NOVEL MECHANISMS OF ANTIBIOTIC RESISTANCE IN SOIL ORGANISMS

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By

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Abstract

The soil is home to a vast number of bacteria and other microorganisms that must compete for resources in order to survive. This is often accomplished by the production of agents that inhibit the growth of competitors. It is not surprising then that soil bacteria, especially the actinomycetes, are responsible for the synthesis of a number of secondary metabolites that find use as antibiotics. This results in evolutionary pressure for the selection of resistance mechanisms to the numerous antibiotics present. The research described here examines the ability of five TetX orthologues in *S. coelicolor* to detoxify tetracycline. Although similar at the primary sequence level, none on these proteins possessed any tetracycline inactivation properties. Additionally, 36 bacterial isolates obtained from various soil samples were screened for resistance to tetracycline, Synercid, and daptomycin. An *in vitro* inactivation reaction utilizing cell lysates was coupled with a disk diffusion assay to identify resistant organisms capable of enzymatic antibiotic inactivation. A significant proportion of the isolates showed resistance to one or more of the antibiotics, and some novel mechanisms of resistance have been identified including unexpected enzymatic inactivation of the lipopeptide antibiotic daptomycin. These isolates, and the antibiotic detoxification reaction, were studied in greater detail using a variety of techniques. It is hoped that a greater understanding of this resistance mechanism and others in the soil will shed light on the type of resistance possible to be observed in a clinical setting.

Acknowledgements

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1. Introduction

1.1 Antibiotics and Resistance Development

It was not so long ago that the discovery and clinical application of early antibiotics such as sulfonamides and penicillins helped create a population that was essentially indestructible by infectious diseases caused by bacteria. The year 1928 marked the beginning of this era when Sir Alexander Fleming accidentally observed the actions of penicillin in petri dishes he had marked for disposal (22). By the 1940's, penicillin and many other antibiotics were the medical community's best weapons against bacterial pathogens. Since then, a number of other classes of antibiotics have been introduced including cephalosporins, aminoglycosides, and fluroquinolones. These three categories of drugs block cell wall biosynthesis (71), protein synthesis (80) and the DNA replication enzyme DNA gyrase and/or topoisomerase (18), respectively. Their ability to cure diseases that at one point were lethal primed antibiotics to become "miracle" drugs and as such, their industrial production and consumption dramatically increased.

As expected, bacterial resistance to antibiotics soon emerged. Currently, there are many clinically relevant pathogens that display multi-drug resistant (Mdr) phenotypes including methicillin resistant *Staphylococcus aureus* (MRSA) and various species of Enterococci (71). In North America, infections caused by these pathogens are commonly treated with the glycopeptide antibiotic vancomycin, although the appearance of vancomycin resistant Enterococci (VRE) has limited its effectiveness (39). Recent additions to the medical arsenal against multi-drug resistant bacteria have included Synercid and linezolid (21) (figure 1.1). Synercid is a combination of the semisynthetic

streptogramin antibiotics dalfopristin and quinipristin. When administered alone, each of these is bacteriostatic in nature however, when provided together in the correct ratio, bacteriocidal activity is observed (30). Unfortunately, resistance to Synercid has been documented in several cases (27, 30, 31, 75).

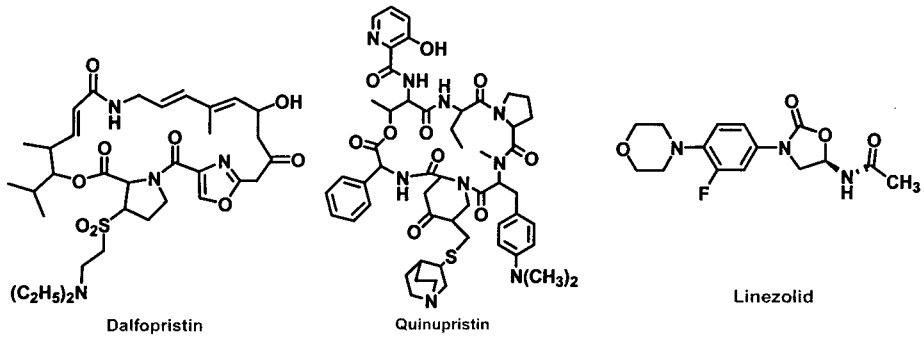


Figure 1.1: Chemical Structures of Synercid and Linezolid. Synercid is a synergistic combination of the semisynthetic streptogramin antibiotics dalfopristin and quinipristin. The completely synthetic compound linezolid belongs to a new class of antibiotics called oxazolidinones.

In 2000, the FDA approved linezolid, a synthetic oxazolidinone, to treat serious Gram-positive infections in the United States. This represents the first new class of antibiotics introduced in several decades. Linezolid inhibits bacterial protein synthesis in a manner similar to tetracyclines and streptogramins however, it is unique in that the initial peptide bond formation involving formyl-met is impaired (40). Although this ensures that cross-resistance is minimized, point mutations in the 23S rRNA of various species have given rise to linezolid resistance (63, 78). The inevitability of bacterial resistance and the speed with which it occurs is of serious concern. In light of this, the need for novel antimicrobials or mechanisms to combat resistance remains of utmost importance.

1.2 Common Mechanisms of Resistance

Antibiotic resistance can be manifested in a number of ways depending on the antibiotic and the bacterial species. The three most common modes of resistance observed in pathogenic bacteria are altered permeability or drug efflux, target modification, and enzymatic detoxification of the antibiotic (figure 1.2) (71).

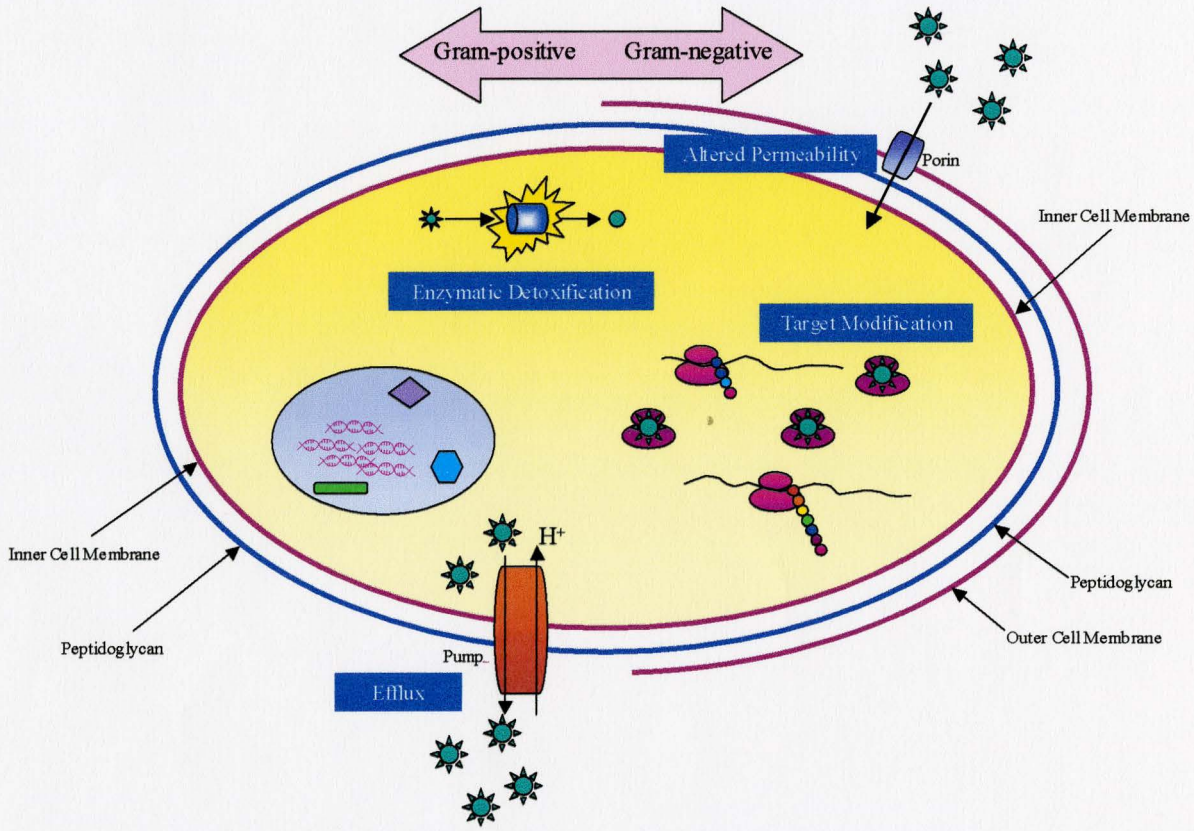


Figure 1.2: Common Modes of Antibiotic Resistance. Bacteria adopt many strategies to evade the action of antibiotics including restricting drug entry, actively pumping antibiotics out of the cell, modifying the drug target or even producing enzymes capable of inactivating antibiotics. Modified from (63).

Protection against antimicrobial agents can be achieved by ensuring that an effective drug concentration is never achieved within the cell. Efflux pumps that actively export

antibiotics against a concentration gradient are often responsible for this type of resistance. Genes encoding efflux pumps have been found in the chromosome of *Pseudomonas aeroginosa* and numerous other bacteria as well as on plasmids or transposons carried by various pathogens (71). The encoded transmembrane pumps can be highly selective, as is the case for tetracycline pumps (12), or display broad specificity and thus confer a Mdr phenotype (71). In addition, pumps can be classified into 4 major groups: the major facilitator subfamily (MFS), the small multidrug regulator (SMR) family, the resistance/nodulation/cell division (RND) family, or the ATP-binding cassette (ABC) family. The first three classes are more common in prokaryotes and utilize the proton motive force (ΔpH) to supply energy while the ABC pumps couple antibiotic export to ATP hydrolysis (71). The classic example of antibiotic resistance mediated by active efflux is resistance to tetracyclines. When tetracyclines enter the cell, they form a complex with Mg^{2+}. This complex binds tightly to the TetR protein, which acts as a negative regulator of *tetA* transcription. Upon tet-Mg^{2+}/TetR complex formation, transcriptional repression is relived, producing the TetA efflux pump. TetA inserts into the membrane where it functions as an antiport to export tetracycline and import protons (12).

In addition to efflux pumps, the highly virulent, multi-drug resistant strain of *E. coli* O157:H7 possesses cell membrane porins, such as OmpF and OmpC, that reduce permeability of many drugs (71).

A second common mechanism of resistance involves the modification of the cellular target such that it becomes insensitive to the antibiotic while maintaining its

essential cellular function. This can occur by point mutations (G2447T in the 23S rRNA confers linezolid resistance (63)), chemical modification (macrolide-lincomycin-streptogramin B (MLS_B) resistance by Erm mediated methylation of A2058 of the 23S rRNA (30)) or by a complete reprogramming of the target as seen in VRE (62). Enterococcal resistance to the glycopeptide antibiotic vancomycin is conferred by the production of peptidoglycan monomers terminating in D-alanyl-D-lactate instead of the natural D-alanyl-D-alanine. Five genes are required for this phenotype and are often plasmid-borne. Three of these gene products, VanH, VanA, and VanX are responsible for the construction of the altered cell wall while VanR and VanS act together as a two-component regulatory system to turn on resistance. VanH is a pyruvate reductase that converts pyruvate to D-lactate. VanA acts as a D-ala-D-lac ligase forming an ester bond between the alanine and lactate. Finally, VanX functions as a highly specific dipeptidase that recognizes D-ala-D-ala but not D-ala-D-lac thereby maintaining a high cellular pool of the latter while minimizing the concentration of the former. The D-ala-D-lac is then incorporated into growing peptidoglycan (62). The resulting structural alteration in the cell wall results in a 1000 fold decrease in vancomycin affinity (9).

Finally, enzymes encoded within bacterial genomes detoxify many classes of clinically important antibiotics (71). Antibiotics can be inactivated by destruction of a core chemical war-head, such as hydrolysis of the β-lactam antibiotics (10) and ring opening of type B streptogramins (51), or by chemical modification of important functional groups on the antibiotic, such as acetylation of chloramphenicol (52) and aminoglycosides (66, 80). Aminoglycoside modifying enzymes are among the best

characterized antibiotic resistance proteins. Detoxification of this class of antibiotics is carried out by three distinctive classes of enzymes: aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-adenyltransferases (ANTs), and aminoglycoside O-phosphotransferases (APHs). Both ANTs and APHs utilize ATP as a cofactor to transfer either a nucleotide or phosphate group to free hydroxyls on the antibiotic, respectively. AACs are responsible for the acetyl-CoA dependant acetylation of primary amine functionalities on the drug moiety (80).

1.3 Origin and Dissemination of Antibiotic Resistance

1.3.1 Introduction

A basic understanding of Darwinian evolution forecasts that given time and selective pressure any species will change to adapt to its environment. Thus, the appearance of antibiotic resistance is not only expected, but also predictable. Over the last 60 years, antibiotics used in both clinical and agricultural settings have forced microbes to evolve in order to survive. What is extraordinary however, is the speed with which bacteria have acquired this immunity. This can, in part, be attributed to human technology. Palumbi (2001) describes humans as "the world's greatest evolutionary force" (55). One of our major impacts has been accelerated evolution of disease causing organisms as a result of over and misuse of antimicrobial agents. For example, in the United States alone, vancomycin use rose by about 9500 kg between 1984 and 1994 (35). This rise in consumption was quickly followed by identification of vancomycin resistant pathogens, most notably among the Enterococci. The Staphylococci were slower to acquire this resistance, but in 2002 the first vancomycin resistant *S. aureus* strain was

reported (1). What was once termed the drug of "last resort" has now become inadequate in many cases. In addition to our mass consumption of antimicrobial agents, it has been estimated that approximately 20-50 % of all antibiotic use in the clinical setting is questionable (13). Unnecessary use of these agents provides an optimal environment in which the bacteria comprising the natural human flora can become resistant to a number of antibiotics. Finally, the extensive use of antibiotics as growth promoters in agriculture have sparked much controversy. Although not in a clinical setting, such application selects for resistance in the environment that can be transferred to human pathogens thus accelerating their evolution (15). The streptogramin antibiotics are an excellent example of this phenomenon. Due to poor solubility, they were not pursued for human use until recently with the introduction of the semisynthetic combination drug Synercid. Regardless of this, resistance mechanisms to streptogramins had been documented well in advance and can perhaps be attributed to the overuse of these compounds in the agricultural sector. Hence, it is clear that the emergence of drug resistant bacteria, while inevitable, has been hastened by human practices.

1.3.2 Intrinsic Resistance

Although antibiotic resistance determinants have been extensively studied, their sources remain poorly understood. A microbe's immunity to antibiotics can be classified as either intrinsic or acquired (71). Intrinsic resistance is characterized by an entire bacterial species being refractory to a given antibiotic in the absence of any change in its genetic material. For example, Gram-negative organisms are intrinsically resistant to a number of antibiotics by virtue of an impermeable outer cell membrane. High intrinsic

multidrug resistance displayed by *P. aeruginosa* can be attributed to a restrictive outer membrane, multidrug efflux pumps, and chromosomally encoded β-lactamases (71). Similarly, many Enterococcal species express aminoglycoside-modifying enzymes that are chromosomally encoded (17). It is not unreasonable to suggest that the inactivating enzymes in these organisms, and others, may serve an alternative function. Thus, the enzyme's ability to detoxify a given antibiotic is a secondary role, one that it has adapted to rather than been accustomed to performing. These inherent genetic advantages permit the microbe to grow in the presence of a given antibiotic without a requirement for the acquisition of additional genetic elements.

1.3.3 Acquired Resistance

A second, and much more common, manner in which bacteria can be resistant to antibiotics involves genetic alterations in the genome of the bacteria itself. This can occur either by mutations or by horizontal transfer of resistance genes on mobile DNA elements. Several pathogens have modified essential components of the cell in order to evade the action of antimicrobials. Modifications, usually a result of deletions, insertions, inversions, or point mutations, can alter a drug target such that the antibiotic is no longer effective (71). This is often observed in organisms resistant to aminoglycosides (81), linezolid (48), and fluroquinolones such as ciprofloxicin (60). In the case of the former two antibiotics, mutations in the ribosome reduce the binding affinity for the drug while maintaining normal protein synthesis within the cell. Ciprofloxicin resistance is achieved by mutations in the target enzyme DNA gyrase.

The ease and frequency with which DNA transfer occurs within the microbial community provides a facile means of acquiring resistance genes (15). Mobile DNA elements, such as plasmids and transposons, are responsible for the movement of antibiotic resistance genes from one species to another In addition, multidrug resistance among the *Enterobacteriaceae* has been linked to the presence of integrons (37). Integrons are stretches of DNA containing a site-specific recombination system and the ability to integrate and express a vast and diverse array of resistance genes in cassette form (figure 1.3). Class I integrons, those most often associated with the multidrug resistance seen in clinical isolates, are incorporated into plasmids or transposons and are thus able to circulate freely within the bacterial population (37).

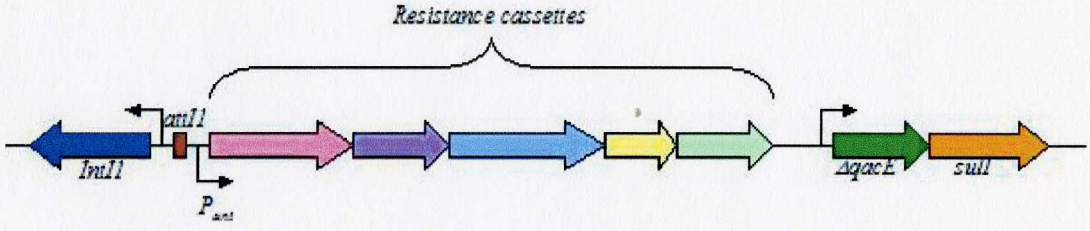


Figure 1.3: Typical Structure of Class I Integrons. Three essential components of integrons are located in the 5' conserved segment. The *intI1* gene encodes the site-specific integrase required for the incorporation of resistance cassettes at the *attI1* site. Immediately following this recombination site is promoter (P_{ant}) that drives transcription of the inserted cassettes. In addition, class I integrons frequently carry genes required for ethidium bromide (*ΔqacE*) and sulfamethoxazole (*sulI*) resistance at the 3' end. A variable region lies between the ends where gene cassettes, often encoding antibiotic resistance determinants, are integrated.

