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Directed molecular evolution of fourth-generation cephalosporin resistance in

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Directed molecular evolution of fourth-generation cephalosporin resistance in
Salmonella and *Yersinia*

by

Wellington Moore

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Biomedical Science (Pharmacology)

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Iowa State University

Ames, Iowa

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ABSTRACT

Cefepime is a fourth-generation cephalosporin approved by the FDA in 1996 (Barlow 2003). Few instances of cefepime resistance have been reported. This research used directed molecular evolution involving error-prone rolling circle amplification on plasmids containing the beta-lactamase gene designated as *bla-cmy-2*. This gene is believed to be responsible for the majority of the nontyphoidal *Salmonella enterica* cephalosporin antimicrobial resistance in this country (Daniels 2007). Plasmids were removed from *Salmonella* and *Yersinia* bacteria exhibiting resistance to the following third-generation cephalosporins: ceftiofur, cefotaxime, cefixime, ceftriaxone, and ceftazidime. The goals of this research were to identify *bla-cmy-2* mutations that confer cefepime resistance. Error-prone rolling circle amplification yielded a mutation encoding cefepime resistance. This mutation altered the secondary structure in the region of the H-10 helix, which is a part of the catalytic site for extended-spectrum beta-lactamases. In summary, *bla-cmy-2* mutations are sources of resistance for extended-spectrum beta-lactamases that can use fourth-generation cephalosporins as substrates in *E. coli*.

CHAPTER 1. INTRODUCTION

Review of beta-lactam antimicrobials

One of the first discovered antimicrobial agents was penicillin, which was isolated by Alexander Fleming in 1928 from *Penicillium notatum*. Penicillin was later discovered to be a beta-lactam drug. Beta-lactam drugs contain a four-member ring as part of their structure. This ring is referred to as the beta-lactam ring (Neu 1986). The bactericidal mode of action of beta-lactams is to bind to and inhibit penicillin-binding proteins (PBPs).

PBPs participate in the synthesis of the bacterial cell wall by catalyzing the maturation and cross-linking of peptidoglycans (Atrih 1999). PBPs are located on the outer surface of the cytoplasmic membranes of bacteria (Chalut 2001). Although all bacteria have these PBPs, there are significant species-dependent variations in their number and morphology. These variations lead to different levels of affinity between species of bacteria for beta-lactam antibiotics (Moya 2009). PBPs have several known enzymatic functions including peptidoglycan transpeptidase activity. The enzymatic function of peptidoglycan transpeptidase is to cross-link the peptidoglycans to form the bacterial cell wall (Mainardi 2005). PBPs are essential for cell survival and are the target of beta-lactam antibiotic therapy. Other enzymatic functions of PBP include carboxypeptidase and endopeptidase activities. Carboxypeptidase function is not fully understood and may be non-essential for bacterial life (Nelson 2001, Ghosh 2008). However, there is research indicating that the enzymatic functions of carboxypeptidase and endopeptidase may be responsible for the overall cell shape (Meberg 2004). When PBPs are identified in bacteria, they are labeled or numbered by their size, which can range from 35 to 120 kDa (Peddi 2009). Using their size, they are divided into low-molecular-mass and high-molecular-mass PBPs (Peddi 2009). The high-molecular-mass PBPs are more likely to have peptidoglycan transpeptidase activity (Peddi 2009).

Several different families of structures are referred to as beta-lactams antibiotics. Note the following structures:

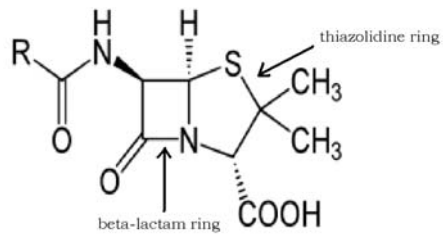


Figure 1. Penicillin, the first beta-lactam drug discovered, consists of a beta-lactam ring and a thiazolidine ring. Side chains attach at “R.”

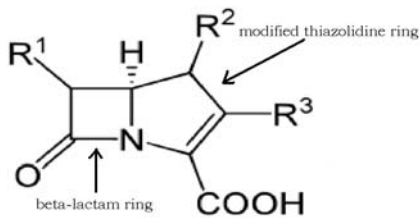


Figure 2. Carbapenems consist of a beta-lactam ring and a modified thiazolidine ring. Carbapenems have three side chains: R¹, R², and R³.

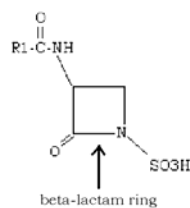


Figure 3. Monobactams consist of a solo beta-lactam ring and one side chain, R¹.

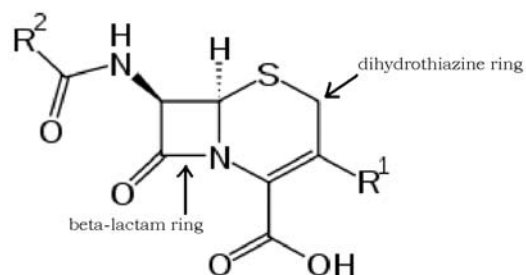


Figure 4. Cephalosporins consist of a beta-lactam ring and a dihydrothiazine ring. Cephalosporins have two side chains: R¹ and R².

The cephalosporin group of beta-lactams was the focus of this research, and thus, they will be emphasized henceforth.

Review of Cephalosporins

Cephalosporin antibiotics originated with the 1945 discovery of *Cephalosporium acremonium* in the sewers of the Mediterranean island of Sardinia (Abraham 1979).

From this fungus, dozens of antimicrobial agents have arisen.

There are five generations of cephalosporin antibiotics currently described. The synthesis of many of them starts with a 7-aminocephalosporanic acid (7-ACA) molecule (Nigam 2005).

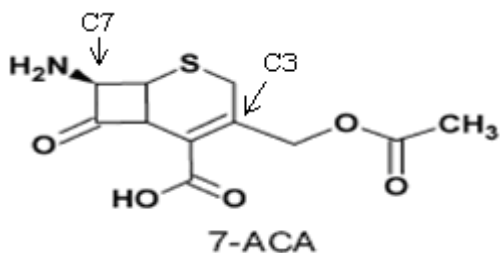


Figure 5. 7-ACA.

7-ACA is synthesized by deacylation of cephalosporin-C (Nigam 2005). Cephalosporin-C was the first cephalosporin isolated. The cephem nucleus consists of a

beta-lactam ring and a dihydrothiazine ring. Side chains that impart individual and unique bactericidal spectral and beta-lactamase resistance characteristics are attached at C-3 and C-7 (Neu 1986). The generational designation of different cephalosporins reflects the spectrum of activity. The first-generation cephalosporin spectrum includes gram-positive organisms (Merk 2009). The second-generation cephalosporin spectrum includes fewer gram-positive organisms and more gram-negative organisms than the first-generation cephalosporin spectrum. The second-generation drugs also have some activity against anaerobes (Merk 2009). The third- and fourth-generation cephalosporin spectra include more gram-negative organisms and fewer gram-positive organisms than the second-generation cephalosporin spectrum. They are recommended clinically for polymicrobial infections caused by gram-negative bacilli and gram-positive cocci (Merk 2009). Table 1 summarizes the generation of cephalosporins including examples and spectra.

Table 1. Drugs from the four generations of cephalosporins and their spectra of activity

First-generation cephalosporins

Cefacetrile
Cefadroxil
Cephalexin
Cefaloglycin
Cefalonium
Cefaloridine
Cefalotin
Cefapirin
Cefatrizine
Cefazaflur
Cefazedone
Cefazolin
Cefradine
Cefroxadine
Ceftezole

Spectrum

Gram-positive:

Penicillinase-producing
methicillin-susceptible staphylococci
and streptococci

Gram-negative:

Including *Proteus mirabilis*,
some *Escherichia coli*, and
Klebsiella pneumoniae

Second-generation cephalosporins

Cefaclor
Cefonicid
Cefprozil
Cefuroxime
Cefuzonam
Cefmetazole
Cefotetan
Cefoxitin

Spectrum

Gram-positive:

Less than first-generation cephalosporins

Gram-negative:

In addition to first-generation spectrum
Haemophilus influenzae,
Enterobacter aerogenes,
and some *Neisseria*

Third-generation cephalosporins

Cefcapene
Cefdaloxime
Cefdinir
Cefditoren
Cefetamet
Cefixime
Cefmenoxime
Cefodizime

Spectrum

Gram-positive:

Some third-generation drugs are
less than first- and second-
generation cephalosporins

Gram-negative:

3rd generation have broader spectrum than 1st
or 2nd generation cephalosporins;

Table 1 continued

Cefotaxime
 Cefovecin
 Cefpimizole
 Cefpodoxime
 Cefteram
 Cefibuten
 Ceftiofur
 Ceftiolene
 Ceftizoxime
 Ceftriaxone
 Cefoperazone
 Ceftazidime

Including *E. coli*, *Klebsiella*, penicillin-resistant *N. gonorrhoeae* and meningitis caused by, pneumococci, meningococci, *H. influenzae*

**Fourth-generation
 cephalosporins**

Cefclidine
 Cefepime
 Cefluprenam
 Cefoselis
 Cefozopran
 Cefpirome
 Cefquinome

Spectrum

Gram-positive:

Similar to first-generation cephalosporins

Gram-negative:

Increase over all spectrum, including CNS infections and *Pseudomonas aeruginosa*

Table 2. Cephalosporin drugs with similar and dissimilar side chains at C-7**Cephalosporin drugs with similar side chains at C-7**

	Group 1	Group 2	Group 3
Generations	↓	↓	↓
First	Cefalotin	Cephradine Cephalexin Cefadroxil	
Second	Cefoxitin	Cefaclor	
Third			Ceftizoxime Cefotaxime Cefpodoxime Ceftriaxone
Fourth			Cefepime Cefpirome

Cephalosporin drugs with dissimilar side chains at C-7

Generations	
First	Cefazolin
Second	Cefotetan Cefuroxime Cefprozil Cefmetazole
Third	Cefoperazone Cefdinir Cefditoren Cefixime Ceftibuten Ceftazidime

(source: Michael E. Pichichero, MD; Cephalosporins can be prescribed safely for penicillin-allergic patients; Journal of Family practice Feb 2006. vol.55, No.2)

Table 3. Cephalosporin drugs with similar and dissimilar side chains at C-3
ibidCephalosporin drugs with similar side chains at C-3

Generations	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
First	↓ Cefadroxil Cephalexin	↓	↓	↓	↓	↓
Second		Cefmetazole Cefotetan			Cefuroxime Cefoxitin	
Third		Cefoperazone	Cefotaxime Cefalotin	Ceftibuten Ceftizoxime		Cefdinir Cefixime
Fourth						

ibidCephalosporin drug with dissimilar side chains at C-3

Generations	
First	Cefazolin
Second	Cefprozil Cefaclor
Third	Cefpodoxime Ceftibuten Ceftriaxone Ceftazidime
Fourth	Cefepime Cefpirome

Cephalosporin bactericidal activity results from a similarity in the structure of the beta-lactam ring and the D-alanyl-D-alanine extremity of nascent peptidoglycan (Mainardi 2005). This similarity in structure creates the competitive inhibition of PBPs, which facilitate the maturation and linking of peptidoglycans in bacterial cell wall synthesis (Mainardi 2005). This results in the inhibition of bacterial wall synthesis. Later generations of cephalosporins target gram-negative bacteria more than the gram-positive bacteria.

The third-generation cephalosporins used in this research include ceftiofur, cefotaxime, cefixime, ceftriaxone, and ceftazidime (Figs 6–11). Large side chains similar

to oxyimino moieties may be responsible for some of the increased resistance third-generation cephalosporins exhibit to enzymatic destruction by beta-lactamases.

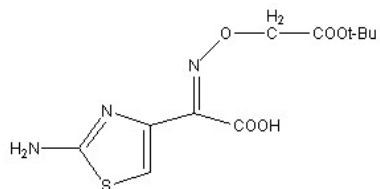


Figure 6. Oxyimino-acetic acid.

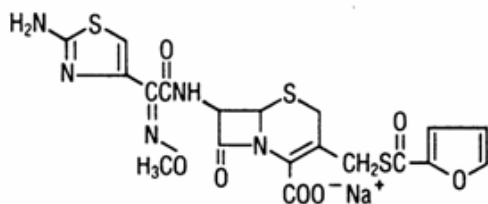


Figure 7. Cefotiofur sodium.

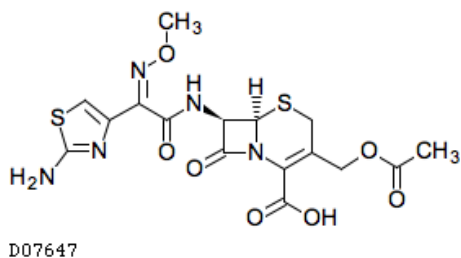


Figure 8. Cefotaxime.

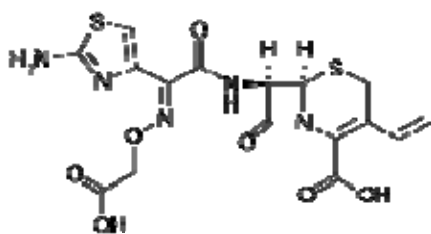


Figure 9. Cefixime.

Cefixime is considered the first third-generation oral cephalosporin (Roche 1989). The structural modification that enabled *per os* administration was the addition of a vinyl group at C-3 (Roche 1989). The third-generation cephalosporin antimicrobial

characteristics possessed by cefixime result from an aminothiazole ring and an R-oxyimino group at C-7 (Roche 1989). Cefixime has been clinically recommended for the treatment of lower respiratory tract, ear-nose-throat, and urinary tract infections (UTIs) (Roche 1989). Cefpodoxime is the only other oral third-generation cephalosporin, and it is structurally similar to cefixime.

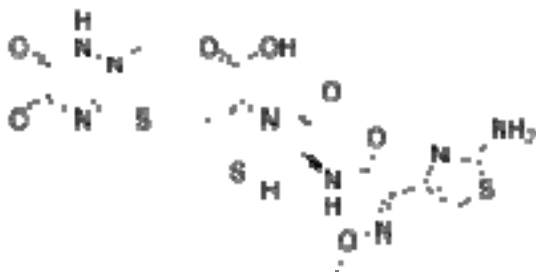


Figure 10. Ceftriaxone.

Ceftriaxone has an oxyimino group at C-7 and a thiadiazolythiomethyl group at C-3 (Reiner 1986). Ceftriaxone has a long half-life of 8 h, and it was the first beta-lactam recommended for once-a-day administration (Reiner 1986).

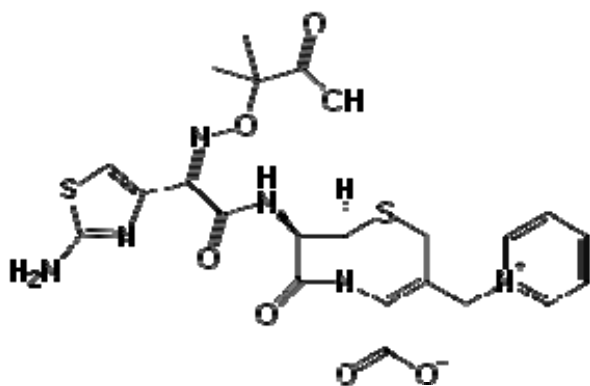


Figure 11. Ceftazidime.

The addition of a quaternary ammonium group at the C-3' position is the modification that distinguishes third-generation and fourth-generation cephalosporins (Garau 2008). Fourth-generation cephalosporins are zwitterionic compounds (Garau

2008). This implies electrical neutrality and polar characteristics (a + end and a – end). These attributes enables fourth-generation cephalosporins to rapidly penetrate the outer membrane of gram-negative bacteria (Garau 2008). In addition, research has demonstrated that beta-lactamase enzymes have lower affinity for fourth-generation cephalosporins (Garau 2008). This low affinity for fourth-generation cephalosporins results in a lower rate of hydrolysis by these enzymes. This fact, together with rapid penetration into the cell, results in a decrease in the rate at which bacteria become resistant to fourth-generation cephalosporins (Giamarellou 1999).

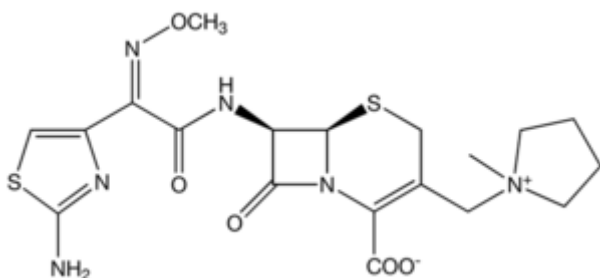


Figure 12. Cefepime -fourth.

