COMPETITION-MEDIATED IDENTIFICATION OF THE FIRST

ENVIRONMENTAL PROTEIN RESPONSIBLE FOR THE DEGRADATION OF THE

LIPOPEPTIDE SURFACTIN

A Thesis

by

KARL VICTOR GORZELNIK

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

MASTER OF SCIENCE

Chair of Committee,	Paul Straight
Committee Members,	Ryland Young
	Jennifer Herman
	Tadhg Begley
Head of Department,	Gregory Reinhart

May 2014

Major Subject: Biochemistry

ABSTRACT

Streptomycetes, as nonmotile microbes, are forced to adapt to environmental conditions they cannot escape. In order to adapt to their environment streptomycetes produce an array of both secondary metabolites to antagonize competitors and degradative enzymes to take advantage of various nutritional sources and to degrade xenobiotics, molecules from foreign organisms. This work shows two instances of streptomycetes adapting to their neighbors. *Streptomyces* sp. Mg1 was determined to be resistant to surfactin, a molecule produced by *Bacillus subtilis* which inhibits aerial hyphae development. After identifying possible enzymatic sources of degradation several candidate enzymes were cloned and expressed in *Escherichia coli*. One candidate, 'secreted hydrolase' was purified under denaturing conditions and refolded. This enzyme was shown to degrade surfactin and another *B. subtilis* metabolite, plipastatin. Upon alteration of assay conditions the enzyme was also able to degrade daptomycin. The other instance of a streptomycete adapting to its neighbors is of *S. coelicolor,* which in the presence of some strains of *B. subtilis* is able to produce undecylprodigiosin earlier than it normally would. The induction of undecylprodigiosin indicates that either *B. subtilis* is altering a neighbor's physiology through a secreted compound or that *S. coelicolor* is able to detect a xenobiotic and responds by producing an antibiotic compound. The inducing compound from *B. subtilis* was fractionated and conditions for its further purification were determined.

Dedicated to my grandmother,

Anna Lukaszewicz

ACKNOWLEDGEMENTS

I appreciate my family's support in my graduate endeavor. I would like to thank my brother for choosing to be assigned to Texas to be closer to me. I am especially grateful that he chose Fort Hood instead of another base in Texas, Fort Bliss, which is eight hours further away, in El Paso…

Most importantly, I am very grateful to my loving girlfriend Keya Mukherjee for her support through tough times. ধন্যবাদ সোনা।

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CHAPTER I

INTRODUCTION

With antibiotic-resistant infections causing health care costs of $20 billion in the United States alone there is an urgent need for new antibacterial agents (Bush, *et al.*, 2011). More than half of all bacterial infections are due to pathogens with one or more drug resistances (Demain, 2009). Soil bacteria from the actinomycete family are the dominant producers of commercially available antibiotics producing over 90% of practically used antibiotics (Hamaki, *et al.*, 2005, Tiwari & Gupta, 2012), with the remainder mostly coming from fungi (Baltz, 2006). The *Streptomyces* genus within actinobacteria is responsible for most antibiotics produced from actinomycetes (Baltz, 2006). Streptomycetes have been extensively mined for antibiotics; they yielded 75 new antibiotics per year from 1960-1980, which diminished to 20 per year between 1980 and 2000 as the discovery rate of 'new' compounds declined (Baltz, 2006). Genome mining of streptomycetes has revealed a vast potential untapped by traditional fermentation, despite many different strategies increase production (Corre, *et al.*, 2008). We have tried a relatively new approach to understand antibiotic production wherein we co-culture streptomycetes with another organism. This will allow us to better understand the roles of antibiotics in nature and possible triggers.

The decreased rate of antibiotic discovery has many causes, reaching all the 'low hanging fruit' of easily attainable antibiotics (Tiwari & Gupta, 2012), the rediscovery of

known compounds which causes the need to screen more organisms (Baltz, 2006), as well as waning research budgets as pharmaceutical companies have shifted their focus towards more marketable drugs (Vicente, *et al.*, 2006). The advent of large-scale combinatorial chemistry and high-throughput screening libraries has further driven drug companies away from traditional natural product discovery (Baltz, 2008). However, more than half of the 'new chemical entities,' compounds with novel motifs, discovered since 1981 came from natural products (Newman & Cragg, 2007). The advantages of natural products as antibiotics, compared to synthesized molecules, range from their scaffold diversity to a high tolerance of functional groups to a much higher occurrence of novel motifs (Ganesan, 2008). Natural products also have an advantage over synthesized compounds in that they have evolved over ~1 billion years for bioactivity (Baltz, 2008). Of the 1184 new chemical entities described by Newman and Cragg from 1981-2006, 52% had a natural product connection.

Within actinomycetes, the genus *Streptomyces* stands as a source of novel molecules, with two thirds of all commercial antibiotics derived from *Streptomyces* secondary metabolites (Bibb & Hesketh, 2009). Genome mining of streptomycetes has shown that the secondary metabolic genetic potential of *Streptomyces* is largely untapped in the laboratory setting (Corre, *et al.*, 2008). Sequenced streptomycetes have at least 20 and sometimes up to 30 gene clusters predicted to encode for secondary metabolites (Baltz, 2011), the vast majority of which are not detected due to insensitive screening techniques (Baltz, 2008, Davies, 2010). Standard fermentation broth screening apparently lacks the chemical or physical signals needed to express most

metabolite gene clusters (Zazopoulos, *et al.*, 2003). This latent genetic potential, for "cryptic metabolites," occupies a large amount of streptomycete genomes, in the larger genomes 5-10% (Baltz, 2008). Secondary metabolites are often highly conserved within strains of an organism, giving credence to the idea that these metabolites confer an adaptive advantage (Borodina, *et al.*, 2005). In *S. coelicolor* at least 23 secondary metabolite gene clusters occupy 5% of the 8.7MB genome (Bentley, *et al.*, 2002). Only 6 metabolites have been isolated, four antibiotics: methylenomycin A, calcium-dependent antibiotic, undecylprodigiosin and actinorhodin; as well as two other molecules: coelichelin, a new siderophore (Challis, 2008, Xu, *et al.*, 2010), and a group of molecules, germicidins, which inhibit spore germination (Aoki, *et al.*, 2011). Varying conditions to mimic environment cues have been used in traditional fermentation screenings to "awaken" the cryptic gene clusters responsible for secondary metabolism.

Traditional approaches include altering nutrient sources (Martin, *et al.*, 2011), over-expressing activators (Baltz, 2010), deleting putative negative regulators (Gottelt, *et al.*, 2010), an extensive amount of single gene knockouts (Castro-Melchor, *et al.*, 2010), heterologous expression of cryptic gene clusters (Widdick, *et al.*, 2003), transposon mutagenesis to either knockout or up regulate various genes (Bibb & Hesketh, 2009), making multiple copies of a particular gene cluster within a genome (Murakami, *et al.*, 2011), knocking out other secondary metabolite gene clusters which compete for precursors (Komatsu, *et al.*, 2010, Gomez-Escribano & Bibb, 2011), or mutagenizing the genome to get higher yields (Inaoka, *et al.*, 2004, Wang, *et al.*, 2008, Inaoka & Ochi, 2011). Secondary metabolites come from the redirection of

intermediaries in primary metabolism into alternative pathways (Murakami, *et al.*, 2011). Thus another approach has been to chemically stimulate various streptomycetes so their primary metabolism is perturbed in such a way that some secondary metabolism is altered or increased (Craney*, et al.*, 2012).

An alternative way of studying secondary metabolism has been to induce a metabolic shift by perturbing one organism with another. This co-culture method has been used to put stress upon an organism, which could occur in the environment but would not otherwise occur in a laboratory. There are tens of studies showing induction of secondary metabolism in fungi and bacteria due to co-culture stress (Patterson & Bolis, 1997, Burgess*, et al.*, 1999, Ueda*, et al.*, 2000, Cueto*, et al.*, 2001, Onaka*, et al.*, 2001, Santamaria*, et al.*, 2002, Trischman*, et al.*, 2004, Yamanaka*, et al.*, 2005, Angell*, et al.*, 2006, Miao*, et al.*, 2006, Oh*, et al.*, 2007, Siemieniewicz & Schrempf, 2007, Schroeckh*, et al.*, 2009, Amano*, et al.*, 2010, Li*, et al.*, 2010, Liu*, et al.*, 2010, Amano*, et al.*, 2011, Li*, et al.*, 2011, Luti & Mavituna, 2011, Luti & Mavituna, 2011, Onaka*, et al.*, 2011, Perez*, et al.*, 2011, Zuck*, et al.*, 2011, Traxler*, et al.*, 2012). The most abundant studies pair marine microbes together to see induction of a metabolite absent in monoculture or either strain. Among the studies that focus on streptomycetes there is a similar pattern, for instance Ueda et al. showed, that from 45 fresh soil isolates of streptomycetes, 98% either increased antibiotic production or triggered early sporulation in other streptomycetes (Ueda*, et al.*, 2000). These studies show that bacteria can and do interact with their neighbors by triggering or responding to chemical stressors, however, there is little mechanistic evidence for the basis of induction of secondary metabolites or

their functions. To study the induction and function of secondary metabolites we decided to investigate their roles through bacterial interactions. By co-culturing in an artificial setting we can observe perturbations in a defined system, allowing us to suggest and test hypotheses.

This work shows two instances of streptomycetes exhibiting features of adaptations to their surroundings which may have arisen from bacterial competition. *Streptomyces* sp. Mg1 was determined to be resistant to surfactin, a molecule produced by *Bacillus subtilis* which inhibits aerial hyphae development. The other story is of a streptomycete adapting to its neighbors is of *S. coelicolor,* which in the presence of some strains of *B. subtilis* is able to produce undecylprodigiosin, a molecule with antibiotic properties, earlier than it normally would.

CHAPTER II

ENZYMATIC RESISTANCE TO THE SECRETED LIPOPEPTIDE SURFACTIN[1]

2.1 Introduction

2.1.1 Observation of surfactin hydrolysis

From a separate matrix associated laser desorption ionization imaging mass

spectrometry (MALDI-IMS) project studying the effects of a molecule produced by

Streptomyces sp. Mg1 (*S.* Mg1 or Mg1) against *Bacillus subtilis*, there was an unrelated

observation of a distinct pattern around *S.* Mg1 (Figure 2.1). The top panel in Figure

2.1A is a representative mass spectrum proximal to *B. subtilis,* with the 1058 *m/z* peak

represented in cyan in 2.1B, the *m/z* is equivalent to an isoform of surfactin plus a

sodium ion. The bottom panel in Figure 2.1A is a representative mass spectrum from the

area proximal to *S.* Mg1, with the 1076 *m/z* peak represented in fuchsia in 2.1B. The

+18 *m/z* mass was an unidentified molecule, but was a possible indication of surfactin

hydrolysis. When looking at the dataset, it was observed that the signal for surfactin was

more intense by *B. subtilis*, but the signal decreased in proximity to *S.* Mg1 while the

+18 *m/z* signal gained intensity near *S.* Mg1. When looking at the IMS data, signals for

surfactin away from *S.* Mg1 were still intact, even if they were an equal distance from *B. subtilis.* Surfactin has multiple isoforms and for each isoform there was a +18 *m/z* signal near *S.* Mg1, but not away from *S.* Mg1, leading us to the conclusion that Mg1 was probably degrading surfactin through hydrolysis. The goal of this research was to identify a mechanism of degradation of surfactin by *S.* Mg1.
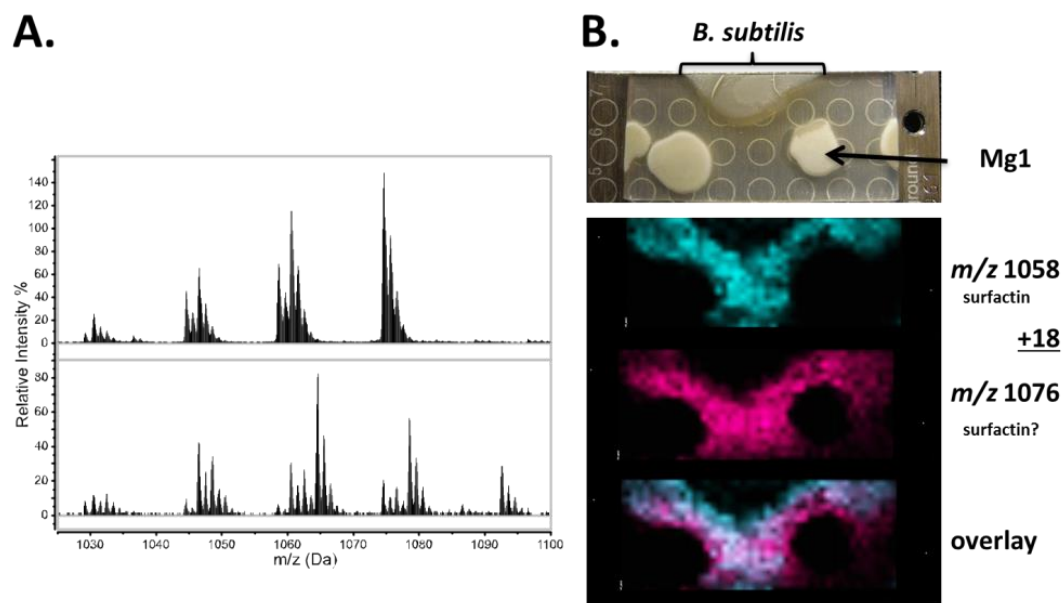
Figure 2.1. Surfactin hydrolysis is proximal to *S.* Mg1. A) Representative mass spectra from the MALDI-IMS experiment, while B) top panel is an image of the co-culture experiment and the bottom panels are false color visualizations of the 1058 *m/z* peak (cyan) and the 1076 *m/z* peak (fuchsia). Work done by Chris Hoefler while visiting the Dorrestein lab, University of California, San Diego.

2.1.2 Background on surfactin

Surfactin is a produced by a number of *Bacillus* species other than *B. subtilis,* including, but not limited to: *B. amyloliquefaciens* (Arguelles-Arias*, et al.*, 2009)*, B.*

thuringiensis (Abderrahmani, *et al.*, 2011), *B. licheniformis* (Thaniyavarn, *et al.*, 2003),

B. mojavensis (Snook, *et al.*, 2009), *B. natto* (Kameda, *et al.*, 1974), and *B. pumilus*

(Morikawa, *et al.*, 1992). Surfactin was discovered in 1968, named surfactin for its

'strong surface active nature' (as a surfactant) and initially characterized as an inhibitor

of fibrin clot formation (hemolytic in nature) (Arima, *et al.*, 1968, Bernheim.Aw &

Avigad, 1970). The structure of surfactin was described the following year, (Figure 2.2)

which classified it as a cyclic lipodepsipeptide (Kakinuma, *et al.*, 1969).
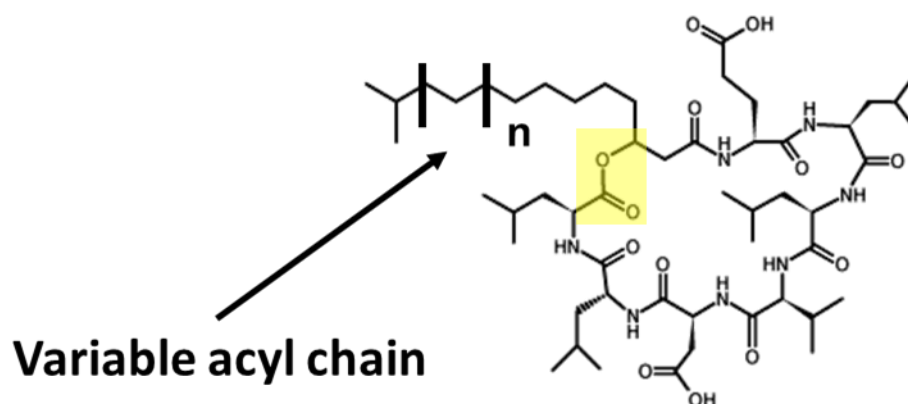
Figure 2.2. Structure of surfactin. Note the variable length acyl chain, there are typically 9-11 'n' -CH$_2$ groups in the acyl chain. Yellow box highlights the ester which is a possible site of hydrolysis.

2.1.3 Chemical properties of surfactin

Surfactin is an amphiphilic molecule with a non-polar acyl tail varying in length

by the addition of -CH$_2$ groups in its tail and it has a peptide head group containing

seven amino acids cyclized by a lactone (Kakinuma, *et al.*, 1969). The cyclized peptide

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moiety of surfactin is composed of D- and L-amino acids, allowing it to fold into a more compact, 'horse saddle' structure which contributes to the stability of the molecule and its 'powerful biosurfactant' properties (Magetdana & Ptak, 1992, Thimon, *et al.*, 1992). The critical micelle concentration has been found to be as low as 7.5µM in Tris·HCl (Ishigami, *et al.*, 1995). Surfactin's micelle forming properties enable surfactin to insert itself into membranes, and in some cases, has been proposed to form pores (Sheppard, *et al.*, 1991, Lopez, *et al.*, 2009).

2.1.4 Applications of surfactin

Surfactin has been described as having anti-foaming activities due to its amphiphilic properties (Razafindralambo, *et al.*, 1998), anti-cancer properties (Kameda, *et al.*, 1974, Seydlova & Svobodova, 2008), anti-mycoplasmic properties (Béven & Wróblewski, 1997), as well as antiviral properties (Kracht, *et al.*, 1999). While these properties allow us to classify it as a useful molecule, *B. subtilis* produces it for a variety of reasons: as an antibiotic (Ongena & Jacques, 2008), as a signal for biofilm formation by causing potassium leakage after puncturing membranes (Lopez, *et al.*, 2009), *B. subtilis* also uses surfactin to lower surface tension allowing it to swarm outward (Kearns & Losick, 2003, Angelini, *et al.*, 2009). The lowering of surface tension has led to surfactin being proposed to be an environmentally friendly way enhance oil recovery compared to artificial compounds (McInerney, *et al.*, 1990), yet this remains elusive due to the high price of commercially available surfactin (Peypoux, *et al.*, 1999).

2.1.5 Surfactin is an antagonistic agent produced by *B. subtilis* against competitors

Importantly, for this work, surfactin acts as an antagonistic agent against other soil organisms (Straight, *et al.*, 2006). When co-culturing *B. subtilis* against *S. coelicolor* there was an inhibition of aerial hyphae development within *S. coelicolor*. A transposon screen of *B. subtilis* revealed one mutant which no longer inhibited aerial growth in *S. coelicolor*, an insertion into the *srfAB* genes (Straight, *et al.*, 2006), the first two genes of the operon responsible for surfactin production (Peypoux, *et al.*, 1999). Upon a deletion of the first gene in the operon, *srfAA*, the phenotype was the same, no inhibition of aerial hyphae development, where there previously was inhibition with wild-type *B. subtilis* (Straight, *et al.*, 2006). Application of purified surfactin exogenously to *S. coelicolor* inhibited aerial hyphae development, further confirming that surfactin was responsible for the phenotype exhibited within *S. coelicolor* (Straight, *et al.*, 2006). Upon further testing, amongst other streptomycetes, it was found that other streptomycetes were also sensitive to the inhibition of aerial development by surfactin (Straight, *et al.*, 2006).

2.1.6 Resistance mechanisms to surfactin have not been identified

Surfactin, being produced by a wide amount of organisms, presumably is degraded by neighboring organisms. There is one report of surfactin, in a mixture of biosurfactants, possibly being degraded by other bacteria (Lima, *et al.*, 2011), however the degradation was implied due to an increase in surface tension, not analytically measuring the compound. Turnover of secondary metabolites in the environment is

common, often beneficial in a clinical mindset, whereby there is limited selective

pressure to develop resistance to the molecule (D'Costa, *et al.*, 2012), as is the case with

clinically relevant antibiotics (Demain & Sanchez, 2009). Surfactin hydrolysis has been

observed before, albeit in an artificial setting, where surfactin was degraded by a

commercially available protease, V8 endoprotease from *Staphylococcus aureus*

(Grangemard, *et al.*, 1999). However, hydrolysis was limited to <15% over an extended

time period (80μM enzyme for 100 hours), which the authors attributed to, 'the

aggregation state of the lipopeptide in solution' or in other words: micelle formation

(Grangemard, *et al.*, 1999).

2.1.7 Surfactin prevents aerial hyphae formation in streptomycetes

Streptomycetes are often isolated with *Bacillus* spp., as they share a common

niche in the soil. Since all streptomycetes previously tested were no longer able to

develop aerial hyphae, complete their lifecycle by forming spores, it was hypothesized

that surfactin acts as a competitive agent by *B. subtilis* (Straight, *et al.*, 2006). With

evidence of hydrolysis, as seen by the +18 *m/z* mass shift proximal to *S.* Mg1 (Figure

2.1), we hypothesized there was an enzyme produced by *S.* Mg1 capable of degrading

surfactin, and thus acting as a resistance mechanism by *S.* Mg1. To see how widespread

this phenomenon was our lab tested purified surfactin against a new set of

streptomycetes and determined that hydrolysis of surfactin by *S.* Mg1 was a unique

phenomenon (Figure 2.3).

DMSO control applied:

Surfactin applied:

S. aizunensis   *S. avermitilis*   *S. pristinaespiralis*   *S. griseoflavus*   *S. Mg1*
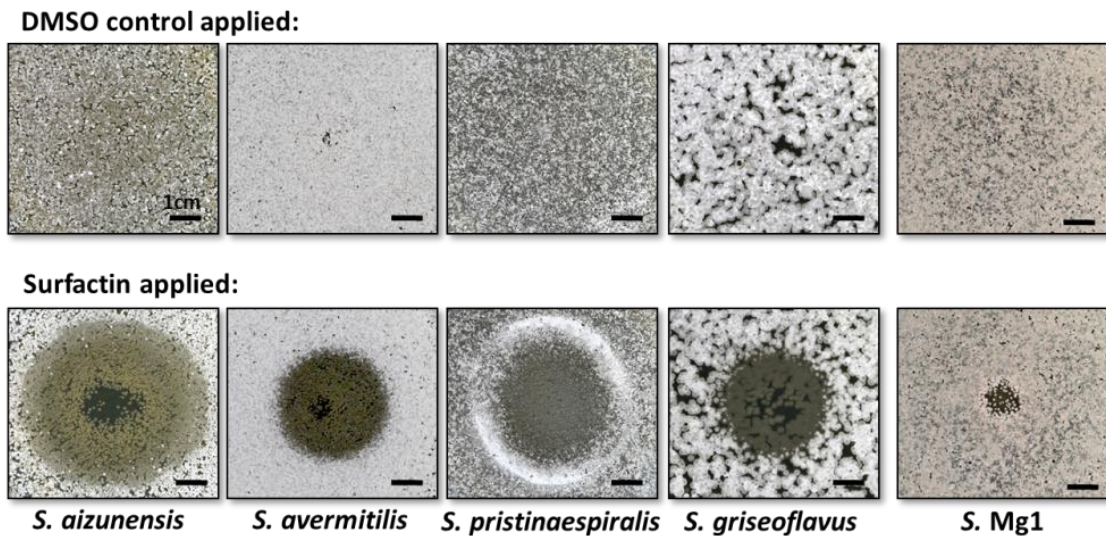
Figure 2.3. Purified surfactin inhibits spore formation in a number of streptomycetes. The top panels have 5μL of DMSO applied to them, while the bottom panels are the same organisms but with 100μg of surfactin dissolved in DMSO applied to them. *Note:* there is killing by surfactin in some of the organisms, but Mg1 is resistant to inhibition of aerial hyphae development seen in the other streptomycetes. Work done by Chris Hoefler.

2.1.8 Initial search for a surfactin degrading enzyme

In order to determine if a secreted enzyme was responsible for surfactin hydrolysis several plates of *S.* Mg1 had the bacterial colonies removed and the agar extracted for secreted proteins. Mass spectrometry was carried out after a trypsin digest of the secreted proteins was subjected to mass spectrometry and a list of possible candidates was determined, prior to the start of this project (Table 2.1 for a list of possible candidates). These secreted proteins formed the initial candidates for a surfactin degrading enzyme.

Table 2.1: Secreted proteins of *Streptomyces* sp. Mg1, identified through a trypsin digest followed by tandem LC/MS/MS. Identified proteins are listed by their putative BLAST annotations, with gi accession numbers of the proteins from *S.* Mg1, and probable molecular weight. Work done by Chris Hoefler and Larry Dangott.

