UNRAVELING THE PHENOTYPE OF COLICIN CYTOPLASMIC IMPORT (CIM) MUTANTS

A Thesis

by

TYSHEENA PERKINS CHARLES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Biology

UNRAVELING THE PHENOTYPE OF COLICIN CYTOPLASMIC IMPORT (CIM) MUTANTS

A Thesis

by

TYSHEENA PERKINS CHARLES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Michael Benedik
Committee Members,	Carlos Gonzalez
	Michael Manson
Head of Department,	Thomas McKnight

December 2008

Major Subject: Biology

ABSTRACT

Unraveling the Phenotype of Colicin Cytoplasmic Import (*cim*) Mutants.

(December 2008)

Tysheena Perkins Charles, B.S., Xavier University of Louisiana

Chair of Advisory Committee: Dr. Michael Benedik

Colicins are a type of bacterial toxin produced by *Escherichia coli* to kill *E. coli* and closely related bacteria. In contrast to the protein secretion pathway, colicins are released into the extracellular environment due to lysis of the cell. The colicins then enter the target bacterial cell by binding to a surface receptor (ex. BtuB) and translocating through an outer membrane channel (ex. OmpF) which is facilitated by the interaction with the Tol proteins. Once inside the target cell, pore-forming colicins form membrane channels in the inner membrane whereas enzymatic colicins enter the cytoplasm to degrade the DNA or RNA. The mechanism used by enzymatic colicins to enter the cytoplasm is still unknown and is the focus of my project.

Cim (cytoplasmic import) mutants were previously discovered in the Benedik lab. These mutants are resistant to enzymatic colicins (E2 and E3) but sensitive to pore forming colicins (A and E1). It was determined that this phenotype was due to a single nucleotide substitution resulting in an amino acid change in *tolB*.

The focus of my project was to understand the phenotype of the *cim* mutants so that I could gain more insight regarding the import of enzymatic colicins. To do this I

constructed colicin hybrids by swapping the T-R domains with the A-I domains of colicins A, E1, and E2. With these hybrids I was able to test whether an enzymatic colicin that was coupled with a pore-forming colicin would be functional or not. The lack of activity in these hybrids may be due to a lack of essential recognition elements used by enzymatic colicins and not possessed by pore-forming colicins.

DEDICATION

To My Husband

ACKNOWLEDGEMENTS

I would like to thank Dr. Benedik, and my committee members, Dr. Manson and Dr. Gonzalez, for their guidance and support throughout the course of this research.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Bacteriocins

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar strains and were first discovered by Gratia in 1925. The colicins of *Escherichia coli* are the most studied bacteriocins, but numerous other types of bacteria produce similar toxins to kill neighboring cells. Bacteriocins that are similar to the structure of colicins include pyocins, cloacins, pesticins, klebocins, marcescins, lumicins, and megacins, however there are numerous others.

1.2 Colicin Structure

Colicin proteins are separated into two groups based on the machinery used by the colicins to enter the cell. Group A colicins are translocated into the cell using the Tol system whereas Group B colicins use the Ton B system. Colicin genes are generally located on a pCol plasmid. The colicin plasmids themselves are also separated into two classes, type I and type II. Type I colicin plasmids are small, about 6-10 kb multicopy plasmids and type II colicin plasmids are about 40 kb monocopy plasmids that can carry either one or two adjacent colicin operons. Group A colicins typically are encoded by the smaller type I plasmids, while group B colicins are generally found on the larger type II plasmids.

Colicins are composed of three domains, the central domain (R) which is

---

This thesis follows the style of Applied and Environmental Microbiology.

involved in receptor binding to the outer membrane of the target cell; the N-terminal domain (T) which interacts with Tol and is required for translocation across the cell; and the C-terminal domain which carries the lethal activity (A). Further downstream in the operon is the immunity gene which confers immunity to the strain that is producing the colicin (Fig. 1). Colicins are separated into subclasses based on their outer cell membrane receptor (6). An example of this are the E colicins which recognize the vitamin B12 receptor to gain entry into the cell.

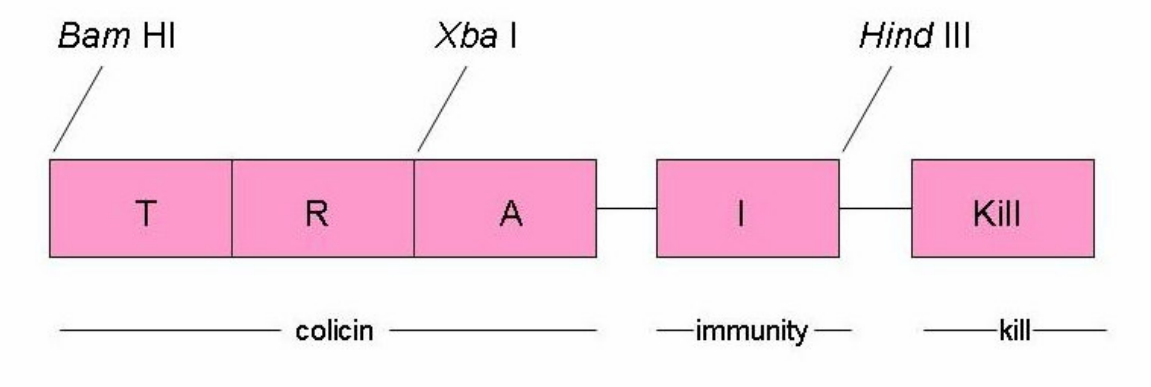


Figure 1. Schematic Represention of the Colicin Operon. The restriction sites indicate the positions used to form the hybrids (discussed in *Construction of Hybrids*)

1.3 Colicin Expression

Colicin transcription is repressed by the LexA protein, the repressor of the SOS regulon. There are two overlapping LexA boxes at the colicin promoter (11). When each LexA dimer binds to the box, it encourages DNA bending and stops transcription of the colicin gene (25). If DNA damage occurs, RecA is activated which causes LexA to

autocleave. This allows the colicin operon to be transcribed. In the laboratory the antibiotic mitomycin C is often used to stimulate the production of the colicin protein.

1.4 Release of Group A Colicins

The lysis protein is involved in the release of colicin proteins into the culture medium, its gene is generally found downstream of the immunity gene. Transcription of the lysis gene relies on read-through from the structural gene promoter across the entire operon, which means the lysis protein is coexpressed with colicin. Due to a transcription terminator between the colicin and the lysis genes, transcription through the lysis gene is much reduced relative to the colicin gene (Fig. 2) (11, 23, 24, 31). The lysis protein is a lipoprotein and when in its mature form is found in the outer membrane.

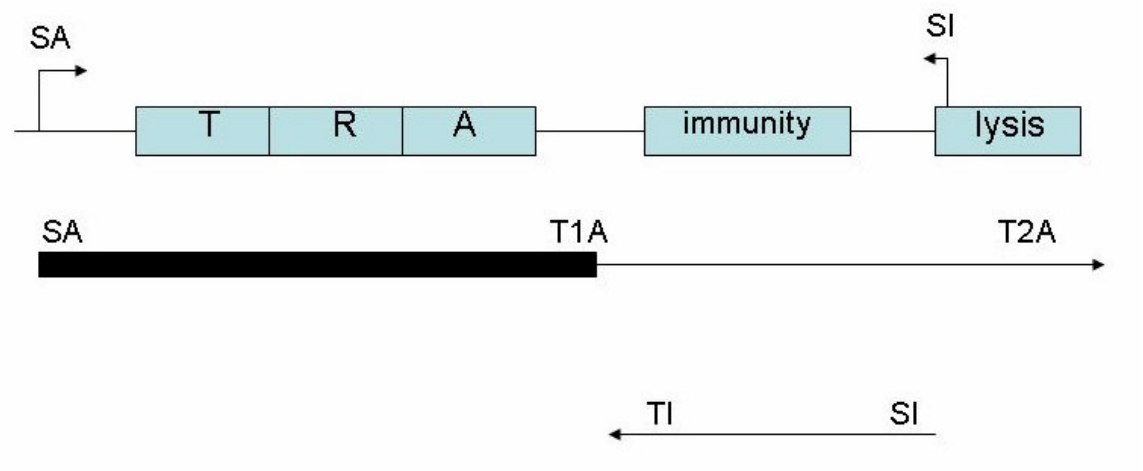


Figure 2. Transcription of the *col* Operon. SA, initiation site for transcription of the colicin gene; SI, initiation site for transcription of the immunity gene; T1A, first termination site for the colicin gene; T2A, second termination site for the colicin operon; TI, termination site for the immunity gene.

Two models have been proposed for the release of colicins by the lysis protein. In both models the lysis protein does not cause true lysis. The first model suggests that the lysis protein provokes quasilysis: modifications of the structure of the cell envelope, activation of the outer membrane phospholipase A (OmpLA), and death of the producing cell. OmpLA is a conserved enzyme that contributes to the virulence of Gram-negative bacteria. The phospholipase activity of this enzyme allows the formation of lysophospholipids, which as a result permeabilizes the outer and inner membranes of the bacterial cell. Because death of the host cell occurs whether or not colicin is expressed, the activation of OmpLA is thought to be the cause of this cell death event (29).

The second model is based on studies with colicin A. These authors tested the role of colicins Au (colicin A interacting with porins in the outer membrane) and their interaction with the lysis protein to cause secretion of the colicin (9). This model addresses a shortcoming of the previous model which did not address if the cells that were lysed during release of colicins were the induced or uninduced cells. Colicins Au were thought to play a role in the secretion of colicin but were disregarded because colicins without porins, which therefore lack colicins Au, still secreted colicin A. Cavard studied this hypothesis further by sampling cell extracts for released and unreleased colicin A at different time points and from strains that had a mutated lysis protein. From this research he was able to propose that membrane proteins, colicin A and the lysis protein may form a complex that enables the colicin to be secreted similar to the protein secretion process of Gram negative bacteria.

1.5 Binding of Colicins to Their Receptor

Group A and B colicins interact with specific outer membrane proteins of the target. Group A, colicins A and E1 to E9, target the vitamin B12 transporter BtuB. There is a second outer membrane protein, OmpF or TolC that is needed for translocation which suggests that the initial binding is for positioning and the secondary binding is required for translocation (7). The BtuB receptor, used by colicins, is an outer membrane pore. It contains a "plug" that stops the passage of molecules when energy is not supplied by the TonB system. Structural studies show that when the receptor domain of colicin E2 binds to BtuB the "plug" still remains (32). This study confirmed that BtuB is only used by Group A colicins as a receptor and not a translocon. Although two receptors are required, there are certain conditions where the initial binding can be avoided. For example with pore-forming colicins, death can still occur when BtuB is bypassed by incubating the cells in buffers of low osmotic strength (10).

