The Role Of Calcium Ions In Signaling And Regulation Of Efflux Pump Genes In Staphylococcus Aureus

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THE ROLE OF CALCIUM IONS IN SIGNALING AND REGULATION OF EFFLUX PUMP GENES IN *STAPHYLOCOCCUS AUREUS*

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by

Amy R. Nava

2021
DEDICATION

This dissertation is dedicated to Dr. Delfina C. Dominguez for her patience, encouragement and mentorship. I also want to thank my family and friends for their kindness and support.
THE ROLE OF CALCIUM IONS IN SIGNALING AND REGULATION OF EFFLUX PUMP
GENES IN *STAPHYLOCOCCUS AUREUS*

by

AMY REBECCA NAVA, BS, MS

DISSERTATION

Presented to the Faculty of the Graduate School of
The University of Texas at El Paso
in Partial Fulfillment
of the Requirements
for the Degree of

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ABSTRACT

Calcium ions (Ca$^{2+}$) play an important role in eukaryote cell signaling and regulation of physiological functions. Although evidence of a similar role for Ca$^{2+}$ in prokaryotes has been difficult to demonstrate, there is mounting evidence that Ca$^{2+}$ acts as a cell regulator in bacteria. The purpose of this study was to investigate Ca$^{2+}$ signaling and the effect of Ca$^{2+}$ on gene expression of various multidrug resistant (MDR) efflux pumps and their regulator MgrA in Staphylococcus aureus and clinically isolated MRSA. We hypothesized that the presence of Ca$^{2+}$ increased gene expression of multidrug resistance pumps, LmrS, NorA, and the regulator, MgrA. This hypothesis was based on our previous evidence of unique intracellular Ca$^{2+}$ concentration signatures in response to various antibiotics and the enhancement of ethidium bromide efflux by Ca$^{2+}$ in S. aureus. The effect of Ca$^{2+}$ on efflux gene expression was evaluated by the addition of increasing concentrations of CaCl$_2$, and the Ca$^{2+}$ chelator, EGTA. Gene expression of the efflux genes and the regulator, MgrA were measured and compared to housekeeping genes, GMK and GyrA under the same conditions. Our results indicate that the presence of Ca$^{2+}$ significantly enhanced gene expression of MDR efflux genes as compared to untreated and EGTA treated cells. The results indicate that the presence of EGTA significantly decreased efflux gene activity as compared to untreated cells and Ca$^{2+}$ treated cells. We conclude that Ca$^{2+}$ may be a signaling molecule for the regulation of gene expression of efflux in S. aureus. Transcriptomic studies are needed to determine the proteins involved in potential calcium transduction pathways for genetic regulation of efflux in S. aureus.
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CHAPTER 1: INTRODUCTION:

1.1 Properties of Calcium

The chemical properties of calcium made it the best candidate as a secondary messenger in the evolution of cell signaling. To fully understand Ca\textsuperscript{2+} homeostasis, one must consider the chemical properties of Ca\textsuperscript{2+} as an explanation of how living cells have evolved signaling mechanisms.

![Figure 1](image.png)

**Figure 1**: (Carafoli, E., 2016) Illustrates the chemical properties that contribute to the binding differences between Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. The higher Ca-O distances allows for a larger variety of binding to proteins with an irregular pattern of binding.

Calcium is the third most abundant metal on earth and was available in large quantities when life began (Carafoli, E., 1987). The ability of metals to bind to ligands depends on hydration energy (this is the easiness of water molecules being stripped off from the metal, polarizability, and the radius of the metal with a hydrogen atom, which determines charge density (Carafoli, E. 2016). Most importantly, Ca\textsuperscript{2+} has a unique coordination flexibility that is much higher than other ions such as Mg\textsuperscript{2+} and Na\textsuperscript{+} that are commonly used in eukaryote functions. The coordination flexibility is defined as the ability of a metal to bind to proteins. (Carafoli, E., 2016). The coordination flexibility of Ca\textsuperscript{2+} is due to how the bond length and angle of Ca\textsuperscript{2+} can easily change to accommodate different ligand binding sites of proteins. In order to be used as a signaling agent, the metal ion must bind to the protein target both tightly and with high specificity. These
properties combined explain why it is easier for Ca\textsuperscript{2+} to bind to complex proteins. The specific chemical properties of Ca\textsuperscript{2+} have more flexibility than Mg\textsuperscript{2+} for proteins to bind and disassociate easily allowing cells to control their intracellular concentration. Hence, allowing for the intracellular concentration to be easily manipulated and to maintain cytosolic Ca\textsuperscript{2+} in the nM range and is crucial for the cell to maintain low levels of intracellular Ca\textsuperscript{2+} concentrations despite higher concentration levels outside the cell. Typically, bacterial cells regulate intracellular Ca\textsuperscript{2+} concentrations in a range between approximately 100-300 nM (Knight et. al., 1991; Torrecilla et. al., 2000). When a signal or environmental stimulus is transmitted cytosolic Ca\textsuperscript{2+} increases (Figure 2) allowing the cell to influx Ca\textsuperscript{2+} through ion transporters or release Ca\textsuperscript{2+} from Ca\textsuperscript{2+} storing compartments. Hence, tight regulation of intracellular Ca\textsuperscript{2+} ions is essential for cell signaling.

![Figure 2: Cell response to many stimuli by transient changes in cytosolic Ca\textsuperscript{2+}](image_url)

1.2 Calcium and Eukaryote signaling:

For many years, calcium signaling was believed only to exist in eukaryotes. However, recently it has been demonstrated that prokaryotes are equipped with similar proteins and sensory mechanisms (Domínguez, et. al., 2004; 2015). Both, eukaryotes, and prokaryotes have transmembrane signaling proteins that lead to effector proteins that either change gene expression or catalyze biochemical pathways (Figure 3). The importance of signal transduction in cells is the ability to sense stress and effect a change to cope with changing environmental pressures. Cells need a variety of different sensors to detect differences in temperature, pH, osmolarity and drought. In all three types of cells: plants, mammalian and bacterial, there is a basic idea governing signal transduction, consisting of a signal being sensed followed by transduction or a conformational change in the sensor protein and finally a cellular response (Verkhratsky, A., 2007). Calcium binding proteins are an integral part of this cellular response. In the following section, the basic mechanism of signal transduction of the three cell types will be discussed.
1.3 Eukaryotic G-Protein Coupled Receptors (GPCRs)

Sensing and transmitting signals are important for the survival of prokaryotes and eukaryotes. The ever-changing environment exposes cells to different insults and conditions. As a result, both eukaryotes and prokaryotes have evolved signaling mechanisms to sense and distinguish each specific environmental condition (Oh, S., 2018). The beginning of the transduction pathway for both eukaryotes and prokaryotes begins with a sensing protein which acts as a transmembrane receptor and signaling device. Signals in the environment are often molecules that bind to the receptor part of the protein. Signals can also be fluctuations in pH, salinity and temperature along with other physiological changes. In mammals, this protein is known as a G-protein coupled receptor (GPCR). GPCRs consist of approximately 7-8 transmembrane helices (Palczewski, K., 2014). GPCRs consist of receptors, which bind to a ligand that binds in a specific pocket of the receptor. The receptor binds to the ligand and a
conformational change occurs in the cytoplasmic domain. The conformational change causes an exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP) and activates a specific transduction pathway depending on the ligand that it binds to. Phosphorylation is crucial for both eukaryote and prokaryote signal transduction (Urao, T, 2000).

**Figure 4**: The basic steps in GPCR transduction signaling (Siderovski, D.P., 2005)

Classical Ca\(^{2+}\) pathways begin with a stimulus of the GPCR. The result increases the inositol triphosphate (IP3) levels when phospholipase C cleaves phosphatidylinositol bisphosphate (PIP2) into IP3 and Diacylglyceride (DAG). Higher concentrations of IP3 result in higher levels of cytosolic Ca\(^{2+}\). When IP3 binds to the IP3 receptor on the endoplasmic reticulum, Ca\(^{2+}\) is released (Palczewski, et al., 2000). Once IP3 binds to the IP3 receptor, restoration of normal Ca\(^{2+}\) concentrations begin by exporting Ca\(^{2+}\) through the plasma membrane through Na\(^+\)/Ca\(^{2+}\) transporters. The Calcium ATPase, sarco endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) then refills the endoplasmic reticulum. Ca\(^{2+}\) acts as a secondary messenger to activate different pathways. For example, calcium signaling occurs when hormones bind to GPCRs. IP3 triggers the release of calcium from storage compartments such as the endoplasmic reticulum.
and is responsible for various functions including muscle contraction and metabolism (Berridge, M., 2003, 2016).

1.4 Plant two component systems (TCS)

Despite the multicellularity of plants, their signaling mechanism is more like prokaryotes than animal GPCRs. Plants have two component regulation systems that upon activation, become phosphorylated at a Histidine residue (Lohrmann, J., 2002). The Arabidopsis plant is perhaps the most well studied plant due to its small and easily manipulated genome. The two-component signaling system ETR1, has been well described and consists of ethylene receptors. There have been two kinds of TCSs described in plants. The simple step TCS is very similar to the bacterial TCS. In a similar way, an input signal is received, and the histidine kinase domain induces autophosphorylation by ATP. The phosphate is then transferred to the response regulator which initiates an output response. In the multistep two-component signaling system, the Histidine containing phosphotransferase (HPt) domain serves as an intermediate step of transferring the phosphate from the kinase to the response regulator (Lohrmann, J., 2002).
Salt stress, mechanical injury and insect damage cause cell depolarization and an increase in cytosolic Ca\(^{2+}\) transients (Gilroy, S., 2016). These signals travel in waves through the cells in the various plant structures (Gilroy, S., 2016). As with prokaryotes, plants also have proteins such as ion exchange transporters, Ca\(^{2+}\) binding proteins, vacuoles for Ca\(^{2+}\) storage and proteins that function as Ca\(^{2+}\) buffers. Many of these proteins have similar motifs as calmodulin or EF hand (Tujeta, N., 2007). Calcium specific pathways in plants have not been fully elucidated as in mammalian cells. However, an overarching hypothesis in plant Calcium signaling is the calcium signature switch hypothesis (Tuteja, N., 2007). The signature switch hypothesis states that specific genetic sequences encoded in DNA determine the type and stimulus of the Ca\(^{2+}\). Further, specific genes determine how to interpret these varied signals. These Ca\(^{2+}\) signals differ in duration, amplitude, and frequency.
1. 5 Prokaryote Two Component Regulatory Systems (TCS) and Transduction

The sensory and response mechanisms of prokaryotes have many similarities to eukaryote GPCRs. The TCS consist of two parts a sensor kinase for sensing changes in the environment and a response regulator to effect change. Like eukaryote GPCRs, bacterial TCS consist of a receptor, transmembrane helices, and a cytoplasmic region. The other component in the TCS is the response regulator and is unique to prokaryotes and plants despite that the functionality is similar to G-proteins. When a ligand binds to the receptor the response regulator becomes phosphorylated by the sensor kinase and due to a conformational change, is considered activated. Phosphorylation is crucial for both eukaryote and prokaryote signal transduction (Urao, T, 2000). Proteins can then bind to DNA to carry out a response to the signal or facilitate biochemical reactions.

The TCS of prokaryotes also exchanges a nucleotide upon activation and conformational change. However, the nucleotide in this case is adenine diphosphate exchanged for adenine triphosphate. The sensor kinase of the TCS becomes auto phosphorylated usually at a Histidine residue but Tyrosine and Serine kinases have been discovered in bacteria. In prokaryotes, the transduction pathway consists only of the TCS and actual gene activators and repressors.

Calcium transduction pathways have not yet been found in bacteria. However, various studies have found a link between Ca$^{2+}$ and physiological processes. These physiological processes include efflux, chemotaxis, and virulence (Nava et al., 2020; Martins, A., 2011; Holland, I., 1991; Alcalde-Rico, M., 2016). Changes in intracellular Ca$^{2+}$ in bacteria as a response to heat and cold shock as well as other environmental changes implies that Ca$^{2+}$ signaling is occurring. Further evidence of this is intracellular Ca$^{2+}$ concentrations in prokaryotes are at very similar ranges as eukaryotes between 100-300nM. Since eukaryotes utilize Ca$^{2+}$ in
signaling for transduction pathways, similarities between eukaryotes and prokaryotes implies prokaryote utilization of Ca\(^{2+}\) for signaling and gene expression. Both prokaryotes and eukaryotes keep tightly controlled intracellular Ca\(^{2+}\) despite environmental signals causing increases cytosolic Ca\(^{2+}\) (Domínguez, D.C., 2018).

![Diagram of Roles of Calcium in Bacteria](Dominguez, D.C., 2018)

**Figure 6**: The potential roles of calcium in the physiological functions of prokaryotes. Retrieved from (Domínguez, D.C., 2018)

1.6 *Staphylococcus aureus*:

*Staphylococcus aureus* is one of many drug resistant bacterial human pathogens. *S. aureus* is a gram-positive, non-motile and non-spore forming cocci that is catalase and coagulase positive. *S. aureus* is found in the normal flora of humans as well as in the environment. According to the Center for Disease Control and Prevention (CDC), methicillin resistant *S. aureus* (MRSA) can be acquired in community settings or in health care settings (Klevens, et al., 2007). *Staphylococcus aureus* is a versatile pathogen that can cause a wide variety of infections and is able to survive in various environments (Onyango et al 2018; Dastgheyb and Otto, 2015; Li, et al., 2015). The unique adaptability of *S. aureus* makes this organism one of the most
important bacterial pathogens worldwide (Hassan et al., 2018; Onyango & Alreshidi, 2018; Dastgheyb et al., 2015; Kobayashi et al., 2015). The survival strategies of this organism are diverse including the ability to replicate in phagosomes, production of a number of virulence factors such as hemolysins, immune evasion factors, and resistance to cationic antimicrobial peptides, which leads to survival in host cells (Kobayashi et al., 2015; Fraunholz & Sinha, 2012). It is important to point out that Ca$^{2+}$ plays an important role in cell adhesion, modulation of biofilm architecture, modulation of $\alpha$-hemolysin and autophagy (Fraunholz & Sinha, 2012; Eichstaedt et al., 2009; Arrizubieta et al., 2004; Thomas et al., 1993).

$S.\ aureus$ can develop resistance to numerous antimicrobial compounds, including antibiotics and biocides (Foster, T.J., 2009; Conceição et al., 2016; Jang, 2016; Santos Costa et al., 2013; Esposito et al., 2011). Although the resistance developed by $S.\ aureus$ strains may be due to different resistance mechanisms, multi-drug resistant efflux pumps (MDREP) play a major role in mediating cross-resistance to antibiotics and biocides (Foster, 2017, T.J.; Conceição et al., 2016; Sapula & Brown, 2016). In this dissertation, we will focus on one of the four major mechanisms of resistance, efflux, and the role of calcium in the regulation of efflux.

