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Thermo-regulation in *Pseudomonas syringae*: Phenotypes and Genetics

by

Kevin Loren Hockett

A dissertation submitted in partial satisfaction of the requirements for the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Steven E. Lindow, Chair
Professor Brian Staskawicz
Professor Peter Quail

Spring 2012

Thermo-regulation in *Pseudomonas syringae*: Phenotypes and Genetics

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by

Kevin Loren Hockett

Abstract

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Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Steven E. Lindow, Chair

Pseudomonas syringae is an important member of the phyllosphere microbial community and has been studied for many decades both as a model saprophyte, residing epiphytically, and a pathogen, living in the plant apoplast. While much is known about the traits that contribute to *P. syringae*'s success as a phyllosphere microbe, one area that is not well understood, is the contribution of temperature dependent gene regulation, or thermo-regulation, to *P. syringae*'s epiphytic colonization and survival strategies.

Flagellar-mediated motility is a trait conserved among plant-associated Pseudomonads. In *P. syringae*, motility contributes both to epiphytic colonization and survival, as well as pathogenicity by allowing cells to invade into the leaf interior. We have found that multiple forms of flagellar-mediated motility, including swarming and swimming motility, are thermo-repressed at around 30 °C. Repression of swarming results from reduced expression of both flagellar genes, including flagellin, encoded by *fliC*, as well genes involved in regulation and biosynthesis of syringafactin, the major surfactant produced by *P. syringae* B728a. Thermo-regulation of the flagellum is context dependent, being influenced by the nutrient status of the agar, which together with temperature contribute to a heterogeneous swimming phenotype at elevated temperatures. The heterogeneous swimming phenotype may represent a so-called "bet-hedging" strategy, which may be an important strategy for colonization of the leaf surface under varying and unpredictable weather conditions.

Investigation into the molecular determinants of thermo-regulation revealed several regulators, which are all conserved across the *Pseudomonas* genus, potentially indicating these are conserved thermo-regulators with in this important group of organisms. *flgM* as well as *fleN* were both involved in thermo-regulation of the flagellum. Additionally, *fleN* appeared to be important for the heterogeneous swimming phenotype, as mutations in Spontaneous Hot Swimming (SHS) mutants, which swim at elevated temperatures with a cool, diffuse swimming phenotype, commonly mapped to this gene, as determined by genome resequencing. Two separate loci, an acyl-CoA dehydrogenase and a nudix hydrolase, together contributed to thermo-regulation of genes involved in the regulation and synthesis of syringafactin. Interestingly, mutations that contributed to thermo-regulation of the flagellum did not affect thermo-regulation of syringafactin, while mutations affecting thermo-regulation of syringafactin did not affect thermo-regulation of the flagellum, indicating that thermo-regulation of these two traits are independently controlled.

For my mother, who taught me that learning is a life-long process

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Introduction

Phyllosphere microbiology

The term "phyllosphere" is used to describe the above-ground parts of plants that are habitats for numerous and diverse microbial inhabitants [1]. Bacteria usually are the most numerically abundant microbial inhabitants of the phyllosphere, and thus have received the most attention, but eukaryotic microbes such as yeasts and filamentous fungi are also commonly found as phyllosphere residents [1]. While the adaptation and ecology of the microorganisms that inhabit the numerous niches of this larger habitat have been extensively studied, an emphasis has often been on the study of plant pathogens that reside on plants prior to causing disease [3]. Additionally, recent interest in phyllosphere microbiology has come from the recognition that plant surfaces can harbor human pathogens [4]. Incidences of human disease linked to contaminated leafy greens such as spinach and lettuce have increased interest in understanding phyllosphere microbiology in an effort to decrease the risks associated with eating uncooked produce.

Factors that influence phyllosphere microbes: what it takes to be an epiphyte

Because the term "phyllosphere" is quite broad, encompassing numerous anatomical parts from the total diversity of plants, which together would include a large number of possible niches, one might expect that adaptations to these niches would be specific. For example, an epiphyte adapted to survive on a bean leaf would not necessarily be well adapted to survive on the needle of a ponderosa pine or in the flower of an apple tree. However, there are certain features of the "phyllosphere" habitat that might be expected to universally impose a selective pressure on colonists, thus leading to general adaptations of epiphytes. The periodic lack of water is likely to one of the most universal stresses encountered by phyllosphere inhabitants [5]. The ability to survive at least transient desiccation stress appears to be a trait common to phyllosphere inhabitants [6-8]. Additionally, temperature and relative humidity appear to strongly influence the diversity and abundance of phyllosphere microorganisms [9, 10]. Nutrient availability also strongly influences the ability of a microorganism to multiply in the phyllosphere [11-13] and plant surfaces are generally carbon limited. High fluxes of UVB and UVA irradiation can impact the survival of microbes, especially those residing on the adaxial leaf surface [14, 15]. Much of our current understanding of phyllosphere microbiology is derived from the study of bacteria residing on leaf surfaces [1, 6, 8]. Despite the stressful environment on leaves certain bacterial species such as *Pseudomonas syringae*, are able to effectively colonize the leaf habitat.

Pseudomonas syringae as a model epiphyte and plant pathogen

Pseudomonas syringae is a group of related, often plant pathogenic, bacteria that display remarkable diversity in both in the types of diseases they cause (ranging from leaf spots, blight, galls, and cankers [16]) as well as their range of host plants including apples, beans, cucumbers, oats, peas, tomato, tobacco, and rice, as well as other ornamental and crop plants [8, 16-18]. This taxa is grouped into five genome species based on multi locus sequence typing (MLST) analysis of four conserved genes [17]. This species is further divided (largely based on plant and *in vitro* phenotypes) into 50 pathovars in which the pathovar designation is defined by the host plant that it can infect [8]. *P. syringae* has been

studied for over half a century both as a model epiphyte and plant pathogen, where many of the traits that contribute to its fitness on plants have been elucidated.

A generalized model of the various stages of plant colonization in the life-cycle of *P. syringae* has been presented [6]: 1) a single cell, or small cellular aggregate arrives at the leaf surface (see below for discussion of dispersal mechanisms); 2) the cells divide and express various traits that enhance their ability replicate and survive on the leaf; 3) the cells reproduce enough to establish a microcolony, or aggregate of cells, that further enhances their ability to survive by increasing their resistance to stresses such as desiccation. Such an aggregate can serve as a reservoir of inoculum, 4) when conditions are permissive and population sizes in the reservoirs on the leaf are high enough, it becomes likely that at least some of the cells will invade the leaf interior, either through natural openings such as hydathodes or stomata, or though damaged sites in the leaf cuticle, 5) after invasion, if the host-pathogen interaction is compatible, the cells multiply and the pathogen grows to high abundance within the leaf, often leading to disease symptoms, 6) following abundant intercellular growth cells egress from the leaf interior and can disperse to other leaves and plants. While this generalized life cycle accounts for much of what is known of the biology and ecology of *P. syringae*, not every step is obligate. For instance, it is possible that a newly arrived cell or cellular aggregate could invade into the leaf interior without first forming an aggregate on the leaf surface (although such an occurrence would be unlikely). Conversely, cells in epiphytic aggregates could disperse to surrounding vegetation and serve as inoculum without first invading the leaf interior.

To be a successful leaf colonist, *P. syringae* possesses numerous traits that enable it to cope with or avoid the stresses encountered in the phyllosphere, including: import and accumulation of plant-derived quaternary ammonium compounds (QAC) [19, 20], as well as elaboration of several exopolysaccharides (EPSs) [21], both of which contribute to its survival in habitats with low water availability. It also has several mechanisms to protect against UV damage, including nucleotide excision repair as well as photo-reactivation mechanisms to prevent DNA damage [22]. *P. syringae* also has several enzymes that destroy free radicals and also produces polymers that protect against oxidative stress [23]. *P. syringae* also exhibits swimming and swarming motility, that might enable it to avoid environmental stresses by residing at protected sites (see below). While there is still much we do not understand about the motility of *P. syringae* its flagellar-mediated motility (a common form of motility in bacteria) has been shown to contribute both to its ability to explore the leaf surface [24] as well as invade the leaf interior [25, 26].

The exploration leaf surface appears to be particularly central to the epiphytic lifestyle of *P. syringae* [27]. *P. syringae* is not distributed evenly over the leaf surface, and is often found in cellular aggregates associated with particular leaf features such as the base of glandular trichomes and intercellular wall junctions [28]. These aggregates, and perhaps also their location at particularly favorable sites for growth and survival, termed "oases", facilitates the survival of *P. syringae* on the leaf surface because these sites provide access to nutrients as well as protection against environmental stresses such as desiccation and UV irradiation [29, 30].

In addition to the importance of survival on the leaf surface, its ability to effectively disperse between leaves or plants is important in the life of *P. syringae*. As *P. syringae* is often found associated with annual crops, it must disperse to these ephemeral host plants to be a successful colonist. *P. syringae* relies on several forms of dispersal: seed

contamination, aerosol dissemination, rain splash, and insect dispersal [8]. *P. syringae* can effectively colonize aerial plant parts when inoculated onto seeds [31, 32]. This mode of vertical transfer likely facilitates its persistence in agricultural settings because it would allow *P. syringae* to gain early access to emergent seedlings, which are relatively free of other microbial colonists, and thus are an open niche [8]. Wind dispersal is a significant contributor to *P. syringae*'s ability to disseminate over large distances and between plant species. *P. syringae* (as well as other phyllosphere bacteria) can be found in relatively high abundance (albeit in variable numbers) over snap bean fields at mid day during the growing season, where the cells both emigrate and immigrate to the plant canopy [33, 34]. Indeed wind dispersal from source plants can account for the abundance and diversity of *P. syringae* arriving at a sink plant [35-37]. The presence of *P. syringae* in precipitation far from obvious source plants suggest that it is capable of long-distance movement, and that it may play a role in the precipitation process whereby it is removed from the atmosphere by serving as ice nucleating agents required for deposition of water from clouds [38]. Rain dispersal involves the physical removal of cells from leaves in the form of splash droplets. Such droplets can easily move to neighboring plants or between leaves on the same plant (reviewed in [39]). While rain can disperse *P. syringae* and is associated with disease outbreaks [40-42], the net movement of bacteria during a rain event is downward and most cells are deposited on the soil [40, 43]. For this reason rain dispersal is probably is unimportant in distributing *P. syringae* over long distances.

The environmental conditions that facilitate dispersal mechanisms such as rain splash and dry aerosol formation are probably quite distinct. Wind dispersal appears to be most prevalent during midday, which is the hottest and driest (lowest relative humidity) part of the day and when wind speeds are maximal [34]. In contrast, rainfall events are associated with cool conditions and with high relative humidity. It would be likely that the traits of *P. syringae* that facilitate dispersal by these two processes would differ. Those conditions that would facilitate wind dispersal would require *P. syringae* to tolerate desiccation stress; such traits would probably not be needed during rain dispersal. While no studies have directly addressed gene expression patterns or the physiological state of cells being dispersed by wind or rain, it seems reasonable that cells being dispersed by wind would be in a more dormant, and stress-tolerant state than those being dispersed by rain. The latter are more likely to exhibit behaviors such as active motility that would enable them to seek out favorable sites on the leaf surface after transfer.

In a more general sense, bacteria living on the leaf surface are likely to monitor the environmental conditions and respond in a way that will allow them to explore the leaf habitat under conditions that enables movement, while exhibiting stress tolerance when exploration is either not possible or would lead to increased stress exposure (i.e. increased UV irradiation or desiccation). Indeed, *P. syringae* appears to be able to acclimate to the plant, thereby increasing its fitness *in planta* [44]. *P. syringae* residing on plants exposed to the variety of environmental conditions seen in the field, better survived shifts from high RH to low RH (i.e. non-desiccating to desiccating) conditions, than did cells residing on plants in controlled (growth chamber) settings, which had not experienced such environmental cues (i.e. increasing solar radiation and temperature) [29, 45].

Circumstantial evidence that the behavior of foliar pathogens, especially those such as *P. syringae* that infect crops in temperate climates is modified by environmental conditions comes from the observations that disease incidence and severity caused by

these pathogens is higher under cool and humid conditions [46]. While this effect could be the result of a direct alteration of the physical environment on plants by high temperatures or dryness thereby inhibiting pathogen invasion, there is also ample evidence that these pathogens up-regulate specific virulence factors in response to environmental cues, such as low temperature [46].

The most thoroughly studied thermo-regulated virulence factor is that of coronatine produced by *P. syringae* pv. glycinea. Coronatine is a molecular mimic of the plant hormone, jasmonyl-L-isoleucine, and is involved in suppressing salicylic acid-mediated plant defenses, including stomatal closure [47, 48]. Elevated growth temperatures (28 °C) inhibit toxin production by both decreasing enzyme activity and suppressing transcription of the genes encoding its biosynthetic enzymes [49]. Pectinase production in *Erwinia chrysanthemi,* cellulase production in *Erwinia carotovora,* and phaseolotoxin production in *P. syringae* pv. phaseolicola [50-52] are other examples of thermo-suppression of virulence factors. While it is clear that thermo-regulation occurs in plant pathogens, nearly all of the research in this area has simply documented it for specific virulence factors, and in most cases the genes involved in the thermo-regulation of these traits are not known.

In this work we sought to both broaden and deepen our understanding of thermo-regulation in plant-associated bacteria by identifying the temperature-dependent traits that are involved in various aspects of plant-microbe interactions. Our determination of the temperature-dependent transcriptome of *P. syringae* has revealed multiple thermo-regulated traits, as well as several genes that are important in different aspects of its thermo-regulation. Prominent among the traits revealed by this approach were those conferring motility. Further investigation of temperature-dependent motility suggests that it plays a major role in tolerance or avoidance of stress on plants. This work sheds light on how *P. syringae* integrates environmental signals in a way that maximizes its fitness as both an epiphyte and pathogen.

Chapter One

Thermoregulation of genes mediating motility and other host interactions in Pseudomonas syringae

1.1 Introduction

The plant pathogen *Pseudomonas syringae* has been studied extensively both as a saprophyte where it lives epiphytically on plant surfaces as well as a pathogen residing within the leaf apoplast. The epiphytic phase is an important part of the disease cycle for plant pathogens such as *P. syringae* since it provides inoculum for subsequent infection. Invasion of plants is apparently a relatively rare event, requiring a large epiphytic population on asymptomatic leaves for disease to be likely to occur [53]. Thus most cells exist as epiphytes prior to invasion into the leaf interior. While an understanding the biology of these pathogenic bacteria on leaf surfaces is of intrinsic scientific interest, it is also of practical significance since the traits that enable these organisms to thrive as epiphytes contribute to diseases of important crop plants.

Leaf surfaces can be relatively hospitable under certain circumstances, but often are a stressful habitat for bacteria due to conditions of desiccation, UV irradiation, and nutrient limitation common on leaves [1, 8]. Furthermore, the conditions on leaves are highly temporally variable, quickly switching between hospitable and inhospitable [1, 8]. Under stressful conditions (high irradiance, low relative humidity), the leaf surface, on balance, is inhospitable. However, some anatomical sites, such as the base of glandular trichomes or epidermal cell junctions, appear to offer protection against environmental extremes and are preferentially colonized by epiphytic bacteria [28]. These sites, often termed "preferred sites", are likely locations where both water and nutrients are more available [30].

Flagellar-mediated motility is an important trait for both the epiphytic and pathogenic lifestyles of *P. syringae* [24-26, 54]. Non-motile *P. syringae* mutants are more sensitive to desiccation and UV exposure than their motile counterparts, presumably because they cannot access preferred sites to escape environmental stresses [24]. Additionally, non-motile mutants are severely reduced in their ability to invade the leaf interior and thus cause disease after topical application to plants [26, 54, 55]. Although motility is apparently beneficial for phyllosphere bacteria, such benefits may be conditional since it would be dependent on at least some free water to be present for flagellar function [56]. The flagellum is a particularly costly organelle to synthesize, estimated to consume 2 % of the cells biosynthetic energy expenditure in *E. coli* [57], so its expression is most likely highly suppressed under conditions that do not permit motility. Additionally, in many bacteria, including Pseudomonads, flagellum synthesis is suppressed when other traits such as the type III secretion system (T3SS)[54, 58], exopolysaccharide (EPS) production [54, 59], or biofilm formation [60] are expressed; all of these traits are critical for plant-pathogen interactions [61, 62].

Given the variable nature of the leaf habitat, the conditional benefit of flagellar-mediated motility, and the inverse regulation between flagellum expression and other plant colonization traits, motility is likely regulated in a way that will maximize the fitness of *P. syringae* on leaves. By understanding the environmental parameters that regulate motility in *P. syringae*, we may gain insight into the conditions that promote invasion by this organism, as well as a sense of how different fitness factors are coordinately regulated under different environmental circumstances. In temperate regions, temperature may be a critical parameter regulating motility because it is a correlate with wetness of leaves. Leaves are unlikely to remain wet for long periods under warm temperatures since this typically occurs during the day and evaporation is rapid. In contrast, leaf wetness from either dew or rain is most likely to persist under cool temperatures that occur at night. Cells might therefore be expected to sense temperature as a surrogate for leaf wetness and perhaps exhibit anticipatory behaviors that would maximize their fitness. Temperature is known to regulate certain virulence factors such as phaseolotoxin in *P. syringae* pv. phaseolicola and coronatine in *P. syringae* pv. glycinea (reviewed in [46]). However, the more general role of temperature as a stimulus for changes in gene expression in *P. syringae* has not been examined. Insights gained from an understanding of the temperature stimulon should illuminate the interactions of this pathogen with host plants.

In this chapter we investigate the effect of incubation temperature on global gene expression in *P. syringae*. Transcriptome analysis revealed that many genes known to be involved in plant colonization are temperature regulated. As we observed that cell motility is particularly strongly affected by incubation temperature, the basis for the regulation of this trait was investigated in some detail.

1.2 Results

Swimming and swarming motility are inhibited at elevated temperatures in *P. syringae*

Swarming motility of *P. syringae* on semi-solid plates was much greater in cells incubated at 20 °C than at temperatures of 28-30 °C (Fig. 1A). As swarming motility in most bacteria involves some form of surfactant production and requires a functional flagellum [63], we assayed swimming motility of *P. syringae* through soft agar (which is flagellum-dependent) as well as the surfactant zone produced by *P. syringae* at 20 °C and 30 °C using the atomized oil assay [64]. Both the swimming area as well as the abundance of surfactant produced by *P. syringae* were significantly reduced at 30 °C compared to 20 °C (Fig. 1B and C), suggesting that biosurfactant production as well as flagellum synthesis or function was suppressed at the warmer temperature.

Genes encoding syringafactin and flagellin are repressed by elevated temperatures

Since swarming motility was strongly temperature-dependent, and temperature appeared to affect both flagellum and surfactant production, we investigated the influence of temperature on expression of genes associated with each trait. We measured the temperature-dependent expression of *fliC*, which encodes flagellin, and *syfA*, which encodes a non-ribosomal polypeptide synthase (NRPS) required for syringafactin (the major biosurfactant produced by *P. syringae* B728a) synthesis [64, 65], using both GFP reporter gene fusions and measurement of RNA abundance using qRT-PCR. The expression of *syfA* and *fliC* decreased greatly in the temperature range of 25 to 28 °C (Fig. 2). While *syfA* is nearly fully repressed at 28 °C the expression of *fliC* is only reduced by about 50% compared to its maximum expression at 19-20 °C, but becomes nearly fully repressed by 30 °C. The transcript abundance of both *syfA* and *fliC* as measured by qRT-PCR were also substantially lower in cells grown at 30 °C compared to 20 °C (Fig. 3).

Transcriptome of *P. syringae* incubated under warm and cool conditions

As the effect of temperature on swimming and swarming motility was linked to a transcriptional response of genes encoding flagellin and syringafactin, we assessed the breadth of temperature-dependent changes in gene expression in *P. syringae* to better understand its influence on the behavior of this species. The messages in cells incubated at 20 °C and 30 °C were compared using Illumina next-generation sequencing of cDNAs. In total, 28.3 % of the putative protein-encoding genes were differentially expressed at these two temperatures; 338 genes had a higher expression at 20 °C than at 30 °C, while 1107 genes were more highly expressed at 30 °C (Table 1). Of the 338 genes that were up-regulated under cool conditions, 205 were expressed more than 2-fold higher. Of the 1107 genes that were up-regulated under warm conditions, 572 were expressed more than 2-fold higher. The differentially-regulated genes were grouped into functional categories to determine if particular processes were more prominently temperature-dependent in *P. syringae*. Tables 2 and 3 list the functional categories in which genes differentially expressed at 20 °C and 30 °C, respectively, were significantly over-represented. More functional categories were enriched in genes more highly expressed at 20 °C than at 30 °C. The differential expression of genes in several functional groups were particularly noteworthy:

Polysaccharide synthesis and regulation. Genes in this functional category were predominantly induced at 20 °C. Noteworthy were genes involved in alginate synthesis (*algE*, 1.9-fold; *algK*, 2.2-fold; *alg44* 2.4-fold; *alg8*, 2.1-fold; and *algD*, 3.0-fold), levan synthesis (*lscC*-1, 4.2-fold), and Psl polysaccharide synthesis (*pslA*, 3.7-fold; *pslB*, 3.5-fold; *pslD*, 2.1-fold; *pslE*, 2.8-fold; *pslF*, 3.4-fold; *pslG*, 2.6-fold; *pslH*-1, 2.3-fold; *pslI*, 3.0-fold; *pslJ*, 2.0-fold). Interestingly, there is a second copy of a gene putatively encoding levansucrase in the *P. syringae* genome (psyr_2103, *lscC*-2) which is induced by 2.1-fold at 30 °C, exhibiting an opposite pattern of temperature-dependent expression than *lscC*-1. A few other genes in this functional category were also more highly expressed at 30 °C, including 3 genes annotated as polysaccharide deacetylases with predicted functions as xylanases or chitin deacetylases (psyr_1809, 1.7-fold; psyr_2692, 2.2-fold; psyr_3937, 1.6-fold). *mucD,* a

negative regulator of AlgT (AlgU/σE) that controls alginate expression, also was more highly expressed at warm incubation temperatures (2.6-fold), which might explain the apparent induction of alginate at 20 °C.

Phage and IS elements. Genes in this functional category were predominantly expressed at a higher level at 20 °C. Prominent among such genes were those in two predicted prophage regions in the *P. syringae* genome corresponding to psyr_2761 to psyr_2832 (prophage region I) and psyr_4582 to psyr_4597 (prophage region II) identified with the Prophinder tool in ACLAME [66]. At least 22 genes in prophage region I were more highly expressed at 20 °C than at 30 °C, with a mean induction of 3.2-fold (range of 1.8 to 6.0-fold). Surprisingly, however, 3 genes in prophage region I were more highly expressed at 30 °C (psyr_2773, 2.0-fold; psyr_2791, 2.7-fold; psyr_2826, 1.7-fold). All 16 genes in prophage region II were more highly expressed at 20 °C with a mean induction of 5.0-fold (range 1.7 to 7.3-fold).

Type VI secretion system. Most genes associated with the recently described type VI secretion system (T6SS) in *P. syringae* B728a [67], including genes encoding structural components of the secretion apparatus as well as *clpV*, encoding an ATPase putatively involved in driving export of secretion substrates were more highly expressed at 20 °C than at 30 °C. T6SS genes had a mean induction of 2.0-fold (range 1.7to 2.6-fold). However, 2 putative *hcp* homologs often associated with T6SS but not co-located with other T6SS genes, were both induced at 30 °C (psyr_1935, 2.1-fold; psyr_4039, 2.9-fold).

Chemosensing & chemotaxis. Many genes in this functional category, including those encoding putative methyl-accepting chemotaxis proteins (15) as well as homologs of *cheV* (2), *cheW* (3), *cheB* (1), *cheA* (1), and an aerotaxis receptor were more highly expressed at 20 °C. The mean induction of these genes at 20 °C was 3.3-fold (range 1.7-fold to 11.9-fold). In contrast, 2 putative methyl-accepting chemotaxis proteins were more highly expressed at 30 °C (psyr_0868 and psyr_1539, 2.0-fold and 1.8-fold, respectively).

Translation. Many genes encoding proteins associated with the 50S and 30S ribosomal subunits, as well as elongation factor Tu and G, were more highly expressed at 20 °C than at 30 °C (average of 2.0-fold induction with a range of 1.6 to 2.3-fold).

Flagellar synthesis and motility. Many genes associated with motility were more highly expressed at cool incubation conditions. For example, *fliC* was expressed 19-fold more at 20 °C than at 30 °C. We thus examined the genes encoding the flagellar cascade in more detail to determine if all were similarly temperature-dependent in their expression. Assuming that regulation of *P. syringae* motility followed the same four-tier system of *Pseudomonas aeruginosa* [68], we found that thermoregulation appeared to be enriched in class III genes, and to a greater extent, class IV genes (Table 4). Late stage (class III and IV) genes were the most significantly temperature-regulated genes in the flagellar cascade, with *fliC* showing the most thermal suppression. Neither of the class I genes (*fleQ* and *fliA*) were temperature regulated. Interestingly, *morA*, a protein involved in c-di-GMP metabolism [69] (possessing both GGDEF and EAL domains) was the only gene in the flagellar motility functional category that was more highly expressed at 30 °C.

Phytotoxin synthesis and transport. Genes involved in the biosynthesis and transport of syringomycin and syringopeptin, as well as the regulators, *salA*, *syrG*, and *syrF* were all more highly expressed at 20 °C than at 30 °C (Table 5).

Transcriptional regulation. The genes encoding numerous DNA-binding transcriptional regulators belonging to the AraC, AsnC, DoeR, GntR, IclR, LacI, LuxR, LysR, MarR, TetR families, as well as putative response regulator partners of two-component systems that also possess DNA binding domains were more highly expressed at 30 °C (Table 6). Very few genes encoding putative transcriptional regulators were most highly expressed at 20 °C, with the exception of four LuxR-type regulators that are involved in syringomycin, syringopeptin, and syringafactin regulation (*salA*, *syrG*, *syrF*, and *syfR*). Interestingly, when examining putative response regulator partner proteins that lack discernible DNA binding domains, more were more highly expressed at 20 °C compared to 30 °C (17.6% vs. 2.9%).

QAC metabolism and transport. Genes encoding enzyme systems involved in choline, glycine betaine (GB), and carnitine import and metabolism were generally more highly expressed at 30 °C than at 20 °C, while the *opuC* transporter, associated with osmoprotection [19], was expressed somewhat higher at 20 °C (1.6-1.8 fold). The *betT* transporter (associated with osmoprotection), along with *betX* and *caiX* solute binding proteins (associated with import of betaine and carnitine for catabolism, respectively) were induced by 2.3 to 2.8- fold at 30 °C [20, 70]. All genes necessary for conversion of choline and glycine betaine to glycine were more highly expressed at 30 °C, including *betAB* (4.3 to 4.9-fold), *gbcAB* (1.8 to2.1-fold), *dgcAB* (2.0 to 2.3-fold), and *soxBDAG* (1.7 to 2.3-fold) [71]. *dhcAB,* predicted to encode the enzymes involved in the second catabolic step in carnitine breakdown, were more highly expressed at 20 °C (1.9 to 2.1-fold), while their regulator (*dhcR*) was up-regulated 2.8-fold at 30 °C. As DhcR induces transcription of *dhcAB* in the presence of 3-dhc (an intermediate in the catabolism of carnitine), these results might suggest that elevated growth temperature increases carnitine catabolism, but that the intermediate required for induction was not present in this experiment.

Hypothetical. Surprisingly, the proportion of genes with no known function that were more highly expressed at high temperatures than at cooler temperatures was much higher than their proportion in the genome. Such genes included both those encoding proteins that possess domains of unknown function, as well as those without such conserved domains.

While as a whole, the expression of genes encoding proteins known to mediate plant-microbe interactions were not preferentially more highly expressed under either cool or warm conditions, several exhibited strong temperature-dependent expression. For example, both homologs of *iaaM* (psyr_1536, psyr_4667) and *iaaH* (psyr_2208, psyr_4268), involved in biosynthesis of 3-indole-acetic acid (IAA) were more highly expressed at 30 °C, as were genes involved in the biosynthesis of tryptophan, a precursor for IAA. Similarly, while most genes encoding components of the type III secretion system (T3SS) were not preferentially expressed at either growth temperature, several, including the master regulator *hrpL*, were more highly expressed at 30 °C.

Temperature affects the ability of *P. syringae* to survive desiccation stress on leaves

Given that motility is expected to be required for cells to access sites where environmental extremes such as desiccation stress could best be avoided, and that high temperatures appeared to strongly suppress the motility of *P. syringae,* we measured the temperature-dependent survival of *P. syringae* on leaves to determine if it was indeed lower at high temperatures. We compared the population size of viable epiphytic cells recovered from plants incubated under cool conditions after inoculation (20 °C, motility-inducing) and warm temperatures (30 °C, motility-suppressing) conditions. To minimize the confounding effect of temperature on the plant itself, as well as any possible differences in population sizes of the bacterium on plants due to different growth rates at various temperatures, bacteria were only incubated under moist conditions for 6 hours on plants held briefly at the test temperature. After this temperature treatment period all plants were moved to a common area where leaves were allowed to dry at the same temperature. Bacterial cells incubated on cool moist leaves survived the subsequent desiccating conditions an average of 3.2-fold better than did cells that had been incubated on hot moist leaves (Fig. 4). Importantly, the *flg*K mutant survived poorly on both cool moist and hot moist leaves and exhibited similar declines in viable population sizes as observed for the wild type strain incubated at 30 °C. This experiment was repeated several times with similar results. These results suggest that motility is required for stress survival on leaves and that the higher motility of *P. syringae* on cool plants facilitated its acquisition of sites where desiccation survival was enhanced. Given that the inoculum for plant inoculations had been grown at 30 °C, conditions that inhibit motility, motility was apparently induced fairly rapidly after arrival of cells onto cool moist leaves. We have also observed that a rapid increase in flagellin production (as estimated using *fliC*::GFP reporter gene fusions) occurred within an hour of transfer of cells from 30 °C to 23 °C (data not shown). In contrast, we found no evidence that syringafactin production contributed to enhanced survival on leaves incubated at cool temperatures under these conditions since the survival of a wild type and a *syfA* mutant were similar on moist leaves incubated at a given temperature (data not shown). Together, these results suggest that bacterial cell behavior during the brief periods that leaves were moist modulated their ability to survive the subsequent desiccation stresses, and motility was a sufficient trait to account for this apparent stress avoidance.

flgM* is required for thermorepression of *fliC

The cascade of flagellar regulation has been elucidated in detail in both *Salmonella typhimurium* and *E. coli*, where the interaction of FlgM (an anti-sigma factor) and FliA (an alternative, flagellar-specific sigma factor) is required for the inhibition of expression of late stage flagellar genes (encoding the flagellar filament, cap, motor proteins, and chemotaxis components) until the hook and basal body has been completed [72]. Since analysis of global patterns of gene expression revealed that the class IV flagellar genes such as *fliC* were the most strongly suppressed by high incubation temperatures, we hypothesized that the FlgM-FliA interaction might also mediate thermo-repression of class IV genes such as *fliC* in *P. syringae*. We thus

compared *fliC* expression in a *flgM* deletion strain, Δ*flgM*, with that in the wild type strain at both 20 °C and 30 °C. While the expression of *fliC* decreased with increasing growth temperature above 25 °C in the wild type strain, its expression in Δ*flgM* was high and independent of growth temperature (Fig. 5A). Over-expression of *flgM in trans* suppressed the expression of *fliC* at both incubation temperatures in both the wild type and Δ*flgM* strains (Fig. 5B). These results indicate that FlgM is required for thermo-repression of *fliC* and that its over-expression on a high copy number plasmid suppresses *fliC* at all temperatures. The transcript abundance of *fliC* measured using qRT-PCR also was much higher in Δ*flgM* compared to the wild type strain in cells grown at 30 °C (Fig. 3). While the abundance of *fliC* in Δ*flgM* grown at 30 °C is less than that in cells grown at 20 °C, this temperature suppression is much less than that seen in the wild type strain. This is inconsistent with estimates of the rate of transcription in these strains made using reporter gene fusions, possibly indicating that the *fliC* transcript is less stable at 30 °C than at 20 °C.

gac-dependent traits are also thermo-regulated

The GacS/GacA two-component regulatory pathway is widely conserved in γ-proteobacteria, where this system generally regulates exported secondary metabolites, among other traits [73]. As biosurfactant production in other Pseudomonads is under *gac*-regulation, we suspected that it also would be in *P. syringae* [74, 75]. Both *syfA* as well as *syfR* (a LuxR-type regulator required for syringafactin expression [64, 65]) expression, as estimated with *gfp* reporter gene fusions, were dependent on *gacS* (Fig. 6). Additionally, *salA*, encoding a LuxR-type transcriptional regulator, which is itself positively regulated by *gacS* and *gacA* [76], was required for expression of *syfR* and *syfA*. Taken together, this data demonstrates that syringafactin expression is under *gac*-regulation via *salA*. As *syfR*, *syfA*, *salA*, as well as genes for syringomycin and syringopeptin production that are *salA*-dependent, were all thermo-regulated, we considered that additional *gac*-regulated traits would also be thermo-regulated. Specifically, we determined whether any *gac*-dependent, but *salA*-independent factors were also thermo-regulated, since all of the thermo-regulated factors we had identified were *salA*-dependent. For example, an extracellular protease in *P. syringae* is *gacS*-dependent, but *salA*-dependent [77, 78]. Psyr_3163, encoding a putative secreted alkaline metalloproteinase, shares 62 % amino acid identity and 73 % similarity with the *gac*-dependent, extracellular protease, AprA, of *P. fluorescens* Pf-5. Additionally, the downstream genes were conserved in these strains, suggesting that psyr_3163 likely encodes the *P. syringae* ortholog of *aprA*. The *aprA* homolog was expressed 2.6-fold more at 20 °C and that 30 °C. *P. syringae* produced a clearing zone in KB medium amended with skim milk at 20 °C but not at 30 °C, indicating that protease was indeed thermo-regulated (Fig. 7). As expected, a *gacS* mutant did not produce a clearing zone at either temperature. These results indicated that thermo-regulation of *gac*-dependent genes was not limited to *salA*-regulated traits.

A *flgM* mutant retains thermo-repression of syringafactin production and extracellular protease

To test whether *flgM* was also required for thermo-repression of syringafactin at high temperatures, we compared syringafactin production in the *flgM* mutant at 20 °C and 30 °C. Reduced surfactant production was observed at 30 °C compared to 20 °C in both Δ*flgM* and the wild type strain (Fig. 8). Additionally, we observed that *flgM* is not required for thermo-regulation of protease production (Fig 7). These results indicate that there are additional thermoregulatory factors in *P. syringae*.

1.3 Discussion

The motility of *P. syringae*, like some other bacteria, is very strongly dependent on temperature. Both swimming and swarming motility are inhibited by incubation at 30 °C and this is apparently mediated by the suppression of flagellum components required for both swimming and swarming motility, as well as reduced expression of syringafactin which contributes to swarming. Temperature-regulated swarming motility and surfactant production has also been observed in *Pseudomonas putida* KT2440, a non plant-pathogenic, soil-associated Pseudomonad, which moves at 22 °C but not 30 °C [79]. Interestingly, biosurfactant production has not been reported for this strain and its swarming motility is not flagellar-dependent, but rather type IV pilus-dependent [79]. In contrast, *P. putida* PCL1445, produces biosurfactants in a temperature-dependent manner, and exhibits surfactant-dependent swarming [75, 80, 81], but neither temperature-dependent flagellar expression nor swimming motility has been reported in this strain. The production of the biosurfactants putisolvin I and II by *P. putida* PCL1445, is dependent on the *gacS*/*gacA* two component signaling system, both of which are more highly expressed at 11 °C than at 28 °C or 32 °C [75]. In contrast to this strain, the transcription of neither *gacS* nor *gacA* in *P. syringae* B728a is influenced by temperature. However, there appears to be some correlation between *gac* regulation and thermo-regulation in *P. syringae*, since genes involved in production of syringafactin, syringomycin and syringopeptin as well as a putative exoprotease, are all *gac* regulated and are also expressed more highly at cool temperatures (Fig. 6, Table 5 [76, 82]). The increased expression of these genes agrees with phenotypic data showing that exoprotease, syringafactin and syringomycin are not produced in *P. syringae* at 30 °C but are produced at lower temperatures (Figs. 1 and 7, [83]). Thermoregulation of the *gac* regulon also occurs in other Pseudomonads. For example, in *Pseudomonas fluorescens* CHA0 thermo-suppression of *gac*-dependent secondary metabolites as well as the regulatory sRNAs, *rsmX*, *rsmY*, and *rsmZ* occurs at 35 °C and is mediated by RetS [84]. Since the *gac* regulon in *P. syringae* is not fully known we cannot determine the extent to which *gac*-regulated genes are also thermo-regulated. A central aspect of *gac*-regulation is repression of translation of target mRNAs mediated by the RNA-binding protein RsmA, which is relieved by expression of small, non-coding RNAs that competitively inhibit RsmA (reviewed in [85]). If thermo-regulation of the *gac*-dependent transcripts described in this chapter is a direct result of temperature on

gac signaling, then it is curious that we observe this effect at the transcriptional level. This could be due either to the coincidental thermo-regulation of *gac*-dependent genes or their transcriptional regulation by components that are not *gac*-dependent. There might also be an intermediate factor(s) that is the direct target of RsmA-mediated translational repression that mediates transcription of the thermo-regulated genes. It is also possible that the rate of degradation of RsmA-bound transcripts is increased due to the inhibition of their initiation of translation, a process known to negatively affect transcript stability [86]. Whatever the case may be, previous groups have successfully investigated *gac*-regulation using transcriptomic approaches [87].