Colicins have the same specificity for the receptor as the natural ligand. In a competition assay it was seen that colicins compete with the corresponding natural ligand for receptor binding, which indicates that there is overlap between the binding site of the colicin and the natural ligand. It has also been observed that there is competition between colicins and metal chelates. For example, cobalamin can rescue colicin (A or E)-treated bacteria, suggesting that this metal chelate displaces a bound colicin from BtuB (8), while ferric enterobactin does not displace colicin B, a group B colicin, on cells (27). Although these results show an overlap between the binding sites, it does not indicate the precise residues involved in binding colicins versus metal chelates.

1.6 Translocation in Tol Dependent Colicins

The Tol system of *E. coli* is found in the cell envelope and is composed of five proteins. All the functions of these proteins are not known but their regulation is linked to cellular stability. Clavel showed that RcsC , which is a sensor that regulates genes encoding a major component of the capsula, induces the *tol-pal* genes in response to stress on the cellular envelope (12). In addition to their role in membrane integrity, the Tol proteins are also involved in the uptake of bacteriophages and group A colicins.

Group A colicins require two cell surface receptors for entry into the target cell: the cell surface receptor, which does not participate in translocation, and either OmpF or TolC, which is used for cell penetration (Figure 3). It has been hypothesized that the N-terminal domain triggers a process whereby the C-terminal domain is able to cross the periplasm and reach the inner membrane or the cytosolic compartment (Figure 3). Although the mechanism of this step in transport is unknown, it has been shown that the N-terminal domain network interacts with components of the cell envelope.

Group A colicins differ in the subsets of Tol proteins used for translocation (Table 1). Guihard *et al.* noticed that the number of colicin A channels increased when Tol proteins were overexpressed. This suggests that Tol proteins are recruited as a result of colicin binding or OM translocation (17). It was also observed that the number of translocation sites per cell correlated with the number of TolA molecules.

Table 1. Receptor, Import Machinery, and Cytotoxic Activity of Group A Colicins. Group A colicins, their outer membrane receptor, the machinery used for import, and their cytotoxic activity

Colicin	**Receptor**	**Import Factors**	**Cytotoxicity**
A	BtuB	OmpF, TolABQR	Pore
E1	BtuB	TolC, TolAQ	Pore
E2-E7-E8-E9	BtuB	OmpF, TolABQR	DNase
E3-E4-E6	BtuB	OmpF, TolABQR	16S RNase
E5	BtuB	OmpF, TolABQR	tRNA-specific RNase
K	Tsx	OmpF, OmpA, TolABQR	Pore
N	OmpF	OmpF, TolAQR	Pore
U	OmpA	OmpF, TolABQR	Pore
Cloacin DF13	LutA	OmpF, TolAQR	16S RNase

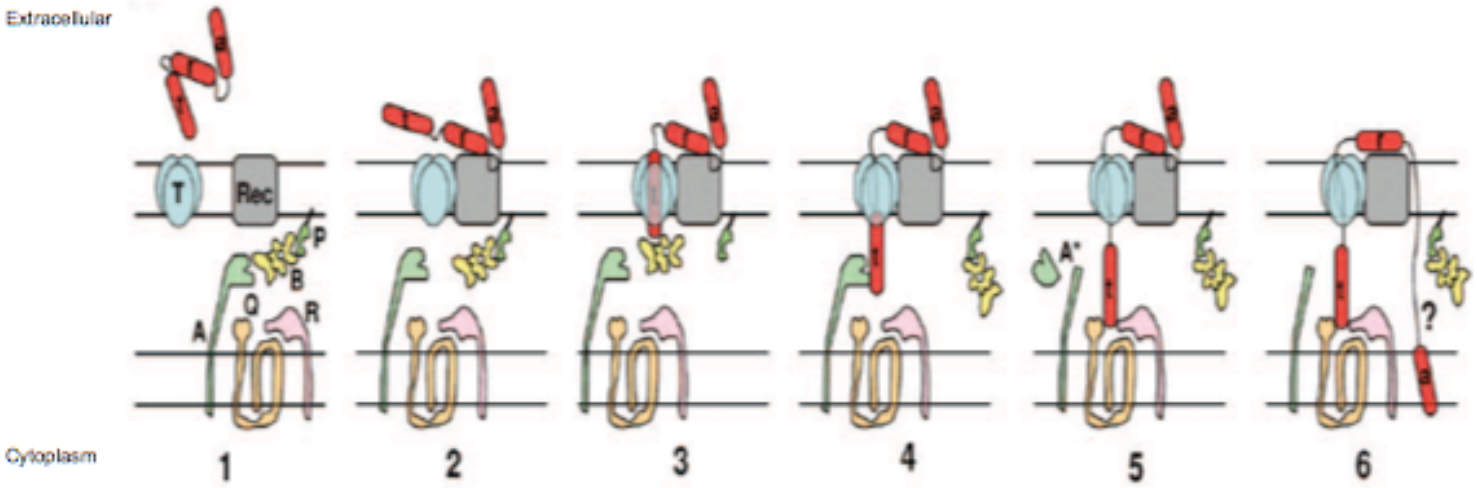


Figure 3. Model for Group A Colicin Import: (1) R domain of colicin binds to the receptor of the target cell, (2) colicin unfolds and recruits Tol system proteins, (3) T domain translocates through the outer membrane and interacts with the Tol B subunit, (4 and 5) interaction with Tol Q and/or Tol R after degradation of Tol A, (6) A domain is translocated by an unknown mechanism.
A, Tol A; A*, degraded product of Tol A; B, Tol B; P, Pal; Q, Tol Q; R, Tol R; Rec, Outer membrane receptor; T, Outer membrane translocon.
Figure taken from Cascales *et al.* (7).

1.7 Translocation in TonB Dependent Colicins

Like Tol, the TonB system in *E. coli* serves to energize active transport across the outer membrane of the cell. This system is parasitized by Group B colicins to gain entry into the cell. The TonB system consists of proteins that are functionally similar to the Tol system, these proteins include an outer membrane transporter and three inner membrane proteins, TonB, ExbB, and ExbD.

Unlike group A colicins, group B colicins are able to bind to and translocate through their receptor. This is mediated by the presence of a TonB box in the R-domain of the colicin. Binding of the R-domain of the colicin to the receptor of the target cell causes an interaction between the TonB box of the receptor and TonB itself, causing the channel to become "unplugged". During import of group B colicins, the R-domain of the colicin binds the TonB-dependent gated channel. The R-domain then recruits the TonB machinery which causes energy input by the ExbBD-TonB complex. Next, the T-domain crosses the outer membrane and interacts with TonB. The N-terminal domain dissociates from TonB and interacts with ExbB and/or ExbD which causes the activity domain to reach the final compartment (Fig. 4) (7). Past experiments have shown that a mutation in either *exbB* or *exbD* does not confer complete tolerance to colicin presumably because TonB can interact with the proteins in the Tol system which substitute (5). Mutations in both *exbB/D* and *tolQ/R* systems confer complete tolerance (4). While the Tol system can substitute for the Ton system in the import of Group B colicins, the reverse is not true, Group A colicins can utilize only the Tol system.

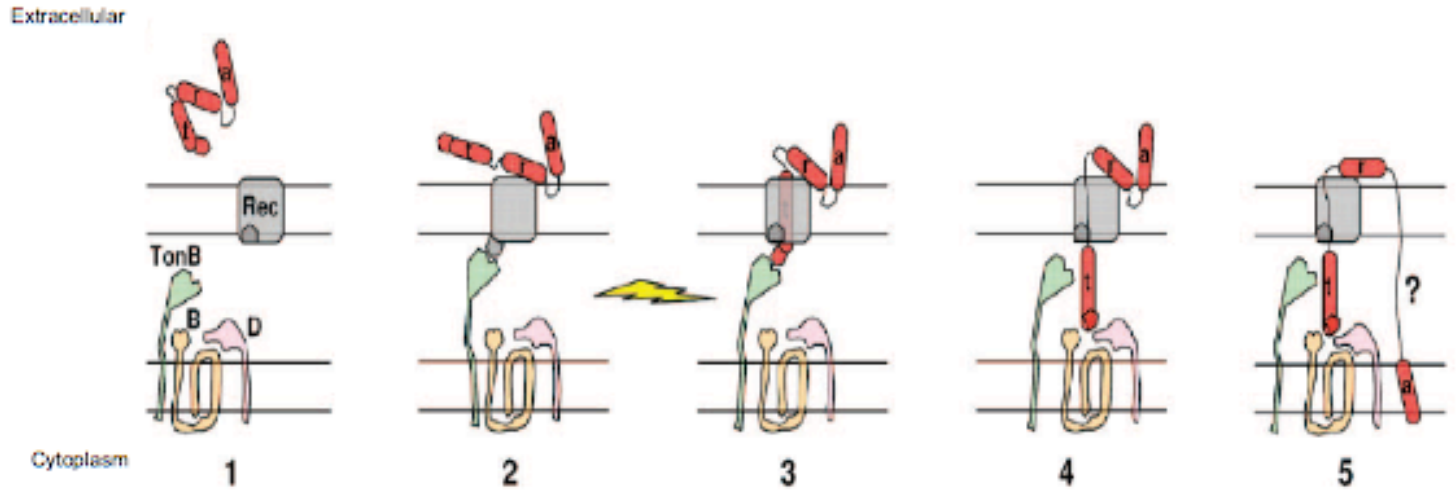


Figure 4. Model for Group B Colicin Import.
B, ExbB; D, ExbD; Rec, TonB-dependent OM-gated channel; yellow flash, energy input by the ExbBD-TonB complex; trapezoids, TonB boxes of the receptor and the colicin. Figure taken from Cascales *et al.* (7).

