling was finished with a 7 minute final extension. The PCR products were
resolved by electrophoresis through a 1.5% agarose gel at 60 volts for 4.5 hours using 1 Kb and 100 bp ladders (Invitrogen).
### III. Experimental Methods

#### Table 6. Genome Walk Primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>GenBank Accession</th>
<th>Binding Location</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMY-7 PE2</td>
<td>GGGTGATGGTGCGATTAACGATATCG</td>
<td></td>
<td>1250-1225</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; GenomeWalk #1 PCR1</td>
</tr>
<tr>
<td>CMY-7 PE1</td>
<td>TGGAGAAGAGGCTGTCAG</td>
<td>JF300163</td>
<td>1190-1172</td>
<td></td>
</tr>
<tr>
<td>CUMC50 GenWalk 2A</td>
<td>CTGCAAGTTATCCACAGAGC</td>
<td>JF300163</td>
<td>864-845</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; GenomeWalk #2 PCR1</td>
</tr>
<tr>
<td>Walk3-Primer1</td>
<td>CTGGTTTCAGTACGGAGATACG</td>
<td></td>
<td>405-383</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; GenomeWalk #3 PCR1</td>
</tr>
<tr>
<td>Walk3-Primer2</td>
<td>GATGAATTCGTTGTTTGAGG</td>
<td>JF300163</td>
<td>295-274</td>
<td></td>
</tr>
<tr>
<td>Walk4 Primer 1</td>
<td>GGATATTTGATACGGGTTTCC</td>
<td>JF300163</td>
<td>36-15</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; GenomeWalk #4 PCR1</td>
</tr>
<tr>
<td>Walk4 Primer 2</td>
<td>GGTTCAGTTTCTTAGG</td>
<td>JF300163</td>
<td>21-3</td>
<td></td>
</tr>
</tbody>
</table>
Regardless of the gel results (because sometimes no product may be visible after the first PCR) the nested PCR was set up using the same reaction contents as PCR 1 except Kit Adapter Primer 2, and gene specific primer 2 were used. First PCR reaction products were diluted 1:49 with nanopure water and used as DNA templates. The nested PCR reactions were amplified with identical temperature parameters except using 5 cycles at 72°C annealing and 20 cycles at 68°C annealing. Nested PCR products were resolved by gel electrophoresis as described for the first PCR.

G. Automated DNA Sequencing and Sequence Analysis

DNA products that were used as template for sequencing were column purified to remove potentially interfering buffers, proteins, and primers. For PCR products, Microcon® YM-50 purification columns (Millipore) were used. For plasmid templates either a QIAGEN plasmid mini kit (QIAGEN, Hilden Germany), or an E.Z.N.A. Plasmid Mini Kit 1 (Omega Bio-TEK, Norcross, GA) was used. Gel purified DNA products were obtained using a QIAGEN QIAquick Gel Extraction Kit.

Automated DNA sequencing of genome walk products, and 5´RACE products was carried out at Creighton University Molecular Biology Core Facility. The IncI1 replicon region, \textit{bla\textsubscript{CMY-2}} coding region, and CUMC-201, CUMC-243 tandem \textit{bla\textsubscript{CMY-2}} sequencing were carried out at the University of Nebraska Medical Center Core Sequencing Facility. With both facilities, sequencing was performed with an ABI Prism\textsuperscript{®} 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). \textit{repZ} promoter region, and all \textit{rob} sequencing was done at ACTG Incorporated (Wheeling, IL) using an ABI Prism\textsuperscript{®} 3730 Genetic Analyzer. All facilities utilized Applied Biosystems DigDye\textsuperscript{®}}
Terminator kits and Agencourt® CleanSEQ® terminator dye removal kits (Beckman Coulter).

Electropherogram data were analyzed with FinchTV version 1.4 (Geospiza, Seattle, WA). Sequence alignments were constructed with Vector NTI® version 10.1 (Invitrogen), ClustalW2 (European Bioinformatics Institute, Cambridge, UK), and DNA Baser v2.91 (Heracle Software, Pitesti, Romania).

For the literature review, a phylogenic tree of available \( \text{bla}_{\text{CMY}} \) sequences was created using Clustal W (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/) with \( \text{bla}_{\text{CMY}} \) sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as noted on the Lahey Clinic \( \beta \)-lactamase classification website (http://www.lahey.org/Studies/).

**H. TA pCR®2.1 Cloning**

An Invitrogen TA Cloning® Kit with a pCR®2.1 vector was used to clone PCR amplification products generated for \( \text{bla}_{\text{CMY-2}} \) upstream sequence of CUMC-201 and CUMC-243, as well as the 5’RACE amplification products generated for CUMC-50 and CUMC-50M.

PCR products used during ligation reactions were amplified using Invitrogen Platinum® Taq HiFi (described in Section II. E). PCR products were purified with Millipore Microcon® YM-50 filters, and DNA concentration was measured using a GeneSpec GeneQuant™ spectrophotometer (Biochrom Ltd., Cambridge, UK). The ligation reactions consisted of a 1:1 molar ratio of PCR product to pCR®2.1 vector determined by Equation 2, with the following contents:
II. Experimental Methods

| 5 µl | Sterile water |
| 1 µl | Purified PCR product (at concentration determined by Equation 2) |
| 1 µl | 10X Ligation Buffer 1 µl [1×] |
| 2 µl | pCR®2.1 vector (25 ng/µl) [5 ng/µl] |
| 1 µl | T4 DNA Ligase (4.0 U/µl) [0.4 U/ng] |
| 10 µl | |

The ligation reactions were incubated overnight in a 14°C water bath, after which 2 µl of each reaction was added directly into a vial of competent One Shot® TOP10 E. coli cells that were thawed on ice. The cells were incubated with the ligation products for 30 minutes on ice, then heat shocked for 30 seconds at 42°C without shaking and transferred back to ice for 5 minutes. Each vial had 250 µl of room temperature S.O.C. (super optimal broth with catabolite repression) medium added and was shaken at 120 rpm for 1 hour at 37°C. After this incubation, 90 µl of each S.O.C. cell suspension was spread on to an LB agar plate containing 50 µg/ml kanamycin (LB-Kan50) and incubated for 18-20 hours at 37°C. Single transformant colonies from the LB-Kan50 plate were lifted with a straight wire needle and plated on to a fresh LB-Kan50 plate, and inoculated into 5 ml of LB broth with 50 µg/ml kanamycin. The plasmid vectors within the cultured transformants were then isolated and evaluated for proper ligation and transformation of the desired DNA sequence using the small plasmid alkaline lysis protocol described in Section I. below.
II. Experimental Methods

**Equation 2.** Determination of Insert Quantity Required for pCR®2.1 TA Cloning Ligation Reaction

\[
\text{ng of DNA insert to add} = \frac{(\text{bp length of insert}) \times (50 \text{ ng})}{3900 \text{ bp}}
\]

50 ng is the weight of the pCR 2.1 vector being added to the reaction, and 3900 bp is its length. The equation seeks to achieve a roughly equal molar ratio of product to vector.

I. Small Plasmid Alkaline Lysis Isolation

All cloning efforts with pCR® 2.1 and pET100 vectors utilized a small plasmid alkaline lysis protocol to isolate transformed plasmids. For this procedure, isolated transformant strains were then grown overnight at 37°C shaking at 120 rpm in 5 ml of LB broth containing selective antibiotic (50 µg/ml kanamycin for pCR®2.1, and 50 µg/ml ampicillin for pET100). The following day, 1.5 ml of overnight culture was transferred into a microfuge tube and cells were concentrated by centrifugation at 5,000 × g for 5 minutes. The supernatant was removed and the cells were suspended in 100 µl of GTE (50 mM glucose, 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). A 200 µl volume of freshly prepared lysis solution (1% SDS, 0.2 M NaOH) was added and mixed by inversion. Within 5 minutes of adding the lysis solution, 150 µl of 5 M potassium acetate was added to the tube to precipitate protein and chromosomal DNA, and renature the small plasmids. The precipitation was allowed to proceed on ice for 10 minutes before the precipitate was concentrated by centrifugation at 15,000 × g for 10 minutes.
II. Experimental Methods

Approximately 400 µl of supernatant containing the soluble plasmids was transferred to a new microfuge tube. Residual protein was removed from the supernatant by adding an equal volume (~400 µl) of phenol-chloroform-isoamyl alcohol (25:24:1) and gently rocking tubes by hand for 30 seconds. The denatured protein was separated by centrifugation at 15,000 × g for 10 minutes and 360 µl of the upper aqueous phase containing the plasmids was placed into a new microfuge tube. The plasmid DNA was precipitated by adding 1.5 volumes (520 µl) of cold 95% ethanol and mixing by inversion. The DNA was allowed to precipitate at room temperature for 10 minutes (or 4°C refrigerator for 1 hour, or -20°C overnight) before being concentrated by centrifugation at 15,000 × g for 10 minutes. The ethanol was removed and the remaining pellet was washed with 500 µl of 70% ethanol and concentrated by centrifugation at 15,000 × g for 5 minutes. The ethanol wash was removed by pipetting, and the DNA was air dried for 10 minutes before being resuspended in 50 µl of TE solution (10 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0) containing 40 ng/µl RNase A and incubated at 37°C for 30 minutes. To visualize plasmids, 3 µl of the extracted plasmid suspension was mixed with loading dye and loaded into a 0.8% agarose gel and resolved by electrophoresis for 20 hours at 35 volts, and stained with ethidium bromide (described in Section II. E.).
J. Modified TRIzol® RNA Isolation

A modified TRIzol® method was used to obtain bacterial mRNA for expression studies. All isolations were performed using dedicated RNA bench space, equipment, and RNase free containers and tips. All centrifugation steps were performed in centrifuges refrigerated at 4°C. RNA was collected from cells in log phase at an OD_{600} of 0.5 grown as described for mutation studies (Section II. D.) with the exception that cells were grown in an Erlenmeyer flask containing 95 ml of MHB. Cultured cells were concentrated by centrifugation for 3 minutes at 15,000 × g and the cell pellet was resuspended in 800 µl of TRIzol® reagent (Invitrogen) and 200 µl of 3 M sodium acetate lysis buffer (0.5 M CH₃COONa, 5% SDS, 250 mM disodium EDTA). Cells were lysed by heating at 94°C for 5 minutes. Each sample was allowed to cool for 5 minutes before adding 200 µl of chloroform. The tubes were inverted six times and the RNA was separated from DNA and cellular proteins by centrifugation at 6000 × g. A 400 µl volume of the supernatant was removed by drawing from the top of the aqueous phase and placed in a new 1.5 ml tube. The RNA within the aqueous phase was precipitated by adding 400 µl of ice cold isopropanol, mixing by inversion, then holding at room temperature for 10 minutes before concentrating by centrifugation at 6000 × g for 10 minutes. The concentrated RNA pellet was washed with 1 ml of 70 % ethanol and the RNA pellet was collected by centrifugation at 6000 × g for 5 minutes. The ethanol was pipetted off, and the RNA pellet was allowed to air-dry for 5 minutes before being resuspended in 50 µl of 1X TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). RNA was quantified by spectrophotometry using a GeneQuant™ spectrophotometer, and 8 µg of total RNA was DNase treated for 2 hours at 37°C using 4 units of RQ1 DNase (Promega, Madison, WI), 4 µl of 10X RQ1
buffer, and RNase free water to a final reaction volume of 40 μl. At the end of the incubation 4 μl of RQ1 Stop Solution was added. The DNase was removed by first suspending the reaction in 156 μl of TE and mixing with an equal volume (200 μl) of phenol-chloroform-isoamyl alcohol (25:24:1). The mixture was separated by centrifugation at 15,000 × g for 15 minutes and 160 μl of the aqueous phase containing the RNA was collected and transferred to a sterile 1.5 ml tube. Twenty microliters of 3 M sodium acetate pH 5.2, and 500 μl of 100% ethanol was added to the collected aqueous phase and mixed by inversion. The suspension was placed at -80 °C for at least 12 hours to allow the RNA to precipitate. The next day the RNA was concentrated by centrifugation at 15,000 × g for 10 minutes. The supernatant was removed and the RNA pellet was washed with 500 μl of 70% ethanol, and the pellet was collected by centrifugation at 15,000 × g for 5 minutes. The ethanol was then pipetted off and the RNA pellet was air-dried for 5 minutes before being resuspended in 50 μl of TE and quantified by spectrophotometry.

K. Real-Time Reverse Transcriptase PCR

Real-time reverse transcriptase (RT) PCR reactions were performed to determine relative transcript levels of bla\text{CMY-2}, E. coli bla\text{ampC}, and IncI1 plasmid repZ. All real-time RT-PCR reactions were set up in MicroAmp® reaction tubes (Applied Biosystems) in 50 μl reactions consisting of 25 μl of Quantitect® SYBR® Green RT-PCR Mastermix (QIAGEN), 0.5 μl of Quantitect® RT mix (QIAGEN), 250 ng of RNA suspended in 19.5 μl volume of RNase free water, and 25 pmol of each forward and reverse primer. 23S rRNA was amplified as an endogenous control for all RT-PCR reactions. All reactions
were run on an ABI Prism® 7000 Sequence Detection System with amplification parameters consisting of a 40 minute hold at 50°C for the reverse transcription reaction, followed by a 15 minute hold at 95°C to deactivate the reverse transcriptase, and activate hot-start Taq polymerase. This was followed by 40 cycles of: 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. All reactions were analyzed with ABI Prism® 7000 SDS Software. Relative quantity was calculated by using the $2^{-\Delta\Delta C_T}$ method (see Equation 3) (563). Primers used for real-time RT-PCR transcript level studies and for RT-PCR gene copy number levels (Section II. L.) are listed in Table 7.

Equation 3. $2^{-\Delta\Delta C_T}$ Equations for Relative Transcript and Gene Copy Number

\[
\Delta C_T = C_T \text{(target gene)} - C_T \text{(endogenous control)}
\]

\[
\Delta\Delta C_T = \Delta C_T - \Delta C_T \text{(calibrator strain)}
\]

Where $C_T$ is cycle threshold and is manually set at a level where all reactions being compared in the assay have clearly entered logarithmic amplification.

L. Relative Gene Copy Number Determination by Real-Time PCR

Relative gene copy number of $bla_{CMY-2}$ and the IncI1 plasmid replicon region were determined by real-time PCR assays. Bacterial cells used in DNA template preparation were obtained from 3 ml of log phase MHB culture grown to an OD$_{600}$ of 0.5 as described for modified TRIzol® RNA Isolation (Section II. J.). DNA was isolated using a QIAGEN DNeasy® Blood and Tissue Kit according to manufacturer instructions. The proteinase K digestion step with this kit was carried out for 20 minutes in a 55°C
water bath, and the optional RNase treatment was employed by adding 4 μl of a 100 mg/ml RNase A to the lysis suspension and incubating for 5 minutes at room temperature. Real-time PCR reactions were performed and analyzed as described for RNA expression assays, except 250 ng of genomic DNA was used as template, and the reverse transcriptase reaction was omitted from the amplification reaction. The single copy chromosomal gene ampD was used as the endogenous control using primers E.coliampD-F3 and E.coliampD-R3 (Table 7). bla\textsubscript{CMY-2} copy number assays used primers CMY-2 RT-F and CMY-2 RT-R, IncI1 copy number assays used primers IncI1-F and IncI1-R.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>GenBank Accession</th>
<th>Binding Location</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMY-2 RT F</td>
<td>CGTTAATCGACCACATCACC</td>
<td>JF300163</td>
<td>1231-1249</td>
<td>Real-time PCR for bla&lt;sub&gt;CMY-2&lt;/sub&gt; gene copy number and transcript level. Probe synthesis for Southern hybridization</td>
</tr>
<tr>
<td>CMY-2 RT R</td>
<td>CGTCTTACTAACCAGATCCTAGC</td>
<td></td>
<td>1402-1381</td>
<td>E. coli Real-time RT-PCR endogenous control</td>
</tr>
<tr>
<td>Frr F1</td>
<td>TGATCGTTCAATGTCTCCG</td>
<td>NC_000913</td>
<td>193081-193099</td>
<td>E. coli Real-time RT-PCR endogenous control</td>
</tr>
<tr>
<td>Frr R1</td>
<td>GCTTGTTCTGCTTACCAC</td>
<td></td>
<td>193242-193224</td>
<td>E. coli Real-time RT-PCR endogenous control</td>
</tr>
<tr>
<td>23S rRNA F</td>
<td>CCCGATCACACAAAATGC</td>
<td>NC_000913</td>
<td>226092-226109</td>
<td>Real-time RT-PCR 23S rRNA endogenous control</td>
</tr>
<tr>
<td>23S rRNA R</td>
<td>GACGATAGTGGACGATCCAC</td>
<td></td>
<td>226200-226221</td>
<td>Real-time RT-PCR 23S rRNA endogenous control</td>
</tr>
<tr>
<td>InclI-F</td>
<td>CTGTTATTAGCTGTGAGAGG</td>
<td>NC_011077</td>
<td>153-173</td>
<td>InclI identification and real-time plasmid copy number assays</td>
</tr>
<tr>
<td>InclI-R</td>
<td>CTCTGACTTACTGCGGATAC</td>
<td></td>
<td>268-248</td>
<td>InclI identification and real-time plasmid copy number assays</td>
</tr>
<tr>
<td>InclI Repz F1</td>
<td>CATATAACCGGATACACTTG</td>
<td>NC_011077</td>
<td>476-496</td>
<td>Real-time RT PCR of repZ transcription</td>
</tr>
<tr>
<td>InclI Repz R1</td>
<td>CATTTGGTTTTGGGTGAGTG</td>
<td></td>
<td>630-610</td>
<td>Real-time RT PCR of repZ transcription</td>
</tr>
<tr>
<td>E. coli AmpD F3 RT</td>
<td>CCTCAACCATACGATGTC</td>
<td>NC_000913</td>
<td>119776-118795</td>
<td>Real-time gene copy number, single copy gene endogenous control</td>
</tr>
<tr>
<td>E. coli AmpD R3 RT</td>
<td>CCTGCAGTCAATAGTTCC</td>
<td></td>
<td>118910-118892</td>
<td>Real-time gene copy number, single copy gene endogenous control</td>
</tr>
<tr>
<td>E. coli AmpC-F</td>
<td>GAAATCCTCAAGCGACTTG</td>
<td>NC_000913</td>
<td>4376545-4376526</td>
<td>Real-time RT-PCR of E. coli chromosomal bla&lt;sub&gt;ampC&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli AmpC-R</td>
<td>GGCACGACGAACTGAGTAC</td>
<td></td>
<td>4376441-4376461</td>
<td>Real-time RT-PCR of E. coli chromosomal bla&lt;sub&gt;ampC&lt;/sub&gt;</td>
</tr>
</tbody>
</table>
M. 5′ RACE

5′ Rapid Amplification of cDNA Ends (5′RACE) was performed to determine the
bla<sub>CMY-2</sub> transcriptional start sites in <i>E. coli</i> strain CUMC-50 and its mutant strain
CUMC-50M using a 5′RACE kit from Invitrogen. The basics protocol for using this kit
is written below. Two separate 5′RACE amplifications were performed to determine the
two start sites for <i>bla<sub>CMY-2</sub></i> using primers listed in Table 8.

M. 1. Obtaining RNA for 5′ RACE:

RNA was obtained using the modified TRIzol<sup>®</sup> method described in Section II. J. However RNA yields from that protocol are at concentrations too low for 5′RACE. The
protocol was modified to resuspend the final RNA pellet in one half the volume of TE
(i.e. 25 µl), or an additional precipitation step was employed and RNA was resuspended
in a volume of TE that would yield the desired concentration. For the additional
precipitation of the RNA, a 1:10 volume of 3 M sodium acetate was added to the RNA
suspension to obtain a 300 mM sodium acetate concentration, and 2.5 volumes of 100 %
ethanol (125 µl) were added. The RNA was allowed to precipitate at 4°C for 1 hour, then
concentrated by centrifugation for 15 minutes at 15,000 × g. The RNA pellet was washed
once with 200 µl of 70% ethanol and resuspended in TE at a volume necessary to achieve
approximately 500 ng/µl.
II. Experimental Methods

M. 2. 5’RACE cDNA Synthesis

For each strain tested, cDNA was synthesized for 5´RACE in the following manner. The components were mixed in a 500 µl PCR reaction tube denoted TUBE #1 under a biological safety cabinet and on ice:

- 2 µl   Gene specific primer #1 (10 pmol/µl) [0.80]
- ## µl   Sterile nanopure H2O (enough to a final volume of 15.5 µl after addition of RNA suspension).

The following components were also mixed in a 500 µl PCR reaction tube denoted TUBE #2:

- 2.5 µl  10X SuperScript™ II reaction Buffer [1X]
- 2.5 µl  25 mM MgCl2 [2.5 mM]
- 1 µl    10 mM dNTP [0.4 mM]
- 2.5 µl  0.1 M DTT [0.01]

On the bench 2 µg of total RNA was added to TUBE #1 and the tube was heated in a thermocycler block for 10 minutes at 70°C to relax RNA, followed by emersion in ice for 1 minute. Condensation at the top of the tube was drawn down by brief centrifugation. The contents of TUBE #2 were then added to TUBE #1, and TUBE #1 was placed in a thermocycler block for 1 minute at 42°C. One microliter of Invitrogen SuperScript™ II Reverse Transcriptase was added to the tube and mixed in by stirring with pipette tip and gently flicking the tube. Identical reactions were set up for each strain without reverse transcriptase to serve as controls in subsequent PCRs for determining if residual DNA was contaminating the RNA. For these controls 1 ml of water was added. The reaction was then allowed to incubate at 42°C in a thermocycler block for 50 minutes.
II. Experimental Methods

to complete the reverse transcriptase reaction. The reaction was stopped with 15 minutes incubation at 70°C. Condensation was drawn down by a brief centrifugation. On a bench (away from other RNA work) and working on ice, 1 µl of kit RNase Mix was added to TUBE#1 and mixed thoroughly by stirring with a pipette tip and gently flicking the tube. The tube was incubated for 30 minutes at 37°C in a thermocycler block. At the end of the incubation condensation was pulled down with brief centrifugation.