Inhibition of flagellar motility occurs at the optimal growth temperature of *P. syringae* as in many bacteria. This phenomenon has been studied most extensively in animal pathogens [88] and we suspect that the biological relevance for such a phenotype differs in bacteria colonizing these different habitats. *flgM*, encoding an anti-sigma factor known to inhibit the activity of FliA in numerous organisms, also was required for thermo-repression of *fliC* in *P. syringae*. The patterns of flagellar thermo-regulation in *P. syringae* seems similar to that in *Yersinia enterocolitica*, a gram-negative bacterium that generally conforms to the three-tiered flagellar regulatory hierarchy established in *E. coli* and *S. typhimurium* [89]. *Y. enterocolitica* exhibits flagellar-mediated motility at 25 °C but not 37 °C, while plasmid-encoded virulence factors are expressed in an opposite pattern [90]. Thermoregulation occurs at the level of *fliA* expression, while expression of other class I and class II flagellar genes are insensitive to incubation temperature [89, 91, 92]. Disruption of *fliA* leads to reduced or abolished thermo-regulation of the plasmid-encoded virulence factors, and therefore is thought to be a key mediator of the temperature response [89]. *fliA* transcription in *P. syringae* was insensitive to incubation temperature, suggesting that FlgM-mediated inhibition is an alternative mechanism thermo-regulating FliA activity. Indeed, the FlgM-FliA interaction in *Campylobacter jejuni* is temperature dependent, being destabilized at 42 °C compared to 32 °C or 37 °C [93]. *Campylobacter* is unique in that it is one of the few genera where motility and flagellin expression is maximal at the growth optimum of the organism (42 °C)[93, 94]. It is not clear whether the FliA-FlgM interaction in *P. syringae* is itself temperature sensitive or if other factors mediate a temperature-sensitive interaction of these two components. It would be very interesting if it is the former since our data would suggest that the interaction is stabilized with increasing temperature, opposite to that in *Campylobacter*. The observation that *flgM* is not required for thermo-repression of *syfA*, coupled with the observation that there are over 1000 genes that are significantly influenced by incubation temperature indicates that there is at least one other, if not several other genes required for thermo-repression in *P. syringae*.

Similar to *P. syringae* B728a, *P. syringae* pv. maculicola ES4326 expresses flagellar synthesis and the type III secretion system (T3SS) in a reciprocal fashion [54]. In this system, AlgW plays a role in suppressing expression of flagellar genes while promoting expression of the T3SS in a medium mimicking the apoplastic environment. AlgW is also involved in reciprocally regulating alginate synthesis and flagellum expression in response to nutritional cues. Interestingly, genes involved in

alginate synthesis were more highly expressed at cool temperatures, which would be coordinated with flagellum syntheis (opposite to *P. syringae* ES4326). These results are consistent with a higher *algD* transcript abundance at 18 °C compared to 28 °C both *in planta* and in culture seen in *P. syringae* pv. glycinea [95]. These results may suggest that alginate synthesis and flagellum synthesis are not generally oppositely regulated, and instead have an expression patterns that are context-dependent. Such a contextual pattern of expression may suggest that there is only an indirect linkage between expression of these traits, implying a strain-specific pattern of regulation.

In addition to alginate production, genes involved in producing other EPS species such as Psl and levan, were, in general, suppressed at high temperatures. In *P. syringae* pv. glycinea, levan production is also thermo-suppressed, accumulating at 18 °C but not 28 °C [96]. Psl is a recently described EPS involved in biofilm formation in *Pseudomonas aeruginosa* [97, 98], and is hypothesized to have this role in *P. syringae* as well [99, 100]. As flagellar-mediated motility has been shown to be important for initial attachment and biofilm formation in numerous systems [101], these results taken together imply the initial steps of biofilm formation in *P. syringae* are enhanced at 20 °C. The expression of EPS would seem to interfere with motility of *P. syringae*, and so we presume that the temporal patterns of expression of such genes are synchronized such that motility might precede EPS production, although both traits probably play an important role in initial colonization of new habitats. Since both traits are optimally expressed at cool temperatures, it is expected that colonization, which presumably involves both exploration of a new habitat and attachment to new sites followed by modification of the site and/or more permanent attachment, would proceed from a motile phase to a subsequent colonization phase involving EPS production, both of which would be optimal under cool conditions. Cells incubated on plants at 20 °C were more tolerant to subsequent desiccation than cells incubated at 30 °C (Fig. 4). We presume that under cool conditions motile cells accessed preferred sites of colonization (base of glandular trichomes, grooves between epidermal cells) that facilitated their subsequent desiccation tolerance or enabled them to escape such stresses. A non-mutually exclusive possibility is that such cool conditions also stimulates cell aggregation and biofilm formation due to their enhanced motility and EPS production, and that cells in such aggregates are more desiccation tolerant [29, 102].

Our findings that genes in the prophage regions were thermally regulated is consistent with previous research that has also found temperature-dependent prophage gene expression and prophage activation, although the specific effect of temperature on gene expression apparently varies between strains and prophage regions [103-105].

The fact that numerous transcriptional regulators as well as many hypothetical genes were more highly expressed 30 °C than at 20 °C is consistent with results from *Shewanella oneidensis* and *Streptococcus thermophilus*, whose transcriptional regulators of various protein families were also altered in expression after heat shock [103, 106]. This indicates that the high temperature response of *P. syringae* as well as other organisms includes a variety of uncharacterized functional

genes whose expression is modulated via changes in abundance of regulatory genes themselves.

We found that all of the known uptake systems for QAC compounds contributing to either their accumulation for osmoprotection or for catabolism were up-regulated at 30 °C, except for the *opuC* import pathway, which was slightly higher at 20 °C. It is possible that the increased import capacity of these compounds would allow the cell to rapidly accumulate compatible solutes by repressing expression of the corresponding catabolic enzymes, should water limitation occur (conditions not present in this study). Alternatively, *P. syringae* might anticipate that QACs would increase at high temperatures as tomato plants were found to accumulate QACs as the temperature increased above 25 °C [107].

One of the most obvious effects of temperature on *P. syringae* that is linked to its behavior on plants is the thermo-regulation of motility. The lower survival of cells of *P. syringae* exposed to warm temperatures during moist periods prior to desiccation stress is consistent with previous work that showed that non-motile mutants of this species were less resistant to desiccation and UV stress *in planta* [24]. While there have been numerous descriptions of thermo-regulated motility in animal pathogens, there have been only two studies to date that have linked temperature with the motility of plant pathogenic bacteria [25, 108]. In *Erwinia amylovora*, the causative agent of fire blight, flagellar motility, which is suppressed at warm temperatures appeared to play little role in the infection and disease process, as incubation at either a warm or cool temperature resulted in similar disease severity. In contrast, symptom development in lilac inoculated with *P. syringae* isolates was suppressed on plants incubated at temperatures above 24 °C [109]. Due to the invasive methods of inoculation used in that study, motility may not have been required for infection, however, and the effect of temperature on the motility of the pathogen was not assessed. While disease symptoms caused by other *P. syringae* strains on plants also has been found to be higher under cool conditions, such effects on virulence cannot be linked to the motility of the pathogen. For example, preincubation of *P. syringae* pv. glycinea PG4180 at 18 °C enhanced subsequent *in planta* multiplication and disease symptoms compared to those preincubated at 28 °C [110]. This interaction was shown to be dependent on production of coronatine, a thermo-regulated toxin known as a virulence factor in this organism. It has been hypothesized that the correlation of increased occurrence and severity of other bacterial plant disease with cool and humid weather in temperate climates is likely the result of increased opportunity for invasion due to increased water availability that coincides with cool temperatures [46]. Our results, in light of previous studies, suggest that temperate pathogens are also more invasive under cool conditions because they more highly express motility-related traits.

This work supports the notion of *P. syringae* as a conservative pathogen that coordinates motility with environmental conditions in a way that will optimize its fitness. It also suggests that it exhibits anticipatory behaviors, using temperature as an indicator that water availability that may soon be limited. The conservative pattern of expression of traits, such as motility, would maximize its fitness over the long run by avoiding risks associated with short term exposure to stresses such as desiccation that could be avoided. *P. syringae* thus shares such anticipatory

behaviors as recently described in *Vibrio cholera* that expresses traits in the human gut that would seem to enhance its survival after dispersal into the marine environment [111].

1.4 Materials and Methods

Bacterial strains, plasmids, culture media, and growth conditions

Pseudomonas syringae pv. syringae B728a [112] was routinely cultured in King's medium B (KB) broth, or on KB plates supplemented with 1.5% (w/v) Difco agar technical (BD, Sparks, MD) at 28 °C [113]. E. coli strains TOP10 (Life technologies, Carlsbad, CA) and S17-1 [114] were cultured in Luria-Bertani (LB) medium broth, or on LB plates supplemented with 1.5% (w/v) Difco agar technical at 37 °C [115]. Antibiotics were used at the following final concentrations: rifampicin, 100 μg/mL; kanamyacin, 50 μg/mL; gentamicin, 15 μg/mL; spectinomycin 20 μg/mL; tetracycline, 15 μg/mL; nitrofurantoin (NFT), 30 μg/mL. Natamycin (antifungal) was used at 21.6 μg/mL. Strains and plasmids used in this work are listed in Table 7. Primers used in this work are listed in Table 8.

Temperature-dependent assays were performed in incubators adjusted to the appropriate temperature as described in the text. Plate temperatures were routinely monitored using a CZ-IR thermometer (ThermoWorks, Lindon, UT). Incubator temperatures and RH were routinely monitored using HOBO data loggers (Onset, Bourne, MA).

Detection of biosurfactants

Biosurfactants were detected with an atomized oil assay similar to previously described [64]. Bacterial cultures were grown overnight and diluted into fresh medium the following morning and grown for 2-4 hours. Cultures were then washed once with 10 mM potassium phosphate buffer (pH 7.5) (KPO_4 buffer) and re-suspended to a concentration of 2.0 x 10^8 CFU/mL, as determined by turbididity (OD_{600}). 5 μL of re-suspended culture was spotted onto KB plates and incubated for 20-30 hours at either 20 °C or 30 °C, and the spots then sprayed with a mist of mineral oil. The diameter of the visible halo of brighter oil drops was measured and the area of the producing bacterial colony was calculated and subtracted from that of the surfactant halo to yield the adjusted halo area.

Swimming and swarming assays

Swimming media (50% KB containing 0.25% agar) was allowed to dry overnight on the bench top (unstacked). Bacterial cultures were incubated overnight (28 °C with shaking at 200 RPM) in KB broth amended with the proper antibiotics. Cultures were then diluted into fresh media and allowed to incubate for 2-4 hours and cells recovered by centrifugation and washed once with KPO_4 buffer before being re-suspended to a final concentration of 2.0 x 10^8 CFU/mL and inoculated by stabbing with a sterile toothpick. Plates were incubated for 20-30 hours prior to observation. Swarming assays were performed similar to swimming assays except 5 ul of

bacterial suspension was spot inoculated singly onto the center of swarming plates (KB containing 0.4% agar).

Transcriptional reporter assays

Transcriptional reporter assays were performed similar to [116]. Briefly, culture spots were re-suspended in KPO_4 buffer and diluted to a final OD_{600} of 0.1-0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with a 486-nm-band-pass excitation filter and a 510- to 700-nm combination emission filter. Relative fluorescence was determined by normalizing the fluorescence arbitrary units by the optical density.

RNA isolation and qRT-PCR

Bacterial cultures were harvested from KB plates and suspended immediately in 1.0 mL of RNA*later* (Life Technologies) and stored at 4 °C for no longer than one week, prior to RNA isolation. Total RNA was isolated using TRIzol® reagent (Life technologies; Carlsbad, CA) similar to the manufacturer's protocol except for differing in the following ways: homogenized cells were incubated at 65 °C for 10 minutes in TRIzol® prior to addition of chloroform, rather than 5 minutes at room temperature (RT). After adding chloroform, samples were incubated at room temperature for 15 minutes, rather than 2-3 minutes. RNA was precipitated in isopropanol at -80 °C for 20 minutes, rather than at room temperature for 10 min. Washed RNA was re-suspended in 30-50 µl of RNAsecure™ (Life technologies), according to the manufacturer's protocol. To remove contaminating genomic DNA, RNA was treated with TURBO DNA-*free*™ (Life technologies) according to the manufacturer's protocol. DNase-treated samples were either cleaned using the DNase-inactivating reagent included in the TURBO DNA-*free*™ kit, or were column purified using RNeasy Mini Kit (QIAGEN; Valencia, CA). The absence of contaminating DNA was confirmed by performing a PCR reaction using Phusion® High-Fidelity DNA Polymerase (New England Biolabs; Ipswich, MA) with either DNase-treated or untreated samples using *rpoD*-RT-S and AS primers (Table 7). RNA purity and abundance was routinely assessed using an ND-1000 spectrophotometer (Thermo Scientific; Lafayette, CO). cDNA was generated from 0.5 or 10 µg of DNase-treated RNA, using SuperScript® II reverse transcriptase (Life technologies) with random primers (Life technologies). qPCR was performed using a 7300-Real-Time PCR System (Life technologies) with QuantiTect SYBR Green I (QIAGEN) on 5 or 10 µl of 1:1000 diluted cDNA. Samples not treated with reverse transcriptase were routinely included, which exhibited no significant increase in fluorescence following 35 cycles. Amplification efficiencies were determined using LinReg [117]. Amplification efficiencies were determined to be 95 % of maximum, and were consistent between gene targets. Relative expression was calculated according to the ΔΔCt method with a base of 1.9 to account for amplification efficiency. *rpoD* and psyr_3981, encoding pseudouridine synthase which was found to be stably expressed under numerous conditions and in numerous mutant backgrounds (R. Scott and K. Hockett, unpublished data), were used as endogenous controls. qRT-

PCR results normalized to either endogenous control gave similar estimates of RNA abundance; results normalized to *rpoD* are shown.

mRNA sequencing

Total RNA was harvested as described above. Following total RNA isolation, 16S and 23S rRNA was removed using Ribo-Zero™ rRNA removal Kit (Gram-Negative Bacteria) (Epicentre, Madison, WI) according to the manufacturer's protocol. Enriched mRNA samples were assayed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA) to confirm removal of rRNA. Quantification of mRNA and dsDNA was routinely performed with qBit RNA and dsDNA HS assays (Life Technologies), respectively, according to the manufacturer's protocol. Ambion fragmentation reagent (Life Technologies) was used to generate 100-200 nucleotide fragments from enriched mRNA. Fragmented RNA was isolated by ethanol precipitation with glycogen amendment, followed by re-suspension in RNase-free water. First strand cDNA synthesis was performed using random primers (Life Technologies) and Superscript II reverse transcriptase (Life Technologies), followed by second strand synthesis using RNase H and DNA Pol I (Life Technologies). dsDNA was routinely purified using AMPure XP beads (Beckman Coulter, Brae, CA) with PEG 6000 added to a final concentration of 6.5% (w/v). cDNA end repair was performed using a combination of Klenow DNA polymerase, T4 DNA polymerase, and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). An A tail was added to end-repaired fragments using Klenow *exo* minus with 250 μM ATP (final concentration) (New England Biolabs). Illumina adapters were ligated to A-tailed dsDNA fragments using T4 DNA ligase (New England Biolabs), with PEG 6000 addition to 5.0 % (w/v) final concentration. Adapter-ligated fragments were amplified using Illumina barcoded primers with Phusion DNA polymerase (New England Biolabs). Amplified fragments were purified using AMPure XP beads without PEG addition. Fragment sizes were confirmed to be in the size range of 200-300 nucleotides using the 2100 Bioanalyzer. Sequencing (50 base pair reads) was performed using the Illumina HiSeq 2000 at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley. Three biological repeats were sequenced per temperature treatment on three separate flow cells. Reads were aligned to the *P. syringae* pv. syringae B728a genome (downloaded from the Integrated Microbial Genomes website) using Bowtie 0.12.7 [118] allowing for a maximum of three mismatches between a given read and the reference genome. The number of reads that overlapped with a given gene was counted using a Python script. Differential expression of genes and statistical significance were assessed using edgeR [119].

Deletion and complementation of *flgM*

A markerless *flgM* deletion strain was constructed using an overlap extension PCR protocol similar to [120]. Regions of approximately one kilobase of sequence upstream and downstream of *flgM* were amplified using 5'-S-*flgM*, 5'-AS-*flgM*, 3'-S-*flgM*, and 3'-AS-*flgM* (Table 8). These fragments were combined with a kanamycin resistance cassette flanked by FLP recombinase target sites (*kan*-FRT) amplified

from pKD13 [121] using primers pKD-13-site-4-AS and pKD4/13-site-1-S. The upstream, downstream, and *kan*-FRT fragments were combined into a single PCR reaction which underwent 15 amplification cycles in the absence of primers, followed by 20 amplification cycles with 5'-S-*flgM* and 3'-AS-*flgM* primers added. The resulting fragment was cloned into the suicide vector pTOK2T [70], creating p*flgM*-KO, which was transformed into the *E. coli* mating strain, S17-1. S17-1 harboring p*flgM*-KO was mated with *P. syringae* B728a overnight on KB, followed by transfer onto KB amended with kanamycin and NFT (counter selection for *E. coli*). Single colonies were streaked onto KB amended with either rifampicin and kanamycin or rifampicin and tetracycline. Colonies displaying kanamycin resistance and tetracycline sensitivity (indicative of double recombination events between p*flgM*-KO and the *P. syringae* B728a genome) were screened for replacement of *flgM* with the kanamycin resistance cassette using PCR with the external and internal primer sets, 5'-S-*flgM* + 3'-AS-*flgM* and *flgM*-coding-S + *flgM*-coding-AS (Table 8), respectively. A spectinomycin-resistant version of pFLP2 [116, 122] was electroporated into the kanamycin resistant mutant Δ*flgM.* pFLP2-containing colonies were screened for kanamycin sensitivity. A single kanamycin-sensitive isolate was grown in KB broth overnight in the absence of selection, yielding a markless Δ*flgM* strain.

To construct p519Mcomp, p519nGFP was digested with *XbaI* and *EcoRI*, followed by treatment with T4 DNA polymerase (to create blunt ends). The digested/blunted vector was ligated to a PCR fragment containing *flgM* with its promoter region, amplified with *flgM*-5'-S-complement and *flgM*-3'-AS-complement. p519empty was constructed by self-ligating the digested/blunted vector.

Plant assays

Three week old bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274) were acclimated to 30 °C for 48 hours on a long day (16 hours light/8 hours dark) light regime prior to inoculation. Bacterial strains were grown overnight at 30 °C on KB to suppress motility. Cultures were re-suspended in KPO_4 buffer and diluted to a final concentration of 10^6 CFU/mL in sterile distilled water. Cultures were spray-inoculated onto plants until dripping wet, and bagged to maintain a high relative humidity. Inoculated plants were incubated at either 20 °C or 30 °C for 6 hours, prior to being un-bagged and moved to a common incubation area (28 °C, 50-60 % relative humidity) for an additional six hours. Three primary leaves were sampled immediately after inoculation, while ten primary leaves were sampled immediately after the plants were un-bagged, and again six hours later. Sampled leaves were individually sonicated in washing buffer (100 mM KPO_4 buffer containing 0.1% peptone) for 5 minutes and appropriate serial dilutions plated onto KB amended with rifampicin (100 µg/mL) and natamycin (21.6 µg/mL). Bacterial abundance was normalized for leaf surface area determined by analysis of digital images of leaves using ImageJ software [123]. The mean log death value was calculated as the difference between the log-transformed, leaf area-normalized population size determined immediately after the plants were un-bagged and that subsequently determined after 6 hours incubation under dry conditions.

Acknowledgments

We thank Adrien Burch for providing the results shown in Figure 6.

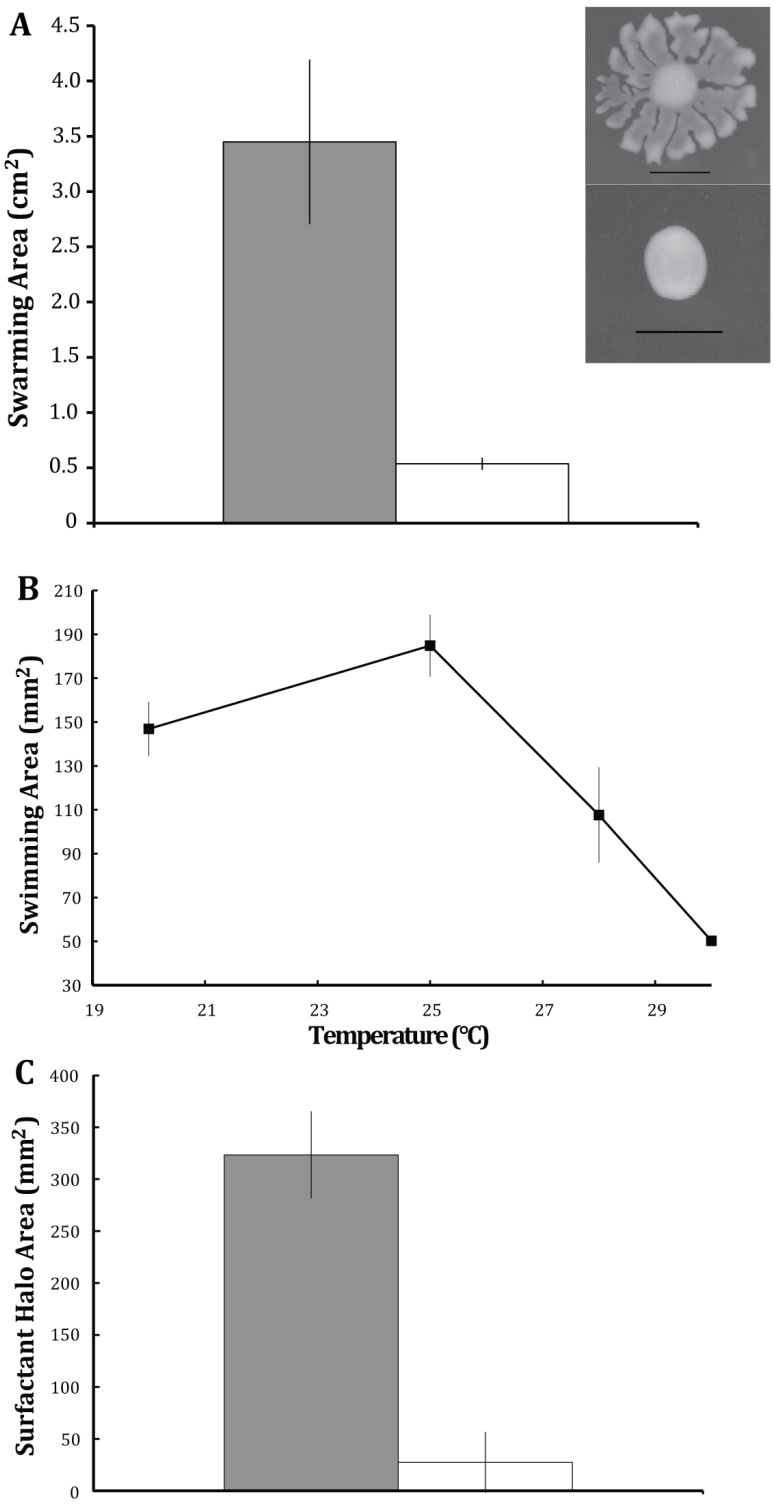

Figure 1.1. Swarming of *Pseudomonas syringae* B728a at 20 °C (gray bar, top photo of inset) and 30 °C (white bar, bottom photo of inset) after 24 hours (A). Area of swimming colonies of *P. syringae* after growth for 24 hours at various temperatures (B). Area of biosurfactant coverage on plates after growth for 24 hours at 20 °C (gray bar) or 30 °C (white bar) (C). The vertical bars represent the standard deviation of the mean.

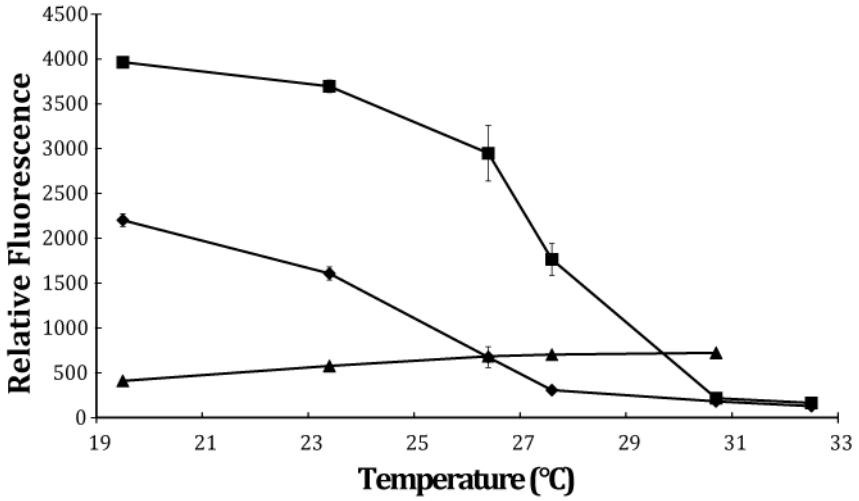

Figure 1.2. Cell-normalized GFP fluorescence of cells of *Pseudomonas syringae* that harbored a fusion of *fliC* (square), *syfA* (diamond), or *nptII* (triangle) with a promoterless *gfp* reporter gene that were grown for 48 hours at various temperatures. The vertical bars represent the standard deviation of the mean.

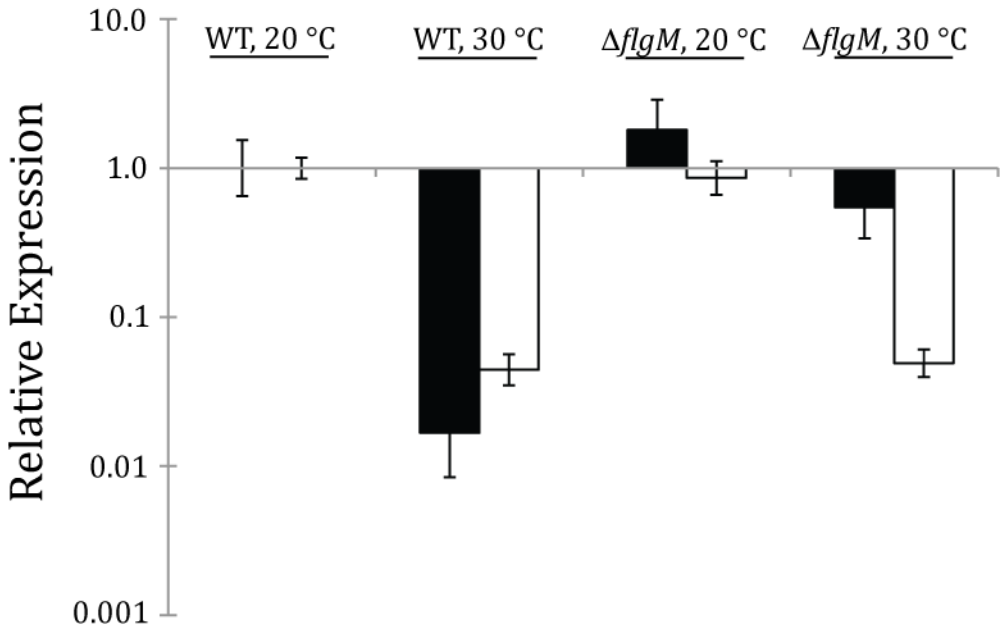

Figure 1.3. Relative expression of either *fliC* (black bars) or *syfA* (white bars) in wild type *Pseudomonas syringae* or a Δ*flgM* mutant grown at either 20 °C or 30 °C. The vertical bars represent standard deviation of the mean.

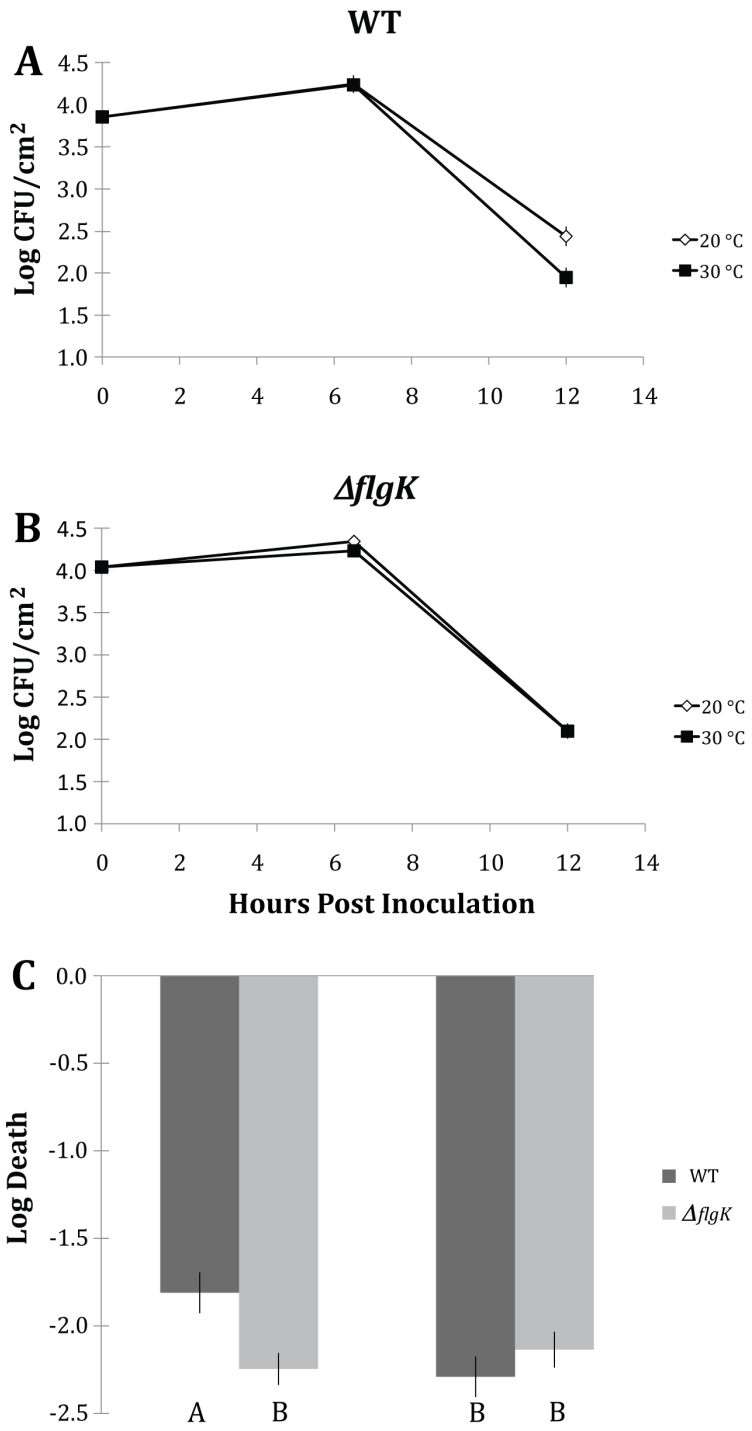

Figure 1.4. Epiphytic population size of wild type *Pseudomonas syrinage* (A) or a Δ*flgK* mutant (B) incubated for 6.5 hours at 20 °C (open diamond) or 30 °C (black square) at 100 % RH immediately following spray inoculation before being exposed to 26-28 °C at 60-65 % RH. Log-transformed population size decline measured during the desiccation conditions that occurred between 6.5 and 12 hours after inoculation (C). Treatments marked with different letters differ significantly, while treatments marked by the same letters do not differ significantly as determined by least significant difference (LSD) test ($p \leq 0.05$). The vertical bars represent the standard deviation of the mean.

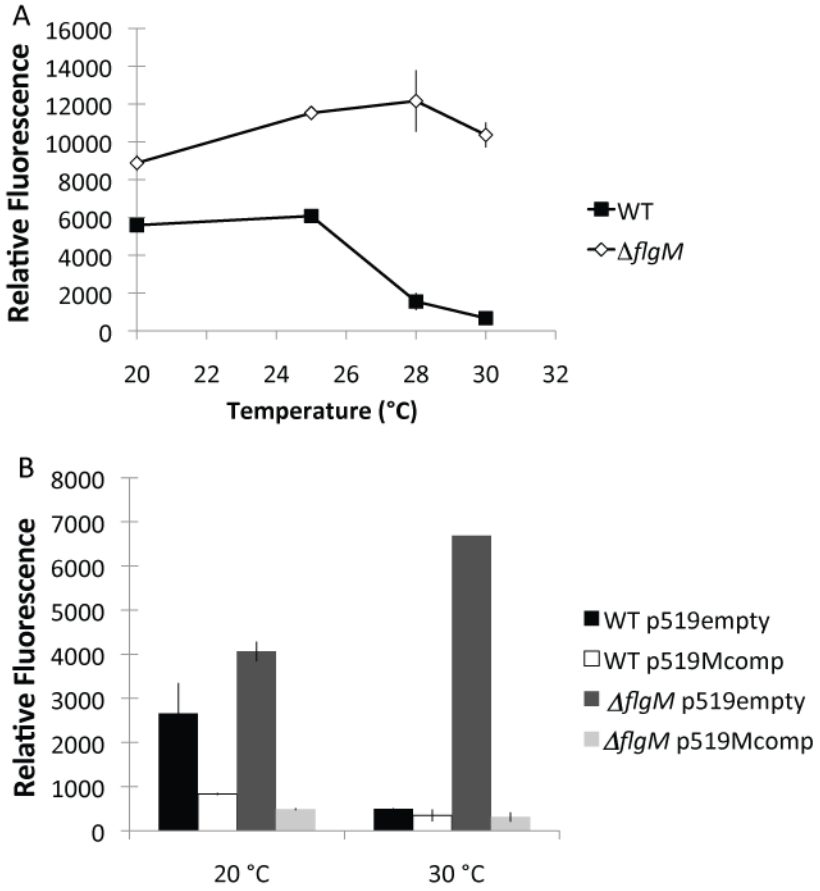

Figure 1.5. Cell-normalized GFP fluorescence of either wild type *Pseudomonas syringae* (black squares) or a Δ*flgM* mutant (open diamonds) harboring a *gfp* reporter gene fusion with *fliC* (A). Cell-normalized GFP fluorescence of wild type *P. syringae* or a Δ*flgM* mutant harboring a *gfp* reporter gene fusion with *fliC* as well as either a *flgM* complementing vector (p519Mcomp) (black and dark gray bar, respectively) or vector control (p519empty) (white and light gray bar, respectively) when grown at either 20 °C or 30 °C (B). The vertical bars represent the standard deviation of the mean.

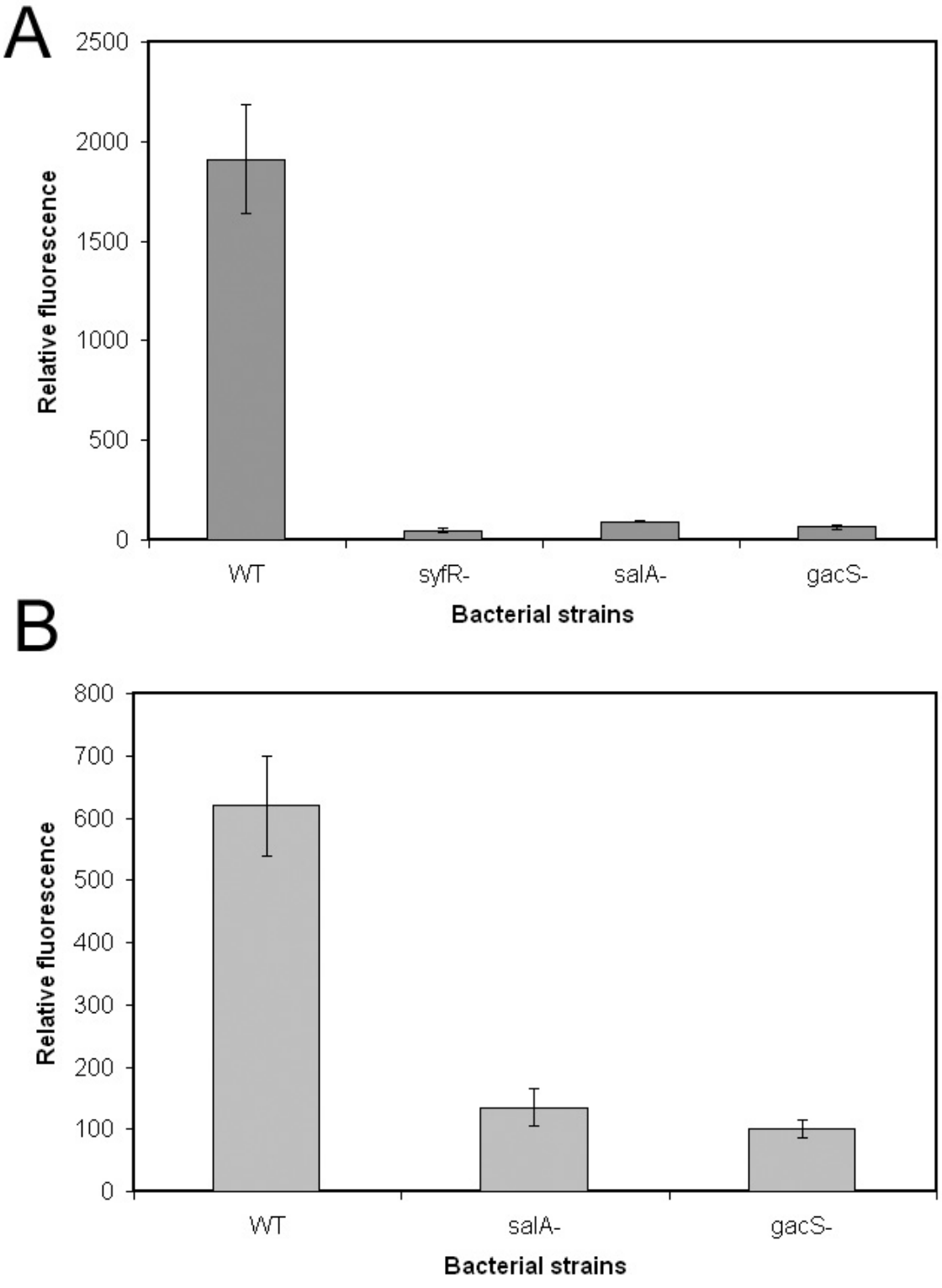

Figure 1.6. Cell-normalized GFP fluorescence exhibited by wild type and various mutants of *Pseudomonas syringae* harboring a *gfp* reporter gene fused to *syfA* (A) or *syfR* (B) when grown on KB medium for 1 day at 20 °C. The vertical bars represent standard deviation of the mean.