1.8 Pore-forming Colicins

Pore-forming colicins contain 10 tightly packed α helices (two are hydrophobic and the other eight are hydrophilic). The hydrophobic region of the colicin remains tucked inside the hydrophilic helices so that it can be water-soluble. The free colicin then binds to the receptor on the susceptible cell (19). The colicin is translocated across the outer membrane and rearranges upon interaction with the inner membrane to become a membrane-bound protein and form ion channels (Fig. 5) (22). This formation resembles an umbrella-like structure by inserting the hydrophobic regions into the membrane while the hydrophilic regions lay on the surface (Fig. 5).

The immunity protein recognizes the C-terminal or A domain of colicins (1, 26). The immunity proteins of pore-forming colicins are 11-18 kDa (15, 16, 22) and are encoded by the same plasmid as colicin. The immunity protein grants the cell protection against the produced colicin but not against colicins of other types, even those with similar modes of action (7). They are constitutively expressed at low levels (23). They reside in the inner membrane (15, 16) and fall into two classes, which are grouped based on the length of their hydrophobic segment: A type and E1 type (7). Colicins A, B, N, and U are A type, while colicins E1, 5, K, Ia, and Ib are E1 type.

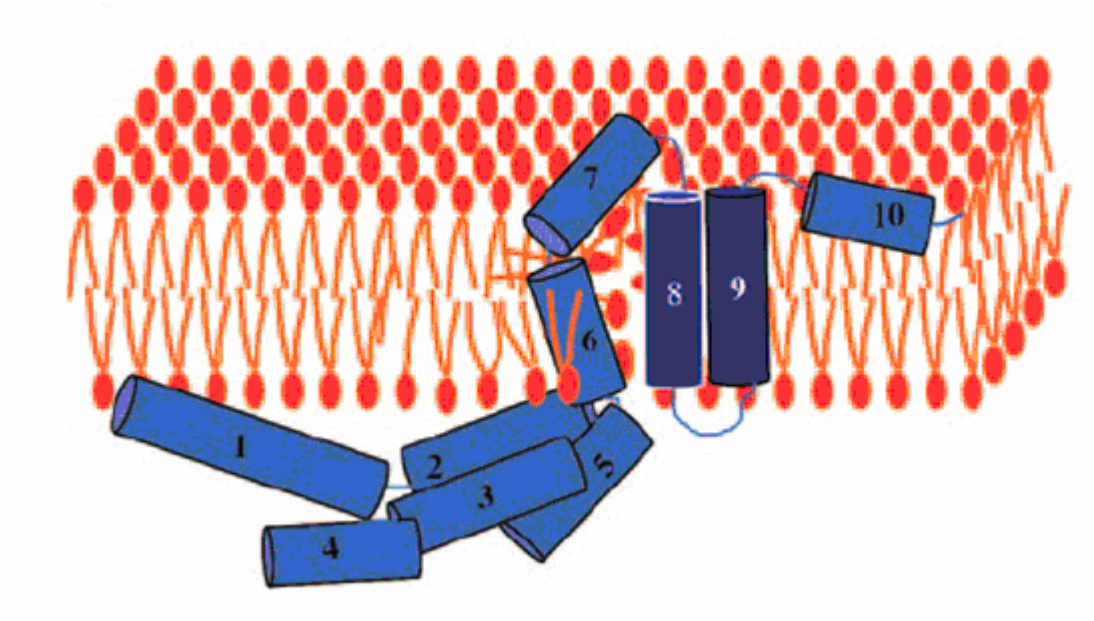


Figure 5. Representation of the Umbrella membrane-bound conformation (21) Adopted by Colicin E1. Figure taken from Cascales *et al.* (7).

1.9 Enzymatic Colicins

Enzymatic colicins enter the cell using either the Tol or TonB systems and then further translocate across the inner membrane, but it is not known how this is accomplished. The cytotoxic domains of the enzymatic colicins differ from the pore-forming colicins in that the domain presumably must cross the cytoplasmic membrane in order to be active in the cytoplasm. This was studied using colicin B, a pore-forming colicin, and colicin D, an enzymatic colicin, where it was seen that enzymatic colicins use LepB, a protein of the general secretory pathway that cleaves the leader sequence from the amino terminus of membrane and secreted proteins (18). *lepB* mutant strains were killed by colicin B, but not by the enzymatic colicins. Sequence comparisons

identified a conserved amino acid sequence in the enzymatic colicins, not found in pore-forming colicins, upstream of the LepB recognition site (Fig. 6).

Unlike pore-forming colicins, the immunity proteins of the enzymatic colicins are released with the protein bound to the activity domain. This is to prevent colicin action on the producing organism before the toxin is released into the environment (Fig. 6). These proteins are approximately 10 kDa (7). Immunity proteins provide immunity either by binding directly to the active site, or by blocking access to the substrate binding site (7). It is still unknown how the immunity protein is lost during translocation but it has been speculated that it must be released before LepB is able to cleave the catalytic domain (13). If the immunity protein is not removed, LepB is not able to cleave the colicin.

The DNase colicins are one subset of the enzymatic colicins, they cleave or degrade DNA in a non-site specific manner. The DNase colicins share about 65% sequence identity and include colicins E2, E7, E8, and E9. There is an H-N-H motif in the DNase colicins that is the core of the active site (7), the N-terminal histidine residue serves as a general base in the hydrolysis mechanism. The asparagine residue forms a stabilizing hydrogen bond backbone across the motif and C-terminal histidine is a metal binding residue. This enzyme most likely causes double-stranded breaks in the DNA and also poorly cleaves single-stranded RNA.

RNase colicins include colicins E3, E4, E5, E6, and D. These colicins cause cell death by inhibiting protein synthesis through a specific phosphodiester bond cleavage in RNA. These enzymes are separated into two groups: rRNases, which attack the 30S

subunit of the bacterial ribosome, (colicins E3, E4, and E6) (2, 3, 30), and tRNases, which cleave single phosphodiester bonds in the anticodon loops of specific transfer RNAs (colicins E5 and D) (7).

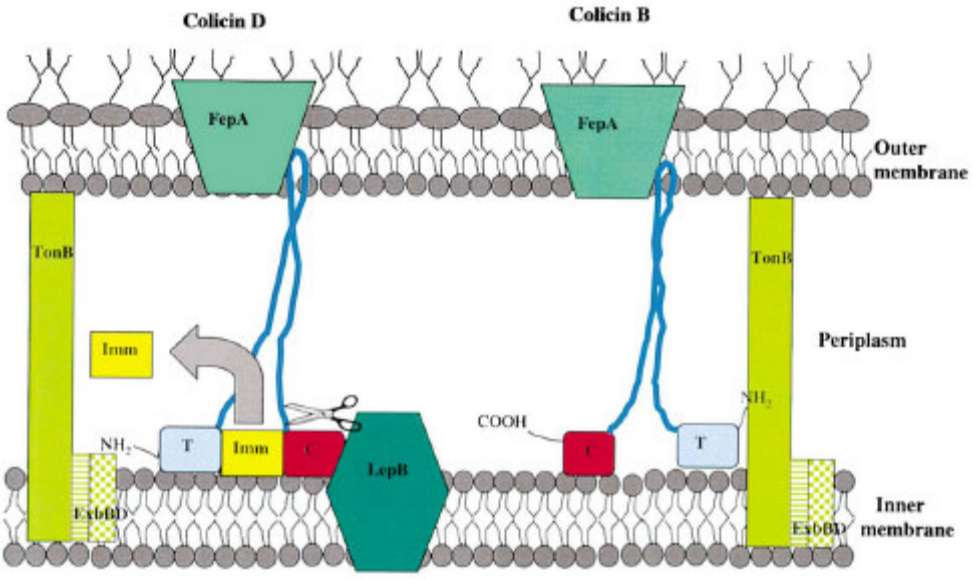


Figure 6. Schematic Drawing of the Colicin B and D Killing Pathways. Dark blue, long coiled-coil receptor; medium green, FepA receptor; light blue, translocation domain; light green, TonB protein; patterned light green, ExbB and D; yellow, immunity protein; dark green, LepB; red, catalytic domain. The receptor interacts with the R domain of both colicins. The T domain the interacts with TonB, ExbB and D. On colicin D, the immunity protein comes off and then LepB cuts upstream the catalytic domain. For colicin B the immunity protein is not bound and it forms channels in the inner membrane. Figure taken from Jakes (18)

1.10 Cytoplasmic Import (*cim*) Mutants

A variety of *E. coli* mutants have been isolated which confer resistance, or tolerance, to colicins. These "tolerant" mutants were how the *tol* genes were identified.

Null mutants within any of the *tol* genes generally confer tolerance to all members of Group A colicins.

In order to identify genes specific to the cytoplasmic import step of enzymatic colicin translocation, a mutant hunt was initiated in our lab. This was done by screening colonies that were resistant to E2 but remained sensitive to E1, to ensure that there was not a mutation in the BtuB receptor or the other common elements. These mutants, which were resistant to the cytoplasmic colicins (E2 and E3) but sensitive to pore-forming colicins (A and E1), were called cytoplasmic import (*cim*) mutants. Mutants were then mapped and sequenced to discover the locus of the Cim phenotype. Sequence analysis revealed that each mutant carried a singe nucleotide change which led to an amino acid change in TolB (unpublished). Of the thirteen mutants sequenced three different substitutions were found (Table 2). The mutant alleles were then cloned and transformed into a *tolB* deleted strain. Performing a colicin spot test demonstrated that the mutations in *tolB* were sufficient to cause the Cim phenotype. This is interesting because although colicins A, E2, and E3 all require TolB, the *cim* mutants are only resistant to colicins E2 and E3. This leads us to believe that there must be a difference, involving TolB, in the specificity of the uptake between pore-forming colicins and enzymatic colicins. Overexpression of the *cim* alleles does not compensate for the defect, which means that the phenotype was not caused by an insufficient amount of TolB (unpublished).

Table 2. Sequences of the *cim* Mutants.

Strain	Resistance Phenotype	Sequence Change	Residue Change
MB1708	Col:$A^S E_1^S E_2^R E_3^R$	TCT → TAT	Ser406 → Tyr
MB1760	Col:$A^S E_1^S E_2^R E_3^R$	TCC → TAC	Ser225 → Tyr
MB2656	Col:$A^S E_1^S E_2^R E_3^R$	TTC → TCC	Phe424 → Ser

1.11 Summary of Aims

Colicins A, E1, and E2 require TolABQR, whereas colicin E1 requires TolACQ. The differences in the requirements for import causes one to question if there is any specificity in the uptake between pore-forming and enzymatic colicins and the usage of Tol proteins.