1.7 Methicillin Resistant $S.\ aureus$ (MRSA) and Mechanisms of Pathogenesis

The rise of MRSA first began in the 1960’s and evolved into two strains that could be acquired nosocomial or from community sources (Abraham, J., 2004). The community acquired MRSA were susceptible to non-$\beta$-lactam antibiotics. Interestingly, these community acquired MRSA strains also carried the virulence factor, Panton-Valentine leucocidin (PVL) and these strains were designated USA300, USA400, USA1000 and USA1100 (Morgan, M.S., 2007). Panton-Valentine leucocidin has been found to be an indicator of the disease-causing strain of
community acquired MRSA. However, a 2006 study by Voyich, et. al. showed that the type and severity of disease-causing community acquired MRSA did not have any correlation to PVL but consented that it might be involved in the epidemiology of necrotizing pneumonia. The nosocomial strains of MRSA were designated as strains USA100, USA200 and USA500 (Klevens, et al., 2007; Murray et. al. 2009). Penicillin binding proteins (PBPs), specifically PBP2, play a major role in methicillin resistance (Hiramatsu, K., 1995; Sekiguchi, K, 1995). MRSA confers resistance to methicillin mainly through the \textit{mecA} gene (Hiramatsu, K., 1995). The \textit{mecA} gene codes for PBP2. PBPs have a high affinity for \(\beta\)-lactams however, PBP2 has a very low affinity for virtually all beta lactams (Hiramatsu, K., 1995; Yao, X., 2012; Bush, K., 2016). Once bound to \(\beta\)-lactams the transpeptidase domain stops the synthesis of peptidoglycan ceases. Moderate resistance to methicillin is achieved through penicillinase, an enzyme that cleaves the lactam ring rendering it harmless. Penicillinase allows for partial resistance by hydrolyzing the antibiotic but is not completely resistant. The acquisition of MecA is important as it confers resistance through modification of the target proteins, PBPs. The \textit{mecA} gene is present in MRSA but not in clinically isolated strains of methicillin susceptible \textit{S. aureus} (MSSA). The acquisition of \textit{mecA} is not solely responsible for resistance but rather the level to which the gene is expressed. According to Hiramatsu, K., 1995, the level of expression can vary the MIC producing ranges from MIC<16 to MIC>2000. This is due in part to the \textit{S. aureus} strain as well. According to the same study, if the \textit{S. aureus} strain has the N315-type mec-region of DNA, the strain is considered susceptible to methicillin.

Biofilm deposition is a key strategy of antimicrobial resistance. Biofilms offer a protective layer that allows for evasion of biocides. Biofilms are a complex matrix of polysaccharides that adhere to surfaces. These biofilms are a community of sessile bacteria and
result from molecular communication between bacteria, known as quorum sensing (Bodelon, et al., 2016; Shirtliff, M., 2002, Verderosa, A., 2019). Quorum sensing is crucial for survival as it is a way for bacteria to communicate in response to cell density and growth as well as changes in the environment (Waters, C., 2005; Bodelon, G., 2016). The phenomenon of quorum sensing also involves the production of small molecules, known as autoinducers (Verderosa, A., 2019) such as acyl-homo serine lactone (Schaefer, et al., 2008). The result of the small molecules moving into the matrix is the simultaneous up-regulation of genes to secrete exopolysaccharides and other polymeric substances for the making of biofilms. Quorum sensing in gram positive bacteria involves the up regulation of a two-component regulatory system (Waters, C., 2005). Gram positive quorum sensing is accomplished through oligopeptides which require transport systems since they do not diffuse across the membrane (Waters, C, 2005; Wai-Leung, N.G., 2015). For S. aureus, communication at high concentrations initiates the release of the autoinducing peptide that secretes toxins and proteases (Ji, G., 1995; Jarraud, et. al., 2002; Peng, et al., 1988). S. aureus secretes an octapeptide which is a ligand for the transduction pathway controlled by the agr gene (Ji et al., 1995).

Biofilms have been studied and have shown structural complexities. These complexities include differences in shape mushroom or pillar (Ben-Ari, E.T., 1999). The biofilm also has many complex channels that allow for nutrient capture and can grow on living or abiotic surfaces. Biofilms are also produced as a response to stressful conditions and often, in addition to other functions, provide a buffer zone from the harsh environmental pressures. Bacteria that are embedded in biofilms have a decided advantage. Biofilms can disperse and form microcolonies in other areas by becoming unattached. The colonization strategy for many bacteria is to simply release motile bacteria into the environment to colonize other surfaces. This allows for a constant
supply of colonizing bacteria and biofilms hence, increasing the density of a species in a particular niche (Nadell, C., 2016). Nested within the matrix of the polysaccharide biofilm, bacteria are protected from immune cells as well as antibiotics which are restricted to the outside of the biofilm. Other advantages include protection from bactericides, a changing environment and effective cell communication for gene expression (Ji, G., 1995).

The importance of efflux in biofilm formation has been studied in various bacteria. There are key roles such as extrusion of exopolysaccharides and quorem quenching molecules (Alav, I., 2018). One of the first studies by Ren et. al., 2003 found that efflux pumps are more highly expressed in biofilm bacteria than in planktonic bacteria. Indicating the importance of efflux pumps in biofilm formation. The efflux genes mdtF and lsrA were both highly expressed during the formation and growth of the biofilm.
Figure 7: retrieved from Alav, I., 2018. The potential roles of efflux pumps in biofilm formation. This is accomplished through influencing aggregation, extrusion of EPS and QS molecules and indirect regulation of biofilm genes through efflux of inducer molecules.

1.8 Bacterial efflux pumps:

Efflux pumps have been identified in both gram-negative and gram-positive pathogens. In the literature, it is understood that efflux pumps have other important physiological roles besides the extrusion of antibiotics and biocides. These roles include biofilm formation, transporting toxins and lipids, as well as cell signaling (Du, et al., 2018). The efflux mechanism is particularly important because bacteria are able to reduce intracellular concentrations of biocides but also increase the potential for mutation allowing for the evolution of resistance (Sun, J., 2014).

There are generally two main classes of efflux pumps in bacteria. The primary active transporters which utilize ATP as an energy source to transport substrates across the membrane. The other class consists of secondary transporters that utilize ion or electrochemical gradients to transport substrates across the membrane (Kumar, et al., 2016). Seven types of multidrug resistance efflux pumps have been identified in S. aureus: the Major Facilitator Superfamily (MSF), ATP-Binding Cassette (ABC) Superfamily, Multidrug and Toxin Extrusion (MATE) family, The Resistance Nodulation Division (RND) and the Small Multidrug Resistant (SMR)
family. More recently, the Proteobacterial Antimicrobial Compound Efflux (PACE) family has been identified as a major family of efflux pumps and the P-aminobenzoyl-glutamate transporter (AbgT) (Hassan, et al., 2015; 2018).

Efflux pumps are thought to be present in all bacteria. This implies that there are other physiological roles for efflux pumps. In general, the primary role of efflux pumps is detoxification (Sun, J., 2014; Jang, S., 2016). This is especially true when bacterial organisms are trying to adapt to harsh environmental conditions. The study of the ArcAB-TolC efflux pumps in clinical strains of *E. coli* have allowed for the assumption that efflux pumps in clinically relevant bacterial pathogens can efflux antimicrobial compounds from the host (Ma, et al., 1995; Okusu, H., 1996). There is an implied connection between bacterial efflux and virulence (Piddock, L., 2006; 2014). Other studies have demonstrated this with mutations of efflux pumps. This was demonstrated in *N. gonorrhea*, which resulted in poor colonization of the mouse urogenital track (Jerse, A.E., 2003).
1.9 Efflux Pumps in Gram Negative and Gram Positive Bacteria

Primary Efflux Pumps Families:

![Diagram of efflux pumps](image)

**Figure 8**: Efflux pumps and substrates from (Andersen, J., 2015) Efflux pumps of Salmonella from four different transporter families. The transport mechanisms, location, families, and substrates are shown. Abbreviations used in the figure indicate the following: aminoglycosides (AMG), novobiocin (NVB), sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), acriflavine (ACF), crystal violet (CV), methylene blue (MB), rhodamine 6G (R6G), benzalkonium chloride (BNKC), nalidixic acid (NAL), tetracycline (TET), chloramphenicol (CLP), norfloxacin (NOR), doxorubicin (DOX), and macrolides (MAC). Key for antibiotics obtained from (Andersen, et al., 2015)

1.9.1 The Major Facilitator Superfamily (MFS)

MFS are found in nearly all living organisms. All of the permeases of the MFS contain a conserved motif of alpha helical spanners (Saier, et. al., 1998). These permeases can extrude or import different substrates from cations, metabolites, drugs, and amino acids by interacting with the various compounds through amino acid residues. The MFS family includes symporters, antiporters, as well as uniporters (Quistgaard, et al., 2016). The MFS transporters found in
bacteria are closely related to eukaryote sugar transporters and have a common mechanism of transport (Kumar, et al., 2016). MFS efflux transporters utilize a proton gradient across the membrane in order to export a large variety of substrates.

Some of the most well studied MFS pumps in *S. aureus* include NorA, and QacA (Dymek, A., 2012; Santos Costa, S., 2013). The NorA pump is resistant to numerous biocides but is especially known for resistance against fluoroquinolones. The increased resistance is due to the overexpression of the NorA gene through mutations in the regulatory proteins of this pump. The mutation occurs approximately 89bp upstream from the initiation codon (DeMarco, et al., 2007). These mutations are in the promoter region and have also led to constitutive expression. Similar to other efflux pumps, the NorA utilizes an H⁺ antiporter (Ng, EY., 1994) and has twelve transmembrane portions (Santos Costa, et al., 2013).

### 1.9.2 Small Molecule Resistant Efflux Pumps (SMR)

The Small Molecule Resistant Efflux Pumps (SMR) family is comprised of genes that are usually obtained horizontally through integrons located on plasmids (Slipski, et. al., 2019). These pumps are usually given the name Qac, due to the ability to pump out molecules in the quaternary ammonia compounds. These genes may have arisen from various species. These specific pumps also provide resistance to guanididium containing biocides (Kermani, et al., 2018).

The SMR family consists of proteins that span the membrane at least four times with helices that have both hydrophilic and hydrophobic properties (Paulsen, et al., 1996). The four transmembrane helices are approximately 150 amino acids in length and where the small designation is derived in name. The hydrophobic transmembrane α-helices allow for the import
of organic solvents (Bay, D., 2008). The SMR family is further divided into two types based on phenotype. These are small multidrug pumps and inhibition of groEL proteins (SUG) (Greener, T., 1993). The SMR homologues comprise a large variety of protein sequences and have been found in both prokaryotes and some eukaryotes. These genes are found on plasmids, integrons and chromosomal DNA (Bay, D., 2008). Interestingly, another sub-class has been found in addition to the ones mentioned above. This subclass is known as the paired SMR protein (PSMR) (Bay, D., 2008).

**Figure 9:** Retrieved from (Bay, D., 2008) The protein topology of E. coli Eco-emrE (A) and C. freundii (B). The figure is a depiction of the transmembrane helices that predict a periplasm and cytoplasmic domain. The residues in red depict amino acids capable of combined drug resistance. The figure demonstrates the homology of the protein both in function and structure among bacterial species.

In gram-positive bacteria, the *Qac* genes C and D, along with *Ebr* gene were among the first SMR genes discovered (Wassenaar, et al., 2015). In *S. aureus*, the Sau-Smr protein has been characterized as an SMR efflux pump. This gene has a remarkable similarity in function to the *M. tuberculosis* gene *Mtu-Smr*. The SMR family of pumps utilizes proton motive force as a means of energy. Simply stated, the energy from one proton entering the cell from a natural gradient is harnessed in order to exchange it for a molecule of antibiotic substance.
1.9.3 Multi Drug and Toxic Compound Extrusion (MATE)

The MATE family confers resistance to a number of dissimilar compounds including biocides, antibiotics and other toxic chemicals. Extrusion is coupled with a Na+. The most well studied *S. aureus* efflux pump from the MATE family is MepA. The efflux pump MepA confers resistance to quaternary ammonium compounds. It was thought that MepA was regulated by MepR and within the mepRAB operon (Katz, et al., 2005). The MepA protein also spans the membrane twelve times. Other MATE family efflux pumps are NorM from *V. cholerae* and *V. parahaemolyticus*, which were among the first to be discovered (Ogawa, et al., 2016). The MATE family transporters consist of 12 transmembrane hydrophobic domains (Kuroda & Tsuchiya, 2009).

1.9.4 ATP Binding Cassette (ABC)

The ABC efflux family utilizes ATP to drive the export or import of various substrates across the membrane. The main mechanism behind efflux is to harness proton motive force to hydrolyze ATP providing the energy to transport any substrate across the membrane. Some of the ABC efflux pumps often affect other transporters that import and export signaling molecules in eukaryote ABC transporters (Oevermann, et al., 2009) This may potentially be true for prokaryote cells as well. The majority of efflux pumps in the ABC family can be divided into two classes, homo- and hetero-dimeric. The literature determined the function of these efflux pumps depends on the conformational changes of the transmembrane efflux protein. It alternates from an open outward to an occluded state then to an open outward confirmation to transport substrates in or out of the cell.
Figure 10: from (Yang, J., 2015) showing the three conformational states of the ABC efflux transmembrane protein. The protein consists of three parts: TMD transmembrane domain, NBD nucleotide binding domain and finally, the Regulatory domain.

1.9.5 Resistance Nodulation Division (RND)

Until very recently, it was thought that RND pumps only existed in gram-negative bacteria. However, recent studies have identified the presence of RND pumps in gram-positive bacteria as well (Schindler & Katz, 2016). Similar to other efflux pumps, the RND family confers resistance to a number of biocides that are dissimilar in terms of chemistry and structure (Bohnert, et al., 2008). In terms of structure, the RND family are usually homotrimers but do have members in the eukaryote organisms that have heterodimeric proteins (Du, et al., 2018). All RND transporters are typically secondary transporters either utilizing PMF or antiport mechanisms (Du, et al., 2018).

The mechanism of the RND efflux pump, ArcB relies on cooperation of the three different structural states of the protein. In the Loose (L) state, the drug accesses the pump in a binding pocket located in the periplasmic space. Once they are further into the binding pocket the conformation changes into the Tight (T) state. Once in the T state, the protein conformation changes into the Open (O) state. In this state, antimicrobials can be moved into a funnel shaped area where they are expelled outside the cell (Wong et. al., 2014)
1.9.6 Proteobacterial Antimicrobial Compound Efflux (PACE)

The PACE family is a recent discovery of transport proteins that give resistance to disinfectants and biocides. More importantly, the PACE family confers resistance to chlorhexidine. Chlorhexidine is considered an important medicine by the World Health Organization as it is present in mouthwashes and hand soaps.

Interestingly, this highly conserved amino acid sequence is in the chromosomal DNA that implies an important function (Hassan, et al., 2018). One of the first discoveries of the PACE pump was a protein isolated from *Acinetobacter baumannii*. This novel protein was later named AceI (Acinetobacter chlorohexidine efflux protein) and other homologues were found in *Klebsiella, Enterobacter, Salmonella* and *Pseudomonas* (Hassan, et al., 2015). These homologues also extended resistance beyond chlorohexidine to other compounds such as: benzalkonium, proflavine and acriflavine among others (Hassan, et al., 2018). The AceI homologous regions are highly conserved across gram-negative bacteria.