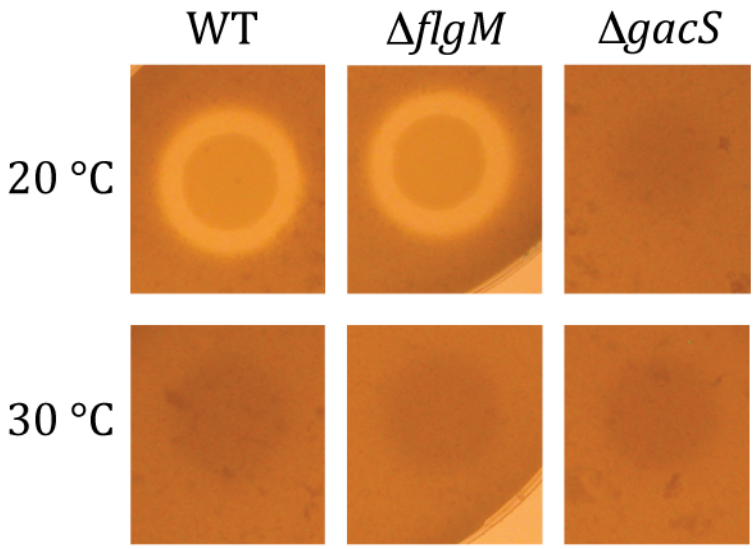

Figure 1.7. Clearing zones indicative of protease activity of wild type *Pseudomonas syringae* and Δ*gacS* and Δ*flgM* mutants incubated for 3 days at 20 °C (top) or 30 °C (bottom) on KB medium amended with 5.0 % skim milk and 100 uM $CaCl_2$.

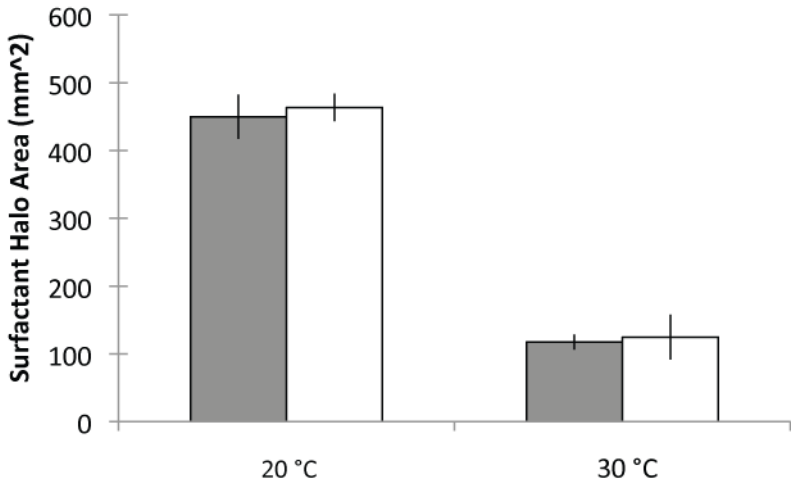

Figure 1.8. Area of biosurfactant-induced halo produced by either wild type *Pseudomonas syringae* (grey bars) or a Δ*flgM* mutant (white bars) when grown at either 20 °C or 30 °C. The vertical bars represent the standard deviation of the mean.

Table 1.1: Functional categories of thermo-regulated genes

Functional Category	# of Genes[a]	% of Total genes	% of Genes Temperature Sensitive[b]	Cold Induced	% of Cold-Induced Genes	Hot Induced	% of Hot-Induced Genes
Type VI secretion system	28	0.55	67.9	18	5.3	1	0.1
QAC metabolism and transport	42	0.82	66.7	5	1.5	23	2.1
Toxin-Antitoxin system	6	0.12	66.7	0	0.0	4	0.4
Phytotoxin synthesis and transport	23	0.45	60.9	14	4.1	0	0.0
Cold shock proteins	5	0.10	60.0	1	0.3	2	0.2
Chaperones/Heat shock proteins	28	0.55	57.1	1	0.3	15	1.4
TAT secretion system	10	0.20	50.0	0	0.0	5	0.5
Plant-associated proteins	11	0.22	45.5	0	0.0	5	0.5
Amino acid metabolism and transport (GABA)	7	0.14	42.9	0	0.0	3	0.3
Phage & IS elements	125	2.45	42.4	37	10.9	16	1.4
Special	12	0.24	41.7	0	0.0	5	0.5
Transport (peptides)	36	0.71	38.9	3	0.9	11	1.0
Siderophore synthesis and transport	76	1.49	38.2	10	3.0	19	1.7
Polysaccharide synthesis and regulation	49	0.96	36.7	11	3.3	7	0.6
Type III secretion system	49	0.96	36.7	0	0.0	18	1.6
Carbohydrate metabolism and transport	117	2.29	35.0	8	2.4	33	3.0
Transcriptional regulation	200	3.92	34.5	0	0.0	69	6.2
Chemosensing & chemotaxis	73	1.43	34.2	23	6.8	2	0.2
Oxidative stress tolerance (Antioxidant enzyme)	15	0.29	33.3	2	0.6	3	0.3
Flagellar synthesis and motility	48	0.94	33.3	15	4.4	1	0.1
Outer membrane proteins	24	0.47	33.3	4	1.2	4	0.4
Fatty acid metabolism	55	1.08	30.9	1	0.3	16	1.4
Hypothetical	1219	23.87	29.9	57	16.9	307	27.7
Transport	101	1.98	29.7	3	0.9	27	2.4
Mechanosensitive ion channel	7	0.14	28.6	0	0.0	2	0.2
Secretion/Efflux/Export	100	1.96	28.0	6	1.8	22	2.0
Sulfur metabolism and transport	61	1.19	27.9	0	0.0	17	1.5
Secondary metabolism	26	0.51	26.9	3	0.9	4	0.4
Organic acid metabolism and transport	105	2.06	26.7	4	1.2	24	2.2
Cyclic di-GMP cyclase proteins	34	0.67	26.5	2	0.6	7	0.6
Compatible solute synthesis	19	0.37	26.3	0	0.0	5	0.5
Unannotated	1007	19.72	26.2	36	10.7	226	20.4
Pili synthesis and regulation	51	1.00	25.5	2	0.6	11	1.0
Transcription - Sigma factor	16	0.31	25.0	1	0.3	3	0.3
Phosphate metabolism and transport	20	0.39	25.0	2	0.6	3	0.3
Amino acid metabolism and transport	238	4.66	24.4	18	5.3	40	3.6

Table 1.2 continued: Functional categories of thermo-regulated genes

Energy generation	83	1.63	24.1	7	2.1	13	1.2
LPS synthesis and transport	38	0.74	23.7	1	0.3	8	0.7
Nitrogen metabolism	57	1.12	22.8	2	0.6	11	1.0
Stress resistance	41	0.80	22.0	0	0.0	9	0.8
Polyamine metabolism and transport	19	0.37	21.1	0	0.0	4	0.4
Transport (inorganic ions)	48	0.94	20.8	1	0.3	9	0.8
Osmosensing & regulation	5	0.10	20.0	1	0.3	0	0.0
Degradation of xenobiotics	15	0.29	20.0	0	0.0	3	0.3
Transcription	10	0.20	20.0	2	0.6	0	0.0
RNA degradation	16	0.31	18.8	3	0.9	0	0.0
Cofactor metabolism	152	2.98	18.4	1	0.3	27	2.4
Transport (organic compounds)	11	0.22	18.2	0	0.0	2	0.2
Light and oxygen sensing	11	0.22	18.2	0	0.0	2	0.2
Proteases	17	0.33	17.6	1	0.3	2	0.2
Signal transduction mechanisms	40	0.78	17.5	4	1.2	3	0.3
Translation	132	2.59	16.7	19	5.6	3	0.3
Post-translational modification	12	0.24	16.7	0	0.0	2	0.2
Glutathione metabolism	18	0.35	16.7	1	0.3	2	0.2
Quorum regulation	6	0.12	16.7	0	0.0	1	0.1
Iron metabolism and transport	27	0.53	14.8	0	0.0	4	0.4
Phospholipid metabolism	36	0.71	13.9	4	1.2	21	1.9
Nucleotide metabolism and transport	83	1.63	12.0	3	0.9	7	0.6
Replication and DNA repair	97	1.90	11.3	0	0.0	11	1.0
Oxidative stress tolerance	11	0.22	9.1	1	0.3	0	0.0
Terpenoid backbone synthesis	12	0.24	8.3	0	0.0	1	0.1
Peptidoglycan/cell wall polymers	29	0.57	6.9	0	0.0	2	0.2
Iron-sulfur proteins	14	0.27	0.0	0	0.0	0	0.0
Oxidative stress tolerance (antioxidant enzyme)	2	0.04	0.0	0	0.0	0	0.0
Cell division	21	0.41	0.0	0	0.0	0	0.0
Total	5106	100.0	28.3	338	100.0	1107	100.0

[a]Total number of genes within functional category

[b]Percentage of functional category that is temperature sensitive

Table 1.2: Functional gene categories enriched in genes more highly expressed under cool growth conditions in *Pseudomonas syringae*

Category	***P*-value**[a]
Polysaccharide synthesis and regulation	8.03E-05
Phage & IS elements	5.70E-14
Type VI secretion system	1.17E-13
Chemosensing & chemotaxis	4.18E-09
Translation	0.02
Flagellar synthesis and motility	9.32E-06
Phytotoxin synthesis and transport	4.77E-10

[a]Bonferroni corrected *P*-value

Table 1.3: Functional gene categories enriched in genes more highly expressed under warm growth conditions in *Pseudomonas syringae*

Category	***P*-value[a]**
Hypothetical	0.04
Transcriptional regulation	8.73E-04
QAC metabolism and transport	1.56E-04
Chaperones/heat shock proteins	0.01

[a]Bonferroni corrected *P*-value

Table 1.4: Thermoregulation of flagellar genes in *Pseudomonas syringae*

	Class I	**Class II**	**Class III**	**Class IV**
Number of thermo-responsive genes[a]	0/2	3/22	6/12	7/13
Mean fold temperature effect[b]	n/a	2.6	2.1	4.8

[a] Genes thermo-regulated within class/total number of genes within class
[b] Determined only for the genes within class that are thermo-regulated

Table 1.5: Phytotoxin regulation, synthesis, and transport genes induced at 20 °C

locus	gene	fold-change[f] (20 °C/30 °C)
Psyr_2601[a]	*salA*	1.8
Psyr_2602[b]	*syrG*	2.5
Psyr_2606		2.1
Psyr_2607[b]	*syrF*	4.2
Psyr_2608[c]	*syrE*	2.6
Psyr_2609[c]	*syrC*	2.4
Psyr_2610[c]	*syrB*2	2.3
Psyr_2611[c]	*syrB*1	3.6
Psyr_2612[c]	*syrP*	8.2
Psyr_2613[c]	*syrD*	7.1
Psyr_2614[d]	*sypA*	2.2
Psyr_2616[d]	*sypC*	2.2
Psyr_2620[e]	*pseA*	4.0
Psyr_2621[e]	*pseB*	2.8

[a] Master LuxR-type regulator of syringomycin and syringopeptin synthesis; [b] LuxR-type regulator of syringomycin and syringopeptin synthesis; [c] Syringomycin synthesis; [d] Syringopeptin synthesis
[e] Syringomycin and syringopeptin export; [f] All values are significant at p-value ≤ 0.05

Table 1.6: Transcriptional regulators influenced by temperature in *Pseudomonas syringae*

Regulator Type	**Annotated in the genome**	**Induced at 30 °C (%)**	**Induced at 20 °C (%)**
LysR	74	21 (28.4)	1 (1.4)
GntR	27	12 (44.4)	0 (0.0)
TetR	27	9 (33.3)	0 (0.0)
LuxR	24	6 (25.0)	4 (16.7)
AsnC	7	2 (28.6)	0 (0.0)
AraC	19	7 (36.8)	0 (0.0)
IclR	5	1 (20.0)	0 (0.0)
DoeR	5	2 (40.0)	0 (0.0)
LacI	15	3 (20.0)	1 (6.7)
MarR	7	1 (14.3)	0 (0.0)
Response Regulator with putative DNA binding domain[a]	28	11 (39.3)	0 (0.0)
Response Regulator with out putative DNA binding domain	34	1 (2.9)	6 (17.6)

[a]This contains 10 Response regulator domains that are paired with LuxR DNA binding domains, 4 of which are induced at 30 °C, none of which are induced at 20 °C

Table 1.7. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (Carlsbad, CA)
S17-1	*recA* RP4-2-Tc::Mu-Km::Tn7	[114]
P. syringae strains		
P. syringae B728a	Wild type, Rf^R, NFT^R	[112]
Δ*flgK*	*P. syringae* B728a with a *flgK* deletion, Rf^R,NFT^R, Km^R	A. Burch (unpublished)
Δ*flgM*	*P. syringae* B728a with a *flgM* deletion, Rf^R, NFT^R	This work
Δ*gacS*	*P. syringae* B728a with a *gacS* deletion, Rf^R, NFT^R	D. Gross (unpublished)
Δ*salA*	*P. syringae* B728a with a *salA* deletion, Rf^R, NFT^R	D. Gross (unpublished)
Plasmids		
p519nGFP	Broad host range vector, GFP$^+$, Km^R	[124]
p519empty	p519nGFP with GFP removed	This work
p519Mcomp	p519n harboring the *flgM* complementing cassette	This work
pFLP2Ω	bhr vector expressing the Flp-recombinase, Sp^R	[116]
pTOK2T	Mobilizable, suicide vector, Tc^R	[70]
p*flgM*-KO	pTOK2T harboring the *flgM*-knock out cassette, Km^R, Tc^R	This work
p$P_{fliC\text{-}gfp}$	pPROBE-GT harboring the *fliC* promoter, Gm^R	[116]
p$P_{syfA\text{-}gfp}$	pPROBE-OT harboring the *syfA* promoter, Sp^R	[64]

[a] Rf, rifampicin; Gm, gentimicin; Sp, spectinomycin; Km, kanamycin; Tc, tetracycline; NFT, nitrofurantoin

Table 1.8. Primers used in cloning and qRT-PCR

Primer	**Sequence**
5'-S-*flgM*	5'-GACCCACCAGCTGAGTTC-3'
5'-AS-*flgM*[a]	5'-GAAGCAGCTCCAGCCTACACACATGATTGAAAAAACCTCTGG-3'
3'-S-*flgM*[a]	5'-GGTCGACGGATCCCCGGAATAACGCTAAGCCAAGGC-3'
3'-AS-*flgM*	5'-CGTCGACCAGTTGCG-3'
pKD4/13-site-1-S	5'-GTGTAGGCTGGAGCTGCTTC-3'
pKD13-site-4-AS	5'-ATTCCGGGGATCCGTCGACC
flgM-coding-S	5'-CCAGAGGTTTTTTCAATCATG-3'
flgM-coding-AS	5'-CAGCCTTGGCTTAGCG-3'
flgM-5'-S-complement	5'-ATCGTCTAGACGTCACCGAAGAAGACG-3'
flgM-3'-AS-complement	5'-ATCGTCTAGACTAGCGTTGGGTTTCG-3'
qRT-PCR primers	
rpoD-RT-S	5'-ACGCGCCATCATGCAGCTGTG-3'
rpoD-RT-AS	5'-GCCAGTGCGTCAGTCCAGCTTTC-3'
3981-RT-S	5'-CAGCGGACGGGTGCAGGTAGAC-3'
3981-RT-AS	5'-CGTCGTTGTCAGGCTCGTCCAG-3'
fliC-RT-S	5'-CGGCGCCTCGAACCAGATCTC-3'
fliC-RT-AS	5'-CGCGGCGATTGAAGCAGAGAAG-3'
syfA-RT-S	5'-CGGCCCGACTGAAACCACTGTG-3'
syfA-RT-AS	5'-CCCCGCACCACCGATGTACAAC-3'

[a] Underlined sequence is complementary to kan-FRT from pKD13

Chapter Two

An Acyl-CoA dehydrogenase and Nudix hydrolase are involved in thermo-regulation in Pseudomonas syringae

2.1 Introduction

Temperature is an important environmental factor that influences many aspects of microbial physiology and profoundly affects an organism's ability to survive and reproduce [125]. Since microorganisms must both perceive and respond to changes in temperature appropriately they possess some form of thermo-regulated gene expression. Certain temperature responses appear to be conserved across diverse bacterial species, such as the cold shock and heat shock responses [126, 127]. In these conserved responses, the stimulatory signals and regulatory mechanisms, are also largely conserved. However, in addition to such conserved 'shock' responses that maintain cellular functions after large and rapid temperature shifts, most bacterial species also possess more specialized forms of thermo-regulation whose role is not necessarily to return the cell to prior homeostasis, but rather to facilitate survival in the altered environment. For example, animal pathogens often use host body temperature as a cue to express virulence factors [128-130]. In this setting many, but not all, animal pathogens suppress the production of flagellar genes since they serve as immune-eliciting antigens (i.e. flagellin) [88]. While the suppression of flagellin at host temperatures is common in such pathogens, the stage within the flagellar hierarchy as well as the mechanism by which such regulation occurs differs between organisms (cf. *E. coli* [131], *Yersinia* [90, 91, 132], *Listeria* [133]). Conserved thermo-regulated traits such as motility therefore do not necessarily have conserved mechanism of regulation. This may be a consequence of the myriad ways in which thermo-regulation can occur (reviewed in [125, 134]). While the central components of thermo-regulated gene expression and their interactions have been described in highly studied organisms, such as *E. coli* [135, 136], 16, 17] much less is known of these processes in most taxa.

While thermo-regulation of certain traits in plant-pathogenic bacteria has been observed in numerous genera [46], our understanding of the mechanisms operative in these organisms remains limited. One of the most thoroughly studied plant pathogen thermo-regulation systems is the CorRS two-component signaling system that regulates coronatine biosynthesis in *Pseudomonas syringae* pv. glycinea [137-139]. While this system is necessary for temperature regulation of coronatine biosynthetic genes, there appear to be additional components necessary for thermo-regulation of this toxin [140]. Furthermore, this two-component system appears to specifically regulate only coronatine biosynthesis. Likewise, phaseolotoxin production is thermo-regulated in *P. syringae* pv. phaseolicola, where the process appears to involve a small, non-coding RNA and potentially a metabolic

intermediate, although the details of this process are currently unknown [141-143]. More generally, it is unknown whether there are any conserved global thermo-regulators in *P. syringae*, similar to those described in *E. coli*. A better understanding of the molecular basis of thermo-regulation in *P. syringae* would not only advance our understanding of common vs. lineage specific thermo-regulation across the eubacterial kingdom, but knowledge of how the temperature signal is integrated into the regulatory networks of *P. syringae* will also help elucidate the adaptations it has made to be a successful epiphyte and pathogen in an environment where temperature regularly fluctuates.

Previously, we demonstrated that production of both the flagellum and the lipopeptide surfactant, syringafactin, were thermo-regulated in *P. syringae* and that this regulation was due, to at least in part, to reduced transcription of *fliC* and *syfA* at 30 °C compared to cooler incubation temperatures (chapter 1). While we found that *flgM* was necessary for such thermo-regulation of *fliC*, it did not appear to play any role in thermo-regulation of *syfA*, suggesting that a more globally-operative means of transcriptional regulation may be functioning in *P. syringae*. In this study we characterized potential thermo-regulators in *P. syringae* identified by assessing *syfA* expression in a large number of random transposon mutants.

2.2 Results

A transposon mutagenesis screen uncovers genes encoding an acyl-CoA dehydrogenase and a nudix hydrolase that over express *syfA* at 28 °C

Since expression of *syfA* is suppressed at 28-30 °C compared to cooler growth temperatures (Chapter 1). We hypothesized that there may be one or more negative regulators that are necessary to establish or maintain such thermo-repression. To test this hypothesis, we screened a miniTn5 library of *P. syringae* harboring a plasmid containing a fusion of the *syfA* promoter (P_{syfA}) with a *gfp* reporter gene. A total of 13 mutants that exhibited a visible increase in GFP fluorescence compared to WT were found in a screen of approximately 30,000 colonies grown at 28-29 °C (Table 1). Six of the mutants were much more fluorescent than the other seven. Identification of the disrupted loci the six highly-fluorescent mutants revealed that two unique genes were each disrupted independently in three mutants; psyr_2474, putatively encoding an acyl-CoA dehydrogenase, and psyr_4843, putatively encoding a nudix hydrolase (Fig. 1). Because these mutants exhibited a pronounced hyper-expression of syfA at high temperatures and were found several multiple times in the collection of mutants, they were further investigated.

Psyr_2474 encodes an acyl-CoA dehydrogenase

Psyr_2474 is annotated as an acyl-CoA dehydrogenase (hereafter referred to as ACDH), predicted to be involved in multiple metabolic pathways by the KEGG database. Generally, acyl-CoA dehydrogenases catalyze the desaturation at positions α,β of CoA-conjugated fatty acids derived from β-oxidation or amino acid metabolism [144]. An operon 5' of ACDH, psyr_2467-2470, encodes likely

homologues of the *liuABCDE* operon in *Pseudomonas aeruginosa*, which is involved in catabolism of leucine as well as acyclic monoterpenes [145-147]. This might suggest that ACDH is involved in the catabolism of a fatty acid, or an acyl-containing amino acid(s). Both psyr_2474 and psyr_2473 (encoding a putative LysR-type transcriptional regulator), are highly conserved across currently sequenced Pseudomonads (Table 2).

Psyr_4843 encodes a putative nudix hydrolase likely involved in mRNA turnover

Psyr_4843 is annotated as a nudix hydrolase, an enzyme widespread throughout the tree of life that catalyzes the general reaction nucleoside diphosphate linked to moiety X converted to nucleoside monophosphate plus phosphate linked to moiety X [148]. psyr_4843 appears to be closely related to *rppH* of *E. coli* based on amino acid conservation (65% identity/80% similarity), as well as its syntenic conservation in the genome (Fig. 2). Additionally, the KEGG database predicts that psyr_4843 is the *P. syringae* B728a ortholog of *rppH*. RppH has recently been demonstrated to stimulate mRNA decay through its action as a de-capping enzyme, whereby it cleaves the 5' pyrophosphate from an mRNA molecule generating a 5'-mono-phosphorylated transcript [149]. The resultant transcript with a 5'-monophosphate subsequently becomes a more favorable substrate for RNase E and its paralog RNase G, resulting in its degradation [150, 151]. While the amino acid and syntenic conservation strongly suggested that psyr_4843 was the *P. syringae* ortholog of *rppH*, more than one nudix hydrolase is often found in bacterial genomes and are capable of acting on a diversity of molecules that have the general structure of nucleotide diphosphate linked to moeity X, which can include mRNA, free nucleotides, sugar nucleotides, among others [148]. We therefore used phylogenetic analysis to determine whether the protein encoded by psyr_4843 was most closely related to RppH of *E. coli* and *Bdellovibrio bacteriovorous*, both of which have been biochemically characterized to be RNA decapping enzymes [149, 152] or whether it was most closely related to other described nudix hydrolases with alternative functions. The *P. syringae* nudix hydrolase was present in the same, moderately-supported clade as RppH from *E. coli*, while the *B. bacteriovorous* nudix hydrolase known to possess RNA de-capping function was found elsewhere in the tree (Fig. 3). This is not necessarily surprising as sequence divergence between the two enzymes has been commented on previously, and the substrate specificities are known to diverge between the RppH of E. coli and *B. bacteriovorous* [152]. In total, these results strongly support the conclusion that psyr_4843 encodes an RNA de-capping enzyme. The named protein most closely related to psyr_4843 is YgdP from *Pseudomonas aeruginosa*, and was an earlier designation for RppH in *E. coli* [153]. We therefore will refer to psyr_4843 as *ygdP*.

ACDH and *ygdP* knockouts require a greater incubation temperature than WT for full thermo-repression of *syfA* but are unaffected in thermo-repression of *fliC*

The contribution of *ygdP* and ACDH to thermo-regulation in *P. syringae* was examined in mutants having individual transposon insertion within these loci

(designated mTn5::*ygdP* and mTn5::ACDH respectively). Both mutants over-expressed *syfA* at 20 °C compared to the wild type strain as well as warmer temperatures, up to ca. 30 - 31 °C; Both mutants expressed *syfA* only marginally more than the wild type strain at the highest temperatures (Fig. 4). Neither mutant appeared to differ substantially from WT in expression of *fliC*, a gene previously shown to be thermo-repressed (Chapter 1) at any temperature, indicating that neither gene contributed to thermo-regulation of *fliC* (Fig. 5).

Deletion mutants ΔACDH and Δ*ygdP* phenocopy the transposon mutants and are complementable

To confirm the genetic basis of the mutant phenotypes described above, we constructed targeted deletion mutants at each locus, as well as complemented them with the full length genes (Figs. 6 and 7). The directed mutants exhibited the same phenotypes as the corresponding transposon mutant; both over-expressed *syfA* at both 20 °C and 29 °C (Fig. 6). Both mutants also produced more surfactant at 29 °C, evident as a larger halo of modified oil drops surrounding colonies when assessed with the atomized oil assay (Fig. 7). The surfactant zones of the mutants were decreased to that of the wild type strain when each gene was expressed *in trans* from its native promoter in pVSP61 (Fig. 7).

The phenotypes of ΔACDH and Δ*ygdP* mutations are additive

While ΔACDH and Δ*ygdP* each over-expressed *syfA* compared to the wild type strain when grown at 29 °C, *syfA* expression was still less than that of the wild type strain grown at 20 °C, indicating that neither mutation individually is sufficient to account for thermo-repression. We therefore tested if simultaneous blockage of both genes would completely abolish *syfA* thermo-repression at these high growth temperatures. The double mutant, ΔACDH /Δ*ygdP*, exhibited higher expression of *syfA* than either individual mutant at both 20 °C and 29 °C and the expression of *syfA* at 29 °C was similar to that of the wild type strain at 20 °C (Fig. 6). However, the expression of *syfA* in a ΔACDH /Δ*ygdP* double mutant was still lower at 29 °C than at 20 °C (Fig. 6), suggesting that there are additional thermo-regulatory inputs for *syfA*.

syfR* is not thermo-repressed in ΔACDH or Δ*ygdP

syfR encodes a LuxR-type regulator that positively regulates *syfA* expression as well as syringafactin production [64, 65]. Like that of *syfA*, expression of *syfR* was suppressed at high growth temperatures (Chapter 1). We measured the thermo-regulation of *syfR* by assessing GFP fluorescence of fusions to a *gfp* reporter gene in various mutant backgrounds to determine whether thermo-regulation of syringafactin production operated through *syfR*. Expression of *syfR* was suppressed at 29 °C compared to 20 °C in the wild type strain while it was much higher in ΔACDH, Δ*ygdP*, and ΔACDH/Δ*ygdP* than in WT at both temperatures. Importantly, a given mutant exhibited similar expression of *syfR* at 29 °C and 20 °C (Fig. 6B).

Genes associated with translation, amino acid import and metabolism, and other processes are not suppressed at high temperatures in a Δ*ygdP* mutant

As *ygdP* appeared to be necessary for suppression of *syfR* expression, and is likely involved in mediating mRNA turnover, we hypothesized it may potentially have a more global role in thermo-regulation of transcript stability in *P. syringae*. A model of how YgdP may function as a thermo-regulator of transcript stability, where elevated temperatures lead to melting of a stem loop structure present in the 5'-UTR of YgdP- sensitive transcripts is illustrated in Figure 8. This model incorporates the observations that a stem loop structure can make a normally YgdP-sensitive transcript resistant to pyrophosphate removal and that 5'-stem-loop structures generally make transcripts resistant to RNase E-mediated degradation [149, 154-156]. To assess the putative role of *ygdP* in global thermo-regulation, we compared the transcriptome of Δ*ygdP* incubated at 20 °C or 30 °C to that of the wild type strain incubated at the same temperatures. In the wild type strain the expression of 1445 genes were significantly different at the two temperatures; 1107 were induced and 338 were repressed at 30 °C compared to 20 °C (Chapter 1). In Δ*ygdP* the expression of 1150 genes were significantly different at the two temperatures; 636 were induced and 514 were repressed at 30 °C compared to 20 °C. A total of 726 genes that exhibited significant differences in expression 30 °C compared to 20 °C in both Δ*ygdP* and the wild type strain (Fig. 9). We also ascertained those transcripts that changed in expression in Δ*ygdP* compared to the wild type strain at either temperature (Fig. 10). When grown at 30 °C a total of 576 genes differed significantly in expression in Δ*ygdP* compared to the wild type strain; 105 genes increased in expression in the mutant, while 471 decreased in expression in the mutant. When grown at 20 °C a total of 175 genes differed in expression in Δ*ygdP* compared to the wild type strain; 42 genes increased in expression in the mutant, while 133 decreased in expression in the mutant. Taken together, these results demonstrated that the knockout of *ygdP* significantly affected the transcriptome of *P. syringae* and that its effect was largely, but not entirely, temperature-dependent. For example, more than three times as many genes were mis-regulated in Δ*ygdP* compared to the wild type strain at 30 °C as at 20 °C. These results also suggested, however, that the *ygdP* knockout had some pleiotropic effects, since not all of the genes that it regulates exhibit temperature-dependent transcription, nor are all of its transcriptomic effects explained by the model presented in Figure 8.

While these results demonstrated a large effect of *ygdP* on the transcriptome of *P. syringae*, its role could be clarified by determining which genes were most significantly altered in expression in Δ*ygdP*, compared to WT. The 20 most significantly induced and repressed genes in Δ*ygdP* compared to the wild type strain at 20 °C and 30 °C are listed in Tables 3 through 6.

Our model of how a *ygdP* knockout mutation would affect the temperature-dependent transcriptome, would have predicted that there would be fewer genes repressed at 30 °C in the mutant than in the wild type strain. Surprisingly, the opposite result was obtained; more transcripts were repressed at 30 °C compared to 20 °C in Δ*ygdP* than in the wild type strain (514 and 338 genes for Δ*ygdP* and WT, respectively). Furthermore, more genes were suppressed than enhanced in expression in Δ*ygdP* when compared to WT at 30 °C (471 and 105 genes suppressed and enhanced, respectively). That is, in Δ*ygdP*, there appear to be more thermo-

supressed transcripts and the *ygdP* knockout appears to generally exert a negative effect on transcript abundance at 30 °C. There are several reasons why these trends might be observed despite the fact that YgdP could thermo-regulate a subset of transcripts in the manner hypothesized. The most likely explanation follows from the fact that RNase E (psyr_1638) was more highly expressed in Δ*ygdP* compared to the wild type strain at 30 °C but not 20 °C. RNase E has recently been shown to stimulate 5'-monophosphate-dependent (i.e. YgdP-dependent) transcript degradation, as well as 5'-monophosphate-independent (i.e. YgdP-independent) transcript degradation. This suggested that Δ*ygdP* may lead to increased turnover of RNase E-dependent, but YgdP-independent transcripts (see discussion). Since RNase E was only over-expressed in Δ*ygdP* at 30 °C but not 20 °C, this effect was temperature dependent. Alternatively, the *ygdP* knockout could lead to an increased abundance of negative regulators or lack of induction of positive transcriptional regulators at 30 °C. We previously found that transcriptional regulators as a functional category were commonly induced at 30 °C in the wild type strain (Chapter 1). A comparison of the expression of these transcriptional regulators in the wild type strain and Δ*ygdP* revealed that the majority were induced in the wild type strain at 30 °C but were not induced in Δ*ygdP* (Table 7). Curiously, while many of these genes were induced in WT but not in Δ*ygdP* when comparing growth at 30 °C with 20 °C, many did not significantly differ in expression in the wild type strain and Δ*ygdP* at a given growth temperature of either 20 °C or 30 °C. We would expect that if a particular gene was significantly differentially expressed at 20 °C compared to 30 °C in the wild type strain but not in Δ*ygdP*, then this gene would also be significantly differentially expressed in the wild type strain compared to Δ*ygdP* at either 20 °C or 30 °C, or both. Many of the transcriptional regulators were less highly expressed in Δ*ygdP* compared to the wild type strain at 30 °C, but the differences were often not sufficiently different to achieve statistical significance.

The likely pleiotropic effects of the *ygdP* mutation that conferred RNase E over-expression at 30 °C, combined with ambiguous effect of the *ygdP* mutation on thermo-regulation of many genes, lead us to be more discriminating in our transcriptome comparisons. To further examine the extent to which our model could explain the observed patterns of gene expression, we focused our analyses on genes that fulfilled two criteria: a) the gene was suppressed at 30 °C compared to 20 °C in WT and b) the gene was over expressed in Δ*ygdP* compared to WT at 30 °C, meaning that the gene was thermo-repressed in WT and that this thermo-repression was dependent on *ygdP*. There were 43 genes that fulfilled these criteria, which lead to enrichment of genes related to the functional categories of translation and amino acid transport and metabolism (Table 8). To assess whether these genes were over-expressed in Δ*ygdP* at both temperatures, indicative of temperature-independent over expression in Δ*ygdP*, we determined whether these 43 genes were up-regulated in Δ*ygdP* compared to WT at 20 °C. Only four genes (psyr_0749, psyr_0750, psyr_2601 [*salA*], and psyr_4175) exhibited such temperature-independent over-expression, whereas the other 39 genes were over expressed in Δ*ygdP* only at 30 °C. Because any given pair-wise comparison of genes was likely to overlap in a way to yield enrichment of at least one category suggesting an

enrichment by chance, we performed the reciprocal comparison as a robust test of such an association. No functional category was found to be enriched in this comparison in contrast to all other pair-wise comparisons that yielded at least one enriched category. Only three genes fulfilled the criteria of being induced (as opposed to repressed) at 30 °C in a *ygdP*-dependent fashion: psyr_2039 (*fimA*), encoding a fimbrial adhesin, psyr_2973 (*gloA*), encoding glyoxalase, predicted to enable the interconvertion of methylglyoxal and lactoylglutathione (both linked to pyruvate metabolism), and psyr_1914 (*talB*), encoding transaldolase B, predicted to be involved in several metabolic pathways by KEGG. *ygdP* affected the expression of 90 genes, regardless of incubation temperature. Two functional categories (cell division and phytotoxin synthesis and transport) were enriched with the 18 genes that were generally up-regulated in Δ*ygdP* compared to the wild type strain (Table 10). The 72 genes that were generally down-regulated in Δ*ygdP* were enriched only for phage and IS elements (Table 10).

Expression of *syfA* and *syfR* is time or growth phase dependent

The finding that a similar suppression of *syfR* expression at 30 °C in both Δ*ygdP* and WT by RNA sequencing (Chapter 1) was not confirmed by assessment of the transcription of the *syfR* promoter using a fusion with a *gfp* reporter gene (Fig. 6B), suggests that other factors may play a role in influencing expression of this important regulator. At least two non-mutually exclusive possibilities exist to explain these dissimilar findings; either *syfR* expression is very strongly dependent on the growth phase of cultures (the transcriptome cultures were collected at 6 hpi, while reporter cultures were collected at 24 hpi), or additional thermo-responsive *cis*-regulatory elements exist that modulate *syfR* expression that are not present in the cloned promoter region in the $P_{syfR:gfp}$ fusions used in this study. We therefore determined if *syfR* or *syfA* expression differed in 12 hour cultures (the earliest time point with sufficient cells to quantify with GFP fluorescence) and 24 hour cultures in the wild type strain or any of the mutants (Fig. 11). *syfR* expression, estimated from GFP fluorescence, increased little in the wild type strain WT from 12 hpi to 24 hpi at either 20 °C or 30 °C. *syfR* was modestly suppressed at 30 °C compared to 20 °C in WT at both time points, similar to what was observed previously (Fig. 6B and Fig. 11). The Δ*ygdP* mutant expressed *syfR* more highly than the wild type strain at all temperatures and time points, however this difference was most pronounced in 24 hour cultures at 30 °C. The double mutant also expressed *syfR* higher at 30 °C after 24 hours growth than the wild type strain. These results suggested that the growth phase of cultures influenced the relative importance of both ACDH and *ygdP* on expression of *syfR* at high temperatures. We also tested the effect of culture age on the relative expression of *syfA*. The expression of *syfA* at 30 °C was similar at all sample times in both the wild type strain and in each of the mutants. At 20 °C, the expression of *syfA* was higher in 24 hour cultures than 12 hour cultures in all strains except ΔACDH. Taken together, these results suggested that only *syfR* expression, and not *syfA*, was affected by growth phase and this effect was most pronounced in the mutants grown at 30 °C. While there was a growth phase dependence on the expression of *syfR* in Δ*ygdP,* even in young cultures Δ*ygdP* over-expresses *syfR*.

The double mutant ΔACDH/Δ*ygdP*, but not either individual mutant exhibits altered thermo-repression of *syfR* and *syfA*

syfR transcript abundance in cultures grown at different temperatures was assessed using qRT-PCR to compare with estimates of rates of transcript production made using *gfp* reporter genes (Fig. 12). Because this assay could quantify RNA in a small number of cells, cells in very young cultures (6 hours after inoculation) were compared with those in older cultures (24 hours). Measures of relative *syfR* transcript abundance in different strains and growth temperatures very similar to those estimated by RNA sequencing, and differed substantially from estimates made using *gfp* reporter genes. Neither ΔACDH nor Δ*ygdP* significantly over-expressed *syfR* or *syfA* at any time compared to WT when grown at 30 °C. ΔACDH/Δ*ygdP* over-expressed both *syfR* and *syfA* compared to WT when grown at 30 °C. Interestingly, young cultures of both single mutants expressed *syfR* and *syfA* at a lower level than the wild type strain at 20 °C. The double mutant displayed a synergistic phenotype in that it expressed both genes by much less than the sum of either gene alone as compared to the wild type strain;(*syfR* expression was 2.1-fold lower in ΔACDH, 1.4-fold in Δ*ygdP* but 36.9-fold lower in ΔACDH/Δ*ygdP*; *syfA* expression was 2.5-fold lower in ΔACDH, 1.6-fold lower in Δ*ygdP*, but 15.7-fold lower in ΔACDH/Δ*ygdP*). These results taken together suggest that either ΔACDH or Δ*ygdP* is sufficient for thermo-regulation of *syfR* and *syfA*.

RsmY, but not RsmZ, is predicted to have a 5' stem-loop that partially melts at 30 °C.

syfR and *syfA* are both regulated by *salA* and thus both are under *gacS/A* control; neither *syfR* nor *syfA* are expressed in either a *gacS* or *salA* mutant (Chapter 1). The *gac* regulatory cascade involves multiple small, non-coding RNAs that relieve translational repression of RsmA-associated transcripts (RsmA binds to the Ribosome Binding Site (RBS), or near to it, and blocks the 30S ribosomal particle from associating with the transcript) [157]. In *P. aeruginosa*, one of the small RNAs, RsmZ, is degraded by RNase G [158]. We thus hypothesized that one of the predicted *gac*-dependent small RNAs in *P. syringae* would exhibit differential folding of its 5' regions predicted at 20 °C and 30 °C. RsmY, but not RsmZ, was predicted to have three nucleotides at the 5' terminus that are paired at 20 °C but not at 30 °C (Fig. 13). The lack of pairing of these three nucleotides would lead to six un-paired nucleotides at the 5' terminus, which would be sufficient to promote degradation by RNAse G [155].