Identified Proteins	Accession Number	Molecular Weight
beta glucosidase	gi|254385125	88 kDa
hypothetical protein	gi|254383925	87 kDa
Trypsinogen	gi|254387105	27 kDa
secreted protein	gi|254385995	76 kDa
extracellular solute-binding protein	gi|254386889	45 kDa
peptidase S8 and S53 subtilisin kexin sedolisin	gi|254381890	51 kDa
serine protease	gi|254382793	115 kDa
conserved hypothetical protein	gi|254386403	53 kDa
leupeptin-inactivating enzyme	gi|254382420	45 kDa
twin-arginine translocation pathway signal	gi|254383653	54 kDa
streptogrisin-B	gi|254387168	30 kDa
sugar hydrolase	gi|254380558	42 kDa
conserved hypothetical protein	gi|297204780	54 kDa
phospholipase D	gi|21912970	58 kDa
conserved hypothetical protein	gi|254385470	29 kDa
ABC transporter solute-binding protein	gi|254386063	38 kDa
ferrichrome ABC transporter substrate-binding protein	gi|254384716	37 kDa
phospholipase D	gi|254384004	36 kDa
secreted tripeptidylaminopeptidase	gi|254384290	58 kDa
secreted hydrolase	**gi|254386602**	**54 kDa**
hypothetical protein	gi|254380900	28 kDa
large secreted protein	gi|254384157	64 kDa
tellurium resistance protein	gi|254382842	20 kDa
branched chain amino acid binding protein	gi|254384241	42 kDa
aconitate hydratase	gi|254386056	97 kDa
glycine betaine ABC transport system permease	gi|254387391	64 kDa
glutamate binding periplasmic protein	gi|254386082	30 kDa
gamma-glutamyltranspeptidase	gi|254382220	62 kDa
Phytase	gi|254381873	59 kDa
peptidyl-prolyl cis-trans isomerase	gi|254380548	27 kDa

2.2 Materials and Methods

2.2.1 Bacterial strains

The strains used in this chapter are listed in Table 2.2. For long term storage *Escherichia coli* strains were grown overnight, spun down, and resuspended in sterile 20% (v/v) glycerol, and stored at -80°C. For streptomycetes the spore suspensions were prepared in sterile mqH$_2$O then stored at -80°C. Spore suspensions of *Streptomyces* sp. Mg1 were prepared using previously established protocols (Kieser, *et al.*, 2000). Briefly, a -80°C stock of the streptomycete was struck out in a square-like pattern onto a GYM plate (see recipe below), once spore formation occurred (~3-4 days) 1mL of sterile mqH$_2$O was applied to the plate and spores were scraped with a cell scraper, removed and 1mL more of sterile mqH$_2$O was applied and taken off with a pipette. The spores were serially diluted in sterile mqH$_2$O and plated on GYM plates to get a working titer for experiments.

Table 2.2. Bacterial strains used in this chapter.

Strain	Relevant Genotype
PDS0066	*Bacillus subtilis* NCIB3610 wild type
PDS0060	*Bacillus subtilis* Δ*srfAA*
PSK0558	*Streptomyces* sp. Mg1 wild type
PDS0366	*Streptomyces* sp. Mg1 Δ*sfhA*
PDS0424	*Streptomyces* sp. Mg1 Δ*sfhA* sfhA ApR
PSK0028	*Streptomyces coelicolor* M145
PSK0495	*Streptomyces canus*
	XL10-Gold (Cloning cell line from Stratagene)
	BL21(DE3)pLysS (*Escherichia coli* for protein expression)
	Rosetta™ 2(DE3)pLysS Novagen (*E. coli* for protein expression)

2.2.2 Media

Bacto media was used, unless otherwise noted. Luria-Bertoni (LB) broth (1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v NaCl) was used for *Escherichia coli* and *Bacillus subtilis* cultures, with LB plates having 1.5% agar added. LB was supplemented with 100μg/mL ampicillin, 25μg/mL kanamycin or 30μg/mL chloramphenicol as appropriate for *E. coli*. All antibiotics used were purchased from Sigma. *Streptomyces* spp. cultures were grown on glucose-yeast-malt extract (GYM) (1% w/v malt extract, 0.4% w/v yeast extract, 0.4% w/v dextrose) with GYM plates having 1.5% agar added. *Streptomyces coelicolor* was cultivated on malt extract-yeast extract-mannitol (MYM) plates (0.4% w/v malt extract, 0.4% w/v yeast extract, 0.4% D-(+)-maltose monohydrate (MP Biomedicals) with 1.5% agar.

2.2.3 Cloning possible hydrolases

Cloning was done using a ligation-free cloning technique to place possible hydrolase genes into a pET28a vector for overexpression in *Escherichia coli*. The possible hydrolase ORFs were amplified from *Streptomyces* Mg1 genomic DNA by using primers listed in Table 2, using standard techniques [1x Phusion GC buffer, 10% DMSO, 0.2mM dNTPs (New England Biolabs), 0.2μM of each primer, 200-400ng genomic DNA, 1 unit of Phusion polymerase (New England Biolabs), all in a 50μL reaction], for the following 5-step program: (1) 98°C for 30 seconds, (2) 98°C for 10 seconds, (3) variable annealing temperature for 30°C, (4) 72°C for 20 seconds, steps 2-4 were repeated for 30 cycles before step (5) 72°C for 2 minutes. The amplified product

was cleaned up using a QIAquick PCR purification kit as per the manufacturer's instructions, and amplified for a second time with primers to extend the overhang regions 40bp further, matching the region around the EcoRI cut site in the pET28a expression vector. The elongated product was gel purified using a QIAquick gel extraction kit as per the manufacturer's instructions, and used as a 'mega primer' for amplification of an EcoRI cut pET28a. pET28a was purified using a QIAprep spin miniprep kit, as per the manufacturer's recommendations, from an overnight culture of *E. coli* XL10-Gold cells with the empty vector. pET28a was cut with 60 units of EcoRI (3µL of 20,000 units/mL) and 1µg of pET28a, in 1x NEB Buffer EcoRI, in a 50µL reaction for 1 hour, then cleaned up using a QIAquick PCR purification kit. The amplification of the cut pET28a was done with ~200ng of the 'mega primer' PCR product, ~100ng of cut pET28a, 2mM dNTPs, and 1 unit of Phusion polymerase for the following program: (1) 98°C for 30 seconds, (2) 98°C for 10 seconds, (3) 72°C for 30 seconds, (4) 72°C for 2 minutes, steps 2-4 were repeated 24 times before step (5) 72°C for 2 minutes. Five microliters of the reaction were transformed without cleaning up into chemically competent XL-10 Gold cells, using standard chemical transformation procedures. Transformations involved letting the newly amplified plasmid mix with chemically competent cells for five minutes on ice, heat shocking at 42°C for 45 seconds, then placing on ice for 1-2 minutes, adding 1mL of LB into the microcentrifuge tube, and allowing the cells to recover at 37°C, shaking at ~200rpm, for 1 hour, then plating on LB plates with appropriate antibiotics. Clones were verified for the correct insert using their initial primers, restriction digests, and finally sequencing. Upon

verification of the correct insert the plasmids were transformed into *E. coli* expression

strains: either BL21(DE3)pLysS or Rosetta2(DE3)pLysS.

Table 2.3. Primers used in this study. Capital letters indicated the overhang for ligation independent cloning.

Annotation	gi #	Size (bp)	Forward Primer	Reverse Primer
sugar hydrolase	194339469	1188	TGGTGCCTCGTGGTA GCCATgtgtctcgattaatcgaa tgcg	CTCAGCTTCCTTTCGGGCTT TGTTActagttgaacggttccagcg
secreted protein	194344857	2160	TGGTGCCTCGTGGTA GCCATatgcctcgcccctcc	CTCAGCTTCCTTTCGGGCTT TGTTActacgggaacgaggtgacg
secreted hydrolase	194345445	1500	TGGTGCCTCGTGGTA GCCATctgtccgccacccagg	CTCAGCTTCCTTTCGGGCTT TGTTAtcagaaccgcccggg
large secreted protein	194343046	1842	TGGTGCCTCGTGGTA GCCATatgcgctcctcgcatc	CTCAGCTTCCTTTCGGGCTT TGTTAttacgggcggaagcg
secreted aminopeptidase	194343178	1614	TGGTGCCTCGTGGTA GCCATctggcggcgagtctgc	CTCAGCTTCCTTTCGGGCTT TGTTActagcggatggccggg
Extender primers			atgggcagcagccatcatcatcatc atcacagcagcggcctggtgcctc gtggtagccat	tatgctagttattgctcagcggtggcagcagcc aactcagcttcctttcgggctttgtta

2.2.4 Expression of possible hydrolases

A starter culture of the expression strain (either BL21(DE3)pLysS or

Rosetta2(DE3)pLysS) was inoculated from a fresh plate and incubated at ~200rpm

overnight at 37 °C. The starter culture was diluted into a larger culture such that the

$OD_{600} = $ ~0.005 (typically a 1:1000 dilution), and the large culture was incubated at

250rpm until $OD_{600} = 0.4 - 0.6$ was reached. The temperature was then lowered to 16 °C

for 1 h, followed by the addition of IPTG to a final concentration of 200μM, which was

determined to be the least amount needed for soluble expression in

Rosetta2(DE3)pLysS. Incubation was continued at 16 °C for 4 h. The cells were pelleted

by centrifugation (8,000 × g for 30 minutes at 4°C). The cell pellet was resuspended in lysis buffer (10mM imidazole, and 100mM sodium phosphate at pH 8.0), and lysed by sonication (Misonix Sonicator 3000) on ice at power 0.5 for small cultures for 10 seconds on, 15 seconds off for 2 minutes total running time. The lysate was centrifuged (13,000 × g for 30 minutes at 4°C), the pellet was saved to run on a gel, and the supernatant saved for further assays and running on a gel.

2.2.5 SDS-PAGE

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used according to standard methods in 12% acrylamide gels. Samples were denatured by heating at 100°C for 5-10 minutes in 1x Laemmli buffer (62.5mM Tris-HCl at pH 6.8, 1.5% w/v SDS, 100mM DTT, 0.005% bromophenol blue, and 8.3% v/v glycerol). For urea-containing samples (below), Laemmli buffer was added, and samples were loaded directly into the gel without heating. Electrophoresis was carried out between 100-200V, as time necessitated. Protein bands were visualized by staining with colloidal Coomassie (Dyballa & Metzger, 2009), which calls for 20% v/v ethanol, 10% w/v $(NH_4)_2SO_3$, 2% w/v H_3PO_4, and 0.1% Coomassie Brilliant Blue G-250, first having the aluminum sulfate dissolved in mqH$_2$O, ethanol added in, mixed with coomassie blue, phosphoric acid added in, then brought up to final volume in mqH$_2$O.

2.2.6 Preliminary surfactin hydrolysis assay

To identify the possible surfactin hydrolase, supernatants with soluble protein had surfactin added to them at a concentration of 200µM, and were allowed to incubate for three hours at room temperature. Afterward 50µL was removed and mixed with five volumes of acetone to precipitate protein. Precipitated protein was pelleted (13,000 x g, for 5 minutes at room temperature) and the supernatant removed to another tube. 1µL of the supernatant was spotted onto a MALDI plate then mixed with 1µL of a saturated solution of Universal MALDI Matrix (Sigma) which was dissolved in a 1:1 mixture of acetonitrile:water with 0.2% TFA added in. The sample/matrix solution was allowed to dry, and MS spectra were collected on a Shimadzu Axima-CFR MALDI-TOF mass spectrometer. The extent of surfactin hydrolysis was determined (semi-quantitatively) by comparing the ratios of intact surfactin ions ([Mass+Na]$^+$) from a control sample to hydrolyzed surfactin in the samples ([M+Na+H$_2$O]$^+$) at different time points.

2.2.7 Optimization of expression conditions and purification of surfactin hydrolase

After expression tests in BL21(DE3)pLysS yielded almost no detectable soluble product (as determined by SDS-PAGE) the pET28a plasmids with various inserts were transformed into Rosetta2(DE3)pLysS competent cells, as described above. Induction of possible hydrolases was tried for varying lengths of induction (1 hour to overnight), varying temperatures (16°C, 25°C, 30°C, and 37°C), and varying concentrations of inducer (0.1mM IPTG – 2mM IPTG). Induction conditions were optimized to levels of highest expression upon lysing the cells: once OD$_{600}$ = ~0.5, lowering the temperature

from 37°C to 16°C, letting the cells adjust for ~1 hour, inducing with 0.2mM IPTG, then

letting the cells grow for ~4 hours before harvesting. However, much of the protein was

insoluble. In order to bypass the solubility problem of the candidate hydrolase, insoluble

protein was unfolded, purified under denaturing conditions, and refolded. After

expression of the protein the cell pellet was resuspended under denaturing conditions

(denaturing lysis buffer: 8M urea, 10mM imidazole, and 100mM sodium phosphate at

pH 8.0, made with fresh urea each time), lysed by sonication on ice (for large

preparations: power 4 for 10 seconds on, 15 seconds off for 10 minutes total running

time), spun down as described above, the pellet discarded, the supernatant run over a

hand-poured Ni^+-NTA Superflow (Qiagen) column (10 mL of bed volume, washed with

2 CV water and equilibrated with 3 CV lysis buffer). The column was washed (8 M

urea, 10 mM imidazole, and 100 mM sodium phosphate at pH 6.3; 3 CV) and then

eluted (8 M urea, 10 mM imidazole, and 100 mM sodium phosphate at pH 5.9; 3 CV,

followed by 3 CV of the same buffer at pH 4.5). Fractions (2 mL) were collected and

screened for total protein by using Bradford's reagent (Sigma). Protein containing

fractions were further screened by SDS-PAGE, and secreted hydrolase (54-kDa band)

containing fractions were pooled. The overexpressed protein was gradually refolded by

dialysis of the urea in steps. A starting dialysis buffer (7 M urea, 500 mM NaCl, 10 mM

imidazole, and 50 mM sodium phosphate at pH 7.5) was prepared (1.5 L), and dialysis

was carried out for 3-4 hours, then the dialysis bag was placed in a new container with

6M urea (all the other components the same), the process repeated with 1M urea steps

down, until all the urea was removed. At the 1M urea step 1mM DTT was added into

the dialysis buffer. As some protein precipitated the final product was spun down, as described previously. The supernatant was applied to a second Ni^+-NTA column for further purification, after the column was equilibrated with binding buffer (50mM sodium phosphate at pH 7.5, 500mM NaCl, and 10mM imidazole; 3 CV). The column was washed (50mM sodium phosphate at pH 7.5, 500mM NaCl, and 80mM imidazole; 3 CV) and then eluted (50mM sodium phosphate at pH 7.5, 500mM NaCl, and 120mM imidazole for 3 CV, followed by 3 CV of the same buffer with 150mM imidazole). Fractions (2 mL) were collected and screened as before. Secreted hydrolase containing fractions were pooled and dialyzed into the final storage buffer (50mM Tris·HCl at pH 8.0, 150mM NaCl, 1mM DTT, and 10% glycerol). The purified protein was stored as an 80µg/mL stock at 4 °C for use in subsequent experiments.

2.2.8 Extraction of metabolites from B. subtilis and various streptomycetes for enzymatic assays

Extraction of surfactin was done from wild-type *B. subtilis* NCIB3610 (PDS0066) as follows: a starter culture was inoculated from a 1 day old plate of *B. subtilis*, after overnight growth the starter culture was used to inoculate a larger culture in LB to $OD_{600} = 0.01$, the large culture was grown for ~20 hours, cells pelleted by centrifugation at 8,000 x *g*, for 30 minutes at 4°C. The pellet was discarded and the supernatant was extracted with 1 volume (equivalent to supernatant volume) of 1:2:3 methanol:methylene chloride:ethyl acetate, with the organic layer saved and the aqueous layer further extracted with 1 volume of butanol. The organic fractions were

concentrated under reduced pressure, resuspended in methanol, and adsorbed onto

Diaion HP-20 resin (Sigma) under reduced pressure. The resin was transferred to a

Büchner funnel, washed with water, then 20% methanol/water, v/v. Surfactin was eluted

with 100% methanol followed by 100% water, then both eluates were dried under

pressure and combined. Extraction of plipastatin was done from a *B. subtilis* Δ*srfAA*

background (PDS0060) to eliminate surfactin background production, and thus

competition for precursor molecules. Extraction was performed in the same way as for

surfactin. Extraction of CDA, calcium-dependent antibiotic, from *S. coelicolor* M145

was done on MYM plates supplemented with trace elements (0.08mg/L $ZnCl_2$, 0.4mg/L

$FeCl_3 \cdot 6H_2O$, 0.02 mg/L $CuCl_2 \cdot 2H_2O$, 0.02mg/L $MnCl_2 \cdot 4H_2O$, 0.02 $Na_2B_4O_7 \cdot 10H_2O$,

0.02 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 2μM thiamine·HCl,

50μg/mL phenylalanine, and 50μg/mL tryptophan) after autoclaving and cooling down

to 50°C. After adding spores the plates were allowed to incubate until aerial hyphae

were observed. The agar was cut into ~1cm pieces, and extracted as surfactin, except

that the organic layer was poured off of the agar pieces instead of using a separatory

funnel. Amphomycin was extracted from 3-day old cultures of *S. canus* (PSK0495)

following a similar procedure surfactin, except the culture was grown in MYM broth,

inoculated with 10^6 spores/mL.

2.2.9 Enzymatic assays with purified surfactin hydrolase

Surfactin was titrated from 10μM to 1mM into a 100μL final reaction volume

(50mM Tris·HCl, 150mM NaCl, 1mM freshly added DTT, all at pH 8.0) with ~60nM of

the enzyme (320ng surfactin hydrolase). The assays were allowed to run for 1 hour at room temperature (20-23°C), before being quenched with five volumes of acetone (500µL). The optimal concentration to get cleavage of surfactin at a high enough intensity compared to the background using MALDI-TOF was found to be 50µM surfactin. 1µL of the quenched reaction was spotted onto a MALDI plate then mixed with 1µL of a saturated matrix solution as described above. The sample/matrix solution was allowed to dry then MS spectra were collected on a Shimadzu Axima-CFR MALDI-TOF mass spectrometer. The extent of surfactin hydrolysis was determined (semi-quantitatively) by comparing the ratios of intact surfactin ions ($[Mass+Na]^+$) from a control sample to hydrolyzed surfactin in the samples ($[M+Na+H_2O]^+$) at different time points. Enzymatic assays with purified compounds (daptomycin, nystatin, iturin a, amphotericin b, erythromycin, and A5145D) were done at 50µM compound, with the other conditions kept the same. Enzymatic assays with crude extracts (surfactin, plipastatin, amphomycin, and CDA) were done in a similar fashion, except the substrates were assayed at several concentrations to get the best signal to noise ratio. After testing CDA, all of the substrates were retested with varying concentrations of Ca^{2+}, from 0mM – 10mM, all other conditions remaining the same. KCl and phosphate buffer (K_2HPO_4/KH_2PO_4 at 50mM, pH 8.0) were also tried but were not found to affect the hydrolysis.

2.2.10 Preparation of hydrolyzed surfactin for NMR

Enzymatically hydrolyzed surfactin and a surfactin control were prepared for NMR in the following way with everything the same between the two samples, except for the surfactin control the enzyme was heat denatured by boiling at 100°C for 20 minutes prior to preparation. The samples were prepared by incubating 3mg of surfactin (~500µM surfactin) with 30nM of surfactin hydrolase (10µg enzyme) in 6mL assay buffer (50mM Tris·HCl, 150mM NaCl, 1mM freshly added DTT, all at pH 8.0) for an overnight incubation with shaking. Surfactin and hydrolyzed surfactin from each sample were isolated by adsorbing the mixture to a pre-wetted Merck LiChrolut RP-18e SPE cartridge (pre-wetted by running 3mL of methanol then 3mL of 20% methanol/water v/v, then 6mL of water), then washed with 6mL of water, 3mL of 10% methanol/water v/v, 3mL of 20% methanol/water v/v, the surfactin and hydrolyzed surfactin from each sample were eluted with ~10mL of methanol, concentrated to dryness under reduced pressure, then dissolved in 600µL of deuterated DMSO-d6. NMR was done by Xiangming Kong in the Biomolecular NMR Laboratory at Texas A&M University, and analyzed for location of hydrolysis by Chris Hoefler, comparing to previously solved structures of intact surfactin (Tang*, et al.*, 2007).

2.2.11 Small scale preparation of Streptomyces gDNA to confirm genetic manipulations

To confirm the deletion of *srfA* and its chromosomal complementation small scale (5-10mL) preparations were done of many isolates from each step. Picked an

isolated colony, ground the mycelia using a sterilized tissue grinder or toothpick,

inoculated a 5-10mL culture of the required media (MYM, see above). Grew cells

overnight at 30°C in a roller drum at ~200rpm (the longer the growth the thicker the cell

wall, making it harder to lyse the cells and get clean DNA). Spun down cells, 8,000 x g

for 5 minutes at room temperature. Washed cells with 1mL genomic lysis buffer (50mM

EDTA, 0.1M NaCl, pH 7.5), spun down as above and resuspended in 700µL of lysis

buffer, with added in lysozyme and ProteinaseK. Lysozyme was weighed out fresh, to

prevent degradation, and added at 5-50mg/mL depending on the difficulty of lysing cells

(for S. Mg1 10mg/mL was used). ProteinaseK was stored at -20°C in a 20mg/mL stock,

20µL were added to a small scale preparation. Incubated at 37°C for >20 minutes,

inverting periodically. After addition of lysozyme and ProteinaseK the solutions were

never vortexed, to prevent shearing of gDNA. The solution turns clear and somewhat

viscous, incubations of two hours worked fine. Added 20µL of RNaseA (20mg/mL),

incubated at 37°C for 10 minutes, inverting periodically. Added 56µL of sarkosyl (20%

w/vol sodium lauroyl sarcosinate in mqH$_2$O), incubated 10 minutes at 37°C, the lysate

becomes more clear and very viscous. Extracted DNA using an equal volume of

phenol/chloroform/isoamyl alcohol (25:24:1), by adding the solvents to the lysate and

inverting periodically for 1 minute. Transferred mixture to 2mL Eppendorf phase lock

tubes and spun down for 5-10 minutes at max speed of bench top microcentrifuge. The

silica resin in these tubes separates the organic layer (on bottom) from the aqueous layer

(on top, above the silica resin). Transferred the aqueous layer to a new phase lock tube

and extracted again as above. Transferred the aqueous layer from the second extraction

to a microcentrifuge tube and added 5M NaCl to a final concentration of 0.8M

(~110μL). Added pre-heated CTAB to 0.1 volumes (~110μL). CTAB,

cetyltrimethylammonium bromide, solutions were 10% w/v in mqH$_2$O with 0.7M NaCl.

The mixtures were incubated at 65°C for 10 minutes and inverted periodically. The

mixtures were cooled down and extracted with an equal volume of chloroform/isoamyl

alcohol, 24:1, in a regular microcentrifuge tube. Spun down for 5 minutes at max speed,

removed aqueous layer to as not to disturb the interface between the aqueous and

organic layer which contained a white layer of proteins or polysaccharides. Repeated

the CTAB extraction as above, but had to add more CTAB than previously (to maintain

the 0.1 volume ratio). After the chloroform/isoamyl alcohol extraction the aqueous layer

was diluted to ~0.48M NaCl with sterile mqH$_2$O, as the phase lock tubes do not separate

out organic from solvent with salt concentrations over 0.5M. Phenol/ chloroform/

isoamyl alcohol extracted with phase lock tubes as described above. Precipitated DNA

from the aqueous layer with 0.7 volumes of isopropanol, removed DNA either by

inverting tube gently and swirling a pipet tip in the solution and removing, or by

centrifuging and decanting the supernatant. In either case the DNA was dried then

resuspended in 200μL TE buffer (10mM Tris·HCl, 1mM EDTA, pH 8.0). Measured

OD$_{230}$, OD$_{260}$, and OD$_{280}$ and ran an aliquot on a gel to test integrity.

2.2.12 Testing surfactin hydrolysis by S. Mg1 and derivatives in liquid culture

In order to ensure that the surfactin hydrolase gene, newly termed *sfhA*, was the

only gene responsible for surfactin hydrolysis the gene was knocked out and then

complemented with the wild-type gene. To detect evidence of hydrolysis, or the lack

thereof, *S.* Mg1 and its two derivatives, *S.* Mg1 Δ*sfhA* and *S.* Mg1 *ΔsfhA* sfhA ApR, each

had 10^6 spores/mL inoculated into a 5mL liquid culture and were grown for 24 hours

growing at 30°C and in a roller drum at ~200rpm, when 50μM surfactin was added to

each culture. The cultures were allowed to grow for another 24 hours before cells were

pelleted by centrifugation (8,000 x *g*, for 10min at 25°C). Supernatants from each

sample were adsorbed onto a pre-wetted Merck LiChrolut RP-18e SPE cartridge (pre-

wetted by running 3mL of methanol then 3mL of 20% methanol/water v/v, then 6mL of

water), then washed with 6mL of water, 3mL of 10% methanol/water v/v, 3mL of 20%

methanol/water v/v, the surfactin and hydrolyzed surfactin from each sample were eluted

with ~10mL of methanol, concentrated to dryness under reduced pressure, resuspended

in 0.5mL. Samples were analyzed via MALDI-TOF as described above.