The 3D structure of PACE has not been solved; however, it is predicted to have four transmembrane α-helices followed by what is known as Bacterial Transmembrane Pair (BTP) domains (Haasan, et al., 2019). There are four highly conserved motifs in the PACE family proteins among genera. The capital letters signify that it is conserved in over 90% of the genera of bacteria while lowercase letters signify around 65% conservation (Hassan, et al., 2018). In Helix 1 they are RxxhaxxfE and in Helix 2 the motif is WNxx or fNxxFd. In Transmembrane 3, the motif is RxxHaxxFe and is like the motif in Helix 1 (Hassan, et al., 2018). In Helix 4, the motif is Ytxxf or ynwxyD. These are similar to the motif in Helix 2. The x signifies any amino acid.
The PACE family of efflux proteins are very different from other efflux families because they appear to be somewhat limited to specific biocides such as chlorohexidine. This is in contrast with the other efflux families that can efflux a wide variety of dissimilar substrates. The presence of PACE family proteins in the genome of bacteria rather than mobile plasmid elements demonstrates a possibly crucial physiological function not yet determined (Hassan, et al., 2015).

1.9.7 p-Aminobenzoyl-Glutamate Transporter (AbgT)

The primary purpose of this transporter is the importation of p-aminobenzoyl-glutamate to synthesize folate. This transporter has distinct features from any other family of efflux pumps (Delmar, J., 2016). Recent X-ray structures of transporters, YdaH from *Alcanivorax borkumensis* and MtrF from *Neisseria gonorrhoeae*, show the bowl-shaped dimers spanning from the cytoplasm through the membrane layer. Each dimer consists of nine transmembrane helices and two hairpins (Su, et al., 2015). The main substrate that MtrF effluxes is PABA.

While experiments reveal that YdaH and MtrF can remove p-aminobenzoic acid from the cell, other assays have demonstrated the ability to efflux sulfonamides. The structure of YdaH contains two hairpins and nine transmembrane helices. The dimer forms a concave liquid filled structure. Interestingly, the MtrF also has nine transmembrane helices and two helical hairpins which closely resemble the YdaH transporter.

A CLUSTAL W alignment revealed that the transporter could exist in other bacteria including pathogens. The alignments revealed over 65% homology in other bacteria including *S. aureus, Escherichia coli, Clostridium difficile, Bacillus halodurans, Vibrio. cholerae, Caulobacter crescentus and Neisseria. meningitidis* among others (Delmar, J., 2016). There are
approximately 13,000 AbgT-like proteins have been found in gram positive, gram negative bacteria as well as yeast (Su, et al., 2015).

Efflux pumps are important for antimicrobial resistance because they can bind to different substrates that are chemically dissimilar. While efflux pumps are present in both susceptible and antimicrobial resistance, it is the expression level that makes them resistant to antibiotics (Webber & Piddock., 2003). The presence of efflux genes in the chromosome of antibiotic susceptible bacteria indicates an ancient ability to adapt and survive in a hostile environment (Lomovskaya, et al., 2001).

1.10 Statement of the Problem:

*S. aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) are significant global health pathogens that remain a burden due to an ability to persist in healthcare and community environments. Some of this persistence is largely due to the ability of MRSA to efflux bactericides, while obtaining sources of energy. Among many mechanisms of antibiotic resistance, efflux pumps are important for survival in harsh environments. Regulation of efflux is not well-known, and the level of expression is directly related to virulence. Although indirectly, Ca$^{2+}$ homeostasis has been shown to be important in bacterial functions including efflux. The role of calcium signaling in efflux pumps is unknown. The study aims to determine if Ca$^{2+}$ regulates efflux activity in *S. aureus*. The direct link between Ca$^{2+}$ and gene regulation of efflux or other important bacterial physiological processes has not been demonstrated. Therefore, this study will focus on how Ca$^{2+}$ are involved in the regulation of gene expression of efflux pumps. The link between Ca$^{2+}$ and gene regulation needs to be established before proteins in a Ca$^{2+}$ signaling transduction pathway can be explored.
1.11 Significance:

Calcium and efflux:

The proposed research seeks to demonstrate that Ca\textsuperscript{2+} regulates efflux of antibiotics in *Staphylococcus aureus*. The present study investigated calcium regulation of efflux pumps in multi-drug resistant *S. aureus* clinical isolates. In addition the present study has advanced our understanding in antimicrobial resistance (AMR) mechanisms and has the potential to contribute to the design of alternative therapeutic approaches by targeting Ca\textsuperscript{2+} signaling pathways in bacteria mitigating the need for antibiotics. In addition, this work will advance knowledge in microbial physiology and will establish Ca\textsuperscript{2+} as the universal regulator. To date, Ca\textsuperscript{2+} and efflux have only been indirectly linked to an enhancement of efflux in several bacteria including *E. coli* and *S. aureus*. Transduction signaling in bacteria is only limited to the two component systems that directly influence genetic expression. Very little is known about other proteins involved between the initial two component regulatory systems (TCSs) response to genetic expression after sensing a stimulus. Some bacterial secondary messengers have been identified such as the cyclins. However, the influence of Ca\textsuperscript{2+} as a secondary messenger in gene regulation of bacterial physiological processes is not known. The present study is the first to establish the link between Ca\textsuperscript{2+} and gene expression of efflux pumps.

1.12 Hypotheses:

1) Antibiotics act by increasing cytosolic Ca\textsuperscript{2+} concentration, which in turn enhance the efflux activity of the *S. aureus* efflux pump LmrS

2) Calcium ions mediate efflux pump expression in *S. aureus*
CHAPTER 2: LITERATURE REVIEW

2.1 Potential role of Ca\textsuperscript{2+} in the regulation of efflux and transport systems in prokaryotes

In a challenging environment, intracellular homeostasis is crucial for bacteria. The ability to transport trace elements and nutrients as well as efflux harmful substances is crucial to survival. Like eukaryotes, the concentration of calcium within prokaryotic cells is tightly regulated. Previous studies have correlated calcium signaling and regulation of various important bacterial physiological functions. Efflux studies in \textit{E. coli} have indirectly shown that Ca\textsuperscript{2+} enhances efflux and that Ca\textsuperscript{2+} homeostasis is maintained in some genera of bacteria. In this review, we discuss the possible role of Ca\textsuperscript{2+} in signaling and regulatory processes, transporters of Ca\textsuperscript{2+}, and the possibility of calcium regulation of bacterial efflux systems.

2.2 Prokaryote Regulation of Calcium and Calcium Signaling in Bacteria

Calcium is important in eukaryote cell signaling and is involved in adenylate cyclase pathways that activate Protein Kinase A (PKA) and also in GPCR signaling. The current research in bacterial transduction pathways is limited to two component regulatory systems which are “GPCR” like and consist of a 5-7 transmembrane domain sensor kinase and a response regulator (Pec, S. 1996). Similar to eukaryote GPCRs, TCs relay a signal from the sensor protein to the response regulator through autophosphorylation. Once a stimulus is intercepted, the cytoplasmic region of the sensor kinase undergoes a conformational change and ATP is exchanged for ADP (Dev, A., 2014). The similarity of this process to eukaryotes seems obvious considering function. However, very little is known about what proteins are involved before gene regulation occurs. Some studies imply arrestin like proteins called antikinases that reset these sensors, once tripped (Pec, S. 1996). Interestingly, stopping this function in eukaryote cells will often re-set GPCR signals.
Various studies have demonstrated that bacteria regulate cytosolic Ca\(^{2+}\) in response to environmental fluctuations (Torrecilla, I., 2000; 2001). Regulation of cytosolic Ca\(^{2+}\) is accomplished through acidocalcisomes, Ca\(^{2+}\) binding proteins, transporters, and the periplasm (Bruni, G., 2017; Jones, H.E., 2002). For example, the NaChBac transporter, isolated from *B. halodurans*, is very similar in functionality to eukaryote voltage gated Ca\(^{2+}\) channels (Ren, D., 2009). It is thought that these low affinity channels are symporters that export Na\(^{+}\) to import Ca\(^{2+}\). In a study by Bruni, et al. (2017), a Ca\(^{2+}\) voltage flux in *E. coli* allowed for the ability to “sense” changes in environmental shear or pulling forces.

Some Ca\(^{2+}\) binding proteins contain the putative EF hand motif present in eukaryote proteins (Sariskova, S.A., 2014; Dominguez et al 2015). Other regulatory proteins such as calmodulin like proteins imply that prokaryotes are regulating cytosolic Ca\(^{2+}\). Many of these strategies such as binding to Ca\(^{2+}\), buffering and storage are like eukaryote strategies and suggests that Ca\(^{2+}\) signaling is occurring.

Intracellular concentrations of Ca\(^{2+}\) under normal circumstances are very similar for both prokaryotes and eukaryotes. This is in the range of about 100-300nM (Naseem, R., 2008; Dominguez, D.C., 2015). The extra cellular Ca\(^{2+}\) can have as much as 1-5mM. The ability of prokaryotes to maintain Ca\(^{2+}\) homeostasis implies that bacteria, like eukaryotes, have Ca\(^{2+}\) binding proteins, storage spaces, buffers and transporters that may be used for maintaining these cytosolic levels that are yet to be discovered. High intracellular calcium levels result in disruption of pH, ion concentration (Na\(^{+}\), K\(^{+}\)) and enzymes in both eukaryotes and prokaryotes. Ca\(^{2+}\) ions are also highly regulated due to their interactions with regulatory proteins, which control cellular metabolic reactions.
In order to maintain low levels of Ca\(^{2+}\) concentration, eukaryote cells rely on three classes of proteins. These are proteins in the cytoplasm that have a high and low affinity for Ca\(^{2+}\) buffering the intracellular Ca\(^{2+}\) concentration to the nM range. The other type of Ca\(^{2+}\) proteins are transporters: ion exchangers, pumps and channels that maintain intracellular Ca\(^{2+}\) concentrations (Carafoli & Krebs, 2016). Calcium ATPases pump out Ca\(^{2+}\) from the cytosol by hydrolysis of ATP (Jones, H.E., 2002). Another calcium transporter are secondary transporters that utilize the energy from the flow of an ion (Na\(^{+}\)) along a concentration gradient in exchange for Ca\(^{2+}\) against the concentration gradient (Cai & Litton, 2004).

For prokaryotes, high intracellular Ca\(^{2+}\) is also toxic and maintenance of Ca\(^{2+}\) concentration is crucial for survival. Bacteria have three types of Ca\(^{2+}\) transport systems. These are ATPases, polyhydroxybutyrate-polyphosphates and low affinity Ca\(^{2+}\) exchangers (Guragain, et al, 2013, Domínguez et al 2015, Dominguez, 2018). These are very similar to eukaryote cells to efflux high levels of Ca\(^{2+}\). Bacteria also apparently have transporters to influx Ca\(^{2+}\) as demonstrated in a study which showed that polyphosphate complexes were able to form Ca\(^{2+}\) channels that were controlled by voltage in \textit{E. coli} (Reusch, RN., 1988). A study by Guragain, et al., 2013 demonstrated that Ca\(^{2+}\) homeostasis was achieved through different transporters. This was apparent when inhibitors of F and P type ATPases and Ca\(^{2+}\) showed impaired Ca\(^{2+}\) homeostasis. Ultimately, the most convincing evidence of this study was that Ca\(^{2+}\) homeostasis was significantly inhibited by the genetic disruption of \textit{P. aeruginosa} P-type ATPases, PA2435 and PA3920 along with the ion exchange transporter, PA2092. This was significant as it demonstrates that bacteria, like eukaryotes, also have mechanisms of Ca\(^{2+}\) influx and efflux to maintain a specific concentration.
Other studies have shown that bacteria utilize ATPases, Ca$^{2+}$ exchangers and proton motif force to efflux calcium. Naseem et. al. (2009) demonstrated that ATP may be regulating Ca$^{2+}$ concentrations. Interestingly, Ca$^{2+}$ exporters utilizing an ion chemical gradient has been described and evidence of Ca$^{2+}$ efflux has been illustrated in various bacteria (Shiner, E. 2006). These exchangers utilize Na$^+$ and H$^+$ to efflux Ca$^{2+}$ (Ivey, et. al., 1993). In these types of transporters, Ca$^{2+}$ is imported against a chemical gradient.

Another method of regulating Ca$^{2+}$ intracellular concentration are vacuoles and the endoplasmic reticulum. As mentioned above, some bacteria store excess Ca$^{2+}$ as a strategy to control Ca$^{2+}$ levels in acidocalcisomes. *Rhodospirillum rubrum* have been described as having acidocalcisomes which utilize an H$^+$ pump and polyphosphates to regulate intracellular concentrations (Seufferheld, M., 2004). Polyphosphates and poly-b-hydroxybutyrate have been known to form Ca$^{2+}$ channels across the membranes of various organisms (Seufferheld, M., 2004). Polyphosphates also provide phosphate energy reserves to help prokaryotes deal with environmental changes (Jones, HE., 2002). Another space for storing Ca$^{2+}$ has been demonstrated in *E. coli* where Ca$^{2+}$ measurements in the periplasmic space (Jones, HE., 2002). In this study, aequorin was targeted to the periplasmic space by using the Outer Membrane protein T (OmpT) signal sequence. The significance of this study was that *E. coli* was able to concentrate Ca$^{2+}$ in the periplasm even when the external concentration was less than 1uM. This suggests that the periplasm may be acting as a store for Ca$^{2+}$ in a similar manner than the endoplasmic reticulum does for eukaryote cells.
2.3 Calcium Binding Proteins in Bacteria

Calcium binding proteins such as calmodulin and Troponin C bind to calcium using the EF hand motif. This helix-loop-helix motif is highly specific to Ca$^{2+}$ due to the six oxygen atoms in the side chains of amino acids like aspartate and glutamate. These negative side chains bind to the positive Ca$^{2+}$. The binding of Ca$^{2+}$ causes a conformational change allowing effector proteins to regulate functions (Fleischer, J., 2009). For bacteria, Michiels, et. al. 2002 did an *in-silico* analysis of potential Ca$^{2+}$ binding proteins. The study identified potential Ca$^{2+}$ binding proteins that may play a possible role in intracellular Ca$^{2+}$ homeostasis. A proteome analysis by Dominguez, D.C., et. al., 2011, revealed total protein transcriptional changes as a result of treatment with Ca$^{2+}$ or EGTA in *B. subtilis*. The study identified six potential Ca$^{2+}$ binding proteins that were made in the presence of Ca$^{2+}$. Interestingly, there was a change on molecular weight of the proteins when incubated with either Ca$^{2+}$ or EGTA. In addition, other studies have identified proteins that have conserved structural domains similar to eukaryote Ca$^{2+}$ binding proteins with the same helix-loop-helix motif and serve a variety of functions including interact with eukaryote cells. A specific example is the highly conserved structural domains in RapA of *Rhizobium leguminosarum*. This protein is responsible for aggregation and adhesion to plant root cells. These conserved structural domains are common bacterial proteins that bare a remarkable similarity to eukaryote cadherins or adhesion proteins (Abdian, et al., 2013). The cadherin like β-sheet conformation in the RapA proteins in Rhizobium interact with glycoproteins of plants in a Ca$^{2+}$ dependent manner. Moreover, this implies possible molecular cross talk between the plant and the symbiont through Ca$^{2+}$ proteins (Abdian, et al., 2013).

