**AN ABSTRACT OF THE DISSERTATION OF**

Cheryl A. Whistler for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on March 1, 2000. Title: Regulation of Antibiotic Production and Stress Response by the Biological Control Organism *Pseudomonas fluorescens* Pf-5.

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Joyce E. Loper / Walter Ream

The soil bacterium *Pseudomonas fluorescens* strain Pf-5 produces the antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), and 2,4-diacetylphloroglucinol (Phl), which contribute to its ability to inhibit plant pathogens. In an effort to understand factors that influence antibiotic production, mutants with altered antibiotic production profiles were studied. A mutant that over-produces Plt and Phl is deficient in $\sigma^S$, which regulates phenotypes associated with stress response. A two-component regulatory system comprised of GacA/GacS was required for normal expression of $\sigma^S$ and development of resistance to oxidative stress. Two mutants that over-produce Plt were characterized in this study. One mutation was in *lon*, which encodes a serine protease. Both Lon and $\sigma^S$ regulated *plt* biosynthetic gene transcription. Lon was induced by heat stress and required for tolerance to ultra-violet irradiation, but appears to regulate Plt production independently of $\sigma^S$. The characterization of the above mutants shows that antibiotic production and stress response are associated. Another mutation that results in Plt over-production was located in *ptsP*, a paralog of *ptsI*, which is involved in carbon utilization. The *ptsP* mutant was comparable to the wild-type strain in its ability to utilize various carbon sources and, in contrast to other Plt over-producing strains was not seriously

compromised in its ability to survive certain stresses. The capacity of Plt over-producers to protect cotton and cucumber seedlings from disease caused by the pathogen *Pythium ultimum* was tested. Although differences were not significant in these studies, trends suggest that overproduction of Plt or Phl can increase biocontrol. To test the ability of Pf-5 to utilize seed exudates that trigger germination of sporangia of *P. ultimum*, two transposon derivatives of Pf-5 were selected that cannot utilize linoleic acid. In one mutant, the disrupted gene *aceB* encodes a malate synthase. The identity of the disrupted gene is unknown in the second mutant. The mutants were impaired in biological control of *P. ultimum* on cucumber suggesting that signal interference may contribute to biocontrol by Pf-5.

# Regulation of Antibiotic Production and Stress Response by the Biological Control Organism *Pseudomonas fluorescens* Pf-5

by

Cheryl A. Whistler

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Cheryl A. Whistler, Author

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## CONTRIBUTION OF AUTHORS

Dr. Alain Sarniguet initiated the research that provided the basis for this dissertation, was involved in completing western blots to detect RpoS protein, and assisted with the analysis and writing of the manuscript presented in chapter 2. Dr. Nathan Corbell isolated the GacA(V203) mutant, perfomed sequence analysis of the wild-type and mutant alleles, and wrote methods, results, and discussion sections pertaining to those studies as presented in chapter 2. Dr. Virginia Stockwell performed UV sensitivity analysis of Lon mutant, provided helpful discussions, and assisted with preparation of the manuscript presented in chapter 3. Kathleen Ferguson assisted with the cloning and sequence analysis of *ptsP*, performed phenotypic analysis of the PtsP mutant, and was involved in the preparation of the manuscript presented in chapter 4. Rachael Andrie constructed transcriptional fusions to the *ptsP* gene and performed ice nucleation assays presented in chapter 4. Dr. Jennifer Kraus was involved in the design, analysis, and writing of chapter 5. Dr. Eric Nelson assisted with the experimental design of chapter 5.

## TABLE OF CONTENTS

## TABLE OF CONTENTS (Continued)

## TABLE OF CONTENTS (Continued)

## TABLE OF CONTENTS (Continued)

Page

## TABLE OF CONTENTS (Continued)

Page

## LIST OF FIGURES

## LIST OF FIGURES (Continued)

## LIST OF TABLES

## DEDICATION

For my parents,

Helen M. Whistler

and

Dr. David P. Whistler

# Regulation of Antibiotic Production and Stress Response by the Biological Control Organism *Pseudomonas fluorescens* Pf-5

## Chapter 1. Introduction:

For millennia, humans have manipulated crops and the physical environment they depend on through technology. However, maintaining crop health under our current practices of large-scale commercial monoculture is challenging. Our lack of understanding of the association of pathogenic and beneficial microorganisms with plants continues to diminish the productivity of agriculture.

Much of what we understand about microorganisms that live in association with plants as well as animals has resulted from research directed towards host-microbe interactions that result in the rare condition of disease (97). This is understandable in light of the obvious impact that disease has on the health of plants and animals. Our current practices of disease management reflect our lack of understanding of beneficial biological interactions. For example, the control of crop diseases typically entails the application of broad-spectrum pesticides that inhibit the growth of microorganisms, both harmful and beneficial, which can create a void in the biological community. Too often, harmful organisms fill this void.

Even when pesticides provide an effective means to manage disease and pests of agricultural plants, they can have detrimental effects on human health and the environment. For example, although fungicides represent less than 10% of the total

application of pesticides per acre in 1987, they are estimated to represent 60% of the total dietary carcinogenic risk (20). In fact, 90% by weight of all fungicides now applied are considered potential carcinogens (20). As public apprehensions increase about groundwater contamination and pesticide residues in food, alternative technology must be developed.

Traditional practices to control disease include crop rotation, fallowing, or the addition of organic amendments that could increase the populations of beneficial bacteria or reduce the number of propagules of certain soilborne pathogens (55), and thus the amount of disease. Other practices such as long-term monoculture favor the propagation of beneficial organisms that contribute to the suppressive nature of some soils (119). These examples demonstrate that a balance exists in the soil between beneficial and harmful organisms and their plant hosts. This balance may favor disease, but may alternatively favor disease suppression, and even enhance plant health. Furthermore, a better understanding of the relationship between beneficial and harmful organisms would empower researchers to manipulate microbial populations and characteristics to the benefit of crops. It is the exploitation of disease suppressive organisms that has spurred the development of biological control.

Although biological control has existed in practice for years, its scientific study and development as an alternative to chemicals has accelerated during the past few decades. Biological control is defined as the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man (21). Biological control has the potential of being both safe and effective. Some notably successful biological control organisms used for the control

of pests and pathogens include ladybugs for the control of aphids, *Bacillus thuringiensis* for the control of harmful insects such as caterpillars and worms, predatory nematodes for the control of soilborne insect pests, and *Pseudomonas fluorescens* A506 for the control of the bacterial pathogen *Erwinia amylovora*, causal agent of fire blight.

One of the chief obstacles impeding the widespread application of biological control agents in agriculture is their variable performance under field conditions (109,159). An understanding of factors that contribute to biological control is likely to be prerequisite to the development of biological control as a viable alternative to chemical pesticides. To this end, the research described herein was initiated to aid in our understanding of factors that influence mechanisms of biological control.

## 1.1 *Pythium ultimum*: a model plant pathogen for suppression by biological control.

*Pythium* spp. are estimated to be the most important pathogens of seeds and seedlings of cultivated crops before their emergence from the soil (94). *P. ultimum* is an oomycete that forms asexual sporangia, which can survive for long periods of time in dry soil and serve as an important source of inoculum. Some isolates of *P. ultimum* also form oospores, thick-walled sexual spores that are resistant to desiccation. Viable oospores have been recovered from dried soil after as long as 12 years (57). Under favorable conditions and in response to seed exudates such as amino acids, carbohydrates, or long-chain fatty acids, spores mature and germinate (94). Due to their production of a large number of exudates, germinating seeds are particularly vulnerable to very rapid infection

by *Pythium* spp.. Seeds can become infected by germ tubes arising from oospores or sporangia within 1 to 14 hours after planting in infested soil (94). Indeed, if infection is not successful within the first few hours or days after planting of seeds, seedlings become increasingly resistant to infection. Commercial practices to control this pathogen include application of chemical pesticides such as metalaxyl, and modification of cultural practices, in particular planting bed preparation and method of irrigation (94).

Many environmental factors influence the severity of seedling diseases caused by *Pythium* spp. directly by influencing the pathogen and indirectly by influencing antagonistic microbes. Of particular importance are soil moisture, temperature, and pH (94). Increased moisture may facilitate the diffusion of exudates from seeds or roots that are needed for germination and growth of the pathogen. Additionally, as soil moisture increases, $O_2$ decreases and $CO_2$ increases, which may favor the proliferation of facultative anaerobic microorganisms such as *Pythium* spp. Temperature is also likely to influence the proliferation of *Pythium* spp. Soil pH is known to influence the bioavailability of many soil minerals or compounds (85). Soil pH also influences the formation of resting structures by *Pythium* spp. as well as their susceptibility to lysis (94). Many of these same environmental factors not only influence *Pythium* spp. but also resident soil microflora, which could inhibit *Pythium* spp.. For example, proliferation of antagonistic microbes may be favored under moisture, temperature, pH conditions that differ from those that favor *Pythium*. The ability of environmental factors to favor the proliferation of suppressive organisms over *Pythium* spp. was exemplified when adjustment of soil pH from 5.3 to 7.4 reduced seed infection. This effect was ameliorated

by the addition of chloramphenicol, which presumably reduced populations of antagonistic bacteria (38).

For the past several decades, much biological control research has focused on Pythium damping off-disease because plant-pathology research has provided a strong foundation in our understanding of the ecology and lifestyle of *Pythium* spp. (94) making it an ideal system for basic research. For example, research demonstrates that native microflora can reduce Pythium damping-off disease providing evidence that the pathogen is amenable to control by biological means. Planting mixes amended with certain mature composts are suppressive against Pythium damping-off presumably due to the activities of suppressive microorganisms present in the compost (16,17,25).

## 1.2 *Pseudomonas* spp. possess multiple attributes for the suppression of phytopathogens

Fluorescent pseudomonads are promising biological control organisms of plant pathogens, such as *P. ultimum*. *Pseudomonas* spp. are ubiquitous soil bacteria that live in association with plant hosts where they inhibit bacterial and fungal plant pathogens (27,91,151). Perhaps one of the most important strengths of *Pseudomonas* spp. as biocontrol organisms is that they posses multiple characteristics that contribute to their suppression of disease. *Pseudomonas* spp. may suppress fungal pathogens directly through the production of antifungal compounds. Biological control may also be achieved indirectly, by influencing either the plant host, making it more resistant to infection, or by altering the environment. In particular, *Pseudomonas* spp. present in the

soil may outcompete pathogens for compounds, such as carbon and iron, needed for growth or they may interfere with plant-derived spore germination signals.

### 1.2.1 Nutrient competition and signal interference

Because sporangia and oospores of *Pythium* spp. require exogenous nutrients for germination, utilization of seed exudates by naturally-occurring *Pseudomonas* spp. is likely to interfere with the pathogen's ability to sense and respond to the presence of seeds. Added to this the limited window of opportunity that *Pythium* spp. have to successfully infect seedlings, and signal interference becomes a promising mechanism for plant protection. On pea and soybean seeds, for example, *Pseudomonas putida* N1R reduces the available carbon which correlated with suppression of Pythium damping-off (113). Furthermore, van Dijk and Nelson have demonstrated that several biocontrol bacteria, including *Pseudomonas* spp., reduced the germination of sporangia of *P. ultimum* by utilizing long chain fatty acids that function as the primary germination stimulants of *P. ultimum* in cotton seed extracts (153).

### 1.2.2 Induced resistance

Exposure of plants to avirulent pathogens (those that are recognized by the plant host which responds with rapid necrosis at the point of contact) induces resistance to a broad range of pathogens, a process known as systemic acquired resistance (SAR)(132). Some non-pathogenic bacteria can also trigger a state of resistance, distinct from SAR,

known as induced systemic resistance (ISR) (73). Although the SAR and ISR both result in a resistant state, the underlying mechanisms resulting in resistance appear to differ. For example, ISR inducing bacteria do not induce necrosis and do not require salicylic acid as a plant signal (156). Additionally, pathogenesis-related (PR) proteins induced during SAR, are not always induced during ISR (115). Regardless of the means by which plants achieve resistance, this resistant state provides protection from a broad range of pathogens, including fungi, bacteria, viruses, and insects, and can increase vigor and productivity of the plant (115).

Many biological control strains of *Pseudomonas* spp. trigger an ISR response in plant hosts (155). ISR was first demonstrated (87,166) by spatially separating the roots of a susceptible plant inoculated with the pathogen from those first exposed to a rhizosphere biocontrol bacterium. Plants exposed to biocontrol bacteria and then challenged with pathogens had reduced disease compared to plants treated with water. Disease suppression by the biocontrol bacterium is thought to be indirect, and due to changes in the plant host. A caveat to these findings is that antifungal compounds produced by biocontrol bacteria may be translocated through plant tissue, where they may directly inhibit pathogens. As advances in research characterize biological mechanisms for ISR and once molecular markers of ISR are identified, the role of ISR in biological control can be more thoroughly demonstrated.

### 1.2.3 Antibiosis

*Pseudomonas* spp. produce many diverse antibiotics (83,90,116,146,150,151) that contribute to their ability to suppress pathogens. Several lines of evidence support the importance of these compounds in disease suppression. For example, purified compounds applied to seed surfaces provide protection from pathogens (58,59). When antibiotic biosynthetic regions are introduced into antibiotic non-producers, thereby conferring production of the compound, the resulting derivatives are improved in their biocontrol capacity (39,50,54). Specific derivatives of biocontrol strains that overproduce one or more antibiotics also have enhanced biological control capacity (136,138). Conversely, mutants devoid of antibiotic production may be impaired although not deficient in biological control (75,22), demonstrating the importance of multiple mechanisms in the suppression of disease. Variability in production of antibiotics on plant surfaces by a biological control organism may contribute to unreliable performance in the field.

## 1.3 *Pseudomonas fluorescens* strain Pf-5: an organism with diverse biosynthetic capability.

*P. fluorescens* Pf-5, a bacterium isolated from the rhizosphere of cotton, is an effective biocontrol organism against several plant pathogens including *P. ultimum*. Strain Pf-5 produces several antifungal compounds, including 2,4-diacetylphloroglucinol (Phl) (105), pyrrolnitrin (Prn) (58), hydrogen cyanide (HCN) (23), and pyoluteorin (Plt)

(59), each having a unique spectrum of activity against plant pathogenic fungi. One of the advantages of Pf-5 as a model system for the study of biological control is the depth of information available about the diverse compounds it produces. Information about antibiotic biosynthesis and biological control activity is available not only from characterization of Pf-5 but also from characterization of similar biological control strains, which produce one or more of the antibiotics produced by Pf-5. *P. fluorescens* strain CHA0 is one such strain. Although Pf-5 was isolated from Texas and CHA0 was isolated from Switzerland, both strains produce the same complement of antibiotics and comparison of sequenced genes shows a high degree of identity between the two strains. For this reason, we have utilized studies of Pf-5 and the similar strain CHA0 to develop our understanding of antibiotic production and regulation by Pf-5.

### 1.3.1 2,4-diacetylphloroglucinol

Phloroglucinols are phenolic metabolites with broad-spectrum activity against fungi, bacteria, viruses, and to some extent plants. Phl is one of many antibiotics produced by *P. fluorescens* Pf-5 and *P. fluorescens* CHA0. For other biological control organisms, including *Pseudomonas aureofaciens* Q2-87 and *Pseudomonas fluorescens* F113, it is the main or sole antibiotic associated with biocontrol activity. Phl produced by various strains of *P. fluorescens* is suppressive to *Thielaviopsis basicola* (68,157), the causal agent of black root rot of tobacco, *Gaeumannomyces graminis* var. *tritici* (80), the causal agent of take-all of wheat, and Pythium damping-off of sugar beet (36), but it does not appear to contribute to suppression of Pythium damping-off of cucumber (80). This

finding demonstrates an important concept in biological control: the plant host may influence the mechanism by which biological control organisms suppress disease. The impact of Phl on biological control capacity has been further demonstrated by introducing the biosynthetic locus extra-chromosomally into 27 different strains that do not produce Phl (50). The resultant strains produce Phl and suppress take-all disease of wheat (50).

### 1.3.2 Pyrrolnitrin

Prn is a chlorinated phenylpyrrole antibiotic derived from tryptophan. Like Phl, Prn is produced by Pf-5 (58) as well as a number of other *Pseudomonas* spp. with biocontrol activity including CHA0 (68) and BL915 (54). Prn inhibits *Rhizoctonia solani*, *Alternaria* spp., *T. basicola*, *V. dahliae*, *Fusarium* spp., and *Pyrenophora tritici-repentis* (58,114). Prn has minimal activity against *P. ultimum* (58). When the Prn biosynthetic locus from strain BL915 was introduced into two strains that do not produce Prn, the resulting strains gained the ability to suppress *R. solani* damping-off of cotton, further demonstrating the potential of this antibiotic to contribute to biological control (39,54).

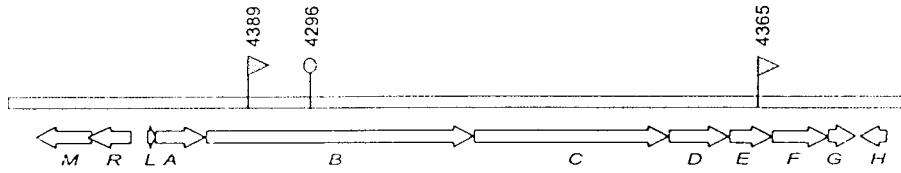
### 1.3.3 Hydrogen cyanide

Many biological control strains of *P. fluorescens* including Pf-5 (75), CHA0 (157), and BL915 (39), produce cyanide from the precursor glycine in the presence of a cofactor such as Fe(III). Cyanide is a general biocide that chelates divalent cations and

interferes with respiration through its interaction with cytochrome c oxidase (157); however, *Pseudomonas* spp. strains are relatively insensitive to cyanide. Cyanide generated by CHA0 inhibits *T. basicola* (157). Introduction of the *hcn* biosynthetic region from CHA0 into cyanide-deficient bacteria increased their suppression against black root rot caused by *T. basicola* (157).

### 1.3.4 Pyoluteorin

Plt is a polyketide antibiotic derived from proline and acetyl CoA (104). Nine clustered genes within a 24-kb genomic region of Pf-5 have been identified that are involved in the biosynthesis of Plt (*pltLABCDEFG* and *pltM*) (Fig. 1.1) (76,104,106). Howell and Stipanovic demonstrated that purified Plt applied to cotton seeds before planting was suppressive to Pythium damping-off (59). However, Kraus and Loper later demonstrated that inactivation of Plt biosynthesis by Pf-5 did not diminish the ability of the strain to control Pythium damping-off of cucumber (75). Two additional studies shed light on the apparent contradiction of these findings. First, Plt derived from strain CHA0 does not contribute to biocontrol of Pythium damping-off of cucumber but it does contribute to the suppression of the disease on cress (95). Secondly, *in situ* expression of pyoluteorin biosynthetic genes by Pf-5 on seed surfaces, assessed with transcriptional fusions, varied between plant species. On cucumber, biosynthetic gene transcription was delayed for over 12 hours after the bacterium was inoculated onto seeds, whereas expression on cotton was induced immediately after inoculation (76). These findings exemplify that differences in plant species can influence antibiotic production, perhaps




| ORF | Size (nt) | Deduced function | Possible function within pathway |
|---|---|---|---|
| *pltA* | 1,347 | halogenase | pyrrole chlorination |
| *pltB* | 7,374 | polyketide synthase | resorcinol synthesis |
| *pltC* | 5,322 | polyketide synthase | resorcinol synthesis |
| *pltD* | 1,632 | halogenase | pyrrole chlorination |
| *pltE* | 1,140 | acyl-CoA dehydrogenase | pyrrole synthesis |
| *pltF* | 1,494 | acyl-CoA synthetase | precursor activation |
| *pltG* | 780 | thioesterase | termination of resorcinol synthesis |
| *pltH* | 669 | transcriptional regulator | pathway specific regulator |
| *pltL* | 264 | unknown | unknown |
| *pltM* | 1,506 | halogenase | pyrrole chlorination |
| *pltR* | 1,029 | transcriptional regulator | pathway-specific regulator |

**Fig. 1.1** Pyoluteorin biosynthetic gene cluster of *P. fluorescens* Pf-5 and description of the putative functions of genes. Triangular flags represent location of transcriptional fusions. Circular tag represents the location of Transposon Tn5 in one strain, JL4296, which was used in these studies.

due to differences in the components of the seed exudates. In liquid culture, Plt production by Pf-5 is repressed by the presence of glucose and favored in the presence of glycerol, demonstrating that exogenous carbon can influence antibiotic production. A derivative of Pf-5 that overproduces Plt and Phl (114,136) enhanced cucumber seedling survival of Pythium damping-off compared to the wild-type strain (136). These results suggest that enhanced or constitutive production of Plt or other compounds by Pf-5 could overcome to some extent the negative influence of plant host on antibiotic production. Taken together, these studies indicate that differences in antibiotic production by a biological control organism on plant surfaces correlate with differences in disease suppression.

## 1.4 Regulators of pyoluteorin production.

Many positive and negative regulators of Plt production have been described in Pf-5 and the similar strain CHA0. Two regulatory genes, *pltR* (104) and *pltH* (13), are linked to the antibiotic biosynthetic gene cluster (Fig. 1.1). Several additional global regulatory genes, including *gacA* (80), *gacS* (23), *rpoS* (136), *rpoD* (138), and *pqqF* (139) influence multiple phenotypes in addition to Plt production. The identification of these diverse regulators demonstrates the complexity of antibiotic regulation. These and yet unidentified regulators are likely to integrate diverse signals such as association with a host, environment, nutrient availability, and growth state, to determine if conditions are appropriate for antibiotic production.

### 1.4.1 PltR

The regulatory gene, *pltR*, is linked to the *plt* biosynthetic operon and encodes PltR, a member of the LysR family of transcriptional regulators. LysR members often require a cofactor for activity, but none has been identified for PltR. When the *pltR* gene was disrupted in Pf-5, the resulting strain did not produce Plt (104). Furthermore, disruption of *pltR* diminished transcription of three biosynthetic genes, *pltB*, *pltE*, and *pltF* (104). These results suggest that *pltR* encodes a transcriptional activator required for Plt production. Upstream of *pltR* is an inverted repeat sequence conforming to the Ebright box motif conserved among many promoters regulated by LysR-type proteins (35,137), suggesting that PltR may activate or amplify its own transcription (104). Binding sites for PltR have not been identified upstream of or within the sequence encoding *plt* biosynthetic genes (104).

### 1.4.2 PqqF

The product of the *pqqF* gene is required for synthesis of pyrroloquinoline quinone (PQQ), which is an essential cofactor in alcohol and glucose dehydrogenase of Gram-negative bacteria, and therefore influences their ability to utilize glucose and alcohol. In strain CHA0, a *pqqF* mutant is unable to utilize alcohol as a sole carbon source and overproduces Plt. Because glucose appears to repress Plt production by Pf-5,

Schnider and colleagues speculate that the repressive mechanism may involve glucose dehydrogenase, and that the glucose dehydrogenase deficiency could alter the flux of precursors such as acetyl CoA into the *plt* biosynthetic pathway (139). However, to date this hypothesis remains untested and the mechanism of glucose repression is still not understood.

### 1.4.3 GacA and GacS

The sensor kinase GacS and its cognate response regulator GacA, products of the *gacS* and *gacA* genes respectively, are members of bacterial two-component regulatory systems (1,112). GacA and GacS homologs are highly conserved among *Pseudomonas* spp. (71), where they regulate the production of extracellular products often in response to association with hosts (Table 1.1). GacS was first characterized as a regulator of virulence factors in the plant pathogen *Pseudomonas syringae* (60,72) and it has since been identified in a number of other plant pathogenic *Pseudomonas* spp. as well as the human pathogen *Pseudomonas aeruginosa*. In Pf-5, GacS is required for production of the antibiotics Plt, Phl, Prn, HCN, as well as several other extra-cellular products including protease(s), and tryptophan side chain oxidase (23). GacA and GacS regulate antibiotic production in a number of other biological control strains including BL915 (39), and CHA0 (80).

