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Biosurfactant Production by Bacteria in the Phyllosphere: Relieving the Tension of Life on a Surface

By

Adrien Yuan Burch

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Steven Lindow, Chair
Professor Mary Wildermuth
Professor Arash Komeili

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Abstract

Biosurfactant Production by Bacteria in the Phyllosphere:
Relieving the Tension of Life on a Surface

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Adrien Yuan Burch

Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Steven E. Lindow, Chair

Biosurfactants are biologically produced compounds that reduce interfacial tensions due to their water- and oil- loving groups. These amphiphilic substances are widely investigated for their potential commercial exploitation, yet little evidence has been assembled for their direct roles in the environment for the bacteria that produce them. In order to better enable the investigation of biosurfactants, we developed an efficient method for biosurfactant detection that is more sensitive than the standard drop collapse assay, as well as capable of detecting surfactants that have low water solubility and would normally be overlooked. A large number of bacteria recovered from different environments were assessed for biosurfactant production using this atomized oil assay. Detectable biosurfactant production was found to be quite common amongst culturable bacteria, with 5 to 13% of all bacteria from various habitats expressing this trait. Furthermore, we deployed the atomized oil assay in two mutagenesis screens to determine the biosynthetic and regulatory pathways of biosurfactant production in *Pseudomonas syringae* pv. syringae B728a.

A recurring theme that emerges in this dissertation is the importance of biosurfactant production for life on a surface. Not only were biosurfactant-producing bacteria more commonly found in terrestrial surface environments such as leaves than in aqueous samples, but the bacteria that produce biosurfactants were also more likely to produce such compounds when grown on a surface compared to planktonically. Furthermore, the patterns of regulation of the biosurfactants produced by *P. syringae* also provide additional support for their importance at surfaces. Syringafactin production is higher in cells grown on agar plates than in broth cultures. Also, an unidentified surfactant is produced in larger quantities when *P. syringae* is grown on hydrated rough surfaces compared to smooth agar plates.

Examination of the control of biosurfactant production in *P. syringae* revealed that *s*yringafactin is regulated by SyfR, a divergently transcribed LuxR-type regulator. SyfR is the mediator of the surface sensing response since in the absence of functional SyfR protein, the SyfR promoter is equally induced in broth and plate cultures, but when present, both the transcription of SyfR and syringafactin is increased on agar plates. A new function for this type of LuxR-type regulator

was thus demonstrated. Furthermore, random mutants with altered surfactant production were identified using the atomized oil assay enabling the investigation of biosynthetic and regulatory genes required for the unidentified biosurfactant produced by *P. syringae* B728a. This surfactant has low water solubility and is synthesized by an acyltransferase that when expressed *in trans* in *E. coli* is sufficient for its production. Production of this surfactant is dependent on proper flagellar assembly and the compound was thus termed BRF (biosurfactant regulated by the flagella). Mutations in genes necessary for early establishment of the flagellar apparatus abolish BRF production, while mutations that stimulate higher flagellin production increase BRF production. Flagellin synthesis is up-regulated at surfaces, and BRF synthesis was co-regulated with flagellin synthesis under conditions of varying agar concentrations in culture media. The induction of BRF production was especially pronounced during growth on hydrated paper discs, where both flagellin and BRF are induced more highly than growth on agar surfaces. BRF was induced even more highly in cells grown in broth cultures, independent of levels of flagellin production. Thus BRF is not restricted to surface production, but its production on a surface appears to be regulated by flagellar surface sensing.

In addition to assembling support from environmental collections and genetic regulation that biosurfactant production is linked to life on a surface, we directly tested its importance *in planta*. Biosurfactant production by *P. syringae* B728a increased the wettability of the leaf surface. While syringafactin production provides a slight increase in the epiphytic fitness of *P. syringae* on the leaf surface, no contribution of BRF could be found under the conditions tested. Syringafactin appears to either increase the colonizable area of the leaf surface (water droplets with lower surface tension have increased surface area), or to increase the local density of bacteria on leaves. Purified syringafactin increased the water permeability of isolated plant cuticles, lending support that its production allows for increased nutrient access on leaves. BRF did not significantly alter the permeability of cuticles, nor do strains defective in its production have lower measurable fitness. BRF thus might function as a flagellar lubricant enabling surface motility. Combined evidence from environmental studies, investigations of genetic regulation, as well as *in planta* experimentation reveals that biosurfactants have multiple roles at surfaces.

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Chapter I.

Introduction

In a world of diminishing natural resources it is becoming increasingly prudent to investigate the natural products produced by microbes. These microscopic powerhouses produce an endless spectrum of compounds, and their rapid multiplication ensures a constant replenishment. A class of bacterial products that is of increasing interest is biosurfactants, which are being commercially exploited for use in pollution degradation, as antibiotics, anti-adhesives, food preparation, and many other purposes (Mulligan, 2005; Rodrigues *et al*., 2006; Nitschke and Costa, 2007). Although biosurfactants have a wide range of potential applications, little is known about the physiological role of biosurfactants in the natural habitats of the producing organisms. The investigation of the natural functions of biosurfactants might reveal important details of bacterial movement and colonization strategies, novel biocontrol methods, as well as unrecognized consequences of biosurfactant application in the field. This dissertation addressed the biosurfactant production by the plant-associated *Pseudomonas syringae* pv. syringae B728a, and what role, if any, this production plays in its life on the leaf surface.

Surfactants and biosurfactants

Surfactants, short for **surf**ace **act**ive age**nts**, are a broad class of amphipathic compounds that demonstrate surface activity by lowering interfacial tension. They are described as having a hydrophobic carbon tail group and a hydrophilic head group, and are generally classified by the composition and charge of the head group. Their hydrocarbon tail groups also vary in length, branching, and saturation. Other properties serve to further characterize surfactants, such as their overall water solubility (hydrophilic-lipophilic balance/HLB), the concentration at which they form micelles (critical micelle concentration/CMC), and the extent to which they lower the surface tension of water (Myers, 2006).

Biosurfactants are surfactants of biological origins, and generally are more biodegradable and non-toxic than their synthetic counterparts which can have undesirable toxicity effects and/or be environmentally persistent (Singh and Cameotra, 2004). Although some biosurfactants are produced by multicellular organisms, such as the pulmonary surfactant produced in human lungs, the majority of classified biosurfactants have microbial origins (D'aes *et al*., 2010). For the remainder of this dissertation, the term biosurfactant will strictly refer to bacterially produced surfactants.

The importance of surfactants to our modern daily lives cannot be understated: most everyone can appreciate the soaps and detergents used for washing bodies, clothing and dishes. But beyond personal hygiene, surfactants have roles in diverse industries from textile production to mining operations to agricultural pesticide application to food formulations (Myers, 2006). Given the broad structural possibilities and variable properties of surfactants, it is little wonder that nature has evolved an immense diversity of biosurfactants. Biosurfactants provide a mostly untapped source of biodegradable and specialized surfactants with potential industrial applications.

Although the potential structures are nearly endless, most characterized biosurfactants can be placed into a few specific classes. Glycolipids have a sugar head group and lipid tail. The most studied example of a glycolipid is rhamnolipid produced by *Pseudomonas aeruginosa*, which has one or two rhamnose head groups linked to a tail composed of a dimer of β-hydroxydecanoate (C_{10}-C_{10}) (Deziel *et al*., 2003). Lipopeptides possess a peptide head group attached to a lipid tail, and the peptide moiety is unique in that it is synthesized non-ribosomally; ie, it is not translated from an mRNA. Rather, lipopeptides are generally synthesized by non-ribosomal peptide synthetases (NRPSs), which are large enzyme complexes that catalyze the sequential assembly of a small peptide, as well as direct the addition of a carbon tail (Schneider and Marahiel, 1998). Other biosurfactants include phospholipids, polymers such as emulsan, as well as whole microbial cells (Nitschke and Costa, 2007). For a more complete discussion of the different types of biosurfactants, there are a number of excellent reviews (Van Hamme *et al*., 2006; Perfumo *et al*., 2010; Satpute *et al*., 2010).

Detecting biosurfactants

The amphipathic properties of surfactants energetically drive them to adsorb at interfaces and to reduce the surface tension between immiscible phases (such as oil/water or water/air). Thus, a number of methods have been developed to measure the amplitude of this effect. Most often, the surfactant's effect on the surface tension at the air/water interface is measured. This can be measured by a variety of methods. The tensiometer, or Du Nouy ring method, measures the force required to lift a thin platinum ring from the surface of a liquid solution (Bodour and Miller-Maier, 1998). Although this is the most traditional method for surface tension measurements, it requires a large volume of water, and thus necessitates large quantities of the test surfactant which are often difficult to obtain from bacterial cultures. Another method that is gaining popularity is the pendant drop method, which only requires a few microliters of a surfactant solution and uses optical analysis of the shape of a hanging aqueous drop to determine the surface tension of the solution (Lin et al., 1990).

Although these methods are precise tools for describing the properties of a surfactant, they are not appropriate for tests of large numbers of biological samples. To address this problem, a number of assays have been developed that exploit the properties of biosurfactants to indicate if a bacterial strain has produced such a material. One of the more direct methods is the drop collapse assay, which qualitatively assesses the effect of a surfactant at the water/oil interface. If a surfactant sufficiently reduces the surface tension of water, then a small droplet of an aqueous solution, when placed on an oily surface, will spread or "collapse" over the oily surface (Bodour and Miller-Maier, 1998). However, this assay requires sufficient quantities of surfactant to reach a threshold surface tension in order for water drop collapse to occur. Another biological assay that is sometimes used is to look for emulsification of insoluble liquids in water, since certain classes of surfactants can emulsify oil into water (Plaza *et al*., 2006). Additionally, some have screened for blood cell lytic activity, since certain surfactants can readily disrupt cell membranes (Youssef *et al*., 2004). However, in cases where multiple biosurfactant detection methods have been compared, both of these approaches were prone to providing false-positive indications of surfactant-like substances, as well as to not respond to certain classes of surfactants (Youssef *et al*., 2004; Chen *et al*., 2007b). Thus, one objective of this dissertation was to develop a new assay for biosurfactant detection that would have less specificity than emulsification or blood lysis, as well as being capable of more sensitive detection than the drop collapse assay.

With an efficient method to detect biosurfactants in hand, a variety of untested assumptions about their production can start to be addressed. Biosurfactant-producing organisms have classically been tested for their ability to emulsify hydrocarbons to enable their use as a nutrient source (Neu, 1996) and thus it is has generally been assumed that there is an enrichment of biosurfactant producers in oil-contaminated environments. Others have speculated that surfactant production may be higher on waxy leaves (D'aes *et al*., 2010). However, there is not yet evidence for enrichment of biosurfactant producers in any tested environment, since the methods used to identify biosurfactant producers vary widely from study to study, and thus no two studies can be directly compared (Table 1). A high-throughput method could enable the comparison of production in different environments, such as explored in chapter 3. Furthermore, rapid and quantitative assessment of biosurfactants enables high throughput screens of random mutants to identify the biosynthetic and regulatory pathways in their producers. I take such an approach to identify the biosurfactants produced by *P. syringae* B728a in chapters 2 and 4.

Table I-1. Proportions of biosurfactant producers observed when collected from different sources and cultured by different methods

Source	Culture conditions	Surfactant producers	Reference
Rain and clouds	Nutrient media, 30 days	50% (70/140)	(Ahern *et al*., 2007)
Soil (wild and contaminated)	MSM + 2% glucose, 7-9 days	3.4% (45/1305)	(Bodour *et al*., 2003)
Soilless cultivation	Nutrient media, 3 days	18.5% (111/600)	(Hultberg *et al*., 2008)
Variety of sources	BH mineral media, 7 days	9.2% (17/185)	(Batista *et al*., 2006)
Unknown	Medium E, 24 hours	>50%	(Youssef *et al*., 2004)
Contaminated soil	7 days	11.7% (7/60)	(Maciel *et al*., 2007)

Bacteria at surfaces

Biosurfactant detection assays will not be effective if the bacterial growth conditions are not suited for their production. Every study listed in Table 1, as well as most other reports of biosurfactant production, have relied on culturing of bacteria in broth media. However, the vast majority of bacteria in nature, even in aquatic environments, are not free-living but rather live within biofilm communities (Costerton *et al*., 1994). Additionally, surfactants are by definition surface active, and thus have their greatest impact at surfaces. Do planktonic growth conditions affect a bacterium's decision to produce biosurfactant? This is one question asked in chapter 3, which describes a broad collection of environmental bacterial isolates and how culture conditions affect the production of biosurfactants. Biosurfactant production is presumably costly for a cell, and bacteria most likely restrict biosurfactant production to conditions where the surfactant will be useful to the cell. Planktonic production could be beneficial to bacteria if it leads to the emulsification of hydrophobic nutrients into the bulk aqueous solution. But if the surfactant is used to maintain biofilms or to move across surfaces, most likely the bacteria will have no need

for its production in broth cultures. Thus, it makes sense that bacteria with multiple habitats should survey their growth environment before committing to production of a biosurfactant.

Large differences in the transcriptomes of bacteria grown planktonically versus on surfaces have been described, with about one-third of genes differentially regulated (Schembri *et al*., 2003; Wang *et al*., 2004). Furthermore, surface culturing is an important cue for many species to transition to a hyper-flagellated swarming phenotpye (McCarter *et al*., 1988; McCarter, 2006). Since it appears that bacteria have phenotypic responses to surfaces, how do they know that they are at a surface? To answer this question, some research has addressed the physical properties of surfaces and the general conditions that surface-associated cells might be responding to. Solutes tend to concentrate on surfaces, and thus cells might respond to the higher osmolarity or concentration of particular ions at surfaces (Goodman and Marshall, 1995). Additionally, bacteria that are situated in biofilms on a surface experience lower oxygen and higher cell density conditions, and might interpret these conditions as location cues (Prigent-Combaret *et al*., 1999). Other modes of surface sensing include responses to physical perturbation of the membrane upon adherence, such as the Cpx two-component system in *E. coli* (Otto and Silhavy, 2002), or responding to the increased torque that appendages such as flagella might encounter upon their interaction with surfaces (Belas and Suvanasuthi, 2005). Thus, it appears that bacteria have a variety of mechanisms with which they can sense surfaces.

Surfactant properties

Different conditions might trigger biosurfactant production in different bacteria, depending on the function of the surfactant to a given species and habitat. However, are there limitations to the tasks a given surfactant can be used for? Although biosurfactant production has been noted for decades, the significance of their different chemical structures is only starting to be appreciated. For instance, it has been found that small changes in peptide components of *Bacillus* surfactants result in large changes of their antifungal and antimicrobial properties (Bonmatin *et al*., 2003). However, as of yet there are no good guidelines on what surfactant structures are appropriate for a given type of bacterial function. This is in contrast to synthetic surfactants, where manufacturers have developed many tools for choosing appropriate surfactant formulations from thousands of synthetic surfactants. One goal of this research is to identify biosurfactants with different physical properties, and determine how these properties affect the biological roles they play to the producing organism.

A particularly important property that was focused on in this study is the water solubility of biosurfactants, a proxy for their hydrophilic lipophilic balance HLB. HLB values are a scalar factor that reflects the degree to which a surfactant is hydrophilic or lipophilic, with a value of zero reflecting a completely lipophilic (hydrophobic) molecule, a value of 10 corresponding to a compound with equivalent hydrophobic and hydrophilic groups, and values over 10 descriptive of predominantly hydrophilic molecules (Adamson, 1982). This value is of great significance commercially since it is used to determine appropriate functions of surfactants. For example, common surfactants such as SDS and Tween 20 have high HLB values and are therefore best suited for emulsifying a hydrophobic substance into the water phase (oil into water). On the other hand, surfactants such as Silwet® L-77 with HLB values near 10 are more suited for wetting, or spreading of a water phase over surfaces such as leaves (Adamson, 1982; Zhang *et al*., 2006). At the other end of the spectrum, lipophilic surfactants are best at forming inverse

emulsions of water into oil. Although biosurfactants were originally proposed to be used by bacteria to solubilize hydrophobic nutrient sources (Neu, 1996), by the HLB classification alone it is obvious that only a small subset of biosurfactants would be optimal for this purpose.

Biosurfactant function

Biosurfactant producers are common in the environment, with around 10% of culturable bacteria in a given environment readily exhibiting this trait (Table 1). Given their prevalence, the general field of microbiology will benefit from a better understanding of biosurfactant production. Additionally, in order for humans to best utilize biosurfactants, it should be informative to discover their natural functions which, in turn, might reveal novel applications for these molecules.

Biosurfactants have been implicated in a large variety of functions beyond hydrocarbon emulsification. In aqueous environments, bacteria might use surfactants to coat themselves and/or surfaces to alter adherence or deherence (Neu, 1996). On the other hand, terrestrial surfaces often only harbor thin films of water; bacteria in such habitats often experience water stress and suffer from low diffusional nutrient fluxes (Or *et al*., 2007). In this circumstance, biosurfactants might prevent evaporation or act as osmotic agents, thus maintaining thicker water films, relieving water stress and increasing microbial access to nutrients (Chen *et al*., 2007a). Their ability to lower the surface tension of water has been implicated in promoting aerial hyphal growth (Straight *et al*., 2006), while their emulsification properties might enable delivery of antagonistic compounds (Perneel *et al*., 2008). Because biosurfactants are amphiphilic, they can insert into membranes, and some surfactants have thus been noted for their potent membrane-disrupting and resultant antimicrobial properties (Hutchison and Gross, 1997). Biosurfactants appear essential for biofilm formation in some bacteria (de Bruijn *et al*., 2007; de Bruijn *et al*., 2008), while they appear to prevent biofilm formation in others (Kuiper *et al*., 2004). Indeed, the anti-adhesive properties of some biosurfactants make them excellent candidates for coating medical devices (Singh and Cameotra, 2004). Additionally, some biosurfactants are proposed to act as autoinducers to signal cellular differentiation (López *et al*., 2009). Obviously all these traits do not apply to a given biosurfactant, but is inclusive of a rather broad spectrum of diverse molecules. Biosurfactant research would greatly benefit from further categorizations of biosurfactants based on their physical properties and demonstration of functions in which they participate.

Biosurfactants have an additional but complicated role in cellular motility. A classic function of biosurfactant activity is its enhancement of bacterial motility across soft agar plates. This motility, termed swarming motility, is an active form of translocation and is generally reliant on flagellar motility and biosurfactant production (Kearns, 2010). Although biosurfactants are necessary for swarming motility in many bacteria, their production provides no benefit to swimming motility, and it is difficult to imagine a natural environment that would support the large local population sizes necessary for swarming motility. Nonetheless, it is widely assumed that biosurfactant production supports bacterial movement *in vivo*. How exactly might biosurfactants be beneficial to motility, and under what natural conditions do they aid motility? This question is addressed in chapter 6.

Biosurfactants in the phyllosphere

Biosurfactant production has been noted in many bacterial species, but few bacterial habitats allow for as easy observation and manipulation of surfactant production as do leaves. Thus, the phyllosphere is an excellent setting in which to test the biological roles of biosurfactant production. Epiphytic bacteria not only survive, but readily flourish on leaves despite the high UV exposure, cycles of desiccation and hydration, rapid temperature fluctuations, and low and heterogeneous nutrient availability found on most leaves (Lindow and Brandl, 2003). It has been shown that growth of surfactant-producing bacteria on a plant can change the wettability of the leaf (Bunster *et al*., 1989). It has previously been postulated that such biosurfactant production might be beneficial to the epiphytic life of bacteria (Hutchison and Gross, 1997; Lindow and Brandl, 2003; Underwood *et al*., 2007) and it is widely assumed that the plant environment is especially enriched with biosurfactant producers for this reason (D'aes *et al*., 2010).

It is already known that once inside the leaf, surfactant production by bacteria such as *P. syringae* is important for the development of disease symptoms, most likely through the induction of plant cell leakage (Raaijmakers *et al*., 2006). However, it remains unclear how biosurfactants specifically aid epiphytic growth of bacteria. Continuous water films may not normally form on such waxy surfaces; by decreasing the interfacial tension between the leaf surface and dispersed water droplets, biosurfactants could increase the wetted surface area of the leaf. Such enlarged water films might increase the distribution of locally abundant nutrients that might be separated by waxy regions of the leaf which would not otherwise be wetted by water. During periods of abundant leaf surface water, it is hypothesized that epiphytes will leave cellular aggregates in which they survive and explore the leaf surface, moving between dispersed nutrient-rich sites (Hirano and Upper, 2000); surfactant-mediated enlarged wetted areas might enable increased regions over which such motility could occur. Furthermore, surfactants might have lubricating properties, and increase bacterial motility on leaves by decreasing potential attractive forces that could immobilize bacteria on surfaces. Besides increasing growth through redistribution of nutrients and bacteria, surfactants might also increase nutrient or water availability in those sites already colonized by bacteria through their plasticizing effect on the cuticle (Schreiber *et al*., 2005).

A number of plant-associated organisms have been studied for biosurfactant production, but few have been directly tested for the role of these compounds *in planta*. When surfactant-deficient mutants have been tested *in planta*, the focus is usually on the contributions of the biosurfactants to virulence or to the membrane-disruptive, phytotoxic properties of these molecules (D'aes *et al*., 2010). A few studies have attempted to include movement in their assessment of biosurfactant roles, but the results are generally mixed; it is difficult to pinpoint the exact cause of a deficiency of colonization of plant surfaces by a mutant (Hildebrand *et al*., 1998; Nielsen *et al*., 2005). Thus, although it has been speculated that the decreased fitness of biosurfactant mutants is due to their decreased motility and/or access to nutrients, neither of these factors have been directly proven on plants.

Although there is a paucity of research on the role of different types of biosurfactants in the phyllosphere, the widespread use of synthetic surfactants in agriculture has provided a large source of information that might be applied to biosurfactants. Surfactants are capable of solubilizing plant epicuticular wax, thus diminishing the barrier of nutrient diffusion from the

leaf onto the surface, although solubilization will only occur at concentrations above the critical micelle concentration (Tamura *et al*., 2001). Biosurfactant production could potentially reach high enough local concentrations in bacterial aggregates to solubilize and strip away adjacent waxes if the biosurfactant is suited for solubilizing hydrophobic substances into water. At lower concentrations, surfactants will have different effects on the cuticle depending on their structures. Hydrophilic surfactants, when adsorbed into the cuticle, will increase the hydration of the cuticle and therefore increase the movement of not only water but also water-soluble molecules. Alternatively, although hydrophobic surfactants readily adsorb into the cuticle, they do not increase the hydration but rather the fluidity of cuticular waxes that, in turn, increases the rate of diffusion of hydrophobic compounds across the cuticle (Hess and Foy, 2000). Additionally, movement of water and bacteria into the apoplast is normally prevented by the high surface tension of water, but can occur spontaneously when the surface tension of the liquid is reduced such as in *Zebrina purpusii* when the surface tension of liquid is less than 30 dyn/cm (Schonherr and Bukovac, 1972). Similarly, during plant invasion, pathogens could be employing a surfactant with high surface tension lowering abilities to facilitate water (and bacterial) entry into stomata and other openings.

Biosurfactants have been implicated in a wide variety of roles, and all of these roles might prove true in specific situations. However, it is important to start defining what types of surfactants are good at achieving a given result. The goal of this dissertation is to examine biosurfactant production in the phyllosphere with an emphasis on the plant-associated *Pseudomonas syringae*, in which several surfactants that it produces will be characterized and studed for their specific roles in the phyllosphere, based on clues from their genetic regulation.

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Chapter II.

Novel high-throughput detection method to assess bacterial surfactant production

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ABSTRACT

A novel biosurfactant detection assay was developed for observation of surfactants on agar plates. Using an airbrush to apply a fine mist of oil droplets, surfactants can be observed instantaneously as halos around biosurfactant-producing colonies. This atomized oil assay can detect a wide range of different synthetic and bacterial-produced surfactants. This method could detect much lower concentrations of many surfactants than a commonly used water drop-collapse method. It is semi-quantitative and therefore has broad applicability for uses such as high-throughput mutagenesis screens of biosurfactant-producing bacterial strains. The atomized oil assay was used to screen for mutants of the plant pathogen *Pseudomonas syringae* pv. syringae B728a that were altered in the production of biosurfactant. Transposon mutants displaying significantly altered surfactant halos were identified and further analyzed. All mutants identified displayed altered swarming motility, as would be expected of surfactant mutants. Additionally, measurements of the transcription of the syringafactin biosynthetic cluster in the mutants, the principle biosurfactant known to be produced by B728a, revealed novel regulators of this pathway.

Author contributions: A.B. and S.L. designed the research, A.B., B.S. and P.B. performed the research**,** A.B. analyzed the data, and A.B. and S.L. wrote the paper.

INTRODUCTION

Biosurfactant-producing organisms have classically been identified by their ability to emulsify and utilize hydrocarbons as a nutrient source (Neu, 1996). It has only been recently appreciated that biosurfactants are produced by bacteria for many reasons other than access to hydrophobic nutrient sources. Among the numerous functions identified, are their use for swarming motility (movement across moist surfaces/ low-percentage agar plates), biofilm structure and maintenance, and delivery of insoluble signals (Ron and Rosenberg, 2001; Van Hamme *et al*., 2006). Biosurfactants have been identified that can either promote biofilms or disperse them on root and abiotic surfaces (Bais *et al*., 2004; Kuiper *et al*., 2004). Additionally, some biosurfactants have been noted for their membrane-disrupting and thus zoosporicidal or antimicrobial activity (de Souza *et al*., 2003; Bais *et al*., 2004; Raaijmakers *et al*., 2006).

An unexplored arena where biosurfactants may prove particularly important is the colonization of waxy leaf surfaces. In order to survive on leaf surfaces, epiphytes must be able to access limited and spatially heterogeneous nutrient supplies and endure daily fluctuations in moisture availability in forms such as dew and rainfall (Hirano and Upper, 2000; Lindow and Brandl, 2003). Continuous water films may not normally form on such waxy surfaces, and surfactants might thus aid in diffusion of compounds across the plant. If the bacteria have a pathogenic life phase, they must first have a method to enter plant tissue after which they create a favorable apoplastic environment for growth (Wright and Beattie, 2004). It is already known that once inside the leaf, bacteria such as *P. syringae* use surfactants to cause plant cell leakage and disease symptoms (Raaijmakers *et al*., 2006). However, some studies have also implicated biosurfactants in the pre-pathogenic stages of plant-associated bacteria (Hutchison and Gross, 1997; Lindow and Brandl, 2003; Underwood *et al*., 2007).

Pseudomonas syringae pv. syringae B728a, a sequenced model organism with a prominent epiphytic lifestyle, produces biosurfactants (Feil *et al*., 2005; Berti *et al*., 2007). A study of the genetic regulation of biosurfactant production should provide insight into its function in this species. The identification of mutants altered in surfactant production would be an important first step in this process. However, an effective method of identifying such mutants needed to be found. Many studies have compared various screening methods to identify biosurfactant producers from limited collections of environmental isolates. Some of the most commonly used methods for analyzing biosurfactant production are drop-collapse, emulsification, and tensiometric evaluation (Bodour and Miller-Maier, 1998; Chen *et al*., 2007). However, when many strains need to be assessed for surfactant production, the drop-collapse assay has been the method of choice (Kuiper *et al*., 2004; de Bruijn *et al*., 2008). While some of the other methods are more sensitive and quantifiable than the drop-collapse method, none of them are practical for high-throughput screening. Unfortunately, even the drop-collapse assay involves a number of steps, including growing each strain in broth culture and testing the supernatant for its ability to collapse a water drop on a hydrophobic surface; this can be highly labor- and time-intensive and thus not suitable for a truly high-throughput screen in which thousands of strains would need to be tested. Furthermore, this test is generally used as a qualitative assay only, and a measurement of the collapsed water droplet under a microscope or many serial dilutions of each sample is required to get a semi-quantitative estimate of surfactant abundance (Bodour and Miller-Maier, 1998; Chen *et al*., 2007). For this reason, high-throughput use of the drop-collapse assay in a

mutagenesis screen would not identify strains which have either increased or incomplete loss of surfactant production.

A novel biosurfactant detection method was developed here in order to quickly screen large numbers of bacteria for surfactant production directly on an agar plate. This atomized oil method is at least as sensitive as the drop-collapse assay, and was found to be useful for all tested biosurfactant-producing strains as well as synthetic surfactants. Additionally, it is semi-quantitative, and is capable of identifying intermediate phenotypes. As an illustration of this method, the atomized oil procedure was used in the context of a high-throughput screen of mutants of *P. syringae* B728a to identify those altered in surfactant production. This method proved very effective, identifying multiple mutations of the gene cluster encoding the non-ribosomal peptide synthetase responsible for syringafactin production, as well as several genes involved in its regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. syringae* pv. syringae B728a (Loper and Lindow, 1987), *P. syringae* pv. tomato DC3000 (Berti *et al*., 2007), and *Pseudomonas fluorescens* SS101 (de Bruijn *et al*., 2008) were maintained on King's medium B (KB) plates with 1.5% technical agar (King *et al*., 1954) and grown at 28 °C. *E. coli* strains DH5α, BW20767 (Larsen *et al*., 2002) and SM10(λpir) (Delorenzo *et al*., 1990), *Bacillus subtilis* 3610 (Kearns and Losick, 2003), and *Pseudomonas aeruginosa* PA14 (Caiazza *et al*., 2005) were maintained on Luria Agar and cultured at 37 °C. Antibiotics were used at the following concentrations (μg/ml): kanamycin (25 for *P. syringae*, 50 for *E. coli*), rifampin (100), gentamycin (75) and spectinomycin (100).

Biosurfactant detection assays. The drop-collapse assay was performed as according to Bodour and Miller-Maier (Bodour and Miller-Maier, 1998). 2 μl 10W-40 Pennzoil® (Pennzoil Products Company, Houston, TX, USA) was applied to delimited wells on the lid of a 96-well plate and allowed to equilibrate at room temperature. Next, 5 μl of either diluted surfactant samples or supernatant from bacterial cultures or resuspended bacterial colonies were pipetted onto the oil surface. Drops which retained a spherical shape were scored as negative for surfactant content, while drops which had a visibly-decreased contact angle with the oil and spread (collapsed) were scored as positive for surfactant content.

The atomized oil assay was conducted as follows: Bacteria were spotted onto LB or KB agar plates using sterile toothpicks and grown overnight. For more uniform inoculation of plates with cells diluted to a common cell concentration, a colony was resuspended in phosphate buffer, the OD_{600} determined in a spectrophotometer, and a small volume of suspension containing the desired number of cells was pipetted onto the plate surface and incubated overnight. Alternatively, if visualizing purified surfactant, 5 μl of diluted surfactant was pipetted onto the plate and allowed to equilibrate for 30 minutes before assaying. An airbrush (Type H; Paasche Airbrush Co., Chicago, IL) was used to apply a fine mist of mineral oil (light paraffin oil, Fisher Scientific) onto the plate with an air pressure between 15 and 20 psi. Depending on the airbrush and setup used, experimenters will need to optimize the appropriate settings in order to deposit a constant and controlled stream of oil droplets. Biosurfactant halos were then immediately visualized with an indirect source of bright light. Halo radii were measured with a ruler from the leading edge of the bacterial colony to the edge of the surfactant halo.

Microscopy. Bright-field microscopy of oil droplets was performed on a Zeiss Lumar V12 microscope using transmitted light at 80x magnification. The microscope was fitted with a CCD camera (QImaging), and images were captured using Ivision software (BioVision Technologies). Images were processed by Adobe Photoshop (Version 6.0).

Extraction of syringafactin, surfactin and rhamnolipids. Crude biosurfactant extracts were prepared with modification to the protocol detailed by Berti *et al*. (Berti *et al*., 2007). Instead of broth cultures, agar plates with confluent lawns of *P. syringae* B728a were grown for 48 hours, while *P. aeruginosa* and *B. subtilis* strains were grown for 24 hours. Cells were harvested from four plates in 90 ml H_2O and centrifuged (5,000 x g, 10 min). This was due to an increased yield of biosurfactant on solid medium, an observation which is being pursued in a separate report. The supernatant was extracted with 150 ml ethyl acetate with 1% (vol/vol) formic acid and the organic fraction was dried to completion. This material was resuspended in 20 ml H_2O, the pH was increased to 8.0 with dilute NaOH, and again dried to completion. This was then resuspended in 4 ml of methanol, filtered though a 0.45 μm Nalgene filter (Fisher Scientific) and dried to completion (Berti *et al*., 2007). The final product was weighed and diluted with deionized water for further testing.

Production of biosurfactant mutants. The production of transposon mutants was done by a method similar to that of Larsen *et al*. (Larsen *et al*., 2002). Briefly, *P. syringae* B728a and one of the two conjugative *E. coli* strains were grown overnight on agar plates with appropriate antibiotics. Strain BW20767 harboring plasmid pRL27 (Larsen *et al*., 2002) has a kanamycin resistance-conferring mini-Tn*5* transposon with a hyperactive Tn*5* transposase, and strain SM10(λpir) harboring pUT mini-Tn*5* Sm/Sp (Delorenzo *et al*., 1990) has a spectinomycin-resistance transposon. Cells were then harvested with a loop, washed and resuspended in potassium phosphate buffer (10 mM, pH 7.5), and then mixed in a ratio of 1:3 (*E. coli* : *P. syringae*) and incubated overnight as a confluent lawn on a KB plate. After incubation, the cells were resuspended in phosphate buffer and 1/10 of the resuspension was plated onto KB medium containing 100 μg/ml rifampin and either 25 μg/ml kanamycin or 100 μg/ml spectinomycin as appropriate, and allowed to grow for three days.

Screening of mutants. *P. syringae* transposon mutants were screened by the following method: Mutants were spotted using sterile toothpicks from selection plates onto KB plates, with spots separated by at least 2 cm. Colonies were allowed to develop overnight and then sprayed with atomized mineral oil drops as described above. Mutants which displayed substantially larger (over 20%) or smaller halos were re-tested. Mutants with phenotypes that were consistently different from the wild-type strain were further investigated. The location of the transposon insertion in these mutants was determined using arbitrarily-primed PCR similar to the method of O'Toole *et al*. (O'Toole *et al*., 1999). To identify mutations generated by the transposon from plasmid pRL27, primers complementary to the 5' end of the transposon were designed. Primer pRLext1, 5'-CGAACTAAACCCTCATGGCTAACG**,** was used in the initial PCR reaction, and the primer pRLint1, 5'-AACAAGCCAGGGATGTAACG, was used in the second reaction to amplify sequences 5' to the insertion site. The PCR product was cleaned (QIAquick PCR Purification kit, Qiagen) and submitted for sequencing with primer pRLint1. When working with the transposon from pUT mini-Tn*5* Sm/Sp, identification of the 5' insertion site followed

the same protocol except the initial PCR primer tn5sm-ext was 5'-GCGCGAGCAGGGGAATTG and the second round primer tn5sm-int was 5'-CGGTTTACAAGCATAAAGCTTGCTC. The locations of the sequenced fragments were determined directly by a BLAST search on the *Pseudomonas* genome database (Winsor *et al*., 2009) and compared to the published sequence of *P. syringae* B728a (Feil *et al*., 2005).

Swarming motility assay. Swarming motility of *P. syringae* B728a was assessed on semisolid KB plates containing 0.4% technical agar as in previous studies (Quinones *et al*., 2005). Cells were grown for 2 days on KB and then harvested and washed in potassium phosphate buffer (10 mM, pH 7.5). Cells were resuspended in buffer to an OD_{600} of 0.27**,** and 5 µl (approximately 2.5 X 10^6 cells) of the appropriate bacterial strain was pipetted onto each plate and incubated for 24 hours at room temperature. Swarming distance was calculated as the average diameter of swarming fronts chosen randomly from two perpendicular vectors for each colony.

Construction of a P*syfA-gfp* transcriptional fusion. The upstream promoter region of the *P. syringae* B728a *syfA* gene was amplified by PCR from genomic DNA with primers syf5-HindIII 5'-TAAGCTTCTTGAGCTTTCCTGATTCCGACCGC and syf3-EcoRI 5'-TGAATTCGGCTCAAGGTCCTTCTTGGCGGG to generate a 289-bp promoter region. PCR conditions were as follows: 28 cycles of 95°C, 59°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. The PCR product was first cloned into pTOPO Blunt (Invitrogen) to generate pTOPO-P*syfA*, and then transformed into *E. coli* DH5α. The insert was sequenced to verify its identity. pTOPO-P*syfA* was digested with *HindIII* and *EcoRI*, and the resulting fragment was cloned into pPROBE-OT (Miller *et al*., 2000) which contains a promoterless *gfp* gene to generate pP*syfA-gfp*.

pP*syfA-gfp* was electroporated into *P. syringae* B728a as well as mutant strains altered in biosurfactant production (Table 3). The appropriate transformed strains were grown overnight on KB plates, then resuspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD_{600} of 0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with a 486-nm bandpass excitation filter and a 510- to 700-nm combination emission filter. A relative fluorescence unit (RFU) was defined as the fluorescence of the suspensions normalized for the suspension turbidity measured as OD_{600}.

Statistical analysis. Most data and regression analysis was carried out using Statistica (StatSoft, Tulsa, OK). Graphs were constructed in CoPlot (CoHort Software, Berkeley, CA).

RESULTS

Detection of biosurfactants with an atomized oil method. A novel surfactant detection assay was developed using *P. syringae* B728a which produces the lipopeptide surfactant syringafactin as a test organism. Syringafactin has previously been demonstrated to be a surfactant by use of the drop-collapse assay; supernatant from *P. syringae* DC3000 collapses on a hydrophobic surface, demonstrating the presence of a surfactant, while supernatants from mutant strains which do not produce syringafactin do not. Although they focused on characterizing the syringafactin extract from *P. syringae* DC3000, the authors also confirmed that syringafactin is produced in strain B728a (Berti *et al*., 2007). We developed a method of surfactant detection involving the misting of oil droplets onto agar plates, hypothesizing that the presence of

surfactants would alter the interaction of the oil with the agar surface. When a fine mist of mineral oil was sprayed over the surface of a KB agar plate on which bacterial colonies of *P. syringae* B728a had grown, a light-diffractive halo was seen around the colonies (Fig. 1B). In contrast, no such halo was observed around *E. coli* DH5α (Fig. 1A), a strain which is not predicted to produce a biosurfactant.

Upon microscopic inspection, it was seen that oil droplets on an un-inoculated agar surface and near DH5α were in energetically unfavorable distorted shapes (Fig. 1E). This was presumably due to random heterogeneity in the hydrophobicity of the agar surface. However, when surfactants spread over the agar surface such as in the vicinity of *P. syringae* B728a, the droplets assumed a more uniform, energetically favorable hemispherical shape (Fig. 1F). Furthermore, the light-diffractive halo observed macroscopically was actually caused by the de-wetting, or beading, of the oil droplets near the surfactant-producing bacteria. The oil droplets, which presumably were in contact with the biosurfactant, stood higher on the plate and appeared more spherical than droplets on the agar surface away from surfactant-producing colonies (Fig. 1J). These raised droplets reflected light at a different angle, making them appear brighter under an indirect source of light.

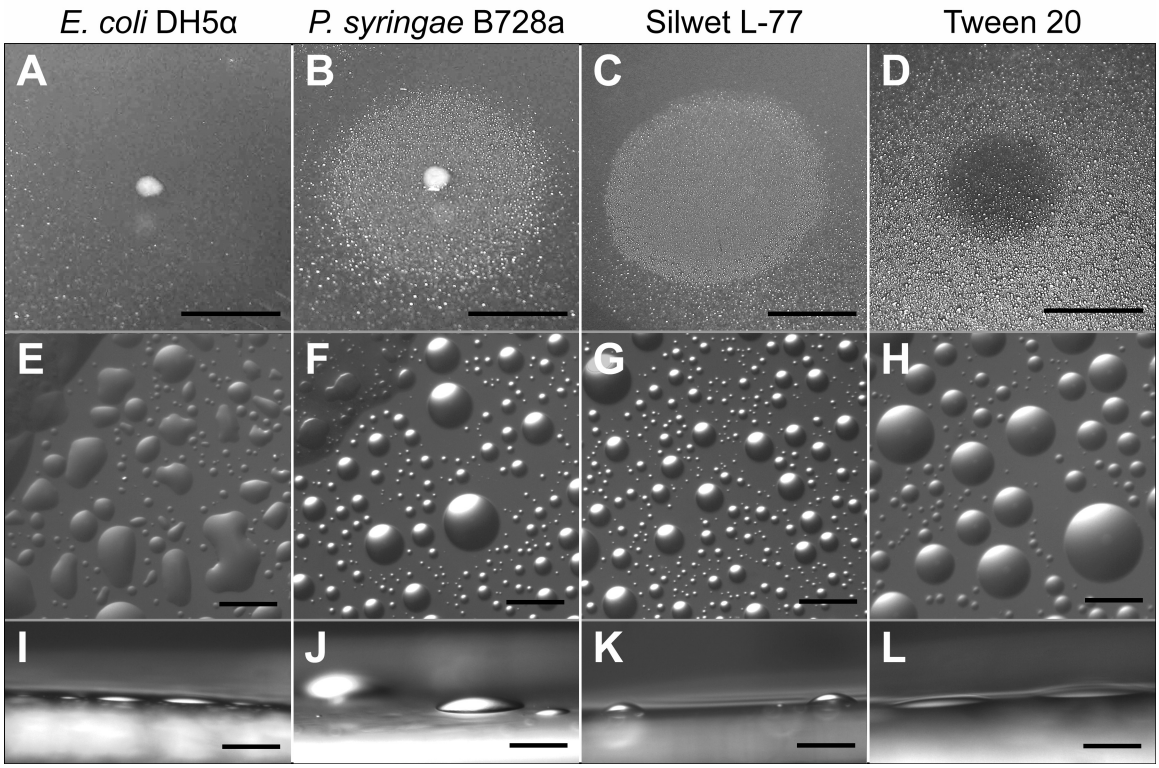

Figure II-1. Atomized oil assay
Comparison of the atomized mineral oil droplets deposited on agar plates around a growing colony of *E. coli* DH5α which does not produce biosurfactant (A,E,I), a growing colony of *P. syringae* B728a which produces biosurfactant (B,F,J), Silwet® L-77 at a 500-fold dilution (C,G,K), and Tween 20 at a 5-fold dilution (D,H,L). Images A-D present overviews of halos seen with this assay, and the bars represent 1 cm. Images E-G are microscopic close-ups of the oil droplets observed within the halos viewed from the top, while images H-L droplets as viewed from the side. Bars represent 0.2 mm for microscopic images E-L.

In order to show that this atomized oil assay was indeed detecting biosurfactant, we obtained a variety of strains with characterized biosurfactant production and for which isogenic strains blocked in biosurfactant production are available. In addition to *P. syringae* DC3000 which produces syringafactin, we tested *Pseudomonas fluorescens* SS101, *Bacillus subtilis* 3610, and *Pseudomonas aeruginosa* PA14. All of the tested biosurfactant-producing bacterial strains produced easily detectable bright halos when sprayed with atomized mineral oil, while none of the biosurfactant mutants exhibited halos in this assay (Table 1). Thus all biosurfactants tested were readily detected with the atomized oil assay and no evidence of false positive indications of surfactant activity was obtained.