2.3 Results

2.3.1 Selection, cloning and expression of candidate enzymes

Secreted proteins isolated from agar surrounding *S.* Mg1 colonies (listed in Table

2.1) were analyzed for the possibility of being the degradative enzyme. In order to

narrow down the probable protein we first eliminated any proteins which were not

enzymes, then focused on enzymes which were annotated as degradative in nature, such

as proteases, peptidases, hydrolases, or lipases. We further narrowed down the list by

looking for proteins with homologues in streptomycetes which were not resistant to

surfactin. Five candidate *S.* Mg1 genes were chosen to be cloned into *Escherichia coli*

to be expressed: sugar hydrolase gi# 194339469, secreted protein gi# 194344857, secreted hydrolase gi# 194345445, large secreted protein gi# 194343046, and secreted aminopeptidase gi# 194343178. These genes were chosen on the basis that their protein products were determined to be secreted, and were either annotated or had homologues that were annotated to be enzymatic and degradative.

After cloning the candidate hydrolase genes into an overexpression vector with an N-terminal 6x-Histidine tag, the plasmids were transformed into BL21(DE3)pLysS cells. After altering a number of variables used in protein expression, such as: alternating lengths of induction, temperature after induction, inducer molecule concentration, and optical density at time of induction, none of the constructs were able to express (data not shown). We theorized this was due to the codon bias in *E. coli*, as streptomycetes have a GC rich genome (van Wezel & McDowall, 2011). To overcome the codon bias, the pET28a plasmids with the various genes inserted into them were transformed into Rosetta2(DE3)pLysS chemically competent cells.

2.3.2 Lysate activity assays of candidate enzymes

Of the four proteins able to express, only two had enough soluble protein to detect via western blot or SDS-PAGE, under a variety of expression conditions tried (data not shown). However, the two constructs, 'secreted protein' gi# 194344857 and the 'secreted hydrolase' gi# 194345445, were expressed on a small scale (100mL for 4 hours), spun down, resuspended in 1mL of lysis buffer (see materials and methods), lysed, spun down, pellets removed, and had surfactin added at a concentration of 1mM

to the supernatant of the lysate. After allowing surfactin to react with the supernatant for one hour at room temperature a 50µL aliquot was removed from each sample, mixed with 250µL acetone, insoluble material pelleted, and analyzed via MALDI-TOF. There was no evident surfactin hydrolysis, however, after allowing the reactions to proceed overnight, and treating the reactions in the same manner as before, there was a faint amount of hydrolysis (Figure 2.4), albeit only in the linear detection mode of detection, not in reflectron mode which has more resolved peaks.
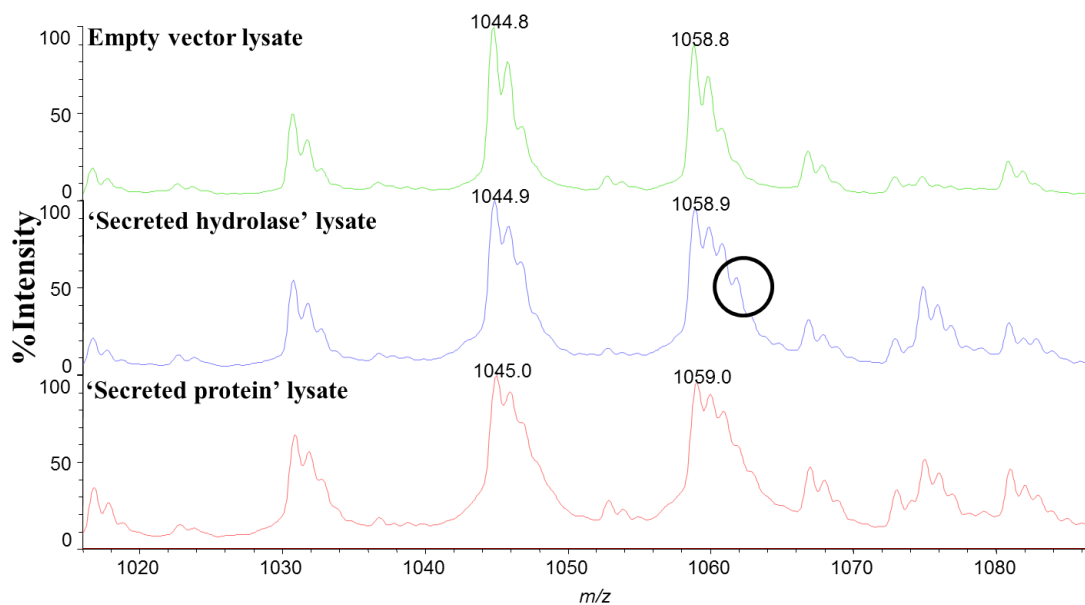
Figure 2.4. Fractionated crude lysates yield a possible hydrolase candidate. Two soluble candidate proteins were lysed, fractionated, and surfactin added to the supernatants. After 22 hours there was faint levels of hydrolysis in the 'secreted hydrolase' lysate (gi# 194345445). Circled peak was not present in either the empty vector lysate or the other expressed gene.

2.3.3 Expression and refolding of 'secreted hydrolase'

The faint level of hydrolysis was enough to pursue the protein annotated as 'secreted hydrolase.' The low levels of hydrolysis were most likely due to only a faint amount of the protein being soluble. Figure 2.5 is a representative gel of further purification of the 'secreted hydrolase', which shows that most of the expressed protein is insoluble (compare supernatant and pellet of expressed hydrolase S vs. P). Low levels of hydrolysis could also be due to increased micelle formation at higher concentrations of surfactin, see below.
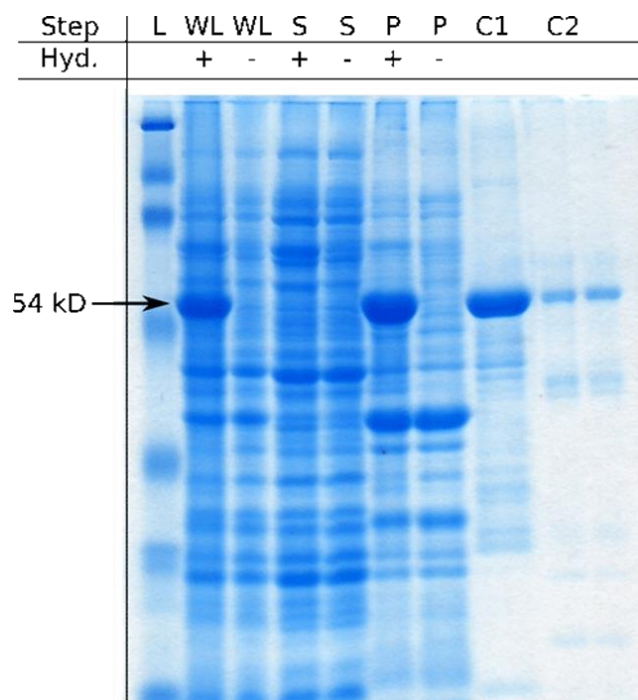
Figure 2.5. Purification of surfactin hydrolase. The 'secreted hydrolase' is a 54kD protein which is overexpressed. Lanes are marked +/- for 'secreted hydrolase' inserted into pET28a or if it was just the empty vector. WL = whole cell lysate, S = supernatant, P = pellet, C1 = Ni^{2+}-NTA column run under denaturing conditions, C2 = the refolded protein from C1 loaded onto a native Ni^{2+}-NTA column, two groups of fractions from the elution step.

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2.3.4 Identification of a surfactin degrading protein from S. Mg1

After obtaining a relatively pure fraction of the 'secreted hydrolase' the enzyme was incubated with 50μM surfactin in a Tris·HCl assay buffer (see materials and methods) for a number of time points, the 10 minute time point is shown to illustrate how fast the surfactin was degraded with 60nM enzyme (Figure 2.6). Assay buffer controls showed no detectable surfactin hydrolysis (data not shown). The 'secreted hydrolase' is hence referred to as 'surfactin hydrolase.'
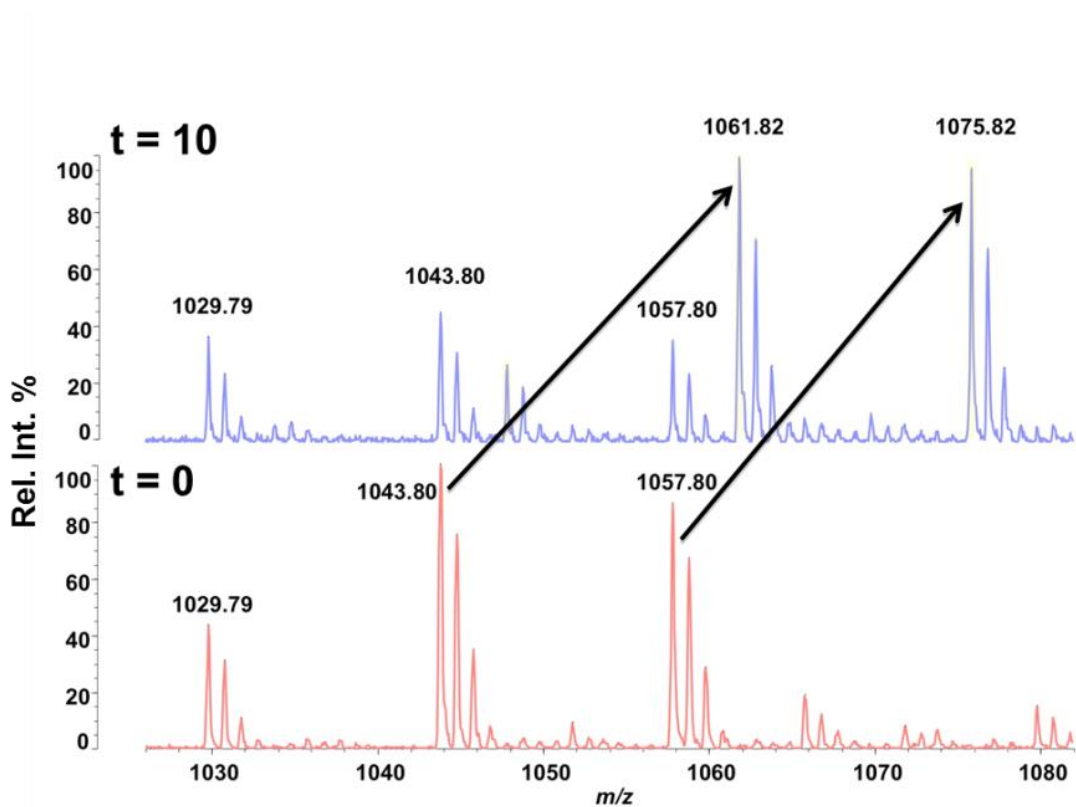
Figure 2.6. MALDI-TOF evidence of surfactin hydrolysis by 'secreted hydrolase.' 50μM surfactin was incubated in a 100μL reaction (50mM Tris·HCl, 150mM NaCl, 1mM freshly added DTT, all at pH 8.0) with ~60nM surfactin hydrolase (320ng) for either 0 or 10 minutes at room temperature (20-23°C), before being quenched with five volumes of acetone (500μL). The arrows indicate where the dominant peaks of each isoform were hydrolyzed (+18 *m/z* peak shifts).

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2.3.5 Preparation of surfactin to determine site of hydrolysis

In an effort to determine where the molecule was degraded by hydrolysis we hydrolyzed 3mg of surfactin and after cleanup over a C18 sep-pak cartridge determined it's structure compared to a non-hydrolyzed control (molecule was mixed with denatured protein and treated the same way) and previously solved structures of various surfactin isoforms (Tang*, et al.*, 2007). The hydrolysis occurred at the ester bond within the lactone which cyclized the peptide backbone of surfactin, confirming tandem MS results from the MALDI-IMS (Hoefler*, et al.*, 2012).

2.3.6 Determining substrate specificity of surfactin hydrolase versus other B. subtilis compounds and daptomycin, another macrolactone

From the initial MALDI-IMS experiments, which first identified surfactin hydrolysis, we had also noticed the same pattern for another molecule *B. subtilis* produced, plipastatin (Hoefler*, et al.*, 2012). We chose to pursue surfactin hydrolysis as surfactin blocks aerial hyphae development in streptomycetes (an observable phenotype), is much better studied, and, importantly, surfactin was commercially available. Since plipastatin was also shown to be degraded in the MALDI-IMS experiments and is also a cyclic lipopeptide with a lactone cyclizing the backbone (Figure 2.7), it was hypothesized that the surfactin hydrolase enzyme identified above could also be the molecule degrading plipastatin.
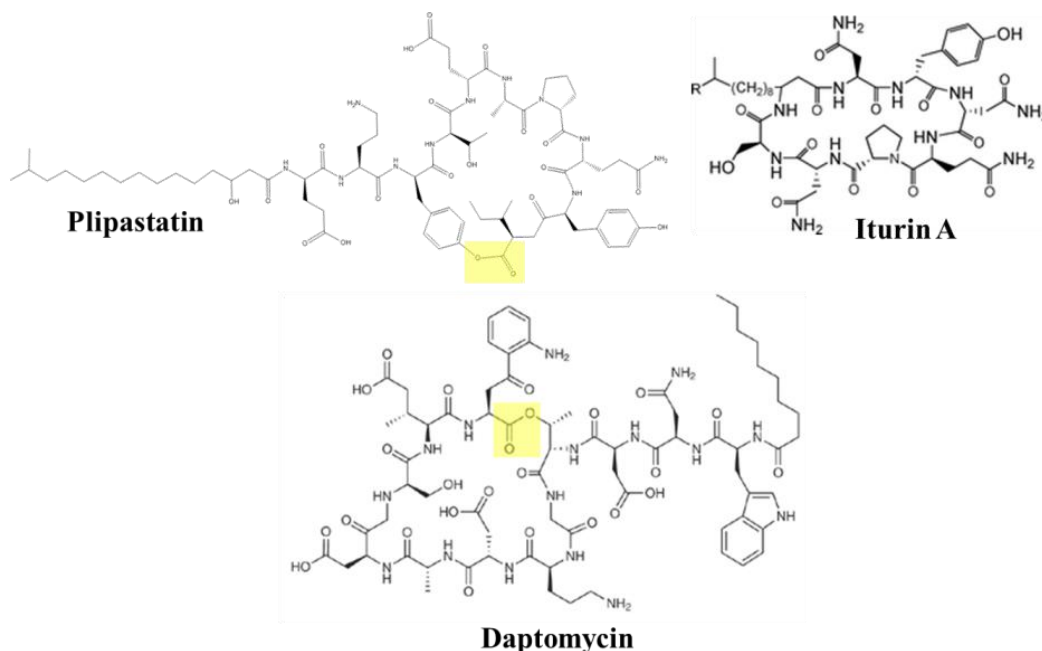
Figure 2.7. Structures of Plipastatin, Iturin A, and Daptomycin. Images adapted from the following sources: Plipastatin (Yang, *et al.*, 2009); Iturin A (Moran, *et al.*, 2009); Daptomycin (Clardy, *et al.*, 2006). Plipastatin and Iturin A are produced by *B. subtilis* spp. while daptomycin is produced by a streptomycete, *S. roseosporus*. Lactones are highlighted in yellow.

Iturins are another class of compounds produced by *B. subtilis* (Romero, *et al.*, 2007). Although iturin is not a cyclic lipopeptide with a lactone, it is a cyclic compound (Figure 2.7) produced by some *B. subtilis* strains. As *S*. Mg1 was isolated with a *B. subtilis* strain, and there is evidence for activity against plipastatin, then it is feasible that the surfactin hydrolase has a broad specificity against *B. subtilis* compounds. *B. subtilis* NCIB3610 (the strain used in the MALDI-IMS experiments) does not produce iturin, so there was no preliminary evidence to support this hypothesis. To examine whether the enzyme was responsible for the observed plipastatin hydrolysis or could hydrolyze iturin, these molecules were tested against the surfactin hydrolase (Figure 2.8). Daptomycin, another lactone cyclized lipopeptide (Figure 2.7), which has 'exceptional'

therapeutic properties against gram-positive multi-drug resistant pathogens (D'Costa, *et al.*, 2012), was also tested to see if there was substrate specificity against lactone cyclized lipopeptides (Figure 2.8).
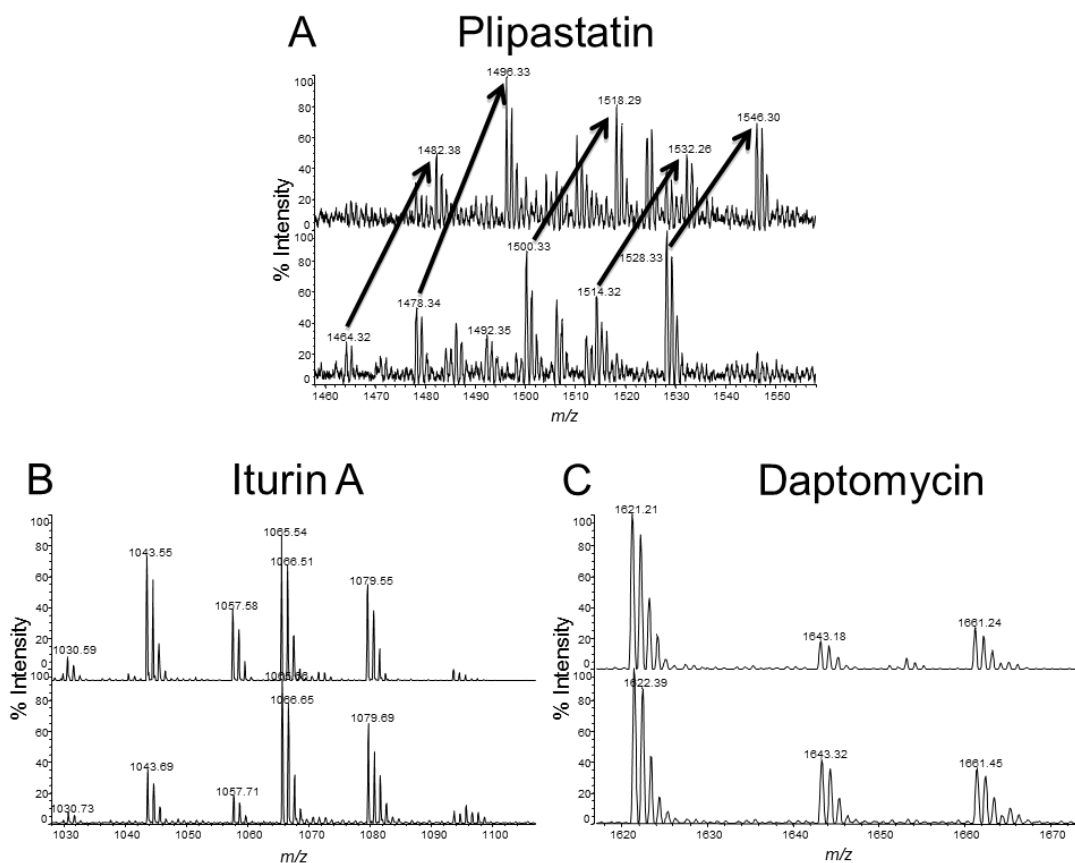
Figure 2.8. MALDI-TOF spectra of the surfactin hydrolase tested against plipastatin, iturin a, and daptomycin. Assay conditions are described in Materials and Methods, Iturin A (panel B), daptomycin (C) were tested at 50µM, while plipastatin (A) was extracted as described in Materials and Methods. The top spectra in each sub-panel was substrate incubated with the hydrolase, while the bottom spectra in each panel was substrate incubated in buffer only. There are only +18 *m/z* peak shifts (characteristic of hydrolysis) observed for plipastatin.

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The surfactin hydrolase was able to degrade plipastatin as is seen through +18 *m/z* peak shifts when incubated with the enzyme versus incubated without the enzyme (Figure 2.8A, top spectra vs. bottom spectra), there is a high level of noise due to the fact that an extract of *B. subtilis* was used, rather than the purified compound, as plipastatin is not commercially available. The hydrolysis confirms the hypothesis that *S.* Mg1 is degrading plipastatin, as was proposed from the MALDI-IMS. However, it cannot be ruled out that another enzyme from *S.* Mg1 could also act on plipastatin. The enzyme was not able to degrade the other *B. subtilis* compound tested, iturin a (Figure 2.8B). While *S.* Mg1 may be resistant to iturin a in the soil, the surfactin and plipastatin degrading enzyme was unable to degrade it. This led to the hypothesis that the surfactin hydrolase was specific to lactone cyclized lipopeptides. However, the lack of daptomycin hydrolysis under conditions that saw surfactin and plipastatin rapidly degraded refuted this hypothesis (Figure 2.8C). After extended incubations with a higher level of enzyme there was a faint amount of hydrolysis that was absent in buffer only controls and reproducible (Figure 2.9). However, there was five times the concentration of enzyme (300µM as opposed to 60nM), incubations were two hours long (12 times the amount of time needed to completely hydrolyze a similar concentration of surfactin with 60nM enzyme) and hydrolysis was at best limited to 1-2% of the pool of daptomycin, so the evidence could be an artifact of adding an excess of the enzyme.
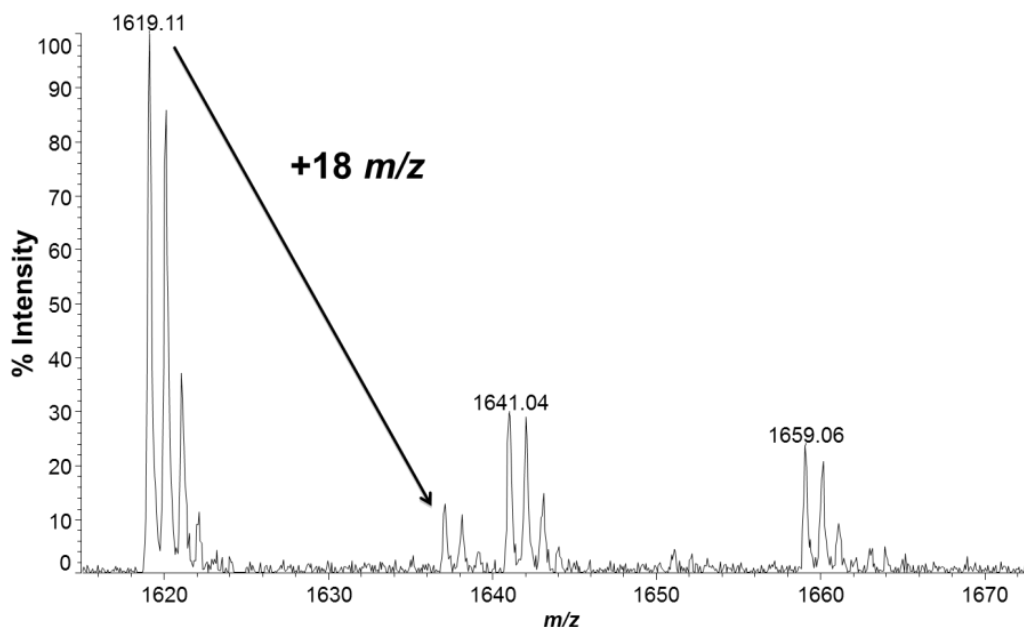
Figure 2.9. MALDI-TOF spectra of an extended incubation of daptomycin with the surfactin hydrolase. Possible faint hydrolysis of daptomycin after a prolonged incubation with a higher concentration of the surfactin hydrolase. The arrow indicates where hydrolyzed daptomycin might have been detected, as seen through a +18 *m/z* peak present from the dominant form of daptomycin.

