Biological roles and regulation of biosurfactants produced by the plant-colonizing bacterium
Pseudomonas syringae B728a

By

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Abstract

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Leaf surfaces are a harsh habitat for microbes since they are subjected to high fluxes of ultraviolet radiation, fluctuations in temperature, low nutrient availability, and frequent drying. Despite this, leaves can be heavily colonized by bacteria. I explored the ability of bacteria to modify their local environment to better tolerate or avoid the water stress they would experience on leaves by addressing the role of the biosurfactant syringafactin produced by the model epiphytic bacterium *Pseudomonas syringae* B728a. Syringafactin is very hygroscopic, binding nearly three times its weight in water, but only in atmospheres of high relative humidity. The water stress status of bacteria differing in syringafactin production was assessed in different settings by measuring the fluorescence of cells harboring a plasmid containing the promoter of *proU*, encoding the synthesis of the compatible solute proline, fused to a promoterless *gfp* reporter gene. The wild-type syringafactin-producing strain experienced less water stress than the mutant strain on membrane filters exposed to high relative humidity, but not on filters exposed to low relative humidity. Syringafactin production was also associated with lower water stress in bacteria on leaves, irrespective of the dryness of the air in which the plants were incubated. The high relative humidity-dependent water-binding capabilities of syringafactin are consistent with a model in which the laminar boundary layer surrounding leaves is nearly saturated with water vapor, irrespective of the dryness of air away from the leaf. Thus, by producing syringafactin, *P. syringae* hydrates its local microenvironment by sequestering liquid water, on an otherwise dry leaf, from the humid air trapped at the leaf surface.

The production of syringafactin requires *syfA*, encoding a synthase necessary for syringafactin production. Therefore, as a readout for syringafactin production, I used a *P. syringae* strain harboring a plasmid containing the *syfA* promoter fused to a promoterless *gfp* reporter gene. GFP fluorescence exhibited by *P. syringae*, was much higher in cells cultured on nutrient agar surfaces compared to in cells in the corresponding broth culture. These observations suggested that expression of this trait was dependent on contact of cells with a solid surface. Immobilization of this strain on various surfaces, including various membranes, plastic surfaces, and on leaves, resulted in rapid increases in apparent expression of *syfA* within two hours compared to that of cells that were maintained in a planktonic state in broth cultures. These findings indicated that immobilization of cells was sufficient to induce the production of this

biosurfactant. These findings also suggested that *P. syringae* might either experience different environmental conditions upon immobilization or might use contact with the surface as a cue to express certain traits in anticipation of their necessity for optimum fitness upon change from a liquid habitat, in which cells might exist in a planktonic state, to a dry habitat, such as the leaf surface. To explore what additional traits besides syringafactin production might be expressed in a contact-dependent fashion in *P. syringae*, I compared the global transcriptome of planktonic cells grown in a broth medium with those immobilized for two hours on a filter surface placed on a similar medium. A large fraction of the genes exhibited either higher (26.63%) or lower expression (33.83%) upon immobilization on the filter surface. Genes in the functional categories of translation, siderophore synthesis and transport, nucleotide metabolism and transport, flagellar synthesis and motility, lipopolysaccharide synthesis and transport, energy generation, transcription, chemosensing and chemotaxis, replication and DNA repair, iron-sulfur proteins, peptidoglycan/cell wall polymers, terpenoid backbone synthesis, iron metabolism and transport, and cell division were significantly more likely to be up-regulated upon immobilization while those in the categories of quaternary ammonium compound metabolism and transport, compatible solute synthesis, carbohydrate metabolism and transport, organic acid metabolism and transport, phytotoxin synthesis and transport, amino acid metabolism and transport, and secondary metabolism were repressed on surfaces compared to in liquid.

While surface contact is increasingly being recognized as an important cue for the differential gene expression in various microorganisms, little is known of the mechanisms by which cells perceive that they are immobilized on such a surface. The mechanisms by which *P. syringae* B728a mediates surface contact-dependent production of syringafactin, and other-contact dependent processes are largely unknown. I identified genes that alter expression of *syfA* on agar surfaces using random transposon mutagenesis of *P. syringae*, harboring a *syfA:gfp* fusion. Putative regulatory genes contributing to activation of *syfA* expression identified by this method included *efeO*, *tsr*, and *mexB* while those contributing to its repression included *psrA*, *cyoC*, and *algW*. Some but not all of these putative regulatory genes also exhibited differential contact-dependent expression in immobilized cells compared to in planktonic cells. The KEGG database was used to analyze the metabolic pathways in which these genes might participate. Furthermore, I formulated possible pathways that could account for the surface-dependent regulation of *syfA* and potentially the regulation of other surface-regulated genes. One of these pathways is a stress sensing pathway that AlgW is part of which may play a role in activating genes involved in adhesion. Another pathway includes Tsr which may regulate genes involved in swarming, a type of bacterial motility that can only occur on a surface. These potential pathways reveal that a variety of mechanisms may be involved in regulating surface-induced genes in bacteria.

To my family

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Chapter 1

Introduction

Despite the fact that leaf surfaces are often heavily colonized by bacteria, fewer studies of the phyllosphere have been conducted compared to other parts of the plant such as the rhizosphere (Hirano and Upper, 2000; Lindow and Brandl, 2003; Vorholt, 2012). In addition, most studies that have examined bacterial colonization of leaves have largely focused on bacterial pathogens (Hirano and Upper, 2000; Lindow and Brandl, 2003). The focus on bacterial plant pathogens is perhaps justified since, although they cause disease only when inside the leaf apoplast, large population sizes of these pathogens develop initially on leaf surfaces (Melotto *et al.*, 2008; Vorholt, 2012). However, the focus on plant pathogens has ignored the many commensal bacteria that typically occupy leaves (Melotto *et al.*, 2008) and it remains unclear whether common strategies of epiphytic colonization occur independent of phylogenetic placement. Despite the fact that most studies of epiphytes have been of plant pathogens, most of our understanding is of their virulence traits leading to disease. Consequently, we still lack fundamental insights into an important part of their life history in which they must grow and subsequently survive as epiphytes on leaf surfaces (Melotto *et al.*, 2008; Vorholt, 2012).

The nature of the leaf surface habitats in which these bacteria live is also poorly understood. Although studies have examined the chemical and physical features of leaf surfaces (Schreiber *et al.*, 2004; Schönherr and Bauer, 1996), the microenvironment that bacteria experience on the leaf is still largely speculative (Beattie and Lindow, 1995; Remus-Emsermann and Leveau, 2010). There is broad appreciation that the surface of the leaf is rather inhospitable to bacteria due to frequent drying, low overall nutrient availability, and high fluxes of ultraviolet radiation (Hirano and Upper, 2000; Leveau and Lindow, 2001; Lindow and Brandl, 2003, Vorholt, 2012). It seems likely, however, that because of their small size and limited mobility, the behavior of bacteria on leaves will be strongly influenced by the local features of the leaf since bacteria are unlikely to perceive habitat features more than a few microns away. Another poorly understood concept is how bacterial epiphytes adapt to the leaf surface immediately after coming into contact with it. Since leaf surfaces are largely devoid of bacteria early in plant development such as soon after seedling emergence or bud break, immigration of bacteria from other sources is an important process in the establishment of epiphytic populations. It therefore seems likely that immigrant bacteria could experience a substantial change in the nature of their habitat upon arriving at a leaf. For example, *Pseudomonas syringae* is now appreciated to be a prominent colonist of aquatic habitats in addition to that of plants, and dispersal of such a species to leaves such as by raindrops etc. could require a substantial change in its behavior in order to colonize leaves after immigration (Morris *et al.*, 2008). Traditionally, microbiological studies have tended to analyze bacteria in liquid culture (Beattie and Lindow, 1995; Kearns, 2010; O'Toole and Kolter, 1998) which disregards the fact that most bacteria colonize surfaces, often as biofilms (O'Toole and Kolter, 1998; Persat *et al.*, 2015). Moreover, as studies of bacteria in biofilms have proliferated, most studies have addressed bacterial physiology and gene expression in mature biofilms (Dötsch *et al.*, 2012; Hickman *et al.*, 2005; Prigent-Combaret *et al.*, 1999). Such studies have revealed interesting and elaborate interactions between bacteria as their local density in a biofilm increases, thereby changing both the physical and chemical environment in which they live and facilitating chemical gradients that dictate their behavior. Unfortunately, very little work has addressed the initial stages of biofilm formation wherein planktonic cells presumably must

respond to the rapid changes in their local environment associated with immobilization on a surface - likely resulting not only in a major change of the environment that they sense, but also requiring rapid compensatory changes in gene expression. In this study I address this important early transition that cells of *P. syringae* experience upon immobilization on surfaces such as leaves.

To better understand life as a bacterium on a leaf surface, this dissertation examines the model organism *P. syringae* B728a and addresses how it adapts to the dry, but humid environment it would experience on a leaf as well as how it adapts to living on a surface as opposed to in an aqueous environment. My studies focus on the production of syringafactin, a biosurfactant that has previously been suggested to be hygroscopic and whose production is apparently dependent on the contact of cells with an agar surface (Berti *et al*., 2007; Burch *et al*., 2011; Burch *et al*., 2014). Biosurfactants have been typically known for their ability to reduce the surface tension of water, allowing the spreading of water across a hydrophobic surface (Bunster *et al*., 1989; Lindow and Brandl, 2003; Vorholt, 2012), as well as for their ability to disperse hydrophobic compounds in aqueous environments (D'aes *et al*., 2010; Van Hamme *et al*., 2006; Neu *et al*., 1996; Ron and Rosenberg, 2001). This work documents a new role for biosurfactants in modulating the local water environment around producing cells and demonstrates their importance in the survival of bacteria on surfaces that become dry. By employing advanced methods for assessing the expression of genes involved in biosurfactant production as well as in response to low water availability of individual bacterial cells in their native habitat on leaves, I provide insight into how bacteria adapt to, and modify, the microenvironment of the leaf surface in the vicinity of their colonization. I also explore how bacteria adjust to life while immobilized on a surface.

Water availability on the leaf surface

The leaf surface is composed of a hydrophobic, waxy cuticle that serves to limit the diffusion of water vapor from the leaf interior (Hess and Foy, 2000; Hirano and Upper, 2000; Knoll and Schreiber, 1998; Lindow and Brandl, 2003; Schönherr and Baur, 1996; Vorholt, 2012). The cuticular surface also serves to limit the diffusion of polar compounds from the leaf interior to the surface, where they might serve as nutrient sources for the proliferation of potentially deleterious microorganisms. In addition, the hydrophobic cuticle prevents water from spreading across the leaf surface as a film and instead causes it to form droplets. Such droplets minimize the contact of water with the hydrophobic leaf surface (Bunster *et al*., 1989; Otten and Herminghaus, 2004). In some cases, water has been deflected off of the leaves of plants having very waxy leaves such as *Tropaeolum majus L.* (Indian Cress) and *Alchemilla vulgaris L.* (Lady's Mantle). Such a phenomenon has been termed the "Lotus effect" (Schreiber, 1996; Otten and Herminghaus, 2004), and is an extreme example of how a limited portion of the leaf is likely to be wetted by water at a given time. Both the patterns of distribution of liquid water on leaves as well as the flux of ultraviolet radiation (Beattie and Lindow, 1995; Hirano and Upper, 2000; Leveau and Lindow, 2001; Lindow and Brandl, 2003; Monier and Lindow, 2003), are thought to be major drivers for the rather heterogeneously dispersed and localized concentrations of bacteria on leaves, which are often most abundant near trichomes and veins (Axtell and Beattie, 2002; Beattie, 2011; Leveau and Lindow, 2001; Lindow and Brandl, 2003). The multiplication of bacteria as epiphytes on leaves presumably would require both the presence of liquid water and a nutrient source. Localized cell aggregates on leaves presumably are a consequence of the

coincidence of such features (Leveau and Lindow, 2001; Lindow and Brandl, 2003; Monier and Lindow, 2003). Axtell and Beattie (2002) have explored the water that is available to bacteria on leaves through the use of a biological water sensor consisting of bacterial cells harboring a gene for the production of proline, a compatible solute required for response to low water availability, linked to a promoterless *gfp* reporter gene. They found that *Pantoea agglomerans* BRT98 cells on a leaf surface began to experience matric stress, a lack of water, within five minutes after bacterial suspensions were applied to dry leaves (Axtell and Beattie, 2002). Presumably, not only was water not provided by the plant to such cells, but they also did not have the capability of modifying the local environment on which they were placed within such a short period of time. A transcriptomic analysis by Yu *et al.* (2013) also revealed that *P. syringae* cells that had been inoculated onto leaves 24 hours or more previously also exhibited higher expression of genes involved in desiccation tolerance than those grown in a liquid broth medium when cells were allowed to dry. These findings further suggest that water availability on the leaf surface is limited. Studies using flow cytometry, however, could not easily resolve the differences in water availability experienced by individual cells on the leaf and thus did not provide insight as to the heterogeneity of the so-called "waterscape" nor any influence of bacterial traits on the levels of desiccation stress that they would encounter (Axtell and Beattie, 2002; Burch *et al.*, 2014). In this study, I expand upon such studies using more quantitative and discriminating methods to show that bacteria can modify their local microenvironment.

Since water is such a valuable resource needed for bacteria to survive, given these observations of limited water availability, it seems counterintuitive that the leaf surface has been found to be highly colonized by as many as 10^6 to 10^7 bacterial cells/cm^2 (up to 10^8 cells/g) (Lindow and Brandl, 2003; Vorholt, 2012). Part of this conundrum may be explained by the fact that the leaf has been shown to be surrounded by a thin layer of still air, known as the laminar boundary layer. This layer, often less than about 1 mm, but whose thickness is inversely proportional to the speed of wind over the leaf, traps water vapor exiting the stomata (Ferro and Southwick, 1984; Parlange *et al.*, 1971; Parlange and Waggoner, 1971; Schuepp, 1993). The relative humidity within the laminar boundary layer at the leaf surface is thus projected to be much higher than the surrounding air (Ferro and Southwick, 1984; Longrée, 1939). While models have long shown that the relative humidity of air near the leaf surface is relatively high, it has been difficult to directly determine such physical characteristics of the laminar boundary layer. Relative humidity is typically measured by pyrometric methods in which the temperature difference between dry and wetted thermometers is assessed (Ferro and Southwick, 1984; Yarwood and Hazan, 1944). Such a wet bulb/dry bulb technique in which thermometers are attached to a leaf (Yarwood and Hazan, 1944), has been applied to measure the relative humidity in the laminar boundary layer of the leaf, but the results were unreliable because this process affected the boundary layer itself (Ferro and Southwick, 1984). Nevertheless, through indirect measurements and theoretical calculations, it has been suggested that the boundary layer is quite humid with a relative humidity of over 90% (Longrée, 1939; Ferro and Southwick, 1984). These measurements are supported by an early study by Longrée (1939) who compared the sporulation of powdery mildew (whose activity/viability is very dependent on high humidity) on rose leaves with that on glass slides exposed to atmospheres with known and constant relative humidities in chambers containing different saturated salt solutions. Such studies revealed the leaf surface to have a relative humidity of 96% or higher. A later study by Ferro and Southwick (1984) developed a model to estimate relative humidity in the laminar boundary layer of leaves and calculated that if the saturated water vapor density in the stomata is 24.4 g/m^3 at 26ºC and if the water vapor

density in the air outside the boundary layer is 13.9 g/m^3, then even at a distance of 3 mm from the stomata air within the laminar boundary layer would have a water concentration of 23.8 g/m^3, corresponding to a relative humidity of 97.54%. It would thus appear that epiphytic bacteria would experience somewhat more humid environments on a leaf surface than would be expected from the relatively dry air in which plants were grown (Beattie, 2011; Monier and Lindow, 2003). In this study, I advance our understanding of the humidity on the leaf using bacterial biosensors similar to the one discussed above. A high humidity environment on the leaf surface however cannot account for bacterial colonization of leaves, since bacterial growth and survival are dependent on liquid water (Axtell and Beattie, 2002; Beattie, 2011; Beattie and Lindow, 1995). In such a humid environment however, epiphytes could be expected to benefit from their production of various hygroscopic materials that could make liquid water available, at least locally, to the cells. While various extracellular polysaccharides such as alginate have been proposed to serve as water binding agents (Beattie and Lindow, 1995; Chang *et al*., 2007), I will demonstrate that other compounds such as biosurfactants are important in such a process.

Biosurfactants and their roles on the leaf surface

Biosurfactants are produced by many microorganisms (Bunster *et al*., 1989; D'aes *et al*., 2010; Van Hamme *et al*., 2006; Ron and Rosenberg, 2001) and are surface active amphiphilic compounds that can reduce the interfacial tension between water and a hydrophobic substance such as oil (Burch *et al*., 2011; Burch *et al*., 2012; Ron and Rosenberg, 2001). Historically the ability of biosurfactants to emulsify hydrocarbons for microbial consumption has been the focus of investigations (Bunster *et al*., 1989; Ron and Rosenberg, 2001; Santos *et al*., 2016). While such processes may not be pertinent to epiphytes, studies have shown that biosurfactants can interact with water droplets on the leaf to enable the spreading of water and the diffusion of soluble nutrients across the leaf surface (Bunster *et al*., 1989; Lindow and Brandl, 2003). Synthetic surfactants such as Triton X-100 have been shown to solubilize the leaf cuticle once applied at relatively high concentrations (Tamura *et al*., 2001), but there is no evidence that biosurfactants produced by epiphytes would have such a function both because of their different properties and the likely presence of lower natural concentrations that would be found on leaves.

While biosurfactants can enable the spreading of water on the leaf surface (Bunster *et al*., 1989; Lindow and Brandl, 2003), such a trait would presumably be dependent on the deposition of macroscopic water in the form of rain or dew. A study by Burch *et al*. (2014) however showed that biosurfactants might interact with water in a different manner. Their study demonstrated that, when subjected to 100% relative humidity, purified, dehydrated syringafactin extracted from *P. syringae* B728a cells absorbed over 240% of its dry weight in water. This demonstrated that syringafactin is hygroscopic, absorbing water from the vapor state. Such a finding suggests that *P. syringae* B728a could use syringafactin to absorb water from the humid laminar boundary layer surrounding leaves, thus making liquid water available to the bacterium. Such a beneficial process would be dependent on syringafactin having sufficient water binding capabilities in atmospheres having the relative humidities characteristic of the leaf surface. Detailed studies of the water binding capabilities of syringafactin in vitro as well as its ability to modulate the apparent water availability experienced by bacteria both under controlled conditions as well as on leaves is explored in a series of studies described in Chapter 2 of this dissertation.

Bacteria and surface attachment

Despite surface colonization being a common phenomenon for most bacteria (Persat *et al.*, 2015; Petrova and Sauer, 2012; Tuson and Weibel, 2013), most studies of bacterial physiology have been of planktonic cells growing in various liquid cultures (Beattie and Lindow, 1995; Kearns, 2010; O'Toole and Kolter, 1998). Only recently have the consequences of bacterial growth on surfaces been fully appreciated, prompting a flood of studies on the nature of bacterial communities found in biofilms (Kearns, 2010; Tuson and Weibel, 2013). As discussed above, the focus of most of these studies has been on later stages of biofilm formation, long after initial attachment of formerly planktonic cells initiates such surface communities (Dötsch *et al.*, 2012; Hickman *et al.*, 2005; Prigent-Combaret *et al.*, 1999). These studies have established a dogma in which a series of events occur in the typical development of a mature biofilm community. The sequence of events following initial reversible attachment to a surface has been described in a variety of bacteria (O'Toole and Wong, 2016; Persat *et al.*, 2015; Petrova and Sauer, 2012). These events include changes leading to irreversible attachment, biofilm maturation, and finally dispersal of cells away from the biofilm (O'Toole and Wong, 2016; Persat *et al.*, 2015; Petrova and Sauer, 2012). The physiological state and gene expression patterns of cells in various states of such a biofilm have also been described (Dötsch *et al.*, 2012; Sauer and Camper, 2001), but most studies have been of bacteria attached to a surface for 24 hours or longer (Dötsch *et al.*, 2012; Pringent-Combaret *et al.*, 1999). There has been a comparative dearth of studies that have addressed the initial interactions of planktonic cells upon their arrival at a surface - the first step in biofilm formation. It might be expected that such a change in lifestyle would be associated with relatively large changes in gene expression, some of which might be anticipatory of subsequent changes in the microenvironment that would be expected after cells form multicellular assemblages on such a surface. Only a few studies have examined transcriptional changes associated with bacteria soon after their attachment to a surface (Bhomkar *et al.*, 2010; Siryaporn *et al.*, 2014). In one notable study, Bhomkar *et al.* (2010) compared gene expression in *Escherichia coli* CSH50 one hour after cells had attached to mannose agarose beads with that of planktonic cells, while Siryaporn *et al.* (2014) examined gene expression in *Pseudomonas aeruginosa* UCBPP-PA14 after cells had been attached to a surface for one hour. In both cases, evidence for a substantial change in the transcriptome very soon after attachment was provided. Although not examined soon after attachment, Yu *et al.* (2013) compared the transcriptome of *P. syringae* B728a, after bacterial growth on leaves for 72 hours, with those of planktonic cells in a minimal medium. As might be expected, they found genes from many different functional categories to be differentially expressed in these two settings. It seems likely that at least some of the genes that were differentially expressed were in response to the immobilization of cells on the surface rather than their planktonic lifestyle, although one might expect substantial differences in the chemical and physical nature of the leaf surface compared to that of broth medium. It was noteworthy however that the genes encoding syringafactin production, *syfA* and *syfB*, as well as the regulator of syringfactin production, *syfR*, were all induced on the leaf surface compared to that of planktonic cells in culture (Yu *et al.*, 2013). Such a finding is consistent with the observations of Burch *et al.* (2011) who found the expression of *syfA* to be higher in the same strain when cells were grown on a surface compared to in a broth culture; such a pattern of expression would be expected of a gene whose transcription was responsive to surface contact. As mentioned previously, since biosurfactants are surface active compounds that can modify the leaf surface (Bunster *et al.*, 1989; Burch *et al.*, 2011; Burch *et al.*, 2012; Lindow and Brandl, 2003; Ron and Rosenberg, 2001), it makes sense that syringafactin would be more highly produced on a surface than in a liquid environment. In Chapter 3, I demonstrate that syringafactin production and expression of *syfA* is solely a contact-dependent phenomenon in *P.*

syringae B728a and that it is apparently not modulated by the chemical environment experienced by the cells. My results showing that expression of *syfA* is very rapid, independent of the nature of any surface on which the cells are immobilized, suggests that *P. syringae* might utilize surface contact as a cue to anticipate transition to a surface environment such as leaves, and thus anticipate the need for expression of a variety of traits in such a new habitat. To test this hypothesis, I determined those genes whose expression was rapidly responsive to the immobilization of *P. syringae* on a surface. The global transcriptome analysis discussed in Chapter 3 comparing gene expression in cells immobilized on a membrane filter on an agar plate for two hours with those simultaneously in a broth culture has provided evidence of a large and rapid response of *P. syringae* to a surface. These results are discussed in the context of models that consider the different chemical and physical features that might be experienced by immobilized and planktonic cells as well as from the perspective of possible anticipatory changes in gene expression whose benefit to the cell would require their production in advance of the new condition.

Bacterial surface sensing mechanisms

While only a few studies have performed detailed analyses of transcriptional changes in bacteria upon mobilization on a surface, a variety of traits have been shown to be surface contact-dependent, particularly traits that lead to virulence of bacteria to various hosts. Therefore, considerable attention has been placed on determining the mechanisms by which bacteria can sense that they are in contact with a surface (Anderson *et al.*, 2010; Belas and Suvanasuthi, 2005; Hickman *et al.*, 2005; McCarter *et al.*, 1992; Otto and Silhavy, 2002; Siryaporn *et al.*, 2014). It has been commonly observed that bacteria in liquid that swim towards a surface loosely attach to the surface via flagella (Belas, 2014; Petrova and Sauer, 2012). This is typically known as "reversible attachment" where the bacterium may be spinning or moving across the surface and can still swim away (Chang, 2017; Li *et al.*, 2011; O'Toole and Wong, 2016; Petrova and Sauer, 2012; Tuson and Weibel, 2013). Bacteria begin to transition to the "irreversible attachment" stage by producing exopolysaccharides or other adhesions (Belas, 2014; Li *et al.*, 2011; O'Toole and Wong, 2016; Persat *et al.*, 2015; Petrova and Sauer, 2012; Tuson and Weibel, 2013). For instance, a study of *P. aeruginosa* PAO1 by Hickman *et al.* (2005) suggested that the chemosensory Wsp pathway plays a role in surface sensing and bacterial attachment since disruption of *wspF* results in increased adhesion to surfaces due to the increased production of exopolysaccharides. The *wspF* mutants also have increased intracellular levels of cyclic diguanylate (cyclic di-GMP), a signaling molecule that has been frequently noted to be involved in the transition to "irreversible attachment" (O'Toole and Wong, 2016; Petrova and Sauer, 2012; Siryaporn *et al.*, 2014; Tuson and Weibel, 2013). Thus, Hickman *et al.* (2005) reasoned that WspF prevents the phosphorylation of the response regulator WspR, but that when WspR is phosphorylated, it enables the production of cyclic di-GMP resulting in increased exopolysaccharide secretion and bacterial attachment. It seems possible that such a pathway could also modulate expression of other surface-dependent genes in this species. Similarly, in a study on *Caulobacter crescentus*, Li *et al.* (2011) noted that "irreversible attachment" was mediated by the stimulation of a polar holdfast adhesive once cells made contact with a surface. Li *et al.* (2011) also observed in *C. crescentus* that once cells made contact with a surface, pili played a role in tethering the cells to the surface and immobilizing flagellar rotation. They went on to demonstrate similar mechanisms of adhesion in *Asticcacaulis biprosthecum* and

Agrobacterium tumefacians. This reveals that similar methods of adhesion may be used in a variety of bacteria.

Another surface sensing mechanism was suggested by Otto and Silhavy (2002) who showed that the Cpx signaling pathway, which is activated via cell wall stress, is induced in *Escherichia coli* K-12 strain MC4100 when cells come into contact with a surface resulting in the activation of genes that may play roles in adhesion. They also showed that the outer membrane lipoprotein NlpE is required for cell-surface interactions and suggested that this protein may be involved in surface sensing by sending an adhesion-specific signal to the Cpx pathway. Siryaporn *et al*. (2014) proposed a somewhat similar surface sensing mechanism in *P. aeruginosa* UCBPP-PA14. As discussed above, Siryaporn *et al*. (2014) performed a transcriptome analysis comparing cells attached to a surface for one hour to that of cells in a broth culture. The study revealed that PilY1, an outer membrane protein was involved in bacterial attachment as well as increased virulence upon attachment. Siryaporn *et al*., (2014) also hypothesized that PilY1 functions as a mechanosensor that detects mechanical signals, resulting from torque on the cell associated with active flagella and an immobilized cell body, resulting in the induction of genes involved in virulence. This phenomenon was especially interesting since it seemed to occur quickly after attachment whereas the Wsp system was only examined around six to seven hours after attachment (Hickman *et al*., 2005).

Because of the considerable interest in the field of microbiology to better understand the mechanisms by which bacteria sense surfaces, I exploited the rapid transcriptional response of many other genes in *P. syringae* B728a to immobilization on the surface as a tool to elucidate the genes necessary for such a response. The simple visual evidence of induction of *syfA* in colonies of cells of *P. syringae* B728a harboring a *syfA* promoter fused with a promoterless *gfp* reporter gene was used to screen random transposon mutants for cells unable to exhibit surface-dependent induction of *syfA*. This powerful strategy revealed several putative regulators of *syfA* and models which propose their function and link them to the expression of other genes found to be contact-dependent are discussed in Chapter 4.

The studies developed in this proposal have the goal to better understand the microhabitats experienced by bacteria on the leaf surface. While there had been previous recognition that the nature of the leaf surface was quite variable, and that bacteria presumably exploited only those microhabitats most conducive to their growth and survival (Beattie and Lindow, 1995, Remus-Emsermann and Leveau, 2010; Remus-Emsermann *et al*., 2012) this study addressed the extent to which bacteria modify their local environment on the leaf to make it more habitable. I thus interrogated the leaf surface water microenvironment at the scale of individual cells and demonstrated the role of biosurfactant production in modifying this microhabitat. Studies of the regulation of biosurfactant production also show that this trait is a powerful tool for analyzing the regulation of bacterial surface adaptation. This dissertation therefore provides insight into bacterial life on a leaf as well as the adaptations, and mechanisms underlying those adaptations, that occur when bacteria come into contact with this habitat.

Chapter 2

Syringafactin production by *Pseudomonas syringae*, affects the local microenvironment on leaves via its hygroscopicity

Abstract

Pseudomonas syringae strain B728a produces the biosurfactant syringafactin which has hygroscopic properties. The water absorbing potential of syringafactin is high (attraction of as much as 250% its weight in water) at high relative humidities (>93%), but is much less hygroscopic at lower relative humidities, suggesting that its benefit to the producing cells is strongly context-dependent. The contribution of syringafactin to the water availability around cells on different matrices on which they were grown was assessed in strains harboring a reporter gene construct consisting of a *proU* promoter fused with a promoterless *gfp* reporter gene. Wild-type cells exhibited significantly less GFP fluorescence on humid but dry filters as well as on leaf surfaces than a syringafactin-deficient strain, indicating that syringafactin made water available to the cells. When vacuum infiltrated into the leaf apoplast, wild-type cells still exhibited less GFP fluorescence than a syringafactin-deficient strain. These results suggest that the apoplast is a dry, but humid environment, and that just as on dry but humid leaf surfaces, syringafactin makes sufficient liquid water available to reduce the water stress exhibited by *P. syringae*.

Introduction

While leaf surfaces support large numbers of both beneficial and detrimental bacteria, leaves are considered a relatively harsh environment. Leaves are a frequently dry environmental habitat that is subject to high ultraviolet fluxes as well as fluctuations in temperature and humidity (Axtell and Beattie, 2002; Burch *et al.*, 2014; Lindow and Brandl, 2003; Melotto *et al.*, 2008; Remus-Emsermann and Schlechter, 2018). However, the leaf surface differs from the surrounding atmosphere in an interesting way. Because of friction with the leaf surface, a thin layer of still air, known as the laminar boundary layer, surrounds leaves. The thickness of this layer is inversely proportional to wind speed. The still air traps water vapor that exits the leaf via its stomata (Beattie, 2011; Burch *et al.*, 2014; Drake *et al.*, 1970; Lindow and Brandl, 2003; Martin *et al.*, 1999; Parlange *et al.*, 1971; Parlange and Waggoner, 1971; Schuepp, 1993; Waggoner, 1965). Thus, air surrounding the leaf surface can exhibit much higher relative humidity (RH) than the surrounding environment (Longrée, 1939; Ferro and Southwick, 1984). However, high RH indicates only a high concentration of water vapor. It does not guarantee liquid water, which is what bacteria, intrinsically aquatic microorganisms, require to avoid desiccation.

The frequent lack of water on leaves would seem to limit the ability of *Pseudomonas syringae* to colonize leaves, yet this bacterium effectively colonizes and survives on the surfaces of leaves, often ultimately causing disease in its host plant bean after it enters the leaf apoplast (Melotto *et al.*, 2008). Though *P. syringae* has mostly been considered to be an epiphyte on leaf surfaces, Morris *et al.* (2008) has found that many strains of this species are commonly found in a variety of aquatic settings such as rivers and lakes. Moreover, Morris *et al.* (2008) found that *P. syringae* can be disseminated via the water cycle since it is able to catalyze ice and supercooled water droplets in the atmosphere, thereby mediating its partitioning between surfaces and airborne

transport mechanisms. These observations suggest that *P. syringae* is quite versatile, which allows it to flourish in both aquatic settings and on stressful leaf surfaces. The traits that convey this versatility, and particularly those that enable *P. syringae* to grow and survive on dry leaf surfaces, are poorly understood.

One of these traits was revealed by Burch *et al*. (2014), who found that the biosurfactant produced by *P. syringae* B728a, known as syringafactin, is hygroscopic. That is, it can absorb water vapor from the air and might also be able to retain water after being wetted by a liquid source. Biosurfactants, which are produced by various microorganisms, are amphiphilic compounds (Burch *et al*., 2011). Studies have historically examined biosurfactants for their ability to disperse hydrophobic compounds, enabling the consumption of these compounds by bacteria (Burch *et al*., 2011; Ron and Rosenberg, 2001). Berti *et al*. (2007) characterized syringafactin as a lipopeptide containing eight amino acids linked to an acyl tail, which makes this molecule amphipathic. The peptide head of this molecule contains several hydroxyl groups capable of hydrogen bonding with water. This observation suggested that syringafactin could interact with and absorb water, a hypothesis that Burch *et al*. (2014) tested by exposing dried, purified syringafactin to a water-saturated environment. With this treatment, the weight of syringafactin increased by over 240%, indicating that it could absorb water from the atmosphere. Burch *et al*. (2014) also showed that, in a water-saturated atmosphere, syringafactin produced by wild-type *P. syringae* B728a cells could wet the hydrophobic surface of Teflon® filters suggesting that cells might also be able to wet the hydrophobic surface of a leaf on which they are growing. After being desiccated on a Teflon® filter, syringafactin could be rewetted when exposed to a water-saturated atmosphere (Burch *et al*. 2014).

Burch *et al*. (2014) found that, in addition to being hygroscopic, syringafactin was apparently not readily dispersed across the leaf surface. A high-pressure liquid chromatography (HPLC) analysis revealed that, when applied to leaves, over 70% of syringafactin was bound to the waxy cuticle of the leaf surface and consequently not easily recovered in water (Burch *et al*., 2014). This observation suggested that syringafactin largely remains in the local environment of the bacterium that secreted it. This hypothesis was further supported when Burch *et al*. (2014) co-inoculated a wild-type *P. syringae* B728a strain and a *syfA* mutant strain, which is deficient in syringafactin production, onto bean leaves in the field. Although the initial population of cells was roughly 50% wild-type, during the 10 days of the experiment the proportion of wild-type cells increased to about 65% of the total population. This shift revealed that even though the *syfA* mutant and wild-type cells were in a mixed population, the *syfA* mutant cells did not seem to benefit from the syringafactin produced by the wild-type cells. Therefore, syringafactin seems to largely affect only the local environment of the cell that produces it rather than serve as a "public good" (Burch *et al*., 2014).

Though they demonstrated the hygroscopicity of syringafactin, Burch *et al*. (2014) did not examine the efficiency of syringafactin in absorbing water at various relative humidities. This is an important question because, even in the high RH environment within a leaf boundary layer, liquid water might not be available to bacteria that cannot acquire water vapor at less than water saturation. Furthermore, the availability of liquid water to syringafactin-producing cells on leaves, and thus whether it could alleviate water stress in this habitat, has not been directly measured. Such information is needed to determine whether syringafactin can improve the

fitness of bacteria growing on the leaf surface, and under what contexts it might benefit the producing cells.

Bacterial cells on the leaf surface may need only a small, localized quantity of water to avoid desiccation stress. This meant that I needed to have a way to indicate how much water would be available to a cell in such a natural habitat (Burkhardt and Hunsche, 2013). Axtell and Beattie (2002) created a whole-cell bacterial biosensor that can be used to quantify liquid water. The biosensor uses the fact that proline is a solute that aids cells under conditions of either matric stress, in which water is not available, or osmotic stress, in which water may be available but it has a high solute concentration resulting in water diffusing out of the bacterial cell (Axtell and Beattie, 2002). Therefore, when the cell produces proline, this solute can aid in maintaining homeostasis between the environment inside of the cell and the environment outside of the cell. The *proU* operon encodes the synthesis of proline. After fusing a *gfp* reporter with the promoter of *proU*, a gene in an operon whose transcription is reflective of proline biosynthesis, Axtell and Beattie (2002) could use GFP fluorescence to find that the *proU* promoter was induced not only under conditions of high osmolarity, but also under conditions when water itself was less available. For example, GFP fluorescence quickly increased when droplets of *Pantoea agglomerans* BRT98 cells harboring this reporter gene fusion were placed on the surface of a bean leaf and allowed to dry. Since it is more likely that water is not present on the leaf surface as opposed to there being the presence of water with a high solute concertation, this result suggests that matric stress can be readily detected by growing cells harboring such a reporter gene system on leaves. In this study I therefore decided to assess water availability in both a wild-type *P. syringae* B728a strain and a *syfA* mutant harboring this reporter gene construct. I thus was able to compare the water availability experienced by these strains while on the leaf surface by quantifying the fluorescence of the *gfp* reporter using epifluorescence microscopy.

In addition to measuring water availability on leaf surfaces, water availability to strains differing in syringafactin production while they grew in the leaf apoplast was compared. The leaf apoplast is comprised largely of air-filled voids between parenchymal cells, which facilitates gas exchange for photosynthesis. Thus, it is a humid, but dry environment (Melotto *et al*., 2008; Xin *et al*., 2016). Since *P. syringae* exists not only on the leaf surface but also multiplies extensively in the apoplast, it seemed reasonable to postulate that syringafactin would contribute to its ability to grow and survive in such a humid yet dry environment (Beattie, 2011; Freeman and Beattie, 2009; Xin *et al*., 2016). By comparing the relative water availability perceived by wild-type and syringafactin-deficient strains harboring the water responsive reporter gene construct, I can test whether the hygroscopic properties of syringafactin enables cells that produce it to acquire more water, both on the leaf surface and in the leaf apoplast, than syringafactin-deficient cells. As described below, my results strongly suggest that syringafactin production by *P. syringae* is a way to avoid the desiccation stress that might be expected both on the leaf surface and in the leaf apoplast. This work thus also reveals an important and previously unrecognized role for microbial biosurfactants in the varied environments in which bacteria are found.

Results

Syringafactin is extremely hygroscopic only at high relative humidities

Since the hypothesized ecological role of syringafactin depends on its ability to interact with water, I examined the conditions over which syringafactin would absorb water. Purified syringafactin dehydrated under a vacuum was exposed to different RH conditions maintained by suspension over different saturated salt solutions (Figure 1). The weight of the syringafactin was determined both before exposure and after three days of exposure to a given RH. The change of weight in syringafactin revealed how much water it had absorbed. Though the water absorbing potential of syringafactin generally increased with increasing RH, it absorbed less than its own weight in water over most levels of water saturation. Importantly, however, its water-binding capacity increased dramatically at relative humidities greater than about 95%, absorbing as much as 250% its weight in water (Figure 1). This indicates that syringafactin is especially hygroscopic at high RH and that its maximum potential ecological value may be at high levels of water saturation.

Syringafactin contributes to water availability to cells at 100% RH on filters

To determine whether syringafactin made enough water available to bacterial cells to alleviate water stress, I compared the water stress exhibited by *P. syringae* cells harboring the water-responsive reporter gene. Wild-type and *syfA* mutant *P. syringae* B728a strains were applied to filters placed on agar plates. As a positive control, some *syfA* mutant strains received exogenous syringafactin. Since they would lack the humid laminar boundary layer of leaves after their removal from an agar surface, filters were used as a more direct means to determine changes in water availability. Syringafactin-producing cells, mutant strains incapable of the production of syringafactin but to which exogenous syringafactin had been applied, and mutant cells alone were placed on filters exposed to various levels of water saturation but lacking water themselves. This was to see if the syringafactin-producing cells and the cells to which syringafactin had been applied, would have greater water availability compared to the mutant cells alone. I hypothesized that when exposed to low water saturation, the strains would not exhibit any significant differences in GFP fluorescence due to the lack of a humid boundary layer. However, I hypothesized that when exposed to high water saturation, the wild-type and *syfA* mutant with the applied syringafactin would exhibit lower GFP fluorescence compared to the *syfA* mutant alone. This is because syringfactin would be capable of absorbing water at a high humidity. To test my hypotheses, the strains were allowed to grow on the filters for eight hours before the filters were transferred to chambers maintaining 52% RH or 100% RH. Filters were incubated in the chambers for four hours and then immersed in a low salt-containing minimal nutrient medium for two hours to resuscitate cells. This resuscitation step enabled cells to translate *gfp* transcripts after being induced by low water availability and enabled any produced GFP to fluoresce. After resuscitation, cells were washed off filters and analyzed for changes in GFP expression by quantifying the fluorescence of individual cells examined using an epifluorescence microscope. At both 52% RH and 100% RH, wild-type cells exhibited GFP fluorescences with means of 945.08 and 797.28 and medians of 928.19 and 784.50 respectively (Figure 2). At 52% RH, GFP fluorescence of the *syfA* mutant strain exposed to exogenous syringafactin (mean = 1,030.90, median = 1,002.50) was similar to the *syfA* mutant strain without syringafactin (mean = 905.25, median = 885.58), which suggests that syringafactin could not make water available to cells at this low RH (Figure 2A). In contrast, at 100% RH, exposure to exogenous syringafactin significantly reduced GFP fluorescence by the *syfA* mutant strain (mean = 769.03, median = 747.01) and brought it into a range similar to that of the wild-type strain (mean = 797.28, median = 784.50) (Figure 2B). The finding that both the wild-type and *syfA* mutant strain with applied

syringafactin exhibited similarly lower GFP fluorescence than the *syfA* mutant strain itself (mean = 905.25, median = 885.58) at 100% RH supports my hypothesis that syringafactin is not only capable of making water more available to cells under high RH conditions, but that wild-type cells produce sufficient amounts of syringafactin to confer this phenotype.