Although much is known about colicins, there is still very little understood about how colicins translocate across the inner membrane to reach the target where their lethal activity takes place. The phenotype of the *cim* mutants showed that there was a difference in how pore-forming and enzymatic colicins used the Tol proteins. My project was designed to probe this by better understanding the *cim* mutants. Characterizing the phenotype of the *cim* mutants would help determine if the *tolB* allelles blocked an import step used by enzymatic but not pore-forming colicins or if the translocation domain itself was not recognized due to the differences in import machinery needed between the colicins. To understand this I attempted to:

a. Build hybrids by swapping the domains of the colicins

b. Compare the hybrids to wild-type strains vs. strains with the Cim defect.

The hybrids were designed to see if the translocation machinery could be changed between colicins or if some type of recognition occurs between the T-R domains and the activity domain. If the results indicated that the phenotype was specific to the activity domain, future experiments would be done to try to understand the role of TolB in the transit across the inner membrane of colicins E2 and E3.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

The bacterial strains and plasmids used throughout this work are described in Table 3.

Table 3. Strains and Plasmids.

Strain/Plasmid	Relevant Characteristics	Reference
Plasmids		
pBC SK^+	Cam^R cloning vector	Stratagene
pC2G	pMALC2G with a deletion of the Maltose Binding Protein domain	Lab stock
Strains		
MB1674	W3110 ColE1	Lab stock
MB1801	ColA-CA31	Lab stock
MB1920	W3110 ColE2-P9	Lab stock
MB3436	MM294 but $lacI^q$ lacZ ΔM15	Lab stock
MB3636	pC2G	Lab Stock
MB3789	TR_{E2} into pC2G *Bam*HI *Xba*I (a.a. 1-448)	This work
MB3791	AI_{E2} into pBC *Xba*I *Hind*III (a.a. 449-581)	This work
MB3811	AI_{E2} cloned into 3789	This work
MB3853	TR_{E1} into pC2G *Bam*HI *Xba*I (a.a. 1-378)	This work
MB3856	TR_A into pC2G *Bam*HI *Xba*I (a.a. 1-393)	This work
MB3858	AI_A into pBC *Xba*I *Hind*III (a.a. 394-592)	This work
MB3872	AI_{E1} into pBC *Xba*I *Hind*III (a.a. 379-522)	This work
MB4018	$TRAI_{E1}$ into pC2G *Bam*HI *Hind*III (a.a. 1-522)	This work
MB4020	$TRAI_{E2}$ into pC2G *Bam*HI *Hind*III (a.a. 1-581)	This work
MB4031	p4018 with *Xba*I insertion between a.a. 345 and 346 (*Xba*I-1)	This work
MB4032	p4018 with *Xba*I insertion between a.a. 356 and 357 (*Xba*I-2)	This work
MB4033	$TRAI_A$ in pC2G *Bam*HI and *Hind*III (a.a. 1-592)	This work
MB4039	p4033 with *Xba*I insertion between a.a. 424 and 425	This work
MB4040	AI_A into pBC *Xba*I and *Hind*III	This work
MB4042	AI_{E1} (4031) into pBC *Xba*I and *Hind*III	This work
MB4044	AI_{E1} (4032) into pBC *Xba*I and *Hind*III	This work
MB4047	AI_{E2} cloned into 4031	This work
MB4049	AI_{E1} (4032) cloned into 4031	This work
MB4051	AI_A cloned into 4031	This work
MB4053	AI_{E2} cloned into 4032	This work

Table 3. (continued)

MB4055	AI_{E1} (4031) cloned into 4032	This work
MB4057	AI_A cloned into 4032	This work
MB4059	AI_{E1} (4031) cloned into 3789	This work
MB4061	AI_{E1} (4032) cloned into 3789	This work
MB4063	AI_A cloned into 3789	This work
MB4065	AI_{E1} (4031) cloned into 4039	This work
MB4067	AI_{E1} (4032) cloned into 4039	This work
MB4069	AI_{E2} cloned into 4039	This work

2.2 Culture Media

All strains were grown either in LB 505 broth, autoinduction media of Studier, which is LB broth containing 0.05% glucose, 0.5% glycerol (0.2% lactose added when necessary to induce protein expression) or Terrific Broth unless otherwise mentioned (33). Antibiotics were used at concentrations of 100 µg/mL ampicillin or 30 µg/mL chloramphenicol for selection of plasmids.

2.3 Preparation of Chemical Competent and Electrocompetent Cells

An overnight culture of MB3436 grown in LB 505 was diluted 1000-fold in 200 mL of LB and grown at 37ºC to a density of OD_{600} 0.2. The cells were chilled on ice for 1 hour and harvested by centrifugation for 10 minutes at 4ºC in 50 mL conical tubes. The cells were then resuspended in transformation and storage buffer (1/10 original volume), aliquoted on dry ice, and stored at -80ºC.

The growth conditions for electrocompetent cells were the same as the chemical competent cells. After the cells were harvested by centrifugation they were resuspended in half the original volume of 10% cold glycerol and centrifuged for 15 minutes at 4ºC.

The second wash was with 2/5 the volume and the cells were finally resuspended in 1 mL 10 % glycerol, aliquoted, and stored at -80ºC.

2.4 Transformation and Electroporation

Transformations were done by thawing competent cells on ice and adding 50µL of the cells to a chilled microcentrifuge tube with 2µL plasmid DNA and incubated on ice for 15 minutes. The tubes were then heat shocked at 43ºC for 45 seconds and returned to ice for 2 minutes, then 500µL of LB was then added to the tube and incubated for 1 hour at 37ºC with agitation. After incubation, 100µL was spread on selective plates and grown overnight.

Electroporations were done when more transformants were needed. Electrocompetent cells were thawed on ice and 50µL were added to a chilled eppendorf tube with 2µL plasmid DNA and kept on ice for 15 minutes. This mixture was then electroporated with an electrical pulse of 1.80 kV. 500µL of LB was then added to the tube and incubated for 1 hour at 37ºC with agitation. After incubation, 100µL was spread on selective plates and grown overnight.

2.5 Plasmid Miniprep

Cultures were grown overnight in LB 505 supplemented with the appropriate antibiotic and plasmid DNA was obtained using the boiling miniprep method. The overnight culture (1.5 mL) was placed in a microcentrifuge tube, centrifuged, and resuspended in 0.5 mL STET (8% sucrose, 5% Triton X-100, 50 mM Tris pH 8) with 25 µL lysozyme (10 mg/mL). The tubes were then vortexed to resuspend the cells, and placed at 100ºC for 3 minutes followed by centrifugation for 20 minutes at full speed.

The pellet was removed and 0.5 mL of isopropanol was added to the supernatant to precipitate the DNA. The tube was again vortexed and centrifuged for 5 minutes following a rinse with 70% EtOH. The EtOH was then removed using a vacuum and once the pellet dried, it was resuspended in 100 µL TE (10 mM Tris-HCl pH 8, 1mM EDTA). For use in site-directed mutagenesis the QIAprep Spin Miniprep protocol was used in order to obtain highly purified DNA.

2.6 Expression of Protein

To express colicin from wild type colicin plasmid carrying cells, 2 mL cultures were grown overnight in LB 505 then diluted 1:100 in 10 mL of LB 505 and grown at 37ºC with agitation until mid-log phase. Mitomycin C (0.2 µg/mL) was added to the cultures and incubated for an additional 3 hours. The cells were removed by centrifuging the cultures at top speed for 10 minutes at 4ºC and the culture supernatant containing colicin stored at -20ºC.

Colicin expression from pC2G clones was done similarly but in Terrific Broth with ampicillin. IPTG (0.1M) was added for induction instead of mitomycin C. To harvest the protein, 1.5 mL of culture was placed in a microcentrifuge tube on ice and sonicated (10 cycles of 30 seconds sonication with 10 seconds of cooling in between). The tubes were then centrifuged at room temperature for 2 minutes to pellet debris, placed on ice, and aliquots of the supernatant were made and stored at -20ºC.

2.7 PCR Amplification, Site Directed Mutagenesis and Cloning

PCR amplification was used in the construction of the hybrids (Figure 1). The T-R (translocation and receptor) domains were amplified with the restriction sites

*Bam*HI upstream of the gene and *Xba*I at the fusion junction (Table 4). The A-I (activity and immunity) domains were amplified with the restriction sites *Xba*I at the fusion junction and *Hind*III downstream of the immunity gene (Table 4). The PCR-amplified T-R and A-I domains were digested with the appropriate enzymes and cloned into the pC2G and pBC vectors, respectively. The resulting recombinant plasmids were then transformed into MB3436.

A second approach used was to amplify the entire colicin and immunity operon. The primers incorporated a *Bam*HI restriction site upstream of the colicin operon and a *Hind*III restriction site downstream of the immunity gene (Table 4). The PCR product was cloned into the pC2G vector digesting both the plasmid and the vector with *Bam*HI and *Hind*III. The resultant plasmid was subjected to site directed mutagenesis to insert an *XbaI* restriction site at the end of the receptor domain and beginning of the activity domain, the fusion junction (Table 4). Mutagenesis was done using the QuikChange® Site-Directed Mutatgenesis Kit (Stratagene, La Jolla, CA). The DNA was digested with *Dpn*I followed by electroporation into MB3436 electrocompetent cells.

2.8 Construction of Hybrids

To combine the two domains a digestion was set up using *Xba*I and *Hind*III for each T-R and A-I containing plasmid. The fragments were purified, ligated together and transformed into MB3436. The transformation was plated on LB plates containing ampicillin plus the colicin appropriate for the A-I domain in order to select for it. Colonies were screened using a triple digestion, *Bam*HI, *Hind*III, and *Xba*I. Following this, the immunity region of the A-I colicins was tested by performing a spot test. Strains

carrying the appropriate plasmids were stored at -80°C for future use, after confirmation by DNA sequencing.

2.9 Sensitivity Testing

Resistance and sensitivity of strains were tested on lawns prepared with overnight cultures, 0.1 mL of the overnight culture was added to 3 mL top agar and poured onto an LB agar plate. Once solidified, 5 µL of each sample was spotted on the plate and incubated at 37ºC overnight. The results were recorded and compared to a control strain.