2.3 Discussion

In this study we used a high throughput screen to identify thermo-regulators of *syfA*, and thus gain insight into the genetic components that mediate responses to

temperature in *P. syringae*. Two genes, ACDH and *ygdP*, both appear to contribute to the proper thermo-regulation of both *syfR* and *syfA*, while *ygdP* appears to be essential for proper thermo-regulation of a large number of other genes that exhibit temperature-dependent transcription. These two genes thus appear to play a role in the temperature-dependent behavior of *P. syringae.*

While both ΔACDH and Δ*ygdP* had higher rates of expression of *syfR* and *syfA* as compared to WT when assessed with reporter gene fusions, the abundance of *syfR* or *syfA* transcripts when assessed with qRT-PCR did not differ. There are several possible explanations for these results. It possible that *cis*-acting, thermo-regulatory elements that control *syfR* and *syfA* expression were not present in the cloned promoter gene fusion (Fig. 14). Temperature-dependent changes in DNA topology (supercoiling and bending) are common mechanisms that mediate thermo-regulation, often in conjunction with nucleoid structuring proteins such as H-NS [125, 159]. It might be expected that the local DNA topology of the promoter regions of *syfR* and *syfA* is different in the clones linked to the reporter genes in plasmid vectors than when present in the chromosome. It is also possible that binding sites for an H-NS-like protein are present within the coding region of *syfR* and *syfA* that was not included in promoter-reporter gene fusions. Some evidence for this possibility is provided by the observation that thermo-repression of *syfR* promoter activity is substantially lower than the suppression of *syfR* transcript abundance in the wild type strain, where GFP fluorescence of the *syfR* reporter gene fusions was 2-fold lower at 30 °C compared to 20 C, while *syfR* transcript abundance was 4.7-fold lower (Fig. 6B and 12). If this model is correct, it would suggest that both ACDH and *ygdP* interact with a structural element(s) within the cloned promoter region, as well as an element(s) outside of the promoter region. Support for the later conjecture is provided by the result that the double mutant over-expressed both *syfR* and *syfA* at 30 °C, which under this model, would require either ACDH or *ygdP* to directly affect the expression or activity of a third, currently unidentified regulator, or alternatively, for the ΔACDH/Δ*ygdP* double mutant to exert a sufficiently strong influence on the *syfR* and *syfA* promoters that the effect of this putative third element is negated. While there remains uncertainty about the cause of the discrepancy in estimates of transcription of *syfA* under various conditions in the single mutants, results provided by analysis of double mutants are more easily explained. GFP reporters and assessment of transcript abundance yielded more consistent patterns of expression under different conditions in the double mutant. Patterns of gene expression in the double mutant differed from that in either single mutant in several striking respects. The ΔACDH/Δ*ygdP* double mutant expressed *syfR* and *syfA* less at 20 °C in young cultures, while it was expressed these genes more highly than the wild type or either single mutant at 30 °C at any culture age. These results demonstrate that both ACDH and *ygdP* contribute to proper thermo-regulation of *syfR* and *syfA*. While we have described the effects of ACDH and *ygdP* on expression of both *syfR* and *syfA*, since *syfR* is a known regulator of *syfA* [64, 65], we hypothesize that thermo-regulation in the wild type strain, as well as the effects of both ACDH and *ygdP* on *syfA* expression is mediated through *syfR*. More work will

be required to determine any interactions between ACDH and *ygdP* that lead to co-modulation of expression of *syfR*.

One of the most striking temperature-dependent phenotypes for which *ygdP* seems to be required was that of expression of ribosomal proteins (Table 10). Over-expression of a ribosomal protein operon, *infC-rpmI-rplT* by increasing their copy number, partially complemented *gacS* and *gacA* mutants in *P. syringae* and *P. fluorescens*, respectively that were deficient in toxin and protease production [160, 161], suggesting that the balance of ribosomal protein composition in the cell can modulate certain phenotypes. The suppression of the *gacA* mutant phenotype in *P. fluorescens* was suggested to be a result of the over-abundance GGA motifs present in the 3' end of the *infC* transcript which bound RsmA, thereby reducing the abundance of the free RsmA needed for repression of translation [157]. This model, however, has not been tested directly, and introduction of clones in which *rpmI* and *rplT*, but not *infC* were deleted could not suppress the mutant phenotype [161], suggesting that more complex interactions may be occurring than initially postulated. If over-expression of certain ribosomal proteins can lead to enhanced expression of *gac*-regulated phenotypes, and this effect is not merely a coincidental blockage of an RsmA-mediated interaction, it would suggest that the ribosome itself is a regulator, and might potentially play some role in mediating temperature-dependent phenotypes. There is some evidence for this conjecture. First, ribosomal function appears to be a regulatory input for both the cold shock and heat shock response given that translation-inhibiting antibiotics can stimulate the cold shock response in *E. coli* and *Bacillus subitilis* at sub-lethal doses [162, 163], while depletion of the 4.5S rRNA or treatment with antibiotics that decrease translational fidelity induce the heat shock response [162, 164]. This suggests that perturbation of ribosome function can mimic the effects of a large temperature shift. Second, one of the prominent effects of heat shock is the down-regulation of a subset of ribosomal proteins [103, 165]. Conversely, expression of ribosomal or ribosome-associated proteins of *B. subtilis* increase following cold shock as well as during prolonged cool incubation [166-168]. It has been hypothesized that modification of ribosomes to function at cooler temperatures is one of the major acclimation processes that occurs when cells experience cool temperatures [169]. More pertinently, RppH/YgdP associates with ribosome precursor particles, and its deletion leads to an increase of the ratio of 30S to 70S ribosomal particles [170], suggesting that disruption of *ygdP* may lead to altered ribosomal function in *P. syringae.* If ribosomal function is in fact altered in a *ygdP* mutant, then we might expect to see an even more pronounced change in the proteome compared to the transcriptome.

While we have hypothesized that a major consequence of the disruption of *ygdP* is altered ribosomal function, the gene may be more directly linked to *syfR* expression by stimulating the abundance of RsmY, a small RNA intermediate regulator in the *gac* regulatory pathway. RsmZ is selectively destabilized by RNase G, a 5'-monophosphate dependent RNase in *P. aeruginosa*. Modeling suggested that RsmY possessed a 5'-terminus that would yield a suitable substrate for YgdP to

decap at high temperatures. Such regulatory interaction will need to be tested more directly.

The role of YgdP in thermo-regulation is complicated for several reasons. First, YgdP is known to catalyze the hydrolysis of a variety of dinucleoside oligophosphate species, Ap_5A in particular, into their constituent mononucleotide mono-, di-, and tri-phosphates [153]. These potential substrates are formed as by-products of amino-acyl tRNA synthesis, and accumulate under heat and oxidative stress conditions [171-173]. While there are several speculative roles of these molecules such as in altering protein function (DnaK, GroEL, ClpB) [174-176] and/or nucleotide metabolism [177] their function(s) within the cell remain to be determined. Second, the mRNA decapping function of YgdP results in RNase E and RNAse G-mediated degradation of messages, and is complicated by the recognition that mRNA turnover can be catalyzed by several independent mechanisms. Most relevant to this work, is the recognition that RNAse E itself can cleave transcripts in a 5'-monophosphate-dependent as well as independent manner, these functions being genetically separable [178, 179]. Our data indicated that the RNase E transcript, like that of transcripts for ribosomal proteins, were more abundant at 20 °C compared to 30 °C in the wild type strain, while Δ*ygdP* expressed these transcripts only more highly at 30 °C than at 20 °C when compared to the wild type strain. There are no previous reports of temperature-regulated RNase E transcript abundance. In *E. coli,* the *rne* transcript is auto-regulated, but in an RppH-independent manner, in that RppH does not cleave pyrophosphate from *rne* transcript [180]. Our results indicated that YgdP is involved in regulation of *rne* in *P. syringae*, but only at warm temperatures. A similar effect was observed in *E. coli*, where deletion of *rppH* led to an apparent increase in RNase E activity (as measured by RNase E autoregulation) [179]. This result suggests that while degradation of transcripts via an YgdP-dependent mechanism may be reduced in a YgdP mutant, it could stimulate the degradation of transcripts via an YgdP-independent but RNase E-dependent mechanism, which would functionally resemble thermo-regulation due to the selective effect at 30 °C (Fig. 15).

Given that YgdP possesses two clear catalytic capabilities that act upon the very distinct, physiologically relevant molecules, mRNA and dinucleoside oligophosphates, there is a real need to understand how YgdP activity is affected by the presence of both potential substrates. For example, could Ap_5A competitively inhibit YgdP decapping of an RNA 5'-triphosphate or vice versa? If such interactions occur at physiological concentrations of the enzyme and its various targets, then these interactions themselves might be regulated by factors such as temperature.

Our understanding of the role of ACDH in thermo-regulation remains limited to its effect on *syfR* and *syfA* expression. However, based on its predicted function and conservation of gene neighbors in different taxa, we can speculate that it modifies the pool of a key metabolic intermediate that regulates expression of *syfR*, likely an amino acid(s) or fatty acid(s). As syringafactin is a family of closely related lipopeptides, consisting of an acyl chain linked to a short peptide head group rich in leucine [65], ACDH could be involved in one or more pathways related to metabolism of syringafactin intermediates. The LysR-type regulator encoded by the

adjacent and oppositely oriented gene psyr_2473, is a likely candidate regulator of such a metabolic intermediate, as this arrangement of genes encoding enzymes flanked genes encoding a LysR-type regulator is common [181]. In addition to fatty acid and branched chain amino acid metabolism, acyl-CoA dehydrogenases related to ACDH are commonly involved in production of secondary metabolites [182, 183].
It is significant that the temperature dependent expression of *syfR* and *syfA* seen in the ACDH/ *ygdP* double mutant was quite different than that seen in either individual mutant. While we are unsure of the nature of the interplay of these two genes, we speculate that together they coordinate alterations in amino acid metabolism since such genes were enriched among those thermo-repressed in a *ygdP*-dependent manner and the fact that ACDH is predicted to be involved in catabolism of branched chain amino acids. Metabolite analysis should be useful in testing this hypothesis.

2.4 Materials and Methods

Bacterial strains, plasmids, culture media, and growth conditions
Pseudomonas syringae pv. syringae B728a [112] and mutant derivatives were routinely cultured in King's medium B (KB) broth, or on KB plates supplemented with 1.5% (w/v) Difco agar technical (BD, Sparks, MD) at 28 °C [113]. *Escherichia coli* strains TOP10 (Life technologies, Carlsbad, CA), S17-1 [114], and SM10(λpir) [184] were cultured in Luria-Bertani (LB) medium broth, or on LB plates supplemented with 1.5% (w/v) Difco agar technical at 37 °C [185]. Antibiotics were used at the following final concentrations: rifampicin, 100 μg/mL; kanamycin, 50 μg/mL; gentamicin, 15 μg/mL; spectinomycin 20 μg/mL; tetracycline, 15 μg/mL; nitrofurantoin (NFT), 30 μg/mL. Natamycin (antifungal) was used at 21.6 μg/mL. Strains and plasmids used in this work are listed in Table 10. Primers used in this work are listed in Table 11.

Temperature-dependent assays were performed in incubators adjusted to the appropriate temperature as described in the text. Plate temperatures were routinely monitored using a CZ-IR thermometer (ThermoWorks, Lindon, UT). Incubator temperatures and RH were routinely monitored using HOBO data loggers (Onset, Bourne, MA).

Transposon mutagenesis
To facilitate a mutagenesis screen using a spectinomycin resistance-conferring transposon, the *syfA* promoter was removed from $pP_{syfA\text{-}gfp}$ (spectinomyin resistance) using HindIII and EcoRI (New England Biolabs, Ipswich, MA) and cloned into pPROBE-gfp[tagless] (kanamycin resistance) [186]. Transposon mutants were generated using a similar approach as [64]. *E. coli* harboring pUT mini-Tn5 Sm/Sp [187] and *P. syringae* ($pP_{syfA\text{-}gfp(K)}$) were grown overnight in either LB or KB broth with appropriate antibiotics, and shaken at 200 rpm at 37 °C or 28 °C, respectively. Cells were mixed in an approximate ratio of 1:3 (*E. coli*:*P. syringae*) and incubated overnight on KB without antibiotic amendment. Culture lawns were re-suspended

in 10 mM potassium phosphate buffer (pH 7.5) (KPO_4 buffer) and spread onto KB plates amended with spectinomycin and kanamycin and incubated at 28-29 °C. After 2-3 days incubation, plates were observed under UV illumination, and colonies displaying an observable increase in fluorescence compared to the majority of the cells were selected and purified to yield single colonies by re-streaking and were re-screened at 28-29 °C to retention of the hyper fluorescent phenotype. Wild type *P. syringae* ($pP_{syfA\text{-}gfp(K)}$) was routinely included for comparison at all stages of screening.

The location of transposon insertions was determined using arbitrarily primed PCR similar to previous studies [64, 188]. Genomic DNA was isolated from transposon mutants using DNeasy Blood & Tissue Kit (QIAGEN; Valencia, CA) and PCR amplified in two sequential reactions using tn5sm-ext (first round of amplification) and tn5sm-int (second round of amplification) (see [64] for primer sequences). Sequenced PCR products were compared to the *P. syringae* B728a genome using BLAST on the Integrated Microbial Genomes website (http://img.jgi.doe.gov).

Targeted deletion of *ygdP* and ACDH and complementation of mutants

ygdP and ACDH deletion mutants were constructed in a fashion similar to that described for *flgM* in Chapter 1. Briefly, approximately 1000bp of genomic DNA both upstream and downstream of the target gene was amplified using Phusion DNA polymerase (Thermo Scientific--previously Finnzymes; Lafayette, CO) with the "KO" primers listed in Table 11. Amplified genomic fragments were combined with a *kan*-FRT PCR fragment amplified from pKD13 (ACDH) or pKD4 (*ygdP*) [121] to conduct three-fragment overlap-extension PCR. The combined fragments were cloned into pTOK2T [70] to create p*ygdP*-KO and pACDH-KO. pygdP-KO and pACDH-KO were electroporated into *E. coli* S17-1, which was mated with *P. syringae* B728a. The structure of the target genes in resultant colonies having kanamycin resistance and tetracycline sensitivity (indicating double cross-over homologous recombination) was verified as having undergone marker exchange using PCR. pFLP2Ω was electroporated into a single isolate of each deletion mutant and the recipient cells were plated on KB amended with spectinomycin. Single colonies were streaked onto KB with or without kanamycin amendment to yield single colonies. Five kanamycin sensitive (indicative of Flp-mediated *kan* cassette excision) colonies in each deletion mutant were grown overnight in KB broth without antibiotic amendment, followed by dilution plating. Ten spectinomycin-sensitive colonies from each deletion were PCR screened for loss of pFLP2Ω. *ygdP* and ACDH deletion mutations were confirmed by sequencing and the strains referred to as ΔygdP and ΔACDH, respectively. To construct a double-deletion mutant, the pACDH-KO construct was mated into the Δ*ygdP* mutant and processed as described above.

ygdP and ACDH complementation vectors, pNcomp and pAcomp, respectively, were constructed by amplifying *ygdP* and ACDH coding sequences along with their upstream promoter regions using Phusion DNA polymerase (Thermo Scientific; Lafayette, CO) and primers "native_comp" primers, followed by blunt-end cloning into pVSP61 [189] which was digested with EcoRI and blunted

with T4 DNA polymerase (New England Biolabs). Complementation constructs were sequenced using the same primers to verify proper insertion.

Phylogenetic analysis of YgdP

A multiple sequence alignment for 337 NUDIX hydrolase homologs was constructed in Geneious Pro 5.5.6 using the Geneious Alignment tool with default settings [190]. Maximum likelihood (ML) searches were used to build a phylogeny for the NUDIX hydrolase homologs with GARLI v0.96 [191] for 50 bootstrap replicates under the WAG+I+G+F model of evolution as determined by the Bayesian and Akaike information criterion in ProtTest v2.4.mac [192]. From these bootstrap trees, a 50% majority-rule consensus tree was constructed using SumTrees v3.0.0 [193] and edited in FigTree v1.3.1 and Adobe® Illustrator®.

Detection of biosurfactants

Biosurfactants were detected as described in Chapter 1. Briefly, bacterial cells were grown over night in KB broth with appropriate antibiotics, and re-suspended to a final concentration of 2 x 10^8 CFU/mL. 5 µL of re-suspended cells was spot-inoculated onto KB amended with appropriate antibiotics. Plates were incubated for about 24 hours at the indicated temperature, and the area of surfactant coverage on the plate was visualized because of the formation of bright oil drops after a mist of mineral oil was sprayed onto the plate was calculated.

Transcriptional Reporter Assays

Transcriptional reporter gene assays were performed similar to [116]. Briefly, spoted cultures were re-suspended in KPO_4 buffer and diluted to a final OD_{600} of 0.1-0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs; Sunnyvale, CA) with a 486-nm-band-pass excitation filter and a 510- to 700-nm combination emission filter. Relative GFP fluorescence was determined by normalizing the fluorescence arbitrary units with optical density. To compare fluorescence in young vs. old cultures (see Results), a 10 mM diameter cork-borer was used to remove cells grown in spots on agar plates as well as the underlying agar. The agar plugs were sonicated for 5 minutes using a Bransonic 5510 sonicator (Branson; North Olmsted, OH) to dislodge cells from the agar surface. The optical density at 600nm of the young cultures were in the range of 0.03 to 0.05.

RNA isolation and qRT-PCR

RNA isolation and qRT-PCR was performed as described in Chapter 1. Briefly, cultures were harvested and suspended immediately in RNA*later* (Life Technologies; Carlsbad, CA) and stored for no longer than one week at 4 °C prior to RNA isolation. Total RNA was isolated using TRIzol® reagent (Life Technologies). Total RNA was DNase-treated with TURBO DNA-*free*™ (Life Technologies) followed by column purification using a RNeasy Mini Kit (QIAGEN). RNA was reverse transcribed using SuperScript® II reverse transcriptase (Life Technologies) with random primers (Life Technologies). qPCR was performed using a 7300-Real-Time PCR System (Life Technologies) with QuantiTect SYBR Green I (QIAGEN) on diluted

cDNA. Samples not treated with reverse transcriptase were routinely included, and showed no amplification following 35 cycles. *rpoD* and psyr_3981 were routinely used as endogenous controls.

mRNA sequencing

mRNA sequencing and analysis was performed as described in Chapter 1.

Predicted RsmX and RsmY folding

The RsmX and RsmY sequences from *Pseudomonas syringae* B728a were modeled using the mfold web server [194] v2.3 energies, with default settings, except for temperature adjusted to 20 °C or 30 °C.

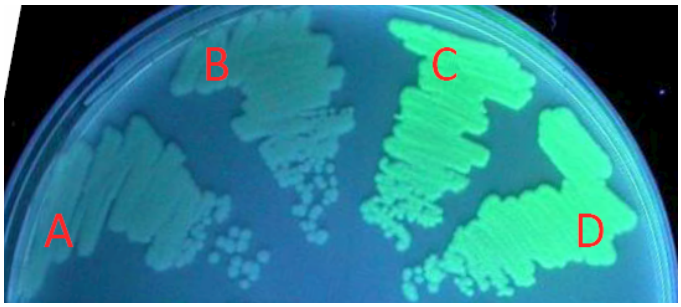

Figure 2.1. GFP fluorescence exhibited by a wild type *Pseudomonas syringae* B728a harboring a plasmid containing a fusion of the *syfA* promoter with a *gfp* reporter gene (A and B) and this strain having a Tn5 mutant insertion in psyr_2474 (acyl-CoA dehydrogenase) (C) or psyr_4843 (nudix hydrolase) (D).

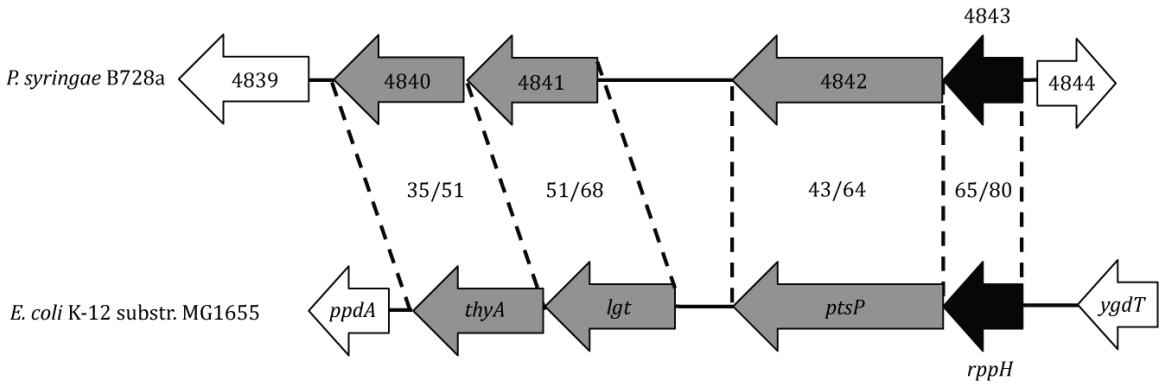

Figure 2.2. Comparison of the genomic region of *P. syringae* B728a containing psyr_4843 encoding a nudix hydrolase (black, top) and a corresponding region in *E. coli* K-12 substr. MG1655. Gray arrows indicate conserved genes between the two strains. White arrows indicate non-homologous genes in the two strains. Black lines between arrows represent non-coding, intergenic sequence. Numbers between arrows indicate % amino acid identity/% amino acid similarity. Arrows and intergenic sequences not drawn to scale.

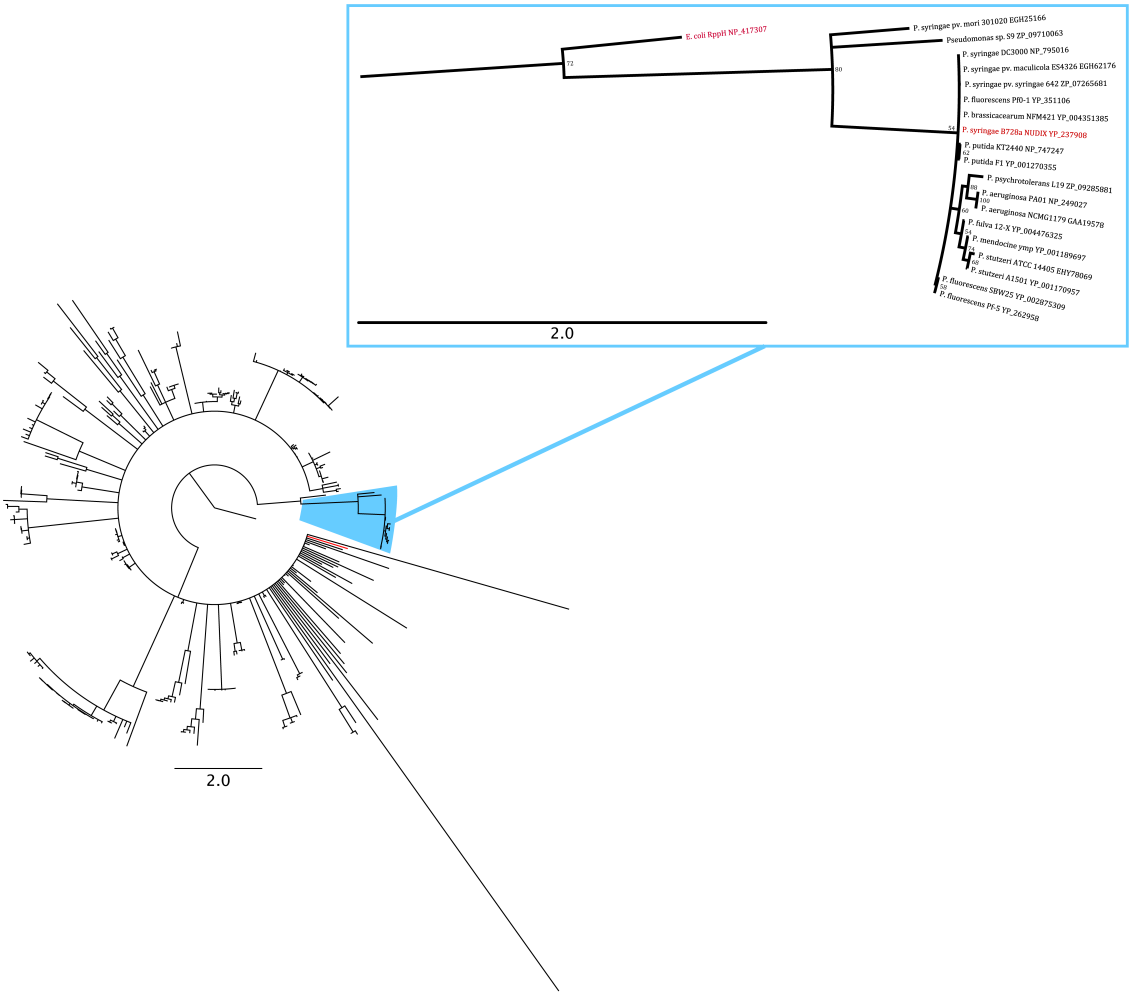

Figure 2.3. 50% majority rule maximum likelihood consensus tree from Garli v0.96 analysis of nudix hydrolase homologs from *Pseudomonas* spp., *E. coli*, *B, bacteriovorous*, and *A. tumefaciens*. Red text indicates *E.coli* RppH and the *P. syringae* NUDIX hydrolase disrupted in this study. Red branch located in polytomy indicates the RppH homolog in *B. bacteriovorous*.

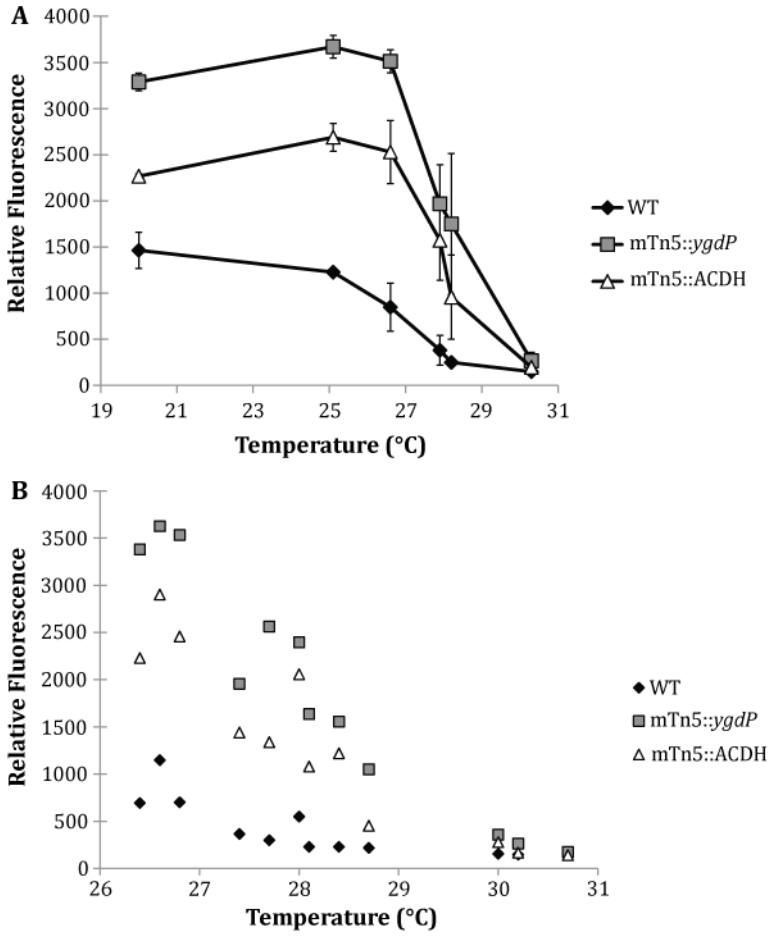

Figure 2.4. Cell normalized GFP fluorescence of wild type *Pseudomonas syringae* B728a (diamonds) and mutant strains containing a Tn5 insertion in a gene encoding ACDH (triangles), or *ygdP* (squares) and harboring a *syfA:gfp* reporter gene fusion when grown at various temperatures (A). Individual colony relative fluorescence values used to calculate the averages in A, indicating the sensitivity of $P_{syfA\text{-}gfp(K)}$ expression over the temperature range of 27 °C to 29 °C in the transposon mutants (B). Vertical bars represent standard deviation of the mean.

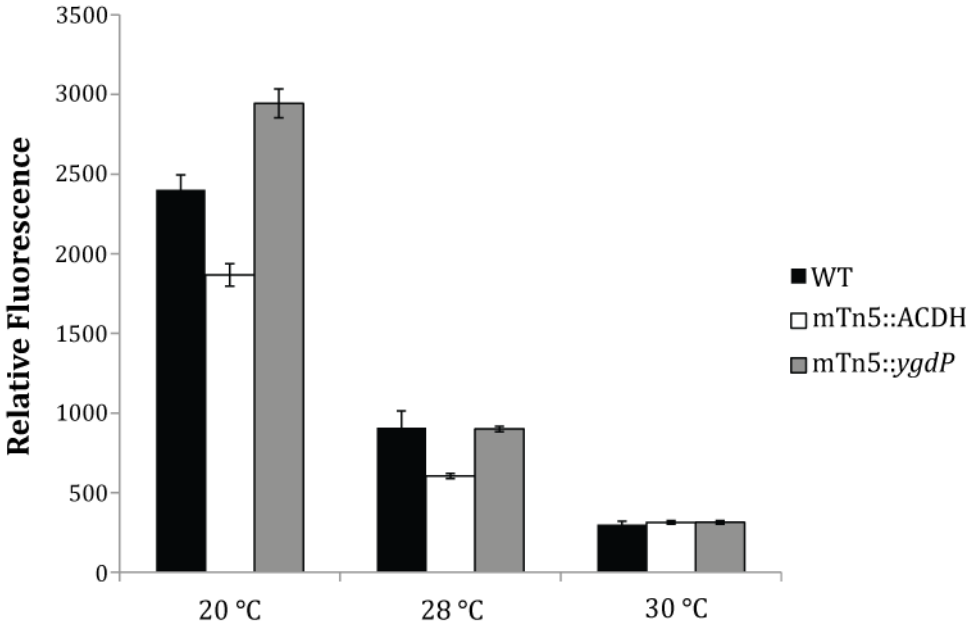

Figure 2.5. Cell normalized GFP fluorescence of wild type *Pseudomonas syringae* B728a (black bars) and mutant strains containing a Tn5 insertion in a gene encoding ACDH (white bars), or *ygdP* (gray bars) and harboring a *fliC-gfp* reporter gene fusion when grown at various temperatures. Vertical bars represent the standard deviation of the mean.

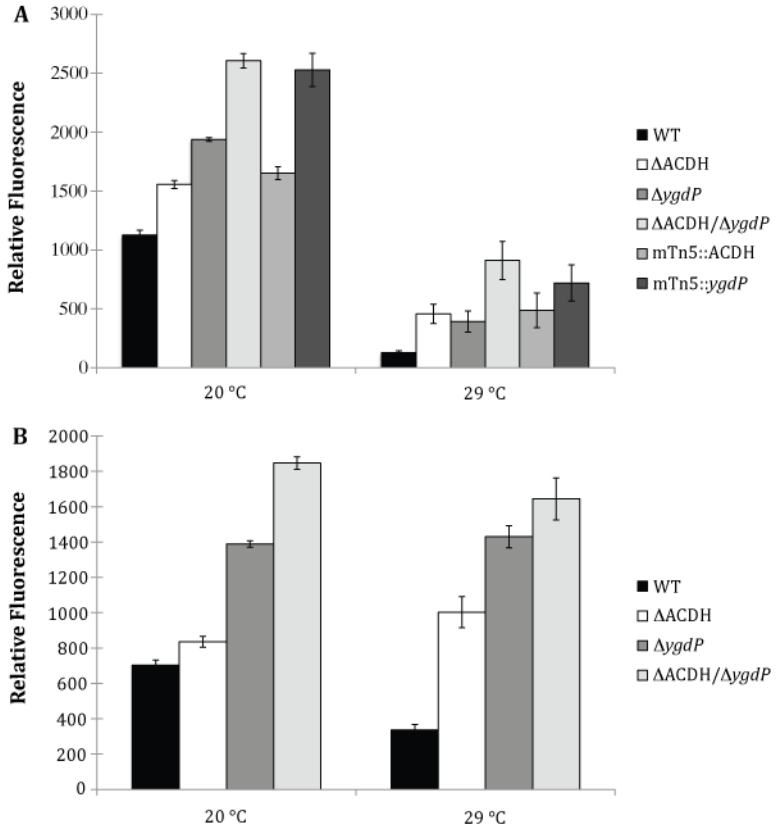

Figure 2.6. Cell normalized GFP fluorescence of wild type *Pseudomonas syringae* B728a (dark squares) and mutant strains containing a knockout of a gene encoding ACDH (white bars), or *ygdP* (dark gray bars) or both ACDH and *ygdP* (light gray squares) and harboring a *syfA:gfp* reporter gene fusion (A) or a *syfR:gfp* reporter gene fusion (B) when grown at various temperatures. Vertical bars represent the standard deviation of the mean.

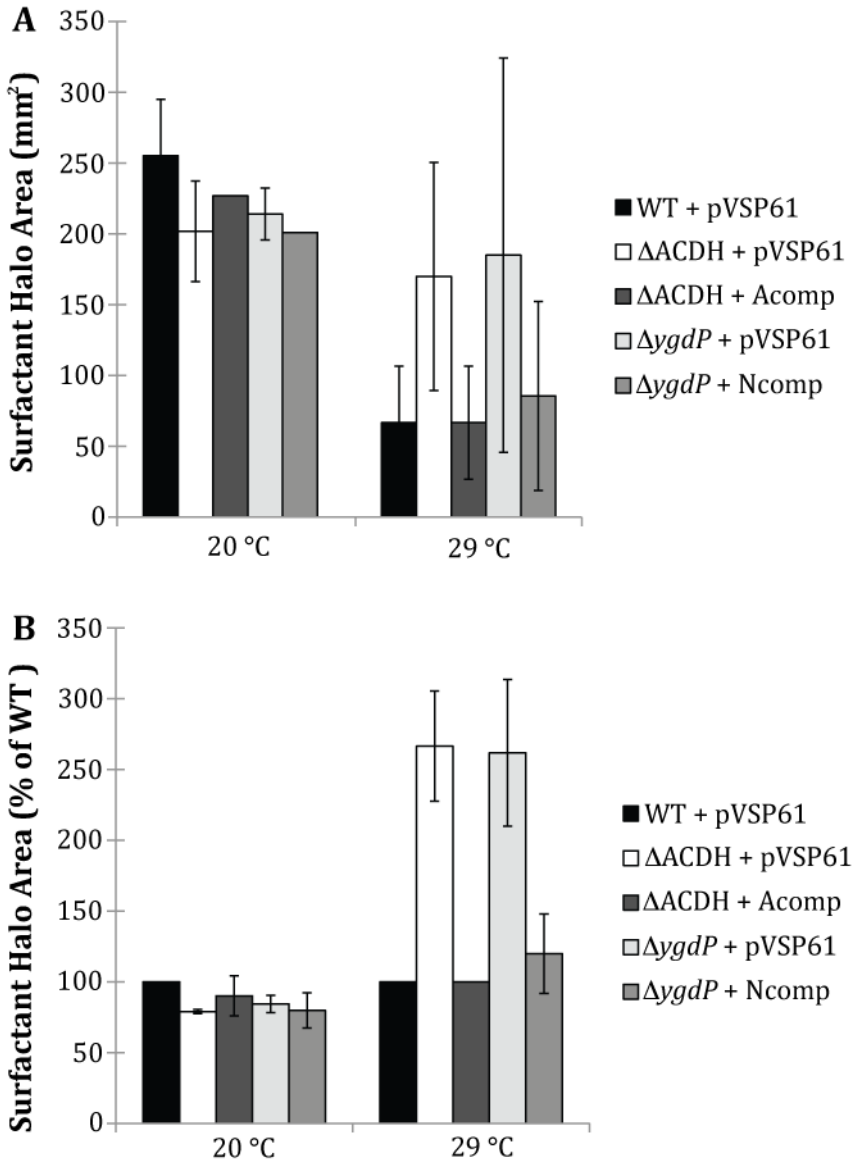

Figure 2.7. Area of surfactant produced by wild type *Pseudomonas syringae* B728a (black bars) and mutant strains containing a knockout of a gene encoding ACDH (white and dark gray bars), or *ygdP* (light gray and medium dark gray bars) harboring either pVSP61 (white and light gray) or pVSP61 containing psyr_2747 (dark gray) or psyr_4843 (medium dark gray) expressed as absolute area (A) or as a percentage of the surfactant area produced by the wild type strain (B). Vertical bars represent the standard deviation of the mean.

