Bacillus mojavensis RRC101 lipopeptide antagonism induces morphologic and metabolic changes echoed by transcriptomic response in *Fusarium verticillioides*

by

ALEX BLACUTT

(Under the Direction of Scott Gold)

Fusarium verticillioides is a mycotoxigenic pathogen of corn, capable of an intimate host association via asymptomatic endophytism and seed infection. Through production of fumonisin mycotoxins, this fungus threatens the industrial and agricultural uses of maize grain. Fumonisin consumption causes lethal diseases in livestock and is linked to esophageal cancers in humans, leading to mandatory regulation. The ubiquity of this pathogen and its close association with the host pose a significant challenge to chemical control strategies, presenting an opportunity for efficacious biological control. The work detailed here characterizes the interaction of *F. verticillioides* with a bacterial endophyte of maize and potential biocontrol agent, *Bacillus mojavensis* strain RRC101. Initial work resulted in the sequencing and annotation of the RRC101 genome for biocontrol-relevant genes, identifying synthetase operons for surfactin and fengycin class lipopeptides. Further work identified the fengycins as primary antifungal secondary metabolites involved in antagonism of *F. verticillioides*. This antagonism generates severe hyphal deformation and lysis, accompanied by increased secondary metabolic activity. Subsequent analysis focused on evaluating the specificity of *F. verticillioides* response to lipopeptide challenge, through transcriptomic analysis of cultures exposed to individual classes and synergistic antagonism. Bioinformatic characterization of lipopeptide-responsive genes

identified differential localization and functional enrichment across lipopeptide classes, suggesting nuanced perception of antagonism in *F. verticillioides*. These transcriptomic data revealed that antagonism induces putative core stress response genes, as well as those involved in maize colonization, indicating common stress pathways involved in microbial antagonism and maize colonization. Additionally, microscopic and biochemical phenotypes of antagonism were corroborated as genes involved in structural components and secondary metabolism were upregulated under antagonistic conditions. These data also provided support for apoptosis-induction as a consequence of lipopeptide antagonism, suggesting an additional mechanism of action underlying this biological control. Together, these data illustrate the differential response of *F. verticillioides* to bacterial lipopeptides and characterize the subsequent response of this phytopathogen to antagonistic conditions.

INDEX WORDS: Fusarium verticillioides, Bacillus mojavensis, bacterial lipopeptides, transcriptomics, biological control, stress

Bacillus mojavensis RRC101 lipopeptide antagonism induces morphologic and metabolic changes echoed by transcriptomic response in *Fusarium verticillioides*

by

ALEX BLACUTT

BS, Virginia Polytechnic Institute & State University, 2009

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

Bacillus mojavensis RRC101 lipopeptide antagonism induces morphologic and metabolic changes echoed by transcriptomic response in *Fusarium verticillioides*

by

ALEX BLACUTT

Major Professor:	Scott Gold
Committee:	Anthony Glenn
	Ronald Walcott
	Shavannor Smith
	Charles Bacon

Electronic Version Approved:

Suzanne Barbour

Dean of the Graduate School

The University of Georgia

May 2017

DEDICATION

Thank you to my parents, who raised me, provided for me and gave me the freedom to follow my imagination, but also made sure I learned algebra and stuff.

Thank you to my major professor, Scott Gold, whose patience, guidance and wisdom were indispensable throughout my graduate career, beyond the research in this dissertation. I hope to carry his lessons in mentorship and sense of humor throughout my life. If you don't like anything in here, he did that too.

ACKNOWLEDGEMENTS

Finally sitting down and listing out the people who have helped me along in my research, it's extremely clear to me that every successful scientific effort is the result of collaboration. Our interpersonal relationships in the lab and beyond shape our lives and our research, and I am extremely fortunate to have shared my time at UGA and the USDA with the following people.

I first have to thank Scott Gold, my major professor. If Scott hadn't taken me on as a student and maintained his unique blend of encouragement and pressure, I doubt that my research would have been as successful as it was. His willingness and ability to act as a sounding board and his (much-taxed) editing skills have been essential to the writing process. He has also always proven his dedication to students, from the students he teaches to those he supervises in the lab (and those who go from one to the other), he's a dedicated educator and every student is fortunate to have worked with him. I hope that I can someday come close to his abilities as a supporting and dedicated mentor, which are only matched (from what I can tell) by his poker skills. Emulating either of those skills would probably be fine by me, really.

Dr. Ron Walcott is an excellent instructor, and has consistently given me advice and confidence regarding my abilities and challenges ahead. His focus on the fundamentals of plant pathology while maintaining a sense of broader context (and a robust sense of humor) is something I will try to maintain going forward. Dr. Shavannor Smith has been inspirational in her knowledge and her willingness to listen to students and support them in their development. If I ever have students of my own, I hope that I will be even half as effective in mentoring them. It has been an honor to have Dr. Anthony Glenn on my committee, to learn from his years of experience working on *Fusarium*, and to have made countless coffee runs to fuel said research. Without Dr. Charles Bacon's foundational work, none of my research would have been possible.

His contribution to my work has not ended there, of course, and as a committee member and encyclopedic resource of knowledge, he has been a tremendous help in my work. I could not possibly thank Dr. Bacon without acknowledging how much I owe to Dorothy Hinton. Beyond her saintly patience and assistance as I started my research, she has always been cheerful and interested in hearing graduate students talk about their lives.

The Gold lab and my research would undoubtedly grind to a halt without Nicole Jozwiak Crenshaw keeping track of our various needs and responsibilities (I'm sorry I wasn't more on top of autoclave responsibilities). Thank you for that and all the great conversation and advice. Thank you to all the undergraduate researchers (especially Garrett – good luck with grad school!) who helped along the way, even if they are all going to end up medical doctors.

As my colleague, coauthor, and friend, Trevor Mitchell provided tremendous support and was an essential resource in developing methods for my work. This work would never have been possible without the expertise and kindness of Dr. Maurice Snook. Without Maurice's expertise working with lipopeptides, my project would not have existed, and his experience with analytical chemistry greatly smoothed the ride of my work.

Thank you to Melinda Vongkunthong, Shawn Cunningham and Cricket Wray for all our conversations and helping me figure out how to actually do things at the USDA. Thank you to Thomas Baldwin for being a friend and showing me the importance of making time in grad school for personal enrichment, but especially disc golf and craft beer.

Thank you to Stephanie Bolton, an excellent researcher and an inspiration in her style and flair in design and presentation. I would like to thank Hugo Gao for being an exemplary lab- and officemate, from the conversations we've had to the food we've shared. Many thanks to Manisha Rath for her support and commiseration throughout our respective student careers. It has been a

pleasure working alongside Shan Gao with her relentless optimism and determination. Many thanks to the Fungal Group at UGA, for the exposure to brilliant researchers and their work, as well as the input on my research throughout my graduate career.

I would also like to thank my fellow plant pathology graduate students, particularly Sidney Everhart and Ansuya Jogi for their guidance over the years. Thank you to the current current SAPPs crew, for inspiring me to be more involved and ambitious as I continue my career.

Double-thanks to the Plant Pathology office staff, Mary Ann, Kisha, Brooke, Judy, Kathy and anyone I've missed, who keep the department running and make sure we graduate students get reimbursed for the conferences we attend. A special thanks to UGA, for instilling in me a great sense of sympathy and solidarity with graduate students around the world. My time here has taught me to never forget the debt every top-tier university owes to its graduate students, whose work is responsible for so much acclaim and prestige.

If you're still reading this, then thank you for taking the time to examine the research I've conducted over these years. I hope that it can help you on your path, or at least that it isn't too boring (I think figure 3.7 looks pretty cool, at least).

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Research Objectives

The purpose of the proposed research was to characterize the molecular underpinnings of the biocontrol interaction between *B. mojavensis* and *F. verticillioides*, two endophytes of maize. Collectively, the experiments conducted were designed to fill knowledge gaps with regards to the mechanisms of antagonism by *B. mojavensis* and the response by *F. verticillioides*. Together, the data gathered should provide a better assessment of the biocontrol potential of *B. mojavensis*, and better inform the development of future biological control agents (BCAs) for pathosystems beyond maize. Broadly, the research described was guided by the following objectives:

1. Evaluate the capability of *Bacillus mojavensis* RRC101 to produce lipopeptides and identify their contribution to *F. verticillioides* antagonism (Chapter 2)

2. Assess the impact of lipopeptide challenge on cellular processes in *F. verticillioides* (Chapters 3 and 4)

Corn (*Zea mays*) is an economically significant crop, averaging an annual value of $66 billion in the US alone from 2010-2014 (National Corn Growers Association, 2015). The world was projected to produce 961 million metric tons of corn from 2015-2016, with the top-producing United States contributing 345 million tons (WAOB, 2016). Domestic corn consumption in the US was projected to be 12.4 billion bushels (299 million tons) from 2015 – 2016, with fuel ethanol and animal feed utilizing approximately 5 billion bushels (140 million

tons) each (ERS, 2016). Beyond animal feed, the grain, in hundreds of cultivars, is a cultural and nutritional staple across Latin America and much of the developing world, making it an invaluable food source for humanity (Nuss and Tanumihardjo, 2010).

Fusarium verticillioides (synonym *F. moniliforme*, teleomorph: *Gibberella moniliformis* Wineland) is a maize pathogen causing *Fusarium* ear and stalk rots as well as seedling blight. Despite its pathogenic habit, *F. verticillioides* can survive as a symptomless endophyte within vegetative tissues and maize kernels (Munkvold et al., 1997). Seed infection is a strong contributor to dissemination and success as a pathogen (Yates et al., 2003). *F. verticillioides* is well known for producing the fumonisin mycotoxins during maize colonization, and while they are known to be virulence factors, they have also been found in significant quantities in asymptomatic maize tissue (Bacon and Hinton, 1996; Bacon et al., 2008; Glenn et al., 2008). The *Fusarium*-maize relationship is further complicated by the fact that, when living endophytically, the fungus can act as a growth promoter or protectant against other pathogens (Yates et al., 1997; Lee et al., 2009).

Quantifying yield losses due to disease caused by *F. verticillioides* is complicated by its ubiquity and insect-vectored nature; maintaining an uninfected control plot in field conditions is difficult. Studies evaluating disease severity and fumonisin concentration in natural- and artificially-inoculated outbreaks have demonstrated a correlation between disease severity and reduced yield, although, due to unintended natural infection, an uninfected control was unavailable (Presello et al., 2008). Further field experiments demonstrated a correlation between disease severity and fumonisin concentration in harvested grain, suggesting that the pathogen reduces both quantity and quality of potential maize harvests (Presello et al., 2007).

Fusarium verticillioides is one of several species of *Fusarium* that are known to produce the fumonisin mycotoxins. This family of toxins exists in a variety of series and isoforms, the most naturally prevalent being the carcinogenic, cytotoxic B series (FB_1, FB_2, FB_3 etc), with FB_1 the most abundant of these isoforms (WHO, 2000). FB_1 is the first discovered inhibitor of sphingolipid metabolism, with significant research applications. In mice, the toxin is both hepatocarcinogenic and nephrocarcinogenic, and fumonisin consumption has been shown to cause other animal diseases such as equine leukoencephalomalacia and porcine pulmonary edema (Kellerman et al., 1990; Haschek et al., 2001). All tested animals are susceptible to fumonisin mycotoxicoses, although swine and horses are especially sensitive due to the high content of contaminated maize in feeds. This is illustrated by outbreaks reported over the past decade (Morgavi and Riley, 2007). Although fumonisin sensitivity varies by animal, all tested species suffered renal or liver toxicity, as well as increased cancer rates.

As maize is a primary feed ingredient for meat and egg chickens, the risks and consequences of mycotoxin contamination have been rigorously evaluated (Murugesan et al., 2015). Although birds in general are less sensitive than mammals to fumonisins, consumption of contaminated grain still poses a threat to poultry production, with turkeys more sensitive than chickens. Symptoms of fumonisin toxicosis in poultry include reduced animal weight and egg production, hepatotoxicity, immunosuppression and increased incidence of intestinal pathogens (D'Mello et al., 1999). As grain for poultry feed can harbor multiple mycotoxigenic species, and a given species may produce multiple mycotoxins, studies and meta-analyses regarding co-contaminated feed are essential to properly evaluate food and feed security (Grenier and Oswald, 2011). These analyses reveal interactions between aflatoxins and fumonisins in poultry, particularly synergistic effects on immunosuppression, reducing body weight gain and increased

pancreas weight. The ubiquity of mycotoxigenic fungi and the economic effects of feed contamination warrant surveillance and, where possible, mitigation of *F. verticillioides* infection and fumonisin contamination.

Relative to the observed deleterious effects in livestock, fumonisins have only been recently associated with human illness. Maize contaminated with *F. verticillioides* has been associated with both esophageal cancer in South Africa and the high rate of neural tube defects along the borders of Texas decades before the structures of the fumonisins were elucidated (Marasas et al., 1981; Sydenham et al., 1990). Both esophageal cancer and neural tube defects are now associated with this class of mycotoxins, which has been found in maize samples worldwide (Chu and Li, 1994; Hendricks, 1999; Marasas et al., 2004; Missmer et al., 2006). Additionally, cell-level cytotoxicity and apoptosis induction from fumonisin exposure have been observed in human and animal tissues, although this varies by tissue (Stockmann-Juvala and Savolainen, 2008). As a staple food, corn represents a major source of nutrition for both industrialized and developing nations; heavy fumonisin contamination of corn could have far-reaching and severe consequences for import/export economics and human health and safety.

Based on the "No Observed Effect Level" for renal toxicity (0.2 mg fb1/kg bodyweight) in rats and a safety factor of 100, the current World Health Organization "provisional maximal tolerable daily intake" for fumonisin B_1 is 2 µg/kg body weight per day (2001). Based on animal toxicity studies, the US Food and Drug Administration suggests that the fumonisin levels in maize should not exceed 2 to 4 ppm (Administration, 2001; Voss et al., 2007). The European Union has imposed regulatory limits for total fumonisin at 4 ppm for whole maize grain, and 0.2 to 2 ppm for any processed maize products (Commission, 2007). These regulatory limits lead to

rejection of maize harvests for food or feed use, and stringent restrictions threaten maize exports, particularly from the US (Miller et al., 1995; Wu, 2004; Dvorak et al., 2007).

Currently, there are no efficient chemical means for managing ear rot caused by *F. verticillioides*, forcing a reliance on cultural methods such as crop rotation and residue removal (Czembor et al., 2010). Studies have shown a correlation between corn resistance to *F. verticillioides* and reduced fumonisin levels, suggesting that resistant lines may mitigate grain contamination (Presello et al., 2007; Presello et al., 2011). A comparison of conventional and organic farming methods revealed a roughly 10% difference in average fumonisin B_1 levels in maize, suggesting that differences between these cultivation practices are not likely major determinants of fumonisin levels in grain production (Agustín et al., 2007). Due to the unclear, potentially beneficial, endophytic relationship between *F. verticillioides* and maize, proposed management strategies include introducing fumonisin-deficient *F. verticillioides* genotypes, or detoxifying fumonisins *in planta* and in postharvest processing, although detoxification strategies are complicated by the structural resilience of fumonisins (Duvick, 2001).

Biological control is generally defined by Eilenberg et al as: "'The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be" (Eilenberg et al., 2001). Biological control strategies can be divided into four categories:

1. Classical – "The intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control"
2. Inoculation – "The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently"

3. Inundation – “The use of living organisms to control pests when control is achieved exclusively by the released organisms themselves”
4. Conservation – “Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests”

In the context of plant pathology, biological control can be defined as, “the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens.” This goal can be achieved using mechanisms that are grouped into three categories (Pal and Gardener, 2006):

1. Direct antagonism includes parasitism or full predation of target organisms by the biological control agent (BCA), as seen in the interaction between nematophagous fungi and their hosts. This type of activity is relatively nonspecific, and highly variable in efficacy, as parasitic or predatory lifestyles may be a response to nutrient deficiency.
2. Mixed-path antagonism refers to the production of either waste products or secondary metabolites as the means of control, primarily seen with bacterial BCAs. These metabolites can act directly as antibiotics or may impair pathogen lifestyles through avenues such as disrupting molecular signals to prevent germination or quorum sensing, thereby lowering pathogen fitness.
3. Indirect antagonism is achieved through actions such as BCA exclusion of pathogens via niche occupation, nutrient sequestration or activation of host defenses.

These mechanisms are not mutually exclusive; it is hypothesized that some BCAs employ multiple strategies in target antagonism, for example producing an antibiotic while physically occupying space on a host root (Mousa et al., 2016).

Bacillus mojavensis, now known to inhabit soil, marine and phyllosphere environments, was first described in 1994 and differentiated from other *Bacillus* species by 16S rDNA data (Roberts et al., 1994). The *B. mojavensis* strain RRC101, isolated from a surface-sterilized maize seed, is known to colonize the maize rhizosphere and apoplast (Bacon and Hinton, 2002). RRC101 also reportedly demonstrates *in vitro* and *in planta* antifungal activity, particularly against mycotoxin-producing species such as *F. verticillioides* (Hinton and Bacon, 1995). Since its discovery, strains of *B. mojavensis* have been shown to also associate with coffee and sugar beet, suggesting a host range beyond grasses (Nair et al., 2002). A broad host range combined with antagonism towards phytopathogenic fungi suggest that *B. mojavensis* is a potential source of efficacious and versatile biocontrol agents.

Bacterial lipopeptides are nonribosomally synthesized, comprised of a peptide ring bound to a variable-length fatty acid chain; the lipopeptides are divided into classes based upon their structure and composition, with further variation within each class (Figure 1.1) (Ongena and Jacques, 2008). These compounds are primarily studied in *Bacillus* and *Pseudomonas*, with fitness impacts such as enhanced motility and antibiosis, as well as a potential for improved nutrient acquisition (Raaijmakers et al., 2010). Lipopeptide antibiosis varies by lipopeptide class as well as target organism, with the working model of antagonism thought to be a result of membrane destabilization and pore formation.

Antibiotic lipopeptides were first discovered in 1953, though their roles in both symbiotic and pathogenic plant-microbe interactions have been a growing research interest over the past decade (Heinemann et al., 1953; Ongena and Jacques, 2008). In addition to antifungal activity, bacterial lipopeptides have been demonstrated to be necessary for swarming motility and biofilm formation, particularly on plant roots (Fall et al., 2004). It is therefore probable that lipopeptide

production plays a significant role in host association and biological control activity of *Bacillus mojavensis*; lipopeptide synthesis has been observed for *B. mojavensis* RRC101 (a surfactin variant), and evidence suggests that one or more non-surfactin metabolites may be responsible for antagonism (Snook et al., 2009).

In the context of plant-microbe interactions, lipopeptides contribute to crop protection through multiple mechanisms of action, with classes acting individually or synergistically (Figure 1.2). These activities correspond with the aforementioned biocontrol mechanisms, but most can be classified as mixed-path or indirect antagonism. These mechanisms are thought to inflict less evolutionary pressure, theoretically prolonging their utility. Lipopeptides are biodegradable, biocontrol strategies incorporating these antibiotics would thus impose minimal disruption to local soil ecology. Additionally, since *B. mojavensis* has been observed to live within plant tissues, there is strong potential for directed activity against *F. verticillioides*. We hypothesize that *Bacillus mojavensis* produces additional lipopeptides, and that these lipopeptides are critical to *F. verticillioides* antagonism and maize colonization.

To confirm lipopeptides as major contributors to *B. mojavensis* antifungal activity, we initially propose to annotate the genome of *B. mojavensis* strain RRC101. Understanding the role of *B. mojavensis* as a potential biocontrol agent requires an appreciation of the mechanism by which it antagonizes *Fusarium verticillioides*, putatively secretion of antifungal lipopeptides. To this end, the antifungal capability of RRC101-produced lipopeptides will be assessed for *Fusarium verticillioides* antagonism.

Antibiosis, though one of the more straightforward biocontrol mechanisms, represents a complex interaction at the cellular level. In *Fusarium verticillioides* specifically, lipopeptide exposure has been shown to decrease secondary metabolite production, notably fumonisins (Hu

et al., 2009). Lipopeptides interfere with life processes in *Podosphaera fusca* and *Sclerotinia sclerotiorum*, particularly germination and sporulation, both of which are critical to plant pathogenesis (Romero et al., 2007). The most conspicuous fungal reaction to antibiotic exposure is one of arrested development (frequently visualized as a zone of inhibition on agar media), but transcriptional processes are not as well described.

Experiments will be conducted using varied levels of lipopeptides to evaluate the existence of dose-dependent morphological responses, with a focus on conidial germination and vegetative growth. In addition, transcriptional analyses will be used to guide construction of deletion mutants to examine the roles of specific differentially activated genes in response to lipopeptide exposure.

REFERENCES

2001. Safety evaluation of certain mycotoxins in food / prepared by the Fifty-sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Geneva : World Health Organization : IPCS, 2001.

Administration, U.S.F.a.D. (2001). Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds; Final Guidance.

Agustín, A., Gloria, E., Teresa, J., and Antonio, H. 2007. Estimation of dietary intakes of fumonisins B1 and B2 from conventional and organic corn. Food Control 18:1058-1062.

Bacon, C.W., and Hinton, D.M. 1996. Symptomless endophytic colonization of maize by Fusarium moniliforme. Can. J. Bot.-Rev. Can. Bot. 74:1195-1202.

Bacon, C.W., and Hinton, D.M. 2002. Endophytic and Biological Control Potential of Bacillus mojavensis and Related Species. Biological Control 23:274-284.

Bacon, C.W., Glenn, A.E., and Yates, I.E. 2008. FUSARIUM VERTICILLIOIDES: MANAGING THE ENDOPHYTIC ASSOCIATION WITH MAIZE FOR REDUCED FUMONISINS ACCUMULATION. Toxin Reviews 27:411-446.

Chu, F.S., and Li, G.Y. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. Applied and Environmental Microbiology 60:847-852.

Commission, E. (2007). Regulation No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards to Fusarium toxins in maize and maize products (Official Journal of the European Union), pp. 4.

Czembor, E., Adamczyk, J., Posta, K., Istvan, S., Oldenburg, E., and Schurch, S. (2010). Prevention of Ear Rots due to Fusarium spp. on maize and mycotoxin accumulation, P.B.a.A. Institute, ed (European Network for the Durable Exploitation of Crop Protection Strategies).

D'Mello, J.P.F., Placinta, C.M., and Macdonald, A.M.C. 1999. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology 80:183-205.
Duvick, J. 2001. Prospects for Reducing Fumonisin Contamination of Maize through Genetic Modification. Environmental Health Perspectives 109:337-342.
Dvorak, N.J., Riley, R.T., Harris, M., and McGregor, J.A. 2007. Fumonisin Mycotoxin Contamination of Corn-Based Foods Consumed by Potentially Pregnant Women in Southern California. REPRODUCTIVE SCIENCES 14:564.
Eilenberg, J., Hajek, A., and Lomer, C. 2001. Suggestions for unifying the terminology in biological control. BioControl 46:387-400.
ERS, U. (2016). Corn: Food, seed and industrial use, FGYearbookTable31.pdf, ed (Feed Grains Database: United States Department of Agriculture).
Fall, R., Vivanco, J.M., and Harsh Pal, B. 2004. Biocontrol of Bacillus subtilis against Infection of Arabidopsis Roots by Pseudomonas syringae Is Facilitated by Biofilm Formation and Surfactin Production. Plant Physiology 134:307-319.
Glenn, A.E., Zitomer, N.C., Zimeri, A.M., Williams, L.D., Riley, R.T., and Proctor, R.H. 2008. Transformation-mediated complementation of a FUM gene cluster deletion in Fusarium verticillioides restores both fumonisin production and pathogenicity on maize seedlings. Molecular Plant-Microbe Interactions 21:87-97.
Grenier, B., and Oswald, I. 2011. Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. World Mycotoxin Journal 4:285-313.
Haschek, W.M., Gumprecht, L.A., Smith, G., Tumbleson, M.E., and Constable, P.D. 2001. Fumonisin Toxicosis in Swine: An Overview of Porcine Pulmonary Edema and Current Perspectives. Environmental Health Perspectives Supplements 109:251.
Heinemann, B., Kaplan, M.A., Muir, R.D., and Hooper, I.R. 1953. Amphomycin, a new antibiotic. Antibiotic Chemotherapy 3:1239-1242.
Hendricks, K. 1999. Fumonisins and neural tube defects in South Texas. Epidemiology (Cambridge, Mass.) 10:198-200.
Hinton, D.M., and Bacon, C.W. 1995. Enterobacter cloacae is an endophytic symbiont of corn. Mycopathologia 129:117-125.
Hu, L.B., Zhang, T., Yang, Z.M., Zhou, W., and Shi, Z.Q. 2009. Inhibition of fengycins on the production of fumonisin B1 from Fusarium verticillioides. Letters in Applied Microbiology 48:84-89.
Kellerman, T.S., Marasas, W.F., Thiel, P.G., Gelderblom, W.C., Cawood, M., and Coetzer, J.A. 1990. Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B1. The Onderstepoort journal of veterinary research 57:269-275.
Lee, K., Pan, J.J., and May, G. 2009. Endophytic Fusarium verticillioides reduces disease severity caused by Ustilago maydis on maize. FEMS Microbiology Letters 299:31-37.
Marasas, W.F.O., Wehner, F.C., Van Rensberg, S.J., and Van Schalkwyk, D.J. 1981. Mycoflora of corn produced in human Esophageal cancer areas in Transkei, South Africa. Phytopathology 71:792-796.
Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martínez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E., and Merrill, A.H., Jr. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. Journal of Nutrition 134:711-716.
Miller, J.D., Savard, M.E., Schaafsma, A.W., Seifert, K.A., and Reid, L.M. 1995. Mycotoxin production by Fusarium moniliforme and Fusarium proliferatum from Ontario and occurrence of fumonisin in the 1993 corn crop. Can. J. Plant Pathol.-Rev. Can. Phytopathol. 17:233-239.

Missmer, S.A., Suarez, L., Felkner, M., Wang, E., Merrill, A.H., Jr., Rothman, K.J., and Hendricks, K.A. 2006. Exposure to Fumonisins and the Occurrence of Neural Tube Defects along the Texas-Mexico Border. Environmental Health Perspectives 114:237-241.

Morgavi, D.P., and Riley, R.T. 2007. Review: An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with Fusarium toxins. Animal Feed Science and Technology 137:201-212.

Mousa, W.K., Shearer, C., Limay-Rios, V., Ettinger, C.L., Eisen, J.A., and Raizada, M.N. 2016. Root-hair endophyte stacking in finger millet creates a physicochemical barrier to trap the fungal pathogen Fusarium graminearum. Nature microbiology 1:16167.

Munkvold, G.P., McGee, D.C., and Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by Fusarium moniliforme. Phytopathology 87:209-217.

Murugesan, G.R., Ledoux, D.R., Naehrer, K., Berthiller, F., Applegate, T.J., Grenier, B., Phillips, T.D., and Schatzmayr, G. 2015. Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies1. Poultry Science 94:1298-1315.

Nair, J.R., Singh, G., and Sekar, V. 2002. Isolation and characterization of a novel Bacillus strain from coffee phyllosphere showing antifungal activity. Journal of Applied Microbiology 93:772-780.

National Corn Growers Association, N. (2015). World of Corn (National Corn Growers Association).

Nuss, E.T., and Tanumihardjo, S.A. 2010. Maize: A Paramount Staple Crop in the Context of Global Nutrition. Comprehensive Reviews in Food Science and Food Safety 9:417-436.

Ongena, M., and Jacques, P. 2008. Review: Bacillus lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16:115-125.

Pal, K.K., and Gardener, B.M. 2006.Biological Control of Plant Pathogens. The Plant Health Instructor. Online, publication/10.1094/PHI-A-2006-1117-02.

Presello, D.A., Iglesias, J., Botta, G., and Eyhérabide, G.H. 2007. Severity of Fusarium ear rot and concentration of fumonisin in grain of Argentinian maize hybrids. Crop Protection 26:852-855.

Presello, D.A., Botta, G., Iglesias, J., and Eyhérabide, G.H. 2008. Effect of disease severity on yield and grain fumonisin concentration of maize hybrids inoculated with Fusarium verticillioides. Crop Protection 27:572-576.

Presello, D.A., Pereyra, A.O., Iglesias, J., Fauguel, C.M., Sampietro, D.A., and Eyherabide, G.H. 2011. Responses to selection of S-5 inbreds for broad-based resistance to ear rots and grain mycotoxin contamination caused by Fusarium spp. in maize. Euphytica 178:23-29.

Raaijmakers, J.M., De Bruijn, I., Nybroe, O., and Ongena, M. 2010. Natural functions of lipopeptides from Bacillus and Pseudomonas: more than surfactants and antibiotics. FEMS Microbiology Reviews 34:1037-1062.

Roberts, M.S., Nakamura, L.K., and Cohan, F.M. 1994. Bacillus mojavensis sp. nov., distinguishable from Bacillus subtilis by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. International Journal Of Systematic Bacteriology 44:256-264.

Romero, D., Kuipers, O.P., Cazorla, F.M., Perez-Garcia, A., Paquot, M., Dufour, S.E., Rakotoaly, R.H., Arrebola, E., and Veening, J.W. 2007. The Iturin and Fengycin Families of Lipopeptides Are Key Factors in Antagonism of Bacillus subtilis Toward Podosphaera fusca [electronic resource]. Molecular plant-microbe interactions : MPMI 20:430-440.

Snook, M.E., Mitchell, T., Hinton, D.M., and Bacon, C.W. 2009. Isolation and characterization of leu7-surfactin from the endophytic bacterium Bacillus mojavensis RRC 101, a biocontrol agent for Fusarium verticillioides. Journal of agricultural and food chemistry 57:4287-4292.

Stockmann-Juvala, H., and Savolainen, K. 2008. A review of the toxic effects and mechanisms of action of fumonisin B1. Human & Experimental Toxicology 27:799-809.

Sydenham, E.W., Thiel, P.G., Marasas, W.F.O., Shephard, G.S., Van Schalkwyk, D.J.K., and Koch, K.R. (1990). Natural occurrence of some Fusarium mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, southern Africa.

Voss, K.A., Smith, G.W., and Haschek, W.M. 2007. Review: Fumonisins: Toxicokinetics, mechanism of action and toxicity. Animal Feed Science and Technology 137:299-325.

WAOB. 2016.World Agricultural Supply and Demands Estimates.

WHO. (2000). Fumonisin B1 (Environmental Health Criteria 219). In Environmental Health Criteria, I.P.o.c. safety, ed (Geneva, Switzerland: World Health Organization).