To date there have been seven different classes of Ca$^{2+}$ proteins identified in prokaryotes. Due to the variety of structures in these proteins it is thought that there are a wide range of
functions. The role of Ca^{2+} appears to play a role in adhesion, regulating biofilm expression, confers resistance and mediates heterocyst formation among many other processes. The following table from (Dominguez, D.C., 2015) lists these proteins, the organisms where they were isolated and the possible role that Ca^{2+} plays in the function.
<table>
<thead>
<tr>
<th>Protein name, accession number organism</th>
<th>Physiological role, relationship to Ca²⁺</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td><strong>Surface proteins</strong></td>
<td></td>
<td></td>
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<tr>
<td>Bap, AAK38834.2, biofilm associated protein, Staphylococcus aureus V129</td>
<td>Mediates Ca²⁺ dependent cellular adhesion; responsible for Ca²⁺ inhibition of biofilm formation; its expression is not affected by Ca²⁺. Bap contains EF-hand motifs; binding Ca²⁺ protects BAP protein from proteolysis.</td>
<td>[155]</td>
</tr>
<tr>
<td>CflA, YP.001331790.1, clumping factor A, Staphylococcus aureus Newman</td>
<td>Promotes binding of bacterial cells to fibrinogen and fibrin; Ca²⁺ and Mn²⁺ inhibit interaction of CflA and fibrinogen; CflA contains EF-hand like motif with Ca²⁺-dependent inhibitory site at residues 310–321.</td>
<td>[153]</td>
</tr>
<tr>
<td>CflB, CAA12115.1, clumping factor B Staphylococcus aureus Newman</td>
<td>Is detectable only in the exponential phase cells and promotes cell clumping in a solution of fibrinogen as well as cell adherence to immobilized fibrinogen in vitro; this activity is inhibited by Ca²⁺ and Mn²⁺.</td>
<td>[154]</td>
</tr>
<tr>
<td>SdrD, CA006651.1, serine-aspartate surface protein, Staphylococcus aureus Newman</td>
<td>Ca²⁺ binding is required for the structural integrity of SdrD B-repeat regions. 15 Ca²⁺-binding sites: five with high and 10 with low binding affinity likely serve for SdrD structural stability and cellular Ca²⁺ homeostasis, respectively.</td>
<td>[152]</td>
</tr>
<tr>
<td>Cah, AIP92899.1, Ca²⁺ binding antigen 43 homolog, E. coli O157:H7</td>
<td>Mediates changes in cell morphology and cellular aggregation; plays role in biofilm formation; reduces adhesion to HeLa cells; EDTA treatment increases cah transcription, but addition of Ca²⁺ has no effect. Ca²⁺ binding is reduced by MgCl₂.</td>
<td>[156]</td>
</tr>
<tr>
<td><strong>Two-component regulatory systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PhoQ, PhoP, PDOM78.1, sensor histidine kinase, PhoP, PDOM80.1, transcriptional regulator, Salmonella enterica Typhimurium</td>
<td>Regulates virulence, cell physiology, entry and survival in the host cells; is negatively regulated by Mg²⁺ and Ca²⁺; mediates Ca²⁺ repression of pho (PhoP-activated genes) loci. Altered sensing of Mg²⁺/Ca²⁺ may lead to avirulence. PhoQ has distinct non-interacting binding sites for Mg²⁺ and Ca²⁺.</td>
<td>[157,194,195]</td>
</tr>
<tr>
<td>VpsR, AAX00610.1, transcriptional regulator CarS, AAF94477.1, Ca²⁺ regulated sensor, CarR, AAF94478.1, Ca²⁺ regulated regulator, Vibrio cholera O1F1</td>
<td>Ca²⁺ decreases the expression of genes required for biofilm matrix production by the decreased transcription of vpsR, which is a negative regulator of biofilm formation. CarSR is negatively regulated by Ca²⁺. Elevated Ca²⁺ represses thermoresistance and decreases heat shock-induced expression of CIRCE regulon, which can be partially attributed to HrcA, since binding of HrcA to CIRCE is modulated by Ca²⁺. Ca²⁺ enhances HrcA stability and promotes its association with GroEL.</td>
<td>[23]</td>
</tr>
<tr>
<td>HrcA, QBDQ92.2, heat inducible transcription repressor, Streptococcus pneumonia CP1200</td>
<td>Elevated Ca²⁺ induces transcription of hrcA, which positively regulates cPHB synthesis</td>
<td>[171]</td>
</tr>
<tr>
<td>AtoSC: AtoS, AIN2630.1, sensory histidine kinase, AtoC, AINS2631.1, Response regulator, E. coli BV25113</td>
<td>Elevated Ca²⁺ induces transcription of atoC, which positively regulates cPHB synthesis</td>
<td>[170]</td>
</tr>
<tr>
<td><strong>Intracellular Ca²⁺ binding protein</strong></td>
<td></td>
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<tr>
<td>CbpB, AAX13998.1 Ca²⁺ binding protein Anaabaena sp. PCC7120 Ca²⁺-binding protein</td>
<td>Negatively regulates heterocyst formation under nitrogen limited conditions; prevents up-regulation of HcrR protease degrading CbpB and increasing intracellular Ca²⁺; contains two Ca²⁺-binding sites with distinct binding abilities; rich in acidic amino acid residues; Ca²⁺-bound form has more compact structure.</td>
<td>[12,42]</td>
</tr>
<tr>
<td><strong>EF-hand proteins</strong></td>
<td></td>
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<tr>
<td>CbcC, NP.631887.1 Ca²⁺ binding protein, Streptomyces coelicolor</td>
<td>Plays role in spore germination and hyphae formation. Contains EF-hand motifs; binds 3 Ca²⁺ per molecule; undergoes small conformational change in the presence of Ca²⁺. CbcC likely acts as a Ca²⁺ buffer and exerts its</td>
<td>[100]</td>
</tr>
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</table>
2.4 The Indirect Association of Calcium and Efflux

Previous research has illustrated that Ca\(^{2+}\) are indirectly associated with efflux in bacteria. To date, some publications have identified Ca\(^{2+}\) as capable of enhancing efflux capacity in *E. coli* (Holland, B. et.al., 1999; Martins, A., 2011). Conversely, Ca\(^{2+}\) channel inhibitors and chelators have been reported as inhibiting efflux activity in some bacteria (Couto, et al., 2008; Sun, J., 2014). For example, the Ca\(^{2+}\) channel blocker Verampil, can inhibit efflux in *Mycobacterium tuberculosis* that confers resistance to many anti-tubercule drugs (Adams, K., 2014). The study identifies efflux pumps such as Rv1258c as a major contributing factor to antibiotic resistance in *M. tuberculosis*. Interestingly, the study showed that Ca\(^{2+}\) blockers also inhibited growth in M. tuberculosis. The results of these studies demonstrate an indirect link between efflux and Ca\(^{2+}\) hence, implying that calcium may be involved in the regulation of efflux.

![Figure 11](image.png)

**Figure 11**: The effects of Ca\(^{2+}\) by proton de-coupler 2,4-dinitrophenol. Illustrating the importance of protons in efflux. (Guragain, M. 2013)

An important association between pH and efflux activity has recently been documented. At lower pH, due to Proton Motive Force (PMF), bacteria are able to efflux without the addition of Ca\(^{2+}\) or metabolic energy (glucose) (Martin, A. 2015; Holland, B et. al., 1999). The preference for Ca\(^{2+}\) decreases with pH. At more alkaline pH, efflux is less efficient as many H\(^{+}\) ions are lost.
in solution, illustrating that hydrolysis of ATP with hydronium ions is crucial for efflux mechanisms as efflux is metabolically expensive (Piddock, L.J., 2006). The same effect of pH is seen in cytosolic Ca\(^{2+}\) levels. In a study by Naseem et. al. (2008), at a higher pH of 9.5, more Ca\(^{2+}\) transients were measured than at pH 5 or 7. The pH did not appear to affect Ca\(^{2+}\) efflux however, the influx was considerably smaller at a more acidic pH of 5. The study cited a competition between Ca\(^{2+}\) and H\(^+\) as the reason as was evident with higher cytosolic Ca\(^{2+}\) levels at higher pH.

Across different species of bacteria Ca\(^{2+}\) transients occur in response to environmental and stressful conditions. Heat and cold shock raise the cytosolic Ca\(^{2+}\) levels of cyanobacteria (Torecilla, et. al. 2000). In response to a repellent, intercellular Ca\(^{2+}\) levels rise, and chemotaxis increases away from a repellent in *E. coli* (Watkins, J., 1995; Tisa, L.S. 1993)). This is a strong implication for cell signaling as there is a Ca\(^{2+}\) transient followed by a physiological response. The photoprotein, aequorin has been used in several bacteria to demonstrate that bacteria are able to sense changes in the environment (Torecilla, I., 2000, 2001; Shiner, E.K., 2006; Guragain, M., 2013). Moreover, an example of calcium signaling exists in the legume-*Rhizobium* symbiosis. The possibility of Ca\(^{2+}\) mediated signaling pathway between legume plants and *R. leguminosarum* was discussed in (Moscatiello, R., 2010). In this work Moscatiello (2010), used aequorin to determine if a calcium pathway was involved in Nod gene transcription. Flavonoids from the plant, known to induce upregulation of Nod genes were added to the aequorin *Rhizobium* strains. Upon addition of flavonoids, the bacterial strains produced calcium transients suggesting a Ca\(^{2+}\) dependent pathway leading to gene up-regulation. Interestingly, other molecules not specific to the symbiosis failed to produce Ca\(^{2+}\) transients. This data suggests that Ca\(^{2+}\) is crucial for sensing and transducing flavonoids from the legume plant.
CHAPTER 3: MATERIAL AND METHODS:

Experimental design overview 3.1:

The project’s main purpose was to explore how Calcium ions (Ca\(^{2+}\)) ultimately influence efflux pump activity and gene expression in *S. aureus* and clinically isolated MRSA. Ca\(^{2+}\) pathways in bacteria are unknown. However, many implications in previous studies suggest that Ca\(^{2+}\) effects or modulates many physiological processes. These include chemotaxis, virulence and cell division (Sarsikova, et al., 2005; Lemovskaya, O. 2001). The process of designing the basic ideas behind this research are to connect the known and implicated knowledge with more direct evidence of Ca\(^{2+}\) regulation of gene expression of efflux. This hypothesis was an extension of theories rooted in eukaryote studies where Ca\(^{2+}\) ions are a fundamental part of transduction pathways and ultimately, gene expression in many cellular functions (Fleischer, J., 2009; Oh, S., 2018). This is important to determine if Ca\(^{2+}\) influences gene expression and to demonstrate that Ca\(^{2+}\) homeostasis is maintained in the cell. Ca\(^{2+}\) homeostasis is simply a spike in intracellular Ca\(^{2+}\) in response to external stimuli followed by a slow return to basal Ca\(^{2+}\) levels. The experiment would need to capture a signal that intracellular Ca\(^{2+}\) has increased in response to a stimulus or treatment. In this case, different concentrations of extracellular Ca\(^{2+}\) ions (CaCl\(_2\)) were added at a specific time to measure a subsequent increase in cytosolic Ca\(^{2+}\). The successful experiment would also test how a Ca\(^{2+}\) specific chelator would affect Ca\(^{2+}\) homeostasis. It is also necessary to control for pH to see if proton availability influences the concentration of Ca\(^{2+}\) being imported into the cell. Finally, our experiments included injections of different antibiotics to measure the response in intracellular Ca\(^{2+}\). This implied the possibility of a transduction pathway. The experiment proceeded starting with the determination of basal levels of cytosolic Ca\(^{2+}\) in *S. aureus*. This was determined using a calcium specific photo-protein which was
transformed into *S. aureus*. These experiments determined the cells response to increasing concentrations of Ca\(^{2+}\), differences in pH and the removal of cytosolic Ca\(^{2+}\) by EGTA.

Evidence of intracellular Ca\(^{2+}\) homeostasis was determined to be manipulated by external concentrations of Ca\(^{2+}\) and also manipulated efflux activity in *S. aureus*. The efflux experiments were designed to measure efflux both indirectly and directly. A dye was chosen that would allow measurement of accumulation and extrusion in real time. The experiments included measuring efflux with treatments including Ca\(^{2+}\), Ca\(^{2+}\) specific inhibitors, with or without metabolic energy (glucose) and finally, adjusting the pH to 5, 7, and 8. Controlling for glucose, pH and Ca\(^{2+}\) would allow for the visualization of the contribution of each. These controls helped distinguish the sole contribution of Ca\(^{2+}\) as compared to the other variables.

Finally, Ca\(^{2+}\) was determined to influence efflux, so that experiments could proceed to the measurement of efflux gene expression. The influence of Ca\(^{2+}\) on gene expression of efflux set a precedence for possible regulation of other physiological processes. In this experiment we demonstrated that efflux regulation increases with added external Ca\(^{2+}\). Total RNA was extracted under the following conditions: when the bacterium was incubated in EtBr with or without Ca\(^{2+}\) and addition of Ca\(^{2+}\) specific chelators. The positive control of efflux regulation would be the EtBr. The negative control would be the *S. aureus* under normal conditions (without EtBr). This represents one main gap in research to date. Once the baseline of efflux is established, manipulation of Ca\(^{2+}\) concentration was assessed by adding different concentrations of Ca\(^{2+}\), Ca\(^{2+}\) inhibitors and chelators to the media. The purpose of this was to observe whether the addition or absence of Ca\(^{2+}\) affects the ability of *S. aureus* to efflux EtBr. *S. aureus* ATCC 25923 and clinical isolate EBSA78 were grown in Brain Heart Infusion (BHI) broth. The *E. coli* strain JM109 containing the expression vector pMMB66EH with the apoaequorin coding sequence was
used to conjugate into *S. aureus* cells. The *E. coli* cells were grown in Luria-Bertani (LB) broth with carbenicillin or ampicillin (100 μg/ml).

### 3.2 Table 2: Bacterial Strains and Media

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Experiment</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>Aquorin and Efflux</td>
<td>BHI, HEPES and PBS</td>
</tr>
<tr>
<td>MRSA EBSA 78 (Clinically Isolated Strain)</td>
<td>Efflux/ Gene expression</td>
<td>BHI, PBS</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>MIC and Direct Efflux</td>
<td>LB, PBS</td>
</tr>
<tr>
<td><em>E. coli</em> LmrS clone</td>
<td>MIC and Direct Efflux</td>
<td>LB, PBS</td>
</tr>
<tr>
<td><em>S. aureus</em> AEQ</td>
<td>IntracellularCa(^{2+}) Measurements</td>
<td>BHI and HEPES</td>
</tr>
<tr>
<td><em>S. aureus</em>/MRSA EBSA 78</td>
<td>Gene expression</td>
<td>BHI</td>
</tr>
</tbody>
</table>
### 3.3 Table 3: Experiment variables and AIMS

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Independent Variables</th>
<th>Dependent Variable</th>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efflux</td>
<td>[Ca$^{2+}$], Ca$^{2+}$ inhibitors, pH (5,7&amp;8)</td>
<td>Efflux and extrusion of EtBr</td>
<td>Ca$^{2+}$ ions modulate efflux capacity in <em>S. aureus</em> and clinically isolated MRSA</td>
</tr>
<tr>
<td>Intracellular Calcium</td>
<td>Ca$^{2+}$ inhibitors and chelators, pHs (5,7, and 8), with and without EtBr incubation, [Ca$^{2+}$]</td>
<td>Cytosolic free Ca$^{2+}$</td>
<td>S. aureus imports Ca$^{2+}$ ions in higher extracellular [Ca$^{2+}$] and under stressful conditions that induce efflux</td>
</tr>
<tr>
<td>Gene Expression</td>
<td>Incubated in EtBr, EtBr + Ca$^{2+}$ Normal + 1mM Ca$^{2+}$, EtBr + Ca$^{2+}$ inhibitor/chelator, EtBr + Ca$^{2+}$ inhibitor/chelator + Ca$^{2+}$ rescue</td>
<td>Gene expression of efflux pump genes as compared to housekeeping genes</td>
<td>Gene expression is higher when Ca$^{2+}$ is added externally. <em>S. aureus</em> can efflux EtBr more efficiently</td>
</tr>
<tr>
<td>LmrS efflux clone</td>
<td>Normal conditions pH 7, With and without Ca$^{2+}$ specific inhibitors, 1mM Ca$^{2+}$</td>
<td>Direct efflux of EtBr under various conditions where the concentrations of Ca$^{2+}$ are manipulated</td>
<td>Ca$^{2+}$ enhances the efflux activity of the LmrS efflux protein.</td>
</tr>
</tbody>
</table>
3.4 Identification of *S. aureus*

*S. aureus* was confirmed after conjugation by culture in Mannitol Salts Agar (MSA), sequencing, testing for coagulase production and latex agglutination. After sequencing, the FASTA files were compared to the *S. aureus* genome using the NCBI BLAST software. Muller-Hinton broth was used to determine the Minimum inhibitory concentration (MIC) for ethidium bromide and inhibitors.