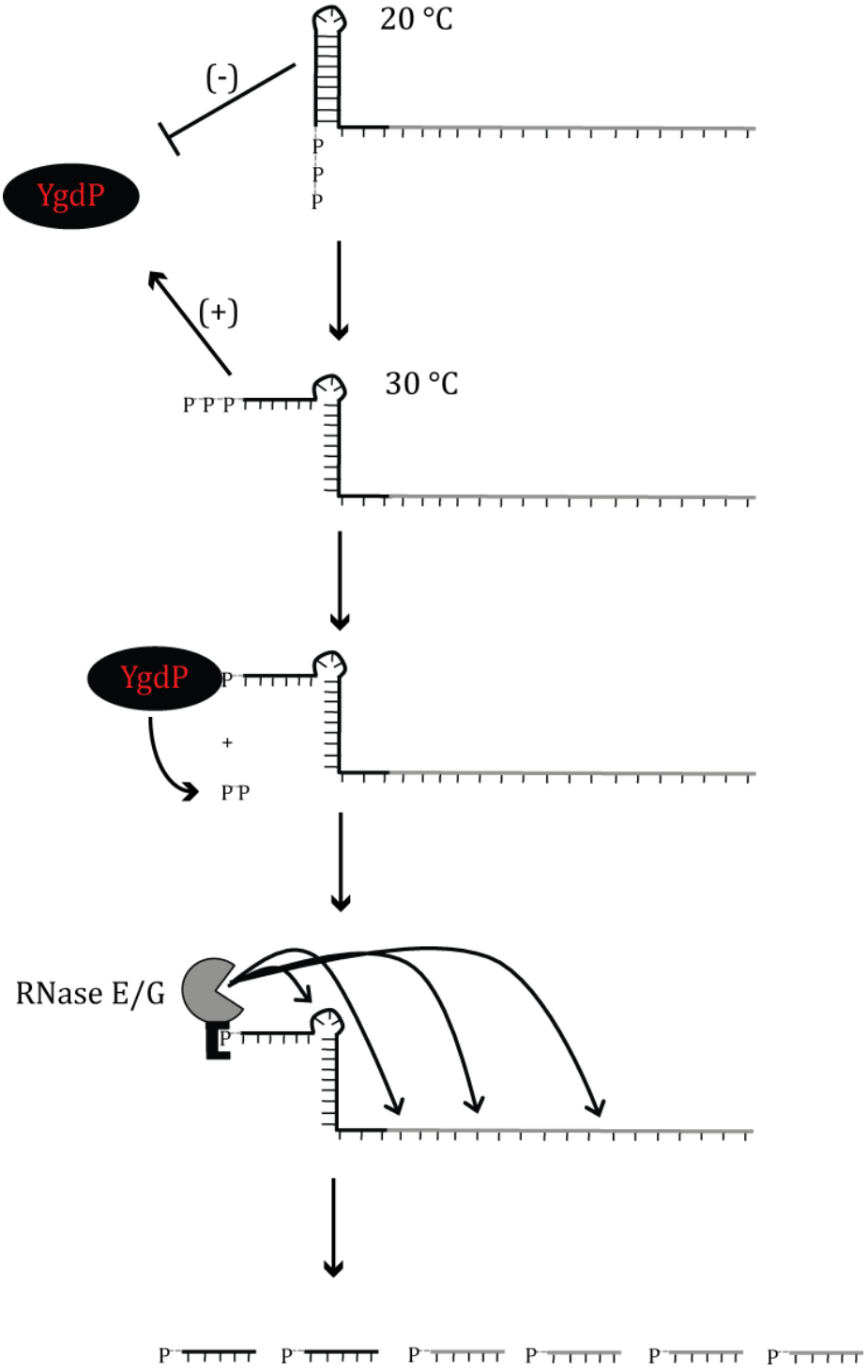

Figure 2.8. Model of YgdP mediation of temperature-dependent transcript degradation. At 20 °C, a 5' UTR stem loop structure forms making the transcript resistant to NUDIX pyrophosphate cleavage (top panel). At 30 °C the stem melts, allowing YgdP access to the 5' nucleotide (2nd panel). YgdP cleaves the pyrophosphate from the transcript, generating a mono-phosphorylated transcript that stimulates nucleolytic cleavage by RNase E and/or RNase G (3rd and 4th panel). Cleavage by either RNase E or RNAse G leads to small, untranslatable fragments that are further degraded by cellular RNases (bottom panel).

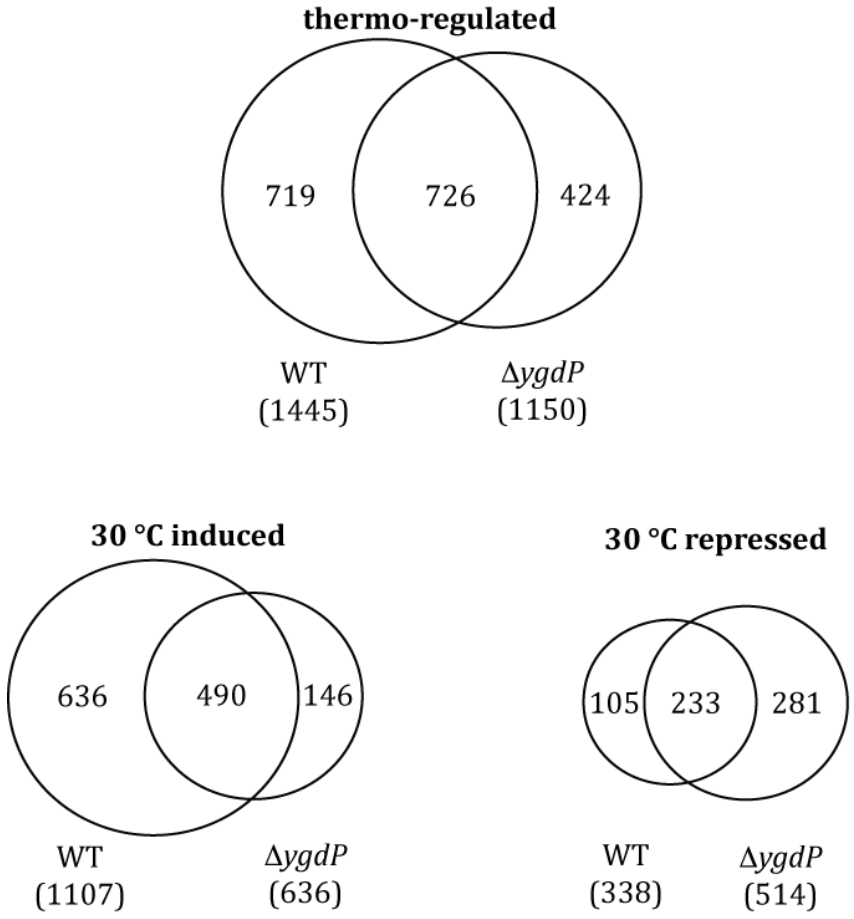

Figure 2.9. Comparison of the number of thermo-regulated genes in a wild type and Δ*ygdP* mutant of *Pseudomonas syringae*.

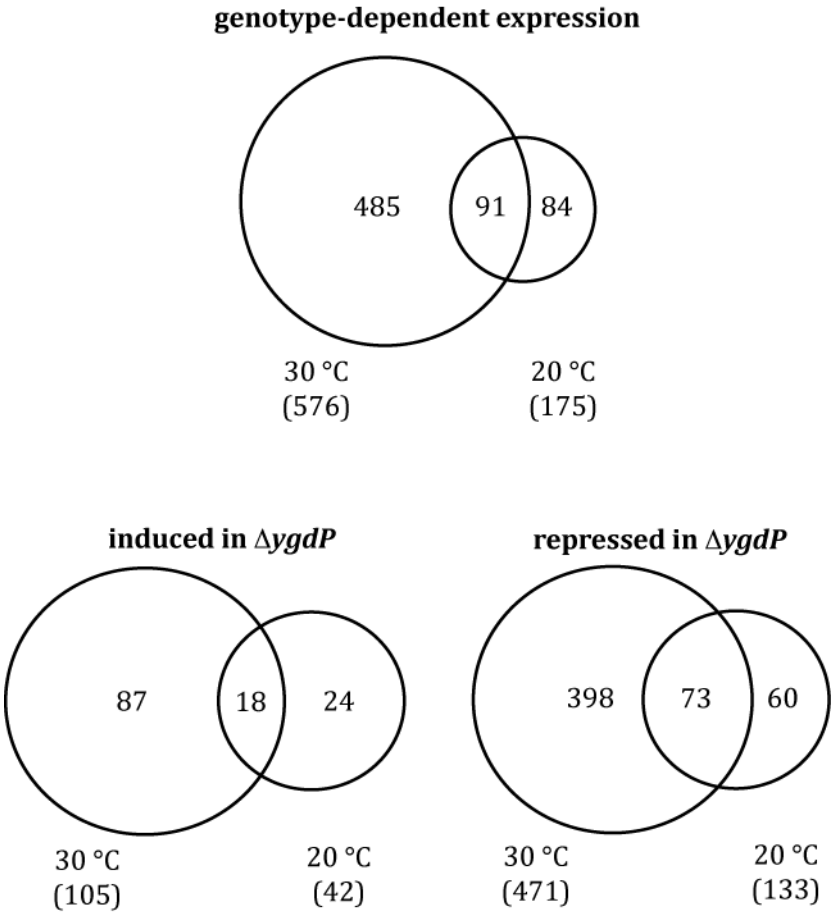

Figure 2.10. Comparison of the number of genes differentially regulated in a wild type and Δ*ygdP* mutant of *Pseudomonas syringae* at different growth temperatures.

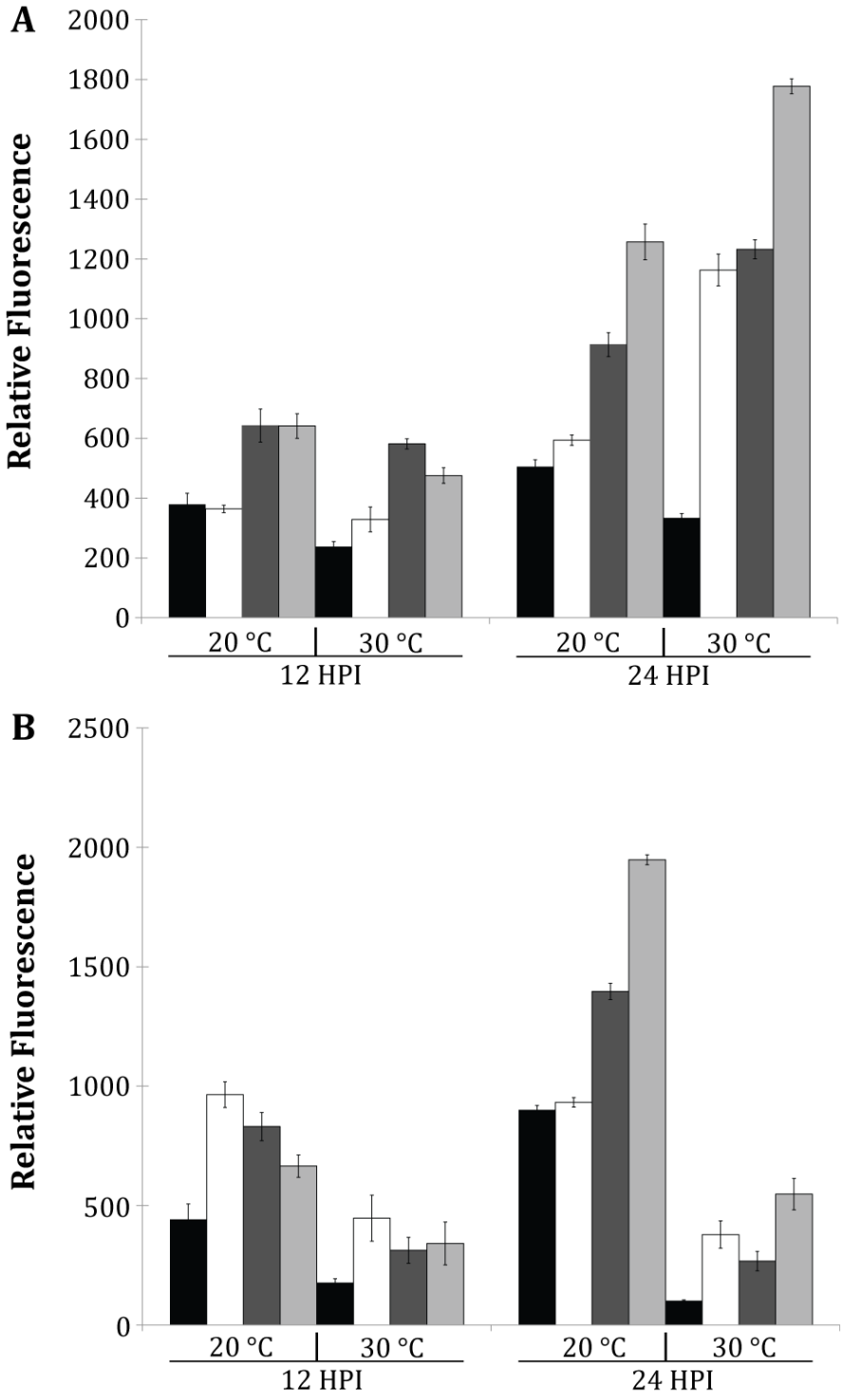

Figure 2.11. Cell normalized GFP fluorescence of wild type *Pseudomonas syringae* B728a (black bars) and mutant strains containing a knockout of a gene encoding ACDH (white bars), or *ygdP* (dark grey bars) or both ACDH and *ygdP* (light grey bars) and harboring a *syfR:gfp* reporter gene fusion (A) or a *syfA:gfp* reporter gene fusion (B) when grown at various temperatures for different period of time. Vertical bars represent the standard deviation of the mean.

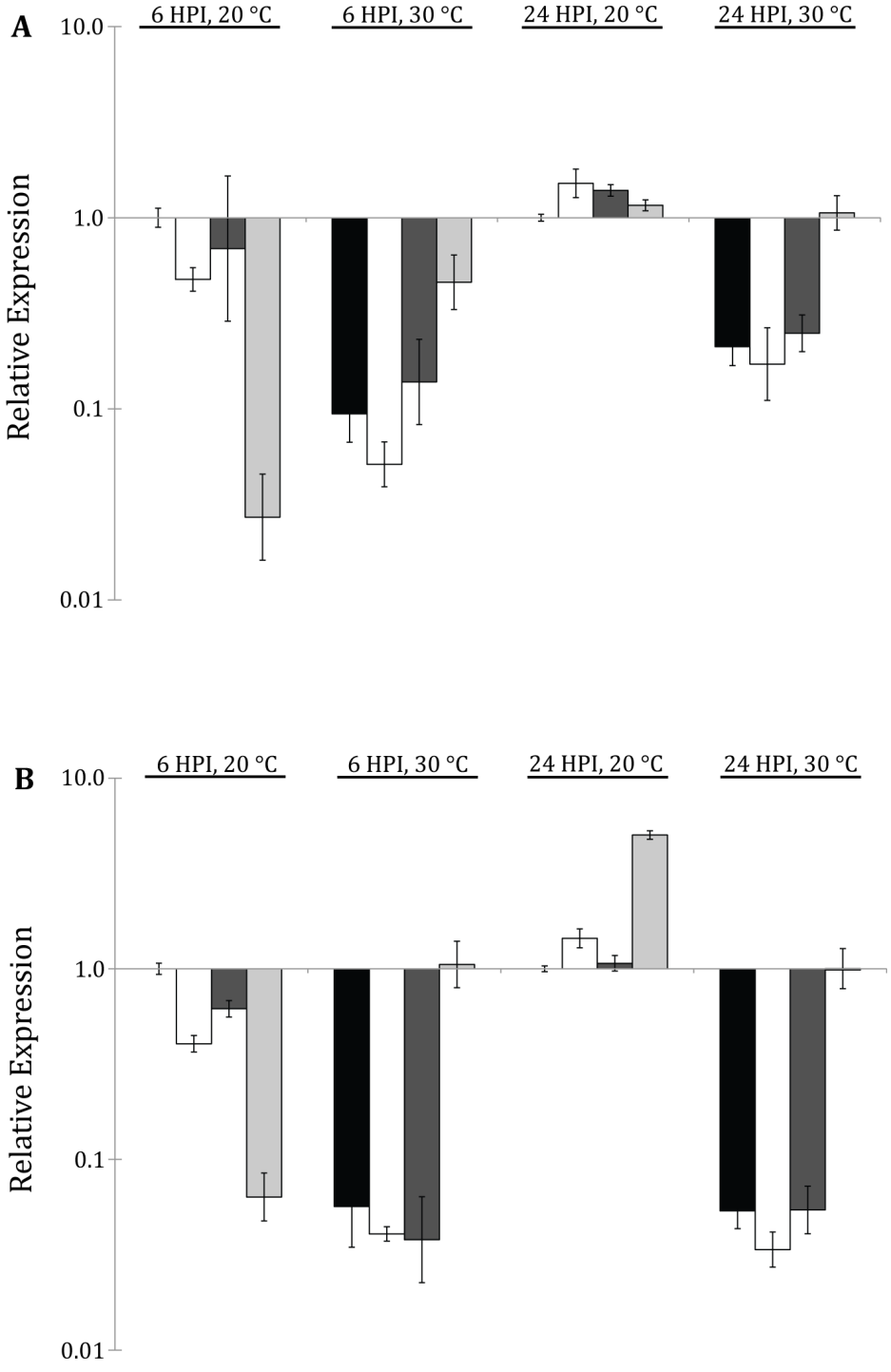

Figure 2.12. Transcript abundance of *syfR* (A) and *syfA* (B) determined by qRT-PCR analysis of wild type strains of wild type *Pseudomonas syringae* (black bars), and mutant strains containing a knockout of a gene encoding ACDH (white bars), or *ygdP* (dark grey bars) or both ACDH and *ygdP* (light grey bars) grown at different

temperatures for different times. All expression values are calculated relative to the wild type strain grown 20 °C at the a given sampling time. Vertical bars represent 95% confidence interval.

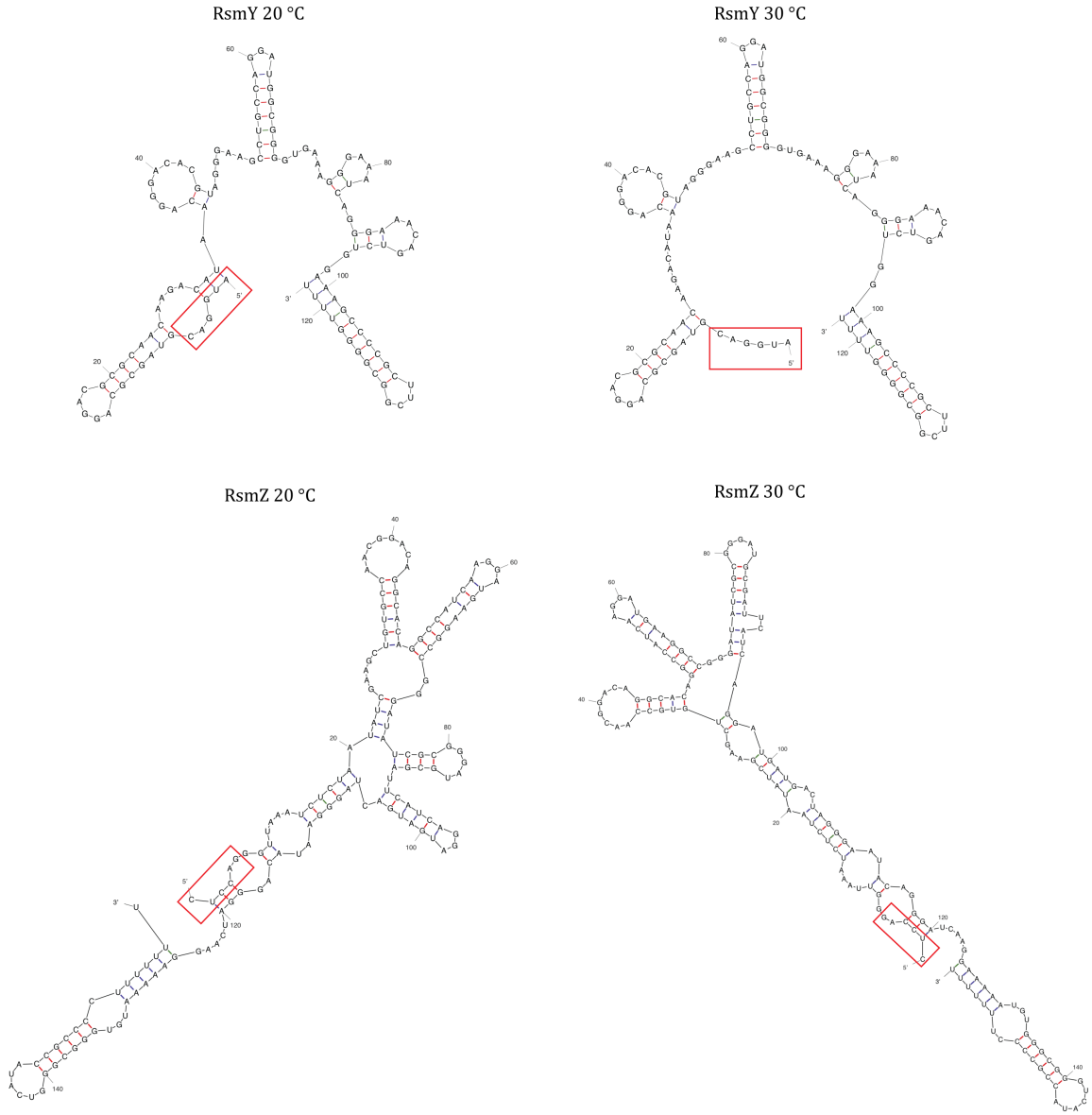

Figure 2.13. Predicted temperature-dependent folding of the small, non-coding RNAs, RsmY and RsmZ from *Pseudomonas syringae*. Red boxes mark the six 5' terminal nucleotides of each sRNA.

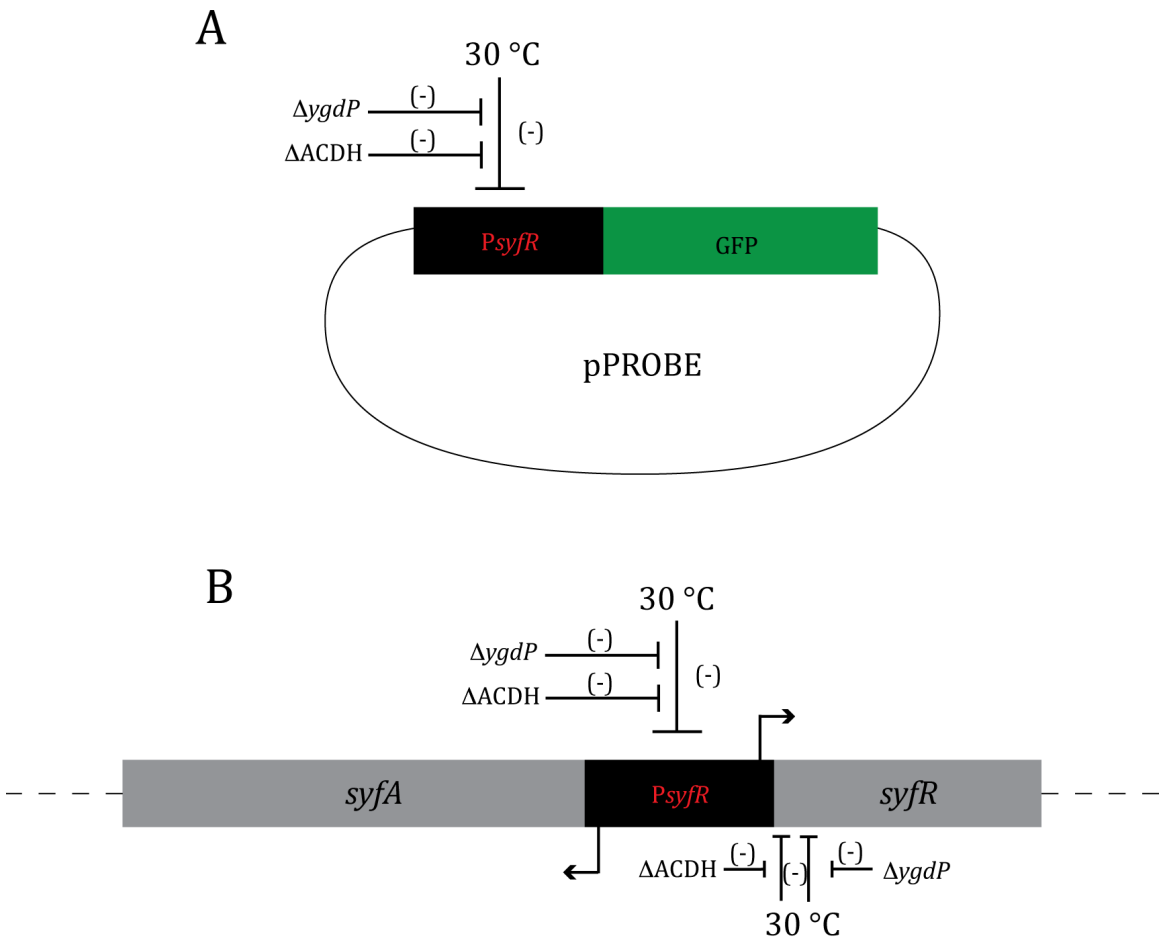

Figure 2.14. Model of thermo-regulation of *syfR in Pseudomonas syringae*. 30 °C incubation down regulates expression of *syfR*, which can be blocked in either Δ*ygdP* or ΔACDH (A). In addition to the effect of temperature directly on the intergenic region upstream of *syfR*, there are additional sites within the *syfR* coding region where incubation at 30 °C exerts a negative influence on *syfR* transcription that is contributed to by both *ygdP* and ACDH.

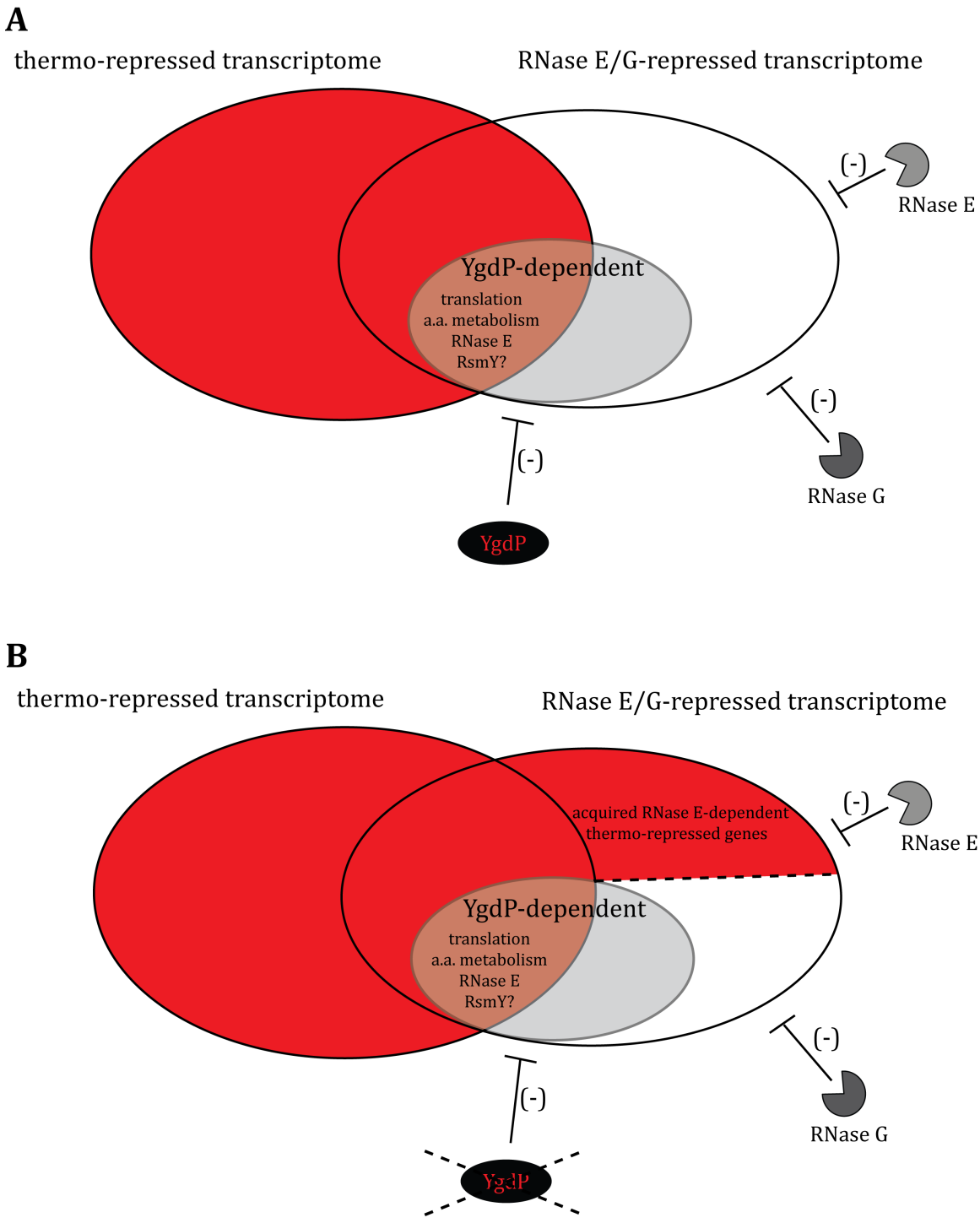

Figure 2.15. Model of the effect of YgdP on the thermo-repressed transcriptome in *Pseudomonas syringae*. In the wild type strain, YgdP mediates thermo-repression of a subset of the total thermo-repressed genes, including transcripts encoding translation-associated proteins, and proteins linked to amino acid metabolism/transport, as well as RNase E, and potentially RsmY (A). In Δ*ygdP*, thermo-repression is relieved on a subset of the thermo-repressed genes, including RNase E. As a consequence of the lack of thermo-repression of RNase E, all or only a

subset of the transcripts that are regulated by RNase E, but are not normally thermo-repressed, are suppressed at high temperatures.

Table 2.1. Genes disrupted in transposon mutants of *Pseudomonas syringae* that over-express *syfA* at 28-29 °C

locus	annotation	fluorescence
Psyr_1083	nucleoid-associated protein	+
Psyr_2474*	acyl-coa dehydrogenase	++
Psyr_2759	eukaryotic-like DNA topoisomerase I	+
Psyr_3575	phenylalanine-4-hydroxylase, monomeric form	+
Psyr_3700	protein of unknown function: DUF306	+
Psyr_4005	hypothetical	+
Psyr_4202	sodium:neurotransmitter symporter	+
Psyr_4493	PAS:GGDEF	+
Psyr_4843*	NUDIX hydrolase	++

*locus was disrupted in multiple, independent isolates

Table 2.2: Similarity of acyl-CoA dehydrogenase (psyr_2474) and linked LysR type transcriptional regulator (psyr_4843) in *Pseudomonas syringae* with homologs from diverse Pseudomonads

Strain	psyr_2474 homolog	psyr_2473 homolog
P. aeruginosa PA01	PA2550 (87/94)*	PA2551 (87/92)
P. fluorescens Pf-5	PFL_3931 (90/96)	PFL_3932 (89/93)
P. putida KT2440	PP_2437 (90/95)	PP_2436 (84/92)
P. syringae pv. phaseolicola 1448A	PSPPH_2632 (95/98)	PSPPH_2631 (95/98)
P. syringae pv. tomato DC3000	PSPTO_2745 (91/97)	PSPTO_2744 (93/97)

* (% aa identity/% aa similarity) when compared to psyr_2474 or psyr_2473.

Table 2.3. Twenty most significantly repressed genes in ΔygdP compared to a wild type strain of *Pseudomonas syringae* grown at 30 °C

locus	gene	predicted function	fold-repressed in ΔNUDIX*
Psyr_1945	*pvdL*	pyoverdine synthesis	5.0
Psyr_1958	*pvdJ*	pyoverdine synthesis	5.0
Psyr_1960	*pvdD*	pyoverdine synthesis	5.0
Psyr_2807	*ninB*	prophage encoded ORF	5.7
Psyr_2808	n/a	hypothetical prophage encoded	9.5
Psyr_2809	n/a	hypothetical prophage encoded	5.7
Psyr_2811	n/a	hypothetical prophage encoded	6.2
Psyr_4587	n/a	prophage encoded	5.0
Psyr_4588	n/a	prophage encoded	5.6
Psyr_4589	n/a	prophage encoded	5.5
Psyr_4590	n/a	prophage encoded	5.4
Psyr_4591	n/a	prophage encoded	5.3
Psyr_4592	n/a	prophage encoded	5.9
Psyr_4594	n/a	hypothetical prophage encoded	7.5
Psyr_4595	n/a	prophage encoded	5.5
Psyr_4596	n/a	hypothetical prophage encoded	6.9
Psyr_4597	n/a	hypothetical prophage encoded	7.5
Psyr_4598	n/a	prophage encoded	5.1
Psyr_4600	n/a	prophage encoded	6.0
Psyr_4605	n/a	PrtN-like transcriptional regulator	5.1

*all genes listed were significant at p-values < 0.05

Table 2.4. Twenty most significantly induced genes in Δ*ygdP* compared to a wild type strain of *Pseudomonas syringae* grown at 30 °C

locus	gene	predicted function	fold-induced in ΔNUDIX*
Psyr_0750	n/a	hypothetical	3.5
Psyr_0915	n/a	NAD-dependent epimerase/dehydratase	3.5
Psyr_0916	n/a	GDP-mannose 4,6-dehydratase	4.2
Psyr_3758	*betX*	betaine import	4.3
Psyr_3909	*gltJ*	glutamate/aspartate transport	3.8
Psyr_3999	n/a	general substrate transporter	5.5
Psyr_4175	n/a	transport associated	10.7
Psyr_4712	*glyA*-1	serine hydroxymethyltransferase	8.3
Psyr_4713	*soxB*-1	sarcosine oxidase, beta subunit	6.6
Psyr_4714	*soxD*-1	sarcosine oxidase, delta subunit	5.0
Psyr_4715	*soxA*-1	sarcosine oxidase, alpha subunit	7.2
Psyr_4716	*soxG*-1	sarcosine oxidase, gamma subunit	7.4
Psyr_4775	*gbcB*	glycine betaine catabolism	3.3
Psyr_4776	*gbcA*	glycine betaine catabolism	8.7
Psyr_4779	n/a	electron transfer flavoprotein, beta subunit	3.3
Psyr_4780	n/a	electron transfer flavoprotein, alpha subunit	5.5
Psyr_4781	*dgcB*	dimethylglycine catabolism	3.7
Psyr_4782	*dgcA*	dimethylglycine catabolism	9.1
Psyr_4783	n/a	hypothetical	9.7
Psyr_4784	n/a	Peptidase M19, renal dipeptidase	11.0

*all genes listed were significant at p-values < 0.05

Table 2.5. Twenty most significantly repressed genes in Δ*ygdP* compared to a wild type strain of *Pseudomonas syringae* grown at 20 °C

locus	gene	predicted function	fold-induced in ΔNUDIX*
Psyr_2782	n/a	hypothetical prophage encoded	5.3
Psyr_2808	n/a	hypothetical prophage encoded	6.3
Psyr_2818	n/a	hypothetical prophage encoded	5.9
Psyr_2819	n/a	hypothetical prophage encoded	6.8
Psyr_2820	*recT*	recombinase prophage encoded	5.5
Psyr_2831	n/a	hypothetical prophage encoded	5.3
Psyr_2845	n/a	hypothetical prophage encoded	5.3
Psyr_4582	n/a	hypothetical	5.2
Psyr_4583	n/a	glycoside hydrolase protein	5.5
Psyr_4587	n/a	baseplate J-like protein	5.6
Psyr_4589	n/a	phage baseplate assembly protein V	5.4
Psyr_4591	n/a	DNA circulation prophage encoded	5.4
Psyr_4592	n/a	phage tail tape measure protein TP901	5.1
Psyr_4595	n/a	bacteriophage Mu tail sheath	5.3
Psyr_4597	n/a	hypothetical	5.1
Psyr_4599	n/a	hypothetical	5.4
Psyr_4600	n/a	glycoside hydrolase, family 5	6.1
Psyr_4605	n/a	PrtN-like transcriptional regulator	6.5
Psyr_5013	n/a	L-carnitine dehydratase	5.5
Psyr_5014	n/a	acyl-CoA dehydrogenase	6.0

*all genes listed were significant at p-values < 0.05

Table 2.6. Twenty most significantly induced genes in Δ*ygdP* compared to a wild type strain of *Pseudomonas syringae* grown at 20 °C

locus	gene	predicted function	fold-induced in ΔNUDIX*
Psyr_0287	n/a	sulfate transporter	2.8
Psyr_0288	n/a	carbonate dehydratase	3.1
Psyr_0487	*gshB*	glutathione synthase	3.0
Psyr_0750	n/a	hypothetical	2.1
Psyr_0915	n/a	NAD-dependent epimerase/dehydratase	2.6
Psyr_0916	gmd	GDP-mannose 4,6-dehydratase	2.8
Psyr_1124	n/a	hypothetical	2.6
Psyr_1704	*slyC*	syringolin A synthesis	2.2
Psyr_1914	*talB*	transaldolase B	2.2
Psyr_2973	*gloA*	glyoxalase I	2.4
Psyr_3770	n/a	peptidase	3.4
Psyr_3870	n/a	SecC motif-containing protein	2.2
Psyr_4175	n/a	transport-associated protein	3.9
Psyr_4232	n/a	hypothetical	2.4
Psyr_4314	n/a	beta-ketoacyl synthase	2.2
Psyr_4315	n/a	asparganine synthase	2.9
Psyr_4316	n/a	hypothetical	3.3
Psyr_4317	n/a	similar to probable taurine catabolism dioxygenase	3.0
Psyr_4466	trx-1	thioredoxin	2.3
Psyr_4813	n/a	sorbitol dehydrogenase	2.4

*all genes listed were significant at p-values < 0.05

Table 2.7. Transcriptional regulators significantly induced in WT at 30 °C.

Locus	Gene	WT (30 °C/20 °C)	NUDIX (30 °C/20 °C)	30 °C (NUDIX/WT)	20 °C (NUDIX/WT)
Psyr_0121	tetR like	**1.9**	**1.9**	1.1	1.1
Psyr_0230	gntR like	**1.6**	1.2	0.7	0.9
Psyr_0299	gntR like	**2.6**	**1.8**	0.6	0.9
Psyr_0421	lysR like	**2.1**	1.1	0.6	1.2
Psyr_0767	lysR like	**1.6**	1.3	0.7	0.9
Psyr_1172	asnC like	**2.0**	1.2	0.6	1.1
Psyr_1435	lysR like	**2.6**	**1.7**	0.6	0.9
Psyr_1437	lysR like	**2.3**	**1.7**	0.7	0.9
Psyr_1558	lysR like	**1.7**	1.5	0.8	0.9
Psyr_1940	luxR like	**2.0**	1.6	**0.6**	0.7
Psyr_1993	araC like	**1.6**	1.5	0.8	0.9
Psyr_2032	*TCS RR	**2.1**	1.3	0.6	0.9
Psyr_2084	gntR like	**1.7**	1.3	1.2	1.6
Psyr_2121	lysR like	**2.0**	1.5	0.6	0.9
Psyr_2131	tetR like	**1.7**	1.1	0.7	1.0
Psyr_2192	tetR like	**1.7**	1.1	0.6	0.9
Psyr_2219	lysR like	**2.2**	1.5	0.6	0.9
Psyr_2268	lysR like	**3.1**	1.6	0.6	1.2
Psyr_2284	nfxB regulator	**2.1**	1.6	0.6	0.8
Psyr_2336	IclR	**1.9**	1.4	0.7	0.9
Psyr_2378	tetR like	**2.1**	1.5	0.7	0.9
Psyr_2397	lysR like	**1.8**	1.4	**0.5**	0.7
Psyr_2441	araC like	**1.9**	**1.8**	1.1	1.1
Psyr_2480	lysR like	**2.1**	1.3	0.7	1.1
Psyr_2675	gntR like	**2.4**	1.4	**0.5**	0.9
Psyr_2713	lysR like	**1.8**	1.7	0.7	0.8
Psyr_2723	lysR like	**2.1**	**1.8**	0.7	0.8
Psyr_2740	ftrA	**2.0**	1.3	0.6	1.0
Psyr_2754	tetR like	**2.1**	**2.1**	1.0	1.0
Psyr_2882	araC like	**1.6**	1.5	0.8	0.9
Psyr_2910	gntR like	**3.4**	**2.2**	**0.6**	0.9
Psyr_2920	gntR like	**4.4**	**3.2**	**0.5**	0.7
Psyr_2927	tetR like	**1.6**	1.4	0.9	1.0
Psyr_2929	lysR like	**1.9**	1.4	0.7	0.9
Psyr_2933	tetR like	**2.2**	1.5	0.7	1.1
Psyr_2945	lysR like	**1.7**	1.3	0.7	0.9
Psyr_3004	lysR like	**2.2**	1.6	0.6	0.8
Psyr_3022	asnC like	**1.7**	1.2	0.7	1.0
Psyr_3051	lysR like	**2.1**	**1.9**	0.8	0.8
Psyr_3057	deoR	**2.1**	1.7	0.7	0.9
Psyr_3073	lysR like	**2.0**	**2.4**	0.7	0.6

Psyr_3084	*TCS RR	**1.7**	1.3	0.8	1.0
Psyr_3091	*TCS RR	**1.8**	1.1	0.6	1.0
Psyr_3118	gntR like	**2.6**	1.4	0.6	1.2

Table 2.7 continued. Transcriptional regulators significantly induced in WT at 30 °C.