Given that bacteria are constantly in an environment containing antibiotics, there remains significant selective pressure to maintain these resistance plasmids. Furthermore, selecting resistance to one antibiotic may actually lead to selection of numerous other resistance genes as well since they are all carried on the same mobile element. It has also been noted that the size of DNA transferred between species is not insignificant. In fact,

the transfer of entire and partial antibiotic biosynthesis clusters, including resistance genes, between species has been observed (19, 20). Perhaps even more alarming, is the identification of DNA in many antibiotic preparations including genes encoding antibiotic resistance elements (15, 72). This puts the genetic material within arms reach of clinical pathogens. With so many potential means of acquiring resistance elements, it is not difficult to see how microbes have built up such a remarkable defense against antibiotics.

1.3.4 Soil Organisms as Reservoirs of Resistance Determinants

The soil environment is home to a vast number of bacteria and other microorganisms that must compete for resources in order to survive. This is often accomplished by the production of agents that inhibit the growth of competitors. It is not surprising then that soil bacteria, especially those of the genus Streptomyces, are responsible for the synthesis of a number of secondary metabolites that find use as antibiotics (32). In addition to genes encoding antibiotic biosynthetic enzymes, the genomes of these organisms must also encode resistance mechanisms in order to protect themselves from the action of the antibiotics produced. This forms the basis of the hypothesis that Streptomyces, and other soil bacteria, serve as reservoirs of resistance determinants that have the potential to become clinically significant (6, 14). This has been supported by the identification of antibiotic resistance genes in producing organisms that are very similar to those found in clinical pathogens. Resistance gene homologues have been identified in producing organisms for aminoglycoside modifying enzymes (15), the vancomycin resistance proteins VanHAX (42, 43, 57), tetracycline efflux pumps (12, 56), and streptogramin detoxifying enzymes (1, 2). In addition, the type B streptogramin

inactivating enzyme, Vgb (51), and the VanHAX genes (43) have also been found in *S. coelicolor* even though this organism is not known to produce any streptogramin or glycopeptide antibiotics. Although these soil organisms rarely act as human pathogens, genes coding for resistance can be transferred to organisms that pose serious clinical concerns. The ability to predict and understand resistance mechanisms that may appear in a clinical setting permits the medical community to be better prepared for this inevitable event. Thus, soil organisms, often an untapped resource, may be the key in aiding scientists to remain one step ahead of bacterial resistance to antibiotics.

This thesis describes research designed to explore this possibility using several different antibiotics. A brief description of each class of antibiotics studied follows to establish the background for this work.

1.4 Tetracyclines

1.4.1 General Characteristics

Among the first antibiotics to be used, tetracyclines are a large family of bacteriostatic agents (figure 1.4) active against various bacteria, including atypical organisms such as chlamydiae, mycoplasmas, rickettsiae, and other protozoan parasites (12). The broad spectrum and lack of major side effects in humans has led to the wide spread clinical use of tetracyclines. The emergence of resistance to this class of antibiotics has prompted the search for chemically modified tetracyclines with greater water solubility and improved activity. One of these groups is the glycylcyclines, represented by the investigational compound tigilcycline, which appears to be a valuable addition to current antimicrobials (54). As well as their extensive clinical use,

tetracyclines have also been used at subtherapeutic levels as growth promoters in animal feed in various countries including the United States (12).

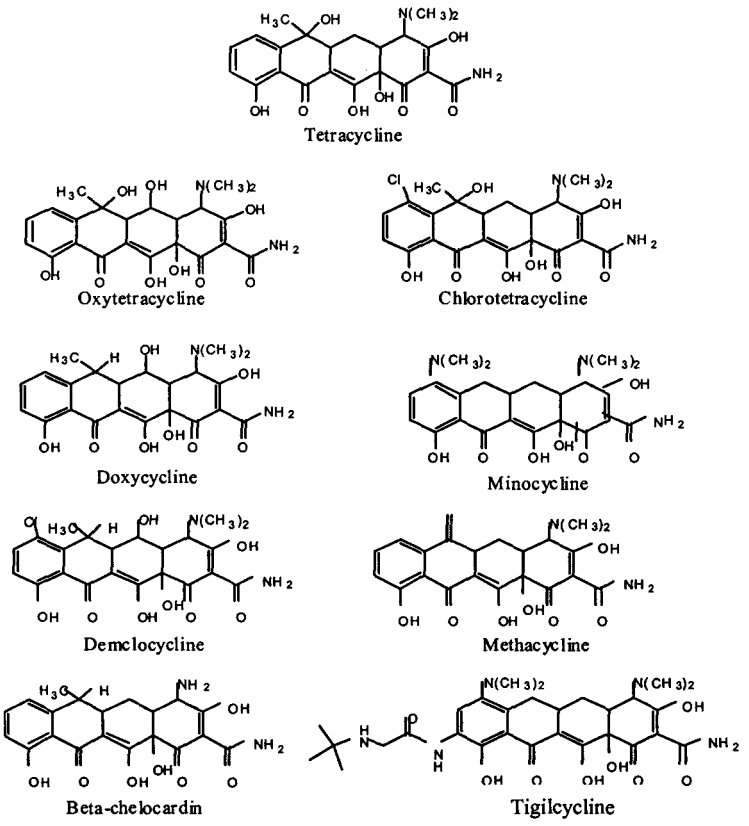


Figure 1.4: Chemical Structures of Some Members of the Tetracycline Family.

As their name implies, tetracyclines are composed of a linear fused tetracyclic core that is decorated with a number of functionalities including hydroxyls, tertiary amines, and methyl groups. Tetracyclines are also strong chelating agents and associate with metal cations (usually Mg^{2+}) to facilitate entry into the cytoplasm where they fulfill their antimicrobial activity (12).

1 4.2 Mode of Action

Tetracyclines are known to inhibit bacterial protein synthesis in both Gram-positive and Gram-negative organisms (figure 1.5). Thus, a discussion on their mode of action warrants an examination as to how tetracyclines enter the cell. In Gram-negative enteric bacteria, tetracyclines travel through the outer membrane via the OmpF and OmpC porins complexed with a metal cation. Once in the periplasmic space, the complex can dissociate and the weakly lipophilic antibiotic can diffuse through the inner membrane, or in the case of Gram-positive bacteria, the cellular membrane. In the cytoplasm, tetracycline is hypothesized to re-associate with a metal cation and bind to both the 30S and 50S subunits of the ribosome (12). A number of tetracycline binding sites have been identified in the small ribosomal subunit (61). The antibiotic was found to interact with both rRNA and protein components of the 30S subunit. The most occupied binding site overlaps with the ribosomal A site hence, tetracyclines interfere with the attachment of aminoacyl-tRNA to the ribosomal attachment site (61).

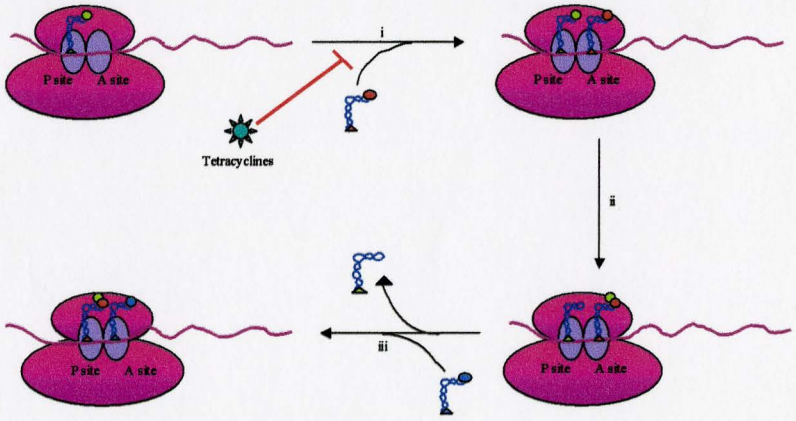


Figure 1.5: Inhibition of Protein Synthesis by Tetracycline Antibiotics. In a healthy bacterial cell, ribosomal protein synthesis occurs by addition of a charged amino-acyl tRNA molecule to the A site (i) followed by peptide bond formation (ii). A subsequent release of the tRNA from the P site and rearrangement of the other tRNA molecule (iii) permits the cycle to start anew. Tetracyclines exhibit their antibiotic activity by blocking the first step in this chain of reactions thus, inhibiting protein synthesis.

1.4.3 Resistance Mechanisms

Due to their overuse, resistance to tetracyclines has developed thereby limiting their applicability. The three main modes of resistance are active drug efflux, ribosomal protection, and enzymatic modification of the drug (figure 1.2) (12).

The best characterized of the tetracycline resistance determinants are the efflux pumps. Efflux proteins belonging to the MFS are found in both Gram-negative and Gram-positive organisms. Transcriptional control of efflux genes is tightly regulated with the antibiotic itself acting as the signaling molecule. Once produced, these membrane-associated proteins confer resistance by exporting a tetracycline/cation complex in exchange for a proton, thereby decreasing the intracellular concentration of the drug (71). As stated previously, many tetracycline efflux pumps are highly specific as suggested by the fact that many of these pumps confer resistance to tetracycline but not minocycline or glycylcylines (12).

Ribosomal protection proteins are cytoplasmic and confer a greater spectrum of resistance in comparison to efflux proteins. These proteins show homology to the EF-Tu and EF-G proteins, especially in the N-terminal GTP-binding domain (12). The best studied ribosomal protection proteins, Tet(M) and Tet(O), have ribosome-dependant GTPase activity. It has been hypothesized that binding of the ribosomal protection protein to the ribosome induces a conformational change in the ribosome such that tetracycline is no longer able to bind. GTP hydrolysis has been proposed to provide the energy required for ribosomal conformational change (12).

Enzymatic inactivation of tetracyclines is the result of a protein encoded by the *tetX* gene isolated from an anaerobic *Bacteroides* species (68). TetX is a 44 kDa cytoplasmic protein that is able to chemically modify tetracycline in the presence of NADPH and oxygen to render the drug inactive (68). Since *Bacteroides* are anaerobic, the presence of the *tetx* does not confer tetracycline resistance in this organism. In addition, recent literature has identified two other *tetx*-like genes, termed *tetx1* and *tetx2*, also on *Bacteroides* transposons (77). TetX2 differs in sequence from TetX by only 1 amino acid, whereas TetX1 appears to be a truncated version of TetX lacking the flavin-binding domain. More recently, the product of a gene cloned from the oral metagenome, *tet(37)* was found to detoxify tetracyclines in a manner similar to TetX (16). Although the mechanism of inactivation appears to be closely related, there is very little primary sequence homology between the two proteins. The identification of *tet(37)* suggests that there may be a number of additional tetracycline inactivating enzymes present in the bacterial community which have yet to be discovered.

1.5 Daptomycin: A Promising Therapeutic Option For Multi-Drug Resistant Pathogens

1.5.1 General Characteristics

Daptomycin was originally discovered and developed by Eli Lily and Company (Lilly) in the early1980s however, adverse skeletal muscle effects in clinical trials halted its progression (69). In light of the current antibiotic resistance paradigm, interest in daptomycin has been renewed. Currently, the antibiotic is in phase III clinical trials with very promising results. Daptomycin shows potent, concentration dependent bactericidal activity against a broad spectrum of Gram-positive organisms including the clinically

relevant MRSA, MRSE, and VRE (59, 79). Daptomycin is a secondary metabolite produced by *Streptomyces roseosporus*, where its peptide backbone, containing nonproteinogenic amino acids, is hypothesized to be assembled by nonribosomal peptide synthetases (NRPS) (46). In addition to N-acylation with a fatty acyl chain, 10 of the 13 amino acids are cyclized through an ester bond between the side chain of threonine at position 4 and the C-terminal kynurenine residue (figure 1.6).

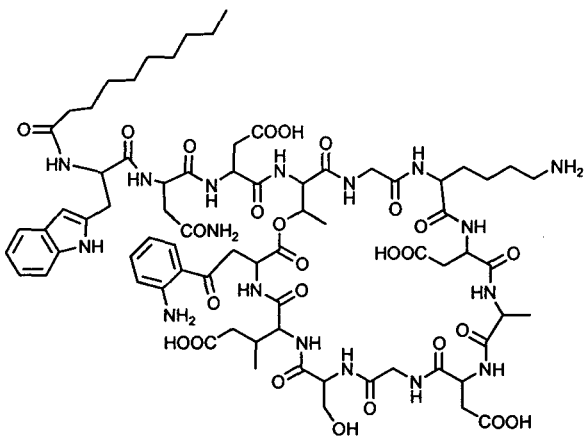


Figure 1.6: Chemical Structure of the Novel Lipopeptide Antibiotic Daptomycin.

1.5.2 Mode of Action

Although the mechanism of its action remains unclear, daptomycin is thought to target the membrane where it interferes with multiple aspects of bacterial plasma membrane function (figure 1.7). The activity of daptomycin is highly dependent on physiological concentrations (1.15 - 1.30 mM) of Ca^{2+} in the media (79). The exact mechanism of this dependency is unknown. Fluorescence studies have indicated that the peptide initially binds to the membrane via its fatty acyl tail. This interaction is enhanced and the antibiotic is drawn further into the bilayer upon the addition of Ca^{2+} (36). Thus, it appears that the divalent cation is necessary for forming a specific, irreversible interaction

with the target. Once associated with the cell membrane, daptomycin has been shown to disrupt the membrane electrical potential ($\Delta\psi$) (2) which in turn, affects amino acid transport as it relies on this gradient (3). Furthermore, Canepari *et al.* (1990) demonstrated that one of the primary targets of daptomycin was lipoteichoic acid biosynthesis, a finding that is supported by the antibiotic's selectivity for Gram-positive bacteria and it's site of action (11). This novel activity displayed by daptomycin aids in validating the lipoteichoic acid biosynthetic pathway as a valid antimicrobial target. Potential daptomycin binding proteins at the cytoplasmic membrane have also been described (8).

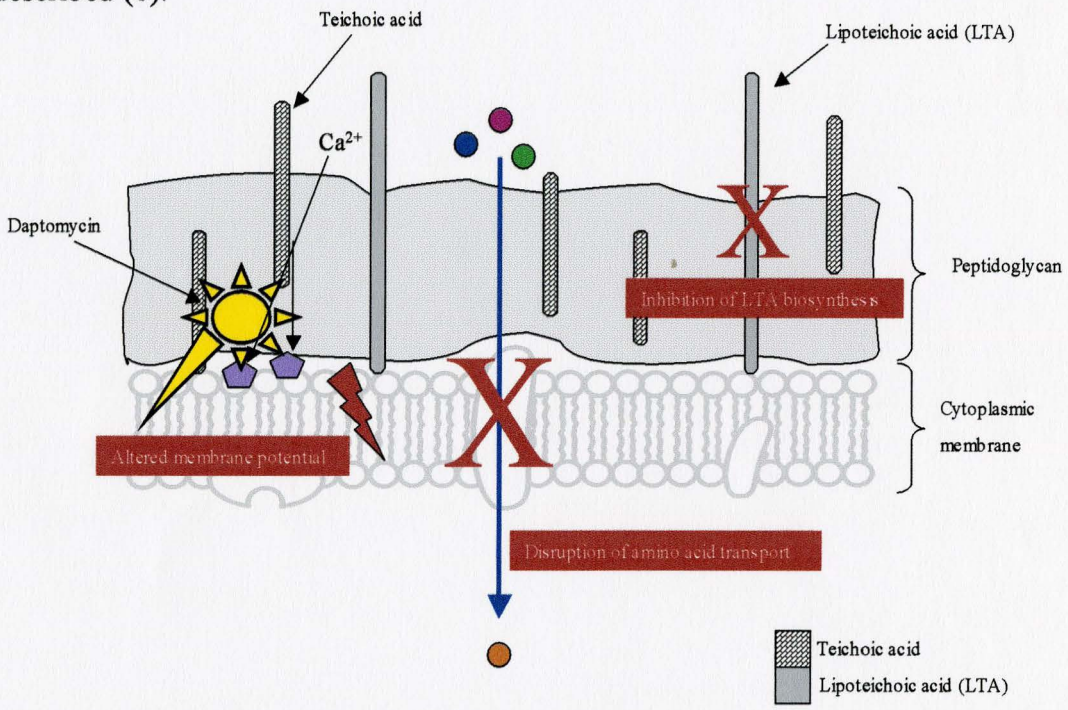


Figure 1.7: Effect of Daptomycin on the Gram-Positive Cell Membrane. The hydrophobic tail on the antibiotic structure and Ca^{2+} ions mediates the interaction of daptomycin and the cellular membrane. This results in a disruption of the membrane potential ($\Delta\psi$), which in turn causes an inhibition of amino acid import. A consequence of reduced amino acids is inhibition of peptidoglycan synthesis. A novel antibacterial target, lipoteichoic acid biosynthesis, has also been suggested for daptomycin.

1.5.3 Resistance Mechanisms

Numerous researchers have undertaken the investigation of daptomycin resistance development (38, 49, 64). Studies investigating the *in vitro* emergence of resistance to daptomycin were unable to identify any spontaneously resistant mutants in any of the organisms examined, including *S. aureus*, *E. faecalis*, and *E. faecium*. Upon passage through increasing drug concentrations, stable *S. aureus* strains were isolated, however they showed marked growth defects and atypical phenotypes. An altered membrane potential was also noted in three mutant strains. In addition mutant strains showed a significant decrease in virulence compared to the wild type (64). Based on the studies conducted thus far, it appears that daptomycin resistance is likely to occur slowly, thus increasing its attractiveness as a novel antimicrobial. Also of importance is the unique cell membrane target of daptomycin, which in theory should reduce the likelihood of cross-resistance development.

1.6 Project Objectives

The necessity of resistance determinants in antibiotic-producing organisms and their unique ecological niche has prompted the hypothesis that soil organisms may serve as reservoirs for resistance determinants observed in clinical settings. The aim of this thesis was to investigate novel mechanisms of antibiotic resistance in soil organisms. This was carried out using both a genetic and microbiology approach. The first approach was used to examine the ability of TetX orthologues in *S. coelicolor* to modify tetracycline. Novel resistance mechanisms in environmental isolates were identified and characterized by employing a microbiological approach.