The contents of TUBE #2 were then added to TUBE #1, and TUBE #1 was placed in a thermocycler block for 1 minute at 42°C. One microliter of Invitrogen SuperScript™ II Reverse Transcriptase was added to the tube and mixed in by stirring with pipette tip and gently flicking the tube. Identical reactions were set up for each strain without reverse transcriptase to serve as controls in subsequent PCRs for determining if residual DNA was contaminating the RNA. For these controls 1 ml of water was added. The reaction was then allowed to incubate at 42°C in a thermocycler block for 50 minutes to complete the reverse transcriptase reaction. The reaction was stopped with 15 minutes incubation at 70°C. Condensation was drawn down by a brief centrifugation. On a bench (away from other RNA work) and working on ice, 1 µl of kit RNase Mix was added to TUBE#1 and mixed thoroughly by stirring with a pipette tip and gently flicking the tube. The tube was incubated for 30 minutes at 37°C in a thermocycler block. At the end of the incubation condensation was pulled down with brief centrifugation.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>GenBank Accession</th>
<th>Binding Location</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' GSP 1</td>
<td>CGCTGAGCTTAATTTTCGC</td>
<td></td>
<td>1457-1440</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; 5'RACE 1</td>
</tr>
<tr>
<td>5' GSP 2</td>
<td>CGTCTTACTAACCCTGCTCCTAGC</td>
<td>JF300163</td>
<td>1402-1381</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; 5'RACE 1</td>
</tr>
<tr>
<td>5' GSP 3</td>
<td>CAGCAATGTGGAGAAAGGAGGC</td>
<td></td>
<td>1199-1178</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; 5'RACE 1</td>
</tr>
<tr>
<td>R2 GSP 1</td>
<td>CTGCAAGTTATCCACAGAAGC</td>
<td></td>
<td>864-845</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; 5'RACE 2</td>
</tr>
<tr>
<td>R2 GSP 2</td>
<td>GCTGTCTGTATTCTGAAGAGTCC</td>
<td>JF300163</td>
<td>667-685</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; 5'RACE 2</td>
</tr>
<tr>
<td>R2 GSP 3</td>
<td>GAAACACGGCTTCATTCGC</td>
<td></td>
<td>612-594</td>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt; 5'RACE 2</td>
</tr>
</tbody>
</table>
M. 3. 5′RACE S.N.A.P. Column Purification

The products of the cDNA synthesis reaction were purified using Invitrogen S.N.A.P. purification columns (cat# 45-0078), with S.N.A.P 40X Wash buffer (cat# 1334717), and S.N.A.P. Binding Solution (cat# Y01320). Prior to beginning the S.N.A.P. purification, the 40X Wash Buffer was diluted to 1X with ethanol (to 52.5% volume) and sterile distilled water (to 45 % volume) and kept on ice. A bottle of 70% ethanol was also kept on ice. The kit Binding Buffer was allowed to warm to room temperature, and a tube of sterile nanopure water was warmed to 65°C. All cDNA reaction samples had 120 µl of Binding Solution added to them before being transferred into a S.N.A.P. column. The non-binding components were passed through the column by centrifugation for 20 seconds at 13,000 × g (flow through from this step was retained and frozen at -20°C as a safety step in case sample cDNA did not bind the column). A 400 µl volume of cold 1X Wash Buffer was then added to the column, and passed through by centrifugation at 13,000 × g for 20 seconds. Flow through was discarded, and the wash was repeated three more times. A 400 µl volume of cold 70% ethanol was then added to the column and passed through by centrifugation at 13,000 × g for 20 seconds. The flow through was discarded into ethanol waste and the ethanol wash was repeated three more times. After completion of the washes, the remaining ethanol in the column was removed from the column by an additional centrifugation at 13,000 × g for 1 minute. The spin column was placed in a sterile 1.5 ml microfuge tube and 50 µl of 65°C nanopure water was added to the column membrane and passed through by centrifugation at 13,000 × g for 20 seconds eluting the cDNA.
II. Experimental Methods

M. 4. 5´ RACE TdT Tailing

A tail of cytosines was added to the 3´ends of the S.N.A.P. purified cDNA product. This allowed for binding of the kit linker primer and also allowed for the 5´ end of the transcript (transcriptional start point) to be identified when sequencing. The tail was added by a kit provided terminal deoxynucleotidyl transferase (TdT). A minus-TdT control reaction was set up to verify that amplifications in PCRs #1 and #2 were not due to mispriming at another location in the cDNA. For each sample, the TdT tailing reaction was set up under a biological safety hood with the following components in a 500 µl PCR reaction tube on ice:

- 6.5 µl Sterile nanopure water
- 5.0 µl 5X Kit tailing buffer [1X]
- 2.5 µl 2mM dCTP [0.2 mM]

On a bench top, 10 µl of S.N.A.P. column purified cDNA was added to each of the tubes and the reactions were incubated for 3 minutes at 94°C on a thermocycler block then chilled on ice for 1 minute. Condensation was drawn down by brief centrifugation, then 1 µl of kit TdT suspension (15 units/µl, final concentration 0.6 U/ µl) was added to the tube for a final reaction volume of 25 µl. The tubes were mixed gently and the reactions were incubated at 37°C in a thermocycler block for 10 minutes, then heated at 65°C for 10 minutes to inactivate the TdT before returning the tubes to ice.
M.  5′RACE PCR of dC-tailed cDNA

The PCR of dC-tailed cDNA (PCR #1) was designed to amplify the product of the cDNA reaction by using a linker primer that binds the 3′ cytosine tailed end of the cDNA product, and using a primer denoted GSP2 that is nested to GSP1 (3′ to GSP1 on the cDNA). All primers were diluted to a 10 pmol/µl concentration. Minus-RT and minus-TdT controls as well as the kit cDNA positive controls were employed. The PCR reactions were set up with the following components in a 50 µl reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Water</td>
<td>31.5 µl</td>
<td></td>
</tr>
<tr>
<td>10 x PCR Buffer [1X]</td>
<td>5.0 µl</td>
<td></td>
</tr>
<tr>
<td>25 mM MgCl [1.5 mM]</td>
<td>3.0 µl</td>
<td></td>
</tr>
<tr>
<td>10 mM dNTP mix [0.2 mM]</td>
<td>1.0 µl</td>
<td></td>
</tr>
<tr>
<td>GSP2 primer 10pmol/µl [0.4 pmol]</td>
<td>2.0 µl</td>
<td></td>
</tr>
<tr>
<td>Abridged Anchor Primer (AAP) 10 µM [0.4 µM]</td>
<td>2.0 µl</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase Invitrogen (Cat# 18038-042) [0.05 U/µl]</td>
<td>0.5 µl</td>
<td></td>
</tr>
<tr>
<td>Poly dC tailed cDNA</td>
<td>5.0 µl</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

The thermocycler block was preheated to 94°C prior to inserting the tubes. Initial denaturation was carried out at 94°C for 2 minutes, and PCR was carried out for 25 cycles under the following conditions: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes 72°C; and a final extension for 7 minutes. Reaction products were separated by electrophoresis for 4 hours at 60 volts through a 2% agarose gel and visualized with ethidium bromide and UV light as described in Section II. E.
M. 6. 5’RACE Nested PCR Amplification

The nested PCR (PCR #2) is designed to amplify products that may have not otherwise been visible in PCR #1. It also verifies the specificity of the first PCR reaction. The PCR #2 reactions were set up with the same contents as PCR #1 (Section II. M. 5.) except the water volume was increased by 2 µl, the primers used were 1 µl of GSP3 and 1 µl of Abridged Universal Anchor Primer (AUAP), and the DNA template consisted of 5 µl of 1:100 diluted PCR #1 product. Thermocycler settings were the same as PCR #1 and products were evaluated the same way with a 2% agarose gel. 5’RACE products were either cloned (see Section II. H.) or gel purified (see Section II. N., below) then submitted for sequencing.

N. Gel Purification of PCR Amplicons.

Gel purification of PCR amplicons was performed using a QIAGEN QIAquick® gel extraction kit according to manufacturer instructions. In all cases before and after photographs of the gel excision were taken to determine that the correct band had been removed.

O. Large Plasmid Isolation

The large plasmid isolation protocol used in this study was based on a method published by Sinnett and Montpetite, but adopted for minipreps in the protocol below (564). A key difference in this procedure from the small plasmid isolation protocol (Section II. I.) is the use of sodium lauroyl sarcosine which allows for the reannealing of
The procedure started with either 5 ml of log phase MHB culture at OD$_{600}$ of 0.5 grown as described for RNA isolation (Section II. J.), or in cases where growth phase was not an issue, 1.5 ml of a fresh 12-16 hour culture from LB or MHB broth was used. For log phase cultures 5 ml aliquots were placed in a 17 x 100 mm polypropylene tube (Fisher, cat # 05-540-6) and centrifuged under refrigeration at 5,000 × g (7995 rpm in SS-34 Rotor) for 8 minutes. For 12-16 hour overnight cultures, 1.5 ml aliquots culture were concentrated by centrifugation at 5000 × g (7500 rpm bench top microfuge) for 5 minutes. The remaining pellet was resuspended completely in 200 µl of Solution I (0.4 µg/µl RNase A, 2.5 mg/ml of lysozyme, in 1X GTE). Often a combination of stirring and vortexing was used to resuspend the cells. For 5 ml samples the suspension was then transferred to a 1.5 ml microfuge tube and kept on ice. A 400 µl volume of Solution 2 (0.2 M NaOH, 1% sodium lauroyl sarcosine) was added to the suspension and mixed gently by inversion 2-3 times. A 300 µl volume of Solution 3 (180 µl of 5 M potassium acetate pH 4.8, with 34.5 µl of 17.4 N glacial acetic acid per 300 µl of prep) was then added within 5 minutes of adding Solution 2, and the suspension was gently inverted 6 times and kept on ice for 15 minutes to allow the plasmids to reanneal and for chromosomal DNA and proteins to precipitate out. The precipitate was then concentrated by centrifugation at 15,000 × g (13,000 rpm in microfuge) for 15 minutes. The supernatant was often cloudy and the precipitate gelatinous, but efforts were made to obtain 900 µl of supernatant (or at least 600 µl) and place it into a new 1.5 ml microfuge tube. A 0.6 volume (540 µl) of cold isopropanol was added to the recovered supernatant.
and inverted slowly 6-10 times. DNA was allowed to precipitate at room temperature for 10 minutes. The DNA was concentrated by centrifugation for 15 minutes at 10,000 × g (10,500 rpm in microfuge). The pellet was washed with 600 µl of 70% ethanol and re-concentrated by centrifugation at 10,000 × g (10,500 rpm in microfuge) for 5 minutes and the remaining ethanol was removed by pipetting. Two hundred fifty microliters of the TE-SLS-Proteinase K solution (1% SLS; 0.1 mg/ml proteinase K, in 1X TE with 20 mM EDTA pH 8.0) was added to the pellet taking care to wash the sides of the tube for plasmid DNA that may have adhered to the side. The suspension was incubated for 60 minutes at 55°C in a heat block. During the incubation the suspension was mixed by gentle rocking or tapping the tube every 10-15 minutes. In many instances the pellet did not completely dissolve but this did not affect the recovery of plasmids. A 250 µl volume of 25:24:1 phenol-chloroform-isoamyl alcohol was added to the suspension and mixed by inversion for 30 seconds, and separated by centrifugation for 15 minutes at 15,000 × g. A 200 µl volume of the aqueous supernatant was collected and put it into a new microfuge tube. DNA in the supernatant was precipitated with the addition of two volumes (400 µl) of 95% ethanol. The suspension was inverted 10 times and allowed to stand at room temperature for 10 minutes. The precipitated DNA was concentrated by centrifugation for 10 minutes at 15,000 × g and washed with 600 µl of 70% ethanol and the pellet was recollected by centrifugation for 5 minutes at 15,000 × g. The ethanol was removed by careful pipetting to remove as much ethanol as possible and the pellet was allowed to air dry for 5-10 minutes. If Plasmid-Safe™ DNase (Epicentre® Biotechnologies) treatment was to be used, 40 µl of the Plasmid Safe™ DNase mix prepared according to manufacturer instructions was added at this point washing the sides of the tube to suspend
all available DNA. The suspension was incubated for 1 hour at 37°C, after which 20 µl of TE (10 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0) was added. If Plasmid-Safe™ DNase was not being used; the pellet was simply suspended in 60 µl of TE. To visualize the plasmids, 20 µl of the suspension was mixed with 5 µl of loading dye and loaded into a 0.5% agarose gel in 1X TAE buffer within an Owl B3 12 × 14 cm mini gel system with built-in recirculation (Thermo Fisher). Note CUMC-214 family isolates required 50 µM thiourea to be in the buffer to eliminate smearing. Plasmids were separated by electrophoresis for 12 hours at 90 volts (or 18 hours 60 volts) at 4°C. The gel was stained in 0.25X – 0.5X SYBR® Gold Nucleic Acid Gel Stain for 1 hour with gentle agitation on a rotary shaker and photographed under UV light using a 535 nm lens filter.

P. Southern Hybridization

Southern hybridization was used to determine the location of $\text{bla}_{\text{CMY-2}}$ in large plasmid preparations, pulsed-field gel electrophoresis (PFGE) studies, and restriction fragment length polymorphism (RFLP) studies. Methods used in this study were based on those described by Sambrook and Russell (565).

P. 1. Southern Hybridization - Transferring DNA to the Membrane

DNA targeted for Southern blotting was separated by PFGE or unidirectional electrophoresis (see Sections II. O. and II. Q. for methods, gel running conditions). Prior to blotting, a photograph of the gel was taken next to a clear plastic UV transparent metric ruler so that blot locations on the membrane could later be compared to DNA bands in the gel photograph. A Roche DNA Molecular Weight Marker VII, DIG-labeled
Ladder (cat# 11669940910) was attempted for some restriction fragment blots but the DIG-label could not withstand the depurination and denaturation methods used in this protocol.

After capturing a pre-blot image, the DNA in the gel was cross-linked by exposure to UV light for 15 minutes on a UV transilluminator. Depurination was carried out by placing the gel in approximately 200 ml of Depurination Solution (0.25 M HCl) for 40 minutes with gentle shaking on a rotary shaker. The solution was exchanged once after 20 twenty minutes. Denaturation was carried out by soaking the gel in Denaturation Solution (1.5 M NaCl, 0.5 M NaOH) for 60 minutes with gentle shaking on a rotary shaker. The Denaturation Solution was exchanged once after 30 minutes. Following denaturation the gel was soaked in Neutralization Solution (1 M NaCl, 0.5 M Tris-HCl, balanced to pH 7.2) for 60 minutes with gentle agitation on a rotary shaker. The solution was exchanged once after 30 minutes.

A Bio-Rad 785 Vacuum Blotter was used to transfer the DNA to Ambion Bright Star® Plus Positively Charged Nylon Membrane (Applied Biosystems). A section of membrane was cut to gel size and pre-soaked for 30 minutes in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). A 35 × 25.5 cm piece of Whatman paper (Fisher) was placed on the vacuum blotter bed, moistened with 2X SSC, and flattened with vacuum pressure. The membrane was then laid on the Whatman paper and overlayed on the edges with a vacuum gasket (Figure 35). The gel was then laid over the gasket/membrane setup with edges of the gel overlaying the gasket to create a vacuum seal. The gel surface was then flooded with 2X SSC and 5-10 inches Hg of vacuum pressure was pulled for 40 minutes for restriction fragment gels, and 60 minutes for plasmid and PFGE gels. The gel
was checked every 10 to 15 minutes to insure that the gel surface remained flooded with 2X SSC. When transfer was complete, the positions of the wells were marked on the membrane with a pencil. This assisted with the determination of gel orientation and lane location. After disassembly of the gel/membrane/gasket assembly, the gel was briefly stained with ethidium bromide as described in Section II. E., and photographed to ensure that no DNA remained in the gel.

The membrane was dried between 4 pieces of Whatman paper (two on each side) compressed between the bottoms of two glass Pyrex® dishes (Figure 35) in a 68°C oven for 1-2 hours.

**P. 2. Southern Hybridization – Probe Synthesis**

A 172 bp DIG-dUTP labeled \( \text{bla}_{\text{CMY-2}} \) specific probe was synthesized with a Roche PCR DIG Probe Synthesis Kit (Cat. # 11 636 090 910) using primers CMY-2 RT-F and CMY-2 RT-R which were the same primers used to amplify \( \text{bla}_{\text{CMY-2}} \) in gene copy number and RT-PCR assays. The probe was synthesized in a PCR reaction containing the following:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.25 µl</td>
<td>Water</td>
</tr>
<tr>
<td>5.00 µl</td>
<td>Roche 10X PCR Buffer with 15 mM MgCl₂ [1X, 0.03 mM MgCl₂]</td>
</tr>
<tr>
<td>5.00 µl</td>
<td>2 mM dNTP Mix [0.2 mM each dNTP except dTTP 0.13 mM and dUTP-dig 0.07 mM]</td>
</tr>
<tr>
<td>1.00 µl</td>
<td>100 pmol/µl Forward Primer (CMY-2 RT-F) [2 pmol]</td>
</tr>
<tr>
<td>1.00 µl</td>
<td>100 pmol/µl Reverse Primer (CMY-2 RT R) [2 pmol]</td>
</tr>
<tr>
<td>0.75 µl</td>
<td>Roche Hi Fidelity Enzyme Mix (3.5 U/µl) [0.0525 U/µl]</td>
</tr>
<tr>
<td>50.00 µl</td>
<td></td>
</tr>
</tbody>
</table>
II. Experimental Methods

The thermocycling parameters consisted of 5 minutes of 94°C initial denaturation, followed by 25 cycles of: 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds. Two dNTP mixes were used in these reactions. A control mix using deoxynucleotidyl phosphates, dATP, dCTP, dGTP, dTTP at 2 mM concentration; and a synthesis reaction mix with dATP, dCTP, dGTP at the same 2 mM concentration but with 1.3 mM dTTP and 0.7 mM DIG-11-dUTP at 1.3 mM. The control and probe synthesis products were evaluated by electrophoresis through a 2% agarose gel. Incorporation of the DIG-UTP nucleotides add an apparent 11% increase in migration size to the PCR product that could be visualized in a 2% gel. The probe synthesis PCR reaction was purified with Millipore Microcon® 50K spin column. The purified probe was then quantified using a GeneQuant™ spectrophotometer and diluted to 100 ng/µl.

P. 3. Southern Hybridization – Probing, Stringency Washing, and Staining:

The Southern blot hybridization protocol below was designed for the Roche DIG Nucleic Acid Detection Kit (Cat # 11175041910) and solution volumes relate to those used for a 12 × 14 cm membrane. The optimum probe hybridization temperature (T_{opt}) was determined using Equation 4. T_{opt} for the bla_{CMY-2} specific probe was 42.7°C. Nylon membranes from gels 12 cm or less were probed in a Fisher Biotech FBH10 Hybridization oven. For larger PFGE gels, hybridization was carried out in an appropriately sized Pyrex® dish placed into a Lab-Line® Shaker Bath set at the T_{opt} hybridization temperature. Membranes were immersed (prehybridized) in of Roche DIG Easy Hyb Buffer at a volume of 10 ml per 100 cm² of membrane area (16.8 ml for a 12 x
14 cm membrane) at $T_{opt}$ for 30 minutes with gentle agitation, or rolling in the hybridization oven. While membrane was prehybridizing, an aliquot of probe was denatured by boiling for 5 minutes then rapidly immersed in ice water. The denatured probe was suspended in Roche DIG Easy Hyb Buffer to a final concentration of 25 ng/ml concentration in a volume of 7 ml per 100 cm$^2$ of membrane area (this equated to 3 µl of a 100 ng/µl probe in 11.76 ml of DIG Easy Hyb buffer for a 12 × 14 cm membrane). When prehybridization was complete, the prehybridization buffer was drained from the membrane, and the Easy Hyb Buffer containing the suspended probe was applied to the membrane and incubated at $T_{opt}$ with gentle agitation for 12 to 24 hours. When probe incubation was complete, the probe-Easy Hyb Buffer solution was poured off and the membrane was washed with 150 ml of room temperature 2X SSC 0.1% SDS two times for at least 5 minutes each time with gentle agitation on a rotary shaker. A 150 ml volume of 68°C pre-warmed 0.5X SSC 0.1% SDS was applied to the membrane and the tray containing the membrane was wrapped in plastic wrap and tinfoil and placed into a rotary water bath at 68°C and incubated for 15 minutes with gentle agitation (125 rpm). After 15 minutes, the 0.5X SSC with 0.1% SDS was exchanged, and the wash was repeated for additional 15 minutes. The membrane was then transferred to a clean glass tray and rinsed for 5 minutes with 100 ml of Washing Solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween® 20) using gentle agitation at room temperature. The membrane was then transferred to a clean glass tray (or roller tube) and incubated with 100 ml (or 30 ml roller tube) of 1X Blocking Solution [provided 10X with the Roche DIG Detection Kit, diluted to1X with Maleic Acid Buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]) and incubated for 30 minutes with gentle agitation. When blocking was complete, 50 ml (10
ml if using a roller tube) of the Antibody Solution (kit anti-digoxigenin alkaline phosphatase conjugated antibody diluted to 150 mU/ml in 1X Blocking Solution) was placed on the membrane and incubated 20 minutes at room temperature with gentle agitation in a Pyrex® dish, or in a roller tube using a hybridization oven rotary.

**Equation 4.** Determination of Southern Blot Optimum Hybridization Temperature ($T_{\text{opt}}$)

\[
T_m = (48.82) + (0.41)(\%\text{GC}) - (600/\text{probe length})
\]

\[
T_{\text{opt}} = T_m - (20^\circ\text{C})
\]

Where $T_m$ is meting temperature of the double stranded DNA probe.
Figure 35. (a) Arrangement of Southern Blot Gasket and Membrane for Vacuum Transfer

Figure 35 (b) Arrangement for Drying Southern Blot Membrane after DNA Transfer.
After incubation, the membrane was transferred to a clean dish and washed two times with 100 ml of Washing Buffer (0.1 M Maleic Acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5) for 15 minutes using gentle agitation. The membrane was again moved to a clean glass dish and equilibrated with 100 ml of Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 minutes using gentle agitation. Afterward the Detection Buffer was poured off, the sides of the dish were wrapped in tin foil to block out light and 400 µl of Color Substrate Solution [kit NBT/BCIP solution (18.75 mg/ml nitroblue tetrazolium chloride, 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt in 67% v/v DMSO)] suspended in 20 ml of Detection Buffer was added to cover the entire membrane and the dish was covered with a plastic lid or aluminum foil and allowed to incubate in the dark for 2 hours to overnight (~16 hours). The color development reaction was stopped by the addition of 50 ml of TE buffer. The membrane was then dried between two pieces of Whatman paper for several hours and imaged with an Epson CX7000 digital scanner (Epson, Long Beach, CA) using the same ruler used to take the initial gel image. Finished blots were stored in a plastic page protector and kept in the dark.