3.5 Efflux Experiments Direct and Indirect

To determine the effect of Ca$^{2+}$ in the efflux systems of *S. aureus*, EtBr was used as a substrate, followed by various treatments with CaCl$_2$, Ca$^{2+}$-chelators and Ca$^{2+}$-dependent inhibitors. The MIC for EtBr was done using the broth dilution method and as recommended by the Clinical Laboratory Standards Institute. A final concentration of 2.5 mg/L EtBr was used for all efflux experiments and was previously published by (Martins et al., 2011) as the MIC for *E. coli* and *S. aureus* respectively. Bacterial strains were cultured overnight in BHI broth at 37°C in a Gyromax rotatory shaker (Amerex Instruments) at 220 rpm. Overnight cultures (1:100) were inoculated into 250 ml triple-baffled flasks containing 50 mL fresh BHI broth and grown to reach an OD$_{600}$ of 0.8. Bacterial cells were harvested by centrifugation at 5000 rpm for 5 min (Beckman Coulter Allegra, rotor C0650). Cells were washed three times in Phosphate buffer solution PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl) adjusting to the different experimental pHs 5.0, 7.0 and 8.0. Bacterial cell concentration was adjusted to an OD$_{600}$ of 0.6 for each pH. Efflux pump activity of EtBr was measured by fluorescence at 585nm in 96 microtiter fluorescent-based plates (Thermo Fischer Scientific) using a Glomax 3000 fluorometer (Promega). Calcium levels in media were manipulated by addition of CaCl$_2$ (Sigma Aldrich) at 1.0 and 5.0 mM. Ca$^{2+}$-
chelators EGTA and EDTA (Thermo Fisher) were added at concentrations of 10 mM for both 1 and 5mM. To investigate Ca$^{2+}$-dependent transport inhibitors, the phenothiazines, CPZ and TFP (Sigma Aldrich), were added at 20 mg/mL (CPZ) and 50μM (TFP). The Ca$^{2+}$ channel blocker, verapamil (Sigma Aldrich) was added at 30 μM. Efflux assays were conducted at the different pHs, 5.0, 7.0 and 8.0. Glucose (.4%) was used as a control for the contribution of metabolic energy to efflux. CCCP (Sigman Aldrich), a proton de-coupler was used to a final concentration 50µM to illustrate proton motive force disruption. Bacterial cells in Aliquots of 100 μL were placed in 96 microtiter plates and fluorescence was recorded at 580nm. For measurement of direct efflux in *E. coli* and *S. aureus*, the cells were inoculated and grown in Luria broth overnight at 37° C. The following morning the cells were sub-cultured and adjusted to 0.6 OD at 600nm. Cells were then incubated in EtBr with or without Ca$^{2+}$ inhibitors, chlorpromazine and calmidazolium or Ca$^{2+}$ chelator, EGTA for 30 minutes. As a control, *E. coli* cells without Ca$^{2+}$ inhibitors and EtBr were measured to understand the effects of Ca$^{2+}$ on efflux. After 30 minutes the cells were pelleted and washed twice with PBS at pH 7 or the indicated pH for the *S. aureus* experiments. Both *E. coli* wildtype and the LmrS clone were also measured for direct efflux as described above.

### 3.6 Cytosolic free Ca$^{2+}$ measurements

**Construction and expression of apoaequorin in *S. aureus*.**

*S. aureus* cells were streaked and transformed by conjugation using *E. coli* cells containing the apoaequorin coding sequence (apoaequorin is the protein without its prosthetic group, coelenterazine). Conjugation was performed according to the two protocols developed in this study. In the first protocol, *S. aureus* cells were grown in BHI broth over night at 37°C at 220 rpm.
$E. \text{ coli}$ cells containing the pMMBEH plasmid with the aequorin gene were grown overnight in LB broth at 37°C at 220 rpms. $S. \text{ aureus}$ and $E. \text{ coli}$ cells were subculture at a 1:100 dilution and grown to about .8OD. The OD was then adjusted to .5 and conjugation of pMMBEH proceeded using either of the following two methods. In the first method, 50µls of Carbenicillin (100ug/ml) was plated onto Chocolate agar plates and allowed to absorb into them for approximately 3 hrs. After absorption of the antibiotic into the agar, the $E. \text{ coli}$ culture containing the aequorin gene was streaked into an X pattern on the agar. The $S. \text{ aureus}$ culture was then streaked on the chocolate agar in a continuous pattern starting at the top and continuing to streak through the X pattern of the $E. \text{ coli}$ streak a several times. Transformed $S. \text{ aureus}$ was isolated by harvesting the white colonies growing next to the $E. \text{ coli}$ X pattern. Half of the colony was inoculated into BHI with 100µg/mL and the other half was streaked onto MSA agar plates and incubated for 48-72 h at 37°C. The $S. \text{ aureus}$ was easily distinguishable from $E. \text{ coli}$ due to the differences in color as compared to the grey color of $E. \text{ coli}$ when grown on chocolate agar. It should be noted that the yellow large colonies are the MRSA strain struggling to efflux carbenicillin due to no natural resistance to the antibiotic. These colonies were not selected because due to lack of presence of the plasmid.

![Figure 12](image-url): A. $S. \text{ aureus}$ (yellow and off white colonies) streaked through the $E. \text{ coli}$ X pattern.
In the second protocol, *E. coli* and *S. aureus* were grown in 250 mL triple-baffled flasks containing 50 ml LB (Becton Dickinson Difco™) broth with 100μg/mL carbenicillin (Sigma Aldrich) and BHI broth (Remel Thermo Fisher) respectively, at 37°C, in a rotatory shaker at 220 rpm overnight. After 18-20 hrs of incubation, *E. coli* and *S. aureus* cultures were adjusted to an OD600 of 0.5. A 1:5 dilution (100 μL of E. coli and 400 μL S. aureus cells) of the bacterial cultures were incubated in 10 mL of BHI with 100 μg/mL carbenicillin and incubated for three hours at 37°C (220 rpm). The culture was dotted (25 μl) onto BHI agar plates containing 100 μg/mL carbenicillin (Remel Thermo Fisher; Sigma Aldrich) and incubated for 48-72 h at 37°C. Transformed *S. aureus* was isolated by subculturing onto MSA (Beckton Dickinson Difco™) for 48-72 hrs at 37°C. Bacterial colonies that fermented mannitol were selected and further tested for coagulase (Beckton Dickinson BBL™) and *S. aureus* latex agglutination (Prolex™ Pro-Lab diagnostics). The presence of the plasmid containing the apoaequorin coding sequence was confirmed by PCR (BioRad iCycler Thermalcycler, BioRad, Hercules CA., USA) after extracting the plasmid. Confirmation of the presence of the aequorin sequence was amplified with the primers:

AQ440LICS: 5’-AAGGAGGAAGCAGGTATGGTCAAGCTACATCAGACTTCGAC-3’
AQ440LICS-CAS 3’-GACACGCACGGGTAGGACAGCTCCACCGTAG-5’ were used (Dominguez, D.C., 2011). The following PCR conditions were utilized to amplify the gene: Initial denaturation 95°C for 4.0 min., 30 cycles of 95° denaturation for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min and final extension for 10 min at 72°C. The aequorin protein was used to monitor the amount of intracellular free Ca²⁺. Aequorin is a protein that luminesces (λmax = 469 nm) when it binds to Ca²⁺. The Ca²⁺ concentration of the cytosol was measured at rest (basal levels) and after adding increasing concentrations of Ca²Cl₂. The
luminescence produced is directly proportional to the concentration of free Ca\(^{2+}\) within the cytosol.

![Figure 13](https://www.tebu-bio.com/blog/2014/06/30/how-to-measure-calcium-in-cell-based-assays/)

**Figure 13:** The function of aequorin and the prosthetic group, coelenterazine to produce luminescence when calcium enters the cell. Retrieved from [https://www.tebu-bio.com/blog/2014/06/30/how-to-measure-calcium-in-cell-based-assays/](https://www.tebu-bio.com/blog/2014/06/30/how-to-measure-calcium-in-cell-based-assays/) Baya, Ali El © copyright 2018

3.7 Expression and Reconstitution of aequorin in *S. aureus* cells

Twenty five mls of transformed *S. aureus* were grown in BHI-carbenicillin broth overnight at 37\(^{\circ}\) (220 rpm). Cells were diluted 1:100 in BHI-carbenicillin broth and incubated (same conditions) until the culture reached an OD\(_{600}\) of .25. The aequorin gene was induced by adding IPTG at 1 mM final concentration and incubated for additional 2 hrs. After two hours, cells were washed twice with 20 mL of ice cold (0-4\(^{\circ}\)C) HEPES buffer buffer (25mM HEPES, 1mM MgCl\(_2\) and 125mM NaCl, pH 7.0 or at the respective experimental pHs (5, 7, and 8) and pelleted by
centrifugation at 4500 rpm rotor C0650. Bacterial cells were re-suspended in 1 mL HEPES and 2.5 μM coelenterazine and incubated in the dark for 1h at room temperature. After incubation, cells were washed twice in 1 mL HEPES buffer and adjusted to an OD600 0.4 at the appropriate pH (according to the experiments performed) and stored on ice for subsequent readings.

3.8 Detection and Quantification of Intracellular Ca^{2+}

Chemiluminescence was measured using a digital luminometer Glomax 3000 (Promega) equipped with two dispensers and allowing the reading on 96-well microfluor microtiter plates (Thermo Fisher Scientific). Measurements were done in triplicates on 100 μL aequorin-loaded cells once every ten seconds for 60 sec to determine resting cytosolic free Ca^{2+} levels. Cells were then injected with a total concentration of 1.0 mM CaCl₂ and chemiluminescence was monitored for an additional 300-500 seconds according to the experiment pHs 5, 7 and 8. At the end of each experiment the remaining amount of aequorin was determined by adding equal volumes of discharge buffer (100mM of CaCl₂, 5.0% v/v Triton X-100) as described by (Dominguez, D.C., 2011). Total lysis of cells was also checked using light microscopy at a magnification of 100X oil immersion. The total chemiluminescence represented by the available aequorin was used to calibrate the concentration of cytosolic Ca^{2+}. Relative light units were converted to Ca^{2+} concentrations utilizing a matrix created by Dr. Anthony Campbell based on the formula: (pCa = 0.612x + 3.745, where x = -log₁₀k) and k = a constant for the rate of decay measured in per sec) (Jones, et. al., 1999). To understand the effect of the calcium chelator, EGTA on intracellular Ca^{2+}, S. aureus cells were injected 1mM final concentration of Ca^{2+} at the indicated time and after 60 seconds of monitoring they were injected with 5mM of EGTA. The luminescence was then tracked for an additional 250seconds. The negative control was S. aureus cells without the
apoaequorin plasmid. To control for differences in Ca\(^{2+}\) a measurement of the baseline luminescence was established by taking 10 consecutive readings without treatment.

3.9 Cloning of *lmrS* gene

For the investigation of the *S. aureus* efflux pump, the *lmrS* gene was amplified and cloned into the expression vector pRMC2 with primer: 5’-GCAAGCTTATGGCTAAAGTTGAATTAACAAC-3’ and 3’-GCGGATCCTTAAAATTTTCCTTCTATTACTTT-5’ (Floyd, J., 2010) and transformed into *E. coli* strain DH5-α. The following thermocycler conditions were used: 95°C 1 min, 35 cycles of 95°C 30 seconds, 51°C for 1 minute followed by 1 minute of extension at 72°C and a final extension step for 5 minutes at 72°C. Efflux activity of both *E. coli-lmrS* and *E. coli* DH5α was measured as previously described. Cells not incubated in 1.5mg/L of EtBr were also used as a control. Direct assays of EtBr efflux were done. Briefly, the cells were incubated for 30 minutes in EtBr and inhibitor. The cells were subsequently pelleted by centrifugation, washed twice and resuspended in PBS buffer. Fluorescence was measured as described above. After 5 minutes cells were injected with 1mM of CaCl\(_2\). Fluorescence was then monitored for an additional 20 minutes. The amount of EtBr to use was determined through other publications that measured fluorescence using *S. aureus* or *E. coli*. 
3.10 Measurement of gene Expression of Efflux Pumps, NorA, NorB, MgrA and Endogenous Control Genes, GyrA and GMK

MRSA clinical isolate, EBSA 78 and S. aureus ATCC 95923 cells were grown in BHI media overnight at 250 rpm. The cells were sub-cultured and grown for an additional 2 hrs at 37º C and 225 rpm so that the cells would reach log phase. The OD was adjusted to 0.6 (600nm) for each sample replicate. Four conditions were tested for gene expression. These are: EtBr (final concentration 2.5mg/mL) alone, EtBr and .5mM of CaCl₂, 1mM of CaCl₂ and EtBr and 5mM of EGTA. The cells were incubated in each treatment for an additional 45 minutes to establish the cell’s genetic response to each treatment. Total RNA was extracted under each condition utilizing an RNA extraction kit, Qiagen Ribopure based on acid guanidinium thiocyanate-phenol chloroform extraction. Briefly, 180µl of trizol was added to the pelleted bacteria. The bacteria was then vortexed using glass beads to break up the membrane. After the addition of chloroform, the upper aqueous layer containing the RNA was put in a column for purification. The RNA was washed with isopropanol and ethanol and finally eluted with TAE buffer. cDNA at a concentration of 1000ng was reverse transcribed as according to specifications for Quantibio XLT cDNA supermix under the following parameters: 5mins at 25º C, 60mins at 42ºC, 5mins at 85ºC and hold at 4ºC.

The real-time PCR was conducted according to the specifications of Forget-Me-Not™ EvaGreen® qPCR Master Mix under the following cycle parameters: of cDNA per sample at 2 min at 95ºC, followed by 40 cycles of 2-5s at 95ºC; 10s at 55ºC; 20s at 72ºC using a Quantstudio 7 real-time PCR system. The annealing temperature was done at 55ºC according to the specifications of the master mix. The treatment conditions were assessed for efflux pump gene expression of NorA and NorB and their regulator MgrA. The gmk and GyrA genes were
used as the endogenous controls to serve as a standard of normal expression levels for comparison.

Primers (shown below) were designed to have the same amplicon length in order for the expression levels of these genes to be accurately measured in comparison to the endogenous controls (housekeeping gene expression). For the eva green studies, the fluoroprobe primer was not added to the reaction. Relative quantification and qualitative analysis were performed to ensure that there are similar concentrations, and that the quality of the RNA was good. The quality was good if it is relatively pure and not containing any contaminants from the extraction process or from genomic DNA. RNA was quantified and the quality were determined using a nanodrop and an RNA denaturing gel.