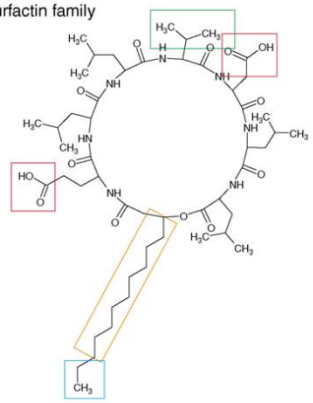
Wu, F. 2004. Mycotoxin Risk Assessment for the Purpose of Setting International Regulatory Standards. Environmental Science & Technology 38:4049-4055.

Yates, I.E., Bacon, C.W., and Hinton, D.M. 1997. Effects of endophytic infection by Fusarium moniliforme on corn growth and cellular morphology. Plant Disease 81:723-728.

Yates, I.E., Arnold, J.W., Hinton, D.M., Basinger, W., and Walcott, R.R. 2003. Fusarium verticillioides induction of maize seed rot and its control. Canadian Journal of Botany 81:422.

Figure 1.1. Structures of representative members of the three predominant lipopeptide families synthesized by *Bacillus* species. [Reprinted with permission from (Ongena and Jacques, 2008)]

Surfactin family



Variants

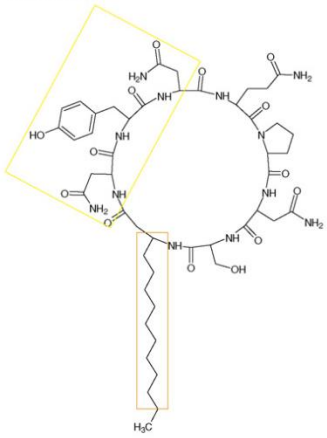
Lenght and branching of the acyl chain

Esperin**	**L-Glu-L-Leu**-D-Leu-**L-Val**-L-Asp-D-Leu-**L-Leu-COOH**	
Lichenysin***	**L-XL_1-L-XL_2**-D-Leu-**L-XL_4**-L-Asp-D-Leu-**L-XL_7**	*i*-C_{13}, *ai*-C_{13}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Pumilacidin	**L-Glu-L-Leu**-D-Leu-**L-Leu**-L-Asp-D-Leu-**L-XP_7**	
Surfactin	**L-Glu-L-XS_2**-D-Leu-**L-XS_4**-L-Asp-D-Leu-**L-XS_7**	*i*-C_{14}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}

** the β-carboxyl of Asp_5 is engaged in the lactone
*** or halobacillin
XL_1 = Gln or Glu ; XL_2 = Leu or Ile ; XL_4 and XL_7 = Val or Ile ;
XP_7 = Val or Ile ;
XS_2 = Val, Leu or Ile ; XS_4 = Ala, Val, Leu or Ile ; XS_7 = Val, Leu or Ile

n, linear
i, iso
ai, anteiso

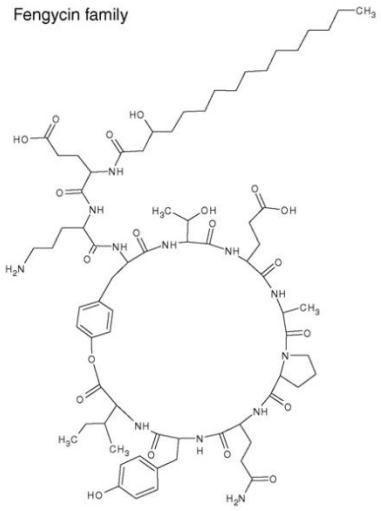
Iturin family



Bacillomycin D	**L-Asn**-D-Tyr-D-Asn-**L-Pro-L-Glu-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin F	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Thr**	*i*-C_{16}, *i*-C_{17}, *ai*-C_{17}
Bacillomycin L	**L-Asp**-D-Tyr-D-Asn-**L-Ser-L-Gln-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin LC*	**L-Asn**-D-Tyr-D-Asn-**L-Ser-L-Glu-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}, *i*-C_{16}
Iturin A	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Iturin A_L	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{16}, *i*-C_{16}
Iturin C	**L-Asp**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Mycosubtilin	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Ser-L-Asn**	*n*-C_{16}, *i*-C_{16}, *ai*-C_{17}

* or bacillopeptin

Fengycin family

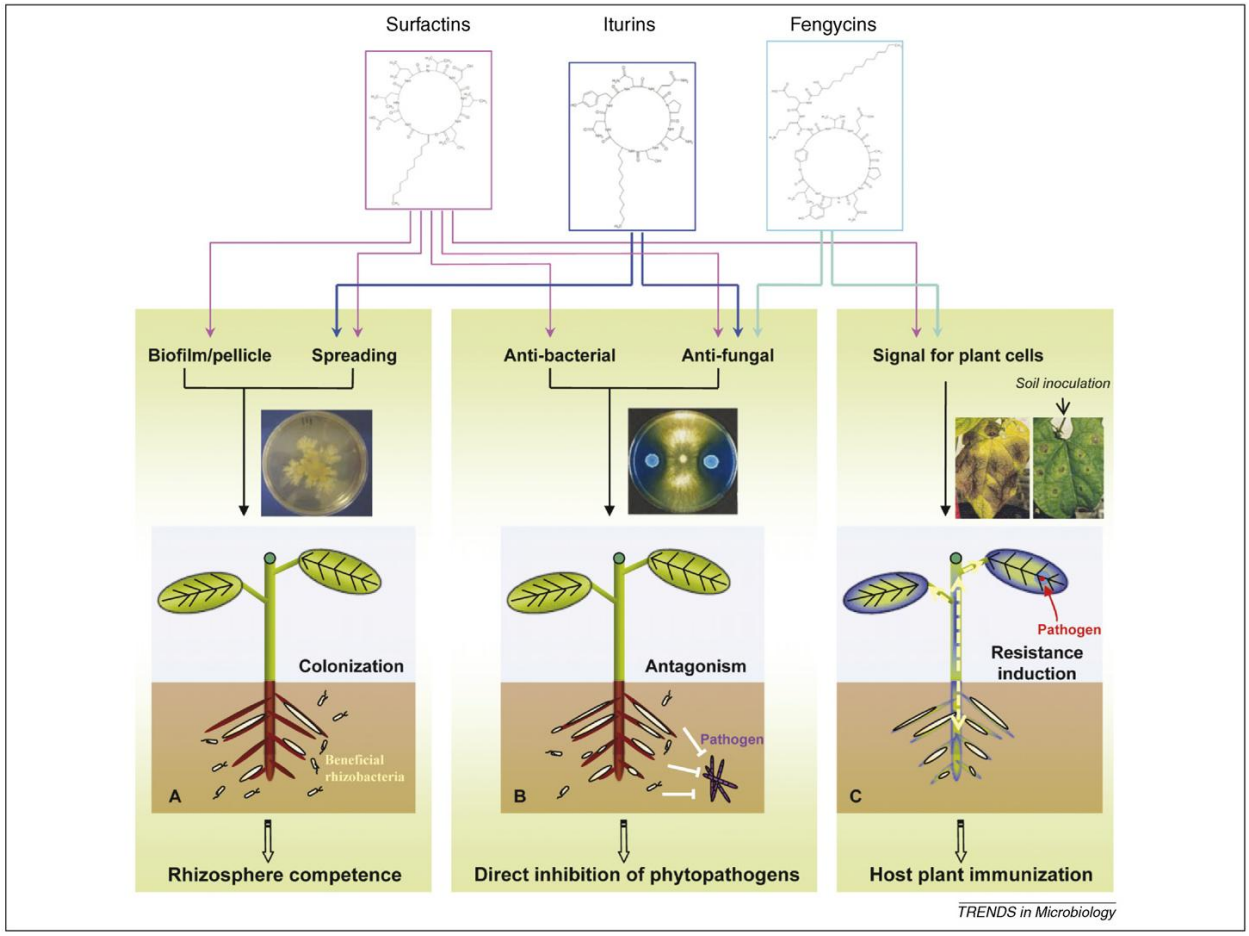


Fengycin A**	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**L-Tyr**-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}
Fengycin B**	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**L-Tyr**-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}, C_{17}
Plipastatin A	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**D-Tyr**-L-Ile	*n*-C_{16}, *ai*-C_{17}
Plipastatin B	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**D-Tyr**-L-Ile	*n*-C_{16}, *ai*-C_{17}

** double bond between carbons 2-3, 3-4 or 13-14 were reported for some acyl chains

TRENDS in Microbiology

Figure 1.2. Overview of *Bacillus* lipopeptide interactions in the context of biological control of plant diseases. From left to right, the three photographs show bacterial spreading, fungal growth inhibition through the production of fungitoxic compounds by bacterial cells (blue) and leaf disease reduction following inoculation of the beneficial bacterium on roots. They illustrate how to get experimental indications about the potential involvement of one particular strain in the three phenomena schematically represented in (A), (B) and (C). Establishment of bacterial biofilms and microcolonies is represented by (A). (B) represents direct antibiosis that can be exerted by the established biocontrol strain toward pathogens sharing the same microenvironment. In (C), the arrows illustrate the emission of a signal following perception of the rhizobacterium at the root level. This signal moves throughout the entire plant leading to some systemic reinforcement allowing pathogen restriction at distal sites of infections (see Box 3). [Reprinted with permission from (Ongena and Jacques, 2008)]


Surfactins
Iturins
Fengycins
Biofilm/pellicle
Spreading
Anti-bacterial
Anti-fungal
Signal for plant cells
Soil inoculation
Colonization
Beneficial rhizobacteria
A
Antagonism
Pathogen
B
Pathogen
Resistance induction
C
Rhizosphere competence
Direct inhibition of phytopathogens
Host plant immunization
TRENDS in Microbiology

CHAPTER 2

WHOLE-GENOME SHOTGUN SEQUENCE OF *BACILLUS MOJAVENSIS* STRAIN RRC101, AN ENDOPHYTIC BACTERIUM ANTAGONISTIC TO THE MYCOTOXIGENIC ENDOPHYTIC FUNGUS *FUSARIUM VERTICILLIOIDES*

S.E. Gold*, A.A. Blacutt*, R.J. Meinersmann, C.W. Bacon. 2014. Genome Announcements. 2(5):e01090-14. Reprinted with permission from the publisher. *Authors contributed equally to this work.

ABSTRACT

Here we report the whole genome shotgun sequence of *Bacillus mojavensis* strain RRC101, isolated from a maize kernel. This strain is antagonistic to the mycotoxigenic plant pathogen *Fusarium verticillioides*, and grows within maize tissue, suggesting potential as an endophytic biocontrol agent.

Bacillus mojavensis strain RRC101, U.S. Patent 5,994,117; ATCC 55732 (1), was isolated from a maize kernel from Northern Italy. It was originally identified as *Enterobacter cloacae* and shown to grow endophytically and enhance the growth of corn seedlings (Hinton and Bacon, 1995). It is antagonistic to *Fusarium verticillioides,* a soil and seedborne mycotoxigenic fungus capable of asymptomatic endophytic growth in maize plants. *B. mojavensis* is very closely related to *B. subtilis* (Roberts et al., 1994), but is distinguishable, among other means, by its 16S rRNA sequence (Roberts et al., 1994; Bacon and Hinton, 2002). The RRC101 genome was prepared as an 8 kb paired-end library and sequenced using 454 technology on a ¼ run through the Georgia Genomics Facility at the University of Georgia. According to RAST automated annotation of the sequence data and visualization in The SEED Viewer, the genome consists of 4,167,987 bp in 4 contigs and includes 4241 coding sequences with 55 possible missing genes. As there are no public *B. mojavensis* sequences included in SEED, the closest related public genome sequence was that of *Bacillus subtilis* subsp. *subtilis* strain 168 (Taxonomy ID: 224308).

Operons for biosynthesis of antifungal compounds surfactin and fengycin, but not iturin were identified. Reciprocal BLASTs for antimicrobial lipopeptides, using *Bacillus amyloliquefaciens* FZB42 as a reference, led to identification (>92% identity) of operons corresponding to the surfactin and fengycin biosynthetic pathways. These identifications were bolstered by conserved synteny in the genes within and surrounding these operons. BLAST against the RRC101 genome using the bacillomycin (iturin) operon failed to identify significantly similar genes outside of the already identified fengycin or surfactin pathways, suggesting that RRC101 lacks the PKS-NRPS genes for bacillomycin synthetase and thus likely produces only the fengycin and surfactin class lipopeptides. Preliminary biochemical analyses

have indicated that, indeed, several molecular variants of surfactin and fengycin are produced by strain RRC101 (Blacutt, A.A., unpublished). Additionally, operons for biosynthesis of a bacillibactin-like siderophore, the antibiotic bacilysin and a number of polyketide synthetase genes similar to those involved in bacillaene production in FZB42 were also identified.

RRC101 possesses a number of genes with likely relevance to rhizosphere competence and endophytism. Specifically, quercetin dioxygenase and acetoin reductase are likely involved in plant-microbe signaling (Chen et al., 2007), with the former degrading plant-produced antimicrobial root exudates (Ramos et al., 2006) and the latter having implications as producing a known plant growth promoting volatile compound likely contributing to the observed enhancement in maize (Hinton and Bacon, 1995) and Arabidopsis (Rath, M., unpublished). Further, while the endophytic trait of *B. mojavensis* RRC101 and other bacteria is likely multifactorial, the presence in the genome of genes typically involved in plant colonization, like pectin lyase, expansin, etc. provides mechanistic clues to endophytic competence (Georgelis et al., 2014).

Nucleotide sequence accession number: The genome sequence of *B. mojavensis* RRC101 has been deposited at NCBI under the accession no. ASJT01000101.1

ACKNOWLEDGEMENTS

We thank Myriam Belanger and Raj Ayyampalayam at the Georgia Genomics Facility at the University of Georgia for technical help in generating the genome sequence. We thank our colleagues Nicole Crenshaw, Anthony Glenn, Trevor Mitchell, and Dorothy Hinton for discussions and technical assistance. This work was funded by the USDA-ARS.

REFERENCES

Bacon, C.W., and Hinton, D.M. 2002. Endophytic and Biological Control Potential of< i> Bacillus mojavensis</i> and Related Species. Biological Control 23:274-284.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, B., Jungblut, P.R., Vater, J., Sussmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G., and Borriss, R. 2007. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium Bacillus amyloliquefaciens FZB42. Nat Biotech 25:1007-1014.

Georgelis, N., Nikolaidis, N., and Cosgrove, D.J. 2014. Biochemical analysis of expansin-like proteins from microbes. Carbohydrate Polymers 100:17-23.

Hinton, D.M., and Bacon, C.W. 1995. Enterobacter cloacae is an endophytic symbiont of corn. Mycopathologia 129:117-125.

Ramos, F.A., Takaishi, Y., Shirotori, M., Kawaguchi, Y., Tsuchiya, K., Shibata, H., Higuti, T., Tadokoro, T., and Takeuchi, M. 2006. Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (Allium cepa) skin. J Agric Food Chem 54:3551-3557.

Roberts, M.S., Nakamura, L., and Cohan, F.M. 1994. Bacillus mojavensis sp. nov., distinguishable from Bacillus subtilis by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. International Journal of Systematic Bacteriology 44:256-264.

CHAPTER 3

BACILLUS MOJAVENSIS RRC101 LIPOPEPTIDES PROVOKE PHYSIOLOGICAL AND METABOLIC CHANGES DURING ANTAGONISM AGAINST *FUSARIUM VERTICILLIOIDES*

Blacutt, A. A., Mitchell, T. R., Bacon, C.W., Gold, S. E. 2016. *Molecular Plant-Microbe Interactions*. Volume 29, Issue 9. 713-723. Reprinted with permission from the publisher.

ABSTRACT

The mycotoxigenic pathogen *Fusarium verticillioides* threatens the quality and utility of maize across industrial and agricultural purposes. Chemical control is complicated by the intimate endophytic lifestyle of the pathogen with its host. *Bacillus mojavensis* RRC 101, a maize-endophytic bacterium, has been observed to reduce *F. verticillioides* disease severity and fumonisin accumulation when co-inoculated to maize. Genome sequencing and annotation identified a number of biocontrol-relevant pathways in RRC 101. Biochemical assays confirmed the presence and activity of surfactin and fengycin type lipopeptides, with fengycins responsible for antifungal activity against *F. verticillioides*. This antagonism manifests as inhibition of filamentous growth, with microscopy revealing hyphal distortions, vacuolization and lysis. *F. verticillioides* secondary metabolism also responds to antagonism, with lipopeptide challenge inducing greater fumonisin production and, in the case of fengycins, eliciting pigment accumulation at sites of inhibition. Together, these data suggest that lipopeptide and mycotoxin production are two opposing components of a biochemical interaction among maize endophytes, one beneficial and one pathogenic.

INTRODUCTION

Fusarium verticillioides is a seed- and soil- borne fungal pathogen of maize, endemic to regions where its host is cultivated (Munkvold et al., 1997). *F. verticillioides* undergoes a period of asymptomatic endophytic growth, reportedly capable of benefitting the host until induced to grow pathogenically – a process theorized to be triggered by environmental stresses such as drought (Lee et al., 2009). Through infection and subsequent production of the fumonisin mycotoxins, *F. verticillioides* threatens all uses of corn: decreased ethanol yields, toxicity to livestock and links to human cancers and birth defects (Marasas et al., 1981; Bothast et al., 1992; USDA, 2001; Voss et al., 2002). Importantly, limits on acceptable fumonisin contamination for maize imports by other countries threaten maize exports from the United States (Miller et al., 1995).

The endemic nature of *F. verticillioides*, its close association with corn and its adaptation as a soil microbe render chemical controls impractical or prohibitively expensive, with little demonstrated control difference between organic and conventional farming practices (Agustín et al., 2007). Thus, the value of potential biological control agents to control *F. verticillioides* is readily apparent, provided that such biorationals are host-benign, persistent and efficacious in their control. With growing consumer awareness of sustainable, low-input agricultural methods, the need for well-understood, effective biological control agents is clear (Batte et al., 2007).

Members of the genus *Bacillus* are heavily represented among biocontrol agents (BCAs), owing to the ubiquity of the genus and its members' resilience and genetic tractability; these are typically strains of *B. thuringiensis*, *B. amyloliquefaciens* and *B. subtilis* (Cawoy et al., 2011). Several *Bacillus* BCAs have been observed to produce lipopeptide-type antibiotics, which are comprised of a peptide ring attached to a hydrocarbon tail of varying length and saturation

(Ongena et al., 2005; Ramos et al., 2006) (Fig. 1). Lipopeptides are amphiphilic molecules, exhibiting varying surfactant potency in addition to antibiotic activity (Ongena and Jacques, 2008). Generally, these antibiotics are stable in a range of pH from 2 to 10 and retain activity after being heated to 100°C for 30 minutes, making them an extremely durable agent of biocontrol (Zhang et al., 2012).

Bacillus mojavensis is a species closely related to *B. subtilis*, with most isolates originating from soil and rhizosphere samples (Roberts et al., 1994; Xu and Cote, 2003). *B. mojavensis* strain RRC 101 was isolated from a surface-sterilized maize kernel and has since been observed to internally colonize maize roots and aerial tissues (Bacon and Hinton, 2002). This endophytism is significant when taken with RRC 101's reported ability to mitigate mycotoxin accumulation and disease inflicted by *F. verticillioides*, with antifungal activity observable *in vitro* (Hu et al., 2009; Bacon and Hinton, 2011a). RRC 101 has also been observed to promote growth in *Arabidopsis*, presumably due to metabolites secreted by the bacterium (M. Rath et al, personal communication).

Collectively, *B. mojavensis* strains have been identified as producing members of each of the three predominant classes of lipopeptides; RRC 101 was previously described as producing surfactins, with initial reports describing them as essential to biocontrol in the maize - *F. verticillioides* pathosystem (Bacon and Hinton, 2011b; Ma et al., 2012). In addition to diverse lipopeptide production, *B. mojavensis* strains have been found to colonize coffee and cacao phyllospheres, suggesting another *Bacillus* species potentially rich in biocontrol agents intimately associated with their hosts, although multi-host colonization data have yet to be published (Nair et al., 2002; Melnick et al., 2008; Jacobsen, Jan. 2010). Endophytic colonization

of host plants could enable targeted, direct delivery of biocontrol activity and crop protection, a useful advance for low-input agriculture and integrated control practices (Batte et al., 2007).

The work described here assesses biocontrol-relevant secondary metabolism genes present in the *B. mojavensis* RRC 101 genome, and identifies fengycins as the principle class of secondary metabolites responsible for *in vitro* antifungal activity against *F. verticillioides*. Under assay conditions, fengycin antagonism is primarily characterized by growth inhibition. Biochemical and microscopic analyses have identified that symptoms of antagonism in *F. verticillioides* involve hyphal swelling and explosive lysis along with significant alterations to secondary metabolism. These data describe the RRC101 – *F. verticillioides* biocontrol interaction as a molecular "conversation" among maize endophytes, with the lipopeptide antagonism from RRC101 prompting a secondary metabolic response from *F. verticillioides'* in the form of pigment and fumonisin production.

RESULTS

The *B. mojavensis* RRC101 genome possesses biocontrol-relevant secondary metabolism pathways.

RAST annotation and subsequent bidirectional BLAST to NCBI GenBank identified a number of biosynthetic clusters for endophytism- and biocontrol-relevant secondary metabolites (Table 1). Genomic regions with >85% identity to known surfactin and fengycin biosynthetic clusters were presumed to be lipopeptide synthetase operons. Other biocontrol-relevant secondary metabolite operons identified through sequence analysis include a catechol-type bacillibactin-like siderophore, the antibiotic bacilysin and a yet-unidentified polyketide synthase cluster resembling that of bacillaene (Weber et al., 2015). In addition to secondary metabolites, the RRC101 genome possessed a number of genes encoding products likely involved in host

colonization; these include several putative cell wall degrading enzymes and an expansin protein with high identity to one promoting root colonization in *B. subtilis* (Kerff et al., 2008). As *B. mojavensis* RRC 101 is currently genetically intractable, biochemical means were employed to assay activity of these secondary metabolite clusters.

B. mojavensis produces diverse fengycin isoforms responsible for _in vitro_ antagonism of _F. verticillioides._

Primary C-18 fractionation of RRC101 culture supernatants yielded several antagonistic fractions, which were confirmed by HPLC-MS to be enriched for fengycin and surfactin lipopeptides (Fig. 2). To assess the antifungal contribution of individual lipopeptide classes, active primary fractions were pooled for secondary fractionation to produce isolated RRC101 fengycins and surfactins. Utilizing the fragmentation patterns and nomenclature assigned by Pathak et al (2012), collision-induced dissociation (CID) fragmentation spectra of fengycins suggests that *B. mojavensis* RRC 101 produces several variants of fengycins A, B, and C (Table 2, Fig. S1). Fengycin-containing preparations exhibited the same *in vitro* antibiosis as crude extracts, with purified surfactins lacking visible antagonism and failing to inhibit *F. verticillioides* growth (Fig. 3, inset). Antagonistic activity of secondary fractions closely followed total fengycin content (Fig. 4).

Fengycin-mediated antagonism of _F. verticillioides_ triggers aberrant hyphal morphology.

Microscopy revealed that the leading edges of fengycin-challenged cultures exhibited hyphal aberrations, even at sub-inhibitory concentrations. The primary microscopic phenotype of fengycin exposure is altered hyphal morphology, characterized by a sinuous growth habit as well as presence of enlarged cell compartments, or “swellings” (Fig. 5). Swellings ranged from engorgement of conidia (average 8.60 ± 2.0 µm, untreated average 3.99 ± 0.67 µm; 100

measurements each) and hyphae to fully swollen intercalary or terminal "bulbs" (average dimensions: 23.22 ± 5.59 µm x 30.69 ± 0.70 µm) (Fig. 5). Swollen hyphal compartments were frequently observed to erupt by violent lysis; conidia were not observed to exhibit this behavior (Fig. 6, Fig. S2, Fig. S3). Symptoms of antagonism were primarily observed on colony surfaces proximal to antagonistic fraction spots, suggesting that such swellings are a direct consequence of fengycin exposure via diffusion. These observed effects are consistent with the current proposed mechanism of membrane permeabilization induced by lipopeptide activity (Deleu et al., 2008).

Fengycin induced hyphal inflation involves cell wall expansion and extensive vacuolization ending in cell rupture.

Observation of fengycin-induced bulbs in *F. verticillioides* strain M3125::GFP under fluorescence microscopy detected strong GFP signal within enlarged hyphae, suggesting that bulb formation involved inflation of the entire hyphal compartment. Fluorescent signal retention within cell wall boundaries suggested that fengycin-induced swelling was not immediately lethal to the cell and that the cell wall and plasma membrane had maintained integrity (Fig. 7c). Bulb formation was consistently accompanied by the presence of one or more enlarged organelles, putatively identified as vacuoles by CMAC staining (Fig. 7f).

While bulbs initially exhibited significant GFP signal and are thus presumed metabolically active, lysis was frequently observed and confirmed using calcofluor and FM4-64 stains. Calcofluor staining of cell walls highlighted post-rupture deformation of *F. verticillioides* compartments (Fig. 7b). Presumptively lysed hyphae frequently exhibited an intense red signal throughout the compartment, suggestive of an influx of FM4-64 owing to compromised membranes (Fig. 7d) (Druzhinina et al., 2011). These lysed structures exhibited little-to-no GFP

fluorescence; as sGFP has a reported half-life of 18 hours, this suggests cytoplasmic leakage and diffusion rather than degradation (Fig. 7c) (Ruijter et al., 2003).

Lipopeptide challenges differentially alter *F. verticillioides* secondary metabolism.

Second to growth inhibition, the most obvious symptom of fengycin-based antagonism is a band of brown-to-purple pigmentation at the antagonized colony edge (Fig. S4). Given that wild type coloration of *F. verticillioides* M3125 on PDA is orange-yellow, the color change is attributable to accumulation of either fusarubins (previously referred to as purple perithecial pigment in *F. fujikuroi*) or bikaverin (Medentsev et al., 2005; Studt et al., 2012).

Lipopeptide-challenged *F. verticillioides* cultures accumulated fumonisins at higher levels than controls in a manner correlated with lipopeptide concentration (Table 3). Despite the lack of antifungal activity, the "high" rate (20x concentration) of surfactins provoked a statistically significant increase in total fumonisin content, whereas the lower (10x concentration) surfactin had no significant effect. To assess whether lipopeptide antagonism disrupted fumonisin export, plugs were compared to those taken directly outside of the colony growth; no statistically significant differences were observed between control and treatment groups for the ratio of colony internal/external fumonisins (not shown). These data suggest that the increased fumonisin accumulation is a genuine increase in production and not an artifact of impaired export. In addition to increased overall fumonisin content, the fengycin-challenged cultures exhibited slightly altered fumonisin isoform composition: The ratio of FB_1 to total fumonisins (sum of FB_1, FB_2 and FB_3) decreased as fengycin concentration increased. While the high-surfactin treatment induced fumonisin accumulation equivalent to that of the low-fengycin (10x concentration) treatment, the distributions of fumonisin isoforms were statistically indistinguishable from controls.

LaeA is a transcription factor identified in *Aspergillus* as controlling secondary metabolism; in *Fusarium*, mutants for the orthologous *LAE1* have previously been described as diminished in fumonisin and bikaverin expression (Bok and Keller, 2004; Butchko et al., 2011). Antagonism assays conducted with a Δ*Lae1* strain of *F. verticillioides* detected fumonisins at concentrations below the limit of quantitation across all treatments and controls (not shown).

DISCUSSION

The genus *Bacillus* contains a number of plant-associated biocontrol agents; *B. mojavensis* is among relatively few described species observed to exhibit endophytic growth traits, strain RRC101 in particular col onizes maize aerial tissues. Genomic data from confirmed endophytes may reveal common trends and elucidate the underpinnings of such associations with plant hosts, such as secretory system losses or the presence of hydrolytic enzymes (Reinhold-Hurek and Hurek, 2011). Sequencing data revealed that the RRC 101 genome is replete with clues to the mechanisms of such associations (Gold et al., 2014). Several annotated genes in RRC101 are predicted to function similar to genes essential to host colonization in phytopathogenic and endophytic bacteria alike, notably pectate lyase and expansin (Table 1). Pectate lyase produced by phytopathogens facilitates host colonization through the primary activity of cell wall digestion; byproducts from such activity are known to modulate host defense pathways (Collmer and Keen, 1986; Wegener et al., 1996; Kovtunovych et al., 1999). Cell wall degrading enzymes and bacterial expansins (observed to enhance pliability of plant cell walls) produced by phytopathogens and *B. subtilis*, respectively, have been demonstrated to act on host cell walls to facilitate colonization; in the case of *B. subtilis*, expansin deletion impaired colonization of maize roots (Kerff et al., 2008; Georgelis et al., 2014). RRC101 possesses genes for synthesis of the hormones indole acetic acid and 2, 3-butanediol; these growth promoting

phytohormones have been observed in both beneficial and phytopathogenic bacteria (Spaepen and Vanderleyden, 2011). Annotation and study of gene repertoires in plant-associated microbes, particularly beneficial endophytes like RRC101, can aid in identifying functions involved in plant colonization strategies common to phyllosphere inhabitants. Knowledge of these colonization strategies will facilitate marker development for screening putative biocontrol agents and may prove useful in designing control strategies against current and future pathogens. Along with putative endophytism factors in the RRC101 genome are operons coding for both surfactin and fengycin synthetases, of which the latter are responsible for the *in vitro* antibiosis observed against *F. verticillioides*.

Bacterial lipopeptide classes have conditionally efficacious antagonism potential, suggesting that activity of lipopeptide-based biocontrol is broadly dependent on both lipopeptide and target membrane compositions. Surfactins have demonstrated hemolytic properties as well as activity against enveloped viruses, some bacteria and mosquito larvae; fengycins and iturins are potently antifungal with varying activities against other organisms (Ongena and Jacques, 2008; Geetha and Manonmani, 2010). Target membrane sterol composition and lipid organization influence these interactions, as do the amino acid composition and lipid tail characteristics of the lipopeptides (Bonmatin et al., 2003). If activity is indeed determined by specific membrane and lipopeptide interactions, these variations could present selective pressure on the underlying synthetases and lead to variable antagonism repertoires at the species or even strain level.