Q. Pulsed-Field Gel Electrophoresis (PFGE) and Restriction Fragment Length Polymorphism Analysis (RFLP).

PFGE and restriction fragment analysis were performed for determining the possible transposition of the $bla_{CMY-2}$ gene in CUMC-50 mutants, and also to compare the genetic relatedness of strains CUMC-201 and CUMC-243. RFLP was performed to determine if the tandem $bla_{CMY-2}$ was the only $bla_{CMY-2}$ arrangement in CUMC-201 and
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CUMC-243; and to determine if the ISEcp1-\(bla_{CMY-2}\) arrangement was the only \(bla_{CMY-2}\) arrangement in CUMC-50 and its mutants. Restriction enzymes used included AvrII, I-CeuI, FseI, XbaI, EcoRI, and NruI. All enzymes were purchased from New England Biolabs (Ipswich, MA). In each case, the DNA of the isolates was prepared in agarose plugs using the protocol below (Section II. Q. 1.) which is based on the CDC Pulse Net protocol for \textit{E. coli} and \textit{Shigella} (566). For analysis of CUMC-50 mutants, strains were grown in log phase cultures in order to keep results comparable to log phase cultures used in gene copy number and transcript level assays (Sections II. K., and II. L.). Also a higher concentration of cells was used per plug than is achieved by the standard CDC protocol (approximately \(1 \times 10^8\) cells per plug instead of \(1 \times 10^7\)). This was done in order to detect the relatively few uncut plasmids that would migrate poorly under pulsed field conditions.

Q. 1. Agar Plug Preparation for PFGE and RFLP

The bacterial cells used to make agar plug DNA preparations were obtained from log phase cultures grown as described for RNA analysis (Section II. J.). When an OD\textsubscript{600} of 0.5 was reached, 10 ml of the culture was transferred to a 17 × 100 mm polypropylene tube (Fisher, cat # 05-540-6). This volume would provide enough cells for 2 plugs. Cells were concentrated by centrifugation at 8,000 rpm (7500 × g) in a Sorvall SS-34 rotor for 8 minutes. The supernatant was decanted and the pellet was resuspended in 1 ml Cell Suspension Buffer (CSB) (100 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0) using a vortex and a serologic pipette tip. When the cells were completely resuspended, they were transferred to a sterile 1.5 ml microfuge tube and concentrated by centrifugation at
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8,000 rpm (5700 × g) for 3 minutes. The supernatant was pipetted off and cells were resuspended in 230 µl of CSB by vortexing. The next few steps were done quickly to avoid premature proteinase K digestion and solidification of agar before the plugs could be cast. A 20 µl volume of proteinase K (20 mg/ml) stock was added to each cell suspension and mixed by inversion 8 to 10 times. Using a pre-warmed pipette, 250 µl of the 1% SeaPlaque® Low Melting Temperature Agarose (Lonza, Rockland, ME) with 1% SDS was added to each tube and mixed quickly with a brief vortex pulse. Then 200 µl volume of the cell/agar suspension was drawn up and 200 µl of the suspension was loaded into one or two 100 µl Bio-Rad agar plug molds. For RFLP the molds were Cat# 170-3713 (10 × 5 × 1.5 mm), and for PFGE Cat# 170-3622 (20 × 9 × 1.2 mm) molds were used. Plugs were solidified 10-15 minutes at room temperature and then deposited into a 50 ml conical tube containing 5 ml of Cell Lysis Buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 1% N-lauroyl sarcosine sodium salt solution) with 20 µl of 20 mg/ml of proteinase K stock. The tubes were placed in a 55°C shaking water bath and the plugs were digested for 2 hours with constant agitation. Cell Lysis Buffer with proteinase K was then poured off, and 10 ml of prewarmed 55°C sterile TE (10 mM Tris-HCl 1 mM EDTA pH 8.0) was added. The plugs were incubated in the TE for 20 minutes at 55°C in the shaking water bath with gentle agitation. Three more TE washes were carried out in this manner before plugs were stored at 4 °C in TE, or immediately processed for restriction endonuclease digestion.

Q. 2. Agar Plug Restriction Digestion

Plugs were cut to 5 × 3 mm in size for PFGE, or 7 × 5 mm for RFLP gels.
III. Experimental Methods

In order to wash out the TE that the plug fragment had been suspended in, the trimmed plug fragment was first immersed in 250 µl of 1X restriction enzyme buffer and incubated for 30 minutes at 37°C prior to enzyme digest. The restriction enzyme digestions were carried out in 250 µl volume for approximately 12 – 16 hours. For AvrII, I-CeuI, and FseI enzymes, 10 units of enzyme were used per digestion. For EcoRI, NruI, XbaI digestions, 50 units of enzyme were used per reaction.

Q. 3. PFGE and RFLP Electrophoresis Settings and Imaging

PFGE was carried out using a BioRad GenePath® System (Bio-Rad) with switch times of 5-15 seconds for 10 hours, followed by 15-60 seconds for 10 hours at 6 volts/cm and a current angle of 120 degrees in 0.5X TBE buffer (40 mM Tris-Base, 40 MM Boric Acid, 1 mM EDTA) recirculating at 14°C. The gel was stained with 1X GelStar® Nucleic Acid Gel Stain (Cambrex, East Rutherford, NJ), and imaged with a Bio-Rad Gel Doc™ XR imaging system.

Unidirectional electrophoresis was carried out in an Owl B3 12 × 14 cm mini gel system with built-in recirculation (Thermo Fisher), at 40 volts for 22 hours at 4°C in 1X TAE buffer. Gels were stained with 10 µg/ml ethidium bromide as described in Section II. E., and imaged with a Kodak EDA 290 (Kodak, Rochester, NY).
III. Experimental Methods

R. Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (gel shifts) were performed to determine if soluble proteins from strains listed in Table 4 were binding to DNA sequences upstream of the -35 sequences of the \( \text{bla}_{\text{CMY-2}} \) transcriptional start sites.

R. 1. Obtaining Soluble Proteins from Lysed Cells

To obtain cell lysate for gel shift experiments, bacterial strains were grown in 300 ml MHB cultures to a log phase OD\(_{600}\) of 0.5 as described for RNA isolation (Section II. J.).

For obtaining 6X-His-Rob protein, BL21 Star™ (DE3) pLys pET-\textit{rob} \textit{E. coli} were grown overnight in 5 ml MHB cultures containing 50 µg/ml of ampicillin. This culture was used to inoculate 295 ml of MHB containing 50 µg/ml of ampicillin. This initial inoculation achieved an OD\(_{600}\) of 0.01. When the culture reached an OD\(_{600}\) of 0.1, IPTG was added to a final concentration of 0.5 mM, and the cells were harvested when the culture reached an OD\(_{600}\) of 0.5 (see Section II. T. for more details).

For all cultures when the desired optical density was reached, 250 ml of the culture was transferred to a 250 ml Nalgene\textsuperscript{®} (Thermo Fisher) centrifuge bottle and cells were concentrated by centrifugation using a Sorvall GSA Rotor (Thermo Fisher) at 7000 rpm (8000 \( \times \) g) for 15 minutes at 4°C. The cell pellet was washed and resuspended three times in 25 ml of 1X PBS then concentrated by centrifugation at 8,000 \( \times \) g for 15 minutes at 4°C. After the final wash, the pellet was resuspended in 1.5 ml of Non-Reducing Sonication Lysis Buffer (10 mM Tris-HCl pH 8.0, 20% glycerol) and transferred to a Fisher 17 \( \times \) 100 mm polypropylene tube that had been cut in half to a height of about 50
mm. The cell suspension was sonicated on ice using a Gallenkamp Soniprep 150 sonicator (MSE, London, UK) for 8 cycles of 8 seconds on, 8 seconds off, at an amplitude of 8 µm. The sonicate was transferred to a 1.5 ml microfuge tube and cell debris was concentrated by centrifugation using a benchtop microfuge at 13,000 rpm (15,000 × g) for 15 minutes. The supernatant was transferred to a 2 ml screw top cryovial (Thermo Fisher) and frozen at -80°C until ready to be analyzed.

R. 2. Protein and β-Galactosidase Concentration Measurements

Protein concentration of sonication lysate was measured using a Bio-Rad Quick Start™ Bradford Protein Assay. Standard curves were established with a Bio-Rad Bovine Gamma Globulin Standard Kit (Cat# 500-0204) using the dilution scheme in Table 9. Non-Reducing Sonication Lysis Buffer was used to make dilutions. Bacterial cell lysates were diluted 1:20 for measurement. Bradford reactions consisted of 5 µl of diluted lysate or protein standard added to 250 µl of Bio-Rad 1X Bradford Dye Reagent (cat# 500-0205). The reactions were vortexed briefly and incubated at room temperature for 5 minutes and analyzed within one hour. A 100 µl portion of the reaction had absorbance measured at 595 nm with a GeneQuant™ spectrophotometer. The absorbance measures of the standards and diluted lysate was entered into a Microsoft® Excel® workbook. A polynomial trend line was drawn through the plots of absorbance (X axis) to protein concentration (Y axis) of each standard. The polynomial line equation was then used to estimate the protein concentration of the lysate based on absorbance.

β-galactosidase levels were measured to ensure that cytoplasmic protein had been obtained. The protocol below was designed for use with a Sigma β-Galactosidase
III. Experimental Methods

Reporter Gene Activity Detection Kit (Sigma, St. Louis, MO, Cat # GAL-A). Dilutions of the kit β-galactosidase standard were made with Non-Reducing Sonication Lysis Buffer as outlined in Table 10. Lysate was diluted 1:100, and 150 µl of diluted lysate or standard was added to 150 µl of the kit 2X Assay Buffer, vortexed briefly, and incubated at 37°C for 30 minutes. When incubation was complete, 500 µl of stop solution was added. All reactions were stopped at the same length of incubation. The contents of the reaction were suspended in 2 ml of deionized water, and absorbance at 420 nm was measured using a Beckman DU-6 Spectrometer. Absorbance was plotted along the X-axis against the β-galactosidase standard concentration, and a straight line was drawn through the absorbance points and used to estimate the β-galactosidase concentration of the lysate.

Table 9. Dilution Scheme for Bio-Rad Bovine Gamma Globulin Standards

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Standard</th>
<th>Standard Volume (µl)</th>
<th>Diluent Volume (Sonication Lysis Buffer) (µl)</th>
<th>Final Concentration (µg/ ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2 mg/ ml</td>
<td>60</td>
<td>60</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>2 mg/ ml</td>
<td>45</td>
<td>75</td>
<td>750</td>
</tr>
<tr>
<td>5</td>
<td>1.5 mg/ml</td>
<td>40</td>
<td>80</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>1 mg/ml</td>
<td>30</td>
<td>90</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>0.5 mg/ml</td>
<td>30</td>
<td>90</td>
<td>125</td>
</tr>
<tr>
<td>2</td>
<td>0.25 mg/ml</td>
<td>36</td>
<td>84</td>
<td>75</td>
</tr>
<tr>
<td>1</td>
<td>0.125 mg/ml</td>
<td>48</td>
<td>72</td>
<td>50</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Dilution scheme for Bio-Rad Bovine Gamma Globulin Standards used as described in Section II.
Table 10. Dilution Scheme for Sigma β-Galactosidase Standards

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Amount of 1 U/µl Standard to Add (µl)</th>
<th>Amount of Carryover to Add (µl)</th>
<th>Beta-Galactosidase Source</th>
<th>Diluent (Sonication Lysis buffer) (µl)</th>
<th>Total Final Volume</th>
<th>Final β-Galactosidase Concentration mU/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td></td>
<td></td>
<td>1998</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>Tube A</td>
<td></td>
<td>450</td>
<td>500</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>Tube 5</td>
<td></td>
<td>30</td>
<td>150</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>Tube 5</td>
<td></td>
<td>60</td>
<td>150</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>Tube 5</td>
<td></td>
<td>90</td>
<td>150</td>
<td>0.04</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td>150</td>
<td>150</td>
<td>0</td>
</tr>
</tbody>
</table>

Dilution Scheme for Sigma β-Galactosidase Standards used as described in Section II. R. 2.

R. 3. Oligonucleotide $^{32}$P Labeling

Six oligonucleotides of 34 to 44 nucleotides in length were designed to represent the 111 and 115 bp upstream of the distal and proximal -35 promoter sequences, respectively, for the $bla_{CMY-2}$ transcriptional start sites of CUMC-50. The oligonucleotides were named 1-1, 1-2, 1-3, from 5′ to 3′ for the proximal start site, and 2-1, 2-2, 2-3, for the distal start site (see Figure 36a.). A similar scheme was used for oligonucleotides representing the distal $bla_{CMY-2}$ promoter regions of $K. pneumoniae$ strain KPVAMC-50 which had IS26 sequence present (Figure 36b). Single stranded oligonucleotides were synthesized and gel purified through a 5% polyacrylamide gel by
III. Experimental Methods

IDT Technologies. Lyophilized oligonucleotides were suspended in TE to 250 pmol/µl. ATP \([\gamma^{32}P]\) 10 Ci/mMol 2 Ci/ml from Perkin Elmer was used to in the labeling reaction. If possible, an effort was made to avoid labeling 5’cytosines which do not react as well with T4 polynucleotide kinase (567). The basic labeling reaction was set up as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 µl</td>
<td>Sterile nanopure water</td>
</tr>
<tr>
<td>2.5 µl</td>
<td>T4 10X polynucleotide kinase buffer [1X]</td>
</tr>
<tr>
<td>2.0 µl</td>
<td>250 pmol/µl Oligonucleotide [100 pmol/µl]</td>
</tr>
<tr>
<td>12.0 µl</td>
<td>ATP ([\gamma^{32}P]) (20-50 µCi) (~0.3 pmol/µl) [~0.16 pmol/µl]</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>T4 polynucleotide kinase (10U/µl) [0.4 U/µl]</td>
</tr>
<tr>
<td>25.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

The reaction was held at 37°C for 40 minutes and stopped by incubating at 65°C for 20 minutes. If there was concern about the efficiency of the labeling, a 2 µl sample the labeling reaction was retained, diluted 1:10, and its activity measured in a scintillation counter. The activity of the sample was then compared to the post column purification sample to determine the percentage of \(^{32}P\) that was incorporated using Equation 5.
**Equation 5.** Determination of $^{32}$P Labeling Efficiency

\[
\text{% label incorporation} = \frac{\text{average cpm for post-column sample } \times (2.17)}{\text{average cpm for pre-column sample } \times (10)} \times 100
\]

Where 2.17 accounts for the dilution of the $^{32}$P labeling reaction with Probe Type Buffer I after the removal of a 2 µl pre-column sample (see section II. R. 3-4). Multiplication by 10 in the denominator accounts for the dilution of the pre-column sample prior to measuring on the scintillation counter.

**R. 4. Removal of Unincorporated ATP[$^{32}$P]**

In order to remove unincorporated ATP[$^{32}$P], GE Healthcare Illustra ProbeQuant™ G-50 Micro Columns were used according to manufacturer’s instructions. Kit Probe Buffer Type 1 was added to the labeling reaction tube to achieve 50 µl total volume. The resin of the G-50 column was briefly vortexed and the suspension buffer was removed by centrifugation at 735 × g (2900 rpm, benchtop microfuge) for 1 minute with cap loosened and the bottom snap cap removed. The column with compacted resin was placed in a 2 ml cryovial and the 50 µl of labeling reaction in Probe Buffer Type 1 was slowly applied to the center of the column. Labeled oligonucleotides and buffer was pulled through the column by centrifugation at 735 × g for 2 minutes. Three microliters of the single stranded column purified labeled oligonucleotides were retained and evaluated for efficiency of annealing reaction (Section II. R. 5. below).
Figure 36. (a) Gel Shift Oligonucleotides for IS\textit{Ecp1} Upstream

ATCC TAA GAC CTGC GAA CCAG TCTCA CAA TTGCA AATTG CTG CAG TAA AA

\textbf{Red Sequence: }\textit{IS}Ecp1
\textbf{Black Sequence: }non-coding intragenic region
\textbf{Blue Sequence: }\textit{bla}_{CMY-2}
\textbf{Grey Highlight: }location of oligonucleotide 2-2

\textbf{Yellow highlight: }putative -35 and -10 sequences and mapped transcriptional start site
\textbf{Underlined Sequence: }Gel-shift oligonucleotide represented region
\textbf{Green Highlight: }translation start codon
**Figure 36. (b) Oligonucleotides for IS26-ISEc1 Upstream of Distal Transcriptional Start Sites of KPVAMC-50**

CTGAAAATGCCGGCCTTTGAATGGGTTTACATGTGCAAGCTCCATCAGCAAAAGGGATGAATTTATCCACCACGGACTATTTGCAACAGTGCCACAAATACTACCCTTG
CTTCTGAAAAGTAGTTATATATCTATGAAAGC

| IS26-1 S | 5’CTGAAAATGCCGGCCTTTGAATGGGTTTACATGTG 3’ |
| IS26-2 S | 5’CAGCTCCATCAGCAAAAGGGATGAATTTATCCACCACGGACTATTTGCAACAGTGCCACAAATACTACCCTTG |
| IS26-3 S | 5’GACTATTTGCAACAGTGCCACAAATACTACC 3’ |

**Blue lettering** - IS26 sequence

**Red Lettering** - ISEc1 sequence

**Yellow Highlight** - Transcriptional start site and putative promoter

**Grey Highlight** – Gel shift oligonucleotide IS26-2 sequence
R. 5. Oligonucleotide Annealing Reactions

The annealing reactions were designed to bind the labeled oligonucleotide with its complementary strand. To ensure that no labeled oligonucleotide remained unbound, a 4-fold higher concentration of complementary strand oligonucleotide was used. Annealing reactions were set up in 75 μl volume as follows:

- 47 μl Column purified single stranded labeled oligonucleotide (10 pmol/μl) [6.27 pmol/μl]
- 15 μl 5x annealing buffer (100mM Tris-HCl, 50 mM MgCl₂). [1X]
- 8 μl Reverse strand oligonucleotide 250 pmol/μl stock [26.7 pmol/μl]
- 5 μl Sterile nanopure water

75 μl

The reactions were heated to 100°C for 5 minutes in a heat block. When incubation was complete, the tubes were floated in a 400 ml beaker of 95°C water within a Styrofoam™ container and allowed to cool 6 hours to overnight at room temperature behind Plexiglas® shielding. The efficiency of the reaction was evaluated by visualizing the migration of the labeled single stranded oligonucleotide and labeled double stranded oligonucleotide through a 7 % polyacrylamide gel. For these test gels approximately 1 × 10⁵ cpm of each product was loaded into the gel for evaluation. If annealing appeared incomplete, the annealing step was repeated with additional complementary strand oligonucleotide.
R. 6. **Protein Binding Reactions**

The exact components of the gel shift binding reaction depended on the interaction that was being tested, the activity of the $^{32}$P label on the oligonucleotide, and the amount of protein present. Below are descriptions of the typical components and methods for the reactions that were used in the different types of gel shift assays.

R. 6. a. **Basic Binding Reaction.**

The basic reaction was used to broadly detect any interaction between the oligonucleotide and the proteins in the bacterial cell lysate. It was also used as a control when competitions or supershifts were attempted. The reactions were carried out in 20 µl volume. Components of the binding reactions were mixed together on ice and consisted of:

- Water necessary for final volume of 20 µl
- 2 µl of 1 µg/µl Poly[d(I-C)] (Roche Cat#10108812001) [100 ng/µl]
- 4 µl of 5X Gel Shift Binding Buffer (10 mM Tris-HCl pH 8.0, 100 mM EDTA, 50 mM KCl, 5% glycerol) [1X]
- 10 pmol of labeled oligonucleotide (1.59 µl of a 6.27 pmol/µl of labeled oligonucleotide, activity $10^5$ to $10^4$ cpm/µl) [0.5 pmol/µl]
- Cell lysate to 40 µg of protein. [2 µg/µl ]

Protein was always added last, and the reaction was incubated at 37°C for 15 minutes. This temperature was used to recreate conditions most closely related to those
found *in vivo* in the cell. However this temperature also activated DNases and it was necessary to have 20 mM EDTA in the final Binding Buffer concentration to avoid degradation of the probe in CUMC-50 family lysates. When the binding incubation was complete, the reaction was mixed with 5 µl of gel shift loading dye. The loading dye was made by first mixing 0.0025 g of bromophenol blue with 1 ml of 5X Gel Shift Binding Buffer. The final loading dye was then made by mixing 5X Gel Shift Binding Buffer containing 0.25% bromophenol blue 1:5 with sterile glycerol. The final bromophenol concentration (0.05%) is about half that of most conventional loading dyes and was kept at this concentration to avoid possible interference with DNA binding proteins. Reaction products were loaded into a 20 × 16 × 0.1 cm 7% polyacrylamide gel in 1X TBE buffer, and separated by electrophoresis at 150 volts for 7 hours chilled at 4°C. The gel was dried onto Whatman paper (Fisher Scientific) using a Fisher FB-GD-45 Gel Dryer at 80°C for 1-2 hours, and its phosphor image was captured with an Amersham Phosphor Image Screen and STORM Phosphor Imager 820, or Typhoon 9410 Imager (Amersham Biosciences, Sunnyvale, CA).

R. 6. b. **Competition Assays.**

Competition assays were used to assess the specificity of observed protein DNA interactions. The assays were performed by using: 1) excess poly [d(I-C)], 2) excess non-specific oligonucleotide, or 3) excess unlabeled specific oligonucleotide (Oligo 2-1). For poly[d(I-C)] competitions, the amount of poly[d(I-C)] was scaled up from 100 ng/µl to 1000 ng/µl. This equated to a 10- to 100-fold weight to weight excess of poly[d(I-C)] over oligonucleotide. For specific and non-specific oligonucleotide competition, the
amount of competing oligonucleotide was also in 10 to 100 molar excess. Competing oligonucleotides were added to the reaction mixture with crude lysate protein being added last.