2.3.7 Determining substrate specificity against a variety of cyclic natural products

To further determine the substrate specificity of the surfactin hydrolase a number of cyclized molecules were screened, which had a variety of scaffolds (Figure 2.10). All the molecules tested were produced by soil organisms, so there is the possibility that *S.* Mg1 evolved the surfactin hydrolase to be active against them as well.
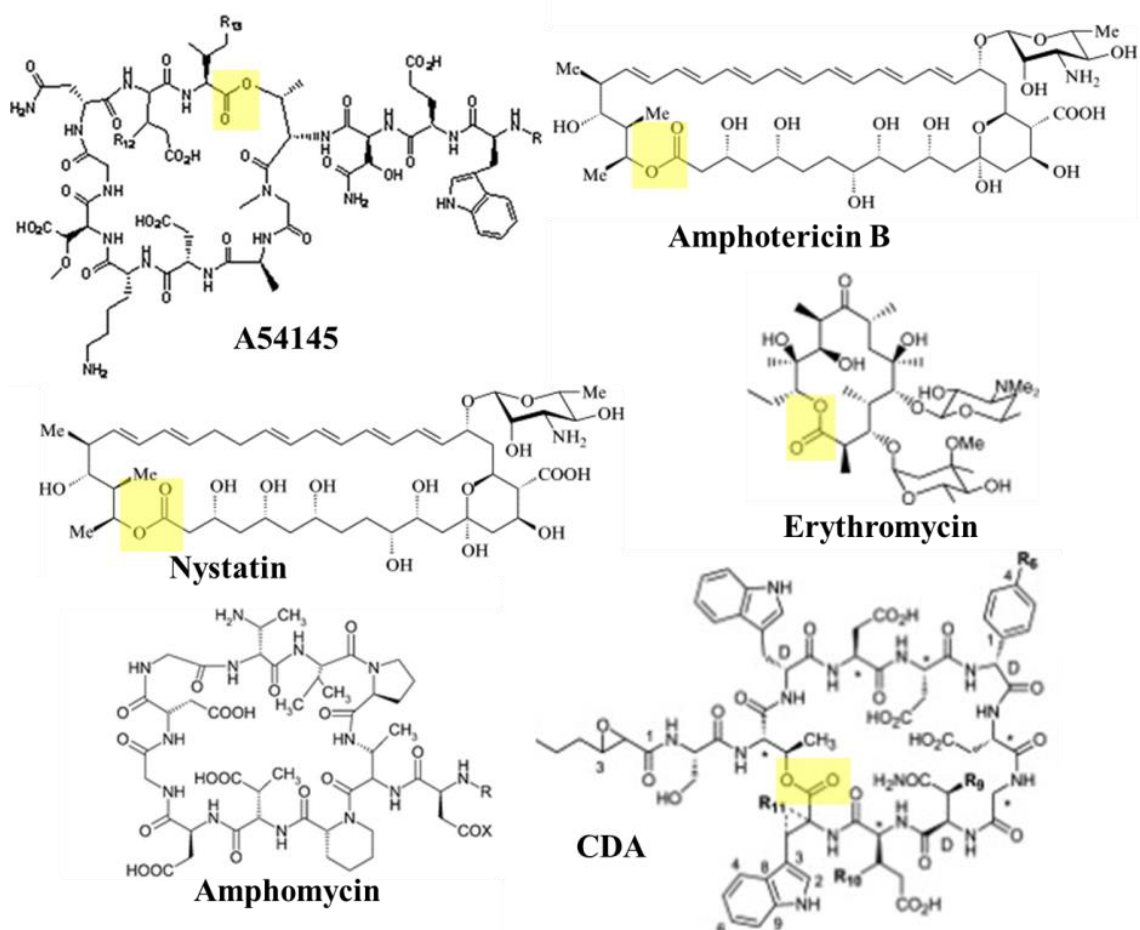
Figure 2.10. Structures of various molecules tested against surfactin hydrolase. A54145, A54145D is a derivative with side groups: R = *anteiso*-undecanoyl, R_{12} = Glu, R_{13} = Ile (Alexander, *et al.*, 2010); Nystatin (Zotchev & Caffrey, 2009); Amphomycin where side groups: X = OH and R = Δ3-*iso*-dodecenoyl (Baltz, *et al.*, 2005); Amphotericin B (Zotchev & Caffrey, 2009); Erythromycin (von Nussbaum, *et al.*, 2006); CDA, calcium dependent antibiotic, has multiple isoforms (Hojati, *et al.*, 2002). Lactones are highlighted in yellow.

A54145D, amphomycin, and CDA (calcium-dependent antibiotic) are all lipopeptides. A54145D is a variant of A54145 which has a lactone cyclizing the peptide backbone and produced by *S. fradiae* (Counter*, et al.*, 1990), amphomycin is produced by *S. canus* (Heinemann*, et al.*, 1953), and CDA is a macrolactone produced by *S. coelicolor* (Hojati*, et al.*, 2002). Amphotericin b and nystatin are both macrolactone polyenes, with amphotericin b produced by *S. nodosus* (Caffrey*, et al.*, 2001) and nystatin produced by *S. noursei* (Fjaervik & Zotchev, 2005). Erythromycin is a macrolide produced by the actinomycete *Saccharopolyspora erythraea* (Donadio*, et al.*, 1991). Conditions used to test the selected compounds were the same as for surfactin, with 60nM enzyme used against 50µM substrate in the Tris·HCl assay buffer (Figure 2.11) with the exception of amphomycin and CDA, which were extracted as described in Materials and Methods.
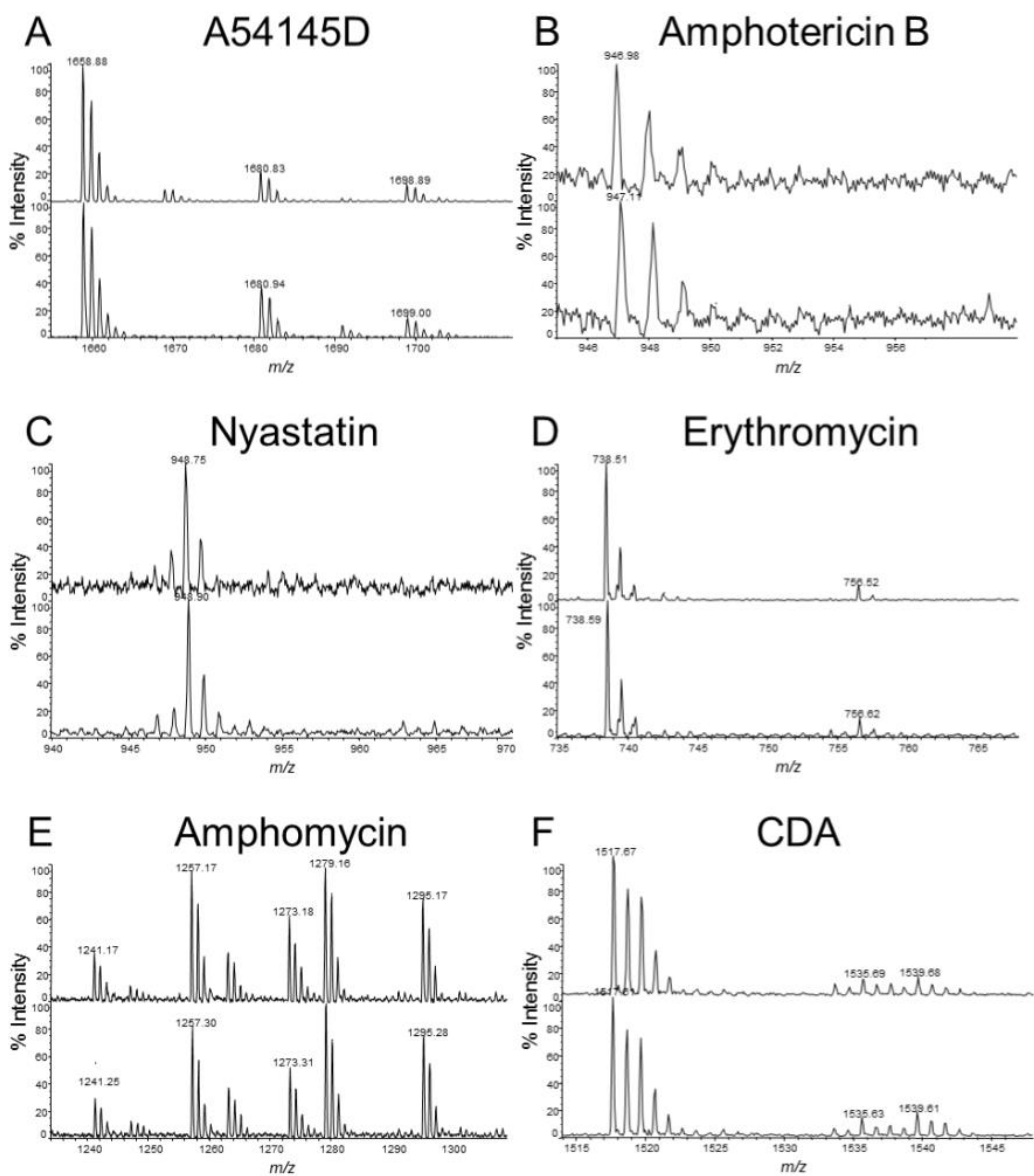
Figure 2.11. MALDI-TOF spectra of cyclic compounds tested against surfactin hydrolase. Assay conditions are described in Materials and Methods. The top spectrum in each panel is substrate incubated with the hydrolase, while the bottom spectrum in each panel is substrate incubated in buffer only. A) A54145D, B) amphotericin b, C) nystatin, and D) erythromycin were at a concentration of 50μM, while E) amphomycin and F) CDA were extracted as described in Materials and Methods, and the lowest dilution which gave the best signal-to-noise ratio using MALDI-TOF was used.

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There was no hydrolysis of any molecules tested. There are peaks arising in spectra from A54145D that differ between the enzyme treated sample and the buffer treated sample, but they were not +18 *m/z*, thus not hydrolysis, and after repeated tests these were shown to be non-reproducible artifacts (data not shown). Surprisingly, the amphomycin and CDA extracts showed very little background while the commercially available amphotericin b and nystatin had a very high signal-to-noise ratio. This is presumably due to the fact that amphotericin b and nystatin used were impure mixtures.

2.3.8 Addition of CaCl$_2$ to assay buffer in substrate specificity trials

While there was no evidence of hydrolysis, this test led to a different set of experiments. CDA, which is only active against gram-positive organisms in the presence of calcium (Lakey, *et al.*, 1983), would presumably only act on *S*. Mg1 in the presence of calcium. Calcium could either act on the molecule changing its conformation or aggregation properties, or calcium could cause a conformational change within the surfactin hydrolase, possibly opening up the active site to new substrates. Initially 10mM calcium was added to the assay buffer (Figure 2.12).
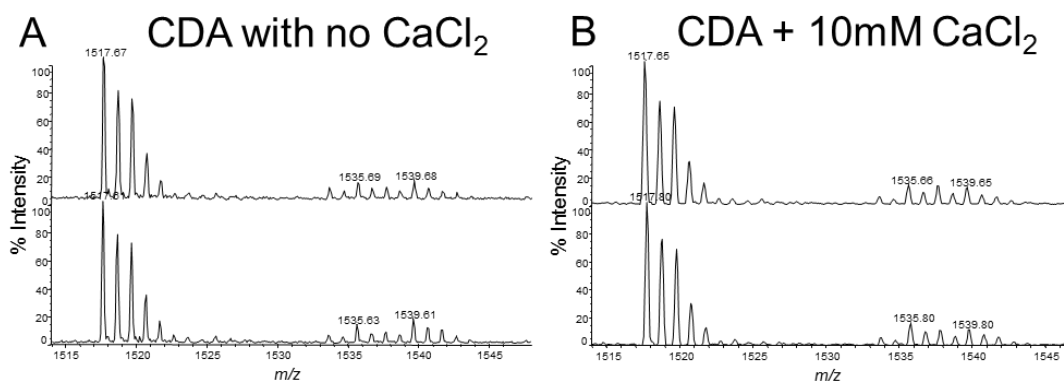
Figure 2.12. MALDI-TOF spectra of CDA tested with or without $CaCl_2$. Assay conditions are described in Materials and Methods. A) CDA tested with no $CaCl_2$ added to the assay buffer and B) CDA tested with 10mM $CaCl_2$ added to the assay buffer. The top spectrum in each panel is substrate incubated with the hydrolase, while the bottom spectrum in each panel was substrate incubated in buffer only. CDA was extracted as described in Materials and Methods, and the lowest dilution which gave the best signal-to-noise ratio using MALDI-TOF was used.

As the CDA used was an extracted compound and an isolated purified molecule, there was a +18 *m/z* peak from the dominant 1517 *m/z* peaks. The +18 *m/z* peak could have just been the molecule getting hydrolyzed during extraction, and was seen in samples with and without the enzyme. There was no detectable change in level of hydrolysis of CDA with the addition of calcium. As the surfactin hydrolase was just stored at 4°C, rather than being flash frozen and stored at -20°C or 80°C, with every new substrate, or set of assay conditions, the enzyme was tested against surfactin to see if it was still viable. After testing surfactin with 10mM $CaCl_2$ in the assay buffer and not seeing any activity, the enzyme was thought to be inactive, except it still hydrolyzed plipastatin (Figure 2.13). Once it was determined that the enzyme was still active, and was just was no longer able to hydrolyze surfactin with calcium in the buffer, other

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substrates were tested to see if their hydrolysis was affected. Substrates which were not

able to be hydrolyzed are not shown.
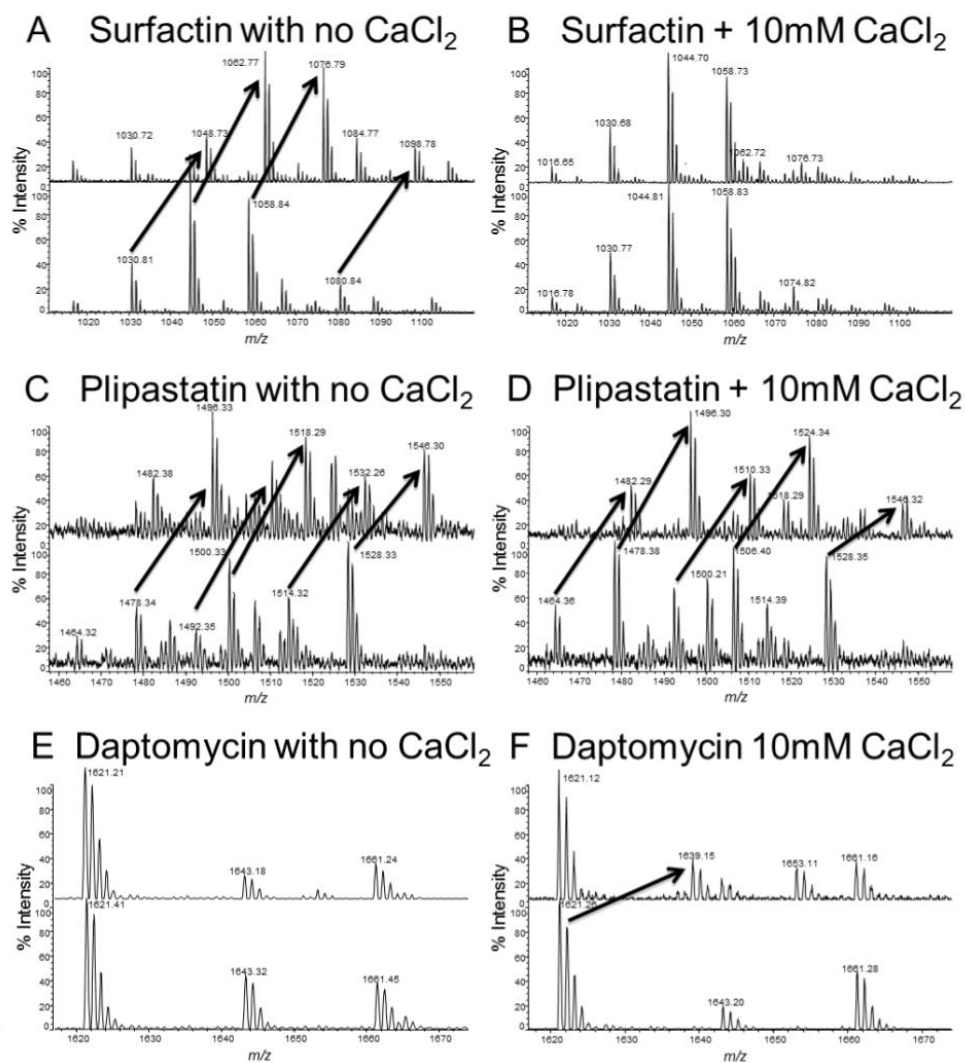
Figure 2.13. MALDI-TOF spectra of surfactin, plipastatin, and daptomycin +/- calcium in the assay buffer. Assay conditions are described in Materials and Methods. A), C), and E) show surfactin, plipastatin, and daptomycin reacted without added calcium in the assay buffer, respectively, while B), D), and F) show the same substrates with 10mM CaCl₂ in the assay buffer. The top spectrum in each panel is substrate incubated with the hydrolase, while the bottom spectrum in each panel was substrate incubated in buffer only. Plipastatin was extracted as described in Materials and Methods, and the lowest dilution which gave the best signal-to-noise ratio using MALDI-TOF was used.

Surfactin hydrolysis was effectively eliminated by the addition of 10mM $CaCl_2$, while control experiments without added $CaCl_2$ showed that the enzyme was still active (data not shown). However, plipastatin hydrolysis remained consistent with the addition of $CaCl_2$. Daptomycin was ~14% hydrolyzed, as calculated by adding the area of the 1639 and 1640 *m/z* peaks divided by the total area of the 1621-1663 *m/z* peaks. The assay conditions were the same as for surfactin (50μM substrate, 60nM enzyme, for 30 minutes). This was a remarkable shift from previous attempts to hydrolyze daptomycin with the enzyme, where a prolonged incubation with five times the amount of enzyme only yielded ~1-2% hydrolysis. The increased daptomycin hydrolysis, coupled with the lack of surfactin hydrolysis, yields some interesting questions about the difference in critical micelle concentration of daptomycin versus surfactin in the presence of Ca^{2+}. No other previously unaffected substrates were able to be hydrolyzed (Table 2.4). To investigate if altering the buffer composition would affect hydrolysis, the buffer was changed from Tris·HCl and NaCl to phosphate buffer and KCl but this did not affect the hydrolysis of any compounds (data not shown).

Table 2.4. Surfactin hydrolase substrates are affected by the addition of $CaCl_2$.

	No added $CaCl_2$	10mM $CaCl_2$
Surfactin	+++	-
Plipastatin	+++	+++
Subtilosin	-	-
Iturin A	-	-
Daptomycin	-	+
A54145D	-	-
Nystatin	-	-
Amphomycin	-	-
Amphotericin B	-	-
Erythromycin	-	-
CDA	-	-

+++ = >95% hydrolysis, + = partial hydrolysis (>10%), - = no detectable hydrolysis.

2.3.9 Titration of calcium into the assay buffer to determine inhibition of surfactin

hydrolysis

The surprising role of calcium in the abolishment of surfactin hydrolysis led to the question of how much calcium does it take to eliminate surfactin hydrolysis. Calcium was added into the assay buffer at 0, 2.5mM, 5mM, 7.5mM, and 10mM to see whether the hydrolase would still hydrolyze surfactin with calcium present (Figure 2.14). This was done to determine if the concentration affecting the hydrolase was a qqphysiological concentration of calcium or a higher concentration less likely to be encountered by the enzyme in nature.
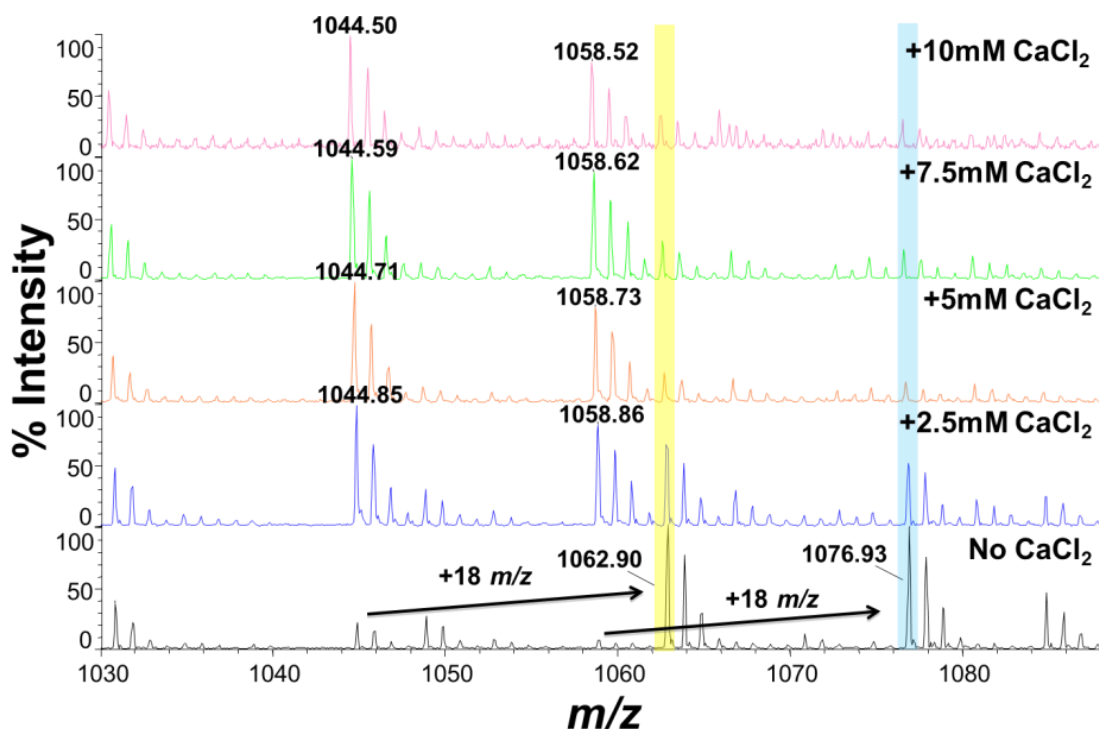
Figure 2.14. MALDI-TOF spectra of surfactin hydrolysis assay with increasing amounts of calcium. Assay conditions are described in Materials and Methods. After a 30 minute assay with the surfactin hydrolase enzyme, from bottom to top there is increasing amounts of $CaCl_2$ added to the assay buffer, from no added $CaCl_2$, to 2.5mM, 5mM, 7.5mM and 10mM $CaCl_2$ in the top spectra. The yellow box indicates where there would be the hydrolysis of the dominant 1044 *m/z* peak, while the blue box indicates where there would be hydrolysis of the next most abundant surfactin isoform, the 1058 *m/z* peak.

2.3.10 Role of surfactin hydrolase in S. Mg1 resistance in vivo

   S. Mg1 was shown to degrade surfactin *in vivo* using MALDI-IMS (Figure 2.1)

and a recombinant *S.* Mg1 protein was shown to hydrolyze surfactin *in vitro* (Figure

2.6), but the hypothesis that the surfactin hydrolase was a resistance mechanism to

surfactin mediated inhibition of aerial hyphae development *in vivo* cannot be addressed

with these methods. To determine whether the enzyme was the sole means of resistance

to surfactin by *S.* Mg1 a knockout of its gene was made and then complemented back.

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When *S.* Mg1, *S.* Mg1 Δ*sfhA,* and *S.* Mg1 Δ*sfhA* sfhA ApR were grown in liquid culture

and had purified surfactin added to them, *S.* Mg1 and *S.* Mg1 Δ*sfhA* sfhA ApR were able

to hydrolyze surfactin, while *S.* Mg1 Δ*sfhA* was unable to hydrolyze surfactin (Figure

2.15). While the complemented deletion mutant (*S.* Mg1 Δ*sfhA* sfhA ApR) was not able

to hydrolyze surfactin as well as wild type *S.* Mg1, it was able to degrade surfactin.
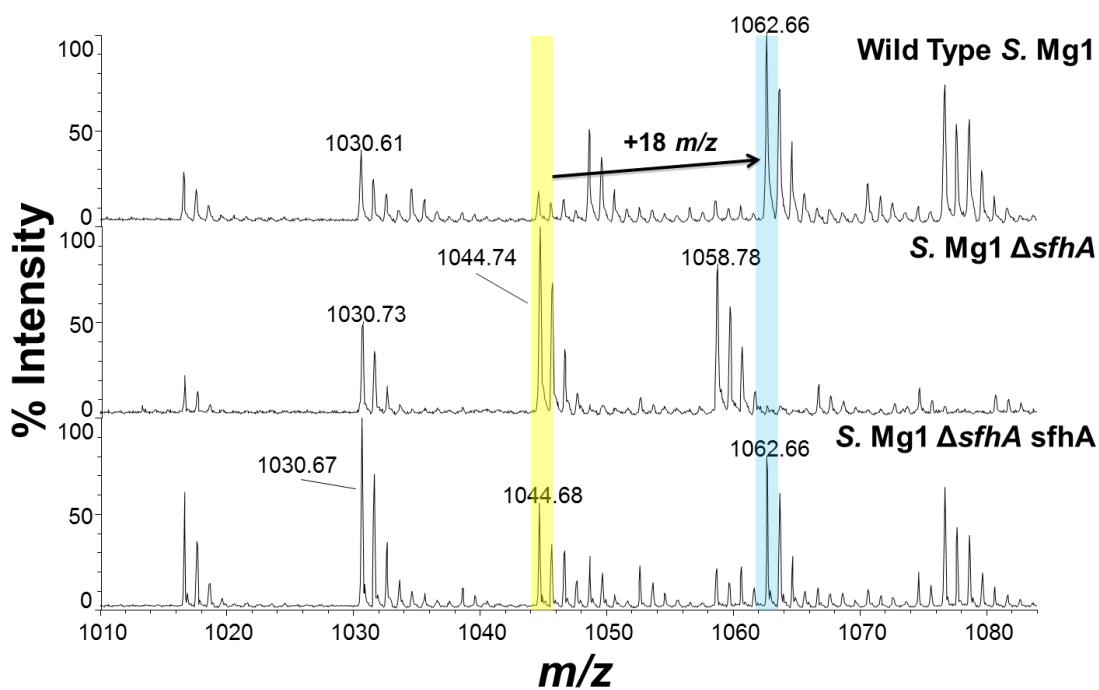
Figure 2.15. MALDI-TOF spectra of *S.* Mg1 and derivatives with exogenous surfactin added. The spectra from top to bottom are wild type *S.* Mg1, *S.* Mg1 Δ*sfhA,* and *S.* Mg1 Δ*sfhA* sfhA ApR. The bacteria were grown in liquid culture and had purified surfactin added to 50μM after 16 hours of growth. The cultures were grown for another 20 hours then extracted as described in Materials and Methods. The yellow box indicates the dominant isoform of surfactin, 1044 *m/z* peak, while the blue box indicates where the dominant isoform of surfactin would be hydrolyzed, the 1062 *m/z* peak, +18 *m/z* away.