Antagonistic potency of RRC101 fengycin extracts against *F. verticillioides* does not perfectly follow the total fengycin content of fractions (Fig. 4). The reverse-phase fractioning, paired with this result, suggest that later eluting (more hydrophobic) fengycin isoforms possess greater antifungal activity against *F. verticillioides* in plate assays (Fig. 3, inset). This may be

due to differences in peptide and/or lipid composition resulting in improved agar diffusion or stronger isoform-specific activity against the fungal membrane. Lipopeptide-membrane specific activity has been observed among surfactins with regards to perception by plant hosts and subsequent defense induction (Jourdan et al., 2009). Given that both systems rely upon lipopeptide interactions with membranes, it is likely that an analogous preferential interaction exists for antifungal activity. Variation in lipopeptide composition is a result of nonribosomal peptide synthetases' substrate promiscuity, which is in turn influenced by sequence diversity at the operon level (Shu et al., 2002; Daren Mueller, 2014). If such specialization is occurring within ecological niches, in-depth genetic and biochemical descriptions of the diversity of lipopeptide synthetases and their products become valuable tools in development of efficacious biocontrol strategies. An experimentally-relevant consequence of lipopeptide variability is added difficulty of separation and characterization of individual isoforms. Without tandem mass spectrometry, for example, a parent ion of 1492.6 m/z could represent C-18 fengycin A or C-16 fengycin B. Though reverse phase HPLC fractioning was capable of separating the two classes of lipopeptides from one another, isoform-level separation would require higher-resolution techniques. Such capability is necessary to better evaluate the utility of specific lipopeptide isoforms, which would in turn enable more nuanced description of their structure-function relationships.

Existing publications refer to surfactin-class lipopeptides as possessing antifungal activity (Maget-Dana et al., 1992; Bacon and Hinton, 2002). However, most available data describes synergistic activity when surfactin is applied in conjunction with antifungal agents, without substantial evidence for standalone efficacy. By contrast, the antifungal activity of fengycins and iturins produced by *B. subtilis* has been shown to compose most if not all of the antifungal

activity of the species' lipopeptide arsenal, as appears to be the case in RRC101 (Ongena et al., 2005). Although isolated RRC101 surfactins lacked detectable antifungal activity against *F. verticillioides in vitro,* evidence in other systems suggests that they may act indirectly in biological control. Surfactin production has been tied to root colonization and biofilm formation by *Bacillus,* and may serve to induce host defenses against pathogens (Fall et al., 2004; Henry et al., 2011).

Hyphal bulbs resulting from fengycin exposure consistently contain at least one putative vacuole, as determined through CMAC staining, often representing the majority of the cell volume. Bulb formation and lysis are consistent with the current model of lipopeptide-based antagonism being a result of membrane destabilization through the formation of mixed micelles and pores. Structures induced by lipopeptides have been referred to as chlamydospores in *F. solani* f. sp. *radicicola* and *F. graminearum* (Li et al., 2012). In contrast, *F. verticillioides'* lack of chlamydospore production is a diagnostic trait of the species (Leslie et al., 2006), and while fengycin-induced swellings bear a strong resemblance to chlamydospores, their irregular shape, heavy vacuolization and tendency to lyse suggest against this possibility. This divergence in *F. verticillioides*' lipopeptide response extends to secondary metabolism responses to both surfactins and fengycins.

Fusarium verticillioides fumonisin production responds differentially when exposed to each class of lipopeptides. The response to both classes tested was an increased production of fumonisins, with surfactins provoking a milder response. Fengycins uniquely affect not only concentration, but fumonisin composition: the increased accumulation of total fumonisins under fengycin challenge is accompanied by a proportional reduction of FB1. The biosynthetic pathway of fumonisins offers a possible explanation here; FB3 serves as a precursor in FB1

production, therefore disproportionate accumulation of FB3 may be a discrepancy in the activities of FUM1 and the converting enzyme, FUM3 (Butchko et al., 2006). This may be explained via enzyme kinetics, where the activity of FUM3 is being outpaced by upstream activity in the fumonisin pathway; however, surfactin-based induction can yield similar concentrations without significant perturbation of isoform composition (Table 3).

Differential induction of secondary metabolite pathways suggests that lipopeptide perception and response in *F. verticillioides* is a nuanced physiological process. Experiments performed by Hu et al. observed a reduction in fumonisin under fengycin challenge, contrary to the relationship observed here (Hu et al., 2009). Differences in assay conditions may explain the discrepancy: the work presented here employed lipopeptide challenges as extracts spotted and allowed to diffuse through agar media, whereas Hu et al. directly applied lipopeptides to a growing liquid culture, followed by incubation. In the experiments where lipopeptide exposure occurs via the gradual process of diffusion, these results suggest that, at lower concentrations, antagonistic lipopeptides induce *F. verticillioides* to mount a response through secondary metabolism, a recognized phenomenon among sub-inhibitory concentrations of antibiotics (Davies et al., 2006). Class-specific responses to lipopeptides suggest divergent mechanisms of perception and response in *F. verticillioides* when compared to observations in other fungi.

Both lipopeptide classes produced by RRC101 induced increased fumonisin levels in *F. verticillioides*, with growth inhibition, pigment accumulation and hyphal distortion observed only following fengycin exposure. *F. verticillioides* colonies antagonized by fengycins accumulate brown-to-purple pigmentation at the leading edge. Pigment production is associated with stress responses in *Fusarium* species; synthesis of bikaverin (a naphthoquinone possessing limited antimicrobial activity) is induced by nitrogen limitation and acidic pH (Limón et al.,

2010; Deshmukh et al., 2014). Genes for bikaverin synthesis in *F. verticillioides* are also coregulated with heat- and oxidative stress response genes through adenylyl cyclase (Medentsev et al., 2005; Choi and Xu, 2010). The species description for *F. verticillioides* states that chlamydospores are not produced, but enlarged hyphae reminiscent of pseudochlamydospores have been observed (Leslie et al., 2006). However, fengycin-induced bulbs resemble inflated bodies observed in *F. graminearum* when induced to produce tricothecenes (Menke et al., 2013). These *F. graminearum* "toxisomes" are themselves morphologically and functionally analogous to structures originally detailed in *Aspergillus parasiticus* during aflatoxin production (Chanda et al., 2009). In fengycin-challenged *F. verticillioides*, presence of these structures correlates with increased fumonisin production, but their lysis and general growth inhibition are incongruent with being a specialized structure, as is their absence during surfactin-induced fumonisin production.

Hyphal vacuolization and death, both symptoms of fengycin antagonism, also resemble the process of autophagy as described by Pollack et al: a central autophagosome-containing vacuole appears and may fill an entire hyphal compartment undergoing cell death (Pollack et al., 2009; Richards et al., 2010). Similarly, senescence in *Podospora anserina* is signaled by increased pigmentation, reduced aerial hyphae and abnormal hyphal morphology (Sharon et al., 2009). As *F. verticillioides* undergoes such processes under fengycin challenge, induced senescence may be part of the response to fengycin antagonism. In *Verticillium dahliae*, cell death pathways were found to be upregulated under iturin antagonism, hinting at a role for such processes in biological control activity (Han et al., 2015). The surfactin-type lipopeptide WH1fungin from *B. amyloliquefaciens* WH1 has also been observed to induce apoptosis in yeast and *Rhizoctonia* (Qi et al., 2010). Induced apoptosis as a mechanism of antagonism has also been

proposed to exist in the case of *Candida albicans* where the fungus-produced isoprenoid quorum sensing molecule farnesol induces cell death in *Fusarium* and *Aspergillus* (Semighini et al., 2008).

Differential responses to lipopeptide challenge in *F. verticillioides* suggest the process of antagonism is more complex than direct lysis following plasma membrane pore-formation. Such nuance is supported by contrasting the fengycin and surfactin-shared response of fumonisin accumulation with the fengycin-specific morphological effects and putative bikaverin production. Both bikaverin and fumonisin biosynthetic genes are regulated by *Lae1*, with fumonisin gene expression dropping in deletants (Butchko et al., 2011). Surfactin challenge induces a more modest accumulation of fumonisin than fengycin, without any other effects of fengycin exposure, suggesting the action of a different perception mechanism rather than overt antagonism (Table 3).

The fengycin-associated upregulation of secondary metabolism observed via fumonisin and pigment accumulation suggest that the response to fengycin antagonism may be at least partially *Lae1*–dependent . Under bioassay conditions, extracts from the tested Δ*LAE1* strain produced no detectable fumonisin across all treatments, but retained pigment accumulation in response to fengycin (not shown). Assays performed by Butchko et al (2012) comparing wild-type and Δ*LAE1* strains of *F. verticillioides* demonstrated a reduction in fumonisin pathway expression at 6 days in liquid culture, but no significant differences in fumonisin production at 6 or 11 days. The discrepancy observed between wild-type and Δ*LAE1* fumonisin production described here may be explained by either alternative regulation of toxin production or the different culture conditions (PDA plates in this study; liquid Glucose-Yeast extract-Asparagine-Malic acid (GYAM) medium used by Butchko et al). These data suggest that antagonism-

induced fumonisin production relies on an *LAE1*-mediated pathway and highlight multifactorial control of fumonisin production.

As specific lipopeptide class production has been shown to respond to the presence of target organisms, it is likely that selection pressures the antibiotic repertoire of a given strain to be effective against commonly-encountered microbes (Leaes et al., 2015). Since *F. verticillioides* and *B. mojavensis* RRC 101 have been observed to colonize maize endophytically, details of their antagonism and underlying mechanisms may produce insight into relationships and interactions of plant microbiota. More detailed studies of the biochemical and transcriptional response of phytopathogens to biocontrol agents are necessary to better understand the mechanisms underlying complex microbial interactions taking place in the phyllospheres of maize and other economically important crops. As lipopeptide-producing *Bacillus* strains have been observed to aid nematodes in defense against pathogens (Iatsenko et al., 2014), such findings as presented may hold significance for microbiotic interactions beyond the realm of the phyllosphere.

MATERIALS AND METHODS

Microbe maintenance and culture conditions.

Bacillus mojavensis RRC 101 (ATCC 55732) and *Fusarium verticillioides* M3125 wild type and M3125::GFP (carrying an integrated pCT74 plasmid conferring GFP expression) were kept at -80°C as 15% glycerol stocks prior to use (Andrie et al., 2005).

Bacillus mojavensis RRC 101 was streaked to isolation on Luria-Bertani agar plates at 30°C. Liquid cultures utilized Liu's Modified medium at 30°C, shaken at 220 rpm for 72 hours (Ma et al., 2012). *Fusarium verticillioides* was cultured at 27°C on Acumedia potato dextrose agar (PDA) plates (Neogen, Lansing, MI).

Genome information.

Details of the sequencing and assembly of the *Bacillus mojavensis* RRC 101 genome are detailed elsewhere; the assembly is available on GenBank under the WGS Project ASJT01 (Gold et al., 2014) and through PubSEED under organism number 1329377.3 (Overbeek et al., 2005). Bidirectional BLAST for secondary metabolism clusters was performed using the *B. amyloliquefaciens* FZB42 genome (CP000560), with supporting evidence provided through the antiSMASH web resource (Weber et al., 2015). Signal peptide identification was conducted through the Phobius web server (Lin et al., 2005).

Preparation of crude extracts.

Liquid cultures of RRC 101 were centrifuged for 30 minutes at 9803 x gand pellets discarded. Supernatants were brought to 50% methanol and applied to a Millipore PrepPak C18 column (1" x 20", packed with 11" Millipore Waters prep-pak 500/C18, 55-105 µm). Primary fractions were eluted with an A:B system of water:methanol + 1% formic acid, starting at 50:50 and progressing in 10% increments (500 mL volumes) to 100% B. Fractions were dried in a Buchi E121 Rotavap and dissolved in methanol at 20X original culture concentration for bioassays and HPLC analysis.

Antifungal fractions were further separated on a Hewlett Packard 1050 HPLC system using the same solvent system, progressing from 20% A (10% methanol + 1% formic acid) to 100% B (methanol + 1% formic acid) over ten minutes and maintaining 100% B for 15 minutes, at a flow rate of 2mL/min and injection volume of 50 µL. Fractionation was conducted using an Agilent ZORBAX Eclipse XDB-C8 column (4.6 x 150 mm, 5 µm), with fractions collected in 30-second intervals using a Gilson fraction collector. Fraction pools were concentrated based on

lipopeptide classes and dried by Rotavap before dissolving in methanol as 100X culture concentration.

Analysis and identification of lipopeptides.

Mass spectrometry analysis of RRC101 fractions was performed using the aforementioned column and solvent system, at a flow rate of 200 µL/min. HPLC-MS analyses of extracts were conducted using a Finnigan Micro AS, Finnigan Surveyor MS Pump Plus, and Finnigan LCQ Duo (Thermo Scientific). This analysis was conducted in positive ion mode utilizing an ESI source with 5kV spray voltage and a capillary temperature of 190°C. Other instrument conditions were maximized for sensitivity by "tuning" to a direct injection of surfactin A (Sigma-Aldrich). Samples were also analyzed on a Dionex UltiMate 3000 AS attached to a Dionex UltiMate 3000 UHPLC^{+} pump, in tandem with a Thermo Scientific LTQ XL. Conditions were the same as above except the instrument was "tuned" by direct injection of iturin A (Sigma-Aldrich). Commercially obtained Surfactin A (Sigma-Aldrich) was employed as a standard for retention and fragmentation patterns of surfactins, with previously published data used to guide fengycin characterization (Bie et al., 2009; Pathak et al., 2012).

Antagonism assays.

Fractions were screened for antifungal activity through a plate growth assay. Lipopeptide extracts (10x and 20x, as well as a methanol blank) were applied as 50 µL spots to the surface of PDA plates, with plates kept open in a sterile laminar-flow hood to fully evaporate the solvent. Five-millimeter plugs of 5-day old *F. verticillioides* plate cultures were placed central to extracts at a distance of 2 cm from any spot. Plates were wrapped with parafilm, inverted and incubated in the dark for 3 days at 27°C before evaluation. For microscopic evaluation, assays were conducted as described, with sterile dialysis membrane (14 kDa MWCO) overlaid prior to

inoculation; at the end of the assay, membranes were lifted from the plates, cut and transferred to glass slides for staining and visualization.

Mycotoxin assays utilized wild-type strain M3125, with 5 mm plugs collected from the leading edges of colonies as well as the immediately adjacent uncolonized agar to account for potential differences in export efficiency as part of antagonism. Fumonisin extractions from 3-day old agar plugs were conducted using in 1:1 acetonitrile:water with 5% formic acid, shaken for 2 hours at room temperature as per Glenn et al (2008). Extracts were then diluted to 25% acetonitrile before quantitative analysis via LC-MS, using FB1, FB2 and FB3 standards for comparison. Statistical analyses were conducted using the R x64 statistical software environment (Ihaka and Gentleman, 1996).

Digital photographs of assay plates were captured in a Protein Simple FluorChem HD2 system, with antagonism assayed digitally. Briefly, photo files were opened *in silico* (The GIMP Development Team, 1995) and the distance from colony center to leading edge proximal to each spot was measured. Antagonism was calculated as the percent difference in radial growth between a negative control (methanol) and the original fraction, with growth reduction from fractions averaged and treated as a percentage of reduction compared to the original antagonistic fraction:

$$100 \times \frac{\text{(Negative control – 2° fraction)}}{\text{(Negative control – original antagonistic fraction)}}$$

Staining and microscopy.

Dialysis membrane sections from antagonism plate assays were transferred to microscope slides, flooded with stain solutions and incubated in the dark for 60 minutes at room temperature. Distilled water was used to rinse excess stain before visualization. Calcofluor white was used at a

concentration of 1 µg/mL to visualize fungal cell walls, CMAC (7-amino-4-chloromethylcoumarin) for vacuole imaging at 100 µM and FM4-64 was used at 17 µM as a vital stain based on visualizing membrane permeability.

Cultures were visualized using a Zeiss Axio Imager.A2 microscope, with an attached AxioCam ERc 5s for digital capture, using Zeiss Zen software for image processing. Fluorescence was observed using a Lumen Dynamics X-Cite 120Q Light source, with Zeiss Filter sets 20 (Rhodamine; for FM4-64), 38 (cGFP) and 49 (DAPI; for Calcofluor and CMAC).

Agustín, A., Gloria, E., Teresa, J., and Antonio, H. 2007. Estimation of dietary intakes of fumonisins B1 and B2 from conventional and organic corn. Food Control 18:1058-1062.

Andrie, R.M., Martinez, J.P., and Ciuffetti, L.M. 2005. Development of ToxA and ToxB promoter-driven fluorescent protein expression vectors for use in filamentous ascomycetes. Mycologia 97:1152-1161.

Bacon, C.W., and Hinton, D.M. 2002. Endophytic and Biological Control Potential of Bacillus mojavensis and Related Species. Biological Control 23:274-284.

Bacon, C.W., and Hinton, D.M. 2011a. In planta reduction of maize seedling stalk lesions by the bacterial endophyte. Canadian Journal Of Microbiology 57:485-492.

Bacon, C.W., and Hinton, D.M. 2011b. Bacillus mojavensis: its endophytic nature, the surfactins and their role in the plant response to infection by Fusarium verticillioides. Pages 21-39 in: Bacteria in Agrobiology: Plant Growth Responses, D.K. Maheshwari, ed. Springer-Verlag, Berlin.

Batte, M.T., Hooker, N.H., Haab, T.C., and Beaverson, J. 2007. Putting Their Money Where Their Mouths Are: Consumer Willingness to Pay for Multi-ingredient, Processed Organic Food Products. Food Policy 32:145-159.

Bie, X., Lu, Z., and Lu, F. 2009. Identification of fengycin homologues from Bacillus subtilis with ESI-MS/CID. Journal of microbiological methods 79:272-278.

Bok, J.W., and Keller, N.P. 2004. LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryotic cell 3:527-535.

Bonmatin, J.-M., Laprevote, O., and Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Combinatorial Chemistry & High Throughput Screening 6:541-556.

Bothast, R.J., Bennett, G.A., Vancauwenberge, J.E., and Richard, J.L. 1992. Fate of Fumonisin B(1) in Naturally Contaminated Corn during Ethanol Fermentation. Appl Environ Microbiol 58:233-236.

Butchko, R.A., Brown, D.W., Busman, M., Tudzynski, B., and Wiemann, P. 2012. Lae1 regulates expression of multiple secondary metabolite gene clusters in Fusarium verticillioides. Fungal Genet Biol 49:602-612.

Butchko, R.A.E., Proctor, R.H., and Plattner, R.D. 2006. Deletion Analysis of FUM Genes Involved in Tricarballylic Ester Formation during Fumonisin Biosynthesis [electronic resource]. Journal of agricultural and food chemistry 54:9398-9404.

Butchko, R.A.E., McCormick, S.P., Busman, M., Tudyznski, B., and MWiemann, P. (2011). Regulation of secondary metabolite production in Fusarium species by the global regulator LAE1 (USDA Agricultural Research Service), pp. 1.

Cawoy, H., Bettiol, W., Fickers, P., and Ongena, M. 2011. Bacillus-based biological control of plant diseases. Pesticides in the modern world—pesticides use and management. InTech, Rijeka:273-302.

Chanda, A., Roze, L.V., Kang, S., Artymovich, K.A., Hicks, G.R., Raikhel, N.V., Calvo, A.M., and Linz, J.E. 2009. A key role for vesicles in fungal secondary metabolism. Proceedings of the National Academy of Sciences 106:5.

Choi, Y.E., and Xu, J.R. 2010. The cAMP signaling pathway in Fusarium verticillioides is important for conidiation, plant infection, and stress responses but not fumonisin production. Mol Plant Microbe Interact 23:522-533.

Collmer, A., and Keen, N.T. 1986. The role of pectic enzymes in plant pathogenesis. Annual review of phytopathology 24:383-409.

Daren Mueller, K.W. (2014). Corn Disease Loss Estimates From the United States and Ontario, Canada - 2012. In Diseases of Corn (Purdue University).

Davies, J., Spiegelman, G.B., and Yim, G. 2006. The world of subinhibitory antibiotic concentrations. Current Opinion in Microbiology 9:445-453.

Deleu, M., Paquot, M., and Nylander, T. 2008. Effect of fengycin, a lipopeptide produced by Bacillus subtilis, on model biomembranes. Biophysical journal 94:2667-2679.

Dertz, E.A., Xu, J., Stintzi, A., and Raymond, K.N. 2006. Bacillibactin-mediated iron transport in Bacillus subtilis. Journal of the American Chemical Society 128:2.

Deshmukh, R., Mathew, A., and Purohit, H.J. 2014. Characterization of antibacterial activity of bikaverin from Fusarium sp. HKF15. Journal of Bioscience and Bioengineering 117:443-448.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V., and Kubicek, C.P. 2011. Trichoderma: the genomics of opportunistic success. Nat Rev Micro 9:749-759.

Fall, R., Vivanco, J.M., and Harsh Pal, B. 2004. Biocontrol of Bacillus subtilis against Infection of Arabidopsis Roots by Pseudomonas syringae Is Facilitated by Biofilm Formation and Surfactin Production. Plant Physiology 134:307-319.

Geetha, I., and Manonmani, A.M. 2010. Surfactin: a novel mosquitocidal biosurfactant produced by Bacillus subtilis ssp. subtilis (VCRC B471) and influence of abiotic factors on its pupicidal efficacy. Letters in Applied Microbiology 51:406-412.

Georgelis, N., Nikolaidis, N., and Cosgrove, D.J. 2014. Biochemical analysis of expansin-like proteins from microbes. Carbohydrate Polymers 100:17-23.

Glenn, A.E., Zitomer, N.C., Zimeri, A.M., Williams, L.D., Riley, R.T., and Proctor, R.H. 2008. Transformation-mediated complementation of a FUM gene cluster deletion in Fusarium verticillioides restores both fumonisin production and pathogenicity on maize seedlings. Molecular Plant-Microbe Interactions 21:87-97.

Gold, S.E., Blacutt, A.A., Meinersmann, R.J., and Bacon, C.W. 2014. Whole-Genome Shotgun Sequence of Bacillus mojavensis Strain RRC101, an Endophytic Bacterium Antagonistic to the Mycotoxigenic Endophytic Fungus Fusarium verticillioides. Genome announcements 2.

Han, Q., Wu, F., Wang, X., Qi, H., Shi, L., Ren, A., Liu, Q., Zhao, M., and Tang, C. 2015. The bacterial lipopeptide iturins induce Verticillium dahliae cell death by affecting fungal signalling pathways and mediate plant defence responses involved in pathogen-associated molecular pattern-triggered immunity. Environmental microbiology 17:1166-1188.

Henry, G., Deleu, M., Jourdan, E., Thonart, P., and Ongena, M. 2011. The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related defence responses. Cellular Microbiology 13:1824-1837.

Hirooka, K., and Fujita, Y. 2010. Excess Production of Bacillus subtilis Quercetin 2,3-Dioxygenase Affects Cell Viability in the Presence of Quercetin. Bioscience, Biotechnology, and Biochemistry 74:1030-1038.

Hu, L.B., Zhang, T., Yang, Z.M., Zhou, W., and Shi, Z.Q. 2009. Inhibition of fengycins on the production of fumonisin B1 from Fusarium verticillioides. Letters in Applied Microbiology 48:84-89.

Iatsenko, I., Yim, Joshua J., Schroeder, Frank C., and Sommer, Ralf J. 2014. B. subtilis GS67 Protects C. elegans from Gram-Positive Pathogens via Fengycin-Mediated Microbial Antagonism. Current Biology 24:2720-2727.

Idriss, E.E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., and Borriss, R. 2002. Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth-promoting effecta. Microbiology 148:2097-2109.

Ihaka, R., and Gentleman, R. 1996. R: A Language for Data Analysis and Graphics. Journal of Computational and Graphical Statistics 5:299-314.

Jacobsen, B.B., MT, US), Zidack, Nina K. (Bozeman, MT, US), Larson, Rebecca (Longmont, CO, US). (Jan. 2010). *Bacillus* isolates and methods of their use to protect against plant pathogens (United States).

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthélemy, J.P., Thonart, P., and Ongena, M. 2009. Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from Bacillus subtilis. Molecular Plant-Microbe Interactions 22:456-468.

Kerff, F., Amoroso, A., Herman, R., Sauvage, E., Petrella, S., Filee, P., Charlier, P., Joris, B., Tabuchi, A., Nikolaidis, N., and Cosgrove, D.J. 2008. Crystal structure and activity of Bacillus subtilis YoaJ (EXLX1), a bacterial expansin that promotes root colonization. Proceedings of the National Academy of Sciences of the United States of America 105:16876-16881.

Kovtunovych, G., Lar, O., Kamalova, S., Kordyum, V., Kleiner, D., and Kozyrovska, N. 1999. Correlation between pectate lyase activity and ability of diazotrophic Klebsiella oxytoca VN 13 to penetrate into plant tissues. Plant and Soil 215:1-6.

Leaes, F.L., Velho, R.V., Caldas, D.G., Ritter, A.C., Tsai, S.M., and Brandelli, A. 2015. Expression of essential genes for biosynthesis of antimicrobial peptides of Bacillus is modulated by inactivated cells of target microorganisms. Research in Microbiology:7.

Lee, K., Pan, J.J., and May, G. 2009. Endophytic Fusarium verticillioides reduces disease severity caused by Ustilago maydis on maize. FEMS Microbiology Letters 299:31-37.

Leslie, J.F., Summerell, B.A., and Bullock, S. 2006. The Fusarium laboratory manual. Wiley Online Library.

Li, L., Ma, M., Huang, R., Qu, Q., Li, G., Zhou, J., Zhang, K., Lu, K., Niu, X., and Luo, J. 2012. Induction of Chlamydospore Formation in Fusarium by Cyclic Lipopeptide Antibiotics from Bacillus subtilis C2. Journal of Chemical Ecology 38:9.

Limón, M.C., Rodríguez-Ortiz, R., and Avalos, J. 2010. Bikaverin production and applications. Applied Microbiology and Biotechnology 87:21-29.

Lin, T.-P., Chen, C.-L., Fu, H.-C., Wu, C.-Y., Lin, G.-H., Huang, S.-H., Chang, L.-K., and Liu, S.-T. 2005. Functional analysis of fengycin synthetase FenD. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1730:159-164.

Ma, Z., Wang, N., Hu, J., and Wang, S. 2012. Isolation and characterization of a new iturinic lipopeptide, mojavensin A produced by a marine-derived bacterium Bacillus mojavensis B0621A. The Journal of Antibiotics 65:5.

Maget-Dana, R., Thimon, L., Peypoux, F., and Ptak, M. 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochimie 74:1047-1051.

Marasas, W.F.O., Wehner, F.C., Van Rensberg, S.J., and Van Schalkwyk, D.J. 1981. Mycoflora of corn produced in human Esophageal cancer areas in Transkei, South Africa. Phytopathology 71:792-796.

Medentsev, A.G., Arinbasarova, A.Y., and Akimenko, V.K. 2005. Biosynthesis of Naphthoquinone Pigments by Fungi of the Genus Fusarium. Appl Biochem Microbiol 41:503-507.

Melnick, R.L., Zidack, N.K., Bailey, B.A., Maximova, S.N., Guiltinan, M., and Backman, P.A. 2008. Bacterial endophytes: Bacillus spp. from annual crops as potential biological control agents of black pod rot of cacao. Biological Control 46:46-56.

Menke, J., Weber, J., Broz, K., and Kistler, H.C. 2013. Cellular Development Associated with Induced Mycotoxin Synthesis in the Filamentous Fungus <italic>Fusarium graminearum</italic>. PLoS ONE 8:e63077.

Miller, J.D., Savard, M.E., Schaafsma, A.W., Seifert, K.A., and Reid, L.M. 1995. Mycotoxin production by Fusarium moniliforme and Fusarium proliferatum from Ontario and occurrence of fumonisin in the 1993 corn crop. Can. J. Plant Pathol.-Rev. Can. Phytopathol. 17:233-239.

Munkvold, G.P., McGee, D.C., and Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by Fusarium moniliforme. Phytopathology 87:209-217.

Nair, J.R., Singh, G., and Sekar, V. 2002. Isolation and characterization of a novel Bacillus strain from coffee phyllosphere showing antifungal activity. Journal of Applied Microbiology 93:772-780.

Ongena, M., and Jacques, P. 2008. Review: Bacillus lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16:115-125.

Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A., and Thonart, P. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of Bacillus subtilis. Applied Microbiology & Biotechnology 69:29-38.

Overbeek, R., Begley, T., Butler, R.M., Choudhuri, J.V., Chuang, H.Y., Cohoon, M., de Crecy-Lagard, V., Diaz, N., Disz, T., Edwards, R., Fonstein, M., Frank, E.D., Gerdes, S., Glass, E.M., Goesmann, A., Hanson, A., Iwata-Reuyl, D., Jensen, R., Jamshidi, N., Krause, L., Kubal, M., Larsen, N., Linke, B., McHardy, A.C., Meyer, F., Neuweger, H., Olsen, G., Olson, R., Osterman, A., Portnoy, V., Pusch, G.D., Rodionov, D.A., Ruckert, C., Steiner, J., Stevens, R., Thiele, I., Vassieva, O., Ye, Y., Zagnitko, O., and Vonstein, V. 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic acids research 33:5691-5702.

Pathak, K., Keharia, H., Gupta, K., Thakur, S., and Balaram, P. 2012. Lipopeptides from the Banyan Endophyte, Bacillus subtilis K1: Mass Spectrometric Characterization of a Library of Fengycins. J. Am. Soc. Mass Spectrom. 23:1716-1728.

Pollack, J.K., Harris, S.D., and Marten, M.R. 2009. Autophagy in filamentous fungi. Fungal Genetics and Biology 46:1-8.

Qi, G., Zhu, F., Du, P., Yang, X., Qiu, D., Yu, Z., Chen, J., and Zhao, X. 2010. Lipopeptide induces apoptosis in fungal cells by a mitochondria-dependent pathway. Peptides 31:1978-1986.

Ramos, F.A., Takaishi, Y., Shirotori, M., Kawaguchi, Y., Tsuchiya, K., Shibata, H., Higuti, T., Tadokoro, T., and Takeuchi, M. 2006. Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (Allium cepa) skin. J Agric Food Chem 54:3551-3557.

Reinhold-Hurek, B., and Hurek, T. 2011. Living inside plants: bacterial endophytes. Curr Opin Plant Biol 14:435-443.

Richards, A., Veses, V., and Gow, N.A.R. 2010. Vacuole dynamics in fungi. Fungal Biology Reviews 24:93-105.

Roberts, M.S., Nakamura, L.K., and Cohan, F.M. 1994. Bacillus mojavensis sp. nov., distinguishable from Bacillus subtilis by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. International Journal Of Systematic Bacteriology 44:256-264.

