The Antimicrobial Activity of *Bacillus subtilis* strain CU12

By

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ABSTRACT

Major drawbacks of synthetic chemicals have led to an increase in biocontrol agents used to control phytopathogens. In this study, we investigated *Bacillus subtilis* strain CU12 and the compounds associated with its antifungal activity. The objectives were to characterize the antifungal activity, to isolate and identify the active compound(s), and to analyze the fungal cellular lipid composition to gain insight into the mode of action of the antimicrobial fengycin. Results showed that *B. subtilis* CU12 was active against a broad range of phytopathogens with varying sensitivities. One of the antifungal compounds found was 3-hydroxypropionaldehyde (3-HPA), which is reported for the first time in *B. subtilis*. Our results also indicated that sterol content and, to a lesser extent, ratio of anionic/zwitterionic phospholipids may be involved in the mode of action of fengycin produced by *B. subtilis* CU12. Results from this study are useful in pursuing field testing of the antagonist and essential in the process of potential registration of *B. subtilis* CU12 as a biocontrol agent.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 . PATHOGENS AND THE AGRICULTURAL SYSTEM

The current human population of approximately 7 billion people is expected to increase by 30% to 9.2 billion in 2050 (Popp *et al.*, 2013). An increase in population of this magnitude will bring about many new problems and challenges. Of the factors to consider with the rising population, the security and sustainability of our food supply is amongst the top of the list. This increase in population could yield up to a 70% increase in food demand (Popp *et al.*, 2013). Past food shortages have been solved by exploitation of new food sources and a concurrent increase in land devoted to agriculture (Godfray *et al.*, 2010). In the past five decades, the demand for grain has almost doubled, yet globally, the amount of arable land has only increased by 9% due to the need of space for other human activities (Godfray *et al.*, 2010). Coupling the need for space and food, it becomes obvious that we must utilize the current lands to the fullest in terms of crop yield. Not only must we attempt to grow food on smaller areas of land, but we must also attempt to use less water, energy, fertilizer, and effectively control pests to reduce losses.

Currently, for fruits and vegetables alone, approximately 35-55% of all food produced is lost prior to consumption (FAO, 2011). Of the numerous steps in the food supply chain, agriculture, post-harvest storage, processing, distribution, and consumption, roughly 80% of all the losses occur post-harvest (FAO, 2011). One of the most significant factors in these losses is phytopathogens.

Crops and overall crop yield have always been affected by phytopathogenic fungi. Crop losses by phytopathogens are difficult to quantify, but it has been estimated that pests (pathogens, insects, and weeds) collectively account for 27-42% of all losses in production from

major crops worldwide, however, without means to protect these crops this value would rise to approximately 48-83% (Glare *et al.*, 2012).

1.2. CURRENT CONTROL MEASURES

Current methods used to control plant pathogens consist mainly of the application of synthetic chemical pesticides both in the field and post-harvest (Tripathi and Dubey, 2004). The current method of pest control suffers two major drawbacks in that the fungus may develop a resistance to the treatment and the chemical may cause adverse effects on human health and the environment (Avis, 2007). Overuse of these chemicals has led to loss or decrease in their efficacy, toxicity to the environment, and harmful effects on the non-target organisms, which collectively affect their commercial applicability (Ongena and Jacques, 2008). Many of the chemical fungicides (antifungal pesticides) used in the past have been deemed harmful, banned, and are not being replaced (Janisiewicz and Korsten, 2002). Currently it takes approximately 10 years and \$250 million to identify, test, and register a new synthetic chemical for use as a pesticide (McDougall, 2010). It is estimated that only 1 in 140,000 chemicals tested actually become acceptable synthetic pesticides (McDougall, 2010). The high cost of developing and registering new synthetic pesticides combined with short term efficacy as a result of increasing rate of resistance in pests has led to a decline in the use of synthetic chemicals to control plant pathogens (Glare *et al.*, 2012; Ongena and Jacques, 2008). With the reduction in the use of synthetic chemicals comes a need for more durable, environmentally-friendly, and safe alternatives. Various alternatives currently exist in the form of disease resistant crops (through traditional breeding programs or as genetically modified crops), cultural practices (such as crop

rotation), and biological control agents. The use of biological control agents has proven one of the more promising alternatives to synthetic chemicals.

1.3. BIOLOGICAL CONTROL METHODS

Biocontrol by definition is the use of living organisms to control pests such as insects, weeds, and plant diseases. Currently about 3.5% of the total global agrichemical market belongs to biopesticides with an expected increase to about 7.7% by 2014 (Lehr, 2010). The biopesticide market is still growing yet slowly because the profit margins are small and large companies are not willing to invest (Glare *et al.*, 2012). Once sales can support a large company, we could see investment into research and development leading to the discovery of new or the improvement of existing products (Glare *et al.*, 2012). Developmental procedures of a new biopesticide can take as little as 3 years and \$3-5 million compared to the 10 years and \$250 million required for the development and commercialization of a novel synthetic antifungal compound (McDougall, 2010).

When screening and selecting for a new biological control agent, there are numerous criteria used to define an ideal candidate. Promising antagonists should be genetically stable, effective at low concentrations, undemanding of nutritional requirements, non-toxic, non-pathogenic to the host, compatible with other treatments, capable of surviving harsh environmental conditions, able to be effectively prepared and stored prior to application, and effective against a broad range of pathogens (Sharma *et al.*, 2009). The commercialization of a biopesticide is comprised of multiple steps (Figure 1).

Figure 1- Steps in the development process involved to discover and commercialize a new biopesticide.

One of the greatest challenges prior to commercialization of a biopesticide is the study of its precise mode of action, which is necessary for registration and important to understand its effectiveness against a limited or wide spectrum of target and non-target organisms. These biocontrol microorganisms may be classified by their mode of action as described below (section 1.4).

1.4. MODE OF ACTION AND ANTIFUNGAL COMPOUNDS

Microbial biocontrol agents will tend to fall under one or more of four main modes of action; (i) competition for space or nutrients, (ii) parasitism, (iii) induced plant resistant, and (iv) antibiosis (Sharma *et al.*, 2009). Antibiosis i.e., the production of antimicrobial compounds is the main mode of action. Antifungal compounds have been found to be produced by plants, fungi, viruses, and bacteria (Ongena and Jacques, 2008). Of the microbial based biocontrol agents, approximately 70% of these belong to bacteria (Ongena and Jacques, 2008).

There is a broad range of diversity in the current naturally-produced antifungal compounds by bacteria. Numerous compounds have been identified with many more being discovered. Many species of bacteria are capable of producing multiple secondary metabolites possessing antimicrobial activity. In some cases, a variety of bacteria can produce the same compound as seen with the production of 3-hydroxypropionaldehyde (3-HPA) by six different genera (Voisenet, 1914; Vollenweider *et al.*, 2003; Wise *et al.*, 2012). Antifungal compounds produced by bacteria can generally be divided into non-peptide- and peptide-containing compounds (Hamdache *et al.*, 2011; Ongena and Jacques, 2008).

1.4.1. Non-Peptide Compounds

Non-peptide compounds include volatile organic compounds (VOC's). VOC's are organic molecules with low vapor pressure and are easily volatilized into the atmosphere. Studies have found an array of volatile compounds produced by various bacteria to be effective against the plant pathogens *Sclerotinia sclerotiorum* and *Rhizoctania solani* (Fernando *et al.*, 2005; Kai

et al., 2009). Another class of antifungal compounds produced by bacteria are the polyunsaturated organic compounds known as polyenes. In addition to antifungal activities, some polyenes have been found to be active against parasites, viruses and prion diseases (Caffrey *et al.*, 2008). Other non-peptide compounds exist however there is greater diversity amongst the peptide compounds produced by bacteria.

1.4.2. Peptide Compounds

Peptides are more prevalent antifungals than non-peptide molecules produced by bacteria. Many peptides appear to be active against a broad spectrum of microorganisms (Lu *et al.*, 2009; Schnürer and Magnusson, 2005; Wong *et al.*, 2008). A few of the antifungal compounds are proteins such as baciain and baciasubin produced by *Bacillus amyloliquefaciens* and *B. subtilis* B-916, respectively (Liu *et al.*, 2007; Wong *et al.*, 2008). Some lactic acid bacteria have also been found to produce proteinaceous antimicrobial compounds, generally named bacteriocins (Schnürer and Magnusson, 2005). Proteinaceous type compounds have been shown to be effective against multiple yeasts and molds (Liu *et al.*, 2007; Schnürer and Magnusson, 2005; Wong *et al.*, 2008).

Glycopeptides and cyclic dipeptides are other classes of peptides known to be produced by bacteria (Lu *et al.*, 2009; Schnürer & Magnusson, 2005). Glycopeptides have a large peptide group and a carbohydrate moiety such as occidiofungin produced by *Burkholderia contaminans* known to have a potent broad spectrum effect against both plant and animal pathogens (Lu *et al.*, 2009). Lactic acid bacteria are one of the main producers of antimicrobial cyclic dipeptides (Schnürer & Magnusson, 2005). Recently, a cyclic dipeptide produced by *Lactobacillus*

plantarum was found to be active against *Aspergillus flavus*, a spoilage fungus known to produce mycotoxins (Yang and Chang, 2010). Numerous peptide classes and compounds exist but currently scientists have been very interested in the antifungal activity of lipopeptides.