Supershift assays were performed to verify the binding of a 6X His (histidine) tagged Rob protein to oligonucleotide 2-2. In these assays an AbCam (Cambridge, UK) mouse Anti-6X His tag® monoclonal antibody (cat # Ab5000) was used to bind a 6XHis-Rob protein expressed from a pET100 vector in BL21 Star™ (DE3) pLys E. coli cells (see Section II. T. below). The Super Shift assays for 6XHis-Rob with Oligo 2-2 were set up in 10 µl reactions with the following components:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.34</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>Oligo 2-2 at 2.5 pmol/µl</td>
<td>[.25 pmol/L]</td>
</tr>
<tr>
<td>1.00</td>
<td>Poly[d(I-C)] at 1000 ng/µl</td>
<td>[100 ng/µl]</td>
</tr>
<tr>
<td>2.00</td>
<td>5 x Gel Shift Binding buffer [1X]</td>
<td></td>
</tr>
<tr>
<td>2.20</td>
<td>6XHis-Rob Protein (3.6 pmol/µl)</td>
<td>[.792 pmol/µl]</td>
</tr>
<tr>
<td>1.46</td>
<td>AbCam Mouse Anti-6X His tag® antibody 1 µg/µl</td>
<td>(6.85 nmol/µl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[0.68 nmol/µl]</td>
</tr>
<tr>
<td>10.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It has been estimated that as little 1/6 of available Rob will bind a given consensus sequence in gel shift reactions (535). The above reactions used a 3-fold molar excess of protein over oligonucleotide. Larger amounts of protein tended to cause smearing. The affinity of the AbCam Mouse Anti-6X His tag® antibody for 6XHis-Rob
bound to DNA was unknown. So for the initial supershift assays a 1250-fold molar excess of antibody over protein was used so that some interaction might be seen even if the affinity was low.

Two strategies were employed for the antibody binding: 1) a “pre-antibody incubation” where antibody was added with protein to the reaction before addition of labeled oligonucleotide. This was done in case the epitope (6XHis) was somehow occluded in the DNA binding, and 2) a post binding antibody incubation was performed where the oligonucleotide and protein were allowed to bind before the addition of antibody. In both cases, the antibody was allowed to incubate with the protein for 1 hour at 4°C with tumbling rotation. Gel shift reaction products were then separated through a 7% acrylamide gel, and visualized as described for basic gel shift reaction (Section II. R. 6. a, above).

S. Streptavidin Magnetic Bead /Biotinylated Oligonucleotide Protein Capture Assays

In order to capture the protein that was binding oligonucleotide 2-2 in gel shift assays, Pierce® Streptavidin Magnetic Beads (Pierce Thermo Fisher, Rockford. IL, cat# 88816) were bound with biotinylated oligonucleotide 2-2, and used to pull down the protein from CUMC-50 cell lysate that was observed binding oligonucleotide 2-2 in gel shift studies. The strategy is simple, but any DNA binding protein recovery method is challenged by steric hindrance and non-specific protein binding (568). To prevent non-specific DNA protein binding, all lysate was first “cleaned-up” by mixing with
streptavidin magnetic beads that had been linked with a biotinylated 40-mer poly[d(I-C)]
oligonucleotide.

After mixing the lysate with poly[d(I-C)] linked beads, the lysate was divided
equally into capture and control aliquots. The capture aliquot was mixed with beads
linked to biotinylated oligonucleotide 2-2, and the control aliquot was mixed with
streptavidin beads linked only to biotin. The proteins bound on the oligonucleotide 2-2
beads could then be compared to proteins bound to the control and the poly[d(I-C)] beads
to determine if a captured protein was indeed specific for that oligonucleotide.

S. 1. Linking Biotinylated Oligonucleotides to Steptavidin Magnetic Beads.

Biotinylated oligonucleotide 2-2(S) and biotinylated-poly[d(I-C)](S) and their
complementary oligonucleotides 2-2(AS) and Poly[d(I-C)](AS) were synthesized by IDT
Technologies and suspended in TE to a 250 pmol/µl concentration. The complementary
oligonucleotide strands were annealed in a suspension containing equal amounts of each
oligonucleotide, in 1X Gel Shift Annealing Buffer as described for gel shift assays
(Section II. R. 5.) yielding 200 pmol/µl double stranded oligonucleotide suspensions.

To determine the amount of oligonucleotide and lysate that would be needed to
capture enough protein to visualize by Coomassie blue staining, some calculations were
performed. The Coomassie blue staining technique used was based on that of Sambrook
and Russell (described below in Section II. S. 5.) and has a minimum protein detection
limit of 150 ng (565). The protein binding oligonucleotide 2-2 in the initial gel shift
assays was estimated to be 34 kDa and at a concentration of 47.5 fmol per 4.17 µl of
CUMC-50 lysate. For a 34 kDa protein, 47.5 fmol would equal about 1.6 ng. Therefore,
the amount of lysate in the capture assay would need to be scaled up 100-fold to 400 µl in order to attain 150 ng. An additional 10-fold scale up was added to account for potential steric hindrance on the beads and error in the above estimations, so a total of 4 ml of lysate was mixed with the capture and control beads.

Both capture and control aliquots (a total of 8 ml of lysate) would first be passed through poly[d(I-C)] linked beads for clean-up of non-specific bead and DNA binding proteins. In the gel shift reactions approximately 500 ng of poly[d(I-C)] was used per microliter of lysate. This could not be achieved with the amount of beads on hand, so instead, 5 ng of poly [d(I-C)] linked to streptavidin magnetic beads per microliter of lysate was used. This was accomplished by binding 1640 pmol (39.1 µg) of the biotinylated 40-mer double stranded poly[d(I-C)] oligonucleotide on to 200 µl of the streptavidin beads.

To bind the biotinylated poly[d(I-C)] oligonucleotide and biotinylated nucleotide oligo 2-2, the binding capacity and characteristics of the beads needed to be considered. According to Pierce® Application Note # 14 by Kaboord and Liermann, the Pierce® Streptavidin Magnetic Beads have a biotin binding capacity of 8.2 pmol/µl for a 25-mer fluorocine tagged oligonucleotide (569). The authors found that 100% binding efficiency was achieved when 45.6 pmol of oligonucleotide per microliter of beads was incubated in a mixing volume 2.5 times the initial volume of beads. So these were the target ratios that were sought. Using 25 µl of beads would provide a theoretical binding capacity for 205 pmol of oligonucleotide and potentially an equal amount of protein. A 205 pmol quantity of a 34 kDa protein would equate to 7.0 µg, which would be more than sufficient for visualization by Coomassie blue staining and identification by mass spectrometry.
III. Experimental Methods

For binding poly[d(I-C)], 200 μl of Pierce® Streptavidin Magnetic Beads (in Tris-HCl suspension as provided by the manufacturer) were placed in a 1.5 ml microfuge tube and pulled with a magnet so suspension buffer could be drawn off. The beads were washed three times with 1X Gel Shift Binding buffer, and all buffer was drawn off before adding the following components:

- 45 μl 200 pmol/μl double stranded poly[d(I-C)] [18 pmol/μl]
- 100 μl 5X Gel Shift Binding Buffer [1X]
- 355 μl Sterile nanopure water
- 500 μl

For binding oligonucleotide 2-2 to streptavidin magnetic beads, a 50.0 μl volume of beads was washed as described above and the following components were added:

- 11.5 μl 200 pmol/μl double stranded oligo 2-2 [18 pmol/μl]
- 25.0 μl 5X Gel Shift Binding Buffer [1X]
- 88.5 μl Sterile nanopure water
- 125.0 μl

The binding components described above were tumbled at 20 rpm overnight at 4°C using a LabQuake® Shaker (rotator) (Cat# 400-110, Lab Industries Inc., Berkeley, CA). Beads were washed three times with 1X Gel Shift Binding Buffer before proceeding to biotin blocking.
III. Experimental Methods

S. 2. Biotin Blocking

After binding oligonucleotides to the beads, biotin was applied to block the available streptavidin binding sites and avoid possible binding of E. coli lysate proteins that had an affinity for streptavidin. According to the Pierce® Streptavidin Magnetic Beads product insert, the beads have a fluorocine tagged biotin binding capacity of 35 pmol/µl of beads. The clean-up, control, and capture beads were each mixed with a total biotin quantity 10-fold above the biotin binding capacity of the beads. Specifically, the 200 µl of poly[d(I-C)] coated beads were mixed with 700 µl of a 100 pmol/µl of biotin suspended in 1X Gel Shift Binding Buffer. Fifty microliters of oligonucleotide 2-2, and control beads were mixed with 175 µl of the biotin suspension. The beads and biotin suspensions were mixed overnight tumbling at 4°C then washed three times with 1X Gel Shift Binding Buffer before performing protein capture experiments.

S. 3. Protein Capture with Oligonucleotide Linked Beads

As described above, it was calculated that 8 ml of E. coli strain CUMC-50 cell lysate would be needed in the protein capture assays. The lysate was prepared as described for gel shift assays (Section II. R. 1.), from 6 separate 300 ml MHB cultures. Once insoluble proteins were removed by centrifugation the soluble lysate of the six aliquots were combined. The lysate had a protein concentration of 8.8 µg/µl, and a β-galactosidase activity 41 µU/µl.

The 200 µl of biotinylated poly[d(I-C)] oligonucleotide bound streptavidin magnetic beads was split into ten 20 µl aliquots and placed into 1.5 ml microfuge tubes (see Figure 37). An 800 µl volume of lysate, and 200 µl of 5X gel shift binding buffer
Figure 37. Schematic for Rob Protein Capture with Biotinylated Oligonucleotide 2-2-Linked Streptavidin Magnetic Beads

For poly [d(I-C)] clean-up, ten tubes containing 20 µl of poly [d(I-C)]-linked Pierce Magnetic Beads were mixed with 800 ul of CUMC-50 lysate, and 200 µl of 5x Gel Shift Binding Buffer and tumbled at 20 rpm for 24 hours at 4°C.

Poly [d(I-C)] Linked Beads

Poly [d(I-C)]-linked beads were retained post-clean-up, washed 5 times with 1x Gel Shift Binding Buffer, then split into two aliquots of 100 µl and stored for use as Poly d(I-C) control.

Control Biotin-only linked Beads

Lysate was added 500 µl at a time to 25 µl of Oligo 2-2-linked beads and to 25 µl biotin-only-linked beads. Each addition of lysate was allowed to tumble for 12 hours at 20 rpm in 4°C cold room.

Oligonucleotide 2-2-Linked Beads

After mixing with lysate, the oligo 2-2 and biotin-only-linked beads were washed five times with 1x Gel Shift Binding Buffer, then suspended in 30 µl of SDS-PAGE Loading Buffer and heated to 100 °C. Beads were pulled with a magnet and loading buffer with captured proteins was loaded into a SDS-PAGE gel.

Proteins captured by oligo 2-2-linked beads but not Poly [d(I-C)]- or biotin only-linked control beads were sought to be identified.
was added to each of the tubes and tumbled together at 20 rpm for 24 hours at 4°C. After this incubation, the beads were pulled with a magnet and the lysate was removed and stored at -80°C. Storage was done in individual 1 ml aliquots to avoid excessive freeze thawing of a pooled lysate. The poly[d(I-C)] linked beads were washed 5 times with 1X Gel Shift Binding Buffer and stored at 4°C until the SDS-PAGE gel was ready to be run.

The ten 1 ml lysate /1X Gel Shift Binding Buffer samples were then split evenly between the control beads and oligonucleotide 2-2 linked beads. The lysate /1X Gel Shift Binding Buffer was added in 500 µl aliquots to the beads and allowed to rotate for 12 hours before being exchanged with fresh 500 µl aliquot. Lysate was stored at -80°C after mixing with the beads.

S. 4. SDS-PAGE of Captured Proteins

After mixing the control and oligonucleotide 2-2 linked beads with their final aliquots of lysate, the beads were washed five times with 1X Gel Shift Binding Buffer. The capture and control beads were then heated for 5 minutes at 100°C in 30 µl of SDS-PAGE loading buffer (10% β-mercaptoethanol, 5% SDS, 37% glycerol, 25 mM Tris-HCl pH 6.8, 100 µg/ml bromophenol blue). Poly[d(I-C)] beads were heated at 100°C in 60 µl of SDS-PAGE loading buffer. After heating, the buffer was drawn away from the beads using a magnet, and 30 µl of each sample was loaded on to the gel. A 1.5 µl volume of Bio-Rad Low Molecular Weight Ladder (Cat# 161-0304) was heated at 100°C in 15 µl of SDS-PAGE loading buffer and loaded into lanes flanking those of the captured protein. Lanes without captured protein or molecular weight ladders were loaded with loading dye to maintain a uniform pH front and prevent distortion of the migrating protein bands. The
proteins were separated by electrophoresis through a 20 × 16 × 0.1 cm SDS-PAGE gel containing a 15% acrylamide resolving portion and a 4% acrylamide stacking gel prepared according to Sambrook and Russell (565). The gel was run in Tris-glycine SDS-PAGE electrophoresis buffer (25 mM Tris-base, 250 mM glycine, 10% SDS) using constant voltage at 80 volts with 25 mA of current, and 13 watts of power. As proteins migrated through the stacking gel and the dye front entered the resolving gel, the voltage was increased to 160 volts, with current and power remaining steady at 25 mA, and 13 watts respectively. The current was applied for 12 hours, and the gel assembly remained attached to a chiller core set to 20°C. This temperature preserved the integrity of the gel and the proteins, but was warm enough to prevent precipitation of the SDS.

S. 5. SDS-PAGE Gel Staining and Imaging

Gels were first imaged by staining with Coomassie blue [0.5 g Coomassie Brilliant Blue R-250 (Bio-Rad), 125 ml methanol, 100 ml deionized water, 25 ml acetic acid, filtered with a Millipore # 4 filter] using gentle agitation (25 rpm) for 12 hours. The gel was then destained with 50% methanol, 40% water, 10% acetic acid using gentle agitation and applying bundled KimWipes® (Kimberly Clark, New York, NY) to the corners of the destaining tray to draw the excess dye away from the gel. Three detaining sessions were used. The first two were at least 90 minutes each and the third was extended until bands were reasonably visible (approximately 45 minutes). No rehydration steps were used for three reasons. One, the protein bands were found to have enhanced visibility and resolution in the dehydrated state. Secondly, the dehydrated gels were more resistant to tearing during the imaging process. And third, the staining with SYPRO®
Ruby requires that the gel be dehydrated before staining, so gels could be readily placed into SYPRO® Ruby stain after the Coomassie destain. Coomassie images were captured using a Dan Hall light box (Dan Hall, Grover Beach, CA) and a Nikon CoolPix 995 digital camera (Nikon, Melville, NY).

Because the yields of captured protein were relatively low, SYPRO® Ruby Protein Gel Stain (Invitrogen) was used to visualize the bands of captured protein in the SDS-PAGE gel. Staining was carried out according to manufacturer directions with an abbreviated wash step. This consisted of dehydrating the gel for 15 minutes in fixing solution (50% methanol, 40% distilled water and 7% acetic acid), then staining with enough SYPRO Ruby to cover the gel completely (approximately 150 ml). The gel was allowed to stain overnight (approximately 16 hours) covered from light with gentle agitation at 25 rpm. The gel was destained with 10% methanol, 7% acetic acid for no more than 15 minutes with one additional destaining wash for 5 minutes, and allowed to remain covered in distilled water until imaging could be completed. Imaging was carried out on a GE Typhoon Imager 9410 using 488 nm excitation laser, and a 610 nm emission filter.

S. 6. **Protein Identification by Mass Spectrometry**

Protein visualized in the SDS-PAGE gel lane representing the oligonucleotide 2-2 captured protein but not visualized in lanes representing the poly[d(I-C)] clean up beads or streptavidin-biotin control beads was excised from the gel with an X-Acto™ knife (Elmers, Westerville, OH) using a fresh new blade. The excised gel band was placed in sterile nanopure water until it could be analyzed. Trypsin digestion and mass spectrometry of the isolated protein was performed by the University of Nebraska.
Medical Center Proteomics Core Facility. Trypsin digests were carried out as described by Schlautman et al. (570). Briefly, the gel plug was cut into 1–2 mm fragments and the pieces were soaked in 100 µl of Wash Buffer I (50% Acetonitrile, 50 mM NH₄HCO₃) for one hour at room temperature. The gel pieces were then dried in a Savant SpeedVac (Thermo Fisher) until completely dry. The dried fragments were rehydrated in 10 µl of 0.1 µg/µl trypsin (Promega) suspended in 10 mM NH₄HCO₃, and tryptic digestion was carried out overnight at 37°C. Peptides were extracted by washing gel pieces twice with Wash Buffer II (0.1% trifluoroacetic acid, 60% acetonitrile) then purified with Millipore ZipTip™ C₁₈ purification tip. Samples were transferred to vials, dried, and resuspended in 15 µl of HPLC grade water with 0.1% formic acid before analyzing with a LTQ Orbitrap XL ETD™ (Themo Fisher) LCMS mass spectrometer. Peptide mass spectrometry data was analyzed with Proteome Discoverer 1.2 (Themo Fisher).

T. pET100 TOPO® N-6X His Tag Rob Protein Expression

No anti-Rob antibodies were commercially available to study the Rob protein in gel supershift assays, or in immunofluorescence microscopy studies, so a Rob protein with an N-terminal 6X His tag was expressed from a pET100 expression vector and an anti-6X His tag antibody was used for detection. The 6X His tag also enabled nickel column purification of the tagged protein for later use in protein specific gel shifts assays.

An Invitrogen Champion™ pET100 Directional TOPO® Expression Kit was used to express the Rob protein with an N-terminal 6X His tag. The system uses a pET100 plasmid with a low copy number (15-20 per cell) pBR322 origin of replication. Gene expression from the in-frame insertion site of the pET100 vector is under the control of a
T7 RNA polymerase which utilizes a unique 15 bp promoter sequence upstream of the insertion site. The pET100 vector with *rob* insert (pET-rob) was transformed into a BL21Star™ (DE3) pLys *E. coli* expression host. This *E. coli* strain encodes the λ DE3 lysogen which carries the gene for the T7 RNA polymerase. Expression of the polymerase is under the control of a *lacUV5* promoter that can be induced by IPTG. The BL21 Star™ strain also lacks functional *lon* and *opmT* protease genes which reduces degradation of heterologous proteins, and produces a truncated RNase E enzyme that increases mRNA stability. The pLysS plasmid also produces a T7 lysozyme which binds to T7 RNA polymerase and reduces basal expression of the pET100 insert. This in combination with the low copy pET100 vector is particularly useful for genes that can be toxic to the host, such as Rob (553).

The Rob protein was amplified for cloning from *E. coli* strain CUMC-50 with PCR primers pET100-rob-start-F, and *rob*-End-R (see Table 11). The PCR reaction was carried out using a Platinum® Pfx DNA polymerase (Invitrogen) which generates blunt end amplicons necessary for the vaccinia topoisomerase I mediated ligation step with the Invitrogen Champion™ pET Directional TOPO® Expression Kit. The forward primer contains a 5´-CACC sequence at the 5´end immediately upstream of the start codon to complement sequence on the pET-100 vector that follows the 5´-CCCTT vaccinia topoisomerase I recognition sequence. Ligation was carried out according to manufacturer instructions with the following components:
III. Experimental Methods

1 µl  Microcon® G50 column purified rob PCR product at 2 ng/µl (3.71 fmol/µl) [0.62 fmol/µl]

1 µl  Kit Salt Solution (1.2 M NaCl, 0.06 M MgCl₂) [0.2 M NaCl, 0.01 M MgCl₂]

3 µl  Kit Sterile water

1 µl  pET-100-TOPO®, vector (4.21 fmol/µl) [.701 fmol/µl]

6 µl
### Table 11. *rob* Cloning Primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>GenBank Accession</th>
<th>Binding Location</th>
<th>Purpose</th>
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<tr>
<td>pET100 rob start F</td>
<td>CACCATGGATCAGCCGGCATATTTCG</td>
<td>NC_000913</td>
<td>4633333 - 4633311</td>
<td>Amplification of <em>E. coli rob</em> gene for cloning with Invitrogen Champion™ pET Directional TOPO® Expression Kit</td>
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<tr>
<td>rob End R</td>
<td>TTAACGACGGATCGGAATCAGC</td>
<td></td>
<td>4632464 - 4632485</td>
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</tr>
</tbody>
</table>
The reaction was limited to 5 minutes at room temperature to avoid excessive TOPO cutting and ligation. Three microliters of the ligation reaction was added to 1 vial of thawed chemically competent One Shot® TOP10 *E. coli* (Invitrogen) and reaction components were allowed to incubate on ice for 15 minutes. Cells were heat shocked as described for TA cloning (Section II. H.) and 160 µl of S.O.C. cell suspension was plated on to a 50 µg/ml ampicillin LB agar plate. Plasmids isolated from the cells by small plasmid isolation methods (described in Section II. I.) and digested with the single cutting BglII endonucelase (New England Biolabs) in 1X NEB 3 buffer for 1 hour at 37°C. The restricted DNA was separated through a 1% agarose gel in room temperature TAE buffer for 12 hours at 35 volts to determine if plasmids had an insert of expected size. Plasmids containing inserts of the correct size were sent to ACGT Inc (Wheeling, Il) for sequencing using T7 forward and reverse primers that flank the TOPO insertion site. When a pET100 vector was found to contain the correct *rob* sequence, it was designated pET-*rob*.