Table II-1. Surfactant production by characterized biosurfactant-producing bacterial strains detected with an atomized oil assay

Organism (and reference)	Surfactant produced	Type of surfactant	Halo radius[a] (mm)
Bacillus subtilis 3610	Surfactin	Lipopeptide	9.5 ± 0.5
B. subtilis mutant *srfAA* (Kearns and Losick, 2003)			0
Pseudomonas aeruginosa PA14	Rhamnolipid	Glycolipid	2.4 ± 0.2
P. aeruginosa mutant *rhlA* (Caiazza *et al*., 2005)			0
Pseudomonas fluorescens SS101	Massetolide A	Lipopeptide	8.3 ± 0.3
P. fluorescens mutant *massA* (de Bruijn *et al*., 2008)			0
Pseudomonas syringae DC3000	Syringafactin	Lipopeptide	3.6 ± 0.2
P. syringae mutant *syfA* (Berti *et al*., 2007)			0

[a] Values are average measured atomized oil halos, ± the standard deviation from triplicate samples

The atomized oil assay can detect a wide variety of surfactants. While this new assay readily detected a variety of both lipopeptides and glycolipids of bacterial origin, we tested the behavior of other types of surfactants with this procedure. All of a variety of commercially available surfactants were detectable by this assay (Table 2). Many of the surfactants behaved similarly to the biosurfactants, causing the oil droplets to assume raised hemispherical shapes that appeared bright when illuminated (Fig. 1C, 1J, 1K). However, a few of the surfactants created a less obvious "dark halo" in which the oil droplets still assumed a circular form, but were less hemispherical and had an increased contact with the water-agar surface (Fig. 1D, 1H, 1L). These "dark halo" droplets, in contrast to the raised droplets in "bright halos," were flat and appeared less bright than the surrounding surfactant-free droplets at certain angles. Interestingly, when the surfactants were ranked by their hydrophilic-lipophilic balance (HLB) values, a common value used to describe surfactants in industry, it was found that surfactants with low HLB values all yielded bright halos while those with higher HLB values resulted in dark halos (Table 2).

Table II-2. Comparison of the detection of a variety of surfactants with a droplet collapse assay and an atomized oil assay.

Surfactant	Hydrophilic-lipophilic balance[a] (HLB)	Type of halo	Limit of detection[b] (g/L)	
			Drop-collapse	Atomized oil
Crude syringafactin[c]	n/a	Bright	0.5	0.01
Crude surfactin[c]	n/a	Bright	15	0.25
Crude rhamnolipid[c]	9.5	Bright	7.5	0.25
Silwet® L-77	8-10	Bright	0.25	0.0125
CTAB	n/a	Bright	0.5	0.001
Tergitol®-7	n/a	Bright	2.5	0.025
Triton® X-100	13.5	Dark	0.25	0.125
Tween 80	15.0	Dark	n/a	0.25
Tween 20	16.7	Dark	10	0.5
SDS	40.0	Dark	2	0.1

[a] HLB values are as given in McCutcheon's Emulsifiers & Detergents, North American Edition. MC Publishing Co., Glen Rock, NJ, with the exception of rhamnolipid (Oberbremer *et al*., 1990) and Silwet® (Jin *et al*., 2008). "n/a" denotes surfactants for which an HLB value has not been conclusively determined.

[b] These values are the lowest dilutions of surfactant which still yielded visual detection by the respective assays. "n/a" denotes samples which were undetectable by the assay at any concentration.

[c] Sample represents ethyl acetate extract of culture supernatant from biosurfactant producing strains

Sensitivity of the atomized oil assay. The sensitivities of the atomized oil and drop-collapse assays to detect a variety of surfactants were compared. Using a range of dilutions of a given surfactant, we determined the lowest concentration of that surfactant that was still detectable by a given assay. Additionally, crude extracts of surfactin, rhamnolipid and syringafactin were prepared, and their limits of detection by the two assays were compared. For all tested surfactants and biosurfactants, the atomized oil assay was found to be more sensitive than the drop-collapse assay (Table 2). In general, the atomized oil assay detected surfactant at concentrations more than 10-fold lower than that of the drop-collapse assay.

In order to relate the size of the observed halo around a source of surfactant to the amount of that surfactant, different dilutions of a syringafactin-containing extract were tested with the atomized oil assay and halo diameters were measured. A log-linear relationship between the amount of surfactant applied to plates and the diameter of the halo was observed (Fig. 2A). Thus a quantitative estimate of the relative difference in amounts of surfactant in different prepared samples can be readily estimated. For each 10-fold increase in concentration of the spotted surfactant, the radius of oil drop alteration increased by about 1.7 mm. Because halo sizes were very consistent for a given amount of surfactant, with standard deviations rarely above 0.25 mm, careful replicate measurements of halos should easily enable the distinction in amounts of surfactant that differ by three-fold or more. However, it must be emphasized that such semi-quantitative estimates are only relevant when comparing samples of the same surfactant on a single medium, since different surfactants will diffuse at different rates.

While it may be possible to quantify the surfactant in a prepared sample by measuring halos, calculating the surfactant produced by a bacterial colony is confounded by the additional parameter of time. The prepared samples discussed above were applied at a distinct time and measured one hour later, but bacterial colonies could produce surfactant over many hours of growth. Given that the distance over which a specified amount of surfactant will spread across an agar surface would be expected to be somewhat dependent on time, we determined the extent to which this factor would influence estimates of surfactant concentration using the atomized oil assay. A fixed concentration of a syringafactin-containing extract from *P. syringae* B728a was applied to agar plates and destructively analyzed by the atomized oil assay at various times after application. Halo radii continued to increase with time, although the rate slowed considerably after about two hours (Fig. 2B). Because of this, in addition to the fact that the bacteria continue to multiply and that the production of many biosurfactants is regulated by cell density (Raaijmakers *et al*., 2006), we concluded that halo measurements could not be used to calculate the absolute amount of surfactant produced by a colony without further investigation. Fortunately for screening purposes, relative amounts of surfactant production should be readily assessed using the atomized oil method unless the growth rate of the strains being compared differs greatly.

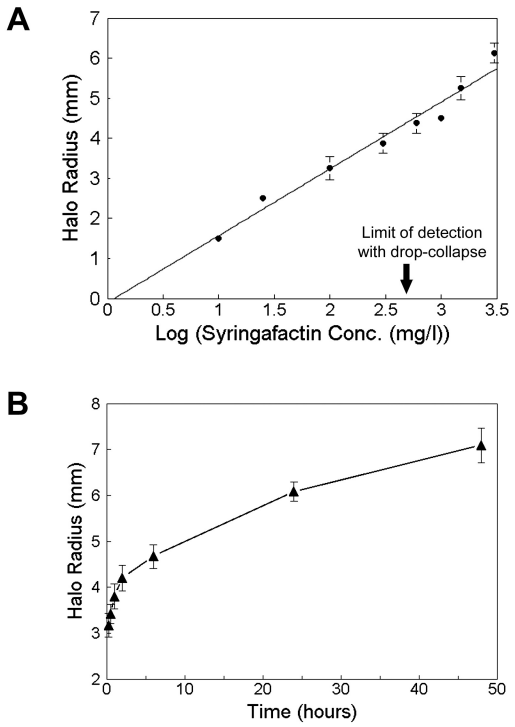

Figure II-2. Effect of concentration and time on atomized oil halo size
Effect of syringafactin concentration (A) and diffusion time (B) on size of halos produced in an atomized oil assay. Vertical bars represent the standard deviation of the mean of 4 replicate measures for each point. The line drawn in A represents the linear relationship $Y= 1.67X – 0.106$ ($R^2= 0.97$; $P<0.0001$). The results are representative of three independent experiments.

Given that a consistent estimate of surfactant production from a given bacterial strain would be needed to compare strains in a high-throughput survey, we estimated variance in estimates of syringafactin production in replicate cultures of wild-type *P. syringae* B728a. Replicate cultures of *P. syringae* were established on plates by toothpick inoculation. On average, about 2.3 ± 0.6 X 10^6 bacteria were applied to a plate using this technique. Radii of halos from the resulting syringafactin production after colony formation were 8.8 ± 0.8 mm. To determine if variations in the number of cells initially deposited to establish spots (colonies) affected the apparent surfactant production, a defined number of cells (10^7) were applied in replicate spots onto the plate and oil was sprayed onto the plates after incubation overnight as in the toothpick-inoculated plates. The radii of oil drop halos around these replicate spots (8.9 ± 0.6 mm) exhibited a similarly small variation as those around colonies established by toothpick inoculation. Application of cells by toothpick therefore results in inconsequential variations in eventual surfactant production as measured by this assay. Due to this limited variation, any strains displaying a halo that differed in radius by 20% or more than a reference strain would likely be significantly different in surfactant production. However, it is important to later confirm the regulation transcriptionally, in the event that a smaller halo is the result of a slower growth rate in a mutant strain.

Mutant analysis of surfactants produced by *P. syringae* B728a on plates. The atomized oil assay was used to individually screen a library of about 7,700 transposon mutants of *P. syringae* for surfactant production. Mutants with a halo radius that differed by more than 1.5 mm from that of wild-type colonies were identified in an initial assessment; this should correspond to an approximate 10-fold increase or decrease in surfactant production. Mutants with large growth defects were discarded based on the logic that fewer cells will produce less total surfactant, although three mutants with slight growth defects were saved for further testing, which includes a cell-normalized measurement of surfactant production. These mutants with visible growth defects were later determined to have insertions in the *suhB* homolog Psyr_1233, the *secA* homolog Psyr_4094, and a PhoH-like protein Psyr_4346. No mutations were observed to cause visible increases in the growth rates.

28 total mutants with significantly altered surfactant production were identified after replicate tests (Table 3). Identification of the sites of transposon insertion revealed that over half of the identified mutants harbored distinct insertions in genes found to be disrupted in at least one other mutant, yielding a total of 12 different genes found to significantly influence surfactant production in strain B728a. The largest number of mutants (9) harbored insertions in the large gene cluster encoding the non-ribosomal peptide synthetase for syringafactin (Berti *et al*., 2007). Given that disruption of this locus in strain B728a greatly decreased surfactant production (Fig. 3), it appears that syringafactin is a major component of the observed surfactant halo in strain B728a. However, the remaining halo suggests that B728a produces a second surfactant in addition to syringafactin, which is in contrast to *P. syringae* DC3000, where disruption of the gene cluster encoding the non-ribosomal peptide synthetase for syringafactin completely blocks all surfactant production as detectable by the atomized oil assay (Fig. 3). If the remaining bright halo corresponds to a biosurfactant with similar diffusional properties as syringafactin, then the observed halo radius of approximately 5.5 mm in a syringafactin knockout (as compared to 8.7

Table II-3. Identification and characteristics of a variety of mutants of *Pseudomonas syringae* strain B728a with altered biosurfactant production identified using an atomized oil assay

Locus of Tn*5* insertion	Predicted function	Individual transposon hits[a]	Surfactant halo radius[b] (mm)	Drop-collapse[c]	Swarming diam[d] (mm)	SyfA expression[e]
WT (no insertion)			8.7 ± 0.2	Yes	24.1 ± 0.5	1716 ± 41
Psyr_1233 (*suhB*)	Inositol monophosphatase	1	5.0 ± 0.2	No	6.7 ± 0.2**	110 ± 12**
Psyr_4094 (*secA*)	Protein secretion	1	5.3 ± 0.5	Yes	12.5 ± 3.2**	2232 ± 116**
Psyr_2576 (*syfA*)	Syringafactin biosynthesis	4	5.3 ± 0.3	No	10.8 ± 0.3**	n/a
Psyr_2577 (*syfB*)	Syringafactin biosynthesis	5	5.8 ± 0.4	No	10.17 ± 0.3**	n/a
Psyr_4346	Unknown	1	6.0 ± 0.4	Yes	14.3 ± 0.7**	900 ± 27**
Psyr_3619	RNA processing and degradation	3	6.2 ± 0.2	No	13.0 ± 0.6**	53 ± 1**
Psyr_3958 (*algT*)	Alginate regulation	1	11.0 ± 0.6	Yes	32.3 ± 0.8**	2691 ± 69**
Psyr_1407 (*pmpR*)	Virulence factor regulation	1	12.0 ± 0.5	No	32.3 ± 3.8**	87 ± 3**
Psyr_1747 (*clpP*)	Posttranslational modification	1	12.3 ± 0.4	Yes	19.3 ± 0.7**	626 ± 64**
Psyr_3957 (*mucA*)[f]	Alginate regulation	1	12.3 ± 0.4	Yes	20.3 ± 1.4**	2378 ± 63**
Psyr_1748 (*clpX*)	Posttranslational modification	5	12.2 ± 0.9	Yes	21.5 ± 1.3*	1595 ± 54
Psyr_1350 (*rseP*)	Alginate regulation	4	12.7 ± 0.3	Yes	32.8 ± 1.7**	2655 ± 71**

[a] Number of times that independent mutants were identified as insertions in the same gene

[b] Halos with significantly smaller or larger radii compared to WT (For all, $P < 0.01$, *t*-test)

[c] Ability to cause drop-collapse of a water droplet on an oil surface

[d] Bacterial motility over semi-solid agar plates: motility was significantly different from wild-type at $P<0.05$ (*) or $P < 0.01$ (**) as determined by a *t*-test

[e] Arbitrary units of relative fluorescence of the P*syfA*::*gfp* reporter in mutant strains: expression was significantly different from wild-type at $P < 0.01$ (**) as determined by a *t*-test

[f] Original mutant strain had a decreased halo size. This strain saved for testing most likely possesses a secondary mutation which has reversed the phenotype of the original mutation.

mm in wild-type) corresponds to a 2-log decrease in total surfactant concentration, implying that the second surfactant is only produced at approximately 1% of the levels of syringafactin production.

In addition to insertions in the syringafactin biosynthetic cluster, a number of other insertions were found to significantly affect surfactant production. In total, 19 additional insertions in a total of 10 genes resulted in strains that consistently produced smaller or larger halos compared to the wild type (Table 3). All of these mutations were within the structural genes noted with the exception of Psyr_3958, the sigma factor AlgT, in which the transposon was inserted less than 30 base pairs upstream of the structural gene, presumably disrupting transcription of the gene. All disrupted genes were under 1,000 amino acids in length, with the exception of the *syfA* and *syfB* homologs which are about 3,000 and 6,000 amino acids in length, respectively. The relatively higher frequency of mini-Tn*5* transoposon insertions into the syringafactin biosynthetic cluster reflects the increased probability of a random insertion event into such a large target, although a few of the smaller genes also had multiple insertions (Table 3).

As a further assessment of surfactant production in the mutants obtained in the screen, their ability to cause a drop-collapse was also evaluated (Table 3). Of the four possible permutations of relative halo size and drop-collapse activity, most mutants were found to fall into the three following categories: 1) Mutants including the syringafactin knockouts which had smaller surfactant halos and no drop-collapse activity, 2) Mutants with smaller surfactant halos but which still conferred drop-collapse, suggesting that syringafactin production has been reduced but not completely blocked, and 3) Mutants with larger surfactant halos (which still produced a drop-collapse). The twelve mutants belonging to this third category were all found to harbor insertions in multiple components of the AlgT extracellular stress pathway: AlgT, MucA, RseP, ClpX and ClpP (Keith and Bender, 1999; Chaba *et al*., 2007). All of the mutants were originally identified as producing a larger surfactant halo, except for one with a disruption of the anti-sigma factor MucA. This mutant was initially noted to confer a smaller halo than the WT strain and to have a highly mucoid phenotype. However, upon retesting this mutant after passage in culture for several generations, it switched to having a large halo and a non-mucoid phenotype (Table 3), a phenomenon that will not be further addressed here.

The most surprising result was the identification of a mutant which fell into the fourth category, having a larger surfactant halo but which did not produce a drop-collapse (PmpR). Most likely this PmpR mutant no longer produces syringafactin but over-produces a second surfactant (Table 3). The discovery of this mutant suggests that the second surfactant is much weaker than syringafactin, such that even when produced in large quantities, it does not lower water surface tension enough to cause a droplet of water to collapse on an oily surface. Alternatively, it could suggest that it has low water solubility (a very low HLB value). Another possibility is that the atomized oil assay could be responding to a substance other than a surfactant, although it is unclear what that substance could be. A surfactant is by definition a surface active agent, and the most probable reason for a change in contact angle of an oil droplet on an aqueous surface would be a change in surface tension.

Because surfactant production is generally required for bacterial swarming ability, the movement of the surfactant mutants was measured. Unlike in DC3000, where a mutant blocked in

syringafactin production could no longer swarm (Berti *et al*., 2007), the syringafactin mutants in B728a were still able to swarm slowly (Fig. 3). This is consistent with the reduced but not eliminated surfactant production in these mutants. Mutants blocked in each of the 12 genes found to alter biosurfactant production each also had altered swarming phenotypes (Table 3). In general, strains with apparently higher surfactant production as evidenced by larger halos in the atomized oil assay swarmed faster, while those with smaller halos swarmed slower.

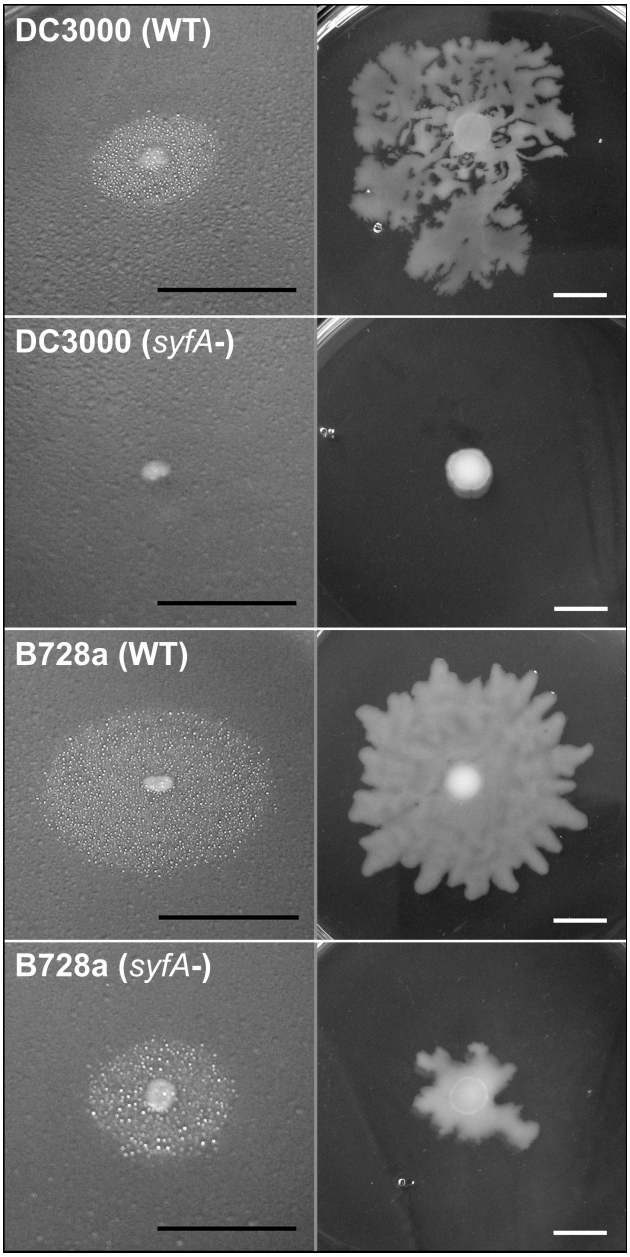

Figure II-3. Surfactant halos and swarming assays of syringafactin mutants
Comparison of surfactant halos (left) and extent of swarming (right) by wild-type *P. syringae* strains DC3000 and B728a and their respective syringafactin mutants (*syfA*-). The bars represent 1 cm.

Regression analysis of the influence of halo size on swarming distance was highly significant (Fig. 4A). Even the PmpR mutant, having a large halo but not conferring a drop-collapse, followed this relationship and swarmed significantly farther than the wild-type strain. In general, the atomized oil assay was much more indicative of swarming ability than the drop-collapse assay. The mutants for which swarming was not predicted based on halo size had insertions in the genes encoding MucA, ClpX and ClpP; all produced large halos but had slightly lower swarming ability. The swarming distance for these mutants is apparently confounded by the phenotypic changes in these strains; these mutants initially swarmed as fast as the other mutants with large halos (first 16 hours) but subsequently had a dry appearance which seemed to suppress their rate of swarming as the colonies aged.

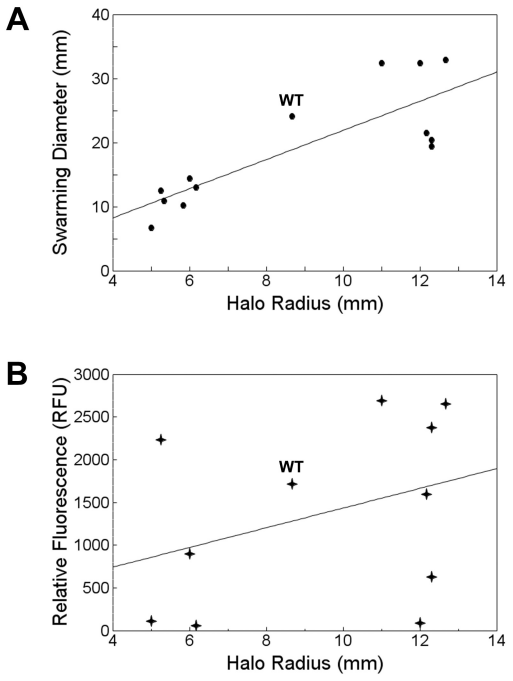

Figure II-4. Halo size correlated with swarming distance and syringafactin transcription
Relationship between surfactant halo sizes produced by mutants of *Pseudomonas syringae* B728a and swarming distance (A) and syringafactin transcription as estimated by GFP fluorescence of a P*syfA-gfp* fusion (B). Wild-type is included in both figures and denoted with a "WT." Coordinates are taken directly from the measurements presented in Table 3. The lines drawn represent the linear relationship Y= 2.275X – 0.8244 (R^2= 0.68; P<0.001) and Y= 115.27X + 282.6 (R^2= 0.12; P<0.3), respectively.

Surfactant production in mutants compared to transcription of syringafactin locus.
Syringafactin appears to be a major surfactant produced by strain B728a since mutants in its biosynthesis exhibit greatly reduced surfactant halos, drop-collapse ability and swarming. Because the atomized oil assay was highly predictive of the effect of surfactant production on swarming but not drop-collapse, we determined how predictive halo measurements would be of syringafactin production. It seemed likely that many of the mutants with altered surfactant

production identified in the atomized oil assay would exhibit altered expression of the genes required for syringafactin production. To test this, a vector in which the promoter-containing region of the syringafactin biosynthetic locus *syfA* was fused to a *gfp* reporter gene was introduced into each of the surfactant mutants and GFP fluorescence was measured. Furthermore, this calculation was cell-normalized, and would therefore identify any mutants with smaller halos resulting from an altered growth rate if they had smaller surfactant halos than wild-type, but similar SyfA transcription.

All putative surfactant mutants identified by the atomized oil assay had altered expression of *syfA* compared to wild-type with the exception of the ClpX mutant (Table 3). However, when considering all of the mutants, no direct correlation between halo size and syringafactin gene expression was found (Fig. 4B), most likely because of the confounding effect of the production of a second surfactant. For instance, the PmpR mutant has a very large surfactant halo and swarms well, but it does not produce syringafactin, suggesting that the putative second surfactant is highly up-regulated in this strain. It appears that in different genetic backgrounds, the two surfactants contribute differentially to the observed halo and swarming phenotypes. Therefore, the apparent presence of a second surfactant readily explains why the sizes of the aggregate surfactant halos are not correlated with the production of just one of the surfactants. The atomized oil assay has thus enabled the identification of promising regulatory genes for biosurfactant production.

DISCUSSION

The discovery of novel biosurfactants and the exploration of the genomics of biosurfactant production would greatly benefit from a quantitative and high-throughput screening method. The features of the atomized oil assay demonstrated here should make it valuable for these purposes. Multiple strains can be simultaneously assayed within a few seconds, thus enabling thousands of strains to be screened for surfactant production in a reasonable time. Although all of our measurements were taken on KB and LB agar plates, we have found that this assay works well on any solid medium which is conducive to bacterial surfactant production. Additionally, given a standard, this assay can provide estimates of surfactant concentrations.

It may seem counterintuitive that biosurfactants cause the oil to bead in our assay, whereas surfactants normally cause water droplets on a hydrophobic surface to collapse. The shape of an oil droplet on an aqueous surface is determined, however, not just by the change of surface tension at the oil/water interface, but also by the counteracting force from the tension change at the air/water interface (Donahue and Bartell, 1952). If an added surfactant lowers the surface tension at the air/water interface more than at the oil/water interface, thermodynamics will favor a decrease of the relative contribution of the oil/water tension, seen as an increased contact angle between the oil and water, and hence a beading of the oil droplet (Fig. 5). In this manner the shape of the oil droplet is determined by the action of the surfactant at the two different interfaces. In general, bright halos such as those conferred by all of the biosurfactants tested result when the predominant effect of the surfactant is on the air/water interface. Although we have arbitrarily classified surfactants as either causing either a “bright” or “dark” halo in oil drops surrounding a surfactant source, it is most probable that there is a spectrum of contact angles for the oil droplet that is dictated by the expected range of change of the various tensions by various surfactants as discussed above. Similarly, while all of our obtained mutants displayed

bright halos, there is a possibility that the contact angles of the oil droplets could be slightly different, especially near mutants unable to produce syringafactin. However, we have not yet found a reliable method for measuring the contact angles of the atomized oil droplets observed with our assay, and no obvious differences in droplet shape were detected during microscopic observation of the droplets.

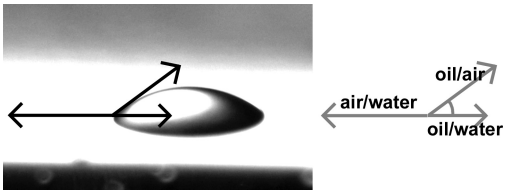

Figure II-5. Diagram of the interfacial tensions acting on an oil droplet
Relationship between interfacial tensions and the contact angle of the oil droplet on the agar-water surface (θ). To keep forces in balance; (tension at air/water) = (tension at oil/water) + (tension at oil/air * Cosine θ). Tension at oil/air interface is a fixed value because it is rarely influenced by surfactants (Myers, 2006). Therefore, as the air/water tension decreases from added surfactant, as does the tension at the oil/water interface, the contact angle θ of the oil droplet will change to compensate for the inequal effect of the surfactant on those two interfaces.

It is not clear if there is an invariant correlation between a surfactant's hydrophilic-lipophilic balance and the shape it imparts to oil droplets on an agar surface. It is, however, tempting to speculate on the utility of this assay in predicting important characteristics of novel surfactants. HLB values are a scalar factor that reflects the degree to which a surfactant is hydrophilic or lipophilic, with a value of zero reflecting a completely lipophilic (hydrophobic) molecule, a value of 10 corresponding to a compound with equivalent hydrophobic and hydrophilic groups, and values over 10 for predominantly hydrophilic molecules. This value is of great significance commercially since it is used to determine appropriate functions of surfactants. For example, common surfactants such as SDS and Tween 20 have high HLB values and are therefore best suited for emulsifying a hydrophobic substance into a water phase (oil into water). On the other hand, surfactants such as Silwet® L-77 with HLB values near 10 are more suited for wetting, or spreading of a water phase over surfaces such as leaves (Adamson, 1982; Zhang *et al*., 2006). These surfactants with balanced water- and oil- loving groups can be very effective as spreading agents, capable of lowering the surface tension of water below 30 mN/m (Lang and Wagner, 1993). Rhamnolipid, with a predicted HLB of 9.5, which can lower the surface tension of water to 28 mN/m, is a highly effective spreading agent involved in bacterial motility (Oberbremer *et al*., 1990). Although there is no consensus on the HLB of surfactin, it is also capable of lowering the surface tension of water to 27 mN/m, suggestive that it may also have an HLB near 10 (Lang and Wagner, 1993; Rosenberg and Ron, 1999). Surfactants like Silwet® L-77 which had lower HLB values conferred bright halos in our assay. The surfactants with HLB values over 13, which are most ideal for emulsification of oil into water, did not cause the oil droplets to bead, resulting in dark halos when tested by the atomized oil assay. It is interesting that none of the biosurfactants tested conferred dark halos, suggesting that their primary roles are not as emulsifiers.

It is noteworthy that the measurements of biosurfactant production using the halo method were strongly correlated with the swarming capability in mutants of *P. syringae* strain B728a. This suggests that the area covered by surfactants at the air/water interface as measured by our assay reflects a similar distance where swarming movement of bacteria across an aqueous agar surface is facilitated. Moreover, it is significant that drop-collapse activity was not a good indicator of the swarming ability of a strain, which raises the question of what specific properties make a surfactant a good lubricant that facilitates bacterial motility. Because the drop-collapse assay only detects surfactants that are able to greatly lower the surface tension of water, this property appears unnecessary for functions such as swarming. In addition, use of the drop-collapse assay in biological screens may cause a wide array of biologically active surfactants to be overlooked. In view of that, it is interesting that a syringafactin mutant of *P. syringae* strain B728a appears to produce a second surfactant that can promote swarming but not cause a drop-collapse. This is in contrast to a syringafactin mutant in *P. syringae* strain DC3000 which does not appear to produce this second surfactant (Fig. 3). It is also striking that no mutants were identified in strain B728a that exhibited a total absence of surfactant halo, pointing to differential regulation of syringafactin and the remaining expressed surfactant (which explains the poor correlation between biosurfactant halos and transcription of the biosynthetic gene cluster for syringafactin production). Furthermore, the disruption of *pmpR* apparently causes the down-regulation of syringafactin while conferring up-regulation of the other surfactant, suggesting its role in regulating (inversely) both surfactants. While both *P. syringae* strains are pathogenic to plants, strain B728a is a much better epiphyte than DC3000 (Boureau *et al*., 2002). Perhaps this second surfactant is particularly useful for the lifestyle of epiphytes such as strain B728a on waxy leaf surfaces. We are actively pursuing the identity and specific properties of this second surfactant. The phytotoxins syringomycin and syringopeptin have been suggested to possess surfactant activities (Hutchison and Gross, 1997), although preliminary results have not yet provided support for the identity of either of these surfactants as the second surfactant (data not shown). It is possible that combining one of the mutations found from this screen with a *syfA* or *syfB* mutation could reveal the identity of the second surfactant

Some, but not all of the genes found to regulate both biosurfactant production and swarming ability in *P. syringae* have homologs that influence swarming in *Pseudomonas aeruginosa*. Disruption of Psyr_3619, encoding an RNA helicase, conferred a similar reduction in swarming as that seen in blockage of its homolog PA2840 in *P. aeruginosa* (Overhage *et al*., 2007). Likewise, disruption of *pmpR* (PA0964) in *P. aeruginosa*, a homolog of Psyr_1407, resulted in enhanced swarming in both species (Table 3) (Liang *et al*., 2008). It is significant that *P. syringae* B728a mutations were not identified in homologs of any of the many other genes found to alter swarming in *P. aeruginosa* (Overhage *et al*., 2007) despite the near completeness of the mutant library, emphasizing that the surfactants that contribute to swarming in these strains differ and/or that many factors other than biosurfactant production contribute to swarming ability. It is also noteworthy that relatively few different genes apparently contribute to biosurfactant production in *P. syringae* B728a. The disruptions of only 12 unique genes, identified from over 7,000 screened mutants, were found to alter biosurfactant production. Assuming random transposon insertion, we predict that we have screened a library of approximately 77% of the *P. syringae* B728a genes. Although we have identified many of the mutations which have an effect on measured surfactant halos, we may have missed a number of mutations which negatively

affected syringafactin production but were masked by a compensatory increase in production of the second surfactant.

For life on the leaf surface, Pseudomonads have been shown to employ a variety of traits to grow and survive despite fluctuating water availability (Lindow and Brandl, 2003). In response to desiccation stress, Pseudomonads produce alginate in order to maintain a hydrated microenvironment (Chang *et al*., 2007). Our finding of multiple components of the AlgT regulatory pathway among mutants of strain B728a with altered biosurfactant production could suggest an intimate relationship between water availability and biosurfactant production. This potential relationship warrants further exploration of either the AlgT pathway or perhaps alginate production itself as a regulator of surfactant production. The role of biosurfactants on the leaf surface is most likely complex, and as such may likely prove to have very complex regulatory networks. The atomized oil assay has revealed a likely diversity of biosurfactants that are produced by strain B728a and their complex patterns of expression, details that would have been difficult to discern using other assays for biosurfactant production. The tools and genetic resources developed here should prove useful in further studies of the roles of surfactants in the interaction of *P. syringae* with plants.

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Chapter III.

Habitat enrichment of contact-regulated biosurfactant production on surfaces

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ABSTRACT

Biosurfactants are diverse molecules with numerous biological functions and industrial applications. A variety of environments were examined for biosurfactant-producing bacteria using a versatile new screening method. The utility of an atomized oil assay was assessed for a large number of bacterial isolates and compared with a commonly-used drop collapse assay from broth and plate cultures. The atomized oil assay detected every strain that produced a biosurfactant detectable by the drop collapse test, and also identified additional strains that were not detected with the drop collapse assay because they produced low levels of surfactant or hydrophobic surfactants such as pumilacidins. Not all strains that produced a biosurfactant detectable by the drop collapse when cultured on agar surfaces produced surfactants detectable by drop collapse when cultured in broth, and vice versa. Many bacterial strains exhibited preferential production of surfactants when grown on an agar surface compared to broth cultures, and such surface enhancement of production could also be stimulated by increasing the viscosity of liquid culture media. Surface induction of surfactant production in *Pseudomonas syringae* was regulated at the transcriptional level. Surfactant production was much more common in bacteria recovered from terrestrial leaf and soil habitats (*ca*. 13% of strains) than in aquatic environments (*ca*. 5%).

Author contributions: A.B. and S.L. designed the research, C.D. and N.P. performed and analyzed mass spectra and surface tension measurements, A.B. and P.B. performed all other research, A.B. analyzed the data, and A.B. and S.L. wrote the paper.

INTRODUCTION

Biosurfactants, or biologically-produced surface active agents, have received wide attention mostly for their potential for hydrocarbon dispersion and remediation. Bacterial biosurfactants were initially proposed to function as emulsifiers of biodegradable hydrocarbons (Neu, 1996). However, a wide variety of roles for biosurfactants have been since described, from biofilm formation to inhibitory activity against pathogenic organisms, sparking a renewed interest in their discovery (Ron and Rosenberg, 2001; Van Hamme *et al*., 2006). Given this interest in biosurfactants, the lack of knowledge of the distribution and frequency of occurrence of surfactant production in the environment is remarkable. Comprehensive examinations of biosurfactant production are lacking, and studies that have addressed this trait in a given environment can seldom be compared with those of other habitats (Perfumo *et al*., 2010); both the screening methods used, as well as pre-screening culturing conditions such as medium and incubation conditions usually vary widely between studies (Ahern *et al*., 2007; Hultberg *et al*., 2008).

In a recent report we described a high-throughput assay which utilizes the application of atomized oil droplets to rapidly detect biosurfactants produced by bacteria on the surface of agar plates (Burch *et al*., 2010). This method has advantages over other common assays such as droplet collapse assays in that it can be performed for many colonies simultaneously after limited growth, does not require sample preparation of culture supernatants, and thus is suited for high-throughput screening for surfactant producing strains. Moreover, this method is capable of detecting much lower concentrations of surfactants than the drop collapse assay, and therefore in principle is capable of identifying biosurfactant producing strains that would escape detection with most other methods. However, since the atomized oil assay has not yet been tested on a broad range of environmental isolates, in this study we address whether the range of strains that it can detect includes all of those detectable by the drop collapse assay. Furthermore, although the atomized oil assay has proven effective at detecting surfactants on agar plates, traditionally broth culture supernatants are screened for biosurfactant activity using the drop collapse assay. Depending on the properties of the surface-active compound and its biological role for the producing strain, its production may depend strongly on whether the producing cells are situated at a surface or not. Since a large difference in the transcriptomes of bacteria grown planktonically versus on surfaces have been described, with about one-third of genes differentially regulated (Schembri *et al*., 2003; Wang *et al*., 2004), it seems likely that biosurfactant production itself may be strongly influenced by cell culture conditions. Surface sensing is an important cue for many species to transition to surface-associated behavior such as swarming, whereby cells move across a moist surface utilizing flagella and surfactant (Kearns, 2010). Although the surface regulation of flagella has been well documented (McCarter *et al*., 1988; McCarter, 2006), the regulation of surfactant production by surfaces has not yet been explored and will be addressed in this report.

Insight into the role of biosurfactants would benefit from a better understanding of the numerical distribution of surfactant producers in different environments. A variety of isolated reports have described collections of biosurfactant producers from aqueous environments, polluted/unpolluted soils, and even clouds, with estimates of their frequency in culturable bacterial communities ranging from less than 3 to as much as 50%, but typically around 10% (Bodour *et al*., 2003; Batista *et al*., 2006; Ahern *et al*., 2007; Maciel *et al*., 2007; Hultberg *et al*., 2008). However, no

encompassing model that describes the selection for such a trait has emerged from these studies, perhaps because few comparative analyses of habitats have been performed. We hypothesize that hydrophobic surfaces are habitats that would be particularly selective for bacteria that produce surface active compounds. The surface of leaves that are usually covered with wax would constitute such a habitat, although surfactant production in this habitat has seldom been investigated (D'aes *et al*., 2010). In order to survive on leaf surfaces, epiphytes must be able to access limited and spatially heterogeneous nutrient supplies and endure daily fluctuations in moisture availability on a water-repellent surface (Hirano and Upper, 2000; Lindow and Brandl, 2003). Epiphytic bacteria could potentially use biosurfactants to increase the wetability of the leaf, to enhance diffusion of nutrients across the waxy cuticle, and/or aid in motility to favorable growth sites. Despite the substantial potential role of biosurfactants on leaves, only a few studies have examined their production in the phyllosphere, all of which have focused on their possible ecological role in only specific strains and have not addressed the frequency of surfactant producers on leaf surfaces (Bunster *et al*., 1989; Hernandez-Anguiano *et al*., 2004; D'aes *et al*., 2010). A comprehensive examination of the phyllosphere inhabitants might reveal strains and biosurfactants not normally encountered in other habitats, and would address the hypothesis of surface enrichment of producing strains.

In this study we compare the frequency of surfactant producers in the phyllosphere to those in soil and water environments. We compare the atomized oil assay with the drop collapse assay to characterize surfactants made by a collection of environmental strains, further demonstrating the usefulness of this assay in high-throughput screening and its much higher sensitivity for all types of biosurfactants encountered, many of which are hydrophobic and poorly detectable by the droplet collapse assay. We also investigate the influence of planktonic versus surface-associated culture conditions on the production of biosurfactants from our environmental isolates, and find evidence for frequent contact-dependent production of surface active compounds.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria were isolated on 10% Trypticase Soy Agar (Difco, Detroit, MI) containing 1.5% agar and natamycin. Both *P. syringae* pv. syringae B728a (Loper and Lindow, 1987) and environmental strains were maintained and screened for surfactant production on King's medium B (KB) (King *et al*., 1954) and grown at 28 °C. Viscous KB broth was produced by amending with polyvinylpyrrolidone (PVP-360) to a concentration of 10% W/V (McCarter *et al*., 1988). Antibiotics were used at the following concentrations (μg/ml): natamycin (21.6), kanamycin (50), and spectinomycin (100).

Environmental isolates. 377 isolates were obtained over the course of one year from diverse locations in California. Half the isolates were from plant samples while the remainder were from soil and water samples collected in native California Chaparral habitats. Only morphologically-distinct taxa from each sample were chosen for testing. A total of 5,196 isolates were obtained in a more extensive sampling made in native California Chaparral habitats in Tilden Regional Park, Berkeley, California over a 4-week period in March and April, 2010. Both California native plants and introduced plant species were present at this site. Samples were taken from five transects, with 10 random plant and soil samples collected at two meter intervals along each transect. Water samples were taken as close to the plant and soil samples as possible, from ephemeral pools, streams, and a lake. 50 colonies representing the most abundant bacteria were

chosen at random from each sample. Samples yielding less than 50 bacterial colonies were discarded.

Biosurfactant detection assays. The drop collapse assay was performed as according to Bodour and Miller-Maier (Bodour and Miller-Maier, 1998). 2 µl 10W-40 Pennzoil® (Pennzoil Products Company, Houston, TX, USA) was applied to delimited wells on the lid of a 96-well plate and allowed to equilibrate at room temperature. Next, 5 µl of either diluted surfactant samples or supernatant from bacterial cultures or re-suspended bacterial colonies were pipetted onto the oil surface. Drops which retained a spherical shape were scored as negative for surfactant content, while drops which had a visibly-decreased contact angle with the oil and spread (collapsed) were scored as positive for surfactant content.

The atomized oil assay was conducted as follows: Bacteria were evenly spotted onto KB agar plates using sterile toothpicks and grown overnight. Alternatively, if visualizing surfactant from broth culture, 1mL of 2-day-old broth culture was centrifuged at 10,000xg for 2 min, and 5 µl of supernatant was pipetted onto the plate and allowed to equilibrate for 30 minutes before assaying. Synthetic surfactants were similarly pipetted onto plates. An airbrush (Type H; Paasche Airbrush Co., Chicago, IL) was used to apply a fine mist of mineral oil (light paraffin oil, Fisher Scientific) onto the plate with an air pressure between 15 and 20 psi. Biosurfactant halos were then immediately visualized with an oblique source of bright light.

Matrix-assisted laser desorption ionization mass spectroscopy. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Omniflex instrument (Billerica, MA) used in reflectron mode, as described previously (Price *et al.*, 2007; Price *et al.*, 2009). Cell-free supernatants and extracted surfactants were mixed with an equal volume of matrix medium (10 mg/ml 2,5-dihydroxybenzoic acid in 70% aqueous acetonitrile. The solution was spotted (0.5 µl) on the sample target and allowed to air dry. Ions were produced with a nitrogen laser (337 nm) and accelerated at 20kV. Each mass spectrum was produced by averaging more than 100 individual laser shots.

Surface tension measurements. The surface tension of cell-free supernatants was determined using the pendant drop method. Cell-free supernatants were analyzed with a FTA 4000 video analysis instrument (First Ten Angstroms Inc., Portsmouth, VA). Droplets were produced using a 22 gauge blunt needle and the values reported represent an equilibrium surface tension determined 60 seconds after drop formation.

Measurement of gene expression. Wild-type *P. syringae* B728a carrying either a plasmid conferring constitutively fluorescence p*519ngfp* (Matthysse *et al*., 1996) or p*PsyfA-gfp* (Burch *et al*., 2010) was grown in KB media overnight, then suspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD_{600} of 0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, CA, USA) with a 486-nm bandpass excitation filter and a 510- to 700-nm combination emission filter. A relative fluorescence unit (RFU) was defined as the fluorescence of the suspensions normalized for the suspension turbidity measured as OD_{600}.

Bacterial identification. Genes encoding 16S rRNA were amplified by colony PCR using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-

TACGGYTACCTTGTTACGACTT) (Lane, 1991). PCR conditions were as follows: an initial denaturation of 10 min at 95°C, 28 cycles of 95°C, 59°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. Products were excised from an agarose gel, extracted (UltraClean GelSpin, MoBio, CA, USA), and submitted for sequencing at the UC Berkeley Sequencing Facility. The 16S rRNA gene sequences were compared to GenBank databases using BLAST (http://www.ncbi.nlm.nih.gov). Sequences having >98% similarity to a known GenBank sequence were assigned to the designated phylotype. These sequence data have been submitted to the GenBank database under accession numbers JF430870-JF430892.