Lipopeptides are a class of peptides known for having a lipid portion within the structure connected to a peptide moiety (Ongena and Jacques, 2008). The most well-known lipopeptides are cyclic in nature and fall under three families known as surfactin, iturin, and fengycin (Ongena and Jacques, 2008). Lipopeptides are becoming one of the more extensively studied groups of antifungal compounds because they have shown low toxicity, high biodegradability, and environmentally friendly characteristics when compared to synthetic chemical fungicides (Chen *et al.*, 2008).

1.4.2.1. Cyclic Lipopeptides

The three families; iturin, fengycin, and surfactin (Figure 2) are known to have an antagonistic effect on phytopathogens by interacting with the membrane and inducing pore formation (Ongena and Jacques, 2008; Pérez-Garcia *et al.*, 2011; Touré *et al.*, 2004). Cyclic lipopeptides are amphiphilic in nature with a hydrophobic fatty acid portion and a hydrophilic peptide ring (Ongena and Jacques, 2008). Lipopeptide biosurfactants have been found to be produced by *Aspergillus*, *Streptomyces*, *Pseudomonas*, and *Bacillus* genera (Raaijmakers *et al.*, 2010). Classification of families is based on the amino acid sequence and fatty acid branching (Romero *et al.*, 2007).

Surfactin is the most studied family of lipopeptides because of its antiviral and antibacterial capabilities as well as its potential to help stabilize biofilms or inhibit competing

biofilms (Bouchoux *et al.*, 2008; Romero *et al.*, 2007). The fatty acid acyl chain inserts into the bilayer and due to repulsion charges between the head groups and surfactin, small micelles are produced (Buchoux *et al.*, 2008). Drawbacks of surfactins in terms of combating phytopathogens include limited fungal toxicity and the ability to disrupt red blood cells (Ongena and Jacques, 2008; Romero *et al.*, 2007).

Figure 2. Representative structures of the three main families of lipopeptides; surfactins, iturins, and fengycins.

Iturin is similar in structure to the other lipopeptides and has shown strong antifungal toxicity *in vitro* but has little to no activity against bacteria, and no antiviral effects have been found to date (Ongena and Jacques, 2008). Interactions with the membrane are still the most likely mode of action and some studies have found insertion of iturin-like molecules into the membrane to be affected by cholesterol (Nasir and Besson, 2012). All families are similar in structure and appear to interact with the membrane, however, interest in the fengycin family has increased because fengycins are less haemolytic yet still retain their antifungal properties (Ongena and Jacques, 2008; Vanittanakom *et al.*, 1986). Little is known of the exact mode of action in which fengycin exhibits its antifungal effect.

1.4.2.1.1. Fengycin

The general structure of fengycin (also known as plipastatins) consists of lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β -hydroxy fatty acid chain that may be saturated or unsaturated (Ongena and Jacques, 2008). Fengycins show strong antifungal efficacy, particularly against filamentous fungi (Kumar and Johri, 2009). A 40-fold reduction in hemolytic activity was found for fengycin when compared to that of surfactin (Deleu *et al.*, 2008; Vanittanakom *et al.*, 1986). The full mechanism of fengycin has not been elucidated, but it is hypothesized that they readily interact with lipid layers (Deleu *et al.*, 2008). There are different variants of fengycin (Fengycin A, Fengycin B, Pliplastin A, and Pliplastin B) however, the mode of action is believed to be similar amongst them (Ongena and Jacques, 2008). Some studies on monolayers with fengycin interaction have shown a concentration dependent mode of action with fengycin (Deleu *et al.*, 2008) whereas other studies indicate an all-or-none

type activity (Patel *et al.*, 2011). Evolutionary constraints on redesigning microbial membrane composition, combined with the proposed mode of action of fengycin could allow delay or elimination of potential resistance in fungal pathogens. Fengycins are known to be produced by various strains of the bacterium including *B. subtilis*.

1.5. BACILLUS SUBTILIS

Bacillus spp. are gram positive endospore-forming rhizobacteria and are naturally found in agricultural soils (Kumar and Johni, 2009). The spore forming ability and resistance to dryness of the spores could lead to a more stable biopesticide formulation (Ongena and Jacques, 2008). A strain of *B. subtilis* (B-3) was the first organism patented as a postharvest biocontrol agent (Pusey and Wilson, 1984). Currently, over half of the commercially available bacterial biological control agents contain *Bacillus* spp. as their active component (Fravel, 2005). *Bacillus* species have long been known as microbial factories. *Bacillus subtilis* is an ideal example of this with approximately 4-5% of its entire genome devoted to the production of over two dozen varieties of antimicrobial compounds (Stein, 2005). *Bacillus subtilis* isolates have been found to produce a wide variety of peptide (Ongena and Jacques, 2008) and/or non-peptide (Hamdache *et al.*, 2011) antimicrobial compounds.

1.9. HYPOTHESES AND OBJECTIVES

Previous work in our laboratory has demonstrated that a *B. subtilis*, named strain CU12, was isolated from a crop growing field with a history of low disease. This strain showed a

marked reduction in the growth of plant pathogens and it was discovered that it produced the CLP fengycin (T.J. Avis, personal communication). The present study is the result of work on its additional antimicrobial compounds and on its precise mode of action with regard to fengycin production. Our hypotheses were:

- *B. subtilis* strain CU12 produces antifungal compounds in addition to fengycin
- Lipid composition in plant pathogens determines sensitivity or tolerance of the pathogen to fengycin

Our first objective was to isolate and identify antifungal compounds in *B. subtilis* CU12. Our second objective was to qualify and quantify the lipids in the membranes of four plant pathogens with varying sensitivity to fengycin in order to correlate lipid composition with fengycin activity and gain insight into the potential mechanism of action of fengycin.

CHAPTER 2

Production and Antimicrobial Activity of 3-Hydroxypropionaldehyde from *Bacillus subtilis*
Strain CU12

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ABSTRACT

Bacillus subtilis strains are known to produce a vast array of antimicrobial compounds. However, some compounds remain to be identified. Disk assays performed *in vitro* with *Bacillus subtilis* CU12 showed a significant reduction in mycelial growth of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum*. Crude *B. subtilis* culture filtrates were subsequently extracted with ethyl acetate and butanol. A bioassay guided purification procedure revealed the presence of one major antifungal compound in the butanol extract. Purification of the compound was performed using a reverse-phase C18 solid phase extraction (SPE) cartridge and flash column chromatography. NMR data showed that the main antimicrobial compound was a cyclic dimer of 3-hydroxypropionaldehyde (HPA). This study demonstrated the antimicrobial activity of *B. subtilis* strain CU12 against phytopathogenic microorganisms mediated at least in part by the production of HPA. It also suggests that this *B. subtilis* strain could be effective at controlling pathogens through protection of its ecological niche by antibiosis.

2.1. INTRODUCTION

The plant phyllosphere and rhizosphere are extremely complex microbial ecosystems containing saprophytes, epiphytes, endophytes, pathogens and other microorganisms. Natural ecosystems tend to contain microbial communities living in relative harmony where all populations balance out in their quest for food and an ecological niche (Bélanger and Avis, 2002). In artificial systems such as agriculture, modifications to the natural balance can drastically alter the microbial community and lead to ingress of or increase in plant pathogens that may have devastating effects on plant health and productivity (Avis *et al.*, 2008). Integration of beneficial microorganisms into agricultural or other disturbed ecosystems has the potential to suppress some of the problems associated with plant pathogens. Among these beneficial microorganisms, some of the most prominent plant pathogen antagonists being studied are members of the *Bacillus* genus.

Bacillus spp. are gram positive endospore-forming rhizobacteria that are widely studied because they are microbial factories for biologically active compounds. The most commonly studied species of this genus, *B. subtilis* (Ehrenberg) Cohn has been found to have approximately 4-5% of its entire genome devoted solely to antibiotic synthesis leading to a vast array of structurally diverse compounds (Stein, 2005). *Bacillus subtilis* isolates produce peptide (Ongena and Jacques, 2008) and/or non-peptide (Hamdache *et al.*, 2011) antimicrobial compounds depending on the strain. However, all possible antimicrobial compounds from *Bacillus* spp. have yet to be elucidated.

Recently, a strain of *B. subtilis* (named strain CU12) was isolated in our laboratory from an agricultural field with a history of low plant disease. However, no work on this strain's ability to produce biologically active molecules has yet been performed. The objectives of this study

were (i) to investigate the antifungal and anti-oomycete ability of *B. subtilis* strain CU12 and (ii) to identify the compound(s) responsible for its antifungal activity.

2.2. METHODS AND MATERIALS

2.2.1. Microbial Material

Bacillus subtilis strain CU12 was isolated from a disease suppressive soil and maintained on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) at 23°C prior to usage. The bacterium was identified as *B. subtilis* by using nucleotide sequence data from the small subunit (16S) of ribosomal RNA (GenBank Accession number JX489167).

The pathogenic fungi *Alternaria solani* Sorauer, *Botrytis cinerea* Pers., and *Fusarium sambucinum* Fuckel and the pathogenic oomycete *Pythium sulcatum* R.G. Pratt & J.E. Mitch were maintained on potato dextrose agar (PDA, Becton Dickinson) at 23°C. *Alternaria solani*, *B. cinerea*, and *F. sambucinum* were obtained from the “Laboratoire de diagnostic en phytoprotection” (MAPAQ, Québec, Québec, Canada). *Pythium sulcatum* was isolated from an infected carrot root and is available at the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada).

