ENTEROBACTIN EXPORT IN *ESCHERICHIA COLI*
VIA P43 (ENTS) AND ASSOCIATED COMPONENTS

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Doctor of Philosophy

by
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ENTEROBACTIN EXPORT IN ESCHERICHIA COLI
VIA P43 (ENTS) AND ASSOCIATED COMPONENTS

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DEDICATION

I dedicate this work to my family, Jennifer & my “chubble-hootmonkey-werewolf” son Caleb. They have put up with my random schedules and working late, and kept me encouraged when I was frustrated. Also, this is dedicated to my mom and dad, Carol & Larry, as well as my grandparents, Al & Betty, for supporting me with constant encouragement, guidance, and financial means all throughout school and life. Finally, to all the friends I have met and kept in graduate school: I've loved being part of the MMI family and look forward to working with all of you in the future as we progress into our science.
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ENTEROBACTIN EXPORT IN *ESCHERICHIA COLI*
VIA P43 (ENTS) AND ASSOCIATED COMPONENTS

Jason Lawrence Furrer

Dr. Mark A. McIntosh, Dissertation Supervisor

ABSTRACT

Ferric iron, critical for the metabolic functions of many microorganisms, is generally insoluble at neutral pH or quickly complexed by host iron storage proteins. To acquire necessary ferric iron against harsh competition in the environment, iron-starved *Escherichia coli* synthesize, excrete and retrieve an iron-scavenging siderophore molecule termed enterobactin. Despite extensive characterization of the enterobactin system, the export machinery allowing enterobactin secretion to the extracellular environment has only recently been identified. *E.coli* membrane protein P43 (*entS*) in the enterobactin gene cluster encodes a Major Facilitator Superfamily (MFS) exporter. A P43 null mutant was unable to efficiently secrete enterobactin to the supernate, but did secrete elevated levels of enterobactin breakdown products as analyzed by TLC, HPLC, and cross-feeding assays. To further evaluate P43 function in enterobactin transport, inverted membrane vesicles were created using French press and incorporated with an iron-binding fluorescent dye, calcein-AM (CA). Differences in siderophore transport were observed between wild-type and the P43-mutant by monitoring CA fluorescence restoration following iron quenching and the addition of enterobactin. Using specific energy poisons in
conjunction with this vesicle system, it was determined that proton motive force energy is utilized for this transport. Additional results demonstrate that siderophore transport from the periplasm to the external environment may be due to contributions from several other identified *E. coli* components, such as the multi-drug export system comprised of the outer membrane protein TolC and the translocase AcrAB. These data all demonstrate P43 provides a critical activity for the *E. coli* enterobactin secretion machinery and establish a mechanism for cellular release of siderophore.
CHAPTER 1: INTRODUCTION

Iron and microbial cells:

Iron is one of the most environmentally abundant and versatile of the transition metal elements. It is required for the growth of nearly all cells, both prokaryotic and eukaryotic, with few exceptions (183, 223). Bacteria have need of iron for a range of metabolic and signaling functions including electron transport, peroxide reduction, amino acid and nucleoside synthesis, DNA synthesis, photosynthesis, and most importantly – some virulence traits (84). It is difficult to overestimate the importance of iron in pathogenic situations. The virulence of *Escherichia coli* (33), *Klebsiella* (221), *Listeria* (137), *Neisseria* (34), *Shigella* (176), *Salmonella* (83), *Vibrio* (41), and *Yersinia* (194) are all increased by excess iron. Quantitatively, virulence of *Yersinia enterocolitica* was demonstrated to be enhanced 10 million-fold by co-injection of ferric desferrioxamine (35, 194). Iron injections administered to iron-deficient children increased *E.coli* bacteremia and meningitis (14). Nonlethal injections of *E.coli* in guinea pigs can become lethal by co-injection of heme (33). It is therefore important to establish the relationships between iron and bacteria.

Iron (Fe) can assume two ionic forms, Fe$^{+2}$ (ferrous) and Fe$^{+3}$ (ferric). Despite the abundance in the environment, it is problematic to acquire. At neutral pH and in aerobic inorganic environments, ferric iron forms insoluble hydroxides (155), which limits free environmental concentrations to approximately 10$^{-18}$ M (189). Iron is also a double-edged sword, causing toxicity in cells even below this concentration due to Fenton reduction reactions (Fe$^{+2}$ + H$_2$O$_2$ $\rightarrow$ Fe$^{+3}$ + OH$^{-}$ + OH) producing harmful hydroxyl radicals. As a result, mammalian cells further sequester iron to prevent these detrimental effects using proteins such as transferrin and lactoferrin, thus reducing the available free iron to an estimated 10$^{-24}$ M in human serum (5, 111, 136, 222). It has been estimated that bacterial
cells require a cytoplasmic iron concentration of $10^{-6}$ M for growth (28), significantly above available amounts. Faced with this iron shortage in a pathogenic situation, bacteria must find alternative means of iron acquisition.

**Siderophores:**

While Fe$^{2+}$ is freely soluble and easily imported to *E.coli* using the *feoAB* system (86), acquisition of Fe$^{3+}$ requires more application. One mechanism developed by bacteria to acquire ferric iron is the use of siderophores. Siderophores, literally meaning “iron carriers” in Greek, are small (generally less than 1000 kD) iron-chelating molecules that have high affinity for Fe$^{3+}$ (156). Siderophores are highly negatively charged. Over 500 different siderophores have been described for bacteria and yeasts (226). Despite their variety, most siderophores are comprised of a peptide backbone with various coordinating iron-ligating groups. The coordinating groups discern siderophores into three families: catechol, hydroxamate, or hydroxyacid.

Perhaps the most studied siderophore system is the enterobactin system of *E.coli*. Enterobactin was isolated in 1970 by two groups independently, one working in *E.coli* and naming the compound enterobactin (166) and one in *Salmonella*, naming the compound enterochelin (179). Due to publishing dates, both designations can be found in the literature. Enterobactin [Fig. 1 A] is a catechol family siderophore and contains a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine. Outside the cell, enterobactin can successfully compete against all known proteins for Fe$^{3+}$ with an unparalleled affinity for iron ($K_d = 10^{-35} \text{ to } 10^{-49}$) (46). Iron coordination involves the hexadentate triscatecholate geometry and upon iron binding and proton loss results in full encapsulation of the iron atom (190) [Fig. 1 B].
**The enterobactin operon and synthesis:**

Many species of gram-negative *Enterobacteriaceae* including *E.coli* (166), *Salmonella* (179), and *Shigella* (118) harbor the ~24 kB gene cluster encoding thirteen proteins that collaborate to synthesize, transport, and process enterobactin (69, 170, 206). It is located at approximately 13 min on the *E.coli* chromosome (18). In this cluster are six genes (*entA-entF*) encoding for enterobactin synthesis components, and several *fep* genes encoding transporter components [Fig. 2]. The three remaining genes – *fes* (ferric enterobactin esterase), P43, and P15 – will be discussed in more detail throughout this manuscript.

Enterobactin is assembled on nonribosomal peptide synthetases using a fork branching from the aromatic amino acid pathway [Fig. 3]. The primary metabolite chorismate is converted to isochorismate, and then diverted to 2,3-dihydroxybenzoic acid (DHB) by the gene products of *entC, entB,* and *entA* (125). The gene products of *entD, entE, entF,* and *entB* then catalyze the amide linkage of DHB and L-serine (73, 74). Serine is activated by adenylation and is then bound to the multifunctional carrier protein EntF which functions as an acyl-S-pantethiene intermediate and terminal thioesterase assembling three DHB monomers into the fully cyclized enterobactin (56, 205).

**The regulation of enterobactin in *E.coli:***

As previously mentioned, both overabundance and scarcity of iron can be detrimental to cells. Hence, *E.coli* practices a tight regulatory control over iron flux. The primary regulator of intracellular iron levels is the ferric uptake regulator (Fur) (89). During iron replete conditions, Fe$^{+2}$ is bound by a Fur dimer, which enhances binding to DNA and subsequent repression of gene transcription and reduced iron uptake (10). Recently, it was demonstrated that two Fur dimers recognize and bind a consensus sequence of two overlapping dyad targets in a Fur Box binding site to produce regulation effects (117). Mechanistically, Fur assembles on the target DNA blocking promoter sequences and
RNA polymerase binding sites to prevent transcription (59, 116). Fur controls transcription of genes involved in oxidative stress, metabolism, virulence, and iron storage, in addition to genes needed for iron uptake, such as those of the enterobactin system (88). Further regulation of the enterobactin system is achieved by several divergent promoters (44, 57, 177, 206) as denoted in [Fig. 2].

**The transport of enterobactin:**

Once released to the external environment, enterobactin scavenges ferric iron and is recovered by the cell. The outer membrane receptor, FepA, actively transports ferric enterobactin into the periplasm (186, 187) using proton motive force energy generated by the TonB protein (104, 184). In the periplasm, FepB binds the complex and shuttles it to the cytoplasmic membrane-bound FepDCG complex of proteins, which functions as a ATP-binding-cassette (ABC) transporter to transduce enterobactin from the periplasm to the cytoplasm (42, 178, 206, 208). Once transported into the cytoplasm, the Fes enzyme cleaves ferric enterobactin complexes and iron is released, ready for cellular purposes (31). Extensive studies have been conducted on the binding and transport of enterobactin by FepA (12, 13) that highlight transport kinetics, key binding residues involved in ferric enterobactin recognition, and protein-protein interactions. The study of enterobactin and its transport has built our knowledge of siderophore systems. The majority of the enterobactin cycle is well characterized and has been the subject of numerous reviews and book summaries (66, 190, 203, 219). Despite this knowledgebase of regulation, synthesis, and recovery, comprehensive mechanisms of siderophore secretion to the extracellular environment are as of yet incomplete.

**E.coli P43 (ybdA) in the enterobactin operon:**

Because the size and charge of enterobactin all but eliminates the possibility of passive diffusion across the membrane, it necessitates the involvement of an exporter or transport pump.
Previous work in *E. coli* (42, 206) uncovered a 43-kD membrane protein (P43) [Fig. 4 A] with strong similarity to the major facilitator superfamily (MFS) class [Fig. 4 B] of proton motive force (PMF) dependent membrane efflux pumps (172). More specifically, P43 would be subclassed in the Drug:H+ Antiporter-3 (DHA3) family (198). These MFS transport proteins are involved in symport, antiport, and uniport of various substrates including antibiotics (134). The DHA3 subfamily is characterized as efflux pumps likely to function by proton antiport mechanisms (198). P43 is a polytopic membrane protein predicted to have 12 helical domains that represent transmembrane segments (TMS). It is encoded by *ybdA*, located in the *ent/fep* gene cluster between *fepD* and *fepB* [Fig. 4 C]. Similarities exist between P43 and other MFS proteins such as the tetracycline efflux pump, TetA (121), especially in four conserved amino acid motifs [Fig. 4 D] predicted to play an important structural or functional role in the transporter. Motif A, between TMS 2 and 3 and conserved in all 17 families of MFS proteins (172), may play a role in conformational changes for opening and closing of the channel (99). Motif C, located in TMS 5, is implicated in direction of transport (82) and is not found in MFS symport proteins, rather only in MFS proteins with multidrug or specific drug efflux activities (82). Motifs D and E, while conserved in P43, have as of yet undetermined functions. Motif B, predicted to be involved in proton transfer (173), is not conserved in P43.

This finding is significant because MFS-type proteins similar to P43 (YbdA) have been reported in other siderophore systems. Results of NCBI nucleotide-BLAST and literature yield P43-like proteins in the yersiniabactin gene island (*ybtX*) of *Yersinia pestis* (65), the alcaligin gene island (*orfX*) from *Bordetella pertussis* (30), the legiobactin operon (*lbtB*) of the human pathogen *Legionella pneumophila* (6), and the ruckerbactin operon (*rucS*) of the fish pathogen *Yersinia ruckeri* (64). Other MFS proteins such as Enb1p, which recognized and transported enterobactin in *S. cerevisiae*, have been reported (95). A complete ABC-type export system (similar to the MFS-class) exists in the *P. aeruginosa*
pyoverdine cluster with a membrane fusion protein (PA2789), an active efflux pump (PA2790), and an outer membrane channel (PA2791).

**Hypothesis and research objectives:**

Despite the extensive body of work done on siderophore systems, in no organism to date has there been a defined export mechanism (219) [Fig. 5]. The goal of this thesis work is to illuminate the mechanisms of enterobactin export. Based on literature and BLAST results, we hypothesize that P43 serves as the export pump for enterobactin. To prove this, we must demonstrate P43 necessity for efficient enterobactin release. In Chapter 2, we create a P43-null mutation and test the ability to export enterobactin by utilizing a nutritional cross-feeding assay to determine released compounds. Further evidence is provided by monitoring cells for enterobactin release under low iron conditions with thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). These results provide evidence for P43’s role in enterobactin export. Chapter 3 seeks additional confirmation of P43 involvement in enterobactin export. We create inverted membrane vesicles and monitor direct transport of enterobactin by fluorescent reporting. These experiments will also provide a means of investigation for energy requirements of enterobactin transport. Chapter 4 addresses the fact that P43, as a predicted inner membrane protein, would likely only allow enterobactin to cross the cytoplasmic membrane, and thus strand enterobactin in the periplasm. We will consider potential interaction partners for P43 and test them experimentally. For this, candidate genes are identified and deleted to observe a similar enterobactin-deficient phenotype. In vivo chemical crosslinking can be used to link and purify the complete enterobactin transport complex.
**Additional goals and results:**

Finally, there is an additional goal to my graduate education. During my graduate training program and with the help of my committee, I have pursued a Graduate Minor degree in College Teaching. Chapter 6 will address education questions such as “how do we best teach microbiology to our students” and “how do we prepare our microbiology graduate students to become teacher-scholars” by considering the place of teacher training in a graduate education, my experiences with teaching and learning to teach, experiences in earning a minor degree in addition to a scientific doctorate, and the additional coursework and preparations incorporated into my training. While this chapter may not contain concrete “data”, I feel it is important to leave a trail for future graduate students that may be interested in teaching and for myself to better understand what it means to be a member of the professoriate involved in the academic pursuits of teaching, research, and service.
CHAPTER 1 FIGURES

FIGURE 1: STRUCTURE OF ENTEROBACTIN.
Structural representations of enterobactin using stick model (A) and space filling model (B).
Enterobactin is a catechol family siderophore and is composed of a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine. Iron coordination involves hexadentate triscatecholate geometry (shown in red) for encapsulation of the iron atom.
FIGURE 2: GENETIC ORGANIZATION OF THE *E.COLI* ENTEROBACTIN LOCUS.

Genes shaded orange (*entD, entF, entC, entE, entB, and entA*) are involved in the synthesis of enterobactin. Genes shaded green (*fepA, fepC, fepG, fepD, fepB*) are involved in recognition and import of ferric enterobactin complexes. Genes shaded red (*fes*) have enzymatic activity necessary for ferric enterobactin function. Genes shaded blue (*P43*) are hypothesized to function in enterobactin export. Genes shaded grey (*fepE, P15*) are partially characterized in regards to function. The bivalent promoter structure of the operon is shown below, represented by arrows showing direction of transcription.
FIGURE 3: BIOCHEMICAL ASSEMBLY OF ENTEROBACTIN.

Assembly of enterobactin is primed on nonribosomal peptide synthetases using the base material chorismate. Chorismate is converted to isochorismate, and then into 2,3-dihydroxybenzoic acid (DHB) by the gene products of entC, entB, and entA (top row). The resulting DHB is then linked to L-serine by EntE and EntD, and finally transferred to the multifunctional carrier protein EntF which functions to assemble three DHB-serine monomers into the fully cyclized enterobactin (right side).
FIGURE 4: E.COLI P43 ANALYSIS.

(A) Predicted topology of the E.coli P43 gene product: a 12-transmembrane segment (TMS) major facilitator superfamily (MFS) protein and (B) comparison to a generic model of the 12-TMS MFS family [adapted from (188)]. Location of conserved motifs of MFS proteins are noted in both figures. (C) Representation of the E.coli chromosomal enterobactin gene cluster showing the gene encoding P43, ybdA, and suggesting a potential role. (D) Alignment of conserved amino acid motifs of MFS-family proteins (CON) versus P43 and a known E.coli MFS protein, the tetracycline pump, TetA.
FIGURE 5: SIDEROPHORE TRANSPORT.

Summary of current knowledge for siderophore excretion and internalization in gram-positive and gram-negative bacteria [adapted from (219)]. The goal of the work presented in this thesis is to define an export mechanism (represented by the ? below) for the siderophore enterobactin in *E.coli*. 
CHAPTER 2: CHARACTERIZATION OF P43 INVOLVEMENT IN ENTEROBACTIN TRANSPORT

Chapter Summary:

The *Escherichia coli* enterobactin iron acquisition system is well understood with the exception of the mechanism of secretion to the extracellular environment. A potential candidate for this role is the *E. coli* membrane protein P43, encoded by *ybdA* in the chromosomal region of genes involved in enterobactin synthesis. The predicted protein shows strong homology to the 12-transmembrane segment major facilitator superfamily (MFS) of export pumps. A P43 null mutation was created and siderophore nutrition assays, performed with a panel of *E. coli* strains expressing one or more outer membrane receptors for enterobactin-related compounds, demonstrated that the P43 mutant was unable to efficiently secrete enterobactin. Products released from the mutant strain were identified with thin-layer chromatography and high-performance liquid chromatography revealing that the P43 mutant secretes little, if any, enterobactin, but elevated levels of enterobactin breakdown products, 2,3-dihydroxybenzoylserine (DHBS) monomer, dimer, and trimer. These data establish that P43 is a critical component of the *E. coli* enterobactin secretion machinery and provide a rationale for the designation of the previous genetic locus *ybdA* as *entS* to reflect its relevant biological function.

Introduction:

Expectations were high for P43 functioning as an enterobactin export pump. However, initial characterizations of a null mutation in the *ybdA* gene produced no assayable enterobactin production or transport defects in siderophore nutrition (cross-feeding) and (Fe$^{55}$)-enterobactin uptake assays despite the similarities of P43 to other MFS proteins. After further consideration, one complication was recognized in the original cross-feeding assays used to define enterobactin
production: no distinction was made between feeding by enterobactin and feeding by other molecules, including enterobactin breakdown products. The activity of ferric enterobactin esterase, Fes (31), results in hydrolysis of enterobactin, whether or not it has chelated ferric iron, into the linear trimer, dimer, and monomer of 2,3-dihydroxybenzoylserine (DHBS). Studies (87) showed that these DHBS molecules could serve as siderophores capable of supporting growth equal to that achieved with enterobactin secreting strains. These breakdown products are transported through the Fiu and Cir receptors [Fig. 6] rather than the enterobactin receptor, FepA (87). The products of Fes activity are thus predicted to diffuse from the cells where they are capable of providing additional siderophore activity.

New crossfeeding assays were developed that account for and differentiate between feeding molecules. A schematic of this assay [Fig. 7] is shown for clarity. During cross-feeding assays as enterobactin and its products are released to the media, the smaller DHBS breakdown products diffuse farther away from the feeder colony and are able to stimulate growth efficiently once they are not in competition with enterobactin for Fe$^{3+}$. A zone of inhibition was expected if the indicator strain is unable to feed on the molecules present. Indicators expressing Cir or Fiu, but not FepA, would then show growth inhibition in a region proximal to enterobactin secreting strains, while growth due to enterobactin products could be observed distally. Against this panel of siderophore receptor mutant strains, distinct differences in the cross-feeding patterns of the wild-type enterobactin producing strains and a P43 mutant derivative were identified. Subsequent chromatographic analysis of culture supernatants was used to identify enterobactin products. The data clearly show that P43 is a membrane protein exporter involved in the release of enterobactin from the cell. We proposed that P43 be denoted EntS in the literature to signify its role in enterobactin secretion and that the ybdA gene now be designated entS.
Materials and methods:

Strains:

*E. coli* BM694 [*E. coli* C1a derived from BM691 (200), *F*, *nalA*] was used as the wild-type strain (113). BM694/MT912 was derived by P1 transduction of the Δ*fepA::kan* locus from MT912 (115). BM694/KS222 was derived by P1 transduction of the *ybdA::kan* locus from KS222 (206). For all experiments described, identical mutations were made in *E. coli* W3110 by P1 transduction and tested in conjunction with BM694 background strains. These strains produced identical results [data not shown].

The following strains were used as indicators in siderophore nutrition (cross-feeding) assays. H1443 [*F*, *araD139 Δ(lacOPZYA)U169 rpsL thiA aroB*], H1728 (H1443, *fiu::MudX cin*), H1875 (H1443, *cir::MudX fepA::Tn10*), H1876 (H1443, *fiu::MudX cir fepA::Tn10*), and H1877 (H1443, *fiu::MudX fepA::Tn10*) were generous gifts of Klaus Hantke. *E. coli* strain RWB18-60 [*F, thi proC leuB trpE entA Δ(entD fepA fes entF) ΔrecA*] was used as a mutant indicator defective for the enterobactin receptor, but expressing both Cir and Fiu.

Media, Chemicals, and Enzymes:

Bacteria were routinely grown in Luria-Bertani (LB) broth (142) or on LB agar plates. MOPS minimal medium (10x MOPS salts: 1 M MOPS pH 7.4, 1.9 M NH₄Cl, 0.276 M K₂SO₄, 0.0005 M CaCl₂, 0.528 M MgCl₂, 5 M NaCl) was supplemented with 0.00132 M K₂HPO₄, 0.2% glucose, 0.05 mg/ml each tryptophan, proline, and leucine, 0.004 M citric acid, and 0.01 M thiamine (55). Additionally, 0.2 mM 2,2-dipyridyl was added to simulate low iron conditions or 0.3% FeCl₃ for high iron conditions. Tris-succinate-EDDA (TSE) (68, 141) medium supplemented with 50 mg/ml each of leucine, proline, and tryptophan, 5 mg/ml thiamine, 0.1 mM CaCl₂, 1 mM MgSO₄, 30 mM sodium succinate, 25 mg/ml each
of phenylalanine, PABA, and tyrosine, and 100 mM EDDA was routinely used for siderophore nutrition cross-feeding experiments. All chemicals and reagents used were purchased from Fisher Scientific (Pittsburgh, PA, USA).

**Cross-feeding Assays:**

Indicator strains were grown with shaking for 18 h in 3 ml LB plus appropriate concentrations of antibiotics if necessary; 300 ml of culture was added to 3 ml TSE top agar (TSE medium + 0.75% agar). The resulting suspension was poured and allowed to solidify on TSE agar plates containing appropriate concentrations of nutrients. Feeder strains were patched onto the top agar and plates were incubated for 24 h at 37°C. Resulting growth and inhibition zones were measured.