Data analysis. Data and regression analysis was carried out using Statistica (StatSoft, Tulsa, OK) and Microsoft Excel. Image analysis to measure the area of water contact on leaves was through ImageJ (NIH; [http://rsb.info.nih.gov/ij/]).

RESULTS

Comparison of surfactant assays. A collection of 377 bacterial strains isolated from a variety of terrestrial and aquatic sources were grown on agar plates and tested for biosurfactant production using the atomized oil assay in which an airbrushed mist of oil droplets was applied to culture plates. Biosurfactant production was evident as a bright zone of de-wetted or raised oil droplets (hereafter referred to as a halo) (Fig. 1A). Additionally, cells of each strain suspended from plates into water as well as drops of broth culture supernatants were tested for drop collapse on an oil surface. A total of 41 of these strains exhibited biosurfactant production in at least one assay. The identities of these strains were determined from partial 16S RNA sequences, and all isolates were assigned to described taxa based on 98% BLAST sequence identity. *Pseudomonas* and *Bacillus* species were the most common genera identified, in line with previous reports of limited surveys (Bodour *et al*., 2003; Chen *et al*., 2007). All biosurfactant producers were members of the Gammaproteobacteria or Firmicutes except for a single *Rhizobium* species (Table 1). After eliminating duplicate taxa from the same sampling location, a total of 23 unique environmental strains that produced surfactant detectable in at least one assay were identified and further characterized (Table 1). All 23 isolates produced surfactant detectable by the atomized oil assay, although only 16 isolates conferred drop collapse of either cells suspended from plates or of broth culture supernatants. Furthermore, cells of only 9 of these 16 isolates conferred drop collapse from both culture conditions. Most of the other 7 strains that conferred drop collapse only under one culture condition did so for suspended plate-grown cells. *P. syringae* strains were typical of this group; cells of four representative isolates conferred drop collapse when suspended in water from plate cultures but not the supernatant of planktonic cultures. While 16 strains of *P. syringae*, *P. fluorescens*, or *B. subtilis* produced biosurfactant that could be detected by both assays, the 7 strains that exhibited biosurfactant activity that was detectable only by the atomized oil assay mostly consisted of a diversity of other taxa (Table 1).

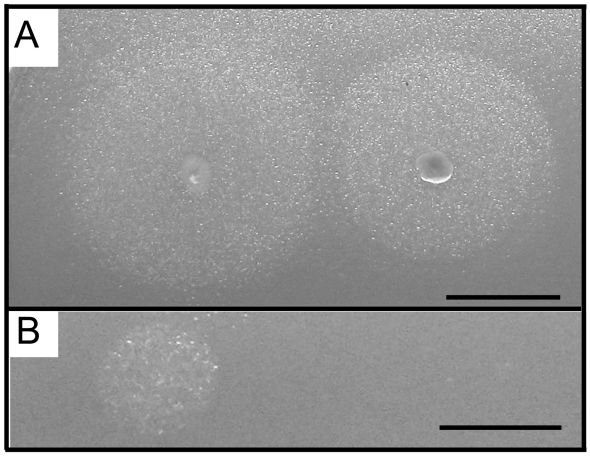

Figure III-1. Atomized oil halos of strains producing surfactants with different properties
Halos of atomized oil droplets modified by surfactants produced by *P. syringae* B728a (left) and *B. pumilis* (right). **A.** Large surfactant-induced halos around colonies of both strains grown on agar plates. **B.** Medium-sized halo conferred by supernatants from water suspensions of plate-grown cells of *P. syringae* B728a but not for *B. pumilis*. In both images the bars represent 1 cm.

Table III-1. Patterns of biosurfactant production among environmental bacteria recovered from different habitats.

Genus	Species	Isolate	Halo	Plate DC	Broth DC
Pseudomonas	*syringae*	PB54	▨		
		PB21	▥	▥	
		PB27	▥	▥	
		PB31	▥	▥	
		PB67	▥	▥	
	fluorescens	PB42	▨		
		PB59	■		■
		PB34	▥	▥	▥
		PB39	▥	▥	▥
		PB52	▥	▥	▥
		PB53	▥	▥	▥
		PB58	▥	▥	▥
		PB63	▥	▥	▥
Xanthomonas	*axonopodis*	PB28	▨		
Pantoea	*ananatis*	PB35	▨		
		PB64	▥	▥	
Cedecea	*davisae*	PB61	▨		
Rhizobium	*Rubi*	PB32	▨		
Bacillus	*subtilis*	PB43	▥	▥	▥
		PB44	▥	▥	▥
		PB57	▥	▥	▥
	pumilis	PB36	▨		
Staphylococcus	*equorum*	PB37	■		■

▨ Strains that produced surfactants detectable with the atomized oil assay as well as conferring drop collapse in cells recovered from plates and in supernatants of broth cultures

▨ Strains that produced surfactants detectable with the atomized oil assay but which did not confer drop collapse irrespective of how cells were cultured

▥ Strains that produced surfactants detectable with the atomized oil assay, but which only conferred drop collapse when cells grown on a plate were suspended in water

■ Strains that produced surfactants detectable with the atomized oil assay, but which only conferred drop collapse when supernatants of broth cultures were assayed

Hydrophobic biosurfactants. Although not appreciated in most biological studies, surfactants differ greatly in their chemical properties in ways that could influence their ability to be detected by various assays. For instance, a fundamental property of a surfactant is its relative solubility in water and oil, which can be broadly described by its hydrophilic-lipophilic balance (HLB) value. Some important synthetic surfactants with low hydrophilicity are not readily dispersible in water, and thus have unique functions such as forming inverse emulsions of water into oil (Tadros, 2005). If a bacterial strain produced a biosurfactant with such low water solubility this could account for its inability to reduce the surface tension of water sufficiently to collapse a water drop. In order for drop collapse to occur on an oil surface, a minimum surface tension reduction at the water/air interface from 72 dyn/cm to around 43 dyn/cm is required (Bodour and Miller-Maier, 1998). Although a surfactant may be present in a sample of interest, it might not be

detected by the drop collapse assay if it is produced in low quantities or has a property preventing it from lowering the surface tension of water. Because the atomized oil assay can detect 10- to 100-fold lower concentrations of surfactant than that of the drop collapse assay (Burch *et al*., 2010), it is reasonable to hypothesize that the atomized oil assay can detect surfactant production in weakly producing strains. Therefore, it was possible that the 7 strains that did not confer drop collapse may simply produce too little surfactant to be detected with this method. Indeed many of these strains exhibited small halos in the atomized oil assay (data not shown), suggestive of low surfactant concentrations. However, a few strains such as *Bacillus pumilis* that did not cause drop collapse produced biosurfactants that conferred halos of de-wetted oil droplets around colonies that were at least as large as many strains whose biosurfactants did confer drop collapse (Fig. 1A). This observation led us to suspect that the surfactant had properties which hindered its ability to be detected by the drop collapse assay.

To address the features of biosurfactants that could be detected by the atomized oil assay but not the drop collapse assay, we distinguished the extent to which the hydrophobicity of the surfactants might limit their detection with the later method or whether the higher sensitivity of the atomized oil assay was responsible for their detection. As a test of the relative hydrophobicity of the surfactant produced by *B. pumilis* we suspended colonies of it as well as *P. syringae* strain B728a in water to identical concentrations, removed the cells by centrifugation, and then tested the supernatant for surfactant activity using the atomized oil assay. The water-soluble material washed from cells of *P. syringae* B728a, which contains syringafactin and readily causes drop collapse (Berti *et al*., 2007; Burch *et al*., 2010), contained sufficient surfactant to produce a large halo of de-wetted oil droplets when placed on an agar surface (Fig. 1B). However, very little biosurfactant was apparently washed from cells of *B. pumilis*, since no zone of de-wetted oil droplets was observed (Fig. 1B). Similarly, the surfactants produced by *Pantoea ananatis* and *Pseudomonas fluorescens* strains which were detected only by the atomized oil assay also appeared to have low water solubility when assayed after washing of cells (data not shown). However, the washings of four other strains (*P. syringae*, *Xanothomonas*, *Cedecea* and *Rhizobium*) that exhibited the ability to de-wet atomized oil droplets but not to collapse water drops, retained the ability to de-wet oil droplets. This suggests that these strains produced only small amounts of a water-soluble surfactant that could be detected by the drop collapse assay if present in higher concentrations. In support of this conjecture was the observation that these later strains exhibited only relatively small halos in the atomized oil assay (data not shown). The low production of water soluble surfactants in these strains was verified for *P. syringae* strain PB54 using mass spectroscopy. This strain was observed to produce the same syringafactins as *P. syringae* B728a, albeit in much lower quantities, confirming that the detection of surfactants in strain PB54 by the drop collapse assay was compromised by its low level of production.

In order to confirm our conjecture that the lack of detection of biosurfactant production in our *B. pumilis* strain in the drop collapse assay was due to its low water solubility, we characterized it using MALDI mass spectroscopy. The mass spectrogram of the material extracted from the cell-free region surrounding colonies on the surface of plates (Price *et al*., 2007; Price *et al*., 2009) revealed a series of prominent peaks in the (*m/z*) range of 1050-1130 (Fig. 2). Several *B. pumilis* strains have previously been shown to produce a family of pumilacidins in this mass range (Naruse *et al*., 1990; Melo *et al*., 2009). The mass spectrogram of our strain shares the same

masses of a sample containing a mixture of pumilacidin A, B, C, and D (Naruse *et al*., 1990). The masses observed in Fig. 2 are a combination of $[M+Na]^+$ and the $[M+K]^+$ adducts commonly seen in MALDI mass spectroscopy. Therefore, we conclude that our strain is producing a mixture of low water solubility pumilacidins that are capable of readily diffusing away from cells on the surface of an agar plate, but which are not sufficiently water soluble to impart drop collapse. In order to demonstrate pumilacidin's surfactant capabilities, the surface tension of a broth culture of *B. pumilis* was measured using a highly sensitive pendant drop analysis. The surface tension of the broth culture supernatant was lowered by production of a surface active compound to 50 dyn/cm; this surface tension is just above the minimum threshold necessary to impart a drop collapse.

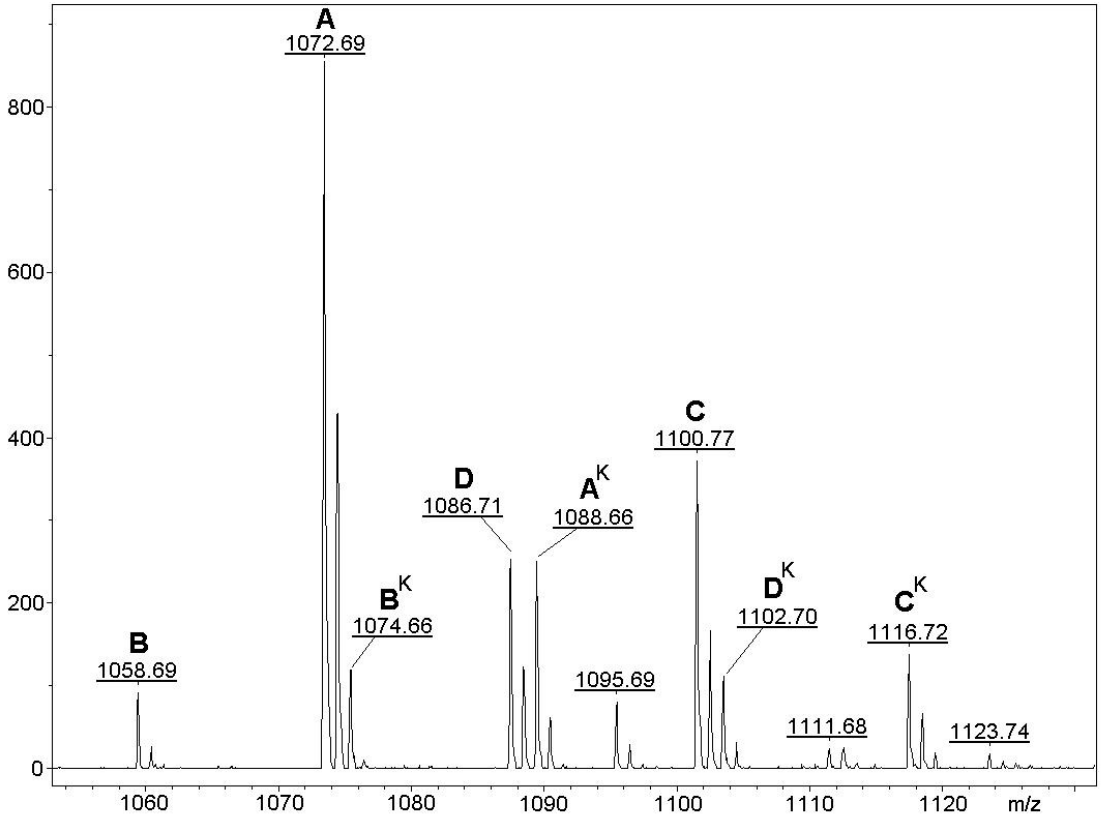

Figure III-2. MALDI-TOF of pumilacidins from an atomized oil assay
Pumilacidins identified by MALDI-TOF mass spectrometry from cultures of *Bacillus pumilus*. **A.** Pumilacidin A (*m/z* 1072.69; $C_{54}H_{95}N_7O_{13}$; calc. accurate masses $[M+Na]^+$ = 1072.6885, $[M+K]^+$ = 1088.6625, $[M+Na_2]^+$ = 1095.6783, $[M+Na+K]^+$ = 1111.6522. **B.** Pumilacidin B (*m/z* 1058.69; $C_{53}H_{93}N_7O_{13}$; calc. accurate masses $[M+Na]^+$ = 1058.6727, $[M+K]^+$ = 1074.6467. **C.** Pumilacidin C (*m/z* 1100.77; $C_{56}H_{99}N_7O_{13}$; calc. accurate masses $[M+Na]^+$ = 1100.7197, $[M+K]^+$ = 1116.6937, $[M+Na_2]^+$ = 1123.7096. **D.** Pumilacidin D (*m/z* 1086.71; $C_{55}H_{97}N_7O_{13}$; calc. accurate masses $[M+Na]^+$ = 1086.7041, $[M+K]^+$ = 1102.6781.

Since the highly hydrophobic pumilacidins were detectable using the atomized oil assay, we further determined the efficiency with which other characterized synthetic surfactants differing in chemical properties could be detected by this method. The assay was performed on synthetic surfactants that possessed a broad range of hydrophobicities. As seen previously, the atomized oil assay readily detected surfactants having more balanced hydrophilic and lipophilic groups, which were also detected by the drop collapse assay (Table 2). On the other hand, the hydrophobic surfactants Span® 85 and Span® 80 each yielded large bright halos in the atomized oil assay, but given their low water solubility, could not be detected in the aqueous phase by the drop collapse assay (Table 2). This is in agreement with our observation that hydrophobic pumilacidins were also only detectable by the atomized oil assay and not by the drop collapse assay. Curiously, the synthetic surfactants not only caused bright halos of de-wetted atomized oil droplets, but those with balanced hydrophilic and lipophilic groups also caused the oil droplets to migrate away from the source of surfactant, traveling at a speed of up to 0.1 mm/minute (Fig. 3). Such expanding halos may result from a strong surfactant gradient, such as explored by Angelini *et al*. (2009), although it is unclear why this should not be also conferred by the hydrophobic surfactants. This property was commonly observed around biosurfactant-producing bacterial colonies and might be used to infer the water solubility properties of the biosurfactants.

Table III-2. Comparison of the behavior of a variety of synthetic surfactants in the atomized oil and drop collapse assays for surfactants.

HLB	Surfactant	0.5% v/v water drop collapse	Atomized oil assay
1.8	Span 85	No	Big halo
4.3	Span 80	No	Big halo
8.6	Span 20	No	Small halo
9.7	Brij 30	Yes	Big "expanding" halo
10	Triton N-57	Yes	Big " expanding" halo
11	Tween 85	No	Small " expanding" halo
11.7	Tergitol NP-7	Yes	Big " expanding" halo
12.4	Triton X-114	Yes	Big " expanding" halo

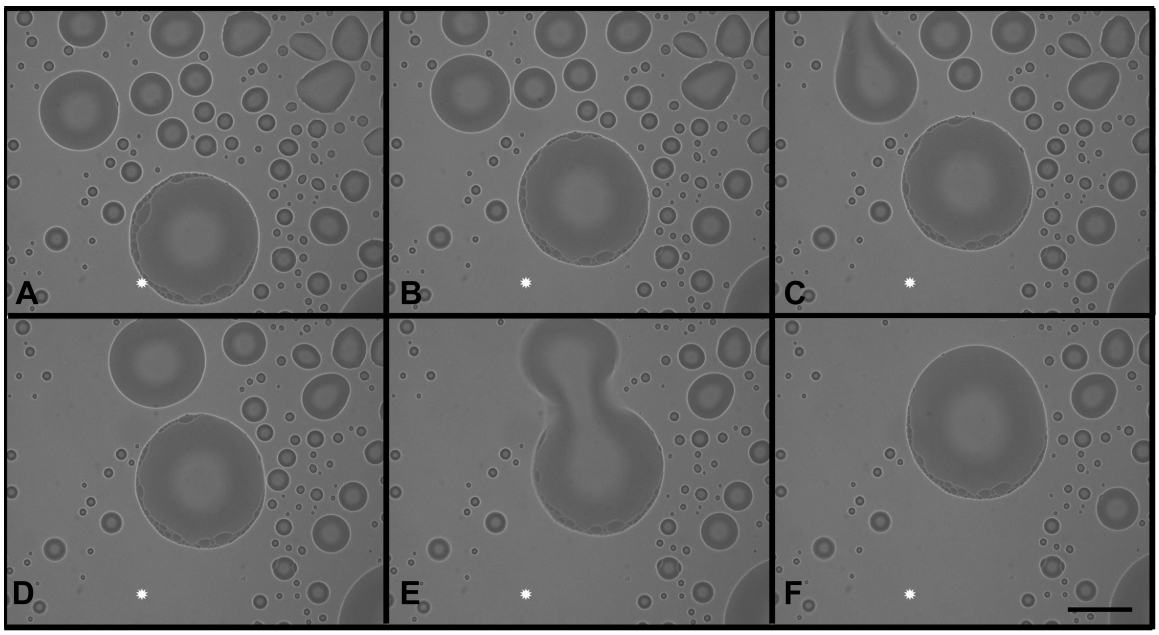

Figure III-3. Time-elapsed photomicrographs of an "expanding" halo
Time-elapsed photomicrographs of oil droplets constituting an "expanding" halo near a droplet of Triton N-57 placed on an agar plate when examined at 0 (A), 1 (B), 2 (C), 3 (D), 4 (E), and 5 (F) minutes after atomizing oil droplets onto the surface of the agar plate. The white spot is a fixed reference point that allows visual orientation of the starting location of one of the moving oil droplets. The bar represents 0.1mm.

Biosurfactants produced at a surface. In addition to the surfactants that were only revealed by the atomized oil assay, we also found that many surfactants were detectable in the drop collapse assay only when cells had experienced a particular growth condition (Table 1). Most prominent among strains exhibiting such growth condition-dependent production of surfactants were strains of *P. syringae*; cultures of this species never conferred water drop collapse when grown planktonically. The factors determining surfactant production in *P. syringae* pv. syringae B728a, typical of this species, was thus investigated. While culture supernatants of this strain did not cause water drop collapse on an oil surface, plate-grown cells suspended to the same concentration as the planktonic culture conferred water drop collapse (Fig. 4A). Suspension of a *syfA*- mutant blocked in production of syringafactin (Burch *et al*., 2010) did not cause water drop collapse, confirming that the drop collapse is due to syringafactin. We thus postulated that enhanced expression of syringafactin production in cells grown on a surface was responsible. In order to link syringafactin production to surface-mediated increases in surfactant production, we examined the transcriptional regulation of *syfA* using a GFP-based bioreporter. Greater than a 10-fold increased expression of *syfA* was observed when cells were grown on an agar surface compared to planktonic growth in broth culture (Fig. 4B). As a control, a strain constitutively expressing GFP exhibited similar levels of fluorescence in both cultures.

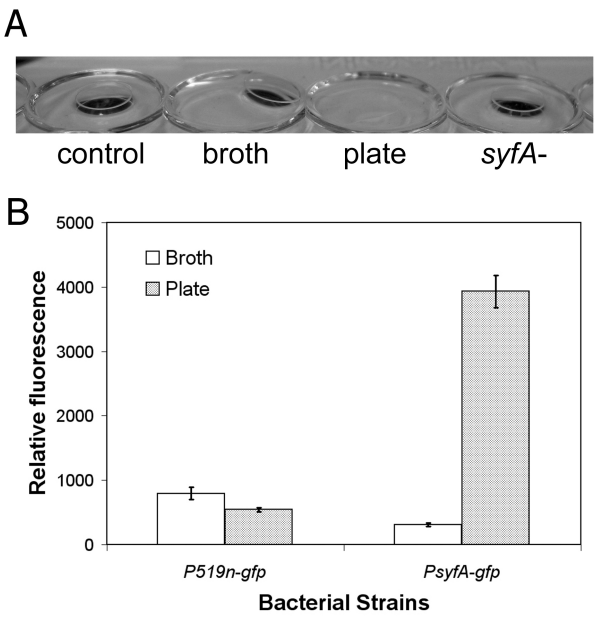

Figure III-4. Surface regulation of syringafactin production
Contact-dependent production of syringafactin. **A.** Drop collapse assay of water alone (control), broth culture supernatant of *Pseudomonas syringae* B728a (broth), supernatant from an equivalent number of cells of *P. syringae* B728a that had been grown on an agar plate but then washed in water (plate), and supernatant from an equivalent number of cells of a *syfA*- mutant of *P. syringae* that had been grown on an agar plate but then washed in water. **B.** Relative GFP fluorescence of cells of *P. syringae* B728a harboring either a constitutively expressed GFP reporter gene (p*519n-gfp*) or a plasmid in which GFP expression is dependent on the promoter of SyfA (p*syfA-gfp*) recovered from broth and plate cultures.

Since there have been reports that production of some surfactants are influenced by growth stage (Lin *et al*., 1994; Ochsner and Reiser, 1995), we examined *syfA* expression at a variety of times for up to 3 days during the growth of both liquid and solid cultures of *P. syringae*. GFP expression was higher in cells recovered from agar plates than broth cultures at all times, indicating that this is not growth-stage dependent phenomenon (data not shown). Additionally, some reports have documented that surfactant production is activated in more dense cultures by quorum sensing (Ochsner and Reiser, 1995; Lindum *et al*., 1998). However, the GFP fluorescence of *P. syringae* harboring the p*syfA-gfp* fusion in the wild-type and a quorum-sensing deficient strain (Quinones *et al*., 2005) was similar both in liquid and solid cultures, indicating that syringafactin production is not dependent on quorum sensing (data not shown). Although not previously connected to surfactant production, one of the ways by which bacteria sense surfaces is apparently through monitoring the viscosity of their environment (McCarter *et al*., 1988). When PVP-360, a viscosifying agent, was added to broth medium, the expression of *syfA* was increased to levels similar to that of cells on agar plates (data not shown). Given this finding, we cultured the strains that had exhibited putative surface-dependent regulation of surfactant production for their ability to induce drop collapse when grown in viscous broth. While *P. syringae* B728a does not produce a surfactant capable of conferring drop collapse from normal broth cultures, it did so when grown in a viscous broth (Fig. 5B). A similar induction of surfactant production was induced by growth of other environmental strains of *P. syringae*, as well as *Pantoea* strain PB64 in viscous broth (Fig. 5C and 5D). Interestingly, *P. fluorescens*

strain PB59, which produced surfactant only in broth media, still produced abundant biosurfactant detectable by drop collapse when grown in viscous broth (data not shown), suggesting that its biosurfactant production is regulated by a different mechanism. Although it is tempting to speculate that the *P. syringae* and *Pantoea* strains are sensing a surface by directly measuring viscosity, growth in viscous broth could be indirectly stimulating biosurfactant production via alteration of growth patterns such as cell aggregation which was stimulated by the reduced turbulent drag of this culture medium. Vigorous shaking of *P. syringae* cultures reduced pellicle formation and resulted in a lower induction of *syfA* (data not shown).

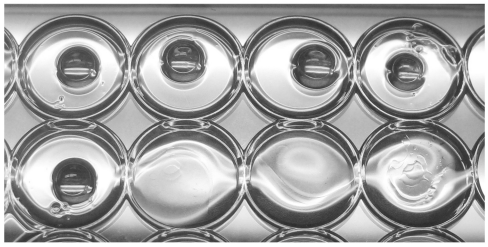

Figure III-5. Stimulation of surface-regulated surfactant production in viscous solution
Drop collapse conferred by culture media alone (A) or cultures of *Pseudomonas syringae* B728a (B), an environmental strain of *Pseudomonas syringae*, PB27 (C), and an environmental strain of *Pantoea ananatis*, PB64 (D) grown in KB broth (top row) or viscous KB broth amended with 10% W/V PVP-360 (bottom row).

Terrestrial environments enrich for surfactant producers. Since leaves are a waxy habitat, we hypothesized that the phyllosphere is enriched for biosurfactant-producing bacterial taxa due to the benefits this phenotype may confer. To test this hypothesis we examined the incidence of this trait in bacteria from different habitats including leaf surfaces using the atomized oil assay. Using this assay, we screened over 5,000 bacteria recovered from leaf surfaces, soil, and freshwater samples in close proximity to each other in the early spring, when there were many ephemeral pools of water and streams. To determine the frequency of surfactant production in bacterial populations this trait was assessed in approximately 50 random strains per sample, and at least 30 samples were collected for each environment. The frequencies at which surfactant producers were found in a community from a given sample ranged from zero to close to 90%. Overall, a much lower frequency of surfactant producers was observed in freshwater samples (*ca*. 5%) than from leaf surfaces or soil (each *ca*. 13%) (Fig. 6). Student's *t*-test with unequal variance comparing the frequencies of surfactant production revealed that leaves and soil harbored significantly higher frequencies of bacteria with this phenotype than water ($P<0.05$ for plant vs water; $P<0.002$ soil vs water). Interestingly, while soil and leaf surfaces harbored a similar average frequency of surfactant producers, there was a much higher deviation in this frequency between samples of leaves than soil; nearly 30% of the leaf samples harbored no surfactant producers compared to 17% and 6% for water and soil samples respectively. Conversely, many leaves also harbored very high proportions of surfactant producing bacteria (data not shown).

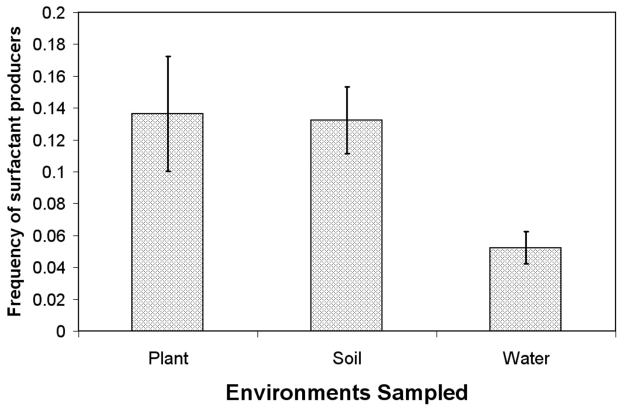

Figure III-6. Proportion of biosurfactant producers in different environments
Proportion of the predominant culturable bacteria in different habitats that produce biosurfactants. Bars represent average proportion of biosurfactant-producing bacteria encountered in a given environment, +/- standard error.

Several features of leaves were examined in an attempt to account for the substantial sample to sample differences in frequency of biosurfactant-producing bacteria. Given that the leaf surfaces of different plant species differ in hydrophobicity, we addressed whether plant species or the degree of water-repellency of leaves was predicative of the fraction of surfactant-producing bacterial strains recovered. There was no correlation between leaf hydrophobicity, measured as the total area covered by a 10 ul droplet of water applied to the leaf, and the frequency of surfactant producers (Fig. 7). Likewise no association between plant species and the frequency of surfactant producers was evident (Fig. 7), although more species would need to be examined to rigorously test this conclusion. Overall, our observations suggest that leaf properties are not the dominant factor that leads to the occurrence of surfactant-producing strains on a given plant. However, since our collections were made in early spring, the leaves examined were all at early stages of growth and thus the microbial communities were also in early stages of colonization. The apparent random patterns of occurrence of bacteria on the leaves therefore suggests that colonization can be described by a neutral theory of competition. As such, the abundance of a given bacterial strain on a leaf is reflective of its early time of arrival on that plant, and largely dependent on chance. In comparison to leaf surfaces, a much more uniform frequency of occurrence of surfactant production was observed in bacteria from soil and water. There was no apparent effect of the source of water on the incidence of surfactant production in these samples, since about 5% of the bacteria in all samples from streams, ephemeral pools and a lake produced biosurfactant (data not shown). Additionally, the frequency of surfactant-producing bacteria found in a given soil sample was not correlated with that from adjacent plant samples (Correlation = -0.1217, data not shown), suggesting that mixing of bacterial members of these two communities was not prominent.

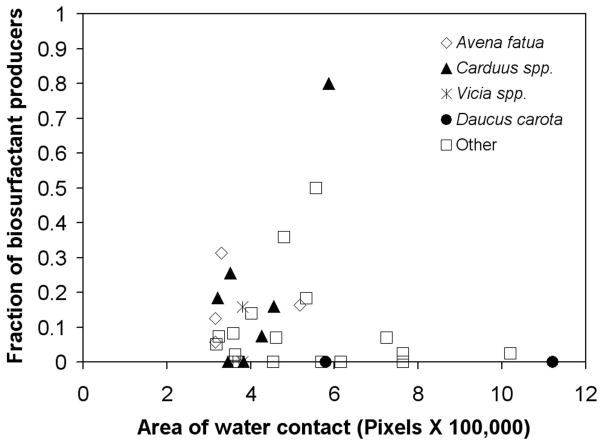

Figure III-7. Proportion of biosurfactant producers from different plant species
Relationship between the proportion of surfactant-producing bacteria in the predominant culturable microflora of different plant species and the wettability of leaves of those species. Leaf wettability was measured digitally as the area of contact of a 10 µl water droplet on the leaf.

DISCUSSION

The application of the atomized oil assay to a wide variety of environmental bacterial strains and synthetic surfactants revealed it to be both more versatile and sensitive than the more commonly used drop collapse assay. The atomized oil assay confirmed surfactant production in every bacterial strain in which surfactants were detected using the drop collapse assay. More importantly, several bacterial strains were identified that produced either low amounts of surfactant or apparently hydrophobic surfactants that were not detectable using the drop collapse assay. The atomized oil assay readily confirmed biosurfactant production in taxa in which it had previously been described. The majority of the strains that produced surfactants detectable by both tests belonged to the genera *Pseudomonas* and *Bacillus* (14/16), both of which have been described in the literature to produce biosurfactants that lower the surface tension of water (Raaijmakers *et al*., 2010). Likewise, the *Pantoea* strain PB64 may produce rhamnolipids as do other members of this genera (Rooney *et al*., 2009), although this was not verified. While surfactant production has not been previously documented in *Staphylococcus*, some species of this genus have been observed to be motile on swarming plates (Dordet-Frisoni *et al*., 2008), suggesting their production of surfactants. The identification of such previously recognized surfactant-producing taxa emphasizes that while the drop collapse assay is suitable for finding such biosurfactant producers, the atomized oil assay may be more readily employed due to its high-throughput capability and higher sensitivity (Burch *et al*., 2010).

The atomized oil assay was particularly useful in identifying biosurfactants in taxa in which this trait had not previously been shown. The surface-active compounds that are produced by the seven strains that were detectable only with the atomized oil assay would have escaped attention in most other studies; these compounds may well have unique biological functions and/or potential industrial applications. For example, our assay detected the hydrophobic pumilacidins produced by *Bacillus pumilis* which have been documented for their potent antibiotic and antiviral properties (Naruse *et al*., 1990), although their surfactant activity has previously been ignored due to their low water solubility (From *et al*., 2007). Likewise, we detected surfactant

production by a *Rhizobium* strain (Table 1); although we have not verified the compound, we suspect it could be similar to the long-chain AHLs produced by *Rhizobium etli*, which cannot be detected with a drop collapse assay but are documented as surfactants with a dual role in quorum sensing and swarming motility (Daniels *et al*., 2006). Furthermore, a biosynthetic gene cluster proposed to synthesize a surface-active lipopeptide virulence factor was identified in the genome sequence of the plant pathogen *Xanthomonas axonopodis* (Etchegaray *et al*., 2004); although incapable of imparting drop collapse, both an authentic culture of *X. axonopodis* pv. glycines as well as a related environmental strain found in this study produced compounds detectable with the atomized oil spray (Table 1, data not shown). Biosurfactants detectable only with the atomized oil assay were also observed in a *Cedecea* strain, a taxon not previously known to produce surfactants; this feature may prove biologically important to its success as an opportunistic pathogen. Therefore it appears that application of the atomized oil assay in environmental surveys might greatly expand our knowledge of novel biosurfactants.

While the atomized oil spray assay has many advantages over other assays there are some limitations that could bias the detection of surfactant producers. This assay best identifies bacterial strains that produce "bright" halos around colonies (Fig. 1), although we have previously shown that some highly hydrophilic synthetic surfactants can modify oil droplets to appear "dark" due to their flattened nature (Burch *et al*., 2010). "Dark" halos are less visibly obvious and no strains that unambiguously exhibited this appearance were found in our survey even though we approached the study with the expectation that we would find biosurfactants of this type. We were surprised that we did not find any biosurfactants that yielded a water drop collapse and such a "dark" halo. Bacteria that produce such compounds must thus be quite uncommon, or it may be that such surfactants are not easily distinguished or detected by either assay. Another limitation of the atomized oil assay, which is shared with any culture-based assay, is that the nutrient medium that we used may have precluded us from detecting production of surfactants by some strains which require specific conditions for surfactant production. Furthermore, our assay is restricted to surfactant production by culturable organisms, although there is evidence that at least on leaves the most common cultured taxa are also among the most prevalent taxa identified by culture-independent methods (Yashiro *et al*., 2011). Metagenomic investigation into the prevalence of biosurfactant production could be fruitful in expanding our understanding of their prevalence in bacterial communities, although advances will be limited until more genetic determinants for their production are described.

An unexpected finding from this study was that the production of surfactants that conferred a reduction of surface tension was very conditional on whether the bacteria were grown on a surface or cultured planktonically. Although a number of studies have connected surface sensing with swarming motility (McCarter and Silverman, 1990; Harshey, 2003), we are only aware of one report, of *Serratia liquefaciens*, which has noted increased biosurfactant production in cells grown on a surface (Lindum *et al*., 1998). In the current work we have shown that a surprisingly large proportion of bacterial strains restrict biosurfactant production to growth on a surface. Although most of these surface-dependent surfactant producers were strains of *P. syringae* isolates, this phenomenon was also seen in a *Pantoea* strain, suggesting that it may be a common trait. Commonly-used methods of screening for biosurfactants by drop collapse employ broth cultures and would likely not identify such strains. On the other hand, two strains were identified that only conferred drop collapse from broth culture and not from cells grown on

plates and subsequently suspended in water drops. However, surfactant production was still detectable in these strains as a small halo of de-wetted oil droplets with the atomized oil spray when cells were grown on plates. The small halo size of these two strains indicates that the amount of surfactant produced by cells grown on plates was probably too low in concentration to be detected by the drop collapse assay; therefore surfactant production was not fully blocked at a surface, but rather dramatically reduced. Although we have not yet encountered such strains, there is the potential for us to overlook biosurfactants which are produced only in broth culture. However, such strains must be uncommon based on our extensive survey, and the high sensitivity of the atomized oil assay should enable even very low production on solid surfaces to be detectable.

Presumably the strong environmental-dependent regulation of surfactant production at surfaces is linked to its role in the habitat of some strains. For example, surfactants contributing to biofilm growth or movement on a surface would be pointless if produced in an aqueous environment. Thus, it makes sense that bacteria with multiple habitats should survey their growth environment before committing to production of a biosurfactant. The surface trigger for surfactant production and its conservation among bacterial taxa remains an active area of research. Bacterial surface sensing has been coined "the 'holy grail' of swarming motility research" (Kearns, 2010). A few specific mechanisms for surface sensing have been investigated, such as two-component systems and flagellar inhibition (Otto and Silhavy, 2002; Belas and Suvanasuthi, 2005). Once a surface is perceived, there is growing evidence that cyclic-di-GMP levels control genes involved in cell surface features that participate in processes such as biofilm growth (Güvener and Harwood, 2007). It is intriguing that increases in viscosity led to increases in surfactant production in this study (Fig. 4), much as it has been shown to induce production of flagella in *Vibrio parahaemolyticus* (McCarter *et al*., 1988). However, our results lead us to believe that it is not viscosity sensing *per se* that is inducing surfactant production, but rather perception of a growth pattern such as cell aggregation that perhaps restricts movement of cells which, in turn is induced by the reduced turbulent drag of a viscous medium. Although it is tempting to speculate that oxygen sensing is involved, neither the high oxygen condition of a shaken culture, nor an oxygen starved broth culture is sufficient to induce syringafactin production (data not shown). We are currently investigating the mechanism behind surface regulation of syringafactin in *P. syringae* B728a, and the biological purpose for restricting syringafactin production to surfaces.

In addition to suggesting that surfactant producers often restrict production to surfaces, our findings also support an inverse conclusion: Surfaces strongly select for surfactant producers. It appears that bacteria cultured from terrestrial surfaces are more likely to produce surfactants than bacteria from aqueous habitats. Previous support for this finding comes from the report that a higher fraction of surfactant producers were associated with the filters of hydroponic systems than in the liquid medium itself (Hultberg *et al*., 2008), although the authors did not explore this linkage. Biosurfactant production is a costly process for a bacterium, and might be an evolutionary disadvantage for aquatic bacteria. Not only is production energetically costly, but the genetic footprint of biosurfactant production can constitute a large portion of the genome; for instance, the biosynthetic locus for syringafactin production is close to 30 kb in length.

If surfaces select for surfactant producers, then it follows that some surfaces might be more selective than others. We tested the hypothesis that the phyllosphere, consisting of waxy leaf

surfaces, would be particularly enriched for surfactant producers. However, on average, leaf surfaces and soil harbored a similar frequency of surfactant producers. There are a number of reasons why our samples may have found no differences between these two habitats. While soil particles on which bacteria reside may not be as hydrophobic as waxy leaf surfaces, surfactants may play an important role in movement and nutrient acquisition in the soil by better enabling water release from soil particles and increasing the thickness of transient water films (Hinsinger *et al*., 2009). Plant and soil samples were collected on relatively young plant tissues growing at the end of the rainy season in California. At least some of the soil bacterial community could consist of epiphytic bacteria that were washed off the leaves into the soil, and thus the two environments would have at least some common bacterial community members during this period. However, if this were true we might have expected to see a correlation between individually paired plant and soil samples, but we did not. It is more likely that the high variability of surfactant producers seen on leaf samples was associated with the relatively immature bacterial communities on the leaves. As epiphytic bacterial population sizes and diversity increase with leaf age (Lindow and Brandl, 2003; Monier and Lindow, 2004; Redford and Fierer, 2009), the relatively young leaves we sampled may not have been at a succession equilibrium, and instead exhibited a strong founder effect where the initial colonizers are able to initially flourish on a leaf even if they are less fit than subsequent immigrants (Hirano and Upper, 2000). Indeed, the incidence of surfactant producers on plant samples had almost twice the variation as in soil or water environments. While surfactant producers constituted a very large proportion of the bacteria recovered from some leaves, they may not yet have immigrated to other leaves from which they were absent. Our large survey of surfactant producers was conducted in a local area during a specific time period, and therefore the results might differ if sampling was made on plants in other environments or experiencing different environmental conditions.

The question remains as to why don't all surface-associated bacteria make surfactants if it is an advantageous trait? Bacteria on leaves are usually present as a mixed community (Monier and Lindow, 2005), and production of extracellular and readily diffusible compounds might confer a similar advantage to both producers as well as neighbors on leaves. Surfactants might thus be considered a "common good" and surfactant producers could be keystones to the population, producing a community resource. An alternative explanation could lie in the heterogeneous nature of the leaf environment; there could be a variety of inhabitable niches on soil and leaf surfaces, only some of which would benefit from production of biosurfactants. Additionally, if biosurfactant production is used to enhance nutrient acquisition, then heterogeneous biosurfactant production might reflect the diversity of nutrients consumed and acquisition strategies employed by bacteria. Although biosurfactants might aid organisms such as Pseudomonads which consume water-soluble substrates, organisms such as methylotrophs which consume volatiles (Sy *et al*., 2005) might not receive any additional benefit from biosurfactant production. Thus, the biological roles of surfactant production in the environment must be better understood in order to explain the prevalence and distribution of producers.

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Chapter IV.

Pseudomonas syringae regulates a motility-enabling surfactant through flagellar-mediated surface sensing

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ABSTRACT

Biosurfactants have diverse and poorly understood roles in natural environments. Although some biosurfactants are produced in large quantities under laboratory conditions, those having less pronounced levels of production are often overlooked. Using a sensitive assay, we detected low levels of an unknown surfactant produced by *P. syringae* pv. syringae B728a that was undetectable with traditional methods. Much larger quantities of this surfactant were produced by bacteria colonizing a porous hydrated paper surface than on agar surfaces. A gene with homology to *rhlA* from *P. aeruginosa* that encodes an acyltransferase responsible for the production of the surfactant HAA was required for production of this surfactant. Analysis of other mutants altered in surfactant production revealed that this acyltransferase is coordinately regulated with the late-stage flagellar gene encoding flagellin, and so we call the surfactant BRF for biosurfactant regulated by flagella. Mutations in genes involved in early flagellar assembly abolish or reduce BRF production, while mutations in flagellin or flagellin glycosylation genes increase its production. However, because a FliA mutation does not abolish production of BRF, nor is the surfactant always regulated with flagellin, we do not conclude that this acyltransferase should be considered a late-stage flagellar gene. When traveling across a rough porous surface, the bacterium increases production of both flagellin and BRF. *P. syringae* was defective in porous paper colonization without functional flagella, and was slightly inhibited in its movement when it lacked BRF production. In contrast, loss of BRF production had no effect on swimming but stopped swarming motility. Growth in broth medium reduced the regulatory control of flagellar assembly on surfactant production. A strain that lacked BRF but could produce syringafactin exhibited dense swarming tendrils, while a strain that overproduced BRF exhibited skinny swarming tendrils; thus it appears that BRF could act as a repellant similar to the HAAs produced by RhlA in *P. aeruginosa*, facilitating bacterial exploration of surfaces by directing the cells away from locations they have already colonized. Based on further analysis of mutants altered in surfactant production, we propose a model of its regulation in *Pseudomonas syringae* B728a.

Author contributions: A.B. and S.L. designed the research, C.D. performed and analyzed surface tension measurements, A.B., B.S. and S.M. performed all other research, A.B. analyzed the data, and A.B. and S.L. wrote the paper.