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To further confirm that the surfactin hydrolase was responsible for resistance to surfactin, and surfactin mediated inhibition of aerial hyphae, wild type *S.* Mg1, *S.* Mg1 Δ*sfhA,* and *S.* Mg1 Δ*sfhA* sfhA ApR were plated as a lawn and had purified surfactin added to them (Figure 2.16).
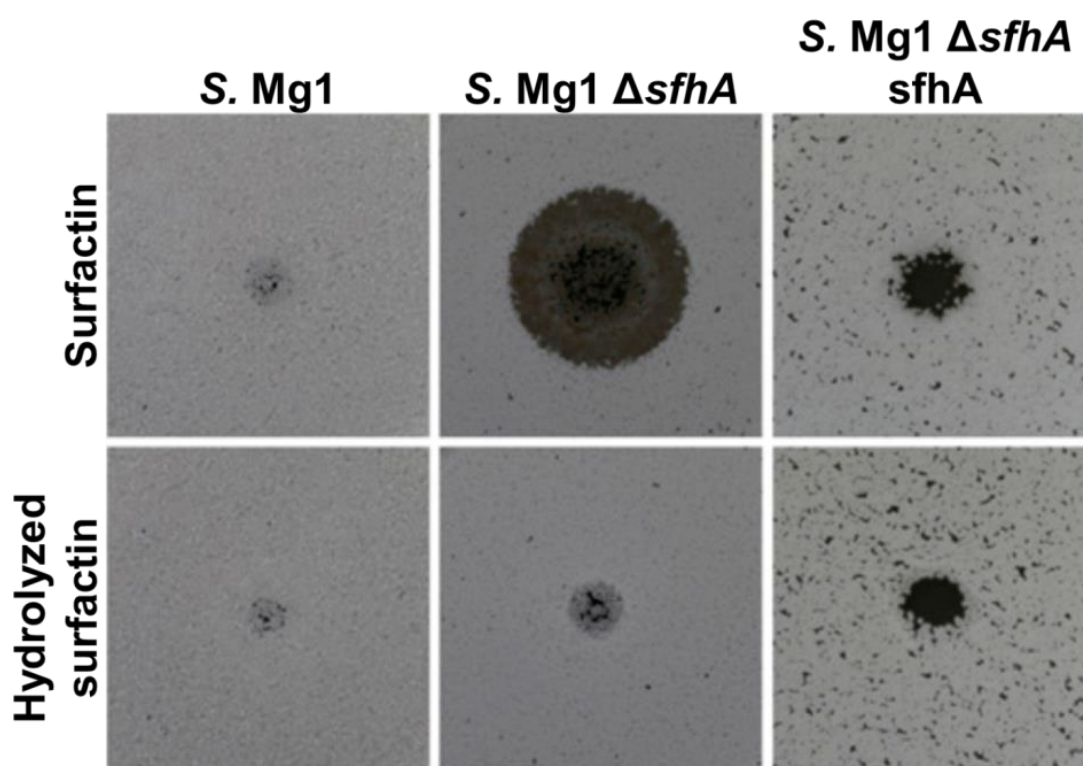
Figure 2.16. Plate assay of *S.* Mg1 and derivatives with surfactin and hydrolyzed surfactin. Wild type *S.* Mg1, *S.* Mg1 Δ*sfhA,* and *S.* Mg1 Δ*sfhA* sfhA ApR were plated with ~100μg of surfactin or hydrolyzed surfactin (assuming 100% recovery). Surfactin was hydrolyzed (or not hydrolyzed) and recovered as described in Materials and Methods.

Once aerial hyphae development and spore formation occurred there is a dramatic phenotypic difference between the wild type and complimented deletion mutant versus the deletion mutant. The inhibition of aerial hyphae in the presence of surfactin is seen through a lack of white pigment (associated with spore formation). If hydrolyzed surfactin is applied then there is no longer inhibition of aerial hyphae development in the deletion mutant (Figure 2.16), further confirming that surfactin inhibits aerial growth and that hydrolyzing surfactin is a defense mechanism against the inhibition of aerial hyphae development. While there is a clearing in the complemented mutant when surfactin and hydrolyzed surfactin were applied, this is inhibition of growth, not inhibition of aerial hyphae development. The inhibition of growth is also seen faintly in the wild type *S.* Mg1 in Figure 2.16 and is more pronounced in the deletion mutant. The inhibition of growth due to surfactin is caused by a combination of the antibiotic properties of surfactin with slightly different titers of spores plated. Figure 2.3 shows a similar inhibition of growth when surfactin was applied to wild type *S.* Mg1. What is clear is that the deletion mutant is inhibited in the formation of aerial hyphae by surfactin but not hydrolyzed surfactin. What little inhibition of aerial hyphae development there is in the deletion mutant could be from incomplete hydrolysis of surfactin. For some reason the 1030 *m/z* peak of surfactin is not hydrolyzed as rapidly or completely as the other isoforms of surfactin during repeated experiments (Figures 2.6, 2.13-2.15).

2.4 Discussion

The overall goal of this project was to identify the cause of surfactin hydrolysis by *S*. Mg1. A proposed mechanism of degradation of surfactin by *S*. Mg1 was that the organism secreted a protein which enzymatically degraded surfactin. By analyzing secreted candidate proteins, cloning and expressing in a heterologous system to get a sufficient quantity protein uncontaminated by other *S*. Mg1 proteins, and *in vitro* trial assays a candidate enzyme was discovered. This study is the first time an enzymatic function has been proposed through MALDI-IMS and then subsequently identified to a protein. In this case the enzymatic mechanism hypothesized was that of degrading a xenobiotic. *S*. Mg1 degrading a foreign compound with antagonistic properties against streptomycetes shows that the surfactin hydrolase enzyme is a resistance mechanism to antibiotics, giving *S*. Mg1 an advantage over other streptomycetes when surfactin is present. A deletion of the gene encoding the enzyme resulted in a strain was no longer able to degrade surfactin, as seen through the lack of hydrolysis in MALDI-TOF analysis of cultures with surfactin added in and as a phenotypically observable trait when surfactin was applied to lawns of the bacteria growing.

Further characterization of the enzyme, as being able to completely hydrolyze plipastatin, hints at the possibility that the enzyme could have evolved specificity against two *B. subtilis* secondary metabolites at once. That the hydrolase changed substrate specificity with the addition of a divalent metal cation is also interesting. Rather than losing substrate specificity for two molecules that it was previously able to hydrolyze, the enzyme lost specificity for one molecule but not the other, surfactin and plipastatin,

respectively. The loss of surfactin hydrolysis activity implies that calcium affects the way surfactin aggregates such that the hydrolase no longer has access to surfactin, or to the ester that is needs to be hydrolyzed. Furthermore, the presence of calcium seems to promote daptomycin hydrolysis. Without calcium in the assay buffer there was barely any detectable daptomycin hydrolysis even after prolonged incubations with high concentrations of the enzyme (Figure 2.9). After addition of calcium, hydrolysis is observed with reduced incubation times and in the presence of a lower concentration of the enzyme. A possible explanation for any hydrolysis of daptomycin without added calcium could be that there were trace amounts of calcium in the buffer used, in the stock of the enzyme, or from contact with the containers used for the assay. This could explain the distinct +18 *m/z* peaks, while still the extremely low rate of hydrolysis without added calcium. Further credence to the role of calcium in the hydrolysis of daptomycin is that daptomycin has little or no activity without calcium (Baltz *, et al.*, 2005) As daptoymcin is only active with calcium present, whenever an organism would need to develop resistance to daptomycin, an enzymatic resistance mechanism would also have to be active in the presence of calcium.

The loss of specificity for one molecule, the expansion of specificity for a second molecule, all while maintaining specificity for a third molecule makes in an interesting enzyme to follow up on. While it is designated as 'surfactin hydrolase,' the enzyme could have promiscuous activities. If there was calcium bound to the enzyme when expressed, after a denaturing purification and refolding of the enzyme presumably any calcium would no longer be bound. When testing the enzyme against surfactin without

calcium then comparing the activity to the enzyme with calcium one proposed explanation for a lack of activity could be that the enzyme was not evolved to have calcium present. However, in the environment there is plenty of calcium around, albeit at lower amounts than tested (for example calcium is present in human serum at ~1.25mM (Barry, *et al.*, 2001)), so there is a good chance that the enzyme likely evolved in the presence of calcium.

Interestingly, there is an extensive literature on surfactin interacting with calcium. One study suggested that the antimicrobial activity of surfactin is dependent on the acyl chain length and also the charge of the peptide backbone, a charge which is altered in the presence of Ca^{2+} ions (Maget-Dana & Ptak, 1995). Surfactin's antimicrobial activity is dependent upon its ability to interact with lipids and insert itself into the cell membrane. Surfactin/Ca^{2+} complexes insert themselves deeper into phospholipid bilayers than surfactin alone (Grau, *et al.*, 1999). When surfactin binds Ca^{2+} the calcium ion forms a bridge between the two acidic residues L-Glu and L-Asp of the peptide backbone (Maget-Dana, *et al.*, 1992, Thimon, *et al.*, 1992). Through the formation of surfactin/Ca^{2+} complexes the antimicrobial properties are increased (Grau, *et al.*, 1999) and it has been proposed that surfactin/Ca^{2+} complexes allow the formation of surfactin dimers which in turn are ion-conducting channels (Sheppard, *et al.*, 1991). The hydrolase must have evolved to deal with some amount of calcium.

Of additional interest is the difference in hydrolysis among surfactin isoforms. The C13 isoform of surfactin, the 1030 *m/z* peak seen throughout this work (which is +22 *m/z* from what the molecule would be due to a Na^+), was less affected by the

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hydrolase than other isoforms (Figures 2.6, 2.13-2.15). One possible explanation is that

the smaller hydrophobic tail allows tighter micelles, possibly preventing access to the

ester by surfactin hydrolase. While the C13 isoform represents a small fraction of

surfactin, it may be important, especially if it is resistant to hydrolysis.

Even though the surfactin hydrolase showed activity after denaturation and

refolding, it is possible that its full activity is not achieved after purification. Therefore,

more attempts need to be made to obtain soluble enzyme without the refolding step.

Different tags like GST, MBP, and SUMO that help with solubility can be tried. If

soluble protein is obtained, inductively coupled plasma mass spectrometry (ICP-MS) can

be done to look for any metals associated with the enzyme.

As the surfactin hydrolase has multiple substrates, it is possible that the substrate-

binding site is large enough that the site can bind multiple ligands at different reaction

coordinates. As surfactin and daptomycin were both able to be hydrolyzed, albeit at

different levels, the enzyme could bind different ligands at higher or lower affinities

which would affect the catalytic rate. Many more ligands need to be examined, some

macrolactones which may be hydrolyzed are: the Antibiotic TA, produced by

Myxococcus xanthus; globomycin produced by a *Streptomyces* sp.; or krisynomycin

produced by *Streptomyces fradiae*.

Finally, *B. subtilis* is frequently used as a biocontrol for bacterial plant diseases

(Ongena & Jacques, 2008, Yanez-Mendizabal*, et al.*, 2012), as an alternative to

fungicide use (Droby*, et al.*, 2009), and is used in about half of the commercially based

biopesticides (Fravel, 2005). The main feature of *B. subtilis* that prevents disease is the

formation of biofilms on plant surfaces (Chen, *et al.*, 2013). Biofilm formation is

mediated by surfactin production and surfactin deficient *B. subtilis* strains are not able to

protect plants from disease (Chen, *et al.*, 2013). It may be interesting to see if the

surfactin hydrolase produced by *S.* Mg1 prevents *B. subtilis* protection from plant

diseases.

CHAPTER III

A POTENTIAL INDUCER OF SECONDARY METABOLISM IN *STREPTOMYCES*

COELICOLOR

3.1 Introduction

3.1.1 The declining rate of discovery of new antibacterial agents

With antibiotic-resistant infections causing health care costs of $20 billion in the United States alone there is need for new antibacterial agents (Bush*, et al.*, 2011). Soil bacteria from the actinomycete family, were the dominant producers of antibiotics, with the remaining coming mostly from fungi (Baltz, 2006). *Streptomyces*, a genus responsible for most antibiotics produced by actinomycetes, yielded 75 new antibiotics per year from 1960-1980, diminishing to 20 per year between 1980 and 2000 as the discovery rate of 'new' compounds declined (Baltz, 2006). The advent of large-scale combinatorial chemistry and high-throughput screening libraries has further driven drug companies away from traditional natural product discovery (Baltz, 2008). However, more than half of the 'new chemical entities' discovered in the since 1981 came from natural products (Newman & Cragg, 2007). The advantages of natural products, compared to synthesized molecules, range from their scaffold diversity to a high tolerance of functional groups to a much higher occurrence of novel motifs (Ganesan, 2008). Natural products also have an advantage over synthesized compounds in that natural products have undergone three billion years of evolution for activity (Baltz,

2008). Of the 1184 new chemical entities described by Newman and Cragg from 1981-2006, 52% had a natural product connection. By applying a set of filters to screen for novel compounds, the authors found that just 24 unique compounds were the starting point for marketed drugs. Of the 24, 5 came from plants and the remaining 19 came from soil bacteria, predominantly from actinomycetes (Ganesan, 2008).

3.1.2 Streptomyces as reservoir of secondary metabolites

Within actinomycetes, the genus *Streptomyces* stands as a source of novel molecules, with two thirds of all commercial antibiotics derived from *Streptomyces* secondary metabolites (Bibb & Hesketh, 2009). Genome mining of streptomycetes has shown that the secondary metabolic genetic potential of *Streptomyces* is largely untapped in the laboratory setting (Corre*, et al.*, 2008). Sequenced streptomycetes have at least 20 and sometimes up to 30 gene clusters predicted to encode for secondary metabolites (Baltz, 2011), the vast majority of which are not detected due to insensitive screening techniques (Baltz, 2008, Davies, 2010). Standard fermentation broth screening apparently lacks the chemical or physical signals needed to express most metabolite gene clusters (Zazopoulos*, et al.*, 2003). This latent genetic potential, for "cryptic metabolites," occupies a large amount of streptomycete genomes, in the larger genomes 5-10% (Baltz, 2008). Secondary metabolites are often highly conserved within strains of an organism, giving credence to the idea that these metabolites confer an adaptive advantage (Borodina*, et al.*, 2005). In *S. coelicolor* at least 23 secondary metabolite gene clusters occupy 5% of the 8.7MB genome (Bentley*, et al.*, 2002). Only

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6 metabolites have been isolated, four antibiotics: methylenomycin A, calcium-dependent antibiotic, undecylprodigiosin and actinorhodin; as well as two other molecules: coelichelin, a new siderophore (Challis, 2008, Xu, *et al.*, 2010), and a group of molecules, germicidins, which inhibit spore germination (Aoki, *et al.*, 2011).

3.1.3 Techniques used to 'awaken' cryptic secondary metabolites

Varying conditions to mimic environment cues have been used in traditional fermentation screenings to 'awaken' the cryptic gene clusters responsible for secondary metabolism. Traditional approaches to awaken cryptic metabolites include altering nutrient sources (Martin, *et al.*, 2011), over-expressing activators (Baltz, 2010), deleting putative negative regulators (Gottelt, *et al.*, 2010), an extensive amount of single gene knockouts (Castro-Melchor, *et al.*, 2010), heterologous expression of cryptic gene clusters (Widdick, *et al.*, 2003), transposon mutagenesis to either knockout or upregulate various genes (Bibb & Hesketh, 2009), making multiple copies of a particular gene cluster within a genome (Murakami, *et al.*, 2011), knocking out other secondary metabolite gene clusters which compete for precursors (Komatsu, *et al.*, 2010, Gomez-Escribano & Bibb, 2011), or mutagenizing the genome to get higher yields (Inaoka, *et al.*, 2004, Wang, *et al.*, 2008, Inaoka & Ochi, 2011). Secondary metabolites come from the redirection of intermediates in primary metabolism into alternative pathways (Murakami, *et al.*, 2011). Thus another approach has been to chemically stimulate various streptomycetes so their primary metabolism is perturbed in such a way that some secondary metabolism is altered or increased (Craney, *et al.*, 2012).

3.1.4 Co-cultures of bacteria to elicit cryptic metabolites

An alternative way of studying secondary metabolism has been to induce a metabolic shift by perturbing one organism with another. This co-culture method has been used to put stress upon an organism, which could occur in the environment but would not otherwise occur in a laboratory. There are tens of studies showing induction of secondary metabolism in fungi and bacteria due to co-culture stress (Patterson & Bolis, 1997, Burgess, *et al.*, 1999, Ueda, *et al.*, 2000, Cueto, *et al.*, 2001, Onaka, *et al.*, 2001, Santamaria, *et al.*, 2002, Trischman, *et al.*, 2004, Yamanaka, *et al.*, 2005, Angell, *et al.*, 2006, Miao, *et al.*, 2006, Oh, *et al.*, 2007, Siemieniewicz & Schrempf, 2007, Schroeckh, *et al.*, 2009, Amano, *et al.*, 2010, Li, *et al.*, 2010, Liu, *et al.*, 2010, Amano, *et al.*, 2011, Li, *et al.*, 2011, Luti & Mavituna, 2011, Luti & Mavituna, 2011, Onaka, *et al.*, 2011, Perez, *et al.*, 2011, Zuck, *et al.*, 2011, Traxler, *et al.*, 2012). The most abundant studies pair marine microbes together to see induction of a metabolite absent in monoculture or either strain. Among the studies that focus on streptomycetes there is a similar pattern, for instance Ueda et al. showed, that from 45 fresh soil isolates of streptomycetes, 98% either increased antibiotic production or triggered early sporulation in other streptomycetes (Ueda, *et al.*, 2000). While these studies are informative to show that bacteria can and do interact with their neighbors by triggering or responding to chemical stressors, there is little mechanistic for the basis of induction.

3.1.5 Co-cultures with model organisms to mechanistically understand elicitation

To study the induction of secondary metabolism through bacterial interactions, we have taken the same approach of co-culturing in an artificial setting. However, we are using two soil-dwelling model organisms, *Bacillus subtilis* and *Streptomyces coelicolor*. We are using model organisms for two reasons: as they coexist in the environment they are likely to interact and evolve against each other; they are also well studied, so deeper mechanistic observations can be made and compared to other closely related organisms (Straight, *et al.*, 2006, Straight, *et al.*, 2007). As model organisms they have both been extensively studied in monoculture, much about how they interact with neighboring organisms is unknown. Previous studies have shown that *S. coelicolor* is affected by the presence of *B. subtilis* (Straight, *et al.*, 2006, Straight, *et al.*, 2007, Liu, *et al.*, 2010, Luti & Mavituna, 2011). One previously studied interaction involves a cyclic lipopeptide, surfactin, produced by *B. subtilis*. Surfactin, required for raising aerial hyphae and thus formation of biofilms in *B. subtilis,* actually inhibits the development of aerial hyphae in *S. coelicolor* (Straight, *et al.*, 2006). By blocking the development of aerial hyphae, and thus spore production, *B. subtilis* is reducing competitors for nutrients for its own offspring spores. This could represent an offensive mechanism by which *B. subtilis* inhibits completion of *S. coelicolor's* lifecycle. However, surfactin is also used by *B. subtilis* for swarming across solid surfaces (Kearns & Losick, 2003). Since surfactin has multiple intraspecific and interspecific roles in culture, a single environmentally relevant function is not defined.

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S. coelicolor and *B. subtilis* have many other chemical interactions. Another example involves a different *B. subtilis* metabolite, bacillaene. When cultured together there is 'a rather unremarkable' phenotype (Straight*, et al.*, 2007), with *S. coelicolor* growing right up to the edge of the *B. subtilis* colony. Transposon mutagenesis of *B. subtilis* revealed bacillaene deficient strains when paired against *S. coelicolor* cause there to be early production of undecylprodigiosin by *S. coelicolor* (Figure 1) (Straight*, et al.*, 2007). Undecylprodigiosin (RED) has immunosuppressive and anticancer activities as well as published antifungal, antibacterial, and antiprotozoal properties (Williamson*, et al.*, 2006). The chemical exchange leading to an early induction of RED is interesting because *S. coelicolor* is able to sense a neighboring organism and turn on production of a secondary metabolite, presumably in defense. Production of RED has since been elicited with living or dead *B. subtilis* cells (Luti & Mavituna, 2011) as well as with *Staphylococcus aureus* heat-inactivated cells (Luti & Mavituna, 2011). But the mechanism of early induction of RED is unknown.
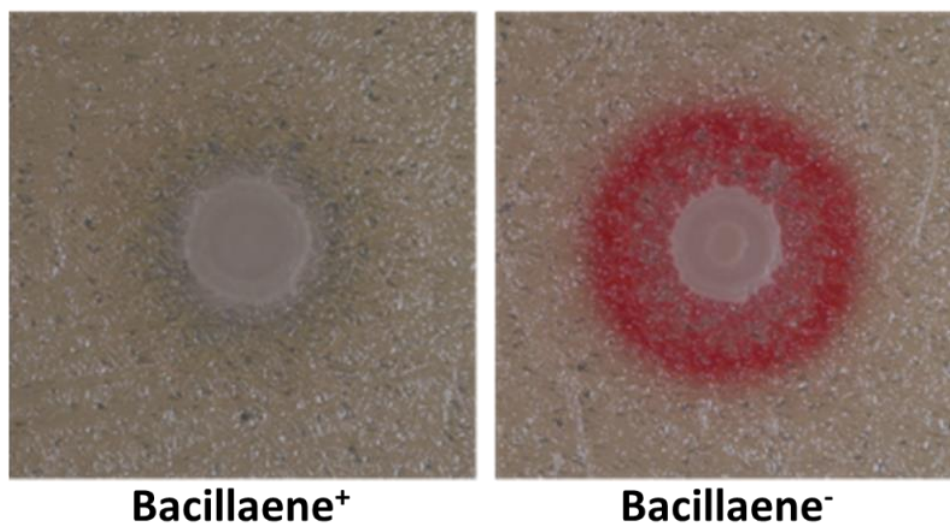
Figure 3.1. RED induction in *S. coelicolor* from bacillaene deficient strains of *B. subtilis*. Left panel: wild-type undomesticated strain *B. subtilis*, NCIB 3610 producing bacillaene, does not induce RED in co-culture with *S. coelicolor*. Right panel: a bacillaene deficient mutant of the 3610 induces early production of RED in co-culture with *S. coelicolor*.

3.1.6 RED induction

RED has antifungal, antibacterial, and antiprotozoal properties (Williamson*, et al.*, 2006), but these roles only apply at the latter end of *S. coelicolor's* lifecycle, to protect the nutrients from degraded vegetative mycelia for the spore forming aerial hyphae from any motile competitors (Bibb, 2005). However since the pigment is largely localized intracellularly, none of these reported functions address a physiological role (Haddix*, et al.*, 2008). Several intracellular roles have been suggested: as an energy spilling function by reducing ATP production (Haddix*, et al.*, 2008), as a metabolic sink for proline or NAD(P)H (Hood*, et al.*, 1992), as a bacterial dispersal agent due to its surface association (Syzdek, 1985), as a UV-protective pigment (Boric*, et al.*, 2011) and most recently as an antioxidant (Stankovic*, et al.*, 2012). RED production is turned on

by a quorum-sensing mechanism and a variety of nutritional conditions as summarized in a recent review (Williamson*, et al.*, 2006). RED itself is also able to be sensed by *S. coelicolor* in a mechanism to coordinate timing of antibiotic production within a colony (Xu*, et al.*, 2010). With regard to its antimicrobial properties, RED has been reported to inhibit growth of *Mycobacterium smegmatis, Streptomyces somaliensis, Staphylococcus aureus* (Gerber, 1975)*, Bacillus* sp. (Staric*, et al.*, 2010), and even *B. subtilis* (Stankovic*, et al.*, 2012), among other microorganisms (Gerber, 1975, Stankovic*, et al.*, 2012). The induction of RED when challenged to *Bacillus subtilis* and *Staphylococcus aureus* implies that *S. coelicolor* is sensing a competitor and responding to it (Straight*, et al.*, 2007, Yang*, et al.*, 2009, Luti & Mavituna, 2011, Luti & Mavituna, 2011). What interests our study is the induction of RED when challenged by another organism, so that we can establish a mechanism of response by the model streptomycete to a competitor. If we were to establish such a mechanism based off a molecular cue, then we will use that cue to elicit cryptic metabolites in other streptomycetes.