2. *Materials and Methods*

2.1 General Materials and Methods

Bacterial cultures were grown in various liquid media such as LB (Luria Broth – 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl per 1.0 L), SOC (10.0 g tryptone, 3.5 g yeast extract, 0.3 g NaCl, 0.1 g KCl, 1.0 g $MgCl_2 \bullet 6H_2O$, 0.6 g $MgSO_4$, 1.8 g glucose per 500 ml), TSB (Tyrptone Soya Broth - 30.0 g Oxoid Tryptone Soya Broth powder per 1 L, contains 17.0 g pancreatic digest of casein, 3.0 g papatic digest of soybean meal, 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose), MHB (Mueller-Hinton Broth – 21 g Difco Mueller-Hinton Broth powder per 1 L containing 300 g infusium from beef, 17.5 g casamino acids, 1.5 g soluble starch per 1 L), NB (Nutrient Broth – 13 g Oxoid Nutrient Broth powder per 1 L containing 1.0 g "Lab-Lemco" powder (beef extract from specially selected raw materials), 2.0 g yeast extract, 5.0 g peptone, 5.0 g NaCl), and SVM (Soygrit Vegetative Media – 1.5 g glucose, 2.0 g potato starch, 1.5 g ground soygrits (Archer Daniels Midland Company), 1.0 g yeast extract, 0.2 g $CaCO_3$, and 1 ml corn steep liquor per 100 ml). Solid media used included LB Agar (LB + 7.5 g agar per 500 ml), TSA (Tryptone Soya Agar – TSB + 15 g agar per 1 L), NA (Nutrient Agar – NB + 15 g agar per 1 L), Streptomyces Isolation Media (0.4 g casein, 1.0 g starch, 0.5 g KNO_3, 0.2 g K_2HPO_4, 0.1 g $MgPO_4$, 0.1 g $CaCO_3$, 15 g agar per 1 L), Transfer Media (10.0 g glucose, 1.0 g yeast extract, 1.0 g KNO_3, 0.1 g $KHPO_4$, 15 g agar per 1 L), Bennet's Agar (5.0 g potato starch, 1.0 g casamino acids, 0.9 g yeast extract, 1.0 ml Czapek mineral mix (10 g KCl, 10 g $MgSO_4 \bullet 7H_2O$, 12 g $NaNO_3$, 0.2 g $FeSO_4 \bullet 7H_2O$, 200 µl concentrated HCl per 100 ml),

7.5 g agar per 500 ml). All media was sterilized by autoclaving at 121°C, 15 psi for 20 min.

Media were supplemented with the appropriate antibiotics to the following final concentrations: 100 μg/ml ampicillin, 50 μg/ml kanamycin, 50 μg/ml apramycin, and 25 μg/ml tetracycline unless otherwise noted. In addition, all experiments involving daptomycin were performed in the presence of at least 50 mg/L (1.25 mM) $CaCl_2$ as per the manufacturer's recommendation.

All bacterial cultures, with the exception of *M. luteus* and *Streptomyces spp.*, were grown at 37°C and 250 rpm (for liquid cultures). *M. luteus* and *Streptomyces spp.* were grown at 30°C and 250 rpm (for liquid cultures). All Streptomyces cultures were grown in spring-baffled flasks. All minimum inhibitory concentration (MIC) determinations were performed according to standard protocols published by the National Committee for Clinical Laboratory Standards (NCCLS) for broth macro and microdilutions unless otherwise specified.

Plasmid DNA was isolated from 3 ml *E. coli* cultures grown in LB overnight at 37°C using the Qiagen QIAprep Spin Miniprep kit (Mississauga, Ontario) according to the accompanying instructions. Restriction digests were carried out in the appropriate New England Biolab (NEB) or MBI Fermentas buffer at the recommended temperature. DNA was analyzed by Tris-acetate- ethylene-diamine-tetraacetic acid (EDTA) (TAE) agarose gel electrophoresis and ethidium bromide staining according to standard protocols (70). When necessary, DNA was excised from agarose gels and purified using the Qiagen QIAEX II Gel Extraction Kit. Ligations were carried out at either room temperature for

two hours or overnight at 16°C. Chemically competent cells were transformed according to standard protocol involving incubation of DNA with cells for 30 min on ice, followed by heat shock at 42°C for 30 sec. 1 ml of SOC was subsequently added to the sample and permitted to grow at 37°C for 1 hr. The cells were plated on LB containing the appropriate antibiotic and grown overnight at 37°C. Electrocompetent cells were transformed using the BioRad MicroPulser and 0.2 mm cuvettes. Following pulse application, 1 ml of cold SOC (4°C) was added and the culture was incubated at 37°C for 1 hr. Cells were plated on LB supplemented with the appropriate antibiotic and incubated overnight at 37°C.

All DNA primers were designed using the Primer Select software (DNAstar) and all sequence alignments were performed using Seqman (DNAstar), unless otherwise noted. All oligonucleotide synthesis and DNA sequencing using an ABI automated sequencer was performed by the Mobix Central Facility at McMaster University (Hamilton, Canada).

2.2 Characterization of TetX Orthologues in S. coelicolor

2.2.1 Cloning, Overexpression, and Purification

A modified version of the salting-out procedure described in Practical Streptomyces Genetics (32) was used to isolate genomic DNA from *S. coelicolor* M145. Briefly, *S. coelicolor* was grown in 200 ml of TSB supplemented with 0.5% (w/v) glycine for 48 – 72 hrs. Mycelia were harvested by centrifugation and resuspended in 10 ml of autoclaved SET buffer (20 mM Tris pH 7.5, 75 mM NaCl, 25 mM EDTA, pH 8.0). Following homogenization using a sterilized glass homogenizer, the cells were pelleted and resuspended in 5 ml of SET buffer. 200 µl of lysozyme (final concentration of 1

mg/ml) was added and incubated at room temperature until the solution became slightly viscous (ca. 20 min.). Proteinase K (140 µl, final concentration of 0.5 mg/ml) and 600 µl of 10% (w/v) boiled SDS (final concentration of 1% (w/v)) were then added. The sample was incubated at 55°C for 2 hrs, inverting occasionally. After the addition of 2 ml of 5 M NaCl (final concentration of 1.25 M) and cooling to 37°C, 10 ml of chloroform was added. The sample was mixed by gentle inversion for 30 min. at room temperature. Following centrifugation at 4500 x *g* at 20°C for 15 min., 0.6 vol of isopropanol was added and mixed by inversion. The precipitated genomic DNA was spooled onto sealed Pasteur pipettes, rinsed in 70% ethanol and air dried for 5-10 min. The isolated DNA was dissolved in ca. 1 ml of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The five genes encoding TetX orthologues, CAB94646, CAC44561, CAC16992, CAC16727, and T36402 were amplified from *S. coelicolor* genomic DNA by the polymerase chain reaction (PCR). PCR reactions (50 µl) contained 500 ng of chromosomal DNA template, 1 µM of each of the forward and reverse primers listed in table 2.1 with engineered restriction sites, 1X Biotools DNA polymerase reaction buffer with 2 mM $MgCl_2$, 0.4 mM of each deoxy-nuleotide 5'-triphosphate (dNTP), and either 5 or 10% dimethoxysulfide (DMSO) and 8 or 10 mM exogenous $MgCl_2$ as outlined in table 2.1. Reactions were incubated at 94°C for 3 minutes prior to the addition of 1 U of Biotools DNA polymerase (Interscience Inc.). Following 30 cycles of 94°C for 1 min., the appropriate annealing temperature (table 2.1) for 1 min., and 72°C for 1.5 mins., the reaction was maintained at 72°C for 10 minutes. The size of the amplified product was

confirmed by TAE-agarose gel electrophoresis and the products were subsequently digested with *Nde*I and *Hind*III in R^+ buffer (MBI Fermentas) at 37°C for 3 hrs.

Table 2.1: Primer sequences and PCR conditions employed to amplify TetX Orthologues from *S. coelicolor*. Restriction sites engineered into the primer sequences are underlined.

Gene	Size (bp)	Primers (5' → 3')	Annealing Temp. (°C)
CAB94646	1122	Forward: GCTCTACACATATGACCACGCACGTCACGA Reverse: CGGAATTCAAGCTTCTACTCCTGGCCGCTGAACAT	50.0
CAC16992	858	Forward: CGTCTAGACATATGCAGCAGCGCACCATCG Reverse: CGGAATTCAAGCTTTCAGGCCGACAGGAGCG	52.0
CAC16727	582	Forward: CGTCTAGACATATGTGGCCAAGTCTCATGGTCG Reverse: CGGAATTCAAGCTTCTACTTCTGTCGGCCCCG	50.0
CAC44561	1191	Forward: CGTCTAGACATATGTTCGTTACGCTGGAGGTGTC Reverse: CGGAATTCAAGCTTTCACGCCTCTGCGCGGCC	46.5
T36402	1167	Forward: CGTCTACACATATGAACTCTCCGACACCCGCC Reverse: CGGAATTCAAGCTTTCACTCGCCGGGTCCGCCGT	48.2

An additional unit of *Nde*I was added after 1.5 hrs. An overnight ligation at 16°C was carried out with the digested PCR products and previously digested (*Nde*I/*Hind*III) and dephosphorylated (using Shrimp Alkaline Phosphatase, SAP) pET28a (Novagen). The ligations were then transformed into chemically competent *E. coli* BL21 (DE3) cells and successful clones were confirmed by restriction digest mapping and sequencing (MOBIX Central Facility, McMaster University).

All of the proteins were over-expressed and purified from a 1L culture of LB, supplemented with 50 μg/ml kanamycin that was inoculated with 10 ml of an overnight culture of the transformed *E. coli* BL21 (DE3). The cells were grown at 37°C until log phase (OD_{600} = 0.6) was reached. At this point the culture was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and grown at 16°C for 20 hrs. Cells were harvested by centrifugation at 5,000 x *g* for 10 mins. Harvested

cells were resuspended in 20 ml of lysis buffer (50 mM N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 0.1 mM dithiothreitol (DTT)) and lysed by three passages through the large cylinder French press at a maximum pressure of 10,000 psi. The lysate was centrifuged at 15,000 x *g* for 15 min to remove the cellular debris. The cleared lysate was applied onto a 1 ml Ni-NTA (nickel-nitrilotriacetic acid) column by using an automated Fast Protein Liquid Chromatography (FPLC) system (Amersham Pharmacia). The protein of interest was eluted with a linear gradient of elution buffer (50 mM HEPES, 250 mM imidazole, pH 7.0). Fractions displaying significant absorbance at 280 nm were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed according to standard protocols (23). The appropriate fractions were pooled and concentrated via centrifugation through a membrane filter with a 10 kDa exclusion limit (Millipore). Protein concentration was determined by the standard Bradford assay.

2.2.2 Assay of Tetracycline Degradation Activity

Reactions (250 µl) containing 1 µg of purified protein, 100 µl co-factor mix, and 50 µl tetracycline (0.25 mg/ml) were incubated at 37°C for 24 hrs. The cofactor mix was prepared in such a way that each reaction would contain final concentrations as follows: 5 mM adenosine 5'-triphosphate (ATP), 5 mM $MgCl_2$, 0.1 mM reduced glutathione (GSH), 0.1 mM Acetyl Coenzyme A, 1 mM oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), 5 mM glucose-6-phosphate (G-6-P), and 2 U G-6-P dehydrogenase. In addition to the five proteins from *S. coelicolor*, TetX2 from *Bacteroides fragilis* (cloned and purified by Gloria Yang in the Wright Lab) was used as a positive control. *M.*

luteus was streaked onto TSA and grown for 48 hrs at 30°C to obtain single colonies. Using sterile 0.9% saline, a suspension having an OD_{625} of 0.08-0.1 (McFarland Standard 0.5) was prepared from the *M. luteus* single colonies. This suspension was swabbed onto TSA to obtain a confluent lawn. Disks containing 20 µl of each reaction were placed onto the plates, which were then incubated at 30°C for 48 hrs. at which time the diameters of the zone of clearance around each disk was compared to the control.

2.3 Novel Mechanisms of Antibiotic Resistance in Environmental Isolates

2.3.1 Isolation of Streptomyces from Soil and Resistance Profiling

Soil samples were collected from various locations including Jamaica, Cuba, Saskatchewan, and Hamilton. The samples were dried at room temperature, in petri plates, for at least 7 days. Subsequently, 1 g of each sample was added to ca. 9 ml of sterile dH_2O and thoroughly mixed by vortexing. Three 10-fold serial dilutions were prepared for each sample giving a final solution that is 1000-fold more dilute than the original. 100 µl of each dilution was plated on *Streptomyces* isolation media supplemented with cyclohexamide (50 µg/ml) to inhibit fungal growth. Plates were incubated, in the dark to mimic natural conditions, at room temperature for 5-7 days. Potential *Streptomyces* colonies were identified by the presence of round, fuzzy colonies, the distinctive "soil-like" odor, and Gram staining performed using the BBL four step Gram-staining kit according to the manufacturer's instructions. From this point onwards, all procedures were carried out in a laminar flow hood to minimize contamination. To obtain pure cultures, well isolated single colonies displaying the previously mentioned characteristics were picked and streaked onto transfer media supplemented with

cyclohexamide (50 μg/ml). These plates were incubated at 30°C for 3-5 days. In this manner, 8 pure cultures have been obtained from each of the locations with the exception of the Saskatchewan sample from which 16 pure cultures have been obtained.

For each pure culture, 1 well isolated colony was resuspended in 100 μl of liquid isolation media (isolation media minus agar). This suspension was used to streak each culture onto solid isolation media supplemented with either 50 μg/ml tetracycline, 50 μg/ml Synercid, or 50 μg/ml daptomycin and 0.05 mg/ml $CaCl_2$. The plates were incubated at 30°C for 5 days, at which point the resistance profile of each strain to the antibiotics tested was defined. From this initial screen, 3 distinct sets of 6 resistant strains for each antibiotic were chosen for further analysis.

Strains were preserved by preparations of spore suspension as described in Practical *Streptomyces* Genetics (32). Briefly, the isolate was grown in 20 mL of SVM in a spring-baffled flask at 30 °C for 2 days. 500 – 700 μl of this culture was then spread evenly onto Benett's Agar to generate a confluent lawn. Following incubation at 30 °C for 5-7 days, the spores were scraped from the surface of the agar, dissolved in 9 ml of dH_2O by vortexing, and gravity filtered through spore filters (prepared as described in 25). The filtrate was centrifuged at 4000 *x g* for 15 min. The pelleted spores were resuspended in 1 ml of sterile 80% (w/v) glycerol prior to storage at –80 °C.

Following standard NCCLS protocols, the MIC of each strain to the appropriate antibiotic was determined using 96 well, round-bottom plates. The experiment was done in duplicate and *S. aureus* (ATCC #29213) and *E. faecalis* (ATCC #29212) were used as controls in each plate. Due to the nature of *Streptomyces* growth, a few minor changes

were made to the accepted protocol. Namely, both the plates used to obtain single colonies of each strain and the MIC plates were grown at 30°C for 3-5 days instead of the standard day to permit sufficient growth of the test organism. The MICs for the control organisms were however, measured after 1 day of growth. For daptomycin, the media was supplemented with $CaCl_2$ to a final concentration of 50 mg/L as per the manufacturer's specifications.

2.3.2 Antibiotic Inactivation Bioassay

Starter cultures of each strain, inoculated with 20 µl of frozen spore suspension, were grown for 48 hrs in 20 ml of SVM at 30°C. 100 µl of this culture was then used to seed 100 ml of TSB supplemented with 0.05% glycine and the appropriate antibiotic (50 µg/ml). These cultures were grown at 30°C for another 48 hrs. Cells were harvested by centrifugation at 5,400 x *g* for 10 min. After two washes with SET buffer, the cell mass was resuspended in 5 ml of SET buffer supplemented with 0.1 mg/ml RNase, 0.1 mg/ml DNase, 1 mM PMSF, 0.1 mM DTT, and 5 mg/ml lysozyme. Following lysis using the small cylinder French press at a maximum pressure of 5,000 psi, the cellular debris was removed by centrifugation at 15,000 x *g* for 15 min and the cleared lysate was used to set up the following reactions: Reaction 1: 100 µl cell lysate, 100 µl co-factor mix, 50 µl of the appropriate antibiotic (0.25 mg/ml stock); Reaction 2: 100 µl cell lysate, 100 µl dH_2O, 50 µl of the appropriate antibiotic (0.25 mg/ml); Reaction 3 (control): 100 µl dH_2O, 100 µl co-factor mix, 50 µl of the appropriate antibiotic (0.25 mg/ml). The co-factor mix was prepared in such a way that each reaction would contain final concentrations as follows: 2 mM Acetyl Coenzyme A, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1.0 mM ATP pH 7.5, and 1.0 mM

GSH. Reactions were incubated at 37°C for 24 hrs. *M. luteus* was streaked on TSA and incubated for 48 hrs at 30°C to obtain single colonies. Using sterile 0.9% saline, a suspension having an OD_{625} of 0.08-0.1 was prepared from the *M. luteus* single colonies. This suspension was swabbed onto TSA to obtain a confluent lawn. Disks containing 20 µl of each reaction were placed onto the plates and incubated at 30°C for 48 hrs. at which time the diameters of the zone of clearance around each disk were compared to the control reaction.

2.3.3 Identification of Daptomycin Inactivating Isolates

Inactivation bio-assays were performed, as described in 2.3.2 for all six of the daptomycin resistant isolates for which MICs were determined. Similar reactions were also performed with the daptomycin-producing organism, *S. roseosporus* NRRL 11379 (National Center For Agricultural Utilization Research), *S. coelicolor* M145, and *S. venezuelae* NRRL B-2277 (National Center For Agricultural Utilization Research).

2.3.4 *Phenotypic Characterization and Identification of Daptomycin Inactivating Isolates*

The three daptomycin inactivating isolates, CA3, J3, and $S4_1$ were streaked on Bennet's agar and grown for 5-7 days at 30°C. The plates were then submitted to the Integrated Microscopy Services (McMaster University, Hamilton, Canada) for scanning electron microscopy analysis. Images, at 5000X magnification, were taken using a JEOL840 Scanning Electron Microscope.

In an effort to determine the identity of these isolates, the gene encoding the 16S rRNA was amplified by PCR and sequenced (Mobix Central Facility, McMaster

University, Hamilton, Canada). PCR reactions (50 µl) contained 500 ng of chromosomal DNA template, 1 µM of each of the forward and reverse primers (listed in table 2.2), 1X Biotools DNA polymerase reaction buffer with 2 mM $MgCl_2$, 0.4 mM of each dNTP, 10% DMSO and 10 mM exogenous $MgCl_2$. Reactions were incubated at 94°C for 3 minutes prior to the addition of 1 U of Biotools DNA polymerase. Reactions were cycled 30 times at 94, 52, and 72°C for 1, 1, and 1.5 minutes, respectively. The ca. 1.5 kb amplified gene was confirmed by TAE-agarose gel electrophoresis.