The side chains that distinguish cefepime are an aminothiadiazolylyl group at C-7' and a quaternary ammonium at position-3 (Watanabe 1996).

Cefepime received FDA approval in 1996 (Barlow 2003). Structurally, it is most similar to a third-generation cephalosporin, ceftazidime (Barlow 2003). These two cephalosporins have noticeably different mechanisms of resistance and susceptibility to bacteria. As would be expected, there are many examples in which cefepime is more resilient than earlier cephalosporins. The greater spectrum of activity of cefepime compared to that of ceftazidime may result from factors including a significantly lower affinity for plasmid-mediated AmpC β -lactamases than ceftazidime and that ceftazidime is hydrolyzed by β -lactamases to a greater degree than cefepime (Endimiani 2008).

Cefepime was approved for the treatment of moderate to severe infections including pneumonia, uncomplicated and complicated UTIs, soft tissue and skin infections, intra-abdominal infections, and febrile neutropenia (Endimiani 2008). A low frequency of spontaneous resistance mutations is described for cefepime (Endimiani 2008). This description resulted from research in which wild-type *P. aeruginosa* strains required an average of 30 passages of cefepime-selected isolation for resistance to

develop, in comparison to 15.2, 18.0, and 3.5 passages for cefpirome, ceftazidime, and cefotaxime, respectively (Endimiani 2008, Carsenti-Etesse 2001).

The mechanisms by which cefepime resistance has developed have rarely been bacteria-specific. *Enterobacteriaceae* such as *E. coli* and *Klebsiella* species have infrequently developed resistance by either producing class A extended-spectrum β -lactamases (ESBLs) or class A or B carbapenemases (Endimiani 2008). *P. aeruginosa* usually acquire cefepime resistance through a combination of tactics. First, *P. aeruginosa* hyper-produce class C chromosomal enzymes, e.g., AmpC. Second, *P. aeruginosa* will upregulate their efflux pumps and remove cefepime (Endimiani 2008). To a lesser extent, there are isolated geographic reports of cefepime-resistant *P. aeruginosa* producing metallo- β -lactamases such as CTX-M (Endimiani 2008). *Acinetobacter baumannii* has exhibited cefepime resistance. There are three mechanisms attributed to *A. baumannii* resistance to cefepime: hyper-production of chromosomal OXA-51/69-like carbapenemases, activation of efflux pumps such as AdeABC, and porin exchange (Endimiani 2008).

Review of β -lactamases

The hope of discovering a panacea felt after the discovery of the antimicrobial action of beta-lactam drugs was soon dashed by the appearance of bacteria possessing innate resistance. The means of resistance to beta-lactam drugs by these bacteria was soon discovered to be the enzymatic destruction of the drugs by a number of different enzymes called beta-lactamases. In keeping with the discovery of new forms of beta-lactam antimicrobial drugs, bacteria have employed or evolved beta-lactamase enzymes that eliminate them.

The first identification of a beta-lactam-destroying enzyme was made in 1940 (Sutherland 1990). These enzymes are as a group referred to as beta-lactamases. The mechanism by which beta-lactamases destroy beta-lactam antimicrobials is to hydrolyze the beta-lactam ring (Bush 1988, Mainardi 2005). Once the ring is destroyed, their antimicrobial activity is lost. In an attempt to overcome the effects of this enzyme, a screening for naturally occurring beta-lactamase inhibitors was performed (Sutherland

1990). From this research, it was found that clavulanic acid from *Streptomyces clavuligerus* is a beta-lactamase inhibitor (Sutherland 1990).

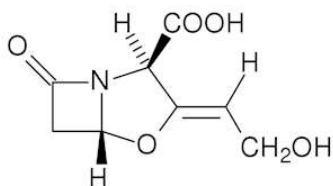


Figure 13. Clavulanic acid.

From the structure in Figure 13, it is clear that clavulanic acid is related to the beta-lactam family (Kim 2009). It, however, has little or no antimicrobial activity (Kim 2009). It functions as a competitive inhibitor of beta-lactamase (Kim 2009). One of the criteria used to categorize different beta-lactamases is whether the beta-lactamase is inhibited by clavulanic acid. Other beta-lactamase inhibitors have been discovered including sulbactam and tazobactam (Hedburg 1992). However, their spectrum of activity is not used in the classification of beta-lactamases (Bush 1995).

The susceptibility of beta-lactamase to clavulanic acid, together with the group of beta-lactams being eliminated and the molecular class of the enzyme, is used as a criterion for classification (Bush 1995). There are three major groupings of beta-lactamases (Bush 1995). Group 1 includes enzymes labeled cephalosporinases; this group hydrolyzes cephalosporins and exhibits resistance to clavulanic acid (Bush 1995). Group 2 includes penicillinases, cephalosporinases, and broad-spectrum beta-lactamases that can potentially eliminate penicillins, cephalosporins, and other beta-lactam groups (Bush 1995). Group 2 beta-lactamases are generally sensitive to clavulanic acid (Bush 1995). Group 3 beta-lactamases include metallo-beta-lactamases and can eliminate penicillins, cephalosporins, and carbapenems (Bush 1995). Group 3 enzymes are resistant to clavulanic acid (Bush 1995).

Table 4. Classification schemes for bacterial β -lactamases

Bush-Jacoby-Medeiros Group	1989 Bush Group (44)	Richmond-Sykes Class (253)	Mirsuhashi-Inoue Type (194) ^a	Molecular Class (2, 121, 132)	Preferred Substrates	Inhibited by		Representative enzymes
						CA	EDTA	
1	1	Ia, Ib, Id	CSase	C	Cephalosporins	-	-	AmpC enzymes bacteria; from gram-negative MIR-1
2a	2a	Not included	PCase V	A	Penicillins	+	-	Penicillinase from gram-positive bacteria
2b	2b	III	PCase I	A	Penicillins, Cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	2b'	Not included except K1 in class IV	CXase	A	Penicillins, narrow spectrum, and extended spectrum cephalosporins, monobactams	+	-	TEM-3 to TEM-26, SHV-2 to SHV-6, <i>Klebsiella oxytoca</i> K1
2br	Not included	Not included	Not included	A	Penicillins	±	-	TEM-30 to TEM-36, TRC-1
2c	2c	II, IV	PCase IV	A	Penicillins, carbenicillin	+	-	PSE-1, PSE-3, PSE-4
2d	2d	V	PCase II, PCase III	D	Penicillins, cloxacillin	±	-	OXA-1 to OXA-11, PSE-2 (OXA-10)
2e	2e	Ic	CXase	A	Cephalosporins	+	-	Inducible cephalosporinases from <i>Proteus vulgaris</i> NMC-A from <i>Sme-1</i> from
2f	Not included	Not included	Not included	A	Penicillins, cephalosporins, carbapenems	+	-	<i>Enterobacter cloacae</i> , <i>Serratia marcescens</i>
3	3	Not included	Not included	B	Most β -lactams, including carbapenems	-	+	I1 from <i>Xanthomonas maltophilia</i> , CcrA from <i>Bacteroides Fragilis</i>
4	4	Not included	Not included	ND ^a	Penicillins	-	?	Penicillinase from <i>Pseudomonas cipacia</i>

^a-Csase, cephalosporinase; Pcase, penicillinase; CXase, cefuroxime hydrolyzing β -lactamase.
^b-CA, clavulanic acid
^c-ND, not determined

(Source: reproduced from Bush K, Jacoby GA, Medeiros AA.1995. "A functional classification scheme for beta-lactamases and its correlation with molecular structure."

Antimicrob Agents Chemother. 1995;39: 1211–33)

This research involved a group 1 beta-lactamase termed *AmpC-bla-cmy-2*.

Interestingly, the first beta-lactamase enzyme discovered was an AmpC enzyme (Jacoby 2009). The genetic sequence of *AmpC* beta-lactamases from *E. coli* was presented in research in 1981 (Jacoby 2009). Since the *AmpC* sequence of *E. coli* was first identified, similar sequences with beta-lactamase activity have been identified and cataloged in different genera and species (Jacoby 2009). However, variability in *AmpC* beta-lactamase genes has been identified even within species (Jacoby 2009). AmpC beta-lactamases can hydrolyze the beta-lactam ring of penicillins but are more effective at eliminating cephalosporins (Jacoby 2009). One of the factors attributed to AmpC activity is its high affinity for beta-lactams (Jacoby 2009). AmpC is classified as a Class C enzyme (Jacoby 2009). Class C enzymes have the advantage of a wider active site for catalysis (Jacoby 2009). This fact is also believed to aid in the increased ability of AmpC to deal with cephalosporins possessing large side chains (Jacoby 2009).

One source stated that a beta-lactamase was first attributed to a sequence on a plasmid in 1976; however, no material remains from this experiment to verify its validity (Phillippon 2002). Subsequently, it has been confirmed that sequences that lead to beta-lactamase synthesis can be transferred between bacteria in plasmids. One grouping of these plasmid-based AmpC beta-lactamases has been classified as *cmy* (Jacoby 2009). *AmpC bla-cmy-2* was used in this research (Jacoby 2009).

Review of Plasmids

Bacteria have several types of DNA. One type is referred to as plasmids. Plasmids are circular pieces of DNA that can be transferred from one bacterial cell to another, thereby spreading antibiotic resistance (Berg 2007). Plasmids are spread between bacteria in two known methods. The first method is by a process referred to as “bacterial conjugation,” which involves the pilus transfer of genes (Nakano 2004). A second method in which a plasmid can be transferred from one bacterium to a second is transformation. In the laboratory, the naturally occurring process of transformation has been elaborated to involve electrocompetent cells and chemically competent cells (Nakano 2004). Cell transformation using electrocompetent cells and chemically competent cells were used in this research.

Review of *Bla-Cmy-2*

The essence of this research is centered on whether a particular beta-lactamase can be induced to confer cefepime resistance to bacteria. This beta-lactamase is *cmy-2*. The *cmy-2* enzyme is of particular interest, as it hydrolyzes cephalosporin beta-lactam drugs. In addition, *bla-cmy-2* has been found on plasmids. This fact means cephalosporin resistance can be transformed between bacteria. This is believed to be responsible for the majority of the nontyphoidal *Salmonella enterica* cephalosporin antimicrobial resistance in this country (Daniels 2007). The sequence of *bla-cmy-2* found in plasmids is thought to have originated from the chromosomal DNA of *Citrobacter freundii* (Daniels 2007). The *bla-cmy-2* sequence in plasmids consists of 1146 nucleotides, leading to 381 amino acids (Bauernfeind 1996). *Cmy-2* is a class C beta-lactamase (Bauernfeind 1996). Transcription of plasmid-mediated *bla-cmy-2* is not activated by a catabolic operon, as it does not contain the AmpR sequence (Nakano2004). There are references suggesting that *bla-cmy-2* transcription may be activated in response to the promoter region *ISEcp1* or *ISEcp1B* (Lartigue 2006).

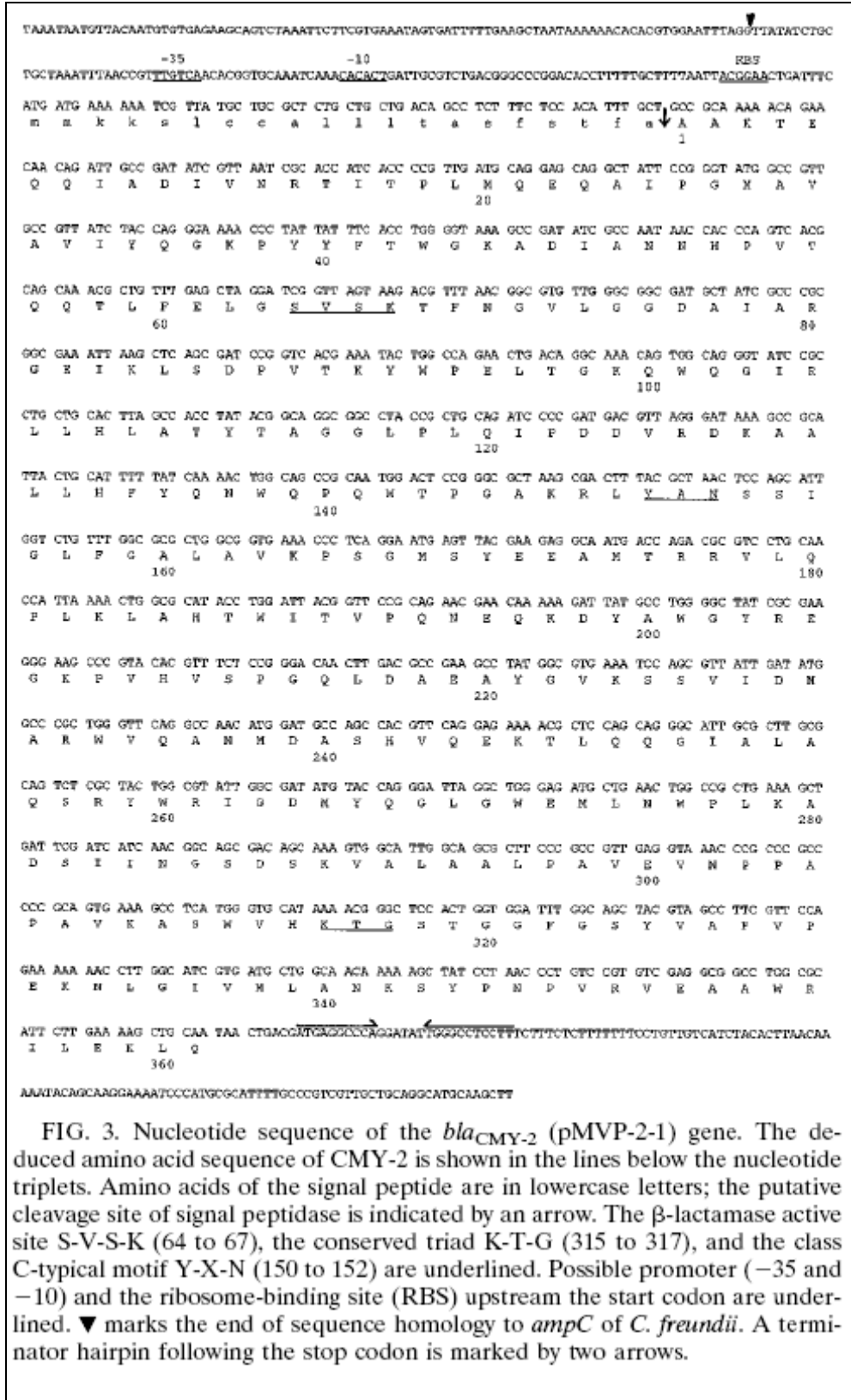


Figure 14. Nucleotide sequence of *bla*_{CMY-2}

(source: reproduced from A Bauernfeind, I Stemplinger, R Jungwirth and H Giamarellou; Characterization of the plasmidic beta-lactamase CMY-2, which is responsible for cephamycin resistance; *Antimicrobial Agents and Chemotherapy*, Jan 1996, 221–224, Vol 40, No. 1)

Review of MnCl₂ interference with DNA polymerase

A 2004 article describing the use of manganese in conjunction with error-prone rolling circle amplification states that manganese ions reduce the fidelity of DNA polymerase by biasing the deoxyribonucleotide concentration (Fujii 2004). Possible causes for this reduced fidelity may result from the observation that in the presence of manganese, the dissociation constant has been reported to decrease 30-fold “such that there is little regard for the instructions provided by the templating base” (Frank 2007). A second possible cause may be that the proofreading capability of DNA polymerase is compromised, which would result in unrepaired mutations. There are three subunits of DNA polymerase III, alpha, theta, and epsilon, with the latter being responsible for the exonuclease proofreading activity (Hamdan 2002). The efficiency of hydrolysis required for exonuclease proofreading activity is altered on the basis of the divalent metal present, namely magnesium versus manganese (Hamdan 2002).

Review of rolling circle amplification

Rolling circle amplification or rolling circle replication is the natural process by which plasmids and some circular viruses are synthesized (Dean 2001). In the 1990s, this natural process had been modified to make it a means of laboratory circular DNA amplification (Fuji 2006). Steps in this process include adding primers to an existing circular piece of DNA (Dean 2001). These primers are resistant to the exonuclease or DNA repair mechanism of the DNA polymerase used (Dean 2001). The DNA polymerase used (psi29 DNA polymerase) is highly processive or strongly attached to the template strand named (Dean 2001). The DNA polymerase attaches at the exonuclease resistant primers on the circular DNA and begins to assemble nucleotides (Dean 2001). As the polymerase reads the circular DNA template, it reaches the next primer (Dean 2001). Other less processive DNA polymerases would stop at this point, but the psi29 DNA polymerase stays attached to the template and continues to assemble nucleotides

until the available nucleotides for assembly are depleted (Dean 2001). The result is a long double-strand of nucleotide repeats of the circular DNA and the primer (Dean 2001). Additional phi 29 DNA polymerases attach at these new primers and start to assemble nucleotides of only the synthesized strand (Dean 2001). This leads to an exponential increase in the repeated strands (Dean 2001). The repeated strands are referred to as concatemers (Fuji 2006). Concatemers reform into a circle by means of intramolecular homologous recombination (Fuji 2006).