2.10 Competition Assay

Competition assays were done to test if protein of nonfunctional hybrids was being produced. This was done by preparing extracts from hybrids to be tested. Constant volumes of the hybrid proteins were spotted along with serial dilutions of the wild type colicins (A, E1, and E2) onto lawns of indicator cells. Diminution of the halo size indicates the hybrid competes with the wild type colicin.

Table 4. Primers Used to Amplify Colicin Domains. Italicized region indicates the restriction sites.

Primers for Amplification of TRAI domains	
Col A-F (TRAI)	cgccc*ggatcc*aggaaagattatgcctggatttaattatgg
Col A-R (TRAI)	gccg*aagctt*agcagcatgatcgccagtaaaatgacacaa
Col E_1-F (TRAI)	cca*gggatcc*aggattttataatggaaaccgcggtagcgt
Col E_1-R (TRAI)	ggtc*aagctt*ccaacaaggaggtttatcgcgaatattcccac
Col E_2-F (TRAI)	tata*ggatcc*agggaatttttatgagcggtggcg
Col E_2-R (TRAI)	ggg*gaagctt*cattcatccataaacaaccggcattcatatttcagccc
Primers for insertion of *Xba*I site between R and A domain	
Col A-F	ggagataaatataaggcg*tctaga*atagcgaaagatattgcggac
Col A-R	gtccgcaatatctttcgctat*tctaga*cgccttatatttatctcc
Col E1-F	ctccttaattcacagatt*tctaga*aaggatgctgttgatgc
Col E1-R	gcatcaacagcatcctt*tctaga*aatctgtgaattaaggag
Col E2-F	ctgctgcaaaagagaagtca*tctaga*gatgctgatgctgcatt
Col E2-R	aatgcagcatcagcatc*tctaga*tgacttctcttttgcagcag
Primers for Amplification of TR or AI domains	
Col A-F (TR)	cgccc*ggatcc*aggaaagattatgcctggatttaattatgg
Col A-R (TR)	ccgg*tctaga*ttccatagcctgacgctgacg
Col A-F (AI)	ctgg*tctaga*gttgcggaaaaagccaaagatgagcgggagc
Col A-R (AI)	gccg*aagctt*agcagcatgatcgccagtaaaatgacacaa
Col E_1-F (TR)	cca*gggatcc*aggattttataatggaaaccgcggtagcgt
Col E_1-R (TR)	ccggg*tctaga*cttatcagcaagttcctgtgcc
Col E_1-F (AI)	ctggg*tctaga*tctaaaggtaagaaaatcggcaatgtgaatgaagctctcgctgc
Col E_1-R (AI)	ggtc*aagctt*ccaacaaggaggtttatcgcgaatattcccac
Col E_2-F (TR)	tata*ggatcc*agggaatttttatgagcggtggcg
Col E_2-R (TR)	gcccgc*tctaga*ctctccttatctaatttatccttagc
Col E_2-F (AI)	gattt*ctaga*taaacggaataagccagggaaggcgacagg
Col E_2-R (AI)	ggg*gaagctt*cattcatccataaacaaccggcattcatatttcagccc

3. RESULTS

3.1 Cloning and Mutagenesis of Wild-type Colicins

To ensure that the cloned colicin genes would function properly in our vectors, the colicin and immunity operon was amplified from each and placed into the pC2G vector. This plasmid allows transcription from the *tac* promoter and carries a copy of the *lacI* gene for regulation. To make these constructs, template DNA was prepared from colicin strains A, E1, and E2. *Bam*HI and *Hind*III restriction sites were inserted by PCR as shown in the Figure 7 below. The PCR product was digested with the appropriate enzymes and the colicin domains were cloned into the pC2G vector and transformed into MB3436 competent cells. The extract produced by each colicin clone strain was tested on lawns of wild-type or colicin immune strains. It was observed that the colicin A extract lysed pColE1 and pColE2 strains, but a pColA strain remained immune. The colicin E1 extract lysed pColA and pColE2 strains, but a pColE1 strain remained immune. The colicin E2 extract lysed pColA and pColE1 strains, but a pColE2 strain remained immune. Based on these results it was concluded that the expressed extracts behaved like the wild-type colicin. Additionally the pC2G clones expressed the appropriate immunity function of the cloned colicin.

An *Xba*I site was then introduced to create a fusion junction in these constructs by site-directed mutagenesis. Clones expressing the *Xba* mutant protein were tested and the results were the same as previously seen by the colicin clones. These results showed

that introducing an *XbaI* site into the colicin gene had no effect and the extract maintained the properties of the wild-type colicin.

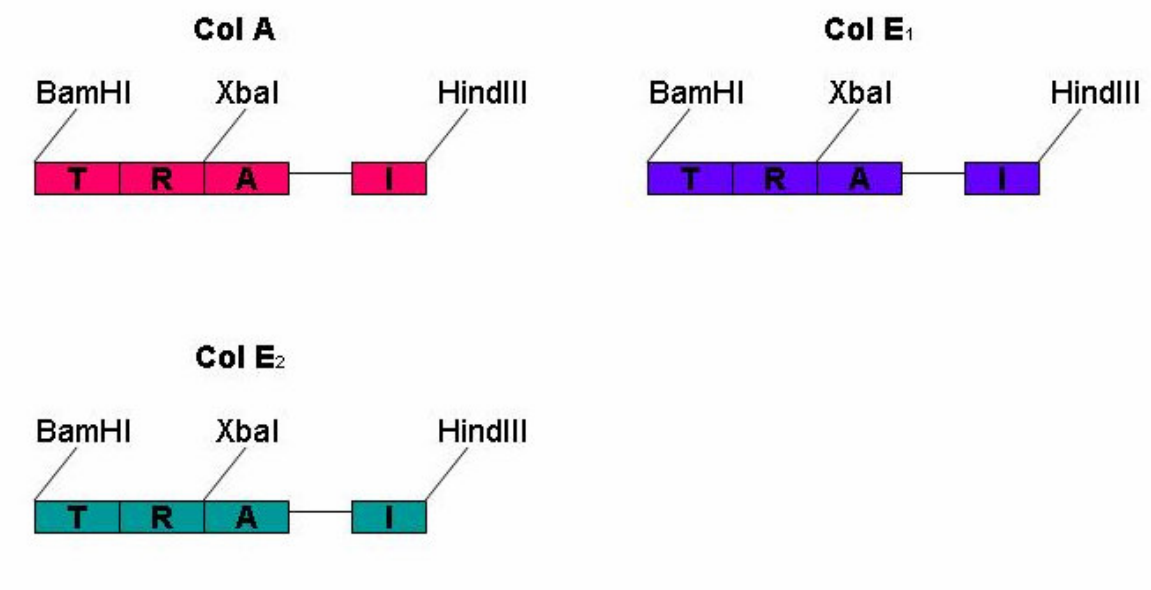


Figure 7. Schematic Representation of Wild-type Colicin Hybrids. Color represents a specific colicin and will be used in the subsequent figures.

In addition to testing the colicin activity, the strains were tested to confirm that the immunity was maintained as well. Indicator lawns were made with the *Xba* mutant strain and wild-type colicin spotted on the plates. The colicin A *Xba* mutant remained immune to colicin A while being lysed by both colicin E1 and E2. The colicin E1 *Xba* mutant remained immune to colicin E1 but was lysed by both colicin A and E2. The colicin E2 *Xba* mutant remained immune to colicin E2 while being lysed by colicin A

and E1. Based on these results it was concluded that the *Xba* mutants maintained their immunity as well and were therefore fully functional.

In order to test whether the location of the fusion junction affected the behavior of the hybrids, an additional *Xba* mutant was made for colicin E1 and E2. For colicin E1 the second *Xba* insertion mutant was eleven residues downstream of the first previously described one. For the colicin E2 mutant, one insertion placed downstream the of *lepB* cleavage site and the other was placed upstream of the cleavage site. These *Xba* mutants were also tested and it was seen that there was no difference in the activity of these mutants.

3.2 Construction and Expression of Hybrids

Colicin A, E2, and E3 all use the same translocation machinery, whereas colicin E1 varies in the proteins used. This causes one to question if there is any specificity in the N-terminus of colicins. To see if the translocation machinery used by the colicins could be interchanged hybrids were constructed. The hybrids were constructed by swapping T-R domains with A-I domains (Figure 8) at the *XbaI* site.

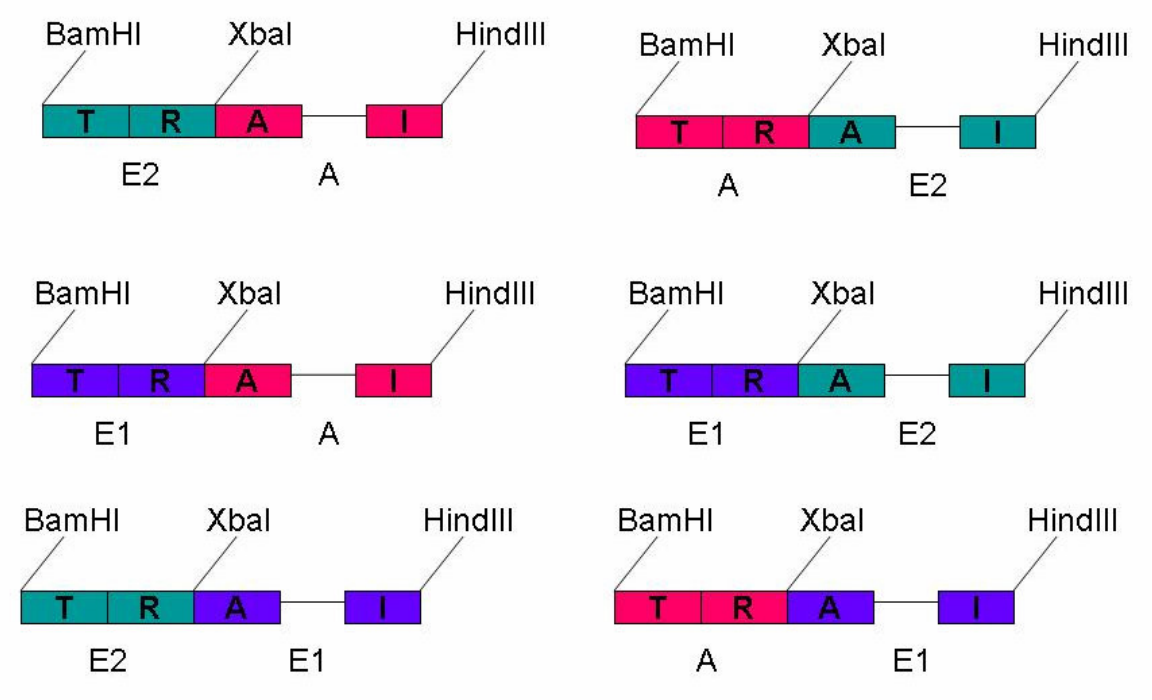


Figure 8. Schematic Representation of Hybrids.