Table 2.2: Primers used to amplify the 16S rRNA gene from the daptomycin inactivating isolates. *EcoRI* sites engineered into the sequences are underlined.

Forward (pA)	5' – CGG AAT TCA GAG TTT GAT CCT GGC TCA G – 3'
Reverse (pH)	5' – CGG AAT TCA AGG AGG TGA TCC AGC CGC A – 3'

The PCR product was excised from the gel and purified before being sequenced. In order to fill in gaps, internal primers specific to each gene had to be designed and synthesized (Mobix Lab). The sequences were submitted as a query in a standard nucleotide-nucleotide BLAST (National Centre for Biotechnology Information, NCBI) search to obtain a number of genes encoding 16S rRNA from various organisms. These sequences were compiled and aligned using the web-based ClustalW program (European Bioinformatics Institute, EBI). An unrooted phylogentic tree was prepared using the Tree View 3.2 software.

2.3.5 Base-Catalyzed Hydrolysis of Daptomycin

The ester bond through which daptomycin is cyclized was cleaved by base-catalyzed hydrolysis as described by Muangsiri *et al.* (2001). Reactions (100 µl) containing

daptomycin (0.3 mM) and NaOH (0.3 mM) were incubated at 60°C for 4 hours. HPLC and Electrospray-Mass Spectrometry (ES-MS) were employed to analyze the reaction products. Furthermore, a disk diffusion assay was performed to confirm that the linearized product was no longer active against *M. luteus*.

2.3.6 Biochemical Characterization of Daptomycin Inactivation by the Soil Isolates

In addition to the bio-assay, reactions in which daptomycin was found to be inactivated were analyzed by high pressure liquid chromatography (HPLC). The reaction components were separated on an analytical 250 x 22 mm, 10 μm particle size, C18 reverse phase column (Alltech Associates Inc.) with 0.05% (v/v) trifluoroacetic acid (TFA) in dH_2O and 0.05% (v/v) TFA in acetonitrile as solvents. Aliquots of the inactivation reactions were also subjected to liquid chromatography-mass spectrometry (LCMS) performed by Amanda Doherty-Kirby at the Biological Mass Spectrometry Laboratory (University of Western Ontario, London, Canada) and Kirk Green at the McMaster Regional Centre for Mass Spectrometry (McMaster University, Hamilton, Canada). In both instances, similar columns and solvents were used. Mass spectral analysis at the University of Western was performed on a Micromass Q-Tof Micro instrument.

2.3.7 Daptomycin Inactivation by Vgb

The ability of daptomycin to act as a substrate for the type B streptogramin inactivating enzyme Vgb was examined via a modification of the bio-assay described in section 2.3.2. Briefly, purified, untagged Vgb protein (cloned by Kari-ann Draker and purified by Tariq Mukhtar in the Wright Lab) was incubated with daptomycin, the

previously determined optimal Mg^{2+} concentration of 2 mM (51), and varying concentrations of Ca^{2+} ranging from 0 to 10 mM. The reaction was incubated at 30°C for 16 hrs. The reaction was then tested for bioactivity by spotting 20 µl of each on disks placed on a lawn of *M. luteus*. The plates were incubated at 30°C for 48 hrs. prior to measuring the diameter of the zone of clearance around each disk.

2.3.8 Daptomycin Inactivation by S. venezuelae

The cofactor requirement of the *S. venezuelae* daptomycin detoxification reaction was studied by performing a modified antibiotic inactivation assay. Each of the cofactors was individually omitted from the cofactor mix added to the inactivation reaction. The essentiality of a cofactor was determined by assessing the zone of clearance around the disk in relation to the control reaction (containing all of the cofactors) and daptomycin itself.

2.3.9 Preparation of Genomic Expression Libraries for the Daptomycin Inactivating Isolates

Genomic DNA from each of the daptomycin inactivating isolates was obtained as described in section 2.2.1. The chromosomal DNA was partially digested with *Dpn*II and 25-35 kb fragments were isolated for cloning into the cosmid shuttle vector pOJ446 (7). In order to determine the optimal partial digestion conditions required to obtain the desired insert size, a number of reactions (10 µl) were set up containing 10 µg of genomic DNA, 1X *Dpn*II buffer, and 0.2 U or 0.4 U of *Dpn*II. Reactions were pre-warmed for 5 min at 37°C prior to the addition of the restriction enzyme and allowed to react for varying amounts of time ranging from 15 min. to 3 hrs. 6X loading dye (0.09%

bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60 mM EDTA) was added (2 µl) in order to terminate the reactions. Fragment lengths were analyzed by 0.5% TAE-agarose gel electrophoresis and compared to Lambda Mix Marker, 19 (MBI Fermentas) standards for size estimation. The optimal reaction conditions for each of the isolates as determined by this analysis are summarized in table 2.3. In order to obtain enough *Dpn*II-digested DNA for library construction, two reactions (50 µl) each containing 50 µg of genomic DNA, 1X *Dpn*II buffer, and 2.5 U of *Dpn*II were reacted as previously described. Reactions were stopped by the addition of sodium acetate to a final concentration of 0.4 M and the DNA was precipitated by the addition of 100% ethanol. The precipitated DNA from both of the reactions was pooled and dissolved in 200 µl of dH_20. The sample was stored at -20°C until further use.

Table 2.3: Optimal conditions for *Dpn*II partial digest reactions to obtain fragments ranging from 25-35 kb

Isolate	Units of *Dpn*II (per 50 µg)	Length of digestion (hrs.)
CA3	1.25	2.5
J3	1.25	1.0
$S4_1$	2.5	0.5

12 ml sucrose gradients from 10 to 40% (w/v) sucrose in 20 mM Tris-HCl pH 7.5, 1 M NaCl, and 5 mM EDTA were prepared in 14 x 89 mm polyallomer SW41 centrifuge tubes (Beckman) using a SG 15 linear gradient maker (Amersham Biosciences) according to the accompanying instructions. The partially digested DNA samples were thawed at room temperature and then incubated at 65°C for 5 min. to separate any aggregates that may have formed. The 200 µl sample was then layered onto a sucrose gradient and

centrifuged at 30,000 rpm for 24 hours at 20°C. The gradient was subsequently fractionated as outlined in Current Protocols in Molecular Biology (74). Fractions (750 µl) were precipitated by the addition of NaCl to 1.25 M and 0.6 volumes of isopropanol. The DNA was permitted to precipitate at -80°C for 1 hr. and then centrifuged at 14,000 x *g* for 30 min. at 4°C. The samples were immediately decanted, removing as much liquid as possible. The pelleted DNA was washed in 70% ethanol, dried using a Savant SpeedVac at room temperature and resuspended in 30 µl of sterile dH_2O. 5 µl of this sample was added to 1 µl of loading dye and analyzed in a 0.5% TAE-agarose gel run at 50V for 16 hrs. at 4°C visualized by ethidium bromide staining.

3. Results

3.1 Characterization of TetX Orthologues in S. coelicolor

3.1 1 Cloning and Purification

The five *S. coelicolor* proteins and TetX2 were over-expressed in *E. coli* BL21 (DE3) as His-tagged fusions thereby facilitating a one step purification protocol using a Ni-NTA column and a linear imidazole gradient. Although expression levels varied among the proteins, sufficient amounts of each could be obtained from a 1 L culture for enzymatic assays. Following purification, the proteins appeared to be relatively pure based on Coomassie blue stained SDS-PAGE (figure 3 1). Moreover, fractions containing T36402 and CAB94646 were light yellow in color suggesting the association of a flavin moiety with the purified protein.

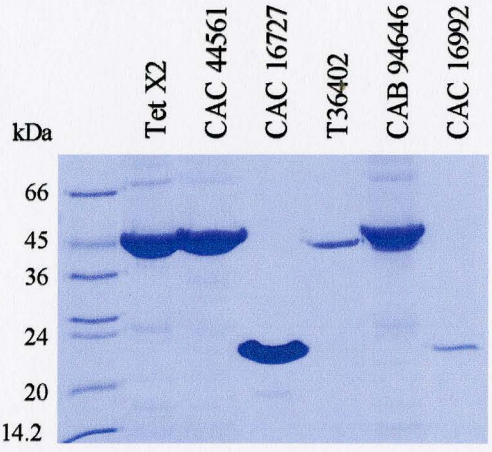


Figure 3.1: Purification of TetX Orthologues in *S. coelicolor*. The proteins were expressed with a N-terminal His-6-tag to facilitate purification on a Ni-NTA column. Purified proteins were analyzed by 15% SDS-PAGE, stained with Coomassie blue and compared to standard low molecular weight markers (lane 1).

3.1.2 Tetracycline Degradation Activity

The ability of the TetX orthologues to detoxify tetracycline was assessed by following inactivation reactions over the course of 24 hrs using a disk assay (figure 3.2).

In the case of TetX2, the positive control, it can be seen that upon 24 hrs of incubation, essentially all the antibiotic in the reaction has been inactivated. In contrast, the *S. coelicolor* proteins are very poor at tetracycline detoxification under these conditions. The amount of antibiotic present after 24 hrs of incubation in reactions containing these proteins does not change substantially from the initial time point, as indicated by the relative zones of clearance.

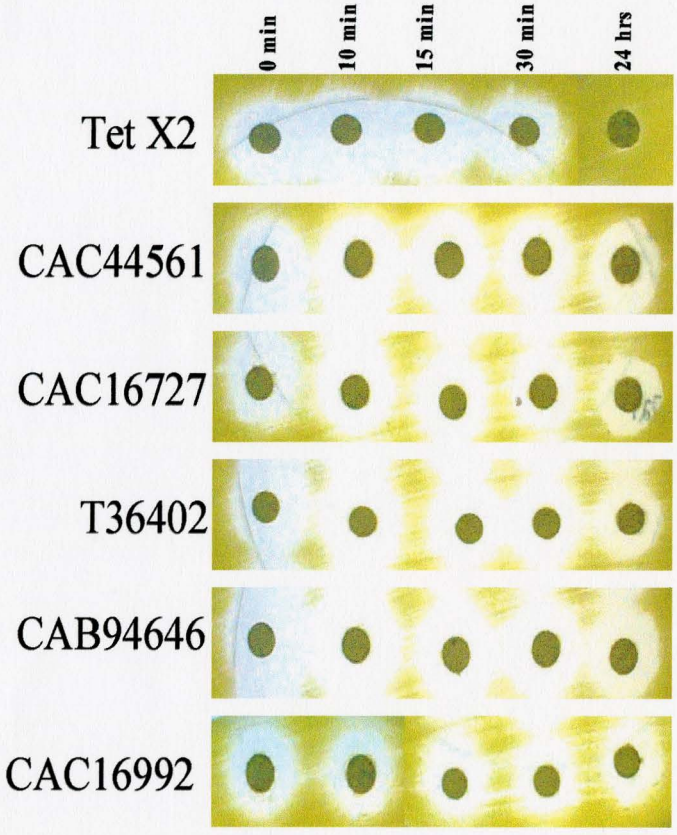


Figure 3.2: Tetracycline Inactivation Assay. The tetracycline detoxification properties of the purified proteins were assayed by following the inactivation reaction over 24 hours. The amount of antibiotic present at time point was compared to the control (TetX2). *M. luteus* was used as a susceptible test organism.

3.2 Novel Mechanisms of Antibiotic Resistance in Environmental Isolates

3.2.1 Isolation and Antibiotic Resistance Profiling of Soil Organisms

Several pure cultures were isolated from soil samples obtained from various locations. Of these, 36 were screened for resistance to Synercid, tetracycline, and daptomycin at a concentration of 50 μg/ml on solid media (figure 3.3). Many of the isolates were able to grow well in the presence of these antibiotics. It is interesting to note that many isolates are resistant to two, or even all three, of the antimicrobials tested.

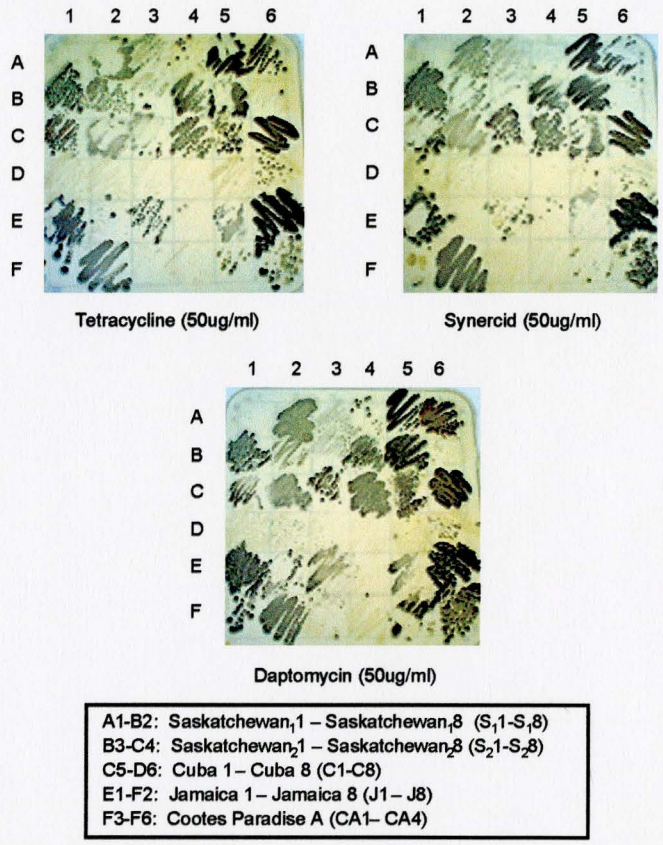


Figure 3.3: Resistance Profiling of Soil Isolates. Pure cultures were isolated from soil samples obtained from various sources. Of these, 36 were screened for resistance to Synercid, tetracycline, and daptomycin at a concentration of 50 μg/ml on Streptomyces isolation media.

The isolates that displayed superior growth in the presence of one drug relative to the others were chosen for further study with that antibiotic. Differential growth may indicate resistance by a mode specific to the drug being tested (i.e. enzymatic modification) rather than a less specific mechanism (i.e. efflux). In this manner, 6 isolates were chosen for each of the three antibiotics being examined. The MIC of the appropriate antibiotic against each of the isolates is summarized in table 3.1. The MIC values are all in the high μg/ml range indicating that many of the isolates display significant resistance to these antibiotics. Also of interest is the apparent decrease in tetracycline MIC upon the transfer from solid isolation media (screened at 50 μg/ml) to liquid MHB for five of the tetracycline resistant isolates. This finding speaks to the importance of media type and composition in relation to expression of antibiotic resistance phenotypes.

Table 3.1: MICs of Daptomycin (dap), Synercid (Syn), and Tetracycline (tet) Against Soil Isolates Identified as Resistant Based on Growth in the Presence of the Antibiotic on Solid Media. *E. faecalis* ATCC# 29212 served as a positive control.

Isolate	Drug	MIC (μg/ml)	Isolate	Drug	MIC (μg/ml)	Isolate	Drug	MIC (μg/ml)
$S4_1$	dap	64	$S8_1$	Syn	256	$S2_1$	Tet	8
J2	dap	>128	$S1_2$	Syn	256	$S5_1$	Tet	32
J3	dap	>128	$S5_2$	Syn	>256	$S3_2$	Tet	16
C8	dap	>128	C1	Syn	>256	$S6_2$	Tet	32
CA1	dap	64	C5	Syn	>256	$S2_1$	Tet	8
CA3	dap	>128	J5	Syn	>256	J1	Tet	>256
E. faecalis	dap	4	*E. faecalis*	Syn	4	*E. faecalis*	Tet	8

3.2.2 Identification of Daptomycin Inactivating Isolates

In a fashion similar to the assay performed for the *S. coelicolor* TetX homologues, the ability of a cell lysate to enzymatically inactivate an antibiotic is indicated by a reduction in the zone of clearance around those reactions containing an active detoxification enzyme. This bioassay has permitted the identification of three isolates that are capable of inactivating the lipopeptide antibiotic daptomycin (figure 3.4).

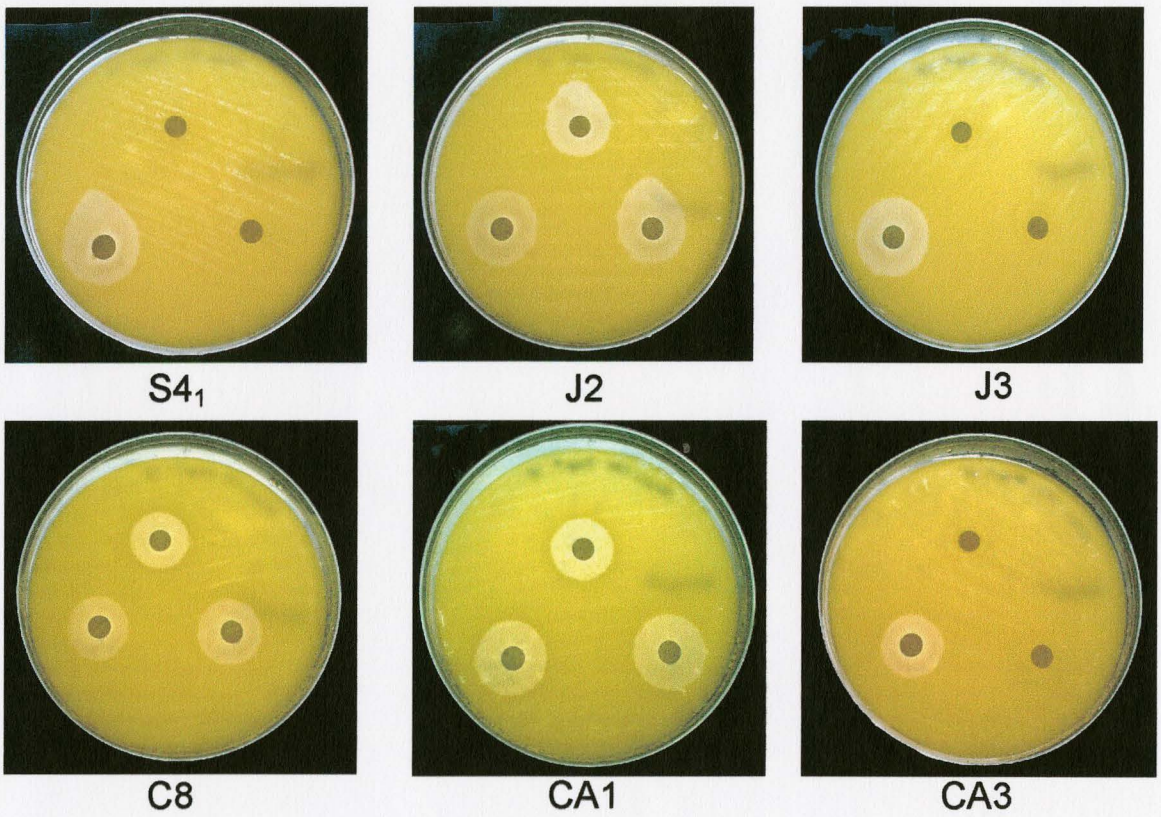


Figure 3.4: Daptomycin Inactivation Bioassay. Cell lysates from the daptomycin resistant soil isolates were incubated at 37°C for 24 hours with daptomycin. Subsequently, an aliquot of each was spotted onto a disk and placed on a lawn of *M. luteus*. On each plate, reaction 1 contained cell lysate, cofactors and daptomycin (50 μg/ml), reaction 2 contained cell lysate and daptomycin (50 μg/ml), and reaction 3 contained cofactors and daptomycin (50 μg/ml).