**Enterobactin Extractions:**

Starter cultures in 2 ml LB were grown for 3 h to an OD$_{600}$ of at least 0.6 (~ 5 x 10$^8$ cells/ml); 1 ml was pelleted and the bacteria were resuspended in 4 ml MOPS minimal medium containing 0.045% FeCl$_3$ and grown for 18 h. The overnight culture (1 ml) was pelleted and the bacteria were then resuspended in 10 ml MOPS minimal media containing either 0.03% FeCl$_3$ or 0.2 mM 2,2-dipyridyl and incubated with shaking for 4 h to an OD$_{600}$ of 0.7. After centrifugation at 20,000 x g for 15 min, culture supernatants were acidified with 50 ml 10 N HCl and extracted twice with a total of 8 ml ethyl acetate. Organic layers were combined and dried in 1 ml aliquots in a SpeedVac Concentrator (Savant Instruments, Farmingdale, NY, USA). Extract residues were resuspended in 50 ml methanol and stored at 4°C if not used immediately.

**TLC Analysis:**
Thin-layer chromatography was performed on either 250 mm layer glass silica gel (20 x 20 cm) 60A plates or on 250 mm layer flexible (20 x 20 cm) PE SIL G/UV\textsubscript{254} plates (Whatman Ltd, Maidstone, Kent, UK). Samples (20 ml) in methanol were spotted in 2 ml aliquots onto plates and dried under a gentle stream of air. Plates were developed with benzene:glacial acetic acid:water (125:72:3 [vol/vol/vol]) in a glass container (Kontes, Vineland, NJ, USA). Plates were then removed from the chamber and allowed to dry briefly, then sprayed with 1% FeCl\textsubscript{3} in water to visualize iron binding compounds.

**HPLC and LC/MS Analysis:**

HPLC methods were based on previously published protocols (225). In brief, enterobactin extract analysis was performed with a Symmetry® Nucleosil C\textsubscript{18}, 5 mm, 4.6 x 250 mm HPLC column (Waters Corp, Milford, MA, USA) using a gradient of 10-50% acetonitrile in water with constant 0.1% trifluoroacetic acid to maintain a pH of 3.0. Injection and separation were accomplished using a Waters 2690 Separations Module and resulting peaks detected with a Waters 996 Photodiode Array Detector (Waters Corp, Milford, MA, USA). Detector wavelength was set at 220 nm and flow rate through the column set to 1 ml/min. 50 ml of each enterobactin extract resuspended in methanol was injected and separated by the gradient. Results were analyzed using Millenium\textsuperscript{32} Chromatography Manager v3.2 software (Waters Corp, Milford, MA, USA).

Identity of extract peaks was confirmed using mass spectrometry with a Finnigan TSQ 7000 triple quadrupole mass spectrometer in the negative ion mode using electrospray ionization (Mass Spectrometry Facility, University of Missouri-Columbia Department of Chemistry). The voltage applied to the capillary was 4 kV. To reduce fractionation, LC peaks were separated with a buffer of methanol and 0.1% formic acid in water (1:1) at a flow rate of 1 mL/min.
Results:

We previously reported (206) that a ybdA::kan strain incapable of P43 expression displayed no obvious phenotypes for enterobactin transport or production based upon uptake and cross-feeding assays, respectively. However, we subsequently showed that purified Fes enzyme preferentially hydrolyzes enterobactin over its ferrated complex (31), suggesting that this enzyme may function to prevent any intracellular accumulation of unliganded siderophore. Evidence was provided (87) that enterobactin hydrolysis products also function as siderophores, using the Fiu and Cir receptors to gain entry. Therefore, since the original cross-feeding assays utilized AN90, an entD strain expressing FepA, Fiu, and Cir, as an indicator strain, re-evaluation of the experiment suggested that it could not distinguish between enterobactin or its breakdown products being used as the iron source. A schematic of the crossfeeding assay experimental design [Fig. 7] is shown to explain our expected results.

We re-examined the P43-null mutant using as indicators a panel of derivatives of the aroB strain H1443 (87) expressing one or more of the enterobactin-related receptors [Fig. 8]. The test strains were BM694, a wild-type isolate (113) that produces and utilizes enterobactin, a fepA::kan derivative (BM694/MT912) that makes but cannot import enterobactin, and the ybdA::kan strain (BM694/KS222). All three of the test strains are capable of stimulating the growth of H1443 [Fig. 8 A, B, & C], similar to the data reported earlier with AN90 (206). The triple mutant H1876 lacking all three siderophore receptors [Fig. 8 D, E, & F] could not be cross-fed. When the indicator strain expressed only a single receptor, distinctly different stimulation patterns were observed. BM694 produced a wide growth zone with all three indicators [Fig. 8 G, J, & M], but if the indicator was deficient for fepA, there was a zone of growth inhibition around the test colony [Fig. 8 G & J]. This inhibition zone was caused by the production of enterobactin, which outcompetes its breakdown products for iron but cannot be transported through Fiu or Cir. When the feeder colony was fepA and could not utilize the enterobactin
it produced, these inhibition zones increased significantly [Fig. 8 H & K]. Spontaneous hydrolysis of the ester linkages in the siderophore produced some breakdown compounds that diffused farther from the test colony and stimulated in the absence of competitor enterobactin. The stimulation zone for H1728, which only expresses FepA, reflects the enterobactin diffusion limits [Fig. 8 N]. The indicator strain RWB18-60, which expresses both Cir and Fiu, showed growth patterns similar to those strains expressing only one of these two receptors.

When the feeder colony was the P43-null mutant (BM694/KS222), no zones of inhibition were observed with H1875 and H1877 [Fig. 8 I & L] indicating that little if any enterobactin was being released from these cells. This was supported by the lack of any stimulation with H1728 [Fig. 8 O] expressing only FepA and thus capable of feeding only on enterobactin. These data suggest that in the absence of P43, primarily enterobactin hydrolysis products are released and support the hypothesis that P43 is a component of an export pathway for enterobactin. In the null mutant, enterobactin would be degraded by the activity of the Fes enzyme and the products released, where they may be utilized as siderophores by strains expressing Fiu or Cir.

Thin layer chromatography (TLC) was used to identify enterobactin and its breakdown products released from strains wild-type or null for P43 expression. Ethyl acetate extracts from 18-hour cultures grown in MOPS minimal medium with iron or the chelator 2,2-dipyridyl were chromatographed on silica gel plates. Neither strain produced any iron-binding compounds when grown in iron-rich medium for 18 hours [Fig. 9 A, lanes 3 & 4]. However, in the iron-depleted medium, BM694/MT912 released significant quantities of enterobactin and its breakdown products [Fig. 9 A, lane 1], corresponding to the monomer (M), dimer (D), and trimer (T) of DHBS as reported previously (201). These products co-migrate with breakdown products in the purified enterobactin sample. In extracts from the P43-null mutant [Fig. 9 A, lane 2], a dramatic decrease in enterobactin release was evident, although some enterobactin was detected. These low levels may reflect cell lysis or an alternative
export process to release enterobactin into the medium after prolonged growth. When the experiment was repeated using a 4-hour growth period [Fig. 9 B], the TLC pattern for BM694/MT912 was very similar to that seen after 18 hours [Fig. 9 B, lane 1], but no detectable enterobactin was found in the extracts from the P43 mutant [Fig. 9 B, lane 2]. In addition, amounts of enterobactin breakdown products, notably the monomer, increased significantly compared to the MT912 strain. Presumably, fewer breakdown products are seen in the 18-hour cultures than in 4-hour cultures since these products serve as siderophores and would be transported during the growth of these strains. Growth in iron-rich media resulted in no detectable products for either strain after the shorter 4-hour growth period [data not shown].

To provide a more quantitative assessment of the release of enterobactin and enterobactin-related products, HPLC was used to examine extracts from isogenic wild-type, P43−, and FepA− strains. Dried ethyl acetate extracts from 4-hour cultures grown in iron-rich (+Fe) or iron-depleted (+Dip) media were resuspended in methanol and fractionated on a C18 reverse phase column. Media blanks for both high-iron and low-iron conditions were extracted using the same protocol [Fig. 10, traces 3 & 4] to establish a baseline that excluded media compound peaks from sample peaks as non-bacterial. Purified enterobactin was run to positively identify its peak [Fig. 10, trace 1]. The enterobactin precursor, 2,3-dihydroxybenzoic acid (DHBA), was run both independently and added to each sample as a marker to evaluate elution times [Fig. 10, trace 2]. Few enterobactin-related products were released into the medium during the iron-rich (+Fe) growth of any strain [Fig. 10, trace 5 and data not shown]. When products from iron-depleted growth extracts were compared, an enterobactin peak (E) was present in all three strains. Seven peaks were detected from the HPLC and labeled as enterobactin (E), DHBA (D), and breakdown product peaks 1, 2, 3, 4, and 5. Two small, unresolved peaks clustered between 14.5-15.5 min were disregarded due to the fact that they could also be detected under high iron growth conditions, suggesting they are unrelated to enterobactin
secretion. Based on similar HPLC studies (225), presumptive identification of the breakdown product peaks were: peak 1, enterobactin monomer; peak 2, linear dimer; and peak 3, linear trimer. With wild-type BM694, which has an intact FepA enterobactin receptor, low levels of enterobactin were recovered in the medium presumably due to its subsequent uptake through FepA [Fig. 10, trace 6]. Extracts from BM694/MT912, which does not express the FepA receptor, contained almost no detectable DHBS monomer and higher levels of enterobactin because this strain is unable to take up the released product [Fig 10, trace 7]. Enterobactin was detected in extracts from the P43 mutant, BM694/KS222 [Fig 10, trace 8], but at a significantly lower level than either BM694 or its MT912 derivative. This trace is also distinctive from the appearance or noticeable increases of presumptive breakdown product peaks. When peak height and area were quantified, the P43 mutant showed an approximate 2-fold decrease in enterobactin levels compared to wild-type and approximately 4-fold less than the receptor mutant. In addition, the P43 mutant produced increased amounts of breakdown product peaks, especially DHBS monomer and dimer, compared to the other strains.

Liquid chromatography/mass spectrometry (LC/MS) was performed with the extracts to confirm the peak identities [data not shown]. For each breakdown product peak (1, 2, and 3), the mass/charge (m/z) ratio was calculated: enterobactin, 668; peak 1 [monomer], 240; peak 2 [linear dimer], 463; peak 3 [linear trimer], 686. These values agreed with previously published data (19). Peak 5 was found in enterobactin extracts from BM694 and BM694/MT912, and in increased amounts in the P43 mutant, but its identity is unknown. Peak 4, despite its increase in both MT912 and KS222 mutants, was also extracted from iron-rich growth cultures, suggesting it may be unrelated to enterobactin. The mass spectra of peaks 4 and 5 were inconclusive, and further investigation of the identities is being conducted using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. The striking increase in breakdown product peaks and sharp decline in enterobactin production in the P43 mutant supports the hypothesis that P43 is a component of the enterobactin export machinery.
In the absence of the principal export device for enterobactin release, it is possible that the P43-null strain accumulates intracellular quantities of enterobactin or, as a result of Fes activity, its breakdown products. Therefore, cell populations of $10^9$-$10^{10}$ cells grown under iron-rich and iron-poor conditions were sonicated, extracted with ethyl acetate, then analyzed by HPLC and cross-feeding assays. No significant HPLC peaks were detected for enterobactin or its breakdown products from wild-type or P43-null strains based on LC/MS identity and these extracts were incapable of feeding H1443 or its receptor mutant derivatives in low-iron growth assays [data not shown]. In contrast, the supernatants from these same cultures gave strong peak signals in HPLC analysis and strong growth responses in the nutrition assays [see Fig. 8 and Fig. 10]. These data indicate that enterobactin or its breakdown products do not accumulate intracellularly during the normal course of synthesis and release. Furthermore, in the absence of P43, enterobactin also does not accumulate intracellularly, suggesting that Fes function can accommodate the synthesis rate of the siderophore and that the breakdown products are rapidly diffused from the cells.

**Discussion and conclusion:**

The MFS-class and other multidrug transporters are involved in efflux of a broad range of structurally unrelated compounds and although they presumably evolved to transport specific physiological substrates (158), they fortuitously protect cells from diverse toxic compounds. Antibiotics, siderophores, and metal transporting agents are produced by many microbes in nature and serve important survival functions for the organisms producing them (49, 54). While structurally quite distinct, the movement of these compounds generally requires an active efflux device. Many of these compounds share common features such as an aromatic ring and the ability to bind cations (181). Enterobactin, like other siderophores, displays both aromatic features and highly charged acidic side chains capable of binding Fe$^{+3}$. These features inhibit the passive diffusion of enterobactin molecules.
through the membrane and suggest that its export is an active process. The identification of a gene within the enterobactin cluster encoding a membrane protein with homology to the MFS-class pumps suggested that the P43 protein functions as an enterobactin exporter.

A P43-null mutant grown under low-iron conditions was expected to be defective in enterobactin secretion and to have growth defects due to intracellular accumulation of toxic levels of the siderophore given that excess of iron-free siderophore would remove essential iron cofactors from the cell component (31, 235). However, in *E. coli*, ferric enterobactin esterase (Fes) activity hydrolyzes the ester linkages of the macrocyclic lactone ring and allows reduction of its chelated iron for metabolic utilization (114). Since Fes activity is 4-fold more effective against the iron-free form of enterobactin (31), it will hydrolyze free enterobactin into DHBS monomer, dimer, and trimer in the cytosol and thus function to prevent interference with iron distribution or cellular metabolism. The breakdown products rapidly diffuse from the cell and can serve as an iron source for *E. coli* by transport through the Fiu and Cir receptors (87). Our original cross-feeding assays utilized indicator strains positive for these receptors as well as the enterobactin receptor, FepA. As a result, the P43 mutant strain fed indicators using these breakdown products and was presumed to secrete enterobactin normally. With these considerations, we modified cross-feeding assays to distinguish between these siderophore molecules.

As mentioned in the introduction, crossfeeding assays are interpreted by a zone of inhibition formed when the indicator strain is unable to feed on the molecules released by the test feeder colony. Strains expressing FepA grew in proximity to enterobactin feeders. Conversely, the P43 mutant stimulated the growth of strains expressing Cir or Fiu in proximity to the feeder colony. Importantly, the P43 mutant produced no zones of inhibition for any indicator and could not feed the strain expressing only FepA, indicative of a deficiency in enterobactin secretion.

TLC and HPLC data showed a severe decline in enterobactin release into the culture medium by the P43 mutant in 4 or 18-hour cultures, although small quantities were detected, and an increase in
the amounts of enterobactin cleavage products: DHBS monomer, dimer, and trimer. The release of small quantities of enterobactin into the supernatant can be explained by cell lysis or by non-specific transport by other export mechanisms. If the cross-feeding plates were allowed to grow for 72-96 hours, BM694/KS222 produced small zones of inhibition with the Cir and Fiu indicators, again suggesting some enterobactin was secreted. TLC and HPLC assays were sensitive enough to detect the minimal amounts of enterobactin in supernatants of the P43 mutant. If we assume that the loss of P43 abolishes normal enterobactin secretion, then Fes activity would be required to prevent the accumulation of toxic levels of enterobactin in the cytosol. HPLC and cross feeding analysis of whole cell extracts failed to detect the intracellular accumulation of enterobactin and its breakdown products strengthening the hypothesis for Fes-mediated degradation of newly synthesized siderophore and the rapid diffusion of its breakdown products from the cells. This would suggest that an \textit{entS fes} double mutant would have difficulty growing in the absence of an alternative means of preventing toxic enterobactin accumulation. Preliminary attempts to create such a double mutant support this concept. Transduction of \textit{entS::kan} into a \textit{fes::cat} background, and the reciprocal transduction, produced viable transductants at a considerably reduced frequency than the transduction of either marker into the wild-type background [data not shown]. Furthermore, the surviving transductants had serious growth defects under low iron conditions, suggesting they may rely on a secondary, less-effective means of exporting the siderophore. Additional genetic analysis of these derivatives is required to identify the basis of their survival under such severe growth conditions.

The imbalance of enterobactin stagnancy is likely to trigger a growth stress reminiscent of conditions that induce non-specific membrane pumps like the AcrAB and EmrAB systems that may assist in transporting excess enterobactin. AcrB is an efflux transporter in the resistance-nodulation-cell division (RND) family (197), and AcrA, a membrane fusion protein family member (51), is thought to connect AcrB to an outer membrane channel (131, 133). The EmrAB system contains a major
facilitator protein class pump (123, 126) that is involved in the efflux of proton conductors and thiolactomycin, but has little effect on antibiotic resistance. Regardless of the identity of the secondary pumps utilized, the levels of secreted enterobactin are significantly less than exported by P43.

This work provides strong evidence that the P43 protein is the primary export device of the secretion apparatus for the *E. coli* siderophore enterobactin. The phenotype of the P43 mutant demonstrates significant decreases in enterobactin detected in culture medium. We proposed that the P43 protein be renamed EntS for enterobactin secretor and the corresponding gene changed from *ybdA* to *entS*. Additionally, these data open the door to the investigation of other siderophore export devices through the examination of linked membrane channels resembling multidrug exporters. The motifs present in such proteins have up to now invited their analysis only as multidrug exporters, resulting in their more relevant function as metabolite exporters to be overlooked. MFS-type proteins similar to P43 (EntS) reported in other siderophore systems mentioned in chapter 1 now have a basis for examination. It is reasonable to speculate that similar systems have evolved to efficiently deliver siderophore molecules to the environment as a primary biological function rather than a generalized stress responder protein.

In conclusion of this chapter, an *E. coli* mutant deficient for the P43 (EntS) protein has implicated the protein as a primary enterobactin exporter, and provided some of the first conclusive evidence of a siderophore export mechanism in a gram-negative bacterium. In addition to its importance in examining the basic mechanisms of siderophore export, the discovery of P43 (EntS) function also leads to further questions of transport and interactions, which will be addressed in the following chapters.
CHAPTER 2 FIGURES

FIGURE 6: SUMMARY OF SIDEROPHORE AND SMALL MOLECULE UPTAKE.

This schematic represents enterobactin (Ent) and siderophore breakdown product (BDP) transport through various outer membrane receptors. The enterobactin outer membrane transporter, FepA, transports both Ent and BDP, while the receptors Cir and Fiu transport only BDP. Because of this redundancy, original crossfeeding assays were unable to distinguish between feeding of indicator strains due to true siderophore versus indirect breakdown product feeding. Consideration of these receptors using new crossfeeding assays allows proper identification of feeding molecules.
FIGURE 7: CROSSFEEDING ASSAY.

Schematic of cross-feeding assays describing expected results due to movement of enterobactin (Ent) and breakdown products (BDP) thru cellular receptors. Due to size and charge, BDP diffuse further from feeder colonies than Ent.  (A) Demonstrates feeding of an indicator strain positive for FepA, Cir, and Fiu by a wild-type enterobactin producer. In this scenario, both Ent and BDP released from the feeder colony feed the indicator, allowing indicator growth both proximally and distally to the feeder colony.  (B) Demonstrates feeding of an indicator positive for Cir and Fiu, but negative for FepA by a wild-type enterobactin producer. In this scenario, the indicator can be fed by BDP, but not Ent.  This produces growth distally from BDP, but a zone of growth inhibition due to Ent diffusion and chelation proximal to the feeder colony.  (C) Demonstrates feeding of an indicator positive for FepA, but negative in Cir and/or Fiu by a wild-type enterobactin producer. In this scenario, the indicator can be fed by Ent, but not BDP.  This produces growth only proximal to the feeder colony. Used in conjunction, these variations of outer membrane receptor indicators can be utilized to accurately detect what siderophore products are being released from colonies.
FIGURE 8: CROSSFEEDING ASSAY RESULTS.

Siderophore product secretion differences as detected by mutations in various outer membrane receptors shows P43-null strains do not efficiently release enterobactin. 300 µL of indicator strain culture (noted at left, with genotype) was mixed with 3 mL of top agar and allowed to solidify on TSE plates. Feeder strains (noted at top, with genotype) were spotted onto the top agar and incubated at 37°C. Feeder strains secrete enterobactin and/or BDP, which diffuse through the media and chelate iron. Zones of growth stimulation occur when siderophores are taken up by indicator strains. Zones of inhibition are observed when indicators are unable to uptake the secreted compounds.

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<th>INDICATOR STRAINS</th>
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Feeder strains (BM694 MT912 fepA, BM694 KS222 P43)
FIGURE 9: THIN-LAYER CHROMATOGRAPHY FOR ENTEROBACTIN.

TLC analysis of culture extracts demonstrates a difference in siderophore release between wild type and P43-null cells. Strains were grown for either 18 hours (A) or 4 hours (B) under high iron (+Fe) or low iron (+Dip) conditions in 10 mL of MOPS minimal medium. Enterobactin and BDP were extracted from culture supernatants with ethyl acetate, dried, and resuspended in 50 µL of methanol. Then, 20 mL was spotted onto TLC plates and developed with benzene:acetic acid:water (125:72:3 v/v/vol). Migration of enterobactin species was visualized by spraying plates with 1% FeCl3. The control enterobactin standard lane is marked for species: enterobactin (Ent), linear monomer (M), linear dimer (D), linear trimer (T).
FIGURE 10: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR ENTEROBACTIN.