INTRODUCTION

Biosurfactants are biologically-produced amphiphilic compounds which display surface activity by lowering the tension at interfaces such as oil/water interfaces. A number of bacterial surfactants have been extensively investigated, but there is still a vast array of biosurfactants probably remaining to be discovered. Even among the best characterized biosurfactants, their true physiological functions have only recently been investigated. Originally, biosurfactants were thought to be produced for the purpose of oil emulsification and degradation (Neu, 1996), most likely because this was a trait used to detect biosurfactant production and also one of the earliest proposals for their utility. However, an increasingly sophisticated understanding of the complexities of bacterial behavior has led to additional hypothesized roles of biosurfactant production including biofilm structure maintenance, pathogenicity, antagonistic activity against other bacteria and/or fungi, and bacterial motility (Ron and Rosenberg, 2001; Raaijmakers *et al*., 2010). It remains to be determined if these roles might also be artifacts of the way in which we currently study bacterial behavior.

One demonstration of biosurfactant activity is its enhancement of bacterial motility across soft agar plates. This motility, termed swarming motility, is an active form of translocation and is generally reliant on flagellar motility and biosurfactant production (Kearns, 2010). Although biosurfactants clearly have a role in bacterial motility in this laboratory setting, because swarming plates are nutrient-rich, homogenous planar surfaces, it is questioned how relevant swarming motility is *in vivo*. Thus there have been efforts to explore more "natural" surfaces than agar plates. One model, the Porous Surface Model (Dechesne *et al*., 2008), has revealed that flagellar motility but not biosurfactant production is necessary for motility over rough porous surfaces (A. Dechesne, personal communication). A rough surface will harbor a heterogeneous range of water film thicknesses, only some of which will be thick enough for bacteria to swim (Dechesne *et al*., 2010). Thus, since most natural surfaces have at least microscale roughness, it may be that biosurfactants do not have a large role in motility. There have been some reports of less effective colonization of natural surfaces by biosurfactant-deficient strains (Hildebrand *et al*., 1998; Nielsen *et al*., 2005), but the question nonetheless remains whether or not bacteria produce biosurfactants for the purpose of movement, and if so, how exactly are they functioning in nature to improve motility.

The biosurfactants produced by *Pseudomonas aeruginosa* serve as an excellent example of the complexity in determining the roles of biosurfactant production. This bacterium produces rhamnolipids, which are a mixture of di-rhamnolipids, mono-rhamnolipids, and HAA, the rhamnose-free lipid precursor (Deziel *et al*., 2003). A wide range of functions have been proposed for rhamnolipids including bacterial access to hydrophobic carbon sources, biofilm structure, biofilm departure, as well as swarming motility (Zhang and Miller, 1994; Davey *et al*., 2003; Boles *et al*., 2005). Curiously, although each of these three surfactants facilitate motility on a swarming plate, more detailed analysis of swarming behavior revealed that HAAs actually have a repellant role while di-rhamnolipids are attractants, suggesting a more complex process by which surfactants enable motility (Tremblay *et al*., 2007). Thus, although HAAs might simplistically appear to aid bacterial motility on a swarming plate by lowering the surface tension, they probably have a more subtle role in *in vivo* motility.

Recently, while investigating the production of syringafactin in the plant-associated bacterium *Pseudomonas syringae* B728a, it was observed that this strain produced a second surfactant detectable on agar plates (Burch *et al*., 2010); mutant strains in which the syringafactin biosynthetic cluster were disrupted still produced surfactant detectable as a halo in an atomized oil assay. This second surfactant, although not produced in sufficient quantities to confer collapse of water drops, enabled swarming motility on a semi-solid agar surface. Since the movement of plant pathogens as well as human pathogens on plants are of biological and practical significance we have characterized this second surfactant in order to better understand the complex roles of surfactants on bacterial behaviors on leaf surfaces. This report addresses the biosynthetic identification of the remaining surfactant, as well as a number of genes that regulate its production. We will show that there is an intimate link between flagellar function and production of this biosurfactant that suggests that it plays a specific role in motility.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. syringae* pv. syringae B728a (Loper and Lindow, 1987) was cultured on King's medium B (KB) plates with 1.5% agar technical (King *et al*., 1954) at 28°C. *E. coli* strains DH5α, S17-1 (Simon *et al*., 1983), and SM10(λpir) (Delorenzo *et al*., 1990) were cultured on Luria Agar at 37°C. Antibiotics were used at the following concentrations (μg/ml): kanamycin (25 for *P. syringae*, 50 for *E. coli*), rifampin (100), gentamycin (75), tetracycline (15), and spectinomycin (100).

Biosurfactant detection assays. The atomized oil assay was performed as in (Burch *et al*., 2010): Bacteria were spotted onto agar plates using sterile toothpicks and grown overnight. An airbrush (Type H; Paasche Airbrush Co., Chicago, IL) was used to apply a fine mist of mineral oil (light paraffin oil, Fisher Scientific) onto the plate at an air pressure of 19 psi. The radius of a "halo" of oil droplets having altered shapes that caused them to appear brighter when visualized with an indirect source of bright light were measured with a ruler from the edge of bacterial colonies to the distal edge of the surfactant halo.

Production of biosurfactant mutants. The production of transposon mutants was done by a method similar to that of Larsen *et al*. (Larsen *et al*., 2002). Briefly, a **Δ***syfA* deletion mutant of *P. syringae* B728a and the conjugative *E. coli* strain SM10(λpir) harboring pUT mini-Tn5 Sm/Sp (Delorenzo *et al*., 1990) were grown overnight on agar plates with appropriate antibiotics. Cells were then harvested with a loop, washed and re-suspended in potassium phosphate buffer (10 mM, pH 7.5), and then mixed in a ratio of 1:3 (*E. coli* : *P. syringae*) and incubated overnight as a confluent lawn on a KB plate. After incubation, the cells were re-suspended in phosphate buffer and 10% of the cell suspension was plated onto KB medium containing 100 μg/ml rifampin and 100 μg/ml spectinomycin and incubated for three days. Putative *P. syringae* transposon mutants were screened for biosurfactant production by the following method: Cells were spotted using sterile toothpicks from colonies on selection plates onto KB plates, with spots separated by at least 2 cm. Colonies were allowed to develop overnight and then sprayed with atomized mineral oil drops as described above. Mutants that exhibited substantially larger (over 20%) or smaller halos were re-tested. Only mutants with phenotypes that were consistently different from the wild-type strain were further investigated. The genes into which the transposon had inserted in these mutants was determined using arbitrarily-primed PCR similar to the method of O'Toole *et al*., (O'Toole *et al*., 1999). Mutations generated by the transposon from pUT mini-Tn5 Sm/Sp,

were characterized using primers complementary to the 5' end of the transposon; primer tn5sm-ext, 5'-GCGCGAGCAGGGGAATTG**,** was used in the initial PCR reaction, and primer tn5sm-int, 5'-CGGTTTACAAGCATAAAGCTTGCTC, was used in a second reaction to amplify sequences 5' to the insertion site. The PCR product was purified (QIAquick PCR Purification kit, Qiagen) and submitted for sequencing using primer pRLint1. The locations of the sequenced fragments were determined directly by a BLAST search on the *Pseudomonas* genome database (Winsor *et al*., 2009) and compared to the published sequence of *P. syringae* B728a (Feil *et al*., 2005).

Motility assays. Swarming motility of *P. syringae* B728a was assessed on semi-solid KB plates containing 0.4% technical agar as in previous studies (Quinones *et al*., 2005). Cells were grown for one day on KB and then harvested and washed in potassium phosphate buffer (10 mM, pH 7.5). Cells were re-suspended in buffer to an OD_{600} of 0.27**,** and 5 µl (approximately 2.5 X 10^6 cells) of the appropriate bacterial strain was pipetted onto each plate and incubated for 24 hours at room temperature. Swimming motility was assessed in 0.25% technical agar plates. Cells were grown for one day on KB and then stab inoculated into the center of the swimming plates. Swimming distance was measured as the distance from the point of inoculation to the bacterial front within the agar. Movement of cells through hydrated paper discs (1/4 in, Schleicher & Schuell, #740-E) was also determined. Bacteria were either inoculated onto the top of the filter disc with a toothpick, or were pipetted onto the discs in 1 µl inocula. More extensive movement through paper was performed by placing filter paper (Whatman #1) cut into 1.5 cm 4.5 cm strips on KB plates and inoculating them at a distance of 0.5 cm from the end by toothpick application. Strips were removed after 16 hours by carefully lifting them so as to minimize manual spreading of the bacteria that had entered the strips. The plates on which the srips had rested were then incubated for at least 2 days at room temperature and the amount of bacterial movement was measured as the most distal extent of bacterial growth.

Construction of biosurfactant deletion mutants. A deletion mutant of the *syfA* gene was constructed by cloning approximately 1 kb fragments upstream and downstream of *syfA* into pENTR/D-TOPO:MCS-Kan (Dulla, 2008). The region downstream of *syfA* was amplified by the primers 5'AACTCGAGGTGAGCATCAACGAACTCTTGGCG (syfAe-xhoF) and 5'AATCTAGACGCGCTGTGCCGGTAGTTGAGC (syfAe-xbaR), digested with *XhoI* and XbaI and ligated into pENTR/D-TOPO:MCS-Kan. The region upstream of *syfA* was amplified by the primers 5'AACCTAGGAATGGATGCGCCGGGTTGGTACC (syfAs-avrF) and 5'GAGGATCCGGCTCAAGGTCCTTCTTGGCGG (syfAs-bamR), digested with *AvrI* and *BamHI*, and ligated into pENTR/D-TOPO:MCS-Kan. The resulting region containing both flanking sequences and *npt2* driving kanamycin resistance were transferred to pLVC/D (Marco *et al*., 2005) via a clonase LR reaction (Invitrogen). The resulting plasmid was isolated and electroporated into *E. coli* S17-1 for conjugal transfer. Both *E. coli* and *P. syringae* were grown individually overnight on plates, then mated overnight. Initial transformants were isolated on KB plates containing rifampin, kanamycin and tetracycline. Deletion mutants were selected which were kanamycin resistant but regained tetracycline sensitivity. Deletions were confirmed by PCR amplification, which verified that the kanamycin cassette had replaced *syfA*.

An unmarked deletion mutant of *brfA* was constructed by a modified overlap extension PCR (Choi and Schweizer, 2005). Briefly, the 5' and 3' regions flanking *brfA* were amplified in a first round of PCR reactions, in addition to a kanamycin resistance cassette flanked with FRT

sites from pKD13 (Datsenko and Wanner, 2000). The primers used were 5'CGGCGCTCGGCATTCGTTG (brfA-F1) and 5'GAAGCAGCTCCAGCCTACACAACAGCCTCCCAGCTAAAATTTGATCCAGC (brfA-R1) used to amplify the region upstream of *brfA*, 5'GGTCGACGGATCCCCGGAATAGCACTCGCCTGCGCC (brfA-F2) and 5'GCAGCGGAGACGATAGGGGTGATT (brfA-R2) used to amplify the region downstream of *brfA*, and 5'GTGTAGGCTGGAGCTGCTTC (FRT-KM-F) and 5'ATTCCGGGGATCCGTCGACC (FRT-KM-R) used to amplify the FRT-flanked kanamycin resistance cassette. In the following PCR reaction, all three fragments were combined and amplified for 15 cycles without added primers, followed by addition of brfA-F1 and brfA-R2 for 20 more PCR cycles to amplify the combined fragment. The resulting fragment was cloned into the suicide vector pTOK2T (Chen *et al*., 2010) and transferred into *P. syringae* by triparental mating (Chen and Beattie, 2007). Initial transformants were isolated on KB plates containing rifampin, kanamycin and tetracycline. Double crossover mutants were selected which were kanamycin resistant but regained tetracycline sensitivity. The *kan* cassette was excised by introduction of the plasmid pFLP2 (Hoang *et al*., 1998) that contained the omega fragment for spectinomycin resistance (R. Scott, unpublished), followed by replica plating to cure the **Δ***brfA* strain of pFLP2-omega. Final markerless deletions were confirmed by PCR. In order to generate a **Δ***syfA*/**Δ***brfA* double deletion mutant, unmarked **Δ***brfA* was first generated before deleting the *syfA* gene in order to avoid redundant kanamycin resistance genes.

Chromosomal disruptions of FleQ, FliA, FliF, and FlgD. Site-directed mutagenesis was performed with single-crossover insertion events. Fragments of the genes of interest were amplified from *P. syringae* genomic DNA by PCR with primers 5'CACCGTCGGGCACTGGCAAGGAG (fleQ-KO-F), 5'GGCGCCATCTCGATCGGGAACAC (fleQ-KO-R), 5'CACCGGCCTGCTTGAAGTCTCC (fliA KO F), 5'CTCACGCTCTGGCAGATTGGC (fliA-KO-R), 5'CACCGAGGTCTCGGCAGTGG (fliF-KO-F), 5'CATTGGCCGCGTTGGTCTTGA (fliF-KO-R), 5'CACCGCTCGTGACGCAGATGAAGAA (flgD-KO-F), and 5'GCCTTCGACCGAGCCTTCAGC (flgD-KO-R). The resulting four inserts were subcloned into pENTR/D-TOPO and subsequently transferred into pLVC/D by clonase LR reactions. Plasmids were isolated and electroporated into *E. coli* S17-1 for conjugal transfer. Both *E. coli* and *P. syringae* were grown individually overnight on plates, then mated overnight. Transformed *P. syringae* were isolated on KB plates containing rifampin and tetracycline. Knockouts were confirmed by PCR amplification.

Construction of pBRF2, a BrfA complementation vector. Full-length *brfA* was amplified by PCR from genomic DNA with primers 5'ACCATGGGCGCACAATCGAAGATTCTAACAATCGG (brfA-nco-F) and 5'TCTCGAGTCAGGCCATCGCGGTG (brfA-xho-R). The 5' primer contained the ATG start codon within the *NcoI* cut site. PCR conditions were as follows: 28 cycles of 95°C, 59°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. The resulting fragment was digested with *NcoI* and *XhoI* and cloned into pMF54-omega, which was a modified version of pMF54 (Franklin *et al*., 1994) that contained the omega fragment for spectinomycin resistance (R. Scott, unpublished). The expression plasmid pBRF2 was electroporated into wild-type and mutant strains of *P. syringae* B728a with selection for spectinomycin resistance.

Construction of pP*brfA-gfp*, pP*fliC-gfp*, pP*fliE-gfp*, and pP*flgB-gfp* transcriptional fusion reporters. Reporter plasmids were constructed similar to described in Burch (2010). The upstream promoter region of the *P. syringae* B728a *brfA* gene was amplified by PCR from genomic DNA with primers 5'AGAAAGCTTCAGGCACTTTCCAAGCC (brfA-pro-F) and 5'AGAATTCAACAGCCTCCCAGCTAAAATTTGATCC (brfA-pro-R) to generate a 495-bp promoter region. The upstream promoter region of the *P. syringae* B728a *fliC* gene was amplified by PCR from genomic DNA with primers 5'TCTCTGCAGTCGCCTTACAAAGAACGCC (fliC-pro-F) and 5'AGGATCCGATGAATTCCTCGGTGGTTTTGG (fliC-pro-R) to generate a 321-bp promoter region. The upstream promoter region of the *P. syringae* B728a *fliE* gene was amplified by PCR from genomic DNA with primers 5'TAGGATCCAGGCACACGGATCGC (fliE-pro-F) and 5'AGGAATTCATCTCTCGTAAGGCCCG (fliE-pro-R) to generate a 235-bp promoter region. The upstream promoter region of the *P. syringae* B728a *flgB* gene was amplified by PCR from genomic DNA with primers 5'TAGGATCCTCAATCGTCAAAGAGACCTTCGGG (flgB-pro-F) and 5'AGGAATTCGGGAAACCTTTGCCGGTTG (flgB-pro-R) to generate a 278-bp promoter region. The PCR products were first cloned into pTOPO Blunt (Invitrogen) and transformed into *E. coli* DH5α. The insert was sequenced to verify its identity. pTOPO-P*brfA* was digested with *HindIII* and *EcoRI*, pTOPO-P*fliC* was digested with *PstI* and *BamHI*, pTOPO-P*fliE* and pTOPO-P*flgB* were digested with *BamHI* and *EcoRI*. The resulting fragments were cloned into pPROBE-GT (Miller *et al*., 2000) which contains a promoterless *gfp* gene in order to generate pP*brfA-gfp*, pP*fliC-gfp*, pP*fliE-gfp*, and pP*flgB-gfp*.

Promoter reporter plasmids were electroporated into *P. syringae* B728a as well as mutant strains altered in biosurfactant production. Unless otherwise indicated, the appropriate transformed strains were grown overnight on KB plates and then suspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD_{600} of 0.2. Cells from hydrated paper discs were inoculated by toothpick onto paper discs, grown overnight, and then the paper discs were transferred into phosphate buffer and vortexed to move the cells into solution. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with a 486-nm bandpass excitation filter and a 510- to 700-nm combination emission filter. A relative fluorescence unit (RFU) was defined as the fluorescence of the suspensions normalized for the suspension turbidity measured as OD_{600}.

Microscopy. Cells were diluted to appropriate concentrations and flash-frozen in a solution containing 2 μg/ml DAPI. DAPI-stained cell samples were washed and spotted onto charged slides (clean glass slides pre-dipped in 0.1% gelatin solution) in 10 μl droplets and air-dried under the hood. Samples were then covered with Aqua PolyMount anti-fade mounting reagent (Polysciences, cat#18606) and cover slips. Bacteria were viewed and photographed at 1000x magnification using a Hamamatsu digital camera attached to a Zeiss AxioImager M1 microscope. Samples were excited using a broad-spectrum mercury arc lamp, and visualized using standard DAPI and EndowGFP filter cubes. Exposure settings were 0.75 seconds for DAPI and 2 seconds for GFP. For each treatment, 5-10 images were acquired using iVision software, using the Multi-D Acquire function for paired DAPI and GFP photomicrographs. For all image pairs, DAPI-stained bacterial cells were masked using the iVision Segmentation function, and the segment masks were copied and pasted onto the tandem GFP images. Mean

GFP pixel intensity for each masked object was quantified. Objects that were less than 10 or more than 200 pixels in size were excluded from the data. Background fluorescence was measured by calculating the pixel intensity from cell-free portions of GFP images.

Extraction of BRF. Crude biosurfactant-containing extracts were prepared using modifications to a general HAA extraction protocol (Deziel *et al*., 2003). Agar plates with confluent lawns of *P. syringae* B728a **Δ***syfA* carrying pBRF2 were grown for 48 hours. Cells were harvested by washing of four plates in 90 ml H_2O and cells were removed by centrifugation (5,000 x g, 10 min). Supernatant was filter-sterilized and appeared opaque even after filtration. The supernatant was brought to pH 2 with concentrated HCl, and mixed with 150 ml chloroform:methanol 2:1. Upon mixing, both organic and aqueous layers became very opaque, and were allowed to separate overnight. The lower organic fraction was later dried to completion. Curiously, there was a fluffy precipitated white substance sandwiched between the aqueous and organic layers, which was also collected and secondarily washed with H_2O pH 2. Upon another wash with chloroform:methanol, the precipitate changed appearance and pelleted whereas it previously had floated on the organic layer. Both the pellet and the chloroform:methanol "powder wash" were saved and tested for surfactant activity, along with the dried organic fraction. The final pellet did not display surface activity, but the powder wash contained large quantities of BRF, as did the original dried organic fraction to a lesser extent. The powder wash was chosen for use in all the experiments requiring BRF extract.

Surface tension measurements

The surface tension of the HAA extract was determined using the pendant drop method. The extract was analyzed with a FTA 4000 video analysis instrument (First Ten Angstroms Inc., Portsmouth, VA). Droplets were produced using a 22 gauge blunt needle and the values reported represent an equilibrium surface tension determined 60 seconds after drop formation.

RESULTS

P. syringae B728a produces two motility-enabling surfactants.

Previously, we observed that transposon mutants with insertions in genes conferring syringafactin biosynthesis in *P. syringae* B728a were still capable of limited swarming and exhibited small surfactant halos when tested with an atomized oil assay (Burch *et al*., 2010). This observation led us to hypothesize that either our insertion mutants were not fully blocked in syringafactin production, or that this strain produces a second motility-enabling surfactant. As a confirmation of this observation, we constructed a deletion mutant of *syfA*, the first of two genes in the syringafactin biosynthetic cluster. As observed in *syfA* and *syfB* insertional mutants, **Δ***syfA* retained the ability to produce a small amount of surfactant detectable with the atomized oil assay (Fig. 1B) and was still capable of limited swarming (Fig. 1E).

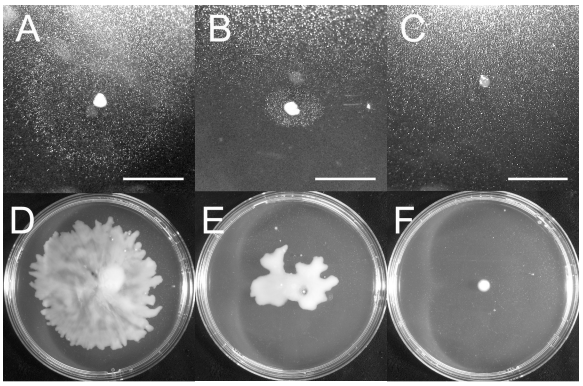

Figure IV-1. Surfactant production and motility phenotypes of surfactant mutants
Comparison of surfactant-induced halos visualized in the atomized oil assay (A-C) and swarming motility on 0.4% soft agar plates (D-F). *P. syringae* WT is in the left column (A and D), a Δ*syfA* strain in the middle column (B and E), and a Δ*syfA*/Δ*brfA* double deletion mutant in the right column (C and E). Bars represent 1 cm (A-C).

Intriguingly, while comparing different methods for bacterial inoculation on agar plates, it was observed that production of this surfactant increased dramatically when the strain was grown on the porous surface of hydrated filter paper discs placed on agar plates (Fig. 2). This was true both when the bacteria were directly applied with a toothpick as a single spot on the paper surface, and somewhat less so when inoculated as a larger patch from an aqueous cell suspension (data not shown). A variety of additional materials other than cellulose such as cotton and polyester fabrics were tested for their stimulation of apparent surfactant production, and all induced production as long as the material was wettable (data not shown). The rough surface-induction of surfactant production led us to the hypothesis that the surfactant might contribute to the colonization of natural surfaces and thus prompted further investigation.

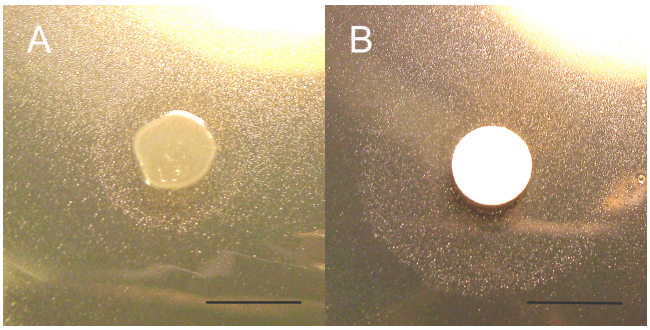

Figure IV-2. Filter paper stimulation of surfactant production
Atomized oil assay after 16 hours of growth of a Δ*syfA* strain grown from a spot of approximately 2.5 X 10^6 cells inoculated by pipette directly onto the plate (A), or inoculated by toothpick onto a filter paper disc placed on an agar plate (B). Bars represent 1 cm.

In order to determine the identity of this surfactant, the atomized oil assay was used to screen a library of Tn*5* mutants created in a **Δ***syfA* background. Over 4,500 independent insertion events were screened for mutants with significantly more or less surfactant production as evidenced by larger or smaller halos on agar plates when assayed with this high-throughput method. After

excluding mutants having obvious growth defects, six strains were identified that exhibited a complete loss of surfactant production. Additionally, 24 other strains were found to consistently produce significantly (P<0.01) more or less surfactant than the **Δ***syfA* parental strain (Table 1). The genes disrupted by transposon insertion were identified, revealing that 6 genes were required for surfactant production, while disruption of 8 different genes conferred less surfactant production and disruption of 6 genes up-regulated production.

Table IV-1. Insertional mutants with altered surfactant production

Locus of Tn*5* insertion	Predicted function	Individual transposon hits[a]	Surfactant halo radius (mm)[b]	Swimming diam (mm)[c]
Psyr_0215 (*xth*)	Exodeoxyribonuclease		0**	6.67 ± 0.6
Psyr_3129[d]	Acyltransferase		0**	8.00 ± 1.0
Psyr_3698 (*gacS*)	Response regulator		0**	8.33 ± 0.6
Psyr_0258 (*ompR*/*amgR*)	Response regulator		0**	6**
Psyr_3461 (*fleQ*)	Flagella sigma factor		0**	0**
Psyr_4446 (*osmE*)	Osmotically-inducible lipoprotein		0**	5.33 ± 0.6**
Psyr_0936	Glycosyl transferase	3	0.67 ± 0.6**	0**
Psyr_0219 (*algC*)	Phosphomannomutase		0.67 ± 0.6**	1**
Psyr_0918 (*wzt*)	ABC transporter	2	1**	0**
Psyr_2083	Unknown		1**	6.33 ± 0.5*
Psyr_0270 (*polA*)	DNA polymerase	2	0.83 ± 0.4**	8.00 ± 0.0
Psyr_1981	PAS:GGDEF		1.17 ± 0.4**	9.00 ± 1.0
Psyr_3669	Outer membrane protein	2	1.33 ± 0.6**	8.67 ± 0.6
Psyr_3480 (*flgC*)	Flagellar assembly		1.67 ± 0.6**	0**
(**Δ***syfA*) No insertion			5.33 ± 0.5	8.33 ± 0.6
Psyr_3466 (*fliC*)	Flagellin		6.67 ± 0.6**	0**
Psyr_3469 (*fgt1*)	Flagella glycosyl transferase	3	7.67 ± 1.2**	2.00 ± 1.0**
Psyr_3468 (*fgt2*)	Flagella glycosyl transferase		7.00 ± 0.9**	8.33 ± 1.5
Psyr_2979 (*gor*)	Glutathione reductase		10.3 ± 1.2**	9.00 ± 1.0
Psyr_0263 (*algB*)	Response regulator	4	11.33 ± 1.2**	9.67 ± 2.5
Psyr_1350 (*mucP*)	Peptidase		14.33 ± 0.6**	9.33 ± 0.6*

[a] Number of times that independent mutants were identified as insertions in the same gene

[b] Halos with significantly smaller or larger radii compared to WT (For all, P < 0.01, t-test)

[c] Bacterial motility through semi-solid agar plates: motility was significantly different from wild-type at P<0.05 (*) or P < 0.01 (**) as determined by a t-test

[d] Insertion is immediately upstream of the gene

An *rhlA* homolog implicated in biosurfactant production.

Of the six mutants identified as being completely blocked in biosurfactant production three of the insertions were into the global regulatory genes *gacS*, *ompR*, and *fleQ*, and thus were deemed to be not specifically responsible for surfactant biosynthesis. GacS is a global regulator of secondary metabolites and extracelullar enzymes (Heeb and Haas, 2001), while an OmpR homolog has recently been hypothesized to be a membrane stress sensor in *P. aeruginosa* (Lee *et al*., 2009), and FleQ is the initial regulatory element of flagellar biosynthesis (Dasgupta *et al*., 2003). Of the remaining genes influencing biosurfactant production, neither Psyr_0215 which is predicted to have general base excision repair activity, nor Psyr_4446 which is an osmotically-induced outer membrane lipoprotein, are likely candidates for contributing to surfactant synthesis. On the other hand, a predicted acyltransferase, Psyr_3129, having 48.5% identity to *rhlA* and 49% identity to *phaG* in *P. aeruginosa* PAO1, seemed likely to be involved directly in surfactant biosynthesis. RhlA is responsible for production of 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursor to rhamnolipids in *P. aeruginosa*, and is independently recognized as a biosurfactant that promotes swarming motility (Deziel *et al*., 2003). PhaG is involved in polyhydroxyalkanoic acid (PHA) synthesis, which is a carbon and energy storage molecule (Rehm *et al*., 1998). Both enzymes divert hydroxydecanoic acids from fatty acid de novo synthesis, and exhibit similar and sometimes overlapping polymerization functions (Soberon-Chavez *et al*., 2005).

Because the transposon insertion was in the promoter region immediately upstream of Psyr_3129, we confirmed that a knockout of this gene also blocked surfactant production by constructing a chromosomal deletion of Psyr_3129 (hereafter called the locus for biosurfactant regulated by flagella – *brfA*) in the **Δ***syfA* background of *P. syringae* (Fig. 1C). This double mutant was also incapable of swarming ability (Fig. 1F). To ensure that disruption of *brfA* and not genomic changes elsewhere was responsible for abrogating biosurfactant production, we complemented this gene *in trans*. Expression of *brfA* under the control of the constitutive *npt2* promoter in plasmid p*519n-gfp*, where *gfp* was replaced with *brfA*, proved to be lethal to *P. syringae* (data not shown). However, when *brfA* was inserted into pMF54 (Franklin *et al*., 1994), to form plasmid pBRF2 where *brfA* is driven by an IPTG-inducible *trc* promoter, this plasmid produced viable transformants. Curiously, when this plasmid is introduced into a **Δ***syfA/***Δ***brfA* double mutant, biosurfactant was produced abundantly without IPTG addition (Fig. 3B), emphasizing the leaky nature of this plasmid. Addition of IPTG did not result in surfactant production beyond that observed in uninduced cells. Thus, either BrfA synthesizes the surfactant or is essential for its expression. Significantly, *rhlA* from *P. aeruginosa* has been shown to be sufficient for HAA production in *E. coli* (Deziel *et al*., 2003; Zhu and Rock, 2008), as well as an *rhlA* homolog in *Serratia* sp. ATCC 39006 which produces an unidentified biosurfactant (Williamson *et al*., 2008). We thus tested if our potential *rhlA* homolog was sufficient to confer biosurfactant production in *E. coli*. *E. coli* DH5α harboring plasmid pBRF2 produced a large amount of surfactant (Fig. 3D).

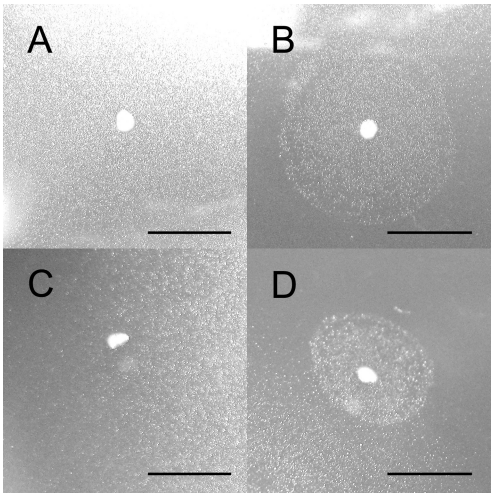

Figure IV-3. Constitutive expression of Psyr_3129 in *P. syringae* and *E. coli*
Atomized oil assay of a *P. syringae* Δ*syfA*/Δ*brfA* deletion mutant (A and B) and E. coli strain DH5α (C and D). Colonies on the right carry pBRF2, a plasmid expressing constitutive BrfA (B and D).

It is important to note that although production of this surfactant in a **Δ***syfA* strain of *P. syringae* is readily detected with the atomized oil assay, it was not detectable with other assays such as the drop collapse assay or by direct chemical detection. This suggested that either the molecule had properties such as low water solubility that prevented its detection with assays such as water drop collapse, or that it was made in relatively low amounts that are not easily detected by assays with lower sensitivity. However, a **Δ***syfA* strain carrying pBRF2 for constitutive BrfA expression was observed to cause a drop collapse (data not shown), thus we presume that low rates of production in native strains explain its lack of detection in a **Δ***syfA* strain with a drop collapse assay. Using a modified protocol for HAA extraction (Deziel *et al*., 2003), we extracted BRF from plate-grown cultures of **Δ***syfA* (pBRF2). The resulting powder yielded an opaque solution in water, indicative of a surfactant with low water solubility exhibiting aggregate formation (Myers, 2006). This concentrated surfactant lowered the surface tension of water to 29 dyn/cm when measured in a pendant drop assay, confirming its potent surfactant activity. It remains to be determined what the chemical structure of BRF is, and if it is HAA.

Coordinate regulation of flagella and biosurfactant production.
An unexpected finding in the analysis of mutants with altered biosurfactant production was the fact that many harbored disrupted genes encoding flagella, which prompted us to named the surfactant BRF (Table 1). While these insertions yielded either increased or decreased surfactant production, they all similarly inhibited flagella-dependent swimming motility, with the exception of the two flagellar glycosylation mutants (Table 1). The inconsistent effects of flagella mutants on surfactant production were, however, associated with the order these genes genes play in flagellar assembly (Table 2).

Table IV-2. Flagellar assembly classes and their effects on surfactant production

Source	Assembly	Gene	Relative halo radius
From screen	Class I	FleQ	0
Targeted mutant	Class I	FleQ	0
Targeted mutant	Class I	FliA	0.36
Targeted mutant	Class II	FliF	0.27
From screen	Class III	FlgC	0.36
Targeted mutant	Class III	FlgD	0.33
From screen	Class IV	FliC	1.22
From screen		Fgt1	1.19
From screen		Fgt2	1.25

Assembly class designations are as according to Dasgupta *et al*. (2003) for *Pseudomonas aeruginosa*. Relative halo measurements are expressed as the average halo size measured for the mutant divided by the average halo size measured for Δ*syfA*, and all averages were calculated from six halo measurements each.

An insertion into the transcription factor *fleQ*, which is involved in the initiation of flagellar assembly results in a total loss of surfactant production. Disruption of *flgC*, a Class III flagellar assembly gene that is involved in formation of the basal body rod in *P. aeruginosa* (Dasgupta *et al*., 2003), also resulted in a large (3-fold) reduction in the surfactant halo. The identification of these two mutants led us to hypothesize that assembly of the flagellar base structure is important for production of BRF. Surprisingly, an insertion in *fliC*, a Class IV structural gene encoding the actual flagellin protein, resulted in enhanced (1.2-fold) surfactant production. Furthermore, insertions in *fgt1* and *fgt2*, two genes involved in flagellar glycosylation that have been shown in *P. syringae* pv. tabaci 6605 to be important for flagellar function (Taguchi *et al*., 2006), both also result in up-regulation of surfactant production. This suggested that once the flagellar base is assembled and flagellin synthesis is initiated, mutations which hinder flagellar assembly or functionality serve to up-regulate the production of BRF. Curiously, even though an insertion in *fgt1* only impaired flagellar swimming motility while an insertion in *fgt2* did not appear to confer any flagellar impairment (Table 1), these mutations both stimulated surfactant production to a similar extent as a loss of flagellin itself. We remain uncertain how these mutations lead to up-regulating surfactant production.

To further support our hypothesis that expression of BRF is dependent on flagellar assembly itself and not merely coincidentally with expression of certain flagellar genes, we constructed targeted knockouts in additional flagellar genes involved at different stages of flagellar assembly (Table 2). A directed knockout mutant of *fleQ* was deficient in surfactant production, confirming our earlier observations of an insertional mutant of this gene. Although the initial screen did not identify any insertions in Class II genes that are important for the initial establishment of the flagellar apparatus, a directed knockout of *fliF* exhibited a dramatic loss of surfactant production. Furthermore, a knockout of *flgD*, a Class III flagellar gene in an operon downstream of *flgC*, resulted in a similar 3-fold reduction in the size of the BRF halo (Table 2). Disruption of *fliA*, encoding the sigma factor responsible for initiating transcription of Class IV genes, also conferred a 3-fold reduction in surfactant production. Thus, although FliA is necessary for

expression of late stage flagellar genes, it does not appear necessary for production of BRF (Fig. 14A).

Because the establishment of the flagellar base appears important for production of this surfactant, we postulated that perhaps the flagellum is in some way necessary for the export of BRF. In order to test this model, we introduced plasmid pBRF2 conferring constitutive BrfA expression into a **Δ***syfA/fleQ*- double mutant strain of *P. syringae*. This strain, despite lacking flagella, exhibited unaltered surfactant production (data not shown), indicating that flagella are not necessary for surfactant export. Thus it appears that the flagellar assembly process most likely influences *brfA* at the transcriptional level.

In order to investigate the contribution of flagellar assembly to transcriptional regulation of *brfA* we linked a *gfp* reporter gene to the promoter containing region 5' to *brfA* in the stable plasmid vector pPROBE-GT (Miller *et al*., 2000) to produce reporter plasmid pP*brfA-gfp*. We introduced pP*brfA-gfp* into the different insertional mutants blocked at different stages of flagellar assembly and observed that, as was indicated by the atomized oil assay, the expression of *brfA* was higher in a **Δ***syfA/fliC*- mutant compared to that in either a **Δ***syfA/fleQ*- or **Δ***syfA/flgC*- mutant (Fig. 4). We also constructed reporter plasmid pP*fliC-gfp* in which a *gfp* reporter gene was fused to the promoter-containing region of *fliC* to provide estimates of the expression of the gene encoding flagellin, a late stage flagellar gene (Fig. 4). Similar to what was observed for expression of *brfA*, the expression of *fliC* was greatly reduced in both a **Δ***syfA/fleQ*- and **Δ***syfA/flgC*- background but was over-expressed relative to that in a **Δ***syfA* background alone in a **Δ***syfA/fliC*- mutant.

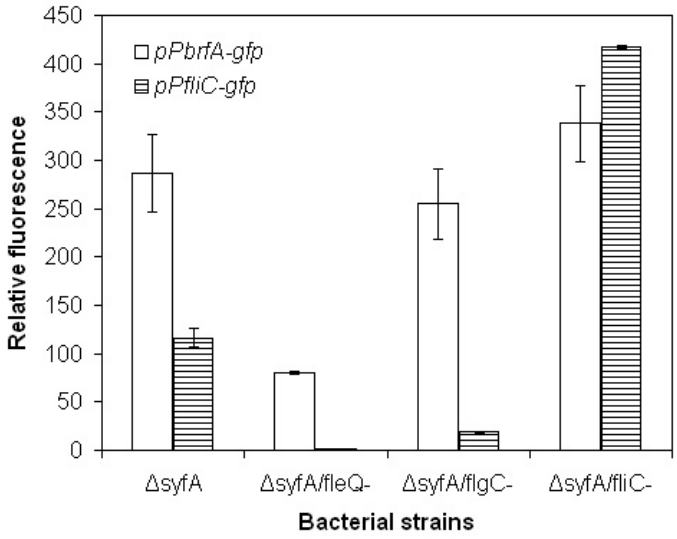

Figure IV-4. Transcriptional regulation of *brfA* and *fliC* by flagellar assembly
Relative GFP fluorescence of different flagellar mutant strains of *P. syringae* B728a harboring either a plasmid in which GFP expression is dependent on the promoter of Psyr_3129 (pP*syfA-gfp*) or a plasmid in which GFP expression is dependent on the promoter of flagellin (pP*fliC-gfp*).

As far as we are aware, flagellar glycosylation has not been documented to have a feedback role in flagellin biosynthesis. Although it is intuitive that a loss of flagellin production might result in constitutive activation of the late-stage flagellar genes through FliA, it is less obvious how flagellar glycosylation mutations might be feeding back to up-regulate flagella production, especially in the case of *fgt2* which does not exhibit any impairment of flagellar function. In order to investigate the feedback process, we constructed transcriptional reporters of both *flgB*, a class II flagellar gene, and *fliE*, a class III flagellar gene, in addition to the *fliC* reporter. Reporter plasmids p*flgB-gfp* and p*fliE-gfp*, respectively, were separately introduced into the original **Δ***syfA* strain as well as a **Δ***syfA*/*fgt2*- strain, so that the effect of flagellar glycosylation on the expression of the three classes of flagella genes could be observed. We clearly observed that a loss of flagellar glycosylation results in up-regulation only of the late stage flagellin gene *fliC* and not of *fliE* or *flgB* (Fig. 5). Loss of glycosylation most likely affects the flagella in such a way as to encourage the export of the anti-sigma factor FlgM, either through increased flagellar breakage or increased export within the flagella, thus releasing FliA from FlgM control.

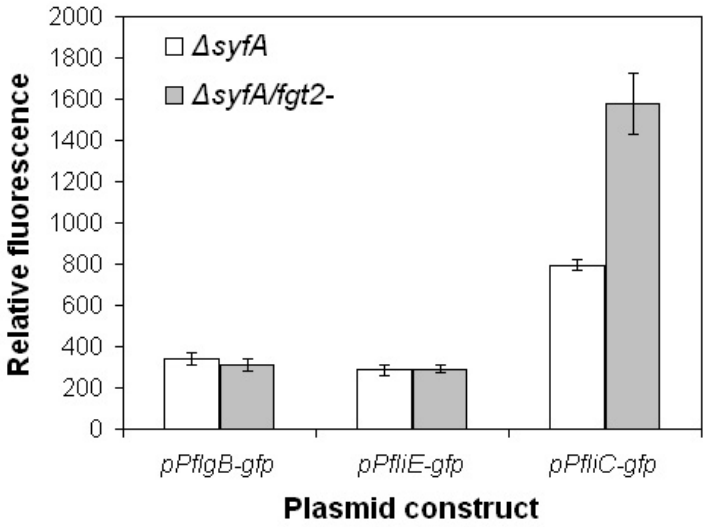

Figure IV-5. Hierarchy of regulatory feedback conferred by a flagellar glycosylation mutant
Relative GFP fluorescence exhibited by either **Δ***syfA* or the flagellar glycosylation mutant **Δ***syfA*/*fgt*2- strain of *P. syringae* B728a harboring plasmids p*flgB-gfp*, p*fliE-gfp*, and p*fliC-gfp* in which GFP expression is dependent on the promoter of flagellar assembly genes *flgB*, *fliE* and *fliC*, which are Class II, Class III, and Class IV flagellar assembly genes, respectively.

Flagellar surface sensing and flagellar control of surfactant production.
To address the process by which paper surfaces up-regulate production of BRF we addressed the expression of *brfA* under various growth conditions. The GFP fluorescence of a WT strain carrying p*brfA-gfp* was compared between when grown on filter paper discs on agar plates and when grown directly on agar plates. While GFP fluorescence exhibited by *P. syringae* harboring plasmid p*519n-gfp* conferring constitutive GFP expression was similar in these two growth conditions, much higher GFP fluorescence was observed after growth on the porous paper in the strain carrying p*brfA-gfp* (Fig. 6). Such apparent paper surface-induced upregulation of *brfA* was observed in both the WT strain as well as a **Δ***syfA* strain (data not shown). No such

induction of *syfA* was observed when strains harboring pP*syfA-gfp* were grown on paper discs (data not shown), indicative that syringafactin is not similarly regulated.

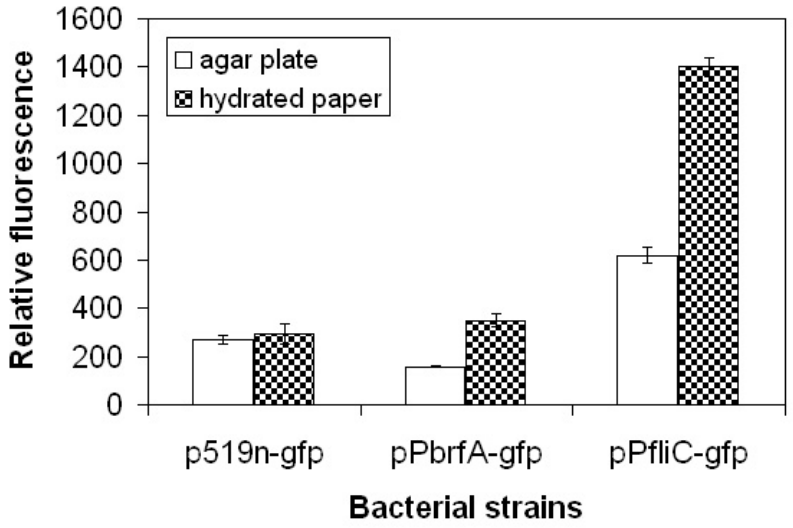

Figure IV-6. Filter paper disc up-regulation of *brfA* and *fliC*
GFP fluorescence of WT *P. syringae* carrying either a constitutively fluorescent plasmid (p*519n-gfp*), a plasmid indicative of *brfA* transcription (pP*brfA-gfp*), or a plasmid indicative of FliC transcription (pP*fliC-gfp*). Strains were tested after overnight growth either on agar plates, or on filter paper discs placed on agar plates. Data is representative of at least two repetitions.