To do this, the DNA from the *Xba* mutant clones discussed previously was digested with *Xba*I and *Hind*III, and the A-I domain replaced by an A-I domain from a different colicin. The hybrids were transformed into MB3436 and the behavior of the colicins was tested after adding IPTG for expression. Because the lysis region is not located on these hybrids, sonication was used to release the protein. The resultant lysates were then tested against sensitive and various immune strains carrying native colicin plasmids. The results were as follows (Table 5):

Table 5. Chart of Hybrid Activity. Protein from the hybrids colicins were spotted onto wild-type and immune strains. -: no clearing +: clearing

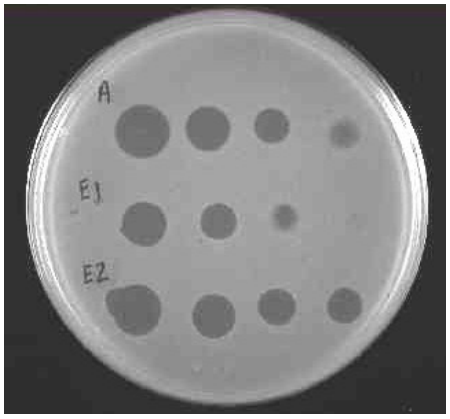
Hybrids	Hybrid Activity			
	Wild-type (MB3636)	Col A (MB4017)	Col E1 (MB1674)	Col E2 (MB1920)
TR_AAI_A	+	-	+	+
TR_AAI_{E1}	+	+	-	+
TR_AAI_{E2}	-	-	-	-
$TR_{E1}AI_A$	+	-	+	+
$TR_{E1}AI_{E1}$	+	+	-	+
$TR_{E1}AI_{E2}$	-	-	-	-
$TR_{E2}AI_A$	-	-	-	-
$TR_{E2}AI_{E1}$	-	-	-	-
$TR_{E2}AI_{E2}$	+	+	+	-

The hybrid extracts that contained either the T-R or A-I domains from colicin E2 were all inactive, however the $TR_{E2}AI_{E2}$ *Xba* mutant (wild-type hybrid) functioned normally. However hybrids between A and E1 pore-forming colicins did function. Based on the results it was concluded that the hybrids that contained domains from the enzymatic colicin (E2) were not able to function when paired with domains from pore-forming colicins (A and E1), however hybrids between A and E1, which differ in their translocation machinery requirements, were functional. All the hybrids, active or not, did express the expected imunity.

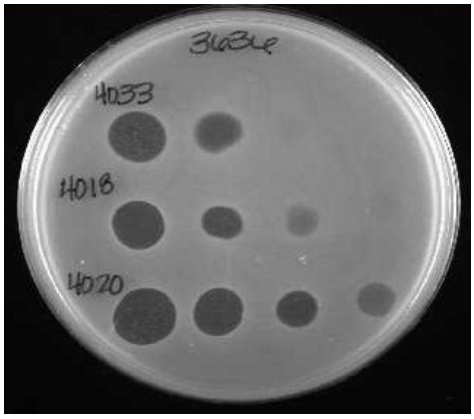
3.3 Stability of Hybrids

Serial dilutions of the wild-type, cloned colicins, *Xba* mutants, and hybrids were done to evaluate if the E2 hybrids were nonfunctional due to instability. This was done by doing a 10-fold dilution series of all the proteins and placing 5 µL onto LB plates seeded with MB3636, which is the pC2G transformed into MB3436. When comparing the cloned colicin and the *Xba* mutants to the wild-type colicins it was seen that that stability of the proteins remained the same except for colicin A (Figure 9). A reduction in the amount of colicin A lysis activity was observed when cloned into the pC2G vector. Next the hybrids were tested and it was seen that the lysis observed decreased approximately 100-fold in all of the hybrids compared to the cloned colicins, and *Xba* mutants (Figure 10).

A.



B.



C.

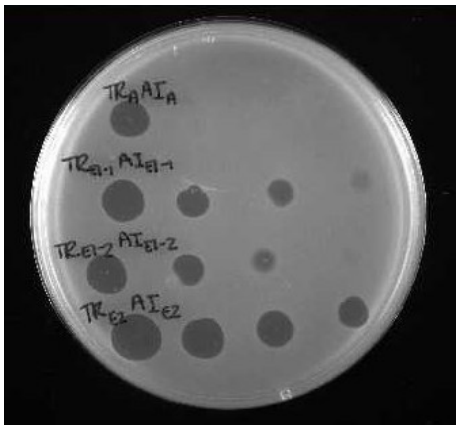


Figure 9. Serial Dilutions of Colicins. Dilutions go from left to right and begin with the undiluted proteins. (A) from left to right: 10-fold dilutions of wild-type colicins; from top to bottom: colicin A, colicin E1, and colicin E2 (B) from left to right: 10-fold dilutions of the cloned colicins; from top to bottom: colicin A (4017), colicin E1 (4018), colicin E2 (4020). (C) from left to right: 10-fold dilutions of the *xba* mutants; from top to bottom: TR_AAI_A, $TR_{E1\text{-}1}AI_{E1\text{-}1}$, $TR_{E1\text{-}2}AI_{E1\text{-}2}$, $TR_{E2}AI_{E2}$

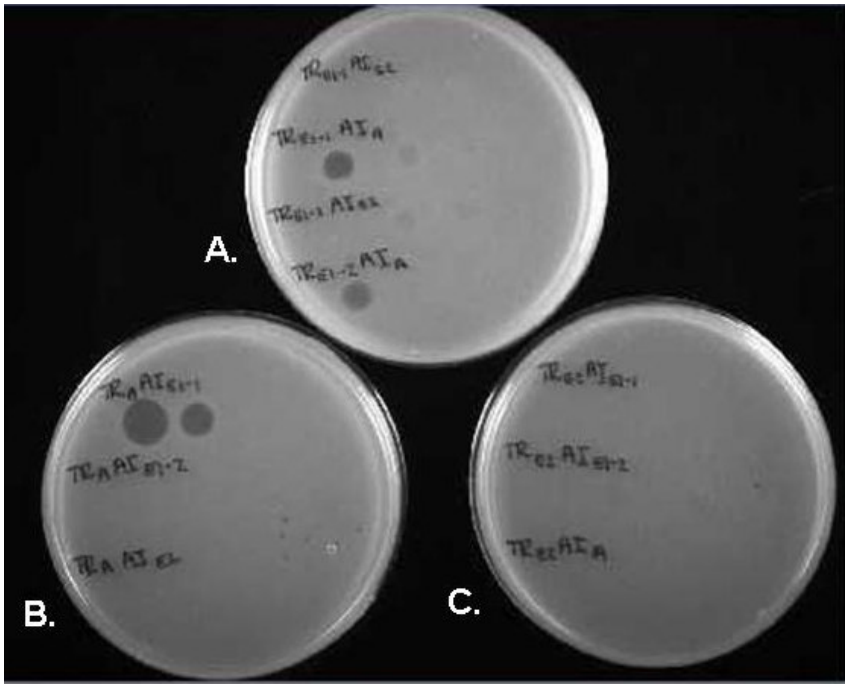


Figure 10. 10-fold Dilutions of the Hybrid Proteins. The dilutions go from left to right and begins with the undiluted protein. (A) from top to bottom: $TR_{E1\text{-}1}AI_{E2}$, $TR_{E1\text{-}1}AI_{A}$, $TR_{E1\text{-}2}AI_{E2}$, $TR_{E1\text{-}2}AI_{A}$ (B) from top to bottom: $TR_{A}AI_{E1\text{-}1}$, $TR_{A}AI_{E1\text{-}2}$, $TR_{A}AI_{E2}$ (C) from top to bottom: $TR_{E2}AI_{E1\text{-}1}$, $TR_{E2}AI_{E1\text{-}2}$, $TR_{E2}AI_{A}$

3.4 Competition Assay

In the previous section the failure of colicin E2 hybrids to have activity was described. From this lack of activity it was unclear whether the protein was present but nonfunctional or not present at all, presumably due to instability. A competition assay was used in an attempt to answer this question. This assay relies on competition between the wild-type colicins and the hybrids in binding to a limited number of receptors present on the cell surface. These were then tested against the wild type colicins in the ratios shown in Table 6.

Table 6. Competition Assay Chart.

1) 10 µl Hybrid + 10µl Col A
2) 10 µl Hybrid + 10µl 10^{-1} Col A
3) 10 µl Hybrid + 10µl 10^{-2} Col A
4) 10 µl Hybrid + 10µl 10^{-3} Col A
5) 10 µl Hybrid + 10µl Col E1
6) 10 µl Hybrid + 10µl 10^{-1} Col E1
7) 10 µl Hybrid + 10µl 10^{-2} Col E1
8) 10 µl Hybrid + 10µl 10^{-3} Col E1
9) 10 µl Hybrid + 10µl 10^{-1} Col E2
10) 10 µl Hybrid + 10µl 10^{-2} Col E2
11) 10 µl Hybrid + 10µl 10^{-3} Col E2
12) 10 µl Hybrid + 10µl 10^{-4} Col E2
13) 10 µl Hybrid + 10µl pC2G
14) 10 µl Hybrid + 10µl 10^{-1} pC2G
15) 10 µl Hybrid + 10µl 10^{-2} pC2G
16) 10 µl Hybrid + 10µl 10^{-3} pC2G

This assay was done by preparing extracts from the colicin E2 hybrids. Next dilutions of the wild-type colicins were made as described in Table 6 and 5 µL of each dilution was spotted onto lawns of pC2G, pCol A, pCol E1, or pCol E2 strains. The dilutions of the wild-type colicins were mixed with one of the four Col E2 hybrids and spotted onto the same lawns. An example of the competition assay is shown below plated onto a lawn of the pC2G control strain, If hybrid protein was present one might expect a reduction in the signal seen when the hybrid was added to the dilutions of the wild type colicins (Figure 11).