2.2.2. Effect of *B. subtilis* CU12 on Mycelial Growth of Pathogens

Bacillus subtilis CU12 was tested for its ability to inhibit the mycelial growth of the pathogens *in vitro*. Petri dishes (100 × 15 mm) containing PDA were inoculated with four 1-cm streaks of *B. subtilis* CU12 at the four cardinal points. In *B. cinerea* and *P. sulcatum* trials, the

streaked bacteria were located 3.5 cm from the center of the dish. In *A. solani* and *F. sambucinum* trials, the streaked bacteria were located 2 cm from the center of the dish. A mycelial plug (5-mm diameter) of each pathogen was deposited individually in the center of the dish. The control treatments consisted of the pathogen inoculations without streaking of the bacterium. The dishes were incubated at 23°C. When all the control mycelia grew to 3.5 cm (*B. cinerea* and *P. sulcatum*) or 2 cm (*A. solani* and *F. sambucinum*) from the center of the dish, the mycelial growth of all treatments were measured. Mycelial growth was assessed as the average of two perpendicular diameters of the thallus. The experiments were performed according to a completely randomized design with four repetitions. Experiments were repeated twice.

2.2.3. Extraction of Antifungal Compounds

A total of 600 ml of tryptic soy broth (TSB, Becton Dickinson) was inoculated with *B. subtilis* CU12 and incubated at 28.5°C under shaking conditions (150 rpm) for 48 h. Following incubation, the culture broth containing *B. subtilis* CU12 was centrifuged at 10,000 rpm for 20 min. The supernatant was recovered and the bacterial pellet was discarded. The supernatant was extracted twice with 300 ml ethyl acetate and the fractions were combined. The aqueous phase was then re-extracted with n-butanol (2 × 300 ml).

The ethyl acetate and butanol fractions were dried using a Heidolph 4000 rotary evaporator (Schwabach, Germany), re-suspended in ethyl acetate or butanol, and transferred to screw-cap tubes. Extracts were further concentrated under a stream of nitrogen and re-suspended in a small volume of the appropriate solvent to yield a concentration 200 times (ethyl acetate fraction) or 100 times (butanol fraction) greater than the initial extracts.

2.2.4. Effect of Extracts on Mycelial Growth of Pathogens

Concentrated extracts were assayed against the four pathogens using a disk diffusion assay. Sterile paper disks (6-mm diam) were inoculated with 25 µl of either the ethyl acetate or the butanol extract and air dried. Twenty-five microlitres of pure ethyl acetate or butanol served as the controls. The centers of the PDA dishes were individually inoculated with a mycelial plug (5-mm diam) of each pathogen. One disk for each treatment was placed individually in the PDA plate. In *B. cinerea* and *P. sulcatum* trials, the disks were located 3.5 cm from the center of the dish. In *A. solani* and *F. sambucinum* trials, the disks were located 2 cm from the center. Dishes were incubated at 23°C. When the control mycelia grew to the disks, the mycelial growth of all treatments was measured. Measurements were noted as the radius of the mycelial growth for each pathogen. The experiment was performed according to a completely randomized design with three repetitions.

The extracts were assayed additionally by using a direct bioassay on a TLC plate. Four microliters of the concentrated ethyl acetate and butanol extracts were spotted on a silica-backed TLC plate with fluorescent marker (Sigma, Mississauga, Ontario, Canada) and left to dry. Pure ethyl acetate and butanol served as controls. To perform the bioassay, 10 ml of ultrapure (Milli-Q) water was mixed with 0.4 g of PDA and heated to the boiling point. A concentrated conidial suspension of *A. solani* was prepared by adding 10 ml ultrapure water to a Petri dish covered with actively growing mycelium of the fungus. The surface of the mycelium was gently scraped to dislodge the conidia. Once the PDA had cooled (50°C), the conidial suspension was added and mixed thoroughly. The resulting suspension was thinly spread over the TLC. The inoculated TLC was placed in a moist chamber (>95% RH) and incubated at 23°C for 48 h. Following

incubation, active fractions were revealed as white inhibition zones on the dark background of the fungus.

2.2.5. Detection and Fractionation of Antifungal Compounds on SPE Cartridge

Extracts presenting antifungal activity were separated using a sterile 1 g (5 ml) Bond Elut C18 SPE cartridge (Varian, Palo Alto, CA, USA). The cartridge was conditioned with two volumes (10 ml) of ultrapure water prior to the application of 60 µl of the concentrated extract. Compounds were sequentially eluted with 1 volume (5 ml) of increasing concentrations of methanol:water as follows: 0:100, 20:80, 50:50, 80:20, 100:0 (v/v). The five collected fractions and the crude extract were separated on TLC using chloroform:methanol 90:10 (v/v). Following migration, the TLC was placed under short (254 nm) and long (365 nm) ultraviolet (UV) wavelengths and the visible compounds were noted. All TLC were bioassayed using the direct bioassay with *A. solani* as described previously.

2.2.6. Purification of Antifungal Compounds

Purification of active compounds from the SPE fraction eluted with methanol:water (80:20) was achieved using flash chromatography with C18 silica gel (Sigma Aldrich, Oakville, Ontario, Canada). The silica gel was soaked in methanol:water (1:1), loaded into the column and equilibrated with 5 column volumes (500 ml) of ultrapure water prior to use. Two millilitres of the concentrated extract were then placed on top of the gel. Compounds were sequentially eluted with 1 volume (100 ml) of increasing concentrations of methanol:water as follows: 0:100, 20:80, 50:50, 80:20, 100:0 (v/v). Fractions (15 ml each) were collected and directly bioassayed on TLC

as described above, and those containing active compounds were combined for spectroscopic analysis.

2.2.7. NMR Analysis

^1H and ^{13}C -NMR experiments were recorded using a Bruker Avance 300 and a Bruker Avance 400 spectrometer (Billerica, MA, USA). Samples were dissolved in methanol- d_4 or chloroform- d . Experiments were performed at room temperature, and the spectra were calibrated based on the residual non-deuterated solvents: δ_{H} 3.31 and δ_{C} 49.1 for methanol, δ_{H} 7.26 and δ_{C} 77.0 for chloroform.

2.2.8. Statistical Analysis

In the *B. subtilis* CU12 and crude extract inhibition trials, ANOVA and/or *t*-tests were performed with SAS software (SAS Institute, Cary, NC, USA) and reported as significant when $P < 0.05$.

2.3. RESULTS

2.3.1. Effect of B. subtilis CU12 on Mycelial Growth of Pathogens

Antifungal activity of *B. subtilis* CU12 was assayed *in vitro* as the inhibition of mycelial growth through the production of diffusible compounds in solid media. ANOVA indicated that there was no significant difference between repetitions of this experiment. Therefore, the data

were combined and analyzed as a single experiment. It was revealed that *B. subtilis* CU12 exhibited significant activity against all tested pathogens, although the degree of inhibition varied considerably (Figure 1) among pathogens. Analysis of the mycelial growth demonstrated that *B. subtilis* CU12 significantly inhibited growth of *A. solani*, *B. cinerea*, *F. sambucinum*, and *P. sulcatum* by 53, 25, 39, and 20%, respectively (Figure 1).

Figure 1. *In vitro* effect of *Bacillus subtilis* strain CU12 on mycelial growth of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum*. Growth was measured as the diameter of the thallus. Error bars are standard errors. For each pathogen, an asterisks (*) above a bacterial treatment denotes significant difference from the control according to a *t*-test ($P < 0.05$; $n = 8$)

2.3.2. Effect of Extracts on Pathogens

The concentrated ethyl acetate and butanol extracts of *B. subtilis* CU12 liquid culture were subjected to a disk diffusion assay to determine their effects on mycelial growth of the four pathogens.

The ethyl acetate fraction did not significantly affect the mycelial growth of any of the tested pathogens when compared to the control (data not shown). Conversely, the concentrated butanol extract significantly inhibited the mycelial growth of *A. solani*, *B. cinerea*, and *P. sulcatum* by 52, 47, and 34%, respectively (Figure 2). *Fusarium sambucinum* mycelial growth was not significantly inhibited by the butanol extract ($P > 0.05$).

Figure 2. Effect of butanol (BU) extract from *Bacillus subtilis* strain CU12 culture filtrates on mycelial growth of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum*. Growth was measured as the radius of the thallus. Error bars are standard errors. For each pathogen, an asterisks (*) above a bacterial treatment denotes significant different from the control according to a *t*-test ($P < 0.05$; $n = 3$)

The *B. subtilis* CU12 ethyl acetate and butanol fractions were also evaluated by using a direct bioassay on TLC with *A. solani*. Result showed that both controls (ethyl acetate and butanol solvents) and the *B. subtilis* CU12 ethyl acetate extract were not inhibitory to *A. solani*. However, the *B. subtilis* CU12 butanol extract showed a lack of *A. solani* growth as revealed by a strong inhibition zone (Figure 3).

Figure 3. Inhibition of *Alternaria solani* growth using a direct TLC bioassay. EAC = Ethyl acetate control; EAX = *Bacillus subtilis* CU12 ethyl acetate extract; BC = Butanol control; BX = *Bacillus subtilis* CU12 butanol extract

2.3.3. Purification of Antifungal Compounds

Many compounds were revealed by analytical TLC under UV lighting for the crude butanol extract and for fractions from reverse-phase chromatography.