Syringafactin improves water availability to cells on the leaf surface only when plants are exposed to high relative humidities

Given that syringafactin could make water more available to cells on abiotic surfaces, I next determined the extent of water stress experienced by bacterial cells on leaves exposed to various environmental conditions and asked whether cells could ameliorate this stress by producing syringafactin. I hypothesized that at a high RH the wild-type cells would exhibit lower GFP fluorescence than the *syfA* mutant cells due to the production of syringfactin. To test this, wild-type and *syfA* mutant strains harboring the *proU:gfp* reporter gene fusion were sprayed onto the leaves of bean plants that were then immediately placed in a 100% RH chamber for two days to enable bacterial growth and production of any extracellular products. As a control, cells from the inoculum for both strains were also measured for GFP fluorescence (Figure 3). This was done in order to determine if there were any differences between the GFP fluorescence exhibited by the strains in a wetter environment. Overall, the wild-type cells (mean = 508.73, median = 469.59) appeared to be more water stressed than the *syfA* mutant cells (mean = 441.45, median = 430.34) (Figure 3). The sprayed leaves initially were covered with many small droplets of bacterial suspension, but after two days, most of the leaf was free of any droplets. Instead, only a few large water droplets persisted on the leaves, suggesting that the water had redistributed to produce large dry areas on the leaf surface.

When examined two days after inoculation, wild-type cells (mean = 457.85, median = 450.00) had lower average GFP expression, indicating that they had experienced greater water availability than the *syfA* mutant cells (mean = 620.14, median = 516.05) (Figure 4). Furthermore, the *syfA* mutant cells exhibited a higher GFP fluorescence than those in the inoculum applied to the plant, indicating that these cells had experienced somewhat dry conditions on the leaf surface. In contrast, the wild-type cells appeared fairly unchanged in their GFP fluorescence. Presumably, the redistribution of water on leaves that were exposed to a water-saturated atmospheric environment was localized at sites on the leaf that were devoid of liquid water.

Initial exposure to a low RH results in the desiccation of cells due to a lack of water absorption by syringafactin

To determine the environmental contexts under which syringafactin could be produced and under which it could confer protection against desiccation stress, I exposed cells of *P. syringae* to drying conditions on leaves both before and after liquid water was made available. Given that leaves are typically exposed to sequential wet and dry conditions, I simulated a situation in which *P. syringae* would immigrate to a dry leaf. I hypothesized that the wild-type strain would be less water stressed than the *syfA* mutant under these fluctuating conditions. This was because Burch *et al*. (2014) had shown that even when syringafactin becomes dry under conditions of low RH, it can become rewetted under conditions of high RH. Therefore, as long as wild-type cells were periodically exposed to conditions of high RH, any syringafactin they produced could

absorb water resulting in less water stress. After the wild-type and *syfA* strains were sprayed onto bean leaves, plants were exposed to 50% RH on the bench for 20 minutes in order to dry. This was enough time to evaporate any water droplets on the leaves, but presumably not enough time to cause death of the cells. Plants were then incubated at 100% RH for two days, during which time the cells were not exposed to liquid water. Under these conditions, the strains did not differ in GFP fluorescence (Figure 5). In another experiment, performed in parallel with the experiment in Figure 5, wild-type and *syfA* strains were sprayed onto bean leaves and plants were subjected to the same treatment as described above, but were then dried for 20 minutes at 50% RH and then returned to a 100% RH chamber for two hours. In this experiment cells also exhibited relatively low GFP fluorescence though cells of the *syfA* mutant strain exhibited somewhat higher GFP fluorescence than that of the wild-type strain (Figure 6). The low GFP fluorescence could have resulted from the death of the cells during their initial exposure to 50% RH since dead cells would be unable to transcribe and translate GFP. Subsequent studies however showed that such brief exposure to such low humidities would have been unlikely to cause sufficient cell death to have accounted for such a population-wide suppression of GFP fluorescence (Axtell and Beattie, 2002; Monier and Lindow, 2003). Alternatively, given that the cells were only very transiently exposed to liquid water on the leaf, they presumably were unable to acquire soluble nutrient resources present on the leaf, and therefore did not exhibit substantial growth or metabolic activity that would have been required for transcription or translation of the *gfp* reporter gene. It thus appears that any benefits of syringafactin-mediated water acquisition are dependent on prior successful growth and metabolic activity of cells on leaves, during which time syringafactin can be made.

Syringafactin helps make water available to cells on the leaf surface when plants are exposed to fluctuating RH conditions following a period of conditions initially conducive to growth

Since previous experiments suggested that cells on leaves could not produce syringafactin unless sufficient water was available for growth and metabolism, I assessed the water availability to bacteria exposed to dry conditions following moist incubation conditions on leaves. These conditions would still be comparable to what bacteria on plants in the field experience due to the fluctuating RH and leaf wetness that bacteria are typically subjected to as a result of dry days and more humid nights (Burch *et al.*, 2014). An experiment was performed where, after spraying the bean leaves, plants were immediately put into a 100% RH chamber for two days in order to enable cells to grow and, in the case of the wild-type strain, produce syringafactin. Plants were then exposed to 50% RH for 20 minutes and then incubated at 100% RH for two hours to allow for the resuscitation of cells. Under these conditions it was evident that both strains experienced considerable water stress: GFP fluorescence was higher than that seen in the parallel experiment in which cells were constantly maintained at 100% RH on leaves (Figure 7). It is noteworthy that nearly all cells of the *syfA* mutant strain exhibited higher GFP florescence than the cells of the wild-type strain, and that the GFP fluorescence of most cells were much higher than that of the wild-type strain (Figure 7). Similarly, when inoculated leaves were incubated under a condition of 100% RH for two days before being exposed to 50% RH for an hour, and then subsequently again being incubated at 100% RH for another two days, cells of the *syfA* mutant again exhibited significantly higher GFP fluorescence than those of the wild-type strain (Figure 8). These results strongly suggest that the *syfA* mutant experienced greater desiccation stress after water availability was reduced by exposure of leaves to a less than fully water saturated environment. This result also supports the hypothesis that cells capable of producing syringafactin must

experience wet conditions that would enable them to produce syringafactin and benefit from its hygroscopicity. However, it appears that once they have produced syringafactin, cells can benefit from it during subsequent exposure to low RH.

Wild-type cells experience less desiccation stress than syfA mutant cells when exposed to fluctuating RH conditions at less than full saturation

Given that leaves frequently experience conditions of less than full atmospheric water saturation (100% RH) (Burch et *al.*, 2014; Lindow and Brandl, 2003), I examined the potential for syringafactin to modulate water availability of cells on the surface of leaves under these conditions. Bean leaves sprayed with either the wild-type or the *syfA* mutant harboring the *proU:gfp* reporter gene fusion were immediately incubated at 100% RH for two days to enable bacterial growth and metabolism and subsequently exposed to 50% RH for an hour to allow liquid water to evaporate from the leaf before being incubated at 97% RH for two days. As was seen when such dry colonized leaves were exposed to 100% RH, the GFP expression of cells of the *syfA* mutant strain were significantly higher than that of the wild-type strain (Figure 9). These results suggest two possibilities. Either the RH experienced by cells on these leaf surfaces was higher than the treatment condition or the amount of water that syringafactin could have acquired at 97% RH was sufficient to alleviate water stress.

Syringafactin helps make water available to bacteria living in the leaf apoplast

After colonizing the leaf surface, cells of *P. syringae* can enter the leaf apoplast through stomata, where they can grow to sufficient numbers to eventually cause disease (Melotto *et al.*, 2008). Given that the apoplast is dry, at least initially after bacterial entry, I determined if syringafactin production could improve cell survival in the leaf apoplast just as it does on the leaf surface. Wild-type and *syfA* mutant cells harboring the *proU:gfp* reporter gene construct were infiltrated under vacuum into bean leaves. Before infiltrating the cells into the leaves, aliquots of cells taken from the liquid culture used as inoculum were assessed for GFP fluorescence to determine if the two strains differed in apparent water availability at time 0. No significant difference in GFP fluorescence of the two strains was seen (Figure 10A). However, when assessed 24 hours after inoculation, cells of both strains exhibited higher GFP fluorescence than at the time of inoculation, indicating that they both experienced higher water stress than in liquid cultures (Figure 10B). Although the difference in GFP fluorescence of the two strains was not significantly different at 24 hours, some cells of the *syfA* mutant seemed to still exhibit slightly higher GFP expression than that of the wild-type strain, suggesting that they were beginning to experience somewhat more desiccation stress than the wild-type strain (Figure 10B). By 48 hours after inoculation, the GFP expression of both strains was higher than after 24 hours, indicating that the apoplast had become even drier during the infection process (Figure 10C). Importantly, by 48 hours after incubation, *syfA* mutant cells exhibited higher GFP fluorescence than the wild-type cells indicating that they experienced a higher water stress than that of the wild-type strain and thus that syringafactin production by the wild-type strain had ameliorated the water stress that it otherwise would have experienced (Figure 10C).

Other factors in addition to syringafactin seem to play a role in cell viability

Given that syringafactin appears to help make water available to cells growing both on the leaf surface and in the leaf apoplast, and that water stress would be expected to detract from cell viability, I tested the conjecture that syringafactin production would increase the viability of cells exposed to desiccation stress. The wild-type strain and a *syfA* mutant of *P. syringae* B728a were grown on filters placed on a King's medium B (KB) agar plate for eight hours and subsequently exposed to 52% RH, 97% RH, and 100% RH. After their removal from the agar plates, the viability of the cells under the different RH conditions was assessed (Figure 11). The viability of both the wild-type and *syfA* mutant cells rapidly and continuously declined when exposed to 52% RH (Figure 11A). The number of viable cells of both strains decreased only slowly over time when incubated at 100% RH (Figure 11B). Curiously, when filters were incubated at 97% RH the number of viable cells initially declined rapidly with time, but then exhibited little additional change for up to 189 hours (Figure 11C). No consistent difference in the number of viable cells of the wild-type and *syfA* mutant strains at a given incubation time for any given RH condition was observed. I noticed that, despite the fact that the cells were incubated on filters under dry conditions, the filters exposed to 97% RH and 100% appeared wet in the vicinity of the cells that had grown on the filters. No such wetness was observed on filters exposed to 52% RH. Such apparent wetness was seen on filters on which both the wild-type as well as the *syfA* mutant had grown. I thus postulated that the apparent wetness was associated with other extracellular products such as water-absorbing exopolysaccharides that appeared to have been produced in abundance by cells growing on the filters that were exposed to high nutrient conditions and the KB agar. To address this model, I determined the influence of alginate production on the viability of *P. syringae* exposed to different relative humidities by using an *algD* mutant since *algD* encodes alginate production, a major exopolysaccharide produced by *P. syringae* B728a (Chang *et al*., 2007). I therefore performed an experiment to determine the viability overtime of both the wild-type and *syfA* mutant strains along with an *algD* mutant, and a *syfA*, *algD* double mutant strain of *P. syringae* B728a harboring the *proU:gfp* reporter gene when exposed to 97% RH or 100% RH. Little difference was seen in the viable cell population size of the various strains when measured at a given incubation time for a given condition. Only after 143 hours after inoculation, did the *syfA* mutant strain exhibit a decrease in cell viability compared to other strains, although it was noteworthy that the *syfA*, *algD* double mutant did not also exhibit such low cell viability (Figure 11B). The apparent wetness of filters upon which the various strains grew when incubated at high relative humidities did not differ substantially, suggesting that alginate production was not associated with either the wetness of the filters or any protection against death during desiccation conditions. It thus would appear that other extracellular factors such as other extracellular polysaccharides may have been associated with both processes.

Discussion

This study was initiated to determine whether syringafactin plays an important role in the epiphytic and endophytic growth of *P. syringae*. It is reasonable to assume that liquid water is required for bacterial growth in and on leaves, since it would be required to mobilize soluble nutrients (Xin *et al*., 2016). However, leaf surfaces are dry much of the time and cells must survive such conditions in order to grow during the brief periods when water might be available. Thus, life on a leaf surface is probably stressful, since it is a dry environment that is only transiently wet (Axtell and Beattie, 2002; Beattie, 2011; Burch *et al*., 2014; Remus-Emsermann and Schlechter, 2018; Schreiber *et al*., 2004).

I found that *P. syringae* B728a can live on dry leaves and in the apoplast by producing syringafactin, a hygroscopic biosurfactant, to attract and concentrate water vapor from the atmosphere. First, I demonstrated that syringafactin is extremely hygroscopic under the high RH conditions expected in both the apoplast and the humid laminar boundary layer immediately above leaf surfaces. Although syringafactin can bind water only in air nearly fully saturated with water vapor, these conditions are consistent with models of the abiotic conditions that prevail on leaves. Such models predict that the air immediately surrounding the leaf, known as the laminar boundary layer, differs greatly from that of air further away from the leaf (Drake *et al*., 1970; Ferro and Southwick, 1984; Schuepp, 1993; Yarwood and Hazan, 1944). At low wind speeds, the laminar boundary layer can be several millimeters thick. Models also predict that air within the laminar boundary layer has a much higher RH than the air in the surrounding environment, since it traps water vapor exiting the leaf via stomata. Thus, a very humid microenvironment is proposed to surround even dry leaves (Beattie, 2011; Burch *et al*., 2014; Drake *et al*., 1970; Lindow and Brandl, 2003; Martin *et al*., 1999; Parlange *et al*., 1971; Parlange and Waggoner, 1971; Schuepp, 1993; Waggoner, 1965). Previous studies have suggested that the laminar boundary layer maintains a RH of 90% or higher (Longrée, 1939; Ferro and Southwick, 1984). By comparing the sporulation of powdery mildew on rose leaves versus glass slides, Longrée (1939) was able to indirectly calculate the leaf surface to have a RH of 96% or higher. Ferro and Southwick (1984) developed a model to estimate RH in the leaf laminar boundary layer and calculated that if the saturated water vapor density in the stomata is 24.4 g/m^3 at 26ºC and if the water vapor density in the air outside the boundary layer is 13.9 g/m^3, then even at a distance of 3 mm from the stomata, air within the laminar boundary layer would have a water concentration of 23.8 g/m^3, which corresponds to a RH of 97.54%. Even though syringafactin absorbs substantial amounts of water only at a RH greater than 97%, cells that exist within a laminar boundary layer apparently experience such a high RH. Even when plants are exposed to a relatively dry environment the lesser water stress exhibited by the wild-type strain compared to the *syfA* mutant strain on dry leaves (Figures 6 - 9) can be attributed to water made available by binding to the syringafactin produced by the wild-type strain. Thus even though the hygroscopicity of syringafactin is strongly dependent on the RH of the atmosphere, cells in and on plants probably grow in a sufficiently humid atmosphere for them to benefit from water-binding by syringafactin.

The observation that syringafactin can mediate absorption of a large amount of water by cells at high RH conditions, but much less in atmospheres that are not close to water saturation, was consistent with patterns of water stress experienced by bacteria growing on an abiotic surface that should not influence the RH of its laminar boundary layer. When immobilized on such a membrane at low RH, there was little difference in water stress experienced by wild-type cells compared to a *syfA* mutant, as evidenced by their expression of a *proU:gfp* reporter gene. Importantly, adding exogenous syringafactin did not affect GFP expression of the *syfA* mutant, suggesting that syringafactin was unable to attract water at such a low RH. However, at 100% RH, the *syfA* mutant expressed significantly more GFP when syringafactin was added, suggesting that syringafactin helped relieve its water stress. Nonetheless, even at 52% RH the wild-type strain experienced less water stress than the *syfA* mutant strain to which exogenous syringafactin had been added. This observation led me to hypothesize that the presence of the *syfA* gene may encode additional properties to deter water stress. However, this will be something that will need to be looked into more in the future.

My study also supports predictions that the physical characteristics of leaves show great spatial heterogeneity. For example, the *syfA* mutant strain harboring the *proU:gfp* reporter gene exhibited greater GFP fluorescence than the wild-type strain even on plants that had been maintained in a water-saturated environment after they were sprayed with bacterial suspensions (Figure 4). These results suggest that many cells of this mutant experienced lesser amounts of water than those of the wild-type strain. The observation that water limitations might occur under such a scenario might initially seem surprising on a seemingly wet leaf, but this observation supports and advances previous models of the movement and distribution of water on leaves. For instance, rather than being evenly distributed across the leaf surface, water is thought to be more prevalent at the base of trichomes or at leaf veins or cracks in the cuticle (Beattie, 2011; Gnanamanickam and Immanuel, 2006; Monier and Lindow, 2003). While there was apparently a very slow removal of water from the leaf surface as a whole, with some of the water evaporating and condensing on walls of the chambers in which the plants were held, the remaining water also seemed to have been redistributed such as in other studies, collecting in preferred sites on the leaf (Beattie, 2011; Gnanamanickam and Immanuel, 2006; Monier and Lindow, 2003). This is quite apparent in the study since leaves that were sprayed with cells harbored many small droplets, but after two days in a water-saturated environment leaves contained fewer but larger water droplets. Most of the leaf surface was apparently devoid of water and none of the leaves harbored films of water. These observations along with my data further support the hypothesis that the hydrophobic leaf surface is very poorly wettable, and that cells emigrating to most of the leaf would experience a relatively dry environment due to the redistribution of water even under relatively humid conditions and a complete lack of water under many conditions (Axtell and Beattie, 2002; Burch *et al*., 2014; Lindow and Brandl, 2003).

My studies of the behavior of both wild-type and *syfA* mutant strains harboring the *proU:gfp* reporter gene after inoculation onto plants under different conditions provides considerable insight into the physiological state of bacteria after immigrating to plants. For example, I observed that when cells were exposed to dry conditions (50% RH) immediately after being sprayed on bean leaves, they exhibited low GFP fluorescence. One would have expected that such cells would have exhibited increased GFP fluorescence since they certainly encountered a dry leaf environment. The lack of expression of the reporter gene however most likely indicates a lack of several processes that must occur before the reporter gene could be expressed. Cells that are allowed to dry on the leaf immediately after application would not be expected to have the opportunity to exhibit motility on the leaf. Such motility has been shown to be a prominent feature of epiphytes, presumably enabling them to explore the leaf in search of spatially localized nutrients (Remus-Emsermann and Leveau, 2010; Remus-Emsermann *et al*., 2012). Given that carbon compounds appear to limit bacterial growth on leaves and that such nutrient resources are heterogeneously distributed across leaves, cells that did not encounter such resources upon direct inoculation or that were unsuccessful in encountering such sites during motility (which might be directed) would likely not be successful in either growth or even survival (Leveau and Lindow, 2001). Studies by Remus-Emsermann and Leveau (2010) and Remus-Emsermann *et al*. (2012), have shown that many cells fail to find such resources and thus have low reproductive success. I would therefore expect that cells that I applied to plants under low RH conditions would have been unlikely to have seen resources, and lacking liquid water, would have also been unlikely to have been sufficiently metabolically active to have initiated transcription of the reporter gene or translation of its transcript. Some cells might also have immediately succumbed to desiccation stress and died. Neither of these situations would produce detectable GFP fluorescence in the

applied bacteria. In contrast when cells were exposed to 100% RH after being sprayed and only subsequently exposed to 50% RH, there was a significant difference in the GFP expression of the wild-type and *syfA* mutant strains, which suggests that they were metabolically active (Figures 7 - 9).

The hypothesis that syringafactin production benefited cells by making water more available, even in leaves exposed to relatively low RH, was supported by comparing the results observed in Figure 4 with those in the parallel experiment depicted in Figure 7. Specifically, as expected, a much higher level of GFP expression was seen in *syfA* mutant strains harboring the *proU:gfp* reporter gene when plants were exposed to 50% RH after growth on the plant for two days (during which time cells were growing and metabolically active) than those on plants which had been in a continually water-saturated environment. Given that a quantitative relationship between GFP expression of cells harboring such a reporter gene construct and the level of either matric or osmotic stress to which cells were exposed to has been observed (Axtell and Beattie, 2002), it seems clear that many of the cells of the *syfA* mutant experienced lower water availability on these drier leaves than those on the plants maintained under humid conditions. Importantly however, a much larger difference in GFP fluorescence exhibited by the wild-type and *syfA* mutants was observed on plants exposed to the low humidity conditions after growth on the plant (Figure 7). Since little relative ability of syringafactin to bind water would have been expected at 50% RH compared to that in water-saturated environments (Figure 1), these results suggest that while the RH of the surface of the leaves exposed to 50% RH was likely lower than that exposed to 100% RH, it was still high enough that a sufficient amount of water could be bound by syringafactin to alleviate water stress in the wild-type strain. Such a result is also consistent with models of the formation of humid laminar boundary layers on leaf surfaces (Longrée, 1939; Ferro and Southwick, 1984). Additional support for this model was also provided by studies in which cells were incubated at 97% RH after the colonization of wetted leaves. Under these conditions the wild-type strain still exhibited less GFP fluorescence compared to the *syfA* mutant strain (Figure 9). These results suggest that after being produced during periods of metabolic activity of cells on leaves, syringafactin benefits cells during their subsequent, and probably inevitable, exposure to periods of low RH by preventing the drying of the cells. This hypothesis is supported by the results of Burch *et al.* (2014) who observed that even though the ability of syringafactin to wet a region of Teflon® filter disappeared at 50% RH, when the filter was re-exposed to 100% RH the syringafactin was able to rehydrate and rewet the region. This suggests that as long as cells are able to produce syringafactin on a leaf surface, even if the leaf is subjected to fluctuating RH conditions, cells under periods of a high RH benefit when the syringafactin absorbs water and makes it more available.

These studies of the water availability to bacteria colonizing the apoplast of bean provided great insight into the important role of syringafactin in this habitat and the nature of the plant apoplast itself. The leaf apoplast has been previously described as "a large, air-filled intercellular space" (Melotto *et al.*, 2008). The amount of free water available in the apoplast is still largely unknown (Beattie, 2011). Earlier studies using a *proU:inaZ* reporter gene had indicated that liquid water is apparently largely absent from the apoplast (Wright and Beattie, 2004). These earlier studies however had not provided any insight as to what the RH in the apoplast might be. It could however be speculated that the water within plant cells would be in close equilibrium to that of water vapor in the apoplast, given the relatively little ventilation that would be expected due to diffusion through the stomata. It would be in such a setting that one might expect syringafactin to

contribute to the fitness of *P. syringae* since its ability to bind water seems to be limited to atmospheres nearly saturated with water vapor. It was important to note therefore that in the apoplast, the wild-type strain apparently experienced less water stress than the *syfA* mutant at 24 hours, and especially 48 hours, after infiltration (Figure 10). Given that *P. syringae* strain B728a is a pathogen of the bean variety used in the study, it was somewhat surprising to find that at least some degree of water limitation was experienced by both the wild-type and *syfA* mutant strains only four hours after inoculation (Figure 10). A recent study has shown that certain effectors such as HopM1 in pathogens such as *P. syringae* mediate the release of water from the plant into the apoplast (Xin *et al*., 2016). Indeed, at least transient water soaking is a typical symptom of the infection of many plants by plant pathogenic bacteria. It is thought that the release of water makes apoplastic nutrients more available to bacteria within this habitat and that, since nutrient limitation probably limits bacterial population sizes in the apoplast, water availability is a determinant of the success of a pathogen. In such a setting, it was somewhat surprising that wild-type *P. syringae* B728a saw lower water availability 24 hours after inoculation than in broth media itself (Figures 10A and B). It is possible that effector-mediated water release in bean is transient. However, it is interesting that the apoplast becomes even drier between 24 hours and 48 hours post-inoculation (Figure 10C). Other studies of incompatible interactions of plant pathogenic bacteria and host plants by Freeman and Beattie (2009) revealed that plants can actively withhold water from bacterial pathogens that enter the leaf apoplast as early as three hours post-inoculation. However this result was observed for incompatible interactions, while the interaction of strain B728a with bean plants is considered compatible. It is nevertheless possible that plant responses to the presence of a compatible pathogen such as strain B728a may be delayed and that such water withholding would occur only later during the interaction. The finding that cells of the wild-type, and particularly the *syfA* mutant exhibited substantial water stress 48 hours after inoculation, is consistent with such a model. Earlier work has also shown that hosts such as bean, which are compatible with *P. syringae*, produce defensive phytoalexins two or more days after the infection process is initiated (Lyon and Wood, 1975). Furthermore, the growth of strain B728a in bean slows with time and typically ceases by two days after inoculation (Wilson *et al*., 1999; Helmann, personal communication) which is consistent with a model of decreasing water availability during the infection process. In such a setting, alleviation of water stress by syringafactin production would benefit *P. syringae*.

Since syringafactin plays an important role in water availability for *P. syringae* B728a on the leaf surface and in the leaf apoplast, and since desiccation stress is a major factor leading to loss of cell viability, I expected that the increased water availability experienced by syringafactin-producing bacteria would be linked to increased cell survival (Lindow and Brandl, 2003). Because of the heterogeneity of leaf surfaces as discussed above, cell viability studies were conducted on membrane filters, which I presumed would present a uniform physical and chemical environment to bacterial cells. I was thus surprised to find that the *syfA* mutant and wild-type strains died at similar rates in all of the RH conditions to which the filters had been exposed (Figure 11). I would not have expected an increased survival of the wild-type strain compared to the *syfA* mutant strain at low relative humidities because syringafactin binds little water at such low RH. Though perhaps little or no lethality of either strain would have been expected in fully water-saturated environments, I would have expected differential survival of these two strains at 97% RH, given the substantial water-binding capability of syringafactin at this RH (Figure 1). These experiments were done under somewhat unnatural conditions, where large amounts of bacterial growth had occurred on filters that had been incubated on a nutrient

rich medium. I speculate that the syringafactin-independent survival under these conditions was associated with other factors, such as copious amounts of various extracellular polysaccharides that had a dominant effect on desiccation tolerance. To test that hypothesis, I used an *algD, syfA* double mutant strain to examine the effect of alginate production, which has been seen to improve desiccation tolerance of other *P. syringae* strains (Yu *et al.*, 1999). However, I found no evidence that alginate production improved cell viability when compared to a *syfA* mutant alone. Various other exopolysaccharides have also been shown to retain water (Beattie, 2011; Chang *et al.*, 2007). *P. syringae* B728a can produce additional exopolysaccharides such as levan, marginalan, and cellulose (Chang *et al.*, 2007). Thus, further work will be needed to determine which of these other factors may play a role in desiccation tolerance and whether they are typically produced on plants under the conditions such as those studied here.

The demonstration that syringafactin helps provide water to *P. syringae* in natural habitats provides support for an important new role for microbial biosurfactants. It seems likely that at least some of the many microorganisms that produce biosurfactants (Ron and Rosenberg, 2001) could similarly benefit. This might be particularly true of those that live in non-water saturated environments such as soil, that produce periodic water stress. In the future, it would also be interesting to link *syfA* gene expression to the expression of other genes that might be involved in water retention in order to gain a better idea of what traits *P. syringae* expresses when on stressful leaf surfaces or in the leaf apoplast.

By better understanding the roles of various biosurfactants produced by bacteria, we can gain more insight into the behavior of biosurfactant-producing microbes and the contribution of such compounds to plant-microbe interactions. We should also gain more insight into the interactions between bacteria and the leaf surface. Since biosurfactant producers occur on edible plants, such as lettuce, they may influence the behavior of human pathogens, such as *Salmonella*, which can coexist with and interact with other epiphytic bacteria (Poza-Carrion *et al.*, 2013). Hence, a better understanding of biosurfactant production, and the use of biosurfactant-producing bacteria as biocontrol agents, may help to mitigate both human and plant pathogens thereby improving both human health and crop yield (Burch *et al.*, 2012).

Acknowledgments

Experimental Procedures

Bacterial strains and growth conditions

Pseudomonas syringae B728a strains were either grown on King's B medium (KB) plates containing 1.5% technical agar or on half-strength 1/2-21C medium plates (Axtell and Beattie, 2002; Halverson and Firestone, 2000; King *et al.*, 1954). Antibiotics were used at the following concentrations (μg/ml): spectinomycin (100), kanamycin (50) and tetracycline (15).

Syringafactin extraction

Syringafactin was extracted using a protocol described by Burch *et al*. (2014) which was modified from a protocol by Berti *et al.* (2007). *P. syringae* B728a strains were grown on agar plates for three days. Cells were then suspended in water and centrifuged for 10 minutes at 5000 x g. The supernatant was poured into a separatory funnel with ethyl acetate (1.5:1). The organic fraction was kept while the aqueous fraction was poured off. The organic fraction was taken to a rotary evaporator (BÜCHI) where the liquid was evaporated off. The remaining powder was resuspended in methanol and dried to completion in a Speedvac (Savant).

Measuring water absorption by syringafactin

Dried, purified syringafactin was added to a pre-weighed 1.5 ml microcentrifuge tube. The tube containing the syringafactin was weighed on an analytical balance (Fisher Scientific) and then placed, with the cap open, in a plastic magenta box. The magenta box contained a given saturated salt solution to keep the environment at a constant RH (Jarrett, 1995). The open tube was left in the magenta box for three days before being taken out, rapidly sealed, and reweighed.

Transformation of P. syringae B728a strains

P. syringae B728a *syfA* mutant cells (Burch *et al*., 2012) were transformed by electroporation with plasmid pPProGreen carrying a fusion of *proU* with a promoterless *gfp* reporter gene (Axtell and Beattie, 2002).

Preparation of in-vitro samples

Wild-type and *syfA* mutant strains were harvested with a loop from half-strength 1/2-21C agar plates that had grown a day on the bench, and were suspended in 1 ml of half-strength 1/2-21C liquid to concentrations of 10^8 cells/ml. In addition, some *syfA* mutant cells were suspended to concentrations of 10^8 cells/ml in 500 µl of half-strength 1/2-21C liquid containing 2.5 mg of purified syringafactin. 10 µl drops of each treatment were then pipetted onto 0.4 µm Isopore® filters sitting on half-strength 1/2-21C agar plates. All filters were left on the plates for eight hours at room temperature. After eight hours, filters were transferred to glass slides in chambers at either 52% RH or 100% RH.

After four hours, filters were put into 1.5 ml microcentrifuge tubes containing 1 ml of half-strength 1/2-21C liquid for resuscitation. After two hours, the tubes containing the filters were vortexed for 20 seconds to remove cells. 5 µl of each solution was then pipetted onto a glass slide and left to dry for 20 minutes. Coverslips were applied to the slides using Aqua PolyMount (Polysciences, cat#18606). Slides were then used for examination of cells under the epifluorescence microscope.

Preparation of epiphytic samples

Wild-type and *syfA* mutant strains were harvested with a loop from half-strength 1/2-21C agar plates that had grown for 24 hours on the bench at room temperature and were suspended in 50 ml of water to a concentration of 10^6 cells/ml. Cells were then sprayed onto the leaves of two-week old plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274, with four to six seedlings per pot). All plants were incubated under varying RH conditions maintained with saturated salt solutions.

Primary leaves were excised from plants (three leaves per replicate) and were immersed in 150 ml of 5 mM KPO_4 buffer in a beaker. Each beaker was placed in a sonicator (Branson 5510MT) for 10 minutes. Buffer containing the leaves was filtered through a 0.4 µm Isopore® filter. Filters were attached to glass slides and coverslips with Aqua PolyMount. Slides were then used for examination of cells under the epifluorescence microscope.

Preparation of apoplastic samples

Wild-type and *syfA* mutant strains were harvested with a loop from half-strength 1/2-21C agar plates that had grown a day on the bench. Each strain was suspended in one L of water to a concentration of 10^6 cells/ml. Cells were vacuum infiltrated into the leaves of two-week old plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274, with four to six seedlings per pot). All plants were stored on the bench at room RH (ca. 50% RH). At time 0, 5 µl of each inoculum was pipetted onto a glass slide and left to dry for 20 minutes. Coverslips were applied to the slides using Aqua PolyMount. Slides were then used for examination of cells under the epifluorescence microscope.

At 24 hours and 48 hours, primary leaves were excised from plants (three leaves per replicate) and cut into strips before being immersed in 45 ml of 10 mM KPO_4 contained in Falcon™ 50 ml conical tubes. Each tube was put in the sonicator for 10 minutes. After sonication, each tube was also vortexed for 20 seconds. The liquid was then decanted from each tube into 50 ml centrifuge tubes and all samples were centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded, and the remaining pellets were resuspended in 10 µl of 10 mM KPO_4. Five µl of each solution was then pipetted onto a glass slide and left to dry for 20 minutes. Coverslips were applied to the slides using Aqua PolyMount. Slides were then used for examination of cells under the epifluorescence microscope.

Quantification of GFP fluorescence

An M2 AxioImager was used for all microscopic analysis. A GFP filter set was used to view cells in all experiments and all images were taken in black and white with a 12-bit Retiga camera. The magnification used for all experiments was 100x. Bitplane Imaris image processing and manipulation software was used to quantify the average GFP expressed by each cell in digital images. Locations were automatically assigned to each cell in a given image, and debris cellular aggregates were identified visually and de-selected. For each location, the program calculated the mean pixel intensity for each cell.

Statistical Analysis

R (R Core Team, 2013) was used to perform the Kolmogorov-Smirnov test (Massey Jr., 1951) to look for significant differences between distributions of data. R was also used to perform the F-test (Fisher, 1925) to compare two variances and Welch's Two Sample t-test (Welch, 1947) to compare unequal variances.

Measuring cell viability

Wild-type *P. syringae* B728a and *syfA* mutant strains harboring a plasmid constitutively expressing *gfp*, an *algD* mutant strain harboring the *syfA* promoter fused with *gfp*, and an *algD*,