HPLC confirms previous data showing distinct differences in siderophore export patterns between wild-type and P43-null cells. Strains were grown for 4 hours under high iron (+Fe) or low iron (+Dip) conditions in 10 mL of MOPS minimal medium. Siderophore products were extracted from culture supernatants with ethyl acetate, dried, resuspended in 50 µL of methanol, and separated by a 10-50% gradient of acetonitrile and water with 0.1% trifluoroacetic acid, then detected at 220 nm. Panels show individual chromatograms of enterobactin and DHBA standards (trace 1 and 2, respectively), media blank extracts (traces 3, 4), P43-null mutant under iron-replete conditions (trace 5), and wild-type, FepA-null mutant, and P43-null mutant under iron-starvation conditions (traces 6, 7, 8, respectively). Detected enterobactin peaks (E) and DHBA (H) are labeled as defined by standards. Numbers mark BDP peaks identified by mass spectrometry: (3) linear trimer; (2) linear dimer; (1) linear monomer. For panels in which enterobactin was detected, retention time, peak height (in µV), and area under the peak (in µV/s) were calculated.
Chapter Summary:

Our experimental TLC and HPLC data demonstrated a role for *E.coli* inner membrane protein P43 (EntS) in transport of enterobactin. However, due to the inherent complexities of transport mechanisms demonstrated in Chapter 2 whereby small amounts of enterobactin are still detected in the absence of P43, a definitive experiment to validate direct P43 involvement is needed. The strategy to provide this proof is to utilize inverted membrane vesicles (IMVs). Wild-type and P43-null cells were used to prepare IMVs, which were loaded with the fluorescent iron-binding dye calcein (CA). When IMVs are exposed to iron, CA fluorescence is quenched. Upon addition of exogenous enterobactin to the system, wild-type IMVs but not the P43-null IMVs are able to restore fluorescence due to internalized enterobactin removing iron from CA. The inability of P43-null IMVs to transport enterobactin in this manner emphasizes the definitive role of P43 in enterobactin export. As mentioned in Chapter 1, P43 is typical of MFS-class transporters and matches many of the classic motifs found in the class. However, motif B, implicated in proton transfer, is not conserved. To discern if the energy requirements necessary for transport of enterobactin differ because of this variation, experiments were conducted with the IMV system and addition of selective energy poisons. Inhibitors of the proton motive force (PMF) abolished transport to varying degrees while inhibitors of ATP had no effect. Taken together, these data convincingly show P43 involvement in transport of enterobactin across the *E.coli* inner membrane and confirm that P43 behaves similarly to other MFS-class transporters with conserved proton motifs, utilizing PMF to convey substrates.
Introduction:

Conclusions made in Chapter 2 of this work implicated *E.coli* membrane protein P43 (EntS) in transport of enterobactin across the inner membrane. The goal of the following set of experiments is to directly demonstrate measurable enterobactin transport in the presence and absence of P43. Based on HPLC experiments in chapter 2, the rapid efflux of enterobactin or breakdown products from cells presents a challenge to directly measure enterobactin release. In order to accomplish this, we have developed an inverted membrane vesicle (IMVs) system that traps transported enterobactin inside the vesicles as opposed to freely releasing it to extracellular environments. Trapped enterobactin can then be quantified to observe P43 contribution in transport.

IMVs (7, 196) have been used previously to examine the activities of numerous membrane exporters for such diverse transport substrates as copper, cobalt, cadmium, and zinc (120, 161, 192), glucose symport (146), and protein translocation (228, 230). In direct relation to our project, IMVs have been used to evaluate transport of the siderophore ferrichrome (154). Vesicles made by the French press method (7, 94) have been shown to be predominantly (80%) inverted, that is, the surface of the cytoplasmic membrane which faces the cytosol in whole cells faces the external medium in such vesicles (7, 72). Most importantly to our work, IMVs prepared from *E.coli* have intact enzyme activity normally associated with the inner surface of the cytoplasmic membrane appearing on the outer surface of these vesicles (72). In this system, enterobactin transport can be monitored in IMVs with the presence or absence of P43.

Tracking the transport of enterobactin can be correlated to quantitative changes in fluorescence. Calcein (CA) is a fluorescent analog of EDTA, with diamine-tetraacetate chelating arms that bind metals, especially Fe$^{3+}$ (38, 58, 145, 214, 232). When bound to metals, CA fluorescence is quenched, but is restored upon removal of the metal (145). Calcein is commercially available as an acetoxyethyl ester derivative (Calcein-AM, CA-AM), which is a neutral molecule capable of passively
permeating cell membranes. Once internalized, non-specific cellular esterases cleave the lipophilic blocking groups to produce a polar (net charge of -4 (45)), actively fluorescent, membrane impermeable dye. This polar form is retained well in cells with intact membranes (145). A potential complication is that Gram-negative bacteria may be poorly permeable to calcein and lack esterases necessary to cleave the CA-AM form (45), suggesting we cannot passively load preformed E.coli vesicles with CA. However, the dye can be activated chemically using KOH (145). Furthermore, when red blood cell ghosts were broken and reformed in the presence of activated CA, it was trapped inside the vesicles (38, 232). Hence, we can load E.coli IMVs by incorporating chemically activated CA during initial IMV formation. Measurement of fluorescence following exposure to varied iron concentrations and chelating molecules will then provide quantitative data about flux across the cellular membrane.

An additional question to address using this system is the energizing mechanism of transport. Multidrug resistance pumps operate via an active efflux mechanism and are typically either ATP- or PMF-dependent. Currently, all known ATP-dependent multidrug efflux systems belong to the ATP Binding Cassette (ABC) superfamily of transporters. One of the first recognized and best studied is the LmrA protein of Lactococcus lactis (217). PMF-dependent multidrug efflux proteins are classified into three major families: the resistance/nodulation/division (RND) (51) (e.g., MexB from P.aeruginosa and AcrB from E.coli), the small multidrug resistance (SMR) (175) (e.g., EmrE from E.coli and Smr from S.aureus), and the major facilitator superfamily (MFS) (172, 198) (e.g., TetA from E.coli). Transport for these families is driven by the transmembrane electrochemical proton gradient (PMF). The PMF is composed of a chemical proton gradient ($\Delta p\text{H}$, inside alkaline) and an electrical potential ($\Delta \Psi$, inside negative) (144, 188). Previous work in our lab (71, 206) places P43 in the MFS family, which would suggest it uses the PMF for energy during enterobactin transport. Specifically, P43 would belong to the
subclass of Drug::H+ Antiporter-3 (DHA3) class of efflux pumps (198). However, MFS motif B, a highly conserved motif implicated in proton transfer (174), is divergent in P43 (206). We will experimentally ascertain whether this impacts the energy mechanisms utilized by P43 for enterobactin transport by addition of selective energy poisons.

**Materials and methods:**

**Strains, Media, Chemicals, and Enzymes:**

_E. coli_ BM694 [E. coli C1a derived from BM691 (200), F−, _nalA_] was used as the wild-type strain (113). BM694/MT912 was derived by P1 transduction of the _ΔfepA::kan_ locus from MT912 (115). BM694/KS222 was derived by P1 transduction of the _ybdA::kan_ locus from KS222 (206). Bacteria were routinely grown in Luria-Bertani (LB) broth (142) or on LB agar plates. MOPS minimal medium (10x MOPS salts: 1 M MOPS pH 7.4, 1.9 M NH₄Cl, 0.276 M K₂SO₄, 0.0005 M CaCl₂, 0.528 M MgCl₂, 5 M NaCl) was supplemented with 0.00132 M K₂HPO₄, 0.2% glucose, 0.05 mg/ml each tryptophan, proline, and leucine, 0.004 M citric acid, and 0.01 M thiamine (55). Carbonyl cyanide _m_-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), D-(_-)-lactate, valinomycin, and nigericin were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals and reagents used were purchased from Fisher Scientific (Pittsburgh, PA, USA).

**Preparation of Calcein-Loaded Inverted Membrane Vesicles:**

Cells were grown for 18 hr with shaking in LB plus 50 µg/mL kanamycin. Cells were subcultured 1:100 into 50 mL MOPS medium and grown to mid-log phase (OD₆₀₀ = 0.6). During growth, Calcein-AM (Molecular Probes) was chemically activated by mixing 100 µL of 1 mg/mL CA-AM in DMSO, 100 µL methanol, and 25 µL of 2 M KOH (145). Activated CA was incubated at 25°C for 1
hr, then transferred to ice until use. Upon activation, CA-containing solutions and cells were kept protected from light at all times.

Upon reaching mid-log phase, pellets were harvested by centrifugation in a pre-chilled rotor and washed once with 5 mL IMV Buffer (10 mM Tris pH 7.5, 140 mM choline chloride, 0.5 mM DTT, 250 mM sucrose) (196) and re-pelleted. Wash buffer was removed by vacuum suction and the final pellet resuspended in 2.5 mL of IMV Buffer. To create CA-loaded spheroplasts, lysozyme and EDTA were added to the resuspended cells at 20 μg/mL and 0.01 M, respectively (20) along with 125 μL of the activated CA solution and incubated on ice for 30 min. IMVs were formed by 3 passages through a French pressure mini-cell at 5000 psi. The crude membranes were incubated for 15 min on ice with 2.5 mM MgCl and 1 μg/mL DNase. Unbroken cells were removed by centrifugation at 10,000 x g for 15 min at 4°C. Supernatants were removed and ultracentrifuged at 100,000 x g for 1 hr at 4°C. IMV pellets were resuspended in IMV Buffer for use. Vesicles were also prepared in identical fashion, but without CA incorporation for use as a baseline control for fluorescence.

**Enterobactin Transport Assay:**

An aliquot of 50 mL of CA-loaded IMVs was added to 1 mL of Transport Buffer (IMV Buffer + 20 mM D(-)-lactate as an energy source) in a fluorescence cuvette. Ferric iron and enterobactin were added at 50 mM as indicated. Suspensions were mixed gently for 1 min and fluorescence measured with a Hitachi F3010 fluoroscope. Settings were as follows: excitation wavelength = 470 nm (slit = 5 nm), emission wavelength = 519 nm, bandpass = 10/10 nm, scan speed = 120 nm/sec, response = 2 sec. Data was gathered using wavelength scans from 475-575 nm or using the scan trace function to quantify fluorescence peak height.
PMF Inhibitor Enterobactin Transport Assay:

Inhibitors were prepared as stock solutions and added to vesicle transport assays after addition of iron at the following concentrations: carbonyl cyanide m-chlorophenylhydrazone (CCCP) = 0.050 mM, 2,4-dinitrophenol (DNP) = 2.0 mM, sodium arsenate = 50 mM, nigericin = 0.010 mM, valinomycin = 0.010 mM. Potassium chloride (KCl) was added at 1 mM during valinomycin inhibition. Inhibitors were mixed with the iron-loaded vesicles and incubated for 15 min. Enterobactin was then added as described above. Fluorescence measurements taken after inhibitor incubations showed negligible changes [data not shown] prior to enterobactin addition.

Results:

In these results, we will specifically address two questions: First, is P43 directly involved in transport of enterobactin across the inner membrane? Secondly, due to the absence of characterized motifs, what is the energy of transport used by P43? A schematic of the experimental design [Fig. 11] is shown for reference. Wild-type or P43-null cells were grown in low-iron MOPS medium to induce the enterobactin system and export machinery. To observe only inner membrane transport, lysozyme and EDTA were used to remove the outer membrane, creating spheroplasts that were then converted to CA-loaded IMVs using a French press. IMVs were resuspended in transport buffer and energized with D(-)-lactate which is able to provide energy to PMF systems by generation of H+ from the electron transport chain. While complete kinetics experiments were not conducted, initial characterization of the system included monitoring of fluorescence as a function of iron added. Calcein concentration was used at the manufacturer’s recommended concentration of 50 µM, a concentration reported in the literature as well (45). As incorporation of CA is a random event in creation of the vesicles, a range of iron concentrations was tried on numerous occasions to establish the optimal concentration of iron needed for quenching CA fluorescence. Because CA binds single iron molecules, the best possible
fluorescence quenching was shown over a variety of trials on different vesicles at equimolar concentration [data not shown]. Addition of higher concentrations of iron was shown to swamp the system and prevent re-fluorescence with enterobactin as free iron was then re-bound by CA after enterobactin chelated initially bound iron. Similar results have been shown in the literature (38, 58). Using wavelength scan measurements, it was determined that an optimal CA fluorescence peak was obtained using an excitation of 470 nm and measurement emission of 519 nm to separate vesicle light scatter from the CA fluorescence peak in both wild-type and P43-null IMVs [Fig. 12 A 1 & B 1]. Fluorescence of CA-loaded IMVs was measured and compared to a baseline of identical IMVs prepared without CA [Fig. 12 A 4 & B 4].

In both wild-type and P43-null IMVs, intravesicular CA fluorescence quenching can be observed by the addition of ferric iron to the IMVs [Fig. 12 A 2 & B 2]. The iron crosses the membrane and decreases fluorescence upon binding to CA. In wild-type cells expressing the P43 protein, the addition of equimolar concentrations of exogenous enterobactin restores CA fluorescence as enterobactin is transported into the vesicles and chelates Fe^{3+} from CA [Fig. 12 A 3]. In contrast, IMVs created from P43-null cells fail to restore fluorescence upon enterobactin addition [Fig. 12 B 3] which implicates P43 as necessary for efficient enterobactin transport. No transport of enterobactin was observed in un-energized IMVs in absence of the electron donor source, D(-)-lactate [data not shown]. Because of potential variations inherent in the IMV preparations (e.g., number of reformed vesicles, orientation, efficiency of CA incorporation), it was important to quantify the transport differences seen between wild-type and P43-null IMVs. To further evaluate the contribution of P43 in enterobactin transport, fluorescence was quantified by calculating a percentage restoration. Numerical values of fluorescence were acquired from scan-trace data at the highest point of the fluorescence peak. The following formulas were then used: Percentages were defined with restoration of total available fluorescence set as the 100% value. Percent of fluorescence quenched (\(\%_Q\)) = (fluorescence after iron
quenching) / (initial fluorescence of IMVs). Restoration = (final fluorescence after enterobactin) –
(fluorescence after iron quenching). Percent restoration (%R) = (restoration) / (%Q). As seen in [Fig.
13], the percentage fluorescence restoration due to enterobactin transport in the presence of P43 (wild-
type IMVs) results in a roughly 10-fold difference compared to P43-null IMVs.

Taking into account the MFS-family and subclassification of P43, it is expected that PMF be
utilized for energy of transport. However, because of P43 divergence in the proton binding motif B, this
prediction may be questioned. The data acquired with our IMV system to track enterobactin transport
allows us to address the question of energy of transport as well. In the following experiments, the
enterobactin transport assays are repeated with the addition of specific energy poisons designed to
interrupt components of energy systems. The ionophores CCCP and DNP dissipate PMF by
equilibrating protons across the membrane and discharging the PMF (185). The stronger ionophore,
DNP, nearly abolished enterobactin transport completely while CCCP produced a decrease of
approximately 5-7 fold in transport [Fig. 14, lanes 2 & 3]. Conversely, if ATP synthesis serves as the
energy source, transport can be inhibited by sodium arsenate (105). Studies have previously
demonstrated arsenate inhibition of ATP-mediated transport (43, 100, 102). Arsenate causes
destruction of high-energy phosphates and stops ATP synthesis. Depletion of the high-energy
phosphate pool occurs as arsenate competes with phosphate in phosphorylation of glycolysis
intermediates, and as substitution of arsenate for phosphate in ADP occurs, it results in rapid and
drastic reduction by spontaneous hydrolysis (105, 129). The addition of sodium arsenate to the system
had no impact on enterobactin transport [Fig. 14, lane 4]. It has been reported that oxidation of lactate
by vesicles is possible and produces ATP through substrate level phosphorylation and proton gradient
production (196). Because arsenate blocks phosphorylation reactions such as the production of high-
energy intermediates, arsenate-insensitive transport is consistent with active transport being
independent of oxidative phosphorylation and suggests ATP is an unlikely energy source for
enterobactin transport (105). This data demonstrates that despite lacking motif B, the MFS protein P43 can utilize PMF for energy of transport.

Susceptibility of the enterobactin transport process to proton conductors suggest that the efflux is energized by a component(s) of the PMF. To investigate which component, $\Delta pH$ or $\Delta \psi$, or both, of the PMF is involved in energizing P43-mediated enterobactin efflux, transport assays were performed in the presence of the ionophores valinomycin and nigericin. In potassium supplemented transport buffer, valinomycin specifically collapses the $\Delta \psi$ by efflux of K+ ions from the IMVs to dissipate membrane potential whereas nigericin, which allows H+:K+ or H+:Na+ exchange, specifically affects the $\Delta pH$. Both nigericin and valinomycin impacted P43 transport of enterobactin [Fig. 14, lanes 5 & 6], albeit to a lesser extent than stronger ionophores. This would suggest that energy from both the $\Delta pH$ and $\Delta \psi$ might be consumed to energize P43. Based on the data acquired with valinomycin, the $\Delta \psi$ may play the larger role in providing transport energy. Taken together, the results presented here provide evidence that the multidrug transport protein P43 interacts directly and specifically with its substrate, enterobactin, and uses elements of the PMF energy to translocate the *E. coli* inner membrane.

**Discussion:**

Intact cells form a proton motive force (alkaline and negative interior) to extrude substrates. The IMVs, conversely, are quantitatively everted, leading to protons pumped inward during electron transport chain activity and respiration of $F_0F_1$-catalyzed ATP hydrolysis, polarizing the vesicles acid and positive interior. As enterobactin is strongly negatively charged (-6 = free ligand or -3 = bound to Fe$^{3+}$), this suggests an energetically unfavorable state for enterobactin inside of normal cells and would agree with our proposed and experimental movements of enterobactin.
Siderophores are highly charged hydrophilic molecules that will resist passive diffusion through the lipid bilayers of microbial membranes. Therefore, it is necessary to provide a suitable energy-driven process to transport them. Secondary active transporters such as the MFS class transporters are defined as coupling a primary source of energy (i.e., electron flow, ion electrochemical gradient) to a process. P43 is classified into the Drug:H+ Antiporter-3 (DHA3) family (198) of MFS proteins. These active transporters can function by uniport, symport, or antiport. Within antiporters are two subcategories: antiport of like molecules (i.e., solute-solute antiport) and antiport of unlike molecules (i.e., solute-cation antiport). It is widely accepted that PMF, specifically proton antiport mechanisms, power the DHA3 family (198). The ionophores DNP and CCCP, both PMF inhibitors, halted enterobactin transport as compared to the ATP inhibitor sodium arsenate. Our results confirm the MFS protein P43 as using PMF to transport enterobactin rather than ATP energy.

Transport of iron across IMV membranes is also not a trivial matter. While IMV leakiness, transport by movement down a concentration gradient, porins, or passive diffusion is all possible, they are not overly likely to move a significant amount of iron across the membrane. There have been several ferrous iron transport systems described that do not require siderophores such as the E.coli Nramp-like ferrous transporter system, feoABC (40), and the metal permease zupT (81). Recent work has implicated transport mechanisms of free ferric iron across the inner membrane that would be intact in an IMV and potentially could serve to deliver the exogenously added ferric iron to CA in the lumen. These systems include the yfeABCD system originally described in Yersinia and shown to rescue an E.coli iron-deficient transport strain (17), the Streptococcus pyogenes mtsABC system (98), the Candida albicans CaFtr1p transporter (61), and the fitABCDE system in E.coli (169). The fit system is induced by iron limitation and encodes an outer membrane protein (FitA), a periplasmic binding protein (FitE), two inner membrane permease proteins (FitC and -D), and an ATPase (FitB). Introduction of the fit system into an E.coli K12 strain increased intracellular iron content and caused an increase in
susceptibility to the intracellular iron-activated antibiotic streptonigrin. While we have not confirmed the presence of these types of transporters in our test strains, E.coli BM694 is a clinical isolate expressing many wild-type genes that are not found in domesticated strains, and it would be reasonable to assume that classes of these transporters could be functional in our assays to move

The membrane potential is typically established by movement of K+ ions through the Na:K ATPase to form a concentration gradient. As K+ moves down the concentration gradient, it leaves anions remaining in the cell and forms a charge separation with the cell membrane interior charged negatively and the cell membrane exterior positive. The ionophores nigericin and valinomycin, which selectively dissipate the $\Delta p\text{H}$ and $\Delta \psi$ of the PMF, respectively, have been valuable tools to study the specific energetics of MFS transporters. Both valinomycin and nigericin decreased enterobactin transport approximately 3-5 fold. However, valinomycin had a greater effect on transport, suggesting $\Delta \psi$ as the most important component of PMF for enterobactin transport. Valinomycin facilitates K+ transport across membranes to near equilibrium and hence dissipates the $\Delta \psi$. The conclusion of using $\Delta \psi$ for enterobactin transport is plausible as it would be favorable to move negatively charged enterobactin towards the positive charge established on the membrane exterior by the $\Delta \psi$. An additional observation that may support our data is that nigericin increases $\Delta \psi$ while simultaneously decreasing the $\Delta p\text{H}$ (160, 191) due to $\Delta p\text{H}$ and $\Delta \psi$ exchangability. Nigericin may have had a lesser effect on transport due to this increase in $\Delta \psi$, allowing the inadvertent increase in membrane potential to compensate for losses in available $\Delta p\text{H}$ energy. However, because nigericin reduces the total cell interior negative charge by conducting protons into the cytoplasm, this may have some effect on enterobactin transport if proton exchange is involved.

The literature has few examples of PMF-driven antiport that would precisely match the predicted P43-mediated enterobactin movement of anion:H+ exchange. Anionic bile acids, possibly in
complex with a multivalent cation, were demonstrated to be actively transported using proton antiport \((\Delta \text{pH})\) (213). Interestingly, it has also been reported that transport of charged compounds in *E.coli* is typically mediated by both components of the PMF, while electroneutral transport is more reliant on \(\Delta \text{pH}\) (122). At pH 7.0, \(\Delta \psi\) constitutes approximately 70% of the PMF, whereas \(\Delta \text{pH}\) constitutes roughly 30% (236). Assuming enterobactin carries a negative charge, this would fit our data of valinomycin and nigericin both having impacts, although \(\Delta \psi\) facilitates the greater role as the more available energy source (236) at neutral pH. It has also been speculated that the net charge of enterobactin may be reduced by post-synthesis protonation or by coupling a chaperone ion such as Mg\(^{2+}\) to the siderophore backbone (91, 92). At neutral pH, the formation constant of enterobactin can be reduced from, owing to proton competition (156) and the weak acid catechol groups that serve as the iron binding sites for enterobactin binding protons at neutral pH. This could make an enterobactin:H\(^{+}\) exchange more electrogenically favorable for the cell.

It is possible to utilize both or either \(\Delta \text{pH}\) and \(\Delta \psi\), as several studies implicate (24, 122, 143, 159). Antiport energetics have been demonstrated for other multidrug export pumps including *Lactococcus lactis* LmrP (164) and *Staphylococcus aureus* QacA (143), which rely on both \(\Delta \text{pH}\) and \(\Delta \psi\). The LmrP (24), Smr (85), and Emr proteins (231) have been shown to use a drug:H\(^{+}\) \((n \geq 2)\) antiport mechanism.