3.10.1 Analysis of gene expression

A comparative method was performed to quantify expression. We determined the threshold cycle \( C_t \) which is the point at which a fluorescent reporter has reached a value that is significantly different from the normalized value. Relative gene expression was quantified using the Livak \( 2^{-\Delta\Delta CT} \) method (Livak, K., 2001). This method is commonly used with a standard that includes an endogenous control gene that would not be affected by the treatments used in the experiment as previously stated. For the preliminary data Syber green fluorescence was used. This may be problematic as software usually automatically removes fluorescent background. Primer dimers can also affect the results since the fluorescent background is usually unknown. Other methods include absolute quantification which estimates gene expression based on a standard curve (Bustin, S., 2009). For this study we will use the \( \Delta\Delta CT \) method as described below.
The statistical analysis for this gene expression experiment was an ANOVA test where the gene of interest was statistically tested for differences in expression levels under the different treatments. Reference genes (Endogenous controls) should include housekeeping genes that are not up regulated during any of the EtBr, Ca\(^{2+}\) or inhibitor treatments. Since more than one condition was analyzed, an ANOVA was used to see how all the genes compare to each other as well as the control. A correlation analysis could also be used to elucidate how a treatment affects multiple efflux genes (Rocha, D., 2015). Normalization of the samples is imperative. It requires analysis of mRNA ratios of target genes to reference genes. Use of one reference gene is not enough when studying expression (Bustin, S. 2009).

The primers were designed in a similar manner as (Patel, D., 2010) to provide a more precise measure of fluorescence in a signature wavelength. Primers with fluorescent TaqMan® probes were designed that target the specific efflux genes, LmrS, NorA and its regulator, MgrA (Truong-Bolduc, Q.C., 2011). Housekeeping genes, GMK and GyrA were used to set up a standard for base expression of genes not associated with efflux. These genes have been previously identified by (Theis, T., 2007; Truong-Bolduc, Q.C., 2011) as suitable for measuring gene expression under EtBr. cDNA dilution curves were utilized by taking known amounts of cDNA and running them under the above cycle parameters for qRT-PCR. The amount of cDNA has a direct proportional relationship to the log fold change in genetic expression of efflux. Relative genetic expression will be determined utilizing the ΔΔCT method as described in (Livak, K., 2001; Xiayu, R., 2014). The change in threshold cycle (ΔCT) is determined by subtracting the CT of the housekeeping gene from the CT of the target efflux gene under each condition.
\[ \Delta \Delta CT = \Delta CT(\text{Average of target efflux gene}_{\text{treatment}}) - \Delta CT(\text{average of reference}_{\text{treatment}}) \] (Livak, K., 2001; Rao, X., 2013; Xiayu, R., 2014). The \( \Delta \Delta CT \) is calculated by the differences in \( \Delta CT \) of the target genes and reference or control condition of each gene under the same treatment minus the average \( \Delta CT \). The control condition was EtBr alone without CaCl\(_2\) or inhibitors and the fold change in gene expression was determined by the formula \( 2^{\Delta \Delta CT} \). (Livak, K., 2001)

Incubation in EtBr links efflux preliminary data to intracellular Ca\(^{2+}\) and genetic expression of efflux. A more direct role that Ca\(^{2+}\) plays in efflux expression was determined by measuring genetic expression under the different conditions described above.
Table 4: Fluoroprobe Primers\(^a\) JOE, 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein; BHQ black hole quencher 534nm, 6-carboxyfluorescein; 6-FAM, TAMRA, 6-carboxytetramethylrhodamine; Source: (IDT technologies)\(^b\) genes published in (this study)

<table>
<thead>
<tr>
<th>Gene</th>
<th>5'-3' Forward</th>
<th>5'-3' Reverse</th>
<th>Taqman Reporter Sequences(^a,b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgrA(^b)</td>
<td>AGC TGA AGC GAC TTT GTC AGA TGC</td>
<td>AGC GTG AAC GTT CCG AAG TCG A</td>
<td>JOE-AATTGTAGCACCACGA CCTGCCCCAGTC-BHQ1</td>
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<tr>
<td>NorA(^b)</td>
<td>GAC ATT TCA CCA AGC CAT CAA</td>
<td>TGC CAT AAA TCC AAC AAT CC</td>
<td>6FAM-AGGCATAACCATACCA GCACACATACACC-BHQ1</td>
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<tr>
<td>LmrS(^b)</td>
<td>GCT AAC GAT GTT GTA GGT GA</td>
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<tr>
<td>Gmk(^b)</td>
<td>GGTGAAACAGACGAA GAG</td>
<td>TTACCAGTTCCGATT GCC</td>
<td>6FAM-ACCGATTGCATCATT GCCATTAT-BHQ1</td>
</tr>
</tbody>
</table>
3.11 Statistical Methods and MIC

For the fluorescent experiments a non-parametric Kruskall-Wallis was conducted to determine the differences in Ca$^{2+}$ treatments (CaCl$_2$ 0.5, 1.0 and 5.0 mM), Ca$^{2+}$ inhibitor treatments (phenothiazines, verapamil and Ca$^{2+}$ chelators) compared to the controls. For the cytosolic free Ca$^{2+}$ measurement experiments, a Kruskal-Wallis non-parametric analysis was used to determine significant differences in intracellular Ca$^{2+}$ levels at concentrations: 0.5mM, 1.0mM and 5.0 mM. To determine significant differences of cytosolic Ca$^{2+}$ at each pH (5.0, 7.0, and 8.0) a one sample t-test and Wilcoxon test was conducted. Both the efflux and cytosolic free Ca$^{2+}$ experiments were analyzed using the statistical and graphics software, Graph Pad 8.0. Gene expression experiments were analyzed using a two-way ANOVA.

3.11.1 MICs

To determine the MIC for each antibiotic, *S. aureus* cells containing the aequorin plasmid were grown overnight in BHI then subcultured into Mueller-Hinton and grown to an OD$_{600}$ 0.8. The culture was then adjusted to a 0.5 McFarland standard (aprox. $10^8$ CFU/mL) in 5 mL. The MIC was determined using MicroScan autoSCAN-4 automated system using MIC panels for Gram positive bacteria PBCP20.

The MIC for the antibiotics for the *E. coli* DH5$\alpha$ and *E. coli-lmrS* was determined using the MicroScan autoSCAN-4 automated system using PBCP-34 panels. Both of the strains were grown in Mueller-Hinton broth overnight and were subcultured and grown to an OD$_{600}$ 0.8 and subsequently adjusted to a 0.5 McFarland standard (aprox. $10^8$ CFU/mL).
The MIC for EtBr and inhibitors CCCP, Ver and CPZ were determined from similar or less concentrations as used in (Couto, et al., 2008) for *S. aureus*. The MIC for the inhibitor trifluoperazine was determined using similar or less concentrations as (Mazumbder et al., 2001). The MIC for inhibitor CDZ was done using the broth dilution method and as recommended by the Clinical Laboratory Standards Institute and also with references using similar concentration. The MIC was determined as the highest dilution where *S. aureus* or MRSA failed to grow. A final concentration of 2.5 mg/L EtBr was used for both direct and indirect efflux experiments.
### 3.12 Chemicals and Biochemicals used in the study

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
<td>EtBr</td>
</tr>
<tr>
<td>ethylene glycol-bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
<td>EGTA</td>
</tr>
<tr>
<td>ethylenediaminetetraacetic acid</td>
<td>EDTA</td>
</tr>
<tr>
<td>isopropyl-β-D-thiogalactoside</td>
<td>IPTG</td>
</tr>
<tr>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
<td>HEPES</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chlorophenylhydrazone</td>
<td>CCCP</td>
</tr>
<tr>
<td>Calmidazolium</td>
<td>CDZ</td>
</tr>
<tr>
<td>Trifluopropazine</td>
<td>TPZ</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Ver</td>
</tr>
<tr>
<td>Phosphate Buffer Solution</td>
<td>PBS</td>
</tr>
<tr>
<td>Tris-Acetate EDTA buffer</td>
<td>TAE</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>CPZ</td>
</tr>
<tr>
<td>coelerenterazine,</td>
<td>CTZ</td>
</tr>
<tr>
<td>Verampamil</td>
<td>Ver</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>TPZ</td>
</tr>
</tbody>
</table>
4.1 The effects of Ca$^{2+}$ and pH on intracellular calcium and calcium homeostasis

The measurement of cytosolic Ca$^{2+}$ is essential in establishing that Ca$^{2+}$ might serve as an intracellular signal. For this reason, the amount of intracellular calcium [Ca$^{2+}$]$_i$ was monitored to establish the possibility that S. aureus is utilizing Ca$^{2+}$ as an intracellular signal to upregulate specific genes. Based on this assumption we determined if S. aureus, was able to maintain [Ca$^{2+}$]$_i$ homeostasis in response to increasing levels of extracellular Ca$^{2+}$ and pH. To accomplish this, we used the photoprotein, aequorin to measure [Ca$^{2+}$]$_i$. Increasing luminescence was directly proportional to increasing concentrations of external CaCl$_2$. The [Ca$^{2+}$]$_i$ values ranged from 6.79, 16.98 and 20.65 µM for .5, 1 and 5mM of extracellular calcium, respectively. This rapid spike was followed by a sharp decline in [Ca$^{2+}$]$_i$ after a few seconds until it reached baseline levels of about .69-.80 µM. This indicated the cells’ ability to maintain calcium homeostasis despite a high concentration of external CaCl$_2$ (Figure 14A) Incubation with EGTA showed a significantly decreased amount of [Ca$^{2+}$]$_i$ at 4.97 µM as compared to cells without EGTA at 16.98 µM. (Figure 14B). Our results are consistent with (Guragain et al., 2013; Dominguez, 2011; Torrecilla et al., 2001, 2000; Holland et. al., 1999) demonstrating the ability of S. aureus to maintain [Ca$^{2+}$]$_i$ despite the addition of of CaCl$_2$ in the range of 0.5-5mM.

The effect of pH was also examined since S. aureus MFS and MATE transporter families have been shown to use electrochemical gradients and proton motive force as the driving force for extrusion of toxic compounds (toxic compound/H$^+$) (Jang, 2016; Santos Costa et al., 2013). We investigated the ability of S. aureus to maintain [Ca$^{2+}$]$_i$ in the pHs 5, 7 and 9 to observe any significant changes due to loss of proton motive force. The cells were adjusted to pH 5, 7 and 8.
(Figure 14C) and baseline [Ca$^{2+}$]$_i$ was monitored for 10s followed by the injection of external CaCl$_2$ to a final concentration of 1mM. After injection a peak was observed in response then followed by a sharp decline in Relative Light Units (RLUs) until baseline [Ca$^{2+}$]$_i$ levels were restored. Significant differences were observed by conducting a student’s T test for each peak produced under the varying conditions and at each pH. At pH 9 [Ca$^{2+}$]$_i$ reached 23µM, at pH 7, 17µM and finally at pH 5 the [Ca$^{2+}$]$_i$ levels rose to 9.5µM (+/- SD.127, .261 and .162, respectively) (Figure 14C). Finally, the effects of EGTA injection on cells to determine the effect of [Ca$^{2+}$]: (Figure 14D). The results show that EGTA induced a peak at 23.1µM before quickly declining to basal levels of about 3µM. Our results also demonstrate that S. aureus mainatin [Ca$^{2+}$]$_i$ homeostasis in the micromolar range
In order to survive, bacteria must be able to sense and respond to different environmental conditions. These environmental conditions illicit unique responses in the form of $[\text{Ca}^{2+}]_i$ changes (Naseem et al., 2009; Torrecilla et al., 2000; Herbaud et al., 1998). Similar to eukaryotic cells, cytosolic $\text{Ca}^{2+}$ transients are very dynamic and vary in shape, amplitude, speed and spatiotemporal patterns. The response to different antibiotics was investigated to determine if *S. aureus* $[\text{Ca}^{2+}]_i$ was capable of sensing each antibiotic (Figure 14). The antibiotics tested were erythromycin, gentamycin, kanamycin, vancomycin, streptomycin and ciprofloxacin. The results
obtained demonstrate unique \([\text{Ca}^{2+}]_i\) responses in terms of amplitude, wavelength, oscillation patterns and peak duration times. Table 6 shows the peak differences in \([\text{Ca}^{2+}]_i\), which ranged from 2.65 to 12.82\(\mu\)M. The lowest was streptomycin at 2.65\(\mu\)M and 40s in length and oscillation pattern. The highest peak at 12.82\(\mu\)M elicited by erythromycin demonstrated the shortest peak length at 20s with no oscillation patterns. All of the antibiotics with the exception of erythromycin, elicited an initial peak followed specific oscillation pattern at different lengths in seconds. Gentamycin, kanamycin and vancomycin induced a specific peak amplitude at 4.01, 5.32 and 3.65\(\mu\)M respectively and differed in peak lengths of 70s, 30s and 130s respectively. Ciprofloxacin induced two large peaks at 3.37\(\mu\)M at a length of 130s each. These results demonstrate that the antibiotics are eliciting unique responses as indicated by the differences in oscillation patterns, speed, amplitude, and spatiotemporal patterns that each antibiotic elicited (Table 6).
Figure 15: Intracellular Ca\textsuperscript{2+} responses to different antibiotics. The results presented represent the mean of biological replicates.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Transient</th>
<th>Average amplitude max. height</th>
<th>Transient pattern</th>
<th>Length of peak</th>
<th>Oscillation length</th>
<th>Transient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td></td>
<td>12.82</td>
<td>Peak</td>
<td>20 s</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
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<td>4.01</td>
<td>Initial peak oscillation</td>
<td>70 s</td>
<td>50 s</td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
<td>5.32</td>
<td>Gradual decrease in Ca^{2+} with oscillation</td>
<td>30 s</td>
<td>60 s</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>3.65</td>
<td>One peak followed by oscillation</td>
<td>130 s</td>
<td>80 s</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td>2.65</td>
<td>Peak followed by oscillation</td>
<td>40 s</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td>Omadroxacin</td>
<td></td>
<td>3.37</td>
<td>Peak followed by one oscillation</td>
<td>130 s</td>
<td>40 s</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Effect of Ca\(^{2+}\) and Ca\(^{2+}\) inhibitors in MRSA efflux of EtBr

To assess the level of efflux activity, we measured EtBr accumulation and extrusion of clinically isolated MRSA cells using fluorometry. For reference, the higher the cells accumulated EtBr, the higher the fluorescence. Conversely, the more the cells extruded EtBr, the lower the fluorescence. Efflux pump activity was measured directly and indirectly in MRSA cells. In the direct method bacterial cells were pre-incubated with EtBr in the presence of an inhibitor of the Ca\(^{2+}\) or pump energy source at the appropriate pH. The cells were then washed and fluorescence was monitored for 5 minutes to establish basal fluorescence levels. After 5 minutes the media was injected with CaCl\(_2\). In the indirect assays, the fluorescence was recorded after the pre-incubation period with EtBr and the treatment.