Transformation of pET-*rob* into the BL21Star™ (DE3) pLys *E. coli* cells was carried out by adding 10 ng (2 µl) of E.Z.N.A. Mini Plasmid Prep (Omega Bio-Tek) purified pET-*rob* to a thawed vial of BL21 Star™ (DE3) pLys *E. coli* cells (Invitrogen). The plasmid was transformed into the cells using chemically competent heat shock techniques described for transformation into TOP10 cells (Section II. H.), except the 37°C incubation after the addition in S.O.C. media was only for 30 minutes. The entire S.O.C. suspension was added to 5 ml of MHB containing 50 µg/ml of ampicillin and grown overnight at 37°C shaking at 120 rpm.
The entire 5 ml of overnight BL21 Star™(DE3) pLys pET-rob MHB culture was used to inoculate 300 ml of MHB containing 50 µg/ml ampicillin. This achieved an initial OD$_{600}$ of 0.02. Cultures were grown at 37°C shaking at 120 rpm, and IPTG was added to the cultures when an OD$_{600}$ of 0.1 was reached. Final IPTG culture concentrations of 1.0 mM and 0.5 mM were attempted, with 0.5 mM working best in terms of yield and growth rate. The cultures were allowed to grow to an OD$_{600}$ of 0.5 (~ 5 hours) before 250 ml of culture was collected and crude extract prepared as described for gel shift lysate experiments (Section II. R. 2.).

U. N-6X His-Rob ProBond™ Elutions

6XHis-Rob was captured from cell lysate and purified using an Invitrogen ProBond™ Purification System. The system utilizes a nickel-chelating resin that is designed for the recovery of recombinant fusion proteins containing six tandem histidine residues. The resin is composed of agarose beads cross-linked to iminodiacetic acid chelated with a nickel atom. The nickel atom forms three hydrogen bonds with the iminodiacetic acid molecule and can form two additional hydrogen bonds with the imidazole rings of two sequential histidine residues. Non-specific nickel binding proteins can be washed from the resin with buffer containing low concentrations (20mM) of imidazole. The 6XHis-tagged proteins are then eluted with buffer containing high concentrations (250 mM) of imidazole.

Working in a 4°C cold room, 1 ml of the resin suspension was poured into a capped 10 ml purification column supplied with the kit. The resin was allowed to settle completely by gravity for 10 minutes and the suspension buffer was drawn off. Three ml
of sterile distilled water was added to the column and mixed with the resin by inversion. The resin was allowed to settle by gravity for 10 minutes and the water was drawn off. The resin was washed three times with 3 ml of Native Purification Buffer (250 mM NaH₂PO₄ pH 8.0, 2.5 M NaCl) each time mixing by inversion and allowing the resin to settle by gravity for 10 minutes. After the final wash, 2 ml of lysate was applied to the column and mixed by rotation at 20 rpm using a small battery operated rotating motor (Red Carpet Studios, Cincinnati, OH) for 60 minutes. When mixing was complete, the resin was allowed to settle by gravity for 10 minutes and the lysate was aspirated and saved at -80°C for SDS-PAGE analysis. The resin was then washed 4 times with 4 ml of Native Wash Buffer (Native Purification Buffer with 20 mM imidazole pH 8.0) each time mixing by inversion, settling the resin by gravity for 10 minutes, and storing the supernatant at -80°C for later SDS-PAGE analysis. The column was then clamped in a vertical position and the bottom snap cap was removed. Three ml of Native Elution Buffer (1X Native purification buffer with 250 mM imidazole pH 8.0) was added to the column and fractions were collected in approximately 1 ml aliquots and stored at -80 °C until they could be evaluated by SDS-PAGE. Approximately 100 µl of sterile glycerol was added to the retained washes and elutions prior to freezing to limit the precipitation of imidazole and protect the captured protein from freeze fracture.

V. Removal of Imidazole by Dialysis

Because imidazole compounds can interfere with the DNA binding of helix-turn-helix proteins, the imidazole was removed from the ProBond™ purified 6XHis-Rob elution by dialysis before using it in gel shift studies (571). Spectra Por® #2 (12 – 14 kDa
porosity) regenerated cellulose ester dialysis tubing (Spectrum Labs, Rancho Dominguez, CA) was used. The tubing was prepared by soaking for 15 minutes in sterile deionized water. The tubing was then pried open with forceps and allowed to soak in a slightly open state for an additional 5 minutes. The entire aliquot of ProBond™ elution fraction #2 was placed in tubing closed off by autoclaved Spectra Por® Standard tubing clips. The contents were dialyzed in 1.5 L of autoclaved 5 mM Tris-HCl pH 8.0 slowly swirled with an autoclaved stir bar in a 4°C cold room. Three exchanges of dialysis were performed at 6-12 hour intervals. Dialyzed proteins were evaluated by SDS-PAGE to assure that no proteases had damaged the purified protein. Glycerol was added until it was 5% final volume, and protein was stored at -80°C until it was analyzed. Dialyzed protein was also concentrated with a Savant SpeedVac® SC110 centrifugal evaporative condenser (Thermo Fisher) for use in gel supershift studies (section II. R. 6. c.).

W. Western Blotting and Dot Blotting with anti-6X His tag Antibody

ProBond™ purified 6XHis-Rob, crude E. coli BL21 Star™ (DE3) pLys pET-rob lysate, and BL21 Star™ (DE3) pLys pET100-empty vector lysate were analyzed by western blot and dot blot to determine the specificity of the anti-6X His antibody that would be used to bind 6XHis-Rob in gel shift assays and immunofluorescent microscopy studies.

SDS-PAGE gels used for western blotting were set up and run as described for streptavidin bead capture assays (Section II. S. 4.), except a 12.5% acrylamide resolving gel was used, and the interface between the resolving and stacking gel was set about 1.5 to 2 cm below the wells. This additional space seemed to improve stacking and resolution.
of protein bands. Gels used for Coomassie blue and western blot evaluation were run separately. Western blotting was performed using methods similar to those described in the Bio-Rad Protein Blotting Guide and the Bartz Laboratory western blot protocol (572, 573). A section of Bio-Rad Immun-Blot® PVDF membrane was cut to gel size (~16 × 16 cm). The membrane was equilibrated in methanol for 5 minutes, and immersed in Wet Transfer Buffer (20 mM Tris-Base, 150 mM glycine, 15% methanol) with gentle agitation for 10 minutes. When SDS-PAGE was complete, one corner of the gel and membrane were cut for orientation, fitted against one another, and assembled into a Bio-Rad Trans-Blot® transfer cell (cat # 170-3939) as illustrated in Figure 38. Protein was transferred at 100 volts for 1 hour in Wet Transfer Buffer at 10°C. When the transfer was complete, the membrane was blocked with 150 ml of 5% Non-Fat Milk Tween Tris-Buffered Saline [100 mM Tris-base, 1.5 M NaCl, 0.5% Tween® 20, 5% W/V dry non-fat milk (Carnation®, Vevey, Switzerland)] using gentle agitation on a rotary shaker for 30 minutes at 4°C. The membrane was then transferred to roller tube and 12 ml of 5% Non-Fat Milk Tween Tris-Buffered Saline containing 6 µl of 1 µg/µl AbCam® mouse Anti-6X His tag® monoclonal IgG2a antibody (Cat# AB5000) (1/2000 dilution, 500 ng/ml final concentration) was added. The membrane and antibody suspension were incubated together overnight in a Fisher 3.5 × 25 cm roller tube using a Fisher FBH10 Hybridization Oven with rolling agitation at 4°C in a cold room. The following day the membrane was transferred to a Pyrex® dish and washed 5 times for 5 minutes with Tween Tris-Buffered Saline (100 mM Tris-base, 1.5 M NaCl, 0.5% Tween® 20) at 120 rpm on a rotary shaker at 4°C. The membrane was then transferred to a clean Pyrex®
Figure 38. Western Blot Bio-Rad Trans-Blot® Assembly Diagram
dish with 50 ml of 5% Non-Fat Milk Tween Tris-Buffered Saline containing 100 ng/ml of Pierce (Thermo Fisher) goat anti-mouse (H+L) horseradish peroxidase conjugated polyclonal secondary antibody (cat# 31430). The membrane was incubated with the secondary antibody for 1 hour at room temperature with gentle agitation under darkness, followed by a wash with Tween Tris-Buffered Saline as described after primary antibody incubation. Pierce SuperSignal® West Femto Chemiluminescent Substrate (Cat# 34096) was applied to the membrane according to manufacturer recommendations to detect the secondary antibody. After 5 minutes incubation, the membrane was placed between two sheets of clear overhead transparency and the chemiluminescent signal was captured immediately with a Kodak Image Station 400R.

Western blotting only tests antibody affinity for denatured protein. In order to assure specificity of the AbCam® mouse Anti-6X His tag® antibody to native 6XHis-Rob, dot blot experiments were performed according to Bio-Rad Immun-Blot® PVDF Membrane Instruction Manual and AbCam® Dot Blot Protocol instructions. (574, 575) A 5 × 4 cm section of Bio-Rad Immun-Blot® PVDF membrane was used with 4 mm diameter circles drawn on the surface in pencil. Spots for lysate from BL21 Star™ (DE3) pLys pET100-empty vector, and BL21 Star™ (DE3) pLys pET-rob E. coli cells were made in duplicate. The membrane was equilibrated as described for western blotting and placed on two layers of paper towels. The first layer was slightly moistened in Wet Transfer Buffer and the one below was left dry to create a wicking effect and pull lysate down into the membrane. The membrane was allowed to rest on the paper towels for 2 minutes so that surface moisture was no longer visible but not dried completely. A 2 µl volume of each lysate was then applied into duplicate circles on the membrane and
allowed to dry for 30 minutes. The application of primary and secondary antibodies was carried out as described for western blotting with a few modifications. Incubation of the primary antibody was carried out in a petri dish on a rotary shaker at 4°C overnight. Secondary antibody was an Invitrogen rabbit anti-mouse IgG (H+L) Alexa Fluor® 568 conjugated antibody diluted 1:2000 (1 µg/ml) in 10 ml of 5% Non-Fat Milk Tween Tris-Buffered Saline. Incubation of the secondary antibody was carried out for one hour in a petri dish on a rotary shaker at room temperature. The membrane was imaged on a Typhoon 9410 imager using the 532 nm excitation laser and the 610 nm band pass emission filter. The photomultiplier tube (PMT) was set at 425 volts which was critical for visualizing the fluorescence. Higher default settings caused too much background signal.

X. 6XHis-Rob Immunofluorescence Microscopy

It was hypothesized that Rob was activated by exposure to β-lactam antibiotics at minimal inhibitory concentration, as activation has been demonstrated with exposure to other oxidative stress agents. Immunofluorescence microscopy was performed to visualize the dispersal activation of the Rob protein pre and post antibiotic treatment as characterized by Griffith et al. (Figure 33) using methods based on those described by the authors and Azam et al. (554, 555).

Strains BL21 Star™ (DE3) pLys pET-rob and BL21 Star™ (DE3) pLys pET100-empty vector (Negative Control) were cultured from frozen stock on to MHA plates without ampicillin. Ampicillin selection was not used in cultures for immunofluorescence microscopy studies due to potential Rob activation, and potential pET-rob high copy
number selection which could overload cells with Rob protein. A few colonies of each strain grown on a MHA plates were used to inoculate 5 ml of MHB and grown overnight (12 hours). One ml of overnight MHB culture was used to inoculate 99 ml of MHB in a side arm flask. This yielded an OD$_{600}$ of 0.01, and the cultures were allowed to grow at 37°C shaking at 120 rpm. While cultures grew, activation inocula (antibiotic or dipyridyl) were prepared to the desired concentration. The 4,4´-dipyridyl (Sigma) was prepared by first dissolving the crystalline solid dipyridyl in ethanol to a concentration 1000 × above the desired final concentration. One hundred microliters of this dissolved stock was added to 900 µl of PBS to achieve an inoculating dose concentration of which 1 ml was used to inoculate the flask to final concentration. Different final concentrations of 4-4´ dipyridyl were tried: 5 mM, 2.5 mM and 0.5 mM. A 5 mM concentration was used to activate Rob in studies by Rosner et al. 2002 and Griffith et al. 2009 (553, 554). However this concentration appeared to be too active for the BL21 (DE3) pLys pET-rob cells in this study, and lower concentrations appeared inactive (see results Section III. M.).

β-lactam antibiotics ceftazidime and piperacillin/tazobactam (Sigma) were used as β-lactam activators. The BL21DE3 pET100-rob cells had a ceftazidime MIC of 0.094 µg/ml and a piperacillin/tazobactam MIC of 0.75 µg/ml. Activation doses of each drug were added to achieve the MIC concentration in the side arm flask cultures. For ceftazidime, drug was suspended in PBS to a 9.4 µg/ml inoculation concentration. For piperacillin tazobactam a 75 µg/ml piperacillin, 400 µg/ml tazobactam PBS suspension was used for the inoculation dose. When cells reached an OD$_{600}$ of 0.3, a 1 ml sample was taken as a -1 minute time sample and immediately fixed in 10 ml of 80 % methanol. The cultures were then inoculated with their activation dose of dipyridyl, β-lactam drug,
or PBS only and swirled briefly to assure mixing before a time zero sample was taken. The culture flasks were then returned to the incubator and additional 1 ml samples were collected at 5, 10, 15 and 30 minutes. All samples were immediately fixed in 80% methanol overnight at 4°C overnight. The following day the fixed cells were concentrated by centrifugation at 2500 × g (4600 rpm on SS-34 rotor) for 5 minutes at 4°C. Methanol was removed and the pellet was suspended in 1.2 ml of PBS-Tween (PBS, 0.05% Tween® 20). The cells were transferred to a 1.5 ml microfuge tube and washed three times with PBS-Tween and reconcentrated by centrifugation at 2500 × g (5300 rpm on microfuge) for 5 minutes each wash. After the last wash cells were resuspended in 0.5 mL of 80% methanol. For each time sample a 12 × 12 mm staining field was drawn on to a Fisherbrand Superfrost® Excel adhesion microscope slide using a Super HT™ Pap Pen (RPI Corp, Beacon Falls, CT). A 10 µl spot of the methanol suspended cells was placed at the center of the staining field and air dried for 20 min. The cells were permeabilized with 100 µl of freshly prepared Lysozyme Solution (1 mg/ml Lysozyme in GTE) for 5 min at room temperature. Slides were then washed four times for five minutes with PBS-Tween in a glass slide carriage or pipette tip box cover using gentle agitation (30 rpm). After the washes, the slides were briefly dehydrated by applying 100 µl of methanol to the staining field. Methanol was removed by inversion and the slides were allowed to air dry completely (10-15 minutes). The staining area was covered with 100 µl of Blocking Buffer (2% bovine serum albumin in PBS-Tween) for 15 min. Blocking buffer was removed by inversion and 20 to 40 µl of AbCam® Mouse Anti-6X His tag® monoclonal IgG primary antibody suspended 1:500 (2ng/µl) in Blocking Buffer was added to the staining field and incubated 16 hours (overnight) at 4°C in a humidity
chamber. The following day the primary antibody was removed by inversion and the slides were washed four times with PBS-Tween. A 50 µl volume of Blocking Buffer was applied to the staining field twice for 10 minutes each time and removed by inversion. A 20 to 40 µl volume of Invitrogen rabbit anti-mouse (H+L) Alexa Fluor® 568 conjugated secondary antibody diluted 1:500 (4 ng/µl) in Blocking Buffer was applied to the slide and incubated in the dark for 1 hour in a humidity chamber at room temperature. After incubation, the antibody was removed by inversion and slides were washed 5 times with PBS-Tween in the dark then covered with 25 µl of Mounting Media [50% PBS, 50% glycerol, containing 10 mg/ml n-propylgallate (Fisher), 1 µg/ml DAPI (4', 6-diamidino-2-phenylindole dihydrochloride)(Sigma)] and covered with a cover slip. Excess mounting media was wiped away from the edges and the cover slips were sealed with Sally Hansen Xtream Wear® clear nail polish (COTY Inc., New York, NY). Nail polish was allowed to dry for 30 minutes in the dark before the slides were viewed with a Nikon ECLIPSE 80i epifluorescence microscope. After viewing, the slides were stored in a closed slide carrier at 4°C.

Y. Growth Curve Estimates and Comparisons

Growth curves for parent and mutant strains during early log phase were estimated from optical density measurements taken during cultures for RNA, DNA and plasmid isolation experiments. OD₆₀₀ measurements were taken with a Beckman DU-6 spectrophotometer (Y-axis) and plotted against the time at which measurements were taken (X-axis). A logarithmic line with a y-intercept (time zero) set to OD₆₀₀ of 0.100 was plotted through the time points using Microsoft Excel. The line equation from the
plotted line was used to estimate the predicted length of time to achieve an $OD_{600}$ of 0.500. Differences in growth curves between parent and mutant strains were also evaluated using ANCOVA (analysis of covariance, Vassarstats.net) (576). For the ANCOVA analysis, $OD_{600}$ measurements taken between 80 minutes from inoculation until the culture reached an $OD_{600}$ of 0.500 or greater were used, and a plot point of time zero $OD_{600}$ of 0.100 was added to the data to anchor the regression line.

Z. RNA Folding Predictions

Predictions of Inc RNA folding were made using RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).
IV. Results

A. Mutation Studies

Mutation studies were carried out as described in Section II. D. to estimate the frequency of piperacillin/tazobactam selected susceptibility mutations in four clinical *E. coli* strains carrying *bla*<sub>CMY-2</sub>. Two of these strains were also evaluated for the emergence of meropenem susceptibility mutants.

A. 1. Piperacillin/Tazobactam Mutation Studies

Strains CUMC-50, CUMC-201, CUMC-214, CUMC-243 (Table 4) were used piperacillin/tazobactam mutation studies. The strains were exposed to piperacillin at 2X, 4X, and 8X their piperacillin/tazobactam MIC with constant 4 µg/ml tazobactam. Rates of putative mutant emergence ranged from $1 \times 10^{-5}$ to $1 \times 10^{-7}$ (Table 12). Lower antibiotic concentrations tended to yield higher rates of putative mutants, but no clear correlation existed ($r^2 = 0.04$). Piperacillin/tazobactam MICs were measured for at least 12 putative mutants from each parent strain as described in Section II. B. Putative mutants with selection antibiotic MICs 4-fold higher than their parent were considered ‘true mutants’. For three of the four strains, the percentage of true mutants among the putative mutants was high, specifically strains CUMC-50 (100%); CUMC-201 (91.7%); and CUMC-243 (100%). For strain CUMC-214 only 50% of putative mutants were true mutants. The correlation between the concentration of piperacillin/tazobactam used for selection, and the piperacillin/tazobactam MIC of the selected mutants was also analyzed.
IV. Results

**Table 12. Piperacillin/Tazobactam Mutation Frequencies**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inoculum</th>
<th>Piperacillin/ Tazobactam Selection Concentration µg/ml</th>
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<tr>
<td></td>
<td>10^9</td>
<td>8</td>
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<td>1.7 x 10^{-6}</td>
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<tr>
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<tr>
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<td>128</td>
<td>2.02 x 10^{-7}</td>
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<td>128</td>
<td>0</td>
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</table>

TNTPC, Too numerous to count
Again, no correlation existed ($r^2 = 0.03$). Likewise, grouping the mutants by their selection level (2X, 4X, and 8X MIC) showed no significant difference in piperacillin/tazobactam MICs among the groups using ANOVA analysis ($p = 0.28$).

A subset of piperacillin/tazobactam agar mutants had susceptibilities assessed for other $\beta$-lactam antibiotics (Table 13). Included in these susceptibility measures was mutant strain CUMC-50M which had been isolated from the elliptical inhibition zone of a piperacillin/tazobactam Etest® assay performed on CUMC-50 in 2004 (see Figure 39 photo). In general, the MICs for all other $\beta$-lactam antibiotics were higher in the mutants than the parent strains. In most cases the mutants exhibited 4-fold changes in MIC to aztreonam, ceftazidime, and cefepime. For CUMC-50, the aztreonam MIC was at a susceptible level according to the 2011 CLSI guidelines (2 $\mu$g/ml), but were 16- to 32-fold higher for the CUMC-50 mutants and at levels considered resistant according to 2011 CLSI breakpoints (130). This was the only instance among the piperacillin/tazobactam mutants where a change from susceptible to resistant phenotype for a drug other than piperacillin/tazobactam was observed. For the carbapenems, imipenem, meropenem, and ertapenem, all mutants were susceptible. Only strains CUMC-50 and CUMC-214 had mutants that exhibited 4-fold changes in MIC to carbapenems (Table 13). Strains that showed a 4-fold increase in carbapenem MIC (CUMC-50M, CUMC-50M2, CUMC-50M8, CUMC-214M1) were also the only mutant strains that demonstrated increased $bla_{CMY-2}$ transcript levels (Table 15).
Figure 39. CUMC-50M Emerging from Etest® Assay Performed on CUMC-50

Red arrow points to CUMC-50M colony growing near piperacillin/tazobactam Etest® strip.
### Table 13. β-lactam Susceptibilities of Select Piperacillin/Tazobactam Mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Piperacillin/Tazobactam</th>
<th>Aztreonam</th>
<th>Cefepime</th>
<th>Ceftazidime</th>
<th>Imipenem</th>
<th>Meropenem</th>
<th>Ertapenem</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUMC-50</td>
<td>4</td>
<td>2</td>
<td>0.12</td>
<td>32</td>
<td>0.12</td>
<td>0.015</td>
<td>0.023</td>
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<td>CUMC-50 M</td>
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<td>64</td>
<td>4</td>
<td>512</td>
<td>0.50</td>
<td>0.06</td>
<td>0.38</td>
</tr>
<tr>
<td>CUMC-50 M2</td>
<td>64</td>
<td>32</td>
<td>0.50</td>
<td>128</td>
<td>0.12&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>CUMC-50 M8</td>
<td>256</td>
<td>32</td>
<td>1</td>
<td>256</td>
<td>0.25</td>
<td>0.06</td>
<td>0.19</td>
</tr>
<tr>
<td>CUMC 201</td>
<td>16</td>
<td>8</td>
<td>0.50</td>
<td>64</td>
<td>0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.38</td>
</tr>
<tr>
<td>CUMC 201 M28</td>
<td>128</td>
<td>64</td>
<td>2</td>
<td>256</td>
<td>0.38&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.50</td>
</tr>
<tr>
<td>CUMC 214</td>
<td>16</td>
<td>8</td>
<td>0.50</td>
<td>32</td>
<td>0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>CUMC 214 M1</td>
<td>128</td>
<td>32</td>
<td>2</td>
<td>128</td>
<td>0.50&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.12</td>
<td>1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>CUMC 243</td>
<td>16</td>
<td>16</td>
<td>0.50</td>
<td>64</td>
<td>0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.50</td>
</tr>
<tr>
<td>CUMC 243 M18</td>
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<td>64</td>
<td>2</td>
<td>256</td>
<td>0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.50</td>
</tr>
</tbody>
</table>

All susceptibilities measured in µg/ml. <sup>e</sup> superscript denotes that susceptibility was measured by Etest<sup>e</sup>. All other β-lactam MICs were determined by agar dilution.
IV. Results

A 2. Meropenem Mutation Studies

Strains CUMC-50, CUMC-50M, CUMC-214, CUMC-214M1, were exposed to meropenem at concentrations 1X, 2X, and 4X, their meropenem MIC. The selection levels were kept lower than those used for piperacillin/tazobactam mutant selection because higher meropenem concentrations had been found not to produce mutants in previous studies with other strains (Ellen Smith-Moland personal communication). No putative meropenem mutants were obtained from strains CUMC-50 and CUMC-50M. However, CUMC-214 yielded putative mutants at a rate of 1.1 x 10^{-6}, and CUMC-214M1 at a rate of 7.1 x 10^{-6}. All four putative mutants from CUMC-214, and twelve putative mutants from CUMC-214M1 had meropenem MICs measured by agar dilution. From this, 75% of the putative mutants from CUMC-214 and 41.7% from CUMC-214M1 were true mutants with meropenem MICs that ranged from 0.25 µg/ml to 2.0 µg/ml. Only one mutant (214M1-MerA) was not susceptible to meropenem according to the January 2011 CLSI interpretation guidelines (130). The susceptibility of the meropenem mutants to imipenem and ertapenem was also evaluated (Table 14). Only one mutant strain (CUMC-214M1 Mer B4) had a 4-fold increase in imipenem MIC above its parent. Seven of the eight meropenem mutants had 4-fold increases in ertapenem MIC.