Our system utilizes D(-)-lactate as the energizing molecule. In *E.coli* membrane vesicles, the respiration-linked transport systems are coupled primarily to oxidation of lactate to pyruvate, catalyzed by a membrane-bound lactate dehydrogenase. The great majority of the individual membrane vesicles catalyze active transport and the generation or hydrolysis of ATP is not involved (103). Although the cytoplasmic membrane may be energized by either electron transport or ATP hydrolysis, the latter is not an obligatory requirement for enterobactin transport in view of the failure of sodium arsenate to
inhibit the process. However, ATP production from D(-)-lactate vesicle energizing may not have reached critical thresholds necessary for enterobactin transport. Future experiments adding exogenous ATP in conjunction with enterobactin may be necessary as some studies (50, 229) demonstrate dual modes of energy coupling for transport. Additionally, while we expected high levels of restoration of fluorescence, we were unable to achieve total (100%) restoration, which is likely due to differences in IMVs. Inherent in the preparation, approximately 80% of vesicles are inverted but may have segments of membrane that are not inverted (7), resulting in bi-directional flux of enterobactin and a balanced overall fluorescence change. An alternative explanation is that IMVs are comprised of smaller (40-110 nm), broken-reformed membrane segments that do not contain a transporter molecule (7).

The results shown here provide some of the first direct evidence for P43-mediated enterobactin transport across the inner membrane. P43-null cells failed to transport enterobactin as determined by the lack of restoration of fluorescence of calcein compared to wild-type cells containing native protein.
FIGURE 11: INVERTED MEMBRANE VESICLE SYSTEM.

Schematic demonstrating rationale of inverted membrane vesicle (IMV) creation and testing. **(Step 1)** chemically produces spheroplasts of intact inner membranes (top) and shows structure (bottom) of the iron-binding fluorescent dye, calcein (CA). **(Step 2)** shows procedure for conversion of inner membranes to IMV using French press (FP) in the presence of activated CA, resulting in approximately 80% CA-loaded IMVs. Used experimentally, **(Step 3)** describes potential IMV differences between enterobactin transport in the presence (MT912) and absence (KS222) of the P43 protein as detected by restoration or lack of CA fluorescence. Our hypothesis is that P43 must be present for enterobactin to cross the membrane and restore fluorescence of CA.
FIGURE 12: TRANSPORT OF ENTEROBACTIN IN INVERTED MEMBRANE VESICLES.

Representative examples of fluorospectroscopic traces of enterobactin transport as measured by calcein refluorescence. CA-loaded IMVs were measured for initial fluorescence in both wild-type (A1) and P43-null (B1) IMVs. To quench CA fluorescence, 50 µM iron was added for 1 min and the IMVs were remeasured for fluorescence, resulting in a decrease of fluorescence in both strains (A2 and B2). Exogenous purified enterobactin at 50 µM was added to the quenched IMVs for 1 min and IMVs were again remeasured for fluorescence. Influx of enterobactin was then correlated to gain of fluorescence attributed to transported enterobactin chelating iron from CA. Wild-type cells produced fluorescence upon addition of enterobactin (A3) while cells lacking P43 and hence unable to transport enterobactin, produced minimal refluorescence (B3).
FIGURE 13: TRANSPORT COMPARISON.

A quantitative comparison of refluorescence in wild-type and P43-null strains. Triplicate samples of IMVs were prepared as described and measured for CA fluorescence either alone, upon addition of iron, or upon addition of iron and enterobactin. Peak heights were calculated and applied to the following formulae to quantitate the effect of P43 on enterobactin transport: Percent of fluorescence quenched\(\%_Q\) = (fluorescence after iron quenching) / (initial fluorescence of IMVs). Restoration = (final fluorescence after enterobactin) – (fluorescence after iron quenching). Percent restoration\(\%_R\) = (restoration) / \(\%_Q\). Data from all trials was averaged and standard deviations calculated to produce a numerical representation of transport. Wild-type IMVs demonstrated approximately 10-fold more enterobactin transport than P43-null IMVs.
FIGURE 14: EFFECTS OF ENERGY POISONS ON ENTEROBACTIN TRANSPORT.

Specific energy poisons demonstrate proton motive force energy is used in transport of enterobactin. Triplicate samples of wild-type IMVs were prepared as in materials and methods and measured for CA fluorescence alone or with iron. After addition of iron, energy poisons affecting proton motive force or ATP-mediated transport were added as listed for 5 min and then re-measured to ensure no change in fluorescence occurred. Finally, exogenous enterobactin (50 μM) was added to attempt to restore fluorescence if transported. Peak heights were calculated and applied to the following formulae to quantitate the effect of energy poisons on enterobactin transport: Percent of fluorescence quenched (\%Q) = (fluorescence after energy poison addition) / (initial fluorescence of IMVs). Restoration = (final fluorescence after enterobactin addition) – (fluorescence after addition of energy poison). Percent restoration (\%R) = (restoration) / (\%Q). Data from all trials was averaged and standard deviations calculated to produce a numerical representation of transport. Strong PMF uncouplers such as (DNP) and (CCCP) impede enterobactin transport, while partial PMF inhibitors such as valinomycin (VAL) and nigericin (NIG) had varied effects on transport. The ATP-inhibitor sodium arsenate (NaArs) had no effect.

![Graph showing determination of transport energy requirements using specific poisons.](image-url)
CHAPTER 4: IDENTIFICATION OF ADDITIONAL COMPONENTS INVOLVED IN ENTEROBACTIN RELEASE

Chapter Summary:

After the identification and characterization of P43, additional components participating in enterobactin transport were tested. Mutations in the outer membrane protein TolC demonstrate a role for its participation, as enterobactin released to the medium is decreased. Likewise, the multidrug export pump complex of AcrAB shows a similar phenotypic pattern, although redundancy in this system is a complicating factor. Based on these data and the work of other groups, a general model of enterobactin transport can be assembled that includes the key players of P43, TolC, and multi-drug resistance complex proteins.

Introduction:

The previous chapters in this thesis describe the E.coli membrane protein P43. Data accumulated to this point demonstrate the role of P43 (EntS) in transporting the siderophore enterobactin (71) across the cytoplasmic membrane using proton motive force energy. The data are incomplete however, as this would strand enterobactin in the periplasmic space. The next puzzle in siderophore transport is to complete the system and identify components moving enterobactin from the periplasm across the outer membrane.

Compounds such as siderophores, antibiotics, and metal transporting agents are produced by many microbes to serve important survival functions for the organisms producing them (49, 54) and generally require an active efflux device due to various cumbersome motifs. Enterobactin is no exception. It displays both aromatic features and highly charged acidic side chains capable of binding
Fe$^{3+}$. These features, coupled with its size of ~700 Da, severely limit the opportunity for enterobactin to passively diffuse through the membrane and suggest that export utilizes an active process. In conjunction with other multidrug transporter classes, MFS-class transporters, such as P43, are responsible for active efflux of numerous structurally unrelated compounds sharing common features such as aromatic ring motifs or cation binding motifs (181). It is presumed that MFS exporters initially evolved to transport specific biological or physiological substrates (158) but because of their relaxed specificity requirements, they are also fortuitously able to respond in protecting cells from diverse toxic compounds.

In an active transport model, enterobactin molecules destined for secretion must cross both the inner (cytoplasmic) and outer membranes and the intervening periplasmic space, believed to be a distance of at least 130Å. While there is no direct evidence that obliges P43 to work in concert with other membrane proteins, transport across the membrane structures in Gram-negative microbes is seldom an isolated event. Furthermore, the final destination of siderophores is the external environment where they can chelate ferric iron. This likely leaves numerous hypothetical components of the enterobactin secretion system yet to be elucidated. The release of charged molecules by MFS-class pumps typically requires three components: an active efflux pump, a membrane fusion protein (MFP) that links the pump to the outer membrane, and an outer membrane channel for release (162, 188). While the genes for these components are often in close proximity to each other in operons, the entS gene has no such affiliations, complicating the search for cooperating proteins. Another consideration is that at least two multiple export systems in *E.coli* are known to share outer membrane channels (63) adding an additional complication to the identification of an outer membrane channel that releases enterobactin to the external environment from the periplasmic space. Finally, overlapping specificities for the outer membrane channels interacting with independent export pumps have been reported (123). While this may seem a complex task, the P43 mutant has provided us a reliable
phenotype that is easily identified by siderophore nutrition assays and tracked with TLC and HPLC. We predict that disrupting the other components of the system will produce a phenotype almost identical to the P43 (EntS) mutation seen in the preceding chapters, assuming a lack of redundancy in export mechanisms.

Based on the current literature of MFS-class systems, we hypothesized that cells would require a channel to convey enterobactin from P43 to cross the outer membrane and began a targeted search for the additional components of the enterobactin release system. In considering possible cooperative molecules, the *E. coli* outer membrane channel-tunnel TolC was a logical candidate based on evidence that it can function as a shared channel between multiple exporter proteins (163). TolC is involved in bacterial 'Type-I' transport and is the outer membrane channel acting with various inner membrane translocases and periplasmic junction proteins to export pyocyanin, hemolysin, tetracycline, doxorubicin, and other antibiotics and toxic metabolites (107, 109, 218, 220). Several of these are similar in size, aromaticity, charge, or toxicity to enterobactin. TolC is the most common outer membrane channel for tripartite efflux systems (75, 107) and is constitutively present at a relatively high copy number of ~ 1500 per cell (215). TolC assembles trimers to form the complete \( \beta \)-barrel structure of an outer membrane channel and has a large curtain domain of \( \alpha \)-helices that extends for approximately 100Å and could potentially span the periplasm to dock with a transporter (110). Finally, TolC contains an equatorial domain, surrounding the middle of the protein, that may be involved in periplasmic junction protein docking (110).

Membrane translocase complexes containing TolC are formed dynamically upon the presence of the respective substrate. TolC recruitment to an inner membrane translocase has been shown to occur in response to substrate engagement and is likely coupled with a periplasmic junction protein to stabilize the completed channel (107). The primary partner and best-characterized efflux pump
complex interactions with TolC are the AcrAB proteins from *E.coli*. This system is known to extrude a wide variety of antimicrobial compounds including antibiotics, detergents, dyes, and organic solvents (132). Located in the inner membrane, AcrB is a drug:proton antiporter and contains 2 large periplasmic hydrophilic domains that could dock with alpha-helix structures at the base of TolC. The top of these domains forms a funnel, which has dimensions that would fit with the tip of the periplasmic helical barrel of TolC (110). Loading of substrate for AcrB takes place within the phospholipid bilayer (233) and export is stimulated through TolC. Alternatively, the crystal structure of AcrB has invited speculation that periplasmic loading of substrates may be possible due to three vestibule regions leading to the central cavity (127) mimicking a ‘Type-II’ or ‘Type-IV’ secretion system. AcrA, a periplasmic junction protein, mediates cooperation between AcrB and TolC and is likely the molecule that recruits TolC to the prepared transport complex (108). All three components are required for efficient transport as disruption of any of the three genes results in hypersusceptibility of *E.coli* to toxic compounds (168).

We hypothesized that TolC may be involved in movement of enterobactin from the periplasm by interacting with P43. In this first section of the report, we examined several *tolC* mutations obtained from the *E.coli* Genetic Stock Center (New Haven, CT) to evaluate the involvement of TolC in enterobactin release. In all strains containing *tolC* mutations, effects on enterobactin release were noted. However, communications with other TolC groups suggested complications with this simple interpretation, as it has been known for some time that *E.coli* mutants in *tolC* exhibit pleiotropic phenotypes and have heightened sensitivity to environmental stresses (108). Consistent with our results, we were contacted by another group with results on TolC and enterobactin release (22). Reports have also considered that the multidrug export system AcrAB may be involved in siderophore transport (181), perhaps functioning as a “relief” system to alleviate toxic accumulation of enterobactin (71). Similar to previous conclusions regarding P43, results suggested that AcrAB may also participate
in normal physiologic functions, such as membrane assembly of PHB(Ca\(^{2+}\)polyP) channel complexes (101) in addition to serving as an antibiotic efflux system. Because of these points and our previous data with P43, we desired to test AcrAB for involvement in enterobactin release. Data subsequently obtained may lend itself to a different interpretation and model compared to those drawn in the literature.

**Materials and Methods:**

**Strains:**

*Escherichia coli* BM694 [E.coli C1a derived from BM691 (200) [F\(^-\), *nalA*] was used as the background strain (113). BM694/MT912 was derived by P1 transduction of the ∆fepA::kan locus from MT912 (115). BM694/KS222 was derived by P1 transduction of the *entS::kan* locus from KS222 (206). Strain ZK796 [Tet\(^r\), same as MC4100 but *tolC::Tn10* (77, 96)] was a generous gift from Dr. Helen Zgurskaya. Strains MN115 [W3110 *acrA::TnPhoA*], MN116 [W3110 *acrB::TnPhoA*] and MN102 [W3110 ∆acrAB] were generous gifts from Dr. Kevin Bertrand. The following strains were provided by the *E.coli* Genetic Stock Center for our use: A586 [F\(^-\), *thr-1, leuB6* (Am), *fhuA21, lacY1, glnV44* (AS), *λ\(^-\)*, *rfbD1, tolC3, thi-1, pro-42* (148)]; EW1b [F\(^-\), *lacY1 or lacZ4, tsx-64, glnV44* (AS), *gal-6, λ\(^-\)*, *hisG1* (Fs), *tolC5* (del), *argG6, rpsL8 or rpsL104 or rpsL17, *malT1* (λR), *mtlA2* (224)]; PB3 [F\(^-\), *lacY1 or lacZ4, gal-6, hisG1* (Fs), *tolC5* (del), *uxaC201, rpsL8 or rpsL104 or rpsL17, *malT1* (λR), *mtlA2* (182)].

**Media, Chemicals, and Enzymes:**

Bacteria were routinely grown in Luria-Bertani (LB) broth (142) or on LB agar plates. MOPS minimal medium (10x MOPS salts: 1 M MOPS pH 7.4, 1.9 M NH\(_4\)Cl, 0.276 M K\(_2\)SO\(_4\), 0.0005 M CaCl\(_2\), 0.528 M MgCl\(_2\), 5 M NaCl) was supplemented with 0.00132 M K\(_2\)HPO\(_4\), 0.2% glucose, 0.05 mg/mL
each tryptophan, proline, leucine, histidine, arginine, glutamine, and threonine, 0.004 M citric acid, and 0.01 M thiamine (55). For MT912 and KS222, kanamycin was used at a concentration of 50 µg/mL. All other reagents and chemicals were obtained from Fisher Scientific. (Pittsburgh, PA, USA)

**Periplasmic Siderophore Assays:**

Osmotic shock cracking experiments were adapted from previous published methods (157). In brief, bacterial strains were subcultured at 1:50 and grown for approximately 4 hours in 50-100 mL MOPS medium. Cells were harvested by centrifugation at 10,000 xg (~7,000 rpm) in a Sorvall SS-34 rotor at 4°C for 15 min. After discarding the supernatant, the cell pellet was resuspended in 8 mL of 20% w/v molecular biology grade sucrose containing 0.03 M Tris-HCl, pH 8.0 and 2 mL of 5 mM EDTA, pH 8.0. Cell suspensions were mixed at room temperature on a rotation shaker at 180 rpm for 15 min. Bacteria were pelleted at 13,000 xg (~10,500 rpm) at 4°C for 15 min. Pellets were then resuspended in 10 mL of ice-cold water and mixed as before in a 4°C cold-room. A final 10 mL of ice-cold water was added after 15 min and the sample was allowed to continue mixing for an additional 15 min to release periplasmic contents.

**Enterobactin Extractions:**

Extractions were done as previously described (71). In brief, bacterial cultures in 3 mL LB were grown for approximately 18 h to late stationary phase with appropriate antibiotics. Cells were subcultured into 10 mL MOPS minimal medium at 1:100 and starved for 3 h with shaking at 37°C. After starvation in low-iron medium, OD$_{600}$ were taken of each culture to ensure approximately equal numbers of cells were present in each culture. Bacteria were pelleted by centrifugation at 20,000 x g for 15 min and culture supernatants were removed. Supernatants were acidified with 50 µL 10 N HCl
and extracted twice with a total of 8 mL ethyl acetate. Organic layers were removed to clean glass tubes, combined and dried in 1 mL aliquots in a SpeedVac Concentrator (Savant Instruments, Farmingdale, NY, USA). Extract residues were resuspended in 100 µL methanol and stored at 4°C if not used immediately. Periplasmic siderophore assay samples were extracted and resuspended in the same manner.

HPLC:

HPLC methods were based on previously published protocols (71, 225). In brief, enterobactin extract analysis was performed with a Symmetry® Nucleosil C₁₈, 5 mm, 4.6 x 250 mm HPLC column (Waters Corp, Milford, MA, USA) using a gradient of 10-50% acetonitrile in water with constant 0.1% trifluoroacetic acid to maintain a pH of 3.0. Injection and separation were accomplished using a Waters 2690 Separations Module and resulting peaks detected with a Waters 996 Photodiode Array Detector (Waters Corp, Milford, MA, USA). Detector wavelength was set at 220 nm and flow rate through the column set to 1 mL/min. Each enterobactin extract or periplasmic extract was resuspended in 100 µL methanol and aliquots were injected and separated by the gradient. Results were analyzed using Millenium³² Chromatography Manager v3.2 software (Waters Corp, Milford, MA, USA).

Cloning of TolC and P43:

\textit{E.coli} tolC and entS were amplified from BM694 using PCR with the following primers: TolC TOPO F (5'-caccatgcgaatgaaatggctcc-3') and TolC TOPO R (5'-gttaoggaaggttatgacc-3'), P43 TOPO F (5'-caccatgcgaatgaaatggctcc-3') and P43 TOPO R (5'-gttaoggaaggttatgaccg-3'). The reaction was performed in an Eppendorf MasterCycler Gradient PCR Cycler (Eppendorf, Westbury NY, USA) using a Touchdown PCR program (53). The thermocycler was programmed for the first
cycle at 94, 65, and 72°C for 30 sec, 30 sec, and 1 min each. The second cycle was set at 94, 64, and 72°C for 30 sec, 30 sec, and 1 min each. The annealing temperature was lowered by 1°C in each of the following steps, with a final annealing temperature of 50°C after 15 cycles. An additional 15 cycles were subsequently run (94°C for 30 s, 50°C for 30 sec, 72°C for 1 min), ending with a final 7 min extension at 72°C. The resulting PCR products were ligated into the arabinose-driven expression vector pBAD102 Directional TOPO (Invitrogen, Carlsbad CA, USA) to create the plasmids pBAD:TolC and pBAD:P43. This vector contains an in-frame C-terminal V5 epitope tag (GKPIPNPLLGLDST) and a polyhistidine (6xHis, HHHHHH-COOH) epitope tag for detection of expressed proteins. Ligated plasmids were transformed into the provided competent cells following manufacturer’s recommendations. Plasmids were then isolated and transformed into BM694 KS222 (P43-null) and ZK796 (tolC::Tn10) E.coli strains for further experimentation.

**Protein Expression Rescue:**

*E.coli* ZK796 containing the pBAD:TolC construct was grown in MOPS low-iron medium at 37°C under antibiotic selection to an OD<sub>600</sub> of 0.5 to 0.6. As an uninduced control, 1 mL of cells was removed from the culture. The remaining culture was then induced with arabinose and allowed to continue growth for 2 h. After induction, cells were harvested by centrifugation (15,000 x g), washed in PBS, and sonicated to release protein. Expression was evaluated on SDS-PAGE gels by Coomassie blue staining. Supernatants were extracted for siderophore products as before.

**Protein Crosslinking:**

Chemical *in vivo* crosslinking was carried out based on previous literature (234). In brief, BM694 KS222 cells containing the pBAD:P43 construct were grown in MOPS low-iron medium at 37°C under antibiotic selection to an OD<sub>600</sub> of 0.5 to 0.6. As an uninduced control, 1 mL of cells was
removed from the culture. The remaining culture was then induced with arabinose and allowed to
continue growth for 2 h. After induction, cells were harvested by centrifugation (15,000 x g), then
washed and resuspended in PBS. Dithiobis[succinimidylpropionate] (DSP) (Pierce Biotechnology, Inc.,
Rockford IL, USA) at 400 µM was added as a crosslinking agent. Crosslinking reactions were
incubated at 37°C for 30 min and then transferred to ice. Crosslinking was terminated by addition of
Tris-HCl to a final concentration of 75 mM. Crosslinked cells were then lysed by sonication. Expression
was evaluated on SDS-PAGE gels by Coomassie blue staining and western blotting with anti-his
antibody (Invitrogen, Carlsbad CA, USA) to detect complexes containing P436xHis. Reduction of
crosslinks was achieved by addition of 10 mM DTT to proteins for 30 min at 37°C.

**Results:**

We hypothesized that because of its ability to function with a variety of export systems,
including MFS-class transporters, the *E.coli* outer membrane protein TolC might be able to interact with
the P43 inner membrane protein to form a complete channel for export of enterobactin. Four strains,
each harboring a different mutation in *tolC* were tested for enterobactin release by HPLC. Strains
A586, Ew1b, and Pb3 are varied length C-terminus deletion mutations in the *tolC* region while strain
ZK796 harbors a Tn10 interruption of *tolC*. Dried ethyl acetate extracts from 4-hour cultures grown in
iron-depleted medium were resuspended in methanol and fractionated by HPLC on a C18 reverse
phase column. Purified enterobactin was run as a standard [Fig. 15, trace 1]. As in previous HPLC
traces shown in Chapter 2, the enterobactin precursor, DHBA, was run independently as a marker to
evaluate elution times and comparisons of iron-rich (+Fe) growth if any strain prevented enterobactin-
related product release into the medium [data not shown]. When products from iron-depleted growth
extracts were separated, identified peaks were labeled based on the previous LC/MS data:
enterobactin peak (E), DHBS linear trimer (T), DHBS linear dimer (D), and DHBS linear monomer (M),
DHBA standard (H). Consistent with published data (71), wild-type cells release enterobactin to the medium [Fig. 15, trace 2] while P43-null cells show a sharp decline in detectable enterobactin and increases in DHBS breakdown products (BDP) [Fig. 15, trace 3]. Extracts from the ΔtolC strains produced traces consistent to our hypothesized expectations. All ΔtolC traces demonstrated a low level of enterobactin released to the external medium, with enterobactin peaks equal to or lesser than the P43-null strain. There was a varied release of breakdown products from the TolC mutants, following an overall trend of releasing slightly diminished breakdown product peaks compared to the P43-null cells [Fig. 15, traces 4-6]. The final tolC strain extract, ZK796 (tolC::Tn10), which was tested at a separate time, also agrees with this trend on subsequent HPLC runs [data not shown]. These results were corroborated by the work of Grass et al (22), which demonstrated that a tolC-deletion releases no detectable enterobactin. Taken together, the absence of TolC results in a pronounced decrease in enterobactin release similar to P43-null cells and supports the hypothesis of TolC participation in enterobactin transport.