Because we observed both enhanced production of BRF and elevated expression of *brfA* in cells grown on hydrated paper discs, as well as a dependence of BRF production on flagella assembly, we hypothesized that genes for flagella for motility would be up-regulated on the paper discs coincidently with those for BRF production. To test this, we compared the GFP fluorescence of cells harboring the *fliC* reporter plasmid pP*fliC-gfp* when grown on agar plates and paper discs. As hypothesized, we observed an up-regulation of genes encoding flagellin when the strain is exploring the porous paper surface (Fig. 6). This implies that flagellar motility is important for growth on this rough porous surface. In order to examine the necessity of flagella for movement through hydrated paper, we compared the lateral spread of a WT strain and a *fleQ*- mutant on paper discs. While flagellated strains quickly moved both into and along the length of the paper discs, the non-flagellated strains remained at the site of inoculation and formed colonies only on top of the paper (Fig. 7A and B). This requirement of motility for colonization of paper disks appears very similar to that observed for exploration of a porous ceramic surface (Dechesne *et al*., 2010).

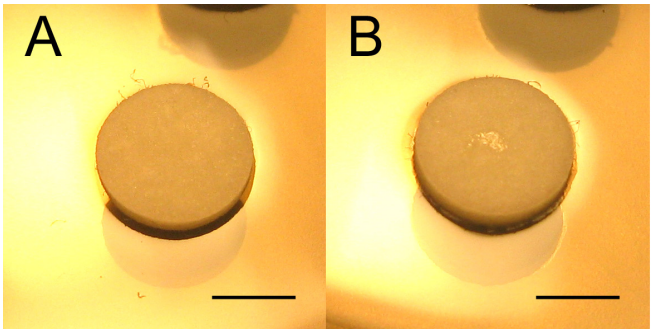

Figure IV-7. Flagella and paper disc motility
Pictures are of WT (A) and *fleQ*- (B) strains 16 hours after toothpick inoculation of bacterial strains onto paper disc surfaces. Bars represent 0.5 cm.

To better determine the relative rate of movement of different strains along paper, we increased the distance over which the bacteria were allowed to move. After inoculating the bacteria by toothpick onto large filter paper strips on an agar plate, we could observe the distance the bacteria were able to travel by removing the paper at chosen times and allowing the growth of the bacteria that had penetrated through the paper. While a **Δ***syfA* mutant strain progressed at a rate of 0.18 cm/hr and a *fleQ*- mutant moved at a rate of only 0.06 cm/hr, a **Δ***syfA*/**Δ***brfA* strain moved at a rate of only 0.14 cm/hr. All of these surfactant mutants moved much slower than the WT strain (0.29 cm/hr), which suggests that both syringafactin and BRF contribute to the form of motility that enables movement through porous materials. Although the surfactants are not necessary for motility through porous paper, they strongly facilitate the process. This is quite distinct from swimming motility to which neither surfactant contributes to the process, and swarming motility where one or the other surfactant are essential.

Because it appeared that BrfA and flagellin determinants were expressed in a similar fashion under several different growth conditions, we tested a variety of media conditions to determine whether this coordinated response was always linked. We thus examined the transcriptional response of both *fliC* and *brfA* in cells grown in nutrient broth alone (non-shaken culture), nutrient broth amended with a variety of agar concentrations (0.25% for swimming, 0.4% for swarming, and 1.5% for solid plates), as well as paper discs on solid plates. For all of the solidified plates and the paper, the relative levels of expression of *fliC* and *brfA* were highly similar in cells grown on a given solid surface (Fig. 8). However, *brfA* was induced, while *fliC* was down-regulated when cells were grown in a plate containing still liquid media with no added agar (Fig. 8). Flagellin has been previously found to be synthesized at lower levels in liquid medium compared to a similar solidified medium (McCarter and Silverman, 1990; Kearns, 2010). Since flagella apparently serve as surface sensors, it appears that flagellar surface sensing might also be contributing to the regulation of *brfA*. It is curious, however, that in liquid medium BRF production is enhanced, leading us to hypothesize that the flagella might not be the sole signal inducing surfactant production in this condition.

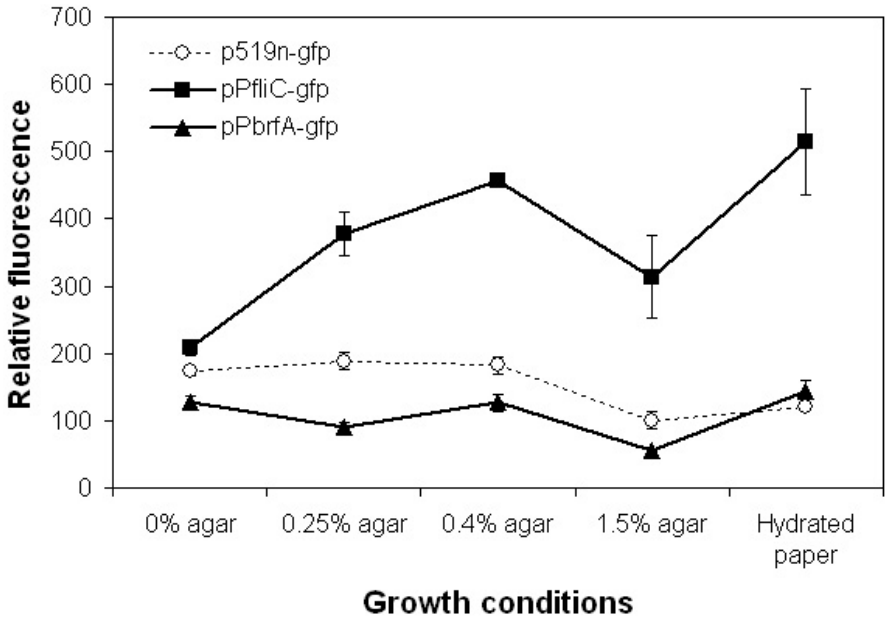

Figure IV-8. Coordinate regulation of *fliC* and *brfA* at surfaces
Relative GFP fluorescence of WT *P. syringae* carrying either a plasmid conferring constitutive fluorescent (pP*519n-gfp*), a plasmid indicative of *brfA* transcription (pP*brfA-gfp*), or a plasmid indicative of *fliC* transcription (pP*fliC-gfp*). Strains were tested after overnight growth on agar plates having various amounts or no added agar. Bars represent standard deviations.

Because it appeared that expression of *fliC*-encoded flagellin and *brfA* was unlinked in cells grown in planktonic conditions, we further evaluated whether this was true under all conditions. In other bacteria it has been noted that flagellar surface-sensing on both hard agar and softer swarming plates can be mimicked by growth in a broth in which the viscosity is increased with the addition of polyvinylpyrrolidine 360 (PVP-360) (McCarter *et al*., 1988). We therefore tested the expression of *brfA* and *fliC* in still and agitated broth cultures containing 10% PVP-360. Although PVP-360 induces flagella production in some other bacteria, we found equivalent levels of expression of *fliC* as in unamended broth culture and presume that this reflected a similar accumulation of flagellin in *P. syringae* (Fig. 9). Additionally, the addition of PVP-360 did not appear to have a large effect on *brfA* transcription. In agreement with earlier results, broth culture conditions reduced the transcription of *fliC* while increasing the expression of *brfA*. Futhermore, it appeared that agitation of the broth cultures further increased expression of *brfA* while further decreasing expression of *fliC*.

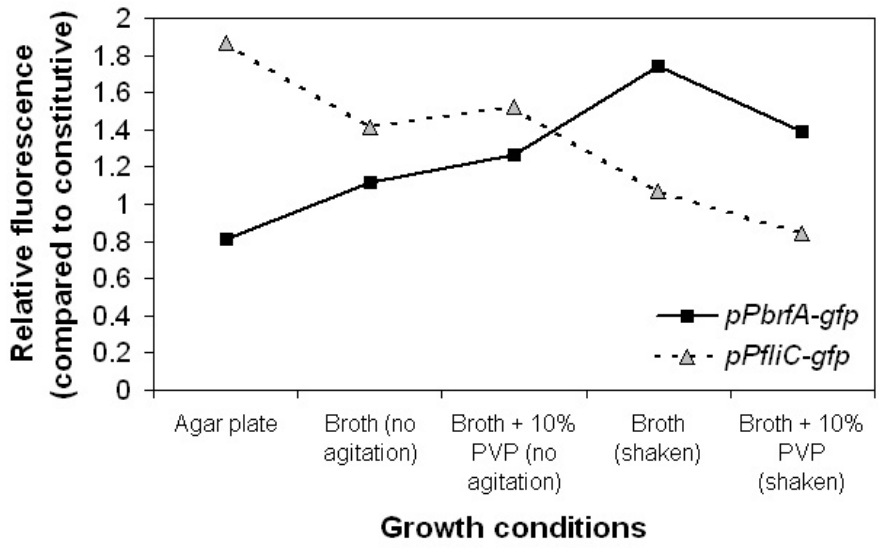

Figure IV-9. Lack of coordinate expression of *fliC* and *brfA* in broth cultures of *Pseudomonas syringae*.

Relative GFP fluorescence determined by quantitive microscopy of WT *P. syringae* carrying either a plasmid indicative of *brfA* transcription (p*brfA-gfp*), or a plasmid indicative of *fliC* transcription (p*fliC-gfp*). Fluorescence intensities were normalized with a *P. syringae* strain harboring plasmid p*519n-gfp* and expressing *gfp* fluorescence constitutively exposed to each of the same conditions. Each measurement is the mean of at least 200 cells.

Although we did not observe an induction of transcription of *fliC* upon addition of PVP-360 as has been noted in other taxa, a dramatic effect of increasing broth medium viscosity on cell shape was apparent. Curiously, cells that were grown with agitation in media containing 10% PVP-360 exhibited a hyper-elongated state similar to that associated with the swarming phenotype in other bacteria (Kearns, 2010). In this culture condition the cells grew up to 20 times the length of a normal cell and appeared multinucleate (Fig. 10). Although in most bacterial taxa this phenotype is linked with swarming motility, we found no evidence that cells of *P. syringae* were elongated when cultured on low agar swarming plates. Likewise, no elongated cells were seen in cultures in non-agitated KB broth amended with 10% PVP-360, nor any other culturing conditions tested.

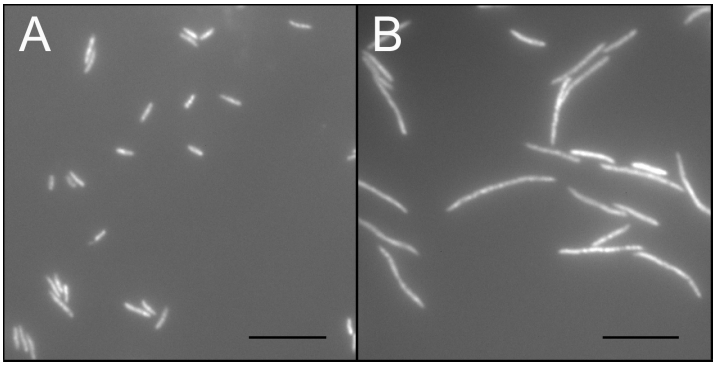

Figure IV-10. Elongated cells of *Pseudomonas syringae* in broth media amended with 10% PVP-360

DAPI stained cells of WT *P. syringae* cells cultured either in shaken KB medium (A) or shaken KB medium amended with 10% PVP-360 (B). Bars represent 10 um.

Relaxed flagellar control of BRF production in planktonic cultures

Given that culturing of *P. syringae* in broth medium induced expression of *brfA* and production of BRF we determined whether this condition also eliminates the influence of flagellar assembly on production of this surfactant. We measured the expression of *brfA* in **Δ***syfA*/*fleQ*-, **Δ***syfA*/*fliC*-, and **Δ***syfA* mutant backgrounds when cells were grown in broth cultures. We observed a dramatic up-regulation of GFP fluorescence in all mutant strains harboring p*brfA-gfp* in shaken broth media, and the expression of *brfA* appeared similar in **Δ***syfA*/*fliC*- compared to **Δ***syfA*. This suggests that flagellin does not play a role in sensing the liquid environment in broth media (Fig. 11). In contrast, although *brfA* expression in a **Δ***syfA*/*fleQ*- strain was higher in cells cultured in broth media compared to on agar plates, its level of expression was only about as high as that of a motile strain cultured on plates (Fig 11). Thus, although the inability to establish the flagellar base has some role in transcriptional repression of *brfA* expression in broth medium, it appears that other factors play a larger role in its regulation. It remains to be seen if this regulation is operative at the level of surfactant production, or whether it only affects transcription of *brfA*, since supernatants of broth cultures do not exhibit water drop collapse.

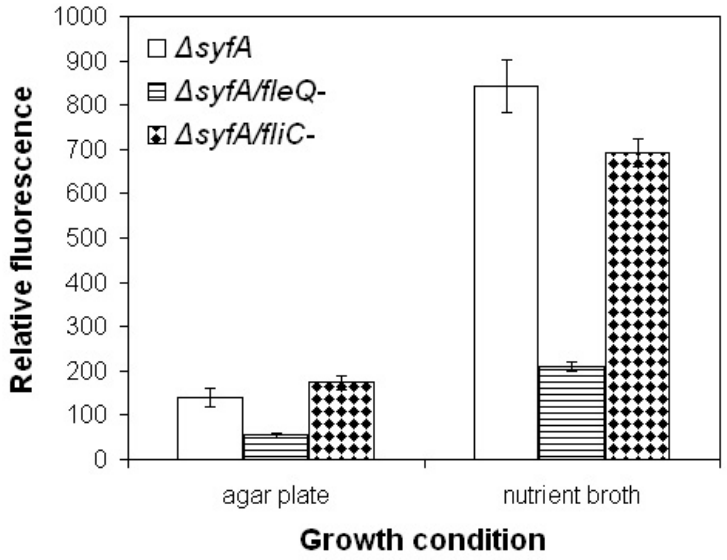

Figure IV-11. Reduced flagella-dependent regulation of *brfA* in broth cultures of *Pseudomonas syringae*.

Relative GFP fluorescenceexhibited by either the Δ*syfA* or the flagellar mutants Δ*syfA/fleQ*- or Δ*syfA/fliC*- of *P. syringae* B728a carrying a plasmid that reports on *brfA* transcription (pP*brfA-gfp*). Strains were grown overnight on agar plates or in shaken broth cultures. Bars represent standard deviations of the mean GFP fluorescence.

Given that broth culture conditions greatly affected expression of *brfA*, we investigated what features of such a culture influenced this regulation. We observed a higher level of expression of *brfA* in shaken compared to still broth medium. However, less than 2% as many cells were produced in still broth compared to shaken broth medium after a given time of incubation. This suggested that cell density might contribute to the high levels of *brfA* transcription in shaken broth cultures and that expression might increase concomitantly with cell density and thus time in cultures. We therefore compared levels of GFP fluorescence in strains harboring pP*brfA-gfp* after one and two days of growth in broth medium and on plates. While similar levels of GFP fluorescence were seen at all sampling times and growth conditions in a constitutively fluorescent strain harboring pP*519n-gfp*, the expression of *brfA* apparently increased in both broth and plate cultures over time (Fig. 12). This induction with age of culture appeared to be much greater in broth compared to plate cultures, which might reflect the preferential accumulation of a signal in broth culture. Thus, although cell density might contribute to *brfA* expression independently from flagella surface sensing, it appears that this effect also is conditional on growth conditions and may involve another signal.

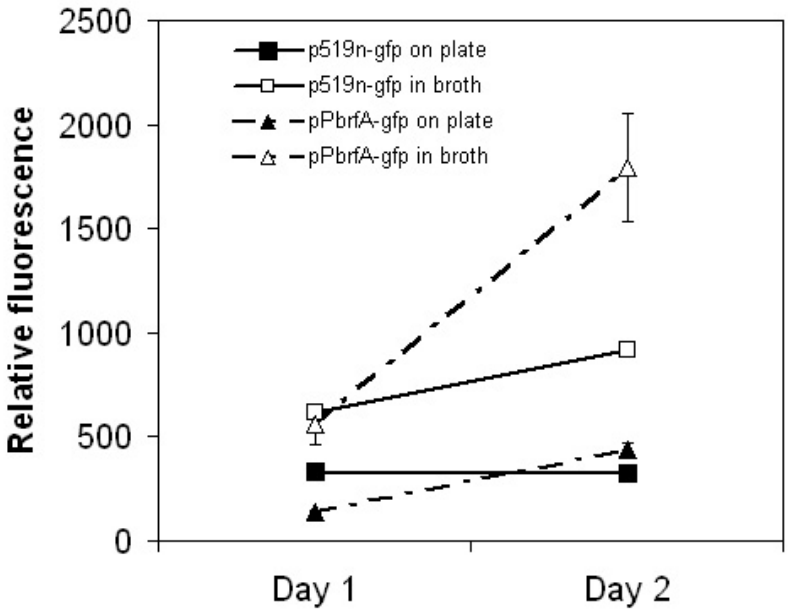

Figure IV-12. *brfA* transcription increases over time in cultures of *Pseudomonas syringae*. Relative GFP fluorescence of WT *P. syringae* carrying either a plasmid conferring constitutive gfp fluorescence (pP*519n-gfp*) or a plasmid indicative of *brfA* transcription (pP*brfA-gfp*). Strains were tested after either one or two days of growth on agar plates and shaken broth cultures. Bars represent standard deviations.

The function of BRF

While BRF apparently aids motility both on low-agar swarming plates and on hydrated porous papers these behaviors were always observed in a **Δ***syfA* mutant incapable of producing syringafactin. We therefore wanted to ascertain whether there was a role for BRF production in a WT background. A **Δ***brfA* strain did not differ from the WT strain in its speed of movement through porous paper (data not shown). However, this strain did differ from the WT strain in the manner in which it moved on swarming plates. The **Δ***brfA* strain produced tendrils of cells that moved away from the point of inoculation that were much broader than the WT strain. Such apparent movement was initially as fast as that of the WT strain, but unlike the WT strain, this mutant failed to fully explore the swarming plate; even after four days, a colony of **Δ***brfA* had not covered the agar surface, whereas the WT had fully covered the swarming plate by day 2. As a further test of the role of BRF in movement of *P. syringae*, we over-expressed BrfA constitutively in the WT strain and observed its swarming motility. Contrary to the broad but short tendrils of cells produced by the **Δ***brfA* mutant, over-expression of BrfA led to the formation of very long and narrow tendrils which moved and eventually covered the plate at the same speed as the WT strain (Fig.13). These observations are in agreement with observations in *P. aeruginosa*, where the branching and avoidance of other tendrils has been proposed to be due to the repellent effect of HAAs which serves to move the swarm front forwards (Tremblay *et al*., 2007).

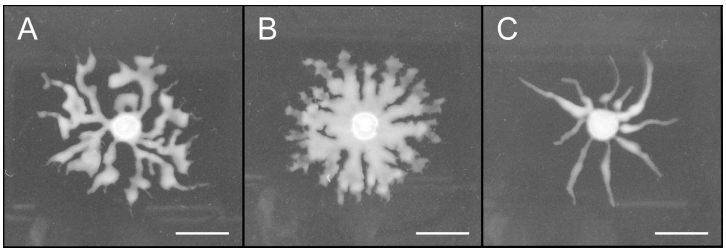

Figure IV-13. Swarming phenotypes of strains differing in expression of *brfA*
Swarming phenotypes of a WT strain of *P. syringae* B728a (A), a **Δ***brfA* mutant, and a WT strain harboring plasmid pBRF2 in which *brfA* is expressed constitutively at a high level (C) after 16 h of incubation on 0.4% swarm agar plates. These images are representative of at least five repetitions. Bars represent 1 cm.

Examination of other genes regulating production of BRF

Given that our mutagenesis screen identified several other genes in addition to flagellar genes that apparently contribute to production of BRF we postulated that at least some of them might be indirectly influencing production of our surfactant through modulation of flagella functioning. All surfactant mutants were therefore tested for swimming ability to determine if any mutations affected flagellar function (Table 1). Mutants blocked in Psyr_0936, Psyr_0219 (*algC*) and Psyr_0918 (*wzt*) had nearly abolished flagellar motility. These mutants were also examined for their ability to express flagellin biosynthesis genes; any mutants that affect *brfA* expression via altered flagella function should exhibit low levels of *fliC* expression. However, only a mutant of Psyr_0936, encoding a glycosyl transferase, exhibited lower *fliC* expression than the WT strain (Table 3). Thus we conclude that Psyr_0936, but not Psyr_0219 (*algC*) or Psyr_0918 (*wzt*), is affecting *brfA* expression indirectly via an inhibitory effect on flagella (Fig. 14B).

Table IV-3. Relationship between the surfactant halos of transposon mutants with the transcriptional activity of *brfA* and expression of *fliC* compared to Δ*syfA*

Locus of Tn*5* insertion	Surfactant halo	p*brfA-gfp*	p*fliC-gfp*
Psyr_0215 (xth)	0**	N/A	*swims*
Psyr_3698 (gacS)	0**	0.60	*swims*
Psyr_0258 (ompR/amgR)	0**	0.15*	*swims*
Psyr_4446 (osmE)	0**	1.09	*swims*
Psyr_0936	0.13**	0.25*	0.004**
Psyr_0219 (algC)	0.13**	1.04	1.40
Psyr_0918 (wzt)	0.19**	0.60	0.92
Psyr_2083	0.19**	0.60	*swims*
Psyr_0270 (polA)	0.16**	0.74	*swims*
Psyr_1981	0.22**	0.87	*swims*
Psyr_3669	0.25**	0.87	*swims*
Psyr_2979 (gor)	1.93**	1.09	*swims*
Psyr_0263 (algB)	2.13**	1.66	*swims*
Psyr_1350 (mucP)	2.69**	2.56*	*swims*

Data for halo sizes is reproduced from Table 1. All reported values were obtained by dividing the average value for the insertion mutant by the average value for **Δ***syfA*. $P<0.05$ (*) or $P < 0.01$ (**) as determined by a *t*-test.

To further test if flagellar function was linked to *brfA* expression we determined if mutants with enhanced BRF production as evidenced by larger surfactant halos had altered expression of flagellar components. In order to test this, we constructed FleQ mutants in Psyr_2979 (*gor*), Psyr_0263 (*algB*), and Psyr_1350 (*mucP*) mutant backgrounds. While a FleQ mutant also blocked in Psyr_2979 produced no detectable BRF, FleQ mutants of both AlgB and MucP, involved in the AlgT extracellular stress pathway, still exhibited BRF production, albeit at a reduced level (data not shown). This demonstrates that the AlgT stress pathway controls BRF production independently of flagellar function (Fig. 14B). We are currently addressing whether this de-repression of the AlgT extracellular stress pathway might also be a response to broth culturing conditions.

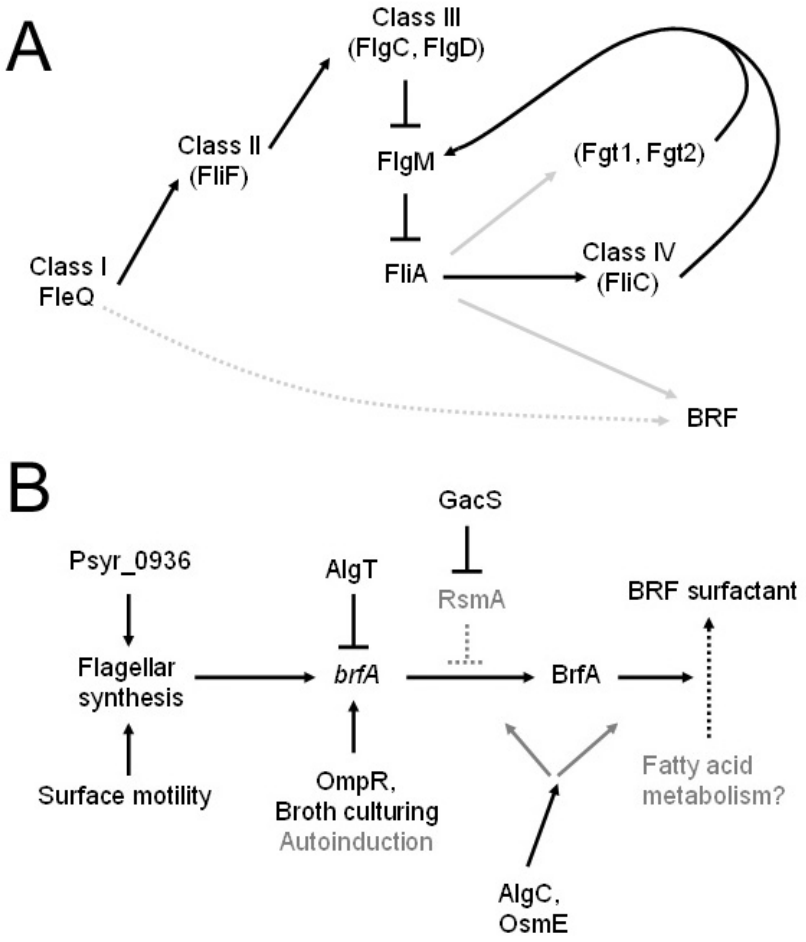

Figure IV-14. Proposed regulatory model of BRF production
Regulatory details for flagellar regulation of BRF (A), and overall regulation of BRF (B). Arrows and words in gray indicate hypothesized roles.

The levels of *brfA* transcription in mutants with reduced surfactant production but having functional flagella was assessed to determine if regulation of surfactant production was mediated

at the transcriptional or post-transcriptional level. Of all such mutants examined, only the mutant in the OmpR homolog had a pronounced effect on *brfA* transcription independent of flagella function. The levels of *brfA* expression in a GacS mutant, although lower than a WT strain, could not account for the absence of any production of BRF. Thus, in agreement with the role of GacS as a post-transcriptional regulator, it appears that it is mainly affecting BRF production post-transcriptionally (Fig. 14B). OsmE and AlgC mutants also did not reduce the transcription of *brfA*, and thus might act post-transcriptionally to reduce surfactant synthesis and/or export of BRF (Fig. 14B). The remaining mutants all had moderately lower levels of expression of *brfA* that could have accounted for their reduced surfactant production, but at this point it is difficult to say how these genes might fit into the regulation of BRF.

DISCUSSION

Although prior research has implicated biosurfactant production in flagellar motility, as far as we are aware, this is the first report to show this as a linear process, where the stages of flagellar assembly are required for proper regulation of surfactant production. We find genetic evidence that biosurfactants are tied to flagellar motility. However, this is not the first report of flagella controlling expression of non-flagellar genes. *Salmonella enterica* ties the expression of some virulence factors to mid-stage flagellar assembly (Iyoda *et al*., 2001). Additionally, Frye *et al*. identified a number of genes that are under the control of flagellar promoters but which have no apparent effect on flagella function in *S. enterica* (Frye *et al*., 2006). Furthermore, expression of virulence factors in *Proteus mirabilis* was found to be tightly co-regulated with FliC expression (Allison *et al*., 1992). Despite these reports of co-regulation, to our knowledge there has been no previous recognition that a flagellin knockout or impairment of function would serve to further up-regulate such non-flagellar genes.

Why does *P. syringae* co-regulate expression of a biosurfactant with flagellar synthesis? Although it is tempting to speculate that BRF could function as a virulence factor, similar to the above-mentioned bacteria, preliminary evidence shows that a strain defective in production of BRF does not have reduced virulence *in planta* (data not shown). A more likely possibility might be that BRF is used for flagellar lubrication. In this scenario, under conditions where there is increased flagella breakage, there will also be increased production of both flagellin and BRF. Production of BRF might help lubricate the sticky surface and/or flagella to minimize breakage. Microscopic and immuno-staining approaches might be utilized in future studies to determine if such a model holds for *P. syringae*.

While BRF may be co-regulated with flagellin because it has protective effects for flagella, it might also be interpreted as a case of regulatory piggy-backing if flagellin synthesis itself is indicative of an external condition for which biosurfactant production is beneficial. One hypothesis that is gaining experimental support is that flagella can function as surface sensors, conveying to the bacterium positional information through the inhibition of flagellar rotation (McCarter *et al*., 1988; Belas and Suvanasuthi, 2005). It is proposed that *V. parahaemolyticus* and *P. mirabilis* can sense surfaces by monitoring flagellar torque, whereby growth on a surface impedes flagellar rotation, which signal upregulates flagellin production (Wang *et al*., 2004). However, in these models PVP addition serves to generate a similar viscous environment that impairs flagellar rotation, and similarly leads to up-regulation of swarming genes. Why do we

instead see a decrease in flagellin synthesis with PVP addition in *P. syringae*? One clue might involve the distinctive requirements for the swarming phenotype in *P. syringae*; whereas cells of *V. parahaemolyticus* and *P. mirabilis* elongate and swarm on 1.5% hard agar, *P. syringae* requires a moister surface (0.4% agar concentration) in order to swarm, and it does not display the elongated cell phenotype during movement. *P. syringae* thus may still use resistance of flagellar rotation to gather positional information, but it might use that information to make different decisions about when to swarm and produce biosurfactant.

Another emerging hypothesis that has experimental support is that flagella act as wetness sensors in bacteria such as *S. enterica* (Wang *et al*., 2005). Bacteria export FlgM, the FliA antisigma factor, through flagella, and it is proposed that this secretion is only possible when the exterior conditions are sufficiently moist. Thus, under wetter conditions, both flagellin and the surfactant should be produced in greater quantities. This model would support our observation that a loss of flagellin does not upregulate surfactant production in broth culture; perhaps the fully hydrated conditions present in a broth culture allow maximal export of FlgM, regardless of flagellar length. However, if FlgM is optimally secreted, it remains unclear why we do not also see an up-regulation of flagellin in broth cultures compared to drier culture conditions such as growth on agar surfaces.

While flagellar function itself might logically be linked to surfactant production, it remains unclear in what way flagellar glycosylation is linked to this process. For instance, why do the glycosylation mutants, especially a mutant blocked in *fgt2*, which has sufficient flagella function to enable unaltered swimming motility, have an equivalent effect on flagellin and surfactant synthesis as a disruption of flagellin production itself? One hypothesis could be that glycosylation blocks FlgM export through the flagella, and without adequate glycosylation, late-stage flagellar genes remain activated. Although this would agree with our observations, it would be surprising if it were true, given that others have not noted this function before. Alternatively, glycosylation has been proposed to function in flagellar stabilization and lubrication in *P. syringae* pv. tabaci, where non-glycosylated flagella formed stiff flagellar bundles (Taguchi *et al*., 2008). If lack of glycosylation makes the flagella more sticky and prone to breakage, then non-glycosylated mutants might still have functional flagella, but these flagella might break more easily, requiring an enhanced supply of fresh flagellin and/or a lubricating surfactant.

Given the co-regulation of BRF with class IV flagellar genes, it was tempting to speculate that FliA, the sigma factor that activates transcription of Class IV genes, might also be directly responsible for regulating *brfA* expression. However, a disruption of *fliA* did not abolish surfactant production, and all Class III mutants still produce at least small quantities of the surfactant, indicating that flagellar regulation of BRF production occurs at multiple levels. It remains to be determined exactly how flagella are acting to affect surfactant production. It is also curious that flagella have less of a role in regulating surfactant production in broth conditions, where the surfactant is relatively highly produced. If the function of this surfactant is to lubricate the flagella at surfaces, then why would *P. syringae* produce such high quantities in broth culture? Although we do not have any evidence of a role for this surfactant in broth cultures, some clues about the surfactant's properties can lead us to hypothesize possible functions. When large quantities of this surfactant are produced in broth culture by

constitutively expressing BrfA we see that this surfactant imparts a milky appearance to the culture supernatants. This is indicative of a surfactant with low water solubility, which most likely associates with surfaces such as the bacterial cell surface, instead of the bulk medium. Therefore, when this surfactant is produced, it likely coats the cells and changes their surface properties. The role of this surfactant in aqueous environments and its effects on cell surfaces and the adhesiveness of cells need to be addressed. As a counter example, syringafactin, a water-soluble surfactant which readily diffuses away from *P. syringae*, is down-regulated in broth cultures (Chapter 3). Thus BRF might best be considered a surface-associating surfactant that modulates the surface properties of either the producing bacterium or the surfaces over which the bacterium must move.

Another important clue for the function of BRF was the finding that multiple stress pathways apparently strongly impact its production. OmpR in most model organisms is responsive to osmotic stress. However, where it has been most studied, high osmolarity environments repress motility, and an OmpR knockout is associated with increased flagellar synthesis, increased motility, and also increased production of virulence factors (Park and Forst, 2006). Such findings are opposite to our observed loss of BRF production in an OmpR (Psyr_0258) mutant of *P. syringae*. Alternatively, the OmpR homolog in *P. aeruginosa*, termed AmgR, has been described to function more like the protein conferring membrane stress response, CpxR in *E. coli* (Lee *et al*., 2009). An examination of the AmgR regulon in *P. aeruginosa* revealed that it had much less in common with that of *E. coli* OmpR regulon than that mediated by CpxR, which has been coined a surface sensor. Anecdotally, we have observed that a mutant in the *ompR* homolog in *P. syringae* grows well on fresh agar media but exhibits impaired growth on relatively old plates with dried surfaces (a higher matric stress environment). In contrast, our mutant screen also revealed the role of two members of the AlgT extracellular stress pathway, both of which when knocked out resulted in an up-regulation of BRF production. The AlgT stress pathway controls the production of alginate in response to membrane stress (Keith and Bender, 1999), and was recently found to similarly influence syringafactin production, with loss of the pathway resulting in up-regulated syringafactin synthesis (Burch *et al*., 2010). It remains unclear why these potentially overlapping stress responses have apparently opposite effects on production of BRF. Further examination of their roles in surfactant production should help elucidate the complex interaction between these two pathways. It might turn out that the combination of these two pathways allows the cell to determine the difference between subtly different stressful situations, only some of which would benefit from surfactant production.

It is significant that BRF is produced by an RhlA homolog, which is responsible for the biosynthesis of the rhamnolipid precursor HAA in *P. aeruginosa*. In *P. aeruginosa*, HAAs serve to repel neighboring tendrils and maintain an outward motility during swarming (Tremblay *et al*., 2007). Such a behavior would tend to maximize the ability of a bacterial colony to explore a given habitat by suppressing inward movement, and thus enhancing only outward movement away from colonized areas, and surfactin in *B. subtilis* has been similarly indicated to have this role (James *et al*., 2009). It appears that BRF shares this ability, but it remains to be determined if the swarm repulsion observation has a true physiological function, or is just a laboratory phenomenon that is merely a result of a fundamental physical property of the surfactant. While a syringafactin mutant of *P. syringae* DC3000 did not apparently make any surfactant and was incapable of swarming motility (Berti *et al*., 2007), we find that such a mutant in strain B728a

produces BRF. An examination of the DC3000 genome reveals a close homolog to *brfA*, but one having a stop codon at the 13th amino acid, apparently accounting for the lack of its production in strain DC3000. *P. syringae* DC3000 is a poor epiphyte, with low rates of survival on the leaf surface (Feil *et al*., 2005); it is intriguing to speculate that BRF is not made in DC3000 because it is primarily useful for epiphytic colonization of plants, or alternatively it might be detrimental and/or induce a host response in the apoplast. Restoration of BRF production in *P. syringae* strain DC3000 should reveal if it can change its virulence or epiphytic fitness.

In this study we have utilized an atomized oil assay to identify the biosynthetic and regulatory pathways leading to production of a biosurfactant expressed in a strongly context-dependent way in *P. syringae* B728a. Observations of the regulation of production of this surfactant in various culture conditions suggest a role for this surfactant primarily in fully hydrated environments. Its coordinated expression with flagella suggests an intimate role between surfactant production and flagellar motility, but the identification of many other regulatory elements reveals a complicated mechanism of regulation. Examinations of the interaction of this surfactant with the bacterial cell, its flagella, and with the surfaces that this bacterium colonizes should illuminate its role in the epiphytic lifestyle of *P. syringae*.

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Chapter V.

SyfR controls a complex pattern of surface-dependent surfactant production in *Pseudomonas syringae*

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ABSTRACT

SyfR is a LuxR-type regulator that is required for syringafactin production in *Pseudomonas syringae*, but lacks an obvious autoinducer-binding or phosphorylation domain for response-mediated regulation. We sought to further characterize this regulator and its role in the preferential production of syringafactin at surfaces. Similar to other LuxR-type regulators, SyfR appears to be active only as a multimer and to autoregulate its own expression. Although SyfR transcription is lower in broth cultures than when cells are gown on agar plates, this transcriptional control is reliant on functional SyfR protein. Thus, when SyfR is not produced, its promoter is only expressed at a baseline level in both culture conditions, demonstrating that it is the mediator of the surface sensing response. Additionally, we demonstrate that GacS and SalA are necessary for basal expression of SyfR. Analysis of a phenotype exhibited by old colonies of **Δ***syfA* mutants but not *syfR*- mutants reveals that SyfR controls more than syringafactin production. We term this phenotype "fried egg" because it exhibits a raised "yolk" of initial colony growth followed by successive expansion of the colony after day three as a thinner fringe or "white." The characterization of mutants insensitive to the development of this phenotype suggest that SyfR also controls production of an additional lipopeptide, syringomycin, causing only a pulse of syringomycin expression that peaks in young (24 hour) colonies. This expression pattern is different from the SyrF-mediated induction of syringomycin by plant signals, and SyfR is not necessary for that response. Although production of syringomycin is necessary for the fried egg phenotype, it is not the signal which activates it. However, we demonstrate that an unidentified self-produced product that is over-expressed in an *algT*- mutant, as well as a range of hydrophobic synthetic surfactants, induce the fried egg phenotype to appear earlier. While the biological significance of the interactions of surfactant-like molecules on the biology of *P. syringae* biofilm development remains unclear, it has allowed us to dissect the SyfR regulatory pathway and expand its regulon to include syringomycin.

INTRODUCTION

Biosurfactants, or biologically-produced surface active agents, are a broad group of natural amphiphilic compounds that are capable of lowering the interfacial tension between two phases. Although many different types of natural products have been identified to function as biosurfactants, one of the most intriguing classes of these compounds is the lipopeptides. Lipopeptides have a peptide head group attached to a lipid tail, and the peptide moiety is unique in that it is synthesized non-ribosomally; ie, it is not translated from an mRNA. Rather, lipopeptides are generally synthesized by non-ribosomal peptide synthetases (NRPSs), large enzyme complexes that catalyze the sequential assembly of a small peptide, as well as direct the addition of a carbon tail (Schneider and Marahiel, 1998). Additionally, these assembly units can specify the incorporation of unusual amino acids, modifications, and/or cyclization (Schwarzer *et al*., 2003). Because of this flexibility and ability to create unique peptides, NRPSs have found widespread use in bacteria and fungi for the synthesis of a variety of compounds including antibiotics, siderophores, pigments and many others (Schwarzer *et al*., 2003). NRPSs specific for lipopeptide synthesis contain an initial condensation domain that catalyzes tail addition to the first amino acid of the peptide, and thus can be readily identified bioinformatically in genomes (de Bruijn *et al*., 2007).

The most familiar example of a lipopeptide is that of surfactin produced by *Bacillus subtilis*. This cyclic lipopeptide is composed of seven amino acids linked to a 12- to 16-carbon tail; the seven amino acids being somewhat variable in composition due to the low fidelity of many NRPSs (Raaijmakers *et al*., 2010). Although generally linked to biofilm formation and motility, surfactin has recently been revealed to act as an autoinducer signal, wherein surfactin production is sensed by non-surfactin producing cells, inducing them to produce an extracellular matrix (López *et al*., 2009a). Other *Bacillus* lipopeptide families include the iturins and fengycins, while Pseudomonads have been found to produce an even broader range of lipopeptides (Raaijmakers *et al*., 2010). Two of the most extensively studied *Pseudomonas* lipopeptides are syringomycin and syringopeptin produced by *Pseudomonas syringae*, which have been noted for their membrane-disrupting and resultant phytotoxic properties (Bender *et al*., 1999). These cyclic lipopeptides contain 9 and 22 to 25 amino acids, respectively, and contribute to the virulence of this microorganism (Bender *et al*., 1999; Scholz-Schroeder *et al*., 2001). Recently, production of syringafactin, an 8-amino acid linear lipopeptide, was also described in *P. syringae* DC3000 and B728a (Berti *et al*., 2007; Burch *et al*., 2010).

With the continued identification of new lipopeptides and the sequencing of their genetic loci, an interesting pattern has emerged; many of the NRPSs for lipopeptide production in Pseudomonads possess divergently transcribed LuxR-type regulators both upstream and often also immediately downstream, of the biosynthetic cluster. When they have been characterized, disruptions in the upstream and sometimes downstream regulator results in blockage of lipopeptide production (Berti *et al*., 2007; Dubern *et al*., 2008; de Bruijn and Raaijmakers, 2009b). These LuxR-type regulators have a characteristic C-terminal helix-turn-helix DNA-binding region, but form a distinct family separate from other characterized LuxR-type regulators (de Bruijn and Raaijmakers, 2009b). Classic LuxR homologs have an autoinducer-binding domain, while other LuxR-type response regulators have receiver domains typical of two-component systems.

However, the NRPS-associated LuxR-type regulators have neither domain, and thus form their own group of regulators (Lu *et al*., 2002; de Bruijn and Raaijmakers, 2009b).

SalA and SyrF are the best characterized of these LuxR-type NRPS regulators; they control and are located in close proximity to the NRPS loci for syringomycin and syringopeptin in *P. syringae* B301D (Lu *et al*., 2002). They have been shown to dimerize, and that a dimerized SalA binds the promoter region upstream of *syrF*, while dimerized SyrF binds to the promoter region of syringomycin (Wang *et al*., 2006a). Thus, similar to *V. fischeri* LuxR, they become active after forming a multimeric complex (Choi and Greenberg, 1992). However, while LuxR must first bind an autoinducer to dimerize and become an activate transcription factor (Nasser and Reverchon, 2007), it is unclear what if any factors contribute to the activation of the NRPS-associated LuxR-type regulators. In addition to SalA and SyrF, a third LuxR-type regulator, SyrG, also exhibits partial control over syringomycin synthesis in *P. syringae*, although it operates independent of SalA and SyrF (Lu *et al*., 2002). Furthermore, *P. syringae* B728a possesses two additional regulators of this type (for a total of five), which flank the syringafactin biosynthetic cluster on both sides. SyfR, the regulator physically upstream of the cluster, was previously demonstrated to be required for syringafactin production in *P. syringae* DC3000. However, the LuxR homolog downstream of the syringafactin biosynthetis cluster had no effect on syringafactin production when deleted, and remains unnamed (Berti *et al*., 2007). No further characterization of SyfR has appeared.