syfA double mutant strain were grown on KB plates containing the appropriate antibiotics for two days. All strains were suspended in one ml to two ml of water to a concentration of 10^6 cells/ml. 10 µl of each suspension was pipetted onto 0.4 µm Isopore® filters sitting on KB agar plates. Plates were left on a laboratory bench at room temperature for six hours (eight hours for the experiment at 52% RH) to allow for growth of the strains and syringafactin production. Filters were then transferred to chambers containing saturated salt solutions to maintain 52%, 97%, and 100% RH. In addition, before being transferred to the 97% RH and 100% RH chambers, filters were briefly dried for approximately five minutes in order to ensure that any water that had been transferred to the filter from the agar would evaporate. This would ensure that the filters were placed into a dry, yet humid environment. Filters being placed into the 52% RH chamber were not dried beforehand since all water associated with the filters evaporated in the chamber relatively quickly. At each time point, filters were added to a microcentrifuge tube containing one ml of water. The tubes were vortexed for 20 seconds each and 10-fold serial dilutions were made in water. 20 µl from each dilution was spotted onto a KB plate. All plates were left at room temperature and countable colonies grew after two days. Colonies were counted, and calculations were made to determine the total number of viable cells at each time point.
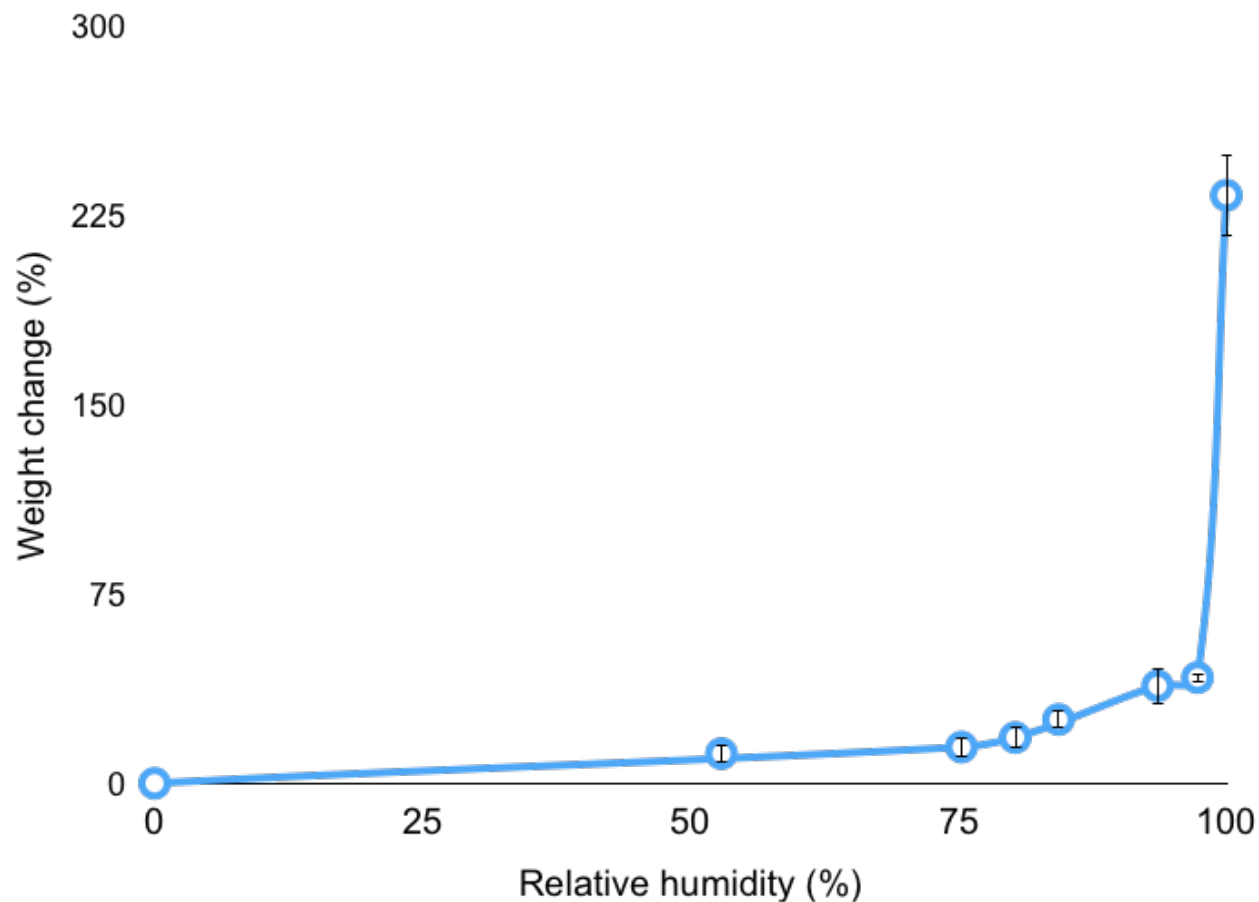
Figure 1. Syringafactin is extremely hygroscopic at high humidities.
Purified, dehydrated syringafactin was subjected to a range of relative humidities to assess weight gained by absorbing water. Water absorption was most pronounced between 97.3% and 100% RH with a weight gain of over 200%. The error bars represent the standard errors of the mean percentage in weight change.
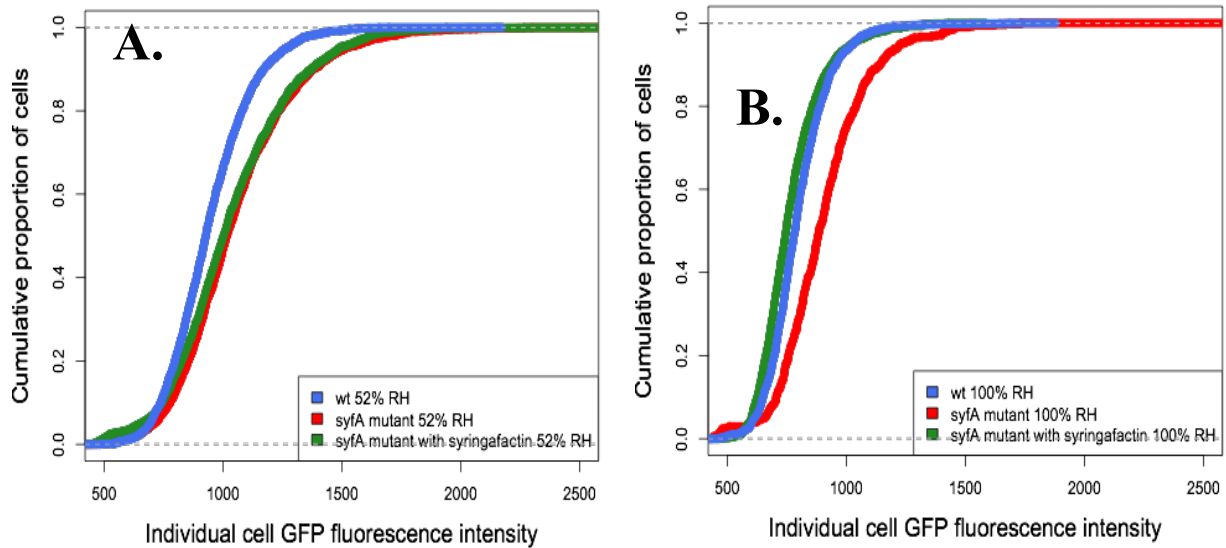
Figure 2. Wild-type cells on filters have more water available at 52% RH and 100% RH than *syfA* mutant cells.
Cumulative distribution plots of GFP fluorescence in wild-type, *syfA* mutant, and *syfA* mutant cells to which exogenous, purified syringafactin was added after exposure to (A) 52% RH and (B) 100% RH. Wild-type and *syfA* mutant strains were grown on half-strength 1/2-21C agar plates for one day before being suspended in 1 ml of half-strength 1/2-21C liquid to concentrations of 10^8 cells/ml. As a positive control, some *syfA* mutant cells were suspended to concentrations of 10^8 cells/ml in 500 µl of half-strength 1/2-21C liquid containing 2.5 mg of purified syringafactin. 10 µl drops of each treatment were pipetted onto 0.4 µm Isopore® filters sitting on half-strength 1/2-21C agar plates. All filters were left on the plates for eight hours at room temperature. After eight hours, filters were transferred to glass slides in chambers at either 52% RH or 100% RH. (A) Cells were exposed to 52% RH for four hours. The wild-type cells had a sample size of 3,206, a mean of 945.08, and a median of 928.19. The *syfA* mutant cells had a sample size of 2,697, a mean of 1,055.38, and a median of 1,016.34. The *syfA* mutant cells with applied syringafactin had a sample size of 2,058, a mean of 1,030.90, and a median of 1,002.50. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.2014, p < 2.2e-16; wt vs *syfA* mutant with syringafactin: D = 0.17746, p < 2.2e-16; *syfA* mutant vs *syfA* mutant with syringafactin: D = 0.043927, p-value = 0.02211). Additional tests performed were the F-test to compare two variances (wt vs *syfA* mutant: p < 2.2e-16, numerator degrees of freedom = 2,696, denominator degrees of freedom = 3,205; wt vs *syfA* mutant with syringafactin: p < 2.2e-16, numerator degrees of freedom = 3,205, denominator degrees of freedom = 2,057; *syfA* mutant vs *syfA* mutant with syringafactin: p = 0.8697, numerator degrees of freedom = 2,696, denominator degrees of freedom = 2,057) and Welch's Two Sample t-test (wt vs *syfA* mutant: p < 2.2e-16, wt vs *syfA* mutant with syringafactin: p < 2.2e-16, *syfA* mutant vs *syfA* mutant with syringafactin: p = 0.001148). (B) Cells were exposed to 100% RH for four hours. The wild-type cells had a sample size of 1,384, a mean of 797.28, and a median of 784.50. The *syfA* mutant cells had a sample size of 511, a mean of 905.25, and a median of 888.58. The *syfA* mutant cells with applied syringafactin had a sample size of 1,250, a mean of 769.03, and a median of 747.01. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.3173, p-value < 2.2e-16; wt vs *syfA* mutant with syringafactin: D = 0.13943, p-value = 1.625e-11; *syfA* mutant vs *syfA* mutant with syringafactin: D = 0.39693, p-value < 2.2e-16). Additional tests performed were the F-test to compare two variances (wt vs *syfA* mutant: p < 2.2e-16, numerator degrees of freedom = 510, denominator degrees of freedom = 1,383; wt vs *syfA* mutant with syringafactin: p = 0.284, numerator degrees of freedom = 1,383, denominator degrees of freedom = 1,249; *syfA* mutant vs *syfA* mutant with syringafactin: p < 2.2e-16, numerator degrees of freedom = 510, denominator degrees of freedom = 1,249) and Welch's Two Sample t-test (wt vs *syfA* mutant: p < 2.2e-16, wt vs *syfA* mutant with syringafactin: p = 5.341e-08, *syfA* mutant vs *syfA* mutant with syringafactin: p < 2.2e-16).
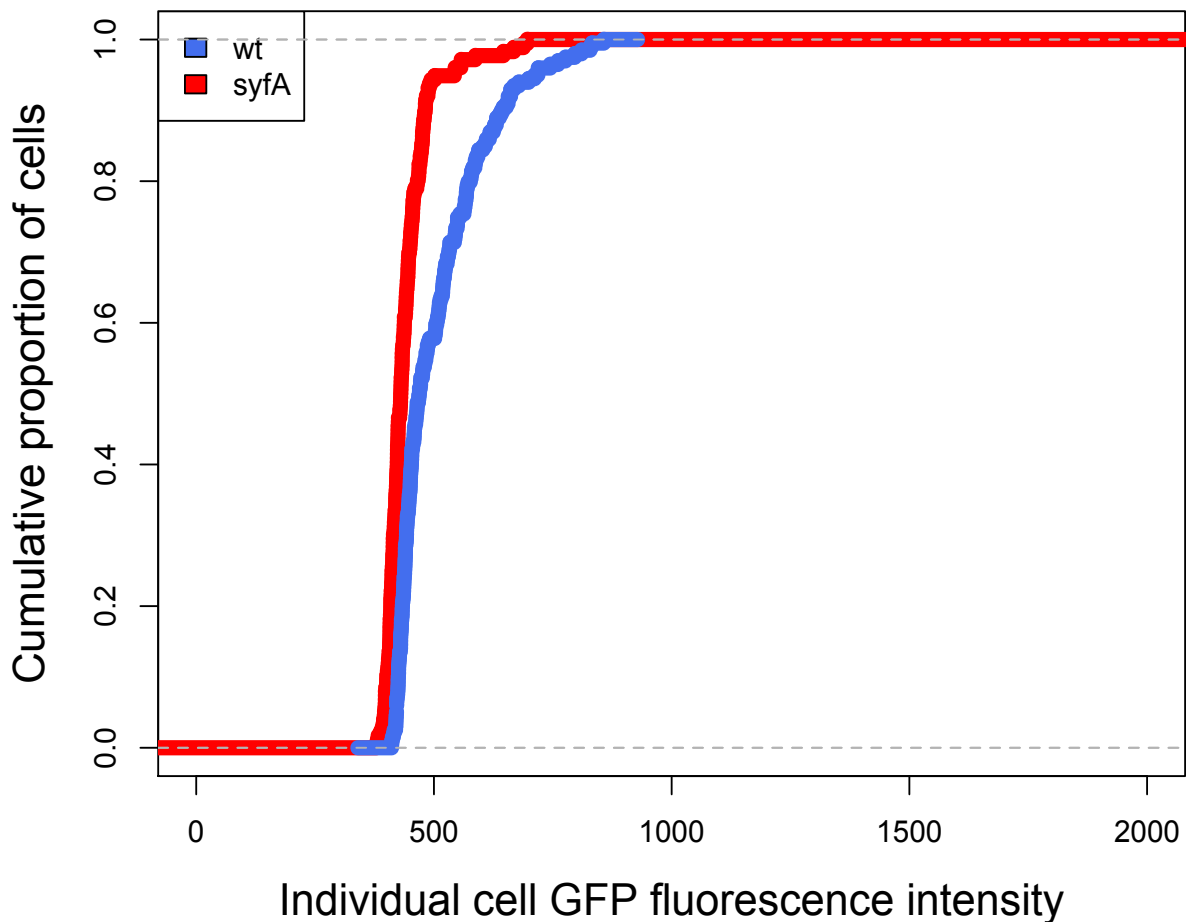
Figure 3. Wild-type used for inoculum exhibit a higher level of GFP fluorescence than the *syfA* mutant cells used for inoculum.
Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter were grown on half-strength 1/2-21C agar plates incubated at room temperature for 24 hours. Cells were harvested with a loop and resuspended in 1 ml of water. 5 µl of each strain was pipetted onto a glass slide and its GFP expression was analyzed under the epifluorescence microscope. The wild-type cells had a sample size of 199, a mean of 508.73, and a median of 469.59. The *syfA* mutant cells had a sample size of 176, a mean of 441.45, and a median of 430.34. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.37097, p-value = 1.37e-11). Additional tests performed were the F-test to compare two variances (p < 2.2e-16, numerator degrees of freedom = 175, denominator degrees of freedom = 198) and Welch's Two Sample t-test (p < 2.2e-16).

Figure 4. On the leaf surface at 100% RH, wild-type cells have more water available than *syfA* mutant cells.

Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter fused with a *gfp* reporter gene were spray inoculated onto bean leaves. Following inoculation, bean plants were immediately placed in a 100% RH chamber for two days. The cells were then washed, filtered, and analyzed for changes in GFP expression under the epifluorescence microscope. The wild-type cells had a sample size of 371, a mean of 457.85, and a median of 450.00. The *syfA* mutant cells had a sample size of 156, a mean of 620.14, and a median of 516.05. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.42707, p-value < 2.2e-16). Additional tests performed were the F-test to compare two variances (p < 2.2e-16, numerator degrees of freedom = 155, denominator degrees of freedom = 370) and Welch's Two Sample t-test (p = 2.813e-09).

Figure 5. Wild-type cells and *syfA* mutant cells experience desiccation when initially exposed to 50% RH on the leaf surface.
Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter fused with a *gfp* reporter gene were spray inoculated onto bean leaves. Following inoculation, bean plants were left on a bench at 50% RH for 20 minutes before being put in the 100% RH chamber for two days. The cells were then washed, filtered, and analyzed for changes in GFP expression under the epifluorescence microscope. The wild-type cells had a sample size of 192, a mean of 507.54, and a median of 486.94. The *syfA* mutant cells had a sample size of 133, a mean of 535.11, and a median of 465.09. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.20528, p-value = 0.002662). Additional tests performed were the F-test to compare two variances ($p < 2.2e-16$, numerator degrees of freedom = 132, denominator degrees of freedom = 191) and Welch's Two Sample t-test ($p = 0.1417$).
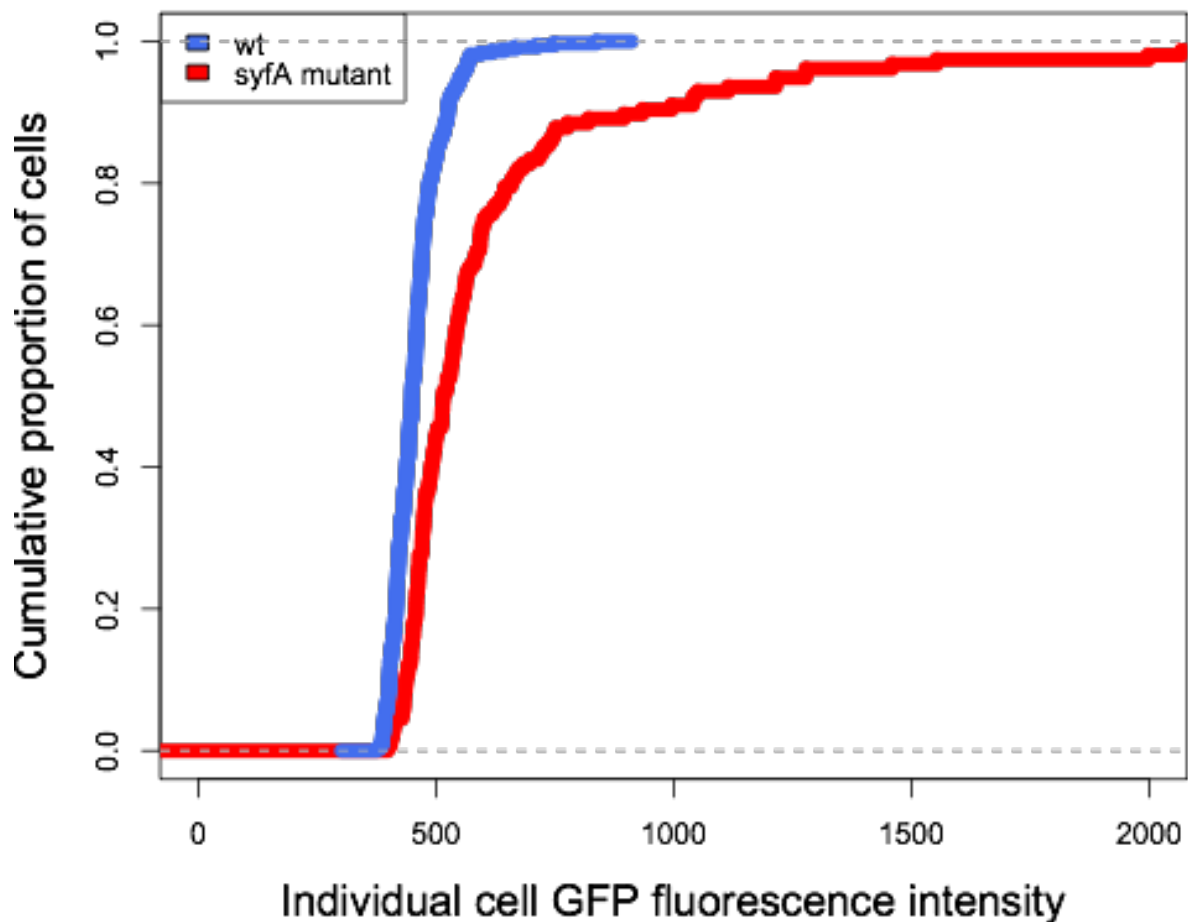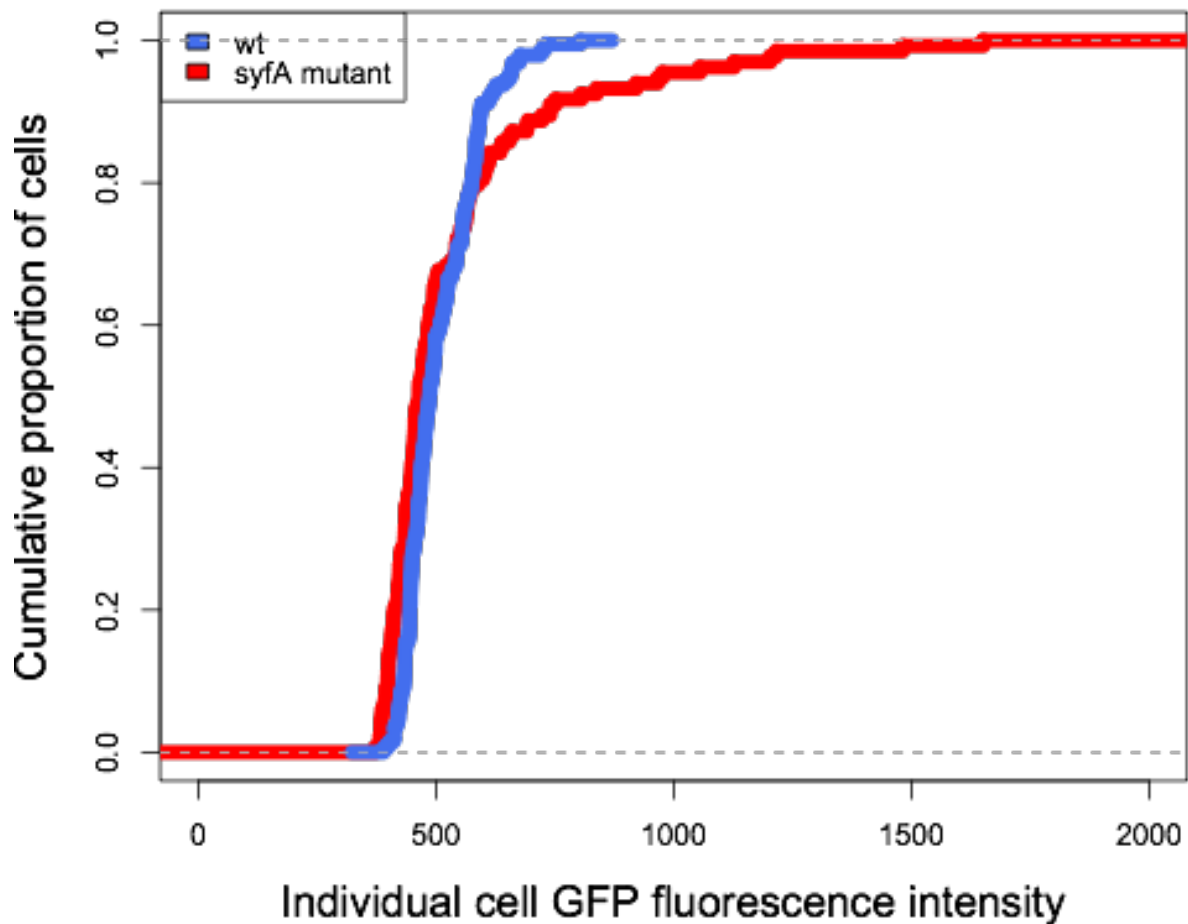
Figure 6. Wild-type cells and *syfA* mutant cells experience desiccation when initially exposed to 50% RH on the leaf surface.

Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter fused with a *gfp* reporter gene were spray inoculated onto bean leaves. Following inoculation, bean plants were left on a bench at 50% RH for 20 minutes before being put in the 100% RH chamber for two days. Plants were then put on the bench at 50% RH for 20 minutes before being put back in the 100% RH chamber for two hours. The cells were then washed, filtered, and analyzed for changes in GFP expression under the epifluorescence microscope. The wild-type cells had a sample size of 186, a mean of 486.29, and a median of 461.30. The *syfA* mutant cells had a sample size of 161, a mean of 628.40, and a median of 502.07. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.25422, p-value = 2.86e-05). Additional tests performed were the F-test to compare two variances (p < 2.2e-16, numerator degrees of freedom = 160, denominator degrees of freedom = 185) and Welch's Two Sample t-test (p = 3.554e-05).

Figure 7. Wild-type cells experience less desiccation stress than *syfA* mutant cells when initially exposed to 100% RH on the leaf surface.

Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter fused to a *gfp* reporter gene were spray inoculated onto bean leaves. Following inoculation, bean plants were placed in the 100% RH chamber for two days. Plants were then put on the bench at 50% RH for 20 minutes before being put back in the 100% RH chamber for two hours. The cells were then washed, filtered, and analyzed for changes in GFP expression under the epifluorescence microscope. The wild-type cells had a sample size of 150, a mean of 1,309.29, and a median of 467.30. The *syfA* mutant cells had a sample size of 282, a mean of 1,942.56, and a median of 1,540.93. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.69291, p-value < 2.2e-16). Additional tests performed were the F-test to compare two variances (p < 2.2e-16, numerator degrees of freedom = 281, denominator degrees of freedom = 149) and Welch's Two Sample t-test (p < 2.2e-16).
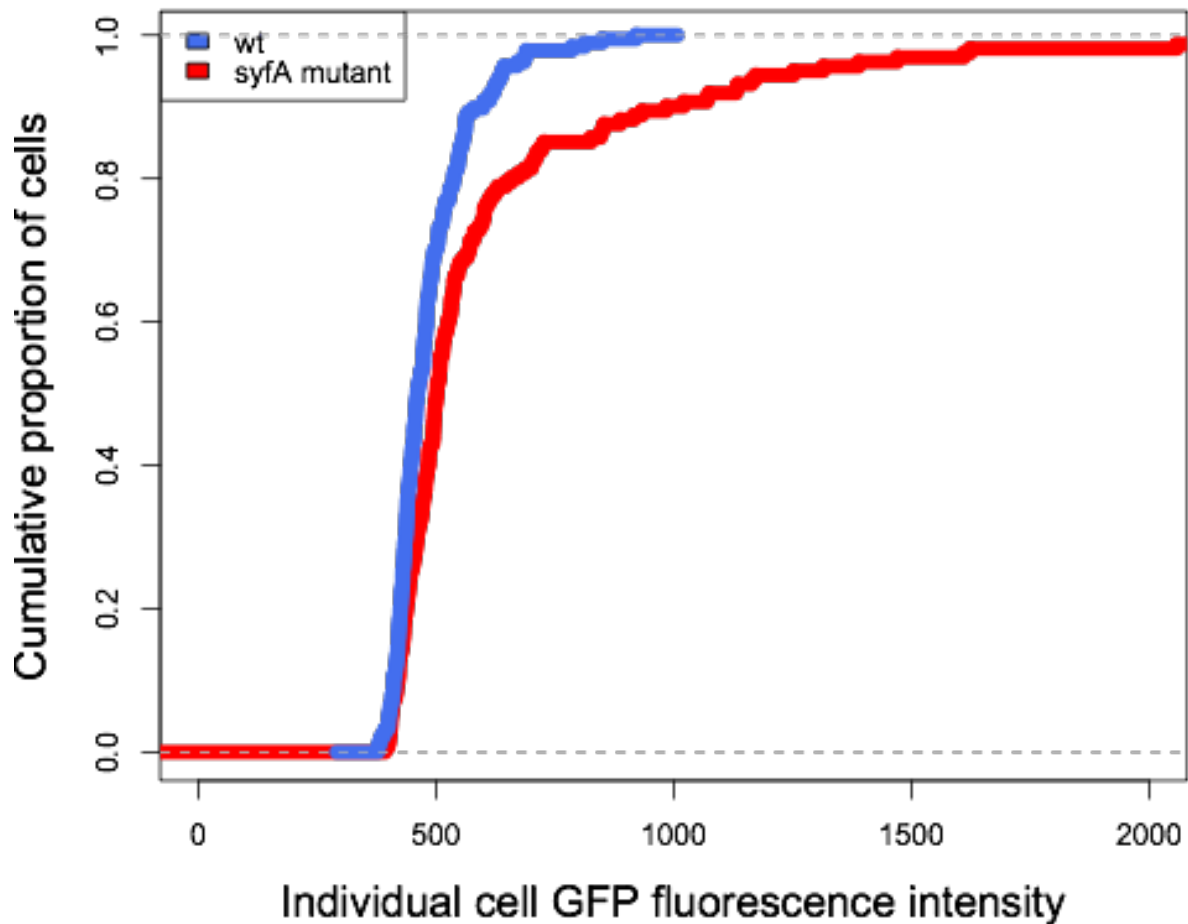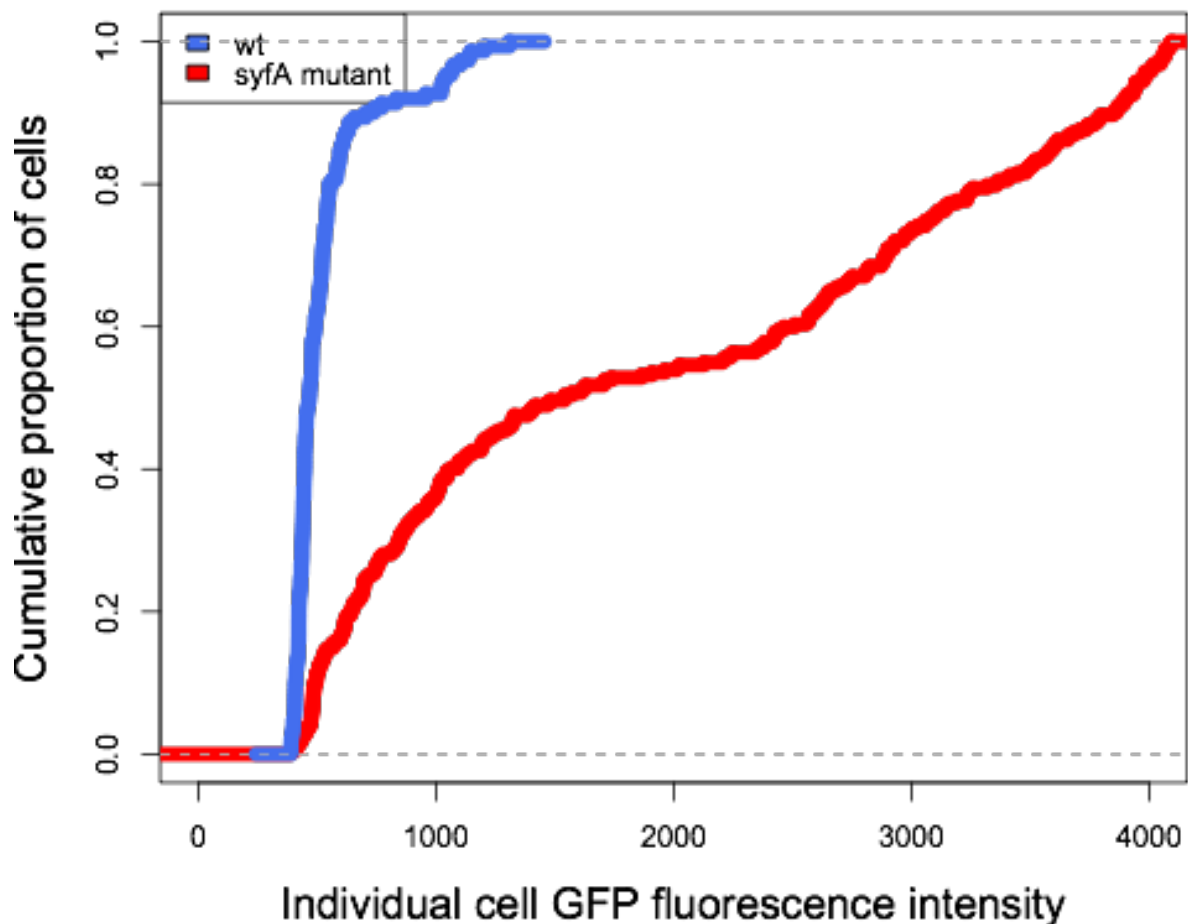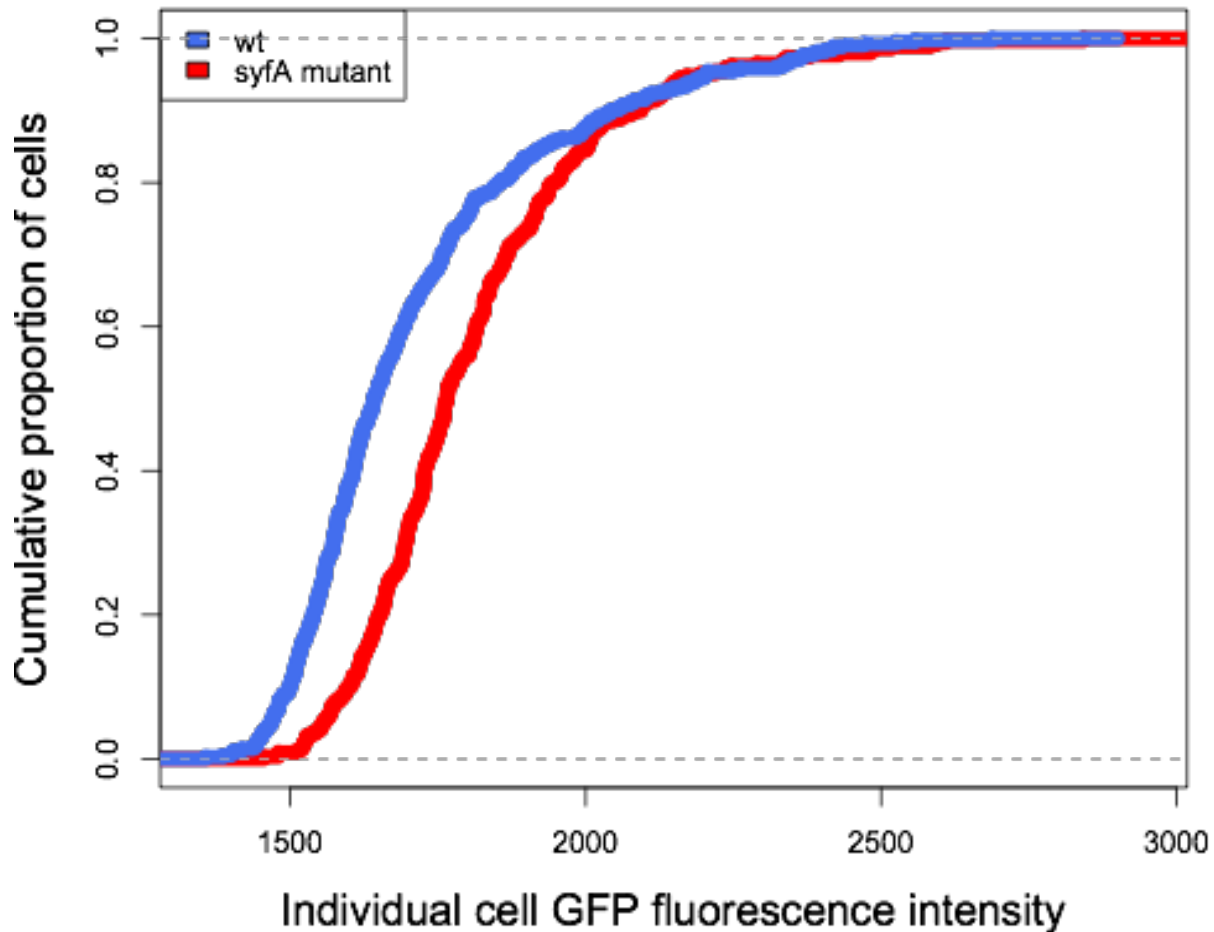
Figure 8. Wild-type cells experience less desiccation stress than *syfA* mutant cells when initially exposed to 100% RH on the leaf surface.
Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter fused with a *gfp* reporter gene were spray inoculated onto bean leaves. Following inoculation, bean plants were placed in the 100% RH chamber for two days. Plants were then put on the bench at 50% RH for an hour before being put back in the 100% RH chamber for two days. The cells were then washed, filtered, and analyzed for changes in GFP expression under the epifluorescence microscope. The wild-type cells had a sample size of 434, a mean of 1,713.15, and a median of 1,641.46. The *syfA* mutant cells had a sample size of 347, a mean of 1,812.62, and a median of 1,765.27. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: $D = 0.32934$, $p < 2.2e\text{-}16$). Additional tests performed were the F-test to compare two variances ($p = 0.03852$, numerator degrees of freedom = 346, denominator degrees of freedom = 433) and Welch's Two Sample t-test ($p = 5.183e\text{-}10$).
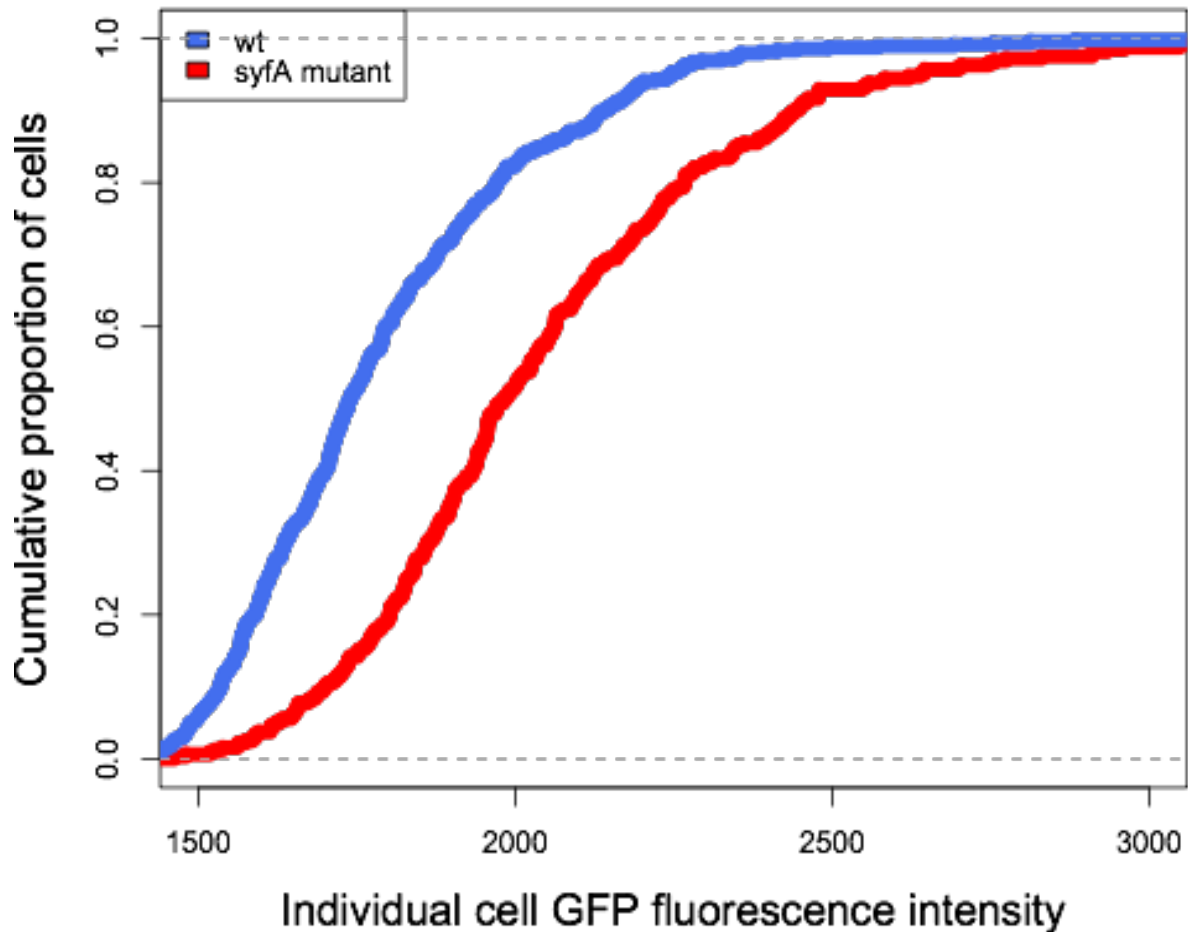
Figure 9. Wild-type cells experience less desiccation stress than *syfA* mutant cells when exposed to 97% RH on the leaf surface.
Wild-type and *syfA* mutant *P. syringae* B728a strains harboring the *proU* promoter fused to a *gfp* reporter gene were spray inoculated onto bean leaves. Following inoculation, bean plants were placed in the 100% RH chamber for two days. Plants were then put on the bench at 50% RH for an hour before being put in the 97% RH chamber for two days. The cells were then washed, filtered, and analyzed for changes in GFP expression under the epifluorescence microscope. The wild-type cells had a sample size of 489, a mean of 1,795.58, and a median of 1,740.89. The *syfA* mutant cells had a sample size of 324, a mean of 2,049.03, and a median of 1,984. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.41296, p < 2.2e-16). Additional tests performed were the F-test to compare two variances (p = 6.083e-07, numerator degrees of freedom = 323, denominator degrees of freedom = 488) and Welch's Two Sample t-test (p < 2.2e-16).
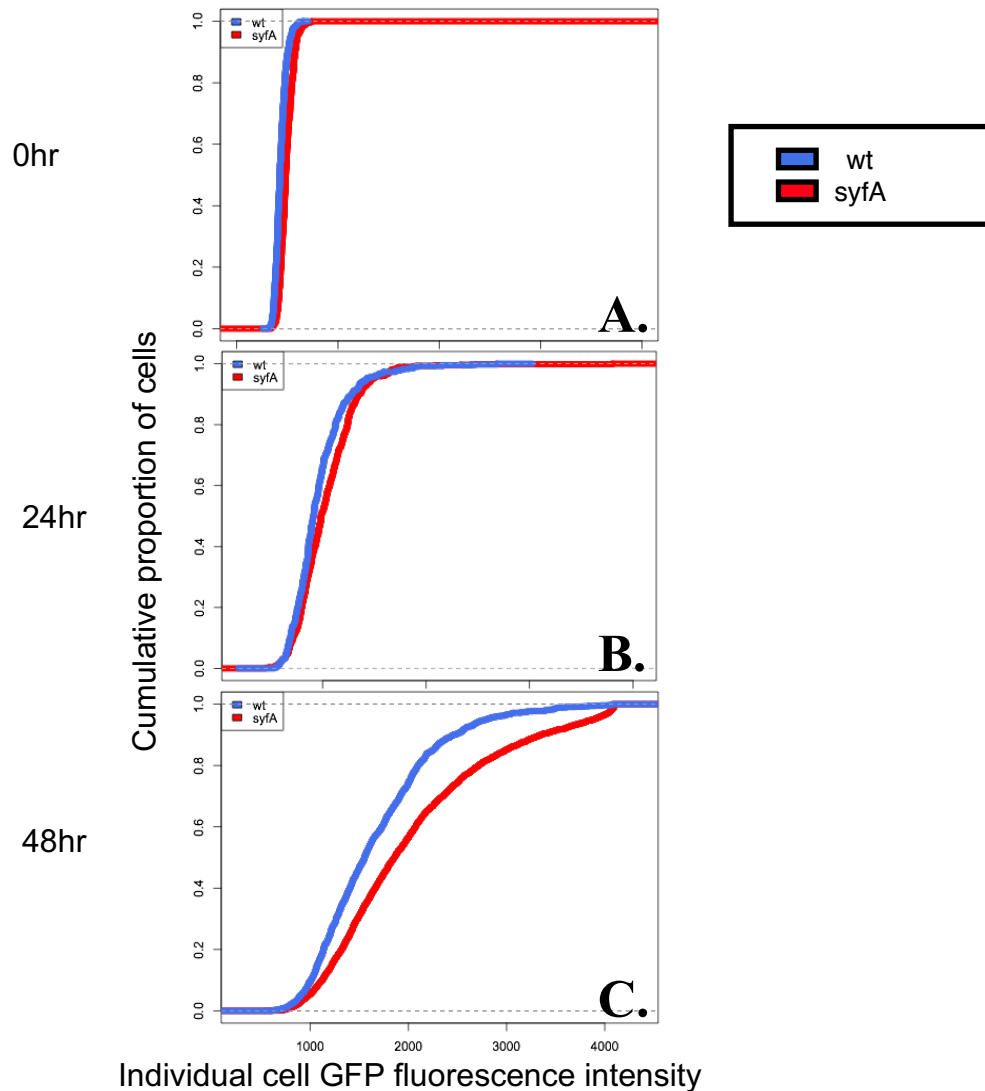
Figure 10. Wild-type cells in the leaf apoplast have more water available than the *syfA* mutant cells at 48hr. (A, B, and C) Cumulative distribution plots were created to display the GFP fluorescence in wild-type and *syfA* mutant cells that were vacuum infiltrated into the apoplasts of bean leaves. The plot shows the percentage of cells on the y-axis as a function of fluorescence on the x-axis. (A) A cumulative distribution plot for wild-type and *syfA* mutant cells at time 0. The wild-type cells had a sample size of 608, a mean of 435.68, and a median of 428.91. The *syfA* mutant cells had a sample size of 441, a mean of 493.17, and a median of 428.91. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.39008, p < 2.2e-16). Additional tests performed were the F-test to compare two variances (p = 3.14e-05, numerator degrees of freedom = 440, denominator degrees of freedom = 607) and Welch's Two Sample t-test (p < 2.2e-16). (B) A cumulative distribution plot for wild-type and *syfA* mutant cells at 24hr post-inoculation. The wild-type cells had a sample size of 406, a mean of 965.69, and a median of 912.66. The *syfA* mutant cells had a sample size of 577, a mean of 1,030.70, and a median of 986.30. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.16279, p-value = 6.537e-06). Additional tests performed were the F-test to compare two variances (p = 0.1123, numerator degrees of freedom = 576, denominator degrees of freedom = 405) and Welch's Two Sample t-test (p = 0.0003635). (C) A cumulative distribution plot for wild-type and *syfA* mutant cells at 48hr post-inoculation. The wild-type cells had a sample size of 1,493, a mean of 1,662.76, and a median of 1,551.92. The *syfA* mutant cells had a sample size of 4,008, a mean of 2,041.35, and a median of 1,857.54. Significance between sample distributions was tested using the Kolmogorov-Smirnov test (wt vs *syfA* mutant: D = 0.18772, p-value < 2.2e-16). Additional tests performed were the F-test to compare two variances (p < 2.2e-16, numerator degrees of freedom = 4,007, denominator degrees of freedom = 1,492) and Welch's Two Sample t-test (p < 2.2e-16).
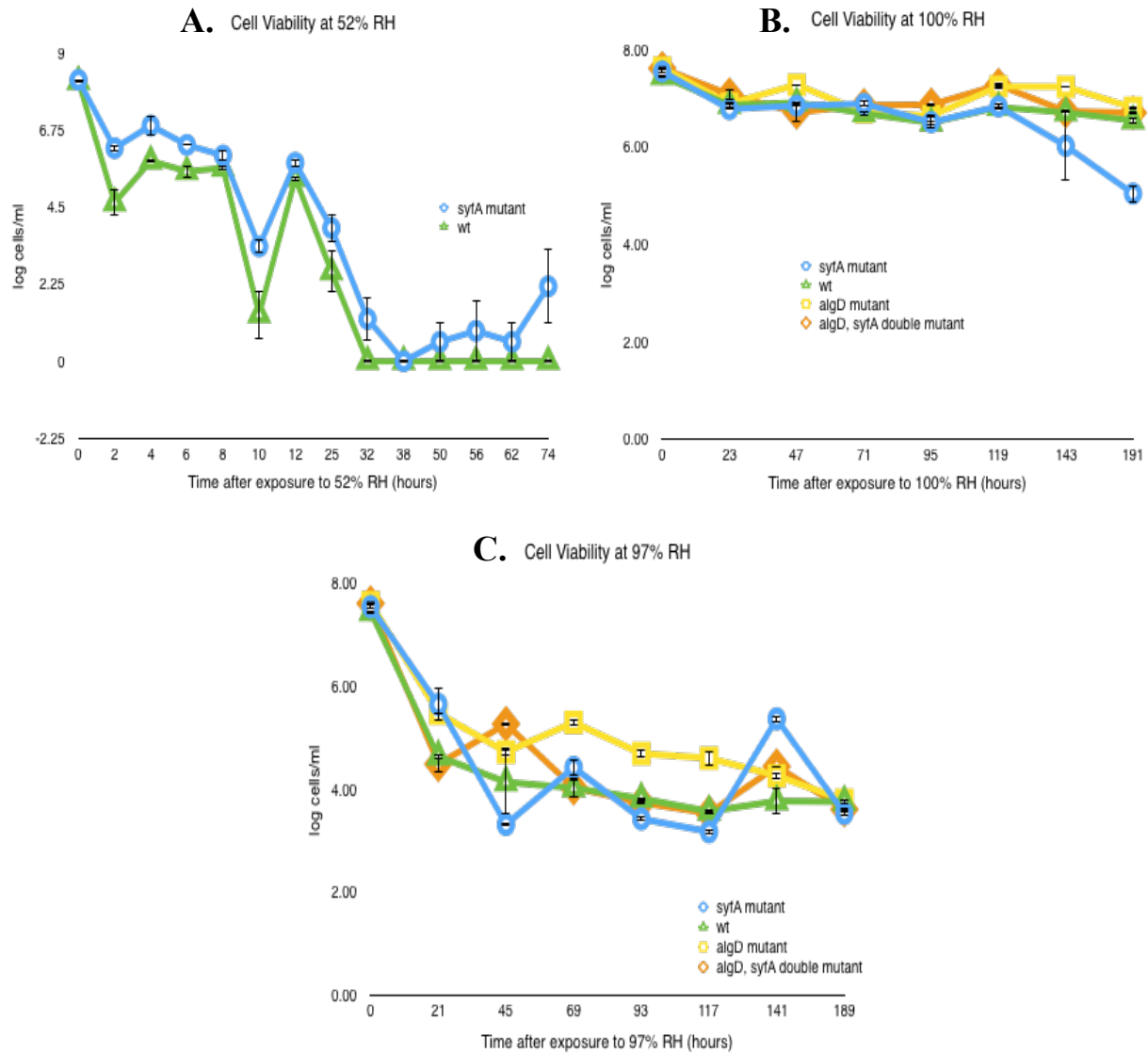
Figure 11. The *algD* mutant is more desiccation tolerant than the other strains when exposed to 97% RH on a filter.