We surmised that if P43 and TolC worked in conjunction to transport enterobactin, the siderophore would be moved to the periplasm by P43 but stranded there in the absence of TolC. Unlike the P43-null strain in which enterobactin is cleaved into BDP by the activity of cytoplasmic ferric enterobactin esterase (Fes), unlinking P43 from TolC might result in intact enterobactin being trapped in the periplasm. To test this, we osmotically shocked cells grown under low-iron conditions to collect periplasmic contents, extracted the contents for siderophores, and separated them by HPLC. As expected, neither wild-type cells nor P43-null cells trapped enterobactin in the periplasm [Fig. 16, traces 3-4] as export is intact in wild-type cells and transport to the periplasm would occur very inefficiently in a P43-null cell. However, none of the four tolC mutant strains had increased or even detectable levels of enterobactin found in the periplasmic contents [Fig. 16, traces 5-7 and data not shown]. When these data were considered in light of our previous data regarding TolC and
enterobactin, it may be that an unknown regulatory or feedback pathway was involved in
downregulation of enterobactin in a tolC mutation strain or that a potentially inefficient TolC homolog
exists. Alternatively, regulation could occur at the level of protein function, if linkage of TolC is required
for proper opening of the P43 channel, whereby absence of TolC may prevent enterobactin transport.

We attempted to restore enterobactin release in a ΔtolC mutant by expressing TolC using a
plasmid-based system. An arabinose-inducible vector, pBAD102 was chosen to allow expression of
TolC at a variety of levels. Additionally, the vector encodes for detectable C-terminus epitopes as we
do not possess an anti-TolC antibody. Despite expression of TolC as detected by Coomassie blue
staining and western blot, HPLC of extracts did not show any restoration of enterobactin release [data
not shown]. One complicating factor in this set of experiments is the presence of the epitope tags. The
V5 and 6x Histidine tags, necessary for detection of the protein, may have interfered with recombinant
TolC function, targeting, or stoichiometry.

To show a direct interaction between P43 and TolC as forming a channel for enterobactin
transport, we undertook crosslinking assays. Using the pBAD102 expression plasmid, a construct of
P43 plus the detectable C-terminus 6x histidine epitope (P43^6xHis) was created and transformed into the
P43-null mutant BM694 KS222. The P43^6xHis construct was induced with arabinose under low-iron
conditions to facilitate formation of enterobactin releasing complexes. Cells were harvested and in vivo
crosslinked using DSP. Proteins were isolated from cells using gentle sonication, so as not to disrupt
complexes. Crosslinked proteins were then separated by SDS-PAGE gel and blotted to PVDF
membranes for immunodetection with anti-6xHis antibodies to observe complexed proteins interacting
with P43^6xHis. While these experiments are still in the preliminary stages, the results are promising.
Crosslinked cell extracts detected with anti-6xHis antibodies show large (> 250 kDa) complexes
formed [Fig. 17 A, lane 2] compared to non-crosslinked cells [Fig. 17 A, lane 1] and to uninduced cells
[Fig. 17 A, lanes 3-4]. Treatment of these same extracts with DTT to sever crosslinks produces loss of
some large complexes and new banding patterns of smaller molecular weight [Fig. 17 A, lanes 2 and 4]. These new bands likely correspond to P43 \(^{6x\text{His}}\) protein species both by size and that they are absent in uninduced samples. Despite that the species comprising the complex are still unknown, this work demonstrates P43 interactions with other proteins and suggests that a large molecular weight complex is involved.

As reported in the literature, one potential model of enterobactin export involves movement across the cytoplasmic membrane to a secondary transporter whereby it is periplasmically loaded into an unknown complex coupled to TolC and release is completed (22). While contrary to our initial model and seemingly energetically unfavorable, results to this point signify this is a valid possibility. We had considered that a relief pump for P43 may exist as well, based on the ability of a P43-null cell to release limited amounts of enterobactin. As data from both groups implicate tolC, we speculated that the relief pump system or secondary transport system could be a known TolC interaction partner, such as AcrAB. We tested whether AcrA, AcrB, or both, might be involved in enterobactin release.

Strains of E. coli harboring mutations in acrA (MN115), acrB (MN116), and \(\Delta\text{acrAB}\) (MN102) were grown in low-iron conditions to stimulate enterobactin release. Dried ethyl acetate extracts from 4-hour cultures were resuspended in methanol and fractionated by HPLC on a C\(_{18}\) reverse phase column.

Purified enterobactin was run to positively identify its peak [Fig. 18, trace 1]. Traces showing enterobactin release from a wild-type cell and a P43-null cell, respectively, matched all previous data [Fig. 18, traces 2 and 3]. Trace 4 and trace 5 represent the acrA and acrB mutants, which show a partial decline in enterobactin release as compared to wild type and P43-null results [Fig. 18, traces 4 and 5]. Conversely, there was no enterobactin release deficiency seen with the \(\Delta\text{acrAB}\) double mutant [Fig. 18, trace 6]. Peak heights and areas were calculated to further evaluate these release phenotypes, and demonstrate that both the acrA and acrB single mutants have an approximately 2-2.5 fold reduction in enterobactin released to the medium, while there is no difference seen in the \(\Delta\text{acrAB}\)
double mutant. While this is not as striking as a tolC or P43-null cell, it is a consistently reproducible difference in our experiments. We would conclude that AcrA and AcrB have some role in enterobactin release, but may not be unique in their ability to transport enterobactin.

**Discussion:**

Mechanisms of siderophore release represent the last few unknown components in understanding bacterial iron acquisition. Our work in the preceding chapters elucidates one part of this release mechanism with identification and characterization of the *E.coli* inner membrane protein P43. Yet, this protein alone is unlikely to be solely responsible for enterobactin transport. This chapter was designed on the premise of identifying cooperating proteins and completing the model of enterobactin export in *E.coli*.

Our initial hypothesis was that the well-characterized and shared multidrug resistance outer membrane protein, TolC, would be involved in enterobactin release from *E.coli*. There are numerous reasons for suspecting TolC involvement including: broad diversity of transported substrates (107, 108) including compounds structurally similar to enterobactin, ability to work with multiple systems such as MFS and RND transporters (75, 233), and high cellular availability (215) for recruitment to enterobactin transport complexes. Consistent with our hypothesis and other works (22), results demonstrate that the absence of TolC impacts enterobactin transport to the medium in a manner equal to or greater than that seen for P43-null cells. However, in our results, enterobactin release was never completely abolished [Fig. 15], nor was release of breakdown products increased in a tolC mutation. Secondly, enterobactin was not confined to the periplasm as demonstrated by assaying periplasmic contents for enterobactin [Fig. 16]. It is for these two reasons that we proposed an initial model of enterobactin transport shown in [Fig. 19]. Based on known MFS-class pumps, inner membrane translocases such as P43, directly interact with a membrane fusion protein and outer membrane channel to form a
complete pore from cytoplasm to external environment (162, 188) and prevent accumulation of substrate in the periplasm (212) [Fig. 19, left panel]. Additionally, this simple model is likely the most energetically favorable mechanism of enterobactin release from the cell. This model however, is incomplete, as it describes only an outer membrane channel and inner membrane transporter. A missing component is a periplasmic junction protein (PJP) that would serve as a linker and/or recruitment protein. As P43 is an “orphaned” MFS-class transporter, another PJP would need to be involved. An E.coli genome database query yielded several PJPs of unknown function (YhcQ, YiaV, YjcR, and b1644) with similarity to known PJPs and in proximity to unknown outer membrane proteins and offer a favorable starting pool. More interestingly, a recent report on the fish pathogen Y. ruckerii siderophore ruckerbactin has a predicted PJP encoded as part of the operon (64) and may be of interest experimentally.

The presence of greatly reduced, but not abolished, enterobactin release in our data could be explained by proteins substituting for TolC in the complex [Fig. 19, middle panel]. E.coli has three predicted TolC homologues that may act in conjunction with any number of the approximately 30 parallel efflux pump systems predicted in the genome (108), albeit less efficiently as they are not the preferred pathway. Alternatively, there may also be an unknown feedback mechanism to slow or halt enterobactin production in the absence of an efficient transporter, as interpreted by the lack of breakdown product increase [Fig. 15] and lack of enterobactin in the periplasm [Fig. 16] in tolC mutants. Such a regulatory loop would benefit the cells by preventing accumulation of potentially hazardous enterobactin in the periplasm or squandering energy creating siderophore that is unable to be transported. Future directions of this work could explore these possibilities using RT-PCR to measure potential changes in the expression levels of tolC homologues or enterobactin synthesis machinery. If no effects are observed at the gene level, it may be beneficial to test protein function as a complete channel may be necessary before P43 is opened to transport substrate.
Attempts to rescue the ∆tolC enterobactin deficient phenotype were conducted, but were unsuccessful, perhaps due to stoichiometric complications or alternatively from the plasmid-encoded tagging proteins V5 and 6x Histidine interference. The presence of epitopes on the C-terminus of TolC may be detrimental to membrane targeting or to functional domains necessary for protein-protein interaction or recruitment to transport complexes. Alternatively, because tolC mutants demonstrate pleiotropic membrane effects (personal communications and (108)), simple rescue of TolC from expression constructs may not provide all the necessary changes or regulatory events to correct other errors as rescue is driven by the pBAD expression promoter rather than native promoters.

Our previous data had always invited speculation regarding the existence of redundant systems that would help relieve high siderophore concentrations or stress conditions and we had suggested that other alternative export mechanisms such as AcrAB or EmrAB may be involved (71). Other groups have taken this to mean more literally that this type of multi-drug resistance mechanism is then primarily responsible for the transport of enterobactin, albeit indirectly, as siderophore is loaded into these systems following transfer to the periplasm by P43 (22). A depiction of this type of transport is shown in [Fig. 19, right panel]. In this scenario, P43 would deliver enterobactin to the periplasm where another transport system that utilizes TolC would then take control and shuttle the siderophore out of the cell. This model is reminiscent of a Type II or Type IV secretion system, both of which have been shown to load substrate via the periplasm. This may help explain previous data from this work whereby a P43-null strain is not devoid of enterobactin release and also why extended growth of a P43-null strain for 48-73 hours will eventually mimic a wild-type enterobactin release phenotype. We would envision a scenario similar to [Fig. 20] to include the notion of an undescribed relief pump in conjunction with TolC. At early timepoints [Fig. 20, left] in a P43-null cell, accumulating enterobactin can be cleaved by Fes to produce BDP that are released to the medium and are able to chelate iron for the cell [see HPLC results, Chapter 2]. As siderophore synthesis continues and potentially builds
cytoplasmically [Fig. 20, right], we would envision that cells in deep starvation conditions would utilize any or all mechanisms at their disposal to export siderophores, such as recruitment of P43-homologs or direct export of the now “toxic” siderophore compounds via multi-drug resistance exporters.

If this description of transport is accurate, it utilizes a multi-drug export system involving TolC. AcrAB is the most characterized of these systems and the “favored” mechanism of TolC as it is more efficient at toxic substance export than other systems based on rescue experiments (132). AcrAB has been shown to move both positively and negatively charged substances such as solvents, dyes, detergents, and lipophilic antibiotics such as novobiocin, erythromycin, fusidic acid, cloxacillin, tetracycline, and chloramphenicol (131-133). Additionally, crystal structure has led to speculation that AcrB can be loaded periplasmically in agreement with the “S-transport” model through three large vestibule regions leading to the central cavity. While not proven experimentally, the region does contain multiple aromatic, polar, and charged residues that would be tempting to a highly-charged compound such as enterobactin for transport. We hypothesized this export system could be involved in enterobactin release and tested the ability of \( \Delta acrAB \) strains to export enterobactin. The single mutations \(acrA::TnPhoA\) and \(acrB::TnPhoA\) demonstrated a slight, but consistent decline in enterobactin release, while an \(acrAB\) double mutant was unaffected in enterobactin release [Acr-HPLC Fig]. These results are again in agreement with previous data, as we propose that the single mutations in \(acrA\) and \(acrB\) create inefficient “mixed” transport complexes as opposed to the complete, high-efficiency AcrAB-TolC complex [Fig. 21, right]. However, in the total absence of AcrAB [Fig. 21, left], other homologs with similar multi-drug export properties such as AcrD, AcrEF, MdtAB, EmrAB, HlyBD, or other as of yet unknown proteins are likely able to substitute efficiently as whole complexes.

Competing groups have also tested AcrB for effects on enterobactin release, but found no significant change from wild-type release (22). There are several differences between their work and ours. Firstly, they are using a “\(\Delta acrB\)” strain compared to our strains, which are TnPhoA interruptions.
Consequently, this may result in either partially functional products or simultaneously affect acrA. As we have shown with our data, a double mutation in both acrA and acrB appears to have little to no effect on release, presupposing compensating mechanisms. Secondly, the background of the strains is very different. Our strains, BM694, a clinical isolate, and W3110 are much closer to wild-type strains and likely to have many such mechanisms in place for multiple compensating export systems as opposed to mostly domesticated strains such as MC4100 and GG199 used by Grass et al that are known to have had additional opportunity to accumulate undefined mutations as well. Finally, the background strains used in all HPLC work by Grass et al are Δfur, in the ferric uptake regulator. The assumption was that this mutation de-regulates the enterobactin operon to control for differences in strain-specific enterobactin release and to constitutively produce enterobactin. However, this mutation produces many other pleiotropic effects (140). In this case, the mutation may be self-defeating as more stress mechanisms would then be on by default. Despite all the above reasons, we see both sets of work to be valid as opposed to contradictory and worthy of consideration in the goal of establishing enterobactin release mechanisms.

There are many future directions for this work. First, with the number of potentially compensating systems, a larger targeted deletion set or library, including a total deletion of known major antibiotic efflux pumps, would be beneficial. We would expect that greater phenotypic differences would be seen if multiple systems are removed simultaneously. Secondly, further characterization of TolC-null strains for non-specific membrane changes and regulatory effects would narrow what effects are directly due to tolC and what are secondary changes. Personal communications with a group at the University of Texas describe preliminary results of gene array data on ΔtolC strains. This information would also help define rescue experiments in the future. Finally, direct interaction studies such as crosslinking and/or pulldown assays seem to be the best opportunity to illuminate interacting complexes. While data may suggest that P43 interacts with a larger complex,
they also do not rule out the direct interaction of TolC with P43 as yet another potential export channel. Our preliminary work is promising, but we lack specific antibodies for other potential interaction partners such as TolC or AcrAB. If previously undescribed proteins are also involved in the efflux complex, this further complicates these experiments. If complexes can be purified and separated in greater concentration, identification of proteins may be conducted with protein mass spectroscopy, available through the Core Facilities located on campus. However, we would speculate that the large complexes represent additional proteins beyond just the simple channel used and include proteins such as coupled synthesis machinery, chaperone/targeting proteins, additional membrane components such as TonB or energy generation proteins, or other unknown factors. Similar large complexes were noted in other experiments with membrane export systems (23, 167).

In summary, the work presented in this chapter does elaborate on the export mechanisms of enterobactin. Despite our initial hypothesis, release of enterobactin may not be as straightforward as first predicted. A summary of transport data is shown in [Fig. 22]. We would predict from the results presented here, in conjunction with other data, that enterobactin is initially moved across the cytoplasmic membrane by P43. From here, enterobactin encounters several possibilities. The great majority of enterobactin would be periplasmically loaded and shuttled by the AcrAB-TolC complex to the external environment. Alternative pathways, such as AcrD, EmrAB, or MdtAB, are likely also functioning, but transport only a small fraction of enterobactin as the most numerous and preferred multi-drug export system, AcrAB-TolC is intact. As conditions deteriorate, such as extended time under low-iron conditions or accumulation of siderophore, stress mechanisms of the cells are activated, which include the assembly of multi-drug export complexes. These active complexes can then be used as direct cytoplasm-to-environmental siderophore trafficking mechanisms to relieve stress on P43 or the cells in general. Despite excellent efforts, there are still details to be discovered about the mechanisms
of enterobactin export, but the key players must include P43, TolC, and multi-drug resistance (MDR) pumps.
CHAPTER 4 FIGURES

FIGURE 15: ENTEROBACTIN RELEASE IN tolC STRAINS.

HPLC demonstrates distinct differences in siderophore export patterns between wild-type, P43-null, and ΔtolC cells. Strains were grown for 4 hours in 10 mL of MOPS minimal medium. Siderophore products were extracted from culture supernatants with ethyl acetate, dried, resuspended in 50 µL of methanol, and separated by a 10-50% gradient of acetonitrile and water with 0.1% trifluoroacetic acid, then detected at 220 nm. Panels show individual chromatograms of enterobactin standards (trace 1), wild-type enterobactin release (trace 2), and P43-null mutant enterobactin release (trace 3) under iron-starvation conditions. These were compared against cells lacking TolC (traces 4-6). Detected enterobactin peaks (E) and DHBA (H) are labeled as defined by standards. Letters mark BDP peaks identified by mass spectrometry: (T) linear trimer; (D) linear dimer; (M) linear monomer. Strains with ΔtolC released distinctly less enterobactin and variable amounts of BDP.
Periplasmic cracking assays were conducted to assess whether a ∆tolC mutation would strand enterobactin in the periplasm. Cells were grown in 50-100mL MOPS minimal medium for 4 hours. Cells were placed in isotonic solutions to release periplasmic contents and siderophore products were extracted from culture supernatants with ethyl acetate, dried, resuspended in 50 µL of methanol, and separated by HPLC. Panels show individual chromatograms of enterobactin and DHBA standards (traces 1 and 2). As expected, no enterobactin was trapped in either wild-type cells (trace 3) or P43-null cells (trace 4) under iron-starvation conditions. These were compared against cells lacking TolC (traces 4-6). None of the ∆tolC strains demonstrated an increase in enterobactin or BDP in the periplasm.
FIGURE 17: CROSSLINKING TO IDENTIFY PROTEINS INTERACTING WITH P43.

Cells containing the P436xHis construct under arabinose expression [Left, lanes 1 & 2] or non-inducing conditions [Left, lanes 3 & 4] were grown in low iron conditions to stimulate enterobactin transport complex formation. In vivo crosslinking was performed with DSP and complexes containing P436xHis were detected by immunoblotting with anti-6xHis. Preliminary crosslinking experiments demonstrate high molecular weight complexes only in induced, crosslinked cells [Left, lane 2]. Treatment of the same arabinose expressed extracts [Right, lanes 1 & 3] (in duplicate) with DTT [Right, lanes 2 & 4] to break crosslinking was performed to observe if complexes would release the P436xHis. Molecular weight markers (in kilodaltons) are shown at far left.
FIGURE 18: IMPLICATION OF AcrA AND AcrB IN ENTEROBACTIN TRANSPORT.

Strains were grown for 4 hours in 10 mL of MOPS minimal medium. Siderophore products were extracted from culture supernatants with ethyl acetate, dried, resuspended in 50 µL of methanol, and separated by a 10-50% gradient of acetonitrile and water with 0.1% trifluoroacetic acid, then detected at 220 nm. Panels show individual chromatograms of enterobactin standards (trace 1), wild-type enterobactin release (trace 2), and P43-null mutant enterobactin release (trace 3) under iron-starvation conditions. These were compared against cells lacking acrA, acrB, or acrAB (traces 4-6). Peak areas and heights are shown at right for comparison. Single mutations in acrA or acrB show a reduction in enterobactin peaks approximately 2-2.5 fold. Strains with ΔacrAB had no phenotypic difference from wild-type for enterobactin release.
FIGURE 19: DIRECT INTERACTION MODEL VERSUS INDIRECT “S-TRANSPORT” MODEL.

[Left] We hypothesized that a direct P43-TolC interaction to transport enterobactin was possible based on known MFS-class transport systems. In this case, an as of yet unidentified periplasmic junction protein would likely be involved. [Middle] Inefficient release of enterobactin in the absence of TolC suggests that there is either compensation by TolC-like homologs or by feedback downregulation of synthesis. [Right] Schematic of indirect “S-transport” model (22) where enterobactin is shuttled to the periplasm and loaded into a multi-drug export complex containing TolC for transport to the extracellular environment.
FIGURE 20: MODEL OF ENTEROBACTIN STRESS RELIEF MECHANISMS.

[Left] At early timepoints after enterobactin synthesis, enterobactin is unable to be transported in a P43-null cell and is cleaved by Fes to produce DHBS breakdown products, which are detected in HPLC as a hallmark of enterobactin transport phenotypes. However, at later timepoints as enterobactin buildup may occur [Right] and cause stress responses, the enterobactin release phenotype appears as wild-type cells, likely due to transport through P43 homologs or direct loading of stress condition exporters, such as the multi-drug resistance class of proteins.
FIGURE 21: INVOLVEMENT OF AcrAB-TolC IN ENTEROBACTIN RELEASE.

As demonstrated, single mutations in acrA or acrB [Right] reduce enterobactin transport presumably through recruitment of homologs that form inefficient “mixed” transport complexes. However, in the absence of both AcrA & B [Left], no enterobactin release phenotype is detected, suggesting compensation through other multi-drug export mechanisms are in place.
Combination of all data to this point suggests multiple conditional export pathways for enterobactin from *E. coli*. Under general release conditions, enterobactin is transported to the periplasm via P43 where it is shuttled primarily to the AcrAB-TolC complex for periplasmic loading and export. However, multiple homologs of AcrAB exist and may serve to export minor amounts of enterobactin or compensate in the case of an *acrA*- or *acrB*-mutation. Under stress conditions when enterobactin can accumulate in the cytoplasm, multi-drug exporters like AcrAB-TolC may serve to prevent toxic metabolite accumulation by direct siderophore transport.
**Chapter Summary:**

This body of work represents a significant advancement in the understanding of siderophore export mechanisms in *E. coli*, with the discovery and preliminary characterization of the first export pump for bacterial siderophores. These data may advance knowledge in many other areas associated with iron transport, bacterial usage of transport proteins, and treatments and applications for disease states.