3.1.7 Transposon screen reveals candidate genes related to the induction of RED

The early onset of RED is most likely due to *S. coelicolor* sensing the chemical presence of a competitor, in this case sensing a compound secreted by *B. subtilis*. If *S. coelicolor* turned on RED as a result of a decrease in nutrients caused by competition with *B. subtilis* then RED induction would always occur in the absence of bacillaene. However, a second transposon mutagenesis screen revealed that there are mutations in *B. subtilis* which lead to a loss of early induction of RED phenotype when the organisms

are co-cultured together (Figure 3.2). This has led to the hypothesis that there is a

secreted metabolite which induces RED (Figure 1 right panel). Alternatively, the

bacillaene deficient strains of *B. subtilis* may be altered in their metabolic flux such that

a molecule normally present in minute quantities is overproduced when bacillaene is not

produced, allowing it to be in a sufficient quantity to be sensed by *S. coelicolor*.
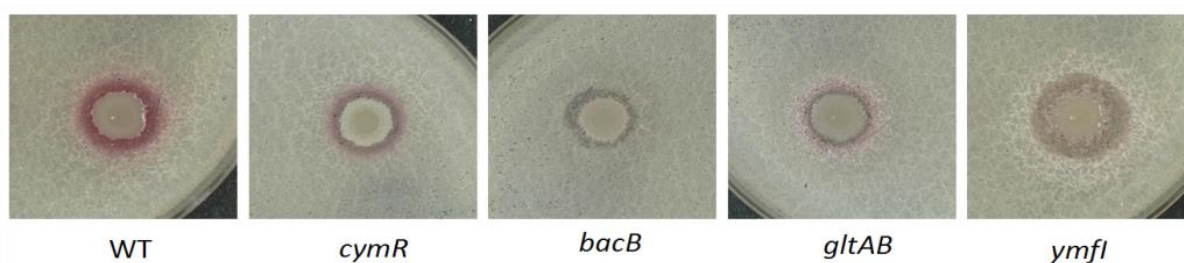
Figure 3.2. Transposon mutagenesis of *Bacillus subtilis* reveals possible inducer molecules of RED in *S. coelicolor*. This is seen through a decreased level of RED production compared to wild type PY79 *B. subtilis* (WT). *CymR*: cysteine-metabolism regulator; *bacB*: bacilysin synthase; *gltAB*: glutamate synthase AB; *ymfI*: alcohol dehydrogenase required for neotrehalosadiamine production.

In the absence of bacillaene there is early induction of RED, presumably because

B. subtilis is producing and secreting more of the inducing molecule. The second

transposon screen used another *B. subtilis* strain, PY79, a domesticated strain that does

not produce a number of secondary metabolites including bacillaene, revealed several

genes which when disrupted altered the early onset of RED.   The best candidate genes

for altered induction of RED (seen in Figure 3.2) include: *cymR*, cysteine-metabolism

repressor, a global regulatory factor for sulfur metabolism (Even*, et al.*, 2006); *gltAB*,

the two of which comprise glutamate synthase (Picossi*, et al.*, 2007); *ymfI*, involved in

swarming and predicted to encode a short chain alcohol dehydrogenase for a precursor of neotrehalosadiamine (Kearns*, et al.*, 2004, Inaoka & Ochi, 2007); and *bacB*, involved in bacilysin biosynthesis (Parker & Walsh, 2012). The effects of each mutation on the metabolic flux within *B. subtilis* causes either the reduction or abolition of production of the molecule(s) which causes *S. coelicolor* to turn on RED early.

3.2 Materials and Methods

3.2.1 Bacterial strains

The strains used in this chapter are listed in Table 3.1. For long term storage *Escherichia coli* strains were grown overnight, spun down, and resuspended in sterile 20% (v/v) glycerol, and stored at -80°C. For streptomycetes the spore suspensions were prepared in sterile mqH$_2$O then stored at -80°C. Spore suspensions of *Streptomyces coelicolor* were prepared using previously established protocols (Kieser*, et al.*, 2000). Briefly, a -80°C stock of the streptomycete was struck out in a square-like pattern onto a R5 plate (Kieser*, et al.*, 2000), once spore formation occurred (~3-4 days) 1mL of sterile mqH$_2$O was applied to the plate and spores were scraped with a cell scraper, removed and 1mL more of sterile mqH$_2$O was applied and taken off with a pipette. The spores were serially diluted in sterile mqH$_2$O and plated on GYM plates to get a working titer for experiments.

Table 3.1. Bacterial strains used in this chapter.

Strain	Relevant Genotype
PDS0066	*Bacillus subtilis* NCIB3610 wild type
PDS0067	*Bacillus subtilis* NCIB3610 *Δpks*
PSK0028	*Streptomyces coelicolor* M145
PDS0288	*Streptomyces coelicolor* M145 *dasR::aacC4* from van Wezel (Rigali, *et al.*, 2006)
PSK0453	*Bacillus subtilis* NCIB3610 *ΔbacB*
PDS0371	*Bacillus subtilis* NCIB3610 *Δpks, ΔscoC, ΔabrB*
PDS0375	*Bacillus subtilis* NCIB3610 *Δpks, ΔscoC, ΔabrB, ΔbacB*
PSK0015	*Bacillus subtilis* 168 wild type
PSK0001	*Bacillus subtilis* PY79 wild type
	Bacillus subtilis PY79 *ΔbacB*

3.2.2 Media

Bacto media was used, unless otherwise noted. Luria-Bertoni (LB) broth (1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v NaCl) was used for *Bacillus subtilis* cultures, with LB plates having 1.5% agar added. LB was supplemented with 10µg/mL kanamycin, 5µg/mL chloramphenicol, 100µg/mL spectinomycin, 10µg/mL tetracycline, and MLS (1µg/mL of erythromycin, 25µg/mL of lincomycin) as appropriate. All antibiotics used were purchased from Sigma. *Streptomyces* spp. cultures were grown on glucose-yeast-malt extract (GYM) (1% w/v malt extract, 0.4% w/v yeast extract, 0.4% w/v dextrose) with GYM plates having 1.5% agar added.

PA media for production of bacilysin (Walker & Abraham, 1970) and the inducing molecule was modified to included 1mM GlcNAc (Sigma) and 100mM morpholinepropanesulfonic acid (MOPS) pH 6.8. GlcNAc addition was to prevent autolysis of *B. subtilis* PY79 as a result of over-production of bacilysin. MOPS was to

maintain pH during growth and production of the inducing compound. PA recipe:

KH_2PO_4 - 1.1g, $MgSO_4 \cdot 6H_2O$ - 0.975g, KCl - 0.55g, monosodium glutamate - 4.4g,

sucrose - 13.7g, 1M MOPS pH 6.8 - 100ml, oligodynamic solution - 1ml, brought to

~992.5ml and filter sterilized. After filter sterilizing 7.5ml of ferric citrate (20mg/ml

ferric chloride, 20mg/ml sodium citrate, filter sterilized) was added. Oligodynamic

solution was prepared as previously described (Pollock & Kramer, 1958).

3.2.3 Co-cultures of B. subtilis and S. coelicolor

Fresh LB plates, plus appropriate antibiotics, were allowed to dry, streaked with

B. subtilis strains from -80°C glycerol stocks. Plates were incubated overnight at 37°C,

removed to room temperature (~22°C) and used within 3-4 days to make overnight

liquid cultures in LB (incubated at 37°C and ~200rpm). 32mL GYM plates were made

using a glass pipette and after all plates were made the lids were removed and plates

allowed to dry for 20 minutes. Plates used for co-cultures were stored for up to a month

at 4°C. Upon use for co-culture the plates were dried for 20 minutes, plated with 100μL

of varying dilutions of *S. coelicolor* using glass beads, glass beads removed and plates

allowed to dry for 20 minutes more. Then 1-2μL of overnight *B. subtilis* cultures were

spotted in the middle of the plate. Plates were incubated at 30°C until desired

development was observed.

3.2.4 Preparation of B. subtilis lyophilized supernatants

B. subtilis cultures were struck out onto fresh LB plates, plus appropriate antibiotics when necessary. Overnight *B. subtilis* cultures were inoculated from 1-4 day old plates and grown at 30°C and ~200rpm, in PA media with 1mM GlcNAc. The overnight culture was used get 1L of the same media to an $OD_{600} = 0.02$ (500ml each in a 2.8l baffled flask). Cultures were grown for 24 or 48hrs, shaking at 200-225rpm. Cells were pelleted at 4,000 x *g* for 30min at 4°C and discarded. Supernatants were flash frozen and lyophilized, then resuspended in mqH_2O at 1:25 of the original volume, then filter sterilized through a 0.2µm filter.

3.2.5 Preparation of B. subtilis crude extracts

Using a previously established protocol for bacilysin production (Parker & Walsh, 2012), extracts were prepared. Overnight *B. subtilis* cultures were grown at 30°C in PA media with 1mM GlcNAc. The overnight culture was used get 1L of the same media to an $OD_{600} = 0.02$ (500ml each in a 2.8l baffled flask). Cultures were grown for 48hrs, shaking at 200-225rpm. Cells were pelleted at 4,000 x *g* for 30min at 4°C and discarded. To the supernatant 1L ethanol was added (to get 50% v/v). The mixture was shaken thoroughly and incubated at 4°C overnight. Precipitated proteins and DNA were pelleted at 4,000 x *g* for 30min at 4°C and discarded. Supernatants were applied to a Dowex 50WX8-200 cation exchange resin (Sigma), pre-equilibrated with 50% ethanol. ~25g resin was used per each column. Washed each column with ~5 column volumes of water (~100ml), eluted each column with 40 mL of 4% NH_4OH in H_2O into 2 50ml

conical cylinders submerged in liquid nitrogen. The elution was lyophilized to dryness, resuspended in smallest volume of mqH$_2$O possible, 1-2mL.

3.2.6 Plating extracts from B. subtilis against S. coelicolor

S. coelicolor was plated as described in section 3.2.3. Filter sterilized extracts were plated after *S. coelicolor* spores had dried and plates were allowed to dry for another 20 minutes.

3.2.7 Fractionation of B. subtilis extracts

Pre-wetted SPE cartridges C8, C18, diol, cyano, NH$_2$, and silica SPE cartridges (Sigma), or their resins were loaded with Dowex eluted *B. subtilis* extracts from wild-type PY79 or Δ*bacB* PY79. C8, C18, and NH$_2$ cartridges were pre-wetted by running 3mL of methanol then 3mL of 20% methanol/water v/v, then 6mL of water. To elute various fractions 10mL fractions were used moving up in steps 10% methanol/water v/v, increasing by 10% methanol steps were used. Samples were concentrated to dryness under pressure then resuspended in mqH$_2$O, filter sterilized through a 0.2μm filter, and plated. Diol, cyano, and silica cartridges were pre-wetted with 3mL of methanol then 3mL of 20% methanol/chloroform v/v, then 6mL of chloroform. Samples were lyophilized to dryness, resuspended in chloroform, then loaded onto the cartridges. Samples were eluted off of the cartridges in 10ml 10% methanol/chloroform steps until the methanol/chloroform ratio was 100:0. Fractions were concentrated to dryness under pressure then resuspended in mqH$_2$O, filter sterilized through a 0.2μm filter, and plated.

3.3 Results and Discussion

3.3.1 Bacilysin as an inducer of RED

From the transposon mutagenesis screen, two genes are directly involved in the production of secondary metabolites: *bacB* and *ymfI*. The other genes which were identified in the screen were more general metabolic or regulatory genes, not associated with a particular secondary metabolite. *ymfI* when plated in a co-culture at varying densities of spores was subsequently shown to induce RED (data not shown). The *bacB* gene is involved in the biosynthesis of bacilysin. When plating lyophilized supernatants of wild type *B. subtilis* and *ΔbacB B. subtilis* there was induction of RED for wild type, but not for *ΔbacB* (Figure 3.3).
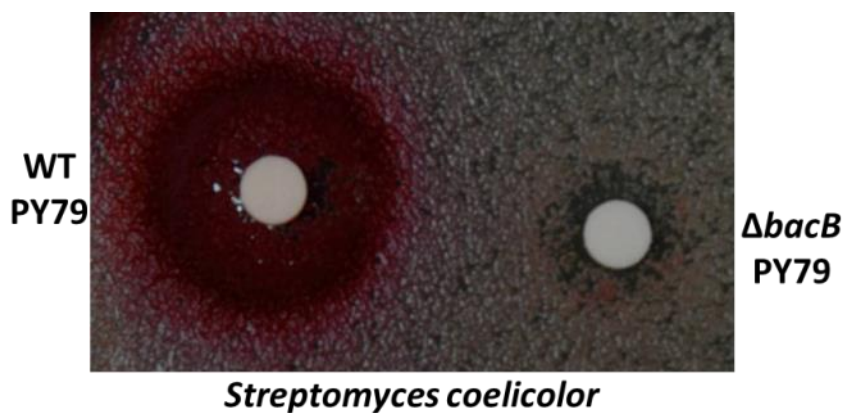
Figure 3.3. Lyophilized supernatants of *B. subtilis* plated against *S. coelicolor*. Wild-type PY79 and *ΔbacB* PY79 were grown in PA media for 48hrs, spun down, the supernatant lyophilized and resuspended in 1/25 the volume and 20μL plated onto filter discs (white circles seen above).

The induction of RED by wild-type PY79, but not *ΔbacB* lyophilized supernatants led us to believe bacilysin was the inducing molecule. The *bacB* gene is directly involved in the biosynthesis of bacilysin (Rajavel*, et al.*, 2009), a dipeptide antibiotic which is taken up into a host and cleaved releasing the non-canonical amino acid anticapsin (Chmara, 1985). Anticapsin, which mimics L-glutamine, irreversibly binds to glucosamine synthase, which is required for cell wall biosynthesis (Chmara, 1985). This is a direct link to a *S. coelicolor* master regulator *DasR*, which controls, among other functions, secondary metabolism (Rigali*, et al.*, 2008). Previously this regulator has been shown to be involved in sensing nutrient stress, as it represses antibiotic production in rich media, but releases the promoters of its regulon when bound by glucosamine-6-phosphate (GlcN-6-P), the product of glucosamine synthase (Chmara, 1985, Rigali*, et al.*, 2008). While halting the production of a molecule which alters the activity of a repressor of antibiotic production would seem counter to elicitation of secondary metabolites, not all of the dynamics of the *DasR* system are fully understood yet (van Wezel & McDowall, 2011, Swiatek*, et al.*, 2012, Swiatek*, et al.*, 2012).

During sporulation, aerial hyphae are raised above the vegetative mycelia and cannibalize the mycelia below for nutrients (van Wezel & McDowall, 2011). Tying sporulation to production of antibiotics protects nutrients from motile organisms which might compete with the spore-forming organism (Chater*, et al.*, 2002). Bacilysin was looked at as an environmental stress, by mimicking a lower level of GlcN-6-P in *S. coelicolor's* overall metabolic flux. This would cause *S. coelicolor* to proceed ahead in its developmental processes, including antibiotic production. We hypothesized bacilysin

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to be the inducing molecule as it a direct link to a global antibiotic regulator, *DasR*, and there was no direct link for our other candidate molecule, NTD. A previous study on the interaction between undomesticated *B. subtilis* and *S. coelicolor* using imaging mass spectrometry did not support the hypothesis of bacilysin as the inducing molecule (Yang*, et al.*, 2009). Experimental conditions have long been known to impact production of secondary metabolites (Gonzalez*, et al.*, 2012). The previous study used different conditions: media, starting inoculums of the two strains, monitored at a different timing, and importantly used different strains (*sfp⁻* instead of Δ*pks* to knock out bacillaene production).

3.3.2 Identification of a B. subtilis strain which over-produces bacilysin

In an effort to purify bacilysin to test for inducing activity against *S. coelicolor* several strains of *B. subtilis* were examined for their bacilysin production characteristics. Reports on bacilysin regulation suggest that *scoC* and *abrB* negatively regulate bacilysin production (Karatas*, et al.*, 2003, Inaoka*, et al.*, 2009). Bacilysin also appears to be produced at higher levels in a *Δpks* background of *B. subtilis*, a strain which no longer produces bacillaene (data not shown). A strain of *B. subtilis* 3610 was constructed with *pks, scoC,* and *abrB* deletions to see if bacilysin was overproduced. This was compared to a similar strain with an additional *bacB* mutation, as a negative control to show the abolishment of bacilysin production. Further comparison against *B. subtilis* strains included the 'undomesticated' parental strain, NCIB 3610, a derivative with *bacB* deleted, again to show abolishment of bacilysin production, and a 'domesticated' strain,

B. subtilis 168. These strains were grown in PA media and lyophilized supernatants

analyzed via LC/MS for bacilysin production as seen in Figure 3.4 (Courtesy of Jared

Parker of the Walsh laboratory, Harvard).
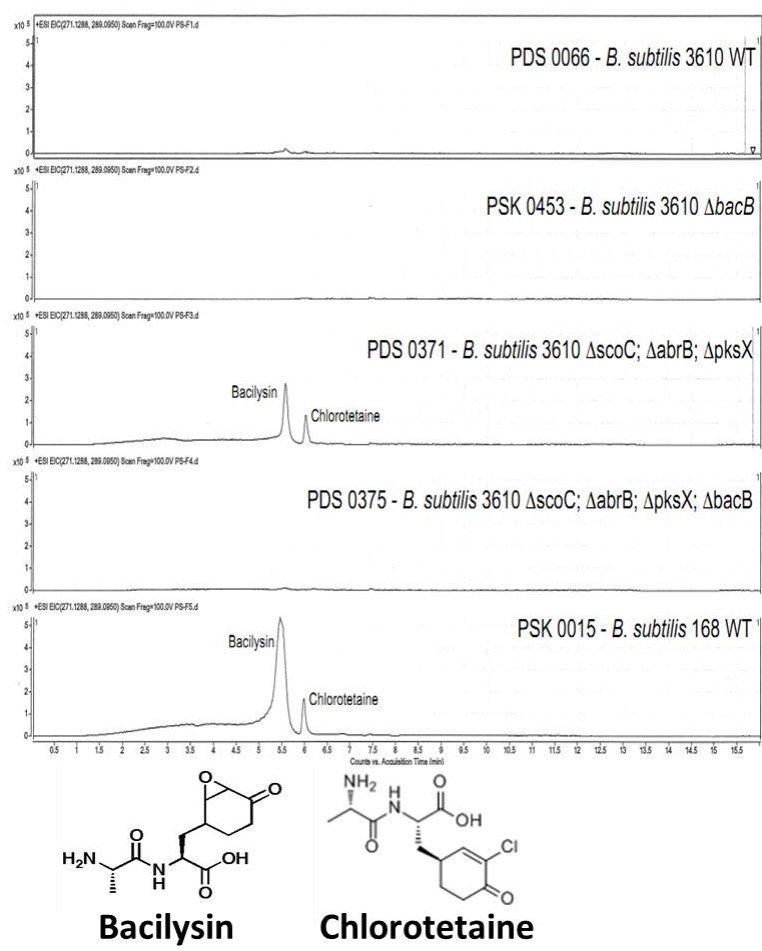
Figure 3.4. Bacilysin production in various *B. subtilis* strains. Strains are listed at the top right for each spectra. Bacilysin, and a similar compound, chlorotetatine are shown when produced in sufficient quantities.

Interestingly, the undomesticated strain of *B. subtilis*, NCIB3610, produced

bacilysin at barely detectable levels in these assays. The triple mutant, *Δpks, ΔscoC,* and

ΔabrB, showed higher levels of bacilysin production, as would be expected with the

deletion of two regulators, *scoC* and *abrB*, combined with the deletion of portions of the

pks gene cluster, responsible for the production of bacillaene. By no longer producing

bacillaene there may be less competition for precursor molecules to bacilysin

biosynthesis. What is intriguing is the overproduction of bacilysin by the domesticated

strain *B. subtilis* 168. *B. subtilis* 168, a laboratory strain derived from NCIB3610, does

not produce the three major secondary metabolites that NCIB3610 produces: surfactin,

plipastatin, or bacillaene.

3.3.3 Determining experimental parameters to see production/ induction of RED

In order to systematically monitor the induction of RED, which is a normal

developmental process of *S. coelicolor*, *S. coelicolor* was plated on several different

media and observed for production of RED. R5 media caused *S. coelicolor* to develop

rapidly such that RED production was seen within 24 hours at some plating dilutions of

spores, and the plates were blue or dark purple shortly thereafter, from actinorhodin

production (data not shown). Actinorhodin is produced at much higher levels than RED

(van Wezel & McDowall, 2011). When the timescale of actinorhodin production is

accelerated it is harder to see induction of RED, so RED induction was monitored on

GYM, to delay induction of ACT (Sanchez*, et al.*, 2010). At the lower plating densities

of spores from *S. coelicolor* RED production takes several days (Figure 3.4). As RED is

a normal development process all colonies of *S. coelicolor* eventually start producing

RED. At plating densities lower than a lawn there are some colonies which turn on

production of RED faster than others, presumably due to factors unrelated to genetics,

note the half of a colony in the top left quadrant of each image which is redder than the

rest of the plate (Figure 3.4).
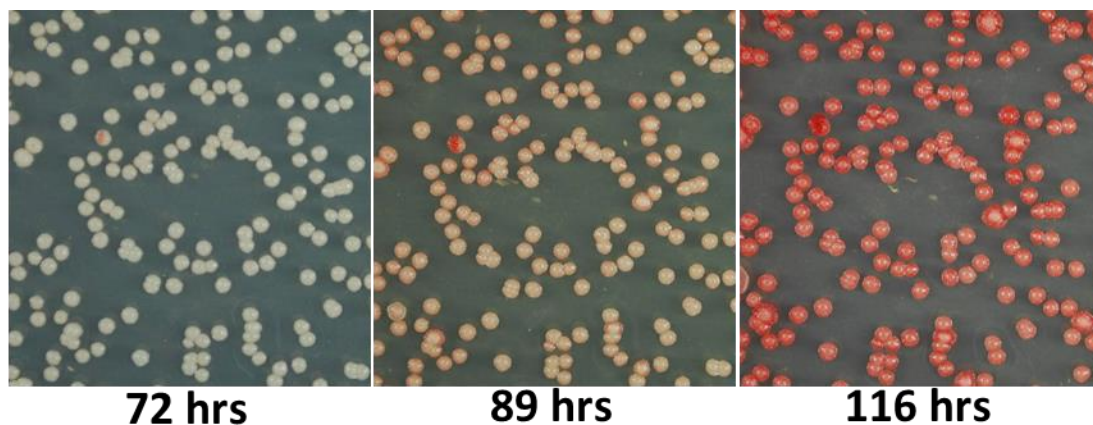
Figure 3.4 Low plating densities of *S. coelicolor* show that all colonies turn on RED production. A plate of *S. coelicolor* with a spore plating density of ~10^2 was monitored for RED production over one week, incubated at 30°C.

In order to determine the fastest time RED is induced, *S. coelicolor* was plated at

different spore titers and observed daily for a week (Figure 3.5). A time course of

images was taken of *S. coelicolor* plated at different spore densities, and a representative

image is shown. This was to determine when RED production normally occurred so as

to be able to monitor early induction of RED. At higher plating densities RED

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production turned on earlier, which is indicative of dependence on the stage of *S.*

coelicolor development (Williamson*, et al.*, 2006).
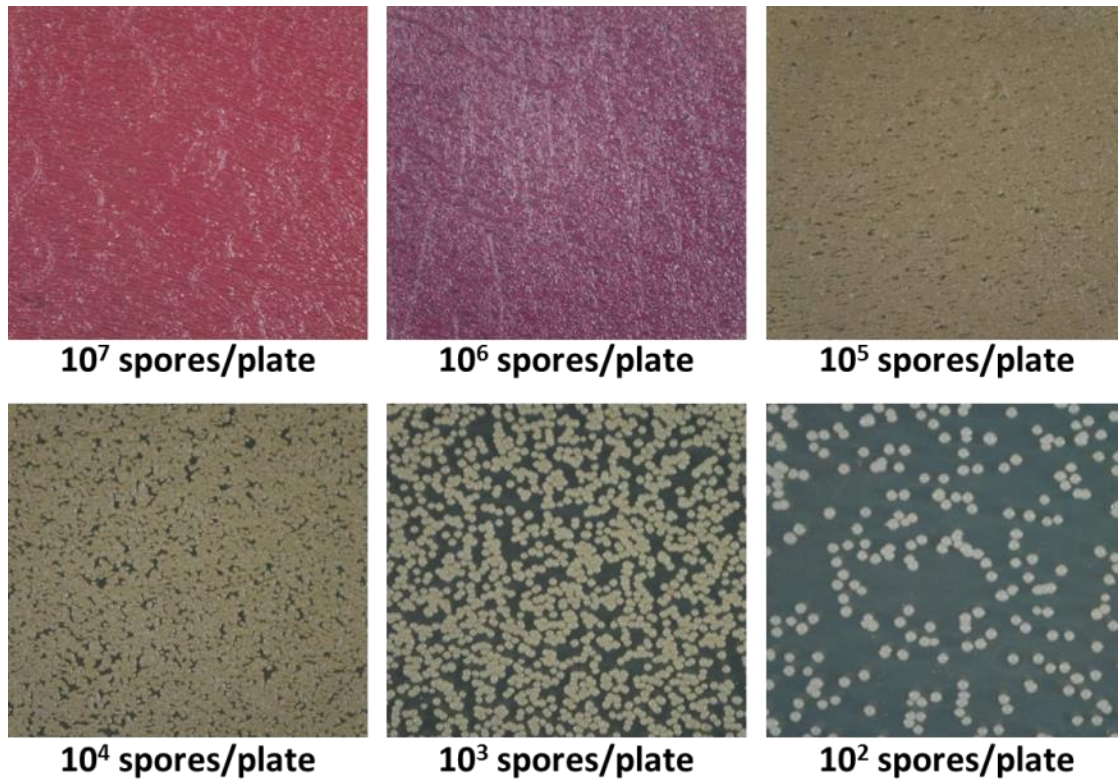
Figure 3.5 Density dependent production of RED. Six different titers of *S. coelicolor* M145 spores were plated and monitored for developmental changes, such as RED and ACT production, as well as spore formation.