Ruijter, N.C.A.d., Verhees, J., Leeuwen, W.v., and Krol, A.R.v.d. 2003. Evaluation and comparison of the GUS, LUC and GFP reporter system for gene expression studies in plants. Plant Biology 5:103-115.
Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper, J.W., and Pare, P.W. 2004. Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol 134:1017-1026.
Semighini, C.P., Murray, N., and Harris, S.D. 2008. Inhibition of Fusarium graminearum growth and development by farnesol. FEMS microbiology letters 279:259-264.
Sharon, A., Finkelstein, A., Shlezinger, N., and Hatam, I. 2009. Fungal apoptosis: function, genes and gene function. FEMS Microbiol Rev 33:833-854.
Shu, H.-Y., Lin, G.-H., Wu, Y.-C., Tschen, J.S.-M., and Liu, S.-T. 2002. Amino Acids Activated by Fengycin Synthetase FenE. Biochemical and Biophysical Research Communications 292:789-793.
Spaepen, S., and Vanderleyden, J. 2011. Auxin and plant-microbe interactions. Cold Spring Harbor perspectives in biology 3:a001438.
Studt, L., Wiemann, P., Kleigrewe, K., Humpf, H.-U., and Tudzynski, B. 2012. Biosynthesis of Fusarubins Accounts for Pigmentation of Fusarium fujikuroi Perithecia. Applied and Environmental Microbiology 78:4468-4480.
The GIMP Development Team. (1995). GNU Image Manipulation Program.
USDA. (2001). Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds; Final Guidance (United States Department of Agriculture).
Voss, K.A., Howard, P.C., Riley, R.T., Sharma, R.P., Bucci, T.J., and Lorentzen, R.J. 2002. Carcinogenicity and mechanism of action of fumonisin B1: a mycotoxin produced by *Fusarium moniliforme* (= *F. verticillioides*). Cancer Detection & Prevention 26:9.
Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H.U., Bruccoleri, R., Lee, S.Y., Fischbach, M.A., Müller, R., Wohlleben, W., Breitling, R., Takano, E., and Medema, M.H. 2015. antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic acids research 43:237-243.
Wegener, C., Bartling, S., Olsen, O., Weber, J., and von Wettstein, D. 1996. Pectate lyase in transgenic potatoes confers pre-activation of defence againstErwinia carotovora. Physiological and Molecular Plant Pathology 49:359-376.
Xu, D., and Cote, J.C. 2003. Phylogenetic relationships between Bacillus species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences. International journal of systematic and evolutionary microbiology 53:695-704.
Zhang, S., Wang, Y., Meng, L., Li, J., Zhao, X., Cao, X., Chen, X., Wang, A., and Li, J. 2012. Isolation and characterization of antifungal lipopeptides produced by endophytic Bacillus amyloliquefaciens TF28. African Journal of Microbiology Research 6:1747-1755.

Table 3.1. Biocontrol-relevant genes in *B. mojavensis* RRC 101

Metabolite/enzyme	Role in Rhizosphere competence/biocontrol	Reference	PEG Number[b]
Surfactin	Biofilm and host colonization, host defense induction	(Ongena and Jacques, 2008)	205 – 208
Fengycin	Antifungal activity	(Ongena and Jacques, 2008)	1874 – 1881
Subtilosin	Bacteriocin antibiotic		3746 – 3753
Bacilysin	Antibiotic		3789 – 3785
Bacillibactin	Siderophore – iron sequestration/mineralization	(Dertz et al., 2006)	3158 – 3162
Quercetin dioxygenase[a]	Protection from host flavonoids, rhizoplane colonization	(Hirooka and Fujita, 2010)	4042, 4043
Acetoin reductase	Putative host growth promotion/defense induction	(Ryu et al., 2004)	474, 1917
Phytase[a]	Nutrient availability, growth promotion	(Idriss et al., 2002)	2048
Expansin[a]	Host wall manipulation, facilitates root colonization	(Kerff et al., 2008)	1914
Pectate Lyase[a]	Facilitates host colonization, potential defense manipulation	(Kovtunovych et al., 1999)	627, 1916

[a] Putatively secreted as designated by Phobius

[b] Genome 1329377.3 on PubSEED

Table 3.2. Observed Fengycin diversity in *B. mojavensis* RRC 101

Fengycin	Major Ions	Peptide Sequence[a]	Lipid chain lengths
A	1080, 966	EOrnYTE**A**PQYI	14 – 17
A2	1066, 952	EOrnYTE**A**PQY**V**	16,17
B	1108, 994	EOrnYTE**V**PQYI	15 – 19
B2	1094, 980	EOrnYTE**V**PQY**V**	16, 17
C	1008, 1122	EOrnYTE**I**PQYI	16 – 18

[a] Bolded amino acid positions indicate differences between isoforms.

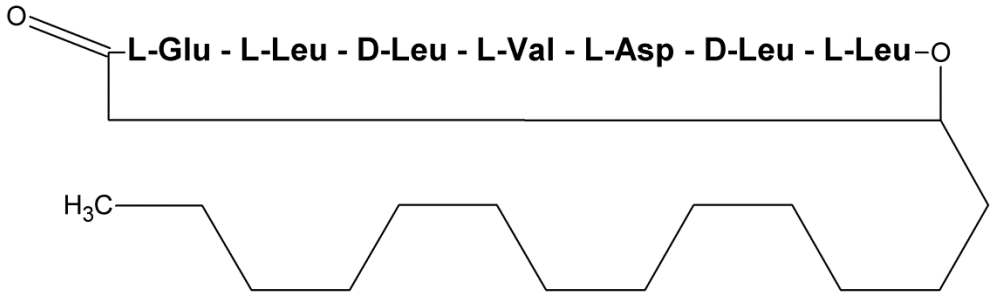
Table 3.3. Fumonisin production by M3125 in response to lipopeptide challenge

Treatment	Experiment 1	Experiment 2	Experiment 3	Average FB1%
Control	19.6 (2.9) ^{C}	18.5 (2.1) ^{D}	17.1 (1.5) ^{B}	60.41 ^{A}
10x Surfactins	20.3 (1.4) ^{BC}	21.5 (1.3) ^{DC}	13.9 (4.0) ^{B}	60.04 ^{A}
20x Surfactins	28.7 (1.2) ^{AB}	32.0 (4.1) ^{C}	16.6 (2.9) ^{B}	59.88 ^{A}
10x Fengycins	30.5 (8.7) ^{AB}	28.3 (2.8) ^{BC}	25.1 (4.4) ^{A}	58.56 ^{B}
20x Fengycins	34.1 (4.2) ^{A}	37.5 (3.1) ^{A}	25.9 (2.0) ^{A}	57.73 ^{B}

Letters indicate groups significantly different via Tukey's HSD at $p < 0.05$. Standard deviations in parentheses.

Figure 3.1. Chemical diagrams of the lipopeptides surfactin and fengycin A, produced by *Bacillus mojavensis* RRC 101. Amino acids comprising the peptide ring are denoted by their three-letter designations.

Surfactin (C-16)



Fengycin A (C-16)

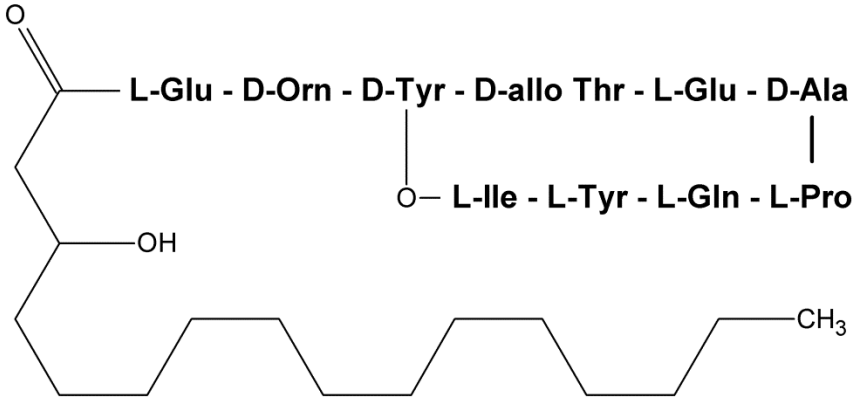


Figure 3.2. Total ion chromatogram of an antagonistic fraction (demonstrated in inset plate photo) as identified through HPLC-MS. Regions corresponding to fengycins and surfactins are underlined and labeled. Inset: Antagonism assay plate demonstrating antifungal activity of the depicted fraction (asterisk), as compared to methanol (Blank) and two inactive fractions (x); the effect of lipopeptide exposure on radial growth.

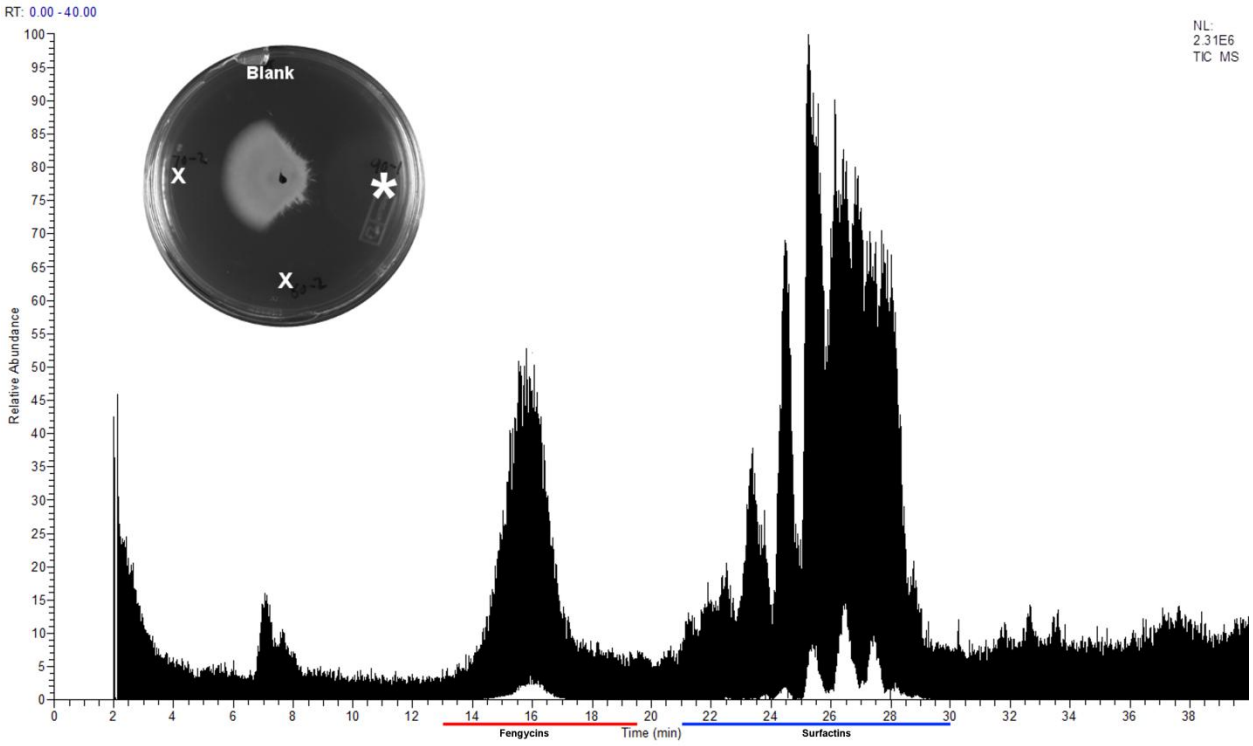
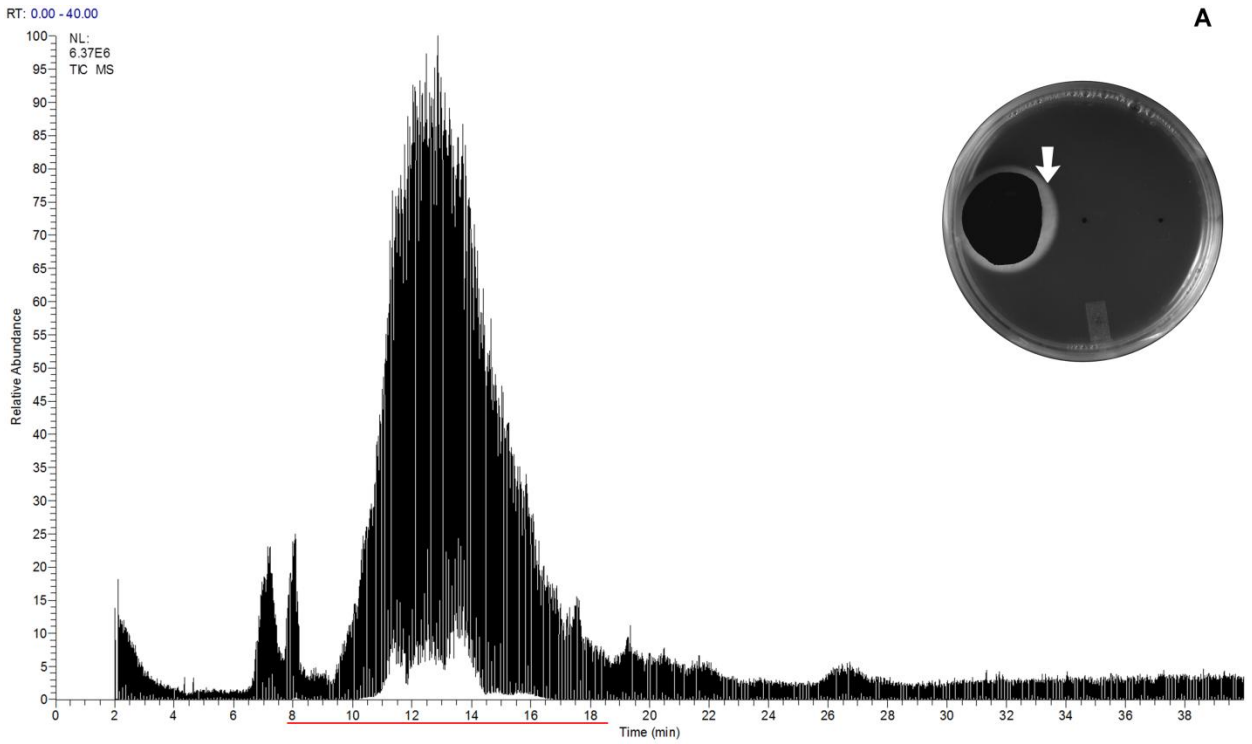

RT: 0.00 - 40.00
NL:
2.31E6
TIC MS
Blank
X
X
*
Relative Abundance
Time (min)
Fengycins
Surfactins

Figure 3.3. HPLC-MS spectra of collected RRC101 a) fengycin and b) surfactin fractions, with individual overlapping peaks representing the varied isoforms in each sample. Representative antagonism plates are inset to each of the spectra, with silhouettes of the respective fractions (in black) superimposed on that of the methanol control (white). The purified surfactins exhibited no significant antagonism, whereas the fengycins produced clear antagonism (indicated by the white arrow).


A
RT: 0.00 - 40.00
NL:
6.37E6
TIC MS
Relative Abundance
Time (min)

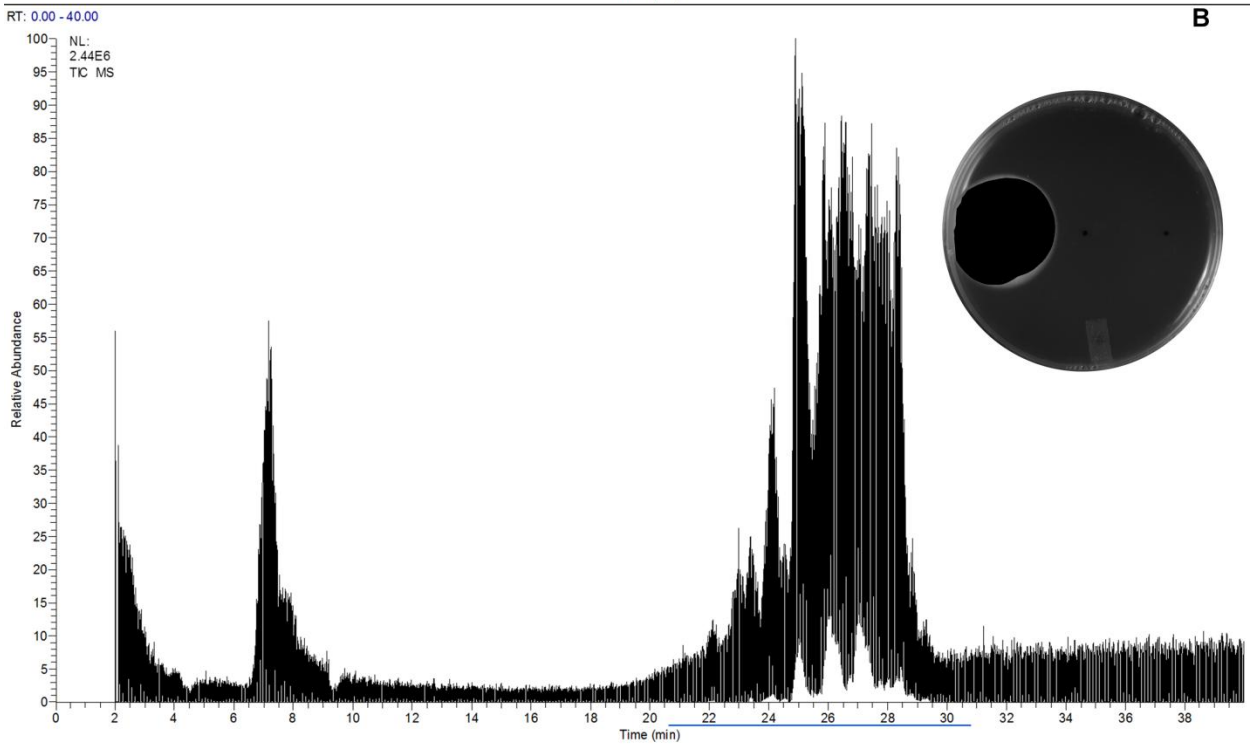

B
RT: 0.00 - 40.00
NL:
2.44E6
TIC MS
Relative Abundance
Time (min)

Figure 3.4. Correlation of antagonism with fengycin lipopeptides in RRC101 extract fractions. Secondary fractions were compared to the parent antagonistic fraction for growth reduction (triangles, solid line) and LC-MS-derived fengycin content as compared to the sum of all fractions (circles, dotted line). Dashed line = 0.

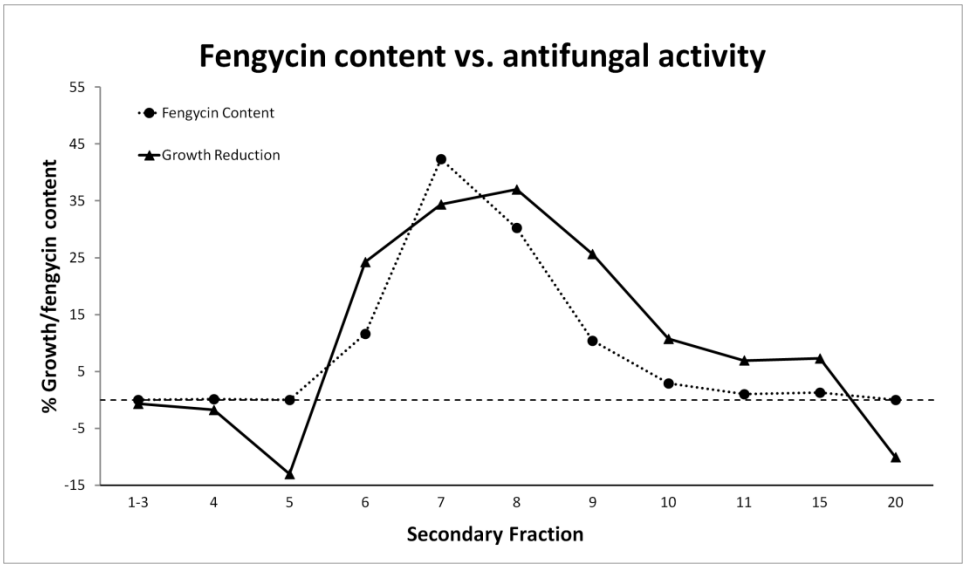

Fengycin content vs. antifungal activity
Fengycin Content
Growth Reduction
% Growth/fengycin content
55
45
35
25
15
5
-5
-15
1-3
4
5
6
7
8
9
10
11
15
20
Secondary Fraction

Figure 3.5. Morphological response of *F. verticillioides* to RRC101 lipopeptide challenge. A-C, Colony edges of *F. verticillioides* challenged by A) Negative control, showing normal hyphal growth and conidiation; B) Surfactin extracts, indistinguishable from controls; C) Fengycin extracts, showing sinuous hyphal growth (arrow) and bulbs (asterisk). Bar = 50 µm. Inset: Wild-type conidia (left); conidia exhibiting fengycin-induced swelling (right) Bar = 10 µm.

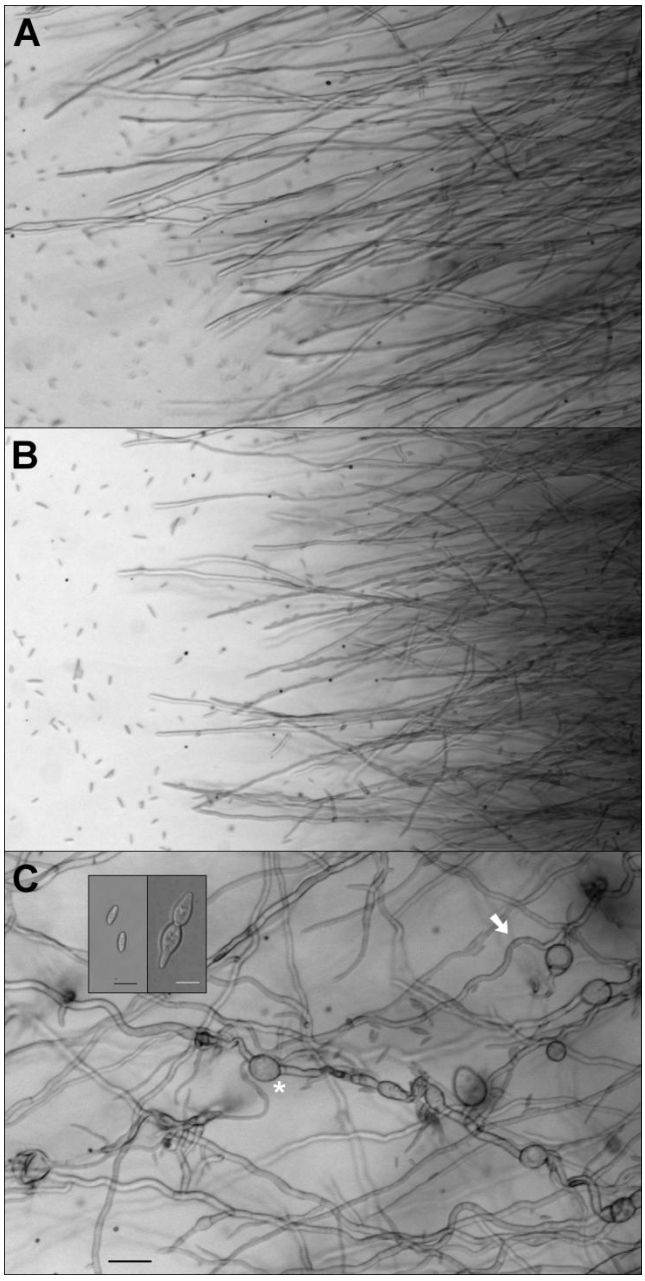

A
B
C
*

Figure 3.6. An example of explosive lysis observed in *F. verticillioides* cultures challenged with RRC101 fengycins, suggestive of cell wall dissolution and membrane failure. Arrow = Hyphal bulb induced by fengycin challenge, asterisk = explosive lysis accompanied by hyphal ejecta.

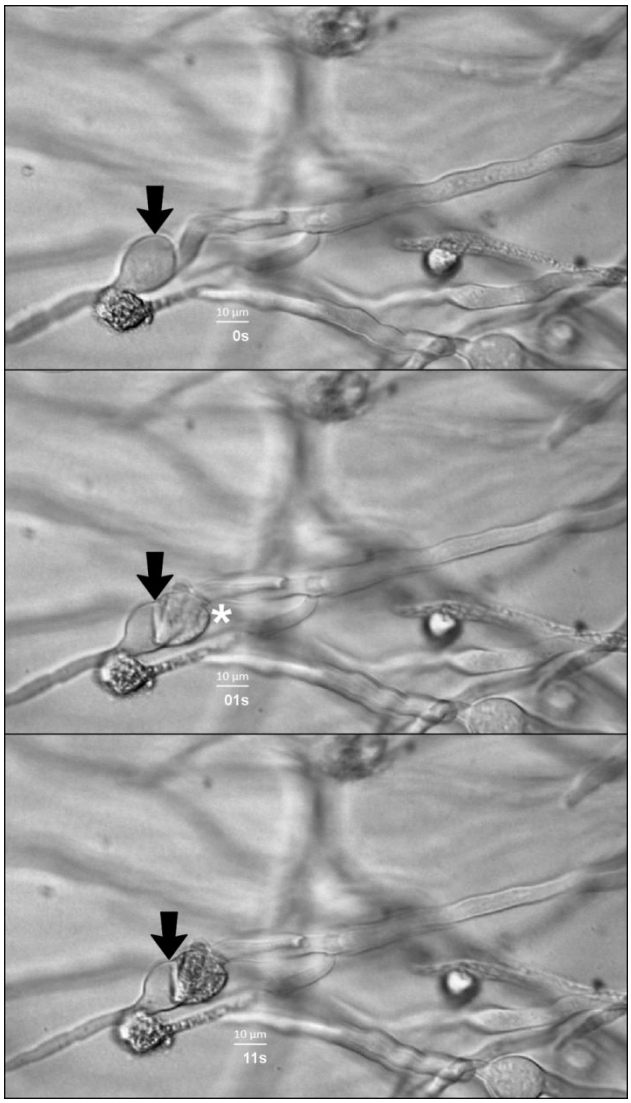
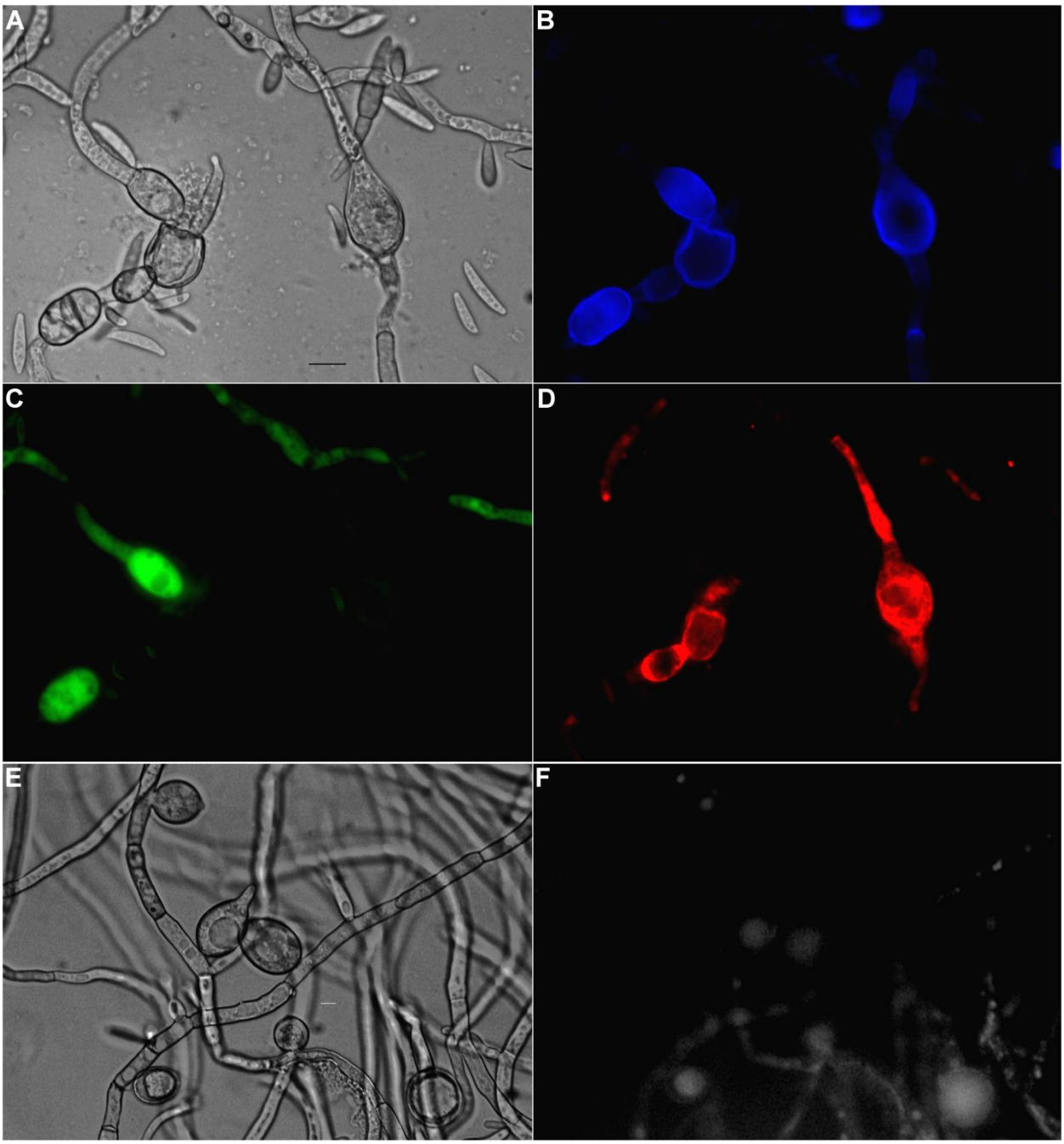

10 µm
0s
10 µm
01s
10 µm
11s

Figure 3.7. Fluorescence microscopy of *F. verticillioides* bulbs produced under challenge from RRC101 fengycins. A) bright field image of hyphae; B) Calcofluor-labeled cell walls; C) Cytosolic GFP expression; D) intense FM4-64 staining indicative of destabilized and hyperpermeable membranes as a result of the fengycin treatment. E&F) *F. verticillioides* hyphae under fengycin challenge exhibit vacuole-like organelle accumulation under DIC and fluorescent visualization of the vacuole-labeling dye CMAC, respectively. Bar = 20 µm.


A
B
C
D
E
F

CHAPTER 4

TRANSCRIPTOMIC ANALYSIS REVEALS *FUSARIUM VERTICILLIOIDES* EXHIBITS DIFFERENTIAL RESPONSES TO *BACILLUS MOJAVENSIS* LIPOPEPTIDE CLASSES, INCLUDING A PUTATIVE STRESS RESPONSOME

Blacutt, A.A., Glenn, A.E., Gold, S.E. For submission to Molecular Plant-Microbe Interactions

ABSTRACT

Fusarium verticillioides is an endophytic mycotoxigenic fungus whose primary threat to maize crop value is production of fumonisin mycotoxins. This threat is compounded by the intimate association of *F. verticillioides* with its host and the cost and limited efficacy and in chemical controls. The nature of this pathosystem is such that an efficacious biocontrol agent would be invaluable for crop protection. *Bacillus mojavensis* RRC101 was isolated from a maize kernel and confirmed to live endophytically in maize roots and aerial tissues. This bacterium is noted for its ability to reduce disease symptoms and toxin accumulation in coinoculation assays, as well as lipopeptide-mediated antifungal activity observed *in vitro*. This antagonism is accompanied by morphological defects and secondary metabolic changes in *F. verticillioides*, suggesting that lipopeptide antagonism provokes a change in the transcriptomic profile. These differential lipopeptide responses were evaluated through RNA sequencing of *F. verticillioides* cultures under lipopeptide antagonism, followed by functional categorization and comparison to deposited transcriptional data from earlier experiments with *F. verticillioides* and *Neurospora crassa*. The data generated from this work illustrate the diversity in lipopeptide response and support apoptosis induction as a mechanism of antagonism. Additionally, these data provide further support that ribosomal proteins act as underrated contributors to fungal success in the phytobiome, against antagonistic hosts and microbes alike. These results suggest fruitful avenues to investigate in fungal pathogen biology in terms of stress responses and subsequent modulation of gene expression.