Psyr_3135	gntR like	**2.0**	1.2	0.6	1.0
Psyr_3156	lysR like	**2.5**	**2.1**	0.8	0.9
Psyr_3188	lysR like	**1.7**	1.5	0.8	0.8
Psyr_3299	luxR like	**2.1**	**1.7**	0.7	0.9
Psyr_3312	lysR like	**1.7**	1.3	0.7	0.8
Psyr_3336	lacI like	**1.6**	1.3	0.7	0.9
Psyr_3357	lysR like	**2.1**	1.1	0.6	1.1
Psyr_3377	marR like	**1.8**	1.5	0.7	0.8
Psyr_3526	lysR like	**2.0**	**1.9**	1.0	1.1
Psyr_3530	gntR like	**2.2**	1.4	0.7	1.1
Psyr_3607	lysR like	**2.0**	**1.8**	0.9	0.9
Psyr_3624	lysR like	**1.6**	1.4	0.7	0.9
Psyr_3634	tetR like	**1.9**	**1.8**	0.9	1.0
Psyr_3793	*TCS RR	**1.8**	1.0	**0.6**	1.0
Psyr_3890	luxR like	**1.7**	**1.7**	0.8	0.9
Psyr_3926	padR like	**1.9**	1.5	0.9	1.2
Psyr_4266	luxR like	**1.9**	**1.8**	0.9	1.0
Psyr_4325	gntR like	**1.6**	1.3	0.7	0.8
Psyr_4376	luxR like	**1.7**	**1.7**	0.9	1.0
Psyr_4463	nrdR like	**1.9**	1.3	0.8	1.1
Psyr_4490	deoR	**1.7**	1.2	0.7	1.0
Psyr_4757	tetR like	**1.7**	1.4	0.7	0.9
Psyr_5050	lysR like	**1.7**	1.2	0.6	0.9
Psyr_5056	gntR like	**2.3**	**1.9**	0.6	0.7
Psyr_5057	negative transcriptional regulator	**2.2**	**2.3**	1.0	1.0

Bold values are significant at a p-value ≤ 0.05.

*Two component system response regulator possessing a DNA-binding domain.

Table 2.8. Thermo-repressed genes in WT that are over expressed in ΔNUDIX at 30 °C

Category	Gene	WT (30 °C/20°C)	ΔNUDIX (30 °C/20 °C)	30 °C (ΔNUDIX/WT)	20 °C (ΔNUDIX/WT)
Translation					
Psyr_4525	*rpsD*	**0.5**	1.2	**2.6**	1.1
Psyr_4526	*rpsK*	**0.5**	1.1	**2.4**	1.1
Psyr_4538	*rplN*	**0.5**	1.1	**2.5**	1.0
Psyr_4539	*rpsQ*	**0.5**	1.1	**2.6**	1.1
Psyr_4540	*rpmC*	**0.4**	1.1	**2.6**	1.0
Psyr_4541	*rplP*	**0.5**	1.1	**2.4**	1.0
Psyr_4542	*rpsC*	**0.5**	1.2	**2.3**	1.0
Psyr_4543	*rplV*	**0.5**	1.1	**2.2**	1.1
Psyr_4544	*rpsS*	**0.5**	1.1	**2.2**	1.1
Psyr_4545	*rplB*	**0.5**	1.1	**2.3**	1.0
Psyr_4546	*rplW*	**0.5**	1.1	**2.7**	1.1
Psyr_4547	*rplD*	**0.4**	1.1	**2.7**	1.1
Psyr_4548	*rplC*	**0.5**	1.1	**2.4**	1.0
Psyr_4550	tuf	**0.5**	1.2	**2.3**	1.0
Psyr_4551	fusA	**0.5**	1.1	**2.3**	1.0
Psyr_4552	*rpsG*	**0.6**	1.1	**2.0**	1.0
Psyr_4557	*rplJ*	**0.6**	1.0	**1.9**	1.1
Amino acid metabolism and transport					
Psyr_1072	*aapJ*	**0.6**	1.6	**1.7**	0.6
Psyr_1073	*aapQ*	**0.4**	1.5	**2.2**	0.6
Psyr_1096	*gcvP*	**0.5**	0.9	**2.0**	1.2
Psyr_1097	*gcvH*-2	**0.5**	0.9	**2.5**	1.4
Psyr_2470	*liuA*	**0.4**	**0.5**	**1.9**	1.5
Psyr_3908	*gltI*	**0.4**	0.9	**2.6**	1.4
Psyr_3909	*gltJ*	**0.3**	0.9	**3.8**	1.3
Psyr_3910	*gltK*	**0.4**	0.9	**3.0**	1.3
Psyr_3911	*gltL*	**0.5**	0.8	**1.8**	1.2
Secretion/Efflux/Export					
Psyr_5134		**0.5**	0.9	**1.9**	1.1
Transport					
Psyr_3999		**0.2**	0.9	**5.5**	1.4
Psyr_4175		**0.2**	0.6	**10.7**	**3.9**
RNA degradation					
Psyr_1638	rne	**0.4**	0.8	**2.0**	1.0
Nitrogen metabolism					
Psyr_4817	*glnA*-1	**0.3**	0.8	**2.0**	0.8

Table 2.8 continued. Thermo-repressed genes in WT that are over expressed in ΔNUDIX at 30 °C

Carbohydrate metabolism and transport					
Psyr_0944	*prsA*	**0.5**	1.1	**1.9**	0.9
Psyr_2440	*mltE*	**0.4**	**0.6**	**2.0**	1.4
Fatty acid metabolism					
Psyr_0749	*fadD*	**0.3**	**0.3**	**1.9**	**2.1**
QAC metabolism and transport					
Psyr_3238	*dhcB*	**0.5**	0.7	**1.9**	1.2
Transcription					
Psyr_4524	*rpoA*	**0.5**	1.2	**2.4**	1
Phytotoxin synthesis and transport					
Psyr_2601	*salA*	**0.5**	0.9	**3**	**1.9**
Transport (peptides)					
Psyr_4235		**0.5**	1.6	**2.7**	0.8
Psyr_4238	*dppA*-1	**0.5**	1.3	**2.1**	0.8
Hypothetical					
Psyr_0750		**0.1**	**4.1**	**3.5**	**2.1**
Psyr_5135		**0.5**	0.9	**1.9**	1.2
Unannotated					
Psyr_2239		**0.4**	1.5	**2.2**	1.4
Psyr_5136		**0.5**	0.9	**1.9**	1.2

Bold values are significant at a p-value ≤ 0.05.

Table 2.9. Gene functional categories enriched over or under expression in Δ*ygdP* at 20 °C and 30 °C

Category	Gene	Annotation	Δ*ygdP*/WT (20 °C)*	Δ*ygdP*/WT (30 °C)*
Cell Division				
Psyr_1611	*minE*	cell division topological specificity factor	1.9	2.5
Psyr_1612	*minD*	septum site-determining protein	1.9	2.7
Phage & IS Elements				
Psyr_1030	*nfrB*	bacteriophage N4 adsorption protein B	0.4	0.6
Psyr_2764	n/a	hypothetical	0.5	0.5
Psyr_2766	n/a	hypothetical	0.4	0.5
Psyr_2805	n/a	hypothetical	0.4	0.5
Psyr_2806	n/a	bacteriophage lambda NinG	0.4	0.3
Psyr_2807	n/a	NinB	0.3	0.2
Psyr_2808	n/a	hypothetical	0.2	0.1
Psyr_2809	n/a	hypothetical	0.3	0.2
Psyr_2810	n/a	hypothetical	0.3	0.3
Psyr_2811	n/a	hypothetical	0.2	0.2
Psyr_2816	n/a	hypothetical	0.2	0.2
Psyr_2817	n/a	hypothetical	0.2	0.3
Psyr_2818	n/a	hypothetical	0.2	0.3
Psyr_2819	n/a	hypothetical	0.1	0.4
Psyr_2820	*recT*	recombinase	0.2	0.3
Psyr_2821	*recE*	promotes recombination by RecT	0.3	0.4
Psyr_2822	n/a	hypothetical	0.3	0.4
Psyr_2823	n/a	hypothetical	0.3	0.5
Psyr_2828	n/a	C-5 cytosine-specific DNA methylase	0.3	0.5
Psyr_2831	n/a	hypothetical	0.2	0.5
Psyr_2832	n/a	phage integrase	0.3	0.4
Psyr_2845	n/a	hypothetical	0.2	0.5
Psyr_2846	n/a	phage integrase	0.3	0.5
Psyr_4586	n/a	hypothetical	0.2	0.2
Psyr_4587	n/a	baseplate J-like protien	0.2	0.2
Psyr_4588	n/a	phage GP46	0.2	0.2
Psyr_4589	n/a	phage baseplate assembly protein V	0.2	0.2
Psyr_4590	n/a	bacteriophage Mu P	0.2	0.2
Psyr_4591	n/a	DNA circulation	0.2	0.2
Psyr_4592	n/a	phage tail tape measure protein TP901	0.2	0.2
Psyr_4595	n/a	bacteriophage Mu tail sheath	0.2	0.2

*all values listed are significant at a p-value ≤ 0.05

Table 2.10. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (Carlsbad, CA)
S17-1	*recA* RP4-2-Tc::Mu-Km::Tn7	[114]
SM10(λpir)	*E. coli* harboring a conjugatable mini-Tn5 (Sm^R/Sp^R) vector	[184]
P. syringae strains		
P. syringae B728a	Wild type, Rf^R, NFT^R	[112]
mTn5::ACDH	*P. syringae* B728a haroboring a mTn5 insertion in ACDH (psyr_2474), Sp^R	This work
mTn5::*ygdP*	*P. syringae* B728a harboring a mTn5 insertion in *ygdP* (psyr_4843), Sp^R	This work
ΔACDH	*P. syringae* B728a harboring a markerless ACDH deletion	This work
Δ*ygdP*	*P. syringae* B728a harboring a markerless *ygdP* deletion	This work
ΔACDH/Δ*ygdP*	Δ*ygdP* harboring a markerless ACDH deletion	This Work
Plasmids		
pFLP2Ω	bhr vector expressing the Flp-recombinase, Sp^R	[116]
pTOK2T	Mobilizable, suicide vector, Tc^R	[70]
p$P_{fliC\text{-}gfp}$	pPROBE-GT harboring the *fliC* promoter, Gm^R	[116]
p$P_{syfA\text{-}gfp}$	pPROBE-OT harboring the *syfA* promoter, Sp^R	[64]
p$P_{syfA\text{-}gfp(K)}$	pPROBE-gfp[tagless] harboring the syfA promoter from p$P_{syfA\text{-}gfp}$, Km^R	This work
pACDH-KO	pTOK2T harboring an ACDH deletion cassette, Km^R, Tc^R	This work
p*ygdP*-KO	pTOK2T harboring a *ygdP* deletion cassette, Km^R, Tc^R	This work
pKD4	Source of kan-FRT fragment for p*ygdP*-KO	[121]
pKD13	Source of kan-FRT fragment for pACDH-KO	[121]
pVSP61	Stable replication vector, Km^R	[189]
Ncomp	pVSP61 harboring a *ygdP* (NUDIX) complementing fragment	This work
Acomp	pVSP61 harboring an ACDH complementing fragment	This work

[a] Rf, rifampicin; Gm, gentimicin; Sp, spectinomycin; Km, kanamycin; Tc, tetracycline; NFT, nitrofurantoin

Table 2.11. Primers used in cloning and qRT-PCR

Primers	
PSYR_4843_5'-native_comp_S	5'-ATCGGAATTCAGGGTTACTCGCTGAAATAG-3'
PSYR_4843_3'-native_comp_AS	5'-ATCGGAATTCTCAGTCGCGCGATAAG-3'
PSYR_4843_5'-KO_S	5'-GCAGCAGCGTGCTTTAC-3'
PSYR_4843_5'-KO_AS[a]	5'-GAAGCAGCTCCAGCCTACACACACGGCAACAACCTC-3'
PSYR_4843_3'-KO_S[a]	5'-TAAGGAGGATATTCATATGCTCAATACGCTGCG-3'
PSYR_2474_native_comp_S	5'-ATCGGAATTCTGATGGGGCGCC-3'
PSYR_2474_native_comp_AS	5'-ATCGGAATTCCTAGGACCTGCTGCGC-3'
PSYR_2474_KO_5'-S	5'-TGGGTCTCGACCGAG-3'
PSYR_2474_KO_5'-AS[a]	5'-GAAGCAGCTCCAGCCTACACACATGGGGTCGCCAC-3'
PSYR_2474_KO_3'-S[a]	5'-GGTCGACGGATCCCCGGAATTAGCTAGCGGTCGATCAC-3'
pKD4_site_2_AS	5'-CATATGAATATCCTCCTTAGTTCCTATTCCG-3'
pKD4/13-site-1-S	5'-GTGTAGGCTGGAGCTGCTTC-3'
pKD13-site-4-AS	5'-ATTCCGGGGATCCGTCGACC
PSYR_2474_KO_3'-AS	5'-AGGAGGCCAAAGATCTG-3'
qRT-PCR primers	
rpoD-RT-S	5'-ACGCGCCATCATGCAGCTGTG-3'
rpoD-RT-AS	5'-GCCAGTGCGTCAGTCCAGCTTTC-3'
3981-RT-S	5'-CAGCGGACGGGTGCAGGTAGAC-3'
3981-RT-AS	5'-CGTCGTTGTCAGGCTCGTCCAG-3'
syfA-RT-S	5'-CGGCCCGACTGAAACCACTGTG-3'
syfA-RT-AS	5'-CCCCGCACCACCGATGTACAAC-3'
syfR-RT-S	5'-GCGCGGGCCTGAAGCATGAC-3'
syfR-RT-AS	5'-CCGCCACGCAGTCAGCCGATAA-3'

[a] Sequence complementary to kan-FRT from pKD4 (Psyr_4843) or pKD13 (Psyr_2474)

Chapter Three

FleN contributes to heterogeneous swimming capabilities at high temperatures in Pseudomonas syringae

3.1 Introduction

Regulation of the bacterial flagellum is a complex process that is achieved by a hierarchy of distinct, interacting regulators [195, 196]. The complexity of regulation is reflective of the elaborate nature of the organelle itself, which requires proper temporal expression and spatial organization of the component proteins to produce a functional flagellum. In addition to the core components of flagellar regulation, certain species, such as Pseudomonads, employ additional levels of regulation that ensure the proper localization of the flagellum, as well as restrict the number of flagella produced [197]. Proper localization and quantitative regulation in polarly-flagellated bacteria appears to be achieved through the interaction of two genes, *flhF* and *fleN/flhG* that are absent in peritrichously flagellated bacteria having multiple flagella distributed over the cell body [198-200].

In addition to the internal molecular complexity of regulation, flagellar synthesis is also responsive to diverse environmental factors, most of which affect expression of the master flagellar regulators, *flhDC* or *fleQ,* depending on the organism [88, 195]. In a few cases, such environmental regulation affects check-points downstream of the master regulator [89, 93]. The fact that a multitude of environmental factors including temperature, carbon source, growth phase, pH, and osmolarity regulate flagellum synthesis (reviewed in [88]), is likely a reflection of the substantial cellular resources that cells must dedicate to their production [57]; bacteria would be expected to limit flagella synthesis only to environmental conditions where motility would be beneficial. Additionally, there is evidence that suggests that synthesis of the flagellum may itself be a risky endeavor, leading to cell death in a fraction of expressing cells, regardless of the environment [201].

Pseudomonas syringae, as a colonist of leaf surfaces, is exposed to highly variable environmental conditions, which fluctuate both regularly (in a diurnal pattern) as well as irregularly in response to prevailing weather conditions [1, 8]. In addition, the resources that *P. syringae* exploits on a leaf are highly spatially variable [13], and the motility of *P. syringae* has thus been found to be an important fitness factor, presumably by enabling the cells to explore the leaf surface in pursuit of such resources and to avoid stressful sites [24]. As such, it would be expected that *P. syringae* is adapted to integrate multiple, sometimes conflicting, environmental signals in a manner to express motility in a way that would maximize its survival and proliferation in this habitat. One such strategy may include the formation of phenotypically distinct sub-populations, some of which are pre-acclimated to respond to sudden and unpredictable environmental fluctuations, enabling a so-called “bet-hedging” life strategy [202]. Such strategies have been documented, and

theoretical as well as empirical work has documented their benefit to cells [203-205].

In this work we describe a novel heterogeneous swimming phenotype exhibited a subset of cells of *P. syringae* incubated at warm temperatures (30 °C), temperatures that are inhibitory to expression of numerous flagellar genes, as well as for normal swimming motility and show that this is due to a transient variation in gene expression within the population of cells. Examination of stable spontaneous mutants that gained the ability to swim at elevated temperatures revealed a role for the polar flagellum-specific regulator FleN, and a model is presented for the apparently novel role of this regulator in modulating cell motility at high growth temperatures.

3.2 Results

P. syringae displays a constellation swimming phenotype when incubated at 30 °C

Previously we showed that *P. syringae* incubated at 30 °C exhibited dramatically less swimming when compared to cooler incubation temperatures. We note, however, that while cells grown at warm temperatures have a much reduced swimming area, those cells that did spread beyond the inoculation site exhibited a qualitative change in their swimming phenotype, whereby they were not uniformly dispersed, but instead formed punctate micro-colonies at different distances from the inoculation stab (Fig. 1). We refer to this phenotype as constellation swimming. Wild type *P. syringae* cells readily formed spatially separated micro-colonies, while a Δ*flgK* mutant did not move at all, indicating that the phenotype required a functioning flagellum and thus reflected active motility (Fig. 2A and B). We tested whether this phenotype could be propagated as well as reversed if the micro-colonies were transferred to fresh media. When isolated micro-colonies were transferred to fresh swimming media, the constellation phenotype was reproduced when the plates were incubated at 30 °C, while they exhibited the normal swimming phenotype when the plates were incubated at 20 °C (Fig. 2C and D). These experiments confirm that the constellation phenotype is likely a genetically-encoded phenotype of the population as a whole and not mis-regulated swimming displayed by a few stable mutants.

We considered that this phenotype might be due to the presence of a sub-population of cells with gene expression patterns that would enable swimming motility that were present in the inoculum, and that prolonged incubation at 30 °C might subsequently suppress motility of all of the cells; such a situation could have allowed a few cells to transiently move beyond the inoculation site until which time as motility was subsequently suppressed at the high temperature, leading them to form colonies at the sites to which they had dispersed. We found, however, that the swimming zone at 30 °C continued to expand with additional incubation time and that the constellation phenotype was maintained; the zone always consisted of many punctate, spatially isolated colonies at the periphery of the swimming colony

(Fig 1 and Fig 7 top panel). This observation seemed inconsistent with a transiently pre-induced sub-population present only at the beginning of the experiment. These observations all suggest that transiently motile cells continually formed in *P. syringae* populations incubated at high temperatures.

A sub-population of cells incubated at 30 °C express *fliC* at levels similar to cells incubated at 20 °C

The nature of the constellation swimming phenotype and the observation that it required functional flagella indicated that there might be heterogeneity in swimming ability within the population of cells. We hypothesized that such behavioral heterogeneity would be manifest at the level of flagellin production, and hence expression of *fliC*. Quantitative single cell microscopy confirmed that after 24 hours of incubation at 30 °C, a subpopulation cells expressed *fliC* at levels similar to those incubated at 20 °C while most cells exhibited very low expression (Fig. 3). We confirmed this result using flow cytometry (Fig. 4). At 30 °C there was a subpopulation of cells that were as fluorescent as the population of cells that were incubated at 20 °C. In this experiment, however, the *fliC*-expressing sub-population was only apparent in cells incubated for 24 hours but not for 12 hours, suggesting that the hyper-expression was dependent on a growth state of cells seen only in older cultures, perhaps in response to low nutrient conditions that would occur only following extensive replication at the point of inoculation. This result also seemed to support the conclusion made above, that the constellation swimming phenotype is not the result of a *fliC*-expressing, motile subpopulation always present in a collection of *P. syringae* cells, but instead, the result of phenotypic heterogeneity that arises only following prolonged incubation at warm temperatures.

***P. syringae* exhibits normal swimming on media with reduced peptone**

Since growth phase appeared to contribute to the appearance of a *fliC*-expressing sub-population of cells at 30 °C, we tested whether reducing the peptone content of the swimming medium could stimulate motility more uniformly within the population of cells. A 50-fold reduction in peptone concentration resulted in a slow but diffuse swimming phenotype at 30 °C (Fig. 5). Taken together, these results suggest that a low proportion of the population of cells attempts to move at relatively high nutrient concentrations, presumably through expression of the flagellum, while that motility is greatly suppressed at high temperatures.

Isolation of Spontaneous Hot Swimming (SHS) mutants following prolonged incubation at 30 °C

To better understand the process by which cells might transiently become motile at high temperatures we explored the characteristics of stable, hyper-motile mutants that also could be observed after several days of incubation at 30 °C. Such mutants were identified as flares that phenotypically resembled the normal swimming phenotype exhibited by *P. syringae* at 20 °C (data not shown). We thought it likely that these flares consisted of spontaneous mutants that were no longer thermo-repressed in motility. Single cells isolated from the flares that were re-inoculated into swimming media at 30 °C no longer exhibited the distinctive constellation

swimming phenotype of the wild type strain, but exhibited the normal diffuse swimming phenotype. All of the putative mutants isolated moved much faster in swimming medium at 30 °C than the wild type strain, but most moved somewhat slower than the wild type strain when incubated at 20 °C. Strains recovered in this way were termed Spontaneous Hot Swimming (SHS) mutants. There appeared to be two general phenotypes of the SHS mutants recovered: mutants such as SHS3 that exhibited normal swimming at both 20 °C and 30 °C (Fig. 1A) and mutants such as SHS1 that exhibited a swimming defect at 20 °C but exhibited normal (non-constellation) swimming at 30 °C (Fig. 6). Isolation of SHS mutants in a wild type strain harboring a fusion of a *gfp* reporter gene with the promoter of *fliC* ($P_{fliC\text{-}gfp}$) on swimming plates at 30 °C revealed that the flares having a normal swimming movement away from the colony exhibited a relatively high expression of *fliC* compared to the wild type strain in the body of the colony (Fig. 7 top panel). A total of 17 putative mutants that exhibited a shift from a constellation to an enhanced, diffuse swimming phenotype at 30 °C were obtained in 3 separate experiments (Table 1).

Lack of correlation between SHS phenotype and increased expression of *fliC* at 30 °C

Since it appeared that the SHS phenotype was associated with increased expression of *fliC* at 30 °C we performed a more quantitative examination of the expression of this gene in the mutants since such a relationship seemed logical given the correlation between *fliC* expression and swimming motility seen earlier (Chapter 1). We thus introduced the $P_{fliC\text{-}gfp}$ vector into several of the SHS mutant backgrounds and measured the fluorescence of cells that had been grown at 20 °C and at 30 °C. Most of the strains that exhibited normal swimming at 30 °C did not significantly over-express *fliC* at 30 °C compared to the wild type strain (Fig. 8). While the average GFP fluorescence of the cell population of the SHS mutants usually differed little from that of the wild type strain, the proportion of individual cells having relatively high fluorescence, and hence *fliC* expression when grown at 30 °C was much higher in the SHS mutants than the wild type strain. The higher proportion of apparently motile cells in populations of SHS mutants than that in the wild type strain at 30 °C apparently accounts for the non-constellation colony phenotype (normal swimming motility) seen in SHS mutants at high growth temperatures (data not shown). In addition to demonstrating that a higher proportion of cells in mutants exhibiting apparently normal swimming at 30 °C do not necessarily all over-express *fliC* at this temperature, it was clear that mutants with similar SHS swimming phenotypes (Table 1) do not necessarily exhibit the same pattern of expression of *fliC*. For example, while SHS4, SHS12, and SHS15 all exhibited similar swimming phenotypes, SHS4 clearly had a higher expression of *fliC* than the others. This result suggested that there may be a diversity of mutations that yield a SHS phenotype.

SHS mutants harbor non-synonymous mutations in *fleN*, or in the promoter region of *fleQ*

The genetic lesions in 17 putative SHS mutants were identified by comparing the genome sequence of these mutants, determined by high-throughput Illumina sequencing, with that of the parental strain and with each other. Because spontaneous mutants often harbor single nucleotide polymorphisms (SNPs), with deletions and insertions being less common, we focused on identification of SNPs in the SHS mutants [206, 207]. In comparison to the parental strain, the majority of the well-supported SNPs in SHS mutants that were not present in every strain (see below) were in a region encoding *fleN* (psyr_3438). Five unique SNPs found in a total of 11 SHS mutants all resulted in a predicted amino acid change in FleN (Table 2, Fig. 6A). FleN (pseudonym of FlhG in non-Pseudomonads) interacts with the flagellar regulators FleQ and with FlhF, which is encoded by the gene immediately upstream of *fleN* (Fig. 10), to regulate the number of flagella produced by cells (see discussion). As we had selected for mutants that exhibited higher motility at high growth temperatures, likely a consequence of altered thermo-regulation of flagellar synthesis, the abundance of mutations in this gene appeared to confirm our approach to studying heterogeneity in motility at high temperatures. Further analysis of the *fleN* mutants revealed that certain identical SNPs arose repeatedly, such as the A to C mutation harbored by SHS1, 2, 5, 14, and 17, that resulted in a substitution of valine for alanine at position 175. While SHS1, 2, and 5 are likely siblings of the same mutation event because they were recovered from the same flare, SHS 14 and 17 were recovered from different flares on separate plates in another experiment. No other SHS mutants isolated with these experiments harbored this particular mutation, indicating that it was not due to an ancestral mutation event present in the founder cells. Likewise, since SHS15 and SHS16, which were isolated from different flares from the same plate, harbored identical mutations they too appear to be a result of independent events.

All five of the unique mutations recovered in *fleN* resulted in a substitution of one aliphatic amino acid for another (A139V, V175A), or a substitution of a non-aliphatic for an aliphatic amino acid (I123S, V84G, V217E). A consequence of these changes is a shift in the hydrophobicity of FleN, potentially determining whether the residue is buried within the protein or exposed on the surface. Four of the five mutations resulted in reduced hydrophobicity of the amino acid at a site, such as when alanine, glutamic acid, serine, or glycine replaced isoleucine or valine. SHS11 harbored the only mutation that resulted in an increase in residue hydrophobicity. We compared the conservation of these five amino acids across putative *fleN* orthologs in strains where it or its interaction partner *flhF* have been studied (Fig. 9B). While none of the particular amino acids were absolutely conserved across all strains examined, each residue position always contained a hydrophobic residue, usually isoleucine, valine, or alanine, but occasionally methionine. A broader analysis performed on the 100 proteins most similar to FleN by Blast analysis, (resulting in an E-value cutoff of -108), revealed that the four highly hydrophobic residue positions (V84, I123, V175, and V217) were highly conserved across all putative *fleN* homologs, and consisted largely of isoleucine or valine residues, but occationally methionine, leucine, and phenylalanine (Table 3). The 139A residue position of FleN was absolutely conserved in this analysis. It was noteworthy that the V175A mutation was seen in all five of SHS mutants having the defective cool

swimming phenotype (Table 1 and Table 2) while none of the mutants not harboring this mutation exhibited the cool swimming defect.

SHS6 and SHS10 harbored neighboring SNPs in the putative promoter region of *fleQ*, the master flagellar regulator (Table 2). Comparison of the *P. syringae fleQ* promoter region with motifs described in its homolog in *P. aeruginosa* [2], revealed that neither mutation occurred within the Vfr binding site (Fig. 11). Using BPROM, an on-line promoter prediction program, we found that both mutations occurred within the putative -10 RpoD/σ70 binding site. The SHS10 mutation altered the predicted -10 site, while the SHS6 mutation did not (*P. syringae* and SHS10 -10 sites in Fig. 11). We also analyzed the promoter region for putative transcription factor binding sites that overlapped with the SNPs using Virtual Footprint [208] (Table 4). Both of these mutations lead to changes in predicted transcription factor binding. However, as the possible role of these transcription factors are only inferred based on their function in *E. coli* and *P. aeruginosa*, their significance in *P. syringae* remains speculative.

SHS7 and SHS8 appeared to be siblings as they were isolated from the same flare and possessed the same two unique SNPs when compared to the parental strain and the other SHS mutants. Both mutants possessed a C to T transition resulting in synonymous codons in psyr_3216. In addition, both strains shared a T to A transversion 12 bp up-stream from the start codon of psyr_3298 in a region between psyr_3297 and psyr_3298. Psyr_3297 encodes a CheW-like protein, predicted to be involved in chemotaxis, while psyr_3298 encodes a hypothetical protein. Neither gene is predicted to be in an operon with their flanking genes.

The sequence analysis of two SHS mutants was not informative; SHS12 harbored a lesion in a gene encoding a hypothetical protein while SHS13 did not contain any well-supported, unique SNPs.

There were 11 SNPs that differed from the sequence of the parental strain that were conserved in all SHS strains (Table 5). Because the identity of these SNPs were conserved across all strains, it seems likely that they accumulated within this particular B728a lineage prior to the mutations that gave rise to the SHS phenotype.

Modeling FleN mutations

To gain insight into the common effect of the five unique SHS mutations, we compared models of the structures of the wild type and mutant FleN. We attempted a template-based homology modeling approach, however, no significant matches were found. Subsequently we used a remote homology-based approach using the Phyre2 pipeline [209]. Greater than 90 % of the residues queried, including all SHS mutations, were modeled with greater than 90 % confidence. This modeling predicted that V84 is located within a coil, I123 and V175 within β-sheets, and A139 and V217 within α-helices (Fig. 9). None of the mutations, however, altered any of these secondary structure predictions. Additionally, none of the mutations were predicted to disrupt the ability of FleN to bind ATP (data not shown). Interestingly, the FleN allele possessing the V175A mutation (cold swimming defect) was predicted to possess a single region of trans-membrane domain-encompassing

residues 138 to 153; none of the other mutant alleles or the native protein was predicted to possess any trans-membrane domains.

3.3 Discussion

It is highly significant that FleN was identified here as playing a role in modulating temperature-dependent motility in *P. syringae* as it is central to regulation of motility in other bacterial species. *fleN* (*P. aeruginosa* homolog of *flhG* in *Vibrio* and *Campylobacter* spp.) contributes to several aspects of flagellar regulation. In *P. aeruginosa*, FleN interacts directly with FleQ, the master flagellar regulator in all *Pseudomonas* species thereby inhibiting its ability to stimulate transcription of class II, III, and IV flagellar genes, but it does not affect the expression of *fleQ* or *fliA* [68, 210]. In *Vibrio cholera*, FlhG negatively regulates the expression of class II, III, and IV flagellar genes, similar to that in *P. aeruginosa*, but it also negatively regulates the expression of *flrA* (*fleQ* homolog in *Vibrio* spp.) [211]. Assuming that FleN in *P. syringae* has a similar effect on flagellar gene regulation as its homolog in *P. aeruginosa* and that modulation of FleN abundance or interaction potential is the determining factor for thermo-regulated flagellar gene expression, the previous observations that neither *fleQ* nor *fliA* expression was influenced by incubation temperature (Chapter 1) would be corroborated.

Unfortunately, there is yet no comprehensive understanding of the residues or domains contributing to the interaction of FleN and FleQ. This interaction appears to involve elements in both the N- and C-terminus of FleN, as all truncations tested (including separate C-terminal and N-terminal truncations) resulted in a loss of this interaction [210]. ATP binding also appears to be necessary for FleN to interact with FleQ, as a change in the residue predicted to disrupt the ATP binding domain abolished their interaction [210]. None of the spontaneous *fleN* mutations of SHS mutants occurred in the putative ATP binding domain, suggesting that the ATP binding capacity of FleN in these mutants was unaltered. Additionally, all FleN variants were predicted to retain the ability to bind ATP as assessed using 3DLigandSite [212]. The V175A mutation resulted in the most significant alteration in FleN structure in that this variant was predicted to have gained a trans-membrane domain. Clearly, this prediction will require experimental verification. The lack of predicted changes in FleN structure is perhaps expected given the limited knowledge of the 3D conformation of FleN homologs. While remote-homology based modeling approaches are fairly successful at predicting core protein structure, they are limited in their ability to predict fine structure of most proteins [209].

In addition to its interaction with FleQ, FleN also physically interacts with another flagellar regulator, FlhF [198]. FlhF is a signal-recognition particle-type GTPase that, along with FleN, is found in polarly flagellated bacteria where it is involved in localization of the flagellum, but not in peritrichously flagellated bacteria such as *E. coli* or *Salmonella* spp. [197, 198, 200, 213-215]. FlhF and FleN reciprocally regulate the number of flagella produced by cells in numerous species.

Deletion of FlhF or over-expression of FleN leads to loss of the flagellar filament, while over-expression of FlhF or deletion of FleN leads to the production of multiple polar flagellar filaments [198, 211, 215-218]. Multi-flagellation is associated with reduced swimming ability, an effect that was clearly observed in SHS1, 2, 5, 14, and 17 at 20 °C. Presumably, the multiple flagellar filaments form bundles that cannot function as well as single filaments. In general, those SHS mutants that did not exhibit a swimming defect at cool temperatures exhibited stronger swimming at 20 °C than at 30 °C. This indicates that while these mutants are capable of normal swimming at 30 °C (as opposed to constellation swimming), they are somewhat defective in flagellum formation, or some other aspect of swimming at 30 °C.

Whether temperature directly influences the structure of FleN is currently unknown. Based on our finding of SNPs primarily in *fleN* in the SHS mutants, we hypothesize that either the overall structure of FleN, or that of a particular FleN domain, is temperature sensitive. While we hypothesize that FleN is the component governing thermal suppression of motility in *P. syringae*, implying that FleN directly senses and responds to temperature, the alternative hypothesis can be formulated that FleN expression itself is thermo-regulated, being more abundant at 30 °C than at 20 °C. If FleN is over-expressed at 30 °C, this is apparently not due to its elevated transcription since similar amounts of *fleN* messages were found in cells grown at different temperatures (Chapter 1). It is possible that *fleN* is translated more efficiently at 30 °C. If FleN is indeed more abundant at 30 °C than 20 °C, then the SHS mutants with altered FleN that are not defective in swimming at 20 °C may exhibit a temperature-sensitive phenotype, resulting in selective loss of FleN activity at 30 °C, but not at 20 °C. Such temperature-sensitive mutations commonly result from single amino acid substitutions that alter the folding, and thus the function, of the protein at the non-permissive temperature [219-221]. It is possible that the V175A substitution in SHS mutants having a swimming defect at 20 °C, conferred a more pronounced change in FleN folding such that it was non-functional even at 20 °C. In yet another model, it remains possible that FleN is neither itself sensitive to temperature, nor is its expression temperature-dependent, but that the mutations in *fleN* negate the effects of an alternative and currently unknown thermo-regulator. In any event, the *fleN* mutations recovered in this work should prove valuable in further investigation of the physical interaction of FleN with flagellar regulatory components such as FleQ and FlhF, as well as the potential effect of temperature on these interactions.

While the majority of SHS mutants supported the role of FleN in thermo-regulation of motility, circumstantial evidence also suggests that other flagellar components also play a role in this process. The two SHS mutants with SNPs in the *fleQ* promoter region are intriguing based on their proximity to each other as well as their co-localization with a predicted σ^{70}-binding site. In *P. aeruginosa* a Vfr binding site overlaps the σ^{70} -10 site [2] (Fig. 11). Vfr is a homolog of CAP (also known as CRP, cAMP receptor protein) in *E. coli;* cAMP being a central mediator of catabolite repression [222]. In *E. coli*, flagellar synthesis, and thus motility, requires CAP to be bound to cAMP, and thus is catabolite repressible [223]. The expression of the *flhDC* operon is also subject to catabolite repression [224]. Certain spontaneous mutants

in *E. coli* that no longer exhibit repressed flagella synthesis at high temperatures also have other pleiotropic defects such as a lack of catabolite repression, while some other spontaneous mutants lacking such catabolite repression are also able to synthesize some flagella at elevated temperatures, indicating either that there is a functional link or common regulator between catabolite repression and thermo-regulation in flagella synthesis in *E. coli* [131, 225, 226]. The contribution of Vfr to flagellar regulation in *Pseudomonas* is poorly understood, and may differ from that in *E. coli*. Over-expression of Vfr in *P. aeruginosa* results in reduced expression of *fleQ* as well as reduced motility [2, 225]. Currently, we have two lines of circumstantial evidence suggesting that nutrient status regulates *fliC* expression and motility in *P. syringae* (Figs. 4 and 5). First, the sub-population of cells in wild type cells that highly expressed *fliC* even at 30 °C only accumulated after a visible colony had formed. Secondly, reduction of the peptone concentration in the medium (the major nutrient source) enabled a normal (diffuse) swimming colony phenotype even when incubated at 30 °C. We speculate that upon the formation of a visible colony there would have been sufficient cell growth to deplete some key nutrient(s) in the local environment that otherwise would have repressed *fliC* expression at 30 °C. Greatly reducing the peptone concentration in the media itself presumably mimics the release from nutrient repression of movement that otherwise would have occurred only near colonies. More study will be needed to better describe the putative linkage between catabolite repression and thermo-regulation of flagella synthesis and motility in *P. syringae*. It is noteworthy that *vfr* (psyr_4577) was stimulated under cool incubation conditions in *P. syringae* (Chapter 1) indicating that if it does bind to the *fleQ* promoter in *P. syringae* (which is currently unknown) then it probably does not inhibit its expression. We previously showed that neither *fleQ* nor *fliA* (the two putative class I regulators of flagellar synthesis in *P. syringae* (see Chapter 1) are thermo-regulated at the level of transcription. Based on the presence of the SNPs of two SHS mutants in the promoter region of *fleQ,* we would expect that these mutations would affect its transcription. These results suggest that, while repression of *fleQ* transcription at high temperatures cannot be the determinant of suppression of flagellar production at high temperatures, over-expression of *fleQ* may be sufficient to mask down-stream thermo-regulation (Fig. 16), potentially by over-expressing *flhF* compared to *fleN*.