Although it is clear that these LuxR-type regulators often control lipopeptide synthesis in Pseudomonads, there has been little investigation of how environmental signals feed into this regulation. Some plant signals have been shown to induce lipopeptide production in plant-associated Pseudomonads, supporting their proposed roles in virulence (Koch *et al*., 2002; Wang *et al*., 2006b). Additionally, lipopeptides are regulated in a manner dependent on quorum sensing and cell density in a few *Bacillus* and *Pseudomonas* species (Raaijmakers *et al*., 2010). We recently found that expression of syringafactin in *P. syringae* is dependent on contact of cells with surfaces (Chapter 3). The current study was undertaken to investigate the role of SyfR in such contact-dependent syringafactin production in strain B728a. We will show that SyfR controls more than syringafactin production, and is involved in a complex web of cross-regulation between other LuxR-type regulators and other lipopeptides in *P. syringae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. syringae* pv. syringae B728a (Loper and Lindow, 1987) was cultured on King's medium B (KB) plates with 1.5% agar technical (King *et al*., 1954) at 28 °C. *E. coli* strains DH5α, S17-1 (Simon *et al*., 1983), and SM10(λpir) (Delorenzo *et al*., 1990) were cultured on Luria Agar at 37 °C. Syringomycin minimal medium (SRM) with 100 μm arbutin and 0.1% fructose was used for syringomycin induction (Mo and Gross, 1991). Antibiotics were used at the following concentrations (μg/ml): kanamycin (25 for *P. syringae*, 50 for *E. coli*), rifampin (100), tetracycline (15), gentamycin (75) and spectinomycin (100).

Biosurfactant detection assays. The drop-collapse assay was performed as according to Bodour and Miller-Maier (Bodour and Miller-Maier, 1998). 2 μl 10W-40 Pennzoil® (Pennzoil Products Company, Houston, TX, USA) was applied to delimited wells on the lid of a 96-well plate and allowed to equilibrate at room temperature. Next, 5 μl of either diluted surfactant

samples or supernatant from bacterial cultures or re-suspended bacterial colonies were pipetted onto the oil surface. Drops which retained a spherical shape were scored as negative for surfactant content, while drops which had a visibly-decreased contact angle with the oil and spread (collapsed) were scored as positive for surfactant content.

The atomized oil assay was performed as in (Burch *et al*., 2010): Bacteria were spotted onto agar plates using sterile toothpicks and grown overnight. An airbrush (Type H; Paasche Airbrush Co., Chicago, IL) was used to apply a fine mist of mineral oil (light paraffin oil, Fisher Scientific) onto the plate at an air pressure of 19 psi.

Chromosomal disruptions of SyfR, Psyr_2578, SyrE, SypA, and AlgT. Site-directed mutagenesis was performed with single-crossover insertion events. Fragments of the genes of interest were amplified from *P. syringae* genomic DNA by PCR with primers 5'CACCTCCAGTACGGGCGACGAGAC (syfR-KO-F), 5'CGTGGCATTGTGGCTGGACTGAG (syfR-KO-R), 5'CACCGCCACGCACCTCTCCTCAC (2578 KO F), 5'AGTCGCTCGGCCTGCTCAA (2578-KO-R), 5'CACCGGGCCGCAACTTCATTACTG (syrE-KO-F), 5'GGGCTGACCGAGGAAAACATACC (syrE-KO-R), 5'CACCGCCCCAATGCCAGCTACAAAAAG (sypA-KO-F), 5'CACCGGGCCGCAACTTCATTACTG (sypA-KO-R), 5'CACCTGCTAACCCAGGAAGAGGAT (AlgT-KO-F), and 5'AGCGCACGGTACCAACAGGACACT (AlgT-KO-R). The resulting five inserts were subcloned into pENTR/D-TOPO and subsequently transferred into pLVC/D by clonase LR reactions. Plasmids were isolated and electroporated into *E. coli* S17-1 for conjugal transfer. Both *E. coli* and *P. syringae* were grown individually overnight on plates, then mated overnight. Transformed *P. syringae* were isolated on KB plates containing rifampin and tetracycline. Knockouts were confirmed by PCR amplification.

Constitutive expression vectors for SyfR. Full-length and truncated expression vectors of *syfR* were generated by replacement of *gfp* in p*519n-gfp* (Matthysse *et al*., 1996). Full-length *syfR* was amplified by PCR from genomic DNA with primers 5'AACATATGAACACGGCGCAACATTTCGAG (syfR-full-F) and 5'AGAATTCTCAATCACCAGCCATCCAGCG (syfR-full-R). Truncated $SyfR_{1\text{-}198}$ was amplified with syfR-full-F and 5'AGAATTCAGTCCTGTGCAAGCCTGCC (syfR-196-R). C-terminal $SyfR_{164\text{-}257}$ was amplified with primers 5'AACATATGCACGCGCAGAGCCTTGCTC (syfR-164-F) and syfR-full-R, with the syfR-164-F primer adding a start site. PCR conditions were as follows: 28 cycles of 95°C, 59°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. The resulting fragments and the p*519n-gfp* vector were digested with *NdeI* and *EcoRI*, and ligated to form plasmids p*519n-syfR*, p$519n\text{-}syfR_{1\text{-}198}$, and p$519n\text{-}syfR_{164\text{-}257}$. Insertions were confirmed by PCR. Expression plasmids were electroporated into wild-type and mutant strains of *P. syringae* B728a with selection for kanamycin resistance.

Construction of a P*syfR-gfp* and P*syrB-gfp* transcriptional fusion. Reporter plasmids were constructed similar to described in Burch (2010). The upstream promoter region of the *P. syringae* B728a *syfR* gene was amplified by PCR from genomic DNA with primers 5'TAAGCTTGGCTCAAGGTCCTTCTTGGCG (syfR-pro-F) and

5'TGAATTCCTTGAGCTTTCCTGATTCCGACCGC (syfR-pro-R) to generate a 289-bp promoter region. The upstream promoter region of the *P. syringae* B728a *syrB1* gene was amplified by PCR from genomic DNA with primers 5'ACAAAGCTTCAAACTCCTGGACCTCAGC (syrB1-pro-F) and 5'AGAATTCGACCAAAGCTCCTGTGTAATAACC (syrB1-pro-R) to generate a 328-bp promoter region. PCR conditions were as follows: 28 cycles of 95°C, 59°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. The PCR products were first cloned into pTOPO Blunt (Invitrogen) and transformed into *E. coli* DH5α. The insert was sequenced to verify its identity. pTOPO-P*syfR* and pTOPO-P*syrB* were digested with *HindIII* and *EcoRI*, and the resulting fragments were respectively cloned into pPROBE-OT and pPROBE-GT, (Miller *et al*., 2000) which contain a promoterless *gfp* gene in order to generate pP*syfR-gfp* and pP*syrB-gfp*.

Reporter plasmids were electroporated into wild-type and mutant strains of *P. syringae* B728a. The appropriate transformed strains were grown overnight (unless otherwise described) on KB plates or in KB broth medium (unless otherwise described), then re-suspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD_{600} of 0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with a 486-nm bandpass excitation filter and a 510- to 700-nm combination emission filter. A relative fluorescence unit (RFU) was defined as the fluorescence of the suspensions normalized for the suspension turbidity measured as OD_{600}.

Generation of mutants. Transposon mutagenesis was performed as in Chapter 4. Briefly, a SyfA- deletion mutant of *P. syringae* B728a and the conjugative *E. coli* strain SM10(λpir) harboring pUT mini-Tn5 Sm/Sp (Delorenzo *et al*., 1990) were grown overnight on agar plates with appropriate antibiotics. Cells were then harvested with a loop, washed and re-suspended in potassium phosphate buffer (10 mM, pH 7.5), and then mixed in a ratio of 1:3 (*E. coli* : *P. syringae*) and incubated overnight as a confluent lawn on a KB plate. After incubation, the cells were re-suspended in phosphate buffer and 10% of the cell suspension was plated onto KB medium containing 100 μg/ml rifampin and 100 μg/ml spectinomycin and incubated for three days. *P. syringae* transposon mutants were spotted using sterile toothpicks from colonies on selection plates onto KB plates, and visually screened after four or more days of growth for absence of the fried egg phenotype. The genes into which the transposon had inserted in these mutants was determined using arbitrarily-primed PCR similar to the method of O'Toole *et al*., (O'Toole *et al*., 1999). Mutations generated by the transposon from pUT mini-Tn5 Sm/Sp, were characterized using primers complementary to the 5' end of the transposon; primer tn5sm-ext, 5'-GCGCGAGCAGGGGAATTG**,** was used in the initial PCR reaction, and primer tn5sm-int, 5'-CGGTTTACAAGCATAAAGCTTGCTC, was used in a second reaction to amplify sequences 5' to the insertion site. The PCR product was purified (QIAquick PCR Purification kit, Qiagen) and submitted for sequencing using primer pRLint1. The locations of the sequenced fragments were determined directly by a BLAST search on the *Pseudomonas* genome database (Winsor *et al*., 2009) and compared to the published sequence of *P. syringae* B728a (Feil *et al*., 2005).

RESULTS

SyfR multimers regulate syringafactin production. Previously, another group identified a LuxR-type regulator oppositely transcribed but immediately upstream of the syringafactin biosynthetic cluster, and termed it *syfR*. Deletion of this regulatory gene abolishes syringafactin production in *Pseudomonas syringae* pv. tomato DC3000 (Berti *et al*., 2007). During a transposon mutagenesis screen to characterize surfactant production in *P. syringae* B728a, although we identified a number of insertions in the syringafactin biosynthetic cluster, we did not identify any insertions in the *syfR* homolog (Burch *et al*., 2010). We thus created a *syfR*- knockout strain in order to confirm its role in regulation of syringafactin production in *P. syringae* B728a (Fig. 1C). Indeed, disruption of *syfR* results in the production of only a very small amount of surfactant as measured by the oil spray assay, equivalent to that seen in a syringafactin-deficient Δ*syfA* strain (Fig. 1B). Furthermore, when we introduced a *syfA:gfp* reporter gene fusion on plasmid p*PsyfA-gfp* into the *syfR*- mutant we observed a 40-fold reduction in fluorescence compared to that in a wild-type strain (data not shown). Similar to *P. syringae* DC3000, disruption of the downstream LuxR-type regulator Psyr_2578 had no effect on syringafactin production (data not shown).

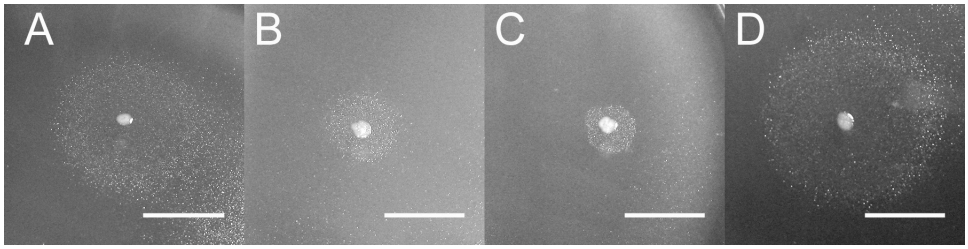

Figure V-1. Surfactant production in *syfA* and *syfR* mutants of *Pseudomonas syringae*
Halos of atomized oil droplets modified by surfactants produced by a wild-type strain of *P. syringae* (A), a Δ*syfA* deletion (B), a *syfR*- mutant (C), and a *syfR*- strain harboring p*519n-syfR* conferring constitutive expression of SyfR (D). Bars indicate 1 cm.

We next complemented the *syfR*- mutant *in trans* by introducing plasmid p*519n-syfR* in which the *npt*2 promoter drives the constitutive expression of *syfR*. This plasmid-borne copy of *syfR* restored syringafactin production to the mutant, with a zone of surfactant production visualized by the oil spray assay as large as that of the wild-type strain (Fig. 1D). GFP fluorescence exhibited when p*PsyfA-gfp* was introduced into this complemented strain was 4-fold higher than that of the wild type strain (Fig. 3).

SyfR belongs to a family of regulators that is not well studied, and so we further examined its mode of function. SyfR was found to have several features in common with the other characterized members of this family. Wang *et al*. (2006a) demonstrated that SalA and SyrF dimerize to form an active regulator. As further evidence of this dimerization, for both SalA and SyrF, as well as LuxR, truncated forms of these regulators that lack the C-terminal DNA binding domain are inactive but can multimerize with functional protein in a dominant negative action (Choi and Greenberg, 1992; Wang *et al*., 2006a). We expressed a truncated version of SyfR that

contained the first 198 amino acids (predicted LuxR dimerizing domain) ($SyfR_{1-198}$) as well as a truncated protein that only included the last 94 amino acids (predicted DNA-binding domain) ($SyfR_{164-257}$) constitutively under the control of the *npt*2 promoter in a *syfR*- mutant (Fig. 2). The later construct was included because a LuxR protein that lacked the dimerizing domain constitutively activated LuxR targets (Choi and Greenberg, 1991).

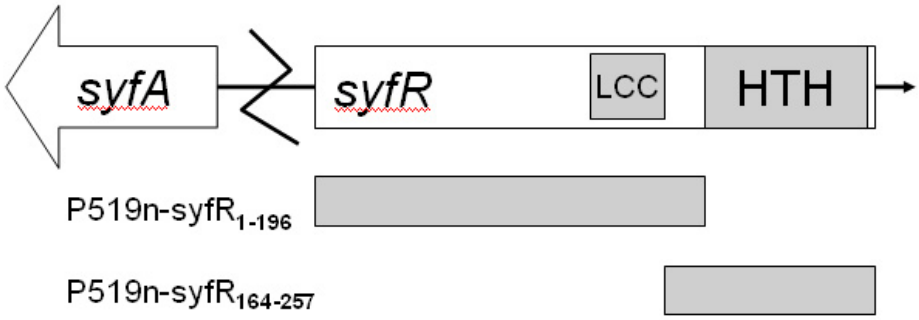

Figure V-2. Genomic context and predicted domain structures predicted for SyfR of *P. syringae* B728a.
The divergently transcribed *syfA* and *syfR* loci are separated by a 288 bp shared promoter region. Regions of SyfR with predicted functions were determined with SMART (Schultz *et al*., 1998). The low compositional complexity (LCC) region is located at aa positions 143-160, while the helix-turn-helix (HTH) DNA-binding domain that is characteristic of LuxR-type regulators is located at aa positions197-254. p*519n-syfR*$_{1-198}$ was designed to exclude the HTH domain (but include the predicted region of dimerization), while p*519n-syfR*$_{164-257}$ was designed to exclude the predicted dimerization domain.

Neither of the truncated SyfR constructs restored syringafactin production in a *syfR*- mutant (data not shown). Either $SyfR_{164-257}$ apparently did not include the correct regions of the DNA-binding domain, or SyfR has a different structural organization than LuxR. We also introduced these truncated SyfR variants into the wild-type strain to test for dominant negative interference which would indicate that SyfR forms multimers similar to SalA and SyrF. While the wild type strain expressing $SyfR_{164-257}$ retained full syringfactin production, the wild-type strain constitutively expressing $SyfR_{1-198}$ produced only the same size small surfactant halo (BRF) as a *syfR*- mutant strain (data not shown). Additionally, when we introduced the p*PsyfA-gfp* reporter fusion into these strains, we observed a similar pattern of GFP fluorescence as production of syringafactin in these strains; overexpression of the SyfR binding domain has a repressive effect on *syfA* transcription (Fig. 3). This supports the hypothesis that this regulator forms a multimeric complex in order to induce syringafactin transcription.

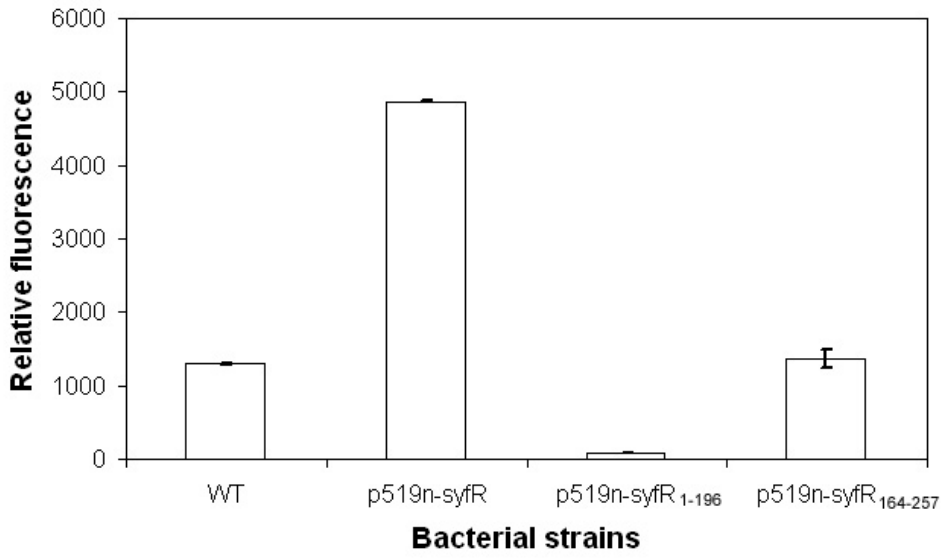

Figure V-3. Dominant negative effect of a truncated SyfR protein on transcription of *syfA* in *Pseudomonas syringae*

Relative GFP fluorescence of cells of *P. syringae* B728a harboring a plasmid in which GFP expression is dependent on the promoter of *syfA* (pP*syfA-gfp*) recovered from plate cultures. Indicated strains contain a second plasmid which confers constitutive expression of full-length SyfR (p519n-syfR), a truncated form of SyfR missing the DNA-binding domain (p*519n-syfR*$_{1\text{-}198}$), or a truncated form of SyfR missing the dimerization domain (p*519n-syfR*$_{164\text{-}257}$). Bars indicate standard deviation.

SyfR autoregulates to induce syringafactin production at a surface.

We tested the hypothesis that SyfR might be involved in conveying the preferential production of the surfactant syringafactin when cells were cultured on agar plates compared to broth cultures. Initially, we determined if constitutive expression of SyfR is sufficient to induce high levels of syringafactin production in broth culture. We grew the wild-type strain, a *syfR*- mutant, and a wild-type strain that over-produced SyfR by expressing *syfR* constitutively on the plasmid p*519n-syfR*, in both plate and broth conditions, and tested for surfactant production by the drop collapse method. Similar to the oil spray assay depicted in Figure 1, the water drop collapse assay indicated that relatively large quantities of syringafactin were produced in both the wild-type and wild-type harboring plasmid p*519n-syfR* strains on agar plates, while a *syfR*- mutant was deficient in surfactant production (Fig. 4). In contrast, while syringafactin production was low in a wild-type strain when these strains were grown in shaken broth cultures, constitutive expression of SyfR induced sufficient syringafactin production to enable drop collapse under these culture conditions (Fig. 4). This suggested that low levels of SyfR might be responsible for the low levels of syringafactin production seen in broth cultures. We thus hypothesized that the surface regulation of syringafactin is at least in part mediated by SyfR.

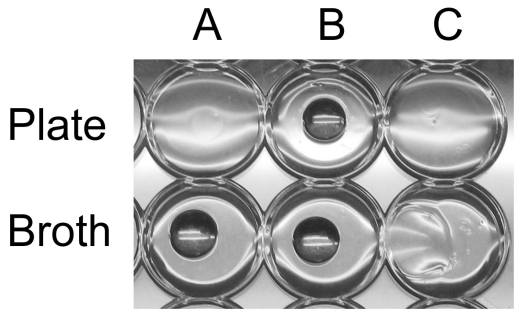

Figure V-4. Constitutive *syfR* expression enables syringafactin production in broth cultures of *Pseudomonas syringae*.
Drop collapse assays of a wild-type strain of *P. syringae* (A), a *syfR*- mutant (B) and a wild-type strain harboring p*519n-syfR* (C). Cell-free supernatant from plate-grown cells suspended to an OD_{600} of 1.0 are in the top row while cell-free supernatant from broth cultures grown and diluted to a similar optical density are in the bottom row. Each well was tested with 5 ul of culture supernatant. The collapsed droplets (not elevated) are indicative of surfactant production.

We determined if the apparently low levels of SyfR in broth culture stemmed from low levels of *syfR* transcription. To test this model we constructed a bioreporter in which a *gfp* reporter gene was expressed under the control of the promoter of *syfR* in plasmid pP*syfR-gfp*. When a wild-type strain carrying pP*syfR-gfp* was grown in broth media, apparent *syfR* transcription was about 3-fold lower than when grown on agar plates (Fig. 5). As a control, similar levels of GFP fluorescence were observed in a strain constitutively expressing the *gfp* reporter gene in these two culture conditions. It should be noted that rates of *syfA* transcription itself were more than 10-fold higher in cells cultured on agar plates compared to broth (Chaper 3). We attribute the larger effect of broth culture on *syfA* expression than on expression of its regulator *syfR* as a consequence of the strong concentration dependence of oligomerization of SyfR that would be expected to contribute to its activation.

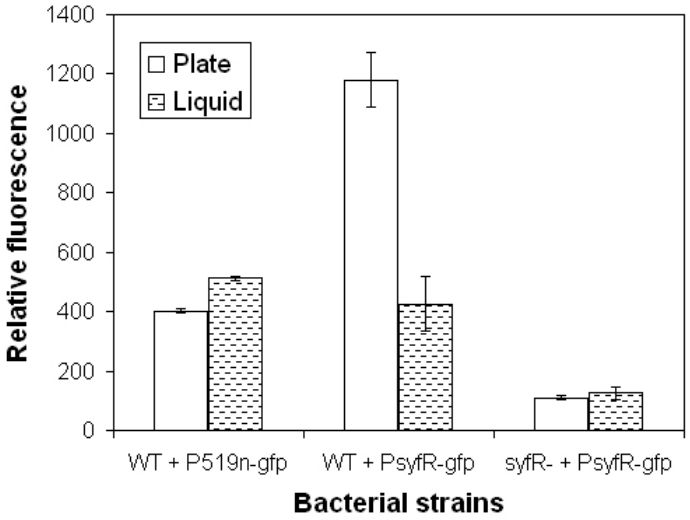

Figure V-5. Surface culture enhanced expression of *syfR* is dependent on SyfR in *Pseudomonas syringae*.
Relative GFP fluorescence of cells of the wild-type or *syfR*- mutant of *P. syringae* B728a harboring a plasmid in which GFP is constitutively expressed (p*519n-gfp*) or a plasmid in which GFP expression is dependent on the promoter of *syfR* (pP*syfR-gfp*) recovered from plate and broth cultures. Bars indicate standard deviation.

We investigated the possibility that *syfR* is subject to autoregulation in *P. syringae* since LuxR induces its own expression at least 2- to 3-fold compared to that in *luxR*- mutant strains (Fuqua *et al*., 1994). Similarly, constitutive SalA expression results in a 2- to 3-fold upregulation of *salA* (Kitten *et al*., 1998). It is noteworthy that this range of autoregulation is of the same magnitude as the differences in *syfR* transcription observed between broth and plate cultures. Therefore, we investigated the transcription of *syfR* in the absence of functional SyfR protein. Surprisingly, we observed equally low GFP fluorescence of a *syfR*- mutant strain harboring pP*syfR-gfp* cultured on both agar plates and in broth media (Fig. 5). This finding suggested two important points. First, it suggests that SyfR is autoregulated, and is necessary for the induction of its own transcription above a low baseline level. Second, it suggests that the surface regulation of both syringafactin production and SyfR abundance are conferred by a post-transcriptional process that affects SyfR levels or activity. Thus, we hypothesize that broth culture conditions reduce the magnitude of SyfR autoregulation, either through degradation of the *syfR* transcript or SyfR itself, or by alteration of SyfR. Further biochemical experimentation will be necessary to determine the mode of this control.

If broth conditions foster the hypothesized destruction or modification of SyfR, then we might expect that constitutive production of SyfR would nonetheless result in lower promoter induction of *syfR* and *syfA* in broth cultures compared to growth on agar plates. We earlier observed that constitutive expression of SyfR enabled syringafactin production even in broth culture, but we did not examine *syfA* expression per se. Apparent *syfA* expression in broth culture, as estimated with the plasmid pP*syfA-gfp* introduced into a strain constitutively expressing SyfR, was slightly below that observed on agar plates (Fig. 6A), which might lend support to our hypothesis. However, the promoter activity of *syfR* in a strain with constitutive expression of SyfR was

slightly higher in broth cultures than in cells recovered from agar plates (Fig. 6B). We have no explanation for why *syfA* expression was lower in broth cultures than on agar plates while *syfR* was higher. Further biochemical work might help elucidate any additional factors that contribute to *syfA* regulation. Nonetheless, the observation that constitutive expression of SyfR results in a further up-regulation of *syfR* further supports our claim that SyfR is autoregulated (Fig. 6B).

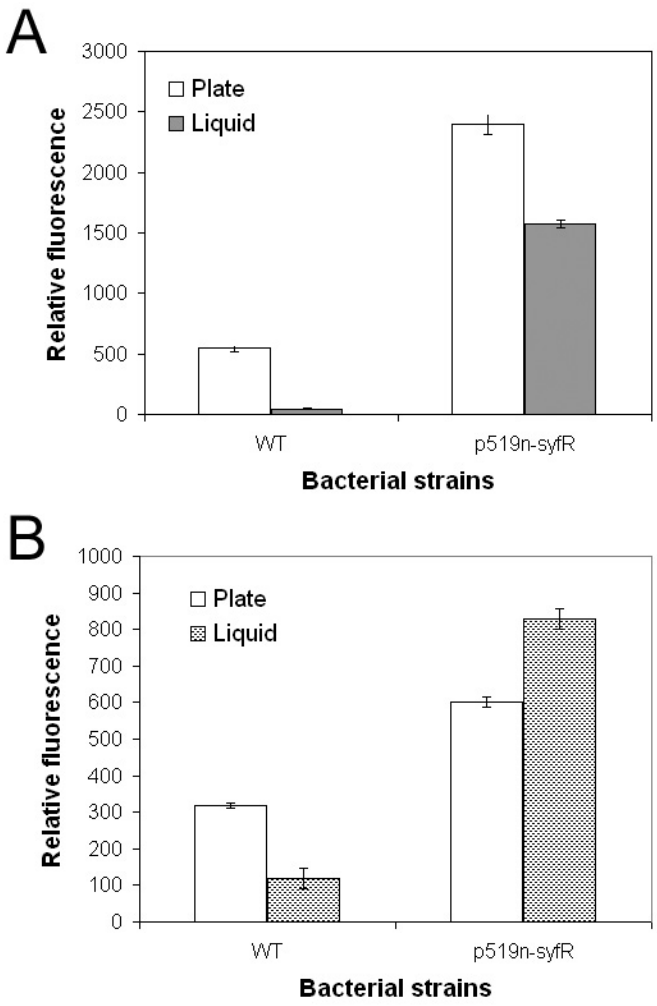

Figure V-6. Effects of constitutive SyfR production on expression of *syfA* (A) or *syfR* (B) in *P. syringae* strains grown under various culture conditions
Relative GFP fluorescence of wild type *P. syringae* B728a or a SyfR-overexpressing strain which carries p*519n-syfR*. Gfp fluorescence intensity reflect rates of *syfA* transcription (A) or *syfR* transcription (B), which is conveyed by plasmids in which GFP expression is dependent on the promoter of *syfA* (pP*syfA-gfp*) or *syfR* (pP*syfR-gfp*), respectively, recovered from plate and broth cultures. Bars indicate standard deviation.

A test of the self-sufficiency of the autoinduction process of *syfR* would be to demonstrate that SyfR is sufficient for *syfA* induction in another bacterial taxa that might lack ancillary components found only in *P. syringae*. Introduction of *luxR* from *V. fischeri* along with its

regulated bioluminescence-encoding operon resulted in expression of of bioluminescence in *E. coli* (Choi and Greenberg, 1991). We sequentially transformed *E. coli* strain DH5α with both p*519n-syfR* and p*PsyfA-gfp*. The resulting *E. coli* strain did not display any GFP fluorescence (data not shown). This indicates either that additional transcription factors are necessary for *syfA* transcription, that processing or some unknown activation of SyfR cannot occur in *E. coli*, that these components were not efficiency transcribed in this *E. coli* host, or that SyfR does not directly regulate *syfA*. Additional investigation to distinguish these possibilities is warranted.

Gac and SalA control SyfR. We investigated the possibility that SyfR functions downstream from other global regulators in *P. syringae*. There have been multiple reports that the GacA/GacS two-component regulatory system controls lipopeptide production (Koch *et al*., 2002; Dubern *et al*., 2005; de Bruijn and Raaijmakers, 2009a). In *P. syringae*, it has been further demonstrated that GacA/S controls lipopeptide production through its regulation of SalA (Kitten *et al*., 1998). We hypothesized that Gac might also control syringafactin production, and thus tested surfactant production in a **Δ***gacS* deletion mutant (D. Gross) using the atomized oil assay as well as determining the expression of various genes involved in syringafactin production using transcription reporters. No surfactant production was observed in a **Δ***gacS* mutant, and *syfR* and *syfA* transcription are nearly abolished (Fig. 7, data not shown). Additionally, we determined whether SyfR operates independently of SalA, or if SalA is upstream from SyfR function. Surprisingly, the **Δ***salA* deletion mutant (graciously provided by D. Gross) also exhibited abolished surfactant production and very low levels of *syfR* and *syfA* transcription (close to the detection level) (Fig. 7, data not shown). This suggests that these genes mediate the baseline expression of SyfR. It also suggests that these pathways are not responsible for the surface-dependent induction of *syfA*, but rather have an epistatic role in syringafactin production.

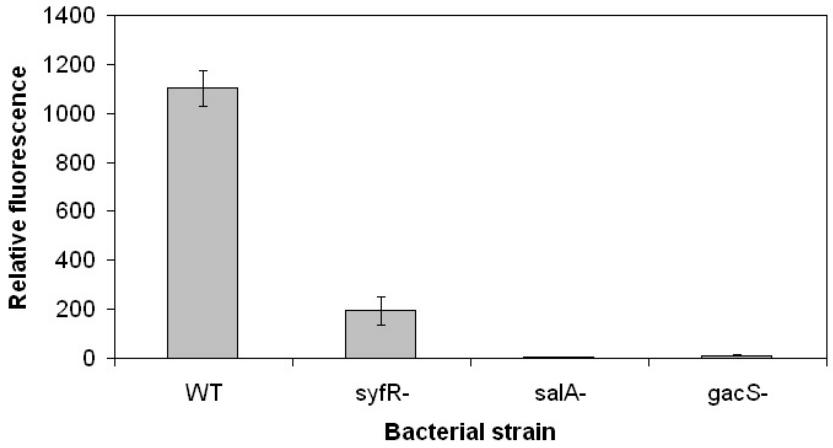

Figure V-7. SalA and GacS both are required for substantial expression of SyfR in *Pseudomonas syringae*

Relative GFP fluorescence of wild-type, *syfR*-, **Δ***salA*, or **Δ***gacS* cells of *P. syringae* B728a recovered from plate cultures that harbored a plasmid in which GFP expression is dependent on the promoter of *syfR* (p*PsyfR-gfp*). Bars indicate standard deviation.

We were surprised to observe that both **Δ***salA* and **Δ***gacS* mutants exhibited abolished surfactant production. We thus questioned if the strains are still capable of producing and secreting syringafactin or whether pleiotropic effects on cell metabolism that made them incapable of this metabolic process was operative in these mutants. To test this, we constitutively expressed SyfR by introducing plasmid p*519n-syfR* into both the **Δ***gacS* and **Δ***salA* mutant strains. These strains produced surfactant detectable both by the atomized oil spray as well as water drop collapse in both broth and plate cultures of these strains (data not shown). These mutants, in which *syfR* is not normally transcribed, thus produce and secrete syringafactin when SyfR is supplied constitutively. This demonstrates that neither GacS nor SalA are necessary for syringafactin transcription, or for supply of necessary intermediates for syringafactin production, but rather exert their influence on syringafactin production solely through their control of the SyfR regulator.

SyfR controls more than syringafactin. While we have demonstrated that GacS and SalA are upstream of SyfR, which itself controls syringafactin production, the question remained as to whether this surfactant is the only product under transcriptional control of SyfR. One clue that SyfR regulates more genes than just those required for syringafactin biosynthesis came from the observation that **Δ***syfA* and *syfR*- mutants did not appear identical in culture. Although the strains initially appear very similar, isolated colonies of the **Δ***syfA* mutant, when allowed to grow undisturbed for four or more days, exhibited a curious "fried egg" phenotype, while the *syfR*- mutant did not (Fig. 8B-C top row). This phenotype appeared as a raised "yolk" of initial colony growth that was typical of the wild type strain, followed by successive expansion of the colony after day three as a thinner wrinkly fringe or "white."

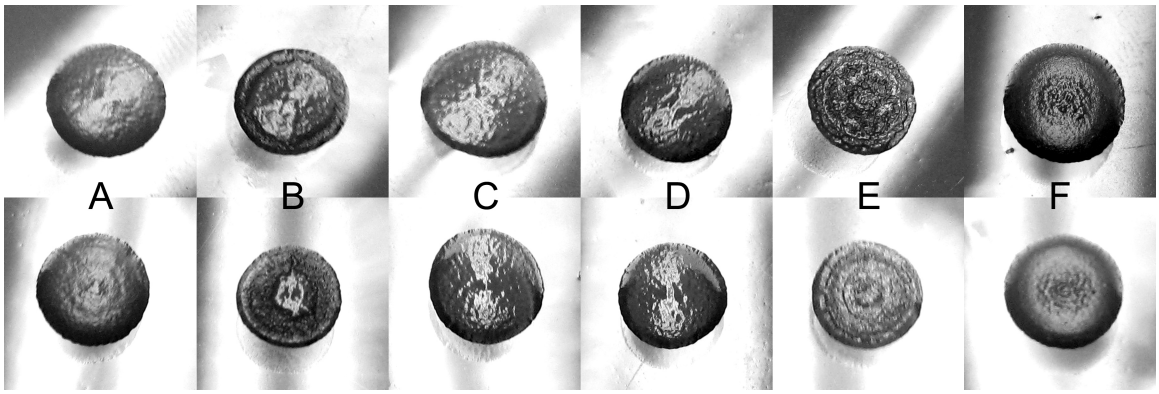

Figure V-8. Fried egg phenotype observed in *syfR*- mutants of *P. syringae* can be blocked by an extracellular, self-produced signal.
Four day old colonies of a wild-type P. syringae strain (A), **Δ***syfA* mutant (B), *syfR*- mutant (C), **Δ***syfA/syrE*- double mutant (D), a **Δ***syfA* mutant harboring p*519n-syfR* (E), and a *syfR*- mutant harboring p*519n-syfR* (F). Single colonies of a given strain grown on an agar plate are depicted in the top row, while colonies of these strains grown in close proximity to a **Δ***syfA/algT*- mutant of *P. syringae* are in the bottom row.

We initially suspected that secondary mutations might have been the cause of what looked to be a poorly growing or stressed strain. However, all independently isolated *syfA*- and *syfB*- transposon mutants as well as the site-directed **Δ***syfA* deletion mutant displayed this same

phenotype, suggesting that it is a direct response to the absence of syringafactin. Nonetheless, in order to rule out the possibility of secondary mutations in our syringafactin-deficient strains, we created a *syfR*- mutation in a **Δ***syfA* deletion strain. Surprisingly, this second mutation abolished the fried egg phenotype normally exhibited by the **Δ***syfA* mutant, and the appearance of this **Δ***syfA/syfR*- double mutant was indistinguishable from that of a *syfR*- mutant alone (picture not shown). This suggested that SyfR transcriptionally regulates more genes than just those enabling syringafactin production, specifically including genes encoding whatever trait triggers development of the fried egg phenotype. We initially hypothesized that perhaps SyfR induced both syringafactin production and also a system involved in either its transport or its perception, and we further postulated that syringafactin served not only as a surfactant, but also as a signaling molecule.

To additionally support the conjecture that SyfR controls more than just syringafactin production, we introduced the plasmid conferring the constitutive expression of SyfR into a **Δ***syfA* strain. Curiously, this strain also exhibited a fried appearance, but one that appeared to be an exaggerated and earlier-onset version of the rough "white" from a **Δ***syfA* strain (Fig. 8E, top). Thus, the strong visual phenotype of this strain provides further evidence that SyfR transcriptionally regulates more than just syringafactin production. In comparison, a wild-type strain capable of syringafactin production and that constitutively expresses SyfR develops a slightly matted appearance, but does not exhibit a fried appearance (Fig. 8F, top). Assuming that this rough fried egg appearance is indeed indicative of stress, we might hypothesize that syringafactin normally plays a protective role for the cell, and that its absence makes the cells somehow more susceptible to other factors induced by SyfR in *P. syringae* itself.

In order to identify genes under the control of SyfR, we screened 2,000 transposon mutants in a **Δ***syfA* mutant background for any that had lost the fried egg phenotype. Several such mutants were identified (Table 1). Prominent among the mutants found were several insertional events in the syringomycin biosynthetic genes and an associated secretion gene (Table 1), prompting further investigation. In order to confirm the requirement for syringomycin to initiate the fried egg phenotype, we constructed a site-directed knockout of *syrE* in a **Δ***syfA* mutant background. This syringomycin deficient mutant was identical in colony appearance to a *syfR*- mutant (Fig. 8D, top). One of the most surprising aspects of this finding was the fact that syringomycin and syringopeptin are assumed to have overlapping roles as plant virulence factors, and are typically co-regulated by SyrF which is downstream of SalA. However, a site-directed knockout in the syringopeptin biosynthetic gene *sypA* did not lead to a loss of the fried egg phenotype (data not shown). These results strongly suggest that syringomycin has a specific role in this phenoptype that is independent from syringopeptin. However, while syringomycin appeared necessary for the fried egg phenotype, this was not proof that it was a factor regulated by SyfR, which was posited to be required for this phenomenon.

Table V-1. Identity of genes disrupted in transposon mutants that could no longer exhibit a fried egg phenotype in a Δ*syfA* mutant background of *Pseudomonas syringae*

Locus of Tn5 insertion	Predicted function
Psyr_0936	Glycosyltransferase
Psyr_1864	Spermidine synthase
Psyr_2603	Secretion protein
Psyr_2608 (syrE)*	Syringomycin
Psyr_2611 (syrB1)	Syringomycin
Psyr_2747	Extracellular ligand-binding
Psyr_3290 (fadB)	Fatty acid oxidation
Psyr_3698 (gacS)	Response regulator
Psyr_5133 (mnmE)	tRNA modification

* Psyr_2608 was identified by two individual transposon insertion events

SyfR controls a pulse of syringomycin production. We postulated that the production of syringomycin in the absence of syringafactin is altering cell physiology in a way that leads to a production of the fried egg phenotype. In order to confirm that syringomycin expression is under the control of SyfR, we developed a plasmid-based transcriptional reporter of *syrB* expression (p*PsyrB-gfp*). Indeed, the GFP fluorescence indicative of *syrB* expression was much lower in cells of a *syfR*- mutant harboring p*PsyrB-gfp* than in either a **Δ***syfA* mutant or the wild type strain; expression in the **Δ***syfA* mutant was similar to that in the wild type strain (Fig. 9). In broth cultures, expression of *syrB* was similarly low in all strains, as further proof that genes downstream of SyfR are not activated in broth conditions (data not shown). This confirms that syringomycin is induced in cells cultured on plates and is under the regulatory control of SyfR.

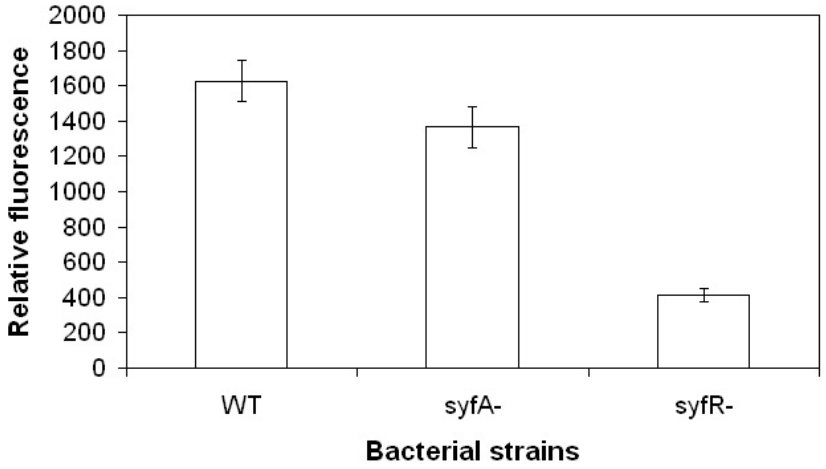

Figure V-9. SyfR controls syringomycin production in *Pseudomonas syringae.*
Relative GFP fluorescence of a wild-type strain, **Δ***syfA* mutant, or *syfR*- mutant of *P. syringae* B728a harboring a plasmid in which GFP expression is dependent on the promoter of *syrB* (p*PsyrB-gfp*), when grown on agar plates. Bars indicate standard deviation.

Because the fried egg phenotype is observed only in colonies older than 3 days, we investigated syringomycin expression in different mutant strains over the course of several days. Surprisingly, although syringomycin was highly expressed in wild-type and **Δ***syfA* strains after one day of growth, only very low levels of expression were detected at any subsequent time (data not shown). To further explore this apparent temporal regulation, we measured the GFP fluorescence of cells of a wild-type strain harboring pP*syrB-gfp* over the course of 48 hours of growth on plates. In agreement with our initial observations, *syrB* expression, and thus presumably syringomycin production, is limited to a short period during initial phases of colony development, peaking after about 24 hours and thereafter diminishing (Fig. 10). This pattern of expression was seen in both the wild-type and a **Δ***syfA* mutant strain, but not in the *syfR*- mutant, in which *syfA* expression was always low (data not shown). This temporal regulation of *syrB* is contrary to that of *syfA* expression, which remains stably induced over this time period (data not shown). Thus the role of SyfR in stimulating syringomycin expression is distinct from its effect on syringafactin expression.

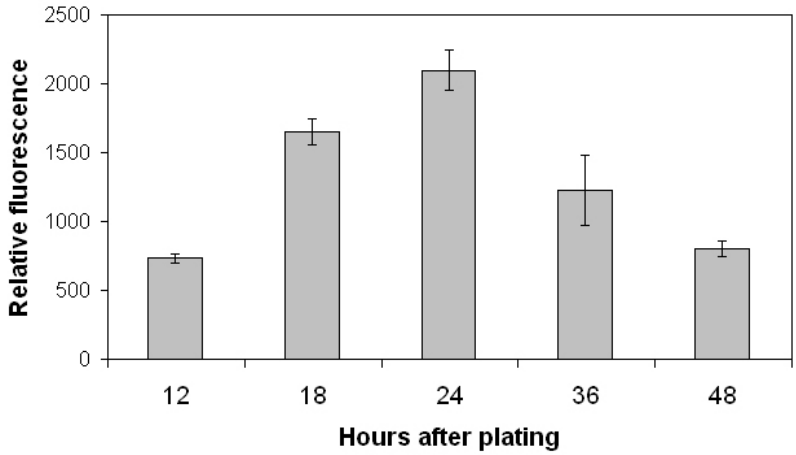

Figure V-10. Temporal dependence of SyfR-dependent expression of *syrB* in *Pseudomonas syringae*.
Relative GFP fluorescence of cells of wild-type *P. syringae* B728a harboring a plasmid in which GFP expression is dependent on the promoter of *syrB* (pP*syrB-gfp*) measured in cells grown on agar plates for various times. Bars indicate standard deviation.