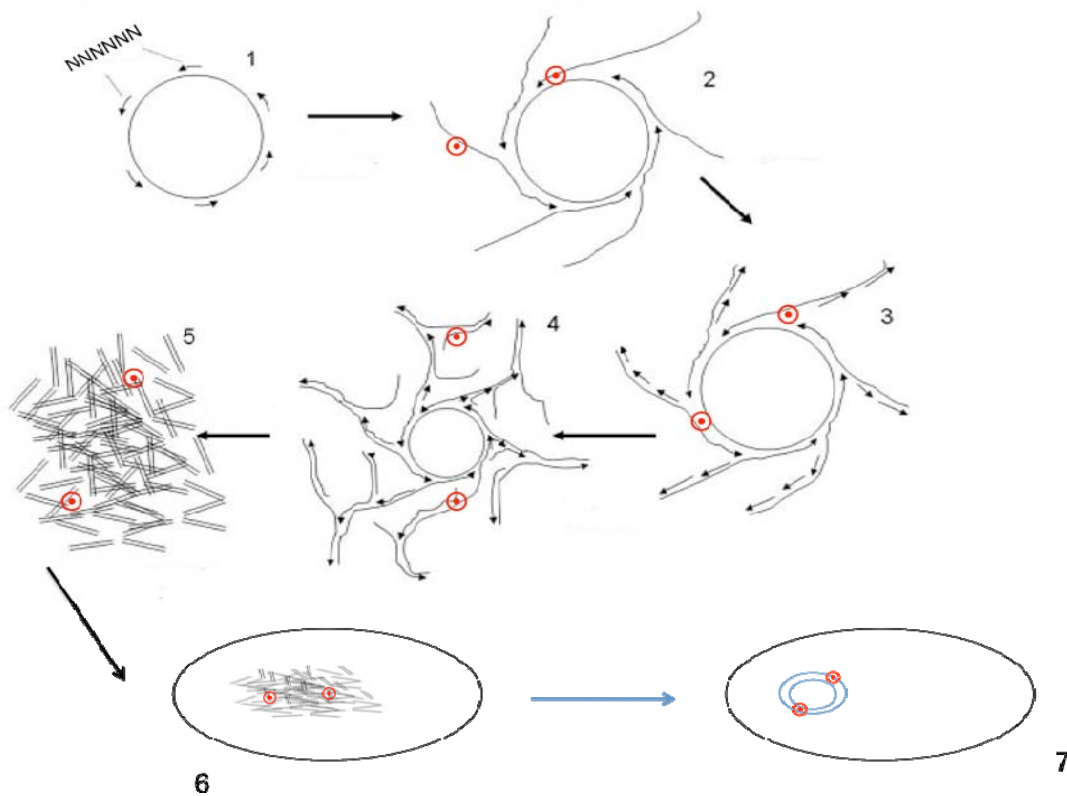


Figure 15. Illustration of rolling circle amplification.

Aims of the project

Bacterial resistance to cefepime, a fourth-generation cephalosporin, has infrequently been documented. The aim of this research is to use directed molecular evolution to predict possible mutations to the beta-lactamase *cmy-2* that will confer bacterial resistance to cefepime and possibly other fourth-generation cephalosporins.

This research involved the use of error-prone rolling circle amplification to modify the genetic sequence of AmpC *Bla-Cmy-2* to anticipate possible mutations resulting from natural evolution that will enable it to hydrolyze fourth-generation cephalosporins. In this pursuit, the AmpC-carrying plasmids that had displayed third-generation cephalosporin resistance were isolated from *S. enterica* var Typhimurium DT104 strains 1441, 3453, 5317, 7330, 7599, 8501, and 9853, *Salmonella* 968, and *S. enterica* var St Paul. Mutagenesis by error-prone rolling circle amplification was performed on these plasmids. Electrotransformation of these plasmids into *E. coli*, *Salmonella*, *Yersinia*, and *Proteus* was followed by the sensitivity testing of a fourth-generation cephalosporin, cefepime, at its breakpoint.

CHAPTER 2. MATERIALS AND METHODS

Bacteria screening sensitivity in 96-well plates

Ceftiofur, cefotaxime, cefixime, ceftriaxone, cefovecin, cefepime, and ceftazidime were attained from commercial sources. Breakpoints were established by CLSI (REF). The following strains were used: *S. enterica* var Typhimurium DT104 strains 196, 247, 799, 1441, 3673, 3453, 4430, 5852, 5317, 5221, 7599, 7356, 7330, 8151, 8501, 9853, and 10452, *Salmonella* 968, and *S. enterica* var St Paul.

Antibiotic sensitivity was assayed using 96-well plates. Bacteria were exposed to the antibiotics at the concentrations given below in lysogeny broth (LB). Incubation occurred for 24 h in an orbital shaker at 37°C.

ceftiofur - 1, 2, 4, 8, 16, 32, 64, 128 µg/ml

cefotaxime - 4, 8, 16, 32, 64, 128, 256, 512 µg/ml

cefixime - 1, 2, 4, 8, 16, 32, 64, 128 µg/ml

ceftriaxone - 1, 2, 4, 8, 16, 32, 64, 128 µg/ml

cefovecin - 1, 2, 4, 8, 16, 32, 64, 128 µg/ml

cefepime - 2, 4, 8, 16, 32, 64, 128, 256 µg/ml

ceftazidime - 1, 2, 4, 8, 16, 32, 64, 128 µg/ml

Cosmid removal–Qiagen large-construct kit

Using the Qiagen large-construct kit, cosmids from the following microbes were removed: *S. enterica* var Typhimurium DT104 strains 1441, 3453, 5317, 7330, 7599, 8501, and 9853, *Salmonella* 968, *S. enterica* var St Paul, and *Yersinia* 968. In addition to the Qiagen large-construct kit, adenosine triphosphate (ATP) disodium salt was purchased. The following procedure was performed individually on each of the above bacteria. Bacteria were statically grown in 500 ml of commercially purchased LB. After 24 h, LB was transferred into 10–50-ml falcon tubes and centrifuged at 4100 rpm (limit of centrifuge) for 15 min. Supernatant was decanted, and tubes left inverted to drain. Ten milliliters of buffer P1 with RNase A were used to re-suspend all 10 bacterial cell pellets (RNase A will digest any RNA present during the alkaline lysis, which is the next step). Twenty milliliters of Buffer P2 were next added. Buffer P2 contains 200 mM NaOH and 1% sodium dodecyl sulfate (SDS; NaOH lyses the bacterial cells, SDS aids in

lysis by solubilizing the phospholipid and protein components of the cell membrane). After 5 min, 20 ml of chilled buffer P3 were added (buffer P3 is acidic potassium acetate; its role is to neutralize the lytic action of buffer P2). After 10 min of incubation on ice, the suspension was centrifuged at 4100 rpm for 30 min. Following centrifugation, the lysate was filtered through a supplied filter (this filtering was done to remove any particulate material that may hinder complete exonuclease action in subsequent steps). The DNA was then precipitated from the lysate by adding 36 ml of isopropanol. This filtered lysate was then centrifuged at 4100 rpm for 30 min. Following centrifugation, the supernatant was carefully decanted. The DNA pellet was then washed by adding 2.5 ml of 70% ethanol to each tube, followed by centrifugation at 4100 rpm for 15 min and careful decantation. This step was repeated. Tubes were then air-dried for 3 min. The resulting DNA pellet dissolved in 4.75 ml of buffer EX. The DNA was then incubated with 200 μ l of ATP-dependent exonuclease and 300 μ l of ATP solution. This step resulted in digestion of all types of DNA other than super-coiled DNA. The tube was placed in a water bath at 37°C for 1 h. Then, its content containing the digested DNA and the intact tightly coiled DNA was transferred to a sterile tube. Ten milliliters of QS buffer then were added to this sterile tube. A Qiagen-tip 500 elution column was primed with 10 ml of QBT buffer. The tube containing the DNA and QS buffer was then applied to the elution column and allowed to run through the column. The DNA is theoretically in the column, and 30 ml of buffer QC were then applied to the column to rinse away any impurities other than the intact tightly coiled DNA. The elution column was placed over a fresh sterile tube, and 15 ml of buffer QF prewarmed to 65°C were applied. The intact DNA was eluted by buffer QF. Isopropanol (10.5 ml) was added, and the tube was centrifuged at 4100 rpm for 30 min. Supernatant from the pellet containing only intact tightly coiled DNA was carefully decanted. Five milliliters of 70% ethanol were added, and the tube was centrifuged at 4100 rpm for 15 min. Supernatant was carefully decanted from the DNA pellet, which was dried for 10 min. One hundred microliters of diethylpyrocarbonate H₂O were added to re-dissolve the pellet.

**Rolling circle amplification w MnCl₂-GE Healthcare Life Science
illustra TempliPhi 100/500 DNA Amplification Kit**

Cosmids from the following bacteria underwent rolling circle amplification with MnCl₂: *Yersinia* 968, *Salmonella* 968, and *S. enterica* var Typhimurium DT104 strains 1441, 3453, 5317, 7330, 7599, and 9853. A GE Healthcare Life Science illustra TempliPhi 100/500 DNA Amplification Kit was used in this project. The amplification kit consists of three components: a sample buffer, a reaction buffer, and an enzyme mix. The sample buffer consists of DNA polymerase primers. These primers are resistant to the exonuclease proofreading activity of DNA polymerase. Their function is to provide an attachment and initiation site for DNA polymerase. The reaction buffer consists of salts and free deoxynucleotides in a solution with a pH that supports DNA polymerase activity. The deoxyribonucleotides are the nucleotides that will be used to assemble new strands. The final part of the kit is the enzyme mix. The enzyme mix has the highly processive DNA polymerase psi 29 and also contains more random hexamers.

Each of the cosmids from the aforementioned bacteria was individually amplified in the following manner. Five microliters of the sample buffer were placed in the reaction tube followed by the addition of 0.5 µl of the individual bacterial cosmid. The reaction tube was then sealed and heated to a temperature of 95°C for 3 min, followed by cooling to 4°C. In a second tube, 5 µl of the reaction buffer and 0.2 µl of the enzyme mix were combined. To this was added 1 µl of 1.5 mM MnCl₂. The two reaction tubes were placed on ice for 5 min, and then their contents were combined. This combination was then kept at 30°C for 18 h (by this time, all of the deoxyribonucleotides are depleted). The reaction tube was then heated to 65°C for 10 min and then cooled to 4°C. This was done to inactivate the psi29 DNA polymerase.

Electrocompetent cell creation

The following electrocompetent cells were synthesized: *Salmonella*, *Yersinia*, *E. coli*, and *Proteus*. Individually, 1 ml of each of the following microbes was added to 1 L of LB. The LB was statically incubated at 30°C for 18 h. After incubation, 40 ml of culture were added to 20–50-ml falcon tubes. Tubes were centrifuged at 4100 rpm for 5 min. The supernatant was decanted, leaving cellular pellets. Cellular pellets were re-

suspended in 40 ml deionized H₂O. Tubes were re-centrifuged at 4100 rpm for 5 min. Supernatant was decanted, leaving cellular pellets. Cellular pellets were re-suspended in 20 ml deionized H₂O. Tubes were re-centrifuged at 4100 rpm for 5 min. Supernatant was decanted, leaving cellular pellets. Cellular pellets were re-suspended in 1 ml of 40% glycerol. Glycerol-suspended cells were aliquoted into 1-ml microtubes and stored at -80°C.

Electrocompetent cell transformation

Using an electroporator, cosmids that underwent error-prone rolling circle amplification were transformed into electrocompetent cells of *Salmonella*, *E. coli*, *Yersinia*, and *Proteus*. Electrocompetent cells and electroporation cuvettes were stored at -80°C. Electrocompetent cells and cuvettes were transferred from -80°C storage to the work bench on ice. To previously aliquoted 50 µl of electrocompetent cells, 2 µl of cosmids from the aforementioned bacteria were added. This combination was transferred into a designated electroporation cuvette. The cuvette was placed inside an electropulsator pre-set at 200 Ω, 2.5 kV, and 25 µF for a 0.2-cm cuvette (Chaveroche 2000). Electricity was discharged. Five hundred-fifty microliters of super optimal broth with Catabolite repression (SOC; bacterial nutrient solution) were rapidly added to the cuvette. The contents of the cuvette were transferred back to the original microtube and incubated for 2 h. Following incubation, 200 µl of transformed cells were transferred into three wells of a 96-well plates containing 200 µl of cefepime (64 µg/ml).

Electrophoresis

Electrophoresis was used twice in this project: first to determine whether a molecule of the size of the cosmids was present following cosmid removal from the bacteria and later determine whether a molecule the size of the desired product was present after polymerase chain reaction (PCR). The size of the expected PCR product is known as the sequence assembled by Taq polymerase and is defined by specific primers. All supplies used here were commercially purchased. For this research, 0.5% gel was used for our electrophoresis. Gel was synthesized by combining 0.2 g of agarose with 40 ml of TAE buffer in a 100-ml Erlenmeyer flask. Thermal agitation aided in dissolving

the agarose. After dissolution was complete, the liquid was cooled at room temperature for 5 min and then transferred to the electrophoresis plastic mold. One drop of ethidium bromide (EtBr) was added. A well mold was placed in the appropriate slot. Following gel solidification, the DNA sample and corresponding ladder were placed in the designated wells. The cap was placed, and the current was set at 88 V for 45 min. After the allotted time, the gel was examined under ultraviolet light, and the extent of molecule migration was determined by the EtBr fluorescence. DNA samples were compared to the ladder. Larger molecules migrate slower, whereas smaller molecules migrate faster.

PCR

PCR was used in this research to amplify the *bla-cmy-2* sequence that conferred cefepime resistance. PCR is a method of amplifying nucleotide sequences utilizing a DNA polymerase that has high temperature tolerance. This is significant because the DNA double-strands are melted to allow DNA polymerase (Taq) access to a single-stranded template. Two microliters each of the established forward and reverse primers were added to 2 μ l of the cosmid. These primers defined the nucleotide sequence for *bla-cmy2*. To this were added 5 μ l of Expand™ 10 \times PCR Buffer, 2 μ l of deoxyribonucleotides, 2 μ l of 2.25 mM MgCl₂, 0.5 μ l of Taq DNA polymerase, and 35.5 μ l of diethylpyrocarbonate H₂O. The thermal cycling protocol is shown in Table 5.

Table 5. PCR thermal cycling protocol

STEP	TIME	TEMPERATURE	CYCLE
Initial			
Denaturation	2 minutes	94°C	1 \times
Denaturation	15 seconds	94°C	
Annealing	1 minute	56°C	25 \times
Extension	5 minutes	68°C	
Final Extension	7 minutes	72°C	1 \times

Denaturation, or melting, of the double strands occurred at 94°C. Taq polymerase attached to the template strand at lower temperatures and produced the nucleotide sequences defined by the primers. The presence of product was confirmed by gel electrophoresis.

Transformation of plasmids using chemically competent cells

The sequence containing the mutated *bla-cmy-2*, after being amplified using PCR, was cloned into a pCR XL-TOPO vector and then transformed into chemically competent cells. To 1 μ l of the PCR-amplified sequence were added 3 μ l of H₂O, 1 μ l of salt solution, and 1 μ l of the pCR-XL-TOPO vector. This mixture was kept at room temperature for 5 min, followed by 5 min on ice. Independently, 2 μ l of 0.5 M β -mercaptoethanol were added to a vial of TOP10 chemically competent cells. To this vial, 2 μ l of the aforementioned combination of the PCR product with the vector were added. This mixture was placed on ice for 30 min. The competent cells were then heat-shocked at 42°C for 30 s. Following the heat shock, the vial was placed in ice for 2 min. Two hundred fifty microliters of SOC were added. Cells were left for 2 h at 37°C under 300-rpm agitation. Cells were then streaked onto a agar plate containing ampicillin (the ampicillin in the agar prevents the growth of invasive bacteria not possessing the pCR-XL-TOPO vector containing the operon that confers ampicillin and kanamycin resistance). Following overnight incubation, 30 colonies were selected. These colonies were placed in 1-ml tubes containing 50 μ g/ml kanamycin. Kanamycin was again added to the tubes in an effort to prevent the growth of invasive bacteria not possessing the pCR-XL-TOPO vector containing the operon that confers kanamycin resistance. In this manner, contaminants or bacteria not expressing the vector with the *bla-cmy-2* sequence of interest were eliminated.