3.3.4 B. subtilis Dowex elutions induce RED

As RED production was first observed at 40-42 hours at the highest plating

density, 10^7 spores per plate, this plating density was chosen for future experiments in

order to speed up the timescale to results. To observe the induction of RED from *B.*

subtilis extracts a dowex elution (see Materials and Methods) was applied to the highest plating density of *S. coelicolor* and observed for one week (Figure 3.6). Since *B. subtilis* 168, the domesticated strain, had the highest levels of bacilysin production, all future experiments are done with another domesticated *B. subtilis* strain, PY79, which is derived from 168 but does not have the auxotrophies of 168 (Zeigler*, et al.*, 2008). There was no discernable difference between *B. subtilis* 168 and *B. subtilis* PY79 in co-cultures with *S. coelicolor* or when their extracts were plated against *S. coelicolor* (data not shown). *S. coelicolor* turned on RED production around 42hrs when untreated. With *B. subtilis* extracts applied to *S. coelicolor* there was induction of RED before 37hrs, as seen by the bright red dot in the center of the 37hr image. Interestingly there was spore formation where the *B. subtilis* extracts were applied to *S. coelicolor*, when *S. coelicolor* rarely forms spores on GYM (as seen through a white pigment, characteristic of spore formation). A developmental link between antibiotic production and spore formation in *S. coelicolor* has been seen previously (Ueda*, et al.*, 2000, Perez*, et al.*, 2011).
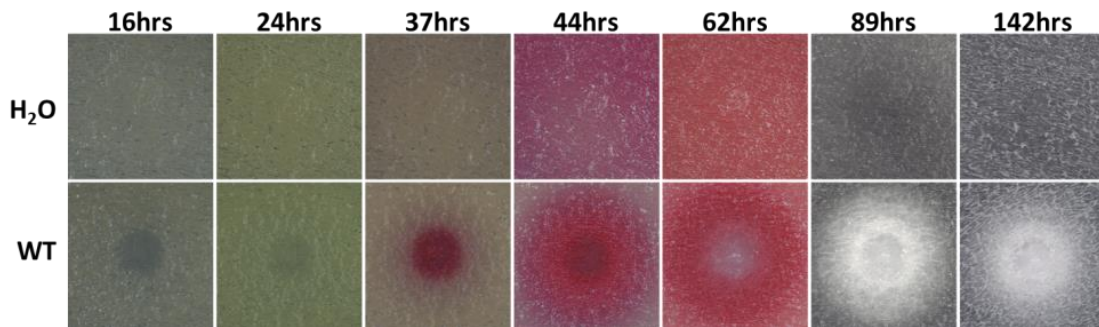
Figure 3.6. Induction of RED and spore formation in *S. coelicolor* when *B. subtilis* Dowex elutions are applied. 10^7 spores of *S. coelicolor* were plated onto GYM and spotted with Dowex elutions from *B. subtilis*.

3.3.5 Determining if bacilysin is the inducer of RED

In order to determine if bacilysin is the inducer of RED, as is hypothesized, purified bacilysin was plated on *S. coelicolor* (Figure 3.7); bacilysin was graciously donated by Jared Parker. When 20µg of purified bacilysin was applied to *S. coelicolor*, there was no induction of RED after 36hrs (Figure 3.7) or at later time points (data not shown). To confirm bacilysin was in an active form, it was also plated against a sensitive organism, as *Staphylococcus aureus* is used as a tester strain in many studies on bacilysin (Walker & Abraham, 1970, Perry & Abraham, 1979). There was killing of *S. aureus* (data not shown), confirming bacilysin was active even though there was no induction of RED in *Streptomyces coelicolor*. As a positive control for induction, Dowex elutions were again applied to *S. coelicolor*, showing induction of RED. To reaffirm the link to bacilysin (or at least *bacB*) Dowex elutions from a *bacB* background in PY79 showed no induction of RED (Figure 3.7).

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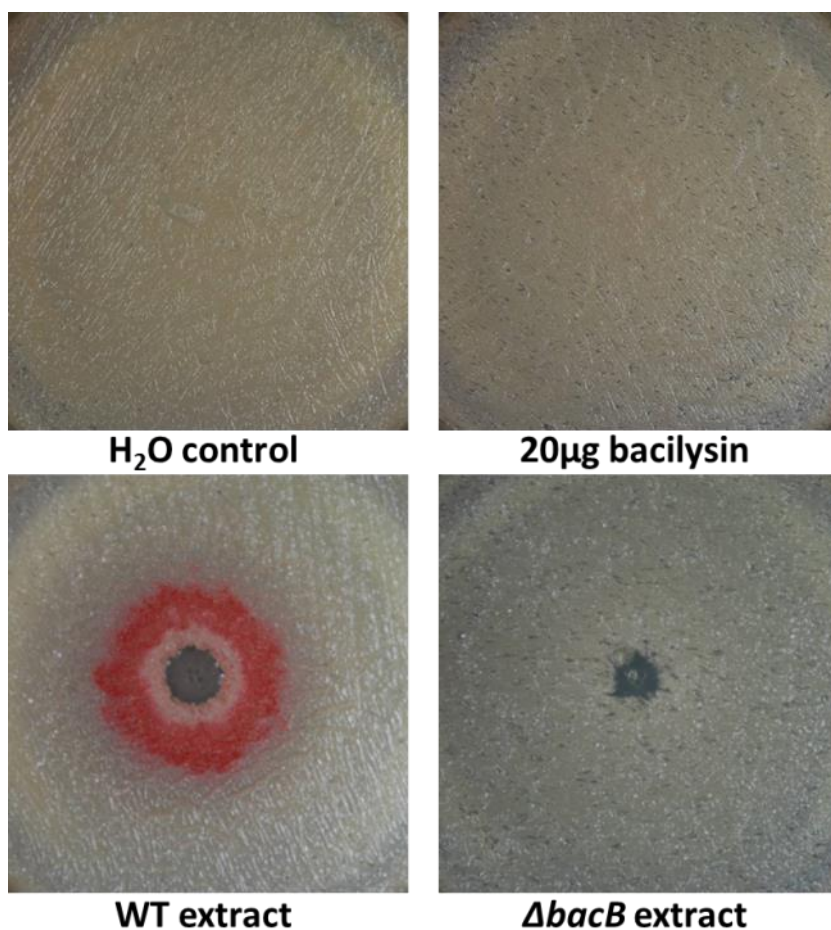
Figure 3.8. Bacilysin does not induce RED but the *B. subtilis* PY79 Dowex elution does. 10^7 spores of *S. coelicolor* were plated onto GYM and spotted with water, 20µg bacilysin (courtesy of Jared Parker) or Dowex elutions from *B. subtilis* PY79 wild type and *ΔbacB*.

Our proposed link between bacilysin and induction of RED was a regulatory gene in *S. coelicolor*, DasR. *DasR*, as previously stated, controls many processes within *S. coelicolor,* including regulation of the four most studied secondary metabolites of *S. coelicolor,* RED, ACT, CDA, and *cpk* (cryptic yellow polyketide) (Rigali*, et al.*, 2008). *DasR* had been shown to be involved in sensing nutrient stress but was repressed when bound by glucosamine-6-phosphate, the product of glucosamine synthase (Chmara,

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1985, Rigali, *et al.*, 2008). While bacilysin inhibited production of GlcN-6-P we

theorized that the link might be more complicated. Once bacilysin was shown to not be

the inducer of RED we still wanted to test the *dasR* connection. When testing the *dasR*

mutant (BAP29 from van Wezel) we saw that RED was still induced by *B. subtilis*

extracts (Figure 3.9), therefore, the master regulator DasR is not involved in the *B.*

subtilis mediated RED induction.
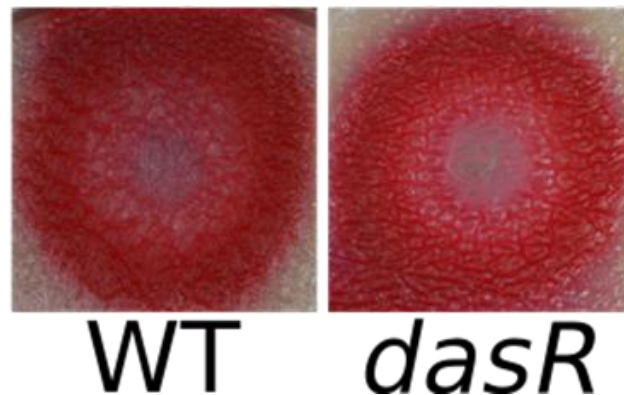
Figure 3.9. Dowex elutions from wild type *B. subtilis* PY79 plated on wild type and *dasR S. coelicolor* M145. There is no loss of induction of RED when the master regulator DasR is disrupted.

      While the master regulator DasR is not involved in induction of RED by *B.*

subtilis, there potentially is a link to a morphogenesis regulator. Observing the same

plates spotted with Dowex elutions from wild type and *ΔbacB B. subtilis* PY79, over an

extended time course, similar to Figure 3.6, showed an interesting result. A sporulation

inducing compound was present in wild type *B. subtilis* extracts, but not in *ΔbacB*

(Figure 3.10). This molecule could be the same compound as the RED inducing

compound or it could be another compound. There have been reports of developmental

links between secondary metabolite production and sporulation (Perez*, et al.*, 2011, van

Wezel & McDowall, 2011).
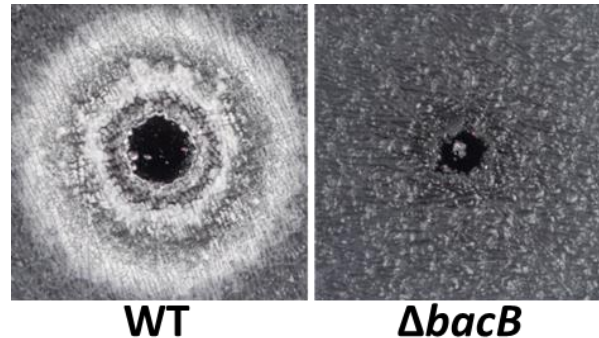
Figure 3.10. *B. subtilis* Dowex elutions contain an inducer of morphogenesis. 10^7 spores of *S. coelicolor* were plated onto GYM and spotted with Dowex elutions from *B. subtilis* PY79 wild type and *ΔbacB*. These are the same plates from Figure 3.8 but after ~140hrs.

As bacilysin is not the inducing molecule there could be multiple reasons why

the inducing molecule is no longer produced in a *bacB* strain. When bacilysin is not

made either precursors of bacilysin are shunted into another metabolic pathway or

byproducts of bacilysin production are also no longer made, such that the inducing

molecule is then deprived of some component. Alternatively bacilysin could have an

intracellular signaling role within *B. subtilis* which has not been elucidated. Bacilysin

inhibits glmS (Chmara, 1985), it has yet to be shown if bacilysin inhibits one or both of

B. subtilis's two glmS enzymes. If bacilysin did inhibit either of the enzymes then this

could cause a developmental transition within *B. subtilis*, possibly when the organism

slows down growth (resulting from depletion of glucosamine-6-phosphate). Due to the genetics in the deletion of *bacB,* another possibility could simply be that either *bacB* or one of the downstream genes in the *bac* operon is involved in the biosynthesis of the inducing molecule. The deletion of *bacB* was done with a resistance marker and multiple transcriptional terminators inserted in place of the gene. As *bacB* is the second gene of the six gene operon, it is impossible to tell which gene downstream of *bacB* could be involved in production of the inducing molecule (Inaoka*, et al.*, 2003, Steinborn*, et al.*, 2005).

3.3.6 Determining purification conditions for the inducer of RED

When comparing wild type and *bacB* Dowex elutions via LC/MS there was no detectable molecule in wild type *B. subtilis* that was not also in *ΔbacB* (work done by Jared Parker, data not shown). In order to purify the inducer of RED a new purification step in the purification strategy needed to be developed before LC/MS to detect a difference between wild type and *ΔbacB*, because there is a phenotypic difference in *S. coelicolor* when plated with the different Dowex elutions (Figure 3.8 and 3.10).

One step which produced an observable difference was growing *B. subtilis* for 48hrs as opposed to 24hrs. Dowex elutions were prepared from wild type and *ΔbacB B. subtilis* PY79 and plated at different dilutions (Figure 3.11). Unlike previous Dowex elutions, there was a faint zone where *ΔbacB* induced RED. The *ΔbacB* strain also produced higher levels of some kind of antibiotic which inhibited the growth of *S. coelicolor*. There is also an inhibition of growth in the wild type Dowex elution, as seen

by a light clearing and lower production of RED where the Dowex elution was applied, which decreases as the elution is diluted (Figure 3.11 WT 1x to 0.25x dilutions, note the intensity of RED at the center). The production of a growth inhibiting factor was seen for wild type as well as *ΔbacB* (Figure 3.8) especially if the Dowex elution was at higher concentrations (data not shown). When plating the Dowex elutions against a lower titer of *S. coelicolor* spores there is killing (or inhibition of growth) for both wild type and *ΔbacB*, although there is no induction of RED in *ΔbacB*. The lack of induction of RED from *ΔbacB* at a lower titer of spores is presumably due to the delayed developmental process, as was seen for lower titers of spores in Figure 3.5.
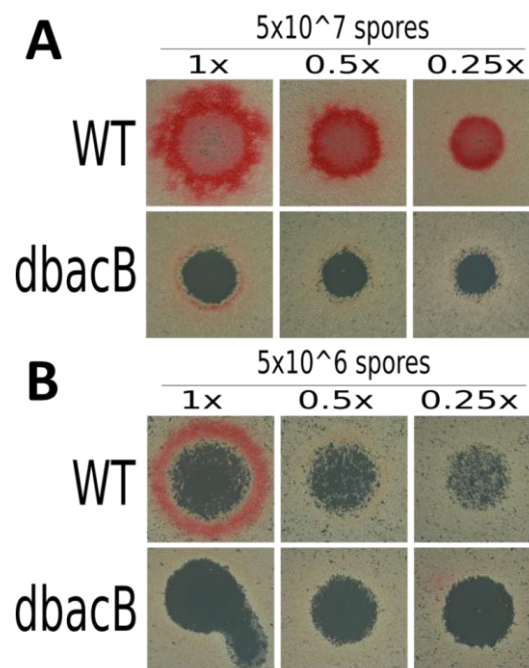
Figure 3.11. Dowex elutions from wild type and *ΔbacB* after 48hrs of *B. subtilis* growth. 10^7 spores (Panel A) or 10^6 spores (Panel B) of *S. coelicolor* were plated onto GYM and spotted with Dowex elutions from *B. subtilis* PY79 wild type and *ΔbacB*. The weird shape in panel B, *ΔbacB* applied with 1x Dowex elution is presumably due to the incomplete drying and the liquid spreading when the plate was inverted.

To better understand the nature of the inducing molecule supernatants from wild type and *ΔbacB B. subtilis* PY79 were extracted with a variety of solvents which ranged in polarity, dried under pressure, filter sterilized and plated (Figure 3.12). When plated against *S. coelicolor* hexanes, butanol, and chloroform extracts of wild type PY79 showed induction of RED, while only the butanol extract of *ΔbacB* showed induction of RED. This is additional evidence that *ΔbacB* produces the inducing molecule, just in concentrations much lower than wild type *B. subtilis*. Of note is that there is again production of some antimicrobial/growth inhibiting compound, and it is produced at higher levels in *ΔbacB* than wild type *B. subtilis*.
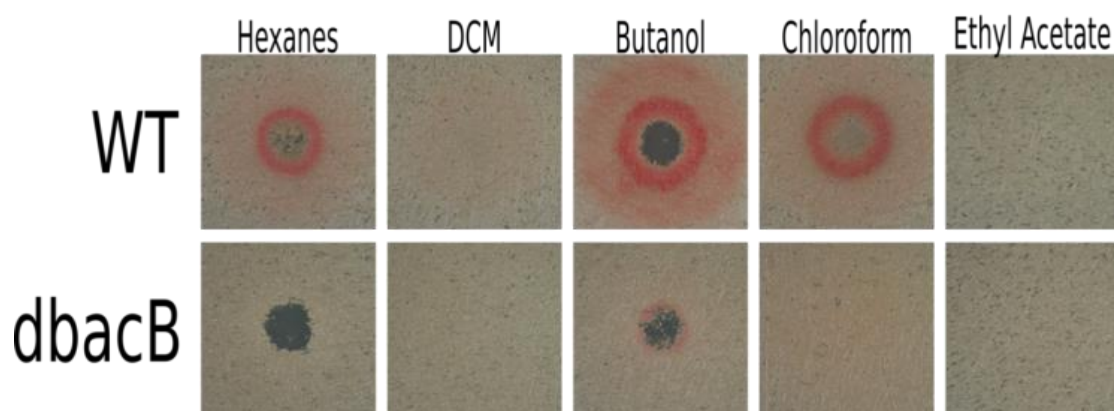
Figure 3.12. Solvent extractions of *B. subtilis* supernatants plated against *S. coelicolor*. 10^7 spores of *S. coelicolor* were plated onto GYM and spotted with solvent extractions of wild type and *ΔbacB B. subtilis* supernatants from cultures grown in PA media.

A series of co-culture experiments between *S. coelicolor* and *B. subtilis* PY79, wild type and *ΔbacB* further showed that *ΔbacB* produces the inducing molecule, as seen

through plating the *B. subtilis* strains against *S. coelicolor* at a high plating density of

spores (Figure 3.13). Decreased levels of the inducing molecule produced by *ΔbacB*

may have not induced RED production at low levels of spores, but at higher levels of

spores there was clearly induction (Figure 3.13 bottom vs. top panels). While there is

less induction of RED in the lower dilution of *S. coelicolor* spores compared to the

higher dilution, there is more induction in co-cultures of wild type *B. subtilis* compared

to *ΔbacB B. subtilis.*
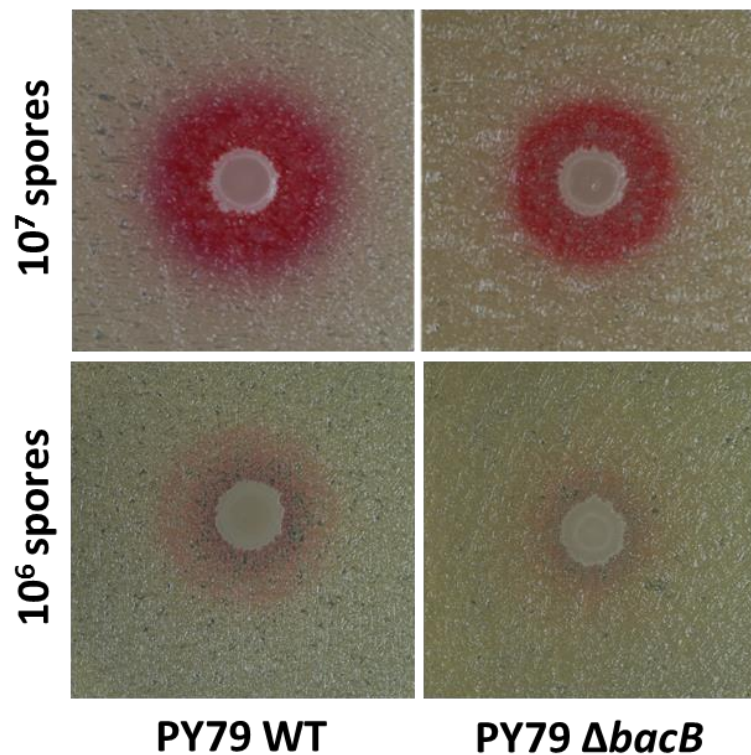
Figure 3.13. Co-cultures of wild type and *ΔbacB B. subtilis* PY79 versus different spore densities of *S. coelicolor.* 10^7 or 10^6 spores of *S. coelicolor* were plated onto GYM and spotted with overnight cultures of wild type and *ΔbacB B. subtilis* grown in PA media.

3.3.7 Fractionation of Dowex elutions to further isolate the inducer of RED

After it was determined that *ΔbacB B. subtilis* still produces the inducing

molecule steps were taken to isolate the inducing molecule from wild type *B. subtilis* but

not *ΔbacB*. In order to deduce some molecular properties of the molecule a variety of

tests were performed on Dowex elutions from wild type *B. subtilis*. The elutions were

boiled and subjected to ProteinaseK treatments to see if the molecule was proteinaceous,

in both cases the inducing molecule was resistant to the treatments, indicating that it was

not a large peptide (data not shown). Dowex elutions brought up to a high pH (>12)

were still able to induce RED (data not shown), indicating that the molecule is somewhat

stable in the presence of basic conditions. Dowex elutions were also dried under

pressure and resuspended in various organic solvents in order to d solubility of the

molecule for later use in extractions. The resuspensions were spun down and the

supernatants removed from the pellets. Both fractions were dried under pressure,

resuspended in water and filter sterilized. When plated against *S. coelicolor* the inducing

molecule appeared to be soluble in only two solvents, chloroform and methanol (Figure

3.14). While the soluble fraction from chloroform shows a purplish color as opposed to

RED, the color could be due to induction of RED and actinorhodin (ACT) or just

induction of ACT. ACT is the compound for which *S. coelicolor* gets its name, as it

changes color between blue and red in a pH-dependent manner (Bystrykh*, et al.*, 1996).

ACT is also a normal developmental process in *S. coelicolor*, but the media used in this

study (GYM) contains glucose, which is known to repress ACT production (Tiffert*, et

al.*, 2008). In Figures 3.5 and 3.6 there is a purple color before RED. In Figure 3.5 the

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purplish color is in the 10^6 spore dilution while in Figure 3.6 the purplish color appears

at the 44hr time point before the plate is completely red at the 62hr time point. As all of

the organic solubility tests were done with the same amount of Dowex elution, they all

should have the same amount of RED inducing activity either in the soluble or insoluble

fractions. The ethyl acetate and butanol fractions appear as if some of the inducing

molecule was degraded, as there is faint inducing activity. The inducing molecule from

the chloroform fractionation test may have been split between the soluble and insoluble

fractions, as there is induction in both of the fractions.
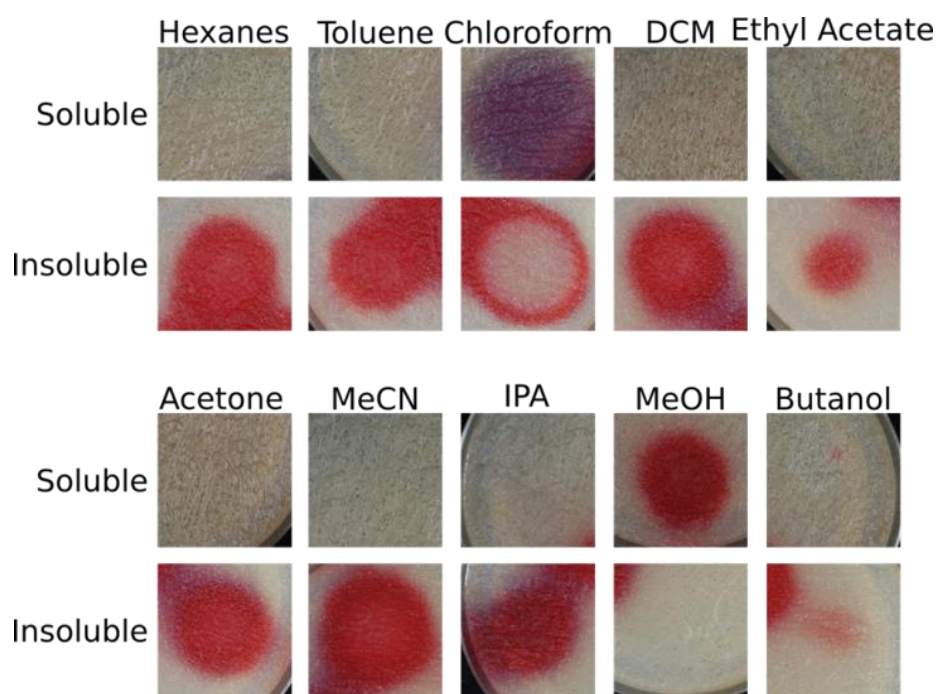
Figure 3.14. Solvent solubility tests of *B. subtilis* Dowex elutions plated against *S. coelicolor*. Ten 100µL aliquots, of a typical wild type *B. subtilis* Dowex preparation, were separately dried and resuspended in 1mL of various organic solvents. The samples were spun down, pellets and supernatants both dried and resuspended in 100µL of water. 20µL of each fraction was plated against 10^7 spores of *S. coelicolor*. DCM – dichloromethane, MeCN – acetonitrile, IPA – isopropyl alcohol, MeOH – methanol.