INTRODUCTION

Fusarium verticillioides is a mycotoxigenic pathogen of maize whose success and ubiquity is largely due to its ability to survive as an asymptomatic or even protective endophyte of its host (Lee et al., 2009). The fungus associates with corn throughout the host's life cycle and colonizes all host tissues, from the rhizosphere to the phylloplane. As a pathogen, *F. verticillioides* causes seedling blight, ear and stalk rots, and is capable of seedborne dissemination via silk channel infection. Disease symptoms resulting from *F. verticillioides* infection threaten maize yields, but accumulation of fumonisin mycotoxins can be devastating to crop value and utility (Wu et al., 2011). Contaminated grain use, including fuel ethanol, animal feed and human consumption, is threatened by fumonisin contamination (Morgavi and Riley, 2007). Endophytism and the hemibiotrophic lifestyle employed by *F. verticillioides* enables it to associate with maize and facilitates disease spread; field stubble is heavily colonized, providing inocula that infect subsequent crops. Given this close association and the challenges inherent to chemical control strategies, *F. verticillioides* management options are limited (Nguyen et al., 2017). Biological control in the context of integrated pest management presents an alternative means of controlling plant disease with potentially reduced costs, environmental or otherwise, compared to chemical control strategies.

Isolated from a maize kernel in 1996, *Bacillus mojavensis* RRC101 has been shown to act as a beneficial bacterial endophyte under controlled environmental conditions. The bacterium can reduce disease severity and mycotoxin accumulation in addition to promoting plant growth (Bacon and Hinton, 2011). Genome sequencing and annotation of RRC101 revealed the presence of operons for cyclic lipopeptide antibiotic synthetases (Gold et al., 2014). While the strain's recalcitrance to transformation has precluded mutant testing, chromatography and bioassay of

culture extracts have allowed isolation and identification of fengycin-class lipopeptides as the primary antifungal metabolite against *F. verticillioides*, whereas previous efforts had credited this activity to surfactins *(Snook et al., 2009; Blacutt et al., 2016)*. Biochemical assays identified differentially increased fumonisin production as a common response to lipopeptide challenges, but fengycins alone induce stronger secondary metabolic response, and a hyphal deformation-and-lysis phenotype suggesting that *F. verticillioides* perceives and responds to lipopeptides in a class-specific manner. These results support commonly observed ranges of activity for lipopeptides, wherein their composition, and that of the target membrane, serve as "lock and key" determinants of disruptive potential (Bonmatin et al., 2003).

The reported primary mechanism of successful lipopeptide antagonism is membrane permeabilization via integration and pore formation, leading to osmotic destabilization and death (Patel et al., 2014). The presence of bulb structures in fengycin-challenged *F. verticillioides* supports this hypothesis, as does their violent lysis. The previously observed secondary metabolic response to lipopeptide antagonism suggests an underlying transcriptomic response. Based on previous experiments, we hypothesized that *F. verticillioides* exhibits differential responses to specific lipopeptide classes, and that these responses are reflected by transcriptomic behavior. In order to better characterize the response to lipopeptide antagonism, RNA sequencing experiments were conducted using *F. verticillioides* cultures under lipopeptide challenge. Lipopeptide challenge in *F. verticillioides* induces structural genes as well as those involved in secondary metabolite synthesis, corroborating the previously observed morphological responses to antagonism. Transcriptomic data identified a proposed core stress response gene set, induced by a range of stressors. This work comprises the first known transcriptomic description of a lipopeptide-challenged fungus, specifically an economically

significant mycotoxigenic phytopathogen, and its short-term response to exposure to lipopeptides common to bacterial biocontrol agents.

RESULTS

MIPS functional enrichment of responsive genes varies across lipopeptide treatments

Fusarium verticillioides cultures were treated with aqueous lipopeptide treatments, either individual classes or as a combined preparation, in order to capture transcriptomic responses to antagonism. Solid-medium cultures were utilized to better reflect the hyphal growth habit exhibited during maize colonization, where these lipopeptides are encountered in biocontrol interactions. Genes determined to be differentially expressed via cummeRbund were compared across treatments to isolate genes unique in response to each class, referred to herafter as curation. *Fusarium verticillioides* transcriptional response varied strongly dependent on specific lipopeptide treatment (Figure 4.1). To better identify transcriptional regulation uniquely responsive to specific lipopeptide classes, these data have been curated into three unique, non-overlapping bins, named for their inducers: Fengycin, Surfactin and Synergism (unique to combined fengycin and surfactin lipopeptide treatment) (Supplemental Figure 4.1).

The *F. verticillioides* genome contains 15,869 open reading frames, of which roughly 50% are unclassified (category 99) in the MIPS FunCat system (Ruepp et al., 2004). Across treatments, most of the lipopeptide-responsive genes belong to this category, with statistically significant enrichment in the fengycin group (Table 4.1, Supplemental Table 4.2). Fengycin antagonism in particular elicits a transcriptomic response with statistically significant enrichment for unclassified genes (MIPS category 99). Functional categories enrichmed under fengycin were

those involved in transport, protein degradation and polyketide metabolism. Downregulated genes under fengycin challenge were enriched for secondary metabolism, xenobiotic detoxification and metabolism of amino acids, (subcategories 01.20, 01.20.37 and 01.01, respectively). Proportionally, surfactin-responsive genes were more involved in cell transport, cell rescue/defense and environmental interactions, categories 20, 32 and 34 respectively. Surfactin-downregulated genes were too few to distinguish meaningful groupings beyond metabolism (Category 01) and unclassified (Category 99) genes.

Genes upregulated uniquely in response to lipopeptide synergism (surfactin and fengycin together) primarily consisted of genes categorized as functioning in structural or catalytic roles (category 16) in addition to protein synthesis and modification (categories 12, 14 and 18). Additionally, numerous categories involved in adaptation to environmental conditions were found among synergism-upregulated genes (categories 10, 30, 32, 34 36, 40, 43). Synergism-downregulated genes were most prominently categorized in metabolism, protein fate and catalytic/structural proteins, cell transport and cell defense (01, 16, 20 ,14 32), with strong representation in energy production and transcription (02 and 11). As with fengycin-response, genes annotated as involved in protein synthesis (category 12) were solely upregulated.

Lipopeptide responsive genes feature enrichment in specific gene ontology (GO) function and process categories

Functional enrichment described the fengycin response as upregulating encoded functions such as endopeptidase, cellulase and oxidoreductase activity (Table 4.2). Most prominent among antagonism-downregulated genes were those annotated as symporters or transporters with several predicted to function in heme and tetrapyrrole binding. Process

enrichment highlighted proteolysis, catabolic (primarily TCA cycle) and structural genes as upregulated, with lipid metabolism and oxidative stress response downregulated.

Synergism-induced genes were enriched for structural molecule function and peptidase and hydrolases, with single genes attributed to both sesquiterpene synthase and trichodiene synthase. Genes downregulated under lipopeptide synergism included those annotated as involved in antiporter activity and signal transduction. Metabolic processes in general, and specifically peptide metabolism, were upregulated, while, in contrast, processes related to lipid metabolism, single-organism processes and redox state were downregulated.

Surfactin-induced gene response was functionally characterized as enriched for genes involved in catalytic activity, signal transduction and redox activity. Surfactin-upregulated genes were enriched in the processes of redox maintenance, stimulus response and signaling, as well as stress response. The most common functional annotations in genes downregulated under surfactin challenge were ion binding and the transfer of acyl groups. Surfactin-downregulated processes were predominantly characterized as involved in amino acid and small molecule biosynthesis.

Localization of lipopeptide-responsive gene products varies by lipopeptide class

Bioinformatically-determined localization patterns for the products of genes upregulated in response to lipopeptide challenge are illustrated in Figure 4.2. Twenty-nine percent of fengycin-induced genes are predicted to encode proteins acting extracellularly, representing an enrichment compared to the genome at large (17.78%). Nuclear-localized protein-encoding genes were more strongly respresented as fengycin-induced compared to other treatments, with very little downregulation of genes predicted to be localized to this compartment. Genes encoding proteins predicted to be secreted and membrane-localized were the most common

among those downregulated under fengycin challenge. Genes of uncertain localization represented 14% of upregulated and 3% of downregulated genes responding to fengycin.

Surfactin-induced genes were mostly predicted to act in the cytoplasm (35%), with secreted proteins being underrepresented as compared to the *F. verticillioides* genome (7.94% vs 17.78%). Surfactin response also possessed a higher proportion of putative membrane-localized proteins than the other two groups (25% compared to 16.6% for fengycin and 16.9% for synergism). Compared to the other gene groups, larger proportions were predicted to be localized to the plasma membrane, mitochondria and peroxisome. Genes downregulated in response to surfactin were mostly cytoplasmic as well, with comparatively strong representation of nuclear, mitochondrial and peroxisomal localization. Products with unknown localizations represented 11% of both up and downregulated genes.

Synergism-induced *F. verticillioides* genes, similar to those that are surfactin responsive, were as a plurality localized to the cytoplasm (39%), whereas regulation of membrane-localized proteins was more remniscient of fengycin response. Synergism-responsive genes were the only treatment group containing predicted vacuolar proteins, with one upregulated (FVEG_09934, hexose aminidase) and the other down (FVEG_11873, oxidoreductase).

Lipopeptide challenge induces secondary metabolite biosynthetic pathways

Across individual lipopeptide treatments, five polyketide synthase (PKS) domain-containing proteins were upregulated (Table 4.3). Fumonisin and bikaverin response to lipopeptide antagonism was previously noted, and the corresponding PKS genes (FVEG_00316 and FVEG_03379, respectively) were found to be moderately upregulated under lipopeptide challenge (Blacutt et al., 2016). Additionally, PKS genes for fusarin C and the antibacterial fusaric acid were both upregulated under antagonistic conditions (either fengycin or combined

synergistic treatment), with stronger induction under fengycin challenge as compared to the combined treatment. Additionally, two NRPS-PKS genes without known products were upregulated under lipopeptide treatment. Beyond PKS genes, MIPS functional characterization of genes upregulated under fengycin, synergism and surfactin conditions, respectively, identified 6, 7, and 8 genes, respectively, categorized under secondary metabolism (MIPS category 1.20).

Lipopeptide exposure induces genes involved in pathogenicity to maize

Genes responsive to each of the lipopeptide treatments were compared to three *F. verticillioides* EST libraries containing genes involved in maize colonization, one for colonization of shoots and roots (FvI) one for colonization of maize kernels (FvJ) and a pair subtracted for genes upregulated in response to maize defense compounds (FvKL, see Supplemental Table 4.1) (Brown et al., 2005). Although no genes from the FvKL set were present in the three curated gene lists, two genes were found in the pre-curation data, responding to both the surfactin and combined treatments: FVEG_13933 (SAM-dependent methyltransferase, downregulated) and FVEG_10103 (cysteine hydrolase ycaC, upregulated).

Of the genes characterized as antagonism-responsive, 32 (28 upregulated, 4 downregulated) were present among EST libraries for shoot and root colonization (FvI) and maize kernel colonization (FvJ) (Tables 4.4 and 4.5, Supplemental Table 4.1). Thirteen of these genes were predicted to encode secreted products, with 11 of those having no known function, and those with predicted functions were mostly hydrolytic enzymes or involved in cell structure or signal transduction, with the exception of FVEG_03831 that encodes a bZIP transcription factor. Genes responding to combined lipopeptide treatment overlapped with 57 (42 upregulated, 15 down) genes in FvI. Induced genes were largely degradative enzymes, with several encoding domains often involved in pathogenesis such as cell wall degrading enzymes. Additionally, a

putative N-acetyltransferase, several ATPases as well as two heterokaryon incompatability proteins and programmed cell death protein 6 were upregulated in response to lipopeptide synergy. The putative bZIP transcription factor FVEG_07194 was also upregulated in both genesets. Among surfactin-responsive genes, 20 were found in the FvI EST library (18 upregulated, 2 down). Genes upregulated under surfactin exposure were primarily dehydrogenase enzymes and signaling proteins but also included a fungalysin-type secreted metalloprotease.

Genes in FvJ, representing those involved in colonizing maize kernels, overlapped with the antagonism and synergism-responsive genes, with none identified as surfactin-responsive (Table 4.5). From the antagonism group, a BYS1-domain containing protein (FVEG_03433), cerato-platanin (FVEG_03005) and a hypothetical protein (FVEG_00611) were upregulated. A protein kinase (FVEG_15889), hypothetical protein (FVEG_11387) and a fatty acid coA ligase involved in fumonisin production (fum10p, FVEG_00321) were all upregulated in response to lipopeptide synergism.

Lipopeptide challenge induces genes in a shared stress response suite

To further characterize lipopeptide responsive genes, comparison was made in FungiDB using data from another filamentous fungus, *Neurospora crassa*, which has numerous expression libraries characterizing abiotic stress responses (Basturkmen et al., 2008). EST data has been collected for *N. crassa* under oxidative, osmotic and heat stresses as well as glucose and nitrogen starvation. Based on syntenic orthology transformation from *N. crassa* data sets, *F. verticillioides* genes responsive to lipopeptide challenge were identified as likely responding to these stressors as well (Table 4.6). These *F. verticillioides* genes in turn overlapped in their putative response to stressors, with a set of 14 identified in the synergism group that were present across all five stress

conditions tested. These 14 genes were slightly elevated in transcript abundance and mostly encode ribosomal proteins (n = 8) along with a putative regulator of chromosome structure and a programmed cell death protein. Antagonism-responsive genes putatively responsive to osmotic and oxidative stress included those encoding structural proteins and a bZIP transcription factor (FVEG_03831) predicted to respond to all but one of the tested stressors (heat). Surfactin-responsive genes showed no specific enrichment across stress categories but were predicted to be functionally involved in respiration and signal transduction.

Apoptosis-related genes are upregulated under lipopeptide antagonism

Genes upregulated in response to lipopeptide treatments were probed using a list of genes potentially involved in fungal apoptotic processes (Fedorova et al., 2005). Fengycin and synergism treatments, but not surfactin, induced additional cell-death related genes such as multiple putative HET protein-encoding genes, programmed cell death protein 6 and oxidative stress response genes (Table 4.7). An ortholog for the *Aspergillus clavatus* starvation-response transcription factor JlbA was found to be encoded by FVEG_07194. JlbA is responds to lipopeptide synergism, and in *A. clavatus* is involved in starvation-induced autophagy (Fedorova et al., 2005).

DISCUSSION

Fusarium verticillioides responds uniquely to lipopeptide classes

Prior to filtering and categorization of genes, the specificity of genes responsive to lipopeptide exposure suggested differential responses to xenobiotic challenge from lipopeptide metabolites across chemical classes (Figure 4.1). Lipopeptides act synergistically, with combined treatment potentiating their membrane-disruptive effects (Patel et al., 2014). Our data show that the combined lipopeptide treatment induces a greater response of unique genes than either

lipopeptide alone. These data are consistent with observations of potentiation and suggest that fungi are capable of perceiving different lipopeptide classes and the degree of antagonism they inflict. Thus, the curation of lipopeptide-responsive genes into unique categories (Fengycin, Surfactin, Synergism, Supplemental Figure 4.1) serves to divide predicted functional responses into categories meaningful in the context of biological control.

Functional diversity of genes responsive to challenge is defined by lipopeptide class

MIPS functional categorization facilitates a limited description of the functions induced under varying lipopeptide challenge (Table 4.1). Based on the sole upregulation of categories concerned with protein expression and cell fate/cycle, *F. verticillioides* exhibits a clear state of stress and response under fengycin antagonism. This regulatory unidirectionality corroborates visual observations of antagonism, wherein culture growth is halted by fengycin-containing extracts and exhibit increased secondary metabolic activity. Antagonism via lipopeptide synergism is similarly stark, with significant enrichment of transport, cell rescue and virulence functions, indicative of a clear perception of antagonism coupled with a phenotype of recovery and retaliation.

REVIGO is a tool that enables condensation of lists of gene ontology (GO) terms, which facilitates discussion by removing redundancy, and annotation data from lipopeptide response was processed in this manner (Table 4.2) (Supek et al., 2011). Among the annotated genes induced under antagonistic conditions (fengycin/synergism), degradative enzymes and those involved in redox maintenance are strongly represented. Notably, fengycin- and synergism responsive genes included those encoding general peptidases and peptidases acting on L-amino acids (GO terms 0008233 and 0070011). Such enzymes may be involved in hydrolyzing lipopeptide rings, which has been shown to abolish antimicrobial activity (Armelle et al., 2015).

This GO term does not appear in surfactin-responsive genes, suggesting these enzymes are directly responsive to antagonistic conditions, lending support to a recover and retaliate model of *F. verticillioides* response to lipopeptide antagonism (Figure 4.3). Functional representation in surfactin response is less diverse but unique in its own right, and while peptidases comprise a significant portion of GO-annotated genes, the response is apparently enriched for structural molecule activity and ribosomal constituents. This difference in composition underscores the lack of visible symptoms upon surfactin challenge, with hyphae visibly unchanged by surfactin exposure. These data suggest that surfactin does indeed inflict a level of stress on *F. verticillioides*, but not to the severity that fengycin is capable, either alone or in concert with surfactin. This potentiation of antagonism is evident in the fold-change data for PKS genes, representative of secondary metabolic involvement. For example, the increase in bikaverin PKS transcripts under combined lipopeptide antagonism suggests an additive effect compared to the individual treatments.

Fengycin antagonism clearly induces a significant number of genes that are currently unclassified, across both MIPS (Category 99, 89 genes) and GO (GO:3674, 60 genes) functional analyses. Whether these genes comprise a range of unknown functions or are involved in competition-relevant pathways is uncertain, but their involvement in both lipopeptide antagonism and maize pathogenicity (Supplemental Table 4.4) suggest that they are a crucial part of *F. verticillioides* survival, and warrant functional characterization. Similarly, the involvement of ribosomal proteins in maize colonization and lipopeptide response is unexpected, although there is precedent.

Ribosomal proteins function as structural components of ribosomes, with work in other systems implicating their differential regulation in stress response pathways (Zhou et al., 2015).

Given the information regarding these proteins in other systems and their involvement in response to antagonism response, ribosomal proteins likely play a subtle but significant role in the survival of *F. verticillioides*. Ribosomal proteins found in the FvI EST library may be involved in modulating *F. verticillioides* lifestyle from endophytic/saprophytic to pathogenic, either early in the process or in coping with the incurred environmental stress.

Phenotypes of lipopeptide antagonism are corroborated by transcriptomic data

As described in our previous work, hyphal deformation and explosive lysis are characteristic symptoms of lipopeptide antagonism, and this violent cell death is illustrative of membrane permeabilization as a mechanism of antagonism (Blacutt et al., 2016). These symptoms are corroborated by the number of structural proteins observed to be upregulated under both fengycin challenge and lipopeptide synergism, suggesting processes of cell wall repair or fortification. Upregulation of genes encoding membrane-integral proteins such as flotillin (FVEG_ 09802, Table 4.6) illustrate a perception of membrane perturbation under lipopeptide antagonism, especially given that this upregulation is not observed under surfactin challenge.

Our previous work described the *F. verticillioides* response to lipopeptide challenge as including secondary metabolite accumulation, with surfactins and fengycins inducing fumonisin accumulation in varying magnitude, with fengycin antagonism accompanied by visible bikaverin accumulation (Blacutt et al., 2016). Here we showed the corresponding PKS genes for these metabolites were upregulated in class-dependent response to lipopeptide challenge. Surfactins and fengycins alike appear to induce accumulation of bikaverin transcripts, but the response to fengycin antagonism included both the fumonisin PKS and the *FUM10* gene. Further, antagonistic conditions, but not surfactin exposure alone, induced PKS genes for the metabolites

fusarin C and fusaric acid, noted for their antibacterial activity (Bacon et al., 2006; Svendsen et al., 2016). These challenge-specific secondary metabolic responses support the hypothesis that these lipopeptides are uniquely perceived and differentiated by *F. verticillioides*, with correspondingly nuanced secondary metabolic responses. As sub-inhibitory concentrations of antibiotics and mycotoxins have been identified as signaling molecules, it's possible that specific lipopeptide classes may act in part as cross-kingdom signaling molecules (Andersson and Hughes, 2014).

Bikaverin's function for *F. verticillioides* is uncertain, however, as a fungal secondary metabolite and potentially valuable natural product it has invited speculation and research (Limón et al., 2010). As a secondary metabolite produced by a pathogen in competitive niches, antimicrobial activity is an expected function. Experiments testing antimicrobial activity of bikaverin determined it is at least as effective as standard antibiotics against a range of clinically-relevant bacterial pathogens (Deshmukh et al., 2014). In a more clinical context, pretreating neuronal cells with bikaverin confers strong protection for plasma membranes and mitochondria against H_2O_2 damage, suggesting a role as a countermeasure against oxidative stresses (and potential pharmaceutical value) (Nirmaladevi et al., 2014). These traits establish a likely bimodal role for bikaverin in response to lipopeptide antagonism, meaning it may function as a defensive molecule against oxidative stress while potentially acting offensively against the source of the insult. Fusarin C, possibly induced by some stress shared by host colonization and lipopeptide antagonism, has limited antibacterial activity but is considered mutagenic and carcinogenic (Svendsen et al., 2016). Fusaric acid possesses activity against some *Bacillus* strains, including those capable of lipopeptide production and biocontrol activity. Induced PKS and secondary

metabolic gene expression further supports the recover and retaliate model of antagonistic lipopeptide perception in *F. verticillioides* (Bacon et al., 2006).

Lipopeptide antagonism induces genes involved in a putative *Fusarium verticillioides* stress responsome

With a dearth of experimental annotation for genes in the *F. verticillioides* genome, much is to be gained through utilization of data from comparable, well-described systems. FungiDB, in addition to being a data repository, allows gene data from one organism to be "transformed" by orthology, with the ability to add stringency via limiting results to syntenic orthologs. Through this syntenic transformation, transcriptional data from other systems could be leveraged in characterizing the responses of *F. verticillioides* to lipopeptide challenge. Specifically, *N. crassa* data concerning response to discrete stressors (oxidative, osmotic, heat, glucose and nitrogen starvations) enabled more comprehensive functional description of the lipopeptide response in *F. verticillioides*.

Stresses evaluated in these EST data can be divided into "extrinsic" and "intrinsic" categories, with glucose- and nitrogen starvation giving rise to intrinsic stress as opposed to the externalized oxidative, osmotic and heat-derived challenges, with some genes putatively acting across all tested stressors. Lipopeptide-responsive genes involved in response to extrinsic stress are more focused on structural maintenance and signal transduction, such as genes encoding cytoskeleton, wall and membrane associated proteins induced under antagonistic (fengycin, combined) treatment. The intrinsic stress response appears to involve degradative enzyme function, although it is unclear whether these enzymes are acting extracellularly or as part of an autophagic process. Given the current mechanistic model of lipopeptide antagonism, it is not surprising that genes induced under such challenge are strongly linked to oxidative and osmotic

stress, with significant overlap in heat-stress response (Deleu et al., 2008). Interestingly, the surfactin-responsive genes do not exhibit significant overlap with any specific stressor. This ambiguity suggests that nonantagonistic lipopeptide challenge, while clearly inflicting stress on *F. verticillioides*, lacks the severity required to induce observable antagonism on its own. As outcomes of lipopeptide-membrane interactions are dependent on specific composition of their participants, it is possible that the comparably mild stress induced by surfactin is due to inefficient or less disruptive membrane interactions.

Across lipopeptide challenge, the osmotic and oxidative stress categories were most highly represented across lipopeptide challenge, with synergism induced genes shared with putative stress response being associated with at least these two stimuli (Table 4.6). In addition, 14 genes present in the synergistic-antagonism set were expressed under all five tested stress conditions. These genes likely comprise part of a core "responsome" for *F. verticillioides*, involved in response to both extrinsic and intrinsic stressors via universal functions. The term "responsome" has been used to refer to the secondary metabolic profile of an organism under a given stimulus (Bachmann, 2014). Our work suggests expanding the definition to encompass a comprehensive modeling of the cell processes underlying responses to environmental stress stimuli. In the spirit of the original definition, it should be noted that products of two of the PKS genes (FVEG_11932, FVEG_05537) induced under fengycin challenge are yet unknown, and may prove to be novel metabolites. Following putative identifications based on *N. crassa* data and differential response to lipopeptide challenge, components of this proposed responsome serve to elucidate nuances involved in fungal perseverance against environmental challenges. Genes responsive to lipopeptide challenge as well as maize colonization represent "multifunctional" stress response genes which may prove valuable targets for management

purposes. For example, FVEG_02548 is a putative component of the "mediator complex" and is induced under fengycin antagonism, four of the five compared stressors (all except nitrogen) and colonization of maize shoots and roots. In *Candida glabrata*, inhibition of the activator-mediator interaction restored azole sensitivity in resistant strains (Nishikawa et al., 2016).

Genes shared in maize colonization and antagonism response imply common environmental stresses

In selecting stress-response genes that may be targeted for management purposes, it is logical to consider those that are also involved in host colonization, such as those present in the FvI EST data set provided by Brown et al. (Brown et al., 2005). The secretion of cerato-platanin, a known phytotoxic peptide, suggests that pathogenicity and stress response are linked in *F. verticillioides.* Levels of oxidative and osmotic stress may serve as inducers of the pathogenic state in the same manner as nitrogen starvation has been demonstrated to induce fumonisin production (Kim and Woloshuk, 2008). Coregulation of stress and pathogenicity genes may also be a result of stress inherent to growth in the host apoplasm, where colonizers contend with nutrient scarcity and oxidative stress. It may be that these putative stress response genes serve to protect fungal growth during the endophytic portion of its lifestyle as it colonizes a comparably inhospitable environment. This may explain the upregulation of genes like FVEG_03433, a homolog of BYS1, in maize colonization EST libraries. This protein is linked to a morphological change in the pathogen *Blastomyces dermatitidis*, whereby higher temperatures prompt BYS1 protein accumulation and differentiation from filamentous to yeast-like unicellular growth (Bono et al., 2001). As FVEG_03433 is upregulated in both FvI and FvJ EST libraries, it is possible that this protein is involved in the endophyte/pathogen "lifestyle switch" in *F. verticillioides*. In *B. dermatitidis*, this change is dependent on temperature, suggesting that external stress stimuli

akin to those inflicted by lipopeptide antagonism may be involved in lifestyle modulation in *F. verticillioides,* for example the oxidative stress inflicted by host-produced H_2O_2. Similarly, the bZIP transcription factor FVEG_03831 may also be involved in determining *F. verticillioides* lifestyle. The bZIP family of transcription factors is associated with stress response and virulence across pathogenic fungi, and the FVEG_03831 is induced by fengycin antagonism and present in the FvI library (Guo et al., 2010). While JlbA is also a bZIP transcription factor, the targets of FVEG_03831 are unknown and, given its presence in both maize colonization and fengycin antagonism, it may be involved in regulating common processes such as the "offensive" protein functions such as secreted hydrolases, secondary metabolism and virulence-associated peptides like cerato-platanin and LysM-related effectors.

LysM domains typically bind fungal cell wall constituents like chitin, and through this activity are thought to act by masking the presence of the fungus to evade host defenses or exogenous chitinase activity (Kombrink and Thomma, 2013). This masking, combined with hydrolase secretion and the secondary metabolic response to antagonism, suggest an 'offensive' expression pattern shared by both antagonism-response and maize colonization. This concept of pathways multitasking across stimuli is further supported by the common accumulation of "defensive" transcripts, for genes involved in coping with environmental stresses, either through detoxification, maintenance of a redox state or the more dramatic apoptotic processes.

Lipopeptide antagonism induces genes putatively involved in programmed cell death

Although the direct mechanism of cyclic lipopeptides is membrane permeabilization leading to lysis, there is significant evidence that antagonism is also accomplished through induction of cell death processes (Table 4.7) (Han et al., 2015). Indeed, previous microscopic examination observed heavy vacuolization under fengycin challenge as part of the antagonism

phenotype, and this is a classical sign of autophagy under apoptotic processes (Saupe, 2000; Blacutt et al., 2016). Heterokaryon incompatibility proteins are classically involved in self/nonself recognition for filamentous fungi, and when incompatible hyphae fuse, genes are induced and lead to the programmed cell death that gives rise to the "barrage zone" phenotype (Paoletti and Clavé, 2007). Functions for many of these genes are unknown, although het-c, het-d and het-e act in an allelic manner to determine anastomosis outcomes, and het-e (upregulated under lipopeptide synergism) at least appears to function in a recognition/signal transduction capacity. FVEG_11873, homologous to apoptosis-inducing factor (AIF2) is downregulated in response to synergistic antagonism. As an AIF ortholog is known to regulate apoptosis in a caspase-independent pathway in yeast, FVEG_11873 is potentially a regulator of apoptotic processes in *F. verticillioides* (Modjtahedi et al.; Wissing et al., 2004).

Ribosomal proteins induced by synergistic antagonism likely play a role in cell death. In higher eukaryote systems these proteins respond to stress and provide a level of regulation for proliferation, differentiation and induction of cell death (Naora and Naora, 1999). Here, cancer research provides illumination as to the roles of these genes: the S14 type proteins, which were expressed in the FvI library and responsive to lipopeptide synergism, are tumor suppressors through activation of p53, which leads to cell cycle arrest and death (Zhou et al., 2015). Filamentous fungi have been found to possess a p53-like gene responsive to nutrient starvation, suggesting this mode of programmed cell death induction may be intact in lower eukaryotes (Katz et al., 2013). Ribosomal proteins upregulated under lipopeptide antagonism and maize colonization are homologous to those causing the cell death-implicated human disease Diamond-Blackfan Anemia, but their involvement with fungal apoptosis cell death pathways is less clear. In addition to HET proteins and the ambiguous involvement of ribosomal proteins, the aptly-

named programmed cell death protein 6 is known to promote apoptosis through interaction with caspase via signaling pathways (Lee et al., 2005). Overall, exposure to fengycin antagonism leads to cell lysis and programmed cell death through clearly observed signaling pathways activated under antagonistic conditions.