After confirming that one plasmid contained the *bla-cmy-2* gene, the plasmid insert was subjected to DNA sequencing by the DNA Facility at Iowa State University.

CHAPTER 3. RESULTS

Transformation of the *bla-cmy-2* sequence-containing plasmids that had undergone error-prone rolling circle amplification from the aforementioned bacteria was repeated until a strain displayed resistance to cefepime 32 µg/ml. Mutated plasmids underwent transformation into electrocompetent cells of *E. coli*, *Salmonella*, *Proteus*, and *Yersinia*. This was done in search of a plasmid containing a mutated *bla-cmy-2* sequence that would confer cefepime resistance. After repeating this transformation 15 times, a bacterium displayed growth at 32 µg/ml. Electrotransformation of the plasmid from *Salmonella* 968 into *E. coli* resulted in cefepime resistance of minimum inhibitory concentration (MIC) > 64 µg/ml. The breakpoint of cefepime is 32 µg/ml. In the initial susceptibility test, the original *Salmonella* 968 had an MIC ≤ 2 µg/ml.

Sequencing of the *bla-cmy2* nucleotide sequence of *E. coli/Salmonella* 968 revealed the five mutations listed in Table 6.

Table 6. Mutations in the *bla-cmy-2* sequence conferring cefepime resistance

<u>Nucleotide</u>	<u>Amino Acid Change</u>	
27	Silent	
150	Silent	
180	Silent	
833	277 arginine→leucine	non-conserved
941	313 alanine→valine	Conserved

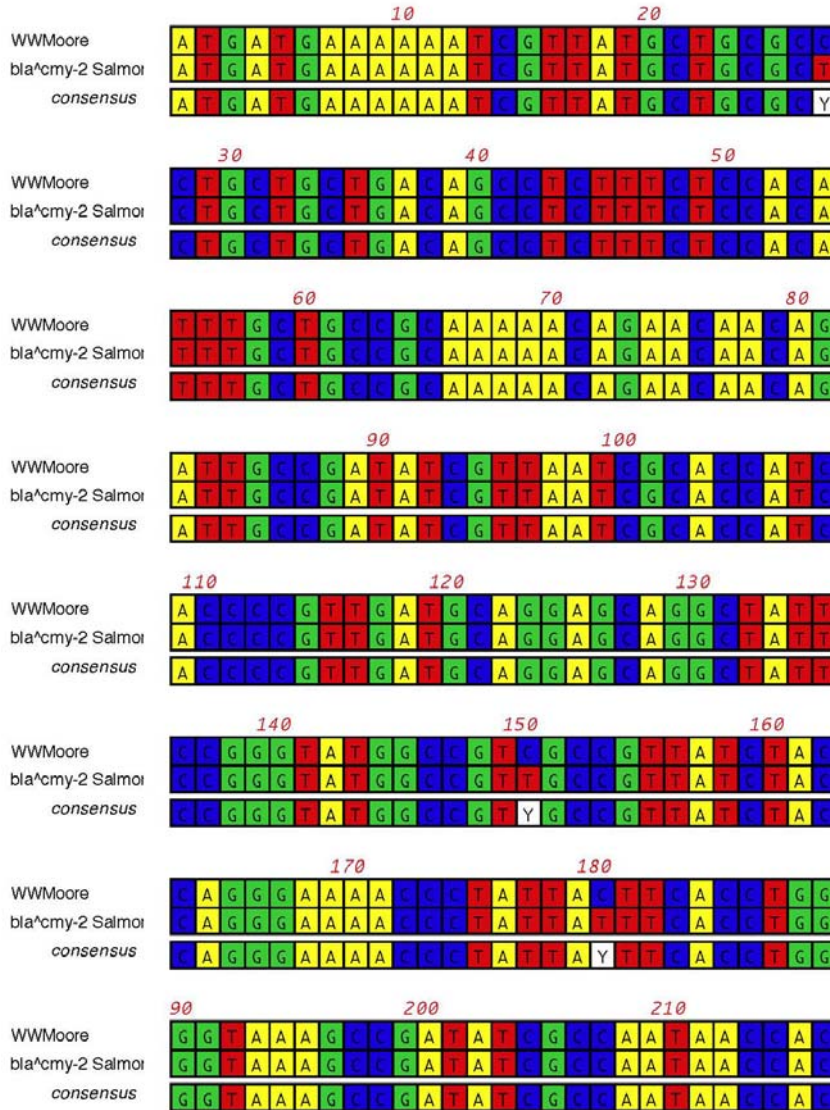
The mutations occurred at nucleotides 27 (silent), 150 (silent), 180 (silent), 833, and 941. Silent mutations do not alter the amino acid sequence of the final protein. The guanine was replaced by thymidine at nucleotide 833 (this resulted in arginine being replaced by a leucine at amino acid 277; this is a non-conserved substitution). The cytosine was replaced by thymidine at nucleotide 941 (this resulted in alanine being replaced by valine at amino acid 313; this is a conserved substitution).

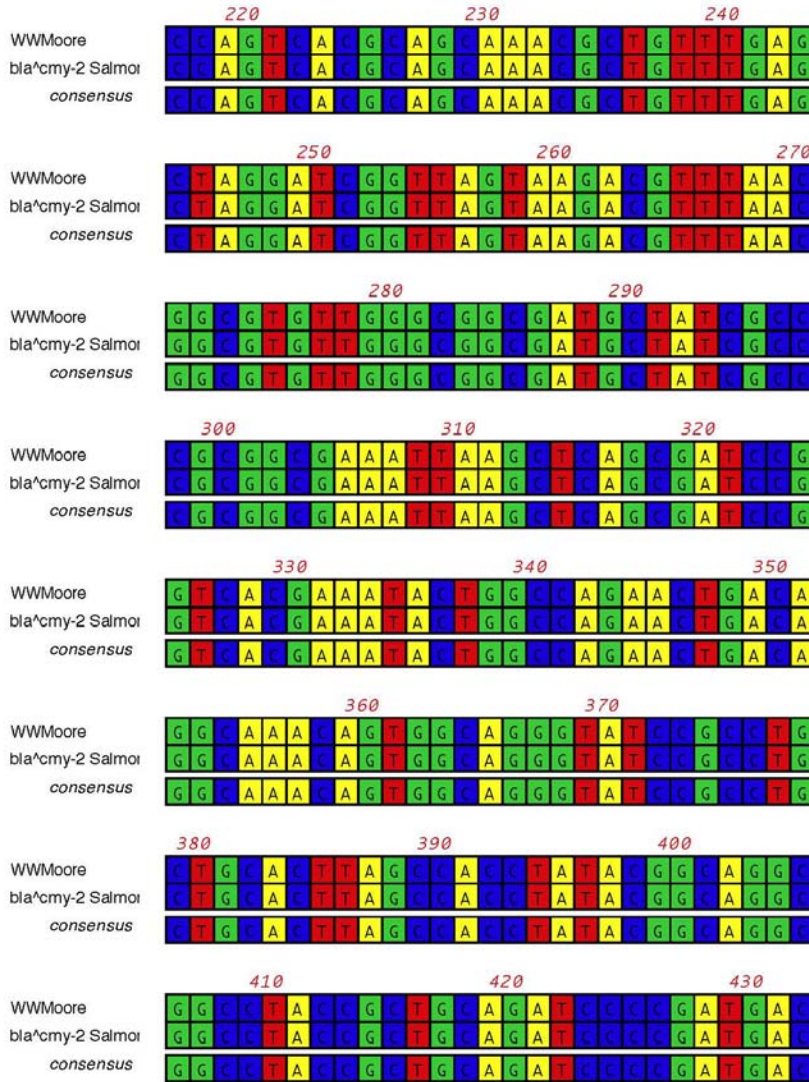
The observation that an amino acid at a particular location in a sequence is common to all strains or varieties of an organism implies that it is vital to the function and survival of that organism.

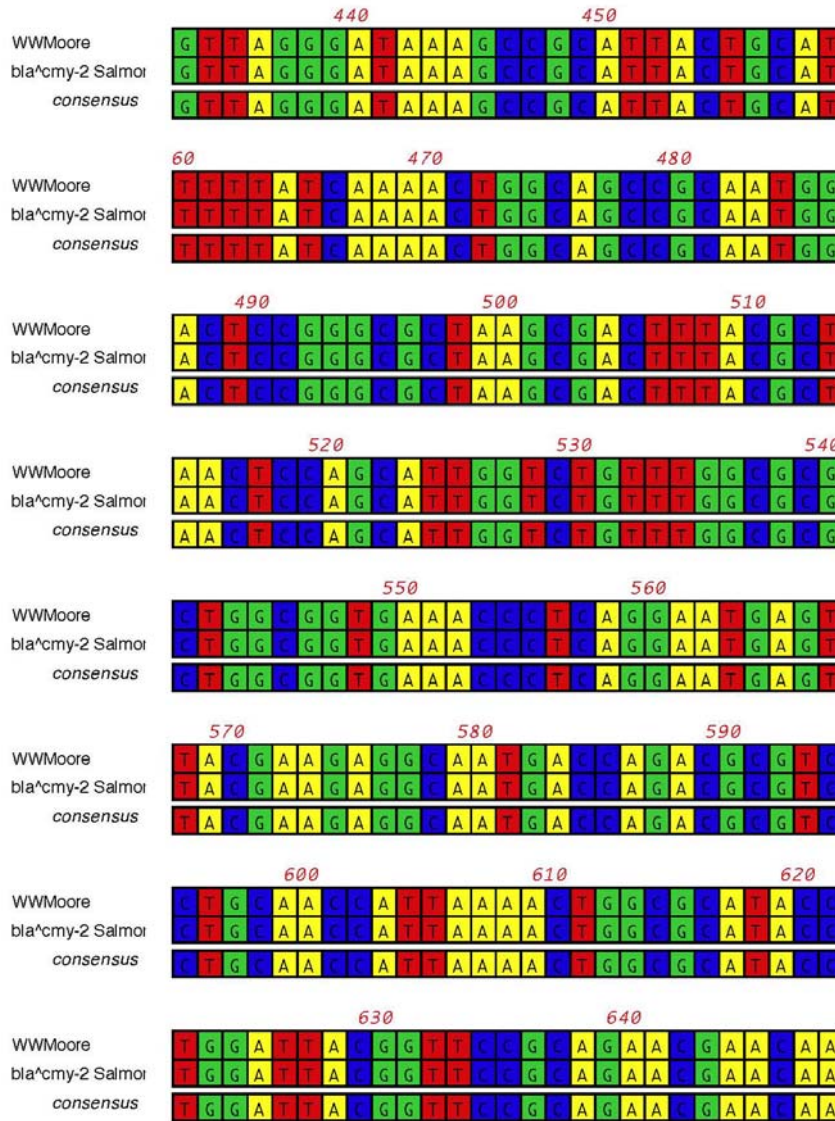
NUCLEOTIDE SEQUENCE OF WILD TYPE BLA CMY 2 AND MUTATED VARIANT

blacmy2WWM v wt (nucleotide)
 Monday, February 15, 2010 4:24 PM

Page 1

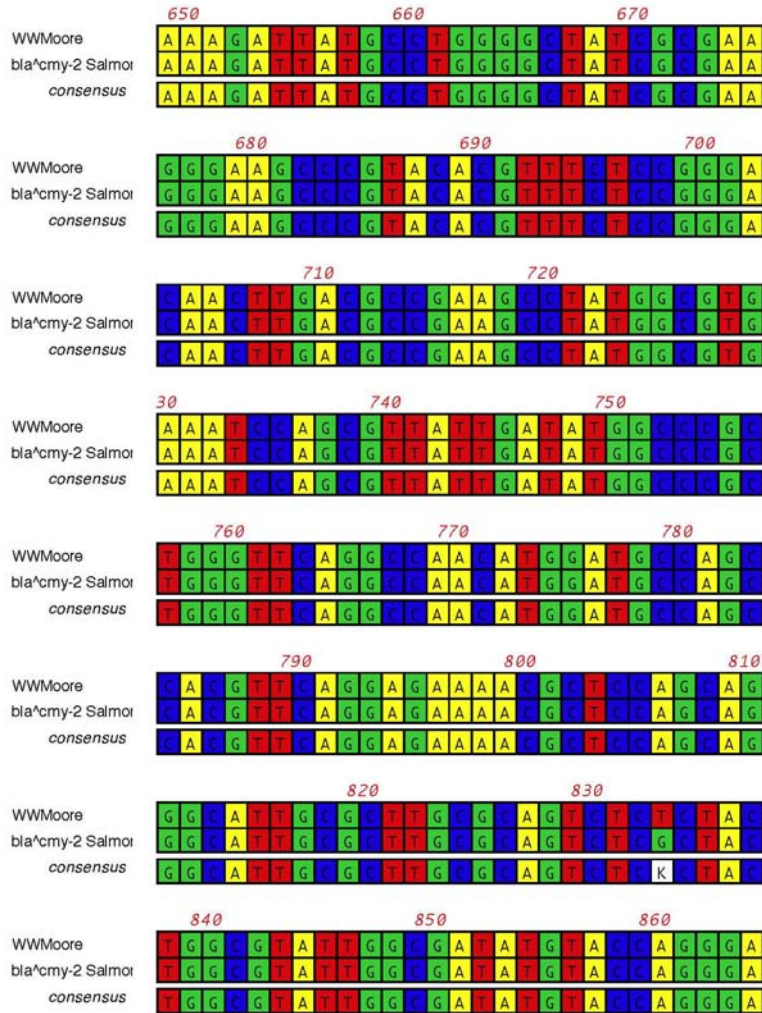






bla^{cmy2}WWM v wt (nucleotide)
 Monday, February 15, 2010 4:24 PM

Page 4



blacmy2WWM v wt (nucleotide)
 Monday, February 15, 2010 4:24 PM

Page 5

	870	880	890
WWMoore	T T A G G C T G G G A G A T G C T G A A C T G G C C G		
bla ^{cmy-2} Salmoi	T T A G G C T G G G A G A T G C T G A A C T G G C C G		
consensus	T T A G G C T G G G A G A T G C T G A A C T G G C C G		
	900	910	
WWMoore	C T G A A A G C T G A T T C G A T C A T C A A C G G C		
bla ^{cmy-2} Salmoi	C T G A A A G C T G A T T C G A T C A T C A A C G G C		
consensus	C T G A A A G C T G A T T C G A T C A T C A A C G G C		
	920	930	940
WWMoore	A G C G A C A G T A A A G T G G C A T T G G T A G C G		
bla ^{cmy-2} Salmoi	A G C G A C A G T A A A G T G G C A T T G G C A G C G		
consensus	A G C G A C A G T A A A G T G G C A T T G G Y A G C G		
	950	960	970
WWMoore	C T T C C C G C G C T T G A G G T A A A C C C G C C C		
bla ^{cmy-2} Salmoi	C T T C C C G C G C T T G A G G T A A A C C C G C C C		
consensus	C T T C C C G C G C T T G A G G T A A A C C C G C C C		
	980	990	
WWMoore	G C C C C C G C A G T G A A A G C C T C A T G G G T G		
bla ^{cmy-2} Salmoi	G C C C C C G C A G T G A A A G C C T C A T G G G T G		
consensus	G C C C C C G C A G T G A A A G C C T C A T G G G T G		
	00	1010	1020
WWMoore	A T A A A A C G G G C T C C A T G G T G G A T T T		
bla ^{cmy-2} Salmoi	A T A A A A C G G G C T C C A T G G T G G A T T T		
consensus	A T A A A A C G G G C T C C A T G G T G G A T T T		
	1030	1040	1050
WWMoore	G G A G C T A G T A G C C T T C G T T C C A G A A		
bla ^{cmy-2} Salmoi	G G A G C T A G T A G C C T T C G T T C C A G A A		
consensus	G G A G C T A G T A G C C T T C G T T C C A G A A		
	1060	1070	1080
WWMoore	A A A A A C C T T G G C A T C G T G A T G C T G G C A		
bla ^{cmy-2} Salmoi	A A A A A C C T T G G C A T C G T G A T G C T G G C A		
consensus	A A A A A C C T T G G C A T C G T G A T G C T G G C A		