It is interesting to note that in the case of all three isolates there does not appear to be a requirement for exogenous co-factors. A hydrolysis reaction, either of a peptide bond or the ester bond, is a possible explanation for this observation.

3.2.3 Phenotypic Characterization of Daptomycin Inactivating Isolates

Studies examining the physical appearance of the isolates were undertaken in a effort to better understand their identity It was found that two of the three daptomycin inactivating isolates, J3 and CA3, were able to sporulate in liquid culture when grown in SVM, without shaking, at room temperature for 3-4 weeks (figure 3.5). This is a characteristic common to numerous species of *Streptomyces* (32).

Figure 3.5: Sporulation in Liquid Culture. When grown in the appropriate conditions, two of the daptomycin inactivating isolates, J3 and CA3, were found to sporulate in liquid SVM.

Furthermore, scanning electron microscopy was used to visualize the isolates at a greater magnification (figure 3.6A-C). It is evident that each appears to have distinctive spore morphology suggesting that they are distinct species. Electron micrographs of *S. venezuelae* (figure 3.6D), and the daptomycin-producing organism *S. roseosporus* (figure 3.6E) were also obtained.

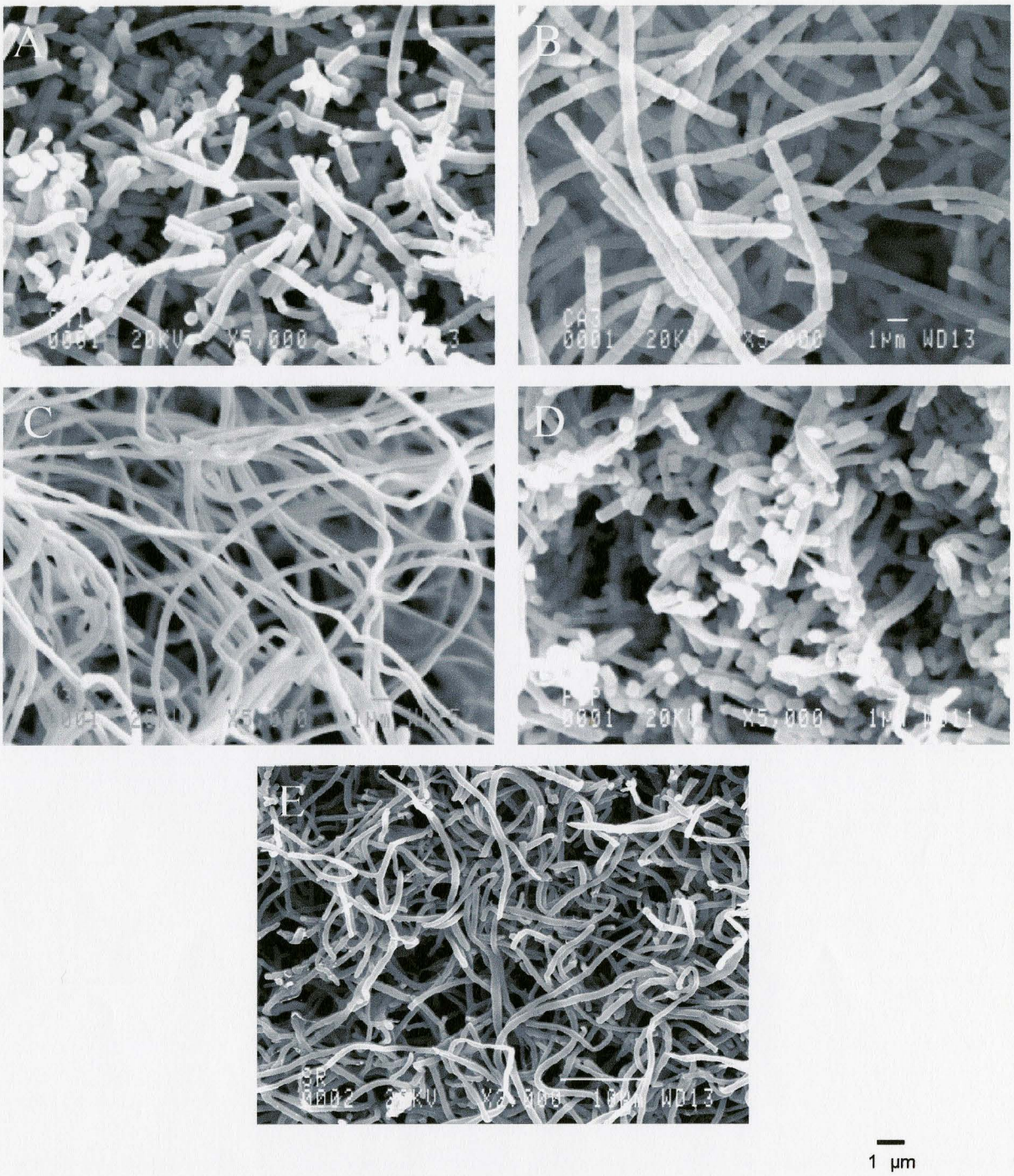


Figure 3.6: Scanning Electron Micrographs of Soil Organisms. The daptomycin inactivating isolates $S4_1$ (A), CA3 (B), and J3 (C) are seen in the sporulating stage of the life cycle. Panel D shows *S. venezuelae*, also at the spourlation stage and the daptomycin-producing organism, *S. roseosporus*, can be seen in panel E. All of the images were obtained at 5000X magnification.

3.2.4 16S rRNA Sequencing and Phylogenetic Tree Construction

In an effort to gain a better understanding of the taxonomic identity of the daptomycin inactivating isolates, the 16S rRNA genes were sequenced. The approximately 1.5 kb gene was PCR amplified from the genomic DNA of each isolate using primers pA and pH described by Mehling (figure 3 7).

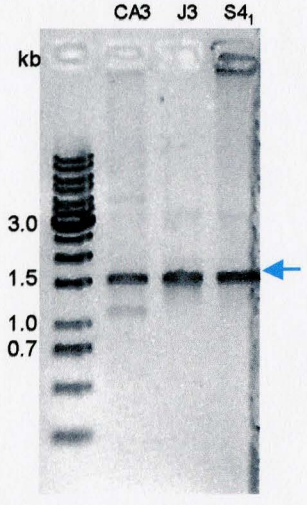


Figure 3.7: PCR Amplification of the 16S rRNA Gene. The gene was amplified using the primers pA and pH to generate a ca. 1.5 kb fragment analyzed by 1% agarose gel electrophoresis. A standard 1 kb ladder was applied to lane 1.

These nucleotide sequences were then entered as a query in a standard nucleotide-nucleotide BLAST search. Shown in figure 3.8 is a phylogenetic tree created by aligning the 16S rRNA sequences obtained in these searches with those determined for the isolates.

Based on this alignment, the daptomycin inactivating isolates are grouped together and in close proximity to the chloramphenicol-producing organism, *S. venezuelae*. A closer examination of the 16S rRNA gene sequences for all three isolates shows that they contain the signature sequence characteristic of Streptomycete species (47).

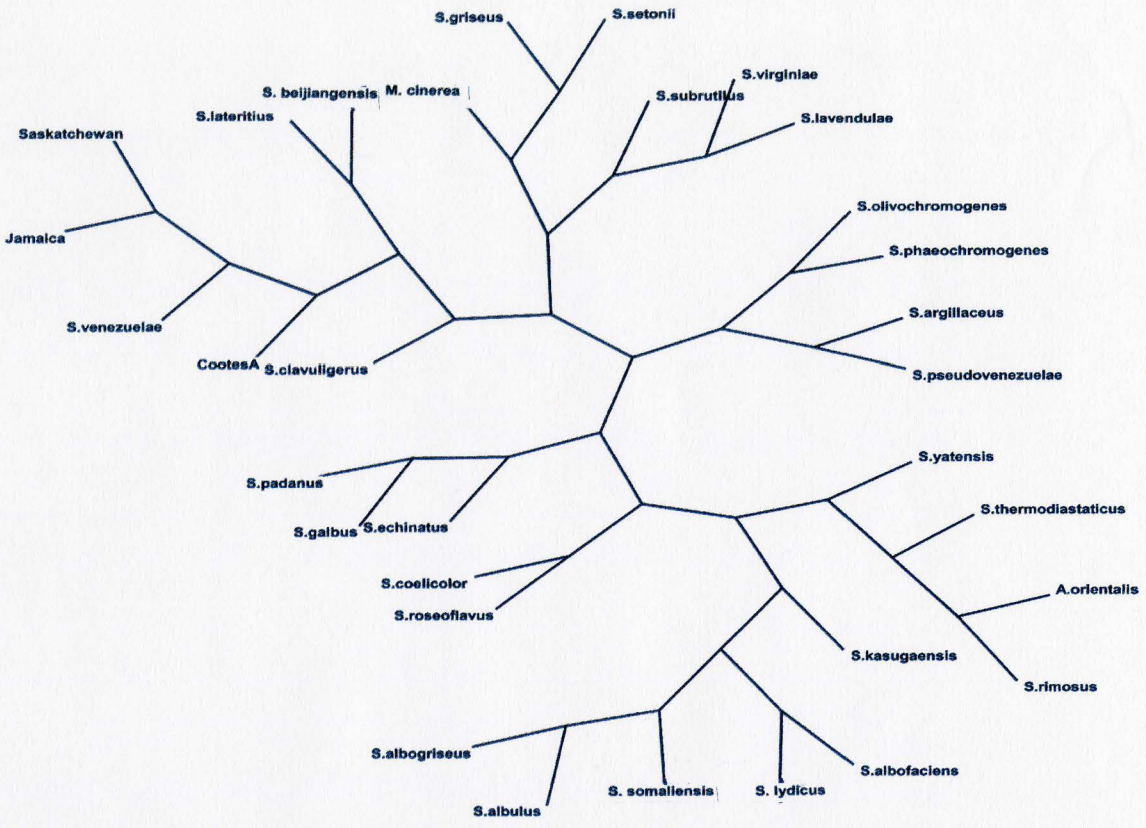


Figure 3.8: 16S rRNA Based, Unrooted Phylogenetic Tree. The sequences of the PCR amplified 16S rRNA genes from the isolates were aligned with numerous other 16S rRNA sequences from soil organisms to generate this tree.

Hence, both the phenotypic and genetic analyses suggest that the isolates are in fact of the genus Streptomyces.

A closer examination of the 16S rRNA gene sequences for all three isolates reveals that they possess characteristics that are specific to *Streptomyces* (figure 3.9). Deletions, of varying lengths, are found within the region of nucleotides 70–90 (*S. coelicolor* numbering) in all of the organisms in comparison to the *E. coli* sequence. This is commonly observed in most Gram-positive organisms. Actinomycetes can be further

distinguished from other bacteria by the presence of a large deletion (ca. 20 nts) at position 450. A prominent feature, conserved only in *Streptomyces* strains, is located at nucleotide position 800. This signature consists of a 16 nt sequence (5'-ACATTCCACGTCGTCG-3') that is found in *Streptomyces* but not in related taxa. Finally, a 5-6 nt insertion in the variable β-region is frequently observed among *Streptomyces spp.* sequences but not among other species (47). These features are conserved in all three of the soil isolates. This finding, and the previous phenotypic characterization studies, indicates that it is highly likely that the isolates belong to the genus *Streptomyces.*

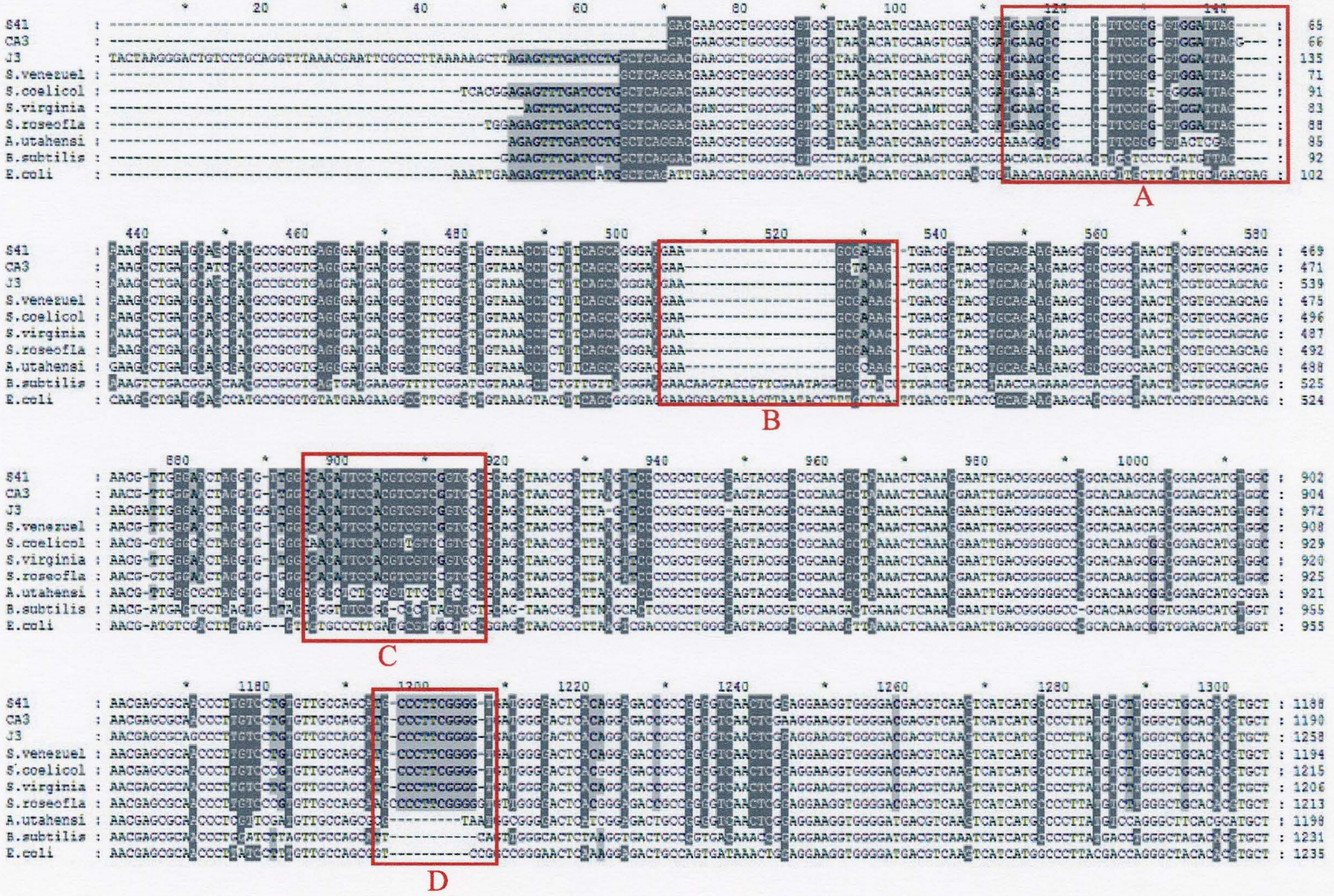


Figure 3.9: Conserved Streptomyces Sequences Within the 16S rRNA Gene. The 16S rRNA genes from the daptomycin inactivating soil isolates display characteristics specific to Streptomyces such as: deletions between nucleotides 70-90 (A), a large deletion around position 450 (B), a highly conserved 20 nt sequence around position 800 (C), and an insertion within the variable β-region (D).

3.2.5 Characterization of Daptomycin Base Hydrolysis

The inactivation bioassays indicated that a hydrolysis reaction may be responsible for detoxification of daptomycin. Thus, prior to biochemical characterization of those inactivation reactions, daptomycin was linearized in a base catalyzed reaction at 60° C. The published procedure followed (50) states that under these conditions, the ester bond of daptomycin is the selective site of hydrolysis. The reaction products were subsequently analyzed by LC-MS (figure 3.10). Although daptomycin and its ester hydrolysed linear form co-elute in the gradient used for chromatography, mass analysis clearly indicates that upon incubation with NaOH, a mass increase consistent with hydrolysis (dap + 18), is observed.

3.2.6 Biochemical Characterization of Daptomycin Inactivation Reactions

The reactions containing cellular extracts of $S4_1$, J3, and CA3, which were identified as possessing daptomycin detoxification properties, were further analyzed by HPLC (figure 3.11) and liquid chromatography-mass spectrometry (LCMS) (figure 3.11). Initial HPLC analysis of reactions containing cellular extracts of J3 (figure 3.11C) showed that a complex reaction, or series of reactions, was occurring. Hence, characterization of this reaction was not pursued further.