Figures 13-17 present models on how we currently view thermoregulation of flagella in *P. syringae*, how constellation swimming might occur, as well as how the *fleN* and *fleQ* SHS mutants might circumvent thermoregulation. At 20 °C (Fig. 13), FleN is present in a conformation that limits its ability to bind to and inhibit either FleQ or FlhF or both, and FleQ and FlhF are thus present largely as free proteins able to direct regulation and localization of flagellar components. Completion of the hook and basal-body (HBB) complex (encoded by class II and III flagellar genes) allows the export of FlgM, freeing FliA to direct transcription of class IV flagellar genes, including *fliC*. At 30 °C (Fig. 14), FleN is present in a conformation that increases its ability to associate with, and inhibit, either FleQ or FlhF or both. This reduces the amount of free FleQ and FlhF in the cell to levels that are insufficient to either drive expression of class II and III flagellar genes or direct their localization to the pole, or both. As the HBB is not completed, FlgM remains bound to FliA, repressing

expression of class IV genes. Constellation swimming at 30 °C (Fig. 15) occurs as the population grows and consumes a key nutrient(s), inducing a starvation-like response that induces flagellum expression. This response includes increased expression of *fleQ* that is sufficient to overcome FleN inhibition, at least in a few cells, and thus enable expression of class II and III genes (including *flhF*). Increased FlhF enables the localization of flagellar components to the pole such that the HBB can be completed, and class IV gene transcription can occur. Alternatively, a starvation-like response may more directly inhibit either the expression or activity of FleN. Cells that are able to complete synthesis of their flagellum are motile and move in a directed fashion away from the colony center toward regions in the media where nutrients are more abundant. Upon reaching a more nutrient-replete region cells multiply and daughter cells are no longer able to initiate another round of flagellar synthesis because the low-nutrient signal is no longer present. As a result the cell remains at a location distal from the point of inoculation, forming a microcolony. In SHS mutants having *fleN* mutations (Fig. 16), FleN is no longer able to respond to temperature, and the distribution of FleN-inhibited and -uninhibited FleQ and FlhF at 30 °C is therefore similar to cells grown at 20 °C, enabling the expression and localization of class II, III, and IV flagellar genes. In SHS mutants harboring *fleQ* promoter mutations (Fig. 17), *fleQ* is over-expressed sufficiently to overcome FleN inhibition and drive expression of class II and III flagellar genes. Over-production of FleQ could either enable sufficient expression of *flhF* that the concentration of FlhF is high enough to overcome FleN inhibition as well, or enough FleQ is synthesized to effectively titrate FleN, allowing FlhF to accumulate and guide localization of the HBB components.

Mutations identified in SHS7, SHS8, and SHS12 were either synonymous, or were likely to affect expression (SHS7 and SHS8) or function (SHS12) of hypothetical proteins. It is quite likely that while disruption of these genes confer the SHS phenotype, their further analysis will be problematic due to a lack of bioinformatic guidance. Given the stability of the SHS mutant phenotypes it seems possible that the causal mutation present in mutants having only synonymous mutations has not yet been identified. More detailed analysis may be required to identify additional SNPs that escaped detection. These strains may also contain mutations such as insertions, deletions, or inversions that would not have been revealed in our analysis. It is also possible that the transcription or translation of these genes may also be altered indirectly due to changes in message stability or conformation.

We might have expected from the outset to observe a more pronounced increase in *fliC* expression in the SHS mutants at 30 °C compared to that in wild type cells, especially given the observation that the diffuse swimming flares that arose in high temperature cultures harboring a *fliC::gfp* fusion exhibited increased GFP fluorescence when compared with the rest of the constellation swimming colony (Fig. 7). There may be several reasons for this discrepancy; mutants having two separate genotypes were isolated, one of which exhibited substantially increased fluorescence at 30 °C (SHS 4) while the other exhibited only slightly more fluorescence (SHS 2). In a flare having a mixture of such mutants, the presence of a highly fluorescent mutant would likely mask the presence of a weakly fluorescent

genotype. Alternatively, incubation of the SHS mutants in swimming media may induce greater expression of flagellin than on the solid media that was used for quantification of relative fluorescence. Such context-dependent *fliC* expression has recently been demonstrated in *P. syringae* [116]. The constellation phenotype appears to be the result of a bimodal distribution of cells, with only a few highly expressing *fliC* and thus motile, and a large majority that do not express *fliC* and thus are non-swimming. The SHS mutations thus probably do not lead to an increase in *fliC* expression in all cells in the population, but instead, only an increase in the proportion of cells highly expressing *fliC* at high temperatures. This might explain why many of the SHS mutations conferred only a small increase in the population-level expression of *fliC* at 30 °C compared to WT. Preliminary studies of the distribution of GFP fluorescence intensity among individual cells of these mutants using quantitative microscopy supports this conjecture, but further work is needed.

Heterogeneity in flagellin expression has been observed in animal pathogens, such as *E. coli* and *Salmonella enterica* serovar Typhimurium [227, 228]. In *Salmonella*, heterogeneous expression of flagellin aids in evasion of the caspase-1 inflammatory response [227]. Heterogeneous flagellin expression *in vitro* is controlled by YdiV, a protein with weak homology to c-di-GMP-degrading EAL domain-containing proteins. Intriguingly, YdiV acts by binding to $FlhD_4C_2$ mediating both stochastic as well as deterministic flagellin expression in response to nutritional cues [229]. Thus similarities to the processes seen to enable heterogeneous motility in *P. syringae* at high temperatures are apparent.

The phyllosphere is characterized by rapid shifts in environmental conditions, such as temperature and water availability [1, 8]. While the duration of water availability is often inversely related to temperature (water is more persistent or likely under conditions of cool temperatures associated with night or rainfall events), it is not a guarantee that an elevated temperature will always lead to reduced water availability. In the event that water availability coincides with elevated temperatures, it may be beneficial for the bacterial population to contain a sub-population of cells that are capable of exploring/exploiting its local habitat. Such exploration would be facilitated by active motility. For example, if water did become sufficiently available even under warm conditions, at least these few cells could explore the leaf surface and exploit un-colonized sites having nutrient abundance. A pre-existing sub-population that was capable of motility because of a lack of catabolite repression, and despite the occurrence of high temperatures could then colonize the leaf during periods where water is not sufficiently available long enough to elaborate the flagellum and chemotaxis system de novo. Such cells could thus be thought of as exceptions to an otherwise conservative strategy of colonization that avoids movement except under cool conditions, when motility will have a higher probability of being successful. The mutants uncovered in this study should prove valuable as tools for investigating how different patterns of thermo-regulated motility as well as heterogeneous motility of *P. syringae* affect its fitness on plants.

3.4 Materials and Methods

Bacterial strains, plasmids, culture media, and growth conditions

Pseudomonas syringae pv. syringae B728a [112] was routinely cultured as described in Chapters 1 and 2. Antibiotics were used at the following concentrations: gentamicin, 15 μg/mL; rifampicin, 100 μg/mL.

Temperature-dependent assays were performed in incubators adjusted to the appropriate temperature as described in the text. Plate temperatures were routinely monitored using a CZ-IR thermometer (ThermoWorks, Lindon, UT). Incubator temperatures and RH were routinely monitored using HOBO data loggers (Onset, Bourne, MA).

Swimming assays

Swimming media was prepared as described in Chapter 1, except in the low peptone swimming media, where only 1.0 % of the normal concentration of peptone was added (0.2 g peptone/L; all other media components were added at 50% of normal concentration.

Quantitative microscopy and flow cytometry

Quantitative single cell microscopy was performed on cells that were grown for 24 hours at various temperatures on KB amended with gentamicin. Following incubation, cells were fixed in 2 % formaldehyde (w/v, final concentration) and stained with DAPI (2.5 μg/μL, final concentration) for 20 minutes. Cells were observed with a Zeiss AxioImager fluorescence microscope with DAPI (325 to 375 nm band pass excitation, and 460 nm long pass emission) and GFP (450 to 490 nm band pass excitation, and 500 to 550 nm band pass emission filter) filter sets (Chroma; Bellows Falls, VT). Images were captured using a Hamamatsu Orca 03 charge coupled device (CCD) camera. The intensity of GFP fluorescence of individual cells was determined using iVision-Mac software. Cumulative probability plots of cell GFP intensities were generated using Statistica (StatSoft; Tulsa, OK).

Cells were similarly prepared (except not stained with DAPI) for flow cytometry, which was performed with a Cytopeia INFLUX Sorter using a 488 nm excitation laser and a GFP detector. Gating was based on cell size, determined by forward scatter and side scatter where significant counts occurred in cell-containing samples but not in buffer controls. Data was plotted with Summit v3.1 (Cytomation, Inc; Fort Collins, CO).

Spontaneous Hot Swimming mutant isolation

Swimming plates were incubated at 30 °C for 3 to 4 days, until robust flares had formed that resembled the normal, diffuse swimming phenotype. A culture from the tip of each flare was streaked onto KB plates to enable isolation of single colonies. These single colonies were used to inoculate fresh swimming medium which was incubated at 30 °C or 20 °C. Wild type *P. syringae* was routinely included for phenotype comparison. Colonies that exhibited the normal swimming phenotype at

30 °C (i.e. non-constellation), regardless of their 20 °C phenotype, were stored at -80 °C.

Sequencing of Spontaneous Hot Swimming mutants

Genomic DNA was isolated from SHS mutants grown overnight in KB broth using a DNeasy Blood and Tissue Kit (QIAGEN; Valencia, CA). Samples were treated with 10 µl RNAse A (10 mg/ml, Fermentas; Glen Burnie, MD) to remove RNA contamination. Isolated genomic DNA was fragmented with a Covaris-S220 adjusted for 300 base pair fragments according to the manufacturer's protocol (Covaris, Inc; Wourn, MA). Genomic fragments were end-repaired and A-tailed similar to cDNA samples used in transcriptomics described in Chapter 1. DNA Samples were routinely purified using AMPure XP beads (Beckman Coulter, Brae, CA). Illumina adapters were ligated onto fragments as described in Chapter 1 and fragment sizes between 300 and 600 nucleotides were selected using various AMPure XP bead-to-sample ratios, which were empirically determined to recover the desired fragment sizes as determined using a 100 bp ladder (Life Technologies; Carlsbad, CA). Following fragment sizing, samples were amplified with bar-coded Illumina primers. Amplified libraries were quantified using a Qbit dsDNA HS assay (Life Technologies) and fragment size distribution was determined with a 2100 bioanalyzer (Agilent; Santa Clara, CA). Sequencing consisted of 100 bp paired-end reads, from a single multiplexed lane using an Illumina HiSeq 2000 at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley.

Reads were aligned to the *P. syringae* B728a genome using bowtie 0.12.7 [118] to identify single nucleotide polymorphisms (SNP) and allowing for a maximum of three SNPs in a given read. Aligned reads were subsequently processed using the SNP detection feature on CLC Genomics Workbench (CLC Bio; Cambridge, MA) with default settings. Predicted SNPs were considered well supported if greater than 80 % of the mapped reads predicted an identical SNP.

Promoter prediction and protein modeling

fleQ promoter site prediction was performed with the Virtual Footprint promoter analysis option, which included all *P. aeruginosa* and *E. coli* position weight matrices [208]. σ^{70} binding sites were predicted using BPROM (available at http://linux1.softberry.com/berry.phtml). FleN modeling and ligand binding predictions were performed using the Phyre2 pipeline with default settings [209] and visualized using MacPyMOL v1.3 (Schrödinger, LLC).

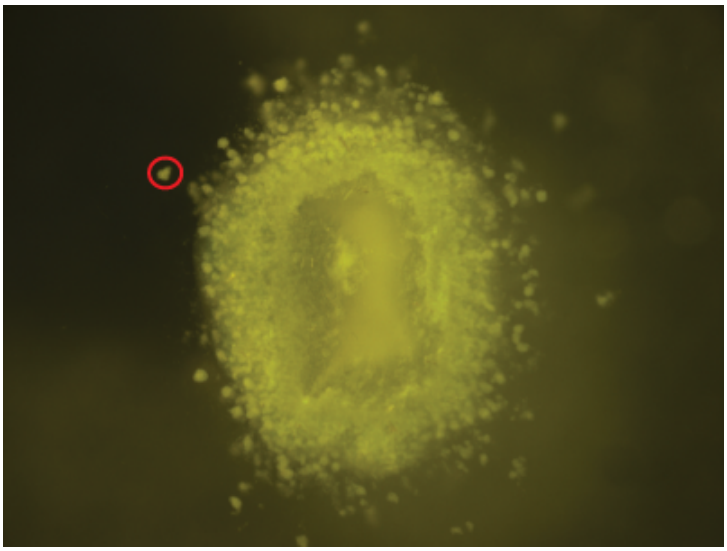

Figure 3.1. Wild type *Pseudomonas syringae* exhibiting constellation swimming at 30 °C. The red circle indicates a microcolony spatially separated from the point of inoculation.

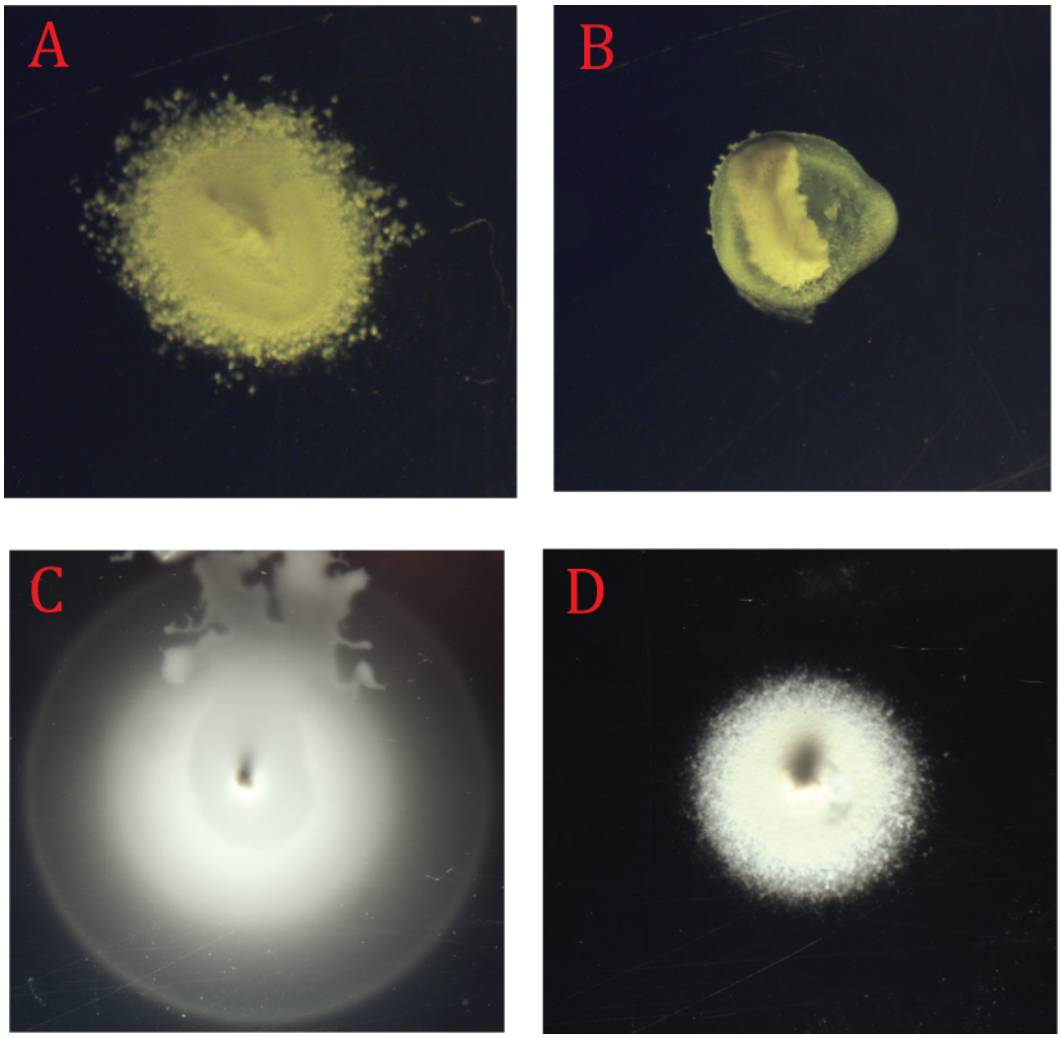

Figure 3.2. Wild type *Pseudomonas syringae* exhibiting constellation swimming at 30 °C (A). Δ*flgK* mutant incubated in swimming media at 30 °C (B). Spatially separated microcolonies from a constellation swimming plate were picked and inoculated into fresh swimming media and incubated at either 20 °C (C) or 30 °C (D).

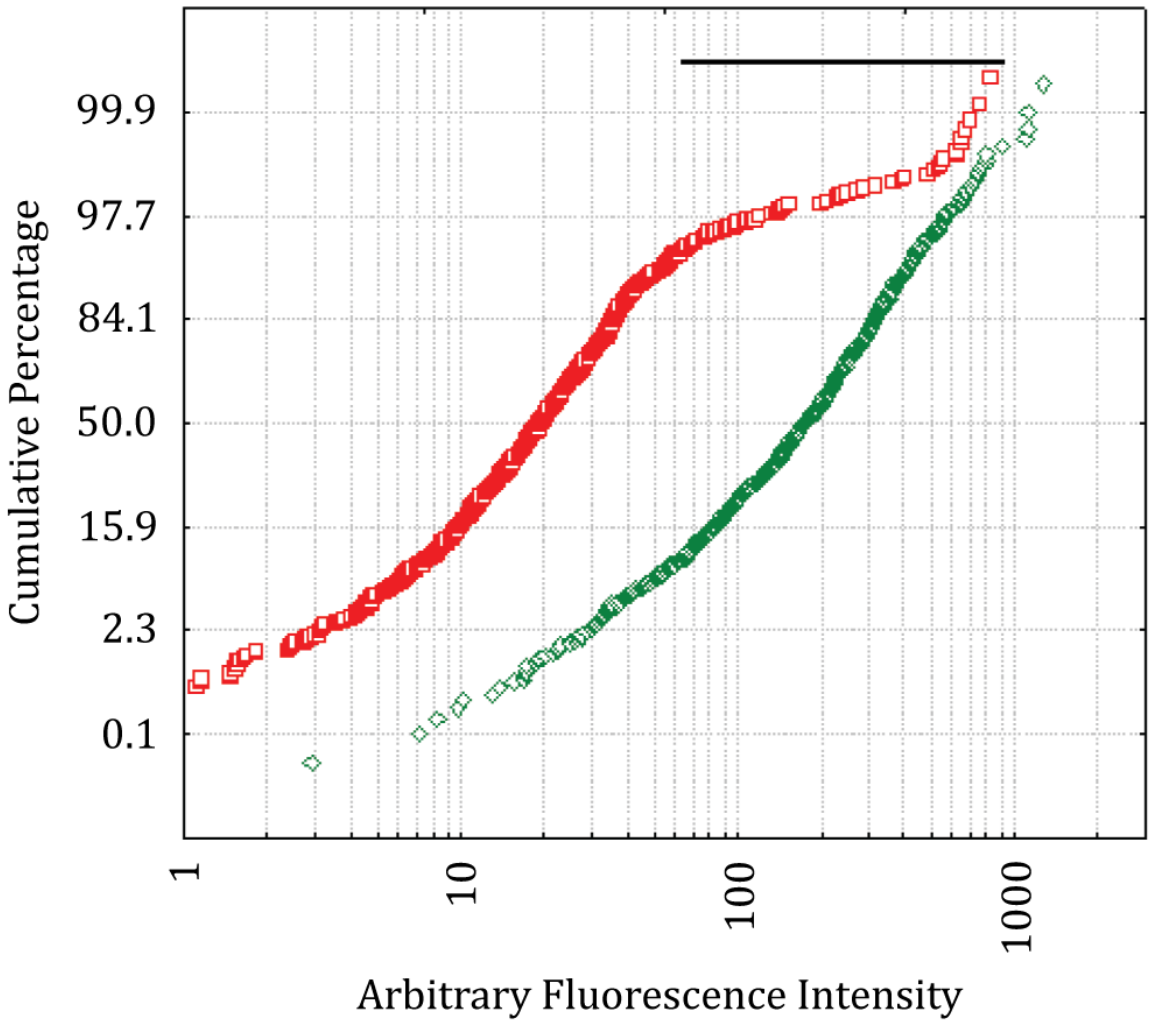

Figure 3.3. Cumulative normal probability plot of *Pseudomonas syringae* harboring $P_{fliC\text{-}gfp}$ incubated at either 20 °C (green diamonds) or 30 °C (red squares) for 24 hrs. The sub population of cells incubated at 30 °C that is expressing *fliC* at a level higher than expected based on an otherwise normal distribution of GFP fluorescence intensity is indicated under the black line.

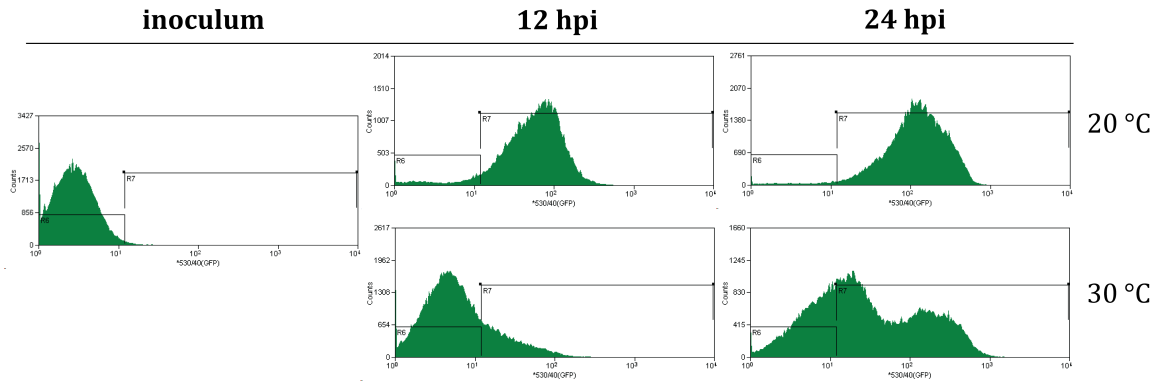

Figure 3.4. Distribution of GFP fluorescence of *Pseudomonas syringae* harboring $P_{fliC\text{-}gfp}$ when incubated at either 20 °C or 30 °C at the indicted times after inoculation. Shown are histograms reflecting the frequency with which cells of a given GFP fluorescence were observed as determined by flow cytometry. Inoculum was incubated over night at 31 °C.

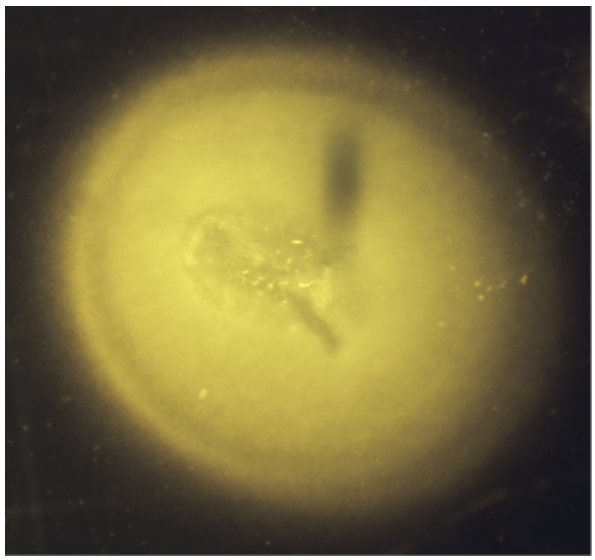

Figure 5. Normal (diffuse) swimming phenotype exhibited by wild type *Pseudomonas syringae* incubated in low-peptone swimming media at 30 °C.

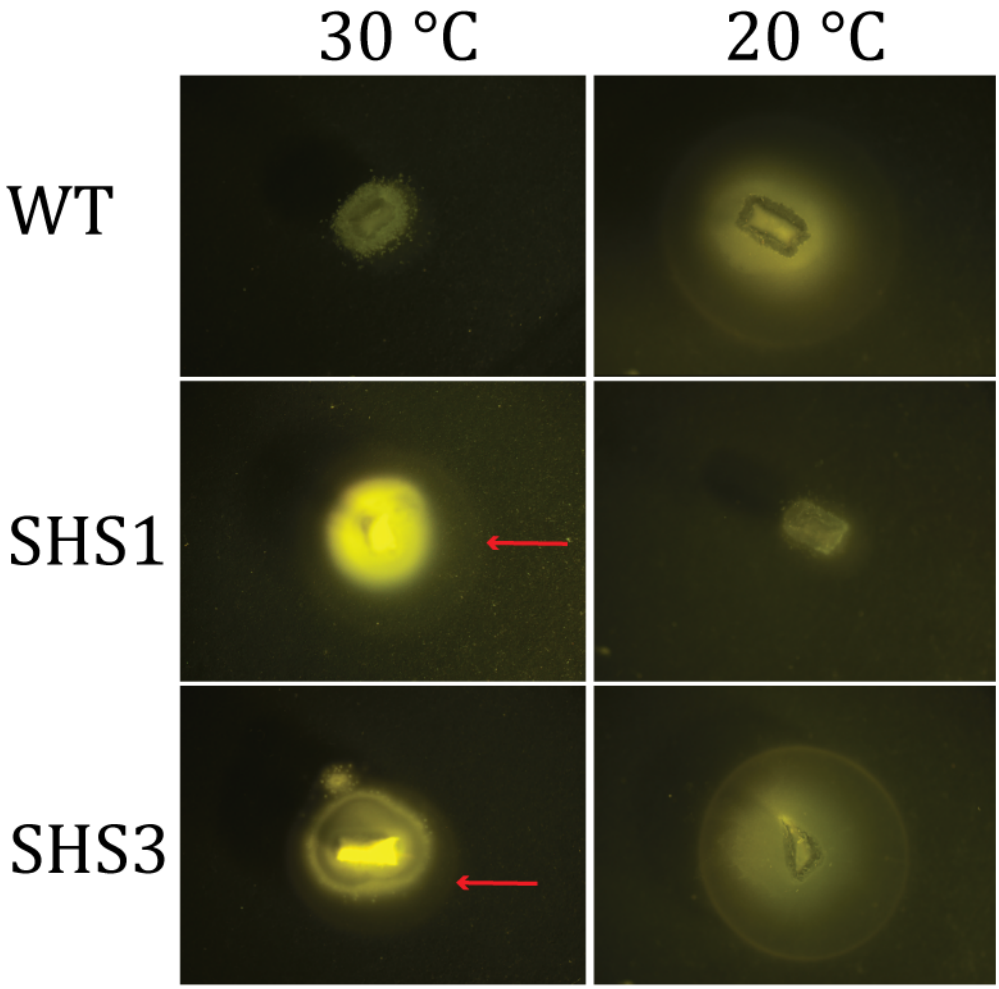

Figure 3.6. Swimming motility exhibited by wild type *Pseudomonas syringae* as well as Spontaneous Hot Swimming mutants SHS1, and SHS3, incubated in swimming medium at 30 °C (left) or 20 °C (right column) for 24 hours. Red arrows indicate the swimming front within the swimming medium.

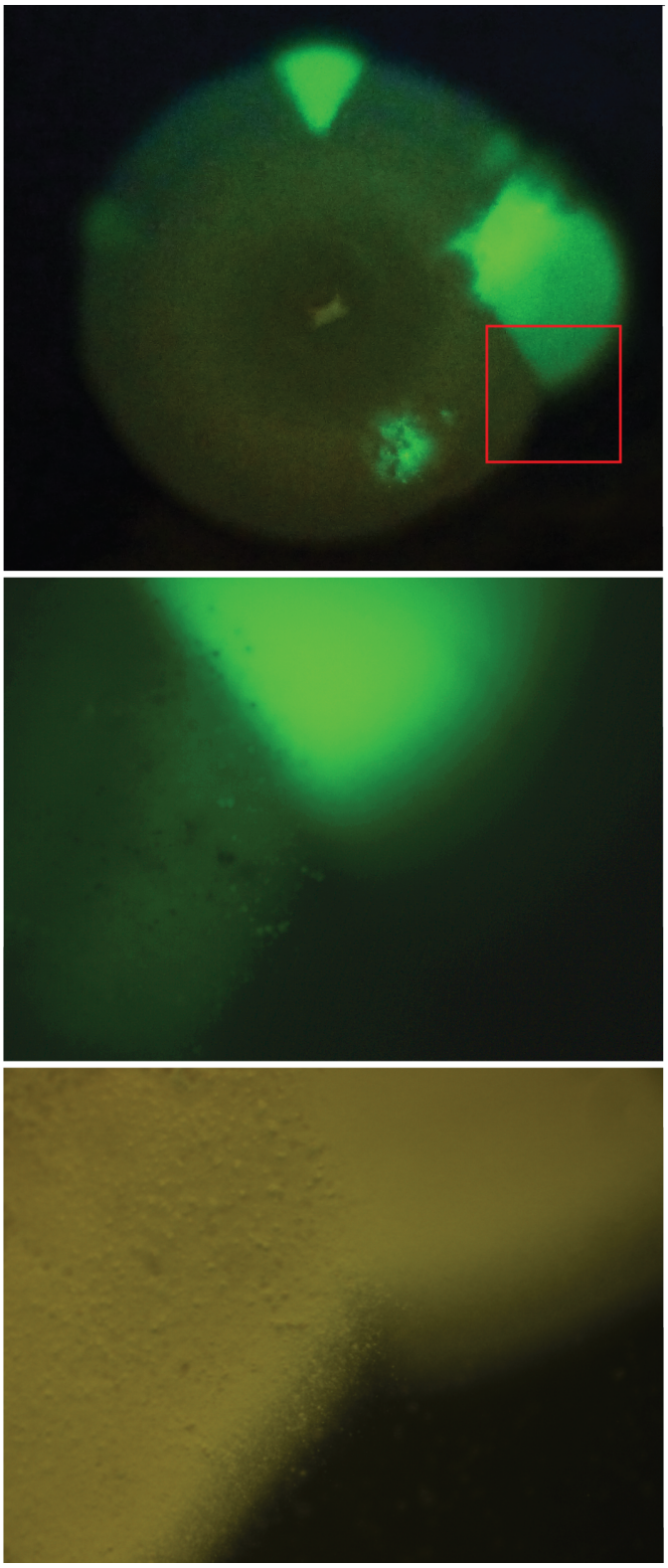

Figure 3.7. Swimming motility of a wild type *Pseudomonas syringae* strain harboring $P_{fliC\text{-}gfp}$ when incubated at 30 °C for 4 days. Green sectors reflect normal swimming (i.e. the swimming phenotype of cells incubated at cooler temperatures) extending from a constellation swimming colony photographed under UV illumination

(top panel). Close up of marked region from top panel (middle panel). Middle panel photographed without UV illumination (bottom panel).

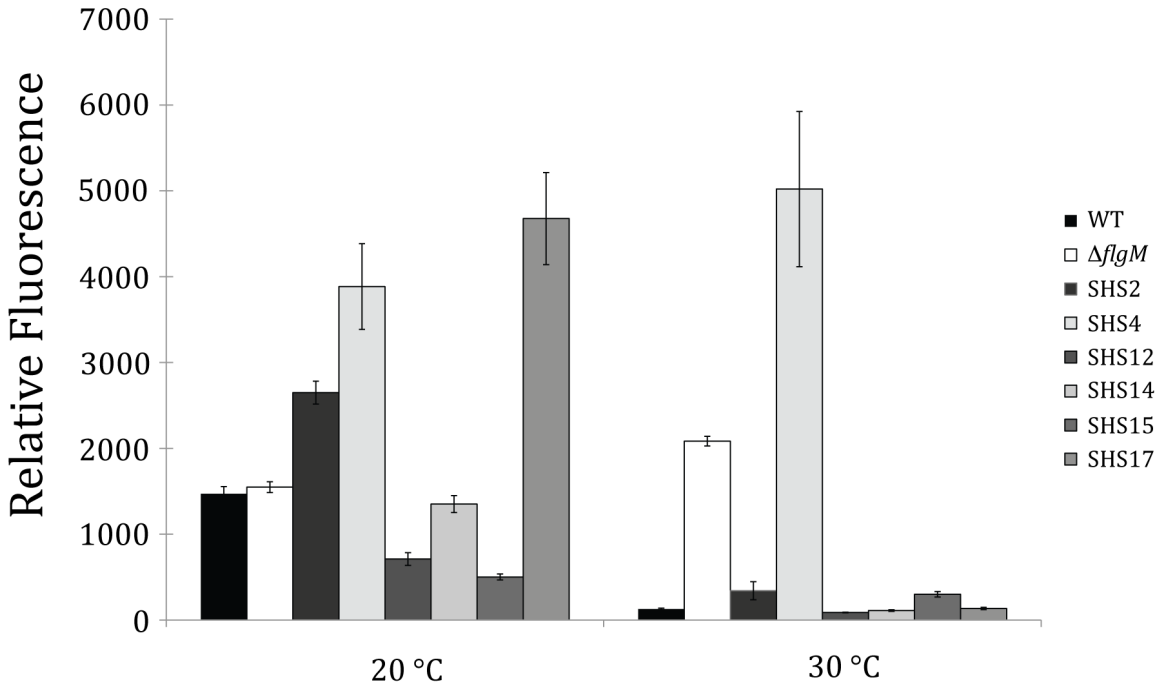

Figure 3.8. Relative GFP fluorescence of wild type *Pseudomonas syringae*, a Δ*flgM* mutant, and selected Spontaneous Hot Swimming mutants harboring $P_{fliC\text{-}gfp}$ when incubated at either 20 °C (left) or 30 °C (right). Δ*flgM* was included because it was previously shown that *fliC* expression in this mutant was insensitive to incubation temperature.

A

```
1                                   25                                  50
MGSMHPVQVIAVTGGKGGVGKTNVSVNLSLALAELGRRVMLLDADLGLAN

51                                  75                                 100
VDVLLGLTPKRTLADVIEGRCELRDVLLQGPGGVRIVPAASGTQSMVHLS
                                    G

101                                 125                                150
PAQHAGLIQAFSEIGDNLDVLVIDTAAGIGESVVSFVRAAQEVLLVVCDE
                                    S                   V

151                                 175                                200
PTSITDAYALIKLLNRDYGMNRFRVLANMAQSPQEGRNLFAKLTKVTDRF
                                    A

201                                 225                                250
LDVALQYVGAVPYDECVRKAVQKQRAVYEAFPRSKCALAFKAIAQKVDTW
                                    E

251                                 277
PLPANPRGHLEFFVERLVHQTSAGPVQ
```

B

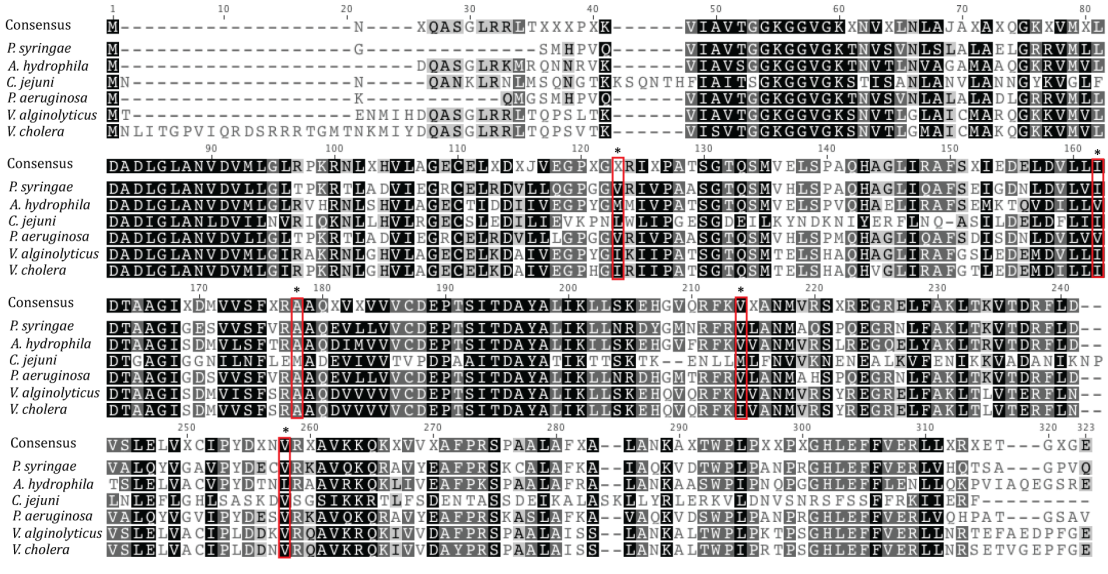

Figure 3.9. Alignment of *Pseudomonas syringae* FleN protein sequence having altered residues in SHS mutants indicated (A). Alignment of FleN orthologs from select proteobacterial species with mutant residue locations indicated by a red box and asterisk (B).

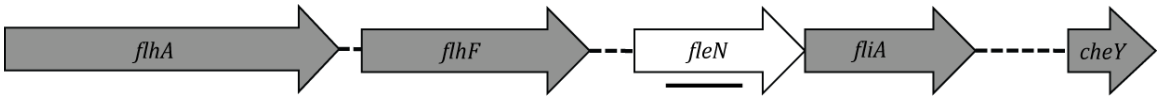

Figure 3.10. Genomic context of *fleN* in *Pseudomonas syringae* B728a. The black bar under *fleN* indicates the approximate range of locations of the Single Nucleotide Polymorphisms observed in Spontaneous Hot Swimming mutants.