GacS is proposed to contain a membrane bound region responsive to unknown signal molecules outside the cell (1,112), but it is equally plausible that it senses intracellular signals. In response to appropriate signal(s), its intracellular kinase domain

**Table 1.1: Characteristics regulated by GacA and GacS homologs of *Pseudomonas* spp.**

| *Pseudomonas* Species | Characteristics regulated | Reference |
|---|---|---|
| *aeruginosa* | Virulence: Homoserine lactone production, pyocyanin, cyanide, lipase | (121, 123) |
| *fluorescens* | Biological control: Pyrrolnitrin, pyoluteorin, HCN, 2,4-diacetylphloroglucinol, extracellular protease, TSO;<br>Stress response: accumulation of RpoS, oxidative stress tolerance | (23, 77, 80, 95, 160) |
| *marginalis* | Virulence: pectate lyase, protease, levan, pyoverdin | (81) |
| *syringae* | Virulence: lesion-forming ability; exoenzyme, tabtoxin, and syrinogmycin production; swarming motility. | (5, 61, 71, 126) |
| *tolaasii* | Virulence: lesion formation; tolaasin toxin, extracellular protease; fluorescent pigment and motility | (47) |
| *viridiflava* | Virulence: pectate lyase, levan | (82) |

autophosphorylates at a conserved histidine residue using ATP as the donor. The phosphoryl group is then transferred to a conserved aspartate residue in the response regulator GacA. Two paradigms currently exist for GacA/S regulation of antibiotic production by fluorescent pseudomonads. The traditional paradigm describes that the phosphorylated GacA binds to DNA by virtue of its typical C-terminal helix-turn-helix DNA binding motif and activates transcription of biosynthetic genes directly (80). Indeed, GacS influences the transcription of *plt* biosynthetic genes assessed with transcriptional fusions to *plt* promoters (22). Unfortunately, attempts to demonstrate that GacA binds upstream of the *plt* biosynthetic operon were not successful (22). GacA influences hydrogen cyanide production by strain CHA0 (8) through a post-transcriptional mechanism, demonstrating a second paradigm for regulation of antibiotic production. With the aid of *hcnA*'-'*lacZ* translational and *hcnA-lacZ* transcriptional fusions carried extrachromasomally on low copy number plasmids, researchers observed that a *gacA* mutation did not alter *hcnA* transcription but translation was reduced by 50-fold (8). Sequence and mutational analysis identified that the same region in the 5' leader sequence of the *hcnA* RNA transcript that is required for GacA activation is also required for repression by RsmA, a molecule that binds to RNA, blocking its translation (8). RsmA in turn is presumed to be antagonized by a non-coding RNA RsmB (8). Researchers suggest that GacA and RsmA bind the same leader sequence (8). An alternative explanation is that GacA positively regulates HCN production indirectly by activating RsmB transcription, consistent with the traditional paradigm of GacA as a transcriptional regulator.

### 1.4.4 RpoS ($\sigma^S$) and RpoD ($\sigma^D$)

RpoD (also called $\sigma^D$) is the sigma subunit of RNA polymerase that is responsible for recognizing promoters and directing the transcription of genes required for growth. Because of its role in transcription of housekeeping functions of the cell, it is indispensable to bacteria. RpoS (also called $\sigma^S$) is an alternative sigma factor. In *Escherichia coli*, it directs the transcription of many genes expressed upon entry into stationary phase (88) and in response to starvation (96,108) or osmotic stress (52). Many of the genes transcribed by the $\sigma^S$ form of RNA polymerase confer stress tolerance on stationary phase cells (96,134).

In Pf-5, an *rpoS* mutant over-produces the antibiotics Plt and Phl and does not produce Prn (75,136). In strain CHA0, multiple copies of *rpoD* led to overproduction of Plt (138), suggesting that there is a relationship between *rpoS* and *rpoD* in the regulation of Plt. The *rpoS* mutant of Pf-5 also is better than the wild-type strain in suppressing Pythium damping-off disease of cucumber (136), most likely because of the overproduction of Plt and Phl. Unfortunately, *rpoS* mutants of Pf-5 are also less tolerant to stresses imposed by exposure to oxidation, salt, starvation, and freezing (136,145).

That *rpoS* influences antibiotic production is clear; however, the mechanism of its repression is unknown. Since sigma factors function by positively directing gene transcription and because *rpoS* negatively influences Plt production, it is likely that *rpoS* influences antibiotic production indirectly. Several mechanisms for indirect regulation of Plt by $\sigma^S$ are possible. $\sigma^S$ could influence Plt post-transcriptionally, by reducing translation or stability of *plt* biosynthetic gene transcripts, or the availability of substrates

required for Plt production. Alternatively, $\sigma^S$ could interfere with the transcription of *plt* biosynthetic genes.

Several indirect mechanisms for $\sigma^S$ repression of *plt* transcription are possible. Because $\sigma^S$ and $\sigma^D$ have opposite affects on pyoluteorin production, it is likely that transcription of the *plt* biosynthetic operon is determined in part by the relative balance of one sigma factor to the other. One possible mechanism proposed in a hypothetical model (Fig. 1.2) is that the $\sigma^D$-form of RNA polymerase holoenzyme directs *plt* transcription and that $\sigma^D$ and $\sigma^S$ compete for binding to core RNA polymerase. Under conditions where $\sigma^D$ is available and any other required regulators such as GacA/S and PltR are functional, *plt* gene transcription would be high and the antibiotic would be produced provided substrates are available. However, under conditions where the level of $\sigma^S$ exceeds $\sigma^D$, as would be expected during the transition between exponential and stationary phase or when cells are exposed to certain stresses, then *plt* gene transcription would be low, even in the presence of GacA/S and PltR. Direct competition between sigma factors is possible but other indirect mechanisms are equally plausible. A second mechanism represented in the model is that $\sigma^S$ may direct the transcription of a repressor that specifically regulates *plt* transcription, whereas $\sigma^D$ directs transcription of an activator.

## 1.5 Statement of research objectives.

The primary goal of my dissertation research was to elucidate the regulatory network in which $\sigma^S$ participates to control antibiotic production by Pf-5. Although the

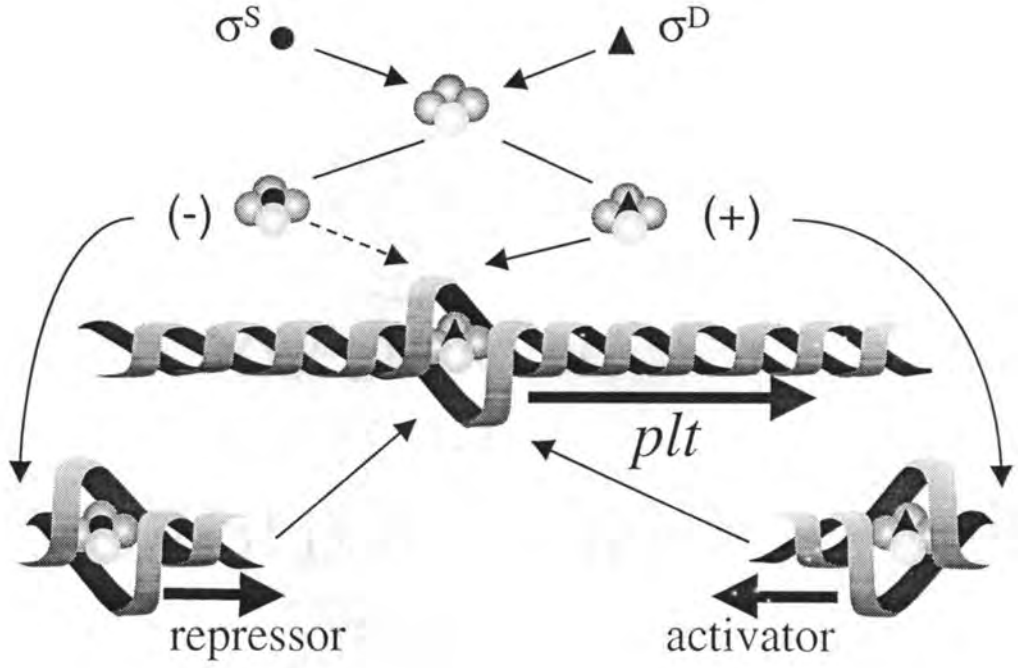



**Figure 1.2:** Model depicting how interaction between sigma factors (σ) for limited core RNA polymerase enzyme could influence transcription of *plt* biosyntheic genes and Plt production. In the event that $\sigma^D$ positively directs transcription core enzyme is limiting, an abundance of $\sigma^S$ would lead to reduced transcription of *plt* biosynthetic genes. Alternatively, $\sigma^S$ may direct transcription of a repressor of *plt* transcription and $\sigma^D$ could direct transcription of an activatior.

research herein tests possible mechanisms of $\sigma^S$ regulation, the purpose of these studies is to understand how regulatory networks within Pf-5 interact to influence antibiotic production. I seek to identify factors that influence Plt production and therefore impact disease suppression. By understanding antibiotic regulatory mechanisms, we may be able to alter the strain to produce antibiotics more reliably, or to predict the conditions under which Pf-5 will suppress disease optimally.

**Objective I**: Determine if RpoS and GacA/S interact to influence antibiotic production.

An initial objective was to determine if $\sigma^S$ interacts with the GacA/GacS regulatory pair to control antibiotic production in Pf-5. Because $\sigma^S$ is a global regulator of many phenotypes expressed during stationary phase and GacA/S appears to influence secondary metabolites that are frequently expressed during stationary phase, I tested the hypothesis that these global regulators are members of the same regulatory network, which is described in chapter 2.

**Objective II**: Determine if $\sigma^S$ represses Plt production by positively regulating a repressor of pyoluteorin production disrupted in the Plt Class VI or VII mutants.

A second objective of my research was to characterize two classes of Pf-5 mutants that overproduce Plt. The Plt-overproduction phenotypes of these Class VI and VII mutants (75) were consistent with those expected if the mutations were in genes encoding repressors of Plt production. Like *rpoS* mutants of Pf-5, these mutants overproduce Plt,

but their sensitivities to stresses were unknown. Therefore, these mutants might provide the superior biological control associated with antibiotic overproduction while lacking the stress sensitivity that compromises the environmental fitness of *rpoS* mutants. Furthermore, even if identified repressors are not intermediates of $\sigma^S$ regulation, they will still provide insight into factors that influence antibiotic production. In chapters 3 and 4, I describe studies characterizing the Plt-overproduction mutants and evaluating the influence of the mutations on *plt* biosynthetic gene transcription. I also tested the hypothesis that the mutated genes were intermediates in the regulatory network through which $\sigma^S$ negatively regulates Plt production.

**Objective III**: Test signal interference as a mechanism for biological control of *Pythium ultimum* by Pf-5.

A secondary goal of my dissertation research was to test signal interference as a mechanism for biological control of Pythium damping-off by Pf-5. It is apparent from studies of Pf-5 that antibiosis is only one mechanism contributing to biological control. The roles of ISR or signal interference in protection of plants from disease are unknown. To test the hypothesis that Pf-5 utilizes plant-derived germination signals, Tn5 was used to mutagenize Pf-5 and derivatives that cannot utilize linoleic acid as a sole carbon source were tested for suppression of Pythium damping-off.

# Chapter 2

## The Two-component Regulators GacS and GacA Influence Accumulation of the Stationary-phase Sigma Factor $\sigma^S$ and Stress Response in *Pseudomonas fluorescens* Pf-5.

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## 2.1 Abstract

Three global regulators are known to control antibiotic production by *Pseudomonas fluorescens*. A two-component regulatory system comprised of the sensor kinase GacS (previously called ApdA or LemA) and GacA, a member of the FixJ family of response regulators, is required for antibiotic production. A mutation in *rpoS*, which encodes the stationary-phase sigma factor $\sigma^S$, differentially affects antibiotic production and reduces the capacity of stationary-phase cells of *P. fluorescens* to survive exposure to oxidative stress. The *gacA* gene of *P. fluorescens* strain Pf-5 was isolated and the influence of *gacS* and *gacA* on *rpoS* transcription, $\sigma^S$ levels, and oxidative stress response of Pf-5 was determined. A GacA$^-$ mutant of Pf-5 was selected that contained a single nucleotide substitution within a predicted α-helical region, which is highly-conserved among the FixJ family of response regulators. At the entrance to stationary phase, $\sigma^S$ content in GacS$^-$ and GacA$^-$ mutants of Pf-5 was less than 20% of the wild-type level. Transcription of *rpoS*, assessed with an *rpoS-lacZ* transcriptional fusion, was positively influenced by GacS and GacA, an effect that was most evident at the transition between exponential growth and stationary phase. Mutations in *gacS* and *gacA* compromised the capacity of stationary-phase cells of Pf-5 to survive exposure to oxidative stress. The results of this study provide further evidence for the co-regulation of stress response and antifungal metabolite production, and for the predominant roles of GacS and GacA in the regulatory cascade controlling stationary-phase gene expression in *P. fluorescens*.

## 2.2 Introduction

Certain strains of fluorescent pseudomonads inhabit root and seed surfaces where they suppress plant diseases caused by soilborne plant pathogens. Antifungal metabolites produced by *Pseudomonas* spp. *in situ* contribute to the suppression of plant disease (151). *Pseudomonas fluorescens* Pf-5 suppresses plant diseases caused by the chromist *Pythium ultimum* (59) and the fungus *Rhizoctonia solani* (58), and produces at least four antibiotic secondary metabolites, including pyoluteorin (59), pyrrolnitrin (58), 2,4-diacetylphloroglucinol (105), and hydrogen cyanide (HCN) (75). Secondary metabolite production by *Pseudomonas* spp. does not occur uniformly in all environments, but is subject to regulation by genes responding to unknown environmental or physiological signals. Mutations in regulatory genes that alter antifungal metabolite production can improve or diminish biological control by *P. fluorescens* (22, 39, 80, 136). Therefore, elucidation of molecular mechanisms regulating antibiotic secondary metabolite production of *P. fluorescens* is likely to provide opportunities for enhancement of biological control.

In *P. fluorescens,* antibiotic secondary metabolite production and biological control is controlled by a two-component regulatory system comprised of GacS and GacA, which are highly conserved among *Pseudomonas* spp. (71, 126). *gacA* (39, 80) encodes a response regulator in the FixJ family, and *gacS* (also called *apdA, lemA, repA,* or *pheN*) (23, 71) encodes the cognate sensor kinase. GacS (for global activator sensor kinase) was renamed recently to reflect the high degree of deduced amino acid sequence similarity and functional conservation among homologues present in various species of

*Pseudomonas* (71). *gacS* and *gacA* are required for production of pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, HCN, extracellular protease(s), and tryptophan side chain oxidase (TSO) by strains of *P. fluorescens*. GacS$^-$ and GacA$^-$ produce none of these secondary metabolites or exoenzymes (23, 39, 80) and are less effective than wild-type strains in suppressing disease (22, 39, 80, 114). Mutants with nucleotide substitutions in *gacA* accumulate in late stationary-phase cultures of *P. fluorescens* (24, 33). The functional *gacA* allele *gacA*(Y49), which specifies a tyrosine residue at position 49 (80), apparently was isolated from such a mutant of *P. fluorescens* strain CHA0. The wild-type *gacA* gene from strain CHA0, termed *gacA*(D49), encodes an aspartate residue at position 49 (14, 123).

The stationary-phase sigma factor $\sigma^S$ is a third regulator of antibiotic production in *P. fluorescens*. In *Escherichia coli*, $\sigma^S$ directs the transcription of many genes expressed upon entry into stationary-phase (88) and in response to starvation (96, 108) or osmotic stress (52). Some genes transcribed by the $\sigma^S$-RNA polymerase holoenzyme confer stress tolerance on stationary-phase cells of *E. coli* (96, 134). In *P. fluorescens* Pf-5, an *rpoS* mutation is pleiotropic, reducing the bacterium's capacity to survive oxidative stress and altering the spectrum of secondary metabolite production (136). An RpoS$^-$ mutant of Pf-5 overproduces pyoluteorin and 2,4-diacetylphloroglucinol, but produces no pyrrolnitrin (114, 136). Characterization of $\sigma^S$-regulated phenotypes of Pf-5 provided the first evidence that a single regulatory gene can control both antibiotic production and stress response in *P. fluorescens* (136).

The research described here was undertaken to determine if the GacS/GacA two-component regulatory system and $\sigma^S$ interact or operate through independent regulatory

circuits in Pf-5. In this study, we describe the nucleotide sequence of the *gacA* gene of Pf-5, and demonstrate that GacS and GacA influence $\sigma^S$ accumulation and *rpoS* transcription in Pf-5. We also demonstrate that *gacS* and *gacA*, like *rpoS*, are required for optimal survival of stationary-phase cells of Pf-5 when exposed to oxidative stress.

## 2.3 Materials and methods

### 2.3.1 Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids are listed in Table 2.1. *P. fluorescens* was grown at 27°C, with shaking at 200 rpm, in King's medium B broth (KB) (70) for routine culturing; in KB broth amended with 4.4 g/L glycine for HCN assays; in Luria-Bertani medium (LB) (135) for transcriptional fusion studies and western analysis; in nutrient broth (Difco Laboratories, Detroit, MI) supplemented with 2% (wt/vol) glucose or 1% (wt/vol) glycerol for antibiotic extractions; in nutrient broth supplemented with 1% (wt/vol) glycerol for TSO assays; or in M9 minimal medium (M9) supplemented with 0.4% glucose (135) for western analysis and oxidative stress tests. Cells of *P. fluorescens* were enumerated by spreading serial dilutions of bacterial suspensions on KB. Cultures of *E. coli* were routinely grown in LB at 37°C.

**Table 2.1: Bacterial strains and plasmids used in this study**

| Strain or Plasmid | Description | Reference |
|---|---|---|
| *P. fluorescens* | | |
| Pf-5 | Rhizosphere isolate | (58) |
| JL3985 | Derivative of Pf-5; *rpoS*::Tn*5* | (136) |
| JL4135 | Derivative of Pf-5, *gacS*::Tn*5* | (23) |
| JL4477 | Derivative of Pf-5; *gacA*(V203) | This study |
| JL4489 | Derivative of Pf-5; *rpoS*::*lacZ* | This study |
| JL4491 | Derivative of JL4135; *gacS*::Tn*5*, *rpoS*::*lacZ* | This study |
| JL4492 | Derivative of JL4477; *gacA*(V203), *rpoS*::*lacZ* | This study |
| *E. coli* | | |
| DH5α | $F^-$, *endA1*, *hsdR17*, ($r_K^-$ $m_K^+$), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*,ϕ80*dlacZ*, ΔM15, λ- | (135) |
| **Plasmids** | | |
| pUC19 | ColE1 replicon, $Ap^r$ | (135) |
| pRK2013 | Mobilizing plasmid, $Tra^+$, $Km^r$ | (37) |
| pRK415 | IncP1 replicon, polylinker of pUC19, $Mob^+$, $Tc^r$ | (69) |
| pME3066 | 1.65-kb *Bam*HI-*Bgl*II fragment containing a *gacA*(Y49) from *P. fluorescens* CHA0 cloned in pLAFR3, $Mob^+$, $Tc^r$ | (80) |

**Table 2.1** (Continued)

| | | |
|---|---|---|
| pMini-Tn*5lac*Z1 | Mini-Tn5 containing promoterless *lacZ* on a 4.1-kb SmaI fragment cloned in pUT. $Km^r$, $Tc^r$ | (26) |
| pJEL01 | Stably maintained in *E. coli* or *Pseudomonas* spp., Replicons from pVSP1 and PACYC184, $Mob^+$, $Tc^r$ | (136) |
| pJEL5649 | 2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pJEL01, $Mob^+$, $Tc^r$ | (136) |
| pJEL5926 | *rpoS-lacZ* transcriptional fusion cloned in pRK415, $Mob^+$, $Tc^r$ | This study |
| pJEL5937 | 1.65-kb *Bam*HI-*Bgl*II fragment containing *gacA* from Pf-5 cloned in pUC19, $Ap^r$ | This study |

Abbreviations: $Ap^r$, $Km^r$, and $Tc^r$; resistance to ampicillin, kanamycin, and tetracycline, respectively.

### 2.3.2 Recombinant DNA techniques

Methods for transformations, digestions with restriction enyzmes, and gel electrophoresis were standard (135). Blunt end ligation was performed by the thermal cycling method (93). Enzymes were from GibcoBRL Life Technologies (Gaithersburg, Md.). Plasmids were purified by an alkaline lysis procedure (135). Plasmids were mobilized from *E. coli* DH5α donors into Pf-5 in triparental matings with helper plasmid pRK2013 (37). Transconjugants were selected on KB containing 200 μg of tetracycline per ml.

### 2.3.3 Derivation of a *gacA* mutant of Pf-5

Strain JL4477, a derivative of Pf-5 containing a point mutation in *gacA*, was selected by the method described by Duffy and Defago (33). Pf-5 was grown in nutrient broth amended with 0.5% yeast extract at 27°C. After 6 days, dilutions of cultures were spread onto LB agar. Colonies that appeared orange in comparison to the wild-type strain after several days incubation at 27°C (a characteristic of GacA$^-$ mutants (33)) were screened for loss of extracellular protease activity on Bacto Litmus milk agar (Difco Laboratories). Protease-deficient mutants were evaluated for antibiotic production by reverse-phase thin layer chromatography, as described previously (75).

### 2.3.4 Cloning of *gacA* from Pf-5

An extant genomic library of Pf-5 (114) was screened by colony hybridization (43) to identify cosmids that hybridized to *gacA*(Y49) of *P. fluorescens* CHA0 (80). The *gacA*(Y49) probe, a 1.65-kb *Bam*HI-*Bgl*II fragment of pME3066, was labeled with $^{32}$P-dCTP or biotinylated-dATP by using a nick translation kit (GibcoBRL Life Technologies), and purified over a D50 column (International Biotechnologies Inc., New Haven, CT). Southern analysis identified restriction fragments in cosmids that hybridized to the *gacA*(Y49) probe. A 1.65-kb *Bam*HI-*Bgl*II fragment that hybridized to the probe was cloned into pUC19 to construct pJEL5937.

### 2.3.5 Sequence analysis of *gacA* alleles

DNA sequencing and oligonucleotide syntheses were performed at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, OR. Sequencing of double-stranded templates was performed on an ABI model 373A Automated DNA Sequencer using a Taq DyeDeoxy (TM) Terminator Cycle Sequencing Kit (Applied Biosystems, Inc. Foster City, CA) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI model 380B DNA synthesizer using phosphoramidite chemistry (2). Sequencing of the *gacA* gene of Pf-5 was performed using primers complementary to pUC19 DNA on either side of the polylinker and by oligonucleotide primers complementary to regions within the 1.65-kb fragment of pJEL5937 containing *gacA*. Sequencing of an allele of *gacA* with a point mutation

[termed GacA(V203)] was performed directly on the PCR product amplified from the genome of JL4477 with primers designed from the sequence of the *gacA* gene cloned in pJEL5937. DNA and deduced protein sequence analyses, and comparisons with sequences in the GenBank database were accomplished with software from the Genetics Computer Group, Inc., Madison, WI (29). Theoretical secondary structures of proteins encoded by alleles of *gacA* were predicted by PepPlot and PlotStructure programs (Genetics Computer Group, Inc.).

### 2.3.6 Antibiotic quantification

Antibiotics were extracted from cells and spent media of cultures grown in triplicate as described (105). Pyoluteorin and pyrrolnitrin concentrations were quantified from cultures grown for 2 days at 20°C in 5 ml of nutrient broth containing 1% glycerol, a medium that favors their production. The concentration of 2,4-diacetylphloroglucinol was quantified from cultures grown for 4 days in 5 ml of nutrient broth containing 2% glucose, a medium that favors its production. Restoration of antibiotic production in JL4477 harboring plasmid pME3066 was assessed in the absence of tetracycline, which decreases growth rate of the strain. Culture supernatants were extracted twice with ethyl acetate and excess water was removed with anhydrous $MgSO_4$. The bacterial pellet was extracted with acetone. Extracts dissolved in MeOH were analyzed by $C_{18}$ reverse-phase HPLC (0.8 × 10cm Waters Nova-pak radial compression cartridge: 45% water/ 30% acetonitrile/ 25% MeOH [vol/vol]; 1.5 ml/min). Antibiotics were detected with a UV photodiode array detector at 225 (pyrrolnitrin), 310 (pyoluteorin) and 278 nm (2,4-

diacetylphloroglucinol) and quantified against authentic standards. Quantification was done twice with similar results.

### 2.3.7 Exoenzyme production

Extracellular protease was assessed visually as a cleared zone around bacterial colonies on Bacto Litmus milk agar (Difco Laboratories, Inc.).

TSO production was quantified from duplicate cultures of *P. fluorescens* grown at 27°C for 48 h with shaking. Cells from 1 ml of culture were harvested, washed, and suspended in 100 µl ice-cold 50 mM potassium phosphate, pH 6.0. Cells were lysed by two sequential cycles of rapid freezing in liquid nitrogen followed by thawing at 45°C. Cell debris was harvested at 4°C and the supernatant was incubated at room temperature in 1.0 mM –acetyl-L-tryptophanamide, 50 mM potassium phosphate, pH 6.0. The production of *N*-acetyl-α,β-didehydrotryptophanamide was monitored spectrophotometrically at 333 nm ($\epsilon_{mM}$ of 19.8 $cm^{-1}$) at 5 min intervals for 30 min, and at 60, 120, and 180 min (102). The rate of production (*N*-acetyl-α,β-didehydrotryptophanamide/min) was determined from the linear portion of a curve relating absorbance at 333 nm to time. TSO production was normalized to CFU, and reported as enzymatic units per $10^7$ CFU. One unit of TSO is defined as the amount of enzyme that catalyzed the formation of 1 µmol of *N*-acetyl-α,β-didehydrotryptophanamide/min (102). Quantification was done twice with similar results.

### 2.3.8 HCN production

To quantify HCN production (44, 48), duplicate cultures were grown at 27°C for 48 h with shaking. A sample from each culture was incubated in the presence of 0.1 N NaOH at room temperature for 3 h in a chamber sealed with paraffin. The NaOH fraction was diluted with 0.1 N NaOH to a concentration within the linear range of a standard curve relating the concentration of an NaCN standard to absorbance at 578 nm. 0.04 ml of the diluted NaOH fraction was added to 1.0 ml of a solution comprised of two parts 0.2 M 4-nitrobenzaldehyde in ethylene glycol monomethyl-ether, two parts 0.1 M *o*-dinitrobenzene in ethylene glycol monomethyl-ether, and one part 0.088N NaOH. After 25 min incubation in the dark at room temperature, absorbance at 578 nm was measured. HCN was quantified against a NaCN standard curve and normalized to CFU. Quantification was done twice with similar results.