(A, B, and C) A cell viability assay was performed in which wild-type, *syfA* mutant, *algD* mutant, and *algD*, *syfA* double mutant cells of *P. syringae* B728a were grown on KB plates for two days. All strains were resuspended in 1 ml to 2 ml of water to a concentration of 10^6 cells/ml and pipetted onto 0.4 μm Isopore® filters placed on KB agar plates. Plates were left on the bench at room temperature for six hours (eight hours for the experiment at 52% RH). Filters were then exposed to 52% RH, 100% RH, or 97% RH in chambers containing saturated salt solutions. At each timepoint, the filters were added to a microcentrifuge tube containing 1 ml of water. The tubes were vortexed for 20 seconds each and 10-fold serial dilutions were made in water. 20 μl from each dilution was spotted onto a KB plate. All plates were left at room temperature and countable colonies grew after two days. Colonies were counted, and calculations were made to determine the total number of viable cells at each timepoint. The error bars represent the standard errors of the mean number of countable colonies.

Chapter 3

Contact-dependent traits in *Pseudomonas syringae* B728a

Abstract

Production of the biosurfactant syringafactin in the plant pathogen *Pseudomonas syringae* B728a is a surface contact-dependent trait. Cells harboring a reporter gene construct consisting of the promoter of the *syfA* gene encoding syringafactin production fused with promoterless *gfp* exhibited GFP fluorescence at low levels in planktonic cells in liquid cultures but over 4-fold higher in cells immobilized on surfaces as varied as glass, plastic, paper, parafilm, agar, membrane filters, and leaves. Similar levels of apparent GFP expression were observed on these various surfaces. Induction of GFP fluorescence was rapid, occurring within two hours after attachment to surfaces. RNA sequencing revealed that, in addition to *syfA*, 3156 other genes were differentially expressed in cells incubated on the surface of a membrane filter placed on a nutrient agar plate versus living planktonically in a broth culture of the same composition. Genes repressed in immobilized cells included those involved in quaternary ammonium compound (QAC) metabolism and transport, compatible solute production, carbohydrate metabolism and transport, organic acid metabolism and transport, phytotoxin synthesis and transport, amino acid metabolism and transport, and secondary metabolism. Genes induced in immobilized cells included *syfA* plus those involved in translation, siderophore synthesis and transport, nucleotide metabolism and transport, flagellar synthesis and motility, lipopolysaccharide (LPS) synthesis and transport, energy generation, transcription, chemosensing and chemotaxis, replication and DNA repair, iron-sulfur proteins, peptidoglycan/cell wall polymers, terpenoid backbone synthesis, iron metabolism and transport, and cell division. That many genes are rapidly differentially expressed upon transfer of cells from a planktonic to an immobilized state suggests that cells experience the two environments differently. It seems likely that surface contact initiates anticipatory changes in *P. syringae* gene expression, which enables appropriate physiological responses to the new environmental conditions. These responses could help cells survive transitions from aquatic habitats to surfaces such as plant leaves.

Introduction

Bacteria such as *Pseudomonas syringae* have adapted to live in a variety of different environments. While most studies of this taxon have focused on its life as a plant pathogen in which it colonizes the surfaces of leaves and subsequently the apoplast of leaves, many strains of *P. syringae* apparently inhabit other habitats. For instance, Morris *et al.* (2008) found *P. syringae* in snow, rain, and in lakes and rivers, suggesting that this bacterium is disseminated through the water cycle. All bacteria are intrinsically aquatic, although many have adapted to live in aggregates, or more commonly biofilms, which are attached to surfaces in contact with liquid environments. The very different chemical and physical properties of aquatic environments versus surfaces, which in the case of *P. syringae* are frequently dry, select for coordinated gene expression that optimizes fitness across the different conditions. Leaf surfaces are relatively harsh environments that exhibit strong spatially and temporally varying conditions. Water and nutrients are unevenly dispersed across leaf surfaces (Leveau and Lindow, 2001; Lindow and Brandl, 2003) and leaves experience rapid temporal fluctuations in temperature, humidity, and liquid water availability (Lindow and Brandl, 2003). Leaf surfaces also experience high

ultraviolet radiation flux (Lindow and Brandl, 2003). In a bacterial population inhabiting such a setting, some cells might exhibit a planktonic existence within water drops containing nutrients, while other cells must contend with relatively dry surfaces.

I have shown (Chapter 2) that *P. syringae* strain B728a produces syringafactin, a hygroscopic biosurfactant whose production is encoded by the *syfA* gene, as one adaptation to the frequent absence of liquid water on leaf surfaces (Berti *et al*., 2007; Burch *et al*., 2014). By binding water vapor trapped within the humid boundary layer and/or by binding liquid water that is occasionally and transiently present on leaves, syringafactin can alleviate water stress by expanding the conditions under which liquid water is available for *P. syringae*. Furthermore, syringafactin does not seem to disperse in water, perhaps due to its strong hydrophobic characteristics, but instead remains immobilized onto leaf surfaces near the cells that produced it. Thus, producing syringafactin is unlikely to benefit cells immersed in water, such as those occurring in the planktonic state in aquatic environments involved in the water cycle (Morris *et al*., 2008). Instead, syringafactin production might only benefit the cells that produce it in non-aquatic settings where it would improve *P. syringae* survival on surfaces - particularly those that are subject to frequent drying.

Interestingly, Burch *et al*. (2011) found that physical contact strongly regulates *sfA* expression. Cells of *P. syringae* B728a harboring a plasmid-borne *syfA* promoter fused to a promoterless *gfp* reporter gene exhibited much higher GFP fluorescence when grown on solidified medium than when grown in a similar liquid broth culture. In support of the hypothesis that syringafactin is a biosurfactant, Burch *et al*. (2011) found that water droplets on an oil surface would collapse when cells grown on agar plates were applied to the surface, whereas applying cells grown in broth culture did not have this effect. Moreover, a *syfA* mutant grown on agar surfaces produced the same effect on water droplets as did normal cells grown in broth cultures. Thus, cells produced syringafactin only when grown on an agar surface. This observation suggests two hypotheses. First, *syfA* gene expression may be regulated by the different chemical or physical conditions found on an agar surface versus a similar broth culture. Alternatively, immobilization of cells on the surface may denote the current or anticipated presence of environmental conditions common to leaf surfaces. Since Burch *et al*. (2011) examined *syfA* expression only after growth on agar plates for 12 hours or more, they could not unambiguously distinguish between these two models of gene regulation.

To distinguish between these two hypotheses, I determined how quickly *syfA* expression increased after planktonic cells of *P. syringae* contacted a surface. As discussed above, it seemed likely that cells of *P. syringae* might benefit from anticipatory patterns of gene expression, where certain cues might be used to indicate a large and rapid change in environmental conditions. Thus, early or prior expression of certain traits would be highly beneficial in adapting to the large change in habitat. In this case, I predicted that changes in gene expression associated with transition from a planktonic to a surface-associated habitat such as a leaf would be linked to perception of a surface by the bacterial cells. While there has been interest in such surface sensing in bacteria, little is known of the mechanism by which cells sense surfaces (Chang *et al*., 2017; Kearns, 2010; O'Toole and Wong, 2016; Petrova and Sauer, 2012; Tuson and Weibel, 2013) and there are few studies that have examined the rapid changes in gene expression associated with perception of a surface (Bhomkar *et al*., 2010). As I will demonstrate here, I have explored the rapid changes in the global transcriptome of B728a soon after planktonic cells

became sessile on a surface. The identified large changes in gene expression associated with the transition from planktonic to sessile life suggest that surface sensing could be a major cue controlling adjustment of *P. syringae* to the leaf surface.

Results

SyfA induction occurs rapidly on a surface

If the *syfA* gene is truly regulated via contact with a surface, I would expect gene induction to occur shortly after surface contact. To test how rapidly *syfA* was induced after planktonic cells were transferred to various surfaces, I monitored the transcription of *syfA* in *P. syringae* B728a harboring a plasmid containing the *syfA* promoter fused to a promoterless *gfp* reporter gene. By assessing the GFP fluorescence of individual cells by epifluorescence microscopy, I could detect induction of the *syfA* promoter at different time points. *P. syringae* B728a cells were grown in broth cultures to a cell concentration of about 10^8 cells/ml. Cells from these cultures were applied to three environments: (1) the surface of agar-solidified King's medium B (KB) media, (2) a 0.4 µm polycarbonate Isopore® filter that was then placed on such an agar surface, and (3) a 0.4 µm polycarbonate Isopore® filter on the surface of a small quantity of KB broth within a plastic petri dish. Planktonic cells in broth were monitored as a temporal control. Presumably, cells were exposed to the same nutrients in all four settings, but experienced different physical conditions. I compared gene expression of cells on Isopore® filters placed on KB agar and KB broth to test whether chemical components of the agar, rather than simply the physical change it elicited, contributed to induction of *syfA* expression as seen by Burch *et al*. (2011). By two hours after transfer to the solid surfaces of the KB agar and Isopore® filters, cells in contact with a surface exhibited significantly greater GFP fluorescence than cells in liquid culture (Figure 1), which shows that *syfA* induction occurs rapidly after cell contact with a surface. Further, similar levels of *syfA* induction occurred on all surfaces. Thus, *syfA* induction on agar appears to be due to a physical change as opposed to any chemical components of the agar.

SyfA is induced by a variety of solid surfaces

Given that *syfA* induction quickly followed transfer of planktonic cells to various surfaces, I explored whether any particular physical parameter could be associated with the induction process by examining how induction varied among surfaces that differed in hydrophobicity and other features. In particular, given that *P. syringae* B728a was first isolated from leaf surfaces (Loper and Lindow, 1987), I tested the hypothesis that leaf surfaces would confer the most rapid or largest contact-dependent induction of this gene. By exposing cells to various types of solid surfaces I also continued to explore whether *syfA* expression was regulated by contact with a surface per se as opposed to a chemical cue. For this experiment, GFP fluorescence was evaluated in *P. syringae* B728a cells harboring the *syfA* reporter gene construct while growing planktonically in KB broth or on surfaces of glass, plastic, paper, parafilm, an excised leaf, agar, and Isopore®, Durapore®, and Teflon® filters. After six hours of incubation, cells applied to the various solid surfaces all exhibited at least 2-fold greater GFP fluorescence than those of the planktonic cells, which exhibited little GFP fluorescence (Figure 2). Little difference in GFP fluorescence was observed among the various solid surface treatments, suggesting that there are probably not strong surface type-dependent chemical cues for induction of *syfA* nor a strong effect of the physical properties of the surface on which cells were immobilized.

RNA sequencing of P. syringae B728a reveals many genes differentially regulated in cells grown on a filter surface versus in liquid culture

Cells exhibited large increases in *syfA* expression within two hours of transfer to a variety of solid surfaces, which suggests that contact-dependent gene expression occurs rapidly, I performed RNA sequencing to determine the extent in which other genes in *P. syringae* B728a exhibit contact-dependent gene expression. For this experiment, the wild-type *P. syringae* B728a strain was grown in broth cultures to a density of 5 x 10^8 cells/ml before aliquots of cells were applied to the surface of 0.4 μm Isopore® membrane filters. The liquid in which the cells had been applied in was removed by applying a vacuum beneath the filters for a few seconds, after which the filters were placed onto the surface of KB agar. As a control, KB broth cultures continued to be incubated with shaking. All cells were incubated at 28°C for two hours before harvesting. After stabilizing RNA, cells were harvested and RNA was isolated and sequenced after reverse transcription.

Three replicates per treatment were submitted for RNA sequencing with labels 1F – 3F denoting the three replicates in which cells were incubated on filters for two hours and labels 1L – 3L denoting the three replicates in which cells were incubated in liquid. RNA sequencing returned over 40 million reads per sample except for samples 3F and 3L which had roughly 20 million and 30 million reads, respectively (Figure 3). Individual genes in each sample also varied in terms of reads per gene, with some genes having more than 1 million reads (Figure 4). A multidimensional scaling (MDS) analysis revealed that replicates 1F – 2F were more similar to one another than to replicates 1L – 3L. However, replicate 3F appeared to differ from replicates 1F – 2F and replicates 1L – 3L (Figure 5). Therefore, except for replicate 3F, this pattern shows that nearly all of the variation in relative expression levels of the average gene was associated with the environment of the cells before RNA was harvested rather than other factors.

Using the p-values of each gene returned from RNA sequencing, I tested whether I could reject the null hypothesis of having an equal distribution of p-values for most of the differentially expressed genes. Among the differentially expressed genes, the differences in relative expression levels between immobilized cells and planktonic cells was highly significant (Figure 6), indicating that for a large proportion of the genes the null hypothesis could be rejected. Expression of 1,390 genes was up-regulated on filter surfaces compared to broth, while expression of 1,766 genes was down-regulated on filter surfaces compared to that in broth. Of the 1,390 genes that were induced on filter surfaces, 881 were induced more than 2-fold while 509 were induced less than 2-fold. Of the 1,766 genes that were repressed on filter surfaces, 1,138 were found to be repressed more than 2-fold while 628 were found to be repressed less than 2-fold. In total, 3,156 genes (60.46% of the *P. syringae* B728a genome) were found to be differentially expressed on the filter surface compared to in the broth culture (Figures 7 and 8). Moreover, a heatmap revealed that while gene regulation was similar among replicates in which cells were in liquid or immobilized on a filter, gene expression differed strongly between treatments (Figure 9). This finding supports that there were more significant differences between the two treatments compared to the differences among replicates.

Functional category analysis

I grouped the differentially regulated genes into functional categories to better establish what processes were potentially more highly expressed on filter surfaces and vice versa. Table 1 shows the functional gene categories that were significantly enriched with genes up-regulated on filters while Table 2 shows those functional gene categories that were significantly enriched with genes down-regulated on filters. Twice as many functional gene categories were enriched in genes significantly up-regulated on the filters than in genes that were down-regulated on the filters. The following gene functional categories were examined in further detail:

Translation. Many genes encoding the 30S and 50S ribosomal protein subunits were induced on the filter surface, as were genes encoding the elongation factor proteins Ts, P, Tu, and G. Many genes encoding for t-RNA synthetases were induced as well. It thus appeared that translation as a whole may have accelerated upon transition of cells from a planktonic to an immobilized state.

Siderophore synthesis and transport. Many genes involved in siderophore synthesis and transport were induced on the filter surface. Many of these genes were involved in pyoverdine regulation and transport including *pvdS, pvdG, pvdL, pvdI, pvdJ, pvdK, pvdD, pvdE, pbdO, pvdN, pvdT*, and *pvdR*. Many genes involved in achromobactin regulation, synthesis, and transport including *acsG, acsD, acsE, yhcA, acsC, acsB, acsA, carA-2, cbrB-2*, and *cbrC-2* were also induced. These results suggest that iron became less available on the filter surfaces than in broth, perhaps due to diffusional limitations associated with the lack of mixing of cells in a liquid medium. Alternatively, iron might commonly be less available on the natural surfaces on which *P. syringae* typically inhabits, such as leaf surfaces, and the filter mimicked physical cues that the bacteria might use to anticipate transition into such low iron environments.

Nucleotide metabolism and transport. Numerous genes involved in nucleotide metabolism and transport were induced on the filter surface. This included many genes involved in purine and pyrimidine metabolism such as *purA, purT, purC, purF, purB, purM, purN, purU-3, purH, purD, purK, purE*, and *pyrB, pyrR, pyrH, pyrG, pyrF, pyrD, pyrC-2* respectively. As with the apparent increase in translational activity seen upon transition of planktonic cells to those on surfaces, increased transcription might be expected to also be linked to such increases, requiring higher rates of nucleotide synthesis.

Flagellar synthesis and motility. Many genes encoding flagellar biosynthesis proteins, flagellar basal body proteins, and flagellar hook-associated proteins were induced on the filter surface. The gene encoding the anti-sigma-28 factor FlgM was also induced more than 2-fold. Genes encoding the flagellar motor proteins MotA, MotB, MotC, and MotD were induced as well. While it would be expected that planktonic cells of *P. syringae* would be motile, higher levels of motility on leaves compared to that in broth cultures has been previously noted (Yu *et al.*, 2013), and would likely require higher levels of flagellar production and repair (Kearns, 2010).

Lipopolysaccharide synthesis and transport. All the genes with significant differential expression involved in lipopolysaccharide (LPS) synthesis and transport were induced on the filter surface except for *arnB* and *arnA*. Most of the induced genes were involved in LPS transport and lipid A biosynthesis.

Energy generation. Many genes encoding proteins involved in oxidative phosphorylation were expressed at a higher level on filter surfaces than in broth cultures. Such genes included *cyoA*,

cyoB, *cyoC*, and *cyoD*, all of which encode cytochrome c oxidase subunits, as well as *ccoN*, *ccoO*, and *ccoP* which encode cytochrome c oxidase cbb3-type subunits. Many genes encoding F0F1 ATP synthase subunits were induced as well.

Transcription. Similar to that seen for nucleotide synthesis, nearly all of the genes involved in transcription that were differentially transcribed were induced on filters compared to that in broth cultures, with the exception being Psyr_4263. Genes up-regulated on surfaces included those encoding the transcription termination factor Rho, the transcription elongation factors GreB and GreA, the transcription anti-termination proteins NusB and NusG, and the DNA-directed RNA polymerase subunits RpoA, RpoC, and RpoB.

Chemosensing and chemotaxis. Many genes involved in chemosensing and chemotaxis appeared to be induced on the filter surface. Many of the induced genes encoded histidine kinases. Interestingly, Psyr_1306, Psyr_1307 and Psyr_1308 which encode homologs to WspD, WspE, and WspF respectively were all down-regulated on the filter surface. Psyr_1309, which encodes the homolog for WspR, was also down-regulated. However, this latter gene was assigned to the cyclic diguanylate (cyclic di-GMP) cyclase proteins functional group.

Replication and DNA repair. Overall, genes involved in replication and DNA repair were induced on the filter surface. This included Pysr_1408, Psyr_1409, and Psyr_1410 which encode RuvC, RuvA, and RuvB respectively. These genes are also involved in homologous recombination in addition to DNA repair.

Iron-sulfur proteins. Many genes encoding iron-sulfur proteins were induced on the filter surface. This included *dsbE* which is involved in cytochrome synthesis.

Peptiodoglycan/cell wall polymers. All of the genes involved in encoding peptidoglycan/cell wall polymers that had significant differential expression on filters compared to broth cultures were induced on the filter surface. Most of these genes were involved in peptidoglycan biosynthesis.

Terpenoid backbone synthesis. Many genes involved in terpenoid backbone synthesis were induced in cells applied to filter surfaces. This included genes that are part of the deoxyxylulose pathway of terpenoid biosynthesis.

Iron metabolism and transport. Many genes involved in iron metabolism and transport were induced on the filter surface. This included the genes *fecE*, *fecD*, *fecC*, *fecB*, *fecA*, *fecR*, and the RNA polymerase ECF sigma factor *fecI*.

Cell Division. Almost all genes with significant differential expression involved in cell division were induced on the filter surface. This included the cell division proteins FtsK, FtsQ, and FtsL as well as the rod-shape determining proteins MreD and MreC. MrdB, a cell cycle protein, was also induced.

Quaternary ammonium compound metabolism and transport. Surprisingly, all genes with significant differential expression involved in quaternary ammonium compound (QAC) metabolism and transport were repressed on the filter surface. This included genes encoding

proteins involved in glycine betaine, choline, and carnitine metabolism and transport. The gene *betI*, which is the transcriptional regulator of choline degradation, was also down-regulated. Such compounds contribute compatible solutes that are part of the cellular response to water stress. It was unexpected that cells on a filter surface would not benefit from compatible solutes and induce their expression.

Compatible solute synthesis. Similar to that seen for genes involved in QAC metabolism, all of the genes that had significant differential expression that are involved in compatible solute synthesis were repressed on the filter surface. Many of these genes contribute to either trehalose or N-acetylglutaminylglutamine amide (NAGGN) synthesis.

Carbohydrate metabolism and transport. Many of the genes involved in carbohydrate metabolism and transport were repressed on the filter surface. This included genes involved in trehalose, mannose, fructose, ribose, arabinose, maltose, manitol, and sorbitol transport as well as genes involved in the pentose phosphate pathway.

Organic acid metabolism and transport. Many of the genes involved in organic acid metabolism and transport were also repressed on the filter surface. This included the genes *phnF*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, *phnM*, *phnN*, and *phnP* which are all involved in phosphonate metabolism and transport. The transcriptional regulator of vanillate metabolism, *vanR*, was also down-regulated.

Phytotoxin synthesis and transport. All of the significantly differentially expressed genes involved in phytotoxin synthesis and transport were repressed in cells applied to filters. These genes included *salA*, which is the regulator of syringomycin, as well as *slyA*, the regulator of syringolin A production. All other genes were involved in syringolin synthesis and transport (*slyB*, *slyC*, *slyD*, and *slyE*), syringomycin synthesis and transport (*syrE*, *syrC*, *syrB1*, *syrP*, and *syrD*), and syringopeptin synthesis and transport (*sypA*, *sypB*, and *sypC*). Syringomycin and syringopeptin secretion proteins PseA and PseB were also down-regulated.

Amino acid metabolism and transport. Many of the genes involved in amino acid metabolism and transport were repressed on the filter surface. This included genes involved in gamma-aminobutyric acid (GABA) metabolism (*gabT-2*, *gabD-2*, *gabT-1*, *gabD-1*, *gabD-3*, and *gabP*).

Secondary metabolism. All of the significantly differentially expressed genes involved in secondary metabolism were repressed on the filter with the noteworthy exception of Psyr_2575, Psyr_2576, and Psyr_2577 which encode SyfR, SyfA, and SyfB, responsible for the regulation of and production of syringafactin, respectively. It was therefore intriguing to find that syringafactin production was the sole example of secondary compounds that were not down-regulated when cells transitioned from a planktonic to a sessile state.

Discussion

The remarkably strong and rapid induction of *syfA* in *P. syringae* B728a in cells transferred from broth culture to any of several different types of surfaces encouraged me to test the hypothesis that a variety of traits in this species would exhibit similar surface-dependent changes in gene expression. The timing and conditions under which *syfA* induction occurred makes it unlikely

that cells modified their local microhabitat in any substantial way. Assessing changes in transcription within two hours of transfer to the solid surface ensured that I was observing contact-specific gene regulation. This makes my analysis different from that of previous approaches, which typically examined bacteria many hours after attachment, usually after a thick biofilm had formed on the surface (Dötsch *et al.*, 2012; Hickman *et al.*, 2005; Prigent-Combaret *et al.*, 1999). By examining regulatory shifts that occurred shortly after contact with a surface, I could isolate initial responses to surfaces from those conditioned by secondary events such as cell-cell contact or cell density-dependent regulatory processes. Given the short exposure of planktonic cells to the filter surface, I was surprised that the analysis revealed differential expression between liquid and solid environments of such a high proportion of the genes in *P. syringae* B728a. In addition to induction of the syringafactin synthesis and regulatory genes *syfA*, *syfB*, and *syfR*, other genes that were differentially expressed were involved in flagellar synthesis and motility, LPS synthesis and transport, chemosensing and chemotaxis, siderophore synthesis and transport, and DNA replication and repair. While coherent arguments could be made for why some of these processes should exhibit contact-dependent expression, the responses of many other processes remain enigmatic.

The fact that many genes involved in the synthesis of peptiodoglycan/cell wall polymers were up-regulated in the presence of surfaces suggests that, like those two other organisms for which surface contact-dependent gene expression has been investigated (Otto and Silhavy, 2002; Siryaporn *et al.*, 2014), *P. syringae* B728a may have a mechanosensitive pathway for sensing a surface. The processes most affected by surface attachment are further described below.

One process that was enriched in surface-induced genes was translation (Table 1). Given that there is a large number of genes whose transcription increases rapidly upon transitioning from a planktonic to sessile state (Figures 7 and 8), this change in overall metabolic processes would require genes involved in translation to become more expressed in order to produce the proteins encoded by the newly induced genes. Other similar processes that were enriched in surface-induced genes were nucleotide metabolism and transport, transcription, and replication and DNA repair (Table 1). As with the apparent increase in translational activity seen upon transition of planktonic cells to those on surfaces, I would expect to see increased transcription to also be linked to such increases, requiring higher rates of nucleotide synthesis. These increases in transcription and nucleotide synthesis would then explain the induction of genes involved in DNA replication since this process would be closely linked to nucleotide metabolism and also play an important role in the transcription of surface-induced genes.

Studies have suggested that cells on leaf surfaces and in the apoplast might experience oxidative stress as a defense response from the plant (Dublan *et al.*, 2014; Fones and Preston, 2012; Kyle *et al.*, 2008). Compounds such as OH radicals, which are created when iron reacts with H_2O_2, can be detrimental to compounds containing iron-sulfur proteins and to cellular DNA (Kyle *et al.*, 2008). Given this, genes involved in iron-sulfur protein synthesis and in DNA repair would be expected to be induced - as I observed here.

Another group of processes enriched in surface-induced genes were siderophore synthesis and transport and iron metabolism and transport (Table 1). Genes in these processes are typically activated under conditions of low iron availability (Yu *et al.*, 2013), which suggests that iron was less available on the filter surfaces than in broth media. Perhaps reduction from a three-

dimensional liquid to two-dimensional surface environment created diffusional limitations. Or, cells fixed to a surface might be unable to move in response to local resource depletion (Bhomkar *et al*., 2010). Alternatively, iron might commonly be less available on the natural surfaces that *P. syringae* typically inhabits, such as leaves, and immobilization on the filter might have mimicked physical cues bacteria might use to anticipate transition into such low iron environments.

Many genes involved in flagellar synthesis and motility and chemosensing and chemotaxis were surface-induced (Table 1). Products of these genes would be important when colonizing leaves with heterogeneously dispersed nutrients on their surface (Leveau and Lindow, 2001). These genes enable motility and chemosensing, both necessary for bacteria to sense and move towards these resources. Moreover, pathogenic bacteria such as *P. syringae* usually eventually colonize the leaf apoplast - a process requiring cells to move towards a stomate or crack in the leaf surface to access this habitat (Melotto *et al*., 2008). The up-regulation of motility and chemotaxis genes was also observed in a study examining the transcriptome of *P. syringae* B728a on the leaf surface and apoplast (Yu *et al*., 2013). Direct support for the importance of cell motility of *P. syringae* on leaves was provided by studies of Haefele and Lindow (1987) who showed that non-motile mutants were much less fit.

Two other functional categories enriched in surface-induced genes were LPS synthesis and transport and peptidoglycan/cell wall polymers (Table 1). Previous studies have suggested that cells experience cell wall damage when they contact a surface (Chang *et al*., 2017; Otto and Silhavy, 2002). Peptidoglycan and cell wall synthesis genes might be induced to repair any damage that resulted from such an encounter. Moreover, the bacterial outer membrane also contains abundant lipopolysaccharides that could also be disrupted during physical binding of cells to a hard surface, which could also make LPS synthesis important (Kearns, 2010; Wood *et al*., 2006; Wood and Ohman, 2009). Interestingly, this initial cell wall stress may also be a cue for the differential expression of other surface-regulated genes as noted above - suggesting that *P. syringae* B728a may possess a mechanosensitive pathway for sensing a surface (Chang *et al*., 2017).

Surprisingly, genes involved in QAC metabolism and compatible solute synthesis tended to be repressed on the surface (Table 2). Such compounds often serve as compatible solutes whose purpose is to maintain equilibrium between the inside of the cell and the outside environment. Thus, genes involved in compatible solute synthesis would be expected to be up-regulated in cells experiencing osmotic or matric stresses (Yu *et al*., 2013). Previous studies have suggested that the leaf surface is frequently sufficiently dry that compatible solute synthesis would be needed in order to combat matric stress (Axtell and Beattie, 2002; Beattie, 2011). However since this experiment was performed on a filter on agar, the water status of cells upon such a surface is uncertain. While one might imagine such a surface to be drier than what cells suspended in a broth medium might experience, it is possible that this would be a moister environment than the leaf surface. The disruption of the gel matrix that would occur by application of a filter on an agar surface might release free water. Indeed – filters immediately appear wet when applied to such a surface. While the leaf surface is composed of a waxy, hydrophobic cuticle to prevent the release of water vapor (Hess and Foy, 2000; Hirano and Upper, 2000), a membrane filter is by definition quite porous and would enable the movement of water onto the filter surface where it could wet cells.

Genes encoding carbohydrate metabolism and transport, organic acid metabolism and transport, and amino acid metabolism and transport were mostly repressed on filter surfaces (Table 2). The down-regulation of these genes is an unexplained puzzle. While I expected nutrient movement from the agar matrix through the filter, it is likely that the rate at which nutrients are replenished by this diffusional process would be slower than that occurring during mixing of cells in a planktonic state in a shaken broth medium. It might thus be expected that the immobilized cells could experience a locally more nutrient-limited environment than those of planktonic cells. The common observation of slower growth of bacteria on the surface of membranes placed on agar media than in shaken broth of the same composition also supports such a model. In this manner, one might expect up-regulation of nutrient transporters – similar to the model developed earlier to explain the apparent iron limitation of cells and thus the stimulation of siderophore biosynthesis. Interestingly it has been noted in other studies that genes involved in amino acid metabolism and transport were generally repressed on the leaf surface, but the reason for this is still unknown (Yu *et al.*, 2013).

Surprisingly, genes involved in phytotoxin synthesis and transport as well as secondary metabolism were generally repressed on the filter surface (Table 2). Such compounds often act as virulence factors when cells encounter hosts. I expected that contact with a surface would cue cells of *P. syringae* that they have encountered a potential host plant after transitioning from a planktonic existence. However, it is thought that the large majority of *P. syringae* cells exist as epiphytes on the surface of leaves rather than in the apoplast during infection. Thus, when *P. syringae* colonizes the leaf surface it is not pathogenic and would not be expected to benefit from the production of various toxins. Rather, *P. syringae* would benefit from producing toxins in the process of causing disease once it has entered the leaf apoplast and reached a high cell density (Melotto *et al.*, 2008). Given this, it seems that genes involved in phytotoxin synthesis and secondary metabolism would be expressed in the leaf apoplast rather than on the leaf surface. I hypothesize that a secondary signal, indicative of conditions within the leaf, would be required for expression of such virulence factors. Such a secondary cue would prevent the costly production of toxins when they would not be beneficial. Indeed, this hypothesis is supported by a previous study of *P. syringae* B728a, in which genes involved in these two categories were more dramatically induced when cells were in the leaf apoplast than on the leaf surface (Yu *et al.*, 2013). Compounds such as arbutin have also been shown to be needed for the induction of toxins such as syringomycin (Mo and Gross, 1991) – and such compounds are not expected to be present on the leaf surface.

Historically, it has been common to study bacteria in broth cultures. While it is presumed that such cultures are more homogeneous and facilitate coordinated patterns of gene expression etc., many aspects of the manner in which microorganisms interact with their environment cannot be studied in such a setting (Petrova and Sauer, 2012; Tuson and Weibel, 2013; Chang *et al.*, 2017). For instance, though *P. syringae* B728a can be found in aquatic environments, it can also colonize the surface of leaves which is a decidedly different environment than the one it experiences in liquid (Lindow and Brandl, 2003; Melotto *et al.*, 2008; Morris *et al.*, 2008). For example, low nutrient conditions in a broth culture are typically associated with high cell densities and thus low oxygen levels. In contrast, cells on the leaf surface would typically experience low nutrient conditions in a fully aerobic environment. Moreover, experiments that have examined how bacteria such as pseudomonads regulate genes to adapt to life on surfaces have focused almost entirely on the process of aquatic biofilm formation (Dötsch *et al.*, 2012;

Hickman *et al*., 2005; Prigent-Combaret *et al*., 1999). Again, the large three-dimensional aggregates of bacteria that form in such flowing fluid environments, in which nutrients are provided from the outside world (dissolved in the fluid), are almost certainly very different from the very thin film (usually monolayer) of bacteria that develops on leaf surfaces (Remus-Emsermann and Leveau, 2010; Remus-Emsermann *et al*., 2012). Delivery of soluble nutrients in liquid is probably an exceptional situation on leaves (Leveau and Lindow, 2001; Lindow and Brandl, 2003). In addition, biofilm formation in a flowing liquid environment is typically not a rapid process. Instead, cells are slowly acquired by, or develop within, a biofilm over a period of many hours, during which the nature of the environment within the biofilm changes dramatically, with large spaceotemporal variations (Dötsch *et al*., 2012). For both practical and other reasons, studies of aquatic biofilms typically have examined gene regulation only 24 hours or later after biofilm initiation (Dötsch *et al*., 2012; Prigent-Combaret *et al*., 1999).

One of the few studies that examined gene expression in bacterial cells soon after surface attachment assessed gene expression in the *Escherichia coli* strain CSH50 by microarray after planktonic cells had attached to glass beads after one, four, and eight hours (Bhomkar *et al*. (2010). Interestingly Bhomkar *et al*. (2010) and I found similar functional genes to be up-regulated soon after cells attached to a surface (Figures 10 and 11). The gene *emrB*, encoding a drug resistance transporter, and *glnH*, involved in the transport of glutamine, were up-regulated in both studies. Other genes that both studies found to be up-regulated on a surface were *emrA*, which binds to *emrB*, and *emrR*, which regulates *emrA* and *emrB*. The genes *marA* and *marR* were also found to be up-regulated on surfaces with *marA* being involved in multi-antibiotic resistance while *marR* regulates *marA*. Both studies also found *ahpF, grxA*, and *katG*, which are involved in oxidative stress tolerance, to be up-regulated on surfaces (Figure 10). Unsurprisingly, the regulatory protein *oxyR*, which regulates *ahpF, grxA*, and *katG* in response to oxygen stress, was also induced on a surface in *P. syringae* B728a. Taken together, these results suggest that both *E. coli* and *P. syringae* experience chemical stresses to which these defensive responses are necessary soon after mobilization on the surface. Alternatively, and perhaps more logically, surface attachment is taken as a cue to alert cells that they have arrived at a surface upon which such chemical stresses could be anticipated. Certainly, *E. coli* can experience chemical stresses associated with innate resistance responses of animal cells (Tagkopoulos *et al*., 2008). Likewise, *P. syringae* will often experience defensive chemicals as well as oxidative stress on both compatible and incompatible host plants (Alcade-Rico *et al*., 2016; Fones and Preston, 2012). Previous studies in *P. syringae* and *E. coli* have shown that cells infecting plants can experience increases in hydrogen peroxide as a host defense response (Dublan *et al*., 2014; Fones and Preston, 2012). To combat this, it has been shown that bacteria adapted to living in and on leaves have high oxidative stress tolerance. This was shown by the up-regulation of genes regulated by *oxyR* in *E. coli* exposed to plant macerates (Kyle *et al*., 2010) as well as homologs in *P. syringae* that were found to be up-regulated when *P. syringae* B728a had colonized plant surfaces (Yu *et al*., 2013). Genes involved in antimicrobial resistance were also found to be up-regulated in a transcriptome analysis conducted by Kyle *et al*. (2010) in which *Escherichia coli* O157:H7 was exposed to romaine lettuce lysates. Similar to Bhomkar *et al*. (2010) and this study, Kyle *et al*. (2010) observed the induction of the genes *ahpF*, *grxA*, and *katG* in the OxyR regulon and the induction of *marR* and *marA* in the Mar regulon. In addition, genes involved in attachment and DNA repair were up-regulated as well. Interestingly, my study also found genes in these categories to be up-regulated. Yu *et al*. (2013), found that genes involved in oxidative stress tolerance were more highly expressed by cells both on the leaf surface and in the apoplast than

by cells growing in minimal broth medium. However, as might be expected from the more intimate association of the cells with the host, bacterial cells attached to surfaces in the apoplast had higher induced expression of these genes compared to cells on the leaf surface. Plants also produce toxic chemicals such as flavonoids in response to pathogens, which suggests that bacteria could adapt to these chemical stresses by increasing the expression of genes involved in efflux transport, such as *emrRAB* and *marRA* upon contact with plant surfaces (Alcade-Rico *et al.*, 2016; Kyle *et al.*, 2010; Vargas *et al.*, 2011). For instance, wild-type *Pseudomonas syringae* pv. *tomato* DC3000 sprayed onto tomato leaves achieved a higher population size than a *mexA* mutant strain deficient in an efflux pump (Vargas *et al.*, 2011). This led Vargas *et al.* (2011) to conclude that efflux pumps improve leaf colonization and survival of *P. syringae* DC3000. My work suggests that many such traits might be induced in *P. syringae* upon contact with the host plant leaf surface. Thus, stress response traits, presumably less important in an open, aquatic environment, are rapidly induced in both *P. syringae* and *E. coli*. when host defenses might otherwise prove lethal.

Bhomkar *et al.* (2010) reasoned that many of the genes induced on beads versus in broth may be expressed because bacteria attached to a surface would experience decreased mobility and a reduction in accessible surface area. These factors would result in the decreased up-take of nutrients and oxygen from media. Consistent with such a model, both studies found that *cysJ* and *cysI*, involved in sulfur metabolism and transport, were up-regulated in cells immobilized on surfaces (Figure 10). Moreover, as discussed above, I found that genes involved in iron metabolism and transport as well as siderophore production and transport were up-regulated on surfaces, suggesting that iron is less available in these settings.