We hypothesized that the strong temporal, and hence cell density-related, regulation of syringomycin synthesis may be due to its suppression by quorum sensing in older cultures. To test this we measured *syrB* expression in a **Δ***ahlR* mutant incapable of quorum sensing (strain courtesy of R. Scott). The temporal expression of *syrB* in this strain was identical to that in a wild type strain, with peak expression at 24 hours. This suggests that quorum sensing does not mediate temporal regulation of syringomycin production (data not shown). Also, these results also cast doubt on the model that syringomycin is directly responsible for inducing the fried egg phenotype, since the colony phenotype appears after about four days of growth, while syringomycin production apparently peaks after only 24 hours.

Although the biosynthetic pathway for syringomycin and regulation of its expression has been extensively investigated, SyfR has never been implicated in its regulation. We thus questioned if SyfR was, in fact, an overlooked necessary regulatory element for syringomycin production. To test this we measured *syrB* expression in a wild-type and a *syfR*- mutant mutant strain on media specifically formulated to induce syringomycin and syringopeptin production (Wang *et al*., 2006b). Although the levels of GFP fluorescence exhibited by a *syfR*- mutant harboring pP*syrB-gfp* were reduced compared to that in the wild type strain, we still see substantial expression of *syrB* in the medium conducive to syringomycin production. This suggests to us that SyfR is not absolutely required for the induction of syringomycin production in this medium that mimics the plant environment, but plays a more ancillary role in its production. This moderate effect on syringomycin production might explain why SyfR has not previously been identified as a regulator of syringomycin.

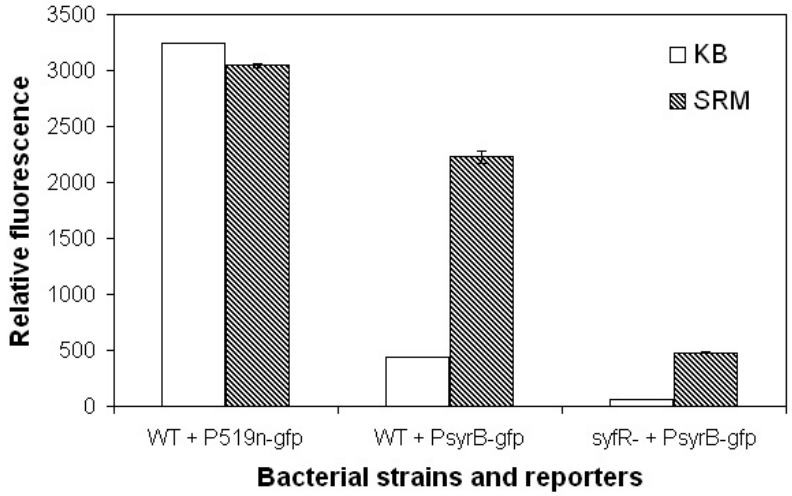

Figure V-11. SyfR contributes only partially to expression of *syrB* in *Pseudomonas syringae*.
Relative GFP fluorescence of cells of a wild-type or a *syfR*- mutant strain of *P. syringae* B728a harboring a plasmid in which GFP is constitutively expressed (p*519n-gfp*) or a plasmid in which GFP expression is dependent on the promoter of *syrB* (pP*syrB-gfp*) recovered from KB plates and plates of SRM containing added arbutin and fructose for maximal syringomycin induction (Mo and Gross, 1991). Bars indicate standard deviation.

An external signal that is not syringomycin triggers the fried egg phenotype. By chance, it was observed that the fried egg phenotype in colonies of **Δ***syfA* mutants appeared much earlier when they were grown near colonies of a **Δ***syfA* mutant blocked in any of various steps in the AlgT regulatory pathway. When colonies of a **Δ***syfA* mutant were grown on the same plate with those of a **Δ***syfA/algT*- mutant (that was previously shown to produce copious quantities of surfactants and perhaps other factors) (Chapter 2 and 4), the timing of the onset of the fried egg phenotype was directly correlated with the distance from the **Δ***syfA/algT*- mutant (Fig. 12). The fried egg phenotype was induced in **Δ***syfA* mutants after as few as 2 days of incubation when cultured near a **Δ***syfA/algT*- mutant. When cultured by an *algT*- mutant that was still capable of syringafactin production, this early-onset property was diminished (data not shown). Premature

induction of the fried egg phenotype occurred only in a **Δ***syfA* mutant, while colonies of wild-type and *syfR*- strains did not change their appearance in response to this signal (Fig. 8A-C, bottom). This observation suggested that the fried egg phenotype must be a response to an extracellular compound that is only sensed by a component of the SyfR regulon. We earlier hypothesized that syringomycin is the compound that induces this fried egg phenotype. If syringomycin directly stresses the cell or otherwise induces this colony phenotype, and if syringomycin is produced in large quantities in an *algT*- mutant, then we should have seen a restoration of the fried egg phenotype in a **Δ***syfA/syrE*- double mutant strain upon exposure to syringomycin. However, when a **Δ***syfA/syrE*- mutant strain is placed in close proximity to a **Δ***syfA/algT*- mutant, there is no restoration of the fried egg phenotype (Fig. 8D, bottom). Furthermore, a **Δ***syfA* mutant still exhibits a strong fried egg phenotype when placed near a **Δ***syfA/algT*-/*syrE*- triple mutant (data not shown). Therefore, it does not appear that syringomycin acts as the direct extracellular signal that invokes this response, but rather is necessary for enabling other factors to induce the response. This evidence, in addition to the finding that syringomycin is only produced during the initial 24 hours of surface growth, lead us to assume that syringomycin instead acts as a signal that primes the colony for the fried egg phenotype that we later observe.

We recently observed that an *algT*- mutant of *P. syringae* produces high levels of a surfactant termed BRF (biosurfactant regulated by flagella), whose production requires an *rhlA* homolog. This surfactant also exhibits a strong temporal pattern of regulation, with production increasing over time (Chapter 4). Therefore, we hypothesized that this surfactant could be responsible for inducing the fried egg phenotype in the **Δ***syfA* mutant. Colonies of a **Δ***syfA*/**Δ***brfA* double deletion mutant did not express the fried egg phenotype at any age. However, this double mutant regained the fried egg phenotype in the presence of a **Δ***syfA/algT*- mutant strain, suggesting that BRF could be a signal that induces this phenotype. Furthermore, neither a **Δ***brfA/algT*- double mutant, nor a **Δ***syfA*/**Δ***brfA*/*algT*- triple mutant is capable of inducing an early fried egg phenotype in a **Δ***syfA* mutant. However, application of a BRF extract near a colony of a **Δ***syfA* mutant does not induce the early appearance of the fried egg phenotype (data not shown). It is thus possible that BRF is modified to gain its activity, or that BRF might play a role in delivering an insoluble signal over the long distances that separate colonies.

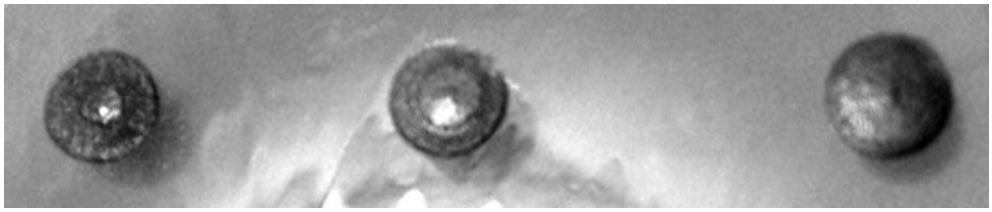

Figure V-12. The fried egg phenotype is a response to a self-produced diffusible signal that is over-expressed in a Δ*syfA/algT*- mutant of *Pseudomonas syringae*.
Fried egg phenotypes exhibited by colonies of a **Δ***syfA* mutant located at different distances from an *algT*- mutant (established on the left side of the plate just outside of the image). Images were taken after four days incubation.

Although none of the biosurfactants produced by *P. syringae* appeared to be sufficient to induce the fried egg phenotype, we investigated a variety of synthetic surfactants for their ability to initiate this trait. Surprisingly, we observed that the hydrophobic surfactant, Span 80 (sorbitol oleate), was capable of inducing this phenotype (Fig. 13).

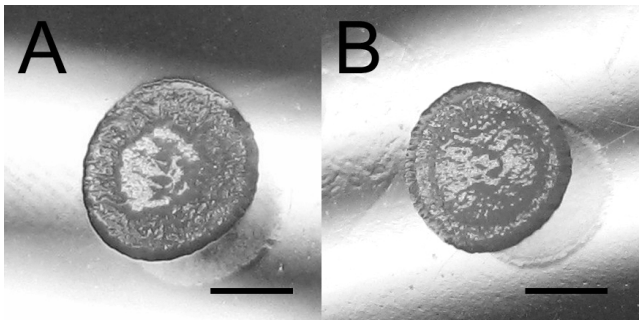

Figure V-13. Induction of the fried egg phenotype in *Pseudomonas syringae* by a hydrophobic synthetic surfactant.
Fried egg phenotypes of colonies of a **Δ***syfA* mutant after growth for three days on agar plates. Span 80 was added to the plates approximately 1 cm from the colony at either day 0 (A) or day 2 (B). Bars indicate 0.25 cm.

DISCUSSION

Similar to the other characterized members of LuxR-type regulators, SyfR appears to form multimers in order to initiate transcription. Furthermore, in keeping with observations of LuxR and SalA, SyfR appears to have an autoregulatory role in its own transcription. However, it is unclear how the activation of SyfR is mediated by external conditions. In the case of LuxR, binding of a quorum signal induces dimerization which then allows LuxR to function as a transcription factor; thus, cell density is conveyed to the cell by increased availability and binding of the autoinducer signal, which stimulates increased LuxR activity (Nasser and Reverchon, 2007). However, SyfR belongs to a class of LuxR-type regulators that do not contain characterized small molecule binding domains, and thus there is no factor that has been determined to limit SyfR dimerization and activity other than its own transcription levels. The observation that SalA, another member of this class of regulators, is up-regulated upon perception of plant signals, makes it appear that the signal perception occurs upstream of SalA (Wang *et al*., 2006b). Our results similarly indicate that the condition of surface sensing stimulates an up-regulation of SyfR. However, we have also demonstrated that this transcriptional activation of *syfR* at a surface is reliant on functional SyfR protein. Therefore, it appears that the SyfR protein itself is involved with perceiving external conditions, and its transcriptional up-regulation is a result of its autoregulatory activity. Thus, we speculate that SalA is similarly self-regulating in response to plant signals, in addition to stimulating transcription of its downstream genes.

If the basal expression of *syfR* is the same in broth cultures as it is on surfaces, then what keeps SyfR from being equally active in both conditions? It is tempting to speculate that an unidentified substrate or modification of SyfR induces its dimerization, activity and perhaps stability, similar to other LuxR-type regulators. However, this model does not agree with a

previous report that SalA and SyrF, when purified from *E. coli*, dimerized *in vitro*, as well as bound to their target DNAs in gel shift assays in the apparent absence of such external factors (Wang *et al*., 2006a). Furthermore, when SyfR is constitutively expressed, we observe an increase in SyfR-mediated transcription of *syfA* and *syfR* under conditions of growth on both plates and in broth, demonstrating that SyfR itself and not SyfR activation (by substrate binding or another means) was the limiting factor in gene expression in broth conditions. On the other hand, although constitutive expression enhances transcription of *syfA* in both growth conditions, we did observe a slightly lower level of *syfA* expression in broth conditions, which might imply lower SyfR activity. Thus we hypothesize that external conditions lead to changes in SyfR activity through changes in protein abundance or modification, or through mRNA degradation, since constitutive expression can apparently overwhelm these processes.

If SyfR activity is mainly controlled through a degradation process, it is curious that *syfR* expression was not sufficient for expression of *syfA* in *E. coli*. Although there are many potential explanations for this observation, we find it most likely that SyfR does not directly regulate *syfA*, but rather controls the expression of another transcription factor that in turn activates *syfA*. Alternatively, SyfR might act in concert with other factors found only in *P. syringae*. Such a model might indicate that degradation of SyfR is not essential for modulation of SyfR activity, and that *E. coli* does not possess the factors necessary for SyfR modification and activation. Another uncertainty is why the C-terminal portion of SyfR was incapable of enabling transcription of *syfA*. We chose to exclude the low compositional complexity region (LCC), which often serves as a flexible linker or a direct binding interface (Coletta *et al*., 2010). While there are many potential reasons why the truncated SyfR did not function, it is likely that the LCC region is necessary for activation of the DNA-binding properties of the HTH domain.

Our findings that SalA regulates *syfR*, as well as the fact that SyfR regulates *syrB* are both surprising. *syfR* is physically located near *syfA* on the genome, while *salA* is physically located near genes encoding syringomycin such as *syrB* and syringopeptin production as well as the other LuxR-type regulators *syrF* and *syrG*. Although these two genetic islands have not previously been functionally associated, our results indicate that there is a great deal of cross-talk between them. The similarities in the given gene names can be confusing, and so in order to better describe a model that accounts for the findings of this study, we present a summary of how SyfR and syringafactin biosynthetic genes fit into the regulation of lipopeptides as a whole in *P. syringae* B728a (Fig. 14). However, many components of this model remain to be determined: Do SyrF and SyfR demonstrate regulatory control over each other? Can these LuxR-type regulators form heterocomplexes? SyrG has previously been demonstrated to have no regulatory association with SalA or SyrF, but does its regulatory pathway intersect with SyfR? Since both up- and down-stream LuxR-type regulators affect viscosin production in *P. fluorescens* SBW25 (de Bruijn and Raaijmakers, 2009b), what is the role of the LuxR-type regulator downstream of syringafactin? Finally, what is the functional significance of the frequent pairing of this class of LuxR-type transcriptional regulators with lipopeptide NRPSs?

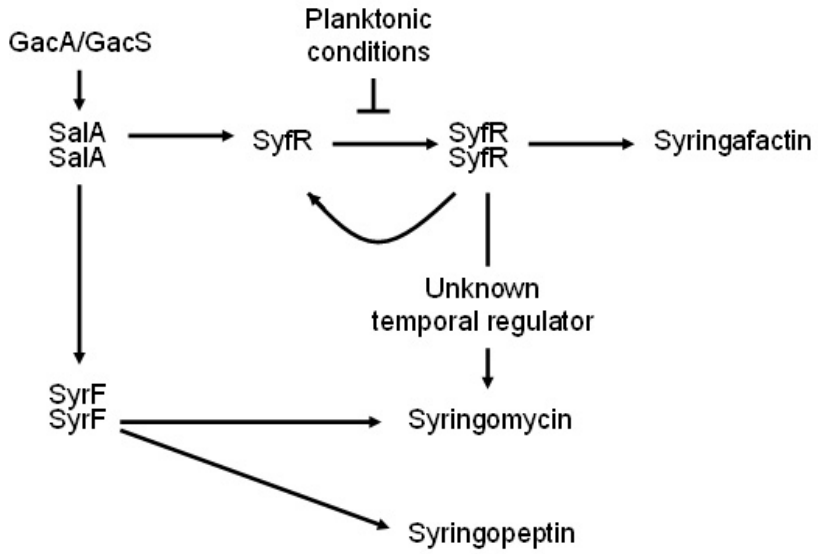

Figure V-14. Model of the role of LuxR-type regulators in the regulation of lipopeptide production in *Pseudomonas syringae*
Diagram of the proposed regulatory hierarchy affecting lipopeptide production in *P. syringae* B728a. SyrG was not included due to lack of information on its regulation

A further mystery generated by this research is the unidentified cascade dependent on SyfR expression that induces an early expression of syringomycin followed by a unique "fried egg" colony appearance phenotype that is dependent on this syringomycin production. Although the "fried egg" trait is only apparent when syringafactin is not produced, it might be indicative of a normal physiological response to aging of cells in colonies that is visually masked when the cells can produce syringafactin. Furthermore, it is intriguing that our response shares some important similarities with surfactin-mediated signaling in *Bacillus subtilis*. *B. subtilis* does not directly sense surfactin, but rather potassium leakage that can be provoked by a variety of other compounds, such as other surfactants (López *et al*., 2009b). This leakage is only perceived by cells that do not produce surfactin, and its perception induces extracellular matrix production (López *et al*., 2009a). We speculate that the fried egg phenotype is due to an altered amount or type of extracellular polysaccharide that is produced in strains of *P. syringae* that lack syringafactin. Although we have no solid evidence of a similar mechanism in *P. syringae*, it is intriguing that a variety of compounds induce this "fried egg" response that is only seen when syringafactin is not produced. However, extracellular syringafactin alone does not induce the response, clearly ruling out it having an identical role to surfactin in such autoinduction.

It should be rewarding to determine the SyfR regulon in order to determine if the observed "fried egg" phenotype is a non-specific response indicative of stress, or rather is a visual manifestation of a regulated response such as altered EPS production. Microarray or RNA sequencing studies comparing transcript abundance of a WT with a *syfR*- mutant strain, as well as that in a **Δ***syfA* mutant at various stages of colonial growth should help elucidate this mystery. Although we have ruled out a role of alginate in formation of this altered colony phenotype (data not shown), other stress-responsive indicators might be observed. Additionally, transcriptome analysis of a

Δ*syfA* mutant in which SyfR is over-expressed (displaying a hyper fried egg phenotype) might reveal components of the SyfR regulon that lead to this phenotype.

Overall, the findings from this study serve to further distinguish the roles of three lipopeptides produced by *P. syringae* B728a. These lipopeptides are structurally different, and they show distinct differences in *in vitro* antimicrobial activities and phytotoxicity (Hutchison and Gross, 1997; Lavermicocca *et al*., 1997). However, little work has been performed to distinguish their individual roles to bacteria inhabiting plant surfaces or in plant pathogenesis. Early evidence that SyrG regulates syringomycin but not syringopeptin provided genetic evidence that certain biological circumstances might warrant syringomycin production specifically, although the consequences of such SyrG regulation was not further investigated (Lu *et al*., 2002). Our evidence that SyfR also has a specific role in syringomycin but not syringopeptin production should further prompt an examination of the roles of these different lipopeptides *in planta*. Perhaps these lipopeptides are used at times in concert for plant virulence, but syringomycin might also have a unique additional signaling role that aids colonization of surfaces. Syringafactin might also contribute to the virulence of *P. syringae*, but have a unique role in motility or biofilm assembly on surfaces. These hypothetical functions of these compounds remain to be tested.

These results raise an important question about how the roles of surfactants on plants should be tested. For instance, what genetic background will yield a truly syringafactin-free strain? Because multiple phenotypes might be regulated by SyfR, it would seem wise to use **Δ***syfA* mutants rather than *syfR*- mutants in experiments to determine the function of syringafactin in natural environments. If we block the expression of a regulator such as SyfR, we might also block other factors that have an important effect separate from the surfactant. However, if the cell normally compensates for production of a surfactant, then just removing the surfactant might alter the cell in other ways we are not aware of. In other words, either of these strain choices might confer reduced fitness for reasons other than the lack of surfactant, and thus over-estimate the role of surfactants *in vivo*. This possibility cannot be easily addressed, but must be considered by those conducting experiments with similar systems.

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Chapter VI.

Multiple roles of biosurfactants produced by *Pseudomonas syringae* in the phyllosphere

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ABSTRACT

Although biosurfactant production by plant-associated organisms in culture has been documented previously, there has been little investigation of their production *in vivo* or demonstration of their contribution to bacterial colonization of plants. This research was designed to demonstrate that biosurfactant production occurs in the phyllosphere, as well as to evaluate the involvement of these compounds in bacterial motility and nutrient acquisition. Growth of *Pseudomonas syringae* pv. syringae B728a on plants resulted in a reduction of leaf surface water tension, mostly attributable to its production of syringafactin. However, production of a biosurfactant regulated by flagella (BRF) in a **Δ***syfA* strain also significantly enhanced the contact of water droplets with the leaf surface, demonstrating its production and activity *in planta*. Biosurfactant production contributed little to motility of *P. syringae* on leaves in various assays, although strains producing syringafactin were more abundant compared to non-producing mutants, measured both by culturing methods, as well as visually at the margin of leaf areas covered by water droplets. While *in planta* motility assays yielded little evidence of a role for biosurfactants in motility, strains producing either surfactant had higher proportions of cells that expressed *fliC* on leaf surfaces compared to a mutant strain blocked in expression of both surfactants. Surfactant production thus apparently results in a higher proportion of motile cells on leaves. Direct application of syringafactin as well as application of cells of WT *P. syringae* to leaves both increased cuticular transpiration. A **Δ***syfA* strain of *P. syringae* induced even higher cuticular transpiration than the WT strain, suggesting that while surfactants are sufficient to alter cuticular permeability, they mediate a complex interaction of bacteria with plant surfaces. Biosurfactant production thus occurs in the phyllosphere, and its potential roles in both motility and nutrient acquisition were assessed.

Author contributions: A.B. and S.L. designed the research, V.Z. and L.S. designed and performed the cuticle permeability experiments, A.B. and C.D. designed and performed the surface tension and contact angle experiments, A.B. and S.M. performed all other research, and A.B. and S.L. wrote the paper.

INTRODUCTION

Epiphytic bacteria regularly contend with high UV exposure, cycles of dessication and hydration, rapid temperature fluctuations, and low and heterogeneous nutrient availability (Lindow and Brandl, 2003). Additionally, the waxy leaf surface presents a physical barrier to water and nutrient availability on plant surfaces due to its impermeable hydrophobic nature (Schreiber, 2010). Despite all these challenges, phyllosphere bacteria have developed adaptations that enable them to persist and multiply, as well as disperse to new regions of the leaf. It is hypothesized that during periods of abundant water availability epiphytes will separate from the cellular aggregates that normally protect them against desiccation stress, and explore the leaf surface, moving between dispersed regions of relatively higher nutrient levels (Hirano and Upper, 2000). Additionally, it is under such conditions of leaf surface moisture when many plant pathogens like *P. syringae* are most successful at causing disease. Such conditions apparently enable invasion of the plant through stomata or other openings and subsequent colonization of the apoplast (Underwood *et al*., 2007). While epiphytes are distinct from most other bacteria in their ability to grow and survive on leaves, the adaptations that they utilize to thrive in this habitat remain largely unknown. Recently, our lab demonstrated that waxy leaf surfaces are enriched in biosurfactant-producing bacteria compared to other habitats (Chapter 3). We and others (Lindow and Brandl, 2003) have previously postulated that biosurfactants might be beneficial to the epiphytic life of bacteria, potentially enhancing their movement and/or ability to acquire nutrients, although such roles remain only conjecture.

Biosurfactants are biologically-produced amphiphilic compounds that exhibit surface activity through the actions of their hydrophilic and hydrophobic groups. Biosurfactants were first noted to enable soil-borne bacteria to access hydrophobic carbon sources, and have since been recognized as having many other functions including biofilm maintenance and as a lubricant required for swarming motility (movement across moist surfaces/ low-percentage agar plates) (Neu, 1996; Ron and Rosenberg, 2001). In plants, biosurfactants have received attention for their possible roles as virulence factors enabling diseases of some waxy plants and as antimicrobial compounds that might contribute to biocontrol of plant diseases (Hutchison and Johnstone, 1993; Hutchison and Gross, 1997; Bais *et al*., 2004; Hernandez-Anguiano *et al*., 2004). However, when tested *in vivo*, the contribution of biosurfactants to biocontrol ability has been generally quite modest (Kruijt *et al*., 2009). With a growing appreciation that human pathogens occur on fresh produce there is renewed interest in the ecological role of these compounds in the phyllosphere.

Previously, it was demonstrated Pseudomonads that produced surfactants *in vitro* changed the wettability of the leaf after they were allowed to multiply *in planta* (Bunster *et al*., 1989). However, it was not determined whether or not this benefitted the bacteria. Biosurfactants can enhance the contact between water and the leaf surface; this enlarged wetted area of the leaf might enable more of the leaf to be colonized by bacteria, and it could increase the distribution of locally abundant nutrients that might be separated by waxy regions of the leaf which would not otherwise be wetted by water. Furthermore, besides increasing growth through redistribution of nutrients, biosurfactants might also increase nutrient or water availability in those sites already colonized by bacteria. Surfactants are capable of solubilizing plant epicuticular wax, although only at concentrations above the critical micelle concentration (Tamura *et al*., 2001). With

reduced epicuticular wax, the diffusion of nutrients from the interior of the leaf to the surface would be enhanced. While waxes are not solubilized at lower surfactant concentrations, surfactants can have a plasticizing effect on the cuticle and enhance diffusion across the cuticle (Schreiber *et al*., 2005). Despite these many hypotheses of biosurfactant function in the phyllosphere, their actual roles have not yet been addressed. Thus we sought to specifically investigate the roles of biosurfactant production in bacterial movement on leaves and cuticle permeability.

We recently reported that *Pseudomonas syringae* pv. syringae B728a produces multiple biosurfactants (Burch *et al*., 2010). Both syringafactin and BRF (a Biosurfactant Regulated by the Flagella) were produced and were required for motility in culture. Although no distinctions are usually made of the features of biosurfactants, extensive characterization of synthetic surfactants has revealed that their physical properties can differ greatly and this will greatly affect the processes that a given surfactant will best participate in. For instance, the overall balance of lipophilic and hydrophilic components (HLB) of a given surfactant is an important descriptor of its useful properties. Hydrophilic, water soluble surfactants are most useful for solubilizing oils into water, while the more hydrophobic surfactants have low solubility in water and are poor at this process. While syringafactin produced by *P. syringae* B728a is relatively hydrophilic, BRF is apparently much more hydrophobic; these two compounds which display different physical properties would be expected to have different biological roles. Additionally, these two surfactants are regulated differently, with syringafactin mainly induced on surfaces, and BRF freely produced in aqueous conditions. This study reports initial explorations of the roles of these two surfactants in the epiphytic lifestyle of *P. syringae* B728a. Because the interactions of bacteria with plants can be easily observed in the phyllosphere we studied the behavior of wild type and surfactant mutants using a variety of techniques including culturing and fluorescent microscopy to address the roles of surfactants in motility and cuticle permeability.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. syringae* pv. syringae B728a (Loper and Lindow, 1987) was cultured on King's medium B (KB) plates with 1.5% technical agar (King *et al*., 1954) at 28°C. Hydrated paper discs were prepared by gently placing individual XX filter discs directly onto agar plates. Antibiotics were used at the following concentrations (μg/ml): kanamycin (25), rifampin (100), natamycin (21.6), gentamycin (75) and spectinomycin (100).

Plant inoculations. For all plant experiments, primary leaves from 2-week old plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274, with 4-6 seedlings per pot) were used. For studies of the wettability of leaf surfaces by bacteria, individual bacterial strains cultured on KB plates were removed by scraping, suspended and washed in sterile phosphate buffer (1 mM, pH 7.5), and adjusted to a final cell concentration of 10^9 cells/ml. Cell suspensions were sprayed using a hand-held perfume mister onto plants and the plants were then enclosed with plastic bags to maintain 100% humidity, and incubated under 16 h light cycles at 25°C for two days. The plants were then allowed to dry on a lab bench at about 60% room humidity for two hours before testing.

For epiphytic fitness assays, individual bacterial strains cultured on KB plates were removed by scraping, suspended and washed in sterile phosphate buffer (1 mM, pH 7.5), and adjusted to a final cell concentration of 10^6 cells/ml. Cell suspensions were sprayed using a hand-held sprayer onto plants and the plants were then enclosed in humidity tents with periodic fogging to maintain 100% humidity, and incubated under 16 h light cycles at 25°C for two days. Leafs were periodically sampled from the plants at 1, 2, and 3 days after inoculation, sonicated for 2 min in phosphate buffer and serially dilution plated in order to obtain CFUs.

For leaf motility assays, individual bacterial strains cultured on KB plates were removed by scraping, suspended and washed in sterile phosphate buffer (1 mM, pH 7.5), and adjusted to a final cell concentration of 2×10^7 cells/ml. 5 µl droplets containing 10^5 cells were applied to leaves on a defined site near the base of the leaf, and the plants were kept in humidity tents with periodic fogging to maintain 100% humidity. After 1-3 days the lower leaf segments collected at 5 cm from the point of inoculation in a direction toward the tip of the leaf were serially plated after sonication in order to determine the populations that had presumably grown as a result of bacterial motility. Alternatively, whole leaves were collected and plated to determine total populations.

For microscopic examination of reporter strains, strains were inoculated onto plants as described above for epiphytic fitness assays, and plants were bagged individually to maintain 100% humidity. For microscopic examination of constitutively fluorescent strains, strains were inoculated onto leaves as individual droplets as described above 4or leaf motility assays.

Surfactant extracts. Crude biosurfactant-containing culture extracts were prepared with modification to the protocol detailed by Berti *et al*. (Berti *et al*., 2007). Agar plates with confluent lawns of *P. syringae* B728a were grown for 48 hours. Cells were harvested by washing four plates with 90 ml H_2O and cells were removed by centrifugation (5,000 x g, 10 min). Filter sterilized supernatant was extracted with 150 ml ethyl acetate and the organic fraction was dried to completion and re-suspended in 4 ml of methanol, filtered through a 0.45 µm Nalgene filter (Fisher Scientific) and dried to completion. Extraction of BRF from a **Δ***syfA* carrying pBRF2 was performed as described in Chapter 4.

Surface tension measurements. The surface tension of cell-free supernatants was determined using the pendant drop method. Cell-free culture supernatants were analyzed with a FTA 4000 video analysis instrument (First Ten Angstroms Inc., Portsmouth, VA). Droplets were produced using a 22 gauge blunt needle and the values reported represent an equilibrium surface tension determined 60 seconds after drop formation.
To determine the contact angle measurements on bean leaves, single leaves were harvested and taped around a 50 ml falcon tube and positioned with the leaf horizon perpendicular to the camera view. A droplet of 5 µl H_2O was deposited onto the horizon of the leaf sample using the FTA instrument, and the image recorded every 100 ms for 15 seconds, by which time the droplet had reached equilibrium. Contact angles were determined using the software provided with the instrument. Leaf areas free of major veins were avoided. Around five droplets for at least four leaves were used to determine the average contact angle of the leaves.

Fluorescence. *P. syringae* B728a strains were transformed with either a pKLN42-tet plasmid conferring constitutive GFP fluorescence (J. Cho, unpublished), pP*syfA-gfp* indicative of syringafactin transcription (Chapter 2), pP*brfA-gfp* indicative of BRF transcription (Chapter 4) or pP*fliC-gfp* indicative of flagellin transcription (Chapter 4). Strains were either inoculated onto plants as described above, or were grown on KB plates overnight and then suspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD_{600} of 0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, CA, USA) with a 486-nm bandpass excitation filter and a 510- to 700-nm combination emission filter. A relative fluorescence unit (RFU) was defined as the fluorescence of the suspensions normalized for the suspension turbidity measured as OD_{600}.

Microscopy. Inoculated leaves were harvested and sonicated in 30 ml potassium phosphate buffer (10 mM, pH 7.5) for 2 minutes. Cells were collected by centrifugation and flash-frozen in a solution containing 2 µg/ml DAPI. DAPI-stained cell samples were washed and spotted onto charged slides (clean glass slides pre-dipped in 0.1% gelatin solution) in 10 µl droplets and air-dried under the hood. Samples were then covered with Aqua PolyMount anti-fade mounting reagent (Polysciences, cat#18606) and cover slips. Bacteria were viewed and photographed at 1000x magnification using a Hamamatsu digital camera attached to a Zeiss AxioImager M1 microscope. Samples were excited using a broad-spectrum mercury arc lamp, and visualized using standard DAPI and EndowGFP filter cubes. Exposure settings for pP*fliC-gfp* were 0.5 seconds for DAPI and 0.5 seconds for GFP. Exposure settings for pP*syfA-gfp* were 0.4 seconds for DAPI and 0.5 seconds for GFP. Exposure settings for pP*brfA-gfp* were 0.2 seconds for DAPI and 2 seconds for GFP. For each treatment, 5-10 images were acquired using iVision software, using the Multi-D Acquire function for paired DAPI and GFP photomicrographs. For all image pairs, DAPI-stained bacterial cells were masked using the iVision Segmentation function, and the segment masks were copied and pasted onto the tandem GFP images. Mean GFP pixel intensity for each masked object was quantified. Objects that were less than 10 or more than 200 pixels in size were excluded from the data. Background fluorescence was measured by calculating the pixel intensity from cell-free portions of GFP images.

Cuticle permeability. Cuticular transpiration was measured using radioactive labelled water (spec. activity: 925 MBq g^{-1}, Hartmann Analytik) and a method described in detail by (Schreiber *et al*., 2005). Enzymatically isolated and UV-sterilized *Prunus* cuticles were mounted into sterile transport chambers filled with $^{3}H_2O$ (6×10^{7} Bq ml^{-1}). Chambers were incubated upside down on scintillation tubes at 100% humidity and 20°C. Water loss was measured for at least 3 d to obtain the initial water flow before inoculation.

For inoculation selected bacterial strains were precultivated on KB plates at 25°C for 2 days. Cuticles were inoculated with 200 µl of surfactant extracts or dense suspensions of bacterial cells at 20°C and 100% humidity. For direct bacterial inoculations, after 24 h bacterial suspension and water were carefully removed with cellulose tissues, leaving a thin layer of adhering cells on the cuticle surface. After inoculation with bacteria or surfactant extracts, water flow was measured again for at least another 8 d. Mean effects of bacteria or extracts on cuticular transpiration were calculated dividing the rate of water flow after inoculation by the rate of water flow before inoculation.

RESULTS

Biosurfactant production on the leaf surface. Since *P. syringae* produces two motility-enabling surfactants on agar plates, syringafactin and BRF, both of the surfactants could contribute to altering the surface tension of leaves. In order to determine the relative contribution of these two surfactants to modifying leaf properties, we initially characterized the relative surfactant activities of these two compounds. Surfactants were extracted from lawns of plate-grown cells of different *P. syringae* strains in order to obtain semi-pure cultures of surfactants. Similar to the wild-type strain, a **Δ***brfA* strain that only produces syringafactin yielded an extract that lowered the surface tension of water to 25-26 dyn/cm (Table 1). On the other hand, extract from a **Δ***syfA* strain that only produces BRF reduced the surface tension of water only slightly below that of an extract from a **Δ***syfA*/**Δ***brfA* strain incapable of producing either surfactant (around 60 dyn/cm). Thus it appears that syringafactin is the main contributor to the surface activity of *P. syringae* in culture. However, as we have observed previously (Chapter 4), when *brfA* is constitutively expressed in a **Δ***syfA* strain (**Δ***syfA* + pBRF2), the extracted BRF surfactant is capable of lowering the surface tension of water to levels near that conferred by syringafactin (Table 1). This indicates that BRF is a potent surfactant, but that its small contribution to surface activity on agar plates stems from its low production levels under these culture conditions on agar plates.

Table VI-1. Surface active properties of surfactants produced by *P. syringae* B728a.

Bacterial strain	Surface tension of water (dyn/cm)
WT	25
Δ*syfA*	55.9
Δ*brfA*	26
Δ*syfA* + pBRF2	29
Δ*syfA*/**Δ***brfA*	59.5

While BRF contributed little to reducing the surface tension of water compared to syringafactin in agar-grown cells we assessed the contribution of both of the surfactants *in planta* since their patterns of production may be quite different on leaves compared to culture media. As a test of the production of surfactants on leaves we measured the water-repellancy of *Phaseolus vulgaris* leaves colonized by *P. syringae* strains differing in their ability to produce these two surfactants. After allowing inoculated bacteria to incubate on moist leaves for two days, we estimated the interfacial tension of water on leaves from the contact angles of water droplets placed on the leaf surface. Plants inoculated with the wild-type strain of *P. syringae* exhibited increased contact of water droplets with the leaf surface, indicated by a reduced contact angle (Fig. 1). The contact angle of water on plants inoculated with a **Δ***syfA*/**Δ***brfA* strain incapable of surfactant production was similarly high as that on non-inoculated plants and much higher than that on plants colonized by the wild type strain of P. syringae (Fig. 1). It is noteworthy that although the amphiphilic virulence factors syringomycin and syringopeptin can still be produced in a **Δ***syfA*/**Δ***brfA* strain, we see little if any contribution of their possible production on the properties

of water on the leaf surface. A **Δ***brfA* strain of *P. syringae* that only produces syringafactin still reduced leaf surface water tension to levels indistinguishable from that conferred by the wild-type strain (Fig. 1). Thus, similar to plate culture extracts, syringafactin is sufficient to account for nearly all of the observed surface activity of *P. syringae* on plants. However, contrary to plate cultures, a **Δ***syfA* strain that is only capable of BRF production significantly reduced the surface tension of water ($P<0.01$) on leaves to a level intermediate between the wild-type and the surfactant-deficient strain (Fig. 1). This suggests that BRF, although not produced at levels comparable to syringafactin, nonetheless is produced in sufficient quantities to demonstrate surface activity on plants and that it is produced at a higher level on leaves than it is in culture on agar plates. This apparent increased production on leaves is consistent with our previous observation that moist, rough surfaces induce *brfA* transcription more than smooth agar surfaces (Chapter 4).

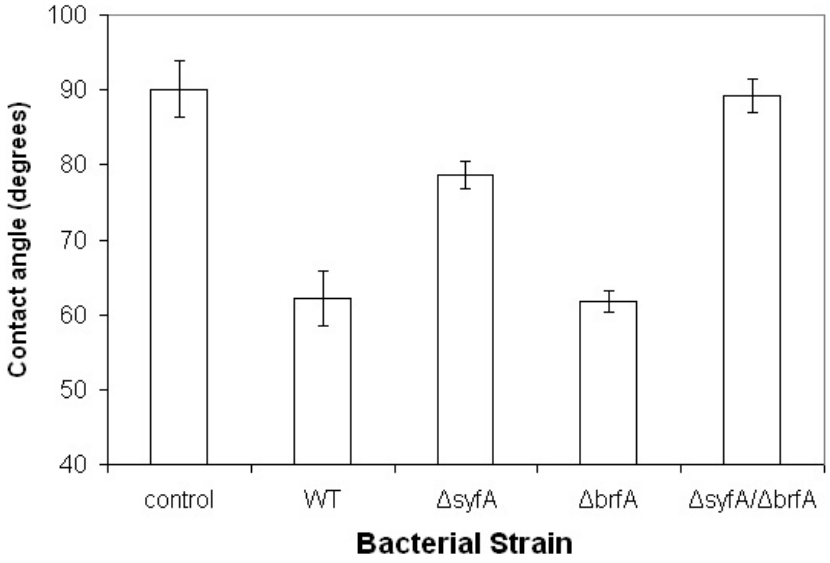

Figure VI-1. Effect of biosurfactant production on leaf surface properties.
Contact angles of individual water droplets on leaves inoculated with a variety of *P. syringae* strains or an un-inoculated control. Each measurement is an average of at least 19 water droplets from at least four different plants from two different pots, ± standard error.

In order to verify production of syringafactin and BRF on the plant surface, we inoculated plants with fluorescent reporter strains indicative of the biosynthesis of each biosurfactant. We recovered the reporter strains after two days of growth on plants in humid conditions, and measured the relative fluorescence of individual cells by calculating their average pixel intensities by quantitative microscopy. Both reporter strains were induced on plants (Fig. 2), each displaying a broad range of induction levels, indicative of varying contributions of surfactant production on the leaf surface. We did not observe distinct tiers of production levels, but rather a semi-normal distribution of production, which suggests that there are not subpopulation of producers and non-producers such as has been observed in *B. subtilis* (López and Kolter, 2010). Thus, in agreement with our detection of biosurfactants on the leaf surface, *P. syringae* induces production of both syringafactin and BRF epiphytically.

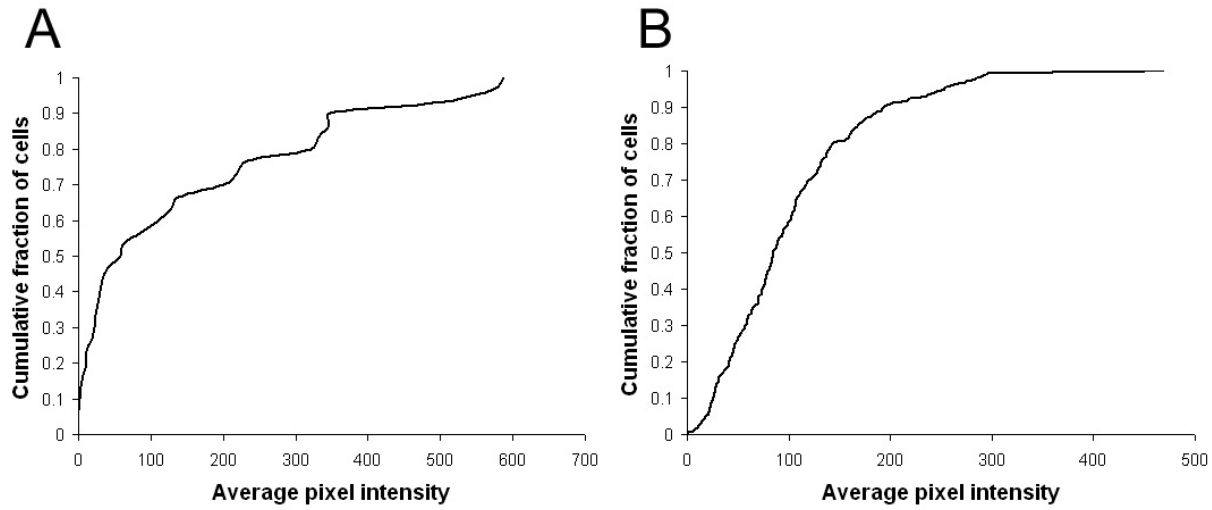

Figure VI-2. Expression of *syfA* and *brfA* from individual cells of *Pseudomonas syringae* recovered from plants

Average pixel intensity of cells harboring the plasmid pP*syfA-gfp* in which GFP expression is dependent on the promoter of syringafactin biosynthesis (A) and the plasmid pP*brfA-gfp* in which GFP expression is dependent on the promoter of BRF biosynthesis (B). The GFP fluorescence of cells was measured by quantitative fluorescence microscopy after recovery of cells from growth on plants. The average pixel intensity of individual cells is plotted against the cumulative proportion of cells having that or lower pixel intensity.