### 2.3.9 Western analysis of $\sigma^S$ and GacS

SDS-PAGE and transblotting for western analysis were performed according to the manufacturer's protocols (Bio-Rad Laboratories, Hercules, Calif.). Exponential-phase cells were obtained from cultures grown to an $OD_{600}$ of 0.2 - 0.4 (for reference see Fig. 2.3A, t=0 to 1 h). Early stationary-phase cells were obtained from cultures grown until the optical density stopped increasing exponentially (see Fig. 2.3A, t=2 to 3 h) and an additional stationary-phase sample was obtained from cultures 4 h later (see Fig. 2.3A, t=6 to 7 h). Optical densities were used to estimate the volume of each culture that would

provide an equivalent number of bacterial cells. Cells from that volume were harvested, immediately frozen in an ethanol and dry-ice bath, and extracted by boiling in protein sample buffer (Bio-Rad Laboratories) containing 5% 2-mercaptoethanol. Proteins were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) for western analysis. Blots were incubated with polyclonal antibodies to *E. coli* $\sigma^S$, generously supplied by K. Tanaka (149), and the antibodies were detected by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham Life Science Inc, Arlington Heights, Illinois). Blots were stripped at 65°C for 30 min in a solution of 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, and 62.5 mM Tris-HCl pH 6.7. Blots were then incubated with polyclonal antibodies to GacS (previously LemA) from *Pseudomonas syringae*, generously supplied by T. Kitten and D. K. Willis (126), and the antibodies were detected by ECL. $\sigma^S$ and GacS were quantified using a Molecular Dynamics Personal Densitometer model SI and ImageQuant software V4.1 (Sunnyvale, CA) and normalized based on Bradford assays for total protein (Bio-Rad Laboratories). In the absence of purified standard for quantification, the linear range of $\sigma^S$ detection was determined using a dilution series of Pf-5 protein extracts. Samples quantified were within the linear range of detection. Cell content of $\sigma^S$ and GacS are reported relative to the amount in stationary-phase cells of Pf-5. Each experiment was done twice with similar results.

### 2.3.10 Transcription of *rpoS*

A transcriptional fusion of *lacZ* to *rpoS* of Pf-5 was constructed by inserting a 4.1-kb blunt-ended *Sma*I fragment from pMini-Tn*5lacZ1* (26) into a blunted *Xho*I site, located at 36 nucleotides from the 3' end of *rpoS*. The *rpoS*::*lacZ* transcriptional fusion cloned in pJEL5926 was exchanged with the genomic copy of *rpoS* in Pf-5 to derive JL4489, in JL4135 to derive JL4491, and in JL4477 to derive JL4492 by marker-exchange mutagenesis as described previously (75). The *rpoS*::*lacZ* mutation in each strain was complemented with pJEL5649, a multicopy plasmid carrying the wild-type *rpoS* gene. From duplicate cultures of each strain grown in LB medium, β-galactosidase activity was determined at 1 h intervals for 8 h as described by Miller (98). Cells were made permeable with sodium dodecyl sulphate and $CHCl_3$, incubated for 10 min at 28°C, and *o*-nitrophenyl-β-D-galactopyranoside was added to a final concentration of 0.66 mg/ml. β-galactosidase was expressed as Miller units (98) and CFUs were determined to verify that optical density was an accurate representation of cell density in all strains. The experiments were done twice with similar results.

### 2.3.11 Stress response

Methods for determining response of *P. fluorescens* to exposure to $H_2O_2$ were as previously described (136) with slight modifications. Stationary-phase cells were harvested at 4 and 8 h after the optical density of cultures stopped increasing in M9 medium with 0.4% glucose. Harvested cells were washed once and suspended in 5 ml of

M9 medium without glucose to obtain an $OD_{600}$ of 0.2. Suspended cells were exposed to 15 mM $H_2O_2$, incubated with shaking at 27 °C for 1 h, and CFUs were enumerated at 20 min intervals. Three replicate cultures were evaluated for each treatment. The experiment was done twice with similar results.

### 2.3.12 Nucleotide sequence accession number

The GenBank accession number for the DNA sequence of the *gacA* gene of *P. fluorescens* Pf-5 is AF065156

## 2.4 Results

### 2.4.1 Sequence analysis of *gacA* alleles

A 1.65-kb *Bam*H1-*Bgl*II fragment that hybridized to *gacA* from *P. fluorescens* CHA0 (80) was identified from a genomic library of Pf-5. The deduced amino acid sequence of a 639-bp ORF present on the fragment is identical to the wildtype GacA(D49) of *P. fluorescens* CHA0 (123), and the ORF was therefore identified as *gacA* of Pf-5. JL4477, a spontaneous mutant of Pf-5 exhibiting the colony morphology described for GacA$^-$ mutants of CHA0 (33), had an allele of *gacA* with a sequence that differs from the *gacA* of the parental strain in a single nucleotide. Nucleotide 607 is a C in *gacA* of Pf-5 and a T in the mutant. Consequently, the deduced amino acid sequence

of the mutant allele, hereafter called *gacA*(V203), has a valine rather than an alanine at position 203. The 314 nucleotides immediately upstream of *gacA* in JL4477 were identical to those upstream of *gacA* in Pf-5.

### 2.4.2 Phenotypic analysis of *gacS*::Tn*5*, *gacA*(V203), *rpoS*::Tn*5*, and *rpoS*::*lacZ* derivatives of Pf-5

Pf-5 produced pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, extracellular protease(s), HCN, and TSO (Table 2.2). JL4135 (*gacS*::Tn*5*) produced no detectable antibiotics, extracellular protease(s), HCN, or TSO. JL4477 [*gacA*(V203)] produced no detectable antibiotics or extracellular protease(s), and it produced less HCN and TSO than Pf-5 produced. Production of pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, HCN, TSO, and extracellular protease(s) by JL4477 [*gacA*(V203)] was restored with plasmid pME3066, containing a functional *gacA*(Y49) allele from CHA0.

Two derivatives of Pf-5 containing insertions in *rpoS* produced more pyoluteorin and HCN, and less pyrrolnitrin than was produced by the wild-type strain (Table 2.2). JL3985 (*rpoS*::Tn*5*) overproduced 2,4-diacetylphloroglucinol, whereas JL4489 (*rpoS*::*lacZ*) produced wild-type levels of this antibiotic. Both JL3985 and JL4489 produced an extracellular protease(s). JL3985 produced no detectable TSO, but JL4489 produced trace levels of TSO. In JL4489, the *lacZ* insertion is located 36 nucleotides from the 3' termunus of *rpoS*, corresponding to domain 4.2 of $\sigma^S$, which is involved in recognition of the -35 region of target promoters (89). JL4489, which exhibited a phenotype intermediate to those of Pf-5 and JL3985, is similar to mutants of *E. coli* that

**Table 2.2: Secondary metabolite and exoenzyme production by *P. fluorescens* Pf-5 and derivatives.**

| Strain | Characteristics | Pyrrolnitrin (μg/ml)[1] | 2,4-diacetylphloro-glucinol (μg/ml)[1] | Pyoluteorin (μg/ml)[1] | HCN (pmol/$10^7$ CFU)[2] | TSO (units/$10^7$ CFU)[3] | Extracellular Protease(s)[4] |
|---|---|---|---|---|---|---|---|
| Pf-5 | wildtype | 3.6 ± 0.4 | 26.2 ± 0.5 | 9.4 ± 1.5 | 30 ± 1 | 2.29 ± 0.29 | + |
| JL4135 | *gacS*::Tn*5* | - | - | - | - | - | - |
| JL4477 | *gacA*(V203) | - | - | - | 9 ± 1 | 0.01 ± 0.02 | - |
| JL4477(pME3066) | *gacA*(V203), *gacA*(Y49) | 1.1 ± 0.4 | 32.3 ± 1.4 | 28.4 ± 1.1 | 38 ± 13 | 0.83 ± 0.16 | + |
| JL3985 | *rpoS*::Tn*5* | - | 60.5 ± 15.6 | 30.9 ± 0.1 | 88 ± 15 | - | + |
| JL4489 | *rpoS::lacZ* | 0.5 ± 0.1 | 29.0 ± 1.1 | 27.8 ± 0.1 | 110 ± 34 | 0.06 ± 0.05 | + |

[1] Values are the means from three replicate cultures ± SD; -, concentration was below the detection limit of 0.1 μg/ml.
[2] Values are the means from two replicate cultures ± SD; -, concentration was below the detection limit of 1 μM.
[3] Values are the means from two replicate cultures ± SD; -, concentration was below the detection limit of 0.01 units. One unit is defined as the amount of enzyme that catalyzed the formation of 1 μmol of *N*-acetyl-α,β-didehydrotryptophanamide/min (36).
[4] +, extracellular protease(s) detected; -, not detected.

produce $\sigma^S$ proteins with altered lengths due to insertions or deletions in domain 4.2; such mutants confer phenotypes that differ quantitatively from the wild-type (63, 165).

### 2.4.3 $\sigma^S$ accumulation

In LB medium, $\sigma^S$ was detected at a low level in exponentially-growing cells of Pf-5. Upon entry into stationary phase, the cellular content of $\sigma^S$ increased by 500% (Fig. 2.1A and B, lanes 1 and 2). $\sigma^S$ was not detected in exponentially-growing cells of JL4135 (*gacS*::Tn*5*) or JL4477 [*gacA*(V203)] (Fig. 2.1A and B, lanes 5 and 7). At the entrance to stationary phase, $\sigma^S$ content in JL4135 and JL4477 was less than 20% of the wild-type level (Fig. 2.1A and B; lanes 2, 6, and 8). Four hours after exponential growth ceased, JL4135 and JL4477 accumulated $\sigma^S$ to a level comparable to that seen in Pf-5 at the entrance to stationary phase (data not shown). Multiple copies of *rpoS* enhanced levels of $\sigma^S$ in derivatives of Pf-5 grown in LB medium (Fig. 2.1A and B; lanes 1, 2, 9, and 10).

$\sigma^S$ was detected in both exponentially-growing cells and in early stationary-phase cells of Pf-5 in M9 medium containing 0.4% glucose (Fig. 2.1C and D; lanes 1 and 2). In contrast, the cellular content of $\sigma^S$ in exponentially-growing cells of JL4135 (*gacS*::Tn*5*) and JL4477 [*gacA*(V203)] was less than 20% the wild-type level (Fig. 2.1C and D, lanes 1, 5, and 7). The cellular content of $\sigma^S$ in early stationary-phase cells of JL4135 and JL4477 was 23% and 49%, respectively, of the wild-type level (Fig. 2.1C and D, lanes 2, 6, and 8). Four hours after exponential growth ceased, the level of $\sigma^S$ increased in JL4135 and JL4477 but did not reach the level observed in Pf-5 (data not shown).

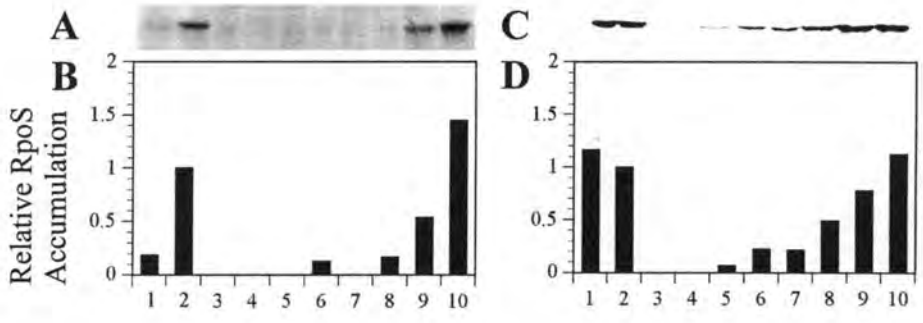


**Figure 2.1**: Relative $\sigma^S$ accumulation. $\sigma^S$ was visualized with antibodies to *E. coli* $\sigma^S$ from western blots of protein extracted from cultures grown in LB (A, B) or M9 minimal medium containing 0.4% glucose (C, D). $\sigma^S$ content in each lane was estimated by scanning western blots with a densitometer. For each sample, $\sigma^S$ content was normalized to total protein content, determined from Bradford assays. The normalized $\sigma^S$ content ($\sigma^S$ content divided by total protein content) for each sample is reported relative to the normalized $\sigma^S$ content of stationary-phase cells of Pf-5 grown in the corresponding medium (B and D, lane 2). Sample numbers correspond to extracts from cells growing exponentially (first of each pair; lanes 1, 3, 5, 7, and 9) or in early stationary-phase (second of each pair; lanes 2, 4, 6, 8, and 10): (1,2) Pf-5, (3,4) JL3985 (*rpoS*::Tn*5*), (5,6) JL4135 (*gacS*::Tn*5*), (7,8) JL4477 [*gacA*(V203)], and (9,10) Pf-5 harboring pJEL5649, a multiple-copy plasmid containing *rpoS* cloned from Pf-5. Scanned images were reproduced for publication using Adobe Photo Shop version 4.0 (Adobe Systems Incorporated, San Jose, Calif.).

### 2.4.4 GacS accumulation

The level of GacS increased slightly (by 20%) in Pf-5 during the transition from exponential growth to stationary phase (Fig. 2.2, lanes 1 and 2). The cellular GacS content was less in JL4477 [*gacA*(V203)] than in Pf-5, for both growth phases and culture media (Fig. 2.2; lanes 1, 2, 7, and 8). In exponentially-growing cells, the GacS content in JL3985 (*rpoS*::Tn*5*) was slightly greater than that in Pf-5 in one experiment (Fig. 2.2; lanes 1 and 3) but no difference was observed in a second experiment (data not shown). Multiple plasmid-borne copies of *rpoS* slightly decreased GacS levels in stationary-phase cells of Pf-5 (Fig. 2.2, lanes 2 and 10) in both experiments.

### 2.4.5 Transcription of *rpoS* assessed with a *lacZ* fusion

β-galactosidase activity conferred by a chromosomal *rpoS-lacZ* transcriptional fusion increased by 300% within a 1 h period when strain JL4489 (*rpoS*::*lacZ*) began the transition from exponential to stationary phase (Fig. 2.3A), reflecting an increase in *rpoS* transcription. In JL4491 (*gacS*::Tn*5*, *rpoS*::*lacZ*) (Fig. 2.3B) and JL4492 [*gacA*(V203), *rpoS*::*lacZ*] (Fig. 2.3C), induction of *rpoS* transcription occurred more gradually and to a smaller magnitude than in strains with functional GacS and GacA proteins (Fig. 2.3A). Multiple copies of *rpoS* decreased β-galactosidase activity of stationary phase cells of JL4489 by 50% (Fig. 2.3C-D).

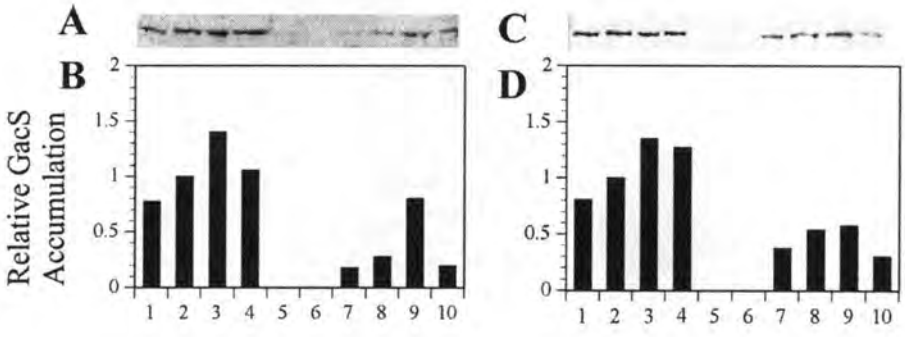


Figure **2.2**: Relative GacS accumulation. GacS was visualized with antibodies to GacS from *P. syringae* (42) on the western blots used for quantification of $\sigma^S$ (Fig. 2). Protein was extracted from cultures grown in LB (A, B) or M9 containing 0.4% glucose (C, D). GacS content in each lane was estimated and reported as normalized values (B and D, lane 2). Sample numbers correspond to extracts from cells growing exponentially (first of each pair; lanes 1, 3, 5, 7, and 9) or in early stationary-phase (second of each pair; lanes 2, 4, 6, 8, and 10): (1,2) Pf-5, (3,4) JL3985 (*rpoS*::Tn*5*), (5,6) JL4135 (*gacS*::Tn*5*), (7,8) JL4477 [*gacA*(V203)], and (9,10) Pf-5 harboring pJEL5649, a mulitple-copy plasmid containing *rpoS* cloned from Pf-5. The truncated form of GacS was not quantified in JL4135 (*gacS*::Tn*5*). Scanned images were reproduced for publication using Adobe Photo Shop version 4.0 (Adobe Systems Incorporated, San Jose, Calif.).

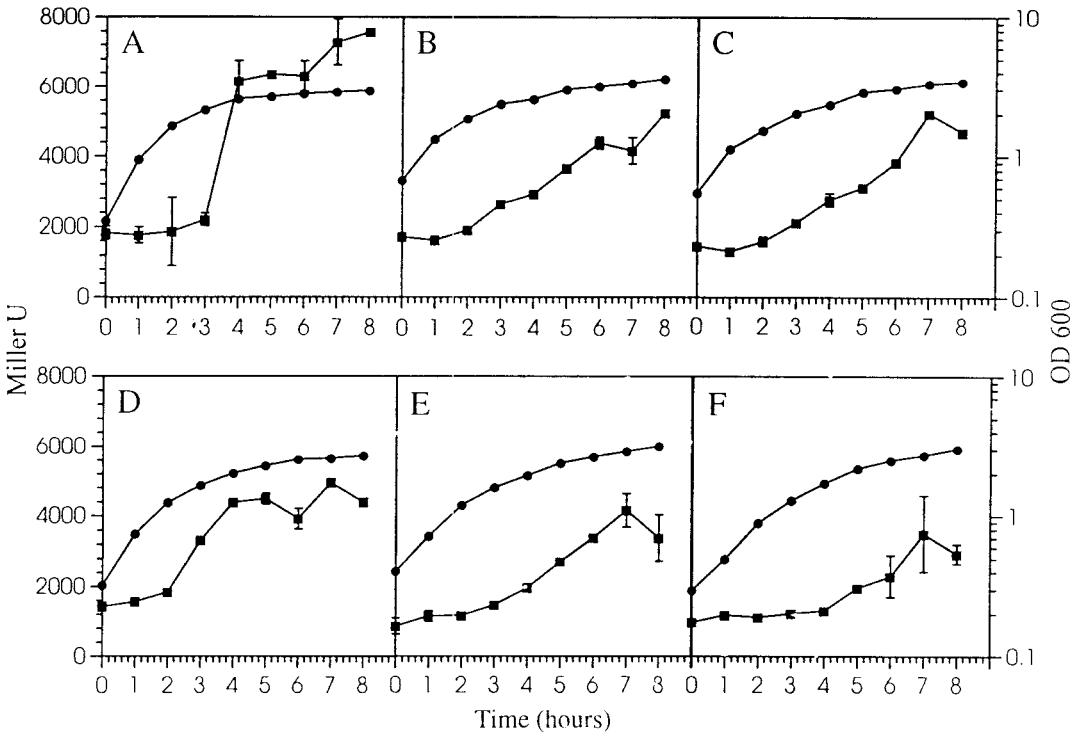


**Figure 2.3**: Growth and β-galactosidase activity of Pf-5 derivatives containing chromosomal *rpoS-lacZ* transcriptional fusions. $OD_{600}$ (•) and β-galactosidase activity expressed in Miller units (▪), was determined at 1 h intervals from duplicate cultures grown in LB medium. Bacterial strains were JL4489 (*rpoS*::*lacZ*) (A), JL4491 (*gacS*::Tn*5*, *rpoS*::*lacZ*) (B), and JL4492 [*gacA*(V203), *rpoS*::*lacZ*] (C). Error bars represent the SD and may be obscured by symbols.

### 2.4.6 Survival of Pf-5, *gacS*::Tn*5*, and *gacA*(V203) derivatives when exposed to oxidative stress

In addition to influencing antibiotic and exoenzyme production, $\sigma^S$ influences the capacity of Pf-5 to survive oxidative stress (136). Stationary-phase cells of JL3985 (*rpoS*::Tn*5*) are more sensitive than stationary-phase cells of Pf-5 to hydrogen peroxide (136). Similarly, JL4135 (*gacS*::Tn*5*) and JL4477 [*gacA*(V203)] harvested from cultures 4 hr (Fig. 2.4) or 8 hr (data not shown) after cell density stopped increasing were more sensitive than Pf-5 to exposure to oxidative stress.

## 2.5 Discussion

This report provides the first evidence that the global regulators GacS and GacA influence the intracellular levels of $\sigma^S$ and stress response of stationary-phase cells of *P. fluorescens*. Mutations in *gacS* and *gacA* reduced $\sigma^S$ accumulation in *P. fluorescens* Pf-5, and compromised the bacterium's capacity to survive exposure to oxidative stress. To date, all known phenotypes of Pf-5 that are positively regulated by *rpoS* also require functional *gacS* and *gacA* genes for optimal expression. These phenotypes are the production of pyrrolnitrin and tryptophan-side-chain oxidase, and the response to oxidative stress. Therefore, our data are consistent with the possibility that the two-component regulatory system comprised of GacS and GacA regulates gene expression, in part, by influencing the levels of $\sigma^S$ in the bacterial cell. Nevertheless, GacS and GacA are required for the expression of certain phenotypes (such as pyoluteorin and 2,4-

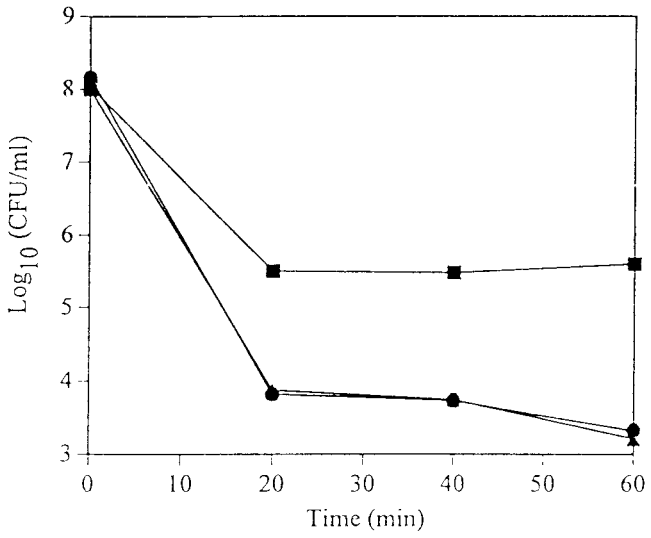


**Figure 2.4**: Survival of oxidative stress. Stationary-phase cells of *P. fluorescens* Pf-5 (■), JL4135 (*gacS*::Tn*5*) (•), and JL4477 [*gacA*(V203)] (▲) were exposed to 15 mM $H_2O_2$, and the numbers of culturable cells were estimated over time. Presented values are the means of three replicate cultures. Error bars representing the SD may be obscured by symbols.

diacetylphloroglucinol production) that are not positively regulated by *rpoS*, indicating that the two-component regulatory system must function through a mechanism other than the control of $\sigma^S$.

Transcription of *rpoS*, assessed with a *lacZ* fusion, was positively influenced by GacS and GacA in Pf-5, which is consistent with the pattern of *rpoS* transcription in *P. aeruginosa* (79, 123). In that species, GacA positively controls the production of the autoinducer *N*-butyryl-homoserine lactone (123), which, through its interaction with the response regulator RhlR, positively influences the expression of *rpoS* (79). An autoinducer involved in quorum sensing has not been found in Pf-5, but it could be among the unknown components of regulatory circuits controlling antibiotic production or stress response in *P. fluorescens*.

GacS accumulation was diminished in a GacA$^-$ mutant and in the presence of multiple plasmid-borne copies of *rpoS*. Positive regulation of GacS content by GacA may be one mechanism by which the relative concentration of the two proteins is controlled. The proper stochiometric balance of other response regulators within the FixJ family and their cognate sensor kinases are required for normal function of these two-component regulatory systems (53). Multiple copies of *gacA*(D49) are not tolerated in *P. fluorescens* CHA0 (123) and multiple copies of *gacA* from Pf-5 can partially compensate for *gacS* mutations in that strain (24), indicating the system is sensitive to relative GacS and GacA contents. The slight negative influence of multiple copies of $\sigma^S$ on GacS accumulation could be mediated non-specifically. For example, $\sigma^S$ could compete for limited pools of core RNA polymerase (64) with other sigma factors that positively regulate GacS, as has been proposed previously as one explanation for the positive effects

of an *rpoS* mutation on pyoluteorin and 2,4-diacetylphloroglucinol production (136, 138). By altering the balance of $\sigma^S$ to other sigma factors, the transcriptional activities of promoters may be altered according to the prevalence of the specific RNA polymerase holoenzyme that transcribes each gene. To date, specific mechanisms by which cellular GacS levels are controlled by GacA and $\sigma^S$ remain speculative, and the above explanations only exemplify possible mechanisms by which such regulation could occur.