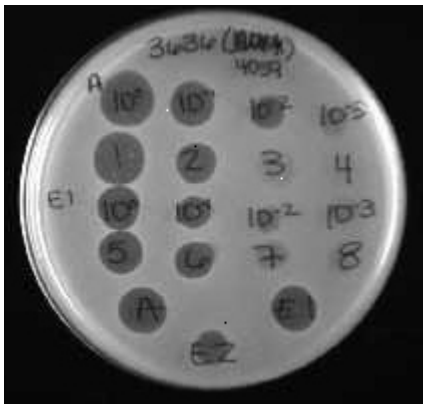
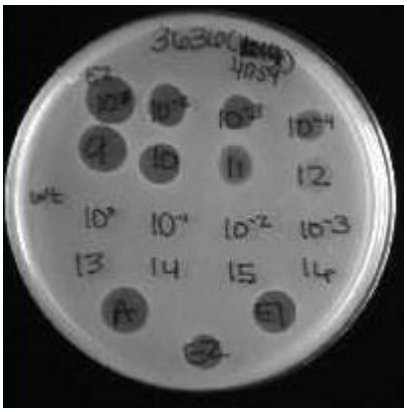
A.  B. 

Figure 11. Competition Assay Between Wild-Type Colicins and $TR_{E2}AI_{E1}$. (A) The first row is a serial dilution of colicin A. The second row is the same colicin A dilution mixed with the hybrid extract. The third row is a serial dilution of colicin E1. The fourth row is the same colicin E1 dilution mixed with the hybrid extract. (B) The first row is a serial dilution of colicin E2. The second row is the same colicin E2 dilution mixed with the hybrid extract. The third row is a serial dilution of MB3636. The fourth row is the same MB3636 dilution mixed with the hybrid extract.

These data show an apparent decrease in halo size with the hybrid colicin. To test whether the reduction seen in Figure 11 was from the hybrid colicin competition and not some other protein in the extract, another control competition assay was set up with the wild-type colicins and an extract from MB3636 (Figure 12). To do this dilutions were made of the wild-type colicins according to Table 6 and 5 µL of each dilution was added onto lawns with either the pC2G, pCol A, pCol E1, or pCol E2 strain. The dilutions of the wild-type protein were mixed with MB3636 extract and spotted onto the same lawns.

Figure 12. Competition Assay Between Wild-Type Colicins and MB3636. (A) The first row is a serial dilution of colicin A. The second row is the same colicin A dilution mixed with MB3636 extract. The third row is a serial dilution of colicin E1. The fourth row is the same colicin E1 dilution mixed with MB3636 extract. (B) The first row is a serial dilution of colicin E2. The second row is the same colicin E2 dilution mixed with MB3636 extract. The third row is a serial dilution of MB3636. The fourth row is the same MB3636 dilution mixed with MB3636 extract.

This assay displayed the same reduction seen previously. Therefore it can be concluded that the observed reduction in the halo size was due to some factor in the extracts and not the hybrid colicin. Therefore this competition assay was not be a suitable test to demonstrate that hybrid protein was present.

3.5 SDS-PAGE Gel

With the failure of the competition assay to show that E2 hybrid protein was present, the next step was to try to visualize the protein on an SDS-PAGE gel. To prepare samples, overnight cultures of MB3636 (negative control), the *XbaI* mutants (positive control), and the hybrids were grown and induced the next day. Next, 200 µL of cells were spun at full speed and resuspended in 100 µL of loading buffer. The samples were boiled for 10 minutes and 15 µL was run on a 10 % SDS-PAGE gel.

The wild-type colicin proteins are approximately 60 kDa. If protein is present there will be a band around 60 kDa in the samples that is not present in MB3636. Based on the gel the only sample that displayed a band was the colicin E2-*Xba* mutant (Figure 13). This is possibly due to the colicin E2-*Xba* mutant being more highly expressed or more stable than the other proteins which was shown previously in Figure 9. The protein gel was also inconclusive in showing whether protein was present in the nonfunctional hybrids probably due to low levels of the protein. A more sensitive method to visualize that the level of protein expression by these hybrids would be a western blot. This would have been the next step but our lab did not have the appropriate antibodies to run the western gels.

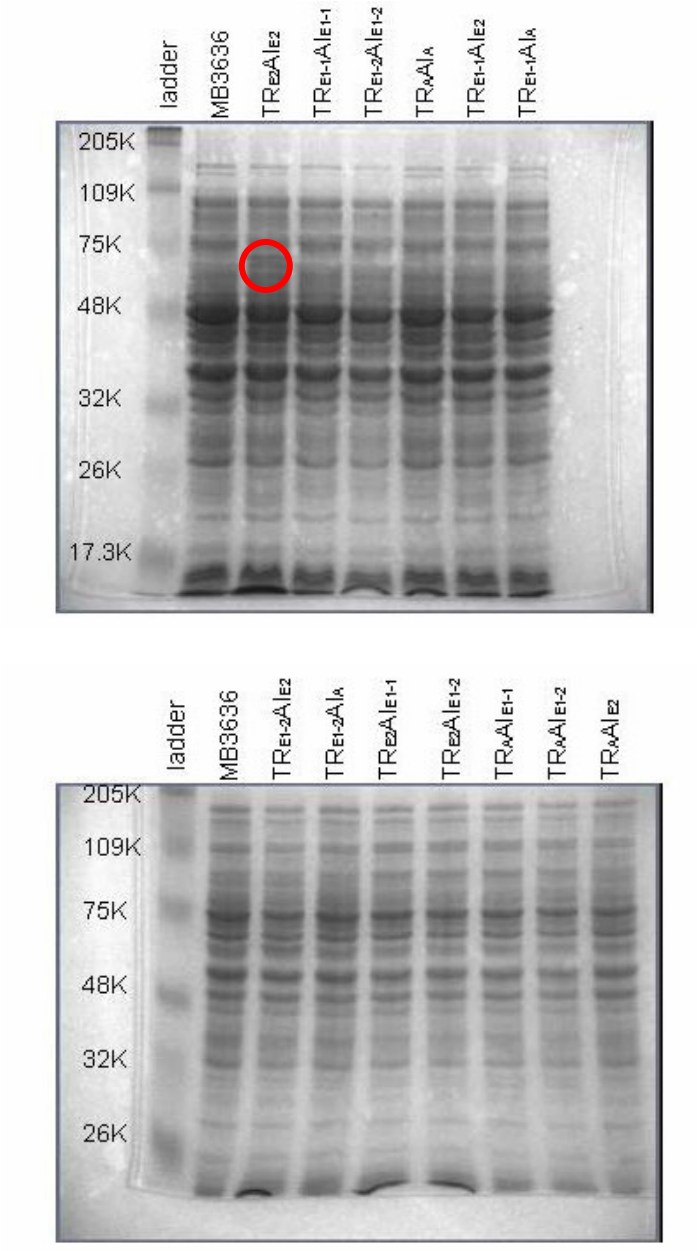


Figure 13. Protein Extracts on a SDS-PAGE Gel. The circle on the top gel indicates the putative colicin E2-*Xba* mutant protein.

4. SUMMARY AND CONCLUSIONS

Colicins are the well-studied bacteriocins of the Gram negative bacterium *E. coli*. There is interest in research on enzymatic colicins, especially in understanding the differences in their mode of entry compared to pore-forming colicins. The focus of this project was to further understand the entry mechanism of enzymatic colicins by understanding the phenotype of *cim* mutants.

Cim mutants were previously discovered by finding "tolerant" mutants to enzymatic colicins but not pore-forming colicins that were due to a mutation in the *tolB* gene. These mutants were shown to have a single base change that resulted in an amino acid change. Although TolB is essential for translocation of colicins A, E2, and E3, cells that display the Cim phenotype are only resistant to colicin E2 and E3.

The first step in this project was to build hybrids by swapping domains between pore-forming (A and E1) and enzymatic (E2) colicins. Many hybrids have been described previously that exchanged domains between pore-forming colicins or attaching segments of other proteins to pore-forming colicins (1, 20). These hybrids were able to function properly and gave insight in understanding the entry of pore-forming colicins. For example, Duche' generated a hybrid in which a signal peptide was attached to the activity domain of colicin A. This hybrid was inactive and the cells grew, which showed that pore-forming colicins can only form channels if they enter the inner membrane from the periplasmic side as opposed to the cytoplasmic side (14). Other hybrids involving enzymatic colicins have been made by exchanging domains between

enzymatic colicins or other enzymatic bacteriocins. Kageyama et al. formed a series of hybrids by swapping domains between pyocins, enzymatic bacteriocins produced by *Pseudomonas*, and colicins E2 and E3. This was done in an attempt to form a pyocin that possesses RNase activity. Like the pore-forming hybrids previously discussed, these hybrids were also functional. Although many hybrids have been made in the past, there have been no published hybrids prior to my work that include domains from both a pore-forming and enzymatic colicin.

My work has shown that hybrids composed of both pore-forming and enzymatic colicins (TR_AAI_{E2}, $TR_{E1}AI_{E2}$, $TR_{E2}AI_A$, $TR_{E2}AI_{E1}$) were nonfunctional, whereas hybrids between only pore-forming colicins (TR_AAI_{E1}, $TR_{E1}AI_A$) functioned as expected. The first set of colicin E2 hybrids I made had the *XbaI* insertion downstream of the *lepB* cleavage site, this would have caused the cleavage site to be carried along with the colicin E2 T-R domains instead of the A-I domains. Because previous research mentioned that the activity domain had to be cleaved by LepB in order for the activity domain of enzymatic colicins to be functional, another colicin E2 *Xba* mutant was constructed in which the *XbaI* insertion was placed upstream of the cleavage site. A complete set of hybrids were created and tested, these still remained nonfunctional. There are a variety of possible explanations that can explain why these hybrids were nonfunctional.

One reason could be that the immunity protein for enzymatic colicins is carried along with the secreted colicin and never released. This is different from pore-forming colicins, where the immunity proteins remain in the producing cells. Because pore-

forming colicins do not need to remove their immunity protein once they enter their target cell, they may not have a recognition element needed to remove the immunity protein from the hybrid colicin and activate it. If the recognition element needed to remove the immunity protein is located in the T-domain this could explain why hybrids TR_AAI_{E2} and $TR_{E1}AI_{E2}$ are nonfunctional.