As the inducing molecule was soluble and stable in methanol a series of fractionations of chloroform and methanol were pursued, so as to be able to fractionate the molecule using normal phase separation (Figure 3.15). Presumably there is no soluble inducing activity in the chloroform fraction due to a lower concentration of the Dowex elution being used in this step than compared to Figure 3.14. After using a ratio of chloroform to methanol of 90/10 there was some solubility of the inducing molecule, while at the 80/20 ratio there was complete solubility of the inducing compound. Of interest is the phenomenon where induction of RED at 37hrs is correlated to accelerated spore formation at 61hrs, which is never seen on GYM. Induction of early secondary metabolite production and sporulation has been seen in streptomycetes (Ueda, *et al.*, 2000, Igarashi, *et al.*, 2001, Onaka, *et al.*, 2001) and *S. coelicolor* in particular (Igarashi, *et al.*, 2001, Perez, *et al.*, 2011). Accelerated development could lead to early induction of secondary metabolites then ultimately sporulation, so it is of interest whether induction of sporulation is directly tied to induction of RED or if the two processes are separated.
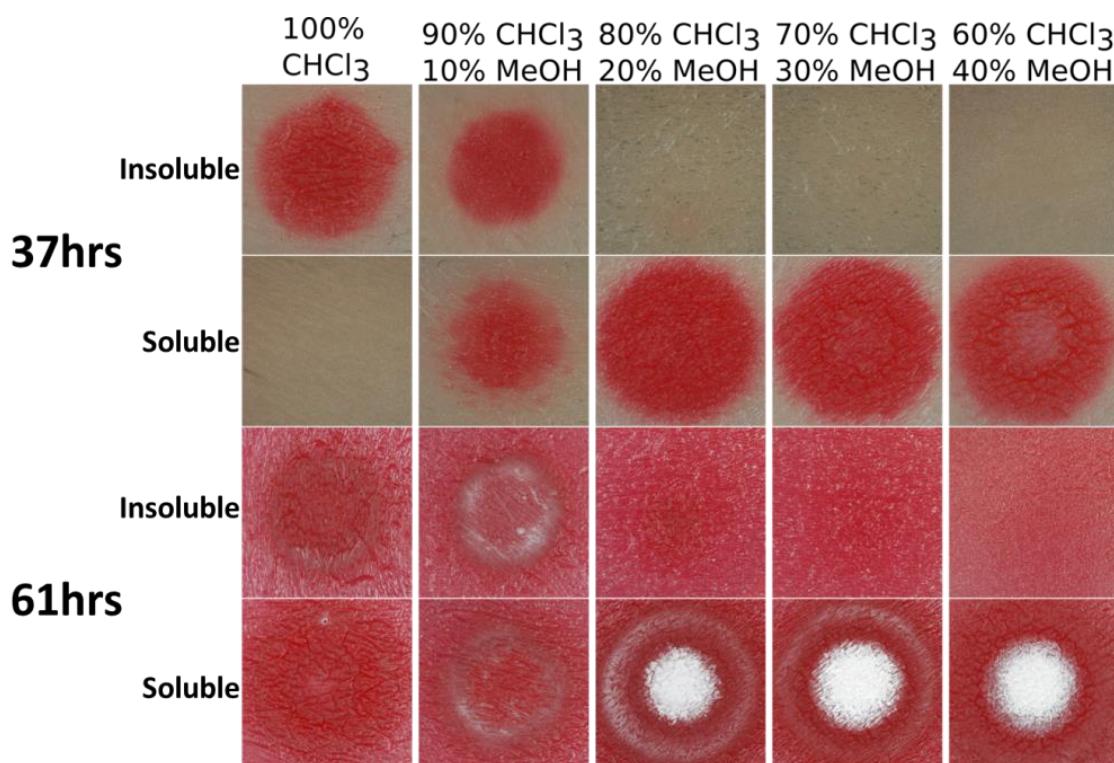
Figure 3.15. Methanol/chloroform solubility of the inducing molecule. Dowex elutions from wild type *B. subtilis* PY79 were reduced to dryness then resuspended in chloroform/methanol at 10% steps from 100% chloroform to 100% methanol. The lower ratios are shown. The 37 and 61 hour time points are shown.

Once conditions were determine to allow reverse phase and normal phase separation a variety of SPE cartridges were used: C8, C18, diol, cyano, NH_2, and silica. C8, C18, cyano, and silica elutions are not shown, the molecule was able to bind the C18 column, but eluted in the first fraction (data not shown). Often times the compound would not bind to the columns if the Dowex elution was too concentrated. To overcome this problem the Dowex elutions were pre-loaded onto the resin by taking the resin out of the cartridge, adding the Dowex elution too it, and drying under pressure. The molecule eluted off of the various resins at different concentrations of solvent for each of the various resins Figures 3.16 (diol and NH_2). The inducing molecule was also able to

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bind to silica resin, but it bound somewhat irreversibly, it did not elute off as well as for

the diol or NH$_2$ resins (data not shown).
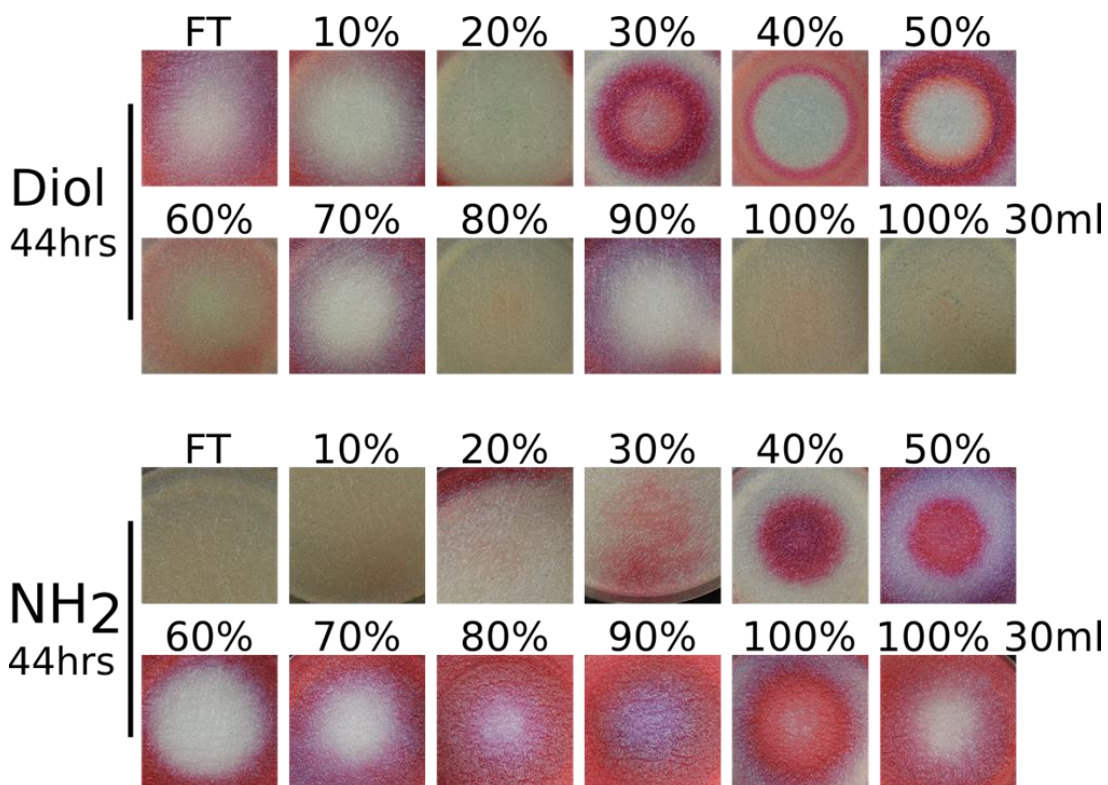
Figure 3.16. Dowex elutions of wild type *B. subtilis* PY79 applied to diol and NH$_2$ resins eluted and applied to *S. coelicolor* M145. After drying the Dowex elutions onto the resin the samples were eluted off in increasing 10ml steps of methanol/chloroform, starting at 0% methanol/100% chloroform (when resin was removed from the rotovap and added into the column, labeled as FT) then 10% increases in the methanol concentration through 100% methanol/0% chloroform. A final 30ml methanol wash of the resin was performed to elute anything stuck to the column.

Figure 3.16 shows elution characteristics of the inducing molecule after a Dowex

elution was pre-adsorbed to the two resins (diol and NH$_2$) under pressure, and then

eluted off. It appears as if the inducing compound eluted off of the diol resin at 30%

methanol/70% chloroform. At the 40% and 50% fractions of methanol/chloroform there

could have been another molecule eluting off which inhibits the production of pigmented

secondary metabolites. Previously we had drawn a link between bacilysin and DasR.

Bacilysin inhibits glmS, responsible for producing Glc-N-6P (Chmara, 1985). Glc-N-6P

binds to DasR, preventing it from repressing secondary metabolism (Rigali, *et al.*, 2008).

By preventing the production of Glc-N-6P bacilysin could be repressing antibiotic

biosynthesis in neighboring organisms.

3.3.8 RED may autoinduce through a quorum sensing mechanism

Finally, through repeated experiments the observation was made that RED

induction spreads throughout a plate of *S. coelicolor*. If multiple samples were spotted

on the same plate of *S. coelicolor* there would often be a larger induction effect than if

the samples were spotted on separate plates. This phenomenon may be a result of a

quorum-sensing mechanism wherein RED triggers production of γ-butyrolactone or if

RED is a quorum-sensing molecule involved in inducing production of itself. Figure

3.17, panel A, shows the flow through, 5% methanol, and 10% methanol fractions from

a wild type *B. subtilis* PY79 Dowex elution applied to a C18 SPE cartridge and overlaid

on the same plate of *S. coelicolor*. There is extensive induction of RED throughout most

of the plate. At the same time, on separate plates of *S. coelicolor,* the same fractions

were also plated but only one sample was spotted on a plate (Figure 3.17, panel B), as

opposed to the three that were spotted on the same plate seen in Figure 3.17A. When

plating the same fractions on separate plates of *S. coelicolor* the induction of RED was

limited to the through flow from the column, not spread throughout the plate. This

phenomenon is not isolated. When looking at plates from the solvent solubility

experiments (shown in Figure 3.14), which had multiple samples spotted on each plate,

there appears to be induction between fractions which had high levels of the inducing

molecule. In Figure 3.17, panel C, there is induction between samples plated which had

the inducer (Figure 3.17C top image) but the induction is not seen between samples with

lower levels of the inducing molecule (Figure 3.17C bottom panel). This could be due to

a quorum sensing mechanism involved in the induction of RED (Williamson, *et al.*,

2006) wherein RED induces production of itself or factors involved in RED induction

are also secreted, inducing RED locally. An alternative explanation could be that the

induction of RED causes what nutrients remain in the media to be used up in the

production of RED. Once streptomycetes sense a lack of nutrients (or unfavorable

conditions) and they undergo morphogenesis to produce spores ensuring further

generations, this process involves the production of secondary metabolites, such as RED.
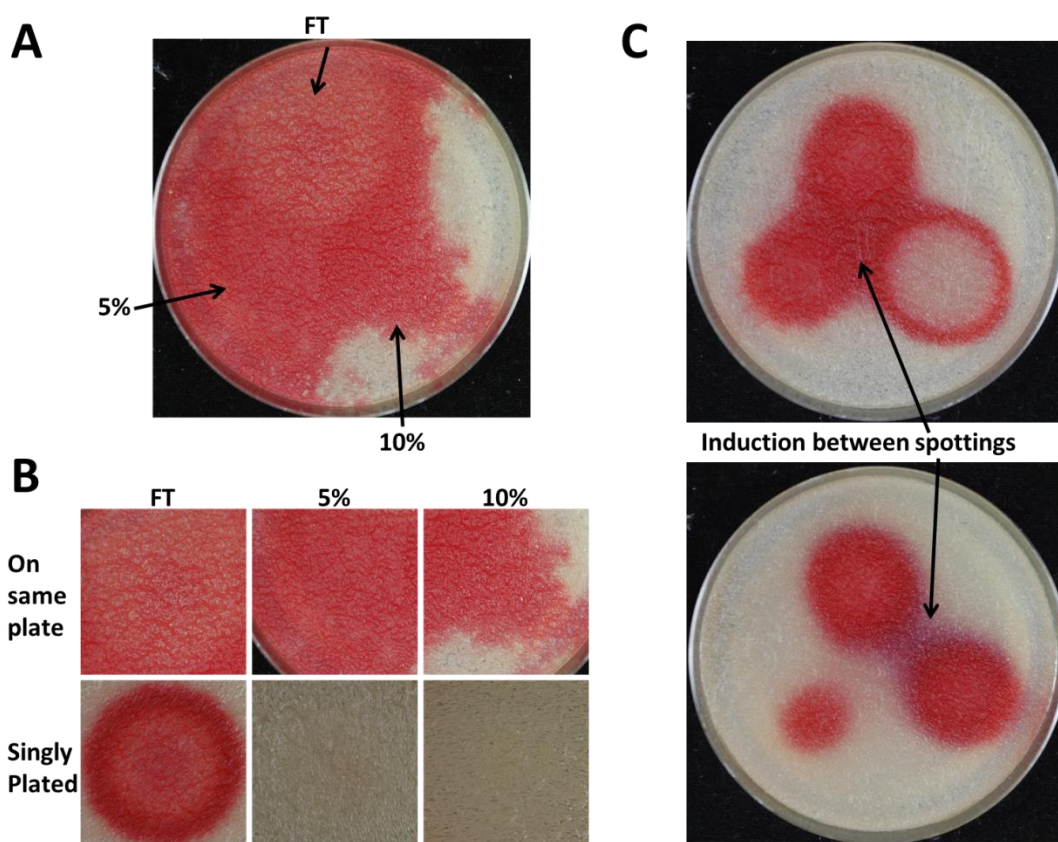
Figure 3.17. Possible quorum sensing induction of RED. Dowex elutions from wild type *B. subtilis* PY79 were applied to a C18 SPE cartridge, and fractions collected, the flow through (FT), 5% methanol/water, and 10% methanol/water fractions are shown plated on the same plate (panel A), and zoomed in on where the samples were spotted (panel B top images). The same samples were plated at the same time on different plates (panel B bottom images). The localized induction of RED between inducing samples is seen on plates from Figure 3.14, when looked at the whole plate.

To avoid quorum-sensing induction of RED as a result of sub-inducing

concentrations of an inducing molecule another strategy to monitor induction of RED

could be employed. A previous study looking at the induction of ACT with a synthetic

chemical compound library used liquid cultures in 96-well plates (Craney*, et al.*, 2012).

This method uses fewer spores, less media, less inducing compounds, and enables

samples to be separated from each other, resulting in a more reliable, less costly

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experiment when screening for the inducing compound. Furthermore, induction could be quantified through colorimetric methods (i.e. using a plate reader to measure absorbance of induced RED compounds). The method would have to be optimized, but in the end would result in cheaper experiments with greater reproducibility.

3.4 Future Directions

While purified bacilysin did not induce RED, the genetics indicated that there is a link between bacilysin production and the inducing molecule (Figure 3.2 shows Δ*bacB* PY79 vs. *S. coelicolor* without induction of RED). When lyophilized supernatants from WT PY79 and Δ*bacB* PY79 were applied to *S. coelicolor*, WT supernatants induced early production of RED in *S. coelicolor* whereas Δ*bacB* supernatants did not (Figure 3.3). To reproduce induction of RED from lyophilized supernatants but without salt, proteins, or other cell debris, the Dowex step from the purification of bacilysin showed that wild type extracts but not *ΔbacB* induced RED. Furthermore, the fractions which induced RED also caused other developmental processes to proceed earlier than normal (Figure 3.6). This includes early production of a blue pigment actinorhodin (ACT) (data not shown) as well as early sporulation, seen through the white pigment characteristic of spores (Figure 3.6). Accelerated morphogenesis causing early production of secondary metabolites (RED and ACT) as well as rapid sporulation could be from a build-up of internal signaling molecules produced early as a result of RED induction, or it could be that the inducer of RED is targeting a regulator which also controls these processes in *S. coelicolor*. Developmental processes in *S. coelicolor* are linked through multiple layers

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of regulation (Bibb, 2005), so induction of RED could conceivably impact other processes. A recent study also reported that *B. subtilis* induced early ACT production and sporulation (Perez*, et al.*, 2011), so the inducing molecule of RED could be tied to a regulator responsible for all three.

Identifying the molecule(s) involved in induction of RED will allow for determining the mechanism of induction in *S. coelicolor*. If the induction is not just a by-product of a metabolic flux, and there is a specific mechanism then it could be conserved amongst streptomycetes. Induction of a specific metabolite (RED) by an unknown molecule from another organism may yield a tool to elicit cryptic metabolites from streptomycetes.

Several studies have noted the increase in RED from the addition of various compounds (Onaka*, et al.*, 2001, Craney*, et al.*, 2012) or conditioned media (Luti & Mavituna, 2011). None of these works address a specific mechanism of induction of RED. Furthermore, there is no report of an identified natural product which induces production of RED. How would *S. coelicolor* respond to a neighboring organism? We hypothesize induction is not caused by affecting a specific regulator of RED. A defense mechanism where *S. coelicolor* turns on RED early against one particular molecule produced probably only by a handful of organisms would be very costly. *S. coelicolor* would have to have tens to hundreds of such regulators to compete with its neighbors. Instead, we hypothesize that induction of RED is due to a stress-sensing mechanism caused by an altered metabolic flux in *S. coelicolor*. An altered metabolic flux could be sensed through a general stress-sensing mechanism, wherein an essential primary

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metabolite is no longer available and the cell turns on developmental processes as a result, similar to the onset of stationary phase in other bacteria. Alternatively the inducing molecule might relieve repression of regulators of developmental processes, affecting a metabolite-responsive *GntR*-like repressor (Hoskisson & Rigali, 2009). Regardless of which metabolite is the inducer of RED, the mechanism of induction will need to be further characterized. Establishing the genetics required for the mechanism of induction will come from mutagenizing *S. coelicolor* then applying the inducer molecule to see which mutations no longer give an early induction of RED.

Many technical issues of transposition in streptomycetes have been overcome in recent years with successful identification of many regulators or other proteins involved in antibiotic biosynthesis in *S. coelicolor* coming from transposon mutagenesis (Hillerich & Westpheling, 2006, Hoskisson*, et al.*, 2006, Ou*, et al.*, 2009, Petzke & Luzhetskyy, 2009, Lamb*, et al.*, 2010, Fernandez-Martinez*, et al.*, 2011, Hull*, et al.*, 2012). Chemical or UV mutagenesis of *S. coelicolor* to search for factors regulating the early induction of RED is one possibility to consider and has been done with other streptomycetes (Yu*, et al.*, 2011). All types of mutagenesis will give a background of mutations in the RED gene cluster, but transposon mutagenesis will allow for the easier identification of where the mutations occur in the genome. This is due to the ability to recover plasmids from mutants with transposon insertions or through the use of arbitrary PCR linked to a sequence in the transposon.

Relatively simple experiments to determine conditions involved in the early induction of RED would be to grow *S. coelicolor* under different nutritional conditions

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then adding the molecule. This could help determine if carbon, phosphorous or nitrogen limitations activate or repress the phenotype induced by the inducing molecule in the initial screen. The induction of RED might be blocked by regulators expressed as a result of different experimental conditions than those used in the initial screen. For instance, actinorhodin is repressed by glucose (van Wezel & McDowall, 2011), since the initial screen was done on GYM (**g**lucose, **y**east extract, **m**alt extract) any induction of actinorhodin would have been suppressed. Determining which nutritional conditions yield induction of RED in response to the metabolite could narrow down the window of regulators involved. This would allow us to limit the potential regulators to those that are expressed under a particular set of conditions which induce RED when the inducer is present but not when the inducer is absent.

Identifying the optimal conditions where early induction of RED is clearly turned off in the absence of the inducer molecule would also make a microarray experiment easier to interpret. A microarray experiment, comparing WT *S. coelicolor* with or without the inducing molecule could identify potential regulators involved in the early production of RED. Up or down regulation of putative regulators, at a time when RED is induced early with the inducing molecule but not without the molecule, would narrow the pool of potential regulators affected by the inducer molecule. A comparison with studies that have already identified altered transcription of putative regulators when RED is turned on could further elicit which ones are involved in the early induction of RED (Huang*, et al.*, 2001, Hesketh*, et al.*, 2007, Lee*, et al.*, 2010). Further experiments comparing altered transcriptional profiles when tested against the metabolite amongst

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various streptomycetes versus a *S. coelicolor* microarray could identify a common regulator within the various species (Kirby, *et al.*, 2011).

There will continue to be a growing need for new bioactive compounds, especially those with novel scaffolds. Furthermore the pharmaceutical industry has left behind natural product discovery and all but abandoned antibiotic research in favor of combinatorial chemistry (Baltz, 2006). Previous drug discovery focused on the low throughput practice of fermentation followed by bioassays for activity. This approach stopped yielding as many new compounds as many of the same compounds were rediscovered. For instance streptomycin is produced by ~1% of streptomycetes, making its rediscovery rate quite high, whereas daptomycin is only produced in ~10^{-7} of streptomycetes (Baltz, 2008). The traditional approach to discovering secondary metabolites has 'plucked the low hanging fruit,' most often coming up with generic compounds like streptomycin while rarely producing unique compounds like daptomycin. With traditional fermentation techniques rediscovering the same metabolites a new approach should be taken. In light of the largely untapped genetic potential of streptomycetes to produce secondary metabolites, there is growing momentum to try to elicit these cryptic metabolites.

The goal through searching for an inducer of RED is ultimately to elicit cryptic metabolites. Two previous studies have identified two molecules produced by streptomycetes which induce other streptomycetes to produce antibiotics, goadsporin and promomycin (Onaka, *et al.*, 2001, Amano, *et al.*, 2010). Our attempt to find a general inducing molecule falls along these story lines yet is different for two reasons.

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First, our inducing molecule is not produced by a streptomycete. Therefore *S. coelicolor* is responding to a neighboring organism rather than an ancient quorum-sensing signal. For this reason we hypothesize that our signaling molecule would affect many streptomycetes. Mounting a defense response to a neighboring organism would seem to be more conserved than retaining a receptor for an ancient signal. The ability to respond to a stress caused by a neighboring organism illustrates that any molecule induced would probably have a bioactive property, either to negate some sort of stress or to have an effect on the neighboring organism. Looking for molecules with bioactive properties would be more productive in terms of drug discovery than looking for the induced production of streptomycete hormones, which may just serve to signal environmental conditions.

The second major difference, unlike the other two studies of inducing molecules, is that the responding strain used in this study, *S. coelicolor,* is a sequenced model organism for streptomycetes. This will allow a mechanism for elicitation of secondary metabolites, to use for rational elicitation of secondary metabolites from other streptomycetes. Once an inducing compound is identified testing it against other streptomycetes would determine if there is induction of metabolites which would not otherwise be produced in monoculture.

CHAPTER IV

CONCLUSIONS

The overall goal of this thesis was to identify the cause of surfactin hydrolysis by *S.* Mg1. By analyzing secreted proteins, cloning and expressing in a heterologous system, followed by *in vitro* assays, a candidate enzyme was discovered. This study is the first time an enzymatic function has been proposed through MALDI-IMS and then subsequently identified to a protein. The enzymatic mechanism hypothesized was that of degrading a xenobiotic. That *S.* Mg1 degrades a foreign compound, with antagonistic properties against streptomycetes, shows that the surfactin hydrolase enzyme is a resistance mechanism to antibiotics, giving *S.* Mg1 an advantage over other streptomycetes when surfactin is present.

Further characterization of the enzyme showed that the hydrolase was capable of degrading multiple compounds and that it changed substrate specificity with the addition of a divalent metal cation is also interesting. Rather than losing substrate specificity for two both molecules that it was previously able to hydrolyze, it lost it for one molecule but not the other, surfactin and plipastatin, respectively. Future characterization of this enzyme could further determine if the enzyme has more substrates, suggesting its evolution against many neighboring organisms. Biofilm formation by *B. subtilis* is required for some plants to be protected against pathogens. As surfactin is required for

biofilm film formation, the surfactin hydrolase could have more roles in the environment than described in this work.

The second project presented was elicitation of undecylprodigiosin by *B. subtilis*. While purified bacilysin did not induce RED, there is a genetic link between the deletion of *bacB* and decreased induction of RED. Fractions of *B. subtilis* extracts which induced RED also caused other developmental processes to proceed earlier than normal. This could be from a build-up of internal signaling molecules produced early as a result of RED induction, or it could be that the inducer of RED is targeting a regulator which also controls these processes in S. coelicolor. Identifying the molecule(s) involved in induction of RED will allow for determining the mechanism of induction in *S. coelicolor,* which could lead to discoveries in inducing cryptic metabolites in other streptomycetes. The identification of new molecules with antibacterial properties is essential for public health, as only the continual discovery of new antibiotics will prevent antibiotic resistance from returning infectious diseases as the number one killer of humans.

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