Figure 4. TLC separation and direct bioassay of *Bacillus subtilis* CU12 crude butanol extract and solid phase extraction cartridge fractions. Direct bioassay with *Alternaria solani* on the TLC: 1 = Crude butanol extract; 2 = 0:100 v/v methanol:water fraction; 3 = 20:80 v/v methanol:water fraction; 4 = 50:50 v/v methanol:water fraction; 5 = 80:20 v/v methanol:water fraction; 6 = 100:0 v/v methanol:water fraction

When the TLC containing the crude butanol extract and the five SPE-recovered fractions were bioassayed, it was shown that a single spot at a retention factor (R_f) of approximately 0.74 was antimicrobial in the crude extract, and from the SPE fraction eluted with 80:20 (v/v) methanol:water (Figure 4). Further purification on flash column chromatography afforded the antimicrobial compound, which was not visible under UV light.

2.3.4. NMR Analysis

Following pooling of pure active fractions from C18 flash chromatography, the purified antimicrobial compound was identified as 3-hydroxypropionaldehyde (HPA) cyclic dimer based on analysis of its 1- and 2-dimensional NMR spectra and comparison with published data (Sung *et al.*, 2003). Proton (^1H) and carbon (^{13}C) data are shown in Figure 5. The ^{13}C NMR spectrum showed six main signals between δ_c 34 and 108 ppm. Each of the signals appeared as doublets and therefore showed that the compound was present in isomeric forms. The ^1H NMR spectrum of HPA showed aliphatic proton signals δ_H 1.20-1.60 ppm as well as signals of protons linked to oxygenated carbons between δ_H 3.50-4.40. Signals at δ_H 4.90 were characteristic of anomeric-type sugar protons. There was no evidence of the presence of HPA monomer.

Figure 5. Carbon-13 (a) and proton (b) NMR spectra of antimicrobial compound in deuterated methanol

2.4. DISCUSSION

Bacillus subtilis strain CU12 was isolated from a disease suppressive soil and potentially could be used as a beneficial bacterial antagonist toward plant disease pathogens. In order to determine its antimicrobial potential, the bacterium was tested against various plant pathogens followed by purification of a chemical responsible for or contributing to the antimicrobial activity. Results showed that *B. subtilis* CU12 provided significant inhibition of mycelial growth of the tested plant pathogens resulting in an inhibition zone. Moreover, butanol extract of culture filtrates of the bacterium equally showed inhibition in pathogen mycelial growth indicating the production of one or more extracellular antifungal compounds.

Following purification, an antifungal compound was successfully isolated from the n-butanol extract and identified as HPA. NMR (^1H and ^{13}C) spectra of the compound were similar to those published by Sung *et al.* (2003). HPA has been previously produced by the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, and *Lactobacillus* (Vollenweider *et al.*, 2003). There is one older report of HPA produced as an acrolein intermediate by *Bacillus amaracrylus* (Voisenet, 1914). The bacterium has since been reclassified as *Bacillus polymyxa* (Holt, 1986) and now as *Paenibacillus polymyxa* (Garrity *et al.*, 2004). To our knowledge, this is the first report of a strain of *B. subtilis* producing HPA.

In aqueous solution, HPA from *Lactobacillus reuteri* is found as a multi-compound mixture (named reuterin) of monomeric, cyclic dimer, and hydrated forms of HPA (Talarico and Dobrogosz, 1989; Sung *et al.*, 2002). The nature and proportion of HPA isomers in solution vary depending on the pH. NMR spectra obtained at pH 4.0 showed that β -hydroxypropionaldehyde was the main compound, while at pH 10.5 the mixture was more complex and contained only traces of β -hydroxypropionaldehyde with more oligomers (Sung *et al.*, 2003). In this study,

NMR data were obtained in both chloroform-*d* (data not shown) and methanol-*d*₄ without pH control. NMR spectra obtained are similar to those published by Sung *et al.* (2003) who reported at pH 10.5 the presence of monomer, cyclic dimer, aldol dimer, hemiacetal and acetal forms of HPA. In a study done by Vollenweider *et al.* (2003), it was observed that the composition of the HPA system is concentration dependent. At concentration levels of 4.9 M, the predominant form present was the HPA dimer while lower concentrations (<1.4 M) showed a significant decrease in dimer concentration (Vollenweider *et al.*, 2003). The main form appeared to be the cyclic dimer as indicated by protons at δ_H 4.90 that were directly linked to oxygenated carbons at δ_C 106-107 based on correlations from the Heteronuclear Multiple Bond Correlation (HMBC) spectrum. The lack of the 3-HPA monomeric form and the presence of the cyclic dimer in the NMR spectra may indicate an elevated concentration of HPA.

At high concentrations, HPA has been found to be active against various microorganisms including some genera that produce them (Vollenweider and Lacroix, 2004). The most intensively studied of the HPA producing bacteria, *L. reuteri* has shown some tolerance to HPA, however, its viability does decrease at very high HPA concentrations (Vollenweider and Lacroix, 2004). In an antimicrobial activity study done by Chen *et al.*, (2002), they determined that an HPA concentration of 20-35 $\mu\text{g/ml}$ can effectively prevent the growth of *B. subtilis* ATCC 6633 and a 30-50 $\mu\text{g/ml}$ concentration was capable of causing death in this test organism. In our work, *B. subtilis* strain CU12 was able to grow in concentrations up to 1,360 $\mu\text{g/ml}$ HPA indicating its high tolerance to its own antimicrobial compound.

HPA and the HPA-based multi-compound reuterin system produced by various microorganisms have been described as broad spectrum antimicrobial agents with antibacterial, antiparasite, antiviral, anticancer, and antifungal properties (Dobrogosz and Lindgren, 1988).

Broad spectrum activity may be related to the mode of action in which HPA/reuterin acts although this has been difficult to study due to the complex nature of the HPA/reuterin system. Two mechanisms have nonetheless been proposed. The first relates to the reaction of the aldehyde group with thiol and other primary amines, which inactivates proteins and small molecules that contain these groups (Vollenweider and Lacroix, 2004) leading to growth inhibition (Schaefer *et al.*, 2010). A second mechanism suggests that the structural similarities between the HPA-dimer and ribose cause it to act as a competitive inhibitor blocking the ribonucleotide reductase enzyme (Vollenweider *et al.*, 2010). The ribonucleotide reductase enzyme is required for the generation of deoxynucleotides that are required for DNA synthesis.

From an ecological point of view, *B. subtilis* strain CU12 would gain an advantage through the production of HPA. Indeed, HPA would provide the antagonist with a means to protect its ecological niche against various microbial competitors including plant pathogens. From a practical point of view, the introduction of *B. subtilis* into agricultural or other artificial systems may protect the microecosystem against unwanted microorganisms such as plant pathogens and could provide a natural means to protect food crops and other plants.

Overall, the identification of the antifungal HPA may explain at least in part, the effectiveness of *B. subtilis* CU12 in inhibiting plant pathogens. Moreover, screening of other *B. subtilis* strains for increased production of HPA could help in selection of strains with superior inhibitory properties. Finally, the discovery of HPA will allow further work in elucidating the molecular, biochemical and ecological phenomena behind the antagonism by *B. subtilis* strain CU12 and help with the prediction of its inhibitory efficacy.

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CHAPTER 3

Analysis of Cellular Lipid Composition in Determining the Mode of Action of Fengycin Against Plant Pathogens

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ABSTRACT

Fungal membranes are responsible for essential functions and maintaining cellular integrity. Energetic costs and evolutionary constraints limit the amount a given fungus may restructure its membrane composition. It is hypothesized that a known antifungal cyclic lipopeptide, fengycin, produced by *Bacillus* spp. interacts with membrane and the mode of action may be more difficult or impossible to overcome. In this study we analyze the lipid content of a variety of plant pathogens looking at sterol content, phospholipid head groups, and Fatty Acid Methyl Esters (FAME). Phytopathogen sensitivities to fengycin indicate that sterol content and to a lesser extent, the ratio of anionic/zwitterionic phospholipids may be involved in the mode of action of fengycin against phytopathogens. Fengycin may have a greater effect on membranes with more neutral lipids. A complete understanding of the mode of action is essential in the process of potential registration of *B. subtilis* CU12 as a biocontrol agent.

3.1 INTRODUCTION

Fungal plant pathogens are accountable for large amounts of both pre- and post-harvest food losses and, absent control methods, these losses would be expected to double (Glare *et al.*, 2012). Current methods to combat these phytopathogens consist mainly of synthetic chemicals. The potential development of resistance in the fungal strain and the harmful effects on the environment and human health are the two major drawbacks to using chemical pesticides (Avis, 2007). A decrease in the use of chemical fungicides has been occurring since 2000 while a concurrent increase in the use of safe and eco-friendly alternatives has been observed (Ongena and Jacques, 2008; Thakore, 2006). Among the alternatives to synthetic chemicals, biocontrol agents i.e., beneficial microorganisms with antagonistic activity against plant pathogens, have been at the forefront. Of these biocontrol agents, those producing antimicrobial compounds with modes of action thought to affect the membrane are of great interest because it is hypothesized that it is too energetically costly for fungus to adapt or re-design their membrane composition to counteract the activity of these antimicrobial compounds and to develop resistance (Avis, 2007). The organisms chosen for this study were *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum*, which are known to target tomatoes, grapes, potatoes, and carrots, respectively. Two of these are soil-borne pathogens (*F. sambucinum* and *P. sulcatum*) and the other two are foliar borne pathogens (*A. solani* and *B. cinerea*). *P. sulcatum* is an oomycete known to have no sterols within the membrane (Bloch, 1983). This range of organisms led to a range of sensitivities with *A. solani* being the most tolerant, followed by *F. sambucinum*, *B. cinerea*, and the most sensitive being *P. sulcatum* (T.J. Avis, personal communication). The differences in sensitivities were believed to be due to differences in membrane composition.