Simultaneous exposure to multiple classes of lipopeptides produces a synergistic effect, potentiating antagonism (Maget-Dana et al., 1992). The transcriptomic response of *F. verticillioides* to fengycin and surfactin synergism suggests that this effect is specifically perceived in target organisms. Synergism-responsive genes were the largest curated set, at 239 genes compared to the 187 of the fengycin group and 81 for surfactin. As these curated sets are all unique, the size of the synergism set illustrates the breadth and specificity of *F. verticillioides* response to lipopeptide antagonism. There are two possible mechanisms by which this perception differs: Synergistic antagonism may be uniquely perceived by *F. verticillioides*, or combined lipopeptide challenge activates a higher threshold of stress response as compared to either the fengycin or surfactin treatments. That all 14 of the proposed responsome genes are induced under synergism response alone suggests that combined lipopeptide exposure inflicts unique stress on *F. verticillioides*, as opposed to an increased level of the same stresses applied by fengycin or surfactin alone. Synergistic lipopeptide antagonism and its effects on the target organism may be better characterized through more fine-tuned experiments, such as those testing the minimum inhibitory concentration for each contributing class. Better understanding of the specific conditions underlying synergistic antagonism will enable characterization of the nature of such antagonism and its value in biological control.

Together, these data illustrate the diversity and conservation in *F. verticillioides* responses to exogenous stressors inherent to the niche of the phytopathogen. The nuances

observed in these data may find application beyond this pathosystem Just as work done in higher eukaryotes and cancer biology contributes to characterization of ribosomal proteins and those involved in apoptotic processes, lipopeptide-response data can also be used to characterize genes currently lacking annotation. As observed in the fengycin- and synergism-responsive gene sets (Table 4.1), unclassified genes comprise the majority of antagonism response in *F. verticillioides*. As the roles of these genes are yet unclear, characterization of their functions contributes to understanding the repertoire of environmental fitness genes in the *F. verticillioides* genome.

Transcriptomic analyses provide a global snapshot of cell processes, potentially describing mechanisms underlying observed phenotypes while providing support and direction for further avenues of investigation. In the described work, RNA-sequencing revealed the global response prompted by microbial antagonism against *F. verticillioides*, a known endophyte and mycotoxigenic pathogen of maize. Curation of lipopeptide-responsive genes enables focused analysis on processes determining *F. verticillioides* survival in the phyllosphere. These processes predictably include stress responsive genes and secondary metabolic pathways, and these data also corroborate apoptosis as a contributor to biocontrol activity. The overlap among genes involved in both antagonism and maize pathogenesis suggests novel targets for management approaches.

Ongoing experiments, guided by functional annotation, focus on deletion of genes likely significant in perception and response to lipopeptide antagonism, specifically structural proteins and a secreted LysM effector potentially responsible for evasion of chitinase activity (Appendix 2). Generation and characterization of these deletants will assist in describing genes essential to *F. verticillioides* success as a pathogen and general maize inhabitant. Deletion targets also

include transcription factors and genes encoding chromatin-modifying enzymes, which may identify central components of the *F. verticillioides* regulatory network involved in rhizosphere competition as well as implicating epigenetic regulation in the survival strategy of this phytopathogen. Such information will hopefully contribute to foundational knowledge for understanding basic microbial ecology applicable beyond the maize phytobiome.

MATERIALS AND METHODS

Extract prep and culture conditions

Lipopeptide treatments were prepared by drying methanolic *B. mojavensis* RRC101 supernatant extracts and resuspending in $1/5^{th}$ concentration Potato Dextrose Broth, to a final concentration of 200 ppm as determined by LC-MS using lipopeptide standards (LipoFabrik, Villeneuve-d'Ascq, France). *F. verticillioides* strain FRC-M3125 5-day liquid cultures were harvested for conidia via cheesecloth filtration, followed by pelleting and washing with reverse-osmosed H_2O. Conidia were finally suspended at 1.5×10^6 conidia/mL. Five microliters of conidial suspensions were spotted onto the center of Potato Dextrose Agar plates before incubation for 5 days, at 27 °C in the dark. Lipopeptide solutions (100 µL) were applied to colony edges and allowed to dry for five minutes in a laminar flow hood. Cultures were then incubated for six hours in the dark at 27 °C. After incubation, treated colony edges were excised and flash frozen in liquid nitrogen for RNA extraction. Each treatment was composed of three biological replicates.

RNA extraction, library preparation and sequencing

One-hundred milligrams of each homogenized culture sample was extracted for total RNA using the Norgen Biotek plant/fungi total RNA kit and manufacturer's protocol (Thorold, ON, Canada). RNA quality (RIN value > 7.0) was determined via Agilent RNA Nano 6000

Chips on an Agilent 2100 bioanalyzer. Messenger RNA was isolated via poly-T magnetic bead purification before libraries were created using the Illumina TruSeq v2 library kit and manufacturer's protocol. Libraries were indexed via available adapters and quantified via fluorimetry before pooling for sequencing. Paired-end reads (length = 36 bp) were generated by the University of Georgia Genomics Facility using an Illumina NextSeq instrument, with a mean 16.8 million reads per sample (Supplemental Table 4.3).

Sequencing data processing

Reads were uploaded to the University of Georgia Galaxy server for trimming, quality control and analysis. The *Fusarium verticillioides* genome file was downloaded in FASTA format from NCBI (Assembly GCF_000149565.1), and the annotations (GCF_000149555.1_ASM14955v1) in GFF (General Feature Format) format. Reads were mapped to the *F. verticillioides* genome via Tophat (v2.0.14); transcript assembly was conducted through Cufflinks (v2.21) (Supplemental Table 4.4). Cuffdiff (v2.2.1.3) output files were downloaded and analyzed via cummeRbund (v2.13.1) (Goff et al., 2012).

Categorization of genes and FungiDB analysis

Through use of the 'getSig' cummeRbund function, genes significantly differentially expressed over control ($q < 0.05$) were compared across treatments to assess potential synergistic or counteracting effects of lipopeptide classes. Preliminary functional annotation was conducted using the MIPS FunCat server (Ruepp et al., 2004). Genes without signal peptides for secretion or transmembrane domains were submitted for localization prediction via the MultiLoc2 (Blum et al., 2009). Localization enrichment was calculated using Fisher's exact test, based on 15869 total genes (as found in FungiDB), 2821 with signal peptides, 3934 membrane proteins (3044 with transmembrane domains, 890 with signal peptides and transmembrane domains) (Rivals et

al., 2007). Gene Ontology analysis was conducted via the FungiDB platform, under the parameters of "Biological Process" and "Molecular Function" with a significance threshold of $q \leq 0.05$.

ACKNOWLEDGMENTS

This work would not have been possible without the instruction and protocol support of Dr. Evelina Basenko of FungiDB and Dr. Zachary Lewis of the Microbiology department at the University of Georgia. This work was funded by the USDA-ARS.

REFERENCES

Andersson, D.I., and Hughes, D. 2014. Microbiological effects of sublethal levels of antibiotics. Nat Rev Micro 12:465-478.

Armelle, T., Anne, C., Katell, B., Jean-Marie, S., Maria, C.U., and Corinne, B. 2015. Characterization by Tandem Mass Spectrometry of Biologically Active Compounds Produced by Bacillus Strains. Journal of Applied Bioanalysis, Vol 1, Iss 1, Pp 19-25 (2015):19.

Bachmann, B.O. (2014). Stimulating and mapping the responsome to accelerate identification of new natural products from actinomyctes. In Annual Meeting and Exhibition 2014 (July 20-24, 2014) (Simb).

Bacon, C.W., and Hinton, D.M. 2011. In planta reduction of maize seedling stalk lesions by the bacterial endophyte. Canadian Journal Of Microbiology 57:485-492.

Bacon, C.W., Hinton, D.M., and Hinton, A., Jr. 2006. Growth-inhibiting effects of concentrations of fusaric acid on the growth of Bacillus mojavensis and other biocontrol Bacillus species. Journal of Applied Microbiology 100:185-194.

Basturkmen, M., Xu, J., Shi, M., Loros, J., Nelson, M., Henn, M., Kodira, C., Lennon, N., Green, L., Galagan, J., Birren, B., Dunlap, J., and Sachs, M.S. (2008). Neurospora crassa EST Sequencing.

Blacutt, A., Mitchell, T.R., Bacon, C.W., and Gold, S.E. 2016. Bacillus mojavensis RRC101 lipopeptides provoke physiological and metabolic changes during antagonism against Fusarium verticillioides. Molecular Plant Microbe Interactions.

Blum, T., Briesemeister, S., and Kohlbacher, O. 2009. MultiLoc2: integrating phylogeny and Gene Ontology terms improves subcellular protein localization prediction. BMC bioinformatics 10:274.

Bonmatin, J.-M., Laprevote, O., and Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Combinatorial Chemistry & High Throughput Screening 6:541-556.

Bono, J.L., Jaber, B., Fisher, M.A., Abuodeh, R.O., O'Leary-Jepson, E., Scalarone, G.M., and Smith, L.H., Jr. 2001. Genetic diversity and transcriptional analysis of the bys1 gene from Blastomyces dermatitidis. Mycopathologia 152:113-123.

Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A.E., Zheng, L., Lee, Y., Utterback, T., Smith, S., Feldblyum, T., Glenn, A.E., Plattner, R.D., Kendra, D.F., Town, C.D., and Whitelaw, C.A. 2005. Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus Fusarium verticillioides. Fungal Genetics and Biology 42:848-861.

Deleu, M., Paquot, M., and Nylander, T. 2008. Effect of fengycin, a lipopeptide produced by Bacillus subtilis, on model biomembranes. Biophysical journal 94:2667-2679.

Deshmukh, R., Mathew, A., and Purohit, H.J. 2014. Characterization of antibacterial activity of bikaverin from Fusarium sp. HKF15. Journal of Bioscience and Bioengineering 117:443-448.

Fedorova, N.D., Badger, J.H., Robson, G.D., Wortman, J.R., and Nierman, W.C. 2005. Comparative analysis of programmed cell death pathways in filamentous fungi. BMC Genomics 6:177.

Goff, L.A., Trapnell, C., and Kelley, D. 2012. CummeRbund: visualization and exploration of Cufflinks high-throughput sequencing data. R package version 2.

Gold, S.E., Blacutt, A.A., Meinersmann, R.J., and Bacon, C.W. 2014. Whole-Genome Shotgun Sequence of Bacillus mojavensis Strain RRC101, an Endophytic Bacterium Antagonistic to the Mycotoxigenic Endophytic Fungus Fusarium verticillioides. Genome announcements 2.

Guo, M., Guo, W., Chen, Y., Dong, S., Zhang, X., Zhang, H., Song, W., Wang, W., Wang, Q., Lv, R., Zhang, Z., Wang, Y., and Zheng, X. 2010. The Basic Leucine Zipper Transcription Factor Moatf1 Mediates Oxidative Stress Responses and Is Necessary for Full Virulence of the Rice Blast Fungus Magnaporthe oryzae. Molecular Plant-Microbe Interactions 23:1053-1068.

Han, Q., Wu, F., Wang, X., Qi, H., Shi, L., Ren, A., Liu, Q., Zhao, M., and Tang, C. 2015. The bacterial lipopeptide iturins induce Verticillium dahliae cell death by affecting fungal signalling pathways and mediate plant defence responses involved in pathogen-associated molecular pattern-triggered immunity. Environmental microbiology 17:1166-1188.

Katz, M.E., Braunberger, K., Yi, G., Cooper, S., Nonhebel, H.M., and Gondro, C. 2013. A p53-like transcription factor similar to Ndt80 controls the response to nutrient stress in the filamentous fungus, Aspergillus nidulans. F1000Research 2:72.

Kim, H., and Woloshuk, C.P. 2008. Role of AREA, a regulator of nitrogen metabolism, during colonization of maize kernels and fumonisin biosynthesis in Fusarium verticillioides. Fungal Genetics and Biology 45:947-953.

Kombrink, A., and Thomma, B.P.H.J. 2013. LysM Effectors: Secreted Proteins Supporting Fungal Life. PLOS Pathogens 9:e1003769.

Lee, J.H., Rho, S.B., and Chun, T. 2005. Programmed Cell Death 6 (PDCD6) Protein Interacts with Death-Associated Protein Kinase 1 (DAPk1): Additive Effect on Apoptosis via Caspase-3 Dependent Pathway. Biotechnology Letters 27:1011-1015.

Lee, K., Pan, J.J., and May, G. 2009. Endophytic Fusarium verticillioides reduces disease severity caused by Ustilago maydis on maize. FEMS Microbiology Letters 299:31-37.

Limón, M.C., Rodríguez-Ortiz, R., and Avalos, J. 2010. Bikaverin production and applications. Applied Microbiology and Biotechnology 87:21-29.

Maget-Dana, R., Thimon, L., Peypoux, F., and Ptak, M. 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochimie 74:1047-1051.

Modjtahedi, N., Giordanetto, F., Madeo, F., and Kroemer, G. Apoptosis-inducing factor: vital and lethal. Trends in Cell Biology 16:264-272.

Morgavi, D.P., and Riley, R.T. 2007. Review: An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with Fusarium toxins. Animal Feed Science and Technology 137:201-212.

Naora, H., and Naora, H. 1999. Involvement of ribosomal proteins in regulating cell growth and apoptosis: Translational modulation or recruitment for extraribosomal activity? Immunol Cell Biol 77:197-205.
Nguyen, P.-A., Strub, C., Fontana, A., and Schorr-Galindo, S. 2017. Crop molds and mycotoxins: Alternative management using biocontrol. Biological Control 104:10-27.
Nirmaladevi, D., Venkataramana, M., Chandranayaka, S., Ramesha, A., Jameel, N.M., and Srinivas, C. 2014. Neuroprotective effects of bikaverin on H2O2-induced oxidative stress mediated neuronal damage in SH-SY5Y cell line. Cellular and molecular neurobiology 34:973-985.
Nishikawa, J.L., Boeszoermenyi, A., Vale-Silva, L.A., Torelli, R., Posteraro, B., Sohn, Y.-J., Ji, F., Gelev, V., Sanglard, D., Sanguinetti, M., Sadreyev, R.I., Mukherjee, G., Bhyravabhotla, J., Buhrlage, S.J., Gray, N.S., Wagner, G., Näär, A.M., and Arthanari, H. 2016. Inhibiting Fungal Multidrug Resistance by Disrupting an Activator-Mediator Interaction. Nature 530:485-489.
Paoletti, M., and Clavé, C. 2007. The Fungus-Specific HET Domain Mediates Programmed Cell Death in Podospora anserina. Eukaryotic Cell 6:2001-2008.
Patel, H., Huynh, Q., Bärlehner, D., and Heerklotz, H. 2014. Additive and Synergistic Membrane Permeabilization by Antimicrobial (Lipo)Peptides and Detergents. Biophysical journal 106:2115-2125.
Rivals, I., Personnaz, L., Taing, L., and Potier, M.-C. 2007. Enrichment or depletion of a GO category within a class of genes: which test? Bioinformatics 23:401-407.
Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Guldener, U., Mannhaupt, G., Munsterkotter, M., and Mewes, H.W. 2004. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic acids research 32:5539-5545.
Saupe, S.J. 2000. Molecular Genetics of Heterokaryon Incompatibility in Filamentous Ascomycetes. Microbiology and Molecular Biology Reviews 64:489-502.
Snook, M.E., Mitchell, T., Hinton, D.M., and Bacon, C.W. 2009. Isolation and characterization of leu7-surfactin from the endophytic bacterium Bacillus mojavensis RRC 101, a biocontrol agent for Fusarium verticillioides. Journal of agricultural and food chemistry 57:4287-4292.
Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. 2011. REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. PLOS ONE 6:e21800.
Svendsen, M., Luelling, S., Adpressa, D., Godbey, A., Belizi, P., Carlson, C., Loesgen, S., Freitag, M., Smith, K., and Gautschi, J. 2016. Development of a Antibacterial Disc Diffusion Assay to Differentiate Fusarin C from Non-Fusarin C activity in Early Screening of Mutant Fusarium graminearum Extracts.
Wissing, S., Ludovico, P., Herker, E., Büttner, S., Engelhardt, S.M., Decker, T., Link, A., Proksch, A., Rodrigues, F., and Corte-Real, M. 2004. An AIF orthologue regulates apoptosis in yeast. The Journal of cell biology 166:969-974.
Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R.L., Munkvold, G.P., Paul, P., Payne, G., and Takle, E.S. 2011. Climate change impacts on mycotoxin risks in US maize. World Mycotoxin Journal 4:79-93.
Zhou, X., Liao, W.-J., Liao, J.-M., Liao, P., and Lu, H. 2015. Ribosomal proteins: functions beyond the ribosome. Journal of Molecular Cell Biology 7:92-104.

Table 4.1. MIPS functional categorization reveal differential trends in genes responsive to specific lipopeptide classes

Functional Category	Fengycin		Synergism		Surfactin	
	Upregulated (157)[x]	Downregulated (30)	Upregulated (148)	Downregulated (91)	Upregulated (63)	Downregulated (18)
01 Metabolism	**28**[y]	**8**	**22**	27	**20**	**6**
02 Energy	3	1		**9**	**6**	2
10 Cell cycle and DNA processing	4		4	5	2	
11 Transcription	2		3	6	3	
12 Protein synthesis	2		**13**[z]			1
14 Protein fate (folding, modification, destination)	**13**		**12**	5	**7**	1
16 Protein with binding function or cofactor requirement	**21**	4	**32**	16	**19**	**4**
18 Regulation of metabolism and protein function	3		**4**	2	**4**	1
20 Cellular transport, transport facilities and routes	**16**	5	12	**16**	**15**	1
30 Cellular communication/signal transduction	**5**		**5**	**5**	**5**	2
32 Cell rescue, defense and virulence	13	**4**	**16**	**16**	**12**	**1**
34 Interaction with the environment	8	**1**	7	**7**	**9**	
36 Systemic interaction with the environment			**2**		1	
38 Transposable elements, viral and plasmid proteins	**1**					
40 Cell fate	6		4		4	
42 Biogenesis of cellular components	13		7	3	5	**1**
43 Cell type differentiation	4		3	**2**	**3**	
99 Unclassified proteins	**89**	19	65	35	24	9

[x] Numbers in parenthesis are total unique genes for a given condition.

[y] Bold numbers indicate subcategories enriched in lipopeptide response as compared to the *F. verticillioides* genome, via Fisher's

exact test.

[z] Underlined numbers are for categories determined by MIPS FunCat to be statistically enriched under lipopeptide response.

Table 4.2. Gene ontology classifications for genes induced by lipopeptide challenge, as abbreviated by REVIGO

Term ID	GO Term (Function)	Genes
Fengycin		
GO:0003674	Molecular_function	60
GO:0043168	Anion binding	14
GO:0036094	Small molecule binding	14
GO:0070011	Peptidase activity, acting on L-amino acid peptides	7
GO:0008233	Peptidase activity	7
GO:0031177	Phosphopantetheine binding	2
GO:0072341	Modified amino acid binding	2
GO:0033218	Amide binding	2
GO:0015095	Magnesium ion transmembrane transporter activity	1
GO:0016639	Oxidoreductase, acting on CH-NH2 donor, NAD/NADP acceptor	1
GO:0003989	Acetyl-coa carboxylase activity	1
GO:0005544	Calcium-dependent phospholipid binding	1
GO:0008810	Cellulase activity	1
GO:0004075	Biotin carboxylase activity	1
GO:0016885	Ligase activity, forming carbon-carbon bonds	1
GO:0004198	Calcium-dependent cysteine-type endopeptidase activity	1
Surfactin		
GO:0003824	Catalytic activity	25
GO:0016491	Oxidoreductase activity	13
GO:0004871	Signal transducer activity	5
GO:0050662	Coenzyme binding	5
GO:0060089	Molecular transducer activity	5
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	4
GO:0016209	Antioxidant activity	3
GO:0004601	Peroxidase activity	3
GO:0016684	Oxidoreductase activity, acting on peroxide as acceptor	3
GO:0008236	Serine-type peptidase activity	2
GO:0016830	Carbon-carbon lyase activity	2
GO:0017171	Serine hydrolase activity	2
GO:0016872	Intramolecular lyase activity	1
GO:0004506	Squalene monooxygenase activity	1
Synergism		
GO:0003735	Structural constituent of ribosome	13
GO:0005198	Structural molecule activity	13
GO:0070011	Peptidase activity, acting on L-amino acid peptides	5
GO:0008233	Peptidase activity	5

GO:0017171	Serine hydrolase activity	3
GO:0045482	Trichodiene synthase activity	1
GO:0004360	Glutamine-fructose-6-phosphate transaminase activity	1

Table 4.3. Lipopeptide challenge induces PKS genes corresponding to secondary metabolites in *Fusarium verticillioides*

		log2(fold change over control)		
Gene ID	**Product**	**Fengycin**	**Surfactin**	**Combined**
FVEG_03379	Bikaverin	0.80	0.66	1.29
FVEG_11086	Fusarin C	1.19	ns*	0.74
FVEG_11932	Unknown	1.20	ns	ns
FVEG_12523	Fusaric acid	1.20	ns	0.45
FVEG_00316	Fumonisin	0.62	ns	ns
FVEG_05537	Unknown	ns	ns	1.04

*ns = Not significantly differentially expressed.

Table 4.4. Lipopeptide-responsive genes with predicted functions found in the FvI EST library[x]

	Gene ID	Annotation	log_2(fold change)
	FVEG_01545	Major Facilitator Superfamily	0.660139
	FVEG_01656	**Glutamate carboxypeptidase**	1.20388
	FVEG_03005	**Cerato-platanin**	1.37928
	FVEG_03607	Proline dehydrogenase	0.75226
	FVEG_03831	bZIP transcription factor	0.845299
	FVEG_03910	WW domain, protein binding	0.537912
	FVEG_06203	**Putative cell wall mannoprotein**	1.43445
	FVEG_06844	Putative cell surface protein	0.72481
Fengycin	FVEG_07480	Lipid binding protein, DUF500	1.41616
	FVEG_08403	**Eukaryotic aspartyl protease**	0.631619
	FVEG_08821	**Glycosyl hydrolase family 61**	1.01654
	FVEG_09406	**putative BYS1**	1.59814
	FVEG_10258	**Cell wall protein phiA**[y]	2.48641
	FVEG_10303	Bacterial NAD-glutamate dehydrogenase	0.836384
	FVEG_10818	Annexin	1.77763
	FVEG_11282	4F5 protein family	0.718699
	FVEG_12879	**Putative phosphatidylinositol phosphate kinase**	1.86405
	FVEG_00381	Ribosomal protein S21e	0.527629
	FVEG_00566	S8e: ribosomal protein S8.e	0.586556
	FVEG_00851	Ribosomal protein S19	0.638235
Synergism	FVEG_00904	Programmed cell death protein 6	0.499845
	FVEG_01326	Glucosamine-fructose-6-phosphate aminotransferase	0.418887
	FVEG_01770	**Type C endoglucanase**	-inf
	FVEG_02245	ATP synthase D chain, mitochondrial (ATP5H)	0.412382

	FVEG_02859	**Putative endoglucanase**	0.713294
	FVEG_04071	**Common central domain of tyrosinase**	0.45377
	FVEG_04370	Carbon-nitrogen hydrolase	0.661581
	FVEG_04403	Dynamin family	0.455198
	FVEG_04675	**Putative hydrolase**	0.420521
	FVEG_04843	**CFEM domain - putatively involved in pathogenesis**	0.698923
	FVEG_05516	Acetyltransferase (GNAT) domain	0.511719
	FVEG_06552	**Amidase**	0.62694
	FVEG_06822	**Polysaccharide deacetylase involved in osmoregulation**	2.21973
	FVEG_07030	Ribosomal protein S10p/S20e	0.57346
	FVEG_07194	bZIP transcription factor JlbA, responsive to starvation	1.54344
	FVEG_07976	Ribosomal protein L4/L1 family	0.483898
	FVEG_07980	Ribosomal protein S14p/S29e	0.66802
	FVEG_08105	Chitin synthesis regulation, resistance to congo red	0.79038
	FVEG_09773	Amino acid permease	0.840669
	FVEG_09796	Serine hydrolase (FSH1)	0.876595
	FVEG_09934	Glycosyl hydrolase family 20, catalytic domain	0.491788
	FVEG_10117	**0048419 Growth Factor Receptor domain**	0.708883
	FVEG_10294	RanBP1 domain	0.462164
	FVEG_10863	**Peptidase inhibitor I9**	1.09508
	FVEG_11169	Ribosomal protein S24e	0.669947
	FVEG_12201	**Domain of unknown function (DUF4267)**	1.35235
	FVEG_12280	**Trypsin-like peptidase domain**	0.496357
	FVEG_13030	ATPase family associated with various cellular activities (AAA)	0.713554
	FVEG_13197	Putative HET-E-1	0.668595
	FVEG_14112	CVNH domain - involved in pathogenesis	0.558782
	FVEG_16675	Heterokaryon incompatibility protein (HET)	1.73185
Surfactin	FVEG_00098	Semialdehyde dehydrogenase, NAD binding domain	0.84792

FVEG_00225	NADH(P)-binding	0.664437
FVEG_02717	Putative cell surface protein involved in stress response	1.14133
FVEG_04017	Putative cell surface protein	0.725479
FVEG_04168	Protein kinase domain	1.17933
FVEG_07303	PAS fold	1.63677
FVEG_07427	Aldehyde dehydrogenase family	0.505239
FVEG_08809	Major Facilitator Superfamily	2.1524
FVEG_09294	Cytochrome P450, putative oxygenase	0.882892
FVEG_10078	Hemerythrin HHE cation binding domain	1.38799
FVEG_11286	C2 domain	0.912736
FVEG_11955	Catalase-related immune-responsive	0.453002
FVEG_12768	GMC oxidoreductase	1.84115
FVEG_13102	short chain dehydrogenase	0.837966
FVEG_13198	Caleosin related protein	0.656462
FVEG_13630	**Fungalysin/Thermolysin Propeptide Motif**	0.570278

[x] Expressed during colonization of maize roots and shoots

[y] Proteins in bold are predicted to be secreted.

Table 4.5. Lipopeptide-responsive genes found in the FvJ EST library[x]

	GeneID	Annotation	log_2(fold change)
Fengycin	FVEG_00611	Uncharacterized hypothetical protein	-0.571
	FVEG_03005	**Cerato-platanin**	1.37928
	FVEG_03433	**BYS1 function unknown, involved in pathogenicity**[y]	1.21124
Synergism	FVEG_00321	Fatty acid coA ligase, FUM10	-0.6495
	FVEG_11387	Uncharacterized hypothetical protein	-0.5217
	FVEG_15889	Protein kinase domain	0.71057

[x] EST library containing genes expressed during maize kernel colonization.

[y] Genes in bold are predicted to encode secreted proteins.

Table 4.6. Lipopeptide challenge induces genes involved in a putative conserved stress 'responsome' in *Fusarium verticillioides*

			***N. crassa* stressor**[x]				
	Gene ID	**Annotation**	**Oxidative**	**Osmotic**	**Heat**	**Nitrogen**	**Glucose**
	FVEG_00522	ATPase family associated with various cellular activities (AAA)			X		
	FVEG_01031	Copper/zinc superoxide dismutase (SODC)	X	X		X	X
	FVEG_01712	Metal transporter, similar to Cu^{+} resistance protein		X	X	X	
	FVEG_02548	Involucrin-like, Med15 signal transducer domain	X	X	X		X
	FVEG_03607	Proline dehydrogenase	X	X		X	X
Fengycin	FVEG_03831	bZIP transcription factor	X	X		X	X
(8.02%)[y]	FVEG_03910	WW domain	X	X	X		X
	FVEG_05556	ATP-grasp domain		X			
	FVEG_06203	Putative cell wall mannoprotein	X				
	FVEG_06789	Ankyrin repeat	X				
	FVEG_06844	SH3 domain, putative cytoskeleton protein	X	X	X		X
	FVEG_07105	MIF4G domain	X	X	X		
	FVEG_09652	Calpain family cysteine protease	X			X	
	FVEG_09802	SPFH domain / Band 7 family (flotillin)	X		X		X
	FVEG_12969	Protein of unknown function (DUF1399)				X	
		Fengycin totals (out of 15)	**11**	**9**	**7**	**6**	**7**
	FVEG_00566	**S8e: ribosomal protein S8e** [z]	X	X	X	X	X
	FVEG_00851	**Ribosomal protein S19**	X	X	X	X	X
	FVEG_00904	**Programmed cell death protein 6**	X	X	X	X	X
	FVEG_01326	**SIS domain**	X	X	X	X	X
	FVEG_01367	**Ribosomal protein L36e**	X	X	X	X	X
	FVEG_01392	Ribosomal protein S28e	X	X	X	X	
	FVEG_02245	**ATP synthase D chain, mitochondrial (ATP5H)**	X	X	X	X	X

	FVEG_04370	Carbon-nitrogen hydrolase	X	X	X		X
Synergism	**FVEG_04519**	**Ribosomal L38e protein family**	X	X	X	X	X
(9.21%)	FVEG_04675	Putative hydrolase	X	X	X	X	
	FVEG_05220	UBA/TS-N domain	X	X			X
	FVEG_05445	Large tegument protein domain PHA03247	X	X			
	FVEG_06021	**Thiazole biosynthesis protein ThiG**	X	X	X	X	X
	FVEG_07976	**Ribosomal protein L4/L1 family**	X	X	X	X	X
	FVEG_07980	**Ribosomal protein S14p/S29e**	X	X	X	X	X
	FVEG_09564	**Ribosomal protein S5, C-terminal domain**	X	X	X	X	X
	FVEG_09801	**Uncharacterized hypothetical protein**	X	X	X	X	X
	FVEG_10294	**RanBP1 domain, regulator of RCC1 chromatin regulator**	X	X	X	X	X
	FVEG_11169	**Ribosomal protein S24e**	X	X	X	X	X
	FVEG_13030	ATPase family associated with various cellular activities (AAA)	X	X		X	X
	FVEG_15956	Protein tyrosine kinase	X	X	X		
	FVEG_15957	Aurora kinase ARK1, regulator of cell shape	X	X	X		
		Synergism totals (out of 22)	**22**	**22**	**19**	**17**	**17**
	FVEG_02123	Serine carboxypeptidase				X	
	FVEG_02125	PAS domain S-box protein	X	X		X	X
	FVEG_02248	Uncharacterized hypothetical protein		X	X	X	
	FVEG_02717	Putative cell surface protein involved in stress response		X	X		
	FVEG_04168	Protein kinase domain	X	X		X	
Surfactin	FVEG_07133	Uncharacterized hypothetical protein		X	X	X	
(14.81%)	FVEG_07303	PAS fold					X
	FVEG_10078	Hemerythrin HHE cation binding domain			X		
	FVEG_10672	FMN-dependent dehydrogenase	X				
	FVEG_10717	Thi4 family				X	X
	FVEG_10718	CarR_dom_SF: lycopene cyclase domain	X				

FVEG_13102	Short chain dehydrogenase		X			
Surfactin totals (out of 12)		**4**	**6**	**4**	**6**	**3**

[x] *F. verticillioides* orthologs for *N. crassa* stress-responsive genes were identified via FungiDB's transformation by orthology tool, limited to syntenic orthologs to increase stringency.