As mentioned previously, a transcriptome analysis was conducted by Kyle *et al.* (2010) in which *E. coli* O157:H7 was exposed to romaine lettuce lysates. It was curious to find that the many responses of *E. coli* to the plant macerates resulted in similar patterns of gene expression observed here for *P. syringae* exposure to surfaces. In particular, Kyle *et al.* (2010) found that genes involved in flagellar motility were up-regulated, much as I found such genes up-regulated in *P. syringae* growing on surfaces (Table 1). Similarly, both this study and Kyle *et al.* (2010) found that genes involved in DNA repair or in the regulation of iron, including iron-sulfur clusters, were up-regulated on surfaces (Table 1). Those authors reasoned that repair genes in these functional categories were up-regulated in order to combat the damage caused by oxidative stress. This is because oxidative stress can damage both iron-sulfur clusters and DNA through the formation of OH radicals. Perhaps *P. syringae* uses surfaces as a cue with which to anticipate OH radicals released by plants during infection. Induction upon perception of the compounds themselves might be too late to prevent cell death, in which case cells that used a correlated cue, such as landing on a surface, might survive and dominate bacterial populations that frequently encounter leaf surfaces.

In another study, Siryaporn *et al.* (2014) examined rapid expression of the *pilY1* gene by *Pseudomonas aeruginosa* UCBPP-PA14 one hour after attaching to a surface. They found that *pilY1* plays a central role in the surface-dependent induction of virulence in *P. aeruginosa* UCBPP-PA14. Although I had used cloning techniques to disrupt the homolog of *pilY1* in *P. syringae* B728a (Psyr_0719), when the mutant strain was examined on a surface the expression of *syfA* was not influenced and it still exhibited the same contact-dependent induction as seen in the wild-type strain (data not shown). RNA sequencing showed that the *pilY1* homolog in *P.*

syringae B728a was not expressed in cells growing on a surface. However the expression of the *P. syringae* homolog *pilW*, another gene in the *pilY1* operon, was expressed at a lower level on filters than in broth cultures. Similarly, the homolog to *pilE* was repressed in *P. syringae* cells on filters while the homolog to *fimU* was induced on the filter surface. Thus while all of the genes in this operon from *P. aeruginosa* were induced on surfaces, their expression with regard to surface attachment was discordant in *P. syringae*. Interestingly, in my work, the functional categories of phytotoxin synthesis and transport and secondary metabolism were both enriched in genes that were down-regulated on a surface (Table 2). This is somewhat consistent with the expected behavior of *P. syringae* since, as discussed above, there is no evidence that various phytotoxins are induced solely by its contact with leaves of host plants. Rather, Yu *et al*. (2013) showed that cells in the apoplast exhibited increased expression of the phytotoxins syringomycin, syringopeptin, and syringolin A compared to cells on the leaf surface - the site of first contact of the bacteria with the plant.

Despite finding little to no surface-mediated induction of most of the genes in the operon that encodes PilY1, my study found other genes induced in *P. syringae* whose homologs were also induced in the study by Siryaporn *et al*. (2014) (Figures 12, 13, and 14). Among these genes were those involved in flagellar synthesis and motility. This finding is consistent with the model of *P. syringae's* lifestyle on the leaf surface, which assumes that a bacterium must move across the leaf to access nutrients or to enter the leaf via open stomata (Lindow and Brandl, 2003). In support of this model, Yu *et al*. (2013) found that genes involved in flagellar synthesis and motility were up-regulated on the leaf surface and more strongly induced on the surface than in broth media. I was surprised that genes involved in motility were more induced on a surface than in liquid, since flagella are required for bacterial swimming. However, when bacteria move in a way known as swarming, the number of flagella the cell possesses tends to increase (Kearns, 2010). For instance, *P. aeruginosa*, which swims with one polar flagellum, produces two or more polar flagella to move across a surface (Kearns, 2010). This phenomenon of cells increasing the number of flagella upon contact with a surface, could explain why genes involved in flagellar synthesis and motility are upregulated when bacteria transition from a liquid to a surface.

Many genes exhibit rapidly increased transcription when cells transition from a planktonic to sessile state (Figures 7 and 8). This rapid change in overall metabolic status of the cell probably requires increased expression by genes involved in translation to produce the proteins encoded by the newly induced genes. As expected, both this study and that of Siryaporn *et al*. (2014) found that ribosomal proteins and the elongation factor, *efp*, were induced in cells growing on a surface (Figure 13).

A variety of genes involved in cell wall synthesis in various bacteria exhibited similar regulatory responses to surfaces. Both this study and Siryaporn *et al*. (2014) found that *mraY* and *murA*, which are involved in peptidoglycan synthesis, as well as *lpxC* and *lpxD*, which are involved in LPS synthesis, were induced when *P. syringae* and *P. aeruginosa* grew on a surface (Figure 13). The gene *ispZ*, involved in cell division, was also induced on surfaces in both studies (Figure 13). Interestingly, genes involved in peptidoglycan synthesis were up-regulated in the Cpx system in *E. coli* when the cell envelope experienced stressful conditions and upon cell surface contact (Otto and Silhavy, 2002; Vogt and Raivio, 2012). This is intriguing because, when the Cpx pathway is activated by cell envelope stress, the cytoplasmic response regulator, CpxR, is activated through phosphorylation by CpxA, an inner membrane histidine kinase. Activation of

this kinase increases phosphorylated binding of CpxR to DNA, which increases expression of a suite of genes, including those involved in adhesion (Otto and Silhavy, 2002). These findings are consistent with a model that surface sensing involves a response (cellular repair) to the physical stresses to the cell envelope that are associated with surface attachment (Chang, 2017; Wood and Ohman, 2009; Wood *et al.*, 2006).

The operon that encodes the *wsp* genes in *P. aeruginosa* has also been examined for its relationship to surface induction (Hickman *et al.*, 2005). In their study, Hickman *et al.* (2005) showed that the *wspF* mutant of *Pseudomonas aeruginosa* PAO1 exhibited enhanced biofilm formation as well as increased attachment when in broth culture. They hypothesized that the phenotypes were due to the absence of *wspF,* causing a conformational change in the protein encoded by the gene immediately downstream of it, WspR, which resulted in WspR being constitutively phosphorylated. This in turn would lead to an increase in intracellular levels of cyclic di-GMP which, in turn, would result in the induction of genes involved in biofilm formation. My study found *wspF* expression to be repressed in cells attached to a surface, but also found *wspD*, *wspE*, and *wspR* to be repressed as well (Figure 15). Moreover, while Hickman *et al.* (2005) found genes involved in exopolysaccharide synthesis to be up-regulated in a *wspF* mutant, my study did not find a significant trend in the up-regulation of exopolysaccharide genes on a surface (Figures 15 and 16). These differences in gene expression may be due to the different methods by which transcriptome analyses were conducted by Hickman *et al.* (2005) compared to this study. Since Hickman *et al.* (2005) examined cell attachment in the *wspF* mutant in cultures grown to an OD_{600} of 0.3, it is very likely that cells had been attached for several hours before RNA analysis. Indeed, Hickman *et al.* (2005) describes attachment assays in microtiter dishes in which cells had been incubated for six to seven hours before analysis. In contrast, I examined cells attached to a surface for only two hours. Bhomkar *et al.* (2010) showed in their transcriptome analysis that many genes that had become induced by one hour of surface attachment exhibited subsequent decreases in expression by four hours of attachment. By eight hours of attachment many of these genes exhibited continued decreases in expression while others only then became induced. There clearly is wide variation in temporal gene expression during such biofilm production. While my study suggests that *P. syringae* B728a experiences increased motility on a surface rapidly after initial attachment, it is likely that genes involved in other processes such as biofilm formation would be up-regulated only later, such as the *wsp* genes.

A thorough global transcriptional analysis of *P. syringae* strain B728a in various conditions was performed by Yu *et al.* (2013) who used microarray analysis to examine the expression of genes in *P. syringae* B728a on the surfaces of bean leaves as well as in other conditions. In addition to the up-regulation of genes involved in flagellar synthesis and motility, Yu *et al.* (2013) and this study both found genes involved in chemosensing and chemotaxis to be induced on a surface (Figure 17). This would be expected since cells on the leaf surface would need to find nutrients which are heterogeneously dispersed (Leveau and Lindow, 2001). However, in contrast with that seen here, Yu *et al.* (2013) found genes involved in QAC metabolism and transport as well as compatible solute synthesis to be up-regulated in epiphytic cells relative to that of cells grown in a minimal broth medium while I found these genes to be down-regulated on filter surfaces relative to a broth medium (Figures 17 and 18). This difference is most likely due to the different conditions operative in our experiments. The leaf surfaces in the experiments by Yu *et al.* (2013) were quite dry, a condition that would create matric stress and thus induce the expression of

genes involved in QAC metabolism and transport and compatible solute synthesis (Axtell and Beattie, 2002). In contrast, the moistened filters on agar plates in my study would be a wetter environment as discussed above, thus negating the need for the production of such compatible solutes.

Interestingly, though all of the previously mentioned transcriptome studies looked at different types of bacteria on different types of surfaces, many of them found induced genes that were similar to the ones found in this study (Figures 10 – 18; Bhomkar *et al.*, 2010; Kyle *et al.*, 2010; Siryaporn *et al.*, 2014; Yu *et al.*, 2013). This reveals that even though I examined bacterial attachment on the surface of a filter placed on agar instead of on glass beads, leaf lysate, microtiter plates, or the leaf surface, there are certain genes that appear to be induced when bacteria come into contact with any kind of surface. Many of these genes seem to aid bacteria in adapting to life on a surface in terms of tolerating oxidative stress and antimicrobial compounds as well as experiencing increased movement and chemosensing.

It is also interesting that I found *P. syringae* B728a genes involved in LPS synthesis and transport and peptidoglycan synthesis to be up-regulated (Table 1). This suggests that the contact of *P. syringae* B728a with a surface may result in stress of the cell wall. In fact, previous studies have suggested that when the cell wall is stressed, which can be due to contact with a surface, not only would genes involved in cell wall recovery be induced such as peptidoglycan synthesis genes, but pathways involved in surface-regulated behaviors could be induced as well (Chang, 2017; Wood and Ohman, 2009; Wood *et al.*, 2006). In particular, Wood *et al.* (2006) showed that by using cell wall-inhibitory antibiotics in *P. aeruginosa* strain PAO1, the sigma factor σ^{22} (AlgU/T) becomes induced resulting in alginate production. As mentioned previously, alginate has been shown to be induced in surface associated processes such as biofilm formation (Hickman *et al.*, 2005). Moreover, Wood *et al.* (2006) also found genes involved in peptidoglycan synthesis and cell division to be up-regulated. These categories were both found to be enriched with surface-induced genes in this study (Table 1). These findings suggest that after *P. syringae* B728a comes into contact with a surface it undergoes cell wall stress which results in the signaling of other genes involved in adapting to a surface such as motility and chemotaxis.

Bacteria such as *P. syringae* B728a can experience different types of environments in their life history including both liquid and surfaces (Melotto *et al.*, 2008; Morris *et al.*, 2008). Though bacteria in liquid have been studied extensively, studies are still examining the changes that bacteria undergo when attached to a surface (Bhomkar *et al.*, 2010; Siryaporn *et al.*, 2014). It is important to continue such analyses in order to understand the different processes that bacteria undergo when on a surface whether it be an increase in virulence or biofilm formation. By better understanding how bacteria interact and adapt to surfaces, we can use this knowledge in a variety of settings from the agricultural to biomedical fields (Tuson and Weibel, 2013).

Experimental Procedures

Bacterial strains and growth conditions

Pseudomonas syringae B728a strains were grown on King's medium B (KB) plates containing 1.5% technical agar (King *et al.*, 1954). Antibiotics were used at the following concentrations (μg/ml): spectinomycin (100), kanamycin (50), and tetracycline (15) as appropriate.

Determining surface regulation of the syfA gene

P. syringae B728a cells harboring a plasmid in with the *syfA* promoter was fused to a promoterless *gfp* reporter gene, were grown in liquid culture until they reached a density of 10^8 cells/ml. 10 μl of cells were then aliquoted onto various surfaces. To remove cells from these surfaces, moistened cotton swabs were used to scrub cells off all surfaces. Cotton swabs were placed into microcentrifuge tubes and vortexed in 100 μl of water to re-suspend cells. 5 μl of cell suspension from each treatment was applied to glass slides for fluorescence microscopy to quantify the GFP fluorescence of individual cells from each treatment.

Quantification of GFP fluorescence in individual bacterial cells

The M2 AxioImager was used for all microscopic analysis. The GFP filter set was used to view cells in all experiments and all images were captured in black and white format using a 12-bit Retiga camera. The magnification used in all experiments was 100x. The software iVision was used to identify all bacterial cells in an image and to quantify the average pixel intensity of each object identified. Clumps of bacterial cells and extraneous particles were identified by visual examination and marked for exclusion before image processing.

RNA Isolation

Bacterial cells were grown in KB liquid medium in a 28°C shaker until cultures reached a cell density of 5×10^8 cells/ml. Three replicate cultures were used. 300 μl of culture was applied to a 0.4 μm Isopore® membrane filter and the liquid was removed by exposing the filter to a vacuum source for 10 seconds. Filters were placed onto KB plates to incubate at 28°C for two hours while broth cultures were returned to a shaker and incubated at 28°C for two hours. Cells in broth cultures were harvested by pipetting 1 ml of cells into a 15 mL conical tube containing 125 μl ice-cold EtOH/Phenol stop solution (5% water-saturated phenol (pH<7.0) in ethanol). Cells on filters were harvested by sonication for 30 seconds in the EtOH/Phenol stop solution followed by vortexing for 20 seconds to ensure complete cell detachment from the filters. All cells were centrifuged at 12,000 rpm (13,800 x g) for five minutes at 4°C. Supernatant was decanted and the cells were frozen in liquid nitrogen and stored at -80°C until RNA isolation. RNA isolation was performed using a Direct-zol™ RNA Kit from Zymo Research. To isolate RNA, 600 μl of TRI Reagent® was added to the pelleted cells to lyse them. An equal volume of ethanol (100%) was added to the cells in TRI Reagent® and mixed thoroughly. The mixture was transferred into a Zymo-Spin™ IICG Column (Zymo Research) in a collection tube and centrifuged. The flow-

through was discarded and the column was transferred into a new collection tube. For DNase I treatment, 400 µl RNA Wash Buffer (Zymo Research) was added to the column which was then centrifuged. 5 µl DNase I (6 U/µl) (Zymo Research) and 75 µl DNA Digestion Buffer (Zymo Research) were then added to the tube and mixed. The mix was added directly to the column matrix and incubated at room temperature (20-30°C) for 15 minutes. 400 µl Direct-zol™ RNA PreWash (Zymo Research) was then added to the column which was centrifuged. The flow-through was discarded and the step was repeated once more. 700 µl RNA Wash Buffer (Zymo Research) was then added to the column which was centrifuged for two minutes to ensure complete removal of the wash buffer. The column was then transferred into a RNase-free tube. To elute RNA, 50 µl of DNase/RNase-Free Water (Zymo Research) was added directly to the column matrix which was then centrifuged. RNA was then stored frozen at -80°C.

mRNA sequencing

1 µl of each sample was diluted into 4 µl of RNase free water and submitted to the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley where Ribo-Zero was used for rRNA removal. RNA abundance and purity were determined using a 2100 Bioanalyzer (Agilent Technologies) and quantified using Qubit (Invitrogen). After reverse transcription, shearing of cDNA, size fractionation, and Illumina library production, the Vincent J. Coates Genomics Sequencing Laboratory samples were sequenced using an Illumina HiSeq4000 platform with 50 base pair, single-end reads. Three biological replicates were sequenced per treatment. Reads were uploaded to Galaxy (Afgan *et al*., 2016) and cleaned up using Trimmomatic (Bolger *et al*., 2014). Reads were aligned to the *Pseudomonas syringae* B728a genome (downloaded from the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov)) using Salmon Transcript Quantification (Patro *et al*., 2017) in Galaxy. The program edgeR in R was then used to assess the differential expression and statistical significance of genes (Robinson *et al*., 2010). Gene expression levels were normalized using a weighted trimmed mean of M values (TMM; where M is the log expression ratio per gene between treatments) (Robinson and Oshlack, 2010). Empirical Bayes estimation and tests based on the negative binomial distribution were then used to determine significance (Robinson and Oshlack, 2010). A gene was considered significantly differentially regulated if the p-value for the difference in relative expression between the filter treatment and the liquid treatment was less than 0.001.

Statistical analysis

The hypergeometric distribution was performed in R (R Core Team, 2013) to test for significance of functional category enrichment (Castillo-Davis and Hartl, 2003). All p-values were adjusted using the Bonferroni correction (Bonferroni, 1936) and the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) in R.
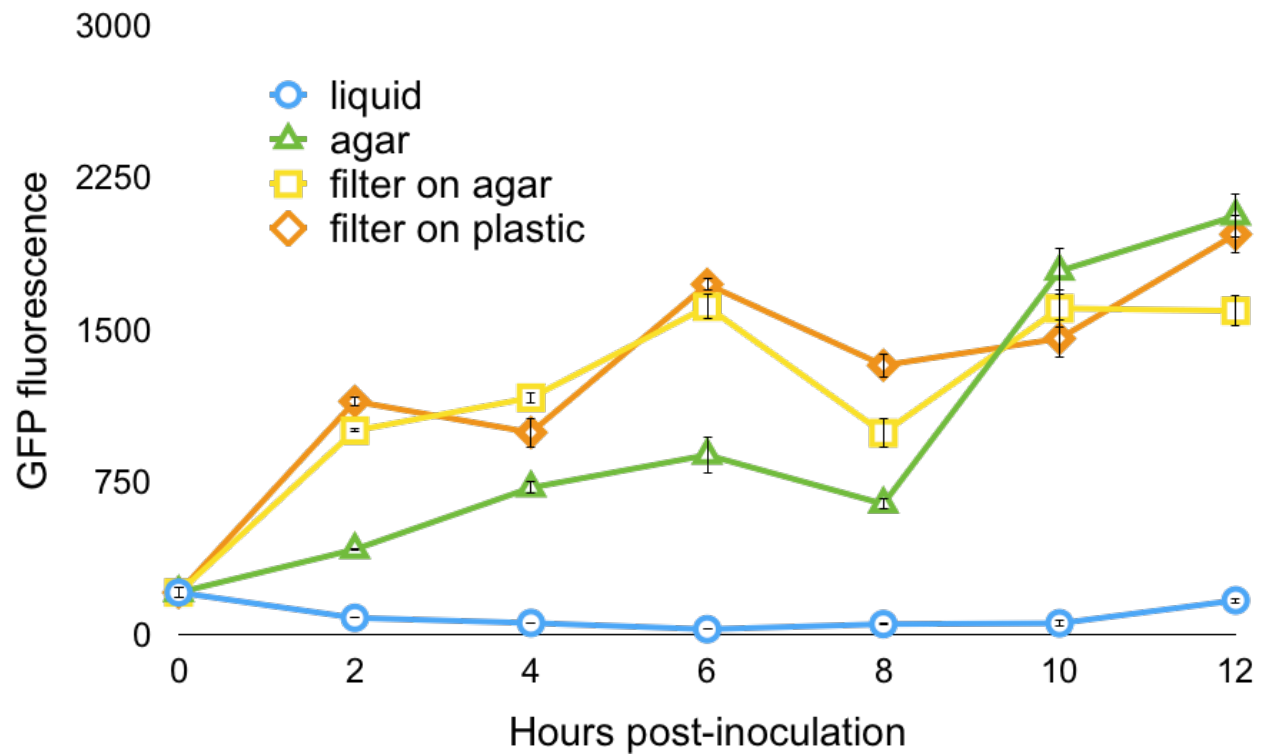
Figure 1. GFP fluorescence increases within 2 hours indicating induction of the *syfA* promoter.

P. syringae B728a cells harboring a plasmid in which the *syfA* promoter was fused to a *gfp* reporter were grown in liquid culture until they reached a density of 10^8 cells/ml. Aliquots of 10 µl of cells were placed onto KB agar, a 0.4 µm Isopore® filter on agar, and a 0.4 µm Isopore® filter on a plastic petri dish containing liquid KB. Every two hours, cotton swabs were used to scrub cells from all surfaces containing cells. Cotton swabs were placed into microcentrifuge tubes and vortexed in 100 µl of water to remove cells. For each treatment, 5 µl of the resulting liquid was added to glass slides so that fluorescence microscopy could be used to quantify the GFP fluorescence of cells from each treatment. 7,038 cells were counted in total for the liquid treatment, 3,068 cells were counted in total for the agar treatment, 10,184 cells were counted in total for the filter on agar treatment, and 3,791 cells were counted in total for the filter on plastic treatment. The error bars represent the standard errors of the mean GFP fluorescence.
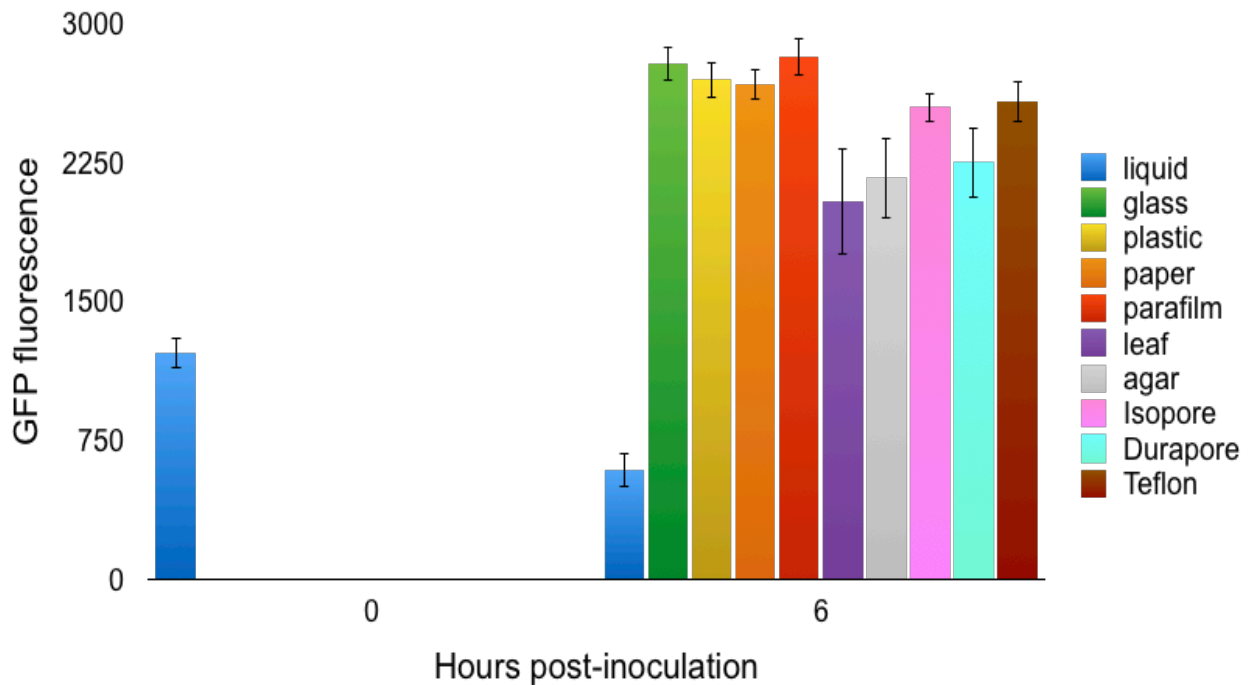
Figure 2. GFP fluorescence increases in cells in contact with a variety of surfaces indicating induction of the *syfA* promoter.

P. syringae B728a cells harboring a plasmid in which the *syfA* promoter was fused to a *gfp* reporter, were grown in liquid culture until they reached a density of 10^8 cells/ml. 10 μl droplets were then aliquoted onto a glass slide, a plastic petri dish, filter paper, parafilm, a bean leaf, KB agar, an Isopore® filter, a Durapore® filter, and a Teflon® filter. All surfaces were put into petri dishes containing KB liquid, which provided nutrients to the cells. Petri dishes also had water droplets on the bottoms of the dishes, as well as wet paper towels taped to the lid, in order to keep cells from drying out. Petri dishes were sealed with parafilm to maintain a humid environment. After six hours, cotton swabs were used to scrub cells off the surfaces they had been placed on. Cotton swabs were placed into microcentrifuge tubes and vortexed in 100 μl of water to remove cells. 5 μl of liquid from each treatment was added to glass slides so that fluorescence microscopy could be used to quantify the GFP fluorescence of cells from each treatment. 2,100 cells were counted in total for the liquid treatment, 1,174 cells were counted in total for the glass treatment, 1,331 cells were counted in total for the plastic treatment, 540 cells were counted in total for the paper treatment, 2,150 cells were counted in total for the parafilm treatment, 537 cells were counted in total for the leaf treatment, 660 cells were counted in total for the agar treatment, 1,283 cells were counted in total for the Isopore® filter treatment**,** 581 cells were counted in total for the Durapore® filter treatment, and 1,106 cells were counted in total for the Teflon® filter treatment. The error bars represent the standard errors of the mean GFP fluorescence.
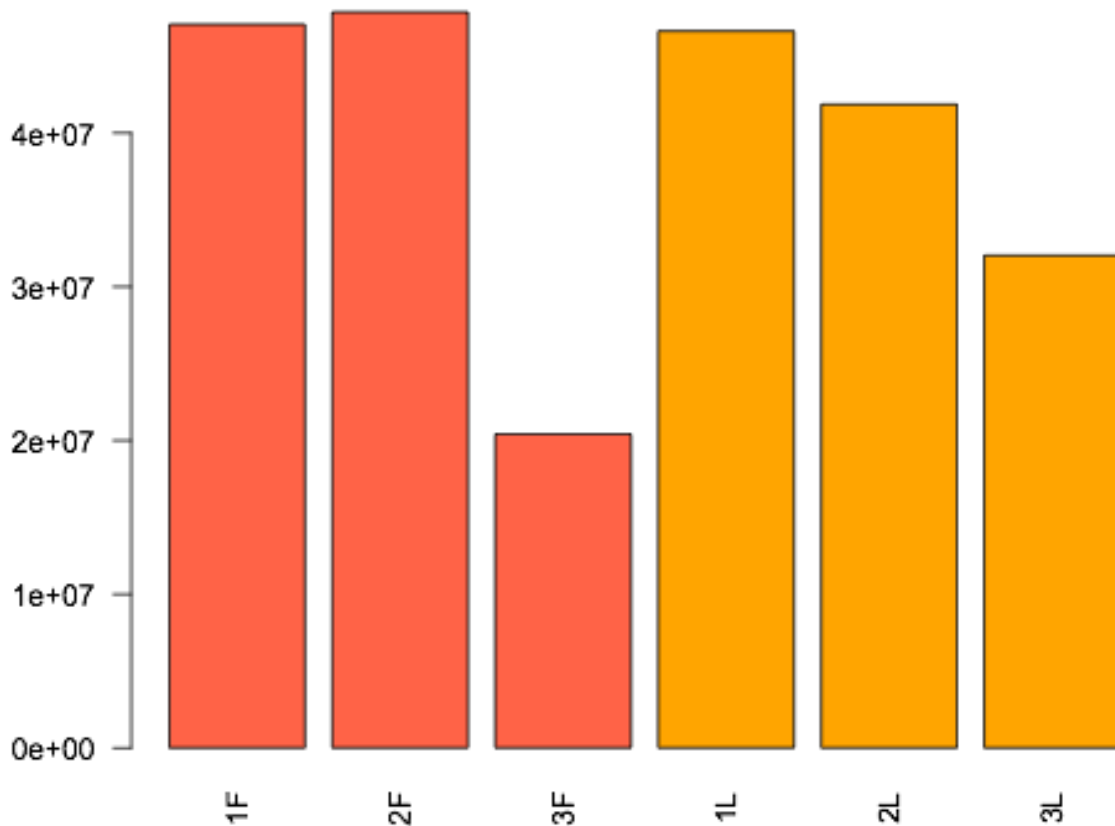
Figure 3. A bar plot of the total number of reads per sample obtained from RNA sequencing.

The R statistical package (R Core Team, 2013) was used to determine the total reads from RNA sequencing per sample. Samples 1F, 2F, and 3F denote the three replicates of bacterial cells added to the surface of a filter placed on an agar plate; samples 1L, 2L, and 3L denote the three replicates of bacterial cells maintained in liquid culture. All cells were incubated at 28°C for two hours before RNA stabilization.
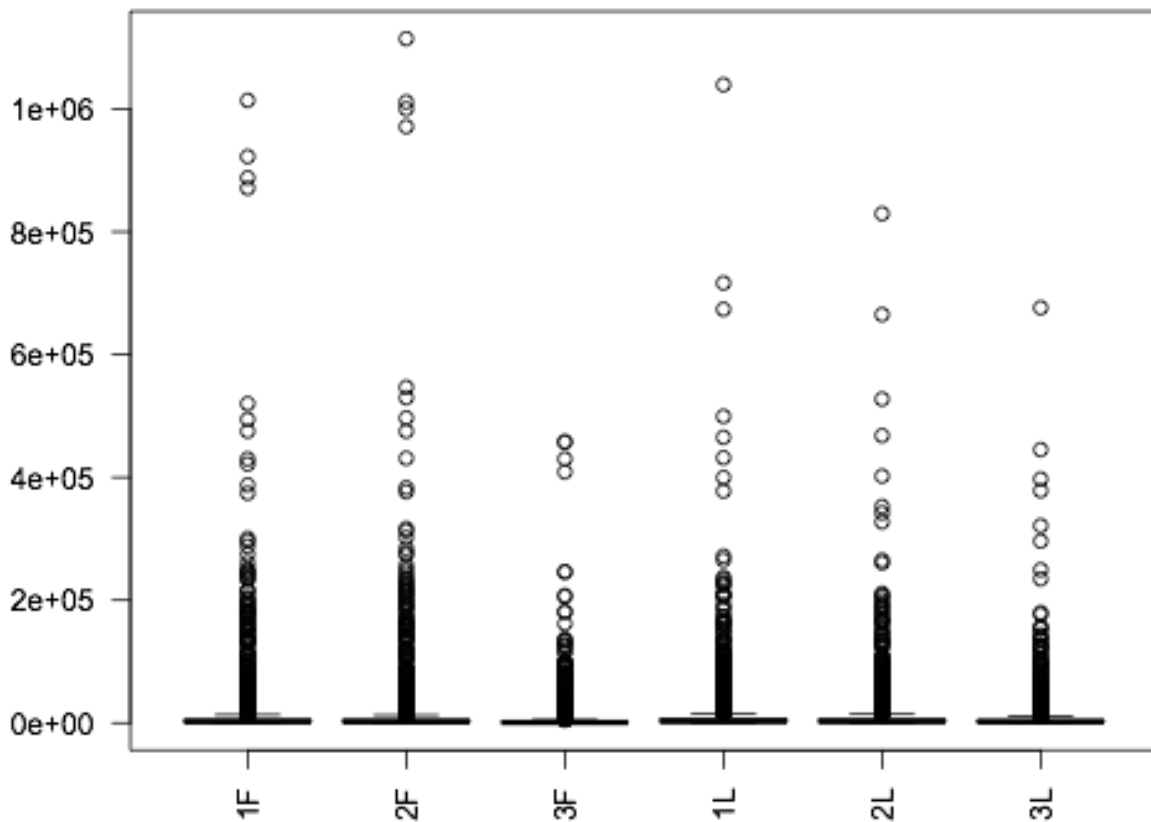
Figure 4. A box plot reveals the reads per gene for each sample used in RNA sequencing. The R statistical package (R Core Team, 2013) was used to determine the reads per gene from RNA sequencing for each sample. Samples 1F, 2F, and 3F denote the three replicates in which bacterial cells were added to the surface of a filter placed on an agar plate and incubated for two hours before RNA stabilization. Samples 1L, 2L, and 3L denote the three replicates in which bacterial cells were incubated in a liquid culture for two hours before RNA stabilization.
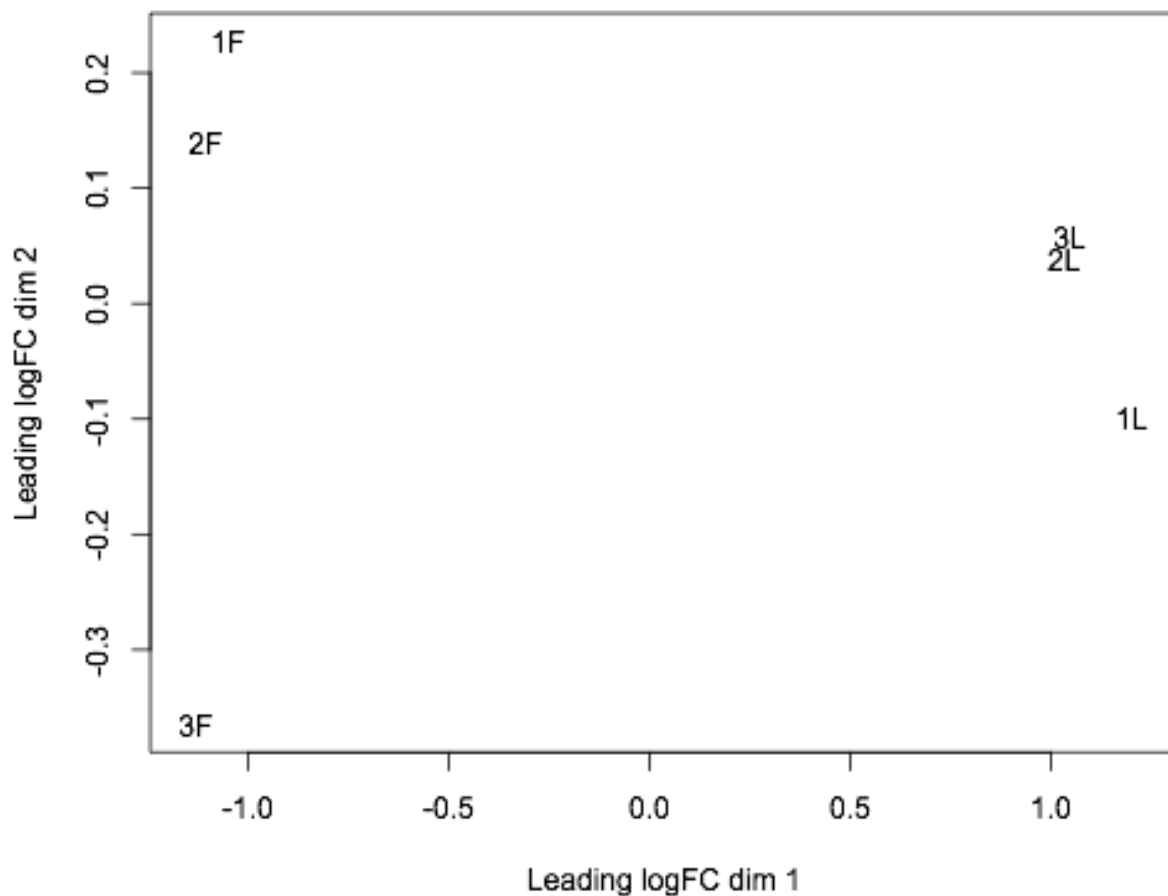
Figure 5. A multidimentional scaling (MDS) plot reveals that overall, *P. syringae* B728a cells on a filter are more similar to one another than *P. syringae* B728a cells in liquid. The R statistical package (R Core Team, 2013) was used to create a MDS plot to compare the similarity among the sample types used for RNA sequencing. Samples 1F, 2F, and 3F denote the three replicates in which bacterial cells were added to the surface of a filter placed on an agar plate and incubated for two hours before RNA stabilization. Samples 1L, 2L, and 3L denote the three replicates in which bacterial cells were incubated in a liquid culture for two hours before RNA stabilization.
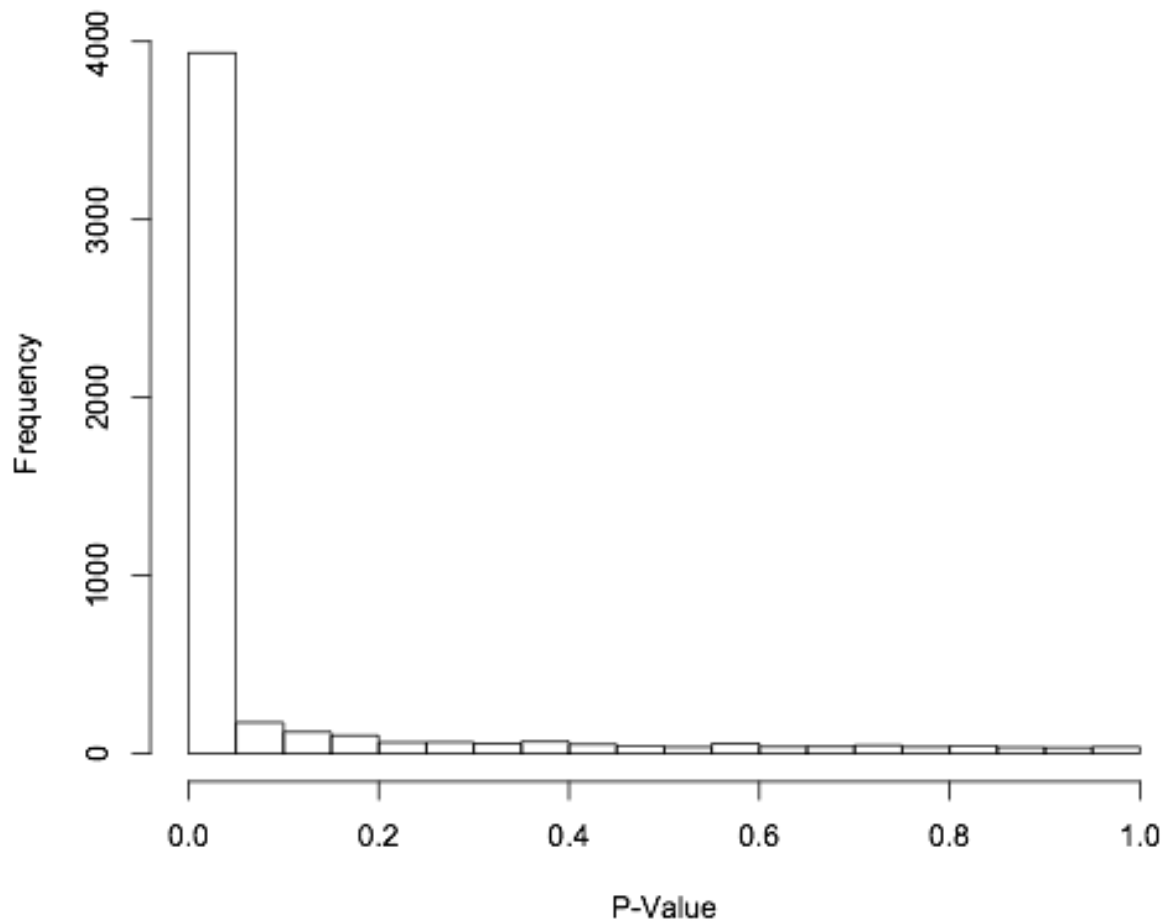
Figure 6. A histogram of the p-values of differentially expressed genes in *P. syringae* B728a reveals that the majority of expressed genes have significant p-values.
The R statistical package (R Core Team, 2013) was used to create a histogram displaying the p-values of the differentially expressed genes identified by RNA sequencing. Most of the genes had small p-values, indicating that the differences in their expression between treatments was near zero.
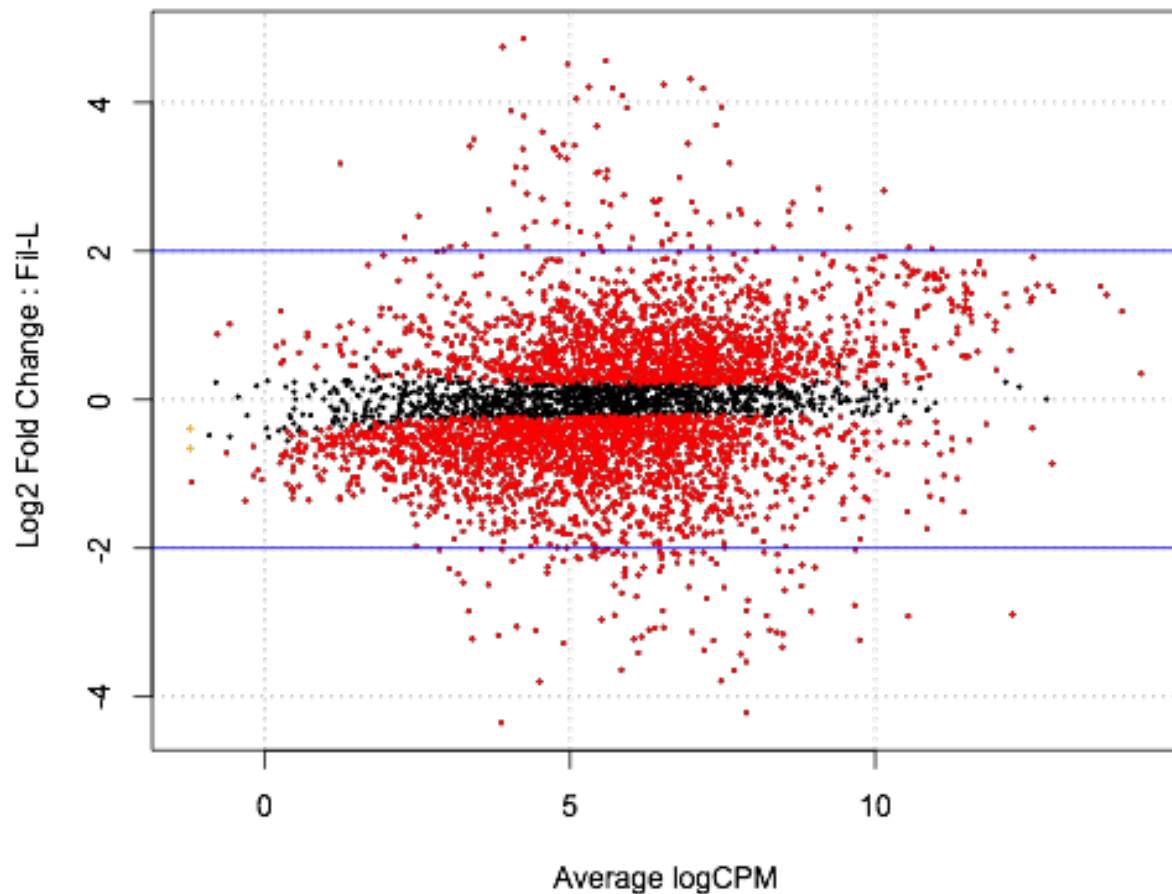
Figure 7. A smear plot shows that many genes in *P. syringae* B728a are differentially expressed on the filter surface.
The R statistical package (R Core Team, 2013) was used to create a smear plot to show the proportion of the genes that were differentially expressed on the filter surface (red) to the genes that were not differentially expressed on a surface (black). Many of the genes were expressed less than 4-fold while a smaller proportion were expressed more than 4-fold as indicated by the blue lines.
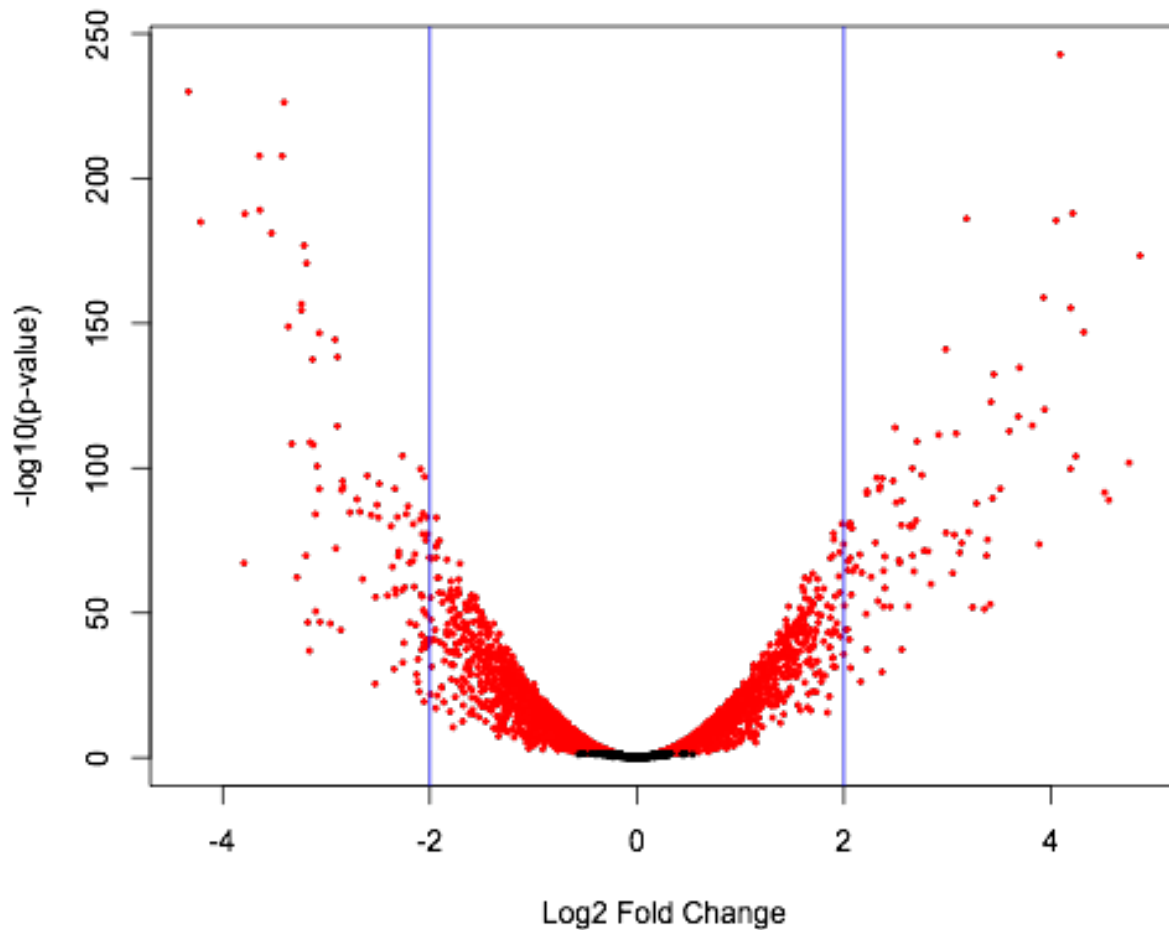
Figure 8. A volcano plot shows that many genes in *P. syringae* B728a are differentially expressed on the filter surface.