For the meropenem mutants of CUMC-214, the increase in MIC resulted in a change from a susceptible to resistant ertapenem phenotype according to the 2011 CLSI guidelines (130). For the meropenem mutants of CUMC-214M1, the ertapenem MICs were 3- to 32-fold over the parent, with all strains (including the parent) interpreted as resistant to ertapenem according to 2011 CLSI guidelines.
### Table 14. Carbapenem Susceptibilities of Selected Meropenem Mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Meropenem</th>
<th>Imipenem&lt;sup&gt;ª&lt;/sup&gt;</th>
<th>Ertapenem&lt;sup&gt;ª&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUMC-214</td>
<td>0.03</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>CUMC-214 Mer C1</td>
<td>0.25</td>
<td>0.50</td>
<td>1.5</td>
</tr>
<tr>
<td>CUMC-214 Mer C2</td>
<td>0.25</td>
<td>0.50</td>
<td>1</td>
</tr>
<tr>
<td>CUMC 214 Mer C4</td>
<td>0.25</td>
<td>0.75</td>
<td>2</td>
</tr>
<tr>
<td>CUMC-214 M1</td>
<td>0.12</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>CUMC-214 M1 Mer A</td>
<td>2</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>CUMC-214 M1 Mer B1</td>
<td>1</td>
<td>0.75</td>
<td>6</td>
</tr>
<tr>
<td>CUMC-214 M1 Mer B2</td>
<td>0.50</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>CUMC-214 M1 Mer B3</td>
<td>0.5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>CUMC-214 M1 Mer B4</td>
<td>1</td>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

Meropenem MICs were determined by agar dilution.
<sup>ª</sup> Susceptibility to imipenem and ertapenem were measured by Etest<sup>ª</sup>.
IV. Results

B. \textit{bla}_{\text{CMY-2}}, and \textit{ampC} Transcript Levels

For selected piperacillin/tazobactam mutants and all meropenem mutants, \textit{bla}_{\text{CMY-2}} transcript levels were measured by real-time RT-PCR. For piperacillin/tazobactam mutants, three mutants from each parent strain were evaluated. All mutants from CUMC-50 were found to have increased \textit{bla}_{\text{CMY-2}} expression 3- to 13-fold above their parent (Table 15). Only one mutant strain from CUMC-214 exhibited an increase in \textit{bla}_{\text{CMY-2}} transcript level. Strain CUMC-214M1 had levels 5-fold above its parent. No increases were seen among the mutants from CUMC-201 and CUMC-243. \textit{bla}_{\text{CMY-2}} transcript levels of CUMC-201, CUMC-214, and CUMC-243 were compared to that of CUMC-50. Strains CUMC-201 and CUMC-243 each had approximately two-fold higher \textit{bla}_{\text{CMY-2}} transcript levels than CUMC-50, and CUMC-214, had levels approximately 3-fold higher (Table 15).

Chromosomal \textit{ampC} transcript levels were also measured to assess the impact the endogenous AmpC \(\beta\)-lactamase may have had on the \(\beta\)-lactam susceptibility of the CUMC-50 mutants, and strains CUMC-201 and CUMC-243. Among the CUMC-50 mutants all \textit{ampC} transcript levels were less than 2-fold above the parent, indicating that changes in AmpC expression were not a factor in \(\beta\)-lactam susceptibility differences. For CUMC-201 and CUMC-243, the level of \textit{ampC} expression was 19- and 25-fold higher than CUMC-50, indicating that chromosomal \textit{ampC} expression may be playing a role in the \(\beta\)-lactam susceptibility differences between these parent strains.

All meropenem mutants demonstrated a less than 2-fold increase in \textit{bla}_{\text{CMY-2}} transcript level than their parent. This indicated that changes in \textit{bla}_{\text{CMY-2}} expression were
IV. Results

not responsible for changes in carbapenem susceptibility. Chromosomal \textit{ampC} expression was not examined for the meropenem mutants.

\textbf{C. \textit{bla}_{CMY-2} and Upstream Sequencing}

The increase in \textit{bla}_{CMY-2} gene expression measured in the mutant strains of CUMC-50, and CUMC-214 could have been due to \textit{bla}_{CMY-2} promoter mutations. To evaluate this possibility, genome walking was performed to determine the sequence upstream of \textit{bla}_{CMY-2} in strain CUMC-50. Sequence identified through genome walking was then used to design primers for amplification and sequencing of DNA 1,138 upstream of the \textit{bla}_{CMY-2} gene for the strains with increased \textit{bla}_{CMY-2} transcript levels (CUMC-50M, CUMC-50M2, CUMC-50M8, CUMC-214, CUMC-214M1). All strains were found to have identical sequence for this region indicating that no promoter mutations had occurred. The entire \textit{bla}_{CMY-2} gene was also sequenced for these strains and each was found to be identical to each other and to published \textit{bla}_{CMY-2} sequence (i.e. GenBank accession no. X91840). This indicated that differences in β-lactam susceptibility between parent and mutant strains were not due to changes in the hydrolytic properties of CMY-2.

For strains CUMC-201 and CUMC-243, \textit{bla}_{CMY-2} sequence was also consistent with published sequence, however when the upstream region of these strains was analyzed, an unusual finding was made. In attempts to amplify the \textit{bla}_{CMY-2} upstream regions of these strains, a 2000 bp product was generated using primer CMY7PE1 as a \textit{bla}_{CMY-2} upstream reverse primer. The product was present when different forward primers were used. Subsequently, the 2000 bp product was gel extracted and cloned into...
a pCR2.1® vector, then sequenced using M13 primers that flank the pCR2.1® insertion site. Sequencing data revealed that each strain possessed two \textit{bla}_{CMY-2} genes in a divergent tandem arrangement centered on an IS5 insertion element (Figure 40). This was confirmed by sequencing PCR products generated from genomic DNA templates and primers listed in Table 5. The IS5 element was flanked by partial \textit{ISEcp1} 3’ end sequence of different lengths (278 bp on 5’ side, and 152 bp on the 3’ side of IS5), with identical 116 bp regions of non-coding DNA of \textit{C. freundii} origin between the \textit{ISEcp1} fragments and the \textit{bla}_{CMY-2} genes (GenBank accession number HQ680722, and HQ680723; Figure 40). An inverted IS5 CWAR target consensus sequence was found flanking the 5’end of IS5 sequence (5’-CTTG-IS5) but was not repeated on the 3’ end (5’-IS5-TTAA). This suggests that the association of IS5 with each \textit{bla}_{CMY-2} gene likely occurred as independent events, and the two genes may have been brought together by a homologous recombination event centered within IS5.
Figure 40. Tandem *bla*<sub>CMY-2</sub> Arrangement

Genetic frameworks for: A. CUMC-50; B. CUMC-201, and CUMC-243. NruI and EcoRI cut sites located as noted. *bla*<sub>CMY-2</sub> specific probe hybridization region located as noted.
IV. Results

D. 5´RACE

Since it was possible that the $\text{bla}_{\text{CMY-2}}$ transcriptional start site changes could have occurred in mutants even without promoter sequence changes, 5´RACE was performed to map the $\text{bla}_{\text{CMY-2}}$ transcriptional start sites for CUMC-50 and CUMC-50M as a representative parent mutant pair. Both strains were found to utilize two identical start sites located within an IS$\text{Ecp1}$ insertion element at 182 and 624 bp upstream of the $\text{bla}_{\text{CMY-2}}$ start codon (Figure 36, GenBank accession number JF300163). These data indicated no changes in promoter sequence or transcriptional start sites were affecting mRNA expression.

Also included in these 5´RACE studies were strains KPVAMC-50 and Misc-341. Both strains used identical $\text{bla}_{\text{CMY-2}}$ start sites as CUMC-50 and CUMC-50M. This was of interest because strain Misc-341 had a six base pair deletion in the intragenic region between IS$\text{Ecp1}$ and $\text{bla}_{\text{CMY-2}}$ immediately 3´of IS$\text{Ecp1}$, and KPVAMC-50 has an IS26 element inserted 12 bp upstream of the putative -35 sequence of the distal transcriptional start site (Figure 36b) (87).

E. $\text{bla}_{\text{CMY-2}}$ Gene Copy Number Assays

An increase in gene copy number could have caused the increase in $\text{bla}_{\text{CMY-2}}$ expression for the mutants of CUMC-50 and CUMC-214. To examine this possibility, gene copy number assays were performed using real time PCR. The mutants of CUMC-50 were found to have 3- to 13-fold increases in $\text{bla}_{\text{CMY-2}}$ gene copy number that directly correlated with the observed increases in $\text{bla}_{\text{CMY-2}}$ gene expression (Table 15).
IV. Results

For mutant CUMC-214M1, a 2-fold increase in \( bla_{CMY-2} \) copy number over its parent was observed (Table 15). This measured copy number increase could not completely explain the 5-fold increase in \( bla_{CMY-2} \) transcript level between the parent and mutant. Interestingly, Southern blot analysis of CUMC-214 and 214M1 plasmids did indicate a copy number change on a scale approximate to \( bla_{CMY-2} \) transcript level (Figure 44), so there may have been some kind of interference occurring with the gene copy number studies.

Parent strains CUMC-201 and CUMC-243 each had \( bla_{CMY-2} \) copy number that was approximately 2-fold higher than that of CUMC-50. This copy number difference was similar to the transcript level differences between these parent strains.
**Table 15.** Gene Transcript Level and Gene Copy Number of Piperacillin/Tazobactam Mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>(bla_{CMY-2}) Transcript Level Relative to Parent Strain</th>
<th>SD</th>
<th>(bla_{CMY-2}) Transcript Level Relative to CUMC-50</th>
<th>SD</th>
<th>(bla_{CMY-2}) Gene Copy Number Relative to Parent</th>
<th>SD</th>
<th>(bla_{CMY-2}) Gene copy Number Relative to CUMC-50</th>
<th>SD</th>
<th>Incl1 Copy Number Relative to CUMC-50</th>
<th>SD</th>
<th>(repZ) Transcript Level Relative to Parent Strain</th>
<th>SD</th>
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<td>50</td>
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<td>4.77</td>
<td>2.47</td>
<td>1.16</td>
<td>10.58</td>
<td>2.30</td>
<td>-</td>
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</tr>
<tr>
<td>214 M5</td>
<td>1.12</td>
<td>0.01</td>
<td>4.02</td>
<td>0.86</td>
<td>-</td>
<td>-</td>
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<tr>
<td>214 M6</td>
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<td>0.15</td>
<td>4.36</td>
<td>1.66</td>
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<td>-</td>
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<td>1.75</td>
<td>0.37</td>
<td>-</td>
<td>-</td>
<td>1.59</td>
<td>0.29</td>
<td>-</td>
<td>-</td>
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<tr>
<td>243 M16</td>
<td>1.01</td>
<td>0.05</td>
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<tr>
<td>243 M18</td>
<td>1.10</td>
<td>0.32</td>
<td>2.01</td>
<td>0.40</td>
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<tr>
<td>243 M28</td>
<td>1.06</td>
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<td>2.05</td>
<td>0.09</td>
<td>-</td>
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</table>

Transcript levels were measured by real-time RT-PCR, and gene copy number measured by real-time PCR. Relative quantities of each were calculated by the \(2^{ΔΔCT}\) method.
IV. Results

F. Southern Blot Assays for $bla_{CMY-2}$ Location.

F. 1. Southern Blots of CUMC-50 and its Mutants

Two mechanisms could have been responsible for the increase in $bla_{CMY-2}$ copy number of the CUMC-50 mutants. Transposition events may have increased the copy number of $bla_{CMY-2}$, or an increase in the plasmid copy number may have occurred for the plasmid carrying $bla_{CMY-2}$. To determine which of these two mechanisms was responsible, a series of Southern analyses were performed.

For the evaluation of plasmid copy number changes, plasmids were isolated from each strain and analyzed by Southern hybridization using a $bla_{CMY-2}$ specific probe (described in Section II. P.). For CUMC-50 strains, the plasmid bearing $bla_{CMY-2}$ was approximately 100 kb in size. Differences in plasmid staining intensity were seen both in agarose gel ethidium bromide staining, and Southern hybridization alkaline phosphatase staining. The intensity differences corresponded with the $bla_{CMY-2}$ copy number differences measured by RT-PCR (Figure 41). This provided visual evidence that a plasmid copy number increase had occurred.

To evaluate if transposition of $bla_{CMY-2}$ had occurred, total genomic DNA from each strain was restricted with XbaI then separated by PFGE and analysed by Southern blot. In preliminary experiments, the 100 kb $bla_{CMY-2}$ carrying plasmid of CUMC-50 was independently subjected to XbaI digestion and appeared not to contain an XbaI restriction site. Since supercoiled plasmids migrate poorly under PFGE conditions, a larger amount of cells than normally used under CDC Pulse Net protocols ($10^8$ CFU instead of $10^7$ CFU per plug) were used in order to visualize the plasmids. This made for a PFGE gel that was slightly overloaded with DNA, but enabled enough plasmid to
Figure 41. Plasmids Isolation and Southern Blot of CUMC-50 Strains

Plasmid isolations and \( \text{bla}_{\text{CMY-2}} \)-specific Southern blot of CUMC-50 and its mutants. Lane L, Epicentre BAC-Tracker supercoiled ladder; Lane 1, CUMC-50; Lane 2, CUMC-50M; Lane 3, CUMC-50M2; Lane 4, CUMC-50M8.

Figure 42. PFGE and Southern Blot of CUMC-50 Strains

PFGE and \( \text{bla}_{\text{CMY-2}} \)-specific Southern blot of XbaI digested plasmid and total genomic DNA preparations of CUMC-50 and its mutants. Lane 1, CUMC-50 plasmid isolation. Lanes 2-5 genomic DNA preparations of: Lane 2, CUMC-50; Lane 3, CUMC-50M; Lane 4, CUMC-50M2; Lane 5, CUMC-50M8.
migrate and be detected by Southern hybridization (Figure 42). Only the band corresponding to the 100 kb plasmid hybridized with the $bla_{CMY-2}$ probe, indicating that the $bla_{CMY-2}$ gene did not transpose to the chromosome of these strains. Again, the intensities of probe signal reflected the differences in $bla_{CMY-2}$ copy number for each strain.

To rule out possible transposition of $bla_{CMY-2}$ to other locations on the 100 kb plasmid, total DNA was digested with the more frequent cutting EcoRI endonuclease, then separated by unidirectional gel electrophoresis and analyzed by Southern hybridization. In these studies, the $bla_{CMY-2}$ probe hybridized to only a single band of approximately 5700 bp in size for all strains, and again, probe binding intensity reflected copy number differences (Figure 43). This indicated that the $bla_{CMY-2}$ had not transposed within the plasmid.

This conclusion was supported by evidence of a possible relationship of the CUMC-50 plasmid with a published 101 kb plasmid called pCVM29188_101 (GenBank accession no. NC_011077) (421). pCVM29188 was obtained by the CDC National Antimicrobial Monitoring System in 2003 from a Salmonella enterica sv. Kentucky strain isolated from a chicken breast product purchased in Atlanta, Georgia (421). The 4th genome walk attempt for the CUMC-50 $bla_{CMY-2}$ upstream region had acquired sequence 2,264 bp upstream of $bla_{CMY-2}$. The distal sequence of this genome walk product contained DNA sequence identical to that found on pCVM29188_101 at the same distance from $bla_{CMY-2}$. Using New England Biolabs NEBcutter V2.0, a projected EcoRI digestion of pCVM29188_101 would yield a 5775 bp fragment containing $bla_{CMY-2}$;
similar to what was seen with EcoRI digests of CUMC-50. In the predicted digestion, the 5′ EcoRI cut site would be located 710 bp upstream of $bla_{CMY-2}$ and the 3′ cut site would be located 3773 bp downstream of $bla_{CMY-2}$. One arm ISEcp1 transposition studies described by Lartigue et al. found that successful one arm transposition of an ISEcp1 element with a downstream β-lactamase did not include more than 2891 bp of DNA downstream of the β-lactamase gene with an average of 1250 bp of downstream DNA being transposed (361). Also, the total size of the transposed fragment in their studies did not exceed 5464 bp. Therefore, transposition of a fragment greater than 5700 may be at the far end of what is possible for this mode of ISEcp1 transposition. Perhaps more importantly, if a fragment larger than 5700 bp did transpose, it would likely visibly alter the migration of the isolated plasmid in gel electrophoresis analysis, and this was not seen for any of the mutant strains (Figure 41).
In the agarose gel an Invitrogen 1Kb ladder flanks the outside lanes. Lanes 1, 3, 5, 7 are unrestricted agar plug DNA preparations. Lanes 2, 4, 6, 8, are EcoRI digest of agar plug DNA preparations. Lane 1, 2 are CUMC-50; Lane 3, 4, are CUMC-50M; Lane 5, 6, CUMC-50M2; Lane 7, 8, CUMC-50M8.
F. 2. Southern Blots for CUMC-214 and CUMC-214M1

In $bla_{CMY-2}$ probe Southern blots of plasmids isolated from CUMC-214 and CUMC-214M1, the probe hybridized with two plasmids of approximately 100 and 75 kb in size. The 100 kb plasmid of the mutant stained more intensely than that of its parent in repeated isolations, and staining intensity corresponded to the 5-fold increase in $bla_{CMY-2}$ transcript level that was measured (Figure 44). Both CUMC-214 and CUMC-214M1 also had probe hybridization present on the 16 kb band that is associated with residual chromosomal DNA and broken plasmid DNA. Excess DNA and probe hybridization at this location may have been due to Plasmid-Safe™ DNase treatment not being used for these particular preparations. Staining of the 16 kb band was also more intense for CUMC-214M1 to about the same level of intensity as the 100 kb plasmid intensity difference. This may have been due to fragments of the 100 kb plasmid migrating with the 16 kb linear DNA band.
Figure 44. Southern Blot of Large Plasmids Isolated from Strain CUMC-214 and CUMC-214M1.

Plasmid isolations and bla<sub>CMY-2</sub>-specific Southern blot. Lane L, Epicentre BAC-Tracker supercoiled ladder; Lane 1, 2, CUMC-214; Lane 3, 4, CUMC-214M1.

Figure 45. Southern Blot of Large Plasmids Isolated from Strains CUMC-201 and CUMC243.

Plasmid isolations and bla<sub>CMY-2</sub>-specific Southern blot. Lane L, Epicentre BAC-Tracker supercoiled DNA ladder; Lane 1, CUMC-50; Lane 2, CUMC-201; Lane 3, CUMC-243.
F. 3. Southern Blots of CUMC-201 and CUMC-243

For CUMC-201 and CUMC-243, the tandem $bla_{CMY-2}$ arrangement was located on a plasmid of approximately 165 kb (Figure 45). It was of interest to determine if this was the only arrangement of $bla_{CMY-2}$ in these cells to further support the findings of a 2-fold higher $bla_{CMY-2}$ transcript and gene copy number level in these strains compared to the single copy strain CUMC-50. To examine this, additional Southern blotting of genomic DNA restricted with NruI and EcoRI was performed. As displayed in Figure 40, the tandem $bla_{CMY-2}$ sequence contains restriction sites for these enzymes that would yield specific fragments containing the binding site of the $bla_{CMY-2}$-specific probe. Restriction with NruI alone would yield a fragment of 3200 bp that hybridized with the probe, and an NruI /EcoRI double digest would yield fragments of 2036 and 1164 bp. If $bla_{CMY-2}$ in these strains only existed in the tandem arrangement, then no other fragments should hybridize with the probe when restricted with these enzymes. Indeed, when Southern blots of these digests were performed, the probe only bound to fragments of these sizes (Figure 46). These data indicated that the tandem $bla_{CMY-2}$ arrangement was the only arrangement of $bla_{CMY-2}$ in these strains.
IV. Results

**Figure 46.** Southern Blot of CUMC-201 and CUMC-243 of EcoRI and NruI single and Double Digests

An Invitrogen 1 kb DNA ladder run with the gel in the corresponding Southern blot is shown on the left. Lanes 1-4 are CUMC-201, and lanes 5-8 are CUMC-243. Lanes 1 and 5, no restriction digest; lane 2 and 6, EcoRI digest only; lane 3 and 7 NruI digest only; lane 4 and 8 EcoRI and NruI double digest.
G. Identification and Copy Number of the IncI1 Plasmid

The Southern analyses of the CUMC-50 mutants indicated that plasmid copy number amplification alone was responsible for the increase in \( \text{bla}_{	ext{CMY-2}} \) copy number. To verify this, the 100 kb plasmid carrying \( \text{bla}_{	ext{CMY-2}} \) was excised from an agarose gel and identified as an IncI1 incompatibility type plasmid through PCR amplification and DNA sequencing of the plasmid replicon region. Real-time PCR gene copy number assays using primers directed toward the IncI1 replicon region were then performed to determine the relative IncI1 plasmid copy number of the mutant strains. It was found that IncI1 copy number of the mutants had increased between 3- and 13-fold, correlating with the increases in \( \text{bla}_{	ext{CMY-2}} \) copy number and \( \text{bla}_{	ext{CMY-2}} \) expression levels that were observed (Table 15).