blacmy2WWM v wt (nucleotide)
 Monday, February 15, 2010 4:24 PM

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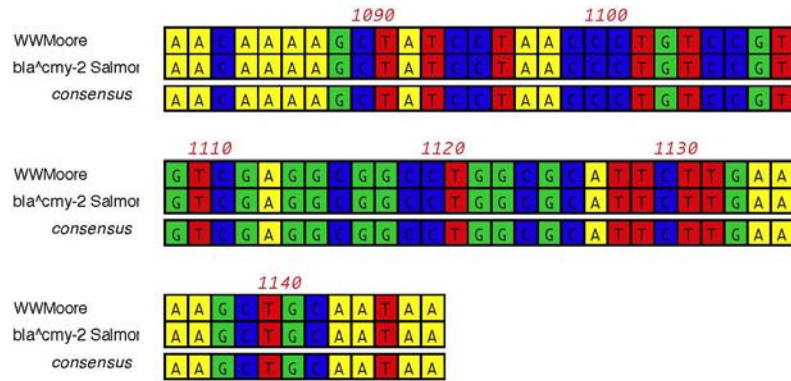
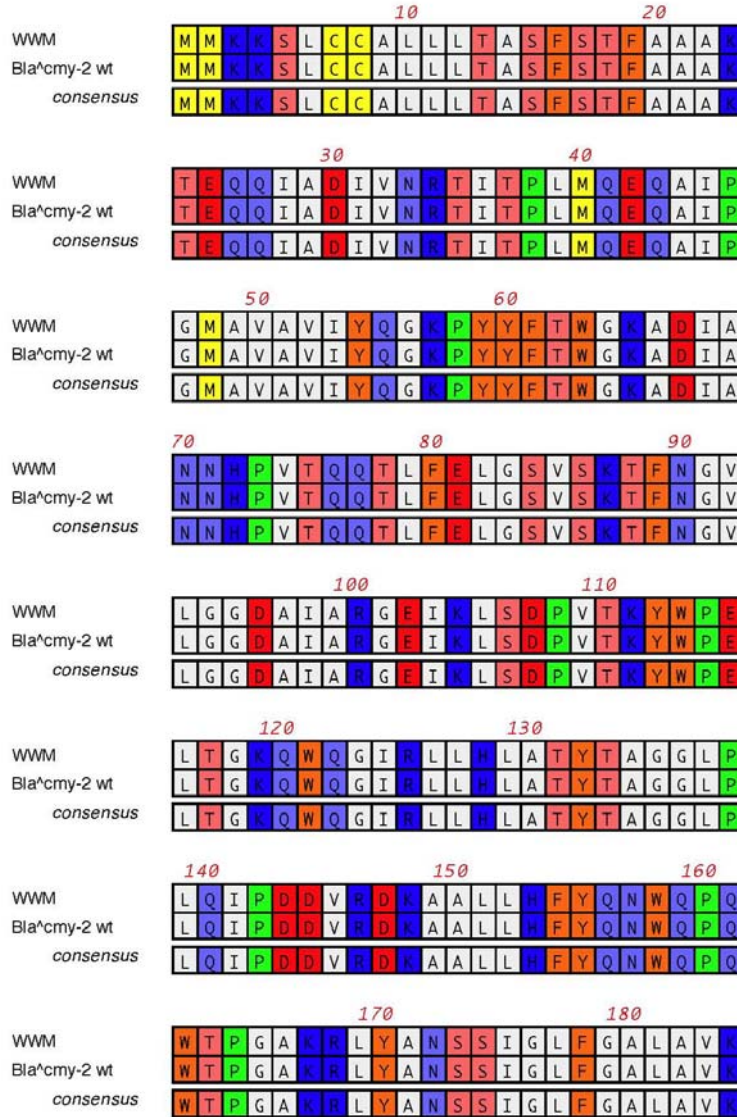


Figure 16. Nucleotide sequence of wild-type BLA CMY 2 and mutated variant.

AMINO ACID SEQUENCE OF WILD TYPE BLA CMY 2 AND MUTATED VARIANT

Bla^{cm}y2 WWM v wt
Monday, February 15, 2010 4:32 PM

Page 1



	190	200	
WMM	P S G M S Y E E A M T R R V L Q P L L A R T		
Bla ^{cmy2} wt	P S G M S Y E E A M T R R V L Q P L L A R T		
consensus	P S G M S Y E E A M T R R V L Q P L L A R T		
	210	220	230
WMM	W I T V P Q N E Q L D Y A W G Y R E G K P V H		
Bla ^{cmy2} wt	W I T V P Q N E Q L D Y A W G Y R E G K P V H		
consensus	W I T V P Q N E Q L D Y A W G Y R E G K P V H		
	240	250	
WMM	V S P G Q L D A E A Y G V S S V I D M A R W		
Bla ^{cmy2} wt	V S P G Q L D A E A Y G V S S V I D M A R W		
consensus	V S P G Q L D A E A Y G V S S V I D M A R W		
	260	270	
WMM	V Q A N M D A S R V Q E V T L Q Q G I A L A Q		
Bla ^{cmy2} wt	V Q A N M D A S R V Q E V T L Q Q G I A L A Q		
consensus	V Q A N M D A S R V Q E V T L Q Q G I A L A Q		
	280	290	
WMM	S L Y W R I G D M Y Q G L G W E M L N W P L K		
Bla ^{cmy2} wt	S L Y W R I G D M Y Q G L G W E M L N W P L K		
consensus	S L Y W R I G D M Y Q G L G W E M L N W P L K		
	300	310	320
WMM	A D S I I N G S D S V V A L V A L P A V E V N		
Bla ^{cmy2} wt	A D S I I N G S D S V V A L A A L P A V E V N		
consensus	A D S I I N G S D S V V A L . A L P A V E V N		
	330	340	
WMM	P P A P A V L A S W V R L T G S T G G F G S Y		
Bla ^{cmy2} wt	P P A P A V L A S W V R L T G S T G G F G S Y		
consensus	P P A P A V L A S W V R L T G S T G G F G S Y		
	350	360	
WMM	V A F V P E L N L G I V M L A N S Y P N P V		
Bla ^{cmy2} wt	V A F V P E L N L G I V M L A N S Y P N P V		
consensus	V A F V P E L N L G I V M L A N S Y P N P V		

Bla^{cm}y2 WWM v wt
Monday, February 15, 2010 4:32 PM

Page 3

	370									380				
WWM	R	V	E	A	A	W	R	I	L	E	K	L	Q	*
Bla ^{cm} y-2 wt	R	V	E	A	A	W	R	I	L	E	K	L	Q	*
consensus	R	V	E	A	A	W	R	I	L	E	K	L	Q	

Figure 17. Amino acid sequence of wild-type BLA CMY 2 and mutated variant.

SECONDARY STRUCTURE OF WILD TYPE BLA CMY 2 AND MUTATED**VARIANT****WILD TYPE BLA_CMY-2**Bla^{cm}-2 wt Protein Toolbox List Page 1

Wednesday, February 16, 2011 4:11 PM

Key:

Argos = Argos transmembrane

vH trns = von Heijne transmembrane

CFhlx = Chou-Fasman Alpha Helix

CFsht = Chou-Fasman Beta Sheet

CFturn = Chou-Fasman Turn

CFval = Chou-Fasman Structure Value

CFtype = Chou-Fasman Probable Type

Pos. AA Argos vH trns CFhlx CFsht CFturn CFval CFtype

1	M	1.420	-0.600	1.470	0.970	0.317	0.65	H
2	M	1.168	-0.600	1.390	0.903	0.361	0.65	H
3	K	1.107	-0.600	1.244	0.886	0.365	0.65	H
4	K	1.107	-0.600	1.233	0.884	0.358	0.65	H
5	S	1.076	-0.600	1.181	0.851	0.390	0.65	H
6	L	1.103	-0.600	1.156	0.841	0.289	0.65	H
7	C	1.146	-0.600	1.166	0.877	0.307	0.65	H
8	C	1.190	-0.600	1.176	0.913	0.331	0.65	H
9	A	1.234	-0.600	1.244	0.923	0.191	0.25	H
10	L	1.267	-0.600	1.177	0.950	0.201	0.65	H
11	L	1.286	0.221	1.203	0.973	0.209	0.65	H
12	L	1.296	0.286	1.161	1.003	0.310	0.65	H
13	T	1.299	0.303	1.130	1.063	0.352	0.40	H
14	A	1.288	0.303	1.061	1.053	0.370	0.40	H
15	S	1.272	0.240	0.994	1.080	0.365	0.65	H
16	F	1.254	0.117	0.961	1.123	0.328	0.65	H
17	S	1.231	-0.036	1.027	1.079	0.351	0.65	H
18	T	1.198	-0.181	1.027	1.079	0.220	0.65	H
19	F	1.155	-0.272	1.094	1.071	0.228	0.40	H
20	A	1.099	-0.370	1.117	0.993	0.266	0.65	H
21	A	1.039	-0.429	1.119	1.030	0.287	0.25	H
22	A	0.974	-0.460	1.206	0.964	0.304	0.25	H
23	K	0.912	-0.484	1.234	0.890	0.338	0.25	H
24	T	0.859	-0.540	1.231	0.876	0.281	0.25	H
25	E	0.822	-0.605	1.186	0.954	0.247	0.65	H
26	Q	0.799	-0.636	1.186	0.954	0.243	0.65	H
27	Q	0.793	-0.682	1.159	0.947	0.224	0.65	H
28	I	0.800	-0.738	1.179	0.981	0.354	0.65	H
29	A	0.818	-0.737	1.103	1.087	0.236	0.40	H
30	D	0.836	-0.706	1.050	1.081	0.298	0.40	H
31	I	0.856	-0.664	1.006	1.109	0.367	0.65	H
32	V	0.874	-0.635	0.986	1.074	0.323	0.65	H
33	N	0.892	-0.602	0.940	1.153	0.388	0.65	H
34	R	0.905	-0.570	0.910	1.223	0.270	0.85	
35	T	0.910	-0.567	0.846	1.107	0.253	0.85	
36	I	0.913	-0.548	0.901	1.040	0.255	1.10	
37	T	0.917	-0.544	0.983	1.070	0.568	0.85	
38	P	0.912	-0.539	1.027	1.043	0.239	1.10	
39	L	0.902	-0.511	1.114	0.977	0.244	0.85	
40	M	0.892	-0.439	1.157	0.884	0.341	0.85	
41	Q	0.894	-0.371	1.223	0.840	0.229	0.65	
42	E	0.907	-0.325	1.287	0.956	0.245	0.85	
43	Q	0.923	-0.259	1.176	0.901	0.231	0.85	
44	A	0.952	-0.187	1.046	0.894	0.280	0.85	
45	I	0.998	-0.164	1.074	0.919	0.679	0.85	T
46	P	1.051	-0.176	1.053	0.940	0.259	0.85	T

47	G	1.101	-0.204	1.001	1.039	0.272	1.10	T
48	M	1.137	-0.210	1.001	1.039	0.228	1.10	T
49	A	1.155	-0.211	0.993	1.044	0.196	0.50	
50	V	1.159	-0.219	1.057	1.160	0.220	0.00	
51	A	1.137	-0.208	1.080	1.207	0.246	0.40	
52	V	1.092	-0.194	1.051	1.183	0.308	0.40	
53	I	1.038	-0.177	0.947	1.186	0.297	0.65	
54	Y	0.976	-0.182	0.993	1.083	0.465	0.85	T
55	Q	0.923	-0.235	0.883	1.046	0.299	1.10	T
56	G	0.880	-0.286	0.856	1.011	0.376	1.10	T
57	K	0.852	-0.358	0.820	0.983	0.685	1.10	T
58	P	0.844	-0.432	0.870	0.993	0.346	1.10	ST
59	Y	0.849	-0.501	0.807	1.051	0.291	0.85	ST
60	Y	0.862	-0.588	0.869	1.083	0.355	0.85	ST
61	F	0.879	-0.643	0.773	1.104	0.383	0.65	S
62	T	0.890	-0.718	0.874	1.123	0.384	0.65	S
63	W	0.896	-0.739	0.956	1.073	0.292	0.65	S
64	G	0.889	-0.750	1.001	0.997	0.333	0.90	S
65	K	0.877	-0.751	0.987	1.016	0.366	0.90	
66	A	0.861	-0.751	1.053	0.971	0.241	0.65	
67	D	0.839	-0.772	1.040	0.917	0.307	0.65	
68	I	0.814	-0.768	1.089	0.894	0.401	0.65	
69	A	0.795	-0.775	1.087	0.939	0.388	0.85	
70	N	0.781	-0.792	0.977	0.901	0.405	1.10	
71	N	0.780	-0.806	0.959	1.011	0.295	1.10	
72	H	0.780	-0.801	0.939	0.977	0.636	1.10	T
73	P	0.784	-0.748	0.936	0.963	0.313	1.10	ST
74	V	0.801	-0.706	0.989	0.969	0.305	1.10	ST
75	T	0.831	-0.674	0.979	1.033	0.300	1.10	ST
76	Q	0.867	-0.626	0.990	1.024	0.307	0.50	S
77	Q	0.906	-0.595	1.069	1.121	0.283	0.00	S
78	T	0.935	-0.545	1.144	1.016	0.240	0.65	S
79	L	0.967	-0.511	1.211	0.989	0.249	0.65	S
80	F	0.998	-0.495	1.110	1.006	0.307	0.65	S
81	E	1.019	-0.480	1.046	1.027	0.377	0.65	S
82	L	1.026	-0.459	1.057	1.067	0.324	0.40	
83	G	1.021	-0.402	0.989	1.057	0.373	0.65	
84	S	1.009	-0.364	1.011	0.979	0.388	0.90	
85	V	1.005	-0.340	0.924	1.044	0.352	0.90	
86	S	1.001	-0.317	0.891	1.087	0.365	0.65	
87	K	0.994	-0.267	0.940	1.064	0.319	0.65	
88	T	0.993	-0.241	0.903	1.060	0.470	0.65	T
89	F	1.000	-0.239	0.903	1.060	0.385	0.65	T
90	N	1.016	-0.266	0.971	1.070	0.342	0.65	T
91	G	1.033	-0.295	0.876	1.091	0.338	0.65	T
92	V	1.041	-0.320	0.837	1.050	0.429	0.65	
93	L	1.039	-0.331	0.833	0.964	0.417	0.90	
94	G	1.041	-0.376	0.889	0.984	0.424	0.90	
95	G	1.033	-0.421	0.947	1.060	0.303	0.65	
96	D	1.016	-0.418	1.001	0.976	0.294	0.90	
97	A	0.988	-0.436	0.953	0.971	0.214	0.90	
98	I	0.957	-0.441	0.953	0.971	0.370	0.90	
99	A	0.925	-0.487	1.079	0.947	0.420	0.65	
100	R	0.900	-0.505	1.069	1.051	0.288	0.40	
101	G	0.870	-0.557	1.060	1.033	0.270	0.65	
102	E	0.844	-0.610	1.107	0.971	0.232	0.65	
103	I	0.822	-0.648	1.040	0.979	0.300	0.65	
104	K	0.805	-0.689	1.051	0.940	0.286	0.85	
105	L	0.792	-0.760	1.046	0.900	0.447	0.85	T
106	S	0.785	-0.792	0.970	1.006	0.317	1.10	T
107	D	0.776	-0.795	0.950	0.971	0.643	1.10	T
108	P	0.769	-0.790	0.950	0.971	0.310	1.10	T
109	V	0.765	-0.815	0.867	1.004	0.367	1.10	T