The fungal membrane is responsible for maintaining cell order, integrity, essential functions, and is imperative to the survival of fungus (Avis, 2007; Deacon, 2006). The fungal membrane is comprised of a phospholipid bilayer with associated enzymes and trans-membrane proteins (Avis, 2007). Animal eukaryotic membranes differ from those of fungal membranes in the type of sterol in the membrane. Ergosterol is the most notable sterol in the fungal membrane (Weete, 1974). The general make-up of constituents in membranes varies among taxonomically different fungal species (Weete, 1974). The overall membrane composition of a given species' membrane has been shown to be affected by the growth conditions such as nutrition, oxygen, and temperature (Pedneault *et al.*, 2007; Suutari, 1995).

Interest in the composition of membranes has increased as information in this field may lead to a better understanding of those antimicrobials with membrane-targeting modes of action. When the membrane becomes too disordered, membrane permeability may occur and could eventually lead to the death of the cell (Avis and Bélanger, 2001). Antibiosis i.e., the antagonistic activity from small antimicrobial compounds produced by antagonists, can involve a major group of antimicrobial compounds known as cyclic lipopeptides, which are thought to interact with membranes (Bouchoux *et al.*, 2008; Deleu *et al.*, 2008). Cyclic lipopeptides are produced by a wide range of organisms, more notably amongst the *Bacillus* spp. (Ongena and Jacques, 2008). *Bacillus subtilis* species synthesize lipopeptides non-ribosomally via large mega-enzymes organized into modules that catalyze various transformational reactions (Ongena and Jacques, 2008). The method in which the lipopeptides are synthesized can lead to a remarkable heterogeneity amongst products (Stein, 2005). Of the three major families; iturin, surfactin, and fengycin, the latter has been found to have a reduced hemolytic effect (Vanittanakom *et al.*, 1986). The amphiphilic nature and small size of the peptide portion of fengycin may aid in the

insertion into the membrane (Deleu *et al.*, 2008). At high enough concentrations fengycin aggregates leading to the creation of large, sustainable pores and a complete efflux of cellular contents (Deleu *et al.*, 2008; Patel *et al.*, 2011). Much remains to be discovered in terms of the exact mode of action and the particular interactions of fengycin with the membrane.

In this study, we analyzed intrinsic sterol content, phospholipid composition (polar head moieties) and fatty acid profiles of various fungal and fungus-like plant pathogens and correlated this data with microbial sensitivity to fengycin in an attempt to elucidate the molecular mode of action of fengycin on the target membranes.

3.2. METHODS AND MATERIALS

3.2.1. Microbial Material

The fungi *Alternaria solani* Sorauer, *Botrytis cinerea* Pers., and *Fusarium sambucinum* Fuckel and the oomycete *Pythium sulcatum* R.G. Pratt & J.E. Mitch were maintained on potato dextrose agar (PDA, Becton Dickinson, Sparks, MD) at 23°C. *Alternaria solani*, *B. cinerea*, and *F. sambucinum* were obtained from the Laboratoire de diagnostic en phytoprotection (MAPAQ, Québec, Québec, Canada). *Pythium sulcatum* was isolated from an infected carrot root and is available at the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada).

3.2.2. Extraction of Fungal Lipids

Alternaria solani, *B. cinerea*, and *F. sambucinum* were grown in 5 × 100 ml of Tryptic Soy Broth (TSB, Becton Dickinson). *Pythium sulcatum* was grown in 5 × 100 ml of V8 medium containing 200 ml/L of clarified V8 juice and 2 g/L of CaCO₃. Each microorganism was grown in triplicate and all flasks were incubated at 28.5°C for 96 h with shaking (180 rev min⁻¹). The microbial biomass was separated from the solvents by filtration (Fisher No.8 filter paper) and frozen (-80°C) prior to lyophilization. Lyophilized cells were ground to a powder and extracted with a chloroform/methanol/water (100:100:50 ml/g of dry weight). The mixture was protected from light and agitated using a stirring bar (400 rpm) for 3 h at 23°C. The solvents were removed by filtration and the biomass was re-extracted with chloroform/methanol/water (200:100:50 ml/g of dry weight) as previously described. The solvents were removed by filtration and the extracts were combined. The aqueous fraction was removed by partitioning the extracts against chloroform and water in a separatory funnel. The organic phases were dried using anhydrous MgSO₄ to remove all traces of water. Collected organic phases were evaporated to dryness using a Heidolph 4000 rotary evaporator (Schwabach, Germany). The residues were re-suspended in chloroform, transferred to screw-cap tubes and dried under a stream of nitrogen gas. The resulting oily residue constituted the total lipid extracts, which were kept at 4°C until use.

3.2.3. Separation of Lipid Classes

Neutral and polar lipids were separated by acetone precipitation. Six ml of acetone was added to the dried total lipids. The tubes were vortexed and placed at -20°C for 1 h. The tubes were then centrifuged at 4500 rpm for 10 min at 4°C. The supernatant was recovered and the

pellet was re-suspended in 1 ml of acetone and the procedure was repeated. The supernatants were combined, evaporated to dryness using a nitrogen drier. The acetone constituted the neutral lipid fraction while the dried pellet represented the polar lipid fraction.

3.2.4. Ergosterol Analysis

Sterols were obtained from alkaline hydrolysis of the neutral lipids. A 1:10 ratio of KOH solution (33% w/v) and ethanol (95%), respectively, was added to the total neutral lipids. The tubes were heated to 90°C for 2 h. Following heating, the mixture was washed with 5 ml of hexane and the hexane fraction containing the unsaponifiable fraction was recovered. Two subsequent 5 ml washes were performed with hexane. The hexane fractions were combined, evaporated to dryness and resuspended in a small volume of hexane for further analysis. Ergosterol in the unsaponifiable fraction was quantified on an Agilent 6890 Series gas chromatograph (Mississauga, ON, Canada) coupled with an Agilent 5973 quadropole mass spectrometer using a 30 m × 0.25 mm ID × 0.25 µm ZB-5 capillary column (5% Phenyl; 95% dimethyl-polysiloxane, Phenomenex, Torrance, CA, USA) in splitless mode. The temperature program was 100°C for 2 min, increased by 20°C min⁻¹ to 200°C, held for one minute, increased by 10°C min⁻¹ to 300°C and held for 5 min. Injector and detector temperatures were 250 and 280°C, respectively. Sterols were quantified using a calibration curve of an authentic ergosterol standard (Sigma, Mississauga, ON, Canada).

3.2.5. Polar Lipid Analysis

Polar lipids (mainly phospholipids) obtained from acetone precipitation were made up in chloroform and divided into two equal volumes. One portion was used for the quantification of phospholipid classes (polar head groups) and the second half was used for quantification of the phospholipid fatty acids.

3.2.5.1. Phospholipid Classes

Individual phospholipids were separated using silica gel preparative TLC (0.5 mm thick) (Analtech, Newark, DE, USA) using a solvent system of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol). Phospholipid classes were located by exposing plates to a minimal amount of iodine vapor. The identification of individual phospholipids was done by comparison of R_f values to authentic standards (Sigma, Mississauga, ON, Canada). Silica gel containing each identified spot was scraped into a clean dry test tube. Bligh-Dyer solution (1:2 ratio of chloroform/methanol by vol) was added to the silica gel and vortexed for 1 min before centrifugation (4000 rpm for 10 min). Supernatants were collected, brought to complete dryness under a stream of nitrogen gas, and stored at 4°C until use.

Phospholipid classes were quantified using a modified colorimetric micro-phosphorus assay (Bartlett, 1958). To each tube, 1 ml of perchloric acid (Caledon, Georgetown, Ontario) was added and the mixture was heated to 130°C for 2 h. The mixture was cooled to room temperature on ice and 7 ml of distilled water, 1.5 ml of ammonium molybdate (2.5% w/v), and 0.2 ml of Fiske and Subbarow reagent (Sigma, Mississauga, ON, Canada) were added to each tube. The

tubes were placed in boiling water for 7 min and allowed to cool for 20 min. Samples were analyzed at λ 830 on a UV-visible spectrophotometer (Varian, Palo Alto, CA, USA). Calibration curves were obtained using potassium phosphate monobasic (BioShop, Burlington, ON, Canada) as a standard.

3.2.5.2. Phospholipid Fatty Acid Analysis

Fatty acid methyl esters (FAME) were prepared by transesterification using 2 ml of BF₃-methanol (14%) for each sample and heating to 70°C for 60 min. Samples were cooled on ice, diluted with 2 ml of water, and extracted with 2 ml of hexane. The hexane fractions were combined and FAME were analyzed on an Agilent 6890 Series gas chromatograph coupled with an Agilent 5973 quadropole mass spectrometer using a 30 m × 0.25 mm ID × 0.25 µm ZB-5 capillary column (5% Phenyl; 95% dimethyl-polysiloxane, Phenomenex, Torrance, CA, USA) in splitless mode. The temperature program began at 100°C and increased by 20°C min⁻¹ to 200°C, held for 5 min, increased by 10°C min⁻¹ to 240°C and held for 5 min. Injector and detector temperatures were 250 and 280°C, respectively. FAME were identified by comparison to the retention times of authentic standards (Supelco, Bellefonte, Pennsylvania, USA) and analysis of MS fragmentation. Peaks were analyzed using ChemStation software.