The 100 kb \( \text{bla}_{	ext{CMY-2}} \) bearing plasmid of the CUMC-214 was also studied and was found not to be an IncI1 plasmid in PCR studies.

H. Identification of Point Mutations in \( \text{inc} \) and Evaluation of \( \text{repZ} \) Expression

Copy number of IncI1 plasmids is controlled in part by Inc antisense RNA transcribed from the plasmid gene \( \text{inc} \) (See section I. D. 2. a). Single nucleotide changes within the loop region of \( \text{inc} \) can affect the loop shape of Inc RNA and its complementary RNA sequence in the transcript of \( \text{repZ} \). Such mutations can hinder the ability of Inc to prevent translation of the RepZ plasmid replication initiation protein, leading to increased IncI1 plasmid replication frequency, and plasmid copy number \( (479, 484, 577) \). To determine if nucleotide changes within \( \text{inc} \) or its promoter region had occurred in the
CUMC-50 mutants, the DNA sequence of *inc* and its flanking regions were analyzed. No changes in *inc* sequence were observed for CUMC-50 and CUMC-50M2 when compared to published sequence (e.g. GenBank accession number CP001121.1, Figure 47). However, strains CUMC-50M and CUMC-50M8 did have point mutations present within the loop region of *inc*. Using the 5′-most *inc* transcriptional start site mapped by Nikoletti *et al.* for *inc* as a reference, an A to G transition at *inc* nucleotide 38, and a G to T transversion at nucleotide 30 were observed for strains CUMC-50M and CUMC-50M8 respectively (Figure 47) (484). When analyzed by RNA folding prediction software, the mutations were found to modify the predicted Inc antisense RNA stem loop structure (Figure 48), and thus could affect Inc mediated plasmid copy number control, and potentially lead to an increase in plasmid copy number as observed in CUMC-50M and CUMC-50M8.

Since a mutation that increases *repZ* transcription could also potentially offset interference by Inc and increase plasmid copy number, relative *repZ* gene transcript levels were measured, and the *repZ* promoter regions were sequenced for all strains. No differences in promoter sequence were observed between CUMC-50 and its mutants, and the *repZ* transcript levels of the mutants were increased 3- to 13-fold, matching the levels observed for *bla*<sub>CMY-2</sub> (Table 15). These data indicated that *repZ* transcript levels were a reflection of plasmid copy number like *bla*<sub>CMY-2</sub> transcript levels were, and therefore were not likely driving plasmid amplification.
Figure 47. Analysis of Inc Sequence and Promoter Region of CUMC-50 Strains

Point mutations in *inc* of CUMC-50M and CUMC-50M8 are denoted by boxed nucleotides. Underlined sequence denotes complementary sequence arms of the Inc RNA stem loop. Gray highlighted sequences indicate the transcriptional start site and putative -35 and -10 promoter hexamers for *inc* as mapped by Nikoletti et al. (477).
Figure 48. Predicted RNA Folding of Inc Anti-sense RNA for CUMC-50 Strains

![Diagram of RNA folding](image)

Arrows indicate point mutation locations. Boxed nucleotides are nucleotides necessary for Inc and SL1 kissing complex as noted by Asano et al. (479).
I. Growth Curve Estimates and Comparisons

Growth curves were estimated from OD_{600} measures taken during early log phase broth cultures for isolating RNA, DNA, and plasmids. Mutant strains with identified \textit{bla}_{\text{CMY-2}} gene copy and plasmid copy number changes (CUMC-50M, M2, M8 and CUMC-214M1) all showed slower growth than their parent. The time required for mutants to achieve OD_{600} 0.5 from an inoculation OD_{600} of 0.1 was estimated to be 4 to 14 minutes longer for these mutants than parent strains. The differences in regression lines for OD_{600} measures plotted against time of collection were evaluated by ANCOVA. Strains CUMC-50M, CUMC-50M2, and CUMC-214-M1 had slopes significantly different from their parents in this comparison (Figures 49-52).
IV. Results

Figure 49. Estimated CUMC-50 Family Growth Curves

<table>
<thead>
<tr>
<th>Strain</th>
<th>Projected Time to Reach OD$<em>{600}$ of 0.500 from OD$</em>{600}$ of 0.100 in Minutes using Logarithmic Line Equation</th>
<th>P-Value for Analysis of Covariance in Growth Curve Regression Line Compared with CUMC-50</th>
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<tr>
<td>CUMC-50</td>
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<td>CUMC-50M</td>
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<tr>
<td>CUMC-50M2</td>
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<td>0.0001</td>
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<td>CUMC-50M8</td>
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<td>0.17</td>
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For Figures 49-52 See Section II. Y. for details of growth curve plots and ANCOVA calculations
IV. Results

**Figure 50.** Estimated CUMC-214 and CUMC-214M1 Growth Curves

<table>
<thead>
<tr>
<th>Strain</th>
<th>Projected Time to Reach OD_{600} of 0.500 from OD_{600} of 0.100 in Minutes using Logarithmic Line Equation</th>
<th>P-Value for Analysis of Covariance in Growth Curve Regression Line Compared with CUMC-214</th>
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</thead>
<tbody>
<tr>
<td>CUMC-214</td>
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<td>CUMC-214M1</td>
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IV. Results

**Figure 51.** Estimated CUMC-201 Family Growth Curves

<table>
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<th>Strain</th>
<th>Projected Time to Reach $OD_{600}$ of 0.500 from $OD_{600}$ of 0.100 in Minutes using Logarithmic Line Equation</th>
<th>P-Value for Analysis of Covariance in Growth Curve Regression Line Compared with CUMC-201</th>
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<td>CUMC-201</td>
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<td>CUMC-201M19</td>
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<tr>
<td>CUMC-201M23</td>
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<td>0.88</td>
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<td>CUMC-201M28</td>
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IV. Results

**Figure 52.** Estimated CUMC-243 Family Growth Curves

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<th>Strain</th>
<th>Projected Time to Reach OD&lt;sub&gt;600&lt;/sub&gt; of 0.500 from OD&lt;sub&gt;600&lt;/sub&gt; of 0.100 in Minutes using Logarithmic Line Equation</th>
<th>P-Value for Analysis of Covariance in Growth Curve Regression Line Compared with CUMC-243</th>
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</thead>
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<td>CUMC-243</td>
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<tr>
<td>CUMC-243M16</td>
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<td>CUMC-243M18</td>
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<td>0.48</td>
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<td>CUMC-243M23</td>
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<td>0.61</td>
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</table>
J. Gel Shift Analysis

J. 1. Basic Binding Reactions

Gel shifts were performed to detect transcription factors that may be binding upstream of the promoter sequences for \textit{bla}_{\textsc{CMY-2}} within \textit{ISEcp1}. Identification of factors acting at these promoter regions could explain the success of \(\beta\)-lactamase genes found downstream of \textit{ISEcp1} and could reveal potential new targets for antibiotic therapies.

The gel shift assays were performed as described in Section II. R. A variety of different migrations were seen among the oligonucleotides (Figures 53, 54). No differences in the gel shift patterns of the parent and mutant strains of CUMC-50 and CUMC-214 were observed (Figures 53, 54), indicating that there were no changes to protein binding for the mutant strains that could be affecting gene expression.

Two oligonucleotides that appeared to yield consistently strong migration shifts were oligonucleotide 1-1, and oligonucleotide 2-2. Additional gel shift assays were performed using these oligonucleotides with lysate from other \textit{E. coli} strains and \textit{Enterobacteriaceae} genera (\textit{Klebsiella}, \textit{Proteus}, and \textit{Salmonella}) in which \textit{ISEcp1} associated \(\beta\)-lactamases have been found. For studies with oligonucleotide 2-2 studies, lysate from \textit{E. coli},
Figure 53. Gel Shift Assays with Lysate from CUMC-50 Strains

Oligonucleotides are described in Section II. R. 3. (p 150) and Figure 36. (p 152). NP represents no protein control; 50, CUMC-50; M, CUMC-50M; M2, CUMC-50M2; M8, CUMC-50M8.
Figure 54. Gel Shifts with Lysate from CUMC-214 and CUMC-214 M1

Oligonucleotides are described in Section II. R. 3. (p 150) and Figure 36. (p 152). NP represents no protein control; 214, CUMC-214; M1, CUMC-214-M1.
Klebsiella pneumonia, Proteus mirabilis, and Salmonella enterica sv. Typhimurim were used. Lysate from all strains except Proteus yielded a similar shift band when incubated with this oligonucleotide (Figure 55a). For oligonucleotide 1-1, lysate from different strains of E. coli and Klebsiella were used, and lysate from all strains yielded shift bands similar to those seen with strains CUMC-50 and CUMC-214 (Figure 55b).

Additional gel shifts were performed using lysate from Klebsiella pneumoniae strain KPVAMC-50. This strain had previously been studied by Mark Reisbig at Creighton University (87). The strain possessed \( \text{bla}_{\text{CMY-2}} \) with an upstream IS\( \text{Ecp1} \) element that was intersected with an IS26 element 12 bp upstream of the distal \( \text{bla}_{\text{CMY-2}} \) -35 promoter sequence. A specific set of oligonucleotides was designed for the IS26-IS\( \text{Ecp1} \) region (Figure 36b.) and used in gel shift studies with KPVAMC-50 lysate. In these studies, oligonucleotide IS26-2, which is positioned a similar distance from the distal transcriptional start site as oligonucleotide 2-2, was found to have its mobility shifted at a similar distance and similar intensity as that seen with oligonucleotide 2-2. It was speculated that the same protein may be binding both oligonucleotides. The oligonucleotide-protein interactions with these oligonucleotides were studied further in competition assays against specific, and non-specific DNA sequences; and against each other as described in Section II. J. 2 below.
IV. Results

**Figure 55.** Gel Shifts with Lysate from Different *Enterobacteriaceae*

(a) Oligonucleotide 2-2

(b) Oligonucleotide 1-1

1. No protein control
2. CUMC-50, *E. coli*
3. CUMC-214, *E. coli*
4. Misc-341, *E. coli*
5. Misc-345, *E. coli*
6. Kleb-249, *K. pneumoniae*
7. PM-Imp-27, *Proteus mirabilis*
8. Sal-100, *Salmonella* sv. Typhimurim

Red arrow indicates the shift of interest
J. 2. **Competition Gel Shifts**

The binding reactions of oligonucleotide 2-2 and IS26-2 were subjected to competition against excess poly[d(I-C)], excess non-specific oligonucleotide (oligonucleotide 1-2), and excess unlabeled specific oligonucleotides. In these competitions the shift seen with oligonucleotide 2-2 appeared to be a specific interaction as it could only be out competed with specific oligonucleotide 2-2 (Figure 56). This specific competition was seen with all lysates tested (CUMC-50, CUMC-214, Kleb-249, Misc-341, Misc-345). The shift with oligonucleotide IS26-2 however could not be completely outcompeted with 100-fold excess unlabeled oligonucleotide IS2-26, and excess poly[d(I-C)] seemed to compete somewhat with the protein-oligonucleotide interaction (Figure 57). When oligonucleotide 2-2 and oligonucleotide IS26-2 were competed against one another, they had no competitive effect on each other, indicating that the shift patterns for these oligonucleotides were not due to the same protein (Figure 58).
Figure 56. Competition Gel Shifts for Oligonucleotide 2-2 with CUMC-50 Lysate

All reactions contain CUMC-50 lysate at 2 μg/μl, and oligonucleotide 2-2 at 0.5 pmol/μl. NP, no protein control; BBR, basic binding reaction.

Yellow triangle indicates reactions competed with increasing poly [d(I-C)] right to left at 2-, 5-, and 10-fold above basic reaction (100- to 20-fold above labeled oligonucleotide conc.).

Green and pink triangles indicate reactions competed with increasing specific and non-specific oligonucleotide right to left at 10-, 25-, 50-, and 100-fold above labeled oligonucleotide concentration in basic reaction.

Red arrow indicates shift of interest
Figure 57. Competition Gel Shifts for Oligonucleotide IS26-2 with KPVAMC-50 Lysate

All reactions contain KPVAMC-50 lysate at 2 μg/μl, and oligonucleotide IS26-2 at 0.5 pmol/μl. NP, no protein control, BBR, basic binding reaction.

Yellow triangle indicates reactions competed with increasing poly[d(I-C)] right to left at 2, 5, and 10-fold above basic reaction (20- to 100-fold above labeled oligonucleotide conc.).

Blue and pink triangles indicate reactions competed with increasing specific and non-specific oligonucleotide right to left at 10-, 25-, 50-, and 100-fold above labeled oligonucleotide concentration in basic reaction.

Red arrow indicates shift of interest
Figure 58. Oligonucleotides IS26-2 and 2-2 Competition Using KPVAMC-50 and CUMC-50 Lysate

Lanes 1-7 are gel shift reactions of oligonucleotide 2-2 with CUMC-50 lysate competed by increasing amounts of oligonucleotide IS26-2 right to left 6.25- to 100-fold above oligonucleotide 2-2.

Lanes 9-15 are gel shift reactions of oligonucleotide IS26-2 with KPVAMC-50 lysate competed by increasing amounts of oligonucleotide 2-2 right to left 6.25- to 100-fold above oligonucleotide IS26-2.

NP, no protein control;

BBR, basic binding reaction.
K. Isolation and Identification of the Rob Protein

To capture the protein binding oligonucleotide 2-2, biotinylated oligonucleotide 2-2 linked to streptavidin coated magnetic beads were used. The strategy and methods of this are described in detail in Section II. S. and in Figure 37. A 34 kDa protein was captured on the oligonucleotide 2-2-linked beads that was not captured on biotin-only-, or poly[d(I-C)]-linked control beads (Figure 59). The protein band was excised from the SDS-PAGE gel, subjected to mass-spectrometry analysis, and identified as Rob (right origin binding protein). A subsequent review of the literature indicated that a 20 nucleotide sequence in oligonucleotide 2-2 had 64% homology to the 20 nucleotide marbox consensus sequence proposed by Martin et al. (541). Within oligonucleotide 2-2 there is a perfect Rob A-box (GAGC) 13 nt from the 5’ end, and a B-box 8 nt further downstream with 67% homology to the YAA B-box consensus as described by Kwon et al. (537). A 23 bp region of oligonucleotide 2-2 had 56.5% homology with the DNase I mapped Rob binding site at the E. coli fumC promoter (540). These findings indicated that the capture of Rob in these pull-down assays was likely due to a specific interaction of the protein with the oligonucleotide 2-2 DNA sequence.
Figure 59. SDS-PAGE Gel SYPRO Ruby Stain Image of Streptavidin Magnetic Bead Captured Proteins

Protein products separated through a 15% SDS-PAGE. L refers to Bio-Rad low molecular weight ladder; poly[d(I-C)], protein from poly [d(I-C)] linked beads; Con, protein from biotin-linked control beads; 2-2 is protein from oligonucleotide 2-2 linked beads. Arrow points to the 34 kDa protein later identified as Rob.
Using the marbox consensus proposed by Martin et al. (523) and the A and B-Boxes proposed by Kwon et al. (521) Oligonucleotide 2-2 aligned at the marbox A-box sequence in red. There is 64% homology with the specific marbox nucleotides.
L. 6X His-tag Rob Supershift Assays

Gel shift and gel supershift assays were performed to verify that Rob was binding oligonucleotide 2-2. The Rob protein was expressed with an N-6X His-tag from a pET100 vector in BL21 Star™ (DE3) pLys E. coli cells and purified using an Invitrogen Probond™ nickel column. Purity of the eluted 6XHis-Rob was assessed by Coomassie blue staining (Figure 61), and specificity of the anti-6X His tag antibody was evaluated by western blot (Figure 62) and dot blot (Figure 63) of BL21 Star™ (DE3) pLys pET-rob lysate. The His-tag served as an epitope for anti-6X His tag antibody in the supershift assays. Controls in these assays included BL21 pET100 (empty vector) lysate, and BL21 Star™ (DE3) pLys pET-rob post IPTG induction lysate. Supershifts were visible with the purified Rob and BL21 Star™ (DE3) pLys pET-rob lysate, and shifts at the 34 kDa level were more intense for the BL21 Star™ (DE3) pLys pET-rob lysate as would be expected with a combination of chromosomal Rob and the induced 6XHis-Rob being present (Figure 64). No supershifts were visible with BL21 Star™ (DE3) pLys pET100 empty vector lysate as expected. It was notable that not all purified Rob supershifted despite a 1000-fold excess of antibody over protein. This may have been due to the 6X His-tag somehow being obscured when the protein bound to DNA. These assays also showed that the purified Rob produced shifts approximately identical to those where only native Rob was present [i.e. BL21 Star™ (DE3) pLys pET100 empty vector lysate].
IV. Results

**Figure 61.** Coomassie Blue Staining of BL21 Star™ (DE3) pLys Lysate, and ProBond™ Nickel Column Elutions

BL21 Star™ (DE3) pLys Lysate
A) with pET-100 Empty Vector.
B) with pET-100-rob 0.5 mM IPTG Induction.
C) with pET-100-rob 0.5 mM IPTG Induction Lysate post-mix with ProBond™ resin.

BL21 Star™ (DE3) pLys with pET-100-rob after 0.5 mM IPTG Induction Lysate
ProBond™ Nickel Column Imidazole Elutions
1) Elution 1
2) Elution 2
3) Elution 3
4) Elution 4
IV. Results

**Figure 62.** Western Blot of BL21 Star™ (DE3) pLys Lysate, and ProBond™ Nickel Column Elutions

BL21 Star™ (DE3) pLys Lysate

A) with pET-100 Empty Vector.
B) with pET-100-rob after 0.5 mM IPTG Induction.
C) with pET-100-rob after 0.5 mM IPTG Induction Lysate post-mix with ProBond™

ProBond™ Nickel Column Imidazole Elutions of BL21 Star™ (DE3) pLys with pET-100-rob Lysate after 0.5 mM IPTG Induction

1) Elution 1
2) Elution 2
3) Elution 3
4) Elution 4
Figure 63. Dot Blot of Lysate of BL21 Star™ (DE3) pLys with pET-100-rob and with pET-100 Empty Vector

A) Lysate of BL21 Star™ (DE3) pLys with pET-100-rob after 0.5 mM IPTG induction.

B) Lysate of BL21 Star™ (DE3) pLys with pET-100 empty vector.
**Figure 64.** Gel Supershift Assays with purified 6X His-Rob, BL21 Star™ (DE3) pLys pET-100 Empty Vector Lysate, and BL21 Star™ (DE3) pLys pET-rob Lysate

Reactions used purified 6XHis-Rob at 3000 fmol/µl or lysate at 4 µg/µl (final concentrations) or Equal volumes of dialysis fluid (5 mM Tris-HCl pH 8.0) or Non-Reducing Sonication Lysis Buffer.

A) Oligo 2-2, no protein (Lysis buffer or dialysis fluid), no antibody.

B) Oligo 2-2, no protein, anti-6XHis antibody

C) Oligo 2-2, protein or lysate, no antibody

D) Oligo 2-2, protein or lysate, with anti-6X His tag antibody added AFTER protein and oligonucleotide binding incubation

E) Oligo 2-2, protein, anti-6XHis tag antibody added to reaction BEFORE addition of oligonucleotide and binding incubation

Orange arrow indicates Oligo 2-2 supershift. With 6XHis-Rob and anti-6XHis tag antibody

Green arrow indicated Oligo 2-2 shift with Rob or 6XHis-Rob
IV. Results

M. Rob Activation Immunofluorescence Microscopy Studies

Past immunofluorescence microscopy studies with Rob specific antibodies have shown that in *E. coli*, Rob is found in clusters of 3-4 foci per cell (555). Work by Griffith *et al.* have presented evidence that Rob exhibits a sequestration-dispersal mechanism of activation (554). Under oxidative stress conditions created by the addition of 4,4’-dipyridyl, they found that a chimeric SoxS-Rob protein disperses from its foci and binds DNA to become a transcription factor (See Section I. F.) (554). Given that β-lactam antibiotics have been found to create oxidative stress conditions in *E. coli* it was hypothesized that these drugs activate (disperse) Rob as well (578). To test this hypothesis BL21 Star™ (DE3) pLys pET-rob *E. coli* were grown in broth culture without IPTG induction and without ampicillin selection to avoid excessive 6XHis-Rob expression and β-lactam activation. The cultures were then treated in early log phase (OD600 0.3) with either a PBS, piperacillin/tazobactam, ceftazidime, or 4,4’-dipyridyl. β-lactam drugs were added to the flask cultures at a dose equal to the minimal inhibitory concentration for the BL21 Star™ (DE3) pLys pET-rob *E. coli* cells.

Dispersal of 6XHis-Rob appeared visible 10 to 15 minutes after the addition of MIC concentrations of ceftazidime and piperacillin/tazobactam (Figures 66 and 67). With ceftazidime treatment, the cells also began to appear elongated at the time of activation. This is a recognized effect of ceftazidime as the drug binds PBP3 in *E. coli*; a protein necessary for cell septa formation and cell division (579). These findings served as a visual internal control that the drug was physiologically affecting the bacteria. After
30 minutes, the 6XHis-Rob protein appeared to begin a return to foci. This was something not previously reported in Rob activation studies by Griffith et al (554).