**Significance of Work:**

Research on siderophores has been conducted for decades, leading to understanding of synthesis, structure and function, metal binding capacity, ferrisiderophore complex uptake, and cellular usage of iron in numerous organisms. However, prior to this work, no export mechanism for siderophores to exert external chelating effects have been described in any organism (71, 219). Working in the model organism *E. coli*, our work establishes that the protein P43 (encoded by *entS*), located within the enterobactin *ent* biosynthetic gene cluster, encodes a membrane protein belonging to the major facilitator superfamily and functions in exporting enterobactin to the periplasm. A variety of techniques including crossfeeding assays and chromatography demonstrated the siderophore species released. The direct relation of P43 to enterobactin transport was explored utilizing a novel inverted membrane vesicle system coupled to the iron-sensitive fluorescent dye calcein. The P43 protein was characterized to utilize proton-motive force (PMF) despite the absence of a conserved proton transfer motif. Overall, the work on P43 represents the first description of a siderophore release mechanism and sets the stage for further investigations of similar systems. Additionally, our work attempted to
complete the circuit of transport from the periplasm to the external environment. Our results strongly suggest that numerous compensating or redundant mechanisms may be involved and that further experimentation is necessary, but provide evidence for involvement of characterized export systems such as the outer membrane protein TolC and multidrug exporters.

A recent report analyzing a library of putative drug transporters in *E.coli* showed a broader resistance spectra than previously thought (163). The MFS-class and other multidrug transporters presumably evolved to transport specific physiological substrates, but fortuitously protect cells from diverse toxic compounds (158). As such, a compound such as enterobactin that shares structural characteristics and synthesis pathway mechanisms with antibiotics should be considered as a “natural” substrate of multidrug efflux pumps and poses a re-evaluation of how to approach characterization of multi-drug resistance (MDR) systems. It is highly possible that these kinds of MDR systems are undescribed, biologically functional transporters that serve redundant efflux functions under stress conditions.

**Broader Impacts of Work:**

As siderophores are nearly ubiquitous in bacterial species, this investigation into siderophore release mechanisms may have broad organism impact as siderophore release mechanisms are undescribed in all such systems. For example, enterobactin, once thought unique to Gram-negative bacteria, has been isolated from two Gram-positive *Streptomyces* species suggesting production of this powerful siderophore may be wider than initially suspected. A protein-protein BLAST search using P43 as the query sequence demonstrates similar proteins in a variety of organisms including species of *Shigella, Salmonella, Serratia, Yersinia, Acinetobacter, Legionella, Streptomyces, Pseudomonas*, and *Corynebacterium*. Additionally, many of these MFS-class proteins can be found in gene regions dedicated to siderophore production or have been consequently shown to be involved in siderophore
transport. Examples include: *Yersinia ruckerii rucS* for ruckerbactin (64), *Vibrio parahemolyticus pvsC* for vibrioferrin (210, 211), *Bordetella pertussis alcS* for alcaligin (29), *Legionella pneumophila lbtB* for legiobactin (6), *Azotobacter vinelandii csbX* for azotochelin (171), and *Fusarium verticillioides FIR1* (128).

Because of the widespread use of siderophores in nature, mammals have evolved countermeasures to block siderophore-mediated iron acquisition as part of their innate immune responses. Molecules such as Secreted lipocalin 2 (Lcn2 or siderocalin) sequesters enterobactin, preventing ferrisiderophore return to the bacterial cell. Neutrophil gelatinase-associated lipocalin (NGAL) is secreted by neutrophils during inflammation and has sufficient affinity to compete with bacterial siderophore uptake mechanisms and hence neutralize iron uptake (78). Siderophores have also been shown to stimulate immune cells directly such as differentiation of monocyte precursors into activated macrophages that release MIP-alpha or triggering release of inflammatory signals such as the chemokine IL-8 in human intestinal epithelial cells (119, 204). These examples demonstrate the concern expressed by hosts in the battle for iron and the importance of understanding siderophore mechanisms.

Concurrent with mammalian “attacks” on siderophores, bacteria have developed methods to avoid immune interference with siderophore function. Siderophores modified by glycosylation, termed salmochelins, have been described (90). It was presumed that enterobactin was poorly effective in the serum because it adsorbs to hydrophobic serum proteins such as albumin (106). But, these additional residues attached to enterobactin reduce hydrophobicity and thereby increase potential interactions with iron (90). It has been demonstrated that Lcn2 does not bind these C-glucosylated derivatives of enterobactin and virulence is increased in a murine model (67). This type of modification has been shown to take place in *E.coli, Salmonella*, and *Klebsiella pneumoniae* along with others. Other pathogens such as *Bacillus anthracis* have developed a siderophore structural variant also able to
avoid Lcn2, termed the “stealth siderophore” petrobactin, which is preferentially released in pathogenic low-iron conditions over the non-modified siderophore bacillibactin (2). Because the siderophores themselves are changing, knowledge of their basic mechanisms will serve to help characterize their roles in disease.

Understanding of siderophore mechanisms also opens the possibility of alternative uses for iron-chelation molecules. Studies have linked antibiotics to siderophores to create “Trojan horse” molecules that would serve as drug delivery agents in treatment of drug-resistant strains of bacteria (32, 195). Improvements on these molecules to create protected, fully differentiated “multiwarhead” siderophores has also been reported and showed great potential in targeting pathogenic microbes (147). Another application for siderophores is during chelation therapy. Imbalances in metal homeostasis has been reported or associated with diseases such as Alzheimer’s disease (4), cardiomyopathy (76), mucormycosis (97), and thalassemia (39, 62). In all these cases, removal of metals with siderophores could prove beneficial to patients. All of these potential applications and interpretations have roots in this work as a basic science foundation for understanding siderophores.
CHAPTER 6: EDUCATIONAL COMPONENT

Introduction:

This chapter is not a typical ending to a molecular microbiology and immunology graduate student’s thesis. As described in the previous chapters, research data and contributions to the greater knowledge of science are central elements of Ph.D. training and serve as a basis on which I will likely be hired for post-doctoral employment. But, the root goal of graduate training is to prepare students for their futures. My career plans are to become an academic researcher at a doctoral or masters-level institution and hence, both teaching and research will be required of me. To this end, I sought teaching preparation as part of my graduate training. Learning and thinking about teaching and practicing teaching have become equally important shaping experiences in my graduate training.

The following chapter is the end result of conversations between my committee and myself. But, by adding an education chapter into a “hard science” graduate thesis, we are heading into uncharted waters, as this (to our knowledge) has never been done at the University of Missouri. As a result, this chapter is likely far from perfect, but may serve as a reasonable framework for future similar endeavors. The goals for this chapter were to consider my experiences in teaching and graduate preparation and to address two questions: 1) How do we best teach microbiology to our students? and 2) How does incorporation of teaching and research in graduate education help microbiology graduate students for their careers in microbiology?

This chapter has no data per se, no experimental outline, no concrete conclusions. I have no guarantees that any of this will be pertinent or appropriate for all readers. I do not consider this to be an all-encompassing manifesto. But, the mentoring and preparation given to me hopefully better prepared me for my desired career in microbiology. My committee and I feel that there is something
that can be learned from my processes and results; just as in bench experiments, sometimes the hypothesis is supported, sometimes disproved, so also some of my educational hypotheses are supported and some need to be altered or discarded. I consider it my duty and privilege to pass on this information to interested parties, to continue this kind of mentoring in my future as a faculty member, and help form the next generation of teacher-scholars.

“Jason’s Experience”

This first section of the education chapter was the simplest place to start, and yet the toughest one to write: my experiences with teaching. It is odd to write in first-person perspective after all the training on scientific writing! Data and concrete conclusions are replaced by frequent subjective quotes of, “I think this” or, “I feel…” My references might be a little sketchy as I am not as familiar with the field and who first proposed ideas. But, by examining my experiences, I can acknowledge problems that may exist and examine my own understanding to see where I need to learn more (termed metacognition). Here I reflect on how I got started in teaching, the classes I have been involved in, and several experiences I have had with graduate education and teaching education.

The Start:

My beginnings into teaching were rather unexpected. When I joined the microbiology program in the summer of 2000, I entered the program to earn a Ph.D. in microbiology. I performed well in my lab work and learned my way around during rotations. But in looking back, that was the only part of training I considered – becoming a scientist at the bench. But as fate would have it, I was literally thrown into teaching within two months of starting the graduate program by being “drafted” to serve as a teaching assistant (TA) for the medical student wet-lab under the guidance of Dr. Arnold Smith and Dr. Louise Thai. Another graduate student decided that he was “too busy” to leave his laboratory to
teach and TA. Dr. Smith called me and asked if I could help. As a brand new graduate student, I figured it could be unwise to turn down the chair of the department when he requested something of me! When I walked into the teaching lab for the first time, I thought I had made a dreadful mistake. All the other TAs were senior graduate students; all of them had done this before. I sat before a table of ten medical students. If the med students in the class could smell fear, they could have tracked me from 50 miles away. What the heck did I know about teaching students? Could they tell that I had never done or even considered teaching before? Having just entered the program, I had to study the material as if I was the one in the class. I was never more than one lecture ahead of the medical students. I admit to being clumsy, inarticulate, confused, and apologetic. Miraculously, I did it. Not only that, but I felt a connection to the students at my table. I liked showing someone the thing I just learned. My evaluations ended up being satisfactory, but I am never one to settle for being just “adequate.” I still cannot place exactly what about that first terrifying experience ended up inspiring me to want to teach more, but I volunteered to teach the med students again the next semester. I think it was then that I realized that I would need to put work into this whole “teaching thing” if I was to become good at it.

The Class:

During my graduate training in the Molecular Microbiology and Immunology (MMI) department, I have been primarily involved in helping to teach one class, the only true undergraduate course offered in MMI: Introduction to Medical Microbiology. I approached the course supervisor, Dr. Louise Thai, about taking a TA position with her. Within two semesters, I was asking for more responsibility in the class and became the Lead Teaching Assistant. I ran the labs. I did lectures. I hired TAs. I made and graded quizzes. I thought I could teach!
When I began, Intro to Med Micro was originally listed as “Micro 205,” a mid-range course number designating that it was for sophomore-level students. Upon the change to course numbers, it was re-listed as “Micro 3200,” now an upper-level class. It is a large (~ 150+ enrollment), lecture-oriented class with a six-week wet lab section. It is heavily content-intensive and is team taught by expert scientists in their respective disciplines (immunology, bacteriology, virology, parasitology). The students enrolled are typically biology-pre-med or pre-nursing, with some other pre-professional majors. This group is heterogeneous in terms of science background coursework. Many biology-pre-meds take the course as juniors or seniors having had significant cell biology, biochemistry, organic chemistry, and other science skills. In contrast, most pre-nursing majors must fit the course into their electives in freshman or sophomore semesters and traditionally lack many upper level science courses beyond introductory biology. Studies have shown that teaching appropriate content to the audience is critical to learning (8, 151). As a result, content in Micro 3200 must be tailored to both groups without sacrificing the interest of the higher-level science groups and without confusing the basic-science group.

The course, like its registration number, underwent restructuring when Dr. Louise Thai assumed control of the class. More technology has been incorporated every year to reach the current format of a large, projected PowerPoint that corresponds to the lecture given. To break up the material into more testable blocks, there are now five shorter exams, lab quizzes, and a comprehensive final given in lecture as opposed to previous semesters that used only three exams and a comprehensive final exam. Assessment is conducted with multiple-choice questions in a case-scenario format to test student knowledge. Wet labs mirror the bacteriology section of the course and provide extra chances for students to put concrete examples together with the abstract concepts from lecture. Previous students from the course conduct regular help sessions. Grades of the students range every semester, with a majority of the class receiving a passing grade; but there are still students who must re-take the class every year.
One final note about this course is that because of its reputation as one of the hardest, but best, courses on campus, it increases the pressure to succeed for both types of students. Among the biology-pre-meds, there can be fierce competition among the top students to earn the highest grade or to receive a letter of recommendation from the instructor. Among the pre-nursing students, the course can be a source of tension due to pre-acceptance to the nursing program (a grade lower than “C” including “C-minus” is considered failing and their enrollment in RN programs or for beginning clinical rotations can be in jeopardy).

My involvement with this class has been going on 6 years, although the teaching requirement for graduate students in MMI is to teach only 2 semesters of the wet lab (a total of 12 class sessions each ~ 1-2 hours in length). I love being a part of this class and I think my enthusiasm for the subject and my loyalty to the students come out in my teaching. But it is not “roses” all the time. I have come to realize that my teaching always needs work. It takes effort to prepare and evaluate my teaching. I try to improve and incorporate new methodologies. I see where students succeed and students fail. But most importantly, from my long-time association with Micro 3200, I can observe different lecturers and content material in the classroom and how my students learn it. This leads into section two of this chapter, where I will take a harder look at the real issues taking place and ask questions. How can we, operating as scientist-teachers, help our students to better learn microbiology?

The Experience:

True story: One day after class, I was in the microbiology office. I was complaining about how difficult it was to explain a particular concept to the class that morning. I said that even for an expert in the field, it is a hard idea to express. As we progressed to talking about teaching methodology in general, a faculty member listening in made the comment that, “all good scientists are good teachers.” I could not believe what I just heard! From there, we were in a fight. I argued that everyone has had
experiences with guest lecturers – brilliant in their respective fields – being invited to address undergraduate classes. Some of these incredibly knowledgeable people in the field are unable to communicate even the simplest idea to an audience. We continued for a few minutes, but neither of us would concede our points. This was one episode I have always remembered and let guide my teaching. Relating this story to the main ideas of this chapter, I state that a degree in one field does not certify you for all tasks. “Professors of physics or biology would not be expected to be experts on the newest and most effective teaching practices in elementary education, nor would one who specialized in education be expected to be expert at all the disciplines of science. Therefore, education and science professors need to work together to create experiences that integrate content and pedagogy” (151, 209).

This experience also started me thinking about future plans, as I realized my job will involve more than just sitting at a benchtop all day. Today’s academics are tasked with three jobs: teaching, research, and service. However, graduate training only effectively prepares students for one of those three. Their forte becomes research, ingrained from their training. In very few programs is teaching preparation emphasized. Pedagogical theories and methodologies are a science in and of themselves to be learned. To me, “learning” is the goal of a Ph.D. By earning the degree, you are stating that you are in constant pursuit of knowledge. If we are increasingly advancing the idea of interdisciplinary sciences, education should be included. Teaching and education theory are bodies of knowledge worth learning. What sense does it make to do experiments without the necessary background? Why should being in a classroom be any different? In my career, I have experienced great teachers and horrible teachers. I have met researchers that I envy and ones that I despise. This experience dedicated me to being remembered as one of the great teachers and great researchers.

The Realizations:
The main driving force in my education preparation came from one class. In the Fall semester of 2003, I enrolled in the College Science Teaching (CST) course at Mizzou. At this point in time, I had been involved in “teaching” the Micro 3200 class for several semesters and considered my teaching to be improving substantially from that first experience. But, what I found instead was a new world of teaching to which I had never been exposed. During my entire undergraduate career, I had become habituated to the traditional lecture format and adopted it for my own teaching style because I was comfortable with it. The CST course forced me to critically evaluate my teaching and determine if my students were actually learning the material presented versus just hearing me lecture. Reflecting on my teaching in the context of CST material demonstrated the difference of active and passive learning and emphasized that my teaching should be understanding of information opposed to transmission of information. Reviewing my evaluations and comments implied that my style still emphasized memorization of factual class material, but could be modified to create real active learning because I was able to frame material into blocks students could understand. Other relevant topics covered in CST included assessment strategies, use of writing in the classroom, learning styles, and misconceptions as barriers to learning. Finally, one of the most useful pieces to my teaching was the creation of my own teaching philosophy statement that has helped guide how I approach and view my teaching in the context of being a scientist.

After completing the course and at the recommendation of the instructor, I attended the Wakonse conference (http://www.wakonse.org) in the summer of 2003. Wakonse is a word from Lakota Indian meaning “to teach, to inspire.” The goal of the foundation was to support, promote, and share the excitement and satisfaction of teaching. This was a joint conference on inspirational aspects of teaching and learning processes for both high school educators and college instructors. Attending Wakonse further made me reconsider my teaching and how I view myself in the classroom, realizing
that other colleagues are also interested in bettering their teaching and finding ways to better prepare incoming college students.

The Committee:

I have to take a very short section to thank my graduate advisory committee for their help. I am certain that many graduate students have horror stories about their committees and graduate requirements. My committee has been nothing but helpful and supportive about my teaching activities above and beyond my bench training as a scientist. As mentioned previously, I have spent much more time out of lab than the typical microbiology graduate student with my classroom duties. This results in extra costs, “lost” time from benchwork, and more. Yet, throughout all of my grad training, the committee has always approved my extra teaching as long as my mentor, Dr. Mark McIntosh, felt I was still accomplishing my research objectives. And because of that, it makes this type of a chapter even more difficult to write. I see things that could be improved in our classes and how our graduate students learn about teaching. But, this is not an “attack” on teaching in our department or division, but rather a suggestion that we could do even better with our graduate training and undergraduate learning than we are already doing! I feel that my example sets a precedent that graduate students can have the “best of both worlds” and be well trained as both good benchtop scientists and good classroom teachers. This relates to the second question posed in the introduction of how to improve graduate training programs to better prepare graduate students for the science of microbiology. This includes more than just grooming graduates for the basic jobs of “teaching, research, and service” of an academic faculty member and instead thinks more broadly about how our science is impacted by things directly related to our teaching: public opinion of science, funding, social learning, and interactions. Perhaps a better way to consider this would be to ask, “what really are the responsibilities
associated with being a Ph.D. researcher-teacher in microbiology?” These questions will be elaborated on in section four of this chapter.

The Comps:

Another aspect of my graduate education that differs from the normal MMI graduate was my comprehensive exams (comps). Traditionally in our department, students complete their comps by designing a set of experimental procedures based on literature of their choosing and write a mock NIH research grant complete with a literature review, specific aims and experimental design, implications, figures, and bibliography. All “preliminary data” for the mock grant comes from previously published work (i.e., the students do no bench work on their grant proposal). The student then defends their proposal in front of their graduate committee with an oral presentation. The aim of these exercises is to give students an opportunity to write a “hard-core” scientific grant and to test knowledge of scientific concepts and experimental design.

For me, this became an opportunity to incorporate my teaching into the process. My committee allowed me to write a different kind of grant called an NSF CAREER grant. Information can be found online (http://www.nsf.gov/home/crssprgm/career/start.htm), but in brief, this grant is designed to support early-career development activities of “teacher-scholars” and is awarded to applicants who effectively integrate both research and education aims into the same funding proposal. Additionally, the activities proposed are to “build a firm foundation for a lifetime of integrated contributions to research and education” (152). I found this to be a highly rewarding experience because I felt it mirrored more closely the types of activities that I felt I would be involved in as a future faculty member and allowed me to challenge myself to think differently about how I do science versus how I act in the classroom. This brings me back to the overarching question I mentioned previously about how we, as scientist-teachers, can help our students to better learn microbiology. This leads me into another set of
problems to discuss in section two that I feel my education training addresses: If I want my students to understand my science, why not tie the science into the teaching? Rather than standing there and talking “at” my students, I should teach the science of microbiology like I research the science of microbiology using scientific methods and inquiry.

The Summary:

I guess I would like to leave you with the two questions I am trying to address in a simple statement that will be the focus of the remaining sections: The better we teach microbiology to our students, the better our students and general public will be, which in turn betters our graduate applicants, interest in microbiology research, and our research in general. How we train our graduate students for research and teaching has direct impact on microbiology instruction.

Problems in Microbiology Teaching

In section one, I alluded to or directly presented several problems related to how the science of microbiology is taught and learned in the descriptions of the courses and activities. The goal of this section is to take the observations from section one and review the educational problems they represent in literature-based review and put them into a research context. These issues, revolving around teaching and learning, have been debated for decades in the educational community and are not unique to only the science of microbiology. However, many of these issues are just recently being addressed in the teaching of the college-level science. As this section attempts to demonstrate, ignorance of them would build a poor future for science.

Teaching and Graduate Preparation:
Incredible achievements have come from doctoral programs in the United States. Trainees of these programs have produced advances in science, medicine, and engineering that were unimagined only a few decades ago and provided understanding of events that affect our daily life and public policy. These next-generation scientists are the product of our higher-education training programs that prepare students for careers primarily in academia or industry. However, while research is an important element in academia, it is not the sole responsibility of a thriving faculty member. A successful lab or graduate program requires creative undergraduate and graduate students under constructive mentoring to produce results. These kinds of creative students are the product of excellent teaching. Yet, excellent teaching is not an inherent ability nor is it emphasized in our graduate training programs.

A recent study of 32,000 graduate students by the National Association of Graduate Professional Students (NAGPS) found that current graduate students desire broader curricula, more career choices, and more information about careers following graduation (47). A survey (79) indicated that most current doctoral students have goals to become faculty members. But one hindering factor to this during training is that the current graduate “culture” is dominated by a research focus where disciplinary research takes precedent over all other types of preparation (1). Of the elements of a graduate education that respondents ranked “most valued” were “experience gained while teaching courses (80%),” “teaching mentoring (67%),” and “courses and seminars (67%)” (25, 60). Yet 50% of respondents felt their preparation to be teaching assistants was inadequate and 59% did not feel that teaching was not emphasized as important (1, 47) and there was a significant deficiency in faculty development for pedagogy (11). The result of this is production of a large number of research post-doctorate fellows with diminished teaching and service skills.

One consequence of this specialized preparation is students potentially leaving graduate programs with only one talent and isolated for only one kind of future employment. Leaders in
business and industry argue that today’s Ph.D.s lack collaborative ways of thinking and claim that student research dissertations are often disconnected from other knowledge and real-world problems (165). The final result of this isolation would be an elitist science that is moderated and understood only by the participating scientists rather than the general populace. As will be discussed later in this section and section four, this could have disastrous consequences for science.

The Mizzou MMI department has produced excellent graduates in recent years and has an outstanding base of current doctoral students in the pipeline. This presents an opportunity and challenge to lead the way in advancing change and innovation by incorporation of educational and teaching components in its graduate training. Because most graduate students are pursuing careers as faculty (47), teaching preparation should be important to graduate training because it mimics the kinds of activities that are done by faculty members. Preparation in teaching may also help in curricular planning, collegiality, and service (60, 165). Teaching is also critical as it drives the science pipeline. If current graduate students are trained in research but not teaching, these trainees then become the next generation of faculty, teaching the sciences and trying to recruit and inspire students to become future scientists. If future faculty members lag in teaching, then potential recruits are dissuaded from entering the pipeline of science. Improvements in graduate education to include teaching could result in increases of applicants who are highly qualified with good reasoning and problem-solving skills.