Based upon sequence similarities to more well-characterized response regulators within the FixJ family, GacA contains two functional domains, an amino-terminal phosphorylation-induced activator domain and a carboxy-terminal output domain characterized by a helix-turn-helix DNA-binding motif (66). Amino acid substitutions within these functional domains typically destroy GacA function, manifested in the loss of multiple phenotypes controlled by the GacS/GacA two-component regulatory system (7). The GacA(V203) mutant evaluated in this study differed from those described previously because it lost only a subset of phenotypes controlled by the two-component regulatory system. Analysis of the theoretical secondary structures of GacA(V203) and GacA indicated that the valine substitution may interrupt an α-helical region, which is downstream of the helix-turn-helix motif and highly conserved within the FixJ family (66). This possibility is consistent with valine's assignment as a strong β sheet-forming residue, whereas the replaced alanine residue is a strong α helix-forming residue (19), (40). Stibitz (144) demonstrated that mutations within this α-helical region in the response regulator BvgA eliminate expression of two genes but have little effect on the expression of a third gene under the control of the BvgS/BvgA two-component system. Thus, the differential effect of the *gacA*(V203) mutation on phenotypes regulated by

GacS/GacA in Pf-5 is not unprecedented, and may reflect the importance of the α-helical region as a specificity determinant for recognition of various promoters by GacA. The binding site(s) of GacA have not been described, however, and further exploration of this possibility would be facilitated by the identification of such target sequences.

The effects of *gacS* and *gacA* mutations on *rpoS* transcription and $\sigma^S$ accumulation were greater during the transition from exponential growth than later in stationary phase. Nevertheless $\sigma^S$-mediated stress response of GacS$^-$ and GacA(V203) mutants was diminished well into stationary phase. Induction of *rpoS* transcription at the transition between exponential and stationary phases may be critical to the process through which cells develop resistance to environmental stress. Consequently, the stress-resistant state could fail to develop fully if the level of $\sigma^S$ increases gradually or later in stationary phase. Although the present study focused on *rpoS* transcription and accumulated levels of $\sigma^S$, GacS and GacA also could influence *rpoS* translation or $\sigma^S$ stability. Indeed, post-transcriptional regulation plays a prominent role in controlling levels of $\sigma^S$ in *E. coli* (78, 167). In the event that GacS and GacA affect *rpoS* translation or $\sigma^S$ stability, the influence of the two-component regulatory system would likely persist beyond the transition period when *rpoS* transcription was most notably influence.

## 2.6 Acknowledgments

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# Chapter 3

## Lon Protease Influences Antibiotic Production and Ultraviolet Tolerance of *Pseudomonas fluorescens* Pf-5

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## 3.1 Abstract

*Pseudomonas fluorescens* Pf-5 is a soil bacterium that suppresses plant pathogenic fungi due in part to its production of the antibiotic pyoluteorin (Plt). Previous characterization of Pf-5 revealed three global regulators, including the stationary phase sigma factor $\sigma^S$, and the two-component regulators GacA and GacS, that influence both antibiotic production and stress response. In this report we describe the serine protease Lon as a fourth global regulator influencing these phenotypes in Pf-5. *lon* mutants over-produced Plt, transcribed pyoluteorin biosynthesis genes at enhanced levels, and were more sensitive to UV exposure than Pf-5. The *lon* gene was preceded by sequences that resembled promoters recognized by the heat-shock sigma factor $\sigma^{32}$ ($\sigma^H$) of *Escherichia coli*, and Lon accumulation by Pf-5 increased after heat shock. Therefore, $\sigma^H$ represents the third sigma factor (with $\sigma^S$ and $\sigma^{70}$) implicated in the regulation of antibiotic production by *P. fluorescens*. Lon protein levels were similar in stationary-phase and exponentially-growing cultures of Pf-5 and were not positively affected by the global regulators $\sigma^S$ or GacS. The association of antibiotic production and stress response has practical implications towards the success of disease suppression in the soil environment where biological control organisms such as Pf-5 are likely to encounter environmental stresses.

## 3.2 Introduction

*Pseudomonas fluorescens* is a ubiquitous soil microorganism that inhabits the surfaces of seeds and roots. Some strains of *P. fluorescens*, when growing in association with plants, can protect them from infection by plant pathogenic fungi (151). One such strain, *P. fluorescens* Pf-5, produces a number of antibiotics including pyoluteorin (Plt) (59), pyrrolnitrin (Prn) (58), and 2,4-diacetylphloroglucinol (Phl) (105). Of the three antibiotics, Plt is most toxic to the Oomycete *Pythium ultimum* (95), which can infect seeds and roots of many plant hosts causing seedling death and root rot (94).

Antibiotic production by *P. fluorescens* is controlled by several global regulatory genes, which influence multiple phenotypes including stress tolerance, and also by regulatory genes linked to antibiotic biosynthetic gene clusters. For example, Plt production by Pf-5 is controlled by *pltR*, a member of the LysR family of transcriptional activators that is linked to the Plt biosynthesis genes (*pltLABCDEFG* and *pltM*) (104), and the global regulatory genes *gacA*, *gacS*, and *rpoS*. GacA and GacS constitute a two-component regulatory system that is required for the production of antibiotics, exoenzymes, and virulence factors by many *Pseudomonas* spp. (23, 71). Derivatives of Pf-5 harboring mutations in *gacA* and *gacS* do not produce Plt, Prn, or Phl (23, 160) and are impaired in their tolerance of oxidative stress (160). The stationary-phase sigma factor $\sigma^S$, encoded by *rpoS*, has a differential effect on antibiotic production by Pf-5; an *rpoS*::Tn*5* mutant does not produce Prn but overproduces Plt and Phl, and is superior to Pf-5 in suppressing Pythium damping-off of cucumber (136). The *rpoS*::Tn*5* mutant is also impaired in its tolerance to oxidative and osmotic stress (136). GacA and GacS are

necessary for the timely expression and accumulation of $\sigma^S$ during the transition between exponential growth and stationary phase, indicating that GacA and GacS influence a regulatory circuit in which $\sigma^S$ is a participant (160).

Characterization of *gacA*, *gacS*, and *rpoS* in Pf-5 has provided a glimpse of the intricate regulatory networks controlling antibiotic production in *P. fluorescens*. In this study, we cloned and sequenced a fourth global regulatory gene influencing antibiotic production by Pf-5. We identified the gene as a homolog of *lon*, which encodes an ATP-dependent serine-protease (45, 46) found in diverse organisms including bacteria, plants, and animals. In *Escherichia coli*, Lon functions in gene regulation by specifically degrading unstable regulatory proteins (45, 46). Lon is also a heat shock protein that non-specifically degrades damaged and mis-folded proteins (45, 46). In *Bacillus subtilis*, *lon* expression is induced by salt and oxidative stress (128), as well as starvation (51). Because resistance to salt, oxidation, and starvation are regulated by $\sigma^S$ in Pf-5 (136, 145), and *lon*::Tn*5* and *rpoS*::Tn*5* mutants are similar in their over-production of Plt (75, 136), we evaluated the influence of $\sigma^S$ on Lon accumulation. Lon protein levels did not increase during stationary phase and were not reduced in *rpoS* mutants of Pf-5, as would be expected for proteins positively regulated by *rpoS*. We also evaluated two stress responses that are influenced by Lon in other bacterial genera. Lon of Pf-5 was required for optimal tolerance of Pf-5 to UV irradiation and Lon protein was induced by heat shock.

## 3.3 Materials and methods

### 3.3.1 Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids are listed in Table 1. *P. fluorescens* was grown at 27°C with shaking at 200 rpm in King's medium B (KB) broth (70) for routine culturing; in nutrient broth (NB) (Difco Laboratories, Detroit, MI) supplemented with 2% (wt/vol) glucose or 1% (wt/vol) glycerol for antibiotic extractions; in NB supplemented with 1% (wt/vol) glycerol for ice nucleation assays; in M9 minimal medium (M9) supplemented with 0.4% glucose (135) for western analysis; or Luria-Bertani medium (LB) (135) for ultraviolet stress tests and western analysis. Cultures of *E. coli* were grown in LB at 37°C. For cultures of *E. coli*, antibiotic concentrations were as follows: 100 μg of ampicillin (Ap) per ml, 12 μg of gentamycin (Gm) per ml, 50 μg of kanamycin (Km) per ml, and 20 μg of tetracycline (Tc) per ml.

### 3.3.2 Recombinant DNA techniques

Genomic DNA was isolated by cetyltrimethylamonium bromide (CTAB) with isopropanol precipitation (4). Plasmids were purified by an alkaline lysis procedure (135). Methods for transformations, digestions with restriction enyzmes, and gel electrophoresis were standard (135). Enzymes were from GibcoBRL Life Technologies (Gaithersburg, MD). Ends of restriction fragments were blunted with the large subunit of DNA Polymerase (135) and thermal cycling was used for blunt-end ligations (93).

**Table 3.1 Bacterial strains and plasmids used in this study**

| Strain or Plasmid | Description | Reference |
|---|---|---|
| *Pseudomonas florescens*: | | |
| Pf-5 | Rhizosphere isolate | (58) |
| JL3985 | *rpoS*::Tn*5* derivative of Pf-5, $Km^r$ | (114)(136) |
| JL4292 | *lon*::Tn*5* derivative of Pf-5, $Km^r$ | (75) |
| JL4389 | *pltB*::Tn*3-nice* derivative of Pf-5, $Plt^-$, $Ina^+$, $Km^r$ | (76) |
| JL4479 | *lon*::Tn*5* derivative of Pf-5 obtained by marker exchange mutagenesis, $Km^r$ | This study |
| JL4490 | *rpoS*::*lacZ* derivative of Pf-5. | (54) |
| JL4590, JL4591 | Δ*hupB*::*aphI* derivatives of Pf-5 obtained by marker exchange mutagenesis, $Km^r$ | This study |
| JL4594 | *lon*::*aacC1* derivative of JL4389 obtained by marker exchange mutagenesis, $Ina^+$, $Gm^r$, $Km^r$ | This study |
| JL4600 | *rpoS*::*lacZ* derivative of JL4389 obtained by marker exchange mutagenesis, $Ina^+$, $LacZ^+$, $Km^r$ | This study |
| JL4601 | JL4389 harboring pJEL5649, $Ina^+$, $Tc^r$ | This study |
| JL4619 | *lon*::*aacC1*derivative of Pf-5 obtained by marker exchange mutagenesis, $Gm^r$, $Km^r$ | This study |
| JL4620 | *lon*::*aacC1*, *rpoS*::*lacZ* derivative of Pf-5 obtained by marker exchange mutagenesis, $Gm^r$, $Km^r$ | This study |
| JL4621 | *lon*::*aacC1*, *rpoS*::*lacZ* derivative of JL4389 obtained by marker exchange mutagenesis, $Ina^+$, $Gm^r$, $Km^r$ | This study |

**Table 3.1** (Continued)

| | | |
|---|---|---|
| ***Escherichia coli:*** | | |
| DH5α | F⁻ *endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1 recA1 gyrA96 relA1* ϕ80*dlacZ*Δ M15 λ- | (135) |
| SG20781 | *lon*⁺, *cps*::*lac* | (12) |
| **Plasmids:** | | |
| pBR322 | ColE1 replicon, $Ap^r$, $Mob^+$,$Tc^r$ | (135) |
| pRK415 | IncP1 replicon, polylinker of pUC19, $Mob^+$, $Tc^r$ | (69) |
| pRK2013 | Mobilizing plasmid, $Tra^+$, $Km^r$ | (37) |
| pLAFR3 | IncP1 replicon; *cos*, $Mob^+$, $Tc^r$ | (143) |
| pMGm | ColE1 replicon, source of *aacC1* ($Gm^r$) cassette on a 2.0-kb *Sma*I fragment, $Gm^r$, $Tc^r$ | (101) |
| pMKm | ColE1 replicon, source of *aphI* ($Km^r$) cassette on a 1.7-kb blunted *Xho*I fragment, $Km^r$, $Tc^r$ | (101) |
| pUC19, pUC18 | ColE1 replicon, $Ap^r$ | (135) |
| pJEL01 | Stably maintained in *E. coli* or *Pseudomonas* spp., replicons from pVSP1 and pACYC184, $Mob^+$ $Tc^r$ | (136) |
| pJEL5500 | 2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pUC19, $Ap^r$ | (136) |
| pJEL5649 | 2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pJEL01, $Mob^+$ $Tc^r$ | (136) |
| pJEL5913 | 9.7-kb *Eco*RI genomic fragment from JL4292 containing Tn*5* and adjacent DNA cloned into pBR322, $Ap^r$, $Tc^r$ | This Study |

**Table 3.1** (Continued)

| | | |
|---|---|---|
| pJEL5922 | 3.6-kb *Hin*dIII-*Eco*RI genomic fragment from pJEL5913 containing 1.1-kb of Tn5 and flanking DNA from JL4292, cloned in pBR322, $Ap^r$, $Tc^r$ | This Study |
| pJEL5926 | *rpoS*::*lacZ* transcriptional fusion cloned in pRK415, $Mob^+$, $Tc^r$ | (160) |
| pJEL6023 | 4.3-kb *Hin*dIII genomic fragment from Pf-5 containing *lon* and *hupB* genes in pUC19, $Ap^r$ | This study |
| pJEL6161 | 5.7-kb *Hin*dIII fragment containing Δ*hupB*::*aphI* cloned in pUC19, $Ap^r$ | This study |
| pJEL6195 | 2.3-kb *Eco*RI genomic fragment from Pf-5 containing the upstream regulatory region of *lon* cloned into pUC18, $Ap^r$ | This study |
| pJEL6197 | 6.3-kb *Hin*dIII genomic fragment containing *aacC1* casette from pMGm inserted into blunted *Sun*I site internal to *lon*, in pRK415, $Mob^+$, $Gm^r$, $Tc^r$ | This study |
| pJEL6206 | Cosmid library clone of Pf-5 containing *lon* and *hupB*, cloned in pLAFR3, $Tc^r$ | This study |

Abbreviations: $Ap^r$, $Km^r$, and $Tc^r$; resistance to ampicillin, kanamycin, and tetracycline, respectively

### 3.3.3 Cloning of *lon* and the linked gene *hupB* from Pf-5

A pLAFR3 genomic library of Pf-5 (114) was screened by colony hybridization (43) to identify cosmids containing wild-type DNA corresponding to the mutagenized locus in the Plt-overproducing mutant JL4292 (Fig. 3.1a). The probe was a 9.7-kb *Eco*RI fragment containing Tn5 and flanking DNA from the genome of JL4292, which was cloned in pJEL5913 (Fig 3.1b). The probe was labeled with digoxigenin-11-dUTP using a nick translation kit (GibcoBRL Life Technologies). Qiabrane filters (Qiagen, Chatsworth, CA) were prepared and hybridized following the methods for the Genius System of Boehringer Mannheim (Indianapolis, IN). Cosmids were isolated from colonies that hybridized to the probe, digested with *Hin*dIII or *Eco*RI, and analyzed in Southern blots. A 4.3-kb *Hin*dIII fragment from a cosmid that hybridized to the probe was identified by Southern analysis (data not shown) and cloned into pUC19 to construct pJEL6023 (Fig. 3.1b). From the same cosmid, an overlapping 2.3-kb *Eco*RI fragment that hybridized to the digoxigenin-11-dUTP labeled 4.3-kb *Hin*dIII fragment from pJEL6023 was cloned into pUC18 to create pJEL6195.

### 3.3.4 Sequence analysis

DNA sequencing and oligonucleotide syntheses were performed at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, OR. Sequencing of double-stranded templates was performed on an ABI model 373A Automated DNA Sequencer using a Taq DyeDeoxy (TM) Terminator Cycle Sequencing

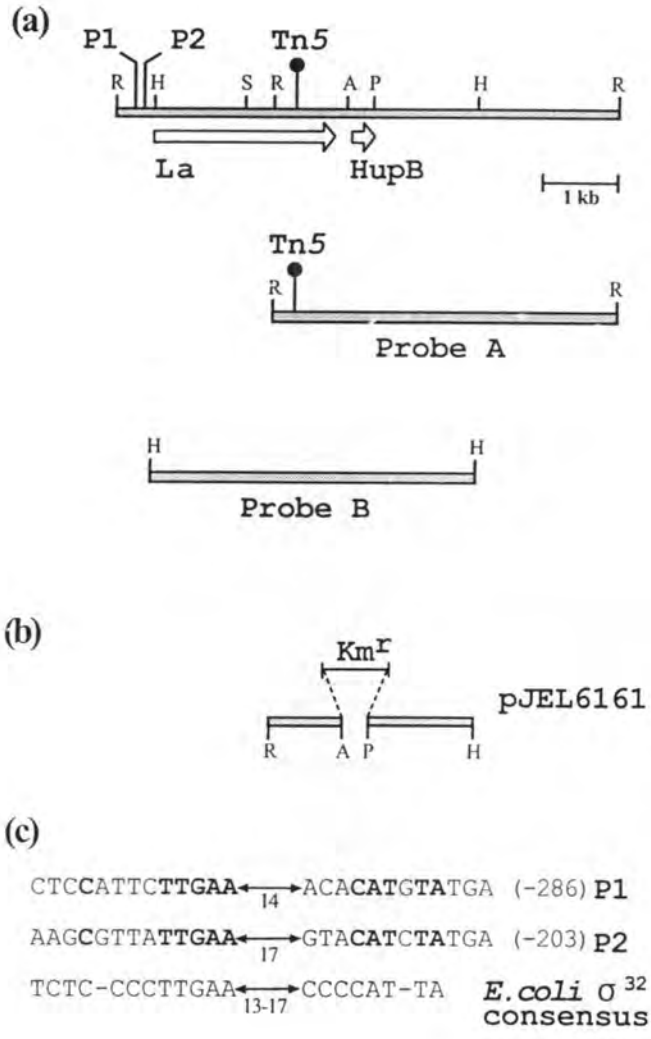


**Figure 3.1:** Schematic representation of the genomic region of Pf-5 containing *lon* and *hupB*. a) Restriction map of the locus and location of Tn5 in the *lon* mutants JL4292 and JL4479. Restriction enzymes: A=*Afl*II, H=*Hin*dIII, P=*Hpa*I, R=*Eco*RI, S=*Sun*I. b) Cloned genomic fragments used in sequence analysis and as probes for Southern blot analysis or for colony hybridization. c) Schematic representation of cloned genomic fragments with insertions in *lon* and *hupB* d) Two potential promoters, P1 at –286 and P2 at –203 relative to the translational start site of *lon* in Pf-5. Bolded nucleotides are identical to $\sigma^{32}$-promoter consensus sequence of *E. coli*. Arrows and numbers represent nucleotides separating the consensus regions.

Kit (Applied Biosystems, Inc. Foster City, CA) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI model 380B DNA synthesizer using phosphoramidite chemistry (2). Sequencing of *lon* and *hupB* cloned in pJEL6023 was initiated using oligonucleotide primers complementary to pUC19 DNA on either side of the polylinker, and was continued using primers complementary to sequenced DNA. Sequencing of the region upstream of *lon* was completed with primers complementary to previously-sequenced DNA. The precise location of Tn*5* in mutant JL4292 was determined from primers complementary to a terminal region of Tn*5* using pJEL5922, a subclone of pJEL5913, as a template. Analyses of DNA and deduced protein sequences and comparisons with sequences in the GenBank database were accomplished with software from the Genetics Computer Group, Inc., Madison, WI (29) and the Staden software package (9).

### 3.3.5 Antibiotic quantification

Antibiotics were extracted from cells and spent media of cultures grown in triplicate by described methods (136). Plt and Prn concentrations were quantified from cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol, a medium that favors their production. The concentration of Phl was quantified from cultures grown for 4 days in 5 ml of NB containing 2% glucose, a medium that favors its production. After centrifugation (5,000 X g, 5 min) of cultures, the bacterial pellet was suspended in 4 ml of acetone, and the suspension was sonicated in an ultrasonic cleaner for 30 sec. Cell suspensions were centrifuged (5,000 X g, 10 min) and the acetone supernatant was

removed and dried under reduced pressure. Culture supernatants were adjusted to pH 2.0 with 1 M HCl and extracted twice with 2 ml of ethyl acetate. The organic phases were combined and dried under reduced pressure. Extracts dissolved in MeOH were analyzed by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Organic extracts were separated on TLC plates (KC18F, Whatman International Ltd, Maidstone, England) in chloroform:acetone (9:1 [vol/vol]) and sprayed with diazotized sulfanilic acid for visualization of compounds (129). Antibiotics were detected by their characteristic colors and $R_f$ values, which conformed to those of authentic standards on TLC plates: Plt, $R_f$=0.32, brown; Prn, $R_f$=0.81, maroon; Phl, $R_f$=0.64, yellow. Antibiotics were separated by HPLC with a 0.8 X 10 cm Waters Nova-pak radial compression cartridge with an 18 min linear gradient from 10% to 100% acetonitrile with 0.1% acetic acid in water at a flow rate of 1 ml/min (Plt and Prn) or with water:acetonitrile:methanol (45:30:25 [vol/vol]) (Phl) at a flow rate of 1.5 ml/min. Antibiotics were detected with a UV photodiode array detector at 310 nm (Plt), 225 nm (Prn), and 278 nm (Phl) and quantified against authentic standards. Antibiotics were quantified by HPLC from two replicated experiments with similar results, and data from one experiment are presented.

### 3.3.6 Derivation of *lon*, *rpoS*, and *hupB* mutants by marker-exchange mutagenesis

A *lon*::Tn*5* mutant. A 9.7-kb Tn*5*-containing *Eco*RI fragment from the genome of JL4292 was cloned into pBR322, which does not replicate in *Pseudomonas* spp., to create pJEL5913 (Fig. 3.1b). pJEL5913 was mobilized from *E. coli* DH5α donors into Pf-5 in a

triparental mating with *E. coli* DH5α containing the helper plasmid pRK2013. Transconjugants from this mating were selected on KB containing 100 μg/ml streptomycin, to counterselect against *E. coli* donors, and 50 μg/ml kanamycin. The resultant marker-exchanged mutant JL4479 had a Tn*5* insertion in the same region as the original mutant JL4292, as determined by Southern analysis (data not shown) with the 9.7-kb *Eco*RI fragment from pJEL5913 as a probe.

*lon*::*aacC1* mutants. We evaluated the effect of a mutation in *lon* on *pltB* transcription using existing fusions of the promoterless ice nucleation reporter gene in Tn*3-nice* to the promoter of *pltB* (*pltB*::Tn*3-nice*) (28, 29, 76, 84). Because Tn*5* and Tn*3-nice* both confer resistance to kanamycin, the 2.0-kb *Sma*I fragment containing *aacC1* from pMGm, which confers gentamycin resistance, was cloned into the blunted *Sun*I site internal to *lon* in pJEL6023 (Fig. 3.1). The resulting 6.3-kb *Hin*dIII fragment, containing *lon*::*aacC1*, was cloned into pRK415, which confers resistance to tetracycline and is not stably maintained in Pf-5, to create pJEL6197. pJEL6197 was mobilized into Pf-5 as described for the *lon*::Tn*5* mutant, and transconjugants were selected on KB supplemented with 200 μg/ml tetracycline, which selects for pRK415 in Pf-5 and counterselects against *E. coli* donors. To allow loss of plasmid, $Tc^r$, $Gm^r$ transconjugants were grown in KB broth without antibiotics at 27° C with shaking for 1-3 days with daily subculturing. The resultant culture was spread on KB containing 40 μg/ml gentamycin and individual $Gm^r$ colonies were screened for loss of pJEL6197 by lack of growth on KB containing 200 μg/ml tetracycline. *lon*::*aacC1* was introduced into Pf-5 and JL4389, which contains *pltB*::Tn*3-nice*. In the resultant marker-exchanged mutants JL4619 and JL4594, respectively, *lon*::*aacC1* replaced *lon*, as determined from Southern analysis

(data not shown) using the 4.3-kb *Hin*dIII fragment from JL6203 as a probe (Fig. 3.1b).

An *rpoS*::*lacZ* mutant. Plasmid pJEL5926, which contains an *rpoS*::*lacZ* fusion (160), was used for marker-exchange mutagenesis of JL4389, which contains *pltB*::Tn*3-nice*, as described for *lon*::*aacC1*. The resultant marker-exchanged mutant JL4600 was confirmed as such by Southern analysis (data not shown) using a 2.9-kb *Eco*RI fragment from JL5500 as a probe.

*rpos*::*lacZ, lon*::*aacC1* double mutants. The *lon*::*aacC1* mutation was exchanged with *lon* in the genome of two *rpoS*::*lacZ* derivatives of Pf-5, strain JL4490 (160), and strain JL4600, which contains *pltB*::Tn*3-nice*, as described above. The resultant marker-exchanged mutants, JL4620 and JL4621 respectively, were confirmed as such by Southern analysis (data not shown) using the 4.3-kb *Hin*dIII fragment from JL6203 as a probe (Fig. 3.1b).