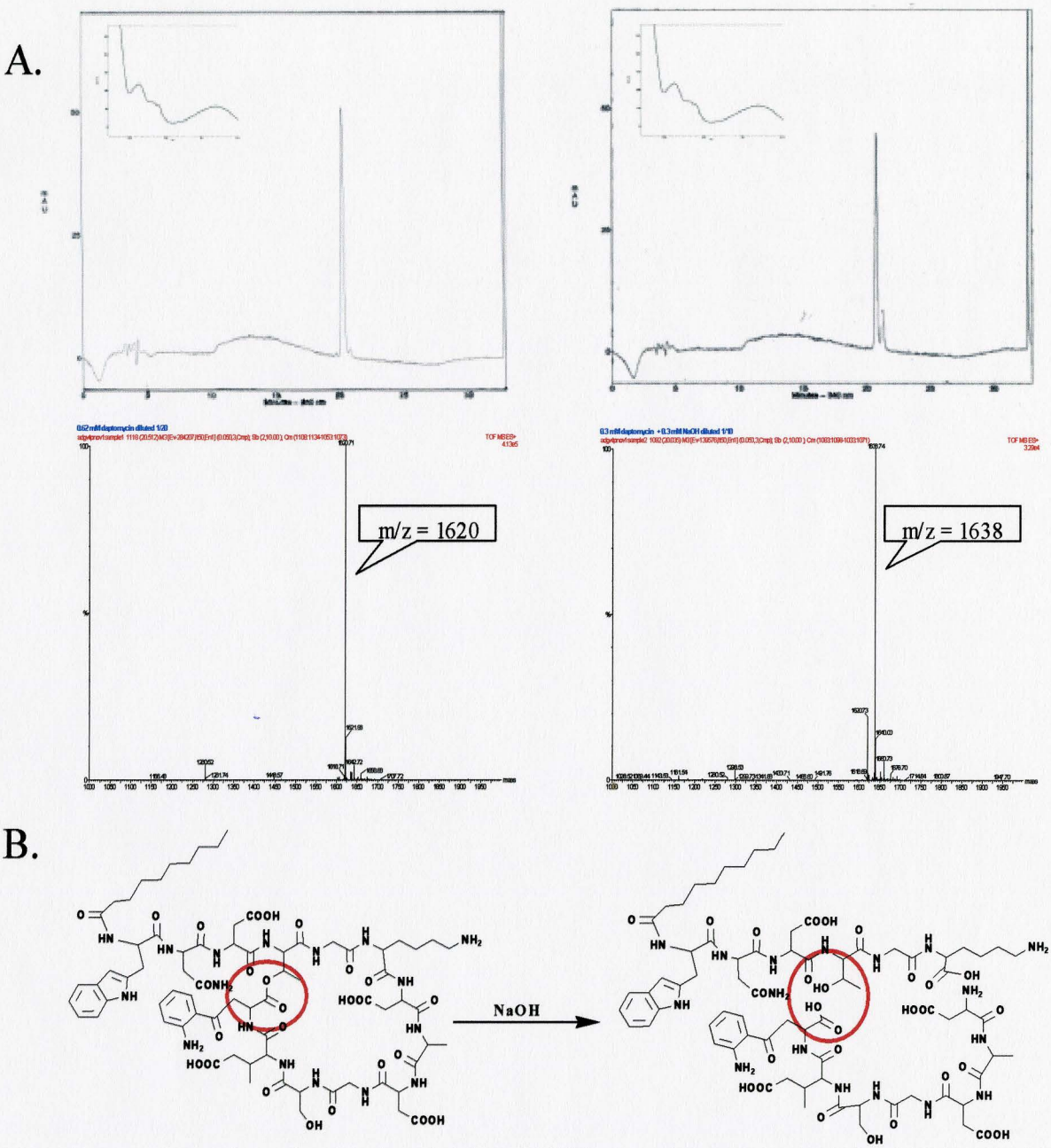


Figure 3.10: Characterization of the Daptomycin Base Inactivation Reaction. Daptomycin was linearized in the presence of NaOH and analyzed by LCMS (A). Although the chromatograms at 340 nm and UV spectrums look similar for daptomycin and its base inactivated form, the mass of the latter has been increased by 18. Depicted in B is the hydrolysis reaction proposed to occur.

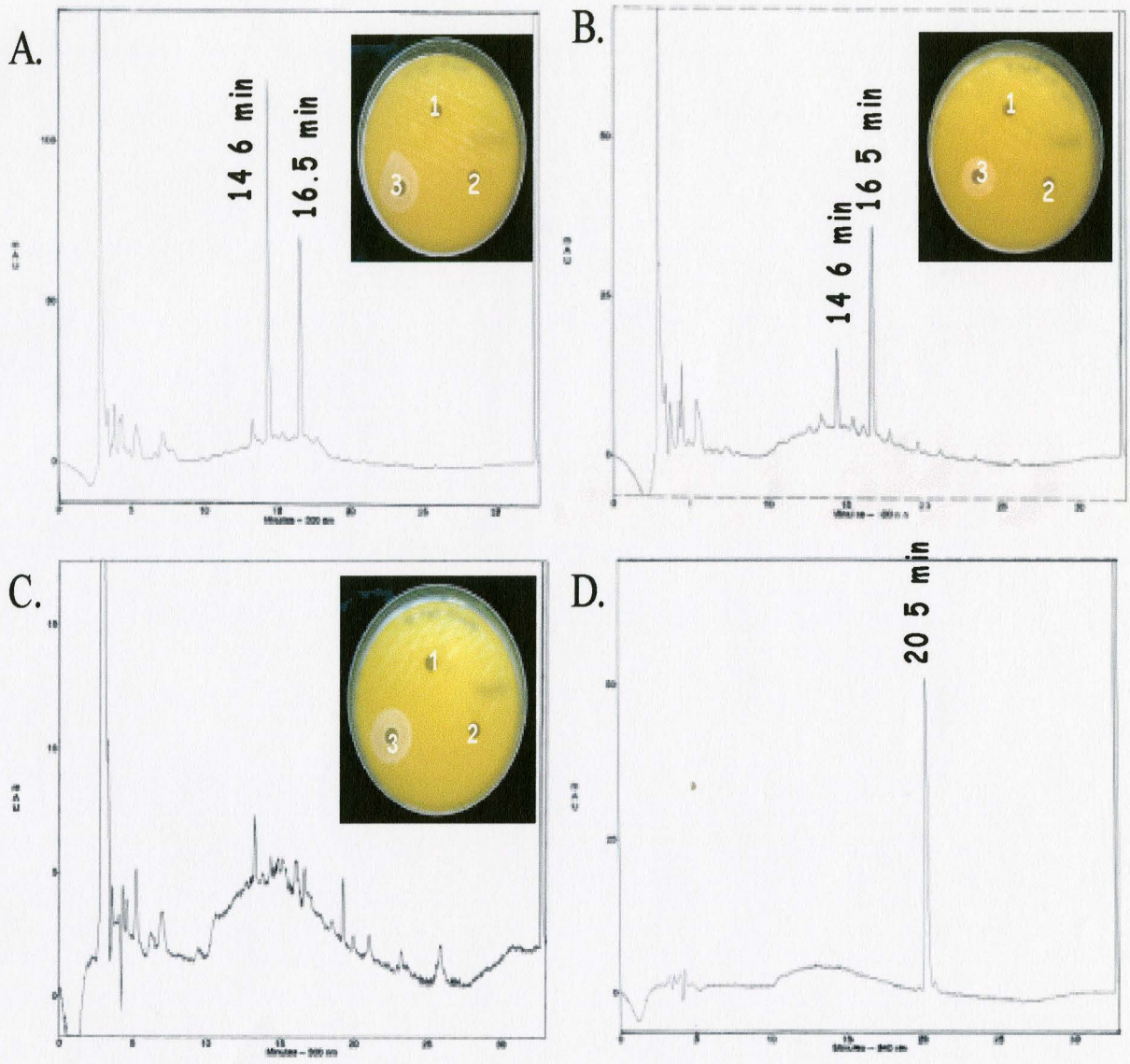


Figure 3.11: HPLC Analysis of the Daptomycin Inactivation Reactions. Reactions that were identified as possessing a potential daptomycin detoxification enzyme based on the bioassay were further analyzed by HPLC on a reverse phase C18 column. The solvent system consisted of 0.05% TFA in dH_2O and 0.05% TFA in acetonitirile. All of the above chromatograms are at 300 nm. Products of the reaction containing cell lysates from $S4_1$ (A) and CA3 (B) elute at 14.6 min and 16.5 min. In contrast, the reaction containing cellular extracts of J3 (C) appears to have a number of products, none of which demonstrates very strong absorbance between 300-450 nm. In a similar HPLC scheme, daptomycin has a retention time of 20.5 min (D).

Mass analysis of the peak at 14.6 min in the reactions containing cellular extracts of either $S4_1$ or CA3 identifies a compound with a mass/charge (m/z) ratio of 1638 (table 3.2), which is the mass of daptomycin plus 18. Thus, this could perhaps correspond to a hydrolysis product. Similar analysis shows that the peak at 16.5 min in both the $S4_1$ and CA3 reactions corresponds to a m/z ratio of 1620 (table 3.2). This is the value expected for daptomycin in its unmodified form or a product linearized via elimination across the ester bond.

Table 3.2: Mass Spectal Analysis of the Daptomycin Inactivation Reactions Catalyzed by $S4_1$ and CA3.

Sample	Retention Time (min)	m/z ratio	Comparison to Daptomycin
Daptomycin	20.5	1620	+0
$S4_1$ Reaction	14.6	1638	+18
$S4_1$ Reaction	16.5	1620	+0
CA3 Reaction	14.6	1638	+18
CA3 Reaction	16.5	1620	+0

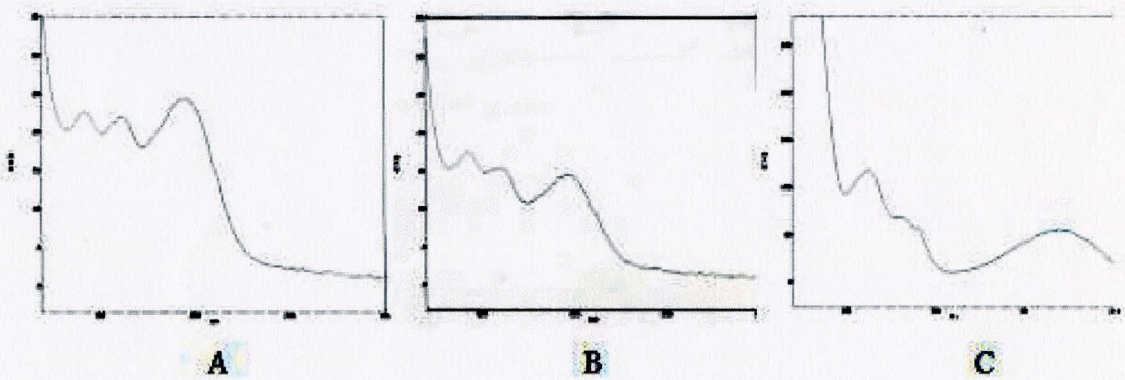


Figure 3.12: UV Spectra of the Peak at 16.5 min in the Daptomycin Inactivation Reactions Catalyzed by $S4_1$ (A), CA3 (B), and NaOH (C). Both of isolate reaction products have local maxima at ca. 300 nm. and comparatively little absorbance at 340 nm. In contrast, the ester hydrolysis product (C) has a greater absorbance at 340 nm than at 300 nm.

Given that neither of these peaks have any antibacterial activity and display a UV spectrum distinct from that seen for daptomycin (figure 3 12), alternative chemical structures can be assigned to these compounds. As suggested previously, hydrolysed daptomycin is likely one of the products. However, the fact that this hydrolysis product has a retention time that is inconsistent with that seen for base hydrolysed daptomycin, suggests that hydrolysis of one of the peptide bonds is occurring. One conceivable modification for the second peak is cleavage of the ester bond via an elimination reaction (figure 3 13). Hence, it would appear that two different detoxification reactions are taking place within one organism.

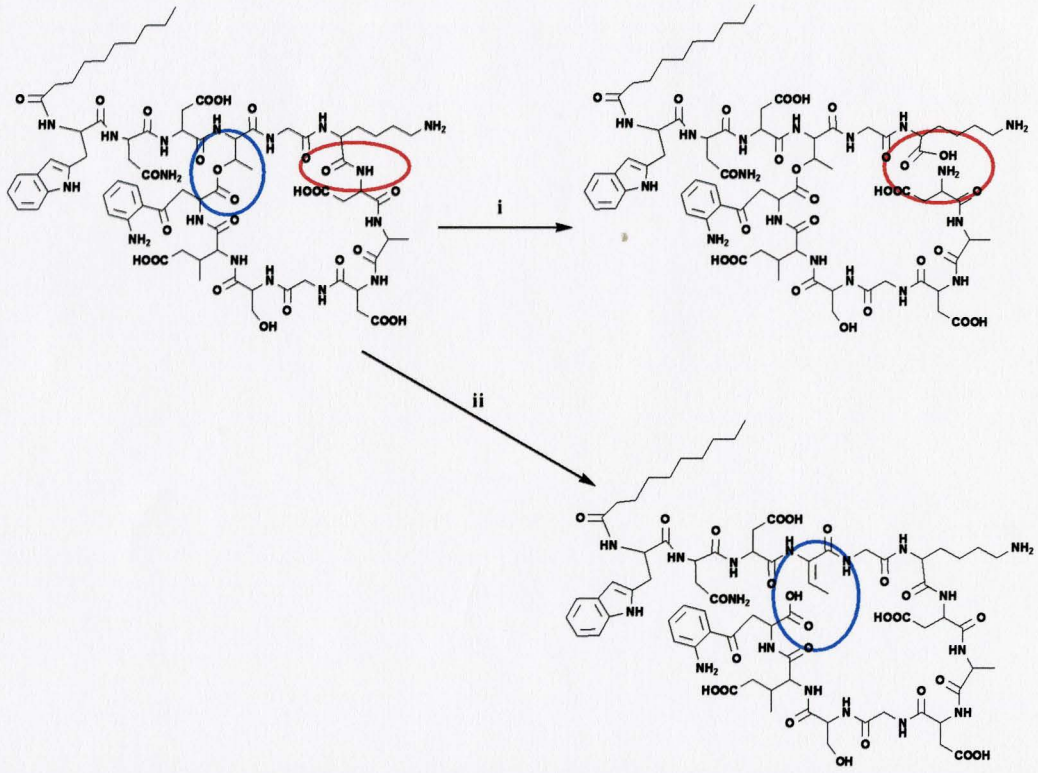


Figure 3.13: Potential Modes of Daptomycin Linearization. The cyclic ring structure can be opened via amide bond hydrolysis (i) or elimination across the ester bond (ii). Amide bond hydrolysis can occur at any site within the ring, thus the scheme shown represents one possibility In addition to elimination, the ester bond can be cleaved via hydrolysis however, mass spectrometry analysis supports the former reaction.

In addition to daptomycin detoxification mechanisms present in soil isolates, the ability of Vgb to modify this antibiotic was examined. Vgb is a B type streptogramin antibiotic lyase that linearizes the depsipeptide by an elimination reaction that cleaves an internal ester bond between a Thr hydroxyl and the C- terminal carboxylate (Mukhtar, 2001). Reactions containing a range of Ca^{2+} concentrations were assessed for enzymatic inactivation of the antibiotic (figure 3 14). Although Vgb is able to cleave the ester bond of quinipristin, it is not capable of detoxifying daptomycin in a similar manner Optimal reaction conditions for quinipristin inactivation require a minimum of 2 mM $MgCl_2$ (51). However, even under these conditions, daptomycin inactivation is not observed.

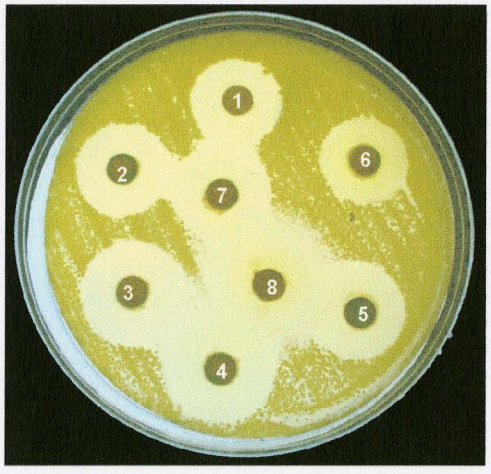


Figure 3.14: Daptomycin Inactivation by Vgb in the Presence of Various Ca^{2+} Concentrations. Reactions containing purified Vgb and daptomycin in the presence of varying amounts of Ca^{2+} were incubated at 30°C for 16 hrs and then spotted on disks placed on a lawn of *M. luteus*. The reactions contained metals as follows:1 – 0 mM Ca^{2+}, 2 – 2 mM Ca^{2+}, 3 – 4 mM Ca^{2+}, 4 – 6 mM Ca^{2+}, 5 – 8 mM Ca^{2+}, 6 – 10 mM Ca^{2+}, 7 – 2 mM Mg^{2+}, and 8 – 2 mM Mg^{2+} in the absence of Vgb.

Based on its proximity to the isolates in the 16S rRNA derived phylogenetic tree (figure 3.8), the activity of daptomycin against *S. venezuelae* was also examined. It was found that this strain was resistant (MIC. 50 μg/ml) and could weakly inactivate

daptomycin. However, unlike the soil isolates, *S. venezuelae* shows a requirement for at least one exogenous cofactor as noted by a zone of inhibition diameter similar to the control when an important cofactor is omitted from the reaction (figure 3 15). When each of the cofactors was left out individually, those reactions lacking metal ions (Mg^{2+} or Mn^{2+}), and to a lesser extent, glutathione were not as effective at detoxifying daptomycin. HPLC analysis shows that the detoxified product has the same retention time and spectrum as daptomycin. In this respect, the product of this reaction is similar to base hydrolysed daptomycin (figure 3 10). Unfortunately, these reactions were difficult to study further The initial three times the reaction was performed, daptomycin detoxification was observed. However, subsequent metal depletion experiments were unsuccessful as the inactivation ability appeared to have been lost.