[y] Parentheses beneath each group name represent the portion putative stress-response genes comprise of the total curated group.

z Bolded genes are proposed to comprise part of the *F. verticillioides* responsome.

Table 4.7. Lipopeptide antagonism induces genes in *Fusarium verticillioides* predicted to be involved in apoptosis

Group	Gene ID	Gene annotation	log2(fold change)
Fengycin	FVEG_01031	Cu/Zn Superoxide dismutase	0.63478
	FVEG_03347	Lipoxygenase	1.56172
	FVEG_05861	Glycine rich, putative stress response (DUF1399)	2.08665
	FVEG_05888	Heterokaryon incompatibility protein (HET)	2.58291
	FVEG_07105	MIF4G domain	0.8171
	FVEG_08793	Ankyrin repeat-containing protein	0.97638
	FVEG_09652	Calpain family cysteine protease	1.54385
	FVEG_11221	Peroxidase	2.21633
	FVEG_11872	Heterokaryon incompatibility protein (HET)	0.98093
	FVEG_11981	Glutathione S-transferase, N-terminal domain (detoxification)	2.19056
	FVEG_17301	Heterokaryon incompatibility protein (HET)	2.50609
Synergism	FVEG_00851	Ribosomal protein S19 - derepressor of PCD	0.63824
	FVEG_00904	Programmed cell death protein 6	0.49985
	FVEG_07194	bZIP transcription factor JlbA	1.54344
	FVEG_07980	Ribosomal protein S14p/S29e - activates p53	0.66802
	FVEG_11822	Heterokaryon incompatibility protein (HET)	2.11144
	FVEG_13197	Putative HET-E-1	0.6686
	FVEG_13265	Heterokaryon incompatibility protein (HET)	inf
	FVEG_16329	Heterokaryon incompatibility protein (HET)	1.76645
	FVEG_16665	Heterokaryon incompatibility protein (HET)	1.96485
	FVEG_16675	Heterokaryon incompatibility protein (HET)	1.73185
	FVEG_16844	Heterokaryon incompatibility protein (HET)	1.14488

FVEG_17256	Heterokaryon incompatibility protein (HET)	1.72683
FVEG_17421	Heterokaryon incompatibility protein (HET)	0.98182

Figure 4.1. Venn diagram illustrating the overlap of genes (prior to curation) differentially expressed (up- and downregulated) in response to fengycin, surfactin and combined lipopeptide challenges. Numbers indicate genes determined to be significantly differentially expressed in respective pairwise comparisons.

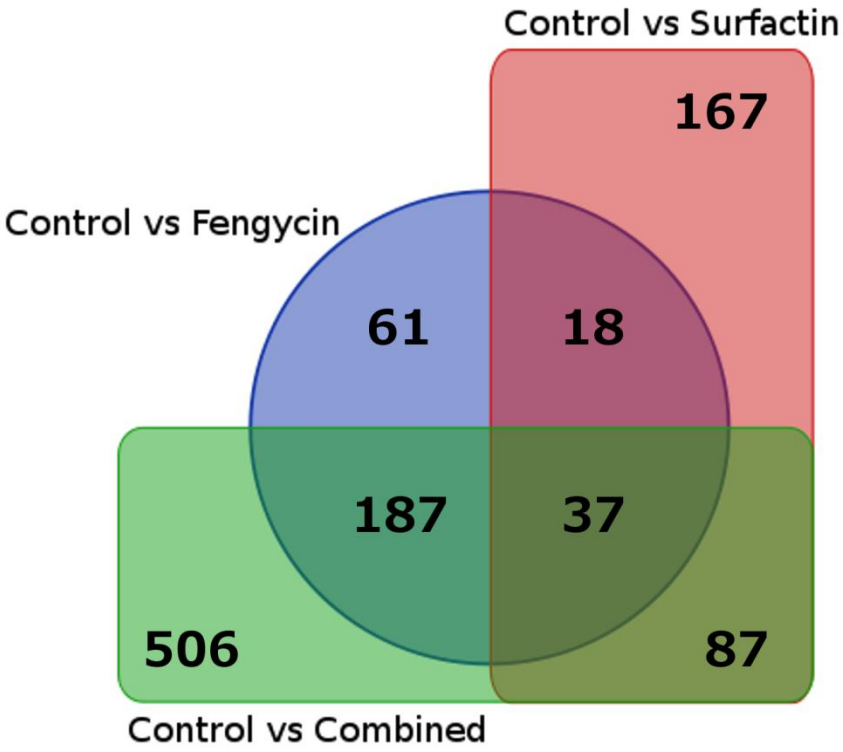
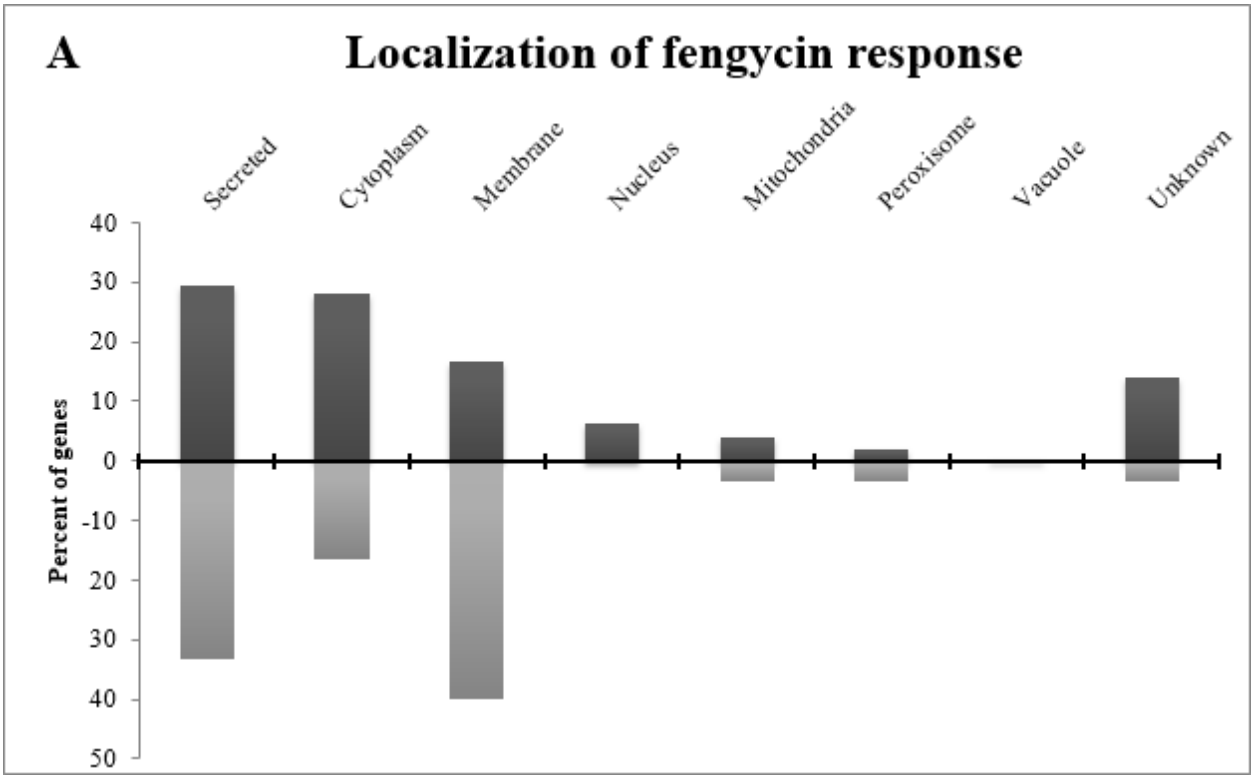
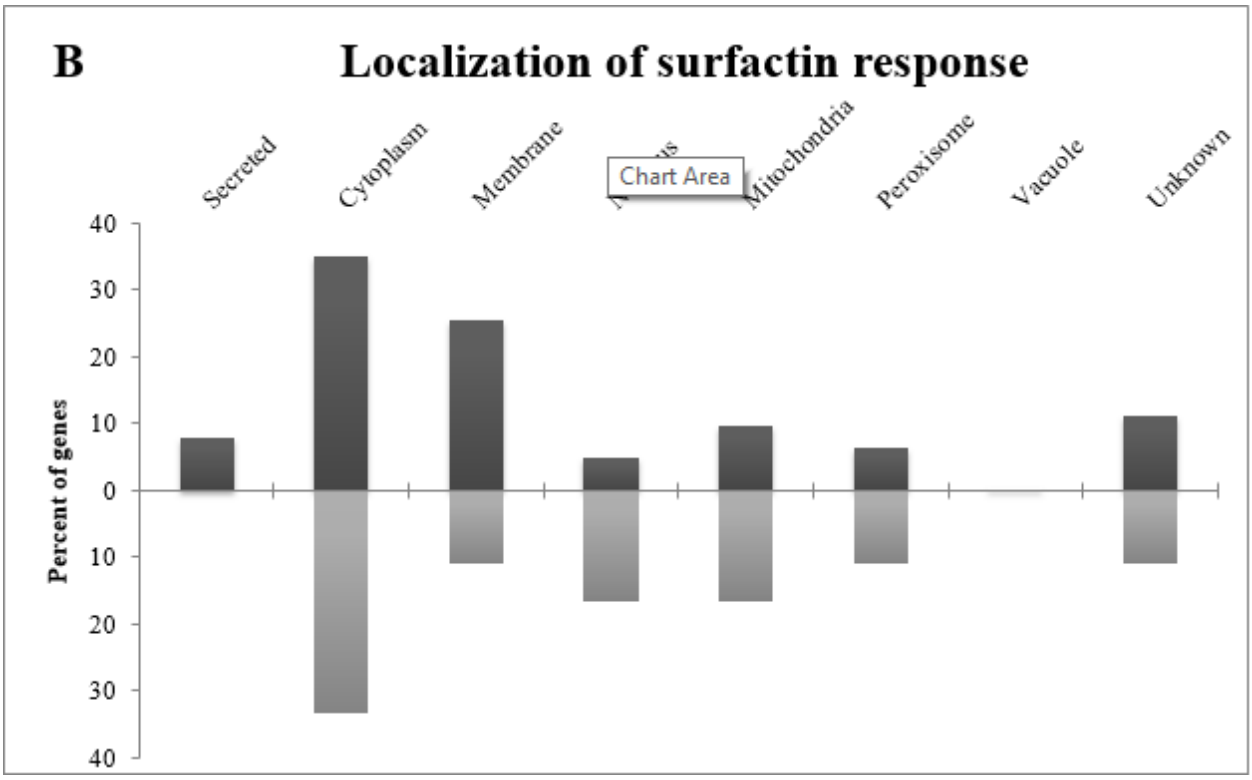

Control vs Surfactin
167
Control vs Fengycin
61
18
187
37
506
87
Control vs Combined

Figure 4.2. Lipopeptide response in *Fusarium verticillioides* varies in putative localization of A) Fengycin-, B) Surfactin-, and C) Synergism responsive gene products. Dark bars indicate percentage of total genes upregulated. Light bars represent percentage of total downregulated genes.


A
Localization of fengycin response
Secreted
Cytoplasm
Membrane
Nucleus
Mitochondria
Peroxisome
Vacuole
Unknown
Percent of genes
40
30
20
10
0
-10
20
30
40
50


B
Localization of surfactin response
Secreted
Cytoplasm
Membrane
Chart Area
Mitochondria
Peroxisome
Vacuole
Unknown
Percent of genes
40
30
20
10
0
10
20
30
40

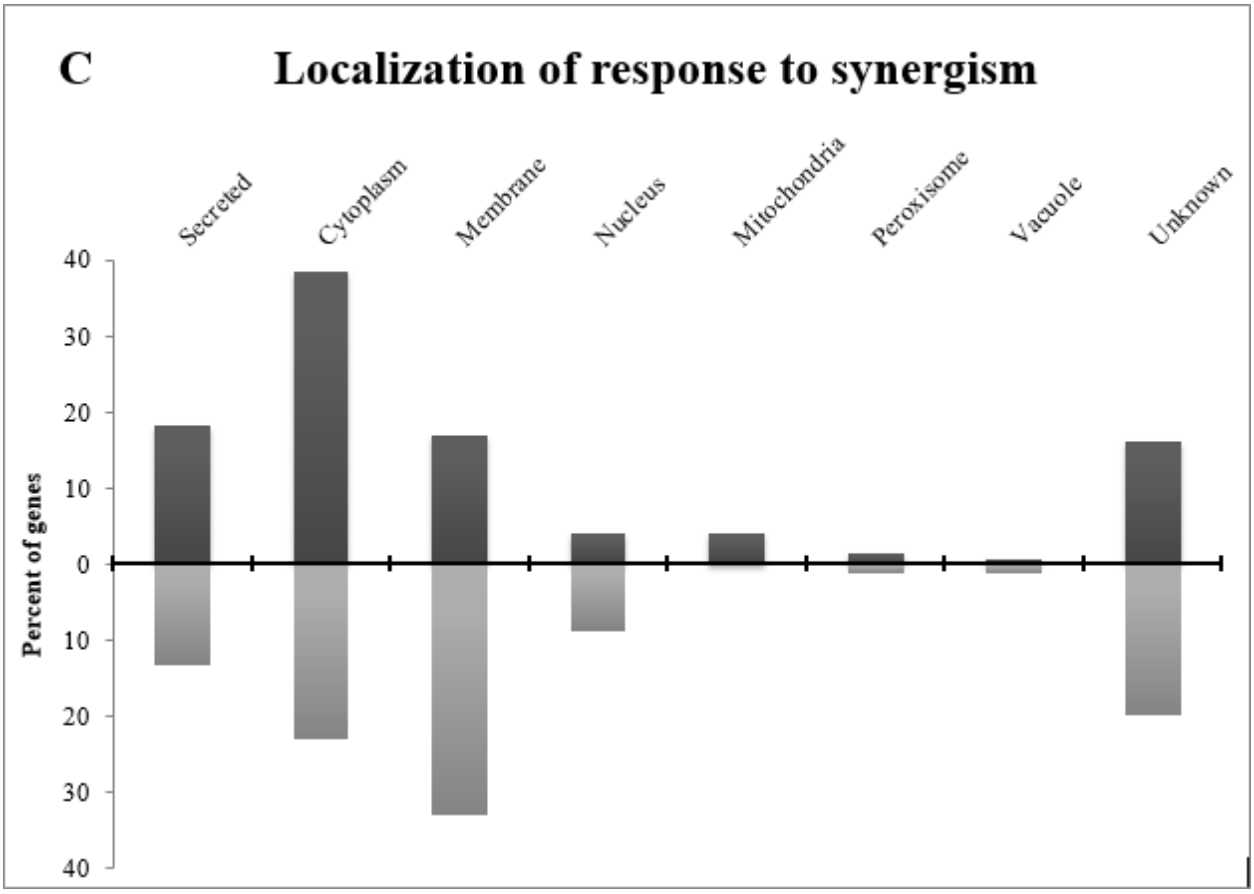
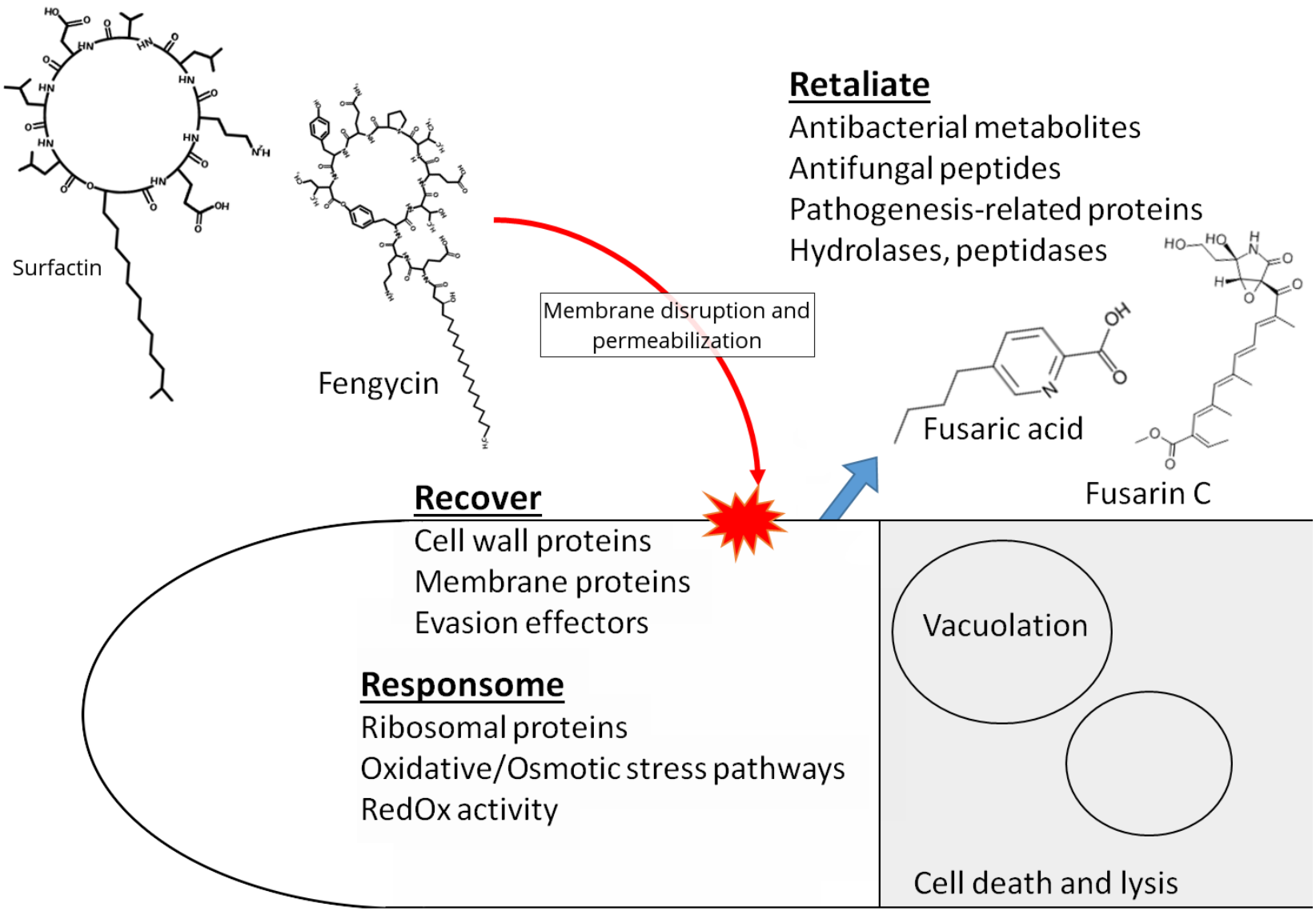

C
Localization of response to synergism
Secreted
Cytoplasm
Membrane
Nucleus
Mitochondria
Peroxisome
Vacuole
Unknown
Percent of genes
40
30
20
10
0
10
20
30
40

Figure 4.3. A graphical representation of the "Recover and Retaliate" model of cLP antagonism response in *Fusarium verticillioides*. Predicted gene functions upregulated under fengycin antagonism are listed under their respective categories, with 'Recover' referring to genes with products acting on structural components, and 'Retaliate' referring to secreted compounds likely involved in counter-antagonism and pathogenesis. The responsome refers to the putative core stress response genes as described through comparison to *Neurospora crassa* EST data. The antagonism phenotype of cell death following vacuolation is included as well (shaded compartment).


Surfactin
Fengycin
Retaliate
Antibacterial metabolites
Antifungal peptides
Pathogenesis-related proteins
Hydrolases, peptidases
Membrane disruption and permeabilization
Fusaric acid
Fusarin C
Recover
Cell wall proteins
Membrane proteins
Evasion effectors
Responsome
Ribosomal proteins
Oxidative/Osmotic stress pathways
RedOx activity
Vacuolation
Cell death and lysis

Supplemental Table 4.1. EST libraries used in analysis of lipopeptide responsive genes in *Fusarium verticillioides*

Library	Details	Reference
FvI	Genes expressed during colonization of maize shoots and roots	(Brown et al., 2005)
FvJ	Genes expressed during colonization of maize kernels	
FvKL	Subtraction library, responsive to maize phytochemicals, specifically BOA	
Oxidative stress	*N. crassa* cultures one hour after exposure to oxidative stress	(Basturkmen et al., 2008)
Osmotic stress	*N. crassa* cultures one hour after growth in 0.68M NaCl	
Heat stress	*N. crassa* cultures after one hour growth at 45 °C	
Glucose deprivation	*N. crassa* after one hour growth in glucose-free medium	
Nitrogen deprivation	*N. crassa* after one hour growth in nitrogen-deprived medium	

Suppplemental Table 4.2. Cuffdiff parameter summary exported from the UGA Galaxy server

Parameter	Setting
Library normalization method	geometric
Dispersion estimation method	per-condition
False Discovery Rate	0.05
Min Alignment Count	7
Use multi-read correct	TRUE
Perform Bias Correction	Yes
Reference sequence data	history
Using reference file	GCA_000149555.1_ASM14955v1_genomic.fna
Include Read Group Datasets	Yes
Include Count Based output files	Yes
apply length correction	cufflinks effective length correction
Additional Parameters for single end reads?	No
Set Advanced Cuffdiff parameters?	No

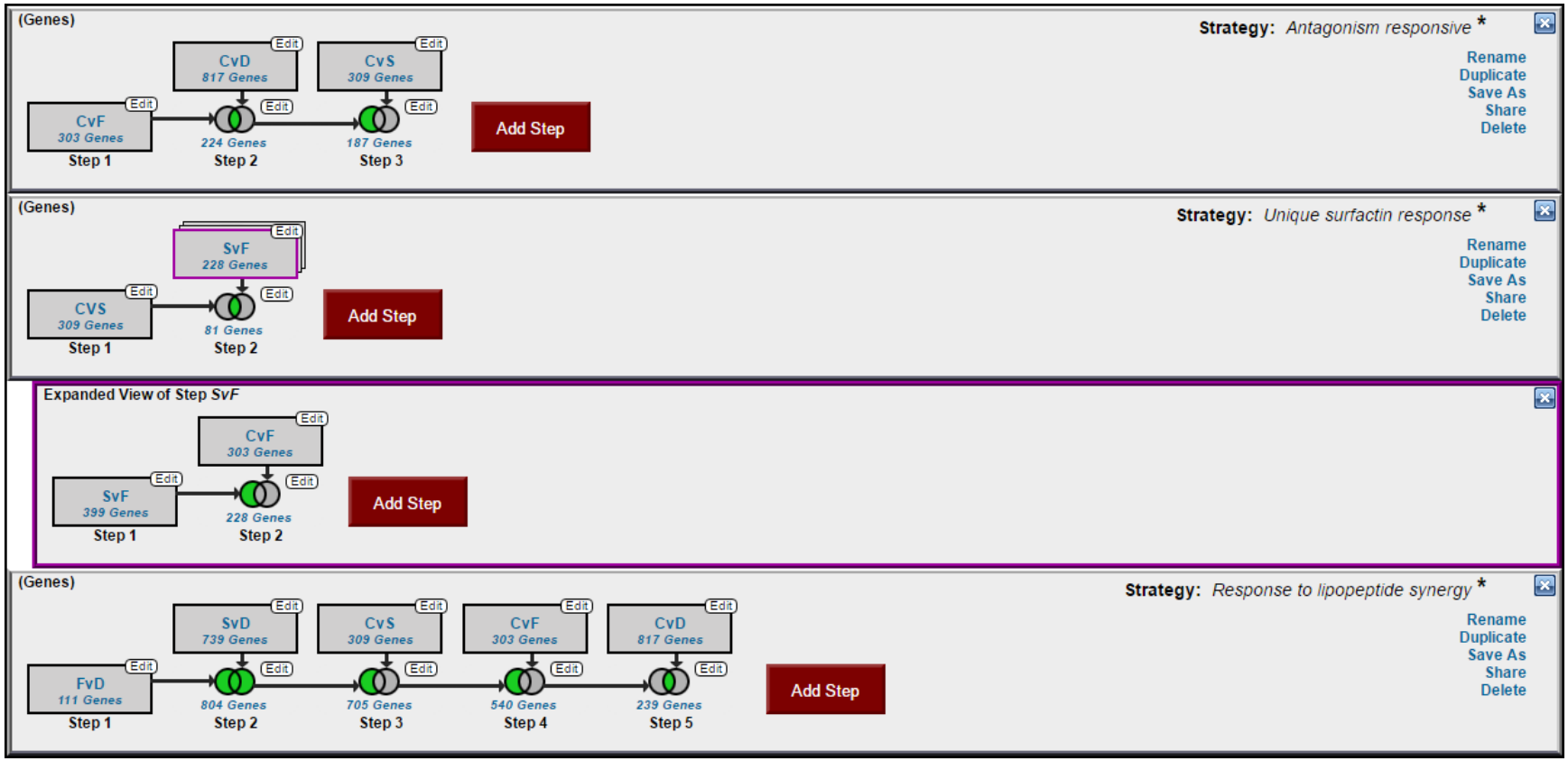
Supplemental Table 4.3. Read information from the cuffdiff output file "read_groups.info"

Condition	Replicate	Total reads	Internal scale
Control	0	1.28E+07	0.852059
Control	1	1.54E+07	1.03167
Control	2	1.66E+07	1.14785
Surfactin	0	1.13E+07	0.651169
Surfactin	1	1.51E+07	0.917635
Surfactin	2	2.33E+07	1.59324
Fengycin	0	9.13E+06	0.438824
Fengycin	1	2.27E+07	1.41889
Fengycin	2	1.70E+07	1.00528
Combined	0	2.38E+07	1.46371
Combined	1	1.84E+07	1.20718
Combined	2	1.64E+07	1.03447

Supplemental Table 4.4. Lipopeptide responsive hypothetical proteins found in the FvI EST library

Fengycin	Surfactin	Synergy
FVEG_03080	FVEG_08139	FVEG_03069
FVEG_03081		**FVEG_03305**
FVEG_04051		**FVEG_05442**
FVEG_06335		FVEG_05579
FVEG_05862		FVEG_06460
FVEG_11065		FVEG_07424
FVEG_08575		FVEG_16568
FVEG_04758		
FVEG_12191		
FVEG_02548		
FVEG_10770		
FVEG_00611		

Supplemental Figure 4.1. FungiDB strategies used for curation of gene lists for downstream analysis. Treatments are referred to by single letter symbols, where C = control, S = Surfactin, F = Fengycin and D = Combined. Gene lists are based on differential response between treatments, where FvD represents genes determined by 'getSig' in cummeRbund to be differentially expressed between fengycin and combined treatment.


(Genes)
CvD
817 Genes
CvS
309 Genes
CvF
303 Genes
224 Genes
187 Genes
Step 1
Step 2
Step 3
Add Step
Strategy: Antagonism responsive *
Rename
Duplicate
Save As
Share
Delete
(Genes)
SvF
228 Genes
CVS
309 Genes
81 Genes
Step 1
Step 2
Add Step
Strategy: Unique surfactin response *
Rename
Duplicate
Save As
Share
Delete
Expanded View of Step SvF
CvF
303 Genes
SvF
399 Genes
228 Genes
Step 1
Step 2
Add Step
(Genes)
SvD
739 Genes
CvS
309 Genes
CvF
303 Genes
CvD
817 Genes
FvD
111 Genes
804 Genes
705 Genes
540 Genes
239 Genes
Step 1
Step 2
Step 3
Step 4
Step 5
Add Step
Strategy: Response to lipopeptide synergy *
Rename
Duplicate
Save As
Share
Delete

CHAPTER 5

CONCLUSIONS

The history of developing biological control agents (BCAs) is fraught with examples that promised early results that then failed to maintain efficacy upon scaling to the less-controlled conditions of the greenhouse or field. Given these challenges, it may be useful to focus less on any specific agent and more on the basic mechanisms underlying successful biological control events. These data can then be leveraged for future development and deployment of BCAs, through improvements in screening and selection, or the more direct route of genetic transformation to literally build a better BCA. The work detailed in this dissertation has thus approached the biocontrol interaction between *B. mojavensis* RRC 101 and *F. verticillioides* with a focus on identifying elements responsible for the interaction as well as the consequences of antagonism. This interaction has been characterized via microscopic, biochemical and transcriptomic analyses.

Building on advancements in affordability and accessibility of -omics technologies, the phytobiomes initiative led by the American Phytopathological Society focuses on knowledge gaps in plant-microbe systems (Leach and APS, 2015). Many of these gaps are specifically applicable to biocontrol interactions, such as those involving phytobiome community interactions and their underlying mechanisms. Initial reductionist experiments that focused on simplification of microbial interactions such as detailing the effects of individual lipopeptide classes and their susceptible targets, are thus useful in filling gaps towards understanding the

complexities of plant-associated microbial interactions overall. These data can be bolstered through incorporating results from experiments in other systems through sequencing repositories.

Transcriptomic analyses facilitate the investigation of phytopathogenically-relevant systems through generating massive datasets. With these data come the inherent challenge of determining significance within a biological system. Biological significance is approximated in this work via direct and indirect comparisons to datasets available through FungiDB, a web-based repository and analysis platform. Specifically, comparing lipopeptide-responsive genes to orthologous stress response genes in *Neurospora crassa*, as well as those involved with maize colonization in *F. verticillioides* confer increased value to all data sets involved. Such data analyses outline basic networks of multifunctional genes as well as generating circumstantial data for which hypotheses can be generated and tested. Through such tools, description and analysis of transcriptomic data becomes streamlined and more accessible for future correlational meta-analysis. Results from those studies, such as the putative identification of the *F. verticillioides* responsome, can then be used to guide experimental design with the goal of confirming and characterizing these biological phenomena.

The work described in this dissertation details the interaction between *Fusarium verticillioides* and one isolate of *Bacillus mojavensis*, RRC101. However, through genomic description of RRC101 and the use of lipopeptide extracts in antagonism assays, these data can be applied across systems in a consistent manner. As illustrated, transcriptional data can be transformed to identify targets in other significant fungi, be they pathogens of plants or animals. Further, the identification of ribosomal proteins as putatively involved in stress responses and apoptosis in *F. verticillioides* suggests a potential system to model cell processes relevant to human diseases. To support this assertion, iturins and fengycins have both been demonstrated to

inhibit tumor cells through oxidative stress and induction of apoptosis, the same mechanisms identified in fungi, including the work described here with *F. verticillioides* (Yin et al., 2013; Dey et al., 2015).