A *ΔhupB*::*aphI* mutant. The *hupB* gene cloned in pJEL6023 was deleted by digesting plasmid DNA with *Afl*II and *Hpa*I, blunting the ends of the digested DNA, and inserting the 1.7-kb *aphI* cassette from pMKm, to derive pJEL6161 (Fig. 3.1c). The resulting 5.7-kb *Hin*dIII fragment was cloned into pRK415 and used to mutagenize Pf-5 as described for *lon*::*aacC1*. Replacement of *hupB* with Δ*hupB*::*aphI* in two independently derived marker-exchanged mutants, JL4590 and JL4591, was confirmed by Southern analysis (data not shown) with the 4.3-kb *Hin*dIII fragment of pJEL6023 as a probe (Fig. 3.1b).

### 3.3.7 Transcription of Plt biosynthetic genes assessed with an ice nucleation reporter gene in Tn*3-nice*

The effect of *rpoS* and *lon* on the transcription of the Plt biosynthesis genes was determined by comparing ice nucleation activity expressed by derivatives of Pf-5 containing insertions of Tn*3-nice* in genomic *pltB* gene (JL4389)(76) (See Figure 1.1) to near-isogenic strains with *rpoS*::*lacZ* (JL4600) (160), with *lon*::*aacC1* (JL4594), with both *rpoS*::*lacZ* and *lon*::*aacC1* mutations (JL4621), or with multiple plasmid-borne copies of *rpoS* (JL4601). Ice nucleation activity was quantified by a droplet-freezing assay at –5°C as described previously (92) from cultures grown for 2 days at 20°C with shaking at 200 rpm in NB amended with 2% glycerol. Cultures were grown in triplicate, the experiment was done twice, and the results of a representative experiment are presented.

### 3.3.8 Western analysis of Lon and $\sigma^S$

SDS-polyacrylamide gel electrophoresis and transblotting of total protein extracts for western analysis were done using the manufacturer's protocols (Bio-Rad Laboratories, Hercules, Calif.) and reported methods (30, 152). Bacterial cultures were grown in LB or M9 supplemented with 0.4% glucose at 27°C with shaking at 200 rpm. For each *P. fluorescens* culture, 30 µg of protein (Biorad DC protein assay) from whole cell extracts was boiled in sample buffer containing 2-mercaptoethanol, and separated on a SDS-8% polyacrylamide gel. 10 µg of protein (Biorad DC protein assay) from whole cell extracts of *E. coli* was loaded on the gel as a positive control. Protein samples were transferred

from the gel onto a nitrocellulose membrane and the membrane was incubated with antibodies to the Lon protein from *E. coli* (30), which were detected with enhanced chemiluminesence as specified by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The amount of Lon in strains of *P. fluorescens* was quantified using a Molecular Dynamics Personal Densitometer model SI and ImageQuant software V4.1 (Sunnyvale, CA) and confirmed to be within the linear range of detection as described previously (160). The amount of Lon is reported relative to the amount detected in Pf-5 growing exponentially at 27°C. $\sigma^S$ was quantified from these and similar blots with 10 µg protein per sample after blots were stripped according to the manufacturer's protocols (Amersham) and re-probed with antibodies to $\sigma^S$ from *E. coli* (149). Each experiment was replicated with similar results.

### 3.3.9 Sensitivity to ultraviolet irradiation

Cultures were grown with shaking at 27°C in LB and stationary-phase cells were obtained 4 h after the optical density ($\lambda$=600 nm) of cultures stopped increasing. Cells were pelleted, washed, serially diluted in 10 mM phosphate buffer (pH 7), and spread onto duplicate or triplicate LB agar plates. Agar plates were exposed to UV irradiation ($\lambda$=254 nm) at a level of 10 erg/mm$^2$ for various durations. Colonies arising from surviving cells were counted following 48 h incubation in the dark. The experiment was done twice with similar results.

### 3.3.10 Nucleotide sequence accession number

The GenBank accession number for the DNA sequence of the *lon* and *hupB* genes of *P. fluorescens* Pf-5 is AF250140

## 3.4 Results

### 3.4.1 Identification of a *lon*::Tn*5* derivative of Pf-5 that over-produces Plt

JL4292, a derivative of Pf-5 obtained following random Tn*5* mutagenesis, overproduces the antibiotic Plt (75). To confirm that the Tn*5* insertion caused overproduction of Plt by JL4292, the transposon was re-introduced into the same site in the genome of Pf-5 by marker exchange mutagenesis to create JL4479. JL4292 and JL4479 each contained a single Tn*5* insertion in a 9.7-kb *Eco*RI fragment of genomic DNA, as determined from Southern analysis (data not shown) using the Tn*5*-containing *Eco*RI fragment from the genome of JL4292 as a probe (Fig. 3.1b). Both JL4292 and JL4479 produced a higher concentration of Plt than did Pf-5 (Table 3.2), confirming that Plt overproduction by JL4292 was associated with the Tn*5* insertion and not due to secondary mutations at other loci. Pf-5, JL4292, and JL4479 did not differ significantly in their production of Phl in two replicate experiments (data not shown).

We began our analysis of the locus disrupted in JL4292 (Fig. 3.1a) by identifying cosmids containing the corresponding wild-type DNA from an extant genomic library of

**Table 3.2: Antibiotic production by *P. fluorescens* Pf-5 and derivatives**

| Strain | Characteristics | Plt (µg/ml)[1,2] | Prn (µg/ml)[1,2] |
|---|---|---|---|
| Pf-5 | Wild-type | 5.3 ± 1.2 | 1.04 ± 0.26 |
| JL4292 | *lon*::Tn*5* | 117 ± 11 | 1.42 ± 0.10 |
| JL4479 | *lon*::Tn*5* | 101 ± 14 | 1.44 ± 0.12 |
| JL4619 | *lon*::*aacC1* | 96 ± 4 | 1.31 ± 0.13 |
| JL4620 | *lon*::*aacC1*, *rpoS*::*lacZ* | 164 ± 13 | 0.32 ± 0.07 |
| JL4490 | *rpoS*::*lacZ* | 145 ± 13 | 0.11 ± 0.02 |
| JL3985 | *rpoS*::Tn*5* | 149 ± 9 | 0.38 ± 0.04 |
| JL4590 | Δ*hupB*::*aphI* | 4.2 ± 0.6 | 0.83 ± 0.18 |

[1] Values are the means from three replicate cultures ± SD.
[2] Plt and Prn were extracted from cells and spent media of cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol.

Pf-5 (114). Cosmids that hybridized to the Tn*5*-containing *Eco*RI fragment from the genome of JL4292 were identified. From one such cosmid, a 4.3-kb *Hin*dIII fragment that hybridized to the probe was cloned (pJEL6023) (Fig. 3.1b) and used a template for sequencing. Within the 4.3-kb *Hin*dIII fragment, an ORF of 2,397 bases was identified with the putative ribosomal binding site GAGG located 8 bases upstream of an ATG start codon (Fig. 3.2). In JL4292, Tn*5* disrupted the ORF at base 1798 of the coding sequence. Once translated, the ORF was predicted to encode a peptide of 798 amino acids, with 70% identity to Lon of *E. coli* (18). Within the deduced amino acid (a.a.) sequence of Lon from Pf-5, we located all of the characteristic Lon protein domains. From a.a. residue 206 to 266, we located a charged region (a.a. 206-221, 53% basic; a.a. 227-249, 52% acidic; and a.a. 251-266, 31% basic). This region is predicted to form a coiled-coil structure in *E. coli* (34), a common motif involved in protein:protein interactions. Within the acidic portion of the charged region, we located a sequence identical to the discriminator activity consensus (KAIQKELGD from a.a. 232-240) from Lon of *E. coli* (34). A mutation within this sequence abolishes activity of Lon towards two specific substrates in *E. coli* (34). ATP binding motifs (41, 158) that matched the corresponding motifs in Lon of *E. coli* (18) were located from a.a. 352 to 359 (GPPGVGKT) and from a.a. 405 to 420 (KVGVRNPLFLLD). A putative catalytic site serine (3) is a.a. residue 674. In JL4292, Tn*5* spatially separates the conserved serine residue from the ATP binding motif and the discriminator activity consensus.

The upstream regulatory region of *lon*, including its promoter, was not located within the 4.3-kb *Hin*dIII fragment cloned in pJEL6023. Therefore, an overlapping *Eco*RI fragment was cloned into pUC18 to create pJEL6195 (Fig. 3.1b), which was a

**Figure 3.2:** Nucleotide and predicted amino acid sequence of the lon and hupB locus from Pf-5. Underlined regions denote two putative promoters (P1 and P2) and a potential ribosomal binding site (RBS).

```
TCGAGATGGAAGGTGTTGACCAGGAGTTCCGACCGATGCCTTGAAGTCCGTCGCCAAGCG  60
                                                    P1
AGCCCTTGAACGCAAGACTGGTGCTCGTGGTCTGCGCTCCATTCTTGAAGGTGTTCTGCT 120
      P2
CGACACTATGTATGAAATCCCCTCGCAGTCCGAGGTGAGTAAAGTAGTGATCGACGAAAG 180

CGTTATTGAAGGCAAGTCCCAGCCACTGATCATCTATGAGAACAGTGAGCCGACGGCCAA 240

GGCAGCGCCAGACGCGTAAGCGTCGCGCTGTTGGAATGAAAGAAGGGGCCTTCGGGCCCC 300

TTTGTTTTTAGCGCCTTAAAGTGCTGTCTTTGAGCTTGTTTTTTTTCCAGGCAGCCCCCA 360
                                                        RBS
ATCTTGGTTTCAAGCTTACTTCCATCTGTTTACGGCCTTACGGCCGCCGTAGAGGCGAAA 420

TCATGAAGACAACCATCGAATTGCCTCTCCTGCCATTGCGTGATGTTGTGGTTTATCCGC 480
  M  K  T  T  I  E  L  P  L  L  P  L  R  D  V  V  V  Y  P  H

ACATGGTTATCCCGCTGTTCGTGGGGCGCGAGAAGTCGATCGAAGCCCTCGAGGCAGCGA 540
  M  V  I  P  L  F  V  G  R  E  K  S  I  E  A  L  E  A  A  M

TGACGGGCGACAAGCAGATTCTCTTGCTGGCGCAGAGAAACCCTGCTGATGACGATCCCG 600
  T  G  D  K  Q  I  L  L  L  A  Q  R  N  P  A  D  D  D  P  G

GTGAAGATGCACTCTATCGCGTAGGTACCATTGCGACTGTCTTGCAGTTGCTCAAATTGC 660
  E  D  A  L  Y  R  V  G  T  I  A  T  V  L  Q  L  L  K  L  P

CTGATGGCACCGTCAAGGTATTGGTTGAAGGCGAACAGCGTGGTGCTGTTGAGCGCTTCA 720
  D  G  T  V  K  V  L  V  E  G  E  Q  R  G  A  V  E  R  F  S

GCGAAGTTGATGGCCATTGCCGTGCCGAAGTCTCCCTGATTGACGAAGTCGATGCTCCTG 780
  E  V  D  G  H  C  R  A  E  V  S  L  I  D  E  V  D  A  P  D

ACCGTGAGTCGGAAGTGTTTGTTCGCAGTCTGCTGTCCCAGTTCGAGCAGTACGTGCAAC 840
  R  E  S  E  V  F  V  R  S  L  L  S  Q  F  E  Q  Y  V  Q  L

TGGGCAAGAAAGTCCCTGCCGAAGTGCTGTCGTCCCTCAATAGCATCGATGAGCCAAGCC 900
  G  K  K  V  P  A  E  V  L  S  S  L  N  S  I  D  D  P  S  R

GCCTGGTGGACACCATGGCTGCGCACATGGCGTTGAAGATCGAGCAGAAGCAGGAAATCC 960
  L  V  D  T  M  A  A  H  M  A  L  K  I  E  Q  K  Q  E  I  L

TCGAGATCATCGATCTGTCCGCTCGGGTCGAGCATGTCCTGGCATTGCTGGACGCCGAGA 1020
  E  I  I  D  L  S  A  R  V  E  H  V  L  A  L  L  D  A  E  I

TCGATCTGCTGCAAGTGGAAAAGCGCATCCGTGGTCGCGTCAAAAAGCAAATGGAGCGCA 1080
  D  L  L  Q  V  E  K  R  I  R  G  R  V  K  K  Q  M  E  R  S

GCCAGCGCGAGTACTACCTGAATGAGCAGATGAAGGCCATTCAGAAAGAACTGGGTGATG 1140
  Q  R  E  Y  Y  L  N  E  Q  M  K  A  I  Q  K  E  L  G  D  G

GCGACGAAGGGCACAACGAAATCGAGGAGCTGAAAAAGCGTATCGATGCCGCAGGCCTGC 1200
  D  E  G  H  N  E  I  E  E  L  K  K  R  I  D  A  A  G  L  P
```

**Figure 3.2** (Continued)

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CAAAAGACGCGATGACCAAGGCTCAGGCCGAACTGAACAAGCTCAAGCAGATGTCGCCGA  1260
  K  D  A  M  T  K  A  Q  A  E  L  N  K  L  K  Q  M  S  P  M

TGTCTGCCGAGGCTACTGTTGTTCGCTCGTATATCGACTGGCTGGTTCAGGTGCCGTGGA  1320
  S  A  E  A  T  V  V  R  S  Y  I  D  W  L  V  Q  V  P  W  K

AGGCACAGAGCAAGGTTCGTCTCGATCTGGCACGTGCCGAAGATATTCTCGATGCCGACC  1380
  A  Q  S  K  V  R  L  D  L  A  R  A  E  D  I  L  D  A  D  H

ACTATGGCCTGGAAGAGGTCAAGGAACGGATTCTCGAATACCTCGCCGTACAGAAACGGG  1440
  Y  G  L  E  E  V  K  E  R  I  L  E  Y  L  A  V  Q  K  R  V

TGAAAAAGATTCGTGGCCCGGTACTGTGCCTGGTGGGGCCTCCTGGGGTGGGTAAGACCT  1500
  K  K  I  R  G  P  V  L  C  L  V  G  P  P  G  V  G  K  T  S

CCCTGGCGGAGTCGATCGCCAACGCTACCAACCGCAAGTTCGTACGCATGGCCCTGGGTG  1560
  L  A  E  S  I  A  N  A  T  N  R  K  F  V  R  M  A  L  G  G

GCGTGCGTGACGAGGCGGAGATTCGTGGTCATCGTCGGACTTATATCGGTTCGATGCCAG  1620
  V  R  D  E  A  E  I  R  G  H  R  R  T  Y  I  G  S  M  P  G

GAAGATTGATTCAAAAGATGACAAAAGTGGGGGTGCGCAACCCACTGTTCTTGCTCGACG  1680
  R  L  I  Q  K  M  T  K  V  G  V  R  N  P  L  F  L  L  D  E

AAATCGACAAGATGGGCAGCGATATGCGCGGTGATCCGGCCTCGGCATTGCTCGAGGTGC  1740
  I  D  K  M  G  S  D  M  R  G  D  P  A  S  A  L  L  E  V  L

TGGACCCCGAGCAGAACCATAATTTCAACGATCACTACCTGGAAGTCGATTACGACTTGT  1800
  D  P  E  Q  N  H  N  F  N  D  H  Y  L  E  V  D  Y  D  L  S

CCGATGTGATGTTCCTCTGCACCTCCAACTCGATGAATATTCCGCCGGCCTTGCTGGACC  1860
  D  V  M  F  L  C  T  S  N  S  M  N  I  P  P  A  L  L  D  R

GGATGGAGGTGATTCGTCTGCCTGGCTACACCGAGGACGAGAAGATCAACATCGCGGTCA  1920
  M  E  V  I  R  L  P  G  Y  T  E  D  E  K  I  N  I  A  V  K

AGTACCTCTCGCCCAAGCAGATTCAAGCCAACGGCCTGAAGAAGGGCGAGCTGGAATTCG  1980
  Y  L  S  P  K  Q  I  Q  A  N  G  L  K  K  G  E  L  E  F  E

AGCCGGATGCGATCCGCGACATCATTCGCTACTACACCCGCGAGGCCGGTGTACGCGGCC  2040
  P  D  A  I  R  D  I  I  R  Y  Y  T  R  E  A  G  V  R  G  L

TGGAGCGGCAGATTGCCAAGGTTTGCCGCAAGGCGGTGAAAGAGCATGCGATGGAAAAGC  2100
  E  R  Q  I  A  K  V  C  R  K  A  V  K  E  H  A  M  E  K  R

GCTTCTCGGTGAAGGTCACTTCCGACCTGCTGGAGCACTTCCTCGGCGTGCGCAAATTCC  2160
  F  S  V  K  V  T  S  D  L  L  E  H  F  L  G  V  R  K  F  R

GCTACGGCCTGGCCGAGCAGCAAGACCAGATCGGTCAGGTGACCGGGTTGGCATGGACTC  2220
  Y  G  L  A  E  Q  Q  D  Q  I  G  Q  V  T  G  L  A  W  T  Q

AAGTGGGCGGTGAGCTGCTGACCATCGAAGCCGCTGTGGTTCCGGGCAAGGGTCAGTTGA  2280
  V  G  G  E  L  L  T  I  E  A  A  V  V  P  G  K  G  Q  L  I
```

**Figure 3.2** (Continued)

```
TCAAGACCGGTTCCCTGGGCGATGTGATGGTCGAGTCGATCACTGCAGCACTGACCGTGG  2340
  K  T  G  S  L  G  D  V  M  V  E  S  I  T  A  A  L  T  V  V

TGCGCAGCCGGGCCAAGAGCCTGGGCATTCCCCTGGACTTCCACGAGAAGCGCGACACCC  2400
  R  S  R  A  K  S  L  G  I  P  L  D  F  H  E  K  R  D  T  H

ATATCCACATGCCTGAAGGGGCGACCCCGAAAGACGGCCCCAGCGCGGGTGTAGGCATGT  2460
  I  H  M  P  E  G  A  T  P  K  D  G  P  S  A  G  V  G  M  C

GCACGGCGCTGGTTTCGGCGCTGACCGGCATTCCGGTGCGGGCTGATGTGGCAATGACGG  2520
  T  A  L  V  S  A  L  T  G  I  P  V  R  A  D  V  A  M  T  G

GCGAGATCACTCTGCGTGGTCAGGTATTGGCTATCGGCGGGTTGAAAGAGAAATTACTCG  2580
  E  I  T  L  R  G  Q  V  L  A  I  G  G  L  K  E  K  L  L  A

CCGCTCATCGGGGTGGAATCAAGACAGTGATCATTCCGGAAGAGAATGTTCGCGATTTGA  2640
  A  H  R  G  G  I  K  T  V  I  I  P  E  E  N  V  R  D  L  K

AAGAAATTCCTGACAATATCAAGCAGGATCTGCAGATTAAACCGGTTAAATGGATTGACG  2700
  E  I  P  D  N  I  K  Q  D  L  Q  I  K  P  V  K  W  I  D  E

AAGTCCTGCAAATTGCGCTGCAATACGCGCCGGAGCCCTTGCCGGATGTGGCTCCCGAGA  2760
  V  L  Q  I  A  L  Q  Y  A  P  E  P  L  P  D  V  A  P  E  I

TAGTCGCAAAGGACGAAAAACGCGAGTCTGACTCTAAGGAAAGAATTAGCACGCATTAGT  2820
  V  A  K  D  E  K  R  E  R  D  S  K  E  R  I  S  T  H  *

ACGCATTAGCCTGGGGGGCTTCCTTGACAGCTTTTTAGAGCCCTTGTTATAAAGCGGCTC  2880

TTAAGTGTCTGTAGGCCATTCAGCACTCGTTTTTGCTTTCACCAAAAAACTTAGAATCAT  2940

ACTCATAGATATATAAGGGGACTTAGAGTGAACAAGTCGGAACTGATTGATGCTATCGCT  3000
                              V  N  K  S  E  L  I  D  A  I  A

GCATCTGCTGATCTCCCGAAAGCTGCTGCTGGCCGTGCGCTGGACGCAGTAATCGAATCC  3060
A  S  A  D  L  P  K  A  A  A  G  R  A  L  D  A  V  I  E  S

GTTACTGGCGCTCTGAAGGCCGGTGACTCTGTGGTGCTGGTTGGTTTCGGTACTTTCTCC  3120
V  T  G  A  L  K  A  G  D  S  V  V  L  V  G  F  G  T  F  S

GTAACTGATCGTCCAGCGCGTATCGGTCGTAACCCACAGACCGGTAAGACTCTGGAAATC  3180
V  T  D  R  P  A  R  I  G  R  N  P  Q  T  G  K  T  L  E  I

GCTGCTGCTAAAAAGCCAGGTTTTAAAGCCGGTAAAGCACTGAAAGAAGCCGTTAACTAA  3240
A  A  A  K  K  P  G  F  K  A  G  K  A  L  K  E  A  V  N  *