To assess the effect of Ca\(^{2+}\) had on efflux of EtBr, the cells were incubated in different concentrations of CaCl\(_2\), with or without glucose (0.4%) and Ca\(^{2+}\) specific inhibitors or chelators. Glucose was used as a control to distinguish the role of Ca\(^{2+}\) from metabolic energy. The Ca\(^{2+}\) inhibitors included the Ca\(^{2+}\)-chelator EGTA and the phenothiazines, chlorpromazine (CPZ) trifluoperazine (TFP), which are known to inhibit efflux pump activity in various pathogens including \textit{S. aureus} (Koul et al., 2009; Martins et al., 2011; Naseem et al., 2008; Pule et al., 2016). Phenothiazines are chemicals known to bind to Ca\(^{2+}\)-binding proteins and target prokaryotic cell membranes (Amaral, Leonard, et al., 2010; Kaatz, et al., 2003). These chemicals affect the bacterial cells by interfering with Ca\(^{2+}\) related processes such as reducing the ability of Ca\(^{2+}\) to bind to transporter proteins as well as Ca\(^{2+}\) dependent enzymes involved in ATP hydrolysis. We also used calmidazolium which inhibits calmodulin, a eukaryote protein and verapamil, a Ca\(^{2+}\) channel blocker (Sunagawa, et al., 2000; Mctavish, D., 1989). We monitored how these inhibitors affected efflux at pHs 5, 7 and 8 to distinguish the contribution of proton motive force (PMF) to efflux. We monitored the efflux activity of \textit{S. aureus} according to the
Martins et. al., 2011 protocols but with modifications. At pH 7 the cells were able to efflux EtBr but significantly less than at pH 5 (Figure 16 A-D) as indicated by fluorescence values between over 1000 as compared to pH 5 with values below 500. The addition of CDZ, Ver, TFP and EGTA showed a significant decrease in the ability of MRSA to efflux EtBr, indicated by the increase of relative fluorescence (RF) significantly higher ranging between 1000 and 1500 at pH 5. This was in comparison to cells treated with CaCl2 and the control cells which were cells that contained EtBr alone (untreated). We also tested efflux activity at pH 7.6 to see if there were any changes with a slight increase in pH (Figure 16A). These cells did demonstrate a significant amount of accumulation at a slightly higher pH and increasing with time from 800 to higher than 2000. The effect of pH was examined since MRSA MFS and MATE transporter families utilize proton motive force as the energy for efflux of toxic compounds at pH 5 (toxic compound/H+) (Jang, 2016; Santos Costa et al., 2013). The cells were also significantly more efficient at EtBr efflux than at pHs 7 or 8 (Figure 17A). This was especially true at pH 8 where proton availability was decreased due to protons being lost in the alkaline solution (Figure 17B). To determine statistical significance, we used a Kruskall-Wallis statistical analysis. S. aureus cells that were treated with either a Ca2+ chelator or inhibitor showed significantly less ability to efflux as compared to cells treated with 1mM of CaCl2.

A rescue assay was conducted to see if efflux could be restored by the addition of Ca2+ after being treated by a Ca2+ chelator at pH 8 and the addition of the Ca2+ antagonist CPZ (0.1µM) (Figure 18). The cells were monitored for accumulation with EDTA at pH 8 and one of the following was added: CPZ, CPZ + 1mM of CaCl2 and CaCl2. Before the addition of the treatment, the cells showed a steady and rapid accumulation of EtBr reaching to over 2000 fluorescent units. The addition of CaCl2 was able to rescue efflux in MRSA at pH 8 as was
evident in the fluorescence values decreasing to 1500 and showing a trend of steadily decreasing in florescence after the addition of CaCl$_2$. This was true for the treatment with both CPZ and CaCl$_2$. In this treatment, the fluorescence values were slightly higher than with CaCl$_2$. The treatment with CPZ continued to increase in fluorescence indicating accumulation of EtBr. Our results are also consistent with Martins et al., 2011 which showed similar results with _E. coli_.

Direct efflux experiments where the cells were pre-incubated in treatment followed by washing of the cells showed similar results (Figure 19 A-C). The experiments were conducted to measure efflux of EtBr within the cells directly by removing excess treatment from the media and resuspending in PBS buffer at the given pH. Similar differences were observed at each pH as with the indirect methods. Here we injected the media with 1mM of CaCl$_2$ and monitored the fluorescence in real time. All of the EtBr experiments demonstrated lower fluorescence values with each concentration of CaCl$_2$ despite the pH. At pH 8, the cells fluoresced at 3076 arbitrary fluorescence values as opposed to 2485 (+/- SD 99) without the addition of EGTA. At pH 7 differences between the the cells at pH 7 or with CDZ were significant at 145.2 and 646.2, respectively (+/- SD 25.4). Finally, at pH 5 significant differences between the cells at pH 5 and with the proton de-coupler CCCP showed a difference of 93.5 and 124.6, respectively (+/- SD 6.5). The results indicate that calcium was important for efflux irrespective of pH.
Figure 16: EtBr efflux and extrusion measured in response to Ca\(^{2+}\) inhibitors at pH 7 (A) The effect of CaCl\(_2\), Glucose (4%), EGTA on efflux of EtBr at pH 7.6 (B) The effect of CDZ, CaCl\(_2\) and glucose on the ability of MRSA to efflux EtBr efflux at pH 7. (C) The effects of CaCl\(_2\), TFP and glucose on EtBr efflux at pH 7. (D) The effects of CaCl\(_2\), Ver and glucose on EtBr efflux at pH 7. The results represent the average of three biological replicates at each time point. Significant differences were seen between calcium, calcium inhibitors and controls (p<.0001) Kruskal-Wallis, non-parametric.

Figure 17: EtBr efflux and extrusion at pHs 5 and 8. (A) S. aureus cells were treated with CaCl\(_2\) 1 and 5mM, CCCP (50µM) and glucose at pH 5 (B) EtBr efflux was measured at pH 8 after the addition of CaCl\(_2\) (1 and 5mM), CPZ (20mL) and EDTA (10mM). The results presented are an average of three biological replicates. A non-parametric Kruskal-Wallis test was conducted to find significant differences between CaCl\(_2\) treatments and inhibitors (p < .0001).
**Figure 18:** Rescue assay where fluorescent measurements were taken at pH 8. The cells were incubated in EtBr and EDTA and fluorescence was measured for 20 minutes. The experiments were then paused and 0.1 µM of CPZ, 0.1 µM of CPZ + CaCl₂ 1mM or CaCl₂ 1mM was added to the media and fluorescent monitoring was resumed for an additional 20 minutes.

**Figure 19:** Direct efflux with MRSA clinical isolate EBSA78. MRSA cells were incubated at pHs 5, 7 and 8 in EtBr and CCCP, CDZ or EGTA. After 5 minutes of recording fluorescence, CaCl₂ injected at a final concentration of 1mM. Fluorescence was then monitored for an addition 20 minutes to observe the effects of calcium on efflux in different pHs and inhibitors and chelators of calcium.

### 4.4 Calcium enhances efflux of Ethidium Bromide (EtBr) in LmrS efflux pump

In order to evaluate the effect of Ca²⁺ in the efflux of EtBr more directly, we cloned the *S. aureus* multi-drug resistant pump *lmrS* gene into *E. coli* cells. We then evaluated if the LmrS pump conferred antimicrobial resistance to the *E. coli* cells (E. coli DH5α-*lmrS* containing the efflux pump and the control E. coli DH5 α, without the pump) by determining the MIC using the microbroth dilution method described in the materials and methods. The resulting MIC illustrated that the *lmrS* gene conferred resistance to all the antibiotics tested (*Table 6*). This data...
was expected considering that the LmrS pump can efflux a wide variety of dissimilar substances across the membrane with the help of pmf/electrochemical gradient. As previously mentioned, the LmrS pump is a member of the MFS family of efflux pumps and relies on pmf to transport substances across the membrane.

4.5 Differences of EtBr Efflux between *E. coli* DH5α and cloned lmrS recombinant *E. coli* (DH5α-*lmrS*)

We assessed the direct efflux of EtBr with both wild type and recombinant *E. coli* cells as indicated in materials and methods. Briefly, the cells were incubated in EtBr (1.5 mg/L) then pelleted and resuspended with PBS to remove any excess EtBr or inhibitors from the media. This allowed for the monitoring of actual efflux and accumulation of EtBr that was already inside the cells. The cells were then monitored for basal fluorescence levels for 5 minutes before injecting the media with CaCl₂ to a final concentration of 1mM. Fluorescence was then monitored for an additional 30 min. Both the recombinant and wildtype cells showed a decrease in fluorescence indicating an immediate enhancement of efflux after the addition of CaCl₂. Differences at each pH were similar to those done with MRSA studies. A Kruskall-Wallis test showed significant differences in starting efflux between the wildtype (DH5α) and recombinant (DH5α-*lmrS*) cells (Figure 21) at 244.6 and 163.3, respectively (SD +/- 11.8) thus demonstrating that the expression of lmrS significantly increases the capacity of efflux.

Direct efflux was also utilized to observe the effects of the Ca²⁺ chelator EGTA and the phenothiazine chlorpromazine (CPZ) as described in materials and methods. The cells were incubated in either EGTA (Figure 21A) or CPZ (Figure 21B) resulting in an increase in accumulation for both the wildtype and recombinant cells as compared to Figure 20 where no inhibitors were used for the experiment. Differences in fluorescence between DH5α-*lmrS* and
wildtype cells incubated in EGTA were 290 and 763 (SD +/- 24.06). The results for CPZ also showed similar differences at 242.5 for recombinant cells and 722 (SD +/- 17.14). As with Figure 19, a Kruskall-Wallis was conducted to determine significant differences in efflux between wild type and recombinant cells. After 5 minutes of recording basal levels of fluorescence, 1mM of CaCl₂ was injected into the media and fluorescence was observed for an additional 30 minutes. The addition of CaCl₂ to the media resulted in a rapid increase of efflux indicated by a decrease in fluorescence.

Figure 19: A Kruskal-Wallis non-parametric test revealed significant differences in efflux rate between wildtype and recombinant cells. (p<.0001)

Figure 20: Direct measurement of efflux and accumulation of EtBr in both wildtype and recombinant cells. The addition of 1mM CaCl₂ demonstrate an increase in efflux. A Kruskal-Wallis non-parametric test revealed significant differences in efflux rate between wildtype and recombinant cells. (p<.0001)
Figure 21: The wildtype and recombinant cells were incubated in either EGTA (A) or CPZ (B) to demonstrate the enhancing effects of CaCl₂. Significant differences in the rate of efflux were observed between wildtype and recombinant cells. The data represents the mean of three biological replicates (p<.0001).

Table 7: Cloning of the LmrS efflux pump gene into *E. coli* resulted in significantly higher resistance to different classes of antibiotics as compared to the wildtype strain.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>LmrS-DH5α (μ g/ml)</th>
<th>Resistant</th>
<th>E. coli DH5α (μ g/ml)</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/Streptomycin (A/S)</td>
<td>10/8</td>
<td>R</td>
<td>8/4</td>
<td>S</td>
</tr>
<tr>
<td>Amikacin (AK)</td>
<td>32</td>
<td>R</td>
<td>16</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin (AM)</td>
<td>16</td>
<td>R</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Ceftazidime (CAZ)</td>
<td>16</td>
<td>R</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>Cefuroxime (CFX)</td>
<td>16</td>
<td>R</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Cefazolin (CFZ)</td>
<td>16</td>
<td>R</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Ciprofloxacin (CP)</td>
<td>2</td>
<td>R</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>Cefuroxime (CRM)</td>
<td>16</td>
<td>R</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Gentamicin (GM)</td>
<td>8</td>
<td>R</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Imipenem (IMP)</td>
<td>8</td>
<td>R</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Levofloxacin (LVX)</td>
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<td>S</td>
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<td>Piperacillin/Tazobactam (P/T)</td>
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<tr>
<td>Tetracycline (TE)</td>
<td>8</td>
<td>R</td>
<td>4</td>
<td>S</td>
</tr>
</tbody>
</table>
4.6 The effects of Ca$^{2+}$ on gene expression of the efflux pump, NorA, LmrS and MgrA

Significant differences in efflux expression were seen with the addition of Ca$_2$Cl to the media as compared to EGTA and trifluoperazine. The NorA gene demonstrated significantly higher upregulation with 0.5 and 1mM of Ca$_2$Cl than with the control expression with EtBr alone. All genes tested showed a significant upregulation with Ca$_2$Cl as compared to calcium specific chelator, EGTA (Figures 22-24). The fold change of expression between 0.5mM CaCl$_2$ and EtBr alone is 1.5 (SD +/- .1) for NorA (Figure 22). The fold change in expression levels for NorA were as follows, 5.79 (SD +/- .78) EtBr control, 18.64 (SD +/- .45) .5mM CaCl$_2$, 11.59 (SD +/- 1.21) 1mM CaCl$_2$ and finally 3.6 (SD +/- .4) EGTA. A one-way ANOVA test revealed significant differences between the means including the control, CaCl$_2$ concentrations and EGTA p<.0001). The greatest differences were seen between the CaCl$_2$ concentrations 0.5mM and 1mM treatments and EGTA which resulted in a 15.04 fold change difference. Significant fold change differences were also observed between CaCl$_2$ concentrations and control resulting in a 12.85 fold change difference. The lowest expression level was observed with the calcium chelator, EGTA indicating the importance of CaCl$_2$ for efflux expression. Both CaCl$_2$ concentrations were also significantly higher that the control (EtBr alone).

The MgrA gene, when phosphorylated modulates the expression both NorA and NorB efflux pumps (Truong-Buldoc, Q., 2010). Here, we measured the expression levels of the MgrA regulator in different calcium concentrations as well as the chelator, EGTA to see if there were differences in expression levels. The fold changes are as follows, The control with EtBr at 3.7 (SD +/- 1.8), .5mM CaCl$_2$ at 9 (SD +/- 1.1), 1mM of CaCl$_2$ (SD +/- .5) and EGTA at .64 (SD +/- 2.1). The highest fold change difference of 8.36 was observed between 0.5 mM of Ca$^{2+}$ and
EGTA (Figure 23). Interestingly, the removal of trace calcium resulted in significant
downregulation as compared to calcium treatments and the control (EtBr alone). The results
indicate modulation of expression of MgrA by calcium and directly links calcium to expression
of the regulator and to the regulation of genes under its control (NorA).

Finally, we measured expression levels under the same conditions where calcium
concentrations were manipulated to understand the influence of calcium on lmrS efflux gene
expression. The results presented show an unprecedented concentration dependent expression
with calcium. The expression level of lmrS went up 2 fold between .5mM and 1mM of calcium
respectively (Figure 24). This result was not seen in NorA or in MgrA. The results for the
expression levels under the four conditions were 3.5 (SD +/- .3) for the control, 6.08 (SD +/- .5)
for .5MM CaCl2, 7.92 (SD +/- .31) for 1mM CaCl2 and finally 2.64 (SD +/- .4) for EGTA. The
expression of lmrS in the presence of EGTA resulted in an almost 5.28 fold reduction from
1mM CaCl2 to 5mM of EGTA.
Figure 22: The NorA gene expression levels with the EtBr control (EtBr alone), 0.5 and 1mM of Ca\(_2\)Cl\(_2\) and 5mM EGTA. The results presented demonstrate significant differences between Ca\(_2\)Cl\(_2\) and inhibitors. An ANOVA was used to analyze the means across treatments. The CT values of three replicates were averaged for each treatment (p<.0001).