As cyclic lipopeptides are antibiotics commonly produced by members of the genera *Bacillus* and *Pseudomonas*, their mechanisms of action should remain consistent across pathosystems, dependent on specific membrane/lipopeptide class interactions, as previously observed. Within lipopeptide classes, differential activity has been confirmed for isoforms, suggesting that the biosynthetic idiosyncracies of a given strain may confer an advantage in antagonism, with practical consequences for BCA development. As the genus *Bacillus* is home to many proprietary BCAs, descriptions of their mechanisms of action are expected to better inform research and development of more efficacious products, such as those capable of inhibiting a diverse range of pathogens. In turn, these products serve to supplement or ideally supplant traditional chemical management in integrated systems, with an end goal of maximal crop protection at a minimal cost to consumers as well as the environment.

REFERENCES

Dey, G., Bharti, R., Dhanarajan, G., Das, S., Dey, K.K., Kumar, B.N.P., Sen, R., and Mandal, M. 2015. Marine lipopeptide Iturin A inhibits Akt mediated GSK3β and FoxO3a signaling and triggers apoptosis in breast cancer. Scientific Reports 5:10316.

Leach, J.E., and APS, P.P.B. (2015). THE PHYTOBIOMES INITIATIVE. In Plant and Animal Genome XXIII Conference (Plant and Animal Genome).

Yin, H., Guo, C., Wang, Y., Liu, D., Lv, Y., Lv, F., and Lu, Z. 2013. Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. Anti-cancer drugs 24:587-598.

APPENDIX A

EVALUATION OF BIOCONTROL POTENTIAL IN THE *BACILLUS MOJAVENSIS* RRC101 GENOME

Sequencing the *Bacillus mojavensis* genome provides new data relevant to the ongoing task of identifying traits desirable in biological control agents. Genes relevant to biocontrol can be divided into functional categories representative of the requirements for persistent and efficacious disease suppression: environmental adaptation, host interactions and antagonism. Currently, *B. mojavensis* RRC101 remains recalcitrant to transformation, preempting mutagenesis-based analysis. Regardless of current limitations, identification and compilation of genes underpinning these traits can provide foundational data for future development of effective biocontrol strategies. In fact, RRC101 genome sequencing revealed the presence of a fengycin biosynthetic operon, leading to the isolation of these lipopeptides, not surfactins as previously thought, as responsible for antifungal activity against *Fusarium verticillioides*.

The Global Biotic Interactions (GloBI) service collects data regarding biological interactions between species. This collected data is easily visualized, demonstrating the range of plant hosts that *B. mojavensis* is known to colonize (Figure A1.1) (Poelen et al., 2014). This host diversity suggest that *B. mojavensis* possesses a robust repertoire of genes facilitating host colonization. Using sequence data and results of studies in other *Bacillus* species, it is possible to select a list of genes likely involved in rhizosphere competence and the establishment of endophytism (Table A1.1).

Establishment of a Biological Control Agent (BCA) in sufficient population in a host system necessitates rhizosphere competence, mechanisms of which can be hypothesized based

on genome annotation data and experimental data in related systems (Table A1.2). Intuitively, given the nature of the rhizosphere, detoxification of xenobiotics, nutrient acquisition and stress compensation are important gene functions. Oxidative stress, specifically, is encountered in both the soil as well as within plant tissues. RRC101 possesses six catalase genes, and this redundancy is characteristic of symbiotic and pathogenic microbes adapting to rhizosphere and phyllosphere colonization. For example, flavohemoproteins such as the RRC101 nitric oxide dioxygenase are known in other systems to scavenge nitric oxide, and during colonization of a host this activity may work to protect the colonizer from stress as well as modulate host defense activity mediated by NO (Boccara et al., 2005). RRC101 is a confirmed endophyte of maize, implying a proficiency at breaching cell walls and gaining access to the apoplasm. Accordingly, RRC101 possesses genes encoding proteins responsible for hydrolysis of cell wall components, including two putative pectate lyase genes. Similarly, RRC101 possesses a cell wall loosening expansin, which is known to promote maize root colonization in *B. subtilis* (Kerff et al., 2008).

Once the host has been colonized, the *B. mojavensis* RRC101 genome suggests potential for strong plant beneficial activity. Growth promotion via phytohormone production is the most direct form of this beneficial activity, with synthases for 2,3-butanediol and IAA common among plant-associated *Bacillus* species. Other indirect forms of benefit include defense induction mechanisms such as quinolinate synthesis and p-aminobenzoic acid; byproducts from enzymatic degradation of the plant cell wall are also known to induce protective defense pathways. Here, again, lipopeptides contribute to biocontrol activity, and the surfactins are known SAR inducers and can mitigate severity of subsequent pathogen attack.

While the majority of my work focused on the antifungal activity of fengycins, the RRC101 genome suggests antagonistic potential beyond these lipopeptides (Table A1.3).

Production of subtilosin, bacilysin and bacillaene all function as tools of antagonism against differing ranges of Gram-positive rhizobacteria, contributing towards establishment of *B. mojavensis* populations necessary for effective biocontrol. RRC101 possesses a putative quorum quenching lactonase; these enzymes are described in other systems as disrupting quorum sensing of gram negative rhizosphere inhabitants; this function defuses virulence gene regulation by a number of plant pathogens (Chen et al., 2013).

Surfactins are antibacterial and well-characterized as essential for swarming motility and host colonization, serving multiple roles in biological control establishment (Ongena and Jacques, 2008). The surfactin operon for *B. mojavensis* RRC101 is 87% identical to that of *B. subtilis* ssp *spizizenii* w23, suggesting potential diversity in the operons for nonribosomally synthesized metabolites. LC-MS analysis of RRC101 supernatants revealed the presence of less common surfactins, specifically a Val7 surfactin and another putative noncanonical surfactin, supporting the hypothesis that genetic diversity in synthetic operons drives secondary metabolic diversity in *Bacillus* (Figure A1.2). This diversity poses a significant avenue for improvement and development of BCA agents, given that functionality is dictated by the composition of secondary metabolites (Schneider et al., 1998).

Genetic modification presents an opportunity for directed improvement of BCAs; these efforts can be aided through the compilation of genetic diversity at loci directly involved in biocontrol-relevant processes (Schneider et al., 1998; Dietel et al., 2013; Wu et al., 2015). Identifying genes relevant to biocontrol can expedite screening assays and selection. Additionally, data regarding genetic diversity around these loci can be used to improve existing BCAs through targeted mutagenesis. Genomic data from demonstrated BCAs like *B. mojavensis* RRC101 is thus capable of supplementing adaptable methods for identifying and “pyramiding”

preferential traits into given BCAs, the end goal being highly customizable tools for disease management across pathosystems.

REFERENCES

Boccara, M., Mills, C.E., Zeier, J., Anzi, C., Lamb, C., Poole, R.K., and Delledonne, M. 2005. Flavohaemoglobin HmpX from Erwinia chrysanthemi confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. The Plant Journal 43:226-237.

Chen, F., Gao, Y., Chen, X., Yu, Z., and Li, X. 2013. Quorum Quenching Enzymes and Their Application in Degrading Signal Molecules to Block Quorum Sensing-Dependent Infection. International Journal of Molecular Sciences 14:17477-17500.

Dietel, K., Beator, B., Budiharjo, A., Fan, B., and Borriss, R. 2013. Bacterial Traits Involved in Colonization of Arabidopsis thaliana Roots by Bacillus amyloliquefaciens FZB42. The Plant Pathology Journal 29:59-66.

Kerff, F., Amoroso, A., Herman, R., Sauvage, E., Petrella, S., Filee, P., Charlier, P., Joris, B., Tabuchi, A., Nikolaidis, N., and Cosgrove, D.J. 2008. Crystal structure and activity of Bacillus subtilis YoaJ (EXLX1), a bacterial expansin that promotes root colonization. Proceedings of the National Academy of Sciences of the United States of America 105:16876-16881.

Ongena, M., and Jacques, P. 2008. Review: Bacillus lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16:115-125.

Poelen, J.H., Simons, J.D., and Mungall, C.J. 2014. Global biotic interactions: An open infrastructure to share and analyze species-interaction datasets. Ecological Informatics 24:148-159.

Schneider, A., Stachelhaus, T., and Marahiel, M.A. 1998. Targeted alteration of the substrate specificity of peptide synthetases by rational module swapping. Molecular & general genetics : MGG 257:308-318.

Wu, L., Wu, H.J., Qiao, J., Gao, X., and Borriss, R. 2015. Novel Routes for Improving Biocontrol Activity of Bacillus Based Bioinoculants. Front Microbiol 6:1395.

Table A1.1. *B. mojavensis* RRC 101 Genes putatively involved in competition and colonization within the rhizosphere * denotes signal peptide)

Gene	Function	Role
pelB	Pectate lyase*	Cell wall degrading enzyme (CWDE)
	Pectate lyase* High homology to *Erwinia sp.*	CWDE
YoaJ	Expansin*	Cell wall loosening
XynA	Xylanase*	CWDE
bglA	Endo-B-1,3-1,4 glucanase (licheninase)*	CWDE
AbnA	Arabinan endo-1,5-alpha-L-arabinosidase*	CWDE
YxiA	Arabinan endo-1,5-alpha-L-arabinosidase*	CWDE
Phy	Phytase	Plant growth promotion
Bglc	Endo-1,4-beta glucanase*	CWDE, cellulase, phytase
3.2.1.8	Endo-1,4-beta xylanase*	CWDE
AbrB	Transition state Regulatory protein	Regulatory Protein
nadA, nadC	Quinolinate synthetase, Quinolinate phosphoribosyltransferase	Plant growth promotion
PabAB	Para-aminobenzoate synthase	Defense induction
alsD	alpha acetolactate decarboxylase	Acetoin synthesis
alsS	Acetolactate synthase	Acetoin synthesis
bdhA	Acetoin reductase	2,3 butanediol synthesis
YsnE	N-acetyltransferase	Indole acetic acid synthesis

* Denotes signal peptide

Table A1.2. *Bacillus mojavensis* genes likely involved in rhizosphere competence

Gene	Function	Role
BesA DhbA-DhbF	Bacillibactin siderophore	Iron sequestration
EfeUOB	Ferrous iron transporter	Iron absorption
YxaG	Quercetin dioxygenase	Detoxification of antibacterial flavonols
VanZ	Teicoplanin resistance family protein	Antibiotic resistance
PhnP	Beta lactamase	Antibiotic resistance
RhaD	Rhamnulose-1-phosphate aldolase	Host-derived carbon utilization
	Manganese superoxide dismutase	Oxidative stress tolerance
	Iron superoxide dismutase	Oxidative stress tolerance
ohrARB	Organic hydroperoxide resistance protein	Oxidative stress tolerance
Multiple (6)	Catalases: clades 1-3 and manganese	Oxidative stress tolerance
hmp	Flavohemoglobin/Nitric oxide dioxygenase	Nitric oxide detoxification

Table A1.3. Secondary metabolites likely involved in competition and biocontrol activity

Genes	Function	Role
AlbA-AlbG, SboA	Subtilosin synthesis	Bacteriocin, limited bactericidal activity
SrfAA-AD	Surfactin synthetase	Antibacterial, essential for swarming motility, defense induction
FenA-E	Fengycin synthetase	Antibiotic, antifungal
Bac	Bacilysin	Antibacterial, limited antifungal activity
BaeA-BaeS	Bacillaene synthesis	Bacteriostatic protein synthesis inhibitor
YtnP	Secreted lactonase	Disruption of quorum sensing among rhizosphere competitors
ESAT/ESX	Type VI Secretion system	Protein secretion, virulence role in animal systems

Figure A1.1. Diversity of hosts from which confirmed *Bacillus mojavensis* has been recovered, as compiled by GloBI.

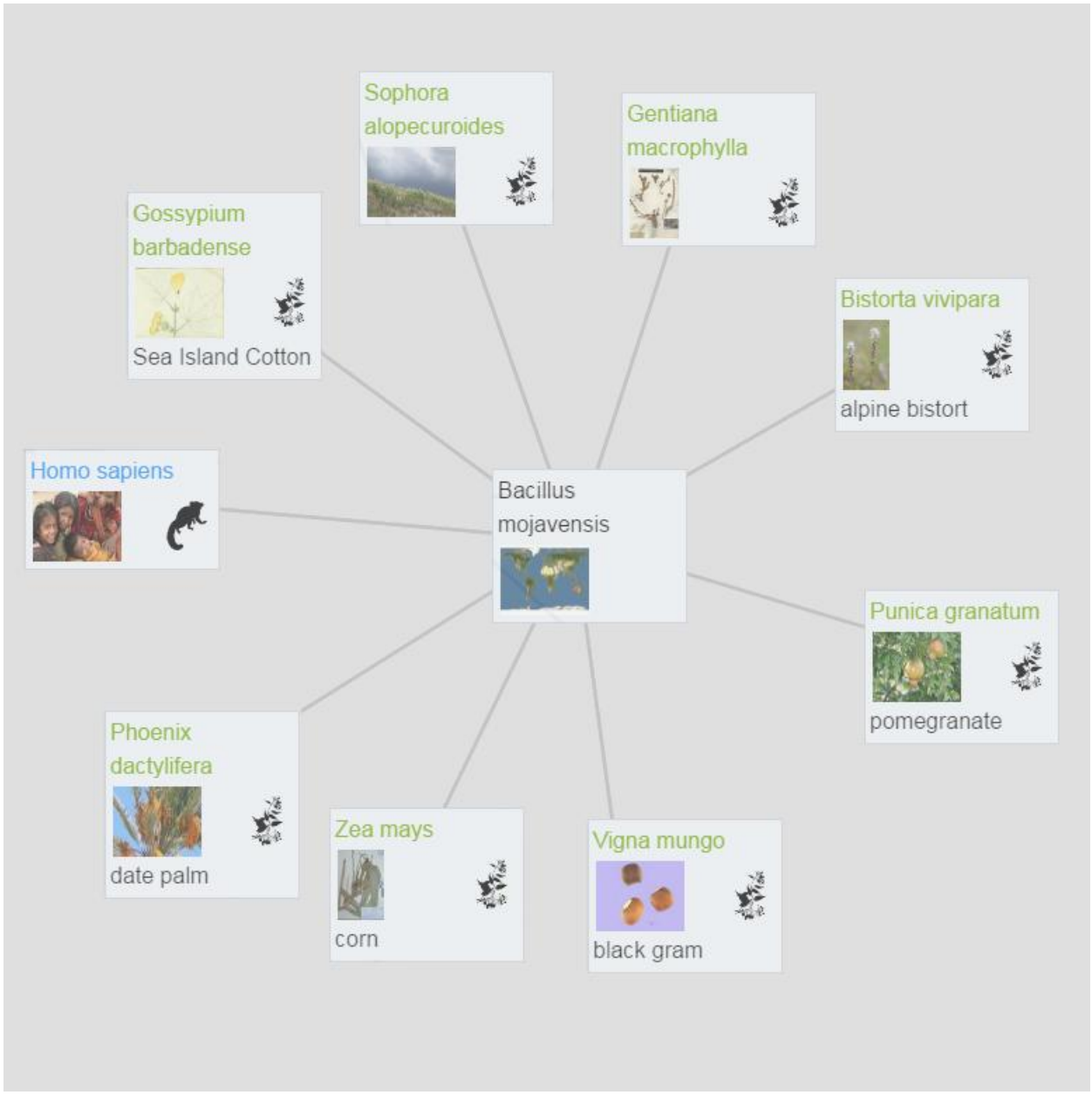
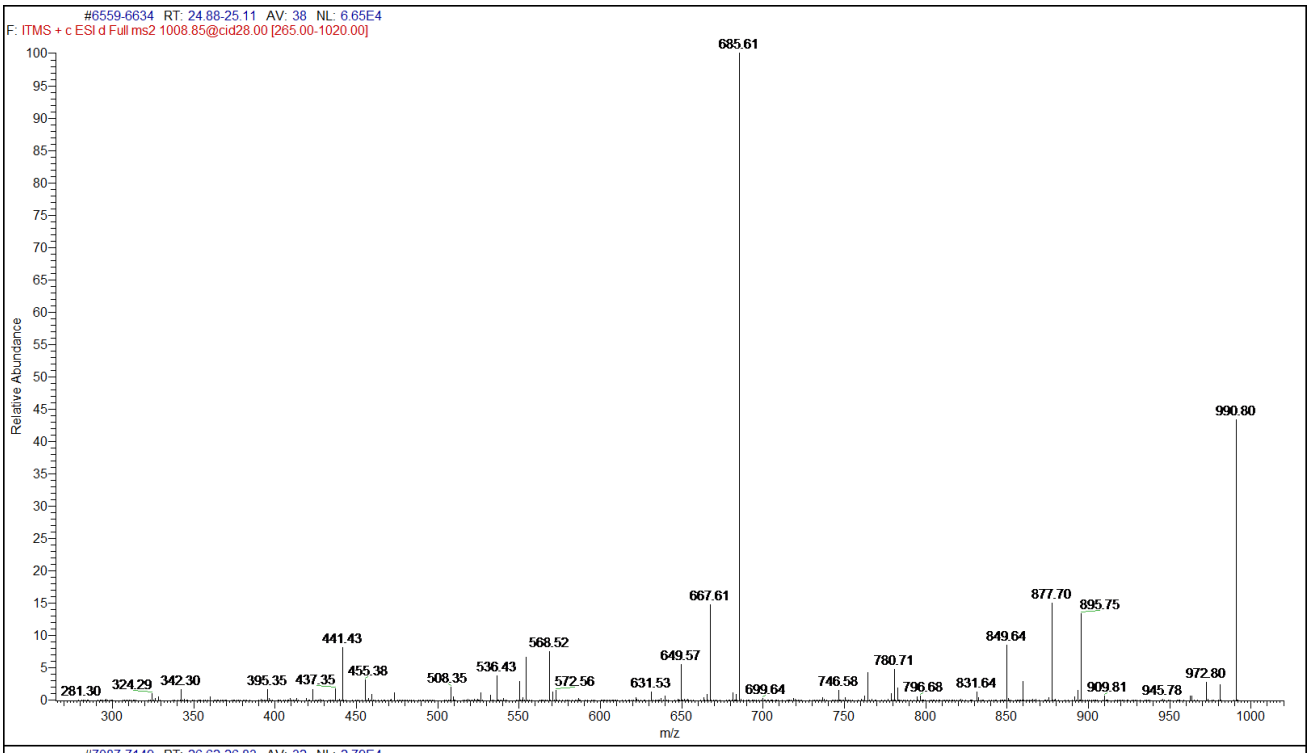
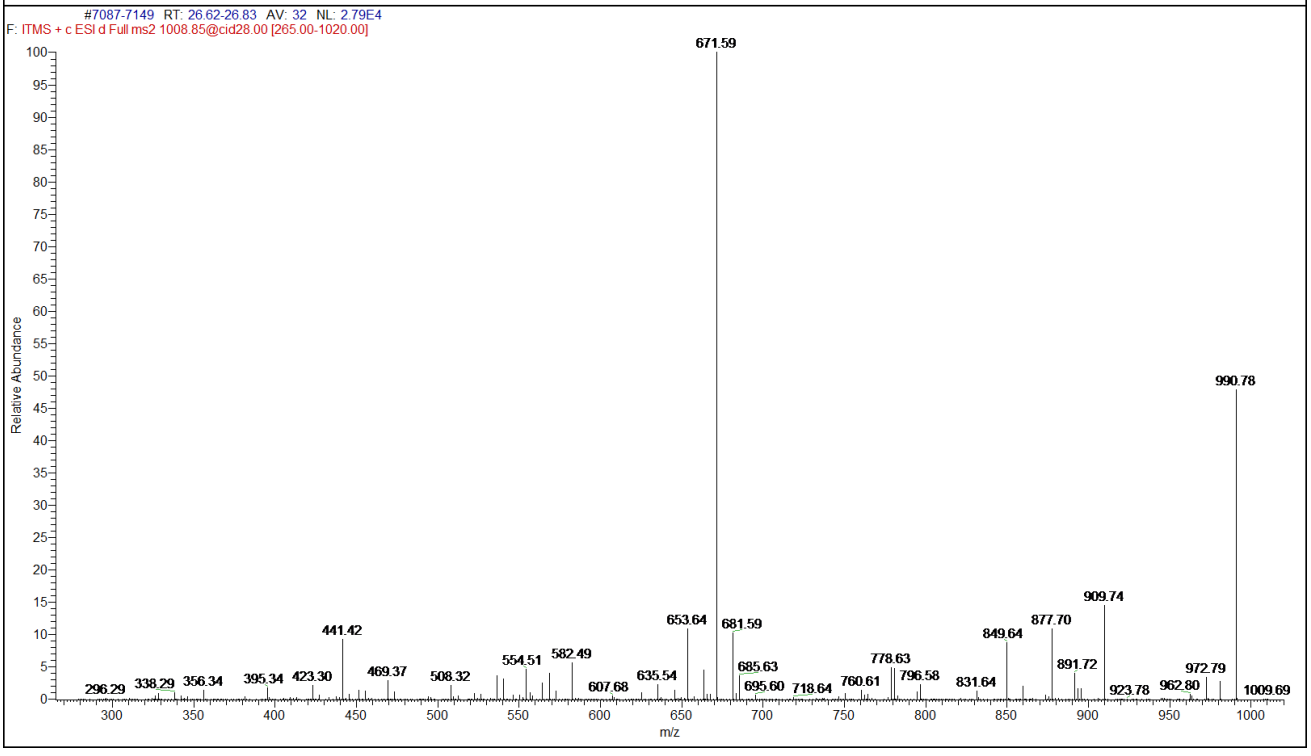

Sophora alopecuroides
Gentiana macrophylla
Gossypium barbadense
Sea Island Cotton
Bistorta vivipara
alpine bistort
Homo sapiens
Bacillus mojavensis
Punica granatum
pomegranate
Phoenix dactylifera
date palm
Zea mays
corn
Vigna mungo
black gram

Figure A1.2. Main ion fragmentation evidence of a [Val7] surfactin produced by *Bacillus mojavensis* RRC101. Based on fragmentation analysis conducted on the typical surfactin, the primary ions (685, 671) are known to represent peptides corresponding to positions 2-7 of the amino ring. The mass shift evident in fragmentation data identifies a substitution of valine for leucine in position 7.


#6559-6634 RT: 24.88-25.11 AV: 38 NL: 6.65E4
F: ITMS + c ESI d Full ms2 1008.85@cid28.00 [265.00-1020.00]
Relative Abundance
m/z
685.61
990.80
667.61
877.70
895.75
441.43
568.52
849.64
649.57
536.43
780.71
455.38
508.35
972.80
281.30
324.29
342.30
395.35
437.35
572.56
631.53
699.64
746.58
796.68
831.64
909.81
945.78


#7087-7149 RT: 26.62-26.83 AV: 32 NL: 2.79E4
F: ITMS + c ESI d Full ms2 1008.85@cid28.00 [265.00-1020.00]
Relative Abundance
m/z
671.59
990.78
909.74
653.64
681.59
441.42
877.70
849.64
554.51
582.49
778.63
891.72
685.63
469.37
972.79
296.29
338.29
356.34
395.34
423.30
508.32
607.68
635.54
695.60
718.64
760.61
796.58
831.64
923.78
962.80
1009.69

APPENDIX B

ANTAGONISM-RESPONSIVE GENES IN *FUSARIUM VERTICILLIOIDES* SELECTED FOR MUTAGENESIS

The wealth of data gathered from transcriptomic analysis provides a snapshot into the cell processes underpinning lipopeptide response in *Fusarium verticillioides*. Further work focuses on characterizing individual genes' contributions to the response, through deletion analysis. Five candidate genes were selected based on their predicted functions and upregulation under antagonism (Table A2.1). Deletants are hypothesized to exhibit readily observable phenotypes in subsequent bioassays. These targeted genes and their predicted phenotypes are detailed below.

FVEG_03803

The putative sirtuin FVEG_03803 belongs to the SIR2 superfamily responsible for deacetylation of proteins, including histones. Acetylation and methylation states of histones have been shown to act in epigenetic regulation of gene expression, with these modifications reliable indicators of transcriptional activation and/or silencing (Li et al., 2011). Histone modifications have been linked to global regulation of secondary metabolism and virulence processes in *F. graminearum* and *F. verticillioides*, and given the functions of other genes induced by lipopeptide challenge, it is possible that such modifications play a role in response to antagonism as well (Visentin et al., 2012). If this protein does indeed act to modify histones, there should be an appreciable phenotype evident in bioassay experiments as disrupted gene silencing would produce a cascade of differential regulation. As FVEG_03803 is a putative class III sirtuin (SIRT5), according to studies in higher eukaryotes it likely functions mitochondrially, with the potential of regulating energy generation or apoptosis induction. In mammals, SIRT5 proteins

colocalize with cytochrome c and mediate “apoptosis inducing factor,” suggesting a role in apoptotic processes (Gertz and Steegborn, 2010). The deletion phenotype for FVEG_03803 may involve apoptotic processes or differential regulation under antagonism. Both can be evaluated under bioassay conditions, with microscopy or MS^2 analysis for secondary metabolites. Localization data, as well as transcriptional evidence for apoptosis markers under antagonistic conditions, may also provide information as to the function of this protein.

FVEG_08663

FVEG_08663, unlike other selected targets, possesses no known functional domains. This gene is conserved in *F. graminearum* and *F. oxysporum*, and is flanked by a hypothetical protein and a MFS transporter (FVEG_08662 and FVEG_08664). The predicted protein bears low identity (<30%) to the *Candida albicans* adhesin-like cell wall protein HYR1, involved in resistance to phagocyte killing (Luo et al., 2010). Phagocytes can kill by exposure to oxidants; the signal peptide suggesting extracellular involvement and the conservation among two plant pathogens suggest this protein may be involved in colonization or other stress response. In addition to microscopy under lipopeptide antagonism, mutants will be assayed for pathogenicity effects and resistance to oxidative stress.

FVEG_08797

FVEG_08797 encodes a putative transcription factor. Given that *F. verticillioides* undergoes global regulatory shifts under lipopeptide challenge, this transcription factor is likely a significant contributor to that response. FVEG_08797 possesses only one syntenic ortholog in *Fusarium* species, FOXG_09669 in *F. oxysporum*, and neither is affiliated with a specific pathway or function. The gene is flanked by an oxidoreductase and a predicted transferase (FVEG_08796 and FVEG_08798), offering no specific clues to the function of this transcription

factor. Deletants will be assayed for differential response to lipopeptide challenge, microscopically and biochemically, with transcriptional data collected to attempt identification of FVEG_08797 targets.

FVEG_10818

Although comparatively little is known about fungal annexins such as the one putatively encoded by FVEG_10818, these proteins are distributed throughout eukaryotes and known to bind Ca^{2+} and phospholipids such as those present in the plasma membrane (Khalaj et al., 2015). Members of this protein family are involved in membrane organization and related processes, most notably the regulation of ion channels. Based on work with deletion mutants in *Aspergillus fumigatus* and *Dictyostelium discoideum*, these proteins may function in conjunction with stress response pathways. This is corroborated by experiments in other eukaryotes. Given that lipopeptide antagonism is thought to involve membrane permeabilization and is accompanied by oxidative and osmotic stresses, this protein is a natural response to fengycin antagonism. Work on orthologous genes in other fungi suggests that deletion of FVEG_10818 will not present a phenotype under normal culture conditions, but rather under stress such as lipopeptide challenge, where mutants will likely exhibit decreased structural stability.

FVEG_12324

LysM domain-containing proteins, such as those encoded by FVEG_12324, function in phytopathogens by binding cell wall components to evade detection by host chitin receptors, but may also act to protect hyphae from chitinase activity (Kombrink and Thomma, 2013). Another potential function involves binding not chitin but peptidoglycan. It is hypothesized that a subset of these effectors could bind bacterial competitors in a manner similar to antibodies, offering a competitive advantage in the rhizosphere. Further, a BLASTP query using FVEG_12324

returned *F. oxysporum* autolysin, belonging to a group of proteins involved in autolytic breakdown of senescent hyphae. The implication of apoptosis in lipopeptide antagonism suggests that this protein may be involved in either response to antagonism or the process of cell death induced by fengycin exposure. If FVEG_12324p is involved in pathogenesis, it is possible that deletants will be impaired in their ability to cause disease on corn. If this protein functions as part of the autophagic process, the phenotype may be evaluated microscopically under antagonistic/apoptotic conditions, with a focus on cell structure in mutants as compared to the wild type.

REFERENCES

Gertz, M., and Steegborn, C. 2010. Function and regulation of the mitochondrial Sirtuin isoform Sirt5 in Mammalia. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1804:1658-1665.

Khalaj, K., Aminollahi, E., Bordbar, A., and Khalaj, V. 2015. Fungal annexins: a mini review. SpringerPlus 4:721.

Kombrink, A., and Thomma, B.P.H.J. 2013. LysM Effectors: Secreted Proteins Supporting Fungal Life. PLOS Pathogens 9:e1003769.

Li, Y., Wang, C., Liu, W., Wang, G., Kang, Z., Kistler, H.C., and Xu, J.-R. 2011. The HDF1 histone deacetylase gene is important for conidiation, sexual reproduction, and pathogenesis in Fusarium graminearum. Molecular Plant-Microbe Interactions 24:487-496.

Luo, G., Ibrahim, A.S., Spellberg, B., Nobile, C.J., Mitchell, A.P., and Fu, Y. 2010. Candida albicans Hyr1p Confers Resistance to Neutrophil Killing and Is a Potential Vaccine Target. Journal of Infectious Diseases 201:1718-1728.

Visentin, I., Montis, V., Döll, K., Alabouvette, C., Tamietti, G., Karlovsky, P., and Cardinale, F. 2012. Transcription of Genes in the Biosynthetic Pathway for Fumonisin Mycotoxins Is Epigenetically and Differentially Regulated in the Fungal Maize Pathogen Fusarium verticillioides. Eukaryotic Cell 11:252-259.

Table A2.1. *Fusarium verticillioides* genes responsive to lipopeptide challenge selected for deletion analysis to characterize the phenotype of antagonism

			log_2(fold change) from Control		
Gene	**PFAM**	**Description**	**Fengycin**	**Surfactin**	**Double**
FVEG_03803	PF02146.12	Putative sirtuin involved in large-scale transcriptional regulation	1.58	ns	1.43
FVEG_08663	Unknown	Conserved in *F. graminearum* & *F. oxysporum*, low identity to adhesin	2.32	ns	1.94
FVEG_08797	PF11951.3	Fungal specific transcription factor domain	1.52	ns	1.03
FVEG_10818	PF00191.15	Annexin, likely involved in stress response	1.85	ns	1.93
FVEG_12324	PF01476.15	Secreted LysM-containing protein, BLAST match to autolysin	3.08	-1.01	2.84