The R statistical package (R Core Team, 2013) was used to create a volcano plot to show the proportion of the genes that were differentially expressed on the filter surface compared to broth cultures (red) to the genes that were not differentially expressed on a surface (black). Many of the genes were expressed less than 4-fold while a smaller proportion were expressed more than 4-fold as indicated by the blue lines. The more expressed genes also tended to have smaller p-values indicating significance.
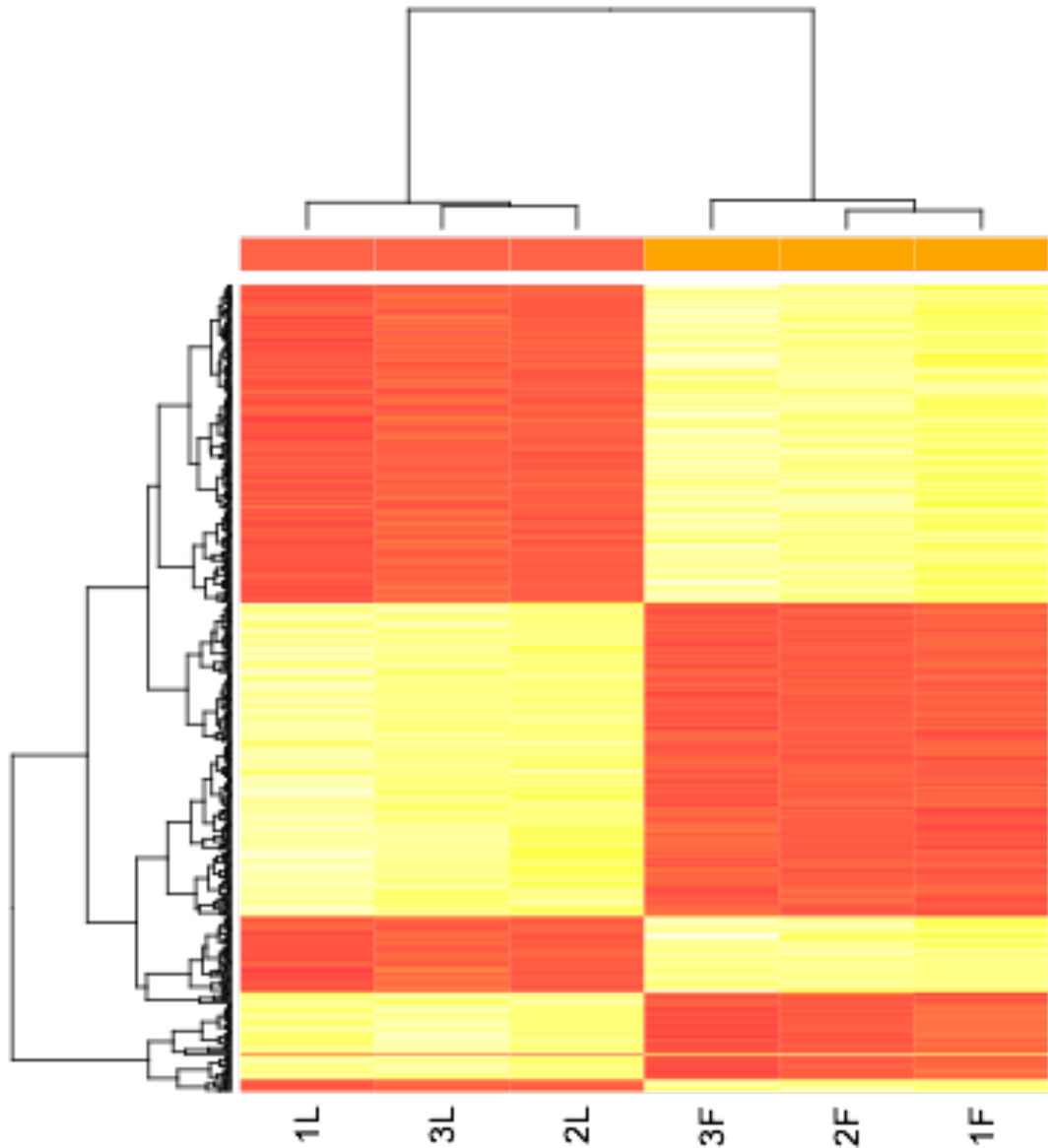
Figure 9. A heat map reveals that different genes are regulated when *P. syringae* B728a cells are in liquid compared to on a filter surface.
The R statistical package (R Core Team, 2013) was used to create a heat map to compare the similarity in expression of a given gene between samples and treatments. Samples 1F, 2F, and 3F denote three replicates of bacterial cells that were added to the surface of a filter placed on an agar plate and incubated for two hours before RNA stabilization. Samples 1L, 2L, and 3L denote the three replicates of bacterial cells incubated simultaneously in a broth culture for two hours before RNA stabilization.

Table 1. Functional gene categories preferentially up-regulated on a filter.

Category	Bonferroni adjusted p-value	Benjamini-Hochberg adjusted p-value
Translation	1.11E-18	1.11E-18
Siderophore synthesis and transport	4.75E-09	2.38E-09
Nucleotide metabolism and transport	1.12E-06	3.73E-07
Flagellar synthesis and motility	2.44E-03	6.11E-04
Lipolysaccharide synthesis and transport	9.16E-03	1.83E-03
Energy generation	0.02	2.63E-03
Transcription	0.04	0.04
Chemosensing and chemotaxis	0.06	7.15E-03
Replication and DNA repair	0.12	0.01
Iron-sulfur proteins	0.19	0.02
Peptidoglycan/cell wall polymers	0.19	0.02
Terpenoid backbone synthesis	0.24	0.02
Iron metabolism and transport	0.25	0.02
Cell Division	0.59	0.04

The significance of functional category enrichment was assessed using the hypergeometric distribution (Castillo-Davis and Hartl, 2003).

Table 2. Functional gene categories preferentially down-regulated on a filter.

Category	Bonferroni adjusted p-value	Benjamini-Hochberg adjusted p-value
Quaternary ammonium compound metabolism and transport	2.19E-08	2.19E-08
Compatible solute synthesis	2.33E-06	1.17E-06
Carbohydrate metabolism and transport	1.72E-05	5.74E-06
Organic acid metabolism and transport	8.84E-05	2.21E-05
Phytotoxin synthesis and transport	1.16E-04	2.32E-05
Amino acid metabolism and transport	0.01	1.77E-03
Secondary metabolism	0.01	1.01E-03

The significance of functional category enrichment was assessed using the hypergeometric distribution (Castillo-Davis and Hartl, 2003).
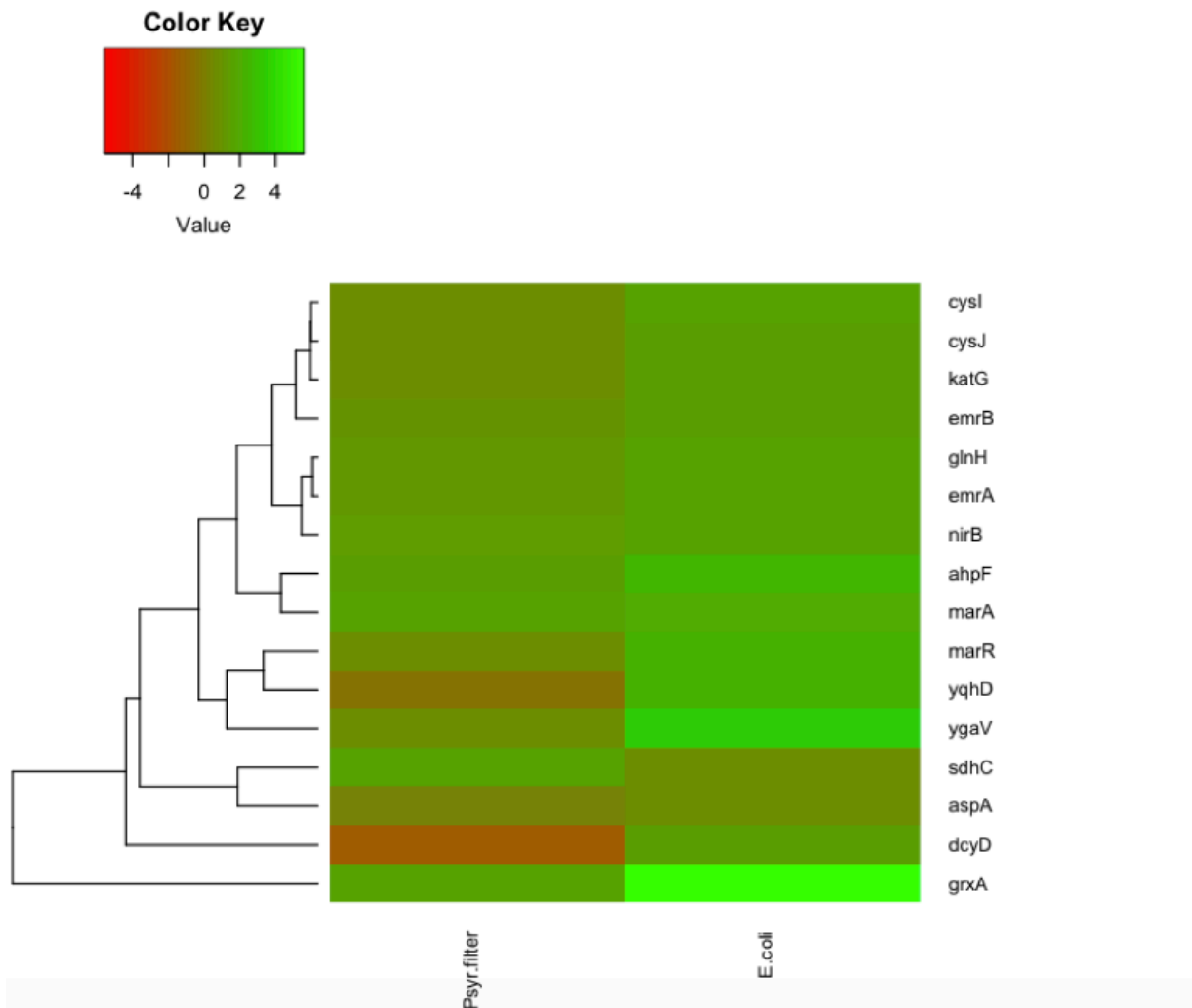
Figure 10. Differential expression in genes of *P. syringae* B728a cells on a filter for 2 hours and *E. coli* CSH50 cells on beads for 1 hour.

Heat map generated via the heatmap.2 program in the R statistical package (R Core Team, 2013) showing differentially expressed genes of *P. syringae* B728a cells on a filter (Psyr.filter) compared to *E. coli* CSH50 cells on mannose agarose beads (E. coli) (Bhomkar *et al*., 2010). A color key at the top shows a range from -4 to 4 (red to green) with red representing down-regulation and green representing up-regulation.

Figure 11. Correlation between the *P. syringae* B728a transcriptome of cells on a filter for 2 hours to the *E. coli* CSH50 transcriptome of cells on mannose agarose beads for 1 hour.
The log(fold-change) values of the transcript levels of genes expressed in *P. syringae* B728a cells on a filter relative to the transcript levels of the same cells in liquid culture (y axes) were plotted against the transcript levels of genes expressed in *E. coli* CSH50 cells attached to beads relative to the transcript levels of the same cells unattached to beads (x axes). Each point corresponds to a shared gene in *P. syringae* B728a and in *E. coli* CSH50.
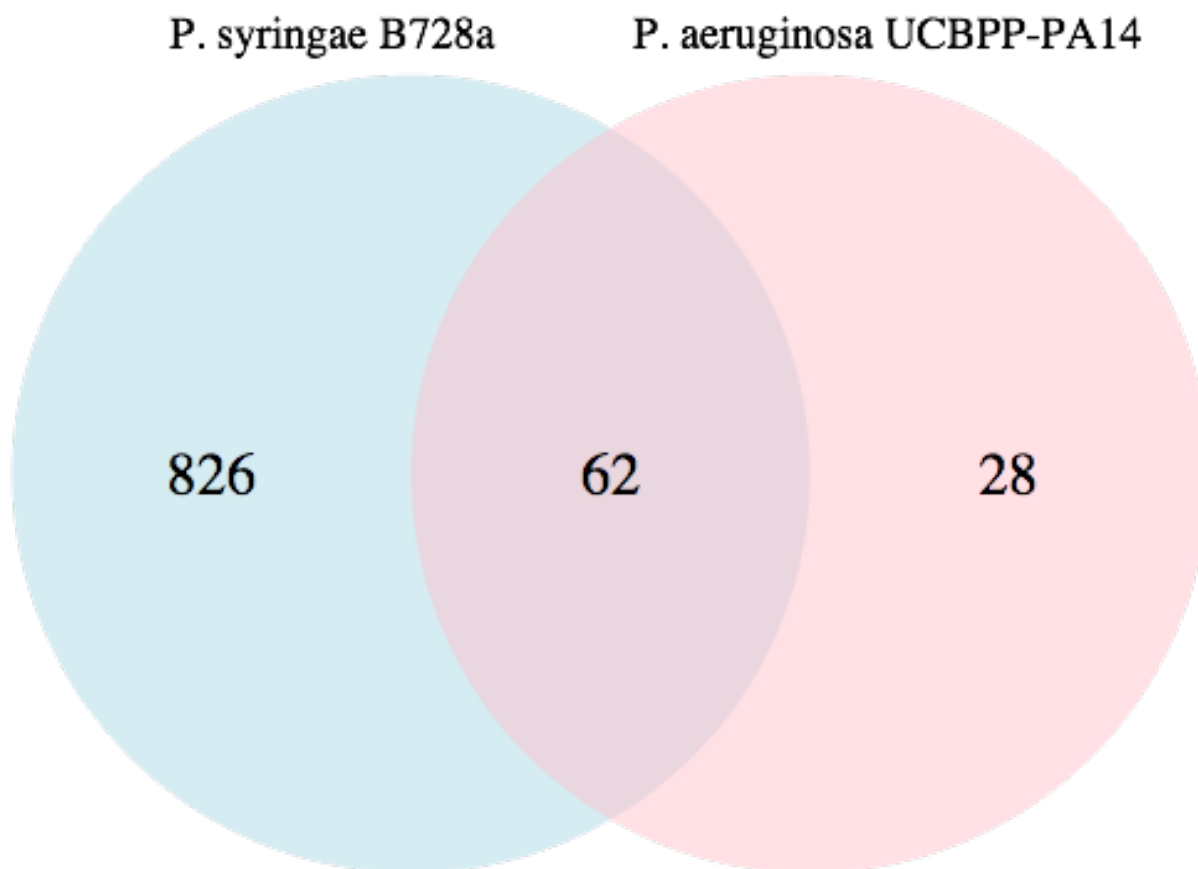
Figure 12. Comparison of the up-regulated genes in *P. syringae* B728a and *P. aeruginosa* UCBPP-PA14.

The R statistical package (R Core Team, 2013) was used to compare the genes that were found to be up-regulated in this study in *P. syringae* B728a, when cells were on a filter on a KB agar surface for two hours, to the genes that were found to be up-regulated in a study by Siryaporn *et al*. (2014) in *P. aeruginosa* UCBPP-PA14, when cells were attached to a glass surface in PS:Development Buffer medium for one hour. Overall, 62 genes were found to be up-regulated in both studies. Using the hypergeometric distribution, this was determined to be significant with a p-value of 1.65E-05 (Castillo-Davis and Hartl, 2003).
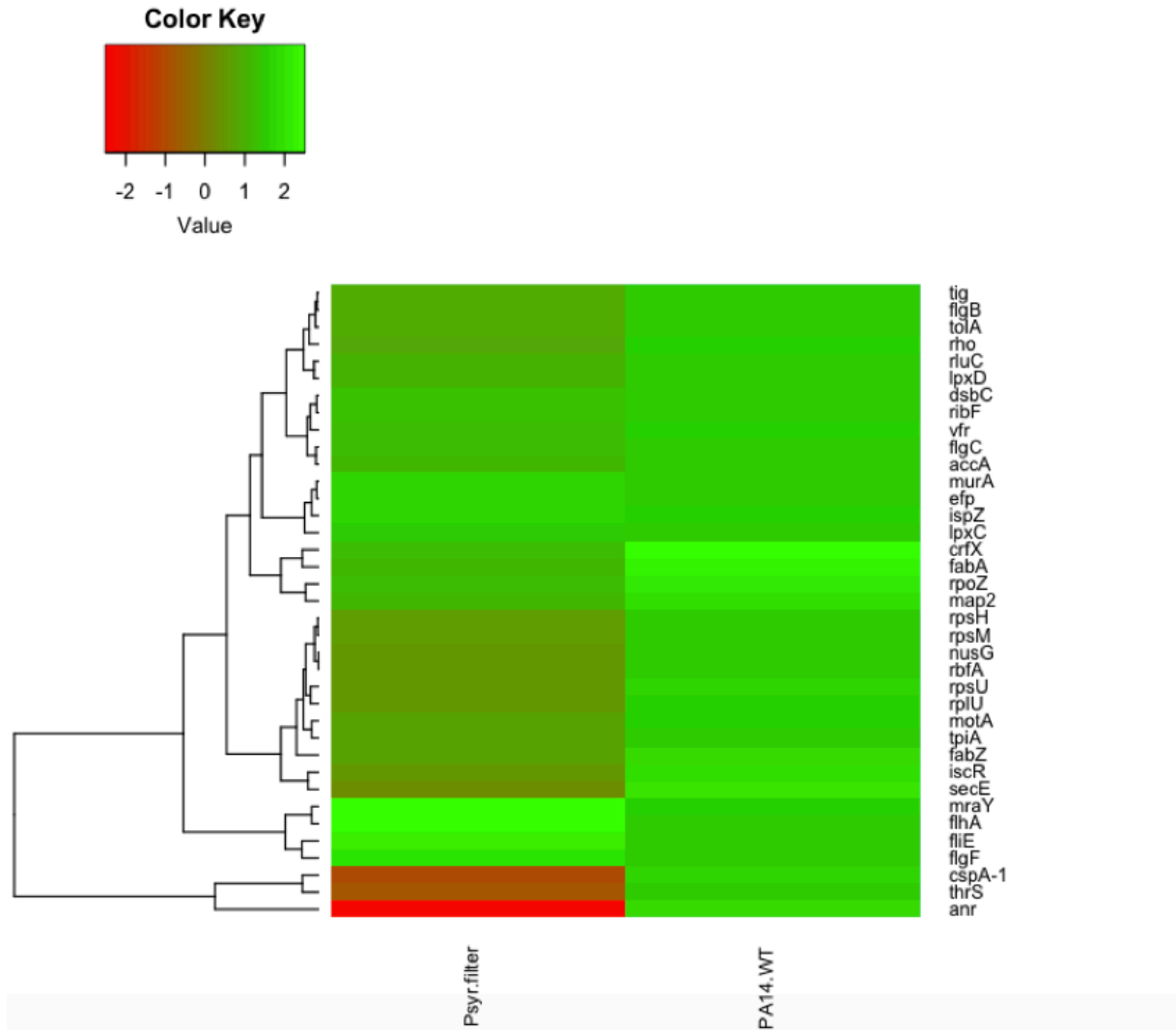
Figure 13. Differential expression in genes of *P. syringae* B728a cells on a filter for 2 hours and *P. aeruginosa* UCBPP-PA14 cells attached to a surface for 1 hour.

Heat map generated via the heatmap.2 program in the R statistical package (R Core Team, 2013) showing differentially expressed genes of *P. syringae* B728a cells on a filter (Psyr.filter) compared to *P. aeruginosa* UCBPP-PA14 wild-type cells attached to a surface (PA14.WT) (Siryaporn *et al.*, 2014). A color key at the top shows a range from -2 to 2 (red to green) with red representing down-regulation and green representing up-regulation.
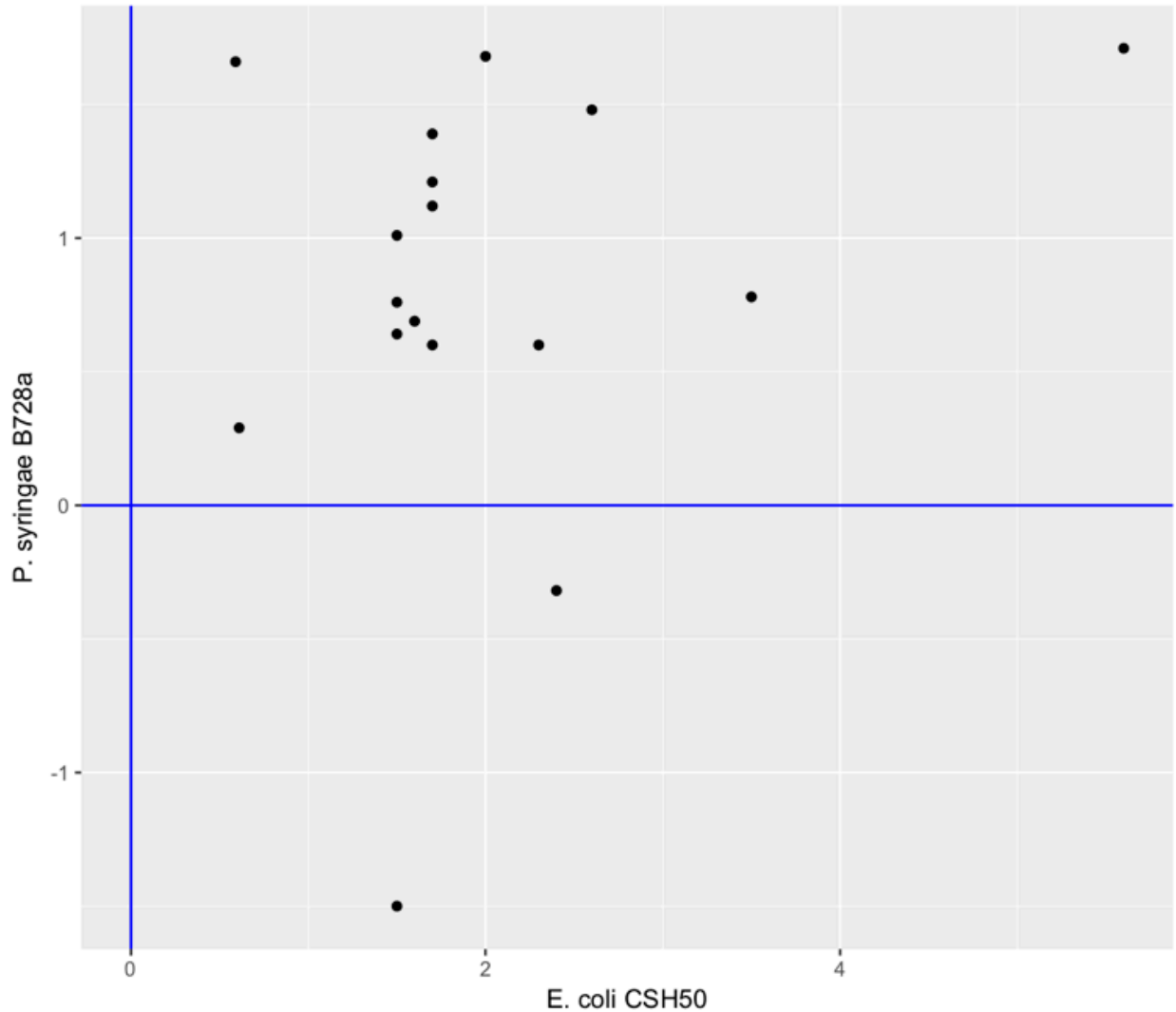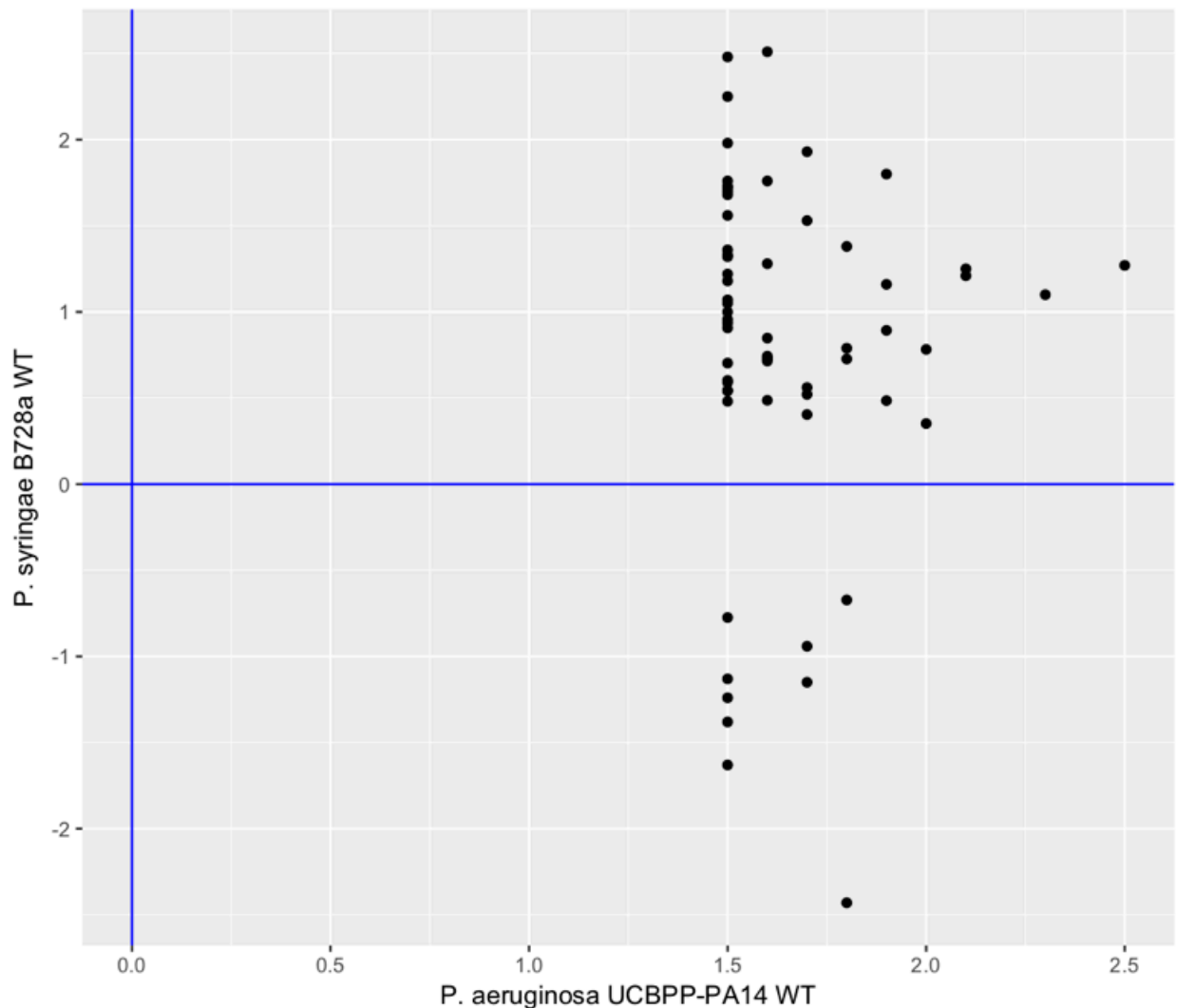
Figure 14. Correlation between the *P. syringae* B728a transcriptome of cells on a filter for 2 hours to the *P. aeruginosa* UCBPP-PA14 wild-type cells attached to a surface for 1 hour.
The log(fold-change) values of the transcript levels of genes expressed in *P. syringae* B728a cells on a filter relative to the transcript levels of the same cells in liquid culture (y axes) were plotted against the transcript levels of genes expressed in *P. aeruginosa* UCBPP-PA14 wild-type cells attached to a surface relative to the transcript levels of *P. aeruginosa* UCBPP-PA14 wild-type planktonic cells from the same culture (x axes). Each point corresponds to a shared gene in *P. syringae* B728a and in *P. aeruginosa* UCBPP-PA14.
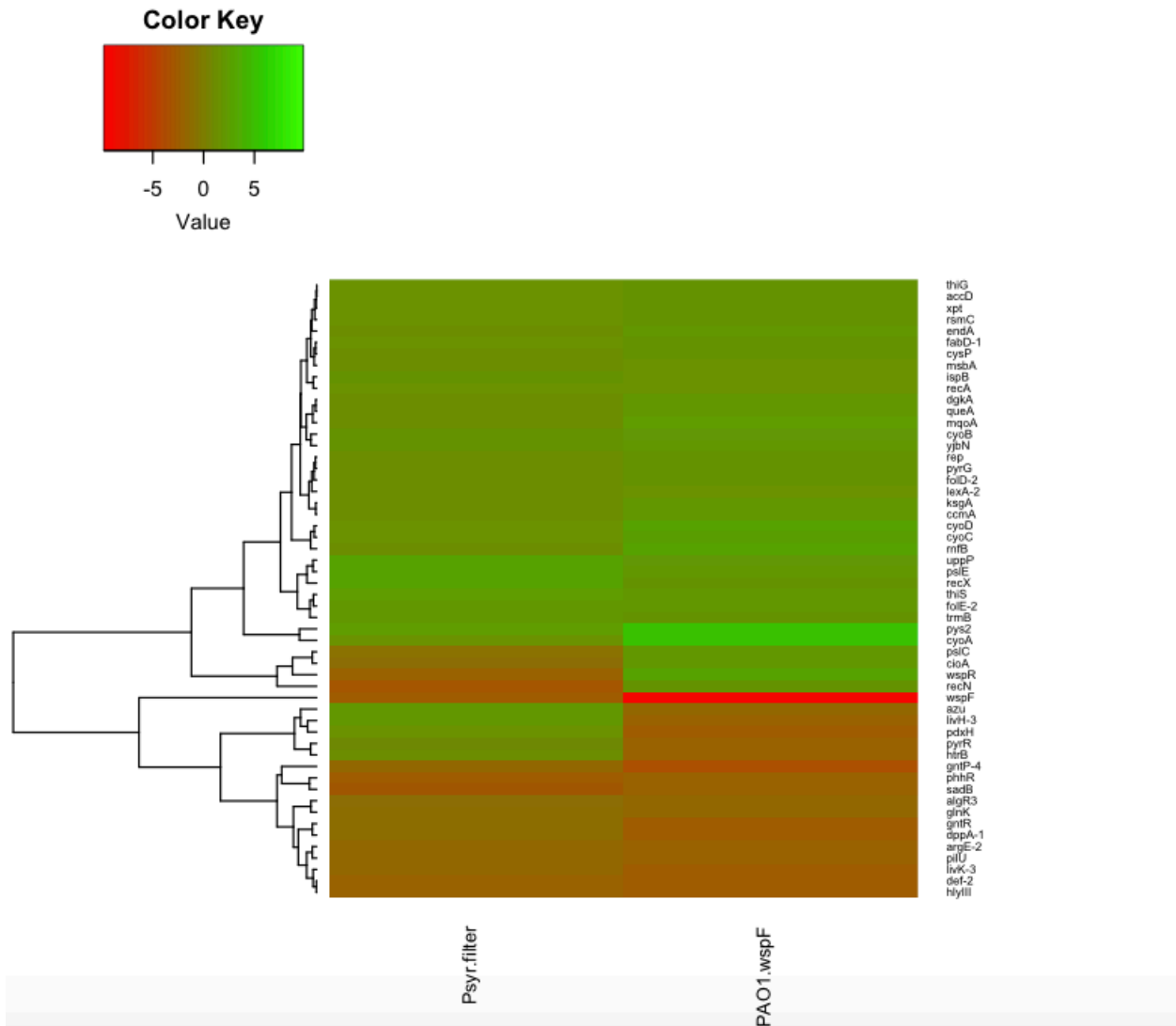
Figure 15. Differential expression in genes of *P. syringae* B728a cells on a filter for 2 hours and *P. aeruginosa* PAO1 *wspF* mutant cells attached to a culture tube for 6 hours.
Heat map generated via the heatmap.2 program in the R statistical package (R Core Team, 2013) showing differentially expressed genes of *P. syringae* B728a cells on a filter (Psyr.filter) compared to *P. aeruginosa* PAO1 *wspF* mutant cells grown in culture (PAO1.wspF) (Hickman *et al*., 2005). A color key at the top shows a range from -5 to 5 (red to green) with red representing down-regulation and green representing up-regulation.
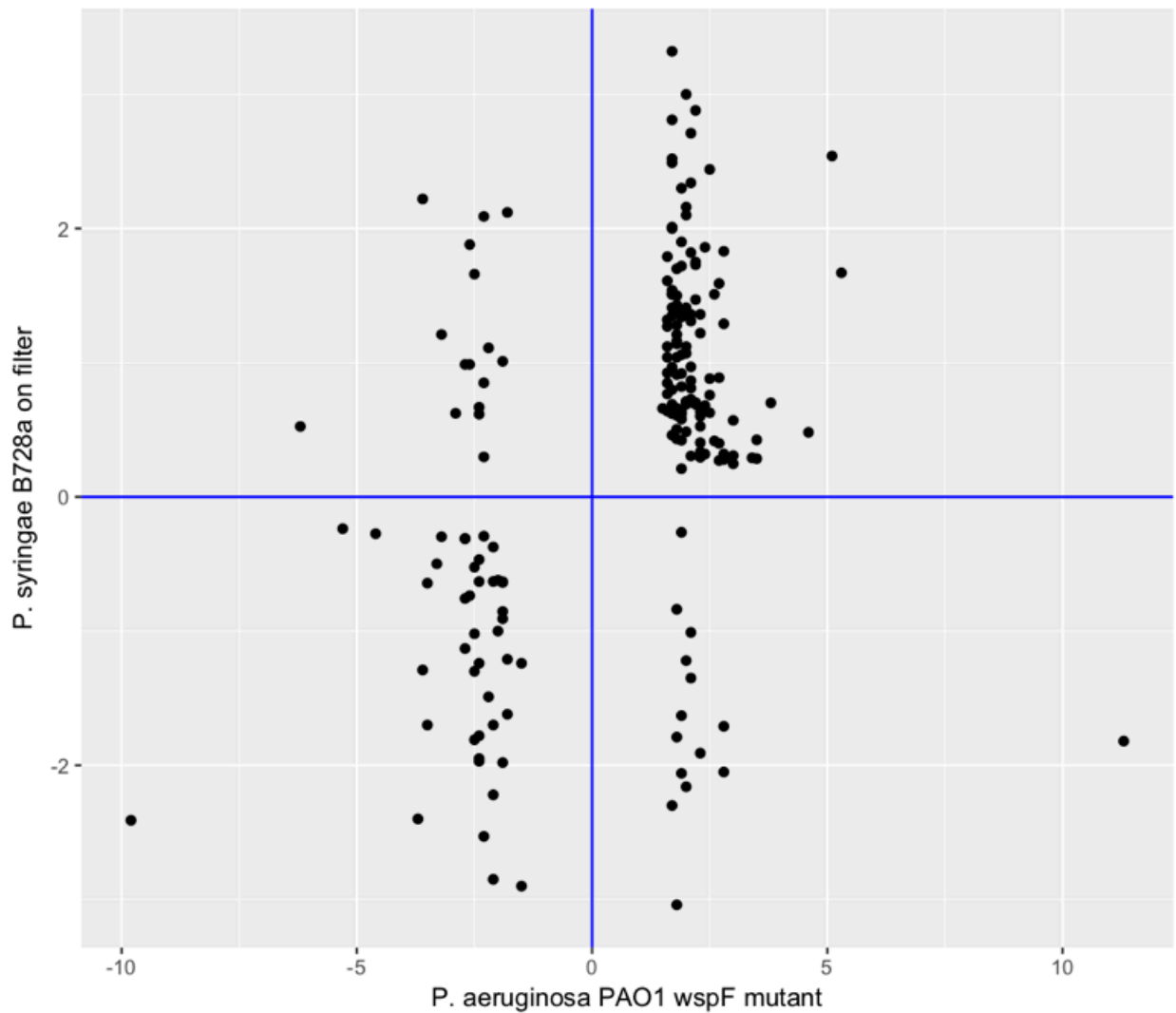
Figure 16. Correlation between the *P. syringae* B728a transcriptome of cells on a filter for 2 hours to the *P. aeruginosa* PAO1 *wspF* mutant cells attached to a culture tube for 6 hours.
The log(fold-change) values of the transcript levels of genes expressed in *P. syringae* B728a cells on a filter relative to the transcript levels of the same cells in liquid culture (y axes) were plotted against the transcript levels of genes expressed in *P. aeruginosa* PAO1 *wspF* mutant cells attached to a culture tube relative to the transcript levels of *P. aeruginosa* PAO1 wild-type cells in culture (x axes). Each point corresponds to a shared gene in *P. syringae* B728a and in *P. aeruginosa* PAO1.
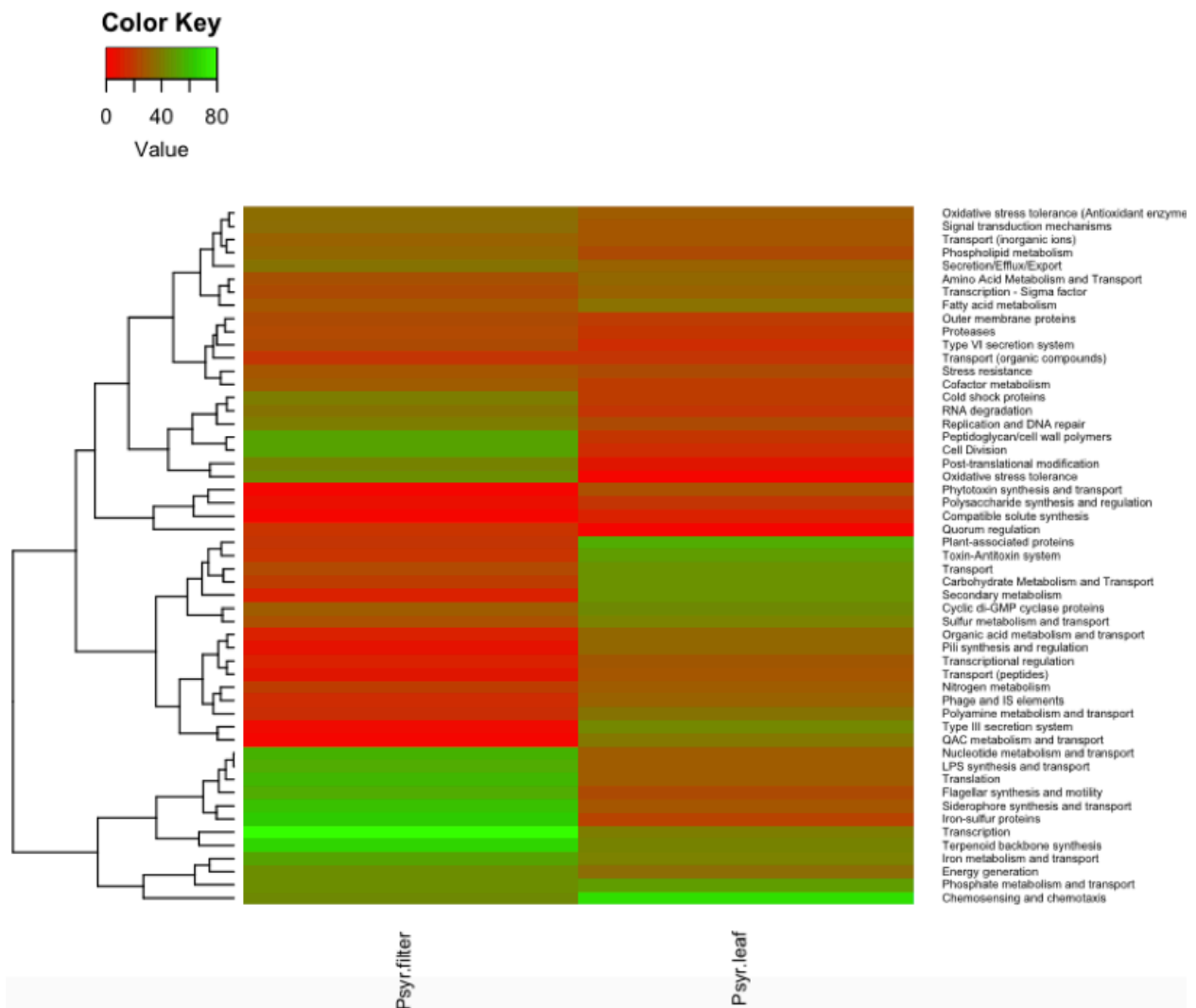
Figure 17. Percent up-regulated gene expression in functional categories of *P. syringae* B728a cells on a filter for 2 hours and *P. syringae* B728a cells on a leaf surface for 72 hours.