As expected, PBS treatment alone had no effect on Rob dispersal (Figure 65). When cells were treated with 5 mM 4,4´-dipyridyl, as had been done by Griffith et al., 6XHis-Rob appeared to disperse immediately at time zero (data not shown) (554). Whereas lower concentrations of 4,4´-dipyridyl (2.5 mM and 0.5 mM) appeared to have little or no discernible dispersal effect on 6XHis-Rob in the BL21 Star™ (DE3) pLys pET-rob cells (Figure 68).
Figure 65. (a) Alexa Fluor® 568 Immunofluorescence Staining of 6XHis-Rob in BL21 Star™ (DE3) pLys pET-rob Cells before and after Exposure to PBS Only

For figures 62-66, microscope fields are at 1000 × and photographed under 400 nm light excitation filter for DAPI, and 560 nm filter for Alexa Fluor® 568
Figure 65. (b) DAPI Staining of BL21 (DE3) pLys pET-rob Cells before and after Exposure to PBS Only
Figure 66. (a) Alexa Fluor® 568 Immunofluorescence Staining of 6XHis- Rob in BL21 Star™ (DE3) pLys pET-rob Cells before and after Ceftazidime Exposure at MIC
Figure 66. (b) DAPI Staining of BL21 Star™ (DE3) pLys pET-rob Cells before and after Ceftazidime Exposure at MIC
Figure 67. (a) Alexa Fluor® 568 Immunofluorescence Staining of 6XHis-Rob in BL21 Star™ (DE3) pLys pET-rob Cells before and after Piperacillin/Tazobactam Exposure at MIC
Figure 67. (b) DAPI Staining of BL21 Star™ (DE3) pLys pET-rob Cells before and after Piperacillin/Tazobactam Exposure at MIC
Figure 68. (a) Alexa Fluor® 568 Immunofluorescence Staining of 6XHis-Rob in BL21 Star™ (DE3) pLys pET-rob Cells before and after 2.5 mM 4,4’-Dipyridyl Exposure
Figure 68. (b) DAPI Staining of BL21 Star™ (DE3) pLys pET-rob Cells before and after 5 mM 4, 4′-Dipyridyl Exposure
N. Effects of Piperacillin/Tazobactam Exposure on \textit{bla}_{CMY-2} Expression

Attempts were made to clone the \textit{bla}_{CMY-2} gene and its promoter sequences into the pET100-\textit{rob} vector and examine the affects that 6XHis-Rob dispersal had on \textit{bla}_{CMY-2} expression. Unfortunately these cloning attempts were unsuccessful. Instead, early log phase cultures of CUMC-50 were exposed to piperacillin/tazobactam at minimal inhibitory concentration, and timed samples were taken over a period of 30 minutes. No changes in \textit{bla}_{CMY-2} transcript level greater than two-fold were seen after the addition of piperacillin/tazobactam, and no significant differences were seen between piperacillin/tazobactam and PBS only treated cultures at the different time points (Table 16).

\textbf{Table 16.} CUMC-50 \textit{bla}_{CMY-2} Transcript Level following Treatment with Piperacillin/Tazobactam at MIC and PBS Only

<table>
<thead>
<tr>
<th>Time Sample</th>
<th>Ave RQ Value</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus 1 PBS</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Minus 1 TZP</td>
<td>1.11</td>
<td>0.37</td>
</tr>
<tr>
<td>Time 0 PBS</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Time 0 TZP</td>
<td>0.88</td>
<td>0.25</td>
</tr>
<tr>
<td>Time 15 min PBS</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Time 15 min TZP</td>
<td>1.24</td>
<td>0.54</td>
</tr>
<tr>
<td>Time 30 Min PBS</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Time 30 Min TZP</td>
<td>0.92</td>
<td>0.21</td>
</tr>
</tbody>
</table>
IV. Discussion

A. Purpose of this Study

Piperacillin/tazobactam is an important first line drug for treating healthcare-associated infections. It has activity against a broad range of Gram-negative pathogens that produce different types of extended-spectrum β-lactamases. Loss of piperacillin/tazobactam from the β-lactam armamentarium incurs a greater reliance on the last remaining β-lactams, the carbapenems; a class that is already being threatened by carbapenem resistant strains. Finding the mechanisms by which Enterobacteriaceae become resistant to piperacillin/tazobactam is an important step toward curbing the loss of this drug and reducing pressure on the carbapenems.

The concept for the piperacillin/tazobactam mutant studies in this dissertation came from anecdotal reports of piperacillin/tazobactam resistant E. coli and Klebsiella pneumoniae being recovered from patients after the initiation of piperacillin/tazobactam therapy. Similar observations were reported in a study by DiNubile et al., who found that patient carriage of piperacillin/tazobactam resistant Enterobacteriaceae increased from 0.6% to 9.3% after a median of 6 days of piperacillin/tazobactam therapy (242). These authors did not evaluate the mechanisms associated with the increased prevalence of resistant isolates.

About the time these reports were published, Mark Reisbig who was a student in the laboratory of Nancy Hanson at Creighton University had observed a piperacillin/tazobactam resistant mutant, CUMC-50M, that was selected from a piperacillin/tazobactam susceptible clinical E. coli strain, CUMC-50 (Figure 39).
V. Discussion

CUMC-50 carried a plasmid-encoded $bla_{CMY-2}$ AmpC β-lactamase gene. CMY-2 is an enzyme that can enable resistance to piperacillin/tazobactam, and it was found that CUMC-50M had increased $bla_{CMY-2}$ expression 13-fold over its parent. It was hypothesized that piperacillin/tazobactam susceptible *E. coli* with $bla_{CMY-2}$ gain piperacillin/tazobactam resistance through an increase in $bla_{CMY-2}$ expression. Such increases could explain the resistant strains that were being reported clinically.

**B. Piperacillin-tazobactam Resistant Mutant Studies.**

To test the hypothesis that increased CMY-2 transcription was the mechanism associated with the piperacillin/tazobactam resistant phenotype, CUMC-50 and three other piperacillin/tazobactam susceptible $bla_{CMY-2}$ carrying clinical *E. coli* strains were exposed to superinhibitory concentrations of piperacillin/tazobactam. Resistant mutants emerged and were evaluated for $bla_{CMY-2}$ expression. Only two strains produced mutants that had increased $bla_{CMY-2}$ expression over their parent strain. Overall only 33% (4 of 12) of the mutant strains tested exhibited $bla_{CMY-2}$ overexpression; so the initial hypothesis was not supported for all resistant mutants. Resistance mechanisms of the mutant strains that did not overexpress CMY-2 were not evaluated, but their resistance phenotype may be due to decreased permeability through porin loss or increased efflux expression.

For all four $bla_{CMY-2}$ overexpression mutants, the increase in $bla_{CMY-2}$ expression was associated with an increase in plasmid copy number as demonstrated by Southern blots of isolated plasmids and $bla_{CMY-2}$ gene copy number studies. The mutants of strain CUMC-50 were studied in depth, and it was found that the IncI1 plasmid on which $bla_{CMY-2}$ was encoded on had increased copy number in the mutants to a level equal to
their increase in \( \text{bla}_{\text{CMY-2}} \) expression. Two mutants with the largest increases in copy number possessed mutations in the \( inc \) antisense RNA gene necessary for IncI1 plasmid copy number control. This is the first time that \( inc \) mutations had been identified as associated with increases in copy number for a 100 kb plasmid (88). Mutant strain CUMC-50M8, which exhibited an 8-fold increase in IncI1 plasmid copy number, had a G to T transversion at \( inc \) nucleotide 30. This same mutation was evaluated in a previous study by Asano \textit{et al.}, where it was predicted to affect stem loop shapes of Inc and SL1, and was associated with a 9-fold increase in plasmid copy number for a ColIB-P9 (IncI1) mini-plasmid construct (479). Strain CUMC-50M possessed an A to G transition at \( inc \) nucleotide 38 and had a 13-fold increase in plasmid copy number. This mutation had not previously been evaluated for its effect on IncI1 copy number. However, Asano \textit{et al.} did find this mutation was associated with a 3-fold increase in the dissociation of Inc and SL1 nucleotides involved in the formation of the kissing complex (479). Like the CUMC-50M8 mutation, the CUMC-50M mutation was predicted to affect the shape of the Inc stem loop structure. Taken together, these findings indicate that the mutation in CUMC-50M would likely impede formation of the inhibition complex and allow for increased plasmid copy number to occur. Strain CUMC-50M2 had a 3-fold higher IncI1 plasmid copy number but had no mutations in \( inc \) sequence. This strain was also the slowest growing CUMC-50 mutant strain. One hypothesis to explain the increase in plasmid copy number in CUMC-50M2 is that there was a mutation in the cell replication mechanism that slowed the rate of chromosomal replication, but did not affect the IncI1 plasmid replication rate, thus allowing the IncI1 plasmid to accumulate in the cell.
The slow growth of the CUMC-50 \( \text{bla}_{\text{CMY-2}} \) mutants in this study should not be overlooked. A number of mutations that enhance bacterial resistance to antibiotics can come at the cost of growth fitness (580). In an environment where other \( E. \ coli \) strains are present and no \( \beta \)-lactam pressure exists, these plasmid copy number mutants may not be able to compete strongly for resources. An interesting finding in the study by DiNubile \textit{et al.} was that two weeks after the cessation of piperacillin/tazobactam therapy, the percent of patients carrying piperacillin/tazobactam resistant \textit{Enterobacteriaceae} dropped from 9.3 \% to 2.6 \%, indicating that resistant strains may not have been able to compete without selection pressure (242). Whereas in a similar study with ceftriaxone treatment, the percentage of patients carrying ceftriaxone resistant \textit{Enterobacteriaceae} actually increased two weeks after treatment (242). The piperacillin/tazobactam mutants of strains CUMC-201 and CUMC-243 had no increase in \( \text{bla}_{\text{CMY-2}} \) expression and did not appear to have slower growth rate than their parent strains. So whatever mechanism enabled piperacillin/tazobactam resistance in these strains, it was not a detriment. These mutants deserve closer examination.

It remains to be seen if the \textit{inc} mutations observed in this study are occurring clinically. IncI1 accounts for about half of all the \( \text{bla}_{\text{CMY-2}} \) carrying plasmids in European and North American studies (404, 430, 432). To date, the presence of increased IncI1 plasmid copy number has not been reported clinically in \textit{Enterobacteriaceae}. And with the exception of a ColIb-P9 cloning construct deposited in GeneBank by Hama \textit{et al} (accession M34837), no IncI1 plasmids with \textit{inc} loop region point mutations are currently reported in GenBank. It should be noted that replicon typing of resistance plasmids in \textit{Enterobacteriaceae} is still not routinely done in most studies of plasmid-encoded
β-lactamase resistance, and plasmid copy number evaluations are almost non-existent, so inc mutations may be occurring but are not being recognized. One study that did evaluate the replicon types of plasmids bearing bla\textsubscript{CMY-2} in \textit{E. coli} isolated from Canadian ICU patients, found IncI1 plasmids to be one of the most common bla\textsubscript{CMY-2} bearing plasmids in their study (9 of 26) (404). All isolates in the study were susceptible to piperacillin/tazobactam, indicating that inc mutations like those seen in the present study were not present.

It is plausible that piperacillin/tazobactam resistance mutations like those seen in this study can be selected clinically. The levels of piperacillin used to generate the mutants (8 – 128 µg/ml), and the number of \textit{E. coli} CFU in the denominator of the mutation rates (1×10\textsuperscript{-5} to 1×10\textsuperscript{-7}) are not beyond that which may be achieved in a patient. Even though not all the resistance mechanisms were elucidated in the mutants, the production of CMY-2 was likely critical to piperacillin/tazobactam resistance in all the mutants observed. Currently there are no CLSI guidelines for detecting plasmid-encoded AmpC β-lactamases like CMY-2 in \textit{Enterobacteriaceae}. This is a potential problem. Most clinicians treat infections based on phenotypic drug susceptibility. Since all parent strains in this study were susceptible to piperacillin/tazobactam, as are up to 91.6% of AmpC producing \textit{E. coli}, it suggests that the drug would be suitable for treatment of infections caused by these strains (243). Such treatment could select for piperacillin/resistant mutants, perhaps not unlike the findings of DiNubile \textit{et al.}, or the anecdotal clinician reports that initiated this study (242).

One optimistic finding in this work was that carbapenem resistance was not so easily selected from parent strains or piperacillin/tazobactam-resistant mutant strains. In
the meropenem mutation studies that were conducted, only strains CUMC-214 and CUMC-214M1 yielded a small number of mutants. Only one of these mutants (CUMC-214M1-MerA) achieved clinical meropenem insusceptibility. None of the meropenem mutants demonstrated changes in \( \text{bla}_{\text{CMY-2}} \) copy number, indicating that some other mechanism was responsible for the development of carbapenem susceptibility changes. A reduction in porin production is a likely cause. Published studies have demonstrated losses in the expression of porins OmpC and OmpF are associated with carbapenem-resistance in \( \text{bla}_{\text{CMY-2}} \) producing \( E. \text{coli} \) (391, 581). Multiple copies of \( \text{bla}_{\text{CMY-2}} \) might also be required for these porin deficiencies to be meaningful. The CUMC-214M1-MerA mutant was estimated to have 11 copies of \( \text{bla}_{\text{CMY-2}} \). In experimental studies by Mammeri et al., a high copy number pUC origin plasmid was used to express \( \text{bla}_{\text{CMY-2}} \) and generate carbapenem resistance in porin deficient \( E. \text{coli} \) (300).

B. Tandem \( \text{bla}_{\text{CMY-2}} \)

Another interesting finding in this work was the discovery of a divergent tandem \( \text{bla}_{\text{CMY-2}} \) arrangement surrounding an IS5 insertion sequence element. This was the first time that such an arrangement has been reported (558). Strains CUMC-201 and CUMC-243 carried this arrangement on a 150 kb plasmid. The strains were isolated in two different healthcare facilities, and both strains appear to have related but distinct (4-band difference) PFGE types which indicates that the strains had diverged, and the tandem \( \text{bla}_{\text{CMY-2}} \) arrangement probably existed for some time prior to being isolated.

IS5 has been known to create multiple tandem genes when two IS5 elements surround a gene (532, 533). The \( \text{bla}_{\text{CMY-2}} \) genes in strains CUMC-201 and CUMC-243 do
not appear to be surrounded by IS5 as outward PCR amplifications did not detect an additional IS5 element in close proximity. Also, gene amplifications caused by flanking IS5 elements do not typically cause divergent arrangements (Herve Nicoloff, personal communication).

IS5 mediated gene amplification is presumed to occur through homologous recombination (532, 533). The nature of these recombinations is unknown. IS5 sequence does have a putative chi recombination site 5’CATGGTGG-3’ at nucleotides 176-183 (P. Kurpiel unpublished observation) which may play a role in recombination. Two $bla_{CMY-2}$ genes with divergent upstream IS5 arrangements likely joined through a homologous recombination event to create the arrangement found in CUMC-201 and CUMC-243 (see Figure 69). What is concerning is that if an IS5 element were to insert itself flanking in close proximity to the divergent tandem arrangement in these strains, then an IS5 mediated tandem $bla_{CMY-2}$ amplification could be possible. Such amplification may provide additional CMY-2 production with less detriment to the host than the plasmid amplifications seen in the CUMC-50 family mutants as observed by their estimated growth curves.
Figure 69. Proposed Formation of the Divergent Tandem $bla_{CMY-2}$ Arrangement through Homologous Recombination


C. Rob Binding $bla_{CMY-2}$ Promoter Region in ISEcp1

In an effort to determine what proteins were assisting $bla_{CMY-2}$ transcription, the transcription factor Rob was captured binding to sequence located upstream of the distal $bla_{CMY-2}$ promoter within ISEcp1. This is the first time that a transcription factor has been found binding to sequence upstream of $bla_{CMY-2}$, or any ISEcp1 promoted $\beta$-lactamase gene. The implications of this are interesting. Rob is known to be activated under conditions of oxidative stress, and it can upregulate $E. coli$ genes whose products respond to oxidative stress and govern cell permeability (292, 321, 553, 554). $\beta$-lactam drugs have been found to induce oxidative stress, so to have a $\beta$-lactamase gene under the influence of Rob would provide the host $E. coli$ with a means to express the $\beta$-lactamase at a time when it is needed most (578). This could potentially explain why the ISEcp1 promoted $\beta$-lactamas $es$ CMY-2 and CTX-M-14, and CTX-M-15 are the most common plasmid-encoded AmpC and extended-spectrum $\beta$-lactamases in the world.

Griffith et al. have provided evidence that Rob resides in insoluble cellular clusters but disperses upon stimulus to behave as a transcription activator (554). Immunofluorescent microscopy experiments in this dissertation support the findings of Griffith et al. Dispersal of 6XHis-Rob in $E. coli$ BL21 Star™ (DE3) pLys cells was observed following exposure to minimal inhibitory concentrations of ceftazidime and piperacillin/tazobactam. The dispersals appeared to be most evident in cells collected 10 to 15 minutes after administration of the drug. An interesting finding in these experiments was that 6XHis-Rob appeared to return to clusters 30 minutes after drug inoculation. This had not been reported by Griffith et al. The findings are intriguing and may add new
insight into the function of Rob. The evolving biological picture of Rob indicates that it is a rapid response protein that disperses from clusters upon a stimulus to bind to promoter region marbox sequences and influences transcription of genes whose products help protect the cell. Its role may be to simply maintain transcription for a short length of time, allowing MarA or SoxS to be translated and bind to the same marbox sequence with greater affinity and specificity for continued activation of gene transcription.

Expression studies were carried out to evaluate $bla_{CMY-2}$ transcript level changes in *E. coli* strain CUMC-50 after treatment with minimal inhibitory concentrations of piperacillin/tazobactam. Only a 0.24-fold average increase in expression was seen at 15 minutes post-treatment. Our laboratory typically uses a 3-fold change as a benchmark for notable transcription level difference. However, most marbox controlled genes have less than 3-fold increase under oxidative stress conditions (582). This calls into question what the true role of MarA, SoxS, and Rob may be for marbox promoters. It is possible that these proteins only provide slight increases in expression or maintain basal levels of expression of genes whose products help the cell respond to oxidative and acid stress conditions. This would allow the cell to respond to environmental stress, but not overburden it with dramatic protein production. This kind of transcriptional maintenance rather than a transcriptional increase may be what is occurring at the distal $bla_{CMY-2}$ promoter within ISEcp1.

It also may be that the marbox identified upstream of the distal promoter in ISEcp1 simply has no effect on $bla_{CMY-2}$ expression. This marbox is different from published functional Rob binding sites, so its functionality is uncertain. The ISEcp1 marbox is a cross between a Class I and a Class II marbox. It is located upstream of the -
35 sequence, like a Class I marbox, but the sequence is aligned in a forward position like a Class II marbox. The zwf promoter has a marbox similar to this, and is sometimes referred to as a “Class I*” marbox (318). The difference is the zwf primary marbox is located -7 bp upstream of the -35 sequence, while the IS\textit{Ecp1} marbox is approximately -48 bp upstream of the -35 sequence. Marboxes have been recognized at this distance previously in other promoter configurations. In particularly, the \textit{micF} and \textit{zwf} promoters have “secondary” marboxes at approximately this same distance and orientation, but their functionality has not been fully examined (551, 583). One of the most distant primary marbox sites recognized to date has been the Class II marbox at the \textit{acrAB} promoter which lies -27 bp upstream of the -35 sequence. Interestingly, \textit{acrAB} is one of the genes for which Rob appears to provide strong transcriptional upregulation, and MarA and SoxS have little or no influence (321). If you consider that SoxS and MarA seem to chaperone RNA polymerase to the promoter, then bringing the polymerase -70 bp or more away from the transcriptional start site may not be helpful (543). Rob on the other hand, is postulated to first bind and bend DNA to make the promoter more favorable for RNA polymerase binding (540, 543). It is quite possible that Rob has influence at the distal \textit{bla}_{\text{CMY-2}} promoter, but further experimentation will be needed to provide evidence of this.

Lastly, it needs to be considered that MIC levels of piperacillin/tazobactam used in the work presented here may not activate Rob in CUMC-50 as seen with 6XHis-Rob in BL21 Star\textsuperscript{™} (DE3) pLys cells. In fact, it must be kept in mind that the Rob sequestration-dispersal activation model proposed by Griffith \textit{et al} is based on a SoxS-Rob chimera, and the work presented here used a N-6XHis-tagged Rob protein. Both of these models
may not represent the true behavior of native Rob. Without an available anti-Rob antibody, the true nature of native Rob remains speculative, and the concentration of piperacillin/tazobactam required to disperse native Rob in CUMC-50 remains unknown.
VI. Future Studies

I.  *bla<sub>CMY-2</sub>* Copy Number Changes in *E. coli* Isolated from Patients

The increased plasmid copy number mutations observed in this work raise the question of whether such mutations are occurring in *E. coli* colonizing/infecting patients during therapy. One approach to answer this question would be through a cohort study of ICU patients receiving piperacillin/tazobactam. Similar to the study by DiNubile *et al.*, enrolled patients could be followed and cultured at regular intervals after treatment to evaluate changes in the β-lactam susceptibility of the *E. coli* colonizing or infecting them (242). Isogenic strains that exhibit changes in the β-lactam susceptibility could be evaluated using genomic and proteomic methodologies to determine the “resistome” of the sequential isolates. In other words, in addition to evaluating β-lactamase expression and plasmid copy number, changes in porin and efflux pump production should be evaluated as well. This way, the contribution of each resistance mechanism in a given strain can be assessed. Knowing the full scope of resistance mechanisms at work will help guide solutions to curbing them.

II. Rob Influence on ISEcp1 Promoted β-lactamases

A number of questions about the Rob protein still remain to be settled. Perhaps most importantly, the dispersal activation of native Rob needs to be confirmed. To do this, experiments utilizing a specific anti-Rob antibody need to be conducted. The antibody would allow Rob activation to be visualized in clinical *E. coli* strains, not just
laboratory constructs. Different concentrations of β-lactam drug and different isolation time points can be assessed. If Rob activation is established in these experiments, then a fundamental understanding of the role that Rob plays in the expression of IS\textit{Ecp1} promoted β-lactamase genes can begin. Isogenic clones with deletions and combination deletions in MarA and SoxS and Rob could be constructed similar to those made by Rosenberg \textit{et al.} to evaluate the impact each transcription factor has at the IS\textit{Ecp1} marbox for promotion of \textit{bla}_\text{CMY-2} or other downstream β-lactamase genes (321). If deletion of Rob is found to have an impact on β-lactam sensitivity of \textit{E. coli} with IS\textit{Ecp1} promoted β-lactamase genes, then experiments can begin to find ways to interfere with Rob activity and perhaps regain the potency of β-lactam drugs against these strains.
VII. Bibliography


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