3.3. RESULTS

3.3.1. Total Lipid Content

Analysis of total lipid content found *P. sulcatum* to have significantly higher intrinsic lipid content (15% lipid dry weight:dry weight) than the other tested fungi. *Alternaria solani* and *F. sambucinum* had statistically similar lipid content (average 9%) while *B. cinerea* had the lowest lipid content (3%). The percentage of neutral lipids (percentage from total lipids) was highest in *A. solani* (84%) which was almost double that of *B. cinerea* (45%). *Fusarium sambucinum* and *P. sulcatum* had comparable percent neutral lipid content (64%). Similar results were observed in the percentage of polar lipids. *B. cinerea* had the highest percentage of polar lipids (54%) while *A. solani* had the lowest (16%). *Fusarium sambucinum* and *P. sulcatum* had comparable percent polar lipid content (37%).

3.3.2. Analysis of Ergosterol Content

In all cases, *A. solani* was found to have the largest ratio of sterols (Table 1) regardless of the basis of comparison (relative to biomass dry weight, total lipids or phospholipids). Sterol content of *B. cinerea* and *F. sambucinum* had significantly less sterols than *A. solani* (10- to 20-fold less depending of basis of comparison) (Table 1). Sterols were not detected in *P. sulcatum* (Table 1).

Table 1- Ergosterol content of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum*.

	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>
S:DW ratio ^a	5.33 a	0.55 b	0.15 b	0.00 c
S:L ratio ^b	5.93 a	0.65 b	0.56 b	0.00 c
S:P ratio ^c	1.79 a	0.12 b	0.06 b	0.00 c

Values in the same row followed by a same letter are not significantly different according to Student's t test ($P < 0.05$).

^a Ratio of sterols:dry weight

^b Ratio of sterols:total lipids

^c Molar ratio of sterols:phospholipids = (ergosterol content x 396) / (phospholipids x 725)

3.3.3. Analysis of Phospholipids

The composition of phospholipids in the membranes varied amongst the tested organisms. Both phosphatidic acid (PA) and phosphatidylglycerol (PG) had statistically similar content across all tested organisms (Table 2). The average amounts of PA and PG were approximately 2 and 36% of total polar lipids, respectively. Phosphatidylcholine (PC) was highest in *A. solani* and *F. sambucinum* (approximately 36%). *Pythium sulcatum* had a significantly lower PC than *A. solani* and *F. sambucinum*, while *B. cinerea* contained the least amount of PC (Table 2). The proportions of phosphatidylethanolamine (PE) had the greatest variation amongst tested organisms (Table 2).

Table 2- Phospholipid content (percentage of total polar lipids) of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum*.

Phospholipid	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>
PA	2.7 a	2.0 a	0.8 a	2.7 a
PC	39.4 a	7.1 c	33.9 a	17.5 b
PE	1.8 c	34.5 b	2.0 c	41.6 a
PG	34.0 a	37.1 a	42.0 a	32.0 a
PS+PI	19.1 a	14.1 a	17.4 a	5.0 b
CL	2.6 b	5.2 a	2.7 b	1.2 b
Unknown	1.3 a	-	3.8 a	-
Anionic	58.6 a	58.4 a	63.6 a	40.9 b
Zwitterionic	41.4 b	41.6 b	36.4 b	59.1 a
A/Z	1.4 b	1.4 b	1.8 a	0.7 c

PA, phosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS+PI, phosphatidylserine+ phosphatidylinositol; CL, Cardiolipin.

Values in the same row followed by a same letter are not significantly different according to Student's t test ($P < 0.05$).

Values are expressed as a percentage of the total phospholipids.

Anionic = percent anionic phospholipids

Zwitterionic = percent zwitterionic phospholipids

A/Z = ratio of anionic:zwitterionic phospholipids

Pythium sulcatum had the highest PE content of all tested organisms. *B. cinerea* had lower PE content, whereas *A. solani* and *F. sambucinum* had the lowest PE content phosphatidylserine (PS) and phosphatidylinositol (PI) co-migrated in the employed solvent system. The tested microorganisms had similar PS+PI content, although PS+PI contents in *A. solani* and *B. cinerea* were significantly different when compared to *P. sulcatum* (Table 2). *B. cinerea* had a

significantly higher proportion of cardiolipin (CL) than all other tested microbial strains. An unknown phospholipid was also identified in statistically similar proportions in both *A. solani* and *F. sambucinum* and was undetectable in *B. cinerea* and *P. sulcatum* (Table 2). *Fusarium sambucinum* was found to have the largest A/Z ratio while *P. sulcatum* had the lowest. *Alteraria solani* and *B. cinerea* had statistically similar A/Z ratios.

3.3.4. Fatty acid profiles

Analysis of the fatty acid (FA) profiles showed marked variability in relative percentage of individual FA between microorganisms (Table 3). Palmitic (16:0), oleic (18:1 $\Delta 9$), and linoleic (18:2 $\Delta 9,12$) were the main FA found accounting for 58.8 (*P. sulcatum*) to 90.9% (*A. solani*) of total FA (Table 3) in each organism. Among these major FA, 16:0 content was highest in *B. cinerea* and *P. sulcatum*. *Fusarium sambucinum* had lower 16:0 content than *P. sulcatum* and *A. solani* had lower 16:0 content than *B. cinerea* and *P. sulcatum*. The 18:1 content was equivalent in *A. solani*, *F. sambucinum*, and *P. sulcatum* (approximately 16.5%). The presence of 18:1 was not detected in *B. cinerea*. *Alternaria solani* and *B. cinerea* showed the highest 18:2 content (approximately 56%). *Fusarium sambucinum* had a significantly lowered 18:2 than *A. solani* and *B. cinerea* while *P. sulcatum* showed the lowest 18:0 content. Among other FA, 12:0, 13:0, 14:1, 18:3, 20:1, 20:2, 20:3, 20:4, and 20:5 were only detected in *P. sulcatum* (Table 3). Of particular note, the highly unsaturated 20:4 and 20:5 were present in relatively high concentrations (Table 3).

In order to better comprehend total unsaturation of the FA, various percentages and ratios were calculated. Saturated FA were highest in *P. sulcatum*. *Botrytis cinerea* and *F. sambucinum* contained statistically lower saturated FA contents, whereas *A. solani* contained the lowest saturated FA content (Table 3). Monounsaturated FA were similar in *A. solani*, *F. sambucinum*, and *P. sulcatum* (approximately 20%) whereas *B. cinerea* had significantly lower monounsaturated FA. Polyunsaturated FA were highest in *A. solani* and *B. cinerea* (approximately 56%). *Fusarium sambucinum* contained significantly lower polyunsaturated FA's. *Pythium sulcatum* contained the least amount of polyunsaturated FA. Overall, the total unsaturated FA were highest in *A. solani* and lowest in *P. sulcatum* (Table 3). A significantly higher ratio of unsaturated to saturated fatty acids was found in *A. solani* (Table 3). The ratio of polyunsaturated to monounsaturated was significantly higher in *B. cinerea* than the other tested fungal strains by approximately 10 fold (Table 3). The degree of unsaturation of the C₁₈ FA was highest in *B. cinerea* whereas *F. sambucinum* and *P. sulcatum* had the lowest degree of C₁₈ FA unsaturation (Table 3). When degree of unsaturation of all detected FA (Δ /mol total) was analyzed, there were no significant differences among all the microorganisms (Table 3).

Table 3- Fatty acid (FA) profiles of polar extracts from *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum* grown at 28.5°C

FA	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>
12:0	- ^a	-	-	0.83
13:0	-	-	-	0.02
14:0	-	0.42 b	0.64 b	12.6 a
14:1 Δ9	-	-	-	0.24
15:0	-	0.22 a	0.19 a	0.07 a
16:0	17.6 c	28.6 ab	23.0 bc	29.7 a
16:1 Δ9	1.44 b	2.18 b	1.97 b	7.48 a
17:0	-	0.14 a	0.22 a	0.06 a
17:1 Δ10	-	0.25	-	-
18:0	3.77 b	2.34 c	7.47 a	0.86 d
18:1 Δ9	19.8 a	-	17.8 a	11.9 a
18:2 Δ9,12	53.5 a	59.1 a	44.8 b	17.2 c
20:0	-	-	0.21 a	0.13 a
18:3 Δ6,9,12	-	-	-	1.20
20:1 Δ11	-	-	-	0.31
20:2 Δ11,14	-	-	-	0.22
22:0	-	0.15 a	0.16 a	0.04 a
20:3 Δ8,11,14	-	-	-	0.81
22:1 Δ9	-	-	-	0.33
20:4 Δ5,8,11,14	-	-	-	3.58
20:5 Δ5,8,11,14,17	-	-	-	8.64
Saturated	21.3 c	31.9 b	31.7 b	44.3 a
Monounsaturated	20.8 a	2.43 b	19.7 a	20.0 a
Polyunsaturated	53.5 a	59.1 a	44.8 b	31.7 c
Total unsaturated	74.2 a	61.6 b	64.6 b	51.9 c
U:S ratio ^b	3.60 a	2.00 b	2.04 b	1.17 b
P:M ratio ^c	2.63 b	25.3 a	2.28 b	1.65 b
Degree of unsaturation (Δ/molC ₁₈) ^d	1.65b	1.92 a	1.53c	1.60bc
Degree of unsaturation (Δ/mol total) ^e	1.33 a	1.28 a	1.14 a	1.23 a

Fatty acid profiles are presented in percent area (% of total area). Values in the same row followed by a same letter are not significantly different according to Student's t test (P < 0.05).