Another possible explanation is that the activity domain may not be cleaved so that it can enter the cytoplasm and carry out its lethal activity. It was shown by de Zamaroczy that in order for enzymatic colicins to become active, the activity domain must be cleaved to allow it to enter the cytoplasm (13). On the other hand, it was seen by Masi that when pore-forming colicins are cleaved they are inactivated, which means that this is a unique step for enzymatic colicins (28). Since cleavage causes pore-forming colicins to become inactive, it would make sense for these colicins to not have the machinery or conserved sequence needed for cleavage. This phenomenon may explain why the hybrids that contain a pore-forming T-R domain and enzymatic A-I domain were inactive. Despite making fusions on either side of the LepB recognition site, there may be contextual information in the T or R domain necessary for its recognition and cleavage.

In addition to the hybrids containing the A-I domain of the enzymatic colicins being nonfunctional, the hybrids that contained the T-R domain of the enzymatic colicins were also nonfunctional. Due to research done in the past, a logical explanation may be that enzymatic colicins contain extra recognition elements in their T-R domain that would cause the activity domain of the pore-forming colicins to be cleaved and

released into the cytoplasm where they would be nonfunctional. Previous research probed whether colicin A could function in the cytoplasm or not (14). This was done by deleting the immunity and lysis domains on the colicin A plasmid. This produced a cell that accumulated colicin A with no immunity protein to protect the cell. It was observed that, although the cell was not protected, it was not lysed by the colicin. If the machinery needed for enzymatic colicin cleavage is found in the T or R domain it would cause the hybrids that contain the T-R domain from the enzymatic colicin and the A-I domains from pore-forming colicins to become inactive, due to the activity domain of the pore-forming colicin being cleaved.

The last plausible explanation for the E2 hybrids being nonfunctional is that the hybrid colicins are not as stable as the wild-type and cloned colicins. I attempted to test this by performing a serial dilution of the wild-type colicins, the cloned colicins, the *Xba* mutants, and the hybrids. The results indicated that the wild-type, cloned colicins and *Xba* mutants were able to be diluted approximately 100-fold times more than the hybrids.

Unfortunately because the E2 hybrids were nonfunctional, we were unable to test the hybrids against the *cim* mutants. Despite this setback we were able to learn more about the differences between pore-forming and enzymatic colicins. One major difference is the need for additional recognition elements by enzymatic colicins, either for removal of the immunity protein or cleavage of the activity domain. Another difference is the ability of the enzymatic colicins to function in the cytoplasm whereas the pore-forming colicins can not. Because of these differences it may be likely that the

Cim phenotype is caused because the T domain of enzymatic colicins must interact with this region of TolB to remove the immunity protein and therefore expose the LepB cleavage site, a step not required with pore-forming colicins.

Future research could include exchanging the translocation or receptor domain between pore-forming and enzymatic colicins instead of exchanging them together. This would allow some insight on whether the translocation or the receptor domain processes the extra recognition elements needed by enzymatic colicins and it could explain why the E2 hybrids designed in my project were nonfunctional. These hybrids could be tested against wild-type colicins and the *cim* mutants to further understand the phenotype of these mutants and the import of enzymatic colicins.

REFERENCES

1. **Benedetti, H., M. Frenette, D. Baty, M. Knibiehler, F. Pattus, and C. Lazdunski.** 1991. Individual domains of colicins confer specificity in colicin uptake, in pore-properties and in immunity requirement. J Mol Biol **217:**429-39.

2. **Boon, T.** 1972. Inactivation of ribosomes in vitro by colicin E 3 and its mechanism of action. Proc Natl Acad Sci USA **69:**549-52.

3. **Bowman, C. M., J. E. Dahlberg, T. Ikemura, J. Konisky, and M. Nomura.** 1971. Specific inactivation of 16S ribosomal RNA induced by colicin E3 in vivo. Proc Natl Acad Sci USA **68:**964-8.

4. **Braun, V.** 1989. The structurally related *exbB* and *tolQ* genes are interchangeable in conferring tonB-dependent colicin, bacteriophage, and albomycin sensitivity. J Bacteriol **171:**6387-90.

5. **Braun, V., and C. Herrmann.** 1993. Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. Mol Microbiol **8:**261-8.

6. **Campbell, A.** 1965. The steric effect in lysogenization by bacteriophage lambda. II. Chromosomal attachment of the b2 mutant. Virology **27:**340-5.

7. **Cascales, E., S. K. Buchanan, D. Duche, C. Kleanthous, R. Lloubes, K. Postle, M. Riley, S. Slatin, and D. Cavard.** 2007. Colicin biology. Microbiol Mol Biol Rev **71:**158-229.

8. **Cavard, D.** 1994. Rescue by vitamin B12 of *Escherichia coli* cells treated with colicins A and E allows measurement of the kinetics of colicin binding on BtuB. FEMS Microbiol Lett **116:**37-42.

9. **Cavard, D.** 2004. Role of Cal, the colicin A lysis protein, in two steps of colicin A release and in the interaction with colicin A-porin complexes. Microbiology **150:**3867-75.

10. **Cavard, D., and C. Lazdunski.** 1981. Involvement of BtuB and OmpF proteins in binding and uptake of colicin A. FEMS Microbiol Lett **12:**311-316.

11. **Chak, K. F., and R. James.** 1985. Analysis of the promoters for the two immunity genes present in the ColE3-CA38 plasmid using two new promoter probe vectors. Nucleic Acids Res **13:**2519-31.

12. **Clavel, T., J. C. Lazzaroni, A. Vianney, and R. Portalier.** 1996. Expression of the *tolQRA* genes of *Escherichia coli* K-12 is controlled by the RcsC sensor protein involved in capsule synthesis. Mol Microbiol **19:**19-25.

13. **de Zamaroczy, M., L. Mora, A. Lecuyer, V. Geli, and R. H. Buckingham.** 2001. Cleavage of colicin D is necessary for cell killing and requires the inner membrane peptidase LepB. Mol Cell **8:**159-68.

14. **Duche, D.** 2002. The pore-forming domain of colicin A fused to a signal peptide: a tool for studying pore-formation and inhibition. Biochimie **84:**455-64.

15. **Geli, V., D. Baty, V. Crozel, J. Morlon, R. Lloubes, F. Pattus, and C. Lazdunski.** 1986. A molecular genetic approach to the functioning of the immunity protein to colicin A. Mol Gen Genet **202:**455-60.

16. **Goldman, K., J. L. Suit, and C. Kayalar.** 1985. Identification of the plasmid-encoded immunity protein for colicin E1 in the inner membrane of *Escherichia coli*. FEBS Lett **190:**319-23.

17. **Guihard, G., P. Boulanger, H. Benedetti, R. Lloubes, M. Besnard, and L. Letellier.** 1994. Colicin A and the Tol proteins involved in its translocation are preferentially located in the contact sites between the inner and outer membranes of *Escherichia coli* cells. J Biol Chem **269:**5874-80.

18. **Jakes, K. S.** 2001. The importance of being cleaved: an essential step in killing by enzymatic colicins. Mol Cell **8:**4-6.

19. **Kadner, R. J., P. J. Bassford, Jr., and A. P. Pugsley.** 1979. Colicin receptors and the mechanisms of colicin uptake. Zentralbl Bakteriol [Orig A] **244:**90-104.

20. **Kageyama, M., M. Kobayashi, Y. Sano, and H. Masaki.** 1996. Construction and characterization of pyocin-colicin chimeric proteins. J Bacteriol **178:**103-10.

21. **Krone, W. J., P. de Vries, G. Koningstein, A. J. de Jonge, F. K. de Graaf, and B. Oudega.** 1986. Uptake of cloacin DF13 by susceptible cells: removal of immunity protein and fragmentation of cloacin molecules. J Bacteriol **166:**260-8.

22. **Latha, K., L. Guynn, M. Berkman, R. Penland, and M. Benedik.** Alleles of *tolB* that block the cytoplasmic import of Colicin E2. University of Houston, Houston, TX.

23. **Lindeberg, M., S. D. Zakharov, and W. A. Cramer.** 2000. Unfolding pathway of the colicin E1 channel protein on a membrane surface. J Mol Biol **295:**679-92.

24. **Lloubes, R., D. Baty, and C. Lazdunski.** 1986. The promoters of the genes for colicin production, release and immunity in the ColA plasmid: effects of convergent transcription and Lex A protein. Nucleic Acids Res **14:**2621-36.

25. **Lloubes, R., D. Baty, and C. Lazdunski.** 1988. Transcriptional terminators in the *caa-cal* operon and *cai* gene. Nucleic Acids Res **16:**3739-49.

26. **Lloubes, R., M. Granger-Schnarr, C. Lazdunski, and M. Schnarr.** 1988. LexA repressor induces operator-dependent DNA bending. J Mol Biol **204:**1049-54.

27. **Mankovich, J. A., C. H. Hsu, and J. Konisky.** 1986. DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. J Bacteriol **168:**228-36.

28. **Masi, M., P. Vuong, M. Humbard, K. Malone, and R. Misra.** 2007. Initial steps of colicin E1 import across the outer membrane of *Escherichia coli*. J Bacteriol **189:**2667-76.

29. **Payne, M. A., J. D. Igo, Z. Cao, S. B. Foster, S. M. Newton, and P. E. Klebba.** 1997. Biphasic binding kinetics between FepA and its ligands. J Biol Chem **272:**21950-5.

30. **Pugsley, A. P., and M. Schwartz.** 1984. Colicin E2 release: lysis, leakage or secretion? Possible role of a phospholipase. Embo J **3:**2393-7.

31. **Senior, B. W., and I. B. Holland.** 1971. Effect of colicin E3 upon the 30S ribosomal subunit of *Escherichia coli*. Proc Natl Acad Sci USA **68:**959-63.

32. **Sharma, O., E. Yamashita, M. V. Zhalnina, S. D. Zakharov, K. A. Datsenko, B. L. Wanner, and W. A. Cramer.** 2007. Structure of the complex of the colicin E2 R-domain and its BtuB receptor. The outer membrane colicin translocon. J Biol Chem **282:**23163-70.

33. **Studier, F. W.** 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr Purif **41:**207-34.

VITA

Name:	Tysheena Perkins Charles
Address:	Department of Biology, Texas A&M University 3258 TAMU College Station, TX 77843
Email Address:	Tysheena@gmail.com
Education:	B.S., Biology, Xavier University of Louisiana, 2005