Heat map generated via the heatmap.2 program in the R statistical package (R Core Team, 2013) showing the percentage of up-regulated genes in functional categories of *P. syringae* B728a cells on a filter (Psyr.filter) compared to *P. syringae* B728a cells on a leaf surface (Psyr.leaf) (Yu *et al*., 2013). A color key at the top shows a range from 0 to 80 (red to green) indicating the percentage of up-regulated genes that are expressed in a category. Categories with less than 40% of up-regulated genes are in shades of red, indicating mostly down-regulated genes, while categories with over 40% of up-regulated genes are in shades of green, indicating mostly up-regulated genes.
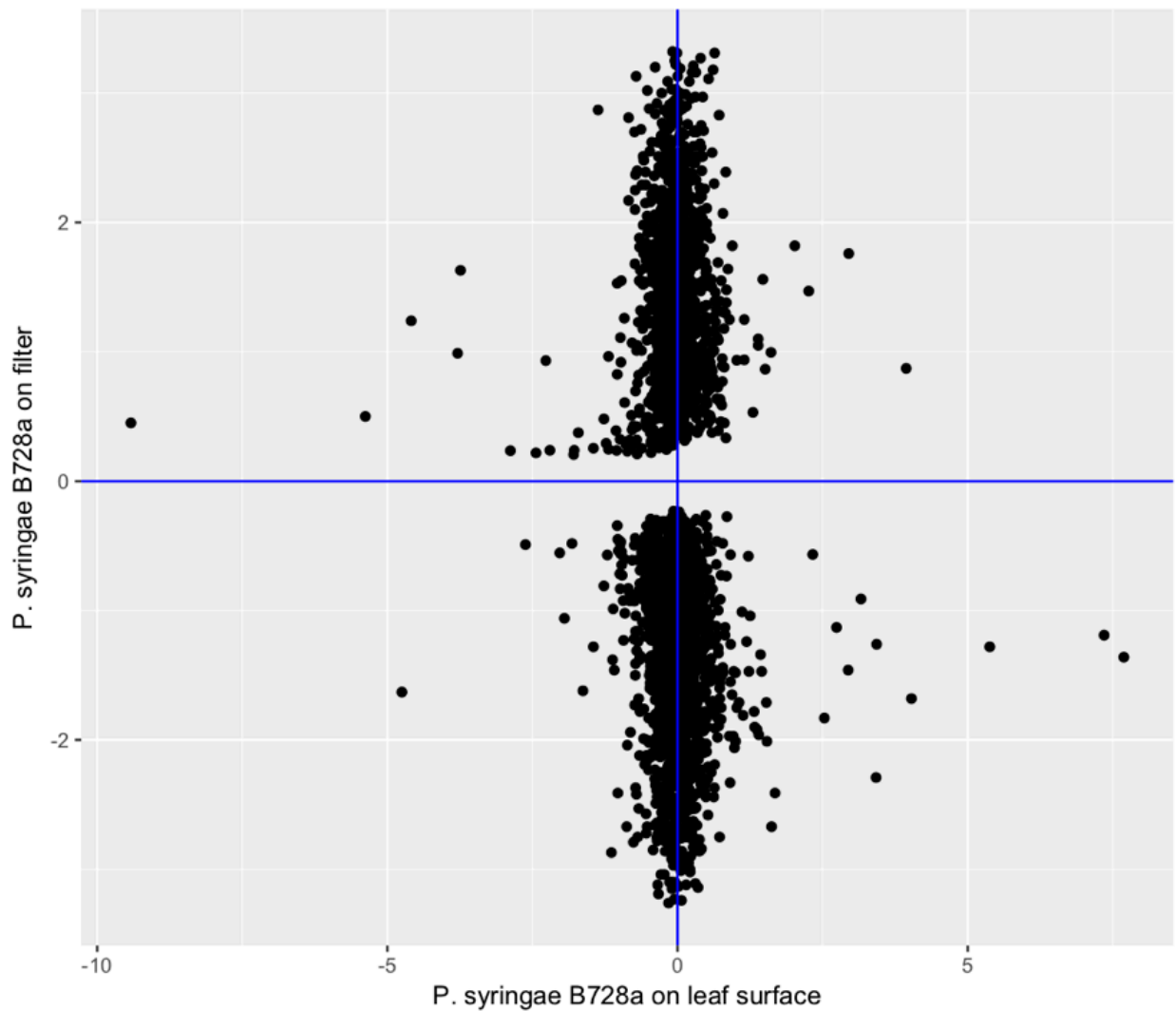
Figure 18. Correlation between the *P. syringae* B728a transcriptomes of cells on a filter for 2 hours compared to cells on a leaf surface for 72 hours.

The log(fold-change) values of the transcript levels of genes expressed in *P. syringae* B728a cells on a filter relative to the transcript levels of the same cells in liquid culture (y axes) were plotted against the transcript levels of genes expressed in *P. syringae* B728a cells on the leaf surface relative to the transcript levels of the same cells in basal medium (x axes). Each point corresponds to a gene in *P. syringae* B728a.

Chapter 4

Regulation of contact-dependent traits in *Pseudomonas syringae* B728a

Abstract

Pseudomonas syringae produces the biosurfactant syringafactin whose production is dependent on contact of cells with various surfaces (Chapter 3; Burch *et al*., 2011). However, it is largely unknown which genes are responsible for the contact-dependent regulation of *syfA* as well as the many other genes in *P. syringae* strain B728a whose expressions are modulated by the contact of cells with surfaces. To determine the identity of regulators mediating contact-dependent transcription, random transposon mutagenesis was performed in *P. syringae* B728a cells harboring a promoterless *gfp* reporter gene fused to the *syfA* promoter. Mutants that experienced either increased or decreased GFP fluorescence compared to the wild-type strain were identified in colonies grown on an agar surface. Mutants that exhibited lower GFP fluorescence, and consequently reduced *syfA* expression, had transposon insertions in Psyr_1001, encoding a basic membrane lipoprotein, Psyr_1491, encoding a methyl-accepting chemotaxis protein orthologous to Tsr in *Escherichia coli* K-12 MG1655, Psyr_2723, encoding a LysR-type transcriptional regulator, Psyr_3368, encoding a hypothetical protein orthologous to the iron transporter EfeO in *Pseudomonas amygdali*, and Psyr_4008, encoding MexB, a component of a multidrug efflux pump. Psyr_2575 encoding SyfR, a positive regulator of *syfA*, also exhibited lower GFP fluorescence than the wild-type strain. Mutants that exhibited higher GFP expression, and consequently increased *syfA* expression, had transposon insertions in Psyr_1143, encoding cytochrome bo_3 quinol oxidase subunit CyoC, Psyr_2474, encoding an acyl-CoA dehydrogenase, Psyr_3282, encoding a TetR transcriptional regulator orthologous to PsrA in *Pseudomonas syringae pv. tomato* DC3000, Psyr_4130, encoding a serine protease that is orthologous to the serine protease AlgW in *Pseudomonas fluorescens* A506, Psyr_4204, encoding a hypothetical protein, and Psyr_4843, encoding the NUDIX hydrolase RppH. Researching the genes found in the transposon mutagenesis revealed some interesting functions. These included that AlgW is involved in a stress sensing pathway which may play a role in activating genes involved in adhesion while PsrA has previously been shown to be important for surface attachment. This suggests that PsrA may play a role in bacterial surface sensing.

Introduction

Bacteria have evolved to adapt to a variety of habitats. While most bacteria are intrinsically colonizers of aqueous habitats, many of them also reside on the surfaces of other organisms such as plants and animals (Morris *et al*., 2008; Tuson and Weibel, 2013). Presumably, such different habitats would require coordination of gene expression to maximize fitness in such different settings. It is still largely unknown, however, how bacteria coordinate gene expression upon the transition from one habitat type to another (Bhomkar et al., 2010; Kearns, 2010; Tuson and Weibel, 2013). *Pseudomonas syringae* B728a, which has been found in aqueous environments, is also a well-studied colonizer of the surface of leaves (Lindow and Brandl, 2003; Melotto *et al*., 2008; Morris *et al*., 2008). This species was therefore deemed to be a good model organism by which such habitat transitions could be studied. RNA sequencing was performed to determine those genes that were differentially expressed by *P. syringae* B728a cells after two hours of immobilization on a surface compared to that in a broth culture of the same chemical environment (Chapter 3). Although a variety of functional categories were found to be enriched

in genes that were up-regulated or down-regulated on the filter surface including flagellar synthesis and motility, chemosensing and chemotaxis, and energy generation, the regulatory pathways controlling the surface-dependent expression patterns of these genes are unknown. While about 60% of the genes of *P. syringae* exhibited differential contact-dependent expression patterns (Chapter 3), it is also unknown to what extent they share common regulators of such habitat-dependent regulation.

The gene *syfA*, which is one of the genes that encodes the enzymes required for production of the biosurfactant syringafactin in *P. syringae* B728a, is much more strongly expressed in cells that are immobilized on surfaces compared to those in liquid culture (Berti *et al.*, 2007; Burch *et al.*, 2011). While previous work by Burch *et al.* (2011) had shown that such up-regulation occurred on agar surfaces compared to that in broth media, studies reported in Chapter 3 revealed that the expression of this gene increased rapidly, irrespective of the nature of the solid surface on which cells were immobilized. These results strongly suggested that *syfA* expression was very responsive to cell immobilization on a surface rather than to chemical cues, since such chemical cues would not have differed in these studies. Given these findings, I reasoned that measurements of the expression of *syfA* cells placed on surfaces would be a good indicator for successful contact-dependent transcriptional regulation in *P. syringae*.

Little is known about the regulation of the *syfA* gene that could provide guidance as to its contact-dependent expression. Berti *et al.* (2007) showed that a LuxR-type regulator, known as SyfR, positively regulates *syfA*. The expression of *syfA* was also shown to be highly temperature dependent, with low levels of expression seen at temperatures above about 27°C (Hocket *et al.* 2014). Previous work by Hockett *et al.* (2014) also found that Psyr_2474, encoding an acyl-coenzyme A (CoA), as well as Psyr_4843, encoding a NUDIX hydrolase known as RppH, both repress *syfA* at elevated temperatures indicating their involvement in thermoregulation. RppH was hypothesized to be involved in high temperature dependent destabilization of messenger RNA (Hockett *et al.*, 2014). Nevertheless, since 1,390 genes in *P. syringae* (26.63% of the *P. syringae* B728a genome) were induced in immobilized cells, this begs the question as to whether the expression of so many genes was influenced by the same regulators as that of *syfA* or by disparate regulators.

To discover what genes are potentially involved in surface regulation, transposon mutagenesis was performed. Since the expression of *syfA* is strongly surface-dependent I utilized this gene as an indicator for surface-dependent gene expression. In this study, *P. syringae* B728a cells harboring a plasmid containing the *syfA* promoter fused to a promoterless *gfp* reporter gene were subjected to random transposon mutagenesis. Since cells of the wild-type strain exhibit GFP fluorescence when grown on an agar surface, it was easy to identify mutants harboring the transposon in genes involved in the regulation of *syfA* since colonies formed by these mutants would exhibit higher or lower levels of GFP fluorescence than that of the wild-type strain. The identity of the gene disrupted by the transposon could be readily found by determining the sequences adjacent to the site of the transposon insertion.

By this approach, 12 genes were found to be involved in *syfA* regulation. Whether these potential regulators were also themselves up- or down-regulated in cells upon immobilization on surfaces could also be determined by review of the transcriptome from studies reported in Chapter 3. SyfR, RppH, and Psyr_2474 were again verified to be involved in *syfA* regulation, providing further validation of this method of screening. However, other genes contributing to expression

of *syfA* on surfaces included Psyr_1001 encoding a basic membrane lipoprotein, Psyr_1143 encoding the cytochrome *bo₃* quinol oxidase subunit, CyoC, Psyr_1491 encoding a methyl-accepting chemotaxis protein orthologous to Tsr in *Escherichia coli* K-12 MG1655, Psyr_2723 encoding a LysR-type transcriptional regulator, Psyr_3282 encoding a TetR transcriptional regulator orthologous to PsrA in *Pseudomonas syringae pv. tomato* DC3000, Psyr_3368 encoding a hypothetical protein orthologous to the iron transporter EfeO in *Pseudomonas amygdali*, Psyr_4008 encoding MexB which is component of a multidrug efflux pump, Psyr_4130 encoding a serine protease orthologous to the serine protease AlgW in *Pseudomonas fluorescens* A506, and Psyr_4204 encoding a hypothetical protein. Further examination of the contact-dependent expression of these genes has revealed putative regulatory pathways contributing to the adaptation of *P. syringae* B728a to life on a surface.

Results

Transposon mutagenesis reveals mutants that positively and negatively regulate the syfA promoter

To determine those genes that are involved in the surface contact-dependent regulation of *syfA,* and potentially other genes that are differentially expressed once cells are immobilized on surfaces, transposon mutagenesis was performed. A suicide plasmid in *Escherichia coli* WM3064 harboring a mariner transposon (Wetmore *et al.*, 2015) was introduced by conjugation into *P. syringae* strain B728a harboring a promoter in which the *syfA* promoter was fused to a promoterless *gfp* reporter gene (P*syfA-gfp*). Mutant cells were identified as colonies on selective media in which only mutants harboring both P*syfA-gfp* and the mariner transposon rescued by insertion into the *P. syringae* genome would grow. Visual examination of colonies illuminated under ultraviolet light revealed colonies that exhibited increased or decreased GFP fluorescence compared to that of the wild-type strain.

A total of 12 mutants were found out of 39,000 screened colonies. Of these mutants, six were more fluorescent than the wild-type strain, indicating that the genes harboring the transposon negatively regulate *syfA*, while six were less fluorescent, indicating that the genes harboring the transposon positively regulate *syfA* (Tables 1 and 2). The genes interrupted by the transposons are described below.

Genes encoding putative positive regulators of *syfA*, with mutants exhibiting lower GFP fluorescence than wild-type strains (Table 1):

Psyr_1001 encodes a basic membrane lipoprotein with no known homology to genes studied in *P. syringae*.

Psyr_1491 encodes a methyl-accepting chemotaxis protein (MCP). MCPs sense environmental signals and transmit them to other proteins involved in chemotaxis (Wuichet *et al.*, 2009). According to the KEGG database (Kanehisa *et al.*, 2004), Psyr_1491 has 26% identity to Tsr in *E. coli* K-12 MG1655. Tsr is an MCP that typically interacts with serine. This gene has also been shown to be important in cell swarming independent of its interaction with serine (Burkart *et al.*, 1998).

Psyr_2575 encodes the LuxR-type regulatory protein, denoted SyfR, which has been shown to be a positive regulator of *syfA* (Berti *et al.*, 2007).

Psyr_2723 encodes a LysR-type regulatory protein. The LysR family of transcriptional regulators consists of proteins that typically become activated in response to a co-inducer and bind to DNA regardless of whether a co-inducer is present or not. However, when a co-inducer is present, this can cause the LysR protein to interact with other parts of the DNA strand, resulting in either the transcriptional activation or repression of genes. LysR proteins also tend to repress their own transcription in order to potentially moderate their own transcript and protein levels (Schell, 1993).

Psyr_3368 encodes a hypothetical protein that has 92.8% identity to the iron uptake protein EfeO in *Pseudomonas amygdali*. EfeO is part of the iron transporter EfeUOB. In *E. coli* this transport system has been shown to be negatively regulated by a ferrous uptake regulator (Fur) and induced under conditions of low iron availability. It is also induced under acidic conditions and is repressed by CpxAR, which is activated in response to cell envelope stress, at a high pH (Cao, 2007).

Psyr_4008 encodes MexB which is a component of the MexAB-OprM efflux pump (Alcade-Rico *et al*., 2016; Martinez *et al*., 2009, Sun *et al*., 2014). The *mexAB-oprM* efflux pump has been shown to play an important role in multidrug resistance in *Pseudomonas* species (Alcade-Rico *et al*., 2016; Martinez *et al*., 2009; Sun *et al*., 2014) including *P. syringae* B728a (Helmann, personal communication). This operon has also been shown to be positively regulated by CpxR in *P. aeruginosa* (Tian *et al*., 2016), but negatively regulated by AefR in *P. syringae* B728a (Scott, 2013).

Genes encoding putative negative regulators of *syfA*, with mutants exhibiting higher GFP fluorescence than wild-type strains (Table 2):

Psyr_1143 encodes the cytochrome *bo₃* quinol oxidase subunit, CyoC. A member of the cytochrome *bo₃* quinol oxidase complex consisting of the *cyoABCDE* genes, CyoC functions under oxygenated conditions and is down-regulated under conditions of low oxygen (Arai, 2011). The gene complex is also induced during iron starvation and repressed by the transcriptional regulator Fur when sufficient iron is present. This complex has been identified as one of the five terminal oxidases in *P. aeruginosa* that functions as part of aerobic respiration (Arai, 2011).

Psyr_2474 encodes an acyl-CoA dehydrogenase. According to the KEGG database (Kanehisa *et al*., 2004), these enzymes are involved in multiple metabolic pathways including fatty acid degradation and metabolism (Hockett *et al*., 2014). This gene was found in a previous transposon screen performed by Hockett *et al*. (2014) who reported that when disrupted in *P. syringae* B728a, the expression of *syfA* was higher at 28°C than in the wild-type strain for which the expression of this gene decreased rapidly with increasing temperatures above about 24°C.

Psyr_3282 encodes a TetR-type regulatory protein (Kojic *et al*., 2001). It has 98.7% identity to the transcriptional regulator PsrA in *P. syringae* pv. *tomato* DC3000. PsrA has been shown to negatively regulate *aefR* in *Pseudomonas aeruginosa* and to positively regulate *rpoS* (Chatterjee *et al*., 2007; Kojic *et al*., 2001). AefR is known to positively regulate quorum sensing in *P. syringae* B728a (Quiñones *et al*., 2003), but negatively regulate the expression of the MexAB-OprM efflux pump (Scott, 2013). RpoS is a regulator involved in expression of those genes operative primarily in the stationary phase of cell growth and has been shown to activate surface-

associated genes such as those involved in chemosensing and chemotaxis (Kojic *et al.*, 2001; Kojic *et al.*, 2002; Kojic *et al.*, 2005; Scott, 2013).

Psyr_4130 encodes a serine protease. It has an 87.7% identity to AlgW in *Pseudomonas fluorescens* A506. In *P. aeruginosa*, Wood and Ohman (2009) stimulated cell wall stress by exposing cells to D-cycloserine which is a peptidoglycan biosynthesis inhibitor. From their observations, they created a model in which a cell that is not experiencing cell wall stress will have the sigma factor, σ^{22}, sequestered by the MucAB complex. However, when cell wall stress is experienced, AlgW plays a role in degrading MucA. This results in the release of σ^{22} which activates genes that promote cell wall recovery. Thus, AlgW has been shown to be essential for cell wall stress response (Wood and Ohman, 2009).

Psyr_4204 encodes a hypothetical protein.

Psyr_4843 encodes a NUDIX hydrolase designated as RppH. This gene was found in a previous transposon screen performed by Hockett *et al.* (2014) who reported that its disruption caused a large increase in expression of *syfA* in cells grown at the elevated temperature of 28°C compared to those grown at cooler temperatures.

All mutants exhibit altered GFP expression indicating altered syfA induction

To verify that the mutants described above, which were selected based on a visual examination of the GFP fluorescence of colonies growing on agar plates, actually exhibited significantly altered *syfA* expression, GFP fluorescence was quantified in each strain using a fluorescence plate reader (Figures 1 and 2). GFP fluorescence was compared to both a wild-type strain harboring the plasmid p*519n-gfp,* in which the *gfp* reporter gene was expressed constitutively as a positive control, and that of a wild-type strain lacking GFP as a negative control. GFP fluorescence was also quantified in the wild-type strain harboring P*syfA-gfp* - which yields contact-dependent GFP fluorescence. GFP fluorescence was compared for each strain grown on an agar surface and in a comparable liquid broth. As expected, the GFP fluorescence exhibited by the positive control strain did not differ when grown on agar surfaces compared to that in broth cultures (Figures 1 and 2). As was noted in visual observations of colonies, the Psyr_1001, *mexB*, *tsr*, *syfR*, *efeO*, and Psyr_2723 mutant strains all exhibited significantly lower GFP fluorescence than the wild-type strain harboring P*syfA-gfp* when grown either on a plate or in liquid culture (Figure 1). These results support the model that these disrupted genes participate in pathways that either directly or indirectly induce *syfA* gene expression, and possibly that of other genes that show increased contact-dependent gene expression.

All mutant strains for which elevated GFP fluorescence was observed by visual inspection, except the *rppH* mutant, exhibited quantitatively higher GFP fluorescence when grown on a solid medium (Figure 2). Neither the *rppH* or *psrA* mutant strains exhibited higher GFP fluorescence than the wild-type strain when grown in liquid broth (Figure 2). Thus, with the exception of the *rppH* mutant, these results corroborate the initial findings made by visual inspection that these latter genes participate in pathways that either directly or indirectly repress *syfA* gene expression.

Mutants exhibited varying levels of biosurfactant production in response to temperature

As the expression of *syfA*, and thus syringafactin production, were both shown to be strongly suppressed in *P. syringae* grown at elevated temperatures (Hockett *et al.*, 2013; Hockett *et al.*, 2014), I explored whether there was an involvement of temperature in the function of the

apparent surface contact-dependent regulators of *syfA* found in the mutagenesis screen. Syringafactin production, normally detected only in cultures of the wild-type strain when grown on agar surfaces, was detected using an atomized oil assay (Burch *et al.*, 2010) for each of the mutants and the wild-type strain, each harboring P*syfA-gfp*, when grown at either 28° or 22°C. In this assay, a halo of raised oil droplets is formed in the vicinity of colonies of syringafactin-producing strains (Burch *et al.*, 2010) and syringafactin production can be readily quantified by measuring the radii of the resultant halos. Of the 12 genes identified in the mutagenesis screen, mutants blocked in seven of these genes exhibited altered syringafactin production compared to the wild-type strain (Table 3). Mutants with an insertion in either Psyr_1001 or *syfR* exhibited lower syringafactin production than the wild-type strain when grown at both 28°C and 22°C (Table 3). This makes sense because these mutants both exhibited decreased GFP fluorescence (Table 1), suggesting that Psyr_1001 and *syfR* both induce *syfA*. Therefore, mutating these genes appears to lead to a decrease in *syfA* induction resulting in a decrease in biosurfactant production. In contrast, mutants with insertions in Psyr_2474 and *rppH*, which both appeared to exhibit increased GFP fluorescence (Table 2)*, both exhibited increased syringafactin production compared to the wild-type strain when grown at 28°C but not at 22°C (Table 3). These results confirm the observations of Hockett *et al*. (2014) that these two genes both negatively regulate *syfA* expression at high temperatures. Therefore, when these genes are mutated, the reduced repression of *syfA* at high temperatures would result in an increase in biosurfactant production. Mutants with insertions in either *algW* or Psyr_4204, which exhibited increased GFP fluorescence (Table 2), also exhibited increased syringafactin production at 28°C but not at 22°C (Table 3) while the mutant with an insertion in Psyr_2723, which exhibited decreased GFP expression (Table 1), exhibited a decrease in syringafactin production when grown at 22°C but not at 28°C (Table 3). These results are further indicators of these genes having an effect on syringafactin production.

Selected genes exhibited varying levels of expression when cells were attached to a surface, suggesting different roles in bacterial adaptation

To further understand the regulatory context of the genes modulating the surface contact-dependent expression of *syfA*, I ascertained whether these genes in cells exhibited differential expression in planktonic cells compared to immobilized cells. The transcript on the analysis of planktonic and immobilized cells (Chapter 3) was analyzed to provide not only descriptions of patterns of gene expression but also the functional categories in which these 12 putative regulatory genes could be grouped (Table 4). Most of the 12 genes exhibited higher levels of expression in cells immobilized on a filter surface compared to that in broth cultures, with *tsr*, *cyoC*, *mexB*, and *algW* being up-regulated more than 2-fold while *syfR* was up-regulated more than 3-fold. *rppH,* Psyr_1001, and Psyr_2474 were up-regulated more than 10-fold. Only Psyr_2723 and Psyr_4204 were expressed at a lower level in cells on a filter compared to that in a broth culture (Table 4); Psyr_2723 was down-regulated more than 3-fold. The differential expression of all of the genes except for *psrA* was highly significant (Table 4). The very high apparent expression of *psrA* in cells on filters compared to that in broth cultures may represent a laboratory artifact as the high p-value (0.49) for observations of this gene suggests high experimental variability.

The 12 genes found to control *syfA* expression in the transposon mutagenesis screen can be placed in a variety of different functional categories. The gene *tsr* can be grouped with other genes involved in chemosensing and chemotaxis, a category that was significantly enriched in

cells immobilized on filters (Chapter 3). *cyoC* can be grouped with genes involved in energy generation, a category that is also up-regulated in immobilized cells (Chapter 3). Other gene categories that included loci found in the transposon mutagenesis screen included those involved in RNA degradation, secondary metabolism, secretion/efflux/export, and transcriptional regulation, while others were unannotated or hypothetical (Table 4). Apparently, many different pathways are directly or indirectly involved in the adaptation of *P. syringae* B728a to life on a surface.

Discussion

Bacteria such as *P. syringae* clearly are able to adapt to life on surfaces in and on plants despite the fact that they likely also inhabit aquatic habitats where they might exhibit a strictly planktonic lifestyle (Melotto *et al.*, 2008; Morris *et al.*, 2008). The many adaptations for life on a surface are likely distinct from those that would optimize fitness in planktonic settings. Furthermore, it might be expected that a common set of adaptations might distinguish planktonic from more sessile life stages. Given that a large number of genes were found to be differentially expressed in cells immobilized on surfaces compared to those in broth media (Chapter 3), it seems likely that they might have common regulators to coordinate expression of such suites of genes. Unfortunately, the regulatory mechanisms coordinating such habitat-specific adaptations were unknown. In this study, monitoring of *syfA* expression using a *gfp* reporter gene construct in conjunction with random transposon mutagenesis revealed that the number of regulators needed for *syfA* expression, and by extension presumably that of many other surface contact-dependent traits, was rather modest. Assuming that the mariner transposon inserted randomly into the genome, the fact that more than one insertion event was found in four of the genes supports the conjecture that my identification of genes having an effect on the surface-dependent expression of *syfA* was nearly complete (Tables 1 and 2).

Of the 12 genes that were found to influence expression of *syfA* upon disruption, half resulted in a decrease in GFP expression indicating that these genes are positive regulators (Figure 1). Among these was *syfR*, a LuxR-type regulator that has already been shown to be a positive regulator of *syfA* (Berti *et al.*, 2007). As expected, the *syfR* mutant exhibited a decrease in GFP expression compared to the wild-type strain as well as a dramatic decrease in syringafactin production (Figure 1 and Table 3). Since this gene is in an operon with *syfA* it is most likely that it is a specific regulator of syringafactin production per se, and not one of the many other genes that exhibited similar expression patterns of *syfA*. In support of this conjecture was the observation that the *syfR* mutant was one of only two strains to exhibit a decrease in syringafactin production in cells grown at both 22°C and 28°C (Table 3). The mutant in which Psyr_1001 was disrupted also was blocked in *syfA* expression at both temperatures (Table 3). This locus putatively encodes a basic membrane lipoprotein, but its role in syringafactin production has not been elucidated and it cannot be easily assigned to any particular functional category (Table 4), indicating that there is still much to be discovered about it. Interestingly, the expression of Psyr_1001 did not differ between cells grown in broth cultures and cells immobilized on filter surfaces (data not shown). Psyr_1001 may encode a structural protein whose abundance may not be expected to rapidly change when cells sense a surface. Instead, since Psyr_1001 encodes a membrane protein, it may function as a surface sensor where it can send signals to other proteins in response to surface contact. Such mechanosensitive proteins have been described in other bacteria including the outer membrane protein PilY1 in *Pseudomonas aeruginosa* which has been shown to play a role in surface sensing and surface

sensing-dependent expression of virulence factors (Siryaporn *et al.*, 2014). In *E. coli* the structural outer membrane protein NlpE is required for surface-dependent expression of the Cpx system which is involved in sensing cell envelope stress (Otto and Silhavy, 2002). In both cases, torque and outer membrane/cell wall disturbance associated with the attachment of cells to an immobile surface is hypothesized to be a component of the mechanosensitive process. Clearly, much more must be done to better understand how such a process operates. It should prove fruitful to understand whether Psyr_1001 contributes only to the surface-dependent expression of *syfA* or, as would be expected if it is a component of a mechanosensitive surface sensing system, would be required for appropriate expression of the many genes I have found to be dependent on immobilization for their expression.

Disruption of Psyr_1491, encoding a MCP, also abolished surface-dependent induction of *syfA*. The KEGG database (Kanehisa *et al.*, 2004) suggests that this gene is orthologous to Tsr in *E. coli* K-12 MG1655 - a MCP that interacts with serine. Interestingly, Tsr has also been shown to be required for bacterial swarming, independent of its interaction with serine (Burkart *et al.*, 1998). It has been suggested that as an outer membrane protein, Tsr communicates signals to other genes to activate swarming, a type of movement that bacteria only exhibit when on a surface (Burkart *et al.*, 1998; Kearns, 2010). Given this putative role, it would make sense that if Psyr_1491 had a similar function as Tsr, it would be expected to be a positive regulator of *syfA* and likely other surface-induced genes. As described in Chapter 3, genes involved in chemosensing and chemotaxis were significantly enriched among those up-regulated on a surface. Such an observation would be expected since chemosensing and chemotaxis are important for adaptation to life on a leaf surface due to the heterogeneous dispersal of nutrients on the leaf surface (Leveau and Lindow, 2001; Yu *et al.*, 2013). Furthermore, genes encoding flagellar synthesis and motility were also typically up-regulated in cells immobilized on surfaces (Chapter 3). Previous studies have indicated that bacterial movement on the leaf surface is very prominent, and likely is required for cells to encounter the spatially disparate sources of nutrients and water on leaves (Leveau and Lindow, 2001; Yu *et al.*, 2013). The likely role of biosurfactants such as syringafactin in the process of swarming on leaves (Kearns, 2010) further supports the logic of a linkage of Tsr to both *syfA* expression, and that of genes contributing to movement on surfaces, and suggests that these genes could logically play a role in expression of the many other surface-dependent genes seen in this study.

Several genes that apparently are negative regulators of *syfA* were discovered in this study. Both Psyr_2474 encoding an acyl-CoA dehydrogenase and Psyr_4843 encoding the RNA degradation enzyme RppH, had been found previously to serve as thermo-dependent repressors of *syfA* at high temperatures (Hockett *et al.*, 2014). My finding of higher levels of syringafactin production in mutants in which these genes were disrupted when assayed at 28°C but not at 22°C supports this earlier observation (Table 3). Surprisingly, while the Psyr_2474 mutant harboring the P*syfA*-*gfp* plasmid exhibited quantitatively increased GFP expression when grown on a plate at 22°C compared to the wild-type strain, the *rppH* mutant exhibited lower GFP expression (Figure 2). This is especially surprising given that *rppH* mutants were originally identified as colonies on a plate that appeared to be more fluorescent than that of wild-type colonies (data not shown). These contrary observations might readily be explained after accounting for the different temperatures used in these various studies and the fact that these two regulators confer thermo-dependent gene expression. Mutant colonies were originally isolated on plates incubated at 28°C to allow colonies to grow, while GFP fluorescence was quantified in cells grown at 22°C. It is

not clear however whether the expression of Psyr_2474 also would have had such a strong and overriding effect of temperature.

Disruption of Psyr_4130, encoding a serine protease, and Psyr_4204, encoding a hypothetical protein, both led to the increased expression of *syfA* in cells grown either on plates or in broth cultures as well as increased syringafactin production at 28°C but not at 22°C (Figure 2 and Table 3). The fact that these strains produce more syringafactin than the wild-type strain at the higher but not at the lower temperature suggests that they, like RppH and Psyr_2474, may be temperature-dependent repressors of *syfA* at higher temperatures. Unfortunately, not much is known about the hypothetical protein encoded by Psyr_4204. However, its expression was found to be lower on cells immobilized on filter surfaces then in cells in broth cultures (Table 4). Clearly, further work is needed to determine the role of this gene.

Although little is known about Psyr_4204, according to the KEGG database (Kanehisa *et al*., 2004) the gene encoded by Psyr_4130 is an ortholog of AlgW in other *Pseudomonas* species. AlgW in *P. aeruginosa* has been shown to be involved in cell wall stress responses (Wood and Ohman, 2009). As mentioned previously, Wood and Ohman (2009) stimulated cell wall stress by exposing *P. aeruginosa* cells to a peptidoglycan biosynthesis inhibitor and from their observations, came up with a model in which a cell that is not experiencing cell wall stress will have the sigma factor, σ^{22}, sequestered by the MucAB complex. However, they hypothesized that when cell wall stress is experienced, AlgW plays a role in degrading MucA which results in the release of σ^{22} resulting in the activation of genes that promote cell wall recovery. Although the cell wall stress response can occur upon exposure of cells to antibiotics, as shown by Wood and Ohman (2009), it has also been shown in studies of the Cpx pathway in *E. coli* that cell wall damage can occur when a cell attaches to a surface (Otto and Silhavy, 2002). The Cpx pathway is activated in response to cell envelope stress in which the cytoplasmic response regulator, CpxR, becomes activated through phosphorylation by CpxA, an inner membrane histidine kinase. The phosphorylated CpxR then binds to DNA resulting in the increased expression of a suite of genes including those involved in adhesion (Otto and Silhavy, 2002). In addition, such stresses tend to also result in compensatory peptidoglycan and lipopolysaccharide synthesis (Chang, 2017; Wood *et al*., 2006; Wood and Ohman, 2009). Therefore, it is noteworthy that genes involved in peptidoglycan and lipopolysaccharide biosynthesis tended to be up-regulated in cells immobilized on filter surfaces (Chapter 3). Given the role of AlgW in degrading MucA so that the stress response signal can be transmitted, it would be expected that this gene would be up-regulated in cells on surfaces, which it is by more than 2-fold (Table 4), so that genes involved in peptidoglycan/cell wall synthesis and lipopolysaccharide synthesis could be induced.

In contrast to mutants exhibiting increased syringafactin production, disruption of Psyr_2723 encoding a LysR-type transcriptional regulator resulted in significantly less syringafactin production at 22°C compared to the wild-type strain (Table 3). Such a phenotype is consistent with the lower apparent transcription of *syfA* grown either on plates or in broth cultures (Figure 1). Surprisingly, the expression of Psyr_2723 was more than 2-fold lower in cells immobilized on a filter compared to that of planktonic cells (Table 4). Given these contradictory results, more studies are needed to decipher the regulatory pathway in which this LysR-type regulator participates.