GTTTCTTCAGGTCTTTGCCCATCCGGGTCGGGGTCATGCCTGATCTGGCAGCGGGGCGCT  3300

AGTCAACCGGTCTACCACCGGTTCACGCCGAAGGTCCGGGTTTAACCTCCTCCGCTCCGC  3360

CAGTTACGAAAA  3372
```

template for sequence analysis. In the sequence upstream of *lon*, we identified two potential promoters (Fig. 3.1d), one 203 bases upstream and the other 286 bases upstream of the start codon. Sequences of both promoter regions resembled the consensus sequence recognized by the $\sigma^{32}$ ($\sigma^{H}$) form of RNA polymerase, which is located upstream of various heat shock-inducible genes in *E. coli* including *lon* (163).

### 3.4.2 A Δ*hupB*::*aphI* derivative of Pf-5 overproduced Phl but did not over-produce Plt

A second ORF with a predicted GTG start codon was identified 148 bases downstream of the *lon* ORF (Fig. 3.2). The deduced protein encoded by the ORF is 90 amino acids in length and is 80% identical to the histone-like protein HU from *Pseudomonas aeruginosa* (28) encoded by *hupB*. The *hupB* gene in *E. coli* encodes one of two subunits of the heterodimeric protein that is involved in regulating transcription by constraining DNA supercoils and DNA accessibility to regulatory proteins (31). In the genome of *E. coli*, *hupB* is located downstream of *lon*, which further supports our designations for the ORFs as *lon* and *hupB* homologs. Within the intergenic region between *lon* and *hupB*, a putative rho factor-independent terminator-like sequence was identified (11) as a run of consecutive thymine residues. However, a discernable region of dyad symmetry that characterizes terminators did not immediately precede the sequence. Without more convincing evidence of a terminator between *lon* and *hupB*, we chose to test the possibility that a polar effect of *lon*::Tn*5* on *hupB* was responsible for Plt overproduction in JL4292 by deleting *hupB* from Pf-5 and testing the resulting strains for Plt production.

Two Δ*hupB*::*aphI* mutants, JL4590 and JL4591, were generated by marker exchange mutagenesis and confirmed not to overproduce Plt, as assessed by TLC (data not shown). Compared to Pf-5, both JL4590 and JL4591 were more mucoid on KB plates and more viscous in NB containing glycerol or glucose. Strain JL4590 was further evaluated by HPLC analysis, which revealed that the Δ*hupB*::*aphI* mutant did not differ from Pf-5 in its production of Plt (Table 3.2). Therefore, the possibility that the Tn*5* insertion into *lon* enhanced Plt production by blocking read-through transcription of *hupB* was discounted. JL4590 produced more Phl than was produced by Pf-5 in parallel cultures (38.6 ± 8.3 and 13.1 ± 0.7 μg/ml, respectively) grown in NB containing 2% glucose.

### 3.4.3 Lon protease and $\sigma^S$ influenced *pltB* biosynthetic gene transcription

The influence of Lon and $\sigma^S$ on the transcription of *pltB* was assessed with transcriptional fusions of the ice nucleation reporter gene in Tn*3-nice* to *pltB* (76). Ice nucleation activity expressed by JL4389, which has an insertion of Tn*3-nice* in the genomic *pltB* gene, was compared to the activity of derivative strains with *lon*::*aacC1* or *rpoS*::*lacZ* mutations, with both the *lon*::*aacC1* and *rpoS*::*lacZ* mutations, or with multiple plasmid-borne copies of *rpoS* (Table 3.3). Ice nucleation activity is expressed as $\log_{10}$(ice nuclei per cell); therefore, increasingly positive values represent higher *pltB* transcription whereas increasingly negative values represent lower *pltB* transcription. In derivatives of JL4389, mutations in *rpoS* and *lon* significantly increased *pltB* transcription compared to strains with functional *rpoS* and *lon*, and transcription in a strain harboring both

**Table 3.3: Influence of *lon* and *rpoS* on transcription of the pyoluteorin biosynthesis gene *pltB*, assessed with an ice nucleation reporter gene in Tn*3-nice*.**

| Strain | Characteristic | Ice Nucleation Activity [log (ice nuclei per cell)][a] |
|---|---|---|
| JL4398 | *lon*$^{+}$, *rpoS*$^{+}$ | -1.7 ± 0.6 |
| JL4594 | *lon*::*aacC1*, *rpoS*$^{+}$ | -0.39 ± 0.1 |
| JL4600 | *lon*$^{+}$, *rpoS*::*lacZ* | -0.67 ± 0.1 |
| JL4621 | *lon*::*aacC1*, *rpoS*::*lacZ* | 0.17 ± 0.3 |
| JL4601 | *lon*$^{+}$, *rpoS*$^{++}$[b] | -3.1 ± 0.4 |

[a] Values followed by ± standard deviation.
[b] *rpos*++ designates the presence of both a genomic copy of *rpoS* and pJEL5649, a plasmid containing the cloned *rpoS* from Pf-5.

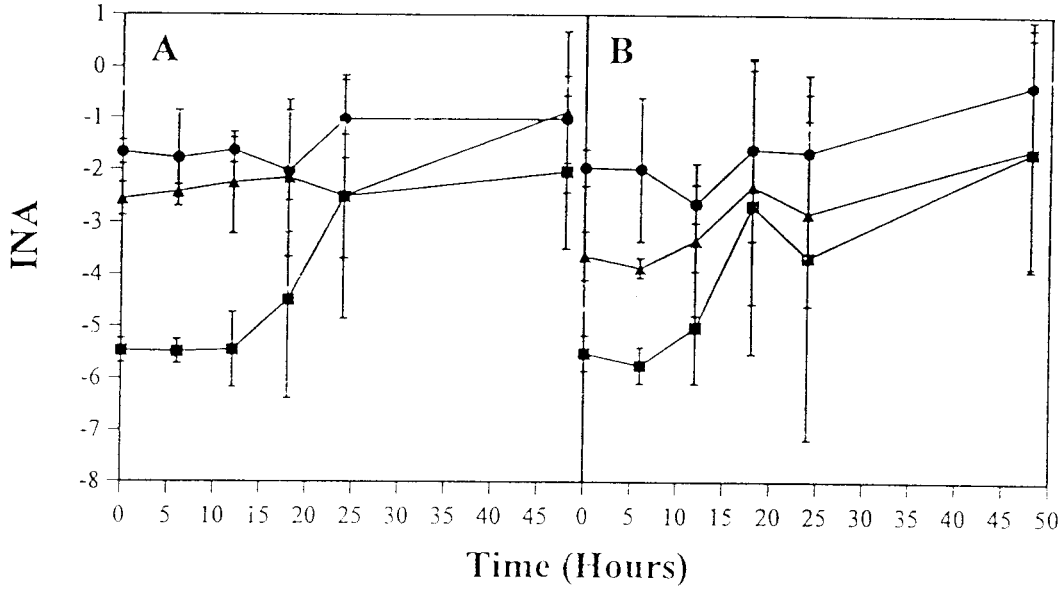


**Figure 3.3:** Transcription of *pltB*::Tn*3-nice* from derivatives of Pf-5 inoculated on cucumber seeds. Washed cells from overnight cultures of wild-type (■), *lon*::*aacC1* (•), and *rpoS*::*lacZ* (▲) derivatives of Pf-5 grown in NB supplemented with glucose, a media repressive to Plt production, were inoculated on seeds that were planted in pasteurized soil and ice nucleation activity (INA) was determined over time from three seeds at each time points. Data from two experiments are presented in (A and B). Error bars represent SD.

mutations was further enhanced. Multiple plasmid-borne copies of *rpoS* significantly reduced *pltB* transcription compared to strains with a single genomic copy of *rpoS*.

Additionally, transcription of *pltB*::Tn*3-nice* was assessed on cucumber seeds (Figure 3.3). Although the seed cultures were grown in glucose-supplemented NB where production of Plt is low, transcription of the *pltB* biosynthetic gene in *lon* and *rpoS* mutants was high when seeds were first inoculated, reflective of either constitutive expression in medium or rapid induction on seeds. In the derivative carrying functional *rpoS* and *lon* genes expression of *pltB* was initially low and increased after 12 to 18 hours of inoculation onto cucumber seeds, similarly to previous studies (76).

### 3.4.4 Lon accumulation increased after heat shock

Western analysis identified the Lon protein in Pf-5 (Fig. 3.4, lanes 2-5) but identified no detectable Lon in JL4292 (lane 10). When exponentially-growing cultures of Pf-5 at an $OD_{600}$ of 0.2 (lane 2) were shifted from 27 to 42°C for 25 min to simulate heat shock (lane 3), Lon accumulation increased. In stationary-phase cultures at an $OD_{600}$ of 2.0 (lane 4) or grown overnight to an $OD_{600}$ of 2.0-4.0 (lane 5), Lon accumulation was not considerably greater than in exponentially growing cultures of Pf-5. An *rpoS*::Tn*5* mutant of Pf-5 still showed heat shock-induction of Lon (lane 6 and 7), and had higher levels of Lon than did Pf-5, both in exponential phase (lane 6) and stationary phase (8 and 9). Lon was detected and induced by heat shock in cells lacking GacS (data not shown). The amounts of $\sigma^S$ in Pf-5 and the *lon*::Tn*5* mutant JL4292 were indistinguishable on

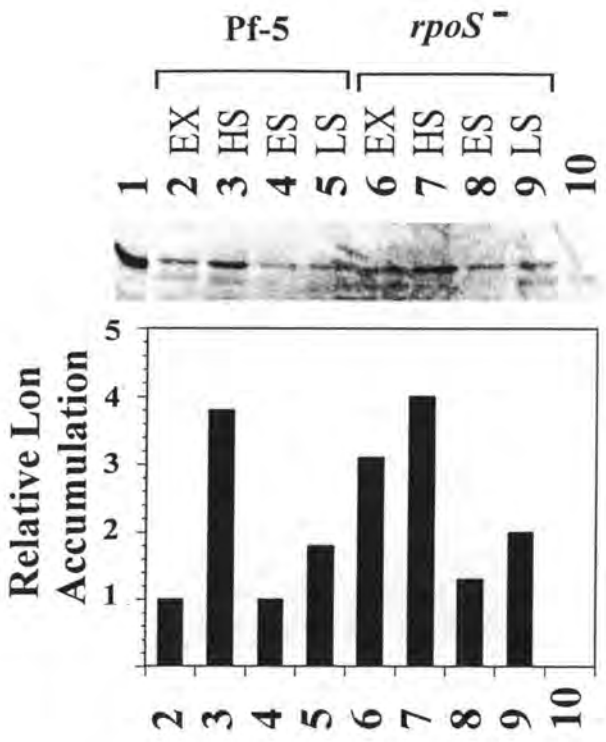


**Figure 3.4:** Relative Lon accumulation. Lon from Pf-5 grown in M9 minimal medium (135) was detected using antiserum to Lon of *E. coli*. Sample numbers correspond to extracts from cells growing exponentially at 27°C at $OD_{600}$ of 0.2 (exponential=**EX**:lane 1,2, and 6), incubated at 42°C for 25 min (heat shock=**HS**:lane 3, 7, and 10); in stationary-phase cultures at an $OD_{600}$ of 2 (early stationary=**ES**:lane 4 and 8); and grown overnight reaching a final $OD_{600}$ of 2-4 (late stationary=**LS**:lane 5 and 9): (1) *E. coli* SG20781, (2-5) Pf-5, (6-9) JL3985 (*rpoS*::Tn*5*), (10) JL4292 (*lon*::Tn*5*). Experiments were repeated with similar results. Scanned images were prepared for publication using Adobe Photo Shop version 4.0 (Adobe Systems Incorporated, San Jose, Calif.).

these and other blots (data not shown). Results were similar when strains were grown in LB (data not shown).

### 3.4.5 The *lon*::Tn*5* derivative was more sensitive than Pf-5 to ultraviolet irradiation

We tested Pf-5 and its *lon*::Tn*5* derivative for their abilities to survive exposure to UV, because *lon* mutants of *E. coli* are more sensitive than *lon*$^+$ strains to UV irradiation (45, 46). The survival ratio of JL4292 was 1000x lower than that of Pf-5 after UV exposure at 206 erg/mm$^2$. Differences in survival ratios of the strains were maintained up to an exposure of 618 erg/mm$^2$ (Fig. 3.5). Cells of JL4292 were also noticeably elongated both before and after UV exposure, consistent with the lethal filamentation phenotype associated with *lon* in *E. coli*.

## 3.5 Discussion

We cloned, sequenced, and partially characterized the *lon* homolog in *P. fluorescens* and demonstrated its role in the regulation of the antibiotic Plt. Like the stationary-phase sigma factor $\sigma^S$ (136), Lon is a global regulator that negatively influences Plt production, so we evaluated the interactions of these regulators in Pf-5. Accumulation of Lon in cells of Pf-5 was not positively influenced by $\sigma^S$ or GacS, a member of a two component regulatory system that controls $\sigma^S$ levels in this bacterium (160). Therefore, Lon does not appear to be an intermediate in the regulatory circuit

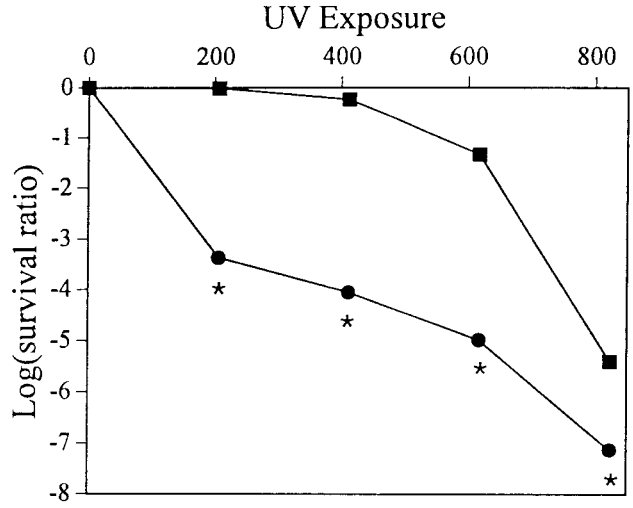


**Figure 3.5:** Sensitivity of cells to ultraviolet irradiation. Stationary-phase cells of *P. fluorescens* Pf-5(■) and JL4292 (●) were exposed to UV radiation ($\lambda$=254 nm) expressed as Ergs/mm$^2$ for various durations. Colonies arising from surviving cells were counted following 48 h incubation in the dark. Data was transformed as the log of the survival ratio. Statistical analysis of variance was completed by SAS statistical software (Version, location). Asterisks indicate data points where Pf-5 and JL4292 differ significantly ($p$=0.05) as determined by Fishers LSD. The experiment was repeated with similar results.

involving GacA, GacS, and $\sigma^S$. Furthermore, levels of $\sigma^S$ were similar in the *lon*::Tn*5* mutant and Pf-5, indicating that Lon and $\sigma^S$ influence *plt* biosynthetic gene transcription and Plt production through separate regulatory circuits. It is possible that these circuits could converge through a *plt* pathway specific regulator, which could integrate signals from diverse sensory transduction pathways.

Competition between sigma factors for limited core RNA polymerase is implicated in regulation of Lon accumulation (110) and Plt production (136, 138). In Pf-5, *lon* is preceded by $\sigma^{32}$-like promoter sequences, suggesting that transcription of *lon* is initiated by the $\sigma^{32}$ homolog $\sigma^H$. Furthermore, Lon accumulation increased after heat shock of Pf-5, as is typical of heat shock proteins transcribed from $\sigma^H$ promoters. In an *rpoS*::Tn*5* derivative of Pf-5, Lon accumulation exceeded wild-type levels, indicating that the stationary-phase sigma factor $\sigma^S$ negatively influences Lon. One possible explanation for this result is that expression of *lon* increases with the concentration of the $\sigma^H$-RNA polymerase holoenzyme, which is likely to be enhanced in the absence of competing sigma factors such as $\sigma^S$. A precident for this explanation exists in *E. coli*, where the induction of heat shock proteins is observed in a strain with decreased levels of the housekeeping sigma factor $\sigma^{70}$ (110). In light of recent evidence that Lon degrades $\sigma^H$ in *B. subtilis* under conditions where the sigma factor has low activity (86), we considered the possibility that Lon repressed Plt production by degrading a sigma factor required for *plt* biosynthetic gene transcription. Close examination of the *plt* biosynthetic operon revealed no $\sigma^{32}$-like promoter sequences; therefore, it is unlikely that $\sigma^H$ initiates *plt* transcription or that Lon represses *plt* transcription by degrading $\sigma^H$ and reducing the amount of $\sigma^H$-RNA polymerase holoenzyme. Previously, two other sigma factors were

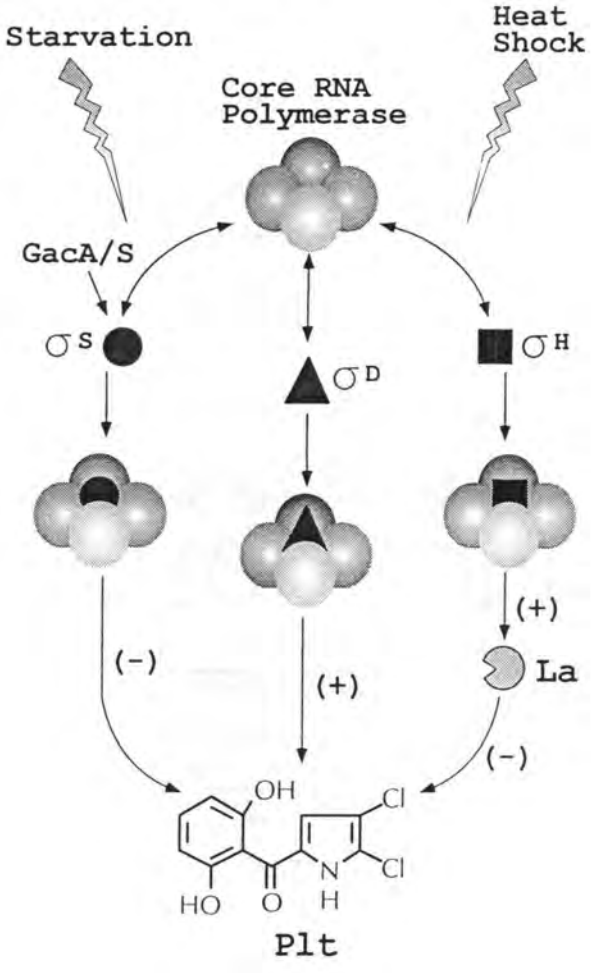


**Figure 3.6:** Proposed model depicting regulation of Plt by several identified global regulators. On one level, competition between $\sigma^S$, $\sigma^D$, and $\sigma^H$ for limited RNA polymerase core enzyme determines the suite of genes expressed and ultimately the level of Plt produced. In the absence of any one sigma factor, gene expression controlled by remaining sigma factors may increase due to decreased competition. In the presence of GacA and GacS, starvation rapidly induces $\sigma^S$ expression (54), which negatively influences Plt production. In response to heat shock, $\sigma^H$ mediates the induction of Lon, which negatively influences pyoluteorin production.

implicated in the regulation of Plt. Multiple copies of *rpoD*, encoding the housekeeping sigma factor $\sigma^D$, enhanced production of Plt in *P. fluorescens* strain CHA0 (138), a phenotype reminiscent of *rpoS* mutations in Pf-5 (136). The identification of Lon as a regulator of antibiotic biosynthesis fits into an evolving model that proposes the involvement of multiple sigma factors in the regulation of Plt production (Fig. 3.6). Direct examination of the role of sigma factor competition in Plt regulation, not included in this study, is warranted by this and previous data.

An alternative mechanism for regulation of Plt by Lon is through degradation of a positive regulator of Plt production, as has been described for many phenotypes regulated by Lon (45, 46). For example, Lon degrades the transcriptional regulator RcsA which, in association with the activator RcsB, positively regulates colonic acid capsular polysaccharide (*cps*) gene expression in *E. coli* (12, 45, 46). In the absence of Lon, RcsA has an enhanced half life resulting in over-expression of *cps* genes (17,18). RcsA and other previously-described targets of Lon degradation are not known to participate in regulatory circuits controlling Plt production in *P. fluorescens*. Nevertheless, Lon could repress Plt production by degrading an activator of *plt* biosynthetic gene transcription. We propose that one possible candidate for Lon degradation is PltR, a transcriptional activator that is linked to the *plt* biosynthetic operon (38). Comparisons between Lon substrates have failed to identify any likely consensus sequence but motifs that are recognized by Lon may be defined by structure (18). Gottesman has proposed that susceptibility of proteins to degradation is controlled by sequestration of target motifs within an active protein or protein complex. If a substrate of Lon that functions in activation of Plt production was identified and target motifs were characterized, the Plt

activator could possibly be altered to be less susceptible to degradation by Lon. This could provide an opportunity to enhance Plt production by Pf-5 when *lon* is induced by certain stresses, consequently improving the activity of Pf-5 as a biological control agent.

In addition to the novel phenotype of antibiotic regulation, two phenotypes of *lon* mutants in *E. coli*, filamentation and enhanced UV sensitivity, were found in *lon* mutants Pf-5. In *E. coli*, Lon specifically degrades SulA, a repressor of cell division that is induced during the cell's SOS response to severe DNA damage (99). When exposed to UV irradiation, *lon* mutants of *E. coli* accumulate SulA and consequently fail to divide, become filamentous, and die. Conservation of Lon function in regulating UV tolerance was observed among the two bacterial species, although direct involvement of SulA in *P. fluorescens* was not investigated.

The influence of *hupB*, located immediately downstream of *lon*, on antibiotic production by Pf-5 was also investigated in this study. Deleting the entire *hupB* gene reduced Plt production, increased Phl production, and resulted in a mucoid morphology. In *P. aeruginosa*, other histone-like proteins, including AlgP (67, 74) and IHF (100, 162), influence alginate production; similarly, the *hupB* product HU is implicated in the mucoidy phenotype of *E. coli* (111). We are uncertain why the Δ*hupB*::*aphI* derivative produced more Phl than Pf-5, or if Phl over-production and mucoidy are related, but it is possible that increased culture viscosity influenced antibiotic production. Indeed, in *P. fluorescens* F113, researchers noted that increasing the viscosity of broth cultures by adding 1.5% agar increases Phl production by ten fold (141). Alternatively, through its influence on DNA conformation, HU may influence transcription of genes required for Phl production.

Lon is the fourth global regulator in Pf-5, along with GacA, GacS, and $\sigma^S$, that influences both stress response and antibiotic production of Pf-5. Both *lon*::*aacC1* and *rpoS*::*lacZ* mutants have enhanced *pltB* biosynthetic gene transcription, implying that transcription of *plt* biosynthetic genes may be repressed under the stress conditions where $\sigma^S$ and $\sigma^H$ are the dominant sigma factors directing transcription. Understanding the regulation of Plt production, both positive and negative, will allow manipulation of the strain to improve its consistency and performance as a biological control organism inhabiting the soil-root interface where many stresses may be encountered.

## 3.6 Acknowledgments

We thank H. Floss, C. Keel and B. Nowak-Thompson for authentic standards of pyrrolnitrin, 2,4-diacetylphloroglucinol, and pyoluteorin, respectively; R. Bonsall, N. Chaney, and M. Brodhagen for optimization of HPLC methods for antibiotic quantification; J. Trempy for the antibody to Lon; and K. Tanaka for antibody to $\sigma^S$. We also thank J. Trempy for helpful discussions and protocols; and L. S. Pierson III, J. Trempy, and C. Yeager for critical reviews of the manuscript.

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# Chapter 4

## Characterization of a PtsP Homolog of *Pseudomonas fluorescens* Pf-5 that Influences Pyoluteorin Production

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Prepared for submission to
Applied and Environmental Microbiology
American Society of Microbiology

## 4.1 Abstract

*Pseudomonas fluorescens* Pf-5 is a soil bacterium that suppresses plant pathogens due in part to its ability to produce the polyketide antibiotic pyoluteorin (Plt). Previous attempts to find derivatives of Pf-5 that overproduce Plt identified regulatory mutants with pleiotropic effects that include reduced tolerance to various stresses. We identified a sugar phosphotransferase enzyme I (PtsI) paralog, PtsP, as a regulator of Plt production by Pf-5. Derivatives of Pf-5 with insertions in *ptsP* overproduced Plt and transcribed *plt* biosynthetic genes at enhanced levels. The C-terminal region of PtsP homologs is similar to PtsI whereas the N-terminal region is similar to NifA, a nitrogen responsive regulator, and SrmR, which governs expression of the polyketide synthase enzyme complex of *Streptomyces ambofaciens*. Because few PtsP homologs are currently characterized, we evaluated a *ptsP* mutant of Pf-5 for phenotypes associated with PtsI (inability to utilize fructose as a sole carbon source) and other regulators of Plt production by Pf-5 (reduced tolerance to environmental stress). The mutant was not impaired in fructose utilization nor was it impaired in tolerance to oxidation or freezing. Therefore, the phenotypes of the *ptsP* mutant contrasts to those of previously-described mutants in *lon* or *rpoS*, which produce more Plt and exhibit enhanced sensitivity to certain stresses.

## 4.2 Introduction

Previous attempts to find derivatives of strain Pf-5 that overproduce Plt, which may enhance biological control capacity of the strain, has led to the identification of the global regulatory genes *rpoS* and *lon* that negatively influence Plt production, but are also required for survival of the bacterium when exposed to certain stresses. In derivatives of Pf-5, mutations in the *rpoS* gene, which encodes the stationary-phase sigma factor $\sigma^S$, results in enhancement of *pltB* gene expression (161), and overproduction of Plt, 2,4-diacetylphloroglucinol (Phl) (75), and hydrogen cyanide (HCN) (160). Derivatives of Pf-5 deficient in $\sigma^S$ also exhibit enhanced biological control of Pythium damping-off of cucumber (136). The strain is impaired in its ability to tolerate exposure to stresses such as oxidation and starvation. Because sigma factors function in directing transcription of genes by RNA polymerase, $\sigma^S$ can only repress genes indirectly. One possibility is that $\sigma^S$ could positively direct transcription of a repressor of Plt transcription. Identification of a repressor downstream of $\sigma^S$ in the Plt regulatory circuit may provide an opportunity to enhance Plt production without compromising stress response.

The search for mutants of Pf-5 that overproduce Plt without pleiotrophic effects on stress tolerance resulted in the characterization of the regulatory gene *ptsP*, first identified by random transposon mutagenesis (75). PtsP homologs have been identified and characterized in only a few genera of bacteria, where they influence diverse and apparently unrelated phenotypes, including virulence in *Pseudomonas aeruginosa* (148), carbon storage in *Azotobacter vinelandii* (140), and antibiotic production by Pf-5. PtsP, also called Enzyme $I^{Ntr}$, is paralogous to the phosphotransferase system (PTS) Enzyme I

(PtsI) (125) involved in the uptake and utilization of many carbohydrates (133)(117). Unlike PtsI, PtsP has an N-terminal domain that is also present in NifA, a nitrogen response regulator (32), and SrmR, which governs expression of the polyketide synthase enzyme complex of *Streptomyces ambofaciens* (42). PtsP along with NPr (120) and Enzyme $IIA^{Ntr}$ comprise a phosphotransferase system distinct from that involved in sugar uptake and utilization. Because of the presence of the NifA-like domain, Reizer and colleagues (125) speculate that PtsP may provide a link between carbon and nitrogen metabolism, although this role for PtsP has not been demonstrated.

In the present study, we evaluated PtsP of Pf-5 for its influence on phenotypes associated with PtsI and negative regulators of Plt production. In Pf-5, the *ptsP*::Tn*5* mutation did not impair fructose utilization, a phenotype associated with *ptsI* in *P. aeruginosa* (148). It did not impair tolerance to oxidation or freezing at –80°C, phenotypes associated with *rpoS* mutations in Pf-5 (136,145). The *ptsP*::Tn*5* mutation appeared to negatively impact tolerance to freezing at –20°C comparable to the effect of an *rpoS* mutation. The *ptsP*::Tn*5* mutation may negatively influence long-term survival of starvation by Pf-5, but not to the extent that an *rpoS* mutation influenced this phenotype. Mutations in *ptsP* increased Plt production, and enhanced *plt* biosynthetic gene transcription, although this enhancement of antibiotic production was modest compared to the previously described *rpoS* and *lon* mutants.

## 4.3 Materials and methods

### 4.3.1 Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids are listed in Table 1. *P. fluorescens* was grown at 27°C, with shaking at 200 rpm, in King's medium B (KB) broth (70) for routine culturing and for freezing tests; in nutrient broth (NB) (Difco Laboratories, Detroit, MI) supplemented with 2% (wt/vol) glucose or 1% (wt/vol) glycerol for antibiotic extractions, ice nucleation assays, and β-galactosidase assays; and in M9 minimal medium (M9) supplemented with 0.4% glucose (135) for oxidative stress tests. Cultures of *E. coli* were grown routinely in Luria-Bertani medium (LB) (135) at 37°C. Culture media were supplemented as needed with 12 µg/ml gentamycin (Gm) for cultures of *E. coli* or 40 µg/ml (Gm40) for *P. fluorescens*, 20 µg/ml tetracycline (Tc) for cultures of *E. coli* or 200 µg/ml (Tc200) for cultures of *P. fluorescens*, 50 µg/ml kanamycin (Km), 100 µg/ml streptomycin (Sm), or 100 µg/ml ampicillin (Ap).

### 4.3.2 Recombinant DNA techniques

Genomic DNA was isolated by cetyltrimethylammonium bromide (CTAB) with isopropanol precipitation (4). Plasmids were purified by an alkaline lysis procedure (135), or with a Perfect-prep Kit (Eppendorf 5', Boulder, CO). Methods for transformations, digestions with restriction enyzmes, gel electrophoresis, and cohesive end ligations were standard (135). Enzymes were from GibcoBRL Life Technologies

**Table 4.1: Bacterial strains and plasmids used in this study**

| Strain or Plasmid | Description | Reference |
|---|---|---|
| *P. fluorescens* | | |
| Pf-5 | Rhizosphere isolate | (58) |
| JL3985 | Derivative of Pf-5; *rpoS*::Tn*5* | (136) |
| JL4292 | *lon*::Tn*5* derivative of Pf-5, $Km^r$. | (161) |
| JL4296 | *pltB*::Tn*5* derivative of Pf-5, $Km^r$. | (75) |
| JL4297 | *ptsP*::Tn*5* derivative of Pf-5, $Km^r$. | (75) |
| JL4365 | *pltE*::Tn*3-nice* derivative of Pf-5, $Plt^-$, $Ina^+$, $Km^r$. | (76) |
| JL4389 | *pltB*::Tn*3-nice* derivative of Pf-5, $Plt^-$, $Ina^+$, $Km^r$. | (76) |
| JL4480 | *ptsP*::Tn*5* derivative of Pf-5 obtained by marker exchange mutagenesis, $Km^r$. | This study |
| JL4617 | *ptsP*::Tn*5* derivative of Pf-5 harboring pJEL6204, $Km^r$, $Tc^r$. | This study |
| JL4518 | *ptsP*::Tn*5* derivative of Pf-5 harboring pJEL6205, $Km^r$, $Tc^r$. | This study |
| JL4622 | *ptsP::aacC1* derivative of Pf-5 obtained by marker exchange mutagenesis, $Gm^r$, $Km^r$. | This study |
| JL4623 | *ptsP::aacC1* derivative of JL4389 obtained by marker exchange mutagenesis, $Ina^+$, $Gm^r$, $Km^r$. | This study |
| JL4624 | *ptsP::aacC1* derivative of JL4365 obtained by marker exchange mutagenesis, $Ina^+$, $Gm^r$, $Km^r$. | This study |
| JL4625, JL4626 | *ptsP::lacZ* derivatives of Pf-5 obtained by marker exchange mutagenesis, $Lac^+$, $Km^r$. | This study |

**Table 4.1** (Continued)

| | | |
|---|---|---|
| JL4627, JL4628 | *ptsP::lacZ* derivative of 3985 obtained by marker exchange mutagenesis, Lac$^+$, *rpoS*$^-$, Km$^r$. | This study |
| ***Escherichia coli*** | | |
| DH5α | F$^-$, *endA1*, *hsdR17*, ($r_K^-$ $m_K^+$), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*,ϕ80*dlacZ*, ΔM15, λ- | (135) |
| **Plasmids** | | |
| pBR322 | ColE1 replicon, Ap$^r$ | (135) |
| pRK415 | IncP1 replicon, polylinker of pUC19, Mob | (69) |
| pRK252 | IncP1 replicon, Mob$^+$, Tc$^r$ | (69) |
| pRK2013 | Mobilizing plasmid, ColE1 replicon, Tra$^+$, Km$^r$. | (37) |
| pUC18, pUC19 | ColE1 replicon, Ap$^r$ | (135) |
| pJEL01 | Stably maintained in *E. coli* or *Pseudomonas* spp., replicons from pVSP1 and pACYC184, Mob$^+$ Tc$^r$. | (136) |
| pMini-Tn*5lac*Z1 | Mini-Tn5 containing promoterless *lacZ* on a 4.1-kb *Sma*I fragment cloned in pUT. Km$^r$, Tc$^r$ . | (26) |
| pMGm | ColE1 replicon, source of *aacC1* (Gm$^r$) cassette on a 2.0-kb *Sma*I fragment, Gm$^r$, Tc$^r$. | (101) |
| pLAFR3 | IncP1 replicon; *cos*, Mob$^+$, Tc$^r$. | (143) |
| pJEL5649 | 2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pJEL01, Mob$^+$, Tc$^r$ | (136) |

**Table 4.1** (Continued)

| | | |
|---|---|---|
| pJEL5917 | 9.3-kb *Eco*RI genomic fragment from JL4297 containing Tn*5* and adjacent DNA cloned into pBR322, $Km^r$, $Amp^r$, $Tc^r$. | This study |
| pJEL5919 | 2.4-kb *Hin*dIII-*Eco*RI genomic fragment from pJEL5917 in pBR322, used for preliminary sequence analysis of *ptsP,* $Km^r$, $Ap^r$, $Tc^r$. | This study |
| pJEL6201 | 4.3-kb *Eco*RI genomic fragment from Pf-5 containing *ptsP* gene in pUC18, $Ap^r$. | This study |
| pJEL6204 | 4.3-kb *Eco*RI genomic fragment from Pf-5 containing *ptsP* gene in pJEL01, $Tc^r$. | This study |
| pJEL6205 | 4.3-kb *Eco*RI genomic fragment from Pf-5 containing *ptsP* gene in pJEL01, $Tc^r$. | This study |
| pJEL6207 | Cosmid library clone of Pf-5 containing genomic DNA corresponding to the *ptsP* locus cloned in pLAFR3, $Tc^r$. | This study |
| pJEL6208 | Cosmid library clone of Pf-5 containing genomic DNA corresponding to the *ptsP* locus cloned in pLAFR3, $Tc^r$. | This study |
| pJEL6216 | 5.2-kb *Eco*RI fragment containing *ptsP::aacC1* cloned into pRK415, $Gm^r$, $Tc^r$. | This study |
| pJEL6219 | 6.8-kb *Eco*RI fragment containing *ptsP-lacZ* cloned in pRK252, $LacZ^+$, $Tc^r$. | This study |

Abbreviations: $Ap^r$, $Km^r$, and $Tc^r$; resistance to ampicillin, kanamycin, and tetracycline, respectively.

(Gaithersburg, Md.). Ends of certain restriction fragments were blunted with the large subunit of DNA polymerase (135) and thermal cycling was used for blunt-end ligations (93).

### 4.3.3 Cloning *ptsP* from Pf-5

A pLAFR3 genomic library of Pf-5 (114) was screened by colony hybridization (43) to identify cosmids containing wildtype DNA corresponding to the mutagenized locus in the Plt-overproducing mutant JL4297. The probe consisted of a digoxigenin-11-dUTP labeled 9.3-kb *Eco*RI genomic fragment containing Tn*5* from JL4297 generated using a nick translation kit (GibcoBRL Life Technologies). Qiabrane filters (Qiagen, Chatsworth, CA) were prepared and hybridized following the methods for the Genius System of Boehringer Mannheim (Indianapolis, IN). Cosmids were isolated from colonies that hybridized to the probe, digested with *Eco*RI and analyzed in Southern blots (data not shown) to identify restriction fragments corresponding to the wildtype DNA. A 4.3-kb *Eco*RI fragment that hybridized to the probe was cloned into pUC18 to construct pJEL6201, which served as the template for sequence analysis and for mutagenesis.

### 4.3.4 Sequence analysis

DNA sequencing and oligonucleotide syntheses were performed at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, OR. Sequencing of double-stranded templates was performed on an ABI model 373A

Automated DNA Sequencer using a Taq DyeDeoxy (TM) Terminator Cycle Sequencing Kit (Applied Biosystems, Inc. Foster City, CA) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI model 380B DNA synthesizer using phosphoramidite chemistry (2). Sequencing of *ptsP* in pJEL6201 was performed using primers complementary to pUC19 DNA on either side of the polylinker and by oligonucleotide primers complementary to regions within the sequenced fragments. The precise location of Tn*5* in JL4297 was determined from sequence initiated from a primer complementary to Tn*5* using pJEL5919 as a template. Plasmid pJEL5919 contains a 2.4-kb *Eco*RI-*Hin*dIII fragment composed of 1.2-kb of Tn*5* and 2.4-kb of genomic sequence flanking Tn5 cloned from JL4297. Analyses of DNA and deduced protein sequence and comparisons with sequences in the GenBank database were accomplished with software from the Genetics Computer Group, Inc., Madison, WI (29) and the Staden software package (9)

### 4.3.5 Derivation of *ptsP*::Tn*5*, *ptsP*::*aacC1*, and *ptsP*::*lacZ* mutants by marker-exchange mutagenesis

A *ptsP*::Tn*5* mutant. The 9.3-kb Tn*5*-containing *Eco*RI fragment from the genome of JL4297 was cloned into pBR322, which does not replicate in *Pseudomonas* spp., to create pJEL5917. pJEL5917 was mobilized from *E. coli* DH5a donors into Pf-5 in a triparental mating with *E. coli* DH5a containing the helper plasmid pRK2013 (37). Transconjugants from this mating were selected on KB Km with Sm, which counterselected against *E. coli* donors. The resultant marker-exchange mutant JL4480

had a Tn*5* insertion in the same region as the original mutant JL4297, as determined from Southern analysis (data not shown) with the 9.3-kb *EcoRI* fragment as a probe.

A *ptsP*::*aacC1* mutant. The 2.0-kb *Sma*I fragment containing *aacC1* from pMGm (101), which confers gentamycin resistance, was cloned into the *Bgl*II site internal to *ptsP* in pJEL6201. The resulting 5.2–kb *Eco*RI fragment, containing *ptsP*::*aacC1*, was cloned into pRK415, which confers resistance to tetracycline and is not stably maintained in Pf-5, to create pJEL6216. pJEL6216 was mobilized into Pf-5 derivatives as described for the *ptsP*::Tn*5* mutant, and putative transconjugants were selected on KB Tc200, which selects for pRK415 in Pf-5 and counterselects against *E. coli* donors. Transconjugants resistant to Tc200 and Gm40 were grown at 27°C with shaking for up to 7 days with daily sub-culturing into fresh KB broth without antibiotics. Loss of pJEL6216 was confirmed by screening for no growth on KB Tc200, and incorporation of marker into the genome of Pf-5 was assessed on KB Gm40. Replacement of *ptsP* with *ptsP*::*aacC1* in the genome of Pf-5, JL4389, and JL4365, resulting in derivatives JL4622, JL4623, and JL4624 respectively was determined by Southern analysis (data not shown).

A *ptsP*::*lacZ* mutant. The 3.5-kb *Sma*I-*Hind*III fragment containing a promoterless *lacZ* from pMiniTn3-LacZ1 (26), was blunted and cloned into the *Bgl*II site internal to *ptsP* in pJEL6201, creating a transcriptional fusion of *lacZ* to the *ptsP* promoter. The resulting 6.8–kb *Eco*RI fragment, containing *ptsP-lacZ*, was cloned into pRK252, which confers resistance to tetracycline and is not stably maintained in Pf-5, to create pJEL6219. pJEL6219 was mobilized into Pf-5 and its *rpoS*::Tn*5* derivative as described for *ptsP*::Tn*5* mutant, and putative transconjugants were selected on KB Tc200, which selects for pRK252 in Pf-5 and counterselects against *E. coli* donors. Additionally,

plates were supplemented with 40 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase (X-gal) (International biotechnologies, Inc. New Haven, CT) and 100 mg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St Louis, MO), to identify clones harboring *lacZ*. To allow loss of plasmid, transconjugants were grown at 27°C with shaking for 3 days with daily subculturing into fresh KB broth without antibiotics. Loss of pJEL6219 was confirmed by screening for lack of growth on KB Tc200, and incorporation of *lacZ* into the genome of Pf-5 was assessed with KB amended with Xgal and IPTG. Replacement of *ptsP* with *ptsP-lacZ* in the genome of two derivatives of Pf-5, creating JL4625 and JL4626, and two derivatives of JL3985 (*rpoS*::Tn*5*), creating JL4627 and JL4628, was confirmed with Southern analysis.

### 4.3.6 Antibiotic quantification

Antibiotics were extracted from cells and spent media of cultures grown in triplicate by described methods (160). Briefly, Plt and Prn concentrations were quantified from cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol, a medium that favors their production. The concentration of Phl was quantified from cultures grown for 4 days in 5 ml of NB containing 2% glucose, a medium that favors its production. Culture supernatants were extracted twice with 2 ml ethyl acetate. The bacterial pellet was extracted with 4 ml acetone. Extracts were dried under reduced pressure dissolved in MeOH, and analyzed by $C_{18}$ reverse-phase HPLC with a 0.8 X 10 cm Waters Nova-pak radial compression cartridge with either 1) an 18 min linear gradient from 10% to 100% acetonitrile with 0.1% acetic acid in water at a flow rate of 1 ml/min

or 2) water:acetonitrile:methanol (45:30:25 [vol/vol]) at a flow rate of 1.5 ml/min. Antibiotics were detected with a UV photodiode array detector at 310 nm (Plt), 225 nm (Prn), and 278 nm (Phl) and quantified against authentic standards. Quantification was done twice with similar results.

### 4.3.7 Visual assessment of HCN, protease, and tryptophan side-chain oxidase

The production of hydrogen cyanide by Pf-5 and its derivatives was determined as previously described (15,136). Protease production was assessed as a zone of clearing on Bacto litmus milk agar (Difco Laboratories, Detroit, Mich). Tryptophan side-chain oxidase activity was assessed by the method of Takai and Hayaishi (147). Each phenotype was determined from two replicates and the experiment was repeated.

### 4.3.8 Growth on various carbon sources

Derivatives of Pf-5 were grown in 5 ml M9 medium containing 0.4% glucose, fructose, or glycerol and incubated at 27°C with shaking at 200 rpm. Optical density was evaluated at 2 h intervals for 14 hours. The experiment was repeated with similar results and one representative experiment is presented.

### 4.3.9 Stress responses

Methods for determining the response of *P. fluorescens* to exposure to $H_2O_2$ were as previously described (160). Briefly, stationary-phase cells were harvested at 4 h after the optical density of cultures stopped increasing in M9 medium with 0.4% glucose. Harvested cells were washed once and suspended in 5 ml of M9 medium without glucose to obtain an $OD_{600}$ of 0.8. Suspended cells were exposed to 15 mM $H_2O_2$, incubated with shaking at 27°C for 1 h, and CFUs were enumerated by spreading dilutions on KB at 20 min intervals. Three replicate cultures were evaluated for each treatment. The experiment was done twice with similar results.

For experiments evaluating the ability of derivatives of Pf-5 to withstand freezing, stationary-phase cells were harvested at 4 h after the optical density of cultures stopped increasing in KB broth. Harvested cells were washed once and suspended in 5 ml of 1mM phosphate-buffered saline (pH 7.0) (135) to obtain an $OD_{600}$ of 0.1. Initial CFUs were enumerated by spreading dilutions on KB, and 0.1 ml of the cell suspensions were aliquotted to microcentrifuge tubes and subjected to temperatures of -80°C, -20°C, 4°C, or room temperature (approximately 25°C) for 12 h. After incubation, 0.9 ml of warm water (42°C) was added to each sample and CFUs were again enumerated by dilution plating. Two replicate cultures were evaluated for each treatment and the experiment was done twice.

Ability of derivatives of Pf-5 to withstand starvation was determined by washing and suspending harvested cells grown in KB broth at an $OD_{600}$ of 0.1 in 25 ml of 1mM phosphate buffer containing 0.8% saline (pH 7.0). Suspended cells were incubated at

27°C with shaking at 200 rpm for four weeks. CFUs were enumerated by plating dilutions on KB for five consecutive days during the first week and three alternate days for each of the three remaining weeks. Two replicate cultures were evaluated for each treatment and the experiment was done twice.

### 4.3.10 Transcription of *pltB* and *ptsP*

The transposon Tn*3-nice* contains a promoterless *inaZ* gene that, when inserted into a gene in the appropriate orientation, generates a transcriptional fusion that confers ice nucleation activity (INA) on its bacterial host (76)(84). The effect of *ptsP* on the transcription of the *plt* biosynthetic genes was determined by comparing INA expressed by derivatives of Pf-5 containing genomic insertions of Tn*3-nice* in *pltB* (JL4389) or *pltE* (JL4365) (76) (See fig. 1.1) to near-isogenic strains also containing mutations in *ptsP*, *lon*, or *rpoS*. INA was quantified by a droplet-freezing assay at –5°C as described previously (92) from cultures grown for 2 days at 20°C with shaking at 200 rpm in NB amended with 1% glycerol. Treatments were done in triplicate, the experiment was done twice, and the results of a representative experiment are presented.

Transcription of *ptsP* was assessed with a transcriptional fusion of *lacZ* to the *ptsP* promoter. From duplicate cultures of JL4625, JL4626, JL4627 and JL4628 grown in NB amended with 1% glycerol, b-galactosidase activity was determined by a standard assay (98) during exponential phase ($OD_{600}$ 0.2-0.7) and during stationary phase defined by the point when the optical density stopped increasing exponentially ($OD_{600}$ 2.5-2.7). Cells were made permeable with SDS and $CHCl_3$, and then incubated for 10 min at 27°C,

after which *o*-nitrophenyl-b-D-galactopyranoside was added to a final concentration of 0.66 mg/ml. β-galactosidase was expressed as Miller units (Miller U) (98).

### 4.3.11 Biological control assay

Acid de-linted cotton seeds (var. Deltapine, Delta and Pine Co., GA, provided by J. Kloepper) were neutralized by washing in 50% sodium carbonate for 15 min. Seeds were then surface disinfested with 30% bleach for 10 min, rinsed with water, placed in 70% EtOH for 1 min, rinsed again with water and air-dried. Inoculum of *P. fluorescens* Pf-5 and derivatives was grown in KB broth, and bacterial cells were washed and suspended in phosphate buffer. Seeds were soaked for 10 min in aqueous cell suspensions of *P. fluorescens* to obtain $10^8$ CFU per seed or mock treated by soaking for 10 min in 10mM potassium phosphate buffer (pH 7.0). Seeds were planted individually in pots containing 50ml of pasteurized Newberg fine sandy loam soil infested with *P. ultimum* var. Tx. Water was added to a final moisture content of 11% (wt/wt) to a matric potential of –0.01 MPa. Pots were maintained at 25° for two weeks and the numbers of emerging and surviving seedlings were recorded daily. Thirty-seven replicate pots per treatment in a complete random block design with one plant per treatment per block were compared with a type III sum of squares multifactor analysis of variance (ANOVA). After square-root transformation, the means were compared using Duncan's multiple range test with a 95% confidence interval (Statgraphics plus version 3 software package, Manugistics, Inc, Rockville MD). Rhizosphere population sizes of Pf-5 and derivatives

were estimated from entire root systems of three healthy plants, grown in the absence of pathogen, by dilution plating of root washings on KB Sm.

Cucumber (c.v. Marketmore 86, Territorial, Cottage Grove, OR) seeds were surface disinfested as described for cotton seeds. Cultures of Pf-5 were prepared as described for cotton and treated seeds were planted individually in pots containing 20ml soil infested with *P. ultimum* var. N1. Water was added to 13% (wt/wt). Pots were maintained at 20°C for ten days and emerging and surviving seedlings were recorded daily. Thirty-six replicate pots per treatment in a complete random block design with five plants per treatment per block were compared. The percentage emerging or surviving seedlings was calculated for each treatment per block. The treatments were compared for influence on seedling survival with a type III sum of squares multifactor analysis of variance (ANOVA). The percent emerging and surviving seedlings per block were square-root transformed and the means were compared using Duncan's multiple range test with a 95% confidence interval (Statgraphics plus software package). Rhizosphere population sizes of Pf-5 and derivatives were estimated from entire root systems of three healthy replicate plants by dilution plating of root washings on KB Sm.

## 4.4 Results

### 4.4.1 Identification of a *ptsP*::Tn*5* derivative of Pf-5 that over-produces Plt

JL4297, a derivative of Pf-5 obtained following random Tn*5* mutagenesis, overproduces the antibiotic Plt in media containing glycerol (75). To confirm that the Tn*5* insertion caused overproduction of Plt, the transposon was re-introduced into the same site in the genome of Pf-5 by marker exchange mutagenesis. Each derivative contained a single Tn*5* insertion in a 9.3-kb *Eco*RI fragment of genomic DNA, as determined from Southern analysis (data not shown). Both JL4297 and the re-created mutant JL4480 produced a higher concentration of Plt than Pf-5 (Table 4.2), confirming that Plt overproduction was associated with the Tn*5* insertion and not due to secondary mutations at other loci. Neither Pf-5 nor JL4297 produced detectable concentrations of Plt in these experiments in media containing glucose.

We began our analysis of the disrupted locus by identifying cosmids containing the corresponding wild-type DNA from an extant genomic library of Pf-5 (114). Cosmids that hybridized to the Tn*5*-containing *Eco*RI fragment from the genome of JL4297 were identified. Within a 4.3-kb *Eco*RI fragment from a library cosmid clone, an ORF of 759 residues was identified with an ATG start codon 14 bases downstream of a putative GAGG ribosomal binding site (Fig. 4.1). Once translated, the predicted protein was > 85% identical to PtsP from *A. vinelandii* (140) and *P. aeruginosa* (148). The C-terminal portion of PtsP was >36% identical to various PtsI homologs in a 515 a.a. residue overlap, whereas the N-terminal domain was 29% identical to NifA of *Azospirillum*

**Table 4.2: Secondary metabolite production by *P. fluorescens* Pf-5 and derivatives.**

| Strain | Characteristics | Plt (µg/ml)[1] | Prn (µg/ml)[1] | Phl (µg/ml)[1] | HCN | TSO | Proteases |
|---|---|---|---|---|---|---|---|
| Pf-5 | wildtype | 5.3 ± 1.4 | 1.20 ± 0.10 | 7.20 ± 0.62 | + | + | + |
| JL4297 | *ptsP*::Tn*5* | 9.8 ± 1.3 | 0.47 ± 0.03 | 4.18 ± 0.01 | + | + | + |
| JL4480 | *ptsP*::Tn*5* | 9.4 ± 0.8 | 0.44 ± 0.07 | 3.03 ± 0.65 | + | + | + |
| JL4622 | *ptsP*::*aacC1* | 10.0 ± 1.4 | 0.45 ± 0.02 | 2.31 ± 0.15 | + | + | + |
| JL4618 | *ptsP*::Tn*5*, *ptsP*$^+$ | 5.7 ± 0.5 | 1.10 ± 0.04 | 8.31 ± 1.30 | + | + | + |

[1] Plt and Prn were quantified from extracts of cells and spent media of cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol.
[2]Phl was quantified from extracts of cells and spent media of cultures grown for 4 days at 20°C in 5 ml of NB containing 2% glucose.
[3] Values are the means from three replicate cultures ± SD. Detection limit defined at 0.01 mg/ml.
[4] (+) = presence of compound assessed qualitatively.
[5] *ptsP*+ represents multiple plasmid borne copies of *ptsP*.

**Figure 4.1:** DNA sequence and deduced protein sequence of the *ptsP* locus.