Audience:

One key to good teaching that happens before the lecture even begins is to understand the background of your audience. This involves not only previous science knowledge but also factors such as ethnicity, socio-economic status, and self-worth. For example, in Micro 3200, the audience ranges from students with high levels of science knowledge to students with a bare basic level. This context demands preparation on the part of the instructor to find a balance of material that all students can
learn without alienating either end of the spectrum. Additionally, many instructors assume that, because classes have prerequisites listed, the students are ready to tackle current material. But the content material from prerequisite classes varies and one should not presuppose that the audience is truly prepared for the current class.

Graduate students who are poorly trained for teaching may misunderstand their audience and present material in a manner consistent with their training: the departmental seminar format. This format is typically above the level of the audience and is completely lost on the listeners. One potential reason for this is that students do not think in the same manner that scientists do (27). Experts organize thought patterns in recallable blocks or nested hierarchies while students organize more into chronological or simple categories (26, 130). Personally, I have “verified” this. I gave a short optional lecture about my bench research in class as a practice run before my seminar. Of the students who stayed for the lecture, many of them were asleep very quickly (including the successful students), despite that I had “dumbed down” the material to what I considered an understandable level.

Considering these issues about the audience in advance will allow for better learning of all students. Additionally, simple pedagogy techniques such as team-based learning, think-pair-share exercises, or audience voting may prove useful to the instructor by providing instant feedback as to the level of understanding of the audience. Flexibility of the instructor to adapt to a varied audience and make appropriate changes to the material or syllabus is also important. On the student end, these kinds of activities help students to assess their own level of knowledge (termed metacognition) compared to that of their peers. By ensuring that students track their own level of knowledge, instructors place more responsibility on the learners to ensure they are staying current with material. Students respond to flexibility as well, seeing that their instructor cares about their learning by adjusting on the fly as students grasp or miss concepts.
Misconceptions:

The level of the audience is not the only factor to consider when planning teaching. The classroom may be filled to the brim with the most intelligent students, but fail in the end due to misconceptions preventing learning. Misconceptions are simply explained as a blending of fact, fiction, and nonsense into a thought process that corresponds to the student's thinking patterns. These misconceptions are generated from varied sources, including unstructured “informal” learning (150) or misinformation (“half-truth”) exposure in advertisements and news stories (180). From a science perspective, student misconceptions are difficult to deal with using standard lecturing. Students modify and refine their knowledge when they acquire new material, but these revisions are not always changed to the “correct” version just because of “sound scientific reasoning” (207). In Micro 3200, students may have heard of some of the abstract concepts discussed in the media and have established thinking patterns regarding them. Personally, I have encountered misconceptions in class about everything from antibiotic mechanisms (“They create antibodies to kill things” – personal interview) to spread of zoonotic diseases (“I’m afraid of West Nile Virus because my mom raises horses and I know that they bite sometimes” – personal communication). Other news events such as bioterrorism, HIV, the *E.coli* O157 spinach outbreak, and bird flu are common examples I have encountered in the class where students have difficulty in learning the true science because of their incoming misconceptions.

Instructors can deal with misconceptions in numerous ways, but must realize that students cling tenaciously to their ideals and will attempt to reconcile new material with their misconceptions rather than abandon them. Perhaps the most effective method to deal with misconceptions is to think about how students would learn the science of microbiology best. The instructor should create lectures that challenge students’ views and force them to consider alternative explanations as rational skeptics (180) by starting with questions such as, “Why hasn’t West Nile virus infected all of us yet?” and forcing
the students to understand and learn versus “informational dumping” and expecting students to memorize facts such as, “West Nile Virus only causes symptoms in every 1:100 people.” Because the science of microbiology is “hot topic” due to potential pandemics, bioterrorism, and cutting-edge research, this presents a unique opportunity for learning microbiology as “teachable moments” rather than a passive experience where class material is disconnected from the real world. It has been shown that ideas are best learned when students see a need or reason for their use, helping them see the relevant uses of the knowledge to make sense of what they are learning (27).

Learning Microbiology:

The question posed from the previous section regarding misconceptions was key. How do students best learn science? The standard format for most microbiology classes, including Micro 3200, is that of a lecture paired with a “cookbook” laboratory. Lectures in microbiology have been described as “boring and old-fashioned” and as a “bug parade” (199). Others have shown that students may retain as little as 20% of the information presented in a traditional lecture class (9). Rather than reflecting the science behind microbiology, instructors rely on the students to memorize inordinate numbers of facts in great detail and regurgitate concepts upon assessment. It is elegantly summed up in the quip, “There are reasons this is called the Age of Information rather than the Age of Understanding…” (202).

Because of the varied level of students, the misconceptions that hinder scientific learning, and teaching style used, this question is ripe for investigation. It has been extensively studied and written about in the educational field for the benefit of improving teaching and learning at the national level. If we want our students to become or think like scientists, why do we not teach them to think like scientists? The term “science” refers to more than a, "body of knowledge" (37). “[Scientist's] knowledge is not simply a list of facts and formulas that are relevant to their domain; instead, their
knowledge is organized around core concepts or ‘big ideas’ that guide their thinking about their domains” (27). The Biological Science Curriculum Studies and Scientific Inquiry programs (http://www.bscs.org) advocate the importance of learning science through activities that emulate real scientists and the efficacy of learning in scientist-like contexts. Many groups including the National Science Teachers Association (NSTA), The American Association for the Advancement of Science (AAAS), and the National Research Council (NRC) have echoed this sentiment. Yet, a majority of the coursework we teach still emphasizes fact over understanding. The challenges facing the science classes to implementing this kind of true science learning are many: how to switch to emphasis to overarching concepts versus factual dumping, how to take more time to “think like scientists” without sacrificing “important” material, and how to conduct this kind of authentic learning in a large lecture setting.

Inquiry:

“If a single word had to be chosen to describe the goals of science educators during the 30-year period that began in the late 1950’s, it would have to be INQUIRY” (48). Many national organizations have concluded that the way to best teach science is through inquiry, defined as learning based on the idea that science education should reflect the way that science itself is carried out. Inquiry should be a learning goal as well as an instructional strategy. Inquiry is an active learning process, asking students to engage, provide evidence, explain, connect knowledge, communicate, and justify (37, 150). Inquiry becomes important as students learn how we do science at the bench. If the goal of the national standards is to learn science in an authentic manner, who better to help them understand the process than a veteran researcher who can help bring the science to life? From this kind of learning, students see that science contains uncertainties and is constantly being re-tooled based on new data and communications. Additionally, classes should be directed towards a more
research-oriented experience with open-ended, collaborative laboratories. This kind of learning not only helps students succeed in the classroom, but also gives them the right tools for becoming proficient researchers and teaches them how scientists communicate and work together. Given that creativity, communication, critical thinking, and self-evaluation are all essential for scientific work, it becomes obvious that teaching students using inquiry methods is important for the future of science.

But is there trust of inquiry methodology in the hard sciences, including microbiology? Prevailing teaching methods use a factual dumping strategy where the attitude of the instructor is analogous to, “if I don’t tell them, how will they ever learn (202)?” This key element of distrust about inquiry stems from the fact that with the body of scientific knowledge available, current teachers feel that “more knowledge is good knowledge.” Instructors fear that material will be lost in inquiry-based classes because students are too busy “thinking” rather than “learning.” Hence, if the students are to “learn,” they need to be given the information directly. This denies that the students have their own sophisticated ways of learning and instead assumes that the instructor’s view is the most important (202). Additionally, instructors fear that if students are allowed to think on their own, they may arrive at incorrect conclusions or form more misconceptions and extra effort must be spent correcting students. However, presentation of material is not essential, as studies have shown that students are capable of learning about relationships they have never been taught about (150). The better role for the instructor in such a situation would be as a facilitator to learning as opposed to a figure of ultimate knowledge.

Finally, there is trepidation about how inquiry is to be run in a classroom. There are varying degrees of inquiry. While inquiry is defined as an active search for knowledge or understanding about phenomena through collection and interpretation of information (93), it does not mean students must be set free in order to learn. Inquiry can run the gamut from controlled to unstructured as long as it begins with curiosity and ends with seeking a solution or explanation. It is puzzling that scientists in the classroom would abandon their tried and true inquiry-based bench methodology and resort to a disjointed lecture.
Personally, I cannot remember the last time an experiment worked exactly as planned or gave me the exact information I was looking for. But I do know that I ended up learning something because of it. Our students can learn the same way.

Research on inquiry-based science teaching has shown it helps to improve student understanding and performance, foster scientific literacy, improve critical thinking, and promote a positive attitude towards science (93, 112, 124, 138, 149). Inquiry learning may help groups that encounter difficulties in science, such as minorities or foreign students, to succeed in classes. Students in K-12 grades using inquiry science classes appear better prepared for science classes in the future. It is for these reasons that inquiry, rather than emphasis on accumulation of knowledge, is good for the science classroom and for the science itself.

Social Aspects of Learning:

The modern science classroom can be a pressure-cooker. The amount of material and potential importance of the class to overall grade point average or as a benchmark pre-requisite for professional schools puts extra stress on students. Many students end up failing because they feel isolated or in constant competition in class. One way to alleviate this is to be social with the students. Many instructors appear distant from their students, showing up only to lecture or hold office hours. But, starting conversations, asking casual questions of students, or asking teaching assistants to be the “eyes and ears” in the classroom can give an instructor a feeling of how the class operates socially.

A classical lecture that utilizes information dumping is perpetuated in our courses, and the overwhelming amount of material discourages students (135). Studies on microbiology classes have shown that using group activities and cooperative learning aid student learning. Specifically, inquiry-based cooperative learning, such as problem-based learning, can promote a relaxed and positive educational environment, improve peer communication and acceptance, increase ability to transfer
acquired knowledge to new contexts, and increase the likelihood of educational success (135, 139, 193). Other studies suggest an approach combining inquiry and cooperative learning enhances scientific literacy and retention, even among students who dislike science (216). Most importantly to the central themes of this chapter, another study showed cooperative learning activities increased student interest in research in microbiology (36). I have also seen this firsthand, having students interested in volunteering in the research lab after experiencing group activities in the Micro 3200 wet lab.

The techniques associated with social learning have been utilized in microbiology since it began – lab exercises. If kept away from “cookbook” types of exercises, group work in lab settings blended with inquiry is an ideal starting point for cooperation. Personally, I try to have all groups share results with other groups to teach what they learned, reinforcing the material for both the presenters and learners. Students can convince each other about information and often have better ways of explaining concepts to their peers than instructors ever considered. Social learning does not have to be confined to lab. In lectures, simple think-pair-share exercises meet the criteria of group learning as students can compare ideas with their neighbors. Dr. Eric Mazur at Harvard has been using think-pair-share “concept questions” in conjunction with large physics classes with excellent results (http://mazur-www.harvard.edu/education/educationmenu.php). Other group exercises are possible, even with very large audiences and provide an excellent way to engage students. This engagement encourages active involvement in thinking about the problem versus the “spoon-feeding” of information typically found in a classical lecture. As students realize what they know or do not know compared to their peers, the metacognition abilities increase. This is very important in my personal experience with a science audience of varied background. Students with less science background are more likely to either “give up” because they feel overwhelmed or not be aware of how little of the material they really grasp. Social learning in lecture and group lab exercises are designed to get students to realize what
they do comprehend and work together to master all of the material. When one student talks about a particular fact, the others that did not know it are stimulated to make sure they find that particular information.

Summary:

Getting students to learn microbiology is a difficult task, and even more so for those unprepared in teaching. It is easy to overwhelm students with information. But how much information is critical to understand microorganism function? Teaching a microbiology lecture course is efficient for the instructor in that it allows the presentation of a large amount of information to many students. The downside of this approach is that it fosters passive learning where students expect to be told what to learn and how to learn it. Students may not develop scientific skills or the interest to learn on their own, and enthusiasm for the course can be low for some learners. All of this is counterproductive to what we know about how students learn and the mission of the university setting. The mission of a university should be to introduce students to research and inspire in them the passion for discovery (80). The end result of poor teaching is a decrease in applicants to graduate schools in the sciences or a decrease in their quality. In the end, without addressing these issues in teaching, microbiology itself will be the biggest loser.

A Reflection on MMI Coursework:

This section aims to compare the current coursework to the problems in section II and my own personal experiences. Despite a good course selection in MMI, there has been discussion about reorganization of coursework. Here, I present several thoughts from a graduate student perspective using what I have learned from my limited education training for what could be done to improve graduate level courses. Additionally, I hope to one day set up my own undergraduate course. My
experiences with Micro 3200 have been very good, but I wondered what an “ideal” undergrad micro
course would entail. Below, I attempt to lay out the “perfect” class based on what I have discussed to
this point.

Why Not Inquiry?:

In the graduate training program for MMI, students are required to take several core courses
and electives. These classes include Molecular Biology I and II, Immunology, Advanced Immunology,
and Pathogenic Mechanisms. While the following may seem a gross oversimplification and may be up
for debate, I have personally experienced all of these classes and was left with the following
impressions: All of these classes are team-taught, so students have different instructors nearly every
week, each experts in the respective field of material to be learned. Lectures are the primary
instructional tool. Additionally, all of these classes, with the exception of Immunology, have no
textbook. Rather, students are to read, memorize concepts and material, and do presentations on
current scientific literature. There is little opportunity for discussion or exploration by the students
outside of the scope of the topics presented. There are no lab or discussion sections. Teaching styles
also vary. Some instructors are enthusiastic, while others impress to the students that it is a hassle to
be away from the bench. While this situation appears bleak, it is not as horrible as it is made out to be.
Rather, I use this to point out that our classes, while solid, are due for an update.

In comparison, the Mizzou Medical School utilizes a hybrid-problem-based-learning (PBL)
format. Medical students are given problems to consider, ask questions, and set learning objectives,
then attend lectures for background material. Students break into groups guided by a PBL tutor to
work on cases related to the material. In a sense, this is a lab exercise where students get to apply
knowledge acquired in class to clinical settings. Core concepts may not be defined, as PBL cases ask
more from the students. However, when students apply lecture material to their case questions, they
define which elements are most relevant to their learning. PBL facilitators act as guides – not experts – in the process. Students drive the learning by identifying key concepts that must be learned to understand the problem and tasking themselves to learn the concepts and teach the rest of the group.

There appears to be a disconnect between these two sets of classes that take place in the same building, although a team of instructors participates in both sets of classes. Our MMI graduate students are successful in their classes and have gone on to very good careers in research. However, research on the effects of PBL in the medical school have shown a trend of increasing scores on board exams and a significant increase from the national average (21). Researchers attribute these outcomes to the style and thought processes of PBL. After the discussion of inquiry (see section II), it would seem bizarre that graduate students in microbiology, who are to be the best and brightest in the research field, are taught with factual information dumping! The group is immersed in inquiry, so why not apply that to the classroom? It would seem a natural fit. The implications for how graduate students could connect teaching, learning, and research is vast because they are constantly reinforced that book theory is intricately tied to their benchwork.

The suggestions I would humbly put forth are fairly simple. Faculty should reconsider how they teach material. Factual dumping of information is lost on microbiology graduate students, who by this time are likely to have a knowledge framework in place and will incorporate only bits of material directly involved to their research versus absorbing all knowledge. Lab exercises or similar activities would be welcome to reinforce concepts and easy to perform with bench-competent graduate students. An alternative is to offer a “methods in microbiology” course designed to develop a broad base of knowledge about techniques, background, and history of the science. Courses like this are offered in other institutions with great success (http://www.udel.edu/chem/white). Additionally, a scientific writing class designed for helping with comprehensive exams, manuscripts, and grant writing would be beneficial as it improves job skills and writing science has been shown to increase critical thinking
ability (16). I would also recommend the addition of two brand-new elective courses to the curriculum of microbiology: A college science teaching course (modeled after the current course at MU), addressing concepts pertinent to microbiology pedagogy, classroom techniques, and utilizing material from ASM (http://www.microbelibrary.com). Secondly, addition of a course in career explorations with discussions of the professoriate, and invited speakers (including past graduates) from business and industry, government, other institutional types to present alternative views of microbiology. All of these would meet the goals of this chapter to better prepare our microbiology graduate students for careers in the future.

Restructuring to Become an “Ideal” Undergraduate Class:

The current class I am involved in teaching is Micro 3200. While having a reputation as one of the best and hardest courses on campus, there is always room for improvement. Sections 1 and 2 described the class and some of the problems that can be associated with it: misconceptions, varied audience, pressure to succeed, and large amounts of material to memorize. Given an opportunity, small changes that incorporate more student-based material or inquiry methods might benefit the course and make it even better. Alternatively, there are things I think would change for the better with the course and its organization if it were restructured in a “perfect” world. The overall learning goals of the course would be for students to appreciate concepts of the complex interactions between microbes and humans that range from beneficial to destructive; understand conserved, basic molecular mechanisms (toxins, invasion, etc) by which organisms can cause disease; and learn how microbial effects have contributed to shaping the world as we know it. Further course objectives are listed below:
**Overall Learning Goal:**

*Students will become aware of the phenomena of microbiology and understand their global impact*

**Learning Goals:**

*Students will...*

- …Appreciate the complex interactions between microbes and humans that range from beneficial to destructive
- …Understand conserved, basic molecular mechanisms (toxins, invasion, etc) by which organisms can cause disease
- …Learn how microbial effects have contributed to shaping the world as we know it.
- …Recognize the global impact (economic, environmental, social, etc) of microbiology

**Critical Thinking Goals:**

*Students will...*

- …Learn to apply knowledge of mechanisms learned in lecture setting to novel terms and organisms.
- …Understand how to evaluate and make an educated opinion on the conclusions based on the facts presented.
- …Develop writing and speaking skills in the context of scientific terminology.

**Other Goals:**

*Students will...*

- …Be encouraged to develop a real and lasting “interest” in science, microbiology, research, and current topics that will influence their thinking in the future
- …Be encouraged to try research firsthand or to consider microbiology as a career

**Teaching Goals:**

*The Instructor will...*

- …Engage students with case-scenarios and develop their desire to learn about microbes
- …Allow time for lecture material related to student-driven concepts and interests
- …Challenge student misconceptions about how microbes function and cause disease by using think-pair-share activities that force students to consider their own knowledge
- …Use social learning methodology to encourage students’ interactions and communication about science
- …Create and use open-ended collaborative activities for both lecture and lab
- …Design lab exercises that mirror how scientists approach problems in the field: by collecting evidence, justifying, connecting to knowledge, and communicating results

My new course would be re-titled Micro 2100: Essential Medical Microbiology for Non-Majors (4 hrs). The enrollment will be capped at 110 students and would be offered in the fall semesters and summer intersession. This section would be a spin-off specifically for those students with a less
extensive science background and I would expect that this would draw a majority of the pre-nursing students from Micro 3200. This would leave Micro 3200 enrollment in the area of 120-150 students, with a strong tendency towards a more homogeneous population of biology-pre-med majors. Micro 2100 would also have lab and discussion sections that run for the entire semester, unlike its predecessor that has no discussion sections and only a short lab (6 sessions). Lectures would be bi-weekly, lab would be conducted one day per week, and discussion sections would be one day per week to review material from the week presented in lecture and lab. Graduate students from microbiology would be selected to teach lab exercises in groups of 10-12 students and be responsible for the same group of students in discussion. The textbook would be changed to a modular form or switched to a text with a strong engagement factor like *Microbiology: Diversity, Disease, and Environment* (199).

The format of the course would not change drastically, with lectures still as the main segment of time in the course. However, all lectures would begin with a clinical case-based scenario where lecture material reveals key points in pathogenesis and cause of disease. Other modifications would include utilizing at least 2-3 audience participations per lecture, including think-pair-share activities and student voting. The largest change would be how ideas are presented and the scope of detail. The new microbiology course would build from the existing framework of Micro3200 and from topics the students themselves find important. Online quizzes would be posted prior to discussion sections, used to ensure students grasp the connection points between lecture material and the case scenarios, and have a chance to discuss them with TAs. I would introduce a “microbe spotlight” where the class as a whole would vote on a particular microbe from each section to be our model organism and work on mini-cases related to material about the spotlight in discussion sections

The first class session would open with a short introductory lecture (30 min) that takes the form of multiple-choice questions for the students to vote on and “test” their knowledge of bacteria. Also in the
lecture will be a brief introduction to types of organisms we will be covering (bacteria, viruses, and parasites) using “buzz organisms” (E.coli O157, HIV, tapeworms) as examples, and facts about worldwide outbreaks. The goal of this lecture is to both pique interest and gauge the level of knowledge in the class. Following the lecture, students will be rearranged into their discussion sections and asked to organize into small groups. I would encourage these groups to sit with each other and work together throughout the semester. To end the class, I would organize an in-class group exercise to design a new pathogen. Students would be asked to work with their teams to brainstorm elements they would imagine as essential to bacterial survival in a pathogenic situation. Students would then be encouraged to share their results with the class and a “master list” of ideas compiled. Additionally, this exercise would be collected and read by the instructor and TAs in preparation for the first set of lectures on immunology and normal flora. The course would include varied topics: the “proud” history of microbiology, disease outbreaks past and future, general structure and functions of microorganisms (replication, some basic chemical principles), cultivation and identification of organisms, microbial genetics, an abbreviated “bug parade” of medically important organisms, conserved virulence factors used to cause diseases, microbes in biotechnology, and vaccines. The hard tradeoff would be the amount of material versus depth of understanding of concepts, as every teacher wants their students to leave knowing as much as possible. The structure of the course would need to be carefully designed so that knowledge necessary for the currently designed licensing exams or professional certification is not jeopardized.

Laboratory exercises would try to reinforce concepts learned in lecture and include exercises such as: an in-class growth curve critical thinking active learning activity, exploring microbial diversity through personal microbe collection, biofilms, fermentation, microbial case studies, isolation of DNA, and genetic transfer and antibiotic resistance. Many of these exercises were designed with active inquiry learning methodology and can be found at the website for the American Society for
Microbiology (ASM) on [http://www.microbelibrary.org](http://www.microbelibrary.org). While involving lecture material, these exercises encourage students to design, test, and evaluate their own experiments rather than complete cookbook exercises based exclusively on lecture material.

Discussion sections would elaborate on lecture and lab material and demonstrate connections to students. Cases based on the “spotlight” organism would be introduced in discussion sections to reinforce material and act as a gauge of student understanding. Discussions will provide a forum to talk microbiology with the graduate TAs and apply student-driven concepts to the “microbial spotlight” organisms. Finally, discussion sections will be used to provide a preview of upcoming lecture material by choosing the next microbial spotlight organism and exploring scenarios involving the organism. These preview scenarios would be brainstormed by students and collected by TAs for presentation to the instructor for final incorporation into lecture material. Weekly meetings with the TAs and instructor would discuss and compare where students are experiencing difficulty with concepts.