Other putative regulators of *syfA* were found that apparently are linked to other aspects of the physiology of *P. syringae*. Psyr_1143 encoding the cytochrome *bo₃* quinol oxidase subunit

known as CyoC, and Psyr_3368 encoding a hypothetical protein that according to the KEGG database (Kanehisa *et al.*, 2004) is orthologus to the iron-uptake protein EfeO in *E. coli,* appear to be a negative regulator and a positive regulator of *syfA* respectively. Both genes, were more highly regulated on cells immobilized on filters compared to in broth, with *cyoC* being up-regulated more than 2-fold (Table 4). Previous studies have also shown that both genes are expressed under aerobic conditions and induced during iron starvation (Arai, 2011; Cao *et al.*, 2007). The functional categories of siderophore synthesis and transport and iron metabolism and transport were significantly enriched for genes up-regulated on filter surfaces (Chapter 3). Moreover, genes in the category of energy generation, which includes *cyoC* and the other genes involved in the cytochrome *bo₃* quinol oxidase complex, were significantly over represented among those up-regulated on a filter as well (Chapter 3). In Chapter 3, it was reasoned that these iron starvation-induced genes were all up-regulated because cells on a surface would be expected to have decreased access to nutrients such as iron. Both on leaves and on a filter, cells would presumably have to rely on local sources of these nutrients through a process of diffusion. In contrast, cells in a liquid environment, especially those subject to mechanical agitation, would see a local replenishment of nutrients and hence diffusion gradients would be unlikely to become established since nutrients overall would be more available than to cells immobilized on a surface. Given these differences in nutrient concentration and mechanisms of access to such nutrients, it is likely that both genes play important roles in the regulation of surface-induced genes, such as *syfA*, that would help cells cope with such local diffusion limited resource acquisition. I am quite confident that EfeO plays an important role in the regulation of *syfA* since it was found three times during the mutagenesis screen (Table 1). The pathway in which EfeO participates seems straightforward in that low-iron conditions induce this gene, leading to the activation of genes involved in iron and siderophore synthesis and transport as well as the potential activation of other surface-related genes such as *syfA* (Figure 3). In contrast, the pathways in which CyoC participates seem to be more complex. While this gene is up-regulated on filter surfaces, it still seems to be a negative regulator of *syfA* due to the increased *syfA* expression exhibited by a *cyoC* mutant (Figures 2 and 3). Further studies are needed to discover the link between this gene and surface-regulated genes such as *syfA*.

A logical linkage can be made for the coordination of Psyr_3282, encoding a TetR family regulatory protein that is orthologous to PsrA in *Pseudomonas*, and Psyr_4008, encoding MexB, a component of an important multidrug efflux pump, in influencing surface-dependent traits in *P. syringae*. While MexB is a positive regulator of *syfA*, PsrA is a negative regulator (Figure 4). Both genes were revealed to be more highly expressed in immobilized cells then planktonic cells although in the case of *psrA* this difference did not reach a level of significance (Table 4). Models accounting for the positive regulation of *syfA* and potentially other surface-induced genes by MexB can be readily constructed. As previously discussed in Chapter 3, efflux pumps seem to play an important role in surface adaptation of both *Pseudomonas* species and *E. coli* since they would be required to contend with toxins made by their hosts (Alcade-Rico *et al.*, 2016). Thus, it is logical that that the *mexAB-oprM* efflux pump would be induced on a surface. MexB might participate in a process by which local chemical composition would change thereby providing a cue that a surface had been sensed by the cells which would lead to expression of a variety of traits that would benefit life in the surface habitat, presumably also including that of *syfA* (Figure 4). Interestingly, PsrA has been shown to negatively regulate AefR which, in turn, negatively regulates the MexAB-OprM efflux pump (Figure 4; Chatterjee *et al.*, 2007; Quiñones *et al.*, 2003; Scott, 2013). AefR has also been shown to positively regulate quorum sensing in *P.*

syringae B728a - a trait shown to be important in the survival of *P. syringae* B728a on leaf surfaces (Figure 4; Monier and Lindow, 2003; Quiñones *et al.*, 2003). Quorum sensing apparently coordinates cell density-dependent gene expression that occurs in bacterial aggregates on leaves, thereby facilitating cell survival within aggregates as opposed to when a cell is solitary (Monier and Lindow, 2003; Quiñones *et al.*, 2003; Scott, 2013). PsrA has also been shown to positively regulate RpoS which serves as a regulator of traits important in stationary phase growth and survival (Figure 4; Kojic *et al.*, 2001). RpoS also activates genes important for surface adaptation such as those involved in chemotaxis (Scott, 2013). Given this, it is likely that PsrA is involved in complex pathways leading to both the potential regulation of, and in the case of *syfA*, repression of genes important in surface adaption. Intriguingly, a study by Gooderham (2008) has shown that *psrA* mutants exhibit decreased attachment to surfaces compared to wild-type cells. This was demonstrated by growing cells in micotiter plates and analyzing how many had attached after a period of 30 minutes. Although it is surprising that PsrA would be a negative regulator of *syfA*, the study by Gooderham (2008) clearly shows that it must play a role in positively regulating some genes involved in surface attachment. Moreover, it seems quite likely that it might be responsible for the surface-dependent induction of at least some of the many genes in *P. syringae* exhibiting this pattern. While more studies of PsrA are needed to better define its linkage to regulation in response to surface attachment, it is clear that it plays an important role in aiding cell adaption to life on a surface.

In conclusion, surface adaption clearly plays an important role in the lifestyle of bacteria such as *P. syringae* B728a since a large proportion of its genes exhibit surface contact-dependent gene expression. However, until this study, little was known about the genes required for such habitat-dependent transcription. This mutagenesis study has shown that a variety of genes having different transcriptional patterns on leaves appear to play a role in both the regulation of *syfA* and potentially other surface-induced genes. These findings further shed light on the complexity of surface adaption and provide candidate genes that should be examined in more detail. In particular, while this study has shown that *syfA* is within the transcriptome of each of these genes, the size of the transcriptomes and the degree of overlap between them for these putative regulators will be important to know. It should be fruitful to perform transcriptomics on these mutants as well as wild-type strains in cells immobilized on surfaces and in planktonic cells. This would provide more verification for a general role of these genes in surface regulation and to decipher the pathways in which these genes participate. With this information, we would be closer to better understanding the phenomenon of surface adaption in bacteria.

Acknowledgments

Experimental Procedures

Bacterial strains and growth conditions

P. syringae B728a and *E. coli* WM3064 strains were grown on Luria Broth (LB) plates (Luria and Burrous, 1957) containing 1.5% technical agar (Difco). As appropriate, 5 µg/ml

Diaminopimelic acid (DAP) was added to the media. Antibiotics were used at the following concentrations (µg/ml): spectinomycin (100) and kanamycin (50).

Production of transposon mutants

P. syringae B728a strains harboring plasmid P*syfA-gfp* in which the *syfA* promoter was fused with a promoterless *gfp* reporter gene (Burch *et al.*, 2010)*,* were grown overnight on LB plates containing 100 µg/ml spectinomycin. *E. coli* WM3064 harboring a mariner transposon (Wetmore *et al.*, 2015), was grown overnight on LB plates containing 50 µg/ml kanamycin and 5 µl/ml of a 60 mM stock of DAP. 10^9 cells/ml were re-suspended in 1 ml of 10 mM KPO4. Three tubes of *P. syringae* B728a and one tube of *E. coli* WM3064 were then mixed. Cells were centrifuged for two minutes at 7000 g and the phosphate buffer decanted. 100 µl of phosphate buffer was then added to each tube to re-suspend the cells*.* 10 droplets of 20 µl each of the mixture were placed on LB plates containing 5 µg/ml of a 60 mM stock of DAP. Plates were incubated overnight at 28°C. Each of the 10 spots of bacterial cells that had undergone mating were re-suspended in 1 ml of 10 mM KPO₄ buffer. Cells were mixed gently using a pipette in 100 µl of buffer and spread onto LB plates, containing 50 µg/ml kanamycin and 100 µg/ml spectinomycin, using glass beads. All plates were incubated at 28°C for two days.

Screening of transposon mutants

Colonies grown on plates were visually examined under illumination with a ultraviolet lamp to identify colonies that exhibited either increased or decreased GFP fluorescence compared to wild-type colonies. Arbitrarily primed PCR was then used to assess the location of the transposon in the mutant colonies (O'Toole *et al.*, 1999). Primer U1 (GATGTCCACGAGGTCTCT), Primer ARB1 (GGCCACGCGTCGACTAGTACNNNNNNNNNNNGATAT), and Primer ARB6 (GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC) were used in the first reaction and Primer U2 (CGTACGCTGCAGGTCGAC) and Primer ARB2 (GGCCACGCGTCGACTAGTAC) were used in the second reaction to amplify the gene that the insertion had occurred in. Primer U1 and Primer U2 were both complementary to the sequence of the mariner transposon while Primer ARB1, Primer ARB2, and Primer ARB6 were used to amplify the regions of the DNA sequence flanking the transposon insertion. PCR products were run on a 1% agarose gel and the largest of several bands that typically resulted from a given amplification with these nested primers were excised and purified using a QIAquick Gel Extraction kit (Qiagen). The purified DNA was subjected to Sanger sequencing (Sanger *et al.*, 1977) using Primer U2 and all returned sequences were analyzed using BLAST on the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov) to determine the homologous chromosomal genes into which the transposon had inserted.

Quantifying GFP fluorescence of mutants to verify changes in syfA expression

P. syringae B728a strains harboring plasmid P*syfA*-gfp or a plasmid conferring constitutive fluorescence (p*519n-gfp*; Matthysse *et al.*, 1996), were grown overnight at 22°C on LB plates containing either 100 µg/ml spectinomycin or 50 µg/ml kanamycin as appropriate for the wild-type and mutant strains respectively. Cells were either re-streaked onto fresh LB agar plates or suspended in LB broth. Plate and liquid cultures were incubated at 22°C overnight. To quantify GFP fluorescence, cells from plates were suspended in LB and 50 µl of the suspended cells or cells grown in broth cultures were added to wells in a 96-well plate (Falcon). Each well also

contained 150 µl of LB broth to reduce cell density. LB broth was used as a blank. Three replications of each treatment were performed. OD_{600} and GFP fluorescence were measured for each well using a plate reader (BioTek). Relative GFP fluorescence was then determined by normalizing the fluorescence intensity (arbitrary units) with the optical density.

Biosurfactant quantification

All *P. syringae* B728a transposon mutants were grown on LB plates, containing 50 µg/ml kanamycin and 100 µg/ml spectinomycin, overnight. Cells were suspended to a concentration of 10^8 cells/ml in 1 ml of LB broth. 5 µl of each suspension was pipetted onto a LB plate containing 50 µg/ml kanamycin and 100 µg/ml spectinomycin. Plates were incubated at either 22°C or 28°C for three days. Light paraffin oil (Fisher Scientific) was applied to the plates using an airbrush (type H; Paasche Airbrush Co., Chicago, IL) operated with an air pressure of about 20 lb/in². A ruler was used to measure the distance of the visible halo formed around each colony as observed visually under oblique light from the edge of the colony.

TABLE 1 Genes disrupted in transposon mutants of *Pseudomonas syringae* B728a with decreased *syfA* expression

Locus of mariner transposon insertion	Predicted function	Number of individual transposon hits
Psyr_3368 (*efeO*)	hypothetical protein	3
Psyr_1001	basic membrane lipoprotein	1
Psyr_1491 (*tsr*)	histidine kinase, HAMP region: chemotaxis sensory transducer	1
Psyr_2575 (*syfR*)	regulatory protein, LuxR	1
Psyr_2723	regulatory protein, LysR:LysR, substrate-binding	1
Psyr_4008 (*mexB*)	hydrophope/amphiphile efflux-1 HAE1	1

TABLE 2 Genes disrupted in transposon mutants of *Pseudomonas syringae* B728a with increased *syfA* expression

Locus of mariner transposon insertion	Predicted function	Number of individual transposon hits
Psyr_3282 (*psrA*)	regulatory protein, TetR	3
Psyr_2474	Acyl-CoA dehydrogenase	2
Psyr_4204	hypothetical protein	2
Psyr_1143 (*cyoC*)	cytochrome c oxidase, subunit III	1
Psyr_4130 (*algW*)	serine protease	1
Psyr_4843 (*rppH*)	NUDIX hydrolase	1
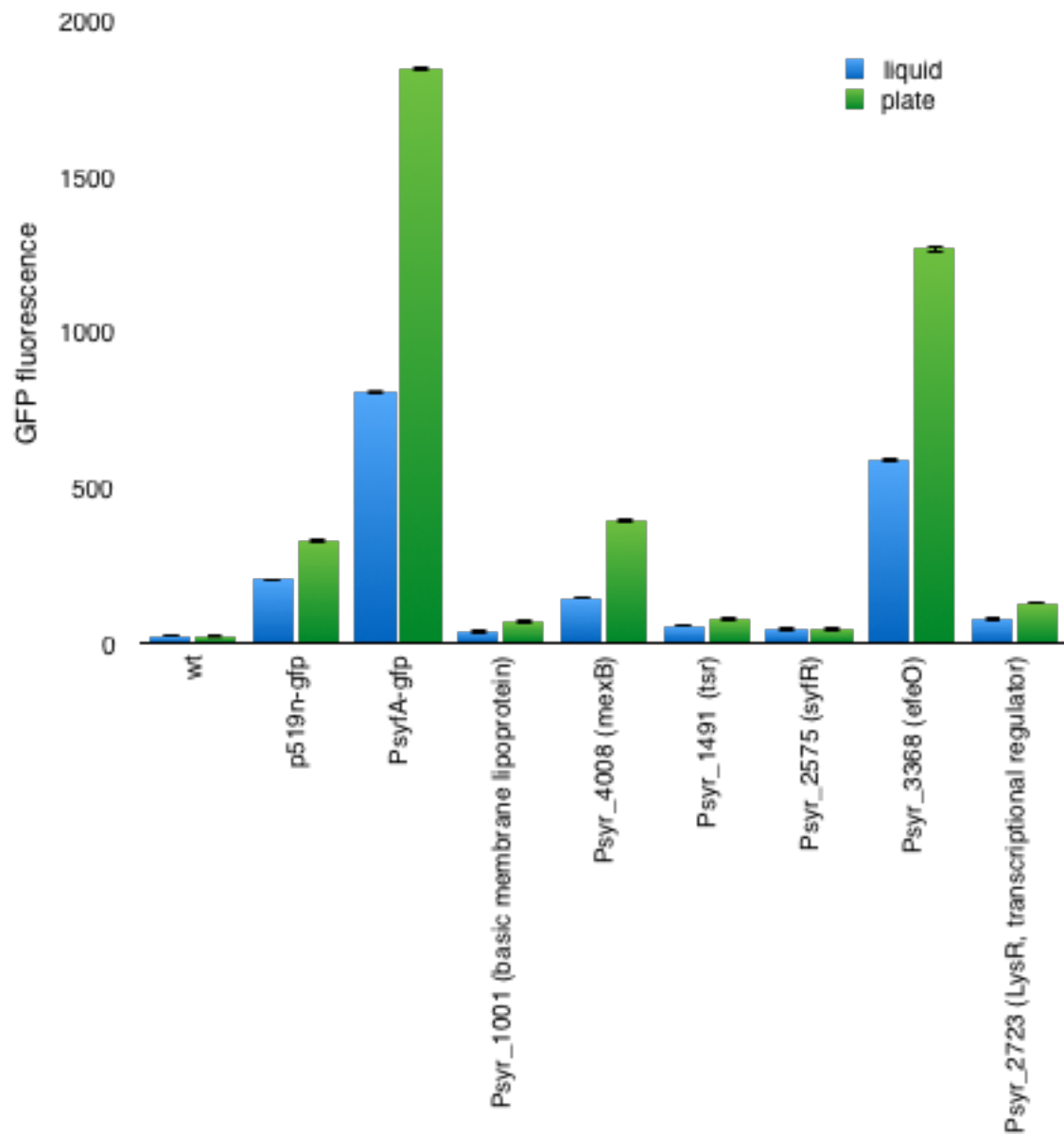
Figure 1. Genes disrupted in transposon mutagenesis exhibit decreased GFP fluorescence.
P. syringae B728a cells harboring a plasmid in which the *syfA* promoter was fused to a *gfp*
reporter (P*syfA-gfp),* were grown in liquid culture and on LB agar plates overnight at 22°C. All
cells were aliquoted into a 96-well plate to measure GFP fluorescence using a plate reader. A
strain harboring a constitutively expressed *gfp* reporter gene (p*519n-gfp*) and a wild-type strain
with no GFP (wt), were used as positive and negative controls respectively. The error bars
represent the standard errors of the mean GFP fluorescence.
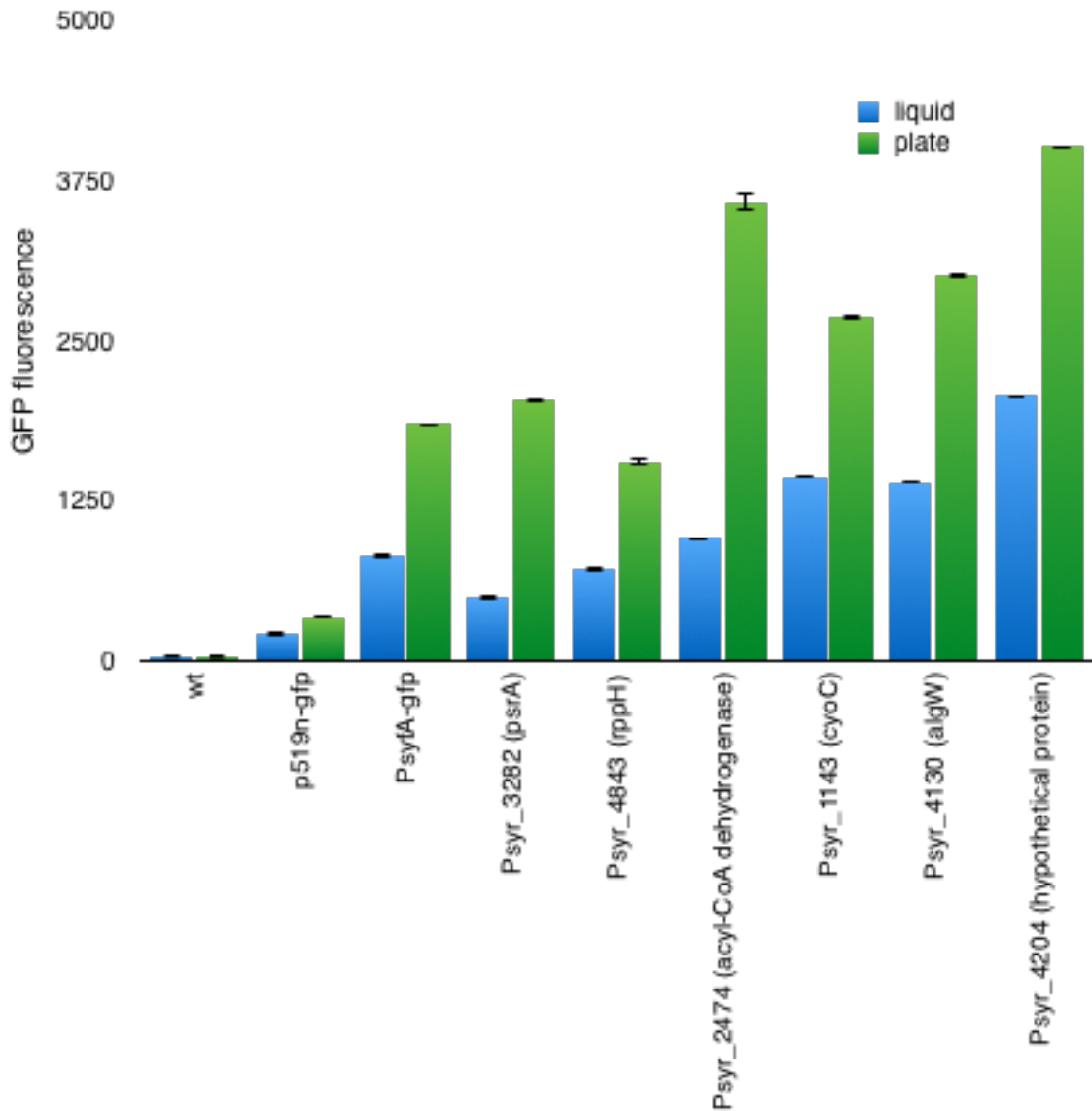
Figure 2. Genes disrupted in transposon mutagenesis exhibit increased GFP fluorescence except for *rppH*.
P. syringae B728a cells harboring a plasmid in which the *syfA* promoter was fused to a *gfp* reporter (P*syfA-gfp)*, were grown in liquid culture and on LB agar plates overnight at 22°C. All cells were aliquoted into a 96-well plate to measure GFP fluorescence using a plate reader. A strain harboring a constitutively expressed *gfp* reporter gene (p*519n-gfp*) and a wild-type strain with no GFP (wt), were used as positive and negative controls respectively. The error bars represent the standard errors of the mean GFP fluorescence.

TABLE 3 Insertional mutants of *Pseudomonas syringae* B728a with altered surfactant production

Locus of mariner transposon insertion	Surfactant halo radius (cm)[a] ± SD at 28°C	Surfactant halo radius (cm)[b] ± SD at 22°C
No insertion (WT harboring P*syfA-gfp*)	1.53 ± 0.03	1.68 ± 0.04
Psyr_1001 (basic membrane lipoprotein)	0 ± 0.00**	0.33 ± 0.06**
Psyr_2474 (acyl-CoA dehydrogenase)	1.82 ± 0.03**	1.67 ± 0.12
Psyr_2575 (*syfR*)	1.13 ± 0.08**	0.98 ± 0.03**
Psyr_2723 (LysR, transcriptional regulator)	1.68 ± 0.10	1.53 ± 0.06*
Psyr_4130 (*algW*)	1.83 ± 0.10*	1.58 ± 0.03
Psyr_4204 (hypothetical protein)	1.82 ± 0.08*	1.83 ± 0.12
Psyr_4843 (*rppH*)	1.77 ± 0.06**	1.37 ± 0.23

[a]Halos with significantly smaller or larger radii than the halo of the WT at 28°C at $P < 0.05$ (*) or $P < 0.01$ (**) as determined by a *t* test.
[b]Halos with significantly smaller or larger radii than the halo of the WT at 22°C at $P < 0.05$ (*) or $P < 0.01$ (**) as determined by a *t* test.

TABLE 4 Expression of genes of *Pseudomonas syringae* B728a when cells were immobilized on a filter surface for two hours compared to in liquid broth.

Category, locus tag	Gene	Log 2 Fold Change (Filter/Liquid)	P-value
Chemosensing and chemotaxis			
Psyr_1491	*tsr*	1.18	2.33E-16
Energy generation			
Psyr_1143	*cyoC*	1.51	1.23E-09
Hypothetical			
Psyr_3368	*efeO*	0.61	1.06E-33
Psyr_4204		-0.50	5.52E-56
RNA degradation			
Psyr_4843	*rppH*	3.33	7.02E-03
Secondary metabolism			
Psyr_2575	*syfR*	1.63	8.16E-08
Secretion/Efflux/Export			
Psyr_4008	*mexB*	1.05	5.23E-21
Transcriptional regulation			
Psyr_2723		-1.85	1.3E-07
Psyr_3282	*psrA*	14.65	4.88E-01
Unannotated			
Psyr_1001		3.55	1.1E-02
Psyr_2474		3.78	1.91E-02
Psyr_4130	*algW*	1.13	4.64E-20
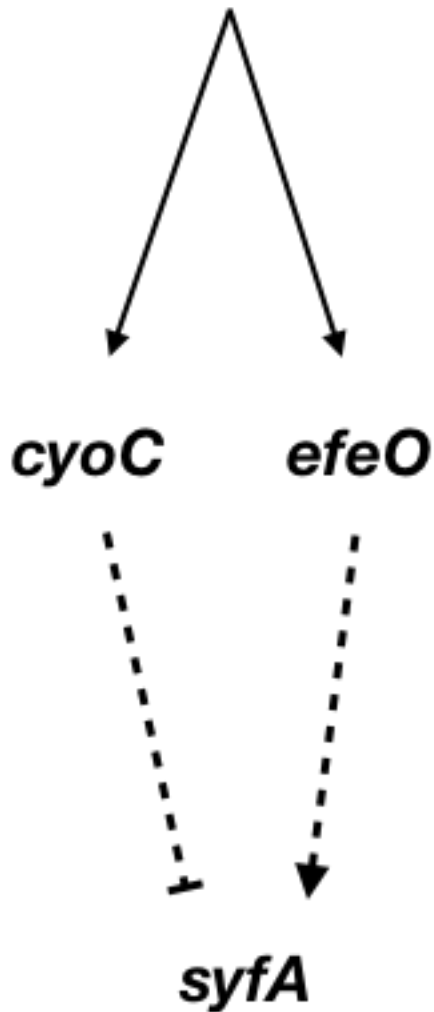
Figure 3. Model depicting the regulation of *syfA* in *P. syringae* B728a.
When iron availability is low *cyoC*, of the *bo₃* quinol oxidase gene complex *cyoABCDE,* and *efeO,* of the iron uptake transporter *efeUOB*, become activated. *cyoC* represses *syfA* whereas *efeO* induces it. These contradictory pathways may be needed in order to regulate the expression of *syfA*.
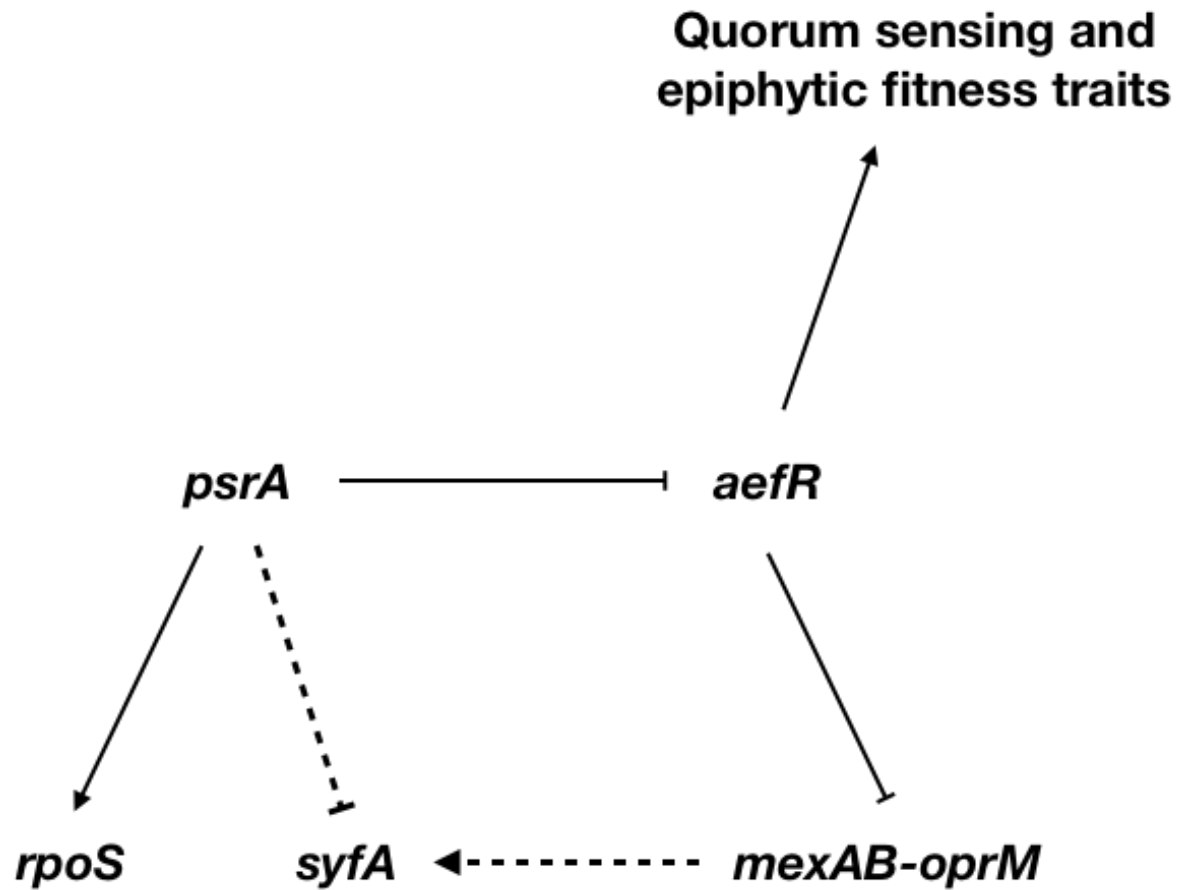
Figure 4. Model depicting the regulation of *syfA* in *P. syringae* B728a.
The gene *psrA* has been shown to induce *rpoS* and repress *aefR*. The regulator *rpoS* activates
genes during stationary phase and has been shown to activate genes when cells are on the leaf
surface. *aefR* activates genes involved in quorum sensing and potentially other epiphytic
fitness traits. *aefR* also represses the *mexAB-oprM* efflux transporter. *oprM-mexAB* induces
syfA while *psrA* represses it.

Chapter 5

Conclusion

To address how bacteria adapt to living on leaf surfaces, I used the model organism *Pseudomonas syringae* B728a which is an excellent epiphyte. It is likely that many of the traits that such a strain would use to colonize surfaces would be shared with other members of the species as well as other epiphytic taxa. The use of GFP-based bioreporters to assess gene expression in individual cells in natural habitats, such as leaf surfaces, provided considerable insight into physiological processes operative in this habitat. Coupled with the use of mutant strains blocked in traits such as biosurfactant production, I was able to make direct measurements of the effect of these traits on bacterial fitness as well as proxy measurements of physiological processes such as the level of water stress that the cells experienced. RNA sequencing also proved to be a very powerful tool to discover other traits relevant for bacterial adaptation. I was surprised to find such a large fraction of the genome of *Pseudomonas syringae* to be so quickly subjected to differential expression upon transition of cells from a planktonic to a sessile state on surfaces. Perhaps it was therefore not surprising to find that several putative regulators of such contact-dependent gene expression were found in a random transposon mutagenesis scheme. While it was not within the scope of this study, I anticipate that most of the genes that exhibited contact-dependent gene expression that I found in my transcriptomics studies are under the control of one or more of these putative regulators. Further work to determine the regulator of each of these putative regulators should prove illuminating, and either verify or refute this hypothesis. Through the use of these various tools and techniques, I was able to validate a new role for biosurfactants as well as provide more insight into the types of traits important for adaption to a surface and the potential surface sensing pathways behind those traits.

Biosurfactants and desiccation tolerance

Given the role of syringafactin in providing water to cells on a leaf surface (Chapter 2), it would be interesting to test this function in biosurfactants produced by other microorganisms, especially those that interact with leaves, in order to determine if this is a common strategy for survival of bacteria that experience periodic desiccation. In addition to organisms on leaf surfaces, most soil organisms are not continuously in an aquatic environment and might also benefit from the retention of water in their vicinity. In a previous study, Burch *et al*. (2011) isolated a variety of bacterial strains from different environmental settings and evaluated their biosurfactant production. 23 of these strains were found to produce biosurfactants. These strains included bacteria recovered from leaf surfaces, soil, and from aquatic environments. It was noteworthy that the highest proportion of biosurfactant production was observed in leaf surface microorganisms, although soil organisms also are much more likely to produce biosurfactants than those isolated from water. Such a finding is consistent with the conjectures that 1) as a public good, biosurfactants would be most logically produced in a setting where they would not be lost to the open environment by diffusion, which would occur in an aquatic habitat and 2) the production of biosurfactants by microorganisms that experience periodic desiccation suggests that a variety of biosurfactants might exhibit the hygroscopic features of syringafactin. It therefore would be fruitful to test biosurfactants for their water-binding capabilities. Such a measure might be readily done by extracting biosurfactants from each strain, dehydrating them,

and then exposing them to a variety of relative humidites to test for weight change by water absorption, a method that was implemented in Chapter 2. As bacteria typically also produce one or more extracellular polysaccharides that might have some ability to bind water, distinguishing the water-binding capabilities of exopolysaccharides from that of biosurfactants might require at least partial purification of the biosurfactants. Since most biosurfactants are much smaller than that of polymeric exopolysaccharides, simple size fractionation using appropriate filters should allow an unambiguous association of small molecular weight biosurfactants with such water-binding. Coupled with the atomized oil assay for quantification of biosurfactants, it should be possible to determine the relative efficacy of water-binding of the biosurfactants. Demonstration of the contribution of biosurfactants to fitness of these strains however would be a more laborious task. The apparent water stress experienced by such strains could be determined by a method similar to that used in this study by using so-called water biosensors. It might be necessary however to develop such biosensors for use in these disparate biosurfactant-producing taxa as they may not all appropriately express *proU* as did *Pseudomonas syringae*. In addition, the fitness effects of biosurfactant production would require that genes involved in biosurfactant production would need to be disrupted so that a comparison could be made between wild-type and mutant strains blocked in biosurfactant production. This obviously would require the identification of such genes - a process that might be accomplished either by random mutagenesis or in a more directed fashion by homologous recombination if the genome sequence of these strains is known. Given that the habitats from which these strains were isolated would differ, and that some of these habitats might be much more difficult to interrogate than the leaf surfaces used in my studies, it would probably prove to be more convenient to compare the fitness of these strains on a model habitat such as filters - much as I did in this study. Such studies should provide insight as to whether hygroscopicity is a common trait for biosurfactants or if it is more restricted to those bacteria that colonize the leaf surface habitat. Indeed, it might be expected that the water-binding capabilities of most biosurfactants would mimic that of syringafactin - with high water-binding restricted to atmospheres with high water content. While the atmosphere surrounding leaf surfaces is apparently highly saturated with water, this may not typically be the case in other habitats such as soil. Hence, selection for hygroscopic biosurfactants may not have occurred in such a setting.

It would also be interesting to determine if there is a link between biosurfactant production and the production of other compounds that assist in desiccation tolerance. It is possible that hygroscopic biosurfactants would be produced together with other hygroscopic materials, such as extracellular polysaccharides, to optimize the amount of water that could be bound. Alternatively, the other possible functions of these molecules might dictate that the production of these classes of compounds could be mutually exclusive. Alginate is produced by almost all *Pseudomonas* species and has been demonstrated to aid in desiccation tolerance in *Pseudomonas* by maintaining a hydrated microenvironment in biofilms (Chang *et al*., 2007). Burch *et al*. (2010), previously demonstrated that biosurfactant production in *P. syringae* B728a is inversely associated with alginate production; mutants with transposon insertions in the alginate regulatory genes *algT*, *mucA*, and *rseP* all exhibited increased biosurfactant production. However, the relationship between the regulation of biosurfactant production and that of other exopolysaccharides such as levan is still unknown. The lack of differences in cell viability among wild-type, syringafactin mutant, alginate mutant, and syringafactin and alginate double mutant strains under different relative humidity conditions in this study further suggests that other molecules that were not addressed also play a role in such a process (Chapter 2). It would

therefore be informative to determine the viability of additional exopolysaccharide mutants under conditions of different relative humidities to ascertain their relative efficacy in contribution to fitness to desiccation stress. It should also be fruitful to perform *in planta* studies using these mutants under different relative humidity conditions to determine their relative contributions to water stress tolerance. Finally, an atomized oil assay could be used to detect biosurfactant production in these various exopolysaccharide mutants in order to gain a better understanding of how the production of these different exopolysaccharides is coordinated with respect to syringafactin production. These experiments should provide insight into what other factors are involved in desiccation tolerance on the leaf surface, and if these are context-dependent traits as is biosurfactant production.

Contact-dependent traits

RNA sequencing proved to be a highly sensitive tool that provided considerable information on contact-dependent gene expression (Chapter 3). Given that cells were only analyzed after two hours on a filter surface, it would be fruitful in the future to perform timecourse studies in which gene expression is determined in cells on a filter at a variety of times after cells are immobilized on a surface. It is possible that there is a cascade of gene expression wherein the expression of some genes changes very rapidly upon immobilization while the expression of other genes is perhaps dependent on the early responding genes. Studies have shown that gene expression changes in cells on a surface overtime (Bhomkar *et al*., 2010; Dötsch *et al*., 2012; Sauer and Camper, 2001). In fact, Bhomkar *et al*. (2010) showed that this can happen rather quickly by analyzing gene expression in *Escherichia coli* cells after being attached to beads for only one, four, and eight hours. Therefore, it would be interesting to track these changes in *P. syringae* B728a in order to provide more insight on which functional gene categories seem to be enriched at different time points. This data could also be used to make comparisons to other contact-dependent mechanisms such as the Wsp system which was found by Hickman *et al*. (2005) to be important for biofilm formation in *Pseudomonas aeruginosa*. My study failed to find any pattern in up-regulation in the Wsp homologs in *P. syringae* B728a nor in exopolysaccharide production which was suggested by Hickman *et al*. (2005) to be positively regulated by the Wsp pathway. Since Hickman *et al*. (2005) looked at gene expression in cells after at least six hours on a surface, it would be interesting to see if a similar mechanism is utilized in *P. syringae* within this timeframe. Likewise, as I have identified a number of putative regulators of contact-dependent gene expression in *P. syringae*, it would be valuable to know not only the extent of their overlapping regulons, but also the extent in which some regulators may play a role in early stages of contact-dependent gene regulation. Since the putative regulators of contact-dependent gene expression identified here were merely shown to be essential for the expression of one such contact dependent gene, *syfA*, after a period of one day or more, we lack information as to whether these regulators mediate a temporal pattern of gene regulation upon sensing surface contact.

Surface sensing mechanisms

Transposon mutagenesis revealed putative regulators of *syfA* as well as potentially other genes whose expression is dependent on surface sensing (Chapter 4). Although bioinformatics analysis of these putative regulators provided the fodder for speculative models for how they might operate, considerable additional research will be required to ascertain the extent to which they

act individually or in concert with one another. While global transcriptome analysis could be performed by comparing gene expression of the wild-type strain with that of mutants of each of these putative regulators, a simpler strategy could also be pursued. By developing bioreporter strains in which the promoter of a particular candidate gene exhibiting surface-dependent gene expression is fused to a promoterless *gfp* reporter gene, I could compare its expression in wild-type strains and mutants in which each of the putative regulators is knocked out. From such results it should be possible to construct a regulatory pathway. Further work might also reveal epistatic relationships between these regulators. These experiments would be especially rewarding to perform for genes such as *psrA*, *algW*, and *cyoC* which seem to operate within complex regulatory pathways, at least for expression of *syfA*. In addition, such studies could better elucidate the potential role of Psyr_1001 in surface sensing since though this gene had a dramatic effect on biosurfactant production, it is unannotated and therefore not much is known about it. Given that it is an outer membrane protein, it is possible that the protein encoded by Psyr_1001 may play a role in surface sensing as has been suggested for the outer membrane protein PilY1 in *P. aeruginosa* (Siryaporn *et al*., 2014) and the outer membrane protein NlpE in *E. coli* (Otto and Silhavy, 2002).

RNA sequencing also revealed that genes involved in lipopolysaccharide synthesis and transport as well as peptidoglycan/cell wall polymers tended to be up-regulated when cells were on a surface, a trait indicative of cell wall damage (Chapter 3). Given that a study by Otto and Silhavy (2002) found the Cpx pathway in *E. coli*, which is activated in response to cell wall damage, to be involved in cell adhesion to a surface, it would be informative to determine if *P. syringae* utilizes a similar surface sensing pathway. One strategy to test such a model would be to expose cells to cell wall inhibitory antibiotics, a method that was used by Wood and Ohman (2009) to characterize a cell wall stress pathway in *P. aeruginosa*. Using this technique along with biological sensors, I could determine if the genes expressed on a surface were also up-regulated by exposure to such an antibiotic. Such a finding would provide further evidence that *P. syringae* also uses a cell wall stress-response mechanism to sense that it has been immobilized on a surface.

As suggested in Chapter 4, many of the regulatory pathways mediating surface-dependent gene expression seem highly complex. Given that many of the putative genes involved in this process are largely unknown it is difficult to speculate on how they might regulate surface sensing traits. It would therefore be valuable to perform more research to determine the functions of these genes. A variety of bioinformatics tools might be used to gauge their potential roles in surface sensing. By conducting studies examining how bacteria sense and adapt to surfaces we will gain greater insight into interactions between bacteria and surfaces as well as more information on how bacteria survive on leaf surfaces. This should be valuable information that could be exploited to combat pathogens in an agricultural setting.

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