110 T 0.765 -0.812 0.891 1.031 0.482 1.10 T
111 K 0.765 -0.797 0.817 1.020 0.252 1.10 T
112 Y 0.765 -0.835 0.949 1.036 0.193 1.10 T
113 W 0.765 -0.844 1.004 0.969 0.615 1.10 T
114 P 0.767 -0.866 1.004 0.969 0.277 1.10 T
115 E 0.769 -0.883 0.909 0.990 0.298 1.10 T
116 L 0.765 -0.904 0.981 0.921 0.454 1.10 T
117 T 0.760 -0.873 1.021 0.873 0.341 0.90 T
118 G 0.761 -0.842 1.089 0.944 0.421 0.65 H T
119 K 0.767 -0.821 1.064 0.951 0.315 0.65 HST
120 Q 0.779 -0.808 0.959 0.937 0.276 0.90 HS
121 W 0.801 -0.734 0.979 0.971 0.421 0.90 HS
122 Q 0.829 -0.678 1.036 0.981 0.257 0.65 HS
123 G 0.873 -0.660 1.046 1.017 0.305 0.65 HS
124 I 0.924 -0.622 1.050 1.049 0.255 0.65 S
125 R 0.970 -0.566 1.083 1.040 0.185 0.25 S
126 L 1.018 -0.497 1.087 1.071 0.249 0.00 S
127 L 1.059 -0.438 1.191 1.069 0.202 0.40 S
128 H 1.091 -0.353 1.171 1.034 0.279 0.65 S
129 L 1.116 -0.264 1.137 1.071 0.327 0.40 S
130 A 1.129 -0.205 1.070 1.099 0.361 0.60 S
131 T 1.133 -0.140 1.069 1.081 0.274 0.60 S
132 Y 1.134 -0.107 0.974 1.059 0.377 0.85 S
133 T 1.125 -0.120 0.869 1.044 0.504 1.10 ST
134 A 1.111 -0.148 0.870 1.061 0.405 0.85 ST
135 G 1.087 -0.180 0.826 0.980 0.291 1.10 ST
136 G 1.057 -0.215 0.909 0.947 0.231 1.10 T
137 L 1.016 -0.311 0.971 0.889 0.586 1.10
138 P 0.966 -0.424 0.926 0.967 0.220 1.10 S
139 L 0.904 -0.502 0.920 0.927 0.240 1.10 S
140 Q 0.835 -0.579 0.989 0.899 0.223 1.10 S
141 I 0.768 -0.652 0.951 0.856 0.694 1.10 ST
142 P 0.715 -0.689 1.007 0.977 0.444 1.10 ST
143 D 0.670 -0.732 0.959 0.973 0.368 1.10 T
144 D 0.644 -0.739 0.926 0.961 0.375 1.10 T
145 V 0.640 -0.760 0.963 0.864 0.442 0.90 T
146 R 0.670 -0.781 1.073 0.901 0.310 0.65 T
147 D 0.722 -0.838 1.109 0.927 0.355 0.65 T
148 K 0.785 -0.904 1.146 0.970 0.236 0.65 ST
149 A 0.844 -0.935 1.201 0.903 0.242 0.65 S
150 A 0.899 -0.957 1.239 0.916 0.175 0.65 S
151 L 0.942 -0.994 1.243 1.001 0.244 0.65 S
152 L 0.960 -1.012 1.170 1.070 0.298 0.60 S
153 H 0.946 -0.976 1.167 1.056 0.393 0.60 S
154 F 0.910 -0.908 1.111 1.036 0.252 0.85 S
155 Y 0.862 -0.847 1.067 1.053 0.538 0.60 ST
156 Q 0.809 -0.806 1.063 1.021 0.319 0.85 ST
157 N 0.762 -0.775 0.963 0.959 0.279 1.10 ST
158 W 0.721 -0.800 0.991 0.884 0.307 1.10 ST
159 Q 0.698 -0.818 1.030 0.869 0.669 1.10 ST
160 P 0.694 -0.871 0.967 0.927 0.343 1.10 ST
161 Q 0.702 -0.916 0.913 0.910 0.220 1.10 ST
162 W 0.720 -0.963 0.851 0.879 0.371 1.10 ST
163 T 0.743 -0.953 0.854 0.893 0.725 1.10 ST
164 P 0.762 -0.900 0.956 0.911 0.317 1.10 ST
165 G 0.781 -0.847 0.911 0.939 0.335 1.10 T
166 A 0.802 -0.779 0.956 0.921 0.344 1.10 T
167 K 0.820 -0.693 0.940 0.927 0.322 0.90
168 R 0.841 -0.591 1.050 0.964 0.267 0.65
169 L 0.863 -0.507 1.099 0.941 0.252 0.65
170 Y 0.891 -0.422 1.031 0.949 0.455 0.65 T
171 A 0.933 -0.362 0.973 0.974 0.374 0.90 T
172 N 0.983 -0.308 0.974 1.040 0.481 0.90 T

173 S 1.032 -0.241 0.869 1.026 0.424 0.90 T
174 S 1.086 -0.190 0.951 0.993 0.414 0.90 T
175 I 1.136 -0.153 0.920 1.053 0.229 0.65 T
176 G 1.180 -0.120 0.871 1.076 0.344 0.65 H
177 L 1.211 -0.094 0.939 1.069 0.350 0.65 H
178 F 1.225 -0.045 1.007 1.079 0.249 0.65 H
179 G 1.221 -0.035 1.053 1.000 0.272 0.65 H
180 A 1.203 -0.025 1.103 1.081 0.173 0.40 H
181 L 1.165 -0.040 1.093 1.046 0.258 0.85 H
182 A 1.119 -0.084 1.014 0.949 0.248 1.10 H
183 V 1.071 -0.131 1.051 0.953 0.317 0.85 H
184 K 1.024 -0.203 0.947 0.956 0.723 1.10 H T
185 P 0.981 -0.278 0.971 0.949 0.486 1.10 H T
186 S 0.945 -0.381 0.904 0.956 0.325 1.10 T
187 G 0.914 -0.484 0.877 0.921 0.434 1.10 T
188 M 0.890 -0.555 0.907 0.919 0.385 0.90 T
189 S 0.872 -0.624 1.039 0.934 0.326 0.65 T
190 Y 0.861 -0.677 1.106 0.927 0.277 0.65 T
191 E 0.854 -0.727 1.236 0.934 0.206 0.65
192 E 0.843 -0.739 1.144 0.969 0.225 0.65
193 A 0.832 -0.748 1.164 0.974 0.292 0.65
194 M 0.825 -0.757 1.199 0.937 0.360 0.65
195 T 0.827 -0.760 1.123 1.043 0.344 0.65
196 R 0.833 -0.795 1.103 1.081 0.272 0.40
197 R 0.832 -0.806 1.100 1.067 0.252 0.60
198 V 0.834 -0.807 0.964 1.020 0.192 1.10
199 L 0.846 -0.769 1.031 0.993 0.263 0.85
200 Q 0.865 -0.738 1.070 0.961 0.596 0.85
201 P 0.892 -0.702 1.119 0.966 0.269 0.85
202 L 0.916 -0.624 1.173 0.881 0.270 0.85
203 K 0.938 -0.560 1.161 0.890 0.169 0.85 S
204 L 0.967 -0.541 1.099 0.949 0.309 0.65 S
205 A 0.991 -0.562 1.166 1.020 0.339 0.65 S
206 H 1.005 -0.580 1.119 1.081 0.368 0.40 S
207 T 1.005 -0.633 1.061 1.144 0.191 0.40 S
208 W 0.983 -0.740 1.006 1.211 0.229 0.85 S
209 I 0.939 -0.830 0.896 1.174 0.245 0.85 S
210 T 0.880 -0.896 0.903 1.134 0.266 0.85 S
211 V 0.808 -0.941 0.913 1.070 0.581 0.85 S
212 P 0.732 -0.962 0.977 1.014 0.455 1.10 ST
213 Q 0.660 -0.960 1.020 0.921 0.332 1.10 T
214 N 0.599 -0.946 1.077 0.859 0.353 0.85 T
215 E 0.558 -0.996 1.096 0.749 0.307 0.65 T
216 Q 0.545 -1.039 1.124 0.836 0.493 0.65 T
217 K 0.557 -1.109 1.127 0.850 0.337 0.65 T
218 D 0.583 -1.203 1.140 0.904 0.414 0.65 T
219 Y 0.615 -1.268 1.014 0.929 0.374 0.90 T
220 A 0.647 -1.315 0.936 0.993 0.388 0.90
221 W 0.672 -1.294 0.897 1.024 0.361 0.90
222 G 0.692 -1.258 0.954 1.029 0.330 0.90
223 Y 0.703 -1.157 0.931 0.981 0.417 0.90 T
224 R 0.703 -1.030 0.923 0.963 0.415 1.10 T
225 E 0.706 -0.944 0.856 0.891 0.281 1.10 T
226 G 0.715 -0.852 0.906 0.973 0.304 1.10 T
227 K 0.732 -0.808 0.977 0.949 0.526 1.10
228 P 0.757 -0.781 0.970 1.020 0.296 1.10
229 V 0.783 -0.802 0.881 1.049 0.241 1.10
230 H 0.805 -0.817 0.876 1.009 0.381 1.10
231 V 0.829 -0.795 0.780 1.030 0.387 1.55
232 S 0.844 -0.778 0.887 1.053 0.799 0.85 T
233 P 0.853 -0.706 0.943 0.986 0.294 1.10 T
234 G 0.855 -0.663 0.917 0.934 0.317 1.10 T
235 Q 0.854 -0.607 0.971 0.850 0.336 1.10 T

236	L	0.852	-0.560	1.060	0.821	0.270	0.65	
237	D	0.857	-0.535	1.170	0.859	0.358	0.65	
238	A	0.863	-0.499	1.193	0.906	0.280	0.65	
239	E	0.876	-0.520	1.091	0.923	0.398	0.65	
240	A	0.888	-0.516	1.036	0.990	0.368	0.65	
241	Y	0.900	-0.481	1.063	0.997	0.288	0.65	
242	G	0.915	-0.502	0.996	1.004	0.328	0.90	
243	V	0.932	-0.509	0.907	1.033	0.408	0.90	
244	K	0.945	-0.522	0.853	1.117	0.372	0.65	
245	S	0.957	-0.510	0.889	1.146	0.341	0.65	
246	S	0.959	-0.497	0.957	1.117	0.262	0.65	
247	V	0.963	-0.523	1.037	1.043	0.330	0.65	
248	I	0.967	-0.514	1.046	1.061	0.225	0.40	
249	D	0.961	-0.551	1.066	1.067	0.349	0.40	
250	M	0.951	-0.559	1.090	1.094	0.410	0.40	
251	A	0.938	-0.573	1.090	1.094	0.283	0.40	
252	R	0.922	-0.609	1.133	1.001	0.207	0.65	
253	W	0.912	-0.629	1.169	1.027	0.220	0.65	
254	V	0.898	-0.677	1.087	0.997	0.286	0.65	
255	Q	0.886	-0.695	1.113	1.007	0.396	0.65	
256	A	0.877	-0.779	1.124	0.969	0.238	0.65	
257	N	0.867	-0.868	1.167	0.934	0.480	0.65	T
258	M	0.855	-0.899	1.154	0.857	0.319	0.65	T
259	D	0.844	-0.941	1.147	0.897	0.402	0.65	T
260	A	0.828	-0.979	1.093	0.981	0.345	0.65	T
261	S	0.807	-0.996	1.146	0.987	0.291	0.65	
262	H	0.780	-0.973	1.141	0.956	0.289	0.65	
263	V	0.758	-0.923	1.169	0.963	0.332	0.65	
264	Q	0.745	-0.902	1.103	1.007	0.285	0.25	
265	E	0.744	-0.846	1.171	1.017	0.306	0.65	
266	K	0.751	-0.784	1.179	0.977	0.297	0.65	
267	T	0.769	-0.743	1.230	0.879	0.246	0.65	
268	L	0.805	-0.715	1.129	0.896	0.348	0.65	
269	Q	0.854	-0.722	1.061	0.996	0.418	0.65	
270	Q	0.902	-0.725	1.070	1.014	0.230	0.65	
271	G	0.942	-0.757	1.137	0.987	0.241	0.65	S
272	I	0.965	-0.727	1.136	0.970	0.213	0.65	S
273	A	0.976	-0.694	1.136	0.970	0.218	0.65	S
274	L	0.973	-0.685	1.071	0.991	0.280	0.85	S
275	A	0.950	-0.645	1.129	1.001	0.368	0.85	S
276	Q	0.916	-0.600	1.093	0.973	0.437	0.85	ST
277	S	0.879	-0.572	1.050	1.007	0.507	0.85	ST
278	R	0.843	-0.593	1.001	1.003	0.284	1.10	T
279	Y	0.819	-0.614	0.956	1.081	0.250	0.85	T
280	W	0.807	-0.609	0.854	1.099	0.348	0.85	T
281	R	0.801	-0.636	0.886	1.066	0.375	0.65	
282	I	0.813	-0.685	0.959	1.063	0.362	0.65	
283	G	0.828	-0.710	0.959	1.063	0.351	0.65	
284	D	0.845	-0.692	0.999	1.014	0.441	0.90	T
285	M	0.872	-0.694	0.941	1.004	0.322	0.90	T
286	Y	0.897	-0.691	0.989	0.943	0.440	0.90	T
287	Q	0.917	-0.653	0.989	0.943	0.347	0.90	T
288	G	0.935	-0.611	0.981	1.003	0.484	0.90	T
289	L	0.944	-0.606	0.977	0.971	0.274	0.90	T
290	G	0.953	-0.608	1.084	0.931	0.247	0.65	
291	W	0.959	-0.619	1.089	0.963	0.221	0.65	
292	E	0.950	-0.628	1.137	0.940	0.265	0.65	
293	M	0.934	-0.631	1.093	0.957	0.451	0.85	
294	L	0.916	-0.622	1.087	0.917	0.276	0.85	
295	N	0.902	-0.608	1.131	0.900	0.278	0.85	
296	W	0.887	-0.620	1.101	0.903	0.599	0.85	T
297	P	0.873	-0.599	1.076	0.893	0.257	0.85	T
298	L	0.861	-0.580	1.039	0.850	0.292	0.85	T

299 K 0.861 -0.592 1.027 0.877 0.416 1.10 T
300 A 0.868 -0.650 1.024 0.921 0.351 0.90
301 D 0.873 -0.653 1.089 1.037 0.355 0.65
302 S 0.871 -0.644 1.031 1.000 0.258 0.65
303 I 0.871 -0.631 0.936 1.021 0.420 1.10
304 I 0.869 -0.624 0.869 1.029 0.422 1.10
305 N 0.867 -0.580 0.869 1.029 0.452 1.10 T
306 G 0.866 -0.495 0.869 1.029 0.526 1.10 T
307 S 0.869 -0.419 0.906 0.931 0.450 1.10 T
308 D 0.887 -0.350 0.897 0.937 0.411 1.10 T
309 S 0.921 -0.295 0.953 0.957 0.319 1.10 T
310 K 0.964 -0.273 1.059 0.971 0.208 0.65 T
311 V 1.012 -0.255 1.126 0.964 0.232 0.65
312 A 1.061 -0.264 1.161 0.990 0.178 0.25
313 L 1.102 -0.296 1.230 1.000 0.242 0.85
314 A 1.129 -0.313 1.129 0.981 0.240 0.85
315 A 1.136 -0.323 1.183 0.897 0.177 0.85
316 L 1.121 -0.270 1.129 0.981 0.540 0.85
317 P 1.086 -0.251 1.149 0.943 0.268 0.85
318 A 1.042 -0.205 1.094 1.027 0.238 0.85
319 V 0.991 -0.193 1.039 1.007 0.239 0.85
320 E 0.940 -0.200 0.927 0.953 0.363 1.10
321 V 0.900 -0.215 0.927 0.953 0.247 1.10
322 N 0.873 -0.272 0.927 0.953 0.644 1.10 T
323 P 0.858 -0.313 0.871 0.831 0.596 1.10 T
324 P 0.863 -0.383 0.850 0.853 0.270 1.10 T
325 A 0.874 -0.455 0.850 0.853 0.539 1.10 T
326 P 0.889 -0.502 0.897 0.854 0.299 1.10
327 A 0.908 -0.551 1.007 0.891 0.238 1.10
328 V 0.925 -0.565 1.050 0.936 0.318 0.85
329 K 0.935 -0.587 1.007 0.970 0.423 0.90
330 A 0.941 -0.578 1.063 1.091 0.316 0.40
331 S 0.936 -0.530 1.053 1.117 0.213 0.40
332 W 0.927 -0.499 1.099 1.014 0.313 0.65
333 V 0.927 -0.462 1.041 1.077 0.260 0.40
334 H 0.929 -0.454 0.937 1.080 0.472 0.65 T
335 K 0.936 -0.422 0.937 1.080 0.459 0.85 T
336 T 0.950 -0.392 0.914 1.090 0.375 0.85 T
337 G 0.970 -0.357 0.864 1.009 0.458 1.10 T
338 S 1.000 -0.317 0.770 0.986 0.570 1.10 T
339 T 1.038 -0.276 0.747 1.064 0.426 0.85 T
340 G 1.070 -0.209 0.709 1.023 0.404 1.10 T
341 G 1.099 -0.162 0.746 1.027 0.439 1.10 T
342 F 1.121 -0.151 0.731 1.070 0.394 0.65
343 G 1.135 -0.167 0.743 1.110 0.408 0.65
344 S 1.138 -0.188 0.847 1.107 0.269 0.65 S
345 Y 1.126 -0.206 0.920 1.164 0.230 0.65 S
346 V 1.097 -0.235 0.897 1.189 0.256 0.85 S
347 A 1.057 -0.263 0.891 1.149 0.195 0.85 S
348 F 1.011 -0.264 0.980 1.120 0.205 0.85 S
349 V 0.966 -0.237 1.053 1.051 0.625 0.60 ST
350 P 0.931 -0.215 1.051 0.947 0.325 0.85 ST
351 E 0.915 -0.211 1.053 0.964 0.432 0.85 T
352 K 0.915 -0.255 0.980 0.907 0.326 1.10 T
353 N 0.932 -0.309 0.989 0.901 0.432 0.90
354 L 0.964 -0.368 1.044 1.023 0.212 0.65
355 G 1.004 -0.444 1.049 1.054 0.217 0.40
356 I 1.038 -0.540 1.059 1.090 0.175 0.40
357 V 1.055 -0.598 1.114 1.110 0.238 0.40
358 M 1.043 -0.580 1.057 1.073 0.219 0.60
359 L 1.011 -0.565 1.153 1.051 0.423 0.60
360 A 0.964 -0.541 1.131 0.980 0.321 0.85
361 N 0.906 -0.531 1.104 0.946 0.526 0.85 T