While the goal of this section was to design a new course strategy, it was not intended to be a fully researchable proposal. Obviously, my expertise is not in designing educational studies and collaboration between science teachers and educational researchers would be of benefit to correctly address correlations between teaching styles and learning processes. The results of these changes would be measurable and expected to be in line with the aims of this chapter to improve our students’ authentic learning of microbiology by incorporation of more inquiry methodology. It is logical to assume that students experiencing microbiology in this manner would be able to work through problems better than their counterparts in other lecture based classes (such as Micro3200) where memorization rather than understanding predominates. An interesting concept test of this would be to provide students from each class a disease state and observe what factors they would deem important to the cause or resolution. Other questions of note would be: correlation of class format and increase of inquiry thinking skills; attitude towards science classes pre- and post-enrollment; sampling students’ understanding of
microbiology immediately after completing the courses versus recall of knowledge 6 months or 1 year post-enrollment; post-enrollment followup to observe the number of students that remained in microbiology, research fields, or related positions.

Furthermore, the incorporation of graduate students as full-semester TAs responsible for laboratory exercises and a discussion section provides information on the second question of this chapter in regards to how incorporation of teaching in graduate education help microbiology graduate students for their careers. Simple standards such as teaching evaluations and student comments offer insight into teaching skills learned and areas for improvement. Working in a class that utilizes inquiry techniques should be comfortable for TAs because it mirrors what they do in bench work and reinforce how teaching and research can be interconnected in the future. Pedagogy training will be available in the form of a graduate elective class offered to prepare graduate students for their discussion sections. The fact that graduate TAs have their own discussion section rather than a set lecture may help to create flexibility in the classroom.

**Becoming More Than “a Ph.D.”:**

The points of section 4 return to the abstract. Here, I consider the true meaning of being a Ph.D. in microbiology from the standpoint of teaching and research. The previous sections laid out a groundwork that earning a Ph.D. obligates one to be a teacher and a scientist who considers the science and the people involved in it (graduate students, undergraduate students, colleagues, and the general public). While some of these points may overlap with previously mentioned ideas, they reinforce what is important.

**Being a Ph.D. in Microbiology:**
So what, exactly, does it mean to hold a Ph.D. in microbiology? The report “Re-envisioning the Ph.D.” describes this “essence” of a doctorate as, “a selective, specialized degree with the singular focus of producing a creative, self-initiation, independent scholar and researcher… who can consider an array of options in terms of careers and contribute to society in many ways outside the academy” (165). Another report tasks Ph.D. awardees, “We should expect holders of the highest academic degree not simply to know a great deal, but to know what to do with what they know, both in the academy (teaching, for instance is one enactment of knowledge) and beyond it” (227). Speaking directly as one who is soon to be in that category… that’s heavy! But, in a time where the supply of Ph.D.s outpaces the demand for faculty positions (165) because of societal pressure for advancement (and requiring more Ph.D.s to do it), it seems reasonable that we as graduating students, should consider what we really stand for in a Ph.D. position and what makes us, as trained microbiologists, special.

The Academy Job (Concrete):

My goal after graduation is to land a tenure-track faculty position at a research (doctoral or masters level) institution. But, graduate students initially attracted by the perceived freedom of the science and university (like myself) now begin to realize what their professors actually do every day. Many doctoral students reported having unclear expectations of the academic career (1, 47, 153). The need to balance teaching and research responsibilities, and the expectations at different types of institutions, are especially unnerving. Comments revealed that exposure to the wide range of faculty members’ roles and responsibilities—committee work, service, teaching across disciplinary lines, faculty governance and institutional politics—often remains unaddressed in traditional TA experiences. As one student summarized, “It’s as if they spent years training me to know everything about the roller coaster. But now I’m in charge of the whole amusement park. I need to know about safety and publicity and all
the other rides. No one had taught me about them...no one had even told me they existed" (227).

Following the recommendations set out in section 3, these kinds of fears can be dealt with by creating graduate students with good science and teaching skills through changes in coursework and teaching that make them better suited for life in or out of the academy. Personally, after experiences in education classes, talking with faculty members, and my research training, I feel confident in my career choice and look forward to the challenge and stimulation of the academy.

**Potential Careers:**

Today’s microbiology graduates have a variety of potential employment opportunities upon finishing a degree. While previously mentioned studies suggest that new Ph.D.s may be ill-prepared for employment, they are still excellent candidates. Changes to graduate education mentioned above may address many of the shortcomings listed. Also, it would be beneficial that active partnerships are formed to benefit all parties. All stakeholders of graduate education: from the entire professoriate (including small colleges, four-year masters and doctoral institutions, and community colleges), to leaders in business, government, cultural institutions, and schools, should participate in graduate education to some degree to encourage a more creative approach to careers. It is important to understand that everyone who obtains a doctorate isn’t going to be emulating the mentor’s career. I need to be creative about letting my students see a broader range of life and career opportunities.

**The Job (Abstract):**

As a Ph.D., I believe I have a responsibility to advance science. To me, this means two things: being interdisciplinary, and continuing to bring people into the pipeline. A recent report, “The Responsive Ph.D.,” sums it up eloquently by describing the next generation as the “Cosmopolitan doctorate” that works by continuing interchange with the worlds beyond academia and engages social
challenges more generously (227). Interdisciplinary work is essential with the rapid explosion of scientific knowledge and material that applies to more than one science. My work and communications may provide key concepts for others, as is evidenced in trying to write this chapter. After all, “Sciences do not exist on separate planets, but in a common enterprise of human knowing” (227). While I think it conceited to say that I am “interdisciplinary” for education and microbiology given my limited encounters, I think this kind of path is open to me having experienced both and I would be more likely to think outside disciplines in approaching my future tasks.

Secondly, my responsibility to science is to ensure its continuity by being an example in my field and drawing in new people to the pipeline. Again, it seems arrogant to consider myself the “shining beacon” to which others are drawn, but I think that is a natural phenomenon for those who teach well. Throughout my graduate career, I am proud to say that I have had many undergrads volunteer in my laboratory because of my enthusiasm in the classroom to see, “what this whole microbiology thing is about.” “Becoming a teacher-scholar does not necessarily limit one’s opportunities for doing science. A thirst for rigorous inquiry, underlying scientific methodology, drives an authentic assessment of student learning. Through teaching, students are encouraged to enter a lab and continue onwards” (52). As a Ph.D., I plan to continue this trend and look forward to mentoring my own students in the same fashion I was treated – with freedom, given choices, and informed about the future. I firmly believe that mentoring is the same as teaching, just not in the classroom. Students should cultivate natural inquiry on their projects and engaged by the problems presented by the mentor. Despite wanting to create “teacher-scholar” clones of myself, it is recommended that students benefit most if I consider myself as guiding the next generation of intellectual leaders (227) and let them make their own informed choices. My preparation with solid research and teaching helps to inspire the next generation of recruits and provides a framework for mentoring in the same fashion.
Pipelining:

Good teaching and being a Ph.D. has broader effects than just in the classroom. A recent report stated that poor teaching was the cause of student unhappiness in undergrad science education (153). Introductory science courses are typically identified as the biggest barrier for pursuing science, technology, engineering, and mathematics (STEM) degrees (1). The experience that the majority of students have after such courses is that of listening to many hours of lecture, reading, and memorizing material from a text. Large courses such as Micro 3200 risk being labeled “boring” (15) or “old-fashioned” (199) if students are not engaged with inquiry or other methods. The end result of this is simple. Students are “turned off” to a science and will look elsewhere. This kind of discouragement is unacceptable because top students that might have entered microbiology graduate programs are lost. Keeping a pipeline open to recruit students into micro requires Ph.D.s with good teaching skills.

Public Understanding:

Subsequent to the last point, students who leave microbiology classes uninterested and misunderstanding the material become members of the general public. While undergraduates are a captive audience because of grade-point averages, the general public has the luxury of ignoring microbiology entirely. At risk of sounding self-important, this is the voting public that controls more of the science than we would like to admit through policy and funding allotment. There is danger in a population that lacks familiarity with microbiology. One need not look any further than recent issues of stem cells on the Missouri ballot or recent *E.coli* O157 outbreaks in spinach to understand that the population needs microbiology knowledge. Instead, ignorance and misconception surrounding microbiology phenomena are prevalent in our society. Scientists may feel that the general public does not care about microbiology, but the opposite is likely true. Topics like bird flu, vaccines, disinfectants, HIV, and anthrax are all nightly news stories watched by millions. The catch is that these topics have
to be presented in a language free of jargon and in a way that emphasizes the big questions and puts the concept in a familiar context. This becomes the challenge for scientists. After all, we are scientists, not public relations professionals, but perhaps we should start. The general public should know how diseases affect them and how to separate fact from fiction when it comes to their health. In the highly likely event of a new outbreak, disease, or bio-terrorism, it is critical that people have a scientifically-based rationale and intelligent approach that will help turn the tide in our favor. This kind of approach stems from the inquiry methods used in science classes.

Furthermore, public influence now holds more power in the changing face of scientific funding. As policy is created that allots tax monies and as private industry/foundation funding increases, the voting public decides on what to spend money researching. To keep the science moving forward, it is essential to have an educated public serving as gatekeepers rather than hindrances to the research of the future. A population with no concept of why research in microbiology is important could devastate scientific progress.

**Reaching Out:**

One major complaint of college professors is that students come to them unprepared for the rigors of science classes at the university level or without proper background in science for advanced classes. Yet, they fail to be proactive about the situation and collaborate with K-12 teachers to better prepare students for the future. Two striking quotes to illustrate the frustration in this impasse: “Today’s schools are the disgrace of the nation. We need everyone’s help. What are Ph.D. programs doing to help us?” and, “We are hoping to become the best urban school district in the world. Are Ph.D.s going to help us do that or are they off in their own worlds, doing other things?” (165). If we desire better students, we should help cultivate them. Over half of doctoral students want to provide community service or outreach programs about their science (79). Ph.D.s and college science teachers should be
willing to reach out and help these programs to develop learning strategies and lifelong learning in students. Partnerships with younger learners foster interest at nearly any age as children are naturally curious. Successful programs with this cooperation have been reported (3).

The “Outsiders”:

I need to be active outside of the academy. My research is easily spread in journals and at scientific meetings, but the rest of my talents and knowledge should be put to use as well. The role of "service" for a faculty member comprises more than just serving on committees. For other institutional types, service incorporates public outreach, and I do not know why that would be any different for me. Because our science is constantly in the news, the general public likely has a greater desire to learn, but does not know the outlet to find information. I can be that outlet. Microorganisms constantly amaze me. Did you know there are bacteria in which it takes fewer than 10 organisms to cause disease? Did you know there are organisms that thrive in boiling water or in 1000 times the radiation necessary to kill a human? Did you ever stop to think how well your “99.9% effective” cleanser works on $1 \times 10^8$ organisms that duplicate every 40 minutes? I could write an entire chapter on how bacteria really may be smarter than humans, even though we consider ourselves the pinnacle of evolutionary achievement. Without bacteria, we would cease to exist. Without us, bacteria would not even notice! I question if the population really comprehends how much of an impact microbiology has on their lives everyday: fossil fuels, health, bread and beer, global oxygen supplies… All microbe-related. My goal is to get people to be as excited as I am about science and microbiology and break the misconceptions and half-truths perpetuated by the media. I can do this by teaching others the information that I have learned. A report tasks us as microbiologists to spread the word through TV, radio, podcasts, and more (199), with which I heartily agree! My efforts create and informed citizenship that I can feel is ready to understand issues like stem cells and E.coli.
I look forward to participating in outreach programs that can spread microbiology. My brother-in-law is a biology teacher at Rock Bridge High School and we have already started talking about how we might be able to arrange a set of lectures in his class. Moreover, the Wakonse conference uniting high school and college teachers mentioned in section one demonstrated to me that schools WANT trained scientists to come and be active in the classroom. If scientists and university professors do not want to have students in their classrooms that are ill-prepared, they should be proactive about wanting to fix it be being part of the change!

**The Future:**

At risk of seeming extraordinarily sentimental, I have to reiterate that these are my convictions and goals as a teacher and scientist: to continue working on my teaching and research to aid learning and understanding of microbiology, to work across disciplines, to teach and do research by example for the continuation of my science, and to seek new ways to apply academic knowledge to social challenges and promote public understanding. My training has produced a competent bench scientist. I have become a researcher in the classroom as well, using my science skills to reach conclusions about my teachings and student learning based on the collection of data from exams and techniques. As a scientist, these skills are not new to me.

**Suggestions for Graduates Interested in Teaching:**

Through my experiences, I think I have gained knowledge beyond my bench training as a graduate student. The goal of this section is to present some suggestions to others interested in following a similar path that includes teaching and research.

**Graduate Minor in College Teaching:**
One of the first things that I feel has helped me immensely was the decision to earn a Graduate Minor in College Teaching (GMCT). This certificate is offered through the graduate school for an additional 12 hours of coursework. More information can be found at (http://gradschool.missouri.edu/mict/degree.htm). There are 6 hours of core courses, one 3-hour elective, and a 3-hour teaching practicum associated with the GMCT. Students interested in a GMCT need to apply to the graduate school and get a letter of approval from their mentor and departmental chair. Coursework to be taken and a plan of action for the teaching practicum must be turned in to the graduate school. Most of the coursework consists of evening classes, usually 1 day per week, so it is not overly disruptive to research if planned for correctly. I would recommend doing the education coursework once finished with all departmental classes and established in a research project.

For core classes, I selected College Science Teaching, taught by Dr. Sandra Abell. I am happy to say that this was one of the best courses I have ever taken at MU because of the instruction and how it forced me to look at my own teaching. College Science Teaching was the course that first made me realize that what I was doing in the classroom was not true “teaching” but rather “talking towards my students” in the hopes they would understand. In the course, students write a statement of teaching philosophy, confront misconceptions, and observe award-winning teachers on campus. Short papers and reading are required for the class. The second core class was also beneficial: The Professoriate, taught by Dr Barbara Townsend. This class was geared to explain the life and times of an academic faculty member that were not always visible. Discussion topics ranged from promotion and tenure, academic freedom, balance of work and personal life, teaching/research/service, institutional types and differences, and the changing face of academia. Students were expected to interview a faculty member about his/her experiences and write several short papers. My elective was The Community College, an exploration of a different academic type from the research university. The course involved learning about the history and future of the community college campus, adjunct faculty, a visit to
Moberly Area Community College, and a final project to create our own community college in an area of our choosing (including background research, courses to be offered, campus, etc) that was done as a presentation to the class. While beneficial, in retrospect I would have chosen a different elective about human learning or the college atmosphere, as it more closely resembles my career goals. In short, it was good I learned that the community college is not for me! The practicum was completed using my teaching experience from Micro 3200, with some additional work. I completed a reflective journal about my teaching experiences and did self-evaluations for each lecture I completed.

Committee:

As mentioned previously, I asked Dr. Abell to join my committee as an education advisor. Because my goal has been to gain experience in education and relate it to microbiology, Dr. Abell has been very helpful in that regard. It was because of her that we decided to add this chapter to my dissertation. This kind of chapter added to a thesis may be difficult to write (and believe me, it is!), but if our graduate training is to change, it will take small steps that need to be compiled into a working body of knowledge. Also, when the time comes to choose a committee, I would advise graduate students to add members who can not only improve your research, but are good teachers that you personally have experienced. If your goals are to work on teaching, it is important that your committee agrees with you.

Mentor:

In regards to the committee, your primary support as a graduate student comes from your mentor. Likewise, he/she must be attuned to your goals of both teaching and research. A long time ago, when I was choosing a lab, I wrote a set of questions for myself to ask each investigator I was interested in. The list included questions about projects available, lab dynamics, mentoring style, and thoughts about teaching. That list was eventually given to the Graduate Student Advisory Committee.
for our department as things to consider when choosing a mentor. Without being specific, there are faculty members who have stated that teaching is a waste of time compared to research training. Obviously, these are people to avoid as mentors if teaching is an important goal for you. A final note about mentors is on finding the time and money to do the extra coursework. Mentors may be responsible for paying the graduate tuition for his/her students. Depending on the funding situation of the lab, this may not be a feasible event for some investigators, in which case tuition must come out of pocket from the student. Mentors may also be supportive of teaching, but demanding about the order of how things get accomplished. My mentor has been wonderful in that I am free to go teach whenever I wish, as long as I can still produce results on my project. Other mentors may not be as flexible. This could be a bad situation if in the middle of the semester, you are called back to the bench for last-minute preliminary data for a grant. Be aware of how a mentor prioritizes.

Comprehensive Exam:

As mentioned in section I, my comprehensive exam was different from the normal path in MMI. Discussion with my committee allowed me to write an NSF CAREER grant as opposed to a NIH R01 research grant. This format of CAREER incorporates an education section integrated with the research proposal. My comps are on file in the MMI office for reference.

To me, this experience was very beneficial. I not only tested my research skills to write a cohesive grant with specific aims, but also attempted to correlate this to an educational aim. I will be honest when I say that each section was a success, but the overall integration was lacking. Discussion with my committee was that the research (biofilms and persistence) was fine and the idea for creating a class based on that research was fine. The problem was that the goal of inquiry and lifelong learning failed. My designed course was the same as every other course: lecture based, exams and papers, with a small lab component. I had designed the same kind of course I have been deconstructing
throughout this chapter! In retrospect, thinking a little further about the designed course in terms of
how this chapter is laid out would have produced better results. To truly design an inquiry-based
course, the crux of the designed class should have revolved around a question rather than explanation
of how the material is tied to the research portion of the CAREER grant. Letting students find their own
biofilm and ask questions about it that then become the lecture topics or discussion/lab activities would
have been a better starting point than a lecture based approach.

**Faculty Influence Graduate Student Attitudes:**

Graduate students are trained as observers. Hence, they see faculty attitudes towards teaching
and often use those as models. In MMI, there are good teachers and there are teachers who need
improvement. Graduate students unfortunately learn that either kind of teaching is acceptable because
the classes always remain the same from semester to semester. This may be due to the fact that most
current faculty were trained and instructed in a “trial by fire” teaching methodology. They continue to
train graduate students in the same mold, where teaching can wait behind research. This kind of
stance is visible in the instructor as they present. As a result, graduate students adopt an attitude that
teaching is a waste of time away from the bench. A personal example of this is what started me on my
teaching path in the beginning: A graduate student decided that his time was better served in remaining
at the benchtop than teaching.

Protection from teaching is another thing graduate students see that may influence their attitudes.
New faculty members are often shielded from the classroom as part of their contract with the university.
The reason behind it is sound: to allow new faculty to get established with bench research and grants
without other distractions and gain a foothold on important promotion and tenure criteria. Yet, it sends
a message that teaching is undervalued. Very few hard science faculty are tenured on teaching alone,
but can be tenured on research alone or with mediocre teaching skills.
Help on Campus:

Being interested in teaching does not place you on an island. There are numerous resources available here at MU to help improve teaching without formal classwork or additional time. The MU Program for Excellence in Teaching (PET) will set up free classroom visits, evaluations, and videotape to critique teaching. The Wakonse group (http://www.wakonse.org) is dedicated to improving teaching. Monthly roundtables on teaching are held by the MU Science Education Center and provide an excellent forum for interdisciplinary exchange and insight into teaching methods others are using on campus. The graduate school also maintains a list of teaching resources on campus and beyond. Finally, do not be afraid to talk about teaching with others in the department. Science does not have to dominate the conversations around the department. I have numerous conversations with faculty members involved in teaching about the techniques they use and how they organize materials and courses. The interested parties are the ones who need to be proactive.

Get In and Do It:

I found this quote in my reading that really made a lot of sense to me: “I really dove into the pedagogical waters as a grad student, teaching labs. It was sink or swim, and I struggled at first, but eventually learned to dogpaddle” (52). The only way to find out if teaching is interesting to you is do it! One thing I will stress is that all the extra work I put into this does not make me a better teacher than others. Some people come into teaching already knowing how to “dogpaddle.” We have numerous graduate students and faculty who have had no teacher training or education classes and are very good teachers who use techniques similar to what I have described here. I feel that what my experiences have done for me is to make me more prepared to deal with challenges I see in the classroom and makes me more flexible in terms of techniques at my disposal.
Reorganization:

Finally, I would suggest a reorganization of the MMI coursework to include some of the above points. The curriculum committee has discussed additional elective courses such as parasitology and virology. Why not add a teaching preparation class to the electives? It could be taught along the same concepts as College Science Teaching, but take a deeper slant on microbiology. The goal of the class would be to introduce graduate students to pedagogy and its applications to inquiry in the classroom and lab. The American Society of Microbiology (ASM) has two of its own education journals as well as resources that could be used in such a class (http://www.microbelibrary.org). Finally, there is a need for more teaching opportunities for graduate students. Suggestions for this would be to re-structure existing classes to have discussion or lab sessions that graduate trainees would be able to participate in, create a new undergraduate class or spin off Micro 3200 as an undergrad non-major class that graduate students could participate in, or open teaching opportunities across campus. One current initiative is to create a doctoral teaching internship program at MU where doctoral students would have a mentored teaching experience (70).

Conclusions & Challenge:

I am very excited to start my career in science as both a researcher and teacher. There are many aspects I feel I have improved in because of my graduate training and educational goals: gaining flexibility in the classroom, being prepared as I approach my teaching, grasping of some pedagogical techniques, understanding mentoring as “teaching, just not in the classroom,” seeing myself as an important part of learning beyond the classroom to both new learners and the general populace, and understanding overall faculty roles and balance. Most importantly, I think that realization of “science as inquiry” makes me reconsider how I teach and do research and how to link them together. This will
improve science overall by cultivating thinking patterns that concentrate not on memorizing facts, but on reasoning through problems based on facts and applying the material to the new microbial situations that will face us in the future. This concept applies to both how I view my future interactions with undergraduates and their learning and how I can better train graduate students as I become a mentor.

I would like to end this chapter with a challenge. As one who feels well-trained leaving the graduate program by incorporation of material outside of my discipline, I think graduate education could be restructured in a similar format to even better prepare our students. Addition of teaching preparation courses covering pedagogical techniques and classroom issues as graduate electives will develop better prepared faculty for future teaching duties. Finally, graduate students should be indoctrinated into more than just their own research by offering and encouraging additional opportunities for teaching in discussion, lab, or lecture settings. Finally, the hard science disciplines, like microbiology, should encourage inquiry methods in all classwork for both graduates and undergraduates to create better prepared candidates for its pipeline and a better educated public for issues important to science.
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