```
    GAATTCGATGGCTGGCGCTGGGTCAGTTATTGGTATCCGTTGGGCCAGGTGGTGAC   56

ATTCAAGCGCGAAGTGTATCGCCGCGCTCTCAAAGAGCTTGCCCCGCGCCTGCTAGTGCG  116

CGACTGACGACGGAGTTCGACCCCGAGCCATGCTCAATACGCTGCGCAAGATCGTCCAGG  176
                                M  L  N  T  L  R  K  I  V  Q  E

AAGTTAACTCCGCCAAGGATCTCAAGGCGGCGTTGGGGATTATTGTATTGCGCGTCAAAG  236
  V  N  S  A  K  D  L  K  A  A  L  G  I  I  V  L  R  V  K  E

AGGCCATGGGCAGCCAGGTCTGCTCGGTCTATCTGCTGGACCCGGAAACCAACCGTTTCG  296
  A  M  G  S  Q  V  C  S  V  Y  L  L  D  P  E  T  N  R  F  V

TCCTGATGGCCACCGAGGGCTTGAACAAGCGCTCCATCGGCAAGGTCAGCATGGCGCCCA  356
  L  M  A  T  E  G  L  N  K  R  S  I  G  K  V  S  M  A  P  N

ACGAAGGCCTGGTAGGCCTGGTGGGCACTCGCGAAGAACCCCTCAACCTGGAAAACGCCG  416
  E  G  L  V  G  L  V  G  T  R  E  E  P  L  N  L  E  N  A  A

CCGACCACCCGCGCTATCGCTACTTCGCCGAAACCGGTGAGGAGCGTTACGCCTCCTTCC  476
  D  H  P  R  Y  R  Y  F  A  E  T  G  E  E  R  Y  A  S  F  L

TCGGTGCGCCGATCATTCACCACCGCCGGGTGGTGGGCGTGCTGGTTATCCAGCAAAAGG  536
  G  A  P  I  I  H  H  R  R  V  V  G  V  L  V  I  Q  Q  K  E

AGCGGCGCCAGTTCGACGAAGGCGAAGAAGCCTTCCTGGTGACCATGAGTGCCCAACTGG  596
  R  R  Q  F  D  E  G  E  E  A  F  L  V  T  M  S  A  Q  L  A

CCGGGGTTATCGCCCATGCCGAGGCCACCGGTTCGATCCGTGGCCTGGGTCGCCAGGGCA  656
  G  V  I  A  H  A  E  A  T  G  S  I  R  G  L  G  R  Q  G  K