362 K 0.842 -0.513 0.969 0.899 0.376 1.10 T
 363 S 0.781 -0.520 0.911 0.861 0.310 1.10 T
 364 Y 0.739 -0.577 0.801 0.824 0.732 1.10 T
 365 P 0.723 -0.661 0.803 0.929 0.272 1.10 T
 366 N 0.721 -0.738 0.764 0.960 0.663 1.10 T
 367 P 0.734 -0.761 0.777 1.037 0.302 1.10 T
 368 V 0.757 -0.771 0.880 0.966 0.258 1.10 T
 369 R 0.794 -0.807 0.990 1.003 0.253 1.10 T
 370 V 0.832 -0.821 1.046 1.023 0.215 0.25
 371 E 0.863 -0.814 1.113 1.094 0.334 0.00 S
 372 A 0.880 -0.801 1.120 1.023 0.285 0.25 S
 373 A 0.895 -0.600 1.121 1.089 0.228 0.00 S
 374 W 0.895 -0.600 1.177 1.021 0.266 0.25 S
 375 R 0.887 -0.600 1.177 1.021 0.204 0.25 S
 376 I 0.868 -0.600 1.169 1.003 0.240 0.25 S
 377 L 0.846 -0.600 1.170 1.020 0.263 0.25 S
 378 E 0.824 -0.600 1.210 0.971 0.305 0.25 S
 379 K 0.816 -0.600 1.073 0.830 0.117 0.25
 380 L 0.797 -0.600 1.048 0.668 0.000 0.25
 381 Q 0.791 -0.600 0.857 0.607 0.000 0.50
 382 * 0.830 -0.600 0.000 0.000 0.000 1.40

MUTATED VARIANT

Bla^{cmv}-2 (WV Moore) Protein Toolbox List Page 1
Wednesday, February 16, 2011 4:09 PM

Key:

Argos = Argos transmembrane

vH trns = von Heijne transmembrane

CFhlx = Chou-Fasman Alpha Helix

CFsht = Chou-Fasman Beta Sheet

CFturn = Chou-Fasman Turn

CFval = Chou-Fasman Structure Value

CFtype = Chou-Fasman Probable Type

Pos. AA Argos vH trns CFhlx CFsht CFturn CFval CFtype

1 M 1.420 -0.600 1.470 0.970 0.317 0.65 H
 2 M 1.168 -0.600 1.390 0.903 0.361 0.65 H
 3 K 1.107 -0.600 1.244 0.886 0.365 0.65 H
 4 K 1.107 -0.600 1.233 0.884 0.358 0.65 H
 5 S 1.076 -0.600 1.181 0.851 0.390 0.65 H
 6 L 1.103 -0.600 1.156 0.841 0.289 0.65 H
 7 C 1.146 -0.600 1.166 0.877 0.307 0.65 H
 8 C 1.190 -0.600 1.176 0.913 0.331 0.65 H
 9 A 1.234 -0.600 1.244 0.923 0.191 0.25 H
 10 L 1.267 -0.600 1.177 0.950 0.201 0.65 H
 11 L 1.286 0.221 1.203 0.973 0.209 0.65 H
 12 L 1.296 0.286 1.161 1.003 0.310 0.65 H
 13 T 1.299 0.303 1.130 1.063 0.352 0.40 H
 14 A 1.288 0.303 1.061 1.053 0.370 0.40 H
 15 S 1.272 0.240 0.994 1.080 0.365 0.65 H
 16 F 1.254 0.117 0.961 1.123 0.328 0.65 H
 17 S 1.231 -0.036 1.027 1.079 0.351 0.65 H
 18 T 1.198 -0.181 1.027 1.079 0.220 0.65 H
 19 F 1.155 -0.272 1.094 1.071 0.228 0.40 H
 20 A 1.099 -0.370 1.117 0.993 0.266 0.65 H
 21 A 1.039 -0.429 1.119 1.030 0.287 0.25 H
 22 A 0.974 -0.460 1.206 0.964 0.304 0.25 H
 23 K 0.912 -0.484 1.234 0.890 0.338 0.25 H
 24 T 0.859 -0.540 1.231 0.876 0.281 0.25 H
 25 E 0.822 -0.605 1.186 0.954 0.247 0.65 H
 26 Q 0.799 -0.636 1.186 0.954 0.243 0.65 H
 27 Q 0.793 -0.682 1.159 0.947 0.224 0.65 H

28	I	0.800	-0.738	1.179	0.981	0.354	0.65	H
29	A	0.818	-0.737	1.103	1.087	0.236	0.40	H
30	D	0.836	-0.706	1.050	1.081	0.298	0.40	H
31	I	0.856	-0.664	1.006	1.109	0.367	0.65	H
32	V	0.874	-0.635	0.986	1.074	0.323	0.65	H
33	N	0.892	-0.602	0.940	1.153	0.388	0.65	H
34	R	0.905	-0.570	0.910	1.223	0.270	0.85	
35	T	0.910	-0.567	0.846	1.107	0.253	0.85	
36	I	0.913	-0.548	0.901	1.040	0.255	1.10	
37	T	0.917	-0.544	0.983	1.070	0.568	0.85	
38	P	0.912	-0.539	1.027	1.043	0.239	1.10	
39	L	0.902	-0.511	1.114	0.977	0.244	0.85	
40	M	0.892	-0.439	1.157	0.884	0.341	0.85	
41	Q	0.894	-0.371	1.223	0.840	0.229	0.65	
42	E	0.907	-0.325	1.287	0.956	0.245	0.85	
43	Q	0.923	-0.259	1.176	0.901	0.231	0.85	
44	A	0.952	-0.187	1.046	0.894	0.280	0.85	
45	I	0.998	-0.164	1.074	0.919	0.679	0.85	T
46	P	1.051	-0.176	1.053	0.940	0.259	0.85	T
47	G	1.101	-0.204	1.001	1.039	0.272	1.10	T
48	M	1.137	-0.210	1.001	1.039	0.228	1.10	T
49	A	1.155	-0.211	0.993	1.044	0.196	0.50	
50	V	1.159	-0.219	1.057	1.160	0.220	0.00	
51	A	1.137	-0.208	1.080	1.207	0.246	0.40	
52	V	1.092	-0.194	1.051	1.183	0.308	0.40	
53	I	1.038	-0.177	0.947	1.186	0.297	0.65	
54	Y	0.976	-0.182	0.993	1.083	0.465	0.85	T
55	Q	0.923	-0.235	0.883	1.046	0.299	1.10	T
56	G	0.880	-0.286	0.856	1.011	0.376	1.10	T
57	K	0.852	-0.358	0.820	0.983	0.685	1.10	T
58	P	0.844	-0.432	0.870	0.993	0.346	1.10	ST
59	Y	0.849	-0.501	0.807	1.051	0.291	0.85	ST
60	Y	0.862	-0.588	0.869	1.083	0.355	0.85	ST
61	F	0.879	-0.643	0.773	1.104	0.383	0.65	S
62	T	0.890	-0.718	0.874	1.123	0.384	0.65	S
63	W	0.896	-0.739	0.956	1.073	0.292	0.65	S
64	G	0.889	-0.750	1.001	0.997	0.333	0.90	S
65	K	0.877	-0.751	0.987	1.016	0.366	0.90	
66	A	0.861	-0.751	1.053	0.971	0.241	0.65	
67	D	0.839	-0.772	1.040	0.917	0.307	0.65	
68	I	0.814	-0.768	1.089	0.894	0.401	0.65	
69	A	0.795	-0.775	1.087	0.939	0.388	0.85	
70	N	0.781	-0.792	0.977	0.901	0.405	1.10	
71	N	0.780	-0.806	0.959	1.011	0.295	1.10	
72	H	0.780	-0.801	0.939	0.977	0.636	1.10	T
73	P	0.784	-0.748	0.936	0.963	0.313	1.10	ST
74	V	0.801	-0.706	0.989	0.969	0.305	1.10	ST
75	T	0.831	-0.674	0.979	1.033	0.300	1.10	ST
76	Q	0.867	-0.626	0.990	1.024	0.307	0.50	S
77	Q	0.906	-0.595	1.069	1.121	0.283	0.00	S
78	T	0.935	-0.545	1.144	1.016	0.240	0.65	S
79	L	0.967	-0.511	1.211	0.989	0.249	0.65	S
80	F	0.998	-0.495	1.110	1.006	0.307	0.65	S
81	E	1.019	-0.480	1.046	1.027	0.377	0.65	S
82	L	1.026	-0.459	1.057	1.067	0.324	0.40	
83	G	1.021	-0.402	0.989	1.057	0.373	0.65	
84	S	1.009	-0.364	1.011	0.979	0.388	0.90	
85	V	1.005	-0.340	0.924	1.044	0.352	0.90	
86	S	1.001	-0.317	0.891	1.087	0.365	0.65	
87	K	0.994	-0.267	0.940	1.064	0.319	0.65	
88	T	0.993	-0.241	0.903	1.060	0.470	0.65	T
89	F	1.000	-0.239	0.903	1.060	0.385	0.65	T
90	N	1.016	-0.266	0.971	1.070	0.342	0.65	T

91 G 1.033 -0.295 0.876 1.091 0.338 0.65 T
92 V 1.041 -0.320 0.837 1.050 0.429 0.65
93 L 1.039 -0.331 0.833 0.964 0.417 0.90
94 G 1.041 -0.376 0.889 0.984 0.424 0.90
95 G 1.033 -0.421 0.947 1.060 0.303 0.65
96 D 1.016 -0.418 1.001 0.976 0.294 0.90
97 A 0.988 -0.436 0.953 0.971 0.214 0.90
98 I 0.957 -0.441 0.953 0.971 0.370 0.90
99 A 0.925 -0.487 1.079 0.947 0.420 0.65
100 R 0.900 -0.505 1.069 1.051 0.288 0.40
101 G 0.870 -0.557 1.060 1.033 0.270 0.65
102 E 0.844 -0.610 1.107 0.971 0.232 0.65
103 I 0.822 -0.648 1.040 0.979 0.300 0.65
104 K 0.805 -0.689 1.051 0.940 0.286 0.85
105 L 0.792 -0.760 1.046 0.900 0.447 0.85 T
106 S 0.785 -0.792 0.970 1.006 0.317 1.10 T
107 D 0.776 -0.795 0.950 0.971 0.643 1.10 T
108 P 0.769 -0.790 0.950 0.971 0.310 1.10 T
109 V 0.765 -0.815 0.867 1.004 0.367 1.10 T
110 T 0.765 -0.812 0.891 1.031 0.482 1.10 T
111 K 0.765 -0.797 0.817 1.020 0.252 1.10 T
112 Y 0.765 -0.835 0.949 1.036 0.193 1.10 T
113 W 0.765 -0.844 1.004 0.969 0.615 1.10 T
114 P 0.767 -0.866 1.004 0.969 0.277 1.10 T
115 E 0.769 -0.883 0.909 0.990 0.298 1.10 T
116 L 0.765 -0.904 0.981 0.921 0.454 1.10 T
117 T 0.760 -0.873 1.021 0.873 0.341 0.90 T
118 G 0.761 -0.842 1.089 0.944 0.421 0.65 H T
119 K 0.767 -0.821 1.064 0.951 0.315 0.65 HST
120 Q 0.779 -0.808 0.959 0.937 0.276 0.90 HS
121 W 0.801 -0.734 0.979 0.971 0.421 0.90 HS
122 Q 0.829 -0.678 1.036 0.981 0.257 0.65 HS
123 G 0.873 -0.660 1.046 1.017 0.305 0.65 HS
124 I 0.924 -0.622 1.050 1.049 0.255 0.65 S
125 R 0.970 -0.566 1.083 1.040 0.185 0.25 S
126 L 1.018 -0.497 1.087 1.071 0.249 0.00 S
127 L 1.059 -0.438 1.191 1.069 0.202 0.40 S
128 H 1.091 -0.353 1.171 1.034 0.279 0.65 S
129 L 1.116 -0.264 1.137 1.071 0.327 0.40 S
130 A 1.129 -0.205 1.070 1.099 0.361 0.60 S
131 T 1.133 -0.140 1.069 1.081 0.274 0.60 S
132 Y 1.134 -0.107 0.974 1.059 0.377 0.85 S
133 T 1.125 -0.120 0.869 1.044 0.504 1.10 ST
134 A 1.111 -0.148 0.870 1.061 0.405 0.85 ST
135 G 1.087 -0.180 0.826 0.980 0.291 1.10 ST
136 G 1.057 -0.215 0.909 0.947 0.231 1.10 T
137 L 1.016 -0.311 0.971 0.889 0.586 1.10
138 P 0.966 -0.424 0.926 0.967 0.220 1.10 S
139 L 0.904 -0.502 0.920 0.927 0.240 1.10 S
140 Q 0.835 -0.579 0.989 0.899 0.223 1.10 S
141 I 0.768 -0.652 0.951 0.856 0.694 1.10 ST
142 P 0.715 -0.689 1.007 0.977 0.444 1.10 ST
143 D 0.670 -0.732 0.959 0.973 0.368 1.10 T
144 D 0.644 -0.739 0.926 0.961 0.375 1.10 T
145 V 0.640 -0.760 0.963 0.864 0.442 0.90 T
146 R 0.670 -0.781 1.073 0.901 0.310 0.65 T
147 D 0.722 -0.838 1.109 0.927 0.355 0.65 T
148 K 0.785 -0.904 1.146 0.970 0.236 0.65 ST
149 A 0.844 -0.935 1.201 0.903 0.242 0.65 S
150 A 0.899 -0.957 1.239 0.916 0.175 0.65 S
151 L 0.942 -0.994 1.243 1.001 0.244 0.65 S
152 L 0.960 -1.012 1.170 1.070 0.298 0.60 S
153 H 0.946 -0.976 1.167 1.056 0.393 0.60 S

154	F	0.910	-0.908	1.111	1.036	0.252	0.85	S
155	Y	0.862	-0.847	1.067	1.053	0.538	0.60	ST
156	Q	0.809	-0.806	1.063	1.021	0.319	0.85	ST
157	N	0.762	-0.775	0.963	0.959	0.279	1.10	ST
158	W	0.721	-0.800	0.991	0.884	0.307	1.10	ST
159	Q	0.698	-0.818	1.030	0.869	0.669	1.10	ST
160	P	0.694	-0.871	0.967	0.927	0.343	1.10	ST
161	Q	0.702	-0.916	0.913	0.910	0.220	1.10	ST
162	W	0.720	-0.963	0.851	0.879	0.371	1.10	ST
163	T	0.743	-0.953	0.854	0.893	0.725	1.10	ST
164	P	0.762	-0.900	0.956	0.911	0.317	1.10	ST
165	G	0.781	-0.847	0.911	0.939	0.335	1.10	T
166	A	0.802	-0.779	0.956	0.921	0.344	1.10	T
167	K	0.820	-0.693	0.940	0.927	0.322	0.90	
168	R	0.841	-0.591	1.050	0.964	0.267	0.65	
169	L	0.863	-0.507	1.099	0.941	0.252	0.65	
170	Y	0.891	-0.422	1.031	0.949	0.455	0.65	T
171	A	0.933	-0.362	0.973	0.974	0.374	0.90	T
172	N	0.983	-0.308	0.974	1.040	0.481	0.90	T
173	S	1.032	-0.241	0.869	1.026	0.424	0.90	T
174	S	1.086	-0.190	0.951	0.993	0.414	0.90	T
175	I	1.136	-0.153	0.920	1.053	0.229	0.65	T
176	G	1.180	-0.120	0.871	1.076	0.344	0.65	H
177	L	1.211	-0.094	0.939	1.069	0.350	0.65	H
178	F	1.225	-0.045	1.007	1.079	0.249	0.65	H
179	G	1.221	-0.035	1.053	1.000	0.272	0.65	H
180	A	1.203	-0.025	1.103	1.081	0.173	0.40	H
181	L	1.165	-0.040	1.093	1.046	0.258	0.85	H
182	A	1.119	-0.084	1.014	0.949	0.248	1.10	H
183	V	1.071	-0.131	1.051	0.953	0.317	0.85	H
184	K	1.024	-0.203	0.947	0.956	0.723	1.10	H T
185	P	0.981	-0.278	0.971	0.949	0.486	1.10	H T
186	S	0.945	-0.381	0.904	0.956	0.325	1.10	T
187	G	0.914	-0.484	0.877	0.921	0.434	1.10	T
188	M	0.890	-0.555	0.907	0.919	0.385	0.90	T
189	S	0.872	-0.624	1.039	0.934	0.326	0.65	T
190	Y	0.861	-0.677	1.106	0.927	0.277	0.65	T
191	E	0.854	-0.727	1.236	0.934	0.206	0.65	
192	E	0.843	-0.739	1.144	0.969	0.225	0.65	
193	A	0.832	-0.748	1.164	0.974	0.292	0.65	
194	M	0.825	-0.757	1.199	0.937	0.360	0.65	
195	T	0.827	-0.760	1.123	1.043	0.344	0.65	
196	R	0.833	-0.795	1.103	1.081	0.272	0.40	
197	R	0.832	-0.806	1.100	1.067	0.252	0.60	
198	V	0.834	-0.807	0.964	1.020	0.192	1.10	
199	L	0.846	-0.769	1.031	0.993	0.263	0.85	
200	Q	0.865	-0.738	1.070	0.961	0.596	0.85	
201	P	0.892	-0.702	1.119	0.966	0.269	0.85	
202	L	0.916	-0.624	1.173	0.881	0.270	0.85	
203	K	0.938	-0.560	1.161	0.890	0.169	0.85	S
204	L	0.967	-0.541	1.099	0.949	0.309	0.65	S
205	A	0.991	-0.562	1.166	1.020	0.339	0.65	S
206	H	1.005	-0.580	1.119	1.081	0.368	0.40	S
207	T	1.005	-0.633	1.061	1.144	0.191	0.40	S
208	W	0.983	-0.740	1.006	1.211	0.229	0.85	S
209	I	0.939	-0.830	0.896	1.174	0.245	0.85	S
210	T	0.880	-0.896	0.903	1.134	0.266	0.85	S
211	V	0.808	-0.941	0.913	1.070	0.581	0.85	S
212	P	0.732	-0.962	0.977	1.014	0.455	1.10	ST
213	Q	0.660	-0.960	1.020	0.921	0.332	1.10	T
214	N	0.599	-0.946	1.077	0.859	0.353	0.85	T
215	E	0.558	-0.996	1.096	0.749	0.307	0.65	T
216	Q	0.545	-1.039	1.124	0.836	0.493	0.65	T