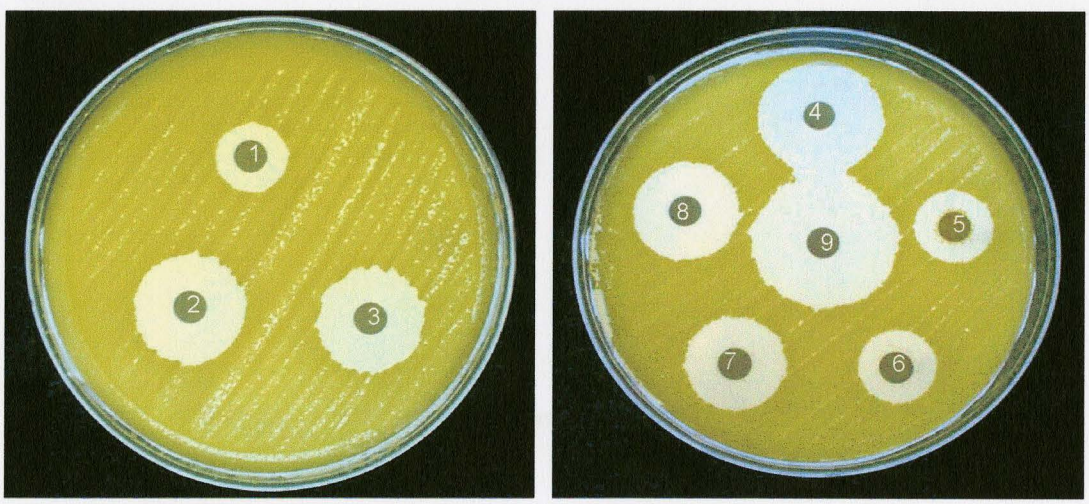


Figure 3.15: Daptomycin Inactivation by *S. venezuelae*. Reactions containing cellular extracts of *S. venezuelae* and daptomycin were incubated at 30°C for 24 hrs. These were subsequently spotted on to disks placed on lawns of the daptomycin susceptible organism *M. luteus*. Reactions contained: 1- cell lysate, cofactors, dap (50 μg/ml), 2 – cell lysate, dap (50 μg/ml), 3 – cofactors, dap (50 μg/ml), 4 – cell lysate, cofactors minus $MnCl_2$, dap (50 μg/ml); 5 – cell lysate, cofactors minus acetyl CoA, dap (50 μg/ml), 6 – cell lysate, cofactors minus ATP, dap (50 μg/ml), 7 – cell lysate, cofactors minus GSH, dap (50 μg/ml), 8 – cell lysate, cofactors minus $MgCl_2$, dap (50 μg/ml), 9 – daptomycin (250 μg/ml)

3.2.7 Genomic Library Construction

In an effort to identify the genetic element(s) responsible for the observed daptomycin resistance phenotype, construction of a genomic library for each of the three isolates has been undertaken. The first step in this process was the identification of a daptomycin susceptible host strain in which selection could be feasible. Table 3.3 lists a number of organisms that were tested in TSB supplemented with 1mM $CaCl_2$ for this purpose.

Table 3.3: MICs for Daptomycin Against a Number of Potential Hosts in Liquid TSB Supplemented With 1.2 mM $CaCl_2$.

Organism	MIC (ug/ml)	Strain Characteristics	Organism	MIC (ug/ml)	Strain Characteristics
E. coli DB10	> 64	Sensitive to type A streptogramins	*S. toyocaensis* NRRL 15009	> 64	A47934 producer
E. coli MC1061	> 64	Sensitive to type A streptogramins	*S. coelicolor* M145	> 50	Wild type
E. coli 901S	> 64	Sensitive to type B streptogramins	*S. coelicolor* Abs$^-$	> 50	Point mutant, little to no antibiotic production
E. faecalis	2	Wild type ATCC strain	*S. coelicolor* pha	> 50	Null mutant, hyper drug production
S. lividans	> 50	Wild type	*B. subtilis* L5087	2	Wild type
S. roseosporus	> 64	Daptomycin producer	*B. subtilis* 168	2	"super" competent

Based on these results, the original strategy had involved selection of positive clones in *B. subtilis* 168. However, it was later discovered that when grown on ONA supplemented with 12 mM $CaNO_3$, *S. coelicolor* M145 was sensitive to daptomycin at 2 μg/ml (figure 3.16) (personal communication, Nodwell Lab). This experiment was repeated in NB and similar results were observed. Thus, *S. coelicolor* M145 can be used as host strain to facilitate the selection of daptomycin resistance genes.

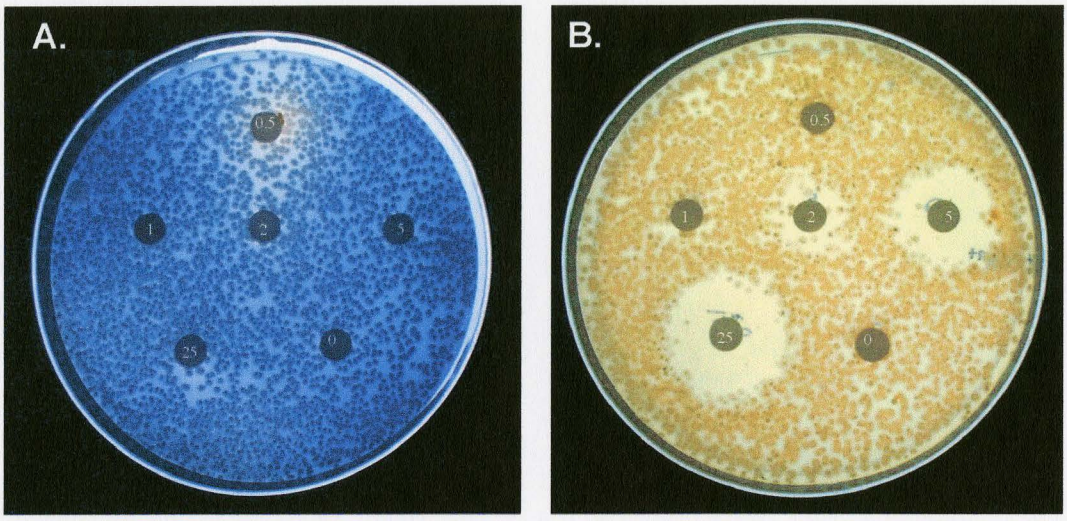


Figure 3.16: MIC of Daptomycin against *S. coelicolor* M145 on TSA versus ONA. *S. coelicolor* M145 expresses a different resistance phenotype when grown in TSA supplemented with 1.5 mM $CaCl_2$ (A) and ONA supplemented with 12 mM $CaNO_3$ (B). The concentration of daptomycin applied is indicated on each disk in μg/ml. On ONA supplemented with 12 mM $CaNO_3$, sensitivity to daptomycin is observed at 2 μg/ml.

*Dpn*II partial digests of the genomic DNA extracted from the isolates were optimized in order to obtain DNA fragments between 25-35 kb for each of the isolates (figure 3 17A). Each of the isolates required different conditions to achieve the greatest concentration of fragments in the desired range. The digestion products of each reaction were subsequently separated by sucrose gradient size fractionation to isolate the fragment sizes of interest from the remainder of the gDNA (figure 3 17B).

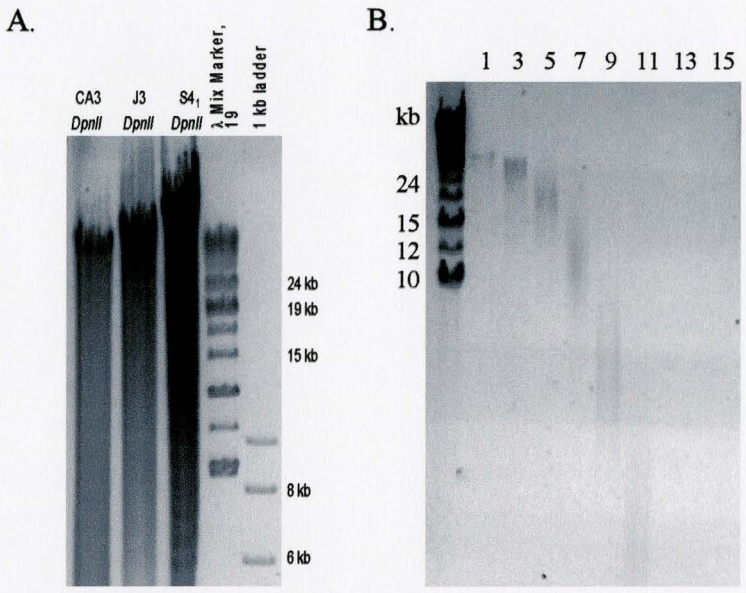


Figure 3.17: Preparation of gDNA Inserts for Cosmid Library Construction. gDNA from each of the isolates was partial digested with *DpnII* (A) and size fractionated on a sucrose gradient from 10 to 40%. Fractions of the sucrose gradient were analyzed on a 0.5% agarose gel and compared to the λ mix marker, 19 (B).

4. Discussion

4.1 Characterization of TetX Orthologues in S. coelicolor

Although resistance to tetracycline antibiotics is a widespread phenomenon, very few enzymatic degradation mechanisms have been described. The best characterized of these, TetX, is encoded by the *tetX* gene isolated from an anaerobic *Bacteroides* species. This 44 kDa cytoplasmic protein is able to chemically modify tetracycline in the presence of NADPH and oxygen to render the drug inactive (68). Given that aerobic conditions are absolutely necessary for tetracycline degradation by TetX, it is possible that the gene has a Gram-positive, aerobic origin, such as *Streptomycetes*

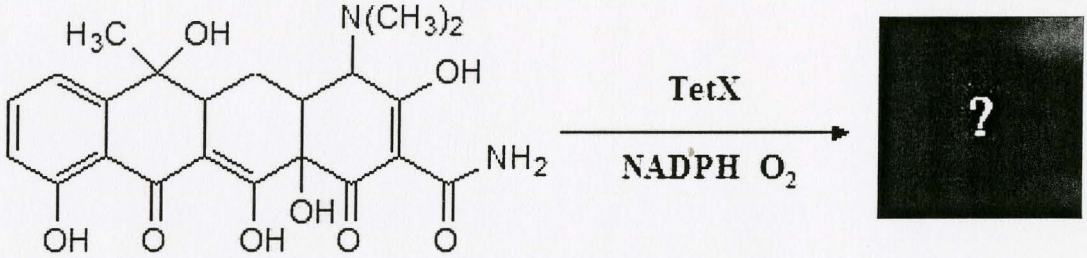


Figure 4.1: TetX Catalyzed Tetracycline Detoxification Reaction. In the presence of NADPH and O_2, TetX modifies tetracycline such that it is no longer active. The products of this inactivation reaction have yet to be characterized. A darkening of the media to greyish-black is also observed when cells expressing TetX are grown in the presence of tetracycline.

The finding that none of the TetX homologues studied here participate in tetracycline inactivation suggests that they may play a role in alternative reactions in the cell. Given that four of the five proteins harbour a predicted flavin binding site, and two were purified with a characteristic yellow chromophore, a reasonable speculation is that these are hydroxylation reactions. The genes located in the vicinity of an unknown can

sometimes aid in suggesting a potential role for the unknown enzyme. A closer examination of the genetic environment surrounding these genes reveals that they are located directly downstream, or upstream in the case of T36402, of a putative TetR homologue, with the exception of CAC16992. TetR is a well-characterized repressor of the tetracycline efflux pump gene, *tetA*. In most cases, *tetR* is located upstream of *tetA* and binding of the signal molecule, tetracycline, to TetR relieves transcriptional repression of *tetA* (71). The organization of the genes studied here suggests that they too may be regulated in a similar fashion. In light of the results described for tetracycline detoxification, it is possible that an alternative signalling molecule is employed in this system. This also suggests that the proteins studied may have the ability to detoxify, or be included in other aspects of, other xenobiotics.

A more recent BLAST search identifies not only these five proteins, but also many additional proteins from *S. coelicolor* and *S. avermitilis*, among others as having homology to TetX (figure 4.2). This finding, taken together with the recent literature on *tet(37)* (77), suggests that the prevalence of tetracycline resistance-like enzymes is much more widespread than originally thought. A greater understanding of the occurrence, and perhaps the origins, of tetracycline resistance via detoxification will be achieved as these proteins, and others like them, are further characterized.

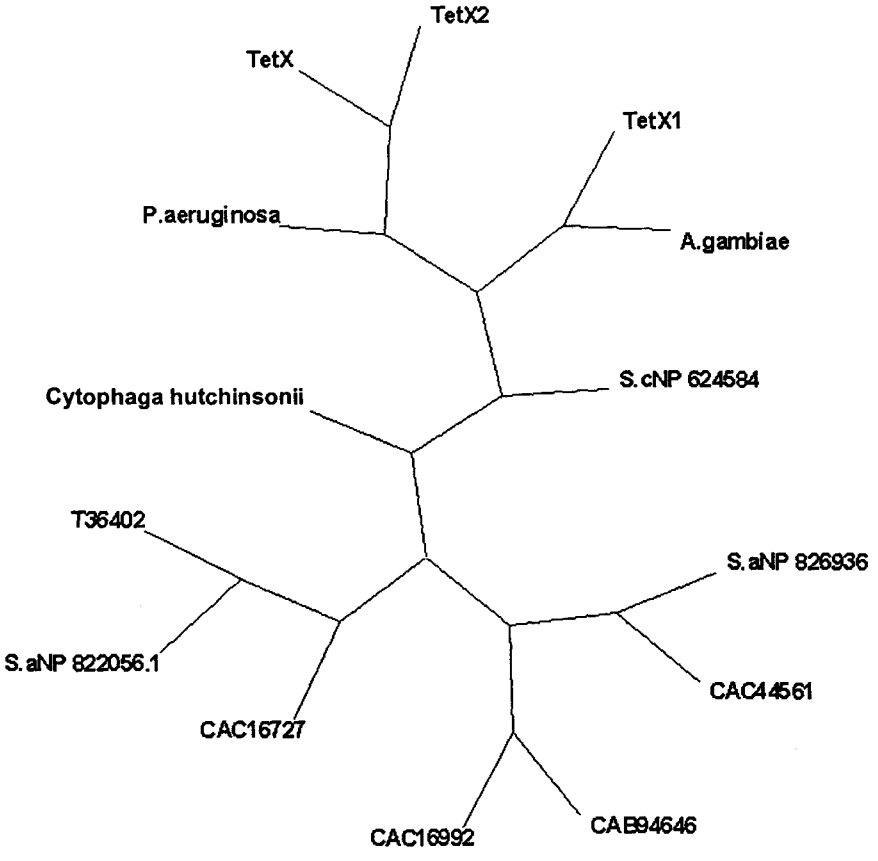


Figure 4.2: Proteins Similar to TetX. In addition to the Bacteroides genes TetX1 and TetX2, a tetracycline detoxification protein has been identified in P. aeroginosa. TetX orthologues can be found in a number of organisms including the malaria mosquito, *A. gambiae*, and the recently sequenced *S. coelicolor* and *S. avermitilis.*

4.2 Novel Mechanisms of Antibiotic Resistance in Environmental Isolates

The process of isolating *Streptomyces spp.* from soil samples was relatively facile, however obtaining pure cultures was often difficult. Many of the organisms isolated required several rounds of streaking in order to obtain pure cultures. The tight association observed between many of these soil organisms may be a reflection of their close co-habitation in the natural environment, perhaps even of a symbiotic nature. It is reasonable

to believe that the isolates live in close proximity to other organisms from which they obtain greater fitness. In return, the antibiotics produced by the *Streptomyces spp.* may help to increase the survival of the other organism.

Screening of pure isolates yielded various organisms that were able to grow well in the presence of the antibiotics selected (figure 3.3). This is likely to be a reflection of the types of antibiotics employed in this study. Tetracycline and daptomycin are naturally produced as secondary metabolites of *S. aureofaciens* (54) and *S. roseosporus* (46) respectively. Similarly, the components of Synercid, quinupristin and dalfopristin, are semi-synthetic derivatives of the natural products pristinamycin I_A and pristinamycin II_A, respectively and are produced by *S. pristinispiralis* (30). Thus, the identification of several soil isolates displaying resistance to these antibiotics is not unexpected. It is highly likely that many, if not all, of the isolates have been exposed to these, or other compounds with similar chemical structures, in the soil. Survival in such an environment would necessitate the acquisition of resistance to these agents. Tetracycline is a member of a large group of antibiotics, many of which are natural products (54). Although the chemical functionalities at various positions can be diverse, the central core structure is highly conserved among all members of this class of antibiotics. Similarly, the streptogramin antibiotics maintain a central backbone structure (30). Furthermore, numerous cyclic peptide antibiotics, resembling daptomycin, have been identified including calcium dependant antibiotic (CDA), which is produced by *S. coelicolor* (32), and ramoplanin, which is produced by an *Actinoplanes spp.* (44). Survival in an environment that is rich in such toxins would necessitate the acquisition of resistance to

these agents. It is conceivable that these resistance mechanisms can be extended to antibiotics that differ slightly in structure but still belong to a similar class as those in an organism's environment. The specific selection of antibiotics that are natural products, or derivatives of such, is likely to have influenced the considerable number of resistant isolates identified. Fewer isolates are likely to be resistant to completely synthetic antibiotics, such as ciprofloxicin or linezolid, as these types of chemicals are not necessarily found in their natural environment. Furthermore, it is interesting to note that many strains are resistant to multiple antibiotics (figure 3.3). Again, this can be attributed to selective pressures in their natural environment.

As an indication of the level of resistance expressed by a selected number of the isolates, MICs were determined. A comparison of the MICs of these antibiotics commonly observed against clinical isolates with those determined for the soil isolates (table 3.1) clearly highlights the elevated level of resistance present in the soil. The constant and enduring presence of these antibiotics in the natural environment has selected for organisms capable of surviving in the presence of high concentrations of these agents.

The importance of media type and composition is emphasized by the change in tetracycline resistance from the initial screening process to the MIC determination study. The initial resistance screening was carried out on solid media rich in the divalent metal cations Mg^{2+} and Ca^{2+} (ca. 100 mg/L), and starch as a C-source. MIC determinations, on the other hand, were performed in liquid media with lower divalent cation concentrations. The activity of certain antibiotics, such as aminoglycosides (53) and daptomycin (26), has

been shown to vary based on the divalent metal cation concentration. The same has also been shown to be true for tetracyclines, whose MIC increased progressively with an increase in metal ion concentration, specifically Mg^{2+} and Ca^{2+} (53). Thus, this could account for the observation that the isolates were able to grow in the presence of 50 μg/ml of tetracycline in the initial screen yet displayed much lower MICs under standard NCCLS protocols (3.3 mg/L for Ca^{2+} and 4.0 mg/L for Mg^{2+}). Moreover, the expression of many genes is regulated by the cellular life cycle. If the resistance phenotype is dependant on the expression of a gene that is temporally regulated, then the nature of the media becomes vitally important to antibiotic resistance determination. Hence, media that support sporulation may display differential antibiotic resistance profiles than those that do not. Thus, the different levels of tetracycline resistance displayed by the isolates may be due to a number of factors however, it remains that these organisms have a level of tetracycline resistance worthy of further examination. In addition, media composition can also influence antibiotic production and susceptibility of certain *Streptomyces spp.* The quantity and type of carbon source available has been shown to affect the production of antibiotics by both *S. coelicolor* and *S. lividans* (33, 34).

The physical characteristics of the three daptomycin inactivating isolates, $S4_1$, CA3, and J3, were studied. Initial Gram-staining and light microscopy had revealed that the isolates were Gram-positive and filamentous. These observations, in combination with the typical round, fuzzy shape and soil-like odour of colonies on solid media, strongly suggested that the isolates belonged to the genus *Streptomyces*.