^a Not detected.

^b Ratio of unsaturated:saturated FA's.

^c Ratio of polyunsaturated:monounsaturated FA's.

^d Δ/molC₁₈ = [(1 x 18:1) + (2 x 18:2) + (3 x 18:3)]/ total C₁₈.

^e Δ/moltotal = [(1 x monoene) + (2 x diene) + (3 x triene) + (4 x tetraene) + (5 x pentaene)]/100.

3.4. DISCUSSION

Fungal membranes primarily act as a barrier to maintain cellular integrity (Avis, 2007). The dynamic of the membrane and its lipid composition play a crucial role in its interactions with the environment and with many membrane-associated proteins (Avis, 2007; Benyagoub *et al.*, 1996). Lipids are the major constituents of the fungal membrane, but the exact composition of lipid content differs between taxonomically different groups (Weete, 1974). It can also be altered based on environmental factors such as temperature, oxygen requirements, and nutritional components (Suutari, 1995; Pedneault *et al.*, 2007).

Many antifungal compounds exist currently, however resistance development in fungi is a great concern. The main reason for the development of resistance is that antifungal compounds generally target one or more specific biosynthetic pathways (Knight *et al.*, 1997). A fungus may become resistant through spontaneous and/or induced genetic mutation, or through the acquisition of resistance genes from other species, leading to the entire bypassing of the affected pathway (McDonald and Linde, 2002). An alternative is to target the general structure of the fungal biomembrane. Many antimicrobial compounds interact with fungal membranes, leading to disorder, disruption or damage to the membrane or its components (Avis and Bélanger, 2001; Benyagoub *et al.*, 1996; Deleu *et al.*, 2008; Xie *et al.*, 2007). As there are evolutionary constraints placed on 'redesigning' biomembranes, it is thought that sensitive fungi would remain sensitive to these compounds (Avis, 2007). It has recently been postulated that fengycin, a known antifungal compound produced by *B. subtilis* strains, targets the membrane, but the exact mode of action has yet to be determined (Deleu *et al.*, 2008). Previous work in our laboratory had determined that the most fengycin-tolerant microorganism was *A. solani*, followed by *F. sambucinum*, *B. cinerea*, and finally *P. sulcatum* (T.J. Avis, personal

communication). In this study, we analyzed the lipid composition of these for fungal and fungus-like microorganisms with different relative sensitivities to fengycin, in order to gain insight into the antimicrobials mode of action.

Sterol analysis showed that ergosterol content correlated well with relative sensitivity of the tested microorganisms. Indeed, the most tolerant fungus (*A. solani*) contained higher ergosterol content than the more sensitive fungi (*F. sambucinum* and *B. cinerea*). The oomycete *P. sulcatum* was included in this study as it does not produce sterols, which correlated well with its higher sensitivity to fengycin. Sterols are known to buffer stress induced changes in membrane fluidity (Bloch, 1983), which may explain our findings. Microorganisms that have little or no sterols are more susceptible to fengycin-membrane interactions, leading to a decrease in viability, whereas those with a larger proportion of sterols are more capable of buffering stress induced changes. A study done by Avis and Bélanger (2001) found that the fungi *Idriella bolleyi* and *Pseudozyma rugulosa* had significantly higher ergosterol contents than all other fungi tested and were the most tolerant to an antifungal compound, *cis*-9-heptadecenoic acid, known to interact with fungal membranes and cause elevate membrane fluidity.

The overall amphipathic nature of glycerophospholipids is the foundation of membranes (Simon, 1974). Characteristics of individual phospholipids such as charge, head groups, and structure are known to vary between organisms and may alter membrane characteristics (Goyal and Khuller, 1994). There was little correlation between the individual phospholipid content and sensitivity to fengycin, however the ratio of anionic (acidic, negatively charged) to zwitterionic (neutral) phospholipids did correlate rather well with fengycin sensitivity. Results showed that the highly sensitive *P. sulcatum* possessed a generally more neutral composition of phospholipids, whereas the more resistant *F. sambucinum* possessed highly anionic

phospholipids. This may indicate that fengycin preferentially interacts with zwitteronic rather than anionic lipids. Fengycin exhibits two negative charges (glutamic acid residues) and only one positive charge (ornithine residue), making it anionic in nature and reducing its miscibility with the phospholipid head groups (Deleu *et al.*, 2008; Eeman *et al.*, 2009). The lipid tail of fengycin inserts readily into the membrane at physiological pH, but the peptide moiety remains outside the membrane (Deleu *et al.*, 2008). Other work has reported that the presence of negative charges on fengycin would result in electrostatic repulsion from the phospholipid headgroups in the membrane (Eeman *et al.*, 2009; Maget-Dana and Ptak, 1995). Our results may therefore indicate that the more anionic phospholipids in the more tolerant fungi reduce fengycin interactions through increased electrostatic repulsion.

It has been found that fatty acid composition and unsaturation in polar lipids can alter membrane fluidity as an adaptive response to temperature fluctuations or other stressors (Avis and Bélanger, 2001). The main FA's found in this study were palmitic (16:0), oleic (18:1 Δ^9), and linoleic (18:2 $\Delta^9,12$) acids. Similar studies on the FA profile of other fungi (*Pleurotus ostreatus* and *P. cornucopiae*) yielded similar results (Pedneault *et al.*, 2007). An increase in palmitic acid correlated well with the sensitivity of the fungi to fengycin. Other individual FA contents did not correlate well with microbial sensitivity. It should however be noted that the highly unsaturated arachidonic (20:4) and eicosapentaenoic (20:5) acid were only detected in *P. sulcatum* and at relatively high concentrations, which might explain its higher sensitivity to fengycin. Elevated FA unsaturation is known to increase membrane fluidity, which may adversely affect membrane order in the presence of an additional membrane affecting compound (Benyagoub *et al.*, 1996). However, while unsaturation of specific FA may indicate high membrane fluidity, the degrees of unsaturation over the major FA (Δ/molC_{18}) or all FA present

(Δ /mol total) is generally a better indicator of membrane fluidity. Of particular note, the average number of unsaturations over all FA (Δ /mol total) indicated that all fungi had equivalent mean unsaturations per moiety (approximately 1.2 unsaturations per FA on average). This suggests that the level of unsaturation of phospholipid fatty acids may therefore not be an important factor in determining microbial sensitivity to fengycin.

Overall, fungal sensitivity appears to be related mainly to low quantities of sterols. Fengycin is known to act in an all-or-none type of action (Patel *et al.*, 2011) and the buffering action of sterols may play a crucial role in the tolerance of a given organism (Deleu *et al.*, 2008). Another non-negligible factor in microbial sensitivity may be a high quantity of zwitterionic phospholipids. The negatively charged fengycin may interact more easily with zwitterionic (neutral) phospholipids rather than anionic phospholipids (Eeman *et al.*, 2009; Maget-Dana and Ptak, 1995). Future work will include the identification of the unknown compounds in the phospholipid fraction as well as an analysis of lipids following exposure to fengycin. Further testing on a larger spectrum of organisms and *in vivo* studies on plants or produce could potentially demonstrate the wide ranging activity of fengycin, as well as its durability and efficacy of *in situ* formulations. The data obtained provides new insight into the antifungal mode of action of fengycin, which is required information for the potential registration of fengycin or a fengycin-producing organism as an alternative control measure of plant pathogens.

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CHAPTER 4

GENERAL CONCLUSIONS

In the search for more durable and environmentally safe methods to control phytopathogens, promising antagonists should ideally be non-toxic to humans and the environment, less prone to resistance, effective against a broad range of pathogens, and able to be prepared and stored prior to application (Sharma *et al.*, 2009). The spore forming ability of *B. subtilis* and the vast array of antimicrobial compounds it can produce make it a valid candidate (Stein, 2005). Some *B. subtilis* strains have been approved for use as biopesticides (Sharma *et al.*, 2009). *Bacillus subtilis* CU12 is a strain isolated by our lab and is a known producer of fengycin (T.J. Avis, personal communication). This study looked at the production of additional antimicrobial compounds produced by *B. subtilis* CU12 as well as to gain insights into fengycin's mode of action on a broad range of phytopathogens.