Figure 23: MgrA is the regulator of NorA and NorB. Here significant differences are seen between the Ca\(^{2+}\) treatments, EGTA and EtBr control. The highest fold change difference of 8.36 was observed between 0.5 mM of Ca\(^{2+}\) and EGTA. The removal of Ca\(^{2+}\) by the chelator, EGTA shows a significant downregulation as compared with the other treatments indicating that Ca\(^{2+}\) may be crucial for efflux regulation. ANOVA (p=.0008)
Figure 24: *lmrS* gene expression is concentration dependent on Ca$^{2+}$. EGTA significantly downregulated gene expression as compared to Ca$^{2+}$ and the control. Significant differences were observed between all the means. ANOVA (p<.0001)
5.1 $[Ca^{2+}]_i$ homeostasis

The results presented in this dissertation demonstrate for the first time that expression of efflux is a Ca$^{2+}$ mediated process in $S. aureus$. We were also the first to demonstrate that the extrusion of EtBr by the multi-drug transporter LmrS is enhanced by Ca$^{2+}$ ions as well as the tight control of cytosolic Ca$^{2+}$ by $S. aureus$ cells. Based on eukaryote studies, Ca$^{2+}$ has been identified as a signaling messenger. Environmental signals cause changes in cytosolic free Ca$^{2+}$ in order to transmit signals, which lead to a physiological response (Torecilla et al., 2000; Naseem et al., 2009). The maintenance of control of cytosolic Ca$^{2+}$ is important because it is a pre-requisite for cell signaling. This was evidenced by the Ca$^{2+}$-mediated responses to different types of antibiotics, which showed unique spatial and temporal oscillation patterns. The results presented here are in agreement with other studies that have measured $[Ca^{2+}]_i$ in other bacteria (Torecilla et al., 2000, 2001; Dominguez, 2004; Bruni et. al., 2017; Gonzalez-Pleiter et al., 2017). In agreement with previous studies, our results show that $S. aureus$ maintains $[Ca^{2+}]_i$ in the micromolar range in the presence of 0.5-5 mM external CaCl$_2$. Maintaining low levels of $[Ca^{2+}]_i$ in the micromolar range protects the cells from the toxic effects of high concentrations of $[Ca^{2+}]_i$. Low levels of cytosolic Ca$^{2+}$ are also needed for signal transduction. Increases in cytosolic Ca$^{2+}$ must quickly disappear for the second signal to take place (Carafoli, 1987; Torrecilla et al., 2000). The implications of this work suggest that the same coordination and signaling transduction pathways observed in eukaryotes are also occurring in bacteria and may be utilized to upregulate specific genes (Campbell, A.K., 2014; Torecilla et al., 2000; Holland, et. al., 1999). The maintenance of low $[Ca^{2+}]_i$ levels after a spike despite flooding the media with
high CaCl₂ concentrations is further evidence of calcium signaling and is also demonstrated by a consistent [Ca²⁺]ᵢ value after the peak subsides for seconds after the initial addition of CaCl₂ to the media.

5.2 The effects of pH on [Ca²⁺]ᵢ

The observation that Ca²⁺ transients occur as a result of pH fluctuations (pH 5,7,9) suggest that *S. aureus* is able to sense pH environmental conditions through changes in cytosolic Ca²⁺. We saw that different pH levels influenced the [Ca²⁺]ᵢ transients. These signals may be important for the organism to adapt and survive in different conditions such as in the human host. It is known that pH is important in various infection processes, wound healing and skin colonization (Foulston et al., 2014). Moreover, the phagosome-lysosome environment is highly acidic implying that *S. aureus* must have sensor systems in response to the acidic conditions to adjust gene expression for survival (M. Martins et al., 2008; Venditri et al., 2003). It is interesting to note that pH triggers gene expression in various bacteria (A. Martins et al., 2009; Perez & Groisman, 2007; Serra-Cardona et al., 2015; Weinrick et al., 2004) including genes involved in cell envelope structure, ion transporters and multidrug transporters (Hayes et al., 2006; Truong-Bolduc et al., 2011; Weinrick et al., 2004). However, the regulatory pathways by which bacteria sense and respond to pH stimuli have not been elucidated.

Another important aspect of pH is that bacteria utilize electrochemical gradients to transport Ca²⁺ ions into the cell exchanging H⁺ or Na⁺ for Ca²⁺ (Dominguez, et al., 2015; Colinet et al., 2016). Our results show an increasing amount of [Ca²⁺]ᵢ directly proportional to the pH. The lowest level of [Ca²⁺]ᵢ at pH 5 (9.5 μM Ca²⁺) and highest for pH 9 (23 μM Ca²⁺). These results demonstrate a potential competition between Ca²⁺ and H⁺ for access into the cell at pH 5.
These results imply that $\text{Ca}^{2+}$ and $\text{H}^+$ are competing for influx into the cell resulting in lower $[\text{Ca}^{2+}]_i$ at pH 5 in comparison to pH 7 and pH8 resulting in increasing levels of $[\text{Ca}^{2+}]_i$ respectively. This also demonstrates the importance of proton motive force in bacterial efflux and influx (Martins, et al., 2011; Griffith et al., 2018; Nové et. al., 2020).

5.3 Antibiotics and Calcium signaling in \textit{S. aureus}

The cells were exposed to different classes of antibiotics, which elicited unique $[\text{Ca}^{2+}]_i$ responses. These cytosolic transients showed differences in peak amplitude, duration of peaks (spatiotemporal) as well as oscillation patterns of $[\text{Ca}^{2+}]_i$. This was more evident when \textit{S. aureus} transients were exposed to antibiotics of the same class. For example, different oscillation patterns were observed in response to gentamycin and kanamycin which belong to the antibiotic class, aminoglycosides. Thus, implying that \textit{S. aureus} is capable of sensing and distinguishing within antibiotic classes. The importance of cell signaling in bacteria is crucial for survival allowing bacteria to sense and respond to environmental stimuli by modulating calcium oscillation patterns in response to different stimuli.

Intracellular calcium oscillations are considered a universal sign of calcium cell signaling in eukaryote cells and increase the efficiency and specificity of gene regulation. This is true for both non-excitable and excitable cells. (Dolmetsch, R., 1998). Oscillation patterns are important for cell signaling because they prevent desensitization of the cell to calcium as well as the toxic effect of high concentrations of calcium inside the cell (Dolmetsch, R., 1998). The results presented in this study are in agreement with other work in the cyanobacteria Anabaena, (Barrán-Berdón et al., 2011; Gonzalez-Pleiter et al., 2017) which demonstrated $[\text{Ca}^{2+}]_i$ specific transients.

To other antibiotics.
5.4 Ca\(^{2+}\) enhancement and the negative effects of Ca\(^{2+}\) inhibitors, antagonists, and chelators on *S. aureus* efflux of EtBr

As previously mentioned, Ca\(^{2+}\) plays a very important role in the regulation of various metabolic processes including transport. Here we utilized various Ca\(^{2+}\) inhibitors such as the phenothiazines and EGTA to study Ca\(^{2+}\) efflux in *S. aureus*. Grimsy and Piddock, 2019 described phenothiazines as having damaging effects on bacteria, such as damaging bacterial membranes, acting as Ca-dependent transporter inhibitors, and antagonists of Ca\(^{2+}\) -binding proteins. For this reason, we tested different phenothiazines to observe any effect on the ability of *S. aureus* to efflux EtBr. The phenothiazines used disrupted Ca\(^{2+}\) dependent process. This was evident in the rapid accumulation of EtBr when these inhibitors or the calcium specific chelator, EGTA were added. The absence of calcium or blocking calcium from being imported into the cell resulted in accumulation of EtBr.

5.5 Proton motive force and Ca\(^{2+}\)

The pH environmental conditions are due to the fact that many efflux pump families rely on proton motive force (pmf) (Griffith, et al., 2018; Martins et al., 2011). This is evident when at low pHs, the bacteria maintained a high degree of efflux as compared to pH 8 where protons are lost in solution. At pH 7, a moderate amount of efflux was evident and major differences were observed between pH 7 and pH 7.6. This indicates that at higher pHs where protons are lost, the bacteria are unable to maintain a higher rate of efflux. This is also reinforced by significant differences in the control and CaCl\(_2\) treatments at pH 5 as compared to pH 8. The addition of Ca\(^{2+}\) resulted in significantly increased efflux enhancement than the control even at pH 5. This implies that the efflux potential is pH dependent due to the availability of protons at lower pHs.
Nové, et al., 2020). In addition, significant EtBr accumulation was observed when the proton uncoupler, CCCP, was added at pH 5. This observation also implies that *S. aureus* sense environmental pH through cytosolic calcium.

5.6 Ca\(^{2+}\) enhancement of the overexpressed efflux pump, LmrS in recombinant strains of *E. coli*.

Cloning of the *lmrS* gene and expressing it in *E. coli* resulted in clinical resistance to a wide variety of antibiotics as compared with the control *E. coli* strain. The evidence in the LmrS experiments show that even under EtBr pressure and with inhibitors, Ca\(^{2+}\) significantly aids in efflux. The reversal of the inhibition of efflux by inhibitors after the addition of Ca\(^{2+}\) further demonstrates this and is in agreement with other studies (Martins, et al., 2009, 2011). This is evidence that efflux is not only an important strategy but also that Ca\(^{2+}\) enhances efflux specifically for the LmrS pump and may be modulating the genetic expression of LmrS.

5.7 The influence of Ca\(^{2+}\), Ca\(^{2+}\) inhibitors and EGTA on gene expression of efflux pumps, LmrS, NorA and MgrA

Significant differences between CaCl\(_2\) treatments, the control with EtBr alone and EGTA, were seen in the expression levels of efflux pumps, NorA, MgrA and LmrS. The highest level of efflux expression differences was observed between treatments of CaCl\(_2\) and the calcium chelator, EGTA for all three pumps. The greater fold change between calcium and EGTA implies a more significant role of calcium in efflux regulation. EGTA chelates available calcium from the media thus taking effect on the system where very trace levels of calcium may exist in
the media. This provides further evidence that calcium is entering the bacteria in order to regulate efflux expression.

Interestingly, the lmrS gene expression was concentration dependent on calcium which was not observed with the other two pumps. The implications here are that some expression of pumps may be completely calcium dependent or that the LmrS pump is in some way involved in calcium translocation. The *L. lactis* MFS efflux pump LmrP, mediates selective H⁺/calcium antiport. Moreover, the purified protein LmrP has a high affinity for calcium binding and is dependent on the membrane proton electrochemical gradient (Schaedler et. al., 2012). The evidence presented here demonstrates that like the LmrP, LmrS may have an affinity for calcium binding and is also dependent on proton motive force. The LmrS protein, as with the LmrP are both members of the MFS superfamily which relies on pmf for cation exchange. More studies on the H⁺/cation exchange properties as well as the affinity for calcium binding in LmrS are needed to derive the same conclusion. However, the calcium concentration dependent expression implies that LmrS may hold similar properties as the LmrP efflux transporter.

5.8 Limitations

Certain limitations exist within the various experiments presented in this dissertation. For both the aequorin and EtBr studies the data presented reflects luminescence and fluorescence values that may be specific to the plate reader used and may vary across the different instruments utilized. For this reason, we labeled our data results as either relative light units or arbitrary fluorescent units to indicate the importance of how the cells responded in comparison to the various treatments rather than the specific fluorescence or luminescence values. Different concentrations of cells were used across experiments to verify that the same significant response
was elicited by the treatments and readers should be aware that the importance of the research is how the cells responded rather than specific values.

For expression studies, the drawback to using syber green is that the instrument also picks up fluorescence from primer dimers. Primer dimers are defined as primers with enough similarity that they anneal to each other. Extraneous fluorescence is inevitable and may skew the results. To address these limitations, the second round of gene measurement would utilize fluorescent probes to tag the efflux genes. Fluorescent tags would eliminate the fluorescent interference from primer dimers and would give more accurate data for gene expression analysis. Due to limitations and lack of access to a qPCR machine with specific sensors that correlated to the different fluorescent probes of each primer, we were unable to conduct the second round of gene expression using the probes. To address this problem the primers were designed to have very minimal primer dimers, if any. They were designed to have the same amplicon size and were checked for the presence of primer dimers on an agarose gel.

5.9 Conclusion

In conclusion the evidence presented here demonstrates that Ca²⁺ homeostasis is maintained in *S. aureus*, antibiotics induce a unique Ca²⁺ mediated response through transients in cytosolic Ca²⁺ and that the LmrS efflux pump is enhanced by Ca²⁺. The likelihood that Ca²⁺ ions are involved in the regulation of efflux is suggested by the expression data and is the first to report a direct link between Ca²⁺ and efflux regulation of the NorA, MgrA and LmrS efflux pumps. More importantly, this research also strongly implies that there may be Ca²⁺ transduction pathways in bacteria that have yet to be discovered.
Further study of global regulation with RNA seq. is needed to determine the proteins involved in efflux regulation with calcium. This would enable researchers to identify potential proteins involved with calcium sensing, the transporters involved in [Ca\textsuperscript{2+}]\textsubscript{i} homeostasis as well as the potential proteins involved in effecting the genes needed to respond to the external environmental signal. The research is significant because understanding how bacteria regulate physiological responses especially those related to virulence and antibiotic resistance mechanisms would enable mitigation of the global crisis of antibiotic resistance. The results presented conjecture the global coordination of calcium transporters in various patterns to elicit an appropriate genetic response from the cell.

The data presented in this study also suggests that further analysis of the effect of Ca\textsuperscript{2+} on the regulation of efflux pumps and the mechanism of action of efflux regulators could help mollify the antimicrobial resistance dilemma. This is evident in different studies that have demonstrated that various physiological processes including antibiotic resistance mechanisms, as presented here with efflux, are regulated by calcium. Moreover, new therapeutic approaches can be developed targeting specific proteins within this pathway that may not have a lethal effect on bacteria thus slowing the evolution of resistance. Perhaps the next generation of antibiotics would more successfully interfere with virulence regulation rather than interfering with vital physiological processes. Moreover, the synergistic effect of an antibiotic coupled with calcium regulation interruption may prove to heighten the antimicrobial effects of antibiotics on bacteria. Thus, increasing the chance for clearance of bacterial infections.
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CURRICULUM VITAE

Growing up in the Las Cruces/El Paso area, I have always felt the desire to contribute to the community in the form of teaching as well as research. I have taught at El Paso Community College, New Mexico State University and The University of Texas at El Paso. Conducting research in the United States/Mexico border town, El Paso, has enabled me to understand how socioeconomic determinants and other factors influence the spread of infectious diseases beyond the lab. This was especially evident after the emergence of COVID-19. For this reason, I have always felt that it is very important to be involved in community outreach and education.

My research history includes morphological and symbiotic characterization of fungal and plant cells using confocal, stereo fluorescence, cryo-electron and scanning electron microscopy. My mentor, Dr. P. Cooke trained me in various microscopy techniques to determine the symbiotic relationship of several systems. This work was one of the first to utilize a lambda scan on the confocal that enabled the differentiation between plant and fungal auto fluorescence allowing us to determine the nature of the symbiosis. During this time, I also mentored students who like myself, were first generation college graduates and children of migrant workers.

Mentoring students has been an immensely rewarding experience and a way to give back to the local community. My belief in interdisciplinary research strongly influenced my decision to pursue a PhD. that merges the fields of clinical and molecular microbiology. During my time as a PhD. student, we published several publications in both the biomedical and microbial bioremediation fields. My permanent email address is amyrnava@yahoo.com