Streptomyces have a complex, multi-stage life cycle that involves numerous cell types. Growth initiates from spores to form a mat of substrate mycelium. The mycelium then give rise to another cell type, the long, filamentous aerial hyphae. Following aerial hyphae production, *Streptomyces* undergo septation to generate thick walled, unigenomic spores. Spores are hearty and are resilient to many harsh conditions such as those found in soil. Generally, spores are cylindrical, but the exact shape can vary from species to species (29). The spore morphology of the isolates was examined by scanning electron microscopy (SEM). From figure 3.6 it is evident that each isolate appears to have distinctive spore morphology suggesting that they are not identical species.

In addition, the phylogenetic identity of organisms is often established by sequencing of the 16S rRNA gene. This gene has various features that make it an ideal marker to aid in determining phylogentic relationships; 16S rRNA is essential to a critical organelle, the bacterial ribosome, and thus is universally distributed. Moreover, the overall sequence of the 16S rRNA gene changes very slowly with time. The presence of variable and conserved sequences within the gene permits the comparison of both distantly related and closely related species (41).

An alignment of the sequences obtained in this work with others obtained by BLAST searches permitted the construction of the tree seen in figure 3.8. Based on this tree, the daptomycin inactivating isolates are grouped together and in close proximity to the chloramphenicol-producing organism, *S. venezuelae*. Interestingly, *S. venezuelae* was also found to be resistant to daptomycin. This clustering effect may be an artifact of the isolation process, however two other organisms isolated using identical techniques do not

lie on the same branch (V. D'Costa, unpublished) suggesting that the observed cluster of daptomycin inactivating organisms is genuine.

Bacteria adapt many strategies to evade the action of antibiotics including active efflux, altered permeability, target modification and even enzymatic modification of the antibiotic itself (71). The primary mode of resistance seen in clinical pathogens can vary based on the antibiotic in question. Enzymes encoded by bacterial genomes detoxify many classes of clinically important antibiotics. An understanding of these enzymes is critical to the continuing battle against bacterial pathogens.

Antibiotic inactivation can be a result of many chemical modifications including acetylation, phosphorylation, and hydrolysis, and can often be dependant on one or more cofactors. Initial experiments indicated that the inactivation reactions catalyzed by the isolates were not dependant on cofactors. It is interesting to note that in the case of all three isolates, there does not appear to be a requirement for any exogenous cofactors. This suggests that daptomycin inactivation is occurring by a mechanism that is independent of cofactors, such as hydrolysis, rather than the addition of a chemical moiety. An examination of the chemical structure of daptomycin, in conjunction with mass spectral analysis, identifies various potential sites of hydrolysis. The most obvious of these are the many peptide bonds in the cyclic portion of the antibiotic (figure 4.4). Hydrolysis of any of these would result in ring-opening and the loss of a structure that is potentially essential for antibiotic activity. Ring-opening can also be achieved by hydrolysis of the ester bond between the carboxy-terminal kynurenin residue and the tyrosine residue at position 4 (figure 4.4). Finally, hydrolysis of the peptide bond

between the acyl chain and the remainder of the molecule could result in the loss of a portion of the antibiotic that is important for interaction with the bacterial cell membrane (figure 4.3). Given the mass analysis, the latter is not likely to be occurring.

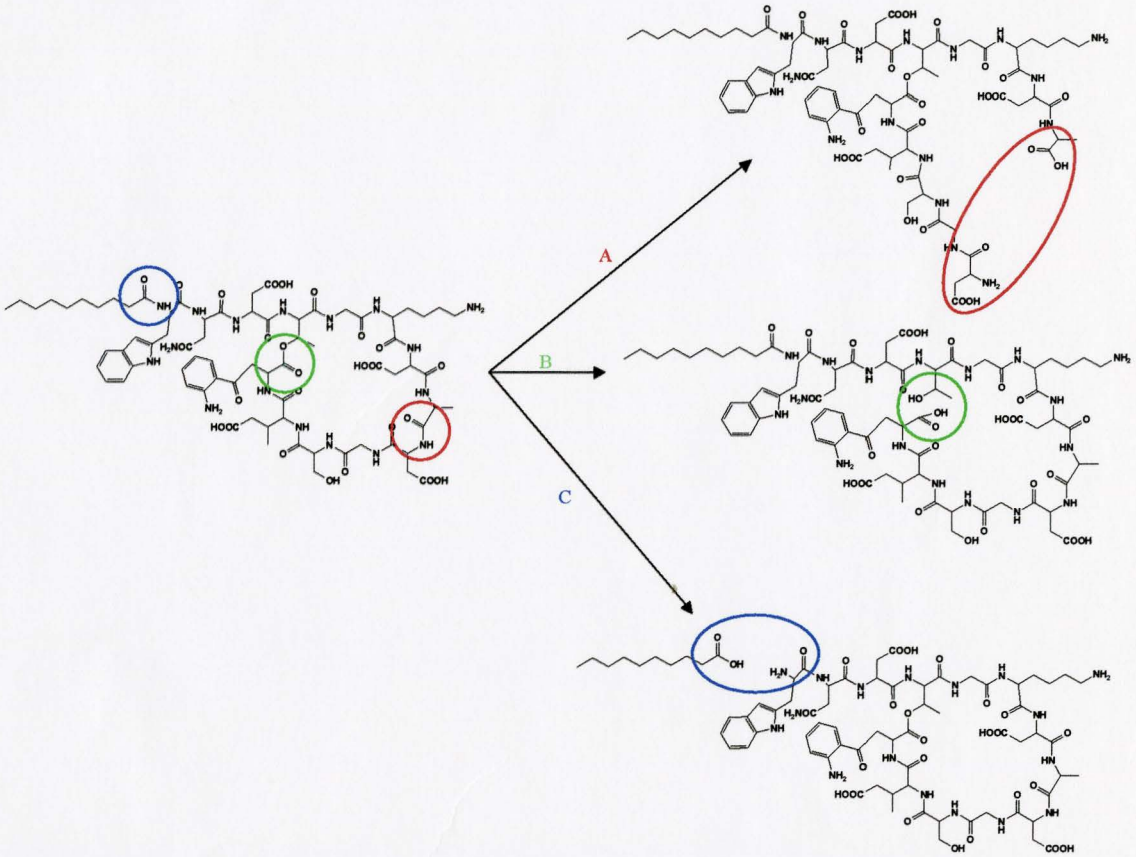


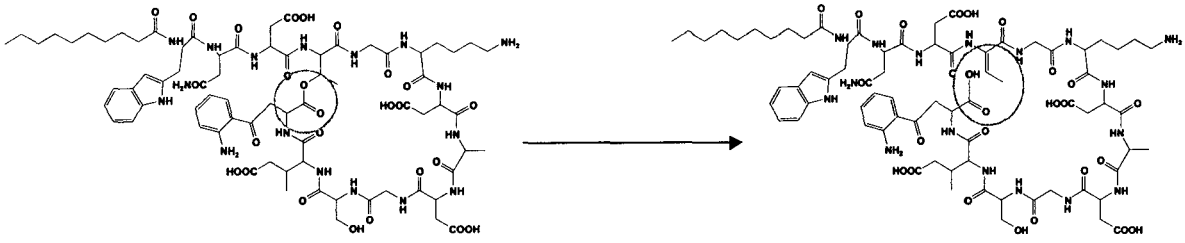
Figure 4.3: Possible Modes of Daptomycin Hydrolysis. Inactivation of the antibiotic can occur via amide bond hydrolysis to generate a ring-opened structure (A), or result in the loss of the fatty acyl chain (C). Additionally, the cyclic structure can be disturbed by hydrolysis of the ester bond (B).

Additionally, linearization of the antibiotic can be achieved via cleavage of the ester bond by an elimination mechanism (figure 4.4). The finding of such a mechanism is exciting because another antibiotic resistance protein studied in the Wright Lab, Vgb from *S. aureus*, catalyzes a similar reaction (51). Inactivation of type B streptogramins is

thought to occur by the action the Vgb enzyme that catalyzes the cleavage of the lactone linkage of the cyclized depsipeptide, resulting in an inactive, linear compound (51). The 33 kDa Vgb protein from *S. aureus* has been biochemically characterized and shown to require divalent metals, preferentially Mg^{2+}, for optimal activity. Until recently, Vgb from this organism was thought to be a result of hydrolysis. However, through a series of careful experiments, Mukhtar *et al.* have identified that the enzyme actually catalyzes type B streptogramin linearization via elimination across the ester bond making it a lyase rather than a hydrolase (figure 4.5). Although the type B streptogramin quinupristin and daptomycin are structurally different, they do share some common features including a peptide backbone cyclized via an ester bond. The inability of Vgb to act on daptomycin may suggest that this enzyme has a fairly narrow substrate specificity. Although type B streptogramins and daptomycin are both depsipeptides, the number of amino acids in the core ring structure is differs between the two with 6 or 7 residues in streptogramins versus 10 in daptomycin. In addition, Mukhtar *et al.* also found that the hyroxypicolynyl group was essential for substrate recognition by Vgb. In daptomycin, a kynurenine residue is substituted at this position. It is possible that these differences are significant enough to prevent Vgb from acting on daptomycin.

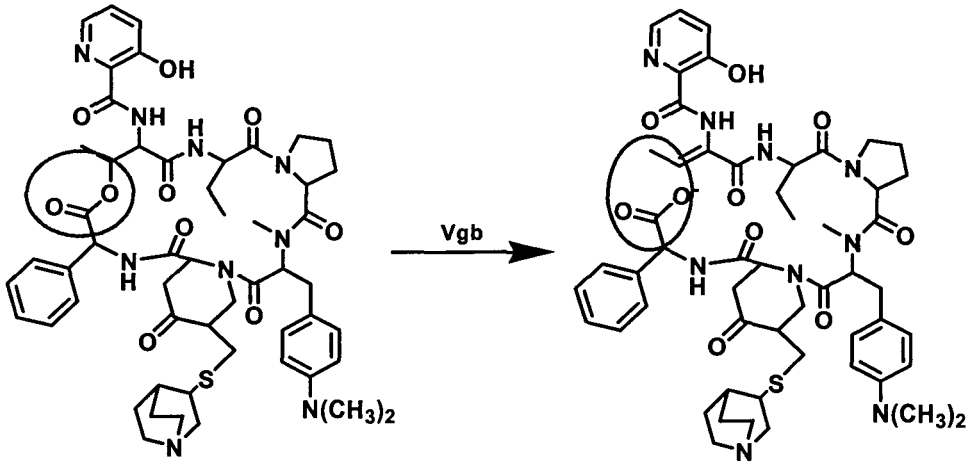
Although the research described here indicates that daptomycin is not a substrate for Vgb, it is interesting to note that homologues of this enzyme have also been identified in *B. pertussis* (the causative agent of whooping cough), the soil bacterium *S. coelicolor*, and many other bacteria (51). Another soil organism, *S. lividans*, has also been shown to harbour a lyase capable of inactivating the type B streptogramin etamycin (4, 5). In many

cases, these genes are chromosomally located (51). Thus, it is plausible that the daptomycin inactivating proteins described here are part of a larger reservoir of lyase-type resistance determinants present in the environment. As discussed previously, a number of antibiotics are cyclized via an ester bond (figure 4.6) and thus have the potential to act as substrates for these enzymes.



Daptomycin (MW = 1619) Elimination Product (MW = 1619)

Figure 4.4: Inactivation of Daptomycin via Elimination. The ester bond through which daptomycin is cyclized can be cleaved by an elimination reaction to generate a linear product. The resulting compound would have a mass identical to daptomycin.



Quinupristin Elimination product

Figure 4.5: Reaction Catalyzed by Vgb. The *S. aureus* enzyme, Vgb, confers type B streptogramin resistance by performing an elimination reaction across the ester bond such that an inactive, linearized product is formed.

One of the most interesting implications of these findings is the suggestion that $S4_1$ and CA3 each possess two distinct mechanisms for enzymatic modification of daptomycin. It is doubtful that these isolates have two proteins specifically dedicated to

daptomycin inactivation. Not only would this be impractical from an energy point of view, but the cellular site of action of daptomycin (cellular membrane) suggests that the inactivation reactions being observed are actually fortuitous. Hence, the detoxification reactions are likely to be a result of housekeeping proteins adopting novel functions upon being supplied with a new substrate. Past precedent for such an activity is observed in *Providencia stuartii* where aminoglycoside acetylation is a secondary role of an enzyme involved in peptidoglycan acetylation (58) and the ability of the aminoglycoside resistance enzyme, AAC (6')-Ii from *E. faecium*, which can also acetylate histones (82).

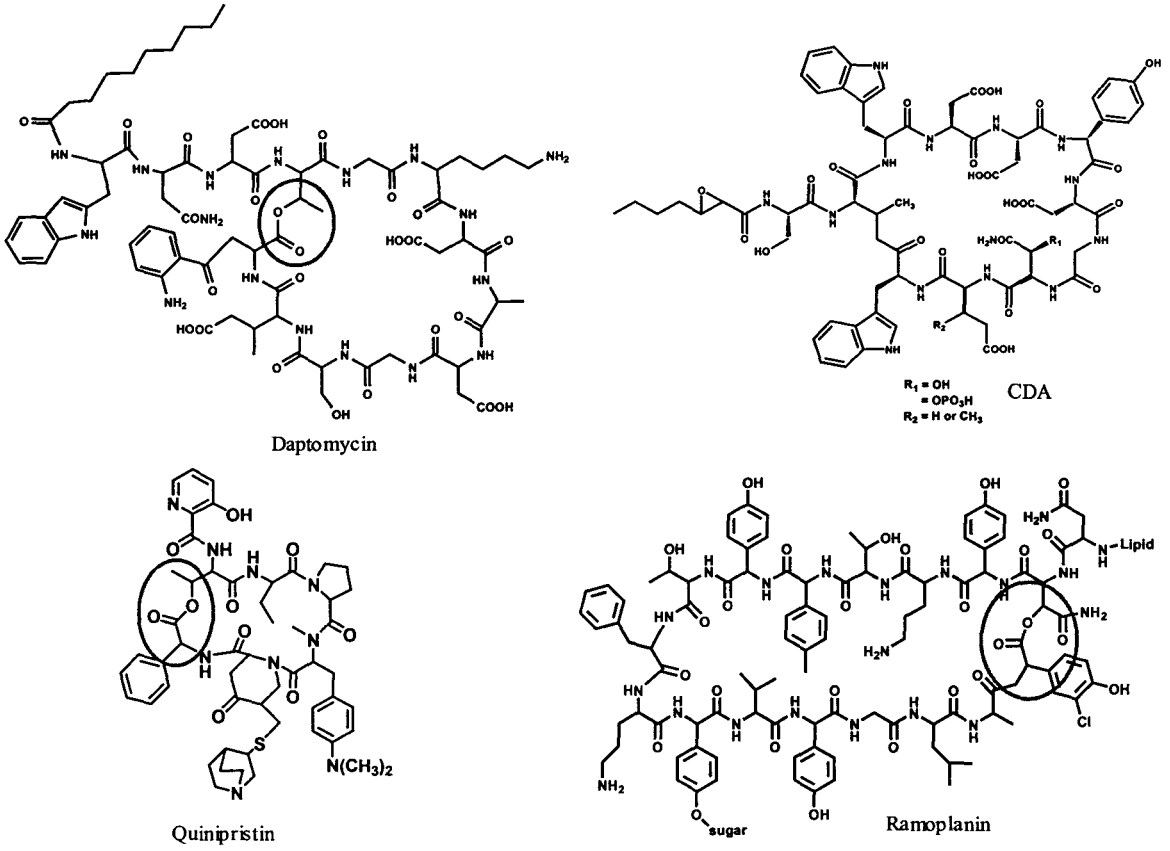


Figure 4.6: Chemical Structures of Daptomycin-like Antibiotics.

Concluding Remarks

The finding of the resistance mechanisms described for daptomycin here, together with those previously described for other antibiotics, lend support for the hypothesis that soil organisms serve as reservoirs of resistance determinants. Agricultural use of antibiotics has been implicated in providing the selective pressure for the maintenance of these determinants in the soil (24, 45, 76). The use of antibiotics in agriculture has long been a point of debate between those in the agricultural sector and scientists in the area of infectious diseases (67). It has been suggested that the use of these agents on livestock breeds for a pool of resistant organisms that have the potential cause human illness upon the ingestion of meat products (25, 65, 67, 76). In the United States, it is estimated that 50% of the antibiotics annually produced are administered to animals. Of this, 2.4 million pounds are given for nontherapeutic purposes; in contrast, only 3 million pounds are given to humans every year (24). The use of antibiotics as animal growth promoters provides selective pressure for the development of resistant microorganisms.

This becomes particularly important for those antibiotics that are identical, or related, to agents used in the treatment of human infections. Virginamycin, a mixture of type A and type B streptogramin antibiotics, has been used extensively in animal production for the last 25 years for the control of clostridial diseases and as a growth promoter (28). An unusually high prevalence of Synercid resistance in *E. faecium* isolated from chickens has been linked to the use of this antibiotic in animal husbandry (28). Similarly, the agricultural use of avoparcin, a glycopeptide antibiotic belonging to the same family as vancomycin, has been associated with increased levels of VRE in poultry in Europe (73).

The use of a combination of avoparcin and virginamycin in European farms has also been implicated in the emergence of the deadly "superbug" displaying resistance to vancomycin and Synercid (73).

In light of the previous studies and the research described here, it becomes obvious that environmental organisms can serve as potentially large reservoirs of antibiotic resistance. Even more alarming is the finding of resistance to an antibiotic that has yet to be available clinically. Although the resistance determinants are currently only described for soil organisms, which, to our current knowledge, are not human pathogens, it is not difficult to conceive that they may eventually reach organisms that pose a human threat. The transfer of genetic material from soil organisms to those residing in or infecting livestock is an event that can occur with relative ease (25). In addition, there is ample opportunity for such an event to take place in an agricultural setting.

The identification of daptomycin inactivating enzymes in soil isolates and the TetX orthologues in both *S. coelicolor* and *S. avermitilis* speaks to the potential reservoirs of resistance determinants present in soil organisms. If nothing else, these findings suggest that these organisms can be a wealth of knowledge regarding antibiotic resistance and that their study is sure to further our current understanding, not only of resistance mechanisms, but also shed light on the origin and dissemination of antibiotic resistance determinants.

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