The antifungal activity of *B. subtilis* CU12 was tested against four different plant pathogens, *A. solani*, *B. cinerea*, *F. sambucinum*, and *P. sulcatum*, which are most notable for the destruction of tomatoes, grapes, potatoes, and carrots, respectively (El-Banna *et al.*, 1984; Hiltunen *et al.*, 2002; Thomma, 2003; Toure *et al.*, 2004). Of the four tested, two are foliar borne pathogens (*A. solani* and *B. cinerea*), while the other two are soil borne pathogens (*F. sambucinum*, and *P. sulcatum*). *Pythium sulcatum* is also an oomycete and does not produce sterols. In a confrontational assay *B. subtilis* CU12 was found to inhibit all four tested pathogens to varying extents. Using this range of pathogens from various origins, we were able to see a small sample of the broad spectrum ability. Purification of the antifungal activity found that the butanol extract significantly inhibited the growth of all pathogens except for *F. sambucinum*. Differences observed in antifungal activity when using the bacteria compared to a more purified product could be an indication of synergistic effects of multiple antimicrobial compounds produced by the bacterium.

Figure 1. HPA system in an aqueous solution

The compound isolated and identified in this study was found to be the HPA system. To the best of our knowledge, this is the first report of a *B. subtilis* strain producing HPA. The production of HPA has been found in other genera such as *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, and most notably, *Lactobacillus* (Vollenweider *et al.*, 2003). The HPA-based multi-compound system has been found to have a broad spectrum of antimicrobial activity, and in some cases it can be active against the compound producing organism (Dobrogosz and Lindgren, 1988; Vollenweider and Lacroix, 2004). Our results found that *B. subtilis* CU12 was able to grow in concentrations up to 1360 µg/mL HPA. Other studies have found that concentrations of 30-50 µg/mL can be lethal to test organisms such as *B. subtilis* ATCC 6633, indicating a high tolerance in our strain. These findings may demonstrate that this strain is capable of producing, and is tolerant to large quantities of HPA.

With an enhanced tolerance, and greater potential to produce large quantities, *B. subtilis* CU12 may be more effective at controlling phytopathogens. Efficacy depends on the target organisms and the mode of action. The mode of action of HPA is difficult to pin point due to the complexity of the HPA system. Researchers have hypothesized two mechanisms of action; one

that interactions of HPA with the target lead to an inactivation of proteins with subsequent growth inhibition or that structural similarities with ribose and competitive inhibition causes a halt in DNA synthesis (Vollenweider *et al.*, 2010; Schaefer *et al.*, 2010). Findings indicate that the monomeric aldehyde of HPA is more chemically reactive and may exhibit a more pronounced antifungal effect than the ribose-like structure of the HPA-dimer or the hydrate (Vollenweider *et al.*, 2010). The forms of HPA in a solution are concentration-dependent with HPA-dimer as the predominant form at higher concentrations and the HPA aldehyde as the main form at lower concentrations (Vollenweider *et al.*, 2003). Thus, further studies should investigate the antifungal effect of various concentrations of HPA to determine if the enhanced production leads to an increase in observed antifungal toxicity.

Studies have investigated the probiotic properties of HPA and also found inhibition of human pathogens indicating little to no toxicity to humans, however, the HPA system and its components can be altered based on the pH, concentration, and temperature of a given system (Dobrogosz and Lindgren, 1988; Vollenweider and Lacroix, 2004). Under long incubation times at 20°C, the production of acrolein has been observed (Vollenweider *et al.*, 2003). Further testing of field conditions is needed to ensure the stability of the HPA system and that harmful byproducts are not produced.

Another area lacking knowledge is the compatibility of HPA with other treatments and compounds. The combination of multiple antimicrobials can sometimes lead to synergistic effects. Also, the use of various antimicrobial compounds with different modes of action may increase the amount of inhibition observed. It would be of interest to compare the inhibitory effects of HPA with other *B. subtilis* antimicrobials, such as fengycin, as well as a combined HPA/fengycin treatment. A much different mechanism of action of HPA from the membrane

interactions and pore inducing all-or-none action of fengycin (Deleu *et al.*, 2008) may lead to synergistic effects.

HPA is not only an antimicrobial, but it is also an important precursor in the production of some industrial chemicals (Vollenweider and Lacroix, 2004). Most current methods of HPA production are chemical based and either expensive or yield harmful side products. The use of a renewable source for the production of HPA coupled with the decreased cost in production (less expensive equipment) make the use of this strain more appealing (Vollenweider and Lacroix, 2004). One of the greatest problems with the bioproduction of HPA is that sensitivity to the compound limits accumulation of the product (Talarico and Dobrogosz, 1989). Methods to bypass this pitfall have had low yields and in order to optimize production, bacterial strains must be discovered or developed that are more tolerant to HPA. Our strain has demonstrated an enhanced tolerance to the compound and further optimization of HPA production with *B. subtilis* CU12 should be investigated for the sake of biotechnological production as well as generating a superior strain with potential inhibitory properties.

In the second part of this study, we more closely studied the cyclic lipopeptide fengycin. The precise mode of action of fengycin has yet to be elucidated, but it has been shown that fengycin interacts with the fungal membranes (Deleu *et al.*, 2008). In this study, the lipid content of various plant pathogens was compared to their relative sensitivities to fengycin to gain insight into the mode of action or mechanism behind its antifungal activity. We determined that sterol content was higher in the more tolerant fungus and lower in the more sensitive fungi. Our results agree with literature that states sterols in the membrane have been shown to buffer stress induced changes in membrane fluidity (Bloch, 1983). The most sensitive microorganism tested, *P. sulcatum*, is an oomycete, a class of fungus-like microorganisms which lack membrane sterols.

Lipopeptide variants produced by *Bacillus* and *Pseudomonas* spp. have been found to have a significant effect on oomycetes (Raaijmakers *et al.*, 2010).

A common way to investigate the mode of action is to perform a comparative analysis of the membrane composition with and without the presence of a sub-inhibitory concentration of an antifungal compound. Such a test is sometimes useful in determining the actual changes observed with the membrane following partial inhibition. A study done by Avis and Bélanger (2001) found that *cis*-9-heptadecenoic acid partitioned into membranes and fungi with a greater sterol content were more resistant because they could buffer the changes in membrane fluidity. Utilization of this type of study may not be appropriate to investigate fengycin because it is reported to act in an all-or-none type fashion where a low concentration does not affect fungal growth or cause membrane pores and higher concentrations will destroy fungal cells leaving little or no biomass to study.

Little correlation was found when analyzing the individual phospholipids, but the relative sensitivities of fungal strains to fengycin did correlate to the ratio of anionic/zwitterionic phospholipids. Our findings found that more anionic phospholipids were present in the tolerant fungi and that electrostatic repulsions may reduce fengycin interactions. At physiological pH, fengycin exhibits two negative charges (glutamic acid residues) and one positive charge (ornithine residue) (Deleu *et al.*, 2008; Eeman *et al.*, 2009). The charges on these amino acid groups can be altered based on the pH of the environment. Studies should be performed on fengycin at different pH levels to assess conformational changes and intermolecular interactions with the membrane. A deeper understanding into the molecular interactions could allow us to define the precise mode of action, optimal conditions for antifungal activity, and potentially help identify sensitive fungi based on known membrane compositions.

The data obtained in this project has defined the membrane composition of the four tested plant pathogens. Using this data we can create model membranes, introduce fengycin, and study changes in model membranes with varying sensitivities to fengycin. We can also use techniques in fluorescent microscopy to visualize changes that occur between treatments and controls to investigate molecular interactions on a smaller scale (the membrane or even the single molecule level).

The forms and quantities of fengycin produced by *B. subtilis* may be subject to change based on environmental conditions which could directly affect efficacy of the antifungal action. Various *Bacillus* spp. have already been approved for use as biocontrol agents, however the potential toxicity to other organisms and the environment must be tested for *B. subtilis* CU12. In terms of toxicity, fengycin is known to have reduced haemolytic activity in comparison to the other cyclic lipopeptides, surfactin and iturin (Ongena and Jacques, 2008; Vanittanakom *et al.*, 1986). Other effects of fengycin *in situ* must also be studied. The application of *B. subtilis* CU12 onto various fruits and vegetables should be investigated, not only for *in situ* antimicrobial effects, but also to test for the production of undesirable traits such as rotting or discoloration on the fruits or vegetables themselves.

Along with production, there is also a need for the study of stability and persistence of fengycin in the environment. Studies by Touré *et al.* (2004) and Romero *et al.* (2007) have both been able to recover fengycin from the test organisms, but yields are low. Surfactins produced by *Bacillus* spp. have been shown to remain in soil for up to 25 days (Asaka and Shoda, 1996). The *in situ* detection of lipopeptides is difficult due to their amphiphilic nature, which allows adsorption to soil as well as irreversible insertion into the membranes of both target and non-target organisms (Raaijmakers *et al.*, 2010). More sensitive methods for detection within target

organisms or in the soil are needed when testing for the persistence of fengycin in the environment (Ongena and Jacques, 2008).

In conclusion, *B. subtilis* CU12 has demonstrated the ability to produce a vast array of antifungal compounds. In the development of biopesticides based on *B. subtilis* CU12, the production of HPA by the bacterium may contribute to the antifungal effects against phytopathogens. With an increased tolerance to the antimicrobial compound, this allows further studies on biotechnological production and potentially enhanced ability to inhibit phytopathogens. Fengycin has a broad range antifungal affect and it was found that both sterol content and anionic/zwitterionic content played a role in the relative sensitivities of the tested plant pathogens. Further studies using fluorescent microscopy and other tests can utilize the information obtained from this work to create model membranes and examine the interactions on a smaller scale.

Both, the production of HPA and a greater understanding of the mode of action of fengycin contribute to our overall knowledge and bring us steps closer to potential registration of *B. subtilis* CU12 as a biocontrol agent.

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