Biosurfactants and leaf motility. While the biosurfactants produced by *P. syringae* on leaves can alter the surface tension of water measured at the scale of individual water droplets, the question remained as to whether such changes in water behavior would affect bacterial behavior at the small scales at which it lives on plants. We thus measured changes in the motility of *P. syringae* strains differing in surfactant production on leaves since we hypothesize that biosurfactant production in the phyllosphere should increase cell motility in this habitat as observed *in vitro*. We previously demonstrated that biosurfactants contribute greatly to the swarming motility of *P. syringae* B728a on moist agar surfaces, but not to either swimming or movement through hydrated paper (Chapter 4). As flagella appeared to be required for most forms of motility in *P. syringae* and since flagellar motility has previously been shown to play a role in movement of *P. syringae* on leaves (Haefele and Lindow, 1987), we also constructed a **Δ***flgK* deletion mutant deficient in flagella production to use as a motility-deficient control strain for comparison purposes. Since cells of *P. syringae* can move several centimeters per day on moist agar surfaces we measured the movement of strains differing in surfactant production and flagellar motility across the distance of entire *P. vulgaris* leaves. Individual leaves were inoculated with a single small (5ul) droplet containing 10^6 cells of a given strain on a defined site near the base of a leaf and the leaves were kept in a humid chamber. After 1-3 days the lower leaf segments at 5 cm from the point of inoculation were quantified by plating after sonication in order to determine the populations that had presumably grown as a result of bacterial motility. Movement of bacteria from the point of inoculation was found to be much more dependent on the environmental condition to which they leaves were exposed than to the features of the bacterial colonists. On relatively dry leaves exposed to non-fully saturated air, bacteria were

rarely detected at distances as far as 5 cm from the point of inoculation. In contrast, when leaves were exposed to highly humid conditions under which moisture does not evaporate from leaf surfaces, we observed similar but highly variable numbers of cells of each strain at the 5 cm distal sampling location, including the flagella knockout (data not shown). Thus it appeared that active motility might not be required for bacterial translocation on wet leaves and that passive processes such as mobile droplets of condensation might be sufficient to disperse bacteria across the leaf.

While long-distance movement of *P. syringae* on leaves was not obviously dependent on surfactant production, we hypothesized that more local movement might benefit from surfactant production. Local motility on leaves was assessed by visualizing *gfp*-marked strains of *P. syringae* that differed in surfactant production within and near droplets of inoculum placed on leaves. Droplets were incubated for one or two days at 100% room humidity, so that the droplets never dried out, nor moved from the point of inoculation. It was not visibly apparent whether the strains differed in their dispersal from the droplets. The large majority of cells remained within the water drops, with small assemblages of cells sometimes visible as satellites of the main droplet, irrespective of genetic background. There appeared to be an increased density of cells at the outer edges of the droplets (Fig. 3), especially in strains which were capable of both flagella and syringafactin production, although the variability between droplets made such observations hard to quantify.

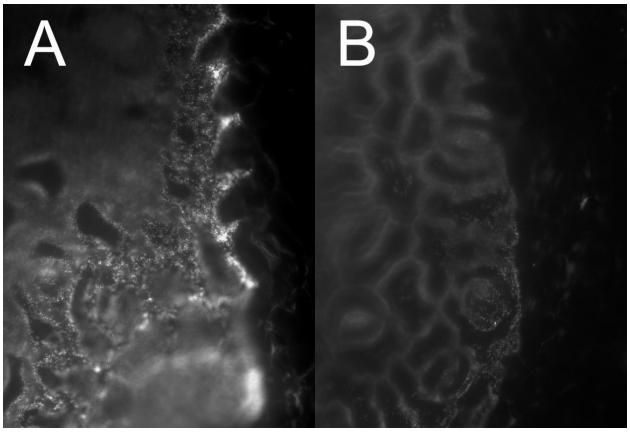

Figure VI-3. Syringafactin enables higher density colonization at the edges of water drops.
Photomicrographs of *gfp*-marked strains of WT (A) and **Δ***syfA* (B) *P. syringae* on leaf surfaces. Strains were inoculated as single droplets of inoculum, and the pictured fields of view focus on the boundary edge of a water droplet containing the inoculum (left portion of view). Excess liquid was removed before imaging.

Because we were unable to accurately quantify the spatial distribution of bacteria on leaves with microscopy observations, we measured the total population of bacteria that had developed on leaves after 24 hours of growth. Given that net growth on leaves would be indicative of successful accessing of nutrients either at the point of inoculation or from nutrients nearby that were accessed by local exploration of the leaf, we reasoned that any effect of surfactant production on either movement of cells on the leaf or increased nutrient diffusion near cells

should result in altered bacterial growth. The results of experiments in which mutants of *P. syringae* altered in surfactant production were applied to leaves were highly variable and therefore the studies were repeated many times. Generally we found that strains capable of producing both flagella as well as syringafactin grow to about twice the population size compared to mutants blocked in syringafactin production (Fig. 4). We hypothesize that this larger population size might be reflective of the higher density of cells observed at the edges of the droplets in the studies discussed above.

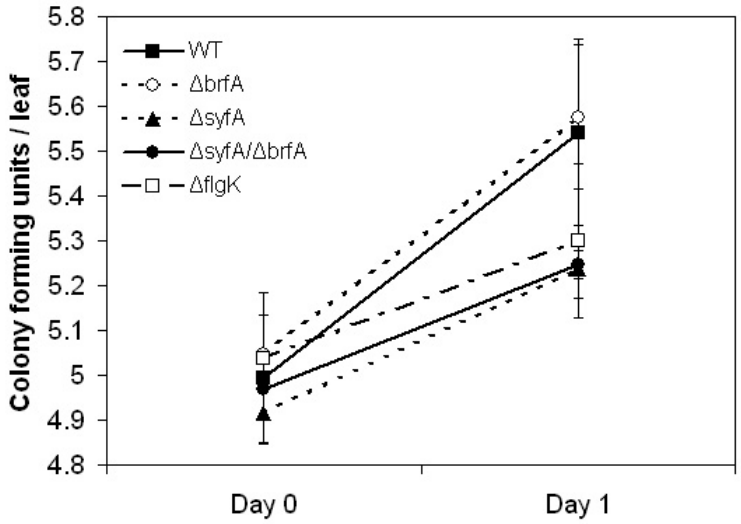

Figure VI-4. Epiphytic multiplication from single spot inocula
Bacterial surface populations on bean leaves after overnight application of spots and measured by CFU.

Since motility of *P. syringae* is dependent on flagella which, in turn, affect the expression of surfactants such as BRF, we investigated the expression of fliC encoding flagellin in mutant strains of differing in surfactant production as an indication of potential for movement in different settings. We first examined the expression of flagellin in the different strains on hard agar plates to determine if flagellin expression differed under conditions when motility is not observed. Indeed, the expression of *fliC* was very similar in all strains, irrespective of their ability to produce surfactant (Fig. 5A). We also examined *fliC* expression in cells that were allowed to colonize hydrated paper discs placed on agar surfaces, a condition that enables active flagellar motility. Although not significantly different, *fliC* expression was higher in a **Δ***brfA* mutant and was lower in a **Δ***syfA/***Δ***brfA* double deletion than the wild-type strain (Fig. 4A). Based on results seen in other studies in culture (Chapter 4), it appears that the **Δ***brfA* mutant produces more flagellin than the wild type strain while on this porous surface, perhaps to compensate for increased flagellar breaking. In contrast, the mutant strain lacking syringafactin production apparently produces slightly less flagellin and also exhibits less motility in these porous surfaces (Chapter 4). Interestingly, the mutant lacking any surfactant production also apparently exhibited reduced flagellin production, perhaps reflecting a response to less favorable conditions for motility.

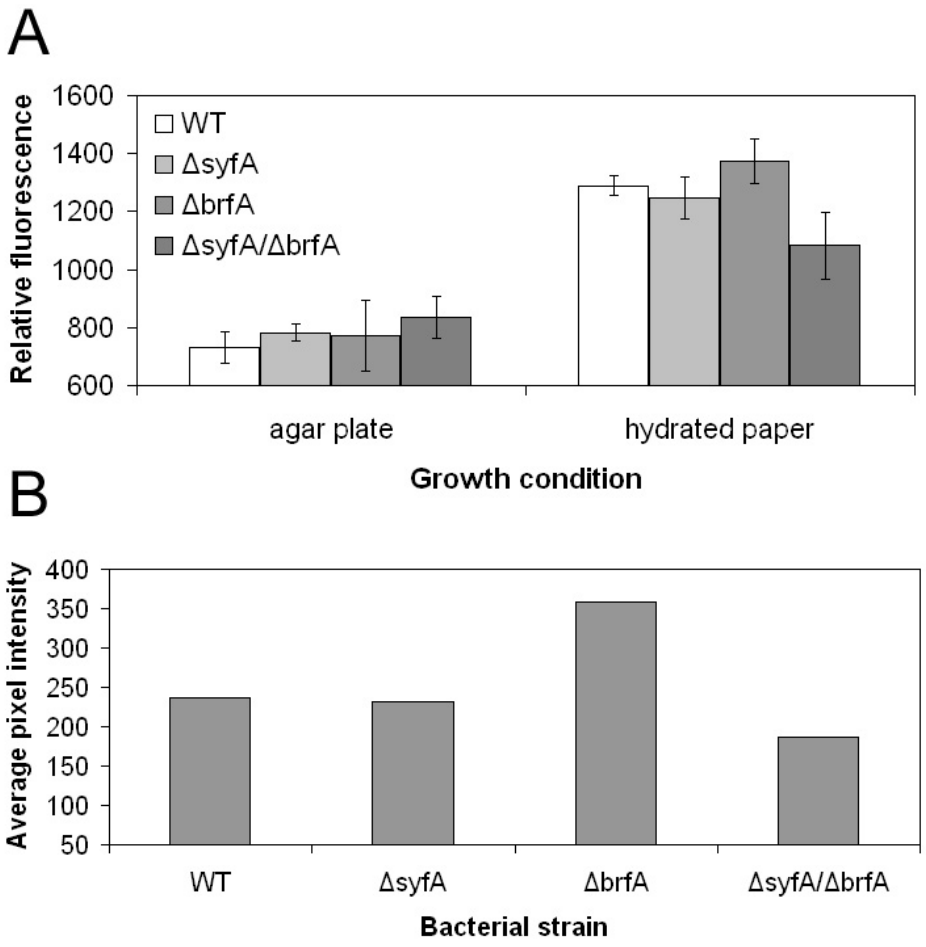

Figure VI-5. *in vitro* and *in vivo* expression of *fliC* in surfactant mutants of *P. syringae*
GFP fluorescence exhibited by the wild-type, **Δ***syfA*, **Δ***brfA*, or the double deletion **Δ***syfA*/**Δ***brfA* strains of *P. syringae* B728a harboring the plasmid p*fliC-gfp* in which GFP expression is dependent on the promoter of *fliC* encoding flagellin synthesis. GFP fluorescence of strains was measured by a fluorimeter after *in vitro* growth on hard agar plates and hydrated paper discs and expressed as relative fluorescence normalized per OD_{600} (A), or the average pixel intensities of those cells which exhibited GFP fluorescence above the level shown by uninduced cells as determined by quantitative fluorescence microscopy after growth on leaves (B).

The expression of *fliC* was also examined in the surfactant mutants recovered from colonized plants. We observed heterogeneous expression of *fliC* in strains recovered from plants indicative of two subpopulations (Fig. 6). An analysis of the variation in GFP fluorescence observed among cells recovered from plants revealed that different proportions of cells of the various strains expressed *fliC* while on leaves. A large number of cells in a given field of view had similarly low GFP fluorescence as cells of *P. syringae* not harboring a *gfp* reporter gene (an average pixel intensity of 15 units at the exposure times used in this study). These cells were assumed to be *P. syringae* cells that lacked any expression of *fliC* since nearly all cells recovered from plants were the inoculated *P. syringae* strains. The remainder of the cells exhibited a range of levels of *gfp* fluorescence detectable by fluorescence microscopy. Assuming that the non-

fliC-induced cells were completely non-motile and that the cells that exhibited at least some detectable *fliC* expression were motile, we quantified the fraction of cells of each strain that were potentially motile on leaves. About 39.2% of WT cells are non-motile on plant surfaces (Fig. 6A) while as many as 44.2% and 47.3% of the cells of **Δ***syfA* and **Δ***brfA* mutant strains respectively were apparently non-motile. Over 53.1% of the cells of a **Δ***syfA*/**Δ***brfA* double deletion were non-motile (Fig. 6B). Contrary to the results of large-scale motility assays, the analysis of the expression of genes related to motility suggests that each of the surfactants do play a significant role in *in planta* motility. However, it remains uncertain how the levels of flagellin synthesis relate to the form or extent of motility.

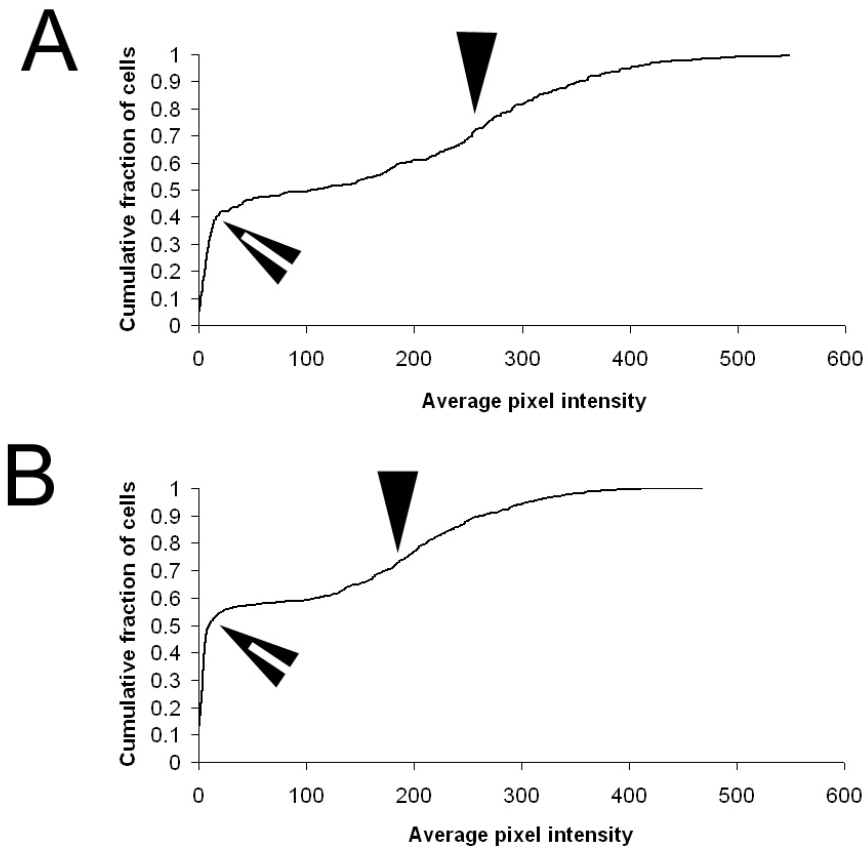

Figure VI-6. Expression of *fliC* of individual cells in individual cells of strains *Pseudomonas syringae* differing in surfactant production after recovery from plants
Average pixel intensity of wild-type (A) or **Δ***syfA*/**Δ***brfA* (B) cells of *P. syringae* B728a harboring the plasmid pP*fliC-gfp* in which GFP expression is dependent on the promoter of *fliC* conferring flagellin synthesis. The GFP fluorescence of cells was measured by quantitative fluorescence microscopy after recovery of cells from growth on plants. The average pixel intensity of individual cells is plotted against the cumulative proportion of cells having that or lower pixel intensity. The open arrow marks the discontinuity in fluorescence intensity that distinguished un-induced and induced cells, and the closed arrow marks the median GFP fluorescence of cells that were considered to have at least some level of induction of *fliC*.

After filtering out the subpopulations of non-*fliC*-induced cells, the average level of GFP fluorescence observed among induced cells was assessed. The relative level of expression of *fliC* in a given strain on plants was similar to that observed on hydrated paper discs in culture. While a **Δ***syfA* mutant exhibited only a slightly lower level of expression of *fliC* compared to the wild-type strain, a **Δ***brfA* mutant enhanced expression. Furthermore, the **Δ***syfA*/**Δ***brfA* double deletion exhibited reduced levels of *fliC* expression.

Biosurfactants and cuticle permeability

To test the hypothesis that the biosurfactants produced by *P. syringae* could act as plasticizers of the cuticle of plants, thereby increasing its permeability to both water and polar solutes, we characterized the effects of culture extracts containing surfactant on isolated cuticles. Using diffusion chambers as described before (Schreiber *et al*., 2005), isolated cuticles from *Prunus laurocerasus* were used to separate two chambers, and the flow of deuterated water from one chamber to the other was measured to estimate cuticular permeability. We applied the extracts directly onto isolated plant cuticles and measured the levels of movement of deuterium across the cuticle. Application of syringafactin (from an extract of a **Δ***brfA* strain) resulted in a 1.5 (± 0.8) – fold increase in the diffusion of deuterated water across the *Prunus* cuticles (Fig. 7A), while extracts from a **Δ***syfA*/**Δ***brfA* mutant had no apparent effect on the cuticular diffusion rate (1.0 ± 0.15) (Fig. 7B). Extracts from a wild-type strain increased cuticular diffusion to a similar extent as that of extracts from a **Δ***brfA* strain, while those from a **Δ***syfA* strain did not alter cuticular diffusion, similar to that of extracts from a **Δ***syfA*/**Δ***brfA* strain (data not shown). The magnitude of these effects of the different mutant strain extracts on cuticular diffusion was similar to their effects on water tension itself (Table 1). While BRF is unlikely to be produced at high levels on the leaf based on our previous results (Fig. 1), we tested the effect of high levels of BRF on cuticle permeability using extracts of a **Δ***syfA* strain which overexpresses *brfA*. Although this extract has high surface activity (Table 1), it induced very little change on the cuticle (data not shown).

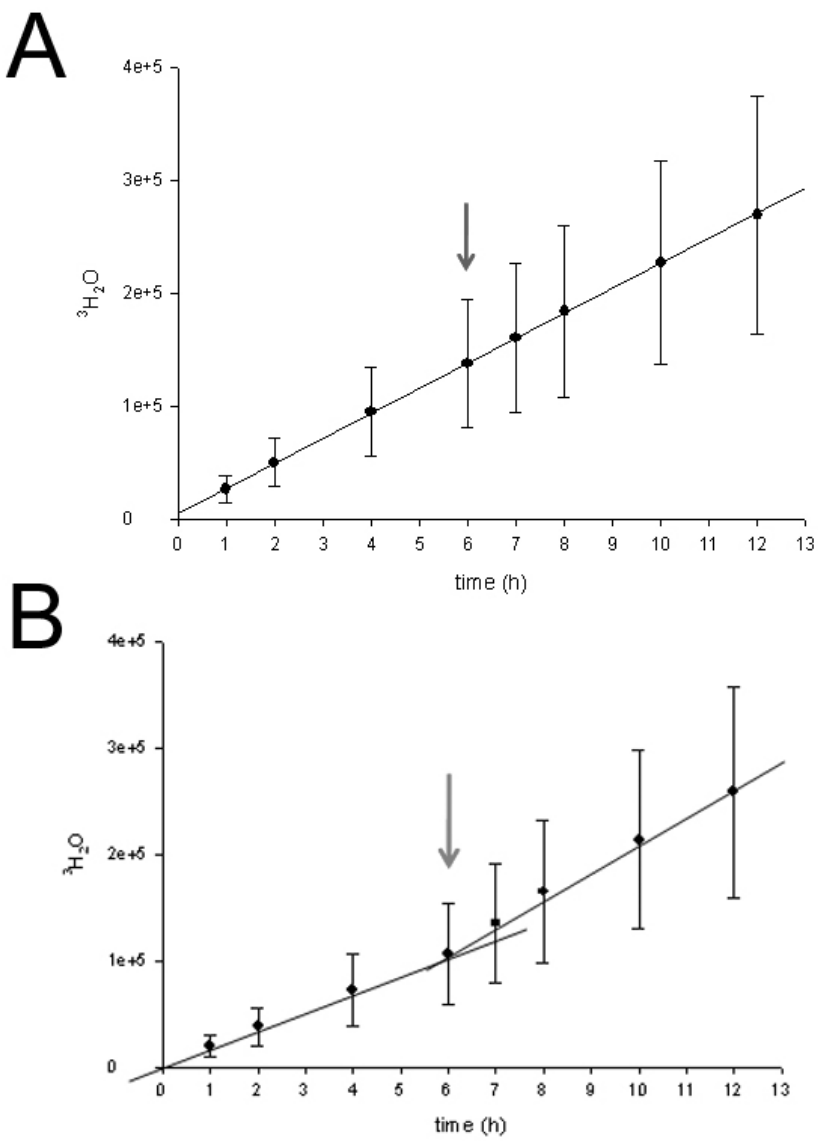

Figure VI-7. Effect of surfactant extracts on cuticular transpiration
Measurements of heavy water that have diffused across the cuticle over time. The arrows indicate the time point where extracts from a surfactant mutant **Δ***syfA*/**Δ***brfA* strain (A) and a **Δ***brfA* strain that produces syringafactin (B). Measurements are an average of at least 9 isolated cuticles.

Given that cuticular permeability was increased upon application of syringafactin we tested whether colonization of leaves with syringafactin-producing bacteria would yield similar changes in cuticular function. Dense cultures of plate-grown cells and their associated surfactant were applied to the cuticles, and diffusion of deuterated water was measured as before. Surprisingly, mutant cells deficient in any surfactant production increased the permeability of the cuticles much more than the cells of the surfactant-producing wild-type strain (Fig. 8).

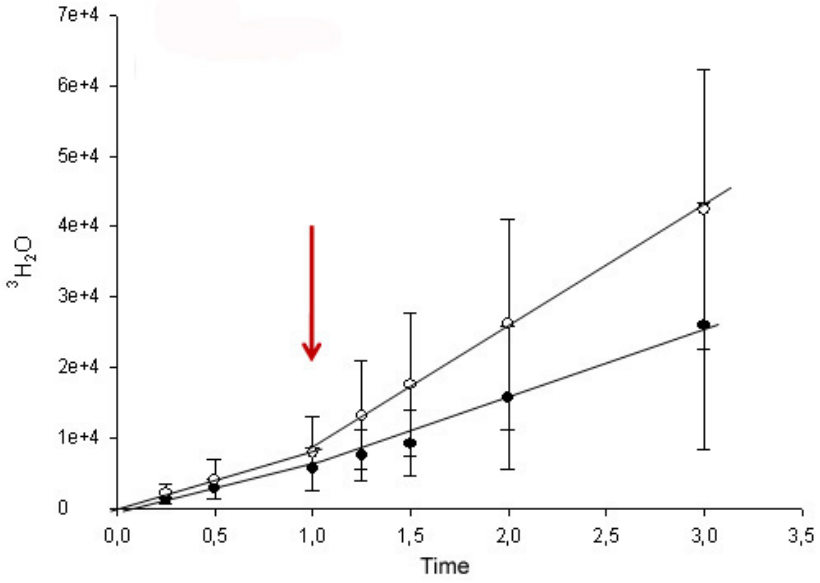

Figure VI-8. Effect of suspensions of cells of *Pseudomonas syringae* strains differing in surfactant production on cuticular transpiration
Measurements of deuterated water that diffused across the isolated cuticle over time. The arrow indicates the time when cell suspensions of a wild-type strain (closed circles) or a surfactant mutant **Δ***syfA*/**Δ***brfA* strain (open circles) of *P. syringae* B728a were applied. Measurements are an average of at least 5 isolated cuticles. The vertical bars represent standard deviations.

DISCUSSION

Pseudomonas syringae pv. syringae has proven to be a very useful model organism by which to study the biological role of surfactant production. While this species includes many strains that are pathogens of a variety of plants it also demonstrates a superior ability to survive and thrive epiphytically on asymptomatic plants compared to most other bacteria (Hirano and Upper, 2000). Biosurfactant production has been noted in many species of Pseudomonads, but few *Pseudomonas* habitats allow for as easy observation and manipulation of surfactant production in these natural habitats as do leaves. Thus, the phyllosphere has proven to be an excellent setting in which to test the biological roles of biosurfactant production. The studies reported here have shown, using a variety of techniques, the different roles of syringafactin and BRF in both bacterial motility and nutrient acquisition in this plant colonist.

Biosurfactants have long been presumed to play a role in bacterial movement. While many reports have shown their contribution to motility in somewhat artificial surfaces such as on agar plates (Kearns, 2010), few studies have addressed this function in more realistic habitats. A clear explanation of how they might aid in motility, especially in complex environments, has not been presented. Our results indicate that biosurfactants play only a modest role in motility on humid leaf surfaces. Although rapid long-distance motility across leaves can be observed, it does not appear that this form of motility resembles biosurfactant- and flagella-mediated swarming motility, at least under the conditions that we explored. Swarming motility relies on a continuous outward expansion of a thick layer of cells. However, such a cell density and

continuity of cell assemblages may not be common on leaf environments, where instead somewhat isolated cell aggregates are much more commonly observed (Monier and Lindow, 2004). In fact, the leaf is considered a spatially heterogeneous environment with scattered sites that are relatively hospitable to bacterial growth. It appears that long-distance travel (movement of more than several centimeters) across a leaf is much more likely to be due to passive movement associated with water movement itself rather than due to active bacterial movement. Previous studies, for example, have also documented that natural processes such as high-velocity rain droplets are important in natural dispersal of bacteria on plants (Hirano *et al*., 1996). Although certain conditions might be envisioned to foster active surfactant-mediated bacterial movement across leaves such as thin, persistent moisture films, such conditions could not be recreated here. Further work will be needed to determine those conditions when surfactants have their maximal effect on bacterial movement on such rough, spatially hetoergenous surfaces such as leaves.

Although biosurfactants might not play a large role in large-scale movement on plants, we find evidence that they enable movement at much smaller spatial scales. Our evidence clearly indicates that the surface tension of individual droplets of water is lowered due to the production of syringafactin on leaves (Fig. 1). The resulting spreading of water droplets across the leaf expands the zones of colonization for the bacteria that produced the surfactant and apparently increases their access to local, but dispersed nutrient-rich colonization sites on the leaf. This process of expanded areas of colonization on leaves might proceed similar to that proposed for swarm expansions in culture (Turner *et al*., 2010). Swarming cells in this model include cells that are stalled at the swarm front that pump water to the edge of the group; the reversal of orientation of their flagella extends them beyond the swarming front which then helps channel water outwards, thereby expanding the swarm front (Turner *et al*., 2010). A surfactant such as syringafactin could lower the tension of the water at the swarm front which would make it easier to perpetuate such thin films of water. Further experimentation with smaller quantities of bacteria in sub-microliter sized water droplets should enable observation of the contribution of surfactants such as syringafactin to such a process.

In addition to enabling local motility and thus the outward expansion of colonized aqueous zones on plants, surfactants may play a role in enabling conditions for flagellar motility itself. The presumably hydrophobic surfactant BRF appears to play a prominent role in such a process. Populations of cells in a mutant of *P. syringae* that could not produce BRF included a larger proportion of cells that did not express *fliC* and thus were presumably non-motile compared to strains that could produce BRF. How might BRF be enabling expression of flagellin and thus presumably enhancing flagellar motility in the phyllosphere? Several lines of evidence and other published reports (McCarter *et al*., 1988; Belas and Suvanasuthi, 2005) suggest that there is a feedback on production of more flagellin and other flagellar components under conditions where flagellar breakage or increased torque occurs. This is especially prominent when bacterial cells are grown on surfaces. We have observed that *fliC* expression is up-regulated in populations of **Δ***brfA* cells (Fig. 5); it appears that BRF probably lubricates flagella, cells and/or surfaces, which increases motility and decreases flagellar breakage. Thus syringafactin and BRF appear to cooperate in different processes that seem to be essential for flagellar-mediated movement on rough surfaces such as leaves. Syringafactin may aid in wetting surfaces to enable movement to take place, while BRF may help protect the flagella themselves to enable the process to proceed.

Alternatively, because we know BRF has relatively low water solubility, it might act as an effective evaporation barrier at the water/air interface, enhancing bacterial access to thin films of water through which to travel. Future examination of water availability in the vicinity of bacteria differing in surfactant production at such small scales should provide much insight into the process of motility.

Although we clearly observe an effect of purified syringafactin on cuticular permeability *in vitro,* it is unclear what role, if any, surfactant production by bacteria has for altering cuticles while they colonize plants. It is puzzling that when a surfactant-deficient mutant was applied to isolated cuticles, it actually increased cuticle permeability to levels higher than those observed for cuticles treated with surfactant-producing cells (Fig. 8). Preliminary evidence suggests that syringafactin has an anti-adhesive property that prevents cells from adhering to the leaf cuticle. This would be in agreement with the many examples of other biosurfactants which prevent the adherence of bacteria to surfaces such as silicone, glass, and stainless steel (Nitschke and Costa, 2007). Perhaps the tighter adherence of surfactant-deficient mutants to the cuticle compared to the wild type strain better enables water loss though another mechanism. Alternatively, the bacteria might produce other unknown plasticizing factors that can increase the permeability of the cuticle, but a film of surfactant on the cuticle might decrease the effect of such compounds and/or disperse these factors and reduce their impact on the cuticle. Further studies will be needed to address these possibilities. Additionally, it should be rewarding to examine the role of these different surfactants in moving substances other than water across the cuticle. Hydrophilic surfactants such as syringafactin, when adsorbed into the cuticle, should increase the hydration of the cuticle, increasing the movement not only of water but also water-soluble molecules. Alternatively, although hydrophobic surfactants readily adsorb into the cuticle, they do not increase the hydration but rather the fluidity of cuticular waxes that, in turn, increases the rate of diffusion of hydrophobic compounds across the cuticle (Hess and Foy, 2000). Although we have not observed an effect of BRF-containing culture extracts on water movement across the cuticle, it might enhance bacterial access to other compounds.

Another potential role of biosurfactant production that we have not explored is their possible contribution to enabling spontaneous bacterial invasion of stomata. This movement of water and bacteria into the apoplast is normally prevented by the high surface tension of water, but can occur when the surface tension of the liquid is reduced such as in *Zebrina purpusii* when the surface tension of liquid is less than 30 dyn/cm (Schonherr and Bukovac, 1972). Both syringafactin and BRF are capable of reducing the surface tension of water to below 30 dyn/cm, and it seems likely that locally concentrated bacterial production of these compounds on leaves could achieve the minimal surface tension reduction necessary for spontaneous infiltration of bean leaves. These surfactants might thus prove to be virulence factors in *P. syringae,* and their role in invasion of plants should be further investigated.

There are certain limitations of the broad extrapolation of the results of this study that will necessitate further study. All studies of bacterial interactions with plants were conducted under humid conditions close to 100% humidity, but many interactions of bacteria and plants occur under much drier conditions. It should be informative to repeat some of these experiments in lower humidity conditions to observe what, if any, role biosurfactants might play in environments having limited hydration. The relative lack of water might concentrate surfactants,

thereby increasing some of their functions, while decreasing others. Furthermore, although we are primarily interested in the role of biosurfactant production in the epiphytic lifestyle of *P. syringae*, it is also pathogenic to our bean plants, and these compounds might facilitate different types of interactions with this bacterium and non-host plants. It would be informative to compare the results of movement and growth of *P. syringae* on a non-host plant with those seen here on the host plant bean. Additionally, because leaf surface waxes have substantial impacts on bacterial colonization and differ greatly between plant species (Marcell and Beattie, 2002), it will be insightful to determine the consequences of biosurfactant production on the epiphytic existence of *P. syringae* on leaves of plants differing in cuticular hydrophobicities.

Our research has mainly focused on syringafactin and BRF on the leaf surface, and has ignored the other amphiphilic compounds syringomycin and syringopeptin that are made by *P. syringae*. It has been postulated that these phytotoxins might also aid in motility of *P. syringae* on the leaf surface (Hutchison and Gross 1997; Bender *et al*., 1999). However, based on our observations that the leaf surface water tension is identical between un-inoculated plants and plants inoculated with a *P. syringae* mutant strain deficient in both syringafactin and BRF production, it does not appear that either of these phytotoxins have a measurable role in altering water relations on leaves. Nonetheless, it remains to be demonstrated that strains blocked in production of these amphiphilic compounds in addition to syringafactin and BRF do or do not a further reduced epiphytic fitness.

Although we have not observed a large reduction in epiphytic fitness of *P. syringae* associated with a loss of biosurfactant production, there have been some clues that both syringafactin and BRF might play specific roles in the phyllosphere. It appears that BRF might contribute more to flagellar movement on the leaf surface than syringafactin, based on its impact on the fraction of cells expressing genes for flagellin production on leaves. On the other hand, we observe that syringafactin might play a larger role in conditioning the waxy leaf surface to enable cuticular permeability as well as fostering expanded aqueous zones and thus enhanced access to favorable growth sites. Further studies of surfactant production on leaves should advance our understanding of the interactions of human and plant pathogens with the plants on which they live.

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Chapter VII.

Conclusion

Our studies reveal that biosurfactant producers are common in the environment; from 5% of bacteria from aqueous habitats and as many as 13% from surface environments produce such compounds (Chapter 3). In fact, we are likely underestimating this prevalence as biosurfactant production was assessed only on a single nutrient medium, while some bacteria might restrict biosurfactant production to other nutrient conditions that more closely mimic the conditions experienced in their natural habitats. Thus, biosurfactant producers are prominent members of bacterial communities, and their production of surfactants can have substantial impacts on the surface properties of their habitats. Human activities also add surfactants to natural environments indirectly as waste, as well as intentionally for agricultural purposes and for oil dispersal. Thus, further investigations of the physiological roles of biosurfactants should reveal important details about bacterial lifestyle and fitness factors, as well as the potential impacts of the addition of surfactants into the environment on bacterial activities.

To address these many questions related to the role of biosurfactants we developed a more highly sensitive method for their detection, as well as collected many biosurfactant-producing bacterial strains from a variety of habitats. Additionally, we developed a number of important tools for the study of biosurfactants, including many mutants altered in their production as well as bioreporter strains in the model bacterium *Pseudomonas syringae* pv. syringae B728a that enable assessment of the conditions which enable biosurfactant production and the interdependency of their expression. Because *P. syringae* produces multiple biosurfactants with different properties, it is an excellent model in which to examine the roles of different types of surfactants within a single system. With these tools, we were able to make substantial progress in investigating the biological roles of different types of biosurfactants using genetic approaches as well as *in planta* experimentation.

Biosurfactant properties and spectrum of activity

The degree to which a surfactant is hydrophilic or lipophilic is not normally used in descriptions of biosurfactants, but it is an industry standard for determining what applications a surfactant can be used for. Not all biosurfactants have the same chemical properties, and if a bacterium produces a biosurfactant for a specific purpose, then that biosurfactant is most likely to have evolved to have the correct physical properties for this function. Biosurfactant researchers would benefit from the development of a variety of physical property-based descriptive assays including the degree to which the surfactant is hydrophilic or lipophilic. Combining our current knowledge of synthetic surfactants with improved property-based classifications of biosurfactants could dramatically improve predictions of biosurfactant functions.

It is interesting that the atomized oil assay appears to be able to differentiate the extent to which a surfactant is hydrophilic or hydrophobic. We observe that hydrophobic and balanced surfactants confer bright halos, while highly hydrophilic surfactants are difficult to detect and confer dark halos. Moreover, balanced surfactants cause the de-wetted oil droplets that form a bright halo to travel along the gradient of surfactant concentration away from its source. It is possible that this gradient-driven passive motility illustrates a biologically-relevant role for

balanced surfactants. Additionally, balanced surfactants specifically induce liquid to be released from agar plates onto the surface; are these surfactants the best candidates for potential osmotic agents? It is interesting that in our isolation of biosurfactant producing bacteria from a variety of environments we did not identify any that conferred dark halos in our assay. This might indicate that the very hydrophilic surfactants best suited for the emulsification of oils into water are not commonly biologically produced. Perhaps a hydrophobic substrate needs to be available to induce production of such emulsifiers.

P. syringae produces both a balanced surfactant with good water solubility, syringafactin, as well as the more hydrophobic surfactant BRF. Hydrophilic surfactants are predicted to increase the hydration of leaf cuticles and increase their permeability to water-soluble substances (Hess and Foy, 2000). Indeed, we observed that syringafactin was capable of increasing permeability across isolated cuticles (Chapter 6). On the other hand, experimentation with hydrophobic surfactants reveals that although they will increase the fluidity of waxes and diffusion of hydrophobic substances across the cuticle, they will not increase the water-permeability of the cuticle (Hess and Foy, 2000). Indeed, when large quantities of BRF were applied to isolated plant cuticles, it results in little if any change in their permeability to water. Because our biosurfactants behaved similarly to synthetic surfactants having similar chemical properties, most likely other biosurfactants could likewise have predictable functions. Not only is this knowledge biologically interesting, but if we identify surfactant-mediated activities that are desirable or undesirable, it would be good to know what properties we might expect the biosurfactants to display in order to effectively screen for them in environmental bacteria.

Regulation of surfactants

Although we would ideally be able to predict the functions of biosurfactants based on their physical properties, we do not yet know the functions of enough known molecules to achieve this goal. Another way we approached the elucidation of biosurfactant functions was to examine their genetic regulation to enable inferences of their roles. It is interesting that we found a wide variety of bacteria to exhibit surface-dependent production of biosurfactants (Chapter 3). This obviously implicates their importance on surfaces. However, how do bacteria such as *P. syringae* sense that they are on surfaces? We demonstrate that it is not through flagellar inhibition, or other factors that are generally recognized at surfaces. At present we have not yet identified the signal, but it is apparent that the surface sensing message is passed to SyfR (Chapter 5). In the future we would like to determine what factor specifically results in lower SyfR activation in broth cultures, namely, whether this is accomplished by degradation of its mRNA or by proteolytic breakdown. Alternately, there could be a regulatory partner that phosphorylates or somehow activates SyfR. Additionally, it would be informative to uncover the hierarchy of regulation linking all of the lipopeptides and LuxR-type regulators in *P. syringae*. These regulators are frequently located near lipopeptide NRPS loci in Pseudomonads; a better understand of how SyfR functions might shed light on the conserved placement of LuxR-type regulators with lipopeptide determinants, as well as what makes them especially suited for their regulation. It will be especially interesting if *Pseudomonas* lipopeptides prove to have autoinductive properties, such as in *Bacillus subtilis* (López and Kolter, 2010).

While investigating SyfR, we focused on a curious “fried egg” phenotype that was displayed specifically by **Δ***syfA* mutants. Although we do not necessarily believe that this phenotype is

biologically relevant, it served as a useful visual phenotype to identify other genes downstream of SyfR. Our results suggest that mutants defective in a surfactant or its regulator could both have unintended consequences on the expression of other genes not directly involved in the synthesis of that biosurfactant. Further analysis of the fried egg phenotype should help inform us whether this is a generalized response to altered cellular physiology or a specific genetic response.

Although BRF is not regulated at surfaces like syringafactin, it is curious that it is regulated by an OmpR homolog in *P. syringae* that is predicted to function similar to the Cpx two-component system in *P. aeruginosa* (Lee *et al.*, 2009). Cpx has been demonstrated to be a method of surface sensing in *E. coli* (Otto and Silhavy, 2002), by virtue of its involvement in detection of misfolded proteins caused by the physical perturbation of surface adhesion. Further work should be done to determine if this OmpR homolog functions as a surface sensor in *P. syringae*, and to uncover how this pathway might contribute to the bacteria's decision to produce BRF.

Another striking finding from both mutagenesis screens (Chapters 2 and 4) was that the AlgT extracellular stress response pathway appears to similarly regulate both syringafactin and BRF production. AlgT, the *Pseudomonas* homolog to SigE, functions in a mode similar to Cpx wherein it senses misfolded proteins in the periplasm (Raivio and Silhavy, 1999). However, while a knockout of the potential Cpx-like system in *P. syringae* abolished BRF production, a knockout in AlgT dramatically up-regulated BRF production. It will be interesting to see how these two pathways differ in their prompts and responses. Furthermore, the ability of *algT* mutants to induce the "fried egg" phenotype (Chapter 5) suggests that an analysis of the compounds secreted by this mutant will yield interesting results.

Finally, regarding BRF, it will be insightful to determine exactly how flagellar function is linked to BRF production. Our finding of the co-regulation of BRF with flagellin production suggests a lubricative role for this biosurfactant. While it is tempting to speculate that BRF production is tied to Class IV flagellar genes and thus to flagellin production, low but significant levels of BRF production in mutants disrupted in Class II and III flagellar genes suggest otherwise (Chapter 4). Furthermore, it is surprising that cells defective in flagellar glycosylation, despite having functional flagella, up-regulate both BRF and flagellin production as much as a flagellin mutant. This suggests a potentially novel role for flagellar glycosylation in the flagellar-mediated sensing mechanism.

Biosurfactants in the phyllosphere

As we postulated, it appears that biosurfactant production is a common occurrence in the phyllosphere (Chapter 3). Further investigation into the factors contributing to surfactant production on leaf surfaces would better elucidate under what conditions biosurfactants might be most useful to their producers in nature. We need to know more of how factors like humidity, leaf age, sun exposure, leaf wax composition, and other parameters affect the prevalence of biosurfactant production. A rigorous analysis of these variables should result in predictive models which would indicate the appropriate conditions for the isolation of large quantities of novel biosurfactant producers. Conversely, in agricultural settings, conditions could be manipulated to minimize or maximize biosurfactant production on crops depending on what roles biosurfactants will prove to have in pathogen motility.

It is interesting that *P. syringae* B728a produces a wider variety of biosurfactants than strain DC3000. It is tempting to speculate that this larger arsenal of surfactants accounts for the ability of B728a to flourish as an epiphyte whereas DC3000 does not. Alternatively, it might indicate that DC3000 has co-opted syringafactin for a wider range of activities including the pathogenic role that is normally assigned to syringomycin and syringopeptin production in B728a, which DC3000 also does not produce. It will be informative to further investigate syringafactin production in DC3000 in light of the fact that this strain does not have the ancillary NRPSs that strain B728a has, and to test its role in the interactions of DC3000 with plants.

Although our *in planta* results might suggest that syringafactin enables larger epiphytic populations to develop by increasing diffusion of nutrients across the plant cuticle, other explanations have not been ruled out. A point of particular concern is that since *P. syringae* is a pathogen to *P. vulgaris*, it might use specific mechanisms to acquire nutrients on the leaf surface that would not be available on a non-host plant. If syringafactin enhances nutrient availability on the leaf surface, is that through general or pathogenic mechanisms? On the other hand, did the pathogenic capabilities of *P. syringae* somehow mask the fitness costs that might otherwise have been seen in surfactant mutations? It will be prudent to test *P. syringae* and its surfactant mutants on non-host plants in the future to see if the surfactant deficient mutants exhibit a larger difference in fitness compared to the wild type strain than seen on beans. We should also expand our assays to measure the ingress of bacteria into leaves, as well as disease incitation and progression in *P. vulgaris*, in order to look into the broader roles of biosurfactant production in the virulence of *P. syringae*.

This field would also benefit from the development of sensitive methods to observe biosurfactants *in vivo*. It might be possible to develop microscopic techniques to track how far surfactants diffuse across the leaf surface from producing cells. It might be possible to either use specific dyes that bind surfactants, or exploit the ability of surfactants to change the surface properties of leaves to visualize their presence. Application of smaller amounts of inocula onto leaf surfaces might also enhance our ability to observe surfactant-aided motility. Additionally, an *in planta* method using intact plants coupled with sensitive nutrient biosensors , similar to the isolated cuticle method, might finally demonstrate the *in vivo* role of surfactant-aided nutrient diffusion.

The widespread prevalence of synthetic and bacterial-produced surfactants in the environment and our food supply should provoke a more thorough investigation into the specific roles these different types of surfactants might have on bacteria. This investigation should not just examine their effects on the lifestyle of the producers, but also what they might do for other resident bacteria that do not produce surfactants. Surfactants, even when bacterially produced, are secreted into the environment and therefore might also affect the interactions of non-producers with their habitats. It seems likely that these surfactants might enable human pathogens to invade plant tissues, for example. Alternatively, they might have a protective effect by preventing the adherence of problematic bacteria. Different surfactants are likely to have varying effects on these processes, and thus it is important that we address these issues for a broad variety of surfactants.

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