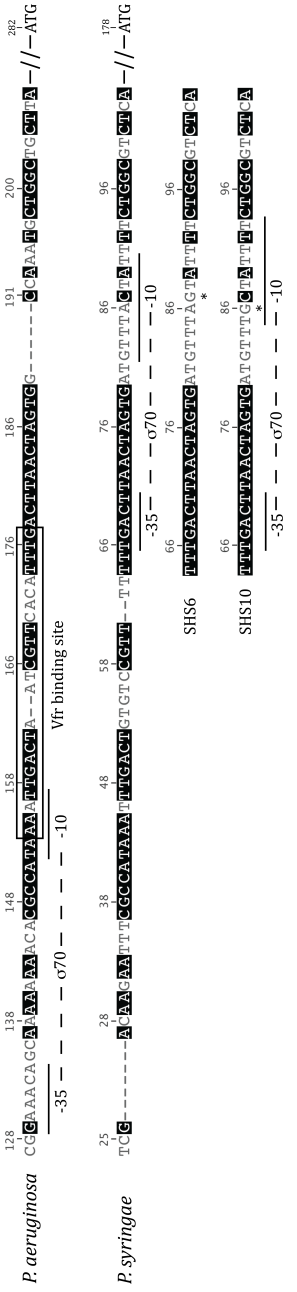

Figure 3.11. Alignment of the *fleQ* promoter regions of *Pseudomonas syringae* and *Pseudomonas aeruginosa* PA01. *P. aeruginosa* Vfr and σ^{70} binding sites were described in [2] while *P. syringae* SHS10 σ^{70} sites were predicted by BPROM (http://linux1.softberry.com/berry.phtml). Asterisks indicate Single Nucleotide Polymorphisms in Spontaneous Hot Swimming mutants.

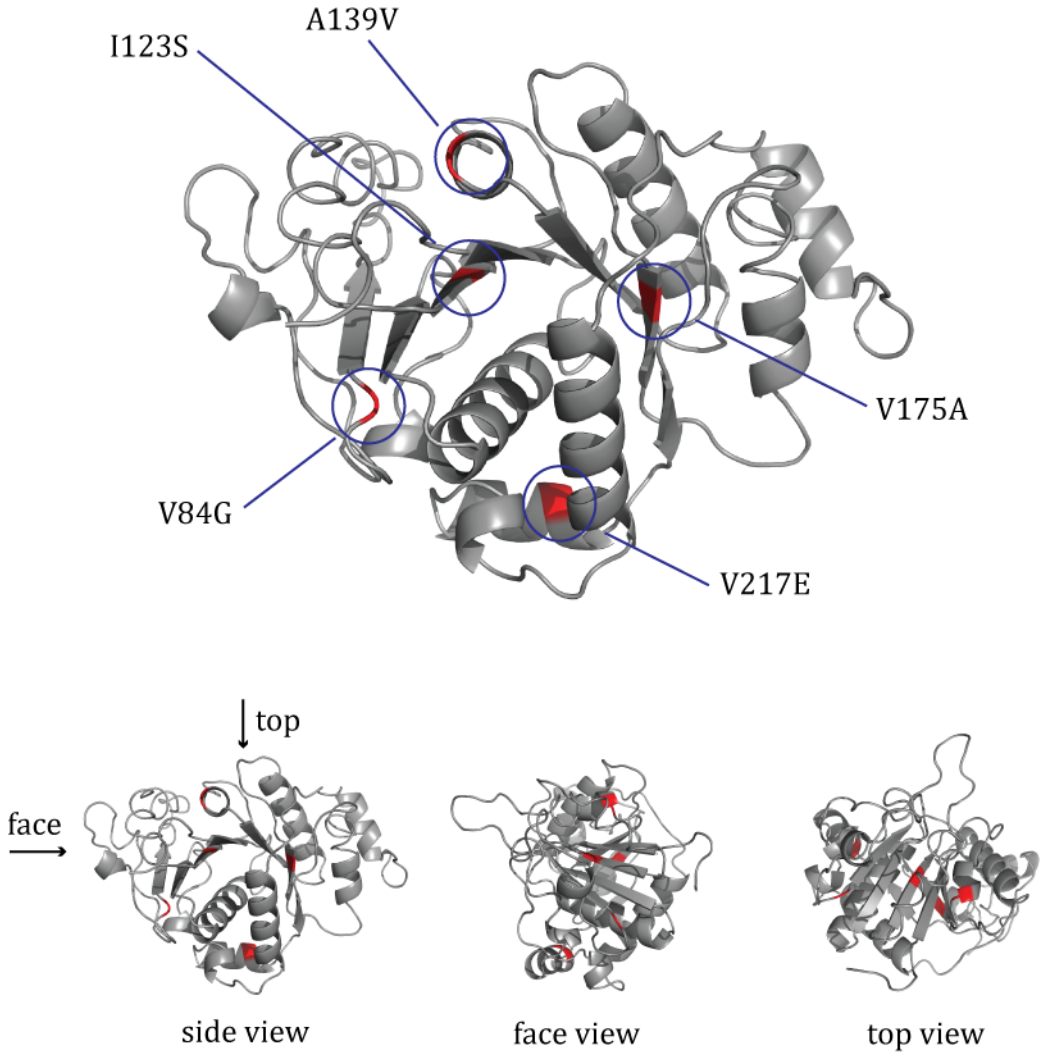

Figure 3.12. 3-D model of FleN from *Pseudomonas syringae* produced using the Phyre2 pipeline and visualized with MacPyMOL. Residues that changed in the mutants are indicated in red in the side view (top panel). Axis of visualization is indicated in the small side view (bottom left panel).

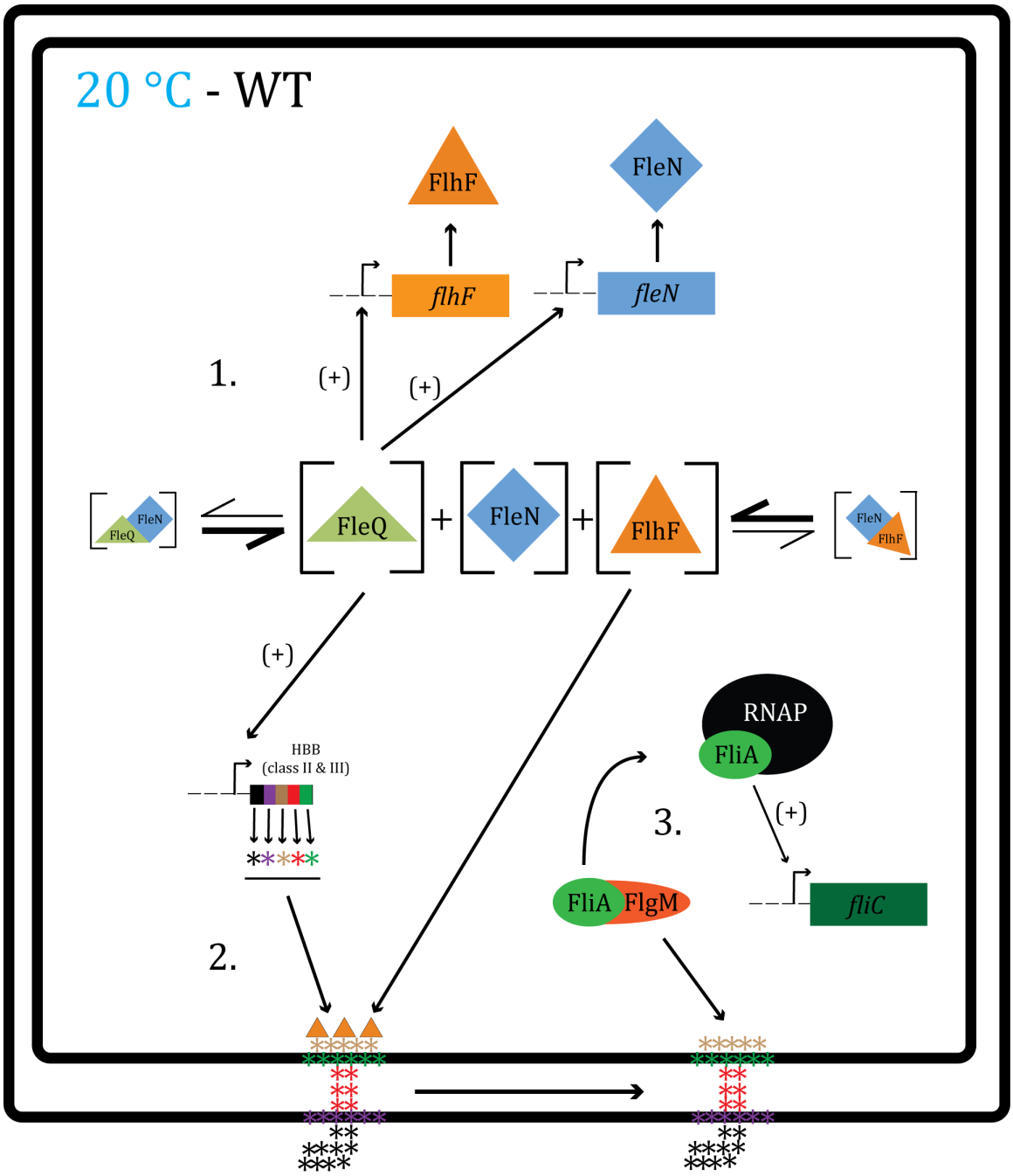

Figure 3.13. Model of flagellar regulation at 20 °C, a permissive swimming temperature. 1) FleN is maintained in a conformation such that both FleQ and FlhF are present in sufficient free abundance (i.e. not bound to FleN) to allow high expression and localization of genes encoding the hook and basal body (HBB) components of the flagellum. Completion of the HBB allows for export of FlgM allowing FliA to pair with RNA polymerase (RNAP) to direct transcription of class IV flagellar genes, including *fliC*. The majority of cells are motile.

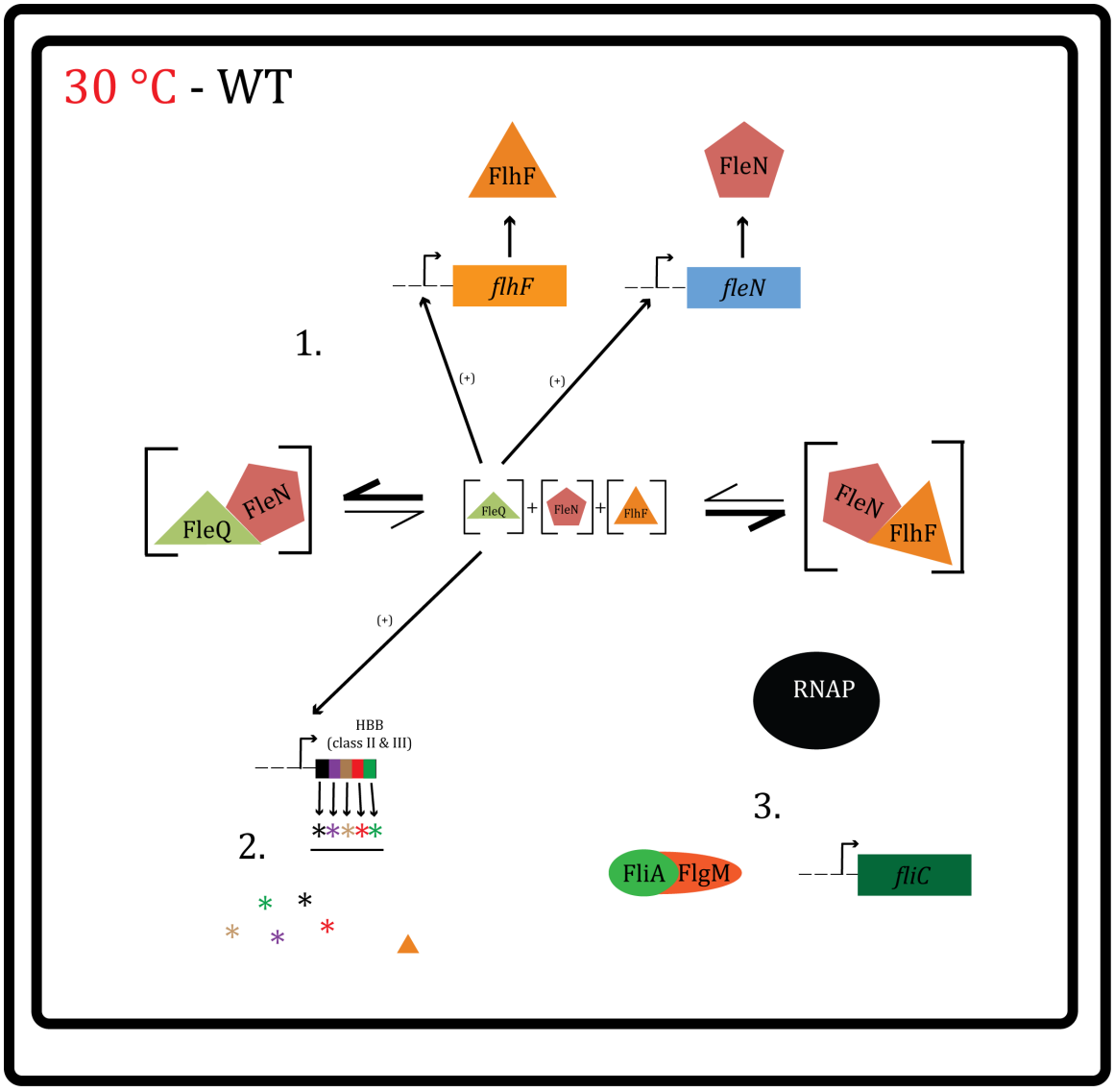

Figure 3.14. The conformation of FleN is altered at 30 °C to make it more likely to bind to and inhibit the activity of either FleQ or FlhF or both. This results in either inadequate expression of class II and III genes, or insufficient FlhF to direct their localization to the pole to form the basal body, or both. Because the HBB complex is not formed, FlgM remains bound to FliA, inhibiting expression of class IV flagellar genes. Cells are non-motile.

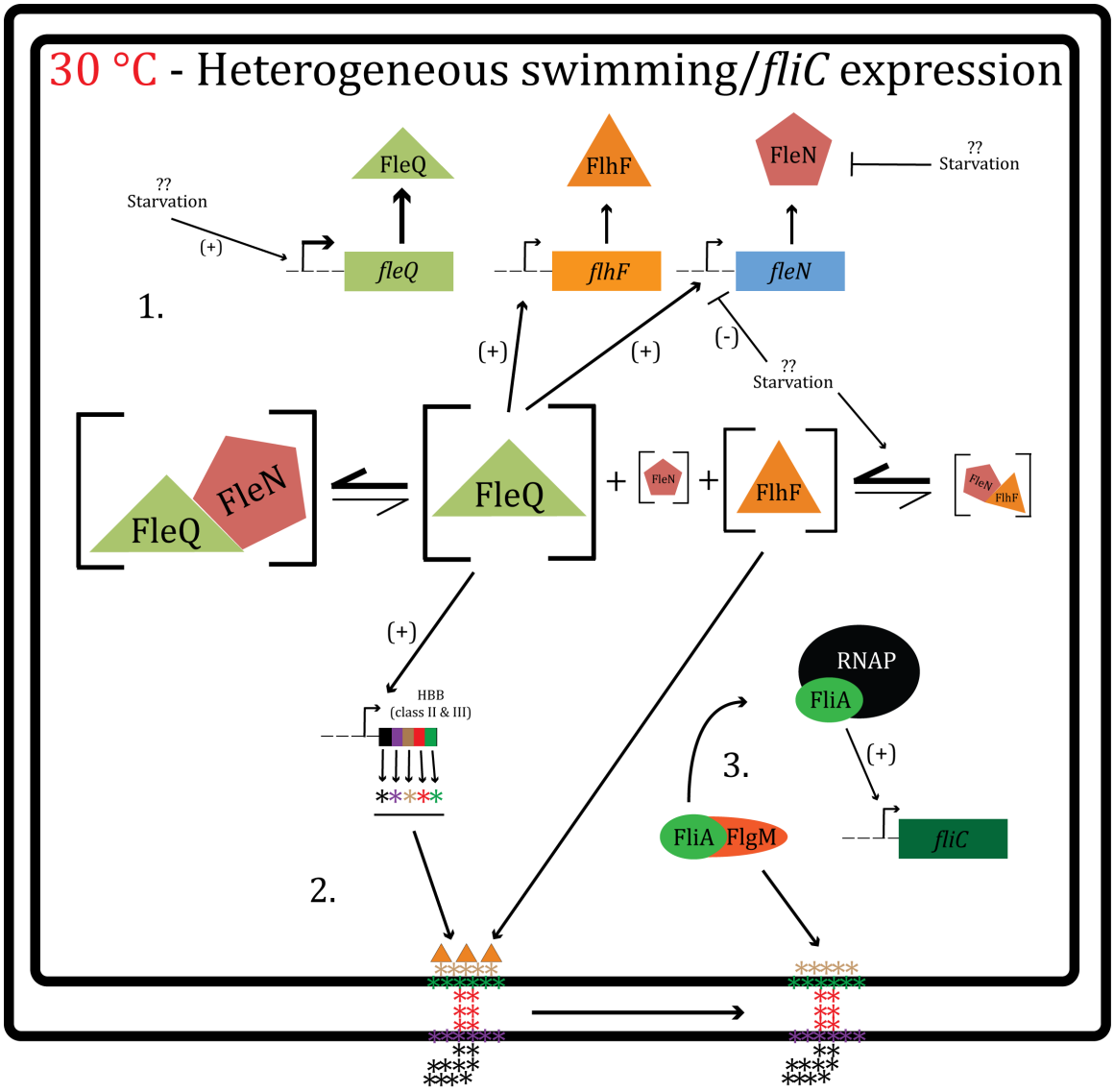

Figure 3.15. Heterogeneous swimming/*fliC* expression at 30 °C in wild type cells of Pseudomonas syringae. As the population of cells in a colony increases, the concentration of a key nutrient(s) decreases and a starvation-like response is triggered. This either leads to increased expression of *fleQ* analogous to that seen in SHS6 and SHS10. Alternatively, a starvation-like response leads to a blockage of the ability of FleN to interact with either FlhF or FleQ, or both. Potentially a secondary messenger, such as cAMP could be produced that would competitively inhibit the ability of FleN to bind ATP. A threshold of starvation-like response is required to effectively inhibit normal thermo-repression of the flagellum, such that only a sub population of cells is able to produce a flagellum. Onl a few cells express *fliC* and are motile while the majority of cells do not express *fliC* and are not motile.

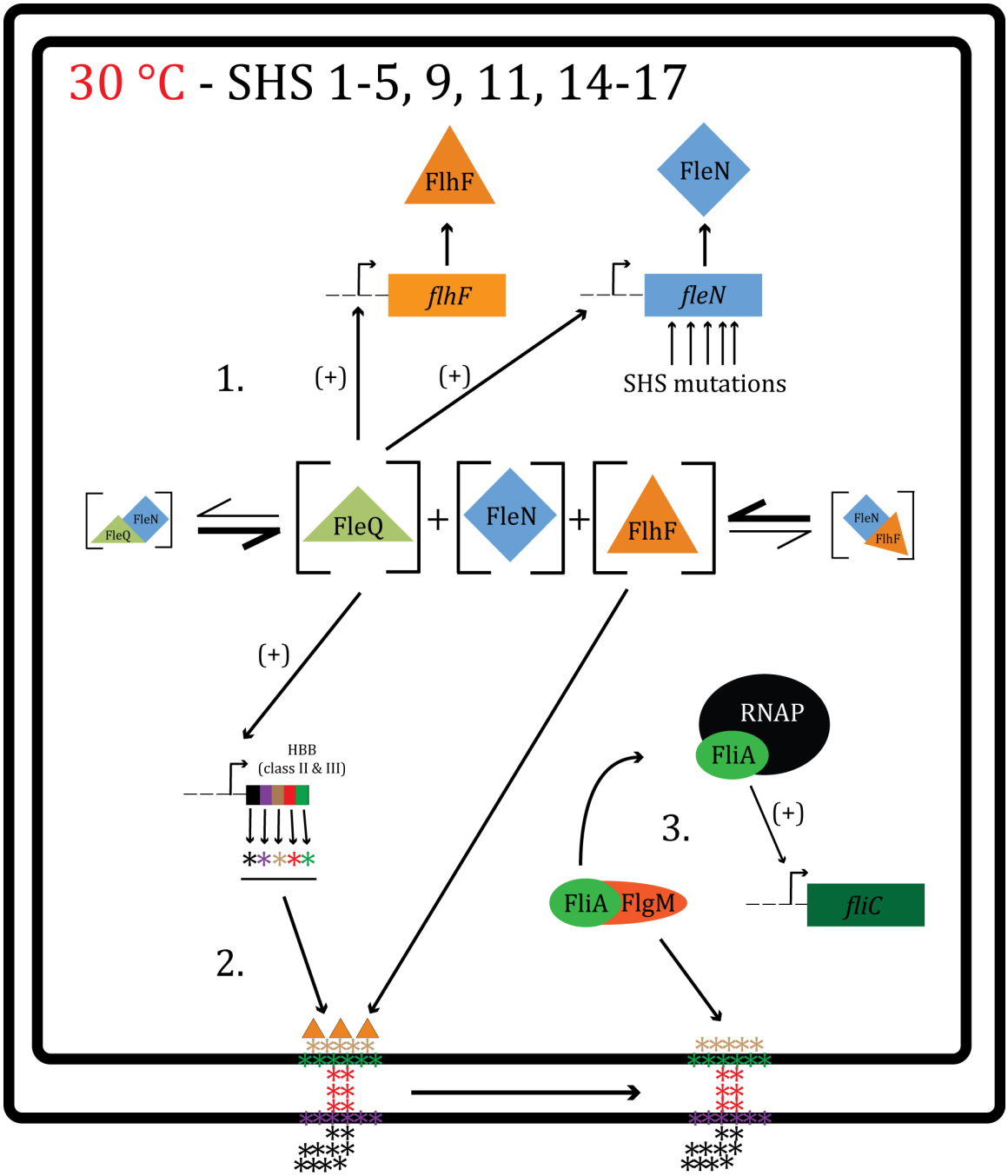

Figure 3.16. SHS mutations in FleN block either its ability to respond temperature or its ability to efficiently bind to FleQ or FlhF. This results in a larger pool of either free FleQ or FlhF or both, enabling formation of the HBB and export of FlgM, triggering expression of class IV flagellar genes. Cells are motile at a normally non-permissive temperature.

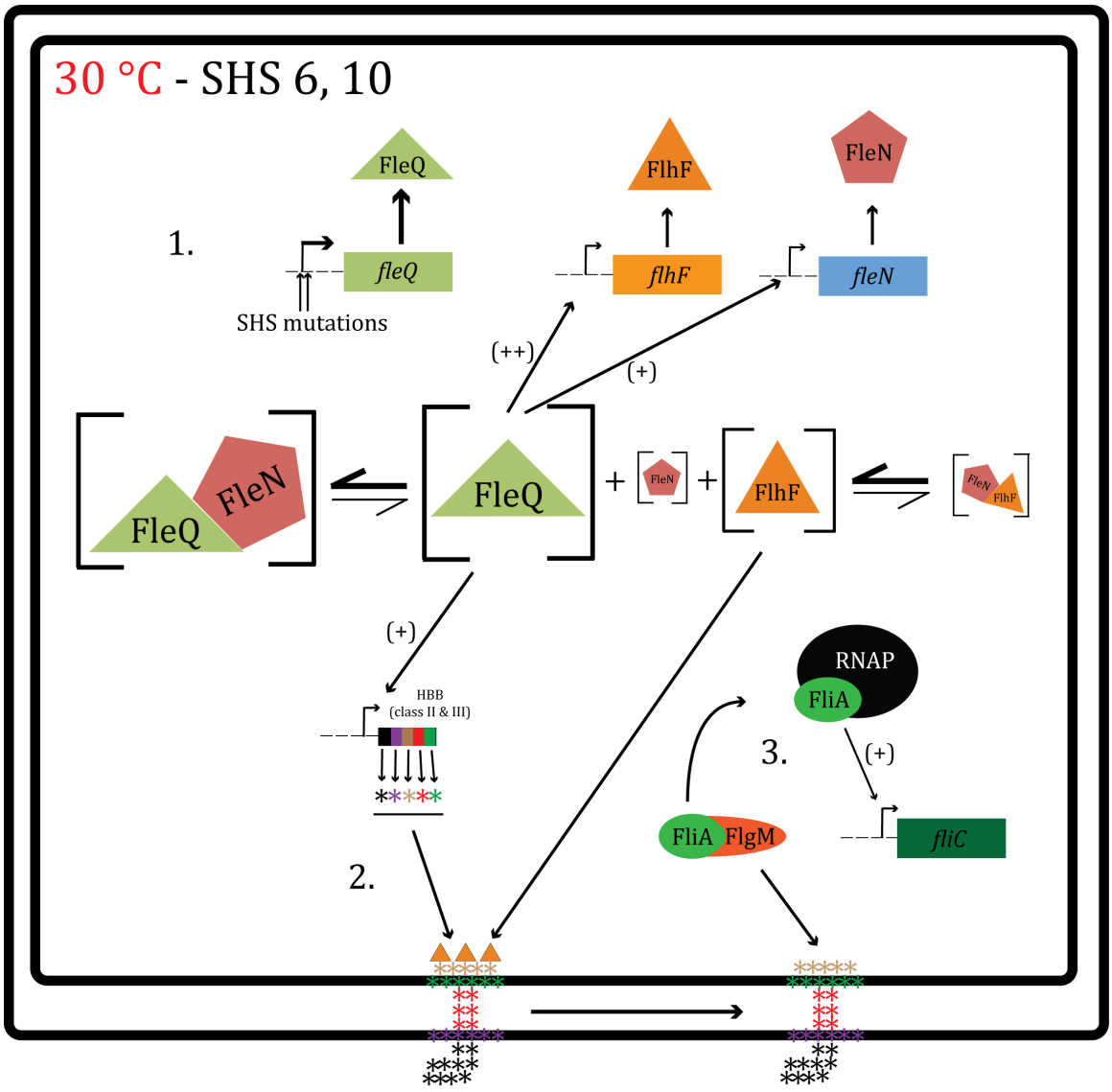

Figure 3.17. SHS mutations in the promoter region of *fleQ* increase its expression to levels higher than the wild type strain at high temperatures. The increased pool of FleQ is sufficient to drive expression of the class II and III flagellar genes, in spite of increased binding by FleN. Additionally, the increased FleQ pool titrates FleN, such that the effective unbound FlhF pool increases to a high enough concentration to guide localization of basal body components to the cell pole. Alternatively, because both *flhF* and *fleN* are class II genes (their expression is FleQ dependent) increased FleQ abundance may lead to an imbalance in their expression (for an unknown reason) such that the effective free pool of FlhF exceeds a threshold concentration to direct localization of basal body components. This leads to expression of class IV flagellar genes. Cells are motile.

Table 3.1. Spontaneous Hot Swimming phenotypes observed in different mutants of *Pseudomonas syringae*

strain	isolation	20 °C swimming phenotype	30 °C swimming phenotype
WT	n/a	normal	Constellation
SHS1	1st	reduced	Normal
SHS2	1st	reduced	Normal
SHS3	1st	normal	Normal
SHS4	1st	normal	Normal
SHS5	1st	reduced	Normal
SHS6	2nd	normal	Normal
SHS7	2nd	normal	Normal
SHS8	2nd	normal	Normal
SHS9	2nd	normal	Normal
SHS10	2nd	normal	Normal
SHS11	2nd	normal	Normal
SHS12	3rd	normal	Normal
SHS13	3rd	normal	Normal
SHS14	3rd	reduced	Normal
SHS15	3rd	normal	Normal
SHS16	3rd	normal	Normal
SHS17	3rd	reduced	Normal

Table 3.2. Single Nucleotide Polymorphisms observed in Spontaneous Hot Swimming mutants of *Pseudomonas syringae* as compared to the wild type strain

mutant*	nucleotide position	mutation**	genetic context	amino acid change
SHS1[a]	4101623	A to C	intragenic, psyr_3438 (*fleN*)	V175A
SHS2[a]	4101623	A to C	intragenic, psyr_3438 (*fleN*)	V175A
SHS3[b]	4101497	A to T	intragenic, psyr_3438 (*fleN*)	V217E
SHS4[b]	4101497	A to T	intragenic, psyr_3438 (*fleN*)	V217E
SHS5[a]	4101623	A to C	intragenic, psyr_3438 (*fleN*)	V175A
SHS6	4126768	G to C	intergenic, psyr_3461 psyr_3462	n/a
SHS7[c]	3852887	C to T	intragenic, psyr_3216 (hypothetical)	silent
SHS7[c]	3947091	T to A	intergenic, psyr_3297 psyr_3298	n/a
SHS8[c]	3852887	C to T	intragenic, psyr_3216 (hypothetical)	silent
SHS8[c]	3947091	T to A	intergenic, psyr_3297 psyr_3298	n/a
SHS9	4101779	A to C	intragenic, psyr_3438 (*fleN*)	I123S
SHS10	4126769	T to C	intergenic, psyr_3461 psyr_3462	n/a
SHS11	4101731	G to A	intragenic, psyr_3438 (*fleN*)	A139V
SHS12	5117165	A to C	intragenic, psyr_4290 (hypothetical)	I141L
SHS13	ND	ND	ND	ND
SHS14	4101623	A to C	intragenic, psyr_3438 (*fleN*)	V175A
SHS15	4101896	T to G	intragenic, psyr_3438 (*fleN*)	V84G
SHS16	4101896	T to G	intragenic, psyr_3438 (*fleN*)	V84G
SHS17	4101623	A to C	intragenic, psyr_3438 (*fleN*)	V175A

*mutants sharing the same letter are likely siblings as they were isolated from the same flare

**mutation description is based on positive strand nucleotide

Table 3.3. Percentage amino acid composition of particular residues of the FleN homolog altered in Spontaneous Hot Swimming mutants of *Pseudomonas syringae* as compared to other taxa

	V84G*/**	**I123S***	**A139V***	**V175A***	**V217E***
isoleucine	59.6	48.0	0.0	45.0	1.0
leucine	11.1	0.0	0.0	2.0	32.0
valine	23.2	52.0	0.0	52.0	55.0
phenylalanine	2.0	0.0	0.0	0.0	0.0
methionine	4.0	0.0	0.0	1.0	12.0
alanine	0.0	0.0	100.0	0.0	0.0

*residue numbers corresponds to psry_3438

**residue is not present in sequence from *P. syringae* Cit7 likely due to lack of available sequence

Table 3.4. Transcription factor binding sites predicted to overlap the promoter region of *fleQ in Pseudomonas syringae* Spontaneous Hot Swimming mutants having Single Nucleotide Polymorphisms in this region

	Transcription Factor	**Organism**	**Start**	**Stop**	**Strand**	**Score**	**Sequence*/****
WT	ArcA	*E. coli*	81	90	+	5.56	TGTTTACTAT
	CytR	*E. coli*	77	88	-	7.34	AGTAAACATCAC
	NarL	*E. coli*	85	91	+	3.76	TACTATT
	PvdS	*P. aeruginosa*	80	88	-	5.87	AGTAAACAT
	RcsAB	*E. coli*	82	89	-	4.86	TAGTAAAC
	YhiX	*E. coli*	85	90	+	2.27	TACTAT
SHS6	ArcA	*E. coli*	81	90	+	5.45	TGTTTAGTAT
	Crp	*E. coli*	73	94	-	6.07	GAAAATACTAA ACATCACTAGT
	NarL	*E. coli*	83	89	-	3.91	TACTAAA
	PvdS	*P. aeruginosa*	80	88	-	5.89	ACTAAACAT
	YhiX	*E. coli*	80	97	-	5.46	CCAGAAAATACT AAACAT
SHS10	CytR	*E. coli*	77	88	-	7.37	AGCAAACATCAC
	YhiX	*E. coli*	80	97	-	5.61	CCAGAAAATAG CAAACAT

*sequence from negative strands is shown as reverse complement

** SNP locations are underlined

Table 3.5. Single Nucleotide Polymorphisms common to all sequenced SHS mutants of *Pseudomonas syringae*

location	mutation*
376070	A to G
376071	G to A
739416	T to C
739417	C to G
739465	T to G
951716	C to A
951765	C to G
951817	C to G
951882	C to G
5423688	C to T
5426993	T to C

*mutation designated based on sense strand

Discussion

Conclusions and Future Directions

This work demonstrated that numerous genes that mediate host-pathogen interactions were temperature sensitive in their expression (Chapter 1), indicating temperature affects numerous aspects of the interaction of *Pseudomonas syringae* with its plant hosts. Most dramatically, we found that both swimming and swarming motility were strongly suppressed by warm incubation temperatures. Surprisingly, motility was suppressed at the optimum growth temperature for the cells, temperatures that this species often encounters on leaves and so should not be considered an exceptional situation. As motility enables *P. syringae* to explore the leaf surface [24] as well as invade into the leaf interior [25, 26], thermo-suppression of this trait would be expected to reduce its ability to cause disease (due to lack of invasion) under warm conditions. Additionally, genes encoding virulence genes such as those conferring synthesis and transport of the phytotoxins syringomycin and syringopeptin, were also more highly expressed at cool temperatures. All of these results correlate well with the observations that many diseases incited by *P. syringae* are more prevalent under cool conditions, leading to the moniker of "cool weather pathogen" often used to describe these pathogens [46]. This work brings more insight into the molecular basis for these behaviors.

Several genes were found to be involved in thermo-regulation in *P. syringae*, including *flgM*, *fleN*, ACDH, and *ygdP*. Interestingly, *flgM*, which was necessary for thermo-regulation of *fliC* and thus motility, did not appear to play any role in thermo-regulation of biosurfactant production via *syfA*. Conversely, *ygdP* and ACDH, which were involved in thermo-regulation of *syfA*, appeared to have no role thermo-regulation of *fliC*. This might suggest that while thermo-regulation of traits in *P. syringae* is common, such regulation involves numerous components that do not necessarily interact. Perhaps such unlinked responses to temperature might allow more contextual behaviors by the cells. While we cannot currently rule out the possibility that *P. syringae* possesses a truly global thermo-regulator such as H-NS which is involved in thermo-regulation of 69 % of the temperature-dependent transcriptome in *E. coli*, [135], our results suggest that thermo-regulation may be "decentralized" in *P. syringae*, meaning that temperature-dependent gene expression is mediated by multiple, independent regulators. Such a dispersed form of thermo-regulation may better enable trait-specific thermo-regulation to occur within the larger environmental stimuli context seen by this pathogen.

Context-dependent thermo-regulation was the main factor leading to the constellation swimming phenotype described in Chapter 3. It appears that some form of nutritional-regulation, linked to the concentration of peptone in the medium, supersedes thermo-regulation, where *fliC*-mediated motility can occur in a

few cells in a population even at elevated temperatures. Specifically, *P. syringae* appears to be hedging its bets that under high temperatures motility of the cells is generally disadvantageous by allowing only a few cells in the population to be motile under these conditions. However, the limited distance that such cells can move into nutrient-rich, warm culture media suggests that the adventurisms of such cells is subsequently suppressed if they encounter high nutrient levels. Thus the overall cell behavior is rather conservative with respect to movement, only when cells are cool (and thus the cells are most likely to experience free moisture to enable movement) will they swim for prolonged periods. As exploration of the leaf surface, is likely a result of seeking out sites of nutrient abundance, suppressing motility once abundant nutrients are encountered, likely contributes to *P. syringae*'s conservative strategy. The nutrient-coupling to temperature regulation of motility, and the fact that thermo-regulation of syringafactin and the flagellum are independent of each other, suggests that context-dependent thermo-regulation of the flagellum is beneficial in the leaf environment, while the same context-dependent regulation of syringafactin biosynthesis is not. Given the cosmopolitan nature of certain sub-clades of *P. syringae,* such as genome group 2 that includes strain B728a, context-dependent thermo-regulation may be an important evolutionary adaptation. Nutritional status or growth-phase and temperature appeared to interact in the regulation of other traits as well, further suggesting a strong contextual behavior in *P. syringae*. In Chapter 2, we showed an apparent growth phase effect on the expression of *syfR* and *syfA*, especially in the context of knockouts of ACDH and *ygdP*. Additionally, the ACDH-encoding gene is probably involved in the catabolism of leucine and other related amino acids, while the comparative analysis of the transcriptome of Δ*ygdP* with the wild type strain revealed that it plays this role in a temperature-dependent manner. Together these results suggest that certain forms of thermo-regulation in *P. syringae* are linked to amino acid metabolism. While more targeted investigations of the specific compounds involved and their interactions with regulatory components will be needed to understand the role of temperature stimuli, there clearly is a great complexity to these interactions. Such interactions also suggest that temperature, by influencing the abundance or composition of nutrients that are leaked onto the leaf surface may indirectly also modify the temperature-dependent responses of *P. syringae* while on plants. Increasing temperature generally increases the permeability of the cuticle, resulting in increased diffusion of water to the leaf surface [230, 231]. The diffusion of both lipophilic [232] as well as polar [233] molecules should also increase. Such temperature-enhanced diffusion may also occur through the "aqueous pores" that coincide with preferred sites of *P. syringae* colonization on the leaf surface. Additionally, choline, which is used by *P. syringae* both as an osmoprotectant as well as a carbon and nitrogen source [19, 70], accumulated to higher concentrations on tomato at warmer temperatures (above 25 °C) than it did at cooler temperatures [107]. Considering that thermo-regulation in *P. syringae* appears to be dependent on the nutritional context of the cells, and that temperature may affect the amount and/or type of nutrients that are leaked onto the leaf surface, future research should be aimed at identifying those particular

compounds that most strongly interact with thermo-regulation. Clearly, more work is also needed to determine how the profile of compounds leaked onto the leaf surface is affected by temperature.

Pseudomonas syringae above (and beyond) the phyllosphere

While the research and interpretation we have presented in this dissertation have been linked to the life-style of *Pseudomonas syringae* as a plant-associated bacterium, certain sub-clades of *P. syringae* such as genomic group 2 have recently been recognized to be cosmopolitan organisms, associated with epilithic (rock-associated) biofilms, river water, snow pack, as well as clouds, and has lead to the proposal that the life history of at least these groups of *P. syringae* is linked to the water cycle [38, 234, 235]. Additionally, since the *P. syringae* communities sampled on different continents are similar in structure, certain *P. syringae* sub-strains apparently migrate on a global scale [234]. Given its global distribution, *P. syringae* is likely to encounter temperature extremes well beyond the range studied in this work, and likely will exhibit additional thermo-regulated traits that are not necessarily linked to the plant habitat. Future research focusing on such traits, and whether their regulation is also linked to other environmental stimuli, should provide great insight into the temperature-dependent behavior of *P. syringae*.

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