217	K	0.557	-1.109	1.127	0.850	0.337	0.65	T
218	D	0.583	-1.203	1.140	0.904	0.414	0.65	T
219	Y	0.615	-1.268	1.014	0.929	0.374	0.90	T
220	A	0.647	-1.315	0.936	0.993	0.388	0.90	
221	W	0.672	-1.294	0.897	1.024	0.361	0.90	
222	G	0.692	-1.258	0.954	1.029	0.330	0.90	
223	Y	0.703	-1.157	0.931	0.981	0.417	0.90	T
224	R	0.703	-1.030	0.923	0.963	0.415	1.10	T
225	E	0.706	-0.944	0.856	0.891	0.281	1.10	T
226	G	0.715	-0.852	0.906	0.973	0.304	1.10	T
227	K	0.732	-0.808	0.977	0.949	0.526	1.10	
228	P	0.757	-0.781	0.970	1.020	0.296	1.10	
229	V	0.783	-0.802	0.881	1.049	0.241	1.10	
230	H	0.805	-0.817	0.876	1.009	0.381	1.10	
231	V	0.829	-0.795	0.780	1.030	0.387	1.55	
232	S	0.844	-0.778	0.887	1.053	0.799	0.85	T
233	P	0.853	-0.706	0.943	0.986	0.294	1.10	T
234	G	0.855	-0.663	0.917	0.934	0.317	1.10	T
235	Q	0.854	-0.607	0.971	0.850	0.336	1.10	T
236	L	0.852	-0.560	1.060	0.821	0.270	0.65	
237	D	0.857	-0.535	1.170	0.859	0.358	0.65	
238	A	0.863	-0.499	1.193	0.906	0.280	0.65	
239	E	0.876	-0.520	1.091	0.923	0.398	0.65	
240	A	0.888	-0.516	1.036	0.990	0.368	0.65	
241	Y	0.900	-0.481	1.063	0.997	0.288	0.65	
242	G	0.915	-0.502	0.996	1.004	0.328	0.90	
243	V	0.932	-0.509	0.907	1.033	0.408	0.90	
244	K	0.945	-0.522	0.853	1.117	0.372	0.65	
245	S	0.957	-0.510	0.889	1.146	0.341	0.65	
246	S	0.959	-0.497	0.957	1.117	0.262	0.65	
247	V	0.963	-0.523	1.037	1.043	0.330	0.65	
248	I	0.967	-0.514	1.046	1.061	0.225	0.40	
249	D	0.961	-0.551	1.066	1.067	0.349	0.40	
250	M	0.951	-0.559	1.090	1.094	0.410	0.40	
251	A	0.938	-0.573	1.090	1.094	0.283	0.40	
252	R	0.922	-0.609	1.133	1.001	0.207	0.65	
253	V	0.912	-0.629	1.169	1.027	0.220	0.65	
254	W	0.898	-0.677	1.087	0.997	0.286	0.65	
255	Q	0.886	-0.695	1.113	1.007	0.396	0.65	
256	A	0.877	-0.779	1.124	0.969	0.238	0.65	
257	N	0.867	-0.868	1.167	0.934	0.480	0.65	T
258	M	0.855	-0.899	1.154	0.857	0.319	0.65	T
259	D	0.844	-0.941	1.147	0.897	0.402	0.65	T
260	A	0.828	-0.979	1.093	0.981	0.345	0.65	T
261	S	0.807	-0.996	1.146	0.987	0.291	0.65	
262	H	0.780	-0.973	1.141	0.956	0.289	0.65	
263	V	0.758	-0.923	1.169	0.963	0.332	0.65	
264	Q	0.745	-0.902	1.103	1.007	0.285	0.25	
265	E	0.744	-0.846	1.171	1.017	0.306	0.65	
266	K	0.751	-0.784	1.179	0.977	0.297	0.65	
267	T	0.769	-0.743	1.230	0.879	0.246	0.65	
268	L	0.805	-0.680	1.129	0.896	0.348	0.65	
269	Q	0.858	-0.652	1.061	0.996	0.418	0.65	
270	Q	0.914	-0.620	1.070	1.014	0.230	0.65	
271	G	0.965	-0.617	1.137	0.987	0.241	0.65	S
272	I	1.004	-0.551	1.136	0.970	0.213	0.65	S
273	A	1.033	-0.483	1.136	0.970	0.218	0.65	S
274	L	1.054	-0.475	1.071	0.991	0.280	0.65	S
275	A	1.058	-0.434	1.177	1.006	0.353	0.65	S
276	Q	1.043	-0.389	1.141	0.977	0.374	0.65	S
277	S	1.018	-0.361	1.099	1.011	0.426	0.65	S
278	L	0.985	-0.383	1.050	1.007	0.275	0.65	S
279	Y	0.957	-0.403	1.004	1.086	0.250	0.65	S

280	W	0.934	-0.398	0.903	1.103	0.348	0.65	S
281	R	0.909	-0.425	0.934	1.070	0.375	0.65	S
282	I	0.894	-0.474	0.959	1.063	0.362	0.65	S
283	G	0.885	-0.499	0.959	1.063	0.351	0.65	S
284	D	0.884	-0.516	0.999	1.014	0.441	0.90	T
285	M	0.895	-0.553	0.941	1.004	0.322	0.90	T
286	Y	0.908	-0.586	0.989	0.943	0.440	0.90	T
287	Q	0.921	-0.583	0.989	0.943	0.347	0.90	T
288	G	0.935	-0.576	0.981	1.003	0.484	0.90	T
289	L	0.944	-0.606	0.977	0.971	0.274	0.90	T
290	G	0.953	-0.608	1.084	0.931	0.247	0.65	
291	W	0.959	-0.619	1.089	0.963	0.221	0.65	
292	E	0.950	-0.628	1.137	0.940	0.265	0.65	
293	M	0.934	-0.631	1.093	0.957	0.451	0.85	
294	L	0.916	-0.622	1.087	0.917	0.276	0.85	
295	N	0.902	-0.608	1.131	0.900	0.278	0.85	
296	W	0.887	-0.620	1.101	0.903	0.599	0.85	T
297	P	0.873	-0.599	1.076	0.893	0.257	0.85	T
298	L	0.861	-0.580	1.039	0.850	0.292	0.85	T
299	K	0.861	-0.592	1.027	0.877	0.416	1.10	T
300	A	0.868	-0.650	1.024	0.921	0.351	0.90	
301	D	0.873	-0.653	1.089	1.037	0.355	0.65	
302	S	0.871	-0.644	1.031	1.000	0.258	0.65	
303	I	0.871	-0.631	0.936	1.021	0.420	1.10	
304	E	0.869	-0.619	0.869	1.029	0.422	1.10	
305	N	0.867	-0.571	0.869	1.029	0.452	1.10	T
306	G	0.866	-0.481	0.869	1.029	0.526	1.10	T
307	S	0.870	-0.400	0.906	0.931	0.450	1.10	T
308	D	0.887	-0.326	0.897	0.937	0.411	1.10	T
309	S	0.922	-0.267	0.953	0.957	0.319	1.10	T
310	K	0.965	-0.244	1.059	0.971	0.208	0.65	T
311	V	1.013	-0.227	1.071	1.049	0.227	0.65	
312	A	1.062	-0.236	1.107	1.074	0.169	0.00	
313	L	1.104	-0.268	1.176	1.084	0.214	0.60	
314	V	1.130	-0.284	1.074	1.066	0.242	0.60	
315	A	1.137	-0.294	1.129	0.981	0.177	0.85	
316	L	1.122	-0.242	1.074	1.066	0.540	0.60	
317	P	1.087	-0.223	1.094	1.027	0.268	0.85	
318	A	1.043	-0.176	1.094	1.027	0.238	0.85	
319	V	0.991	-0.164	1.039	1.007	0.239	0.85	
320	E	0.941	-0.176	0.927	0.953	0.363	1.10	
321	V	0.901	-0.196	0.927	0.953	0.247	1.10	
322	N	0.873	-0.258	0.927	0.953	0.644	1.10	T
323	P	0.858	-0.303	0.871	0.831	0.596	1.10	T
324	P	0.863	-0.379	0.850	0.853	0.270	1.10	T
325	A	0.874	-0.455	0.850	0.853	0.539	1.10	T
326	P	0.889	-0.502	0.897	0.854	0.299	1.10	
327	A	0.908	-0.551	1.007	0.891	0.238	1.10	
328	V	0.925	-0.565	1.050	0.936	0.318	0.85	
329	K	0.935	-0.587	1.007	0.970	0.423	0.90	
330	A	0.941	-0.578	1.063	1.091	0.316	0.40	
331	S	0.936	-0.530	1.053	1.117	0.213	0.40	
332	W	0.927	-0.499	1.099	1.014	0.313	0.65	
333	V	0.927	-0.462	1.041	1.077	0.260	0.40	
334	H	0.929	-0.454	0.937	1.080	0.472	0.65	T
335	K	0.936	-0.422	0.937	1.080	0.459	0.85	T
336	T	0.950	-0.392	0.914	1.090	0.375	0.85	T
337	G	0.970	-0.357	0.864	1.009	0.458	1.10	T
338	S	1.000	-0.317	0.770	0.986	0.570	1.10	T
339	T	1.038	-0.276	0.747	1.064	0.426	0.85	T
340	G	1.070	-0.209	0.709	1.023	0.404	1.10	T
341	G	1.099	-0.162	0.746	1.027	0.439	1.10	T
342	F	1.121	-0.151	0.731	1.070	0.394	0.65	

343	G	1.135	-0.167	0.743	1.110	0.408	0.65	
344	S	1.138	-0.188	0.847	1.107	0.269	0.65	S
345	Y	1.126	-0.206	0.920	1.164	0.230	0.65	S
346	V	1.097	-0.235	0.897	1.189	0.256	0.85	S
347	A	1.057	-0.263	0.891	1.149	0.195	0.85	S
348	F	1.011	-0.264	0.980	1.120	0.205	0.85	S
349	V	0.966	-0.237	1.053	1.051	0.625	0.60	ST
350	P	0.931	-0.215	1.051	0.947	0.325	0.85	ST
351	E	0.915	-0.211	1.053	0.964	0.432	0.85	T
352	K	0.915	-0.255	0.980	0.907	0.326	1.10	T
353	N	0.932	-0.309	0.989	0.901	0.432	0.90	
354	L	0.964	-0.368	1.044	1.023	0.212	0.65	
355	G	1.004	-0.444	1.049	1.054	0.217	0.40	
356	I	1.038	-0.540	1.059	1.090	0.175	0.40	
357	V	1.055	-0.598	1.114	1.110	0.238	0.40	
358	M	1.043	-0.580	1.057	1.073	0.219	0.60	
359	L	1.011	-0.565	1.153	1.051	0.423	0.60	
360	A	0.964	-0.541	1.131	0.980	0.321	0.85	
361	N	0.906	-0.531	1.104	0.946	0.526	0.85	T
362	K	0.842	-0.513	0.969	0.899	0.376	1.10	T
363	S	0.781	-0.520	0.911	0.861	0.310	1.10	T
364	Y	0.739	-0.577	0.801	0.824	0.732	1.10	T
365	P	0.723	-0.661	0.803	0.929	0.272	1.10	T
366	N	0.721	-0.738	0.764	0.960	0.663	1.10	T
367	P	0.734	-0.761	0.777	1.037	0.302	1.10	T
368	V	0.757	-0.771	0.880	0.966	0.258	1.10	T
369	R	0.794	-0.807	0.990	1.003	0.253	1.10	T
370	V	0.832	-0.821	1.046	1.023	0.215	0.25	
371	E	0.863	-0.814	1.113	1.094	0.334	0.00	S
372	A	0.880	-0.801	1.120	1.023	0.285	0.25	S
373	A	0.895	-0.600	1.121	1.089	0.228	0.00	S
374	W	0.895	-0.600	1.177	1.021	0.266	0.25	S
375	R	0.887	-0.600	1.177	1.021	0.204	0.25	S
376	I	0.868	-0.600	1.169	1.003	0.240	0.25	S
377	L	0.846	-0.600	1.170	1.020	0.263	0.25	S
378	E	0.824	-0.600	1.210	0.971	0.305	0.25	S
379	K	0.816	-0.600	1.073	0.830	0.117	0.25	
380	L	0.797	-0.600	1.048	0.668	0.000	0.25	
381	Q	0.791	-0.600	0.857	0.607	0.000	0.50	
382	*	0.830	-0.600	0.000	0.000	0.000	1.40	

Figure 18. Secondary structure of wild-type BLA CMY 2 and mutated variant

CHAPTER 4. DISCUSSION

Reviewing the literature for mutations of *bla-cmy-2* at nucleotides 833 and 941 and amino acids 277 and 313 revealed the following. In an article published in 2003, researchers used “error-prone polymerase Mutazyme in a PCR reaction” in an attempt to find potential mutations in CMY-2 that would confer cefepime resistance (Barlow 2003). Seven strains expressing mutated CMY-2 were published (Barlow 2003). One of these strains that exhibited cefepime resistance at 32 µg/ml had two mutations at nucleotides 932 and 941 (Barlow 2003). Although nucleotide 941 was mutated, it did not cause a mutation at amino acid 313 in this study (Barlow 2003). There was no potential explanation given in this article regarding the increase in cefepime resistance.

A 2009 article may explain some of the increase in cefepime resistance in this research strain. The article described two *bla-cmy-2* variants: *bla-cmy-33* and *bla-cmy-44* (Doi 2009). These two variants have “12- to 24-fold increases in the MICs of cefepime” (Doi 2009). The article attributes this increased cefepime resistance to amino acid deletions in the sequence in proximity to the H-10 helix or R2 loop (Doi 2009). The structure of the beta-lactamase resulting from these sequence alterations allows the enzyme to interact with cephalosporin drugs with larger R2 side chains (Doi 2009). The R2 loop occurs at amino acids 289–307 (Kim 2006). Although the amino acid mutations of this research strain at 277 and 313 are outside of the sequences involved in the loop, their proximity and location on either side of the loop spark interest for further investigation. Reviewing the secondary structure of the research strain indicated that a structural alteration occurred in the sequence corresponding to the mutation at amino acid 277.

In conclusion, mutations occurring from natural evolution are more likely to occur individually (Barlow 2003). The research strain that displayed cefepime resistance contained two mutations, one of which is conserved, implying non-viability under natural conditions. If, however, these mutations were able to occur and persist in nature, it would be reasonable that the non-conserved mutation at amino acid 277 would occur first. Perhaps non-conserved mutations in proximity to the H-10 helix are sentinels of cefepime resistance. In any case, bacterial resistance to cefepime does occur, even though at present, it is only observed in the laboratory.

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