AGGGCATTCAGGAAGCCAAGTTCGTCGGCGTACCGGGCTCGCCCGGCGCGGCGGTGGGCA  716
  G  I  Q  E  A  K  F  V  G  V  P  G  S  P  G  A  A  V  G  S

GCGCGGTGGTGATGCTGCCGCCGGCCGACCTGGATGTGGTGCCGGACAAGCACGTCACCG  776
  A  V  V  M  L  P  P  A  D  L  D  V  V  P  D  K  H  V  T  D

ACATCGACGCCGAACTGGCCCTGTTCAAGAGCGCCCTGGAAGGGGTGCGGGCCGATATGC  836
  I  D  A  E  L  A  L  F  K  S  A  L  E  G  V  R  A  D  M  R

GCGCGCTGTCGGAAAAGCTCGCCACTCAATTGCGCCCTGAAGAGCGCGCGCTGTTCGACG  896
  A  L  S  E  K  L  A  T  Q  L  R  P  E  E  R  A  L  F  D  V
                                        Tn5
                                         ▼
TCTACCTGATGATGCTCGACGACGCCTCCCTGAGCAGTGAAGTGAAGGCCGTGATCAAGA  956
  Y  L  M  M  L  D  D  A  S  L  S  S  E  V  K  A  V  I  K  T

CCGGGCAATGGGCCCAGGGCGCGTTGCGCCAGGTGGTCACCGATCACGTCAACCGTTTCG 1016
  G  Q  W  A  Q  G  A  L  R  Q  V  V  T  D  H  V  N  R  F  E

AACTGATGGACGATGCCTACCTGCGCGAGCGGGCCTCGGACGTCAAGGACCTGGGCCGCC 1076
  L  M  D  D  A  Y  L  R  E  R  A  S  D  V  K  D  L  G  R  R
```

**Figure 4.1** (Continued)

```
GGCTCCTGGCCTACCTGCAGGAAGAGCGCCAGCAGACCCTGGTCTATCCCGACAACACCA  1136
  L  L  A  Y  L  Q  E  E  R  Q  Q  T  L  V  Y  P  D  N  T  I

TCCTGGTCAGTGAAGAACTGACCCCGGCCATGCTCGGCGAAGTGCCGGAAGGCAAGCTGG  1196
  L  V  S  E  E  L  T  P  A  M  L  G  E  V  P  E  G  K  L  V

TGGGCCTGGTCTCGGTGCTGGGTTCGGGCAACTCCCACGTGGCGATCCTGGCACGGGCCA  1256
  G  L  V  S  V  L  G  S  G  N  S  H  V  A  I  L  A  R  A  M

TGGGCATCCCGACGGTGATGGGCCTGGTGGACCTGCCGTACTCCAAGGTCGACGGCATCC  1316
  G  I  P  T  V  M  G  L  V  D  L  P  Y  S  K  V  D  G  I  Q

AGATGATCGTCGACGGCTACCACGGCGAGGTCTACACCAACCCCAGCGACGTGCTGCGCA  1376
  M  I  V  D  G  Y  H  G  E  V  Y  T  N  P  S  D  V  L  R  K

AGCAGTTCGCCGATGTGGTGGAAGAAGAACGGCAACTGGCCCAGGGCCTGGATGCCCTGC  1436
  Q  F  A  D  V  V  E  E  E  R  Q  L  A  Q  G  L  D  A  L  R

GCGACCTGCCCTGCATCACCCCCGATGGCCATCGCATGCCGCTGTGGGTCAACACCGGCC  1496
  D  L  P  C  I  T  P  D  G  H  R  M  P  L  W  V  N  T  G  L

TGCTGGCCGATGTAGCCCGGGCGCAGAAGCGCGGGGCCGAAGGCGTGGGCCTGTATCGCA  1556
  L  A  D  V  A  R  A  Q  K  R  G  A  E  G  V  G  L  Y  R  T

CCGAAGTGCCGTTCATGATCAACCAGCGCTTCCCCAGCGAGAAGGAACAGCTGGCGATCT  1616
  E  V  P  F  M  I  N  Q  R  F  P  S  E  K  E  Q  L  A  I  Y

ATCGCGAACAGCTGGCGGCCTTCCACCCGCAACCGGTGACCATGCGCAGCCTGGACATCG  1676
  R  E  Q  L  A  A  F  H  P  Q  P  V  T  M  R  S  L  D  I  G

GCGGCGACAAGTCGCTGTCGTACTTCCCGATCAAGGAAGACAACCCCTTCCTTGGCTGGC  1736
  G  D  K  S  L  S  Y  F  P  I  K  E  D  N  P  F  L  G  W  R

GCGGGATCCGCGTGACCCTCGACCACCCGGAGATCTTCCTGGTACAGGCCCGGGCGATGC  1796
  G  I  R  V  T  L  D  H  P  E  I  F  L  V  Q  A  R  A  M  L

TCAAGGCCAGCGAAGGCCTGAACAATTTGCGCATCCTGCTGCCGATGATTTCCGGCATCC  1856
  K  A  S  E  G  L  N  N  L  R  I  L  L  P  M  I  S  G  I  H

ACGAGCTGGAAGAAGCCTTGCACCTGATCCACCGGGCCTGGGGCGAAGTGCGCGATGAAG  1916
  E  L  E  E  A  L  H  L  I  H  R  A  W  G  E  V  R  D  E  G

GCACTGACGTACCGATGCCGCCAGTGGGGGTGATGATCGAAATCCCCGCCGCGGTGTACC  1976
  T  D  V  P  M  P  P  V  G  V  M  I  E  I  P  A  A  V  Y  Q

AGACCAAGGAACTGGCGCGCCAGGTGGATTTCCTCTCGGTGGGCTCCAACGACCTGACCC  2036
  T  K  E  L  A  R  Q  V  D  F  L  S  V  G  S  N  D  L  T  Q

AGTACCTGCTGGCGGTGGACCGCAACAACCCACGGGTGGCCGATCTCTACGACTACCTGC  2096
  Y  L  L  A  V  D  R  N  N  P  R  V  A  D  L  Y  D  Y  L  H

ACCCGGCGGTGCTGCAAGCGCTGCAGAACGTGGTGCGTGATGCCCATGCCGAAGGCAAGC  2156
  P  A  V  L  Q  A  L  Q  N  V  V  R  D  A  H  A  E  G  K  P
```

**Figure 4.1** (Continued)

```
CAGTGAGCATCTGCGGCGAGATGGCCGGTGATCCGGCGGCGGCGGTGCTGCTGATGGCGA  2216
  V  S  I  C  G  E  M  A  G  D  P  A  A  A  V  L  L  M  A  M

TGGGCTTCGACAGCCTGTCGATGAACGCCACCAACCTGCCGAAGGTGAAGTGGATGCTGC  2276
  G  F  D  S  L  S  M  N  A  T  N  L  P  K  V  K  W  M  L  R

GTCAGGTGAGCATGACCAAGGCCAAGGAACTGCTGGCGGAAATGATGACCATCGACAACC  2336
  Q  V  S  M  T  K  A  K  E  L  L  A  E  M  M  T  I  D  N  P

CGCAAGTTATCCACAGTTCGCTGCAACTGGCGCTGAAGAAACTCGGGCTGGCGCGGATGA  2396
  Q  V  I  H  S  S  L  Q  L  A  L  K  K  L  G  L  A  R  M  I

TCAATCCGGCTTCGAGCAAGACCCTGTAACCGGGTTGTTTTCTTCGCCGGCAAGCCGGCT  2456
  N  P  A  S  S  K  T  L  *

CCTACAGAAGCGTTCGTGGGAGCCGGCGTGCCGGGGAACGCTTCAGACCTTCAGTTCGAC  2516

CTCTCCCAGATGCCCGCCATAGGGGCCGAAGCTACGCTCCAGCAGATGCCGCGAGCCATC  2576

GGCGTGGACGATCAGTGCCGTGCTGGCTCGTGTGCCGTAGCTGGGGCTGGCGATGAACAC  2636

GCTGGACAGCAGGCTTTCGGTGGCCAAGCCCACGCCGGTGTCCGGCAGGTCGGCGAAGGG  2696

CGCTGTCTGCGGGTCGCTCAACAGCGCCAGCAGTGCCTGGGGCTGTGGATCATGCAACAC  2756

CTCCTGCAACGCCGCCCTGGCCTTGAGCAGCTTGGGCCAGGGCGTATCCAGCCCGGCATT  2816

GGAAAGGCCGTAGACCCCAGGCTCCAGCCGTTGCGGCTGCGGGTCCCGAGCGTTGTAGTG  2876

CCACAGCTGCTCGGCATTGCCCAGCAGCAGGTTGAACCCGGCGTACTCCACCGAGCGCCC  2936

GACTACATCGCGCAAATAATCATCCAGGGGCACGTCGCCGGCGAGAAAGCGCGCCACCAG  2996

TTCACCCCGCGAGCGCCGCGCCGGAGGCTGGTGCGGGTTGCGGATATTGGTCAGGGCGGC  3056

AAAGCGGCCCTCGGCGCCGACCCCGAGCCAGGTGCCGCCGGCTTCCAGGTCGCGACCGGC  3116

GTGGATCTGCGGCAGTTCCGGCCACTGCCCCAGGGGCAGGCTCGGCCGCGCGTAGAATTC  3176
```

*lipoferum* (142) and a putative two-component sensor from *S. coelicolor* A3(2) (122) in a 152 a.a. residue overlap. The *ptsP* gene from Pf-5 was used to complement the *ptsP*::Tn*5* mutation *in trans*, which reduced production of Plt by JL4297 to the level observed in Pf-5 (Table 4.2).

### 4.4.2 *ptsP*::Tn*5* did not impair the ability of Pf-5 to utilize fructose

Since PtsI is required for utilization of fructose by *P. aeruginosa* (148) and because Plt production is enhanced or repressed in response to glycerol or glucose respectively (76), we compared the growth characteristics of Pf-5 and its *ptsP*::Tn*5* derivative in a minimal medium containing glucose, glycerol, or fructose (Fig. 4.2). No apparent growth defect was detected in the *ptsP*::Tn*5* derivative compared to Pf-5.

### 4.4.3 *ptsP*::Tn*5* did not impair the ability of Pf-5 to tolerate oxidative stress or freezing at –80°C

We tested the influence of *ptsP*::Tn*5* on tolerance of Pf-5 to oxidation, freezing, and starvation. Tolerance to these stresses is impaired in the *rpoS*::Tn*5* derivative of Pf-5 (136,145). The *ptsP* mutant survived exposure to oxidation (Fig. 4.3, Table 4.3) and freezing at –80°C (Table 4.3) as well as or better than Pf-5. Although we observed slight differences in tolerance to starvation in one experiment (Fig. 4.4, Table 4.3), these differences were not as substantial as those observed for an *rpoS* mutant. Especially notable, the *ptsP* mutant did not exhibit decline in its population resulting from starvation

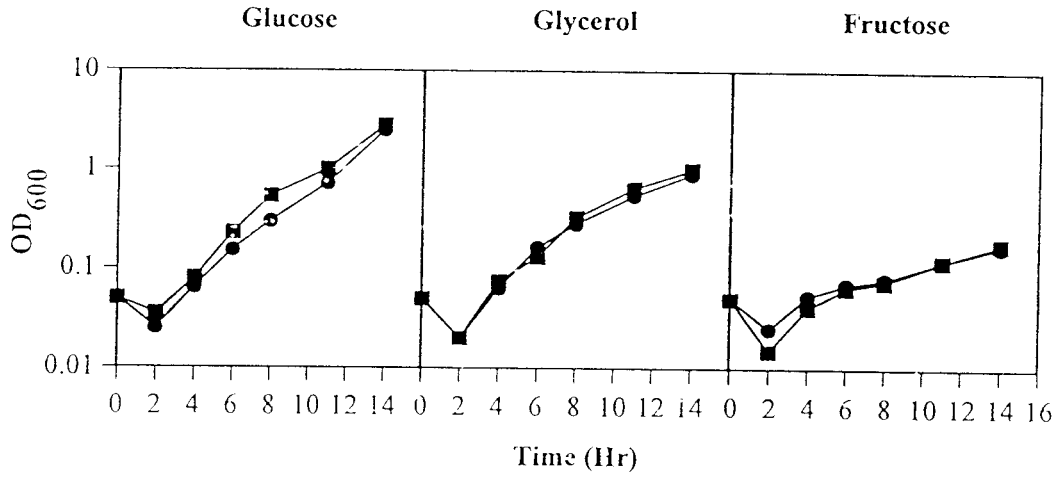


**Figure 4.2:** Growth on various carbon sources. Pf-5 (■) and its *ptsP*::Tn*5* derivative (•) were grown in M9 minimal medium supplemented with glucose, glycerol, and fructose and optical density was monitored over time.

**Table 4.3. Stress tolerance of *P. fluorescens* Pf-5 and derivatives.**

| Strain | Characteristics | Survival ($log[CFU_t/CFU_0]$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Oxidation[1,2] | | Freezing, -20[1,3] | | Freezing, -80 [1,3] | | Starvation[1,4] | |
| | | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| Pf-5 | Wild-type | -1.7 ± 0.1 | -1.0 ± 0.5 | -2.7 ± 1.6 | -2.5 ± 0.8 | -1.0 ± 0.1 | -1.7 ± 0.1 | 0.03 ± 0.2 | 0.4 ± 0.1 |
| JL4297 | *ptsP*::Tn*5* | -0.8 ± 0.1 | -0.8 ± 0.3 | -3.6 ± 0.8 | -4.1 ± 0.1 | -1.0 ± 0.1 | -2.0 ± 0.8 | -0.5 ± 0.1 | 0.3 ± 0.1 |
| JL3985 | *rpoS*::Tn*5* | ND[5] | ND[5] | -2.9 ± 0.1 | -3.2 ± 0.7 | -1.6 ± 0.3 | -2.6 ± 0.4 | -1.1 ± 0.1 | -0.6 ± 0.1 |

[1] Values are expressed as log(survival ratio). Values are the means from two replicate cultures ± SD. Colonies resulting from surviving cells, before and after exposure to stress, were counted after growth on KB. Results from two independent experiments are presented.
[2] Cells were exposed to 15mM $H_2O_2$ for 60 minutes.
[3] Cells were exposed to Freezing for 12 h.
[4] Cells were incubated in phosphate buffered saline for 26 days.
[5] ND= Stress response of JL3985 was not successfully determined in parallel cultures.

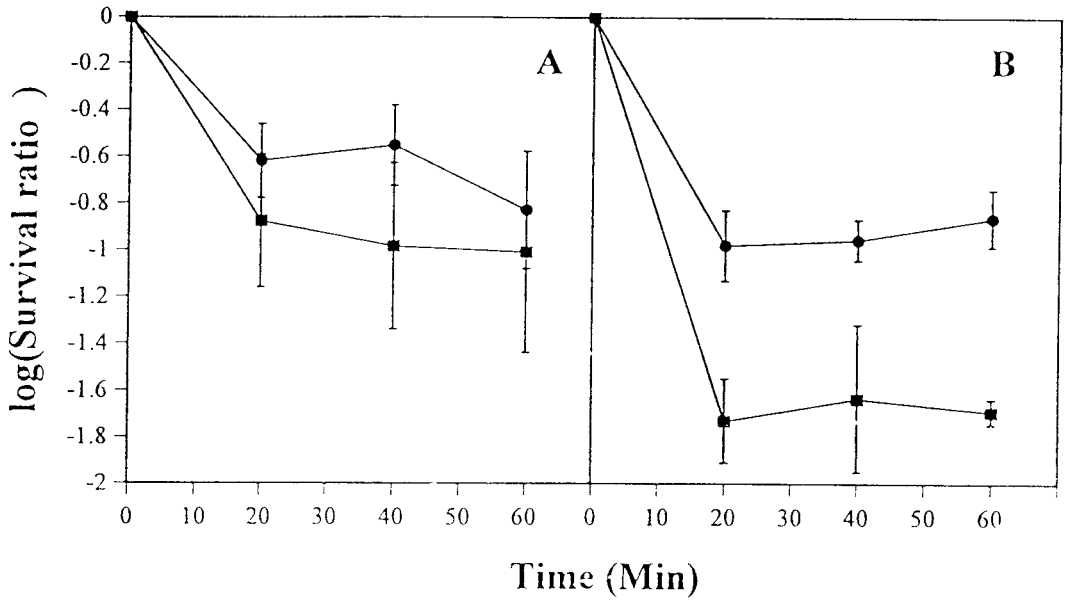


**Figure 4.3:** Survival of oxidative stress. Stationary-phase cells of *P. fluorescens* Pf-5 (▪) and its *ptsP*::Tn*5* derivative (•) were exposed to 15 mM $H_2O_2$, and the numbers of culturable cells were estimated over time. Presented values are the means of three replicate cultures. Two independent experiments are presented, panel A corresponds to Exp. 2 from table 2.4 whereas panel B corresponds to Exp. 1. Error bars represent the SD.

stress between days 5 and 10 whereas the *rpoS* mutant significantly declined. The *ptsP* mutant was less tolerant to freezing at –20°C compared to Pf-5 in one experiment (Table 4.3).

### 4.4.4 *ptsP*::Tn*5* influenced *pltB* and *pltE* gene transcription

The influence of PtsP, Lon, and $\sigma^S$ on the transcription of *plt* biosynthetic genes was assessed with an existing transcriptional fusion of the ice nucleation reporter gene in Tn*3-nice* to *pltB* in strain JL4389, and to *pltE* in strain JL4365. In JL4389 and JL4365, expression of ice nucleation activity is reflective of *pltB* and *pltE* transcription, respectively. Ice nucleation activities expressed by JL4365 and JL4389 were compared to the activities of derivative strains with *ptsP*::*aacC1*, *lon*::*aacC1*, or *rpoS*::*lacZ* mutations, or with both *lon*::*aacC1* and *rpoS*::*lacZ* mutations (Table 4.4). Ice nucleation activity is expressed as $\log_{10}$(ice nuclei per cell); therefore, increasingly positive values represent higher transcription whereas increasingly negative values represent lower transcription of the promoters. A mutation in *ptsP* significantly increased *pltB* and *pltE* transcription compared to the parental strain. However, the influence of the *ptsP* mutation was modest when compared to the affect that mutations in *rpoS* and *lon* had on *pltB* transcription, which differed significantly from both the parental strain and the *ptsP*::*aacC1* derivative.

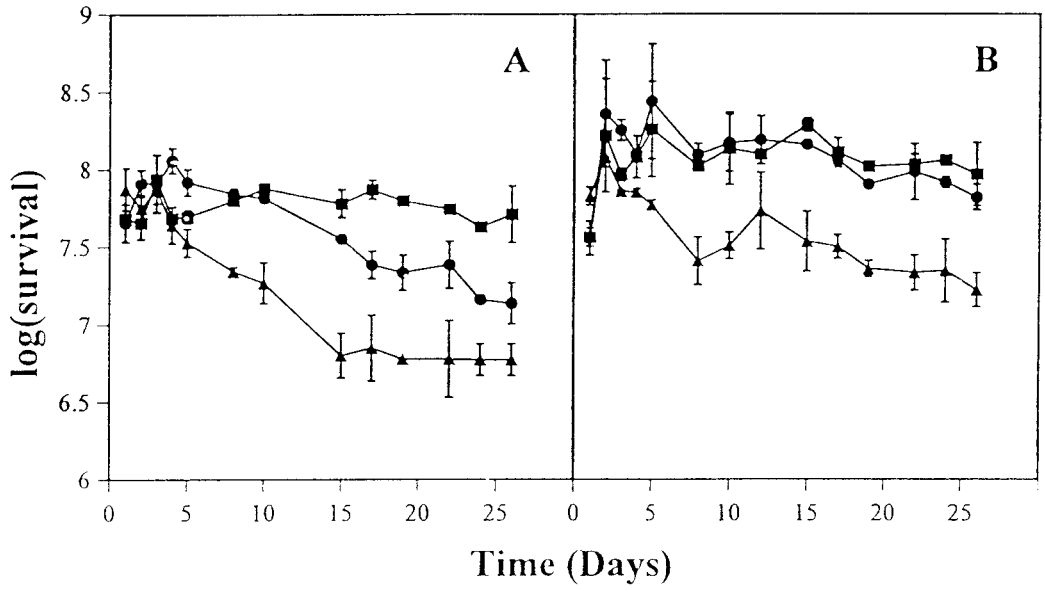


**Figure 4.4:** Tolerance to starvation. Stationary-phase cells of *P. fluorescens* Pf-5 (■) its *ptsP*::Tn*5* derivative (•), and an *rpoS*::Tn*5* derivative (▲) were suspended in phosphate buffered saline and incubated for four weeks. The numbers of culturable cells were estimated over time. Presented values are the means of three replicate cultures. Two independent experiments are presented. Panel A corresponds to Exp. 1 from table 4.3 whereas panel B corresponds to Exp. 2. Error bars represent the SD.

**Table 4.4. Transcription of *plt* biosynthetic genes assessed with ice nucleation reporter gene**

| Characteristics | *pltB*::*inaZ*[1] | *pltE*::*inaZ*[1] |
|---|---|---|
| *lon*$^+$, *rpoS*$^+$, *ptsP*$^+$ | -2.33 ± 0.16 | -2.57 ± 0.19 |
| *ptsP*::*aacC1* | -1.78 ± 0.10 | -1.76 ± 0.16 |
| *lon*::*aacC1* | -0.55 ± 0.15 | ND[2] |
| *rpoS*::*lacZ* | -0.73 ± 0.19 | ND |
| *lon*::*aacC1*, *rpoS*::*lacZ* | -0.15 ± 0.12 | ND |

[1] INA[log (ice nuclei per cell)]Values are the means from three replicate cultures ± SD.
[2] ND= not determined in parallel cultures.

### 4.4.5 A mutation in *rpoS* did not influenced *ptsP-lacZ* gene transcription

The influence of a mutation in *rpoS* on *ptsP* transcription was evaluated by comparing β-galactosidase activity resulting from the LacZ protein expressed from a *ptsP-lacZ* fusion in strains with or without $\sigma^S$ (Table 4.5). Expression of *ptsP-lacZ* did not differ between derivatives of Pf-5 with and without $\sigma^S$. Furthermore, *ptsP-lacZ* transcription decreased somewhat during stationary phase.

### 4.4.6 Enhanced Plt production by derivatives of Pf-5 could improve biological control of Pythium damping-off of cotton and cucumber

Pf-5, one Plt non-producing derivative, and several Plt over-producing derivatives were tested for biological control of Pythium damping-off of cotton (Table 4.6) and cucumber (Table 4.7). Pf-5 did not significantly suppress disease in these experiments. On cotton, the *lon* and *ptsP* mutants suppressed disease, but they did not differ from either Pf-5 or *rpoS* and *pltB* mutants. On cucumber, there is no significant treatment effect as seen from the ANOVA table (Table 4.7).

## 4.5 Discussion

In this report, we described PtsP as a negative regulator of Plt Production by *P. fluorescens* Pf-5, and characterized phenotypes associated with previously identified regulators of Plt. PtsP appears unique among regulators currently described. First, the

**Table 4.5. Transcription of *ptsP-lacZ* in Pf-5 and *rpoS*::Tn*5* derivative**

| Strain | Characteristic | β-galactosidase activity[1] | |
|---|---|---|---|
| | | Exponential-phase[2] | Stationary-phase[3] |
| JL4625 | *rpoS*$^+$ | 747 ± 54 | 416 ± 9 |
| JL4626 | *rpoS*$^+$ | 719 ± 250 | 447 ± 5 |
| JL4627 | *rpoS*::Tn*5* | 877 ± 21 | 414 ± 82 |
| JL4628 | *rpoS*::Tn*5* | 789 ± 250 | 525 ± 110 |

[1] Values are the means from two replicate cultures ± SD, expressed as Miller U.
[2] Cultures grown in NB+1% Glycerol, for 3-6 hr to an $OD_{600}$ of 0.2-0.7.
[3] Cultures grown in NB+1% Glycerol, for 9-12 hr to an $OD_{600}$ of 2.4-2.7.

**Table 4.6. Biological control of Pythium damping-off of cotton by Pf-5 and derivatives**

| Strain | Treatment | Percent emergence[1] | Percent survival[1] |
|---|---|---|---|
| | No Pathogen | 76 | 76 |
| | No Treatment | 13 a | 13 a |
| | Pf-5 | 24 ab | 22 abc |
| JL4292 | *lon*::Tn*5* | 43 b | 43 c |
| JL4297 | *ptsP*::Tn*5* | 38 b | 38 bc |
| JL3985 | *rpoS*::Tn*5* | 35 ab | 32 abc |
| JL4296 | *pltB*::Tn*5* | 27 ab | 22 abc |

[1] values followed by a common letter designate that the square-root transformed means did not differ significantly at the 5% level as determined by Duncan's multiple range test

**Analysis of variance, emergence**

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | *F* Ratio | *P*- Value |
|---|---|---|---|---|---|
| Block | 41 | 1.541 | 0.038 | 0.65 | 0.946 |
| Treatment | 5 | 0.561 | 0.112 | 1.95 | 0.088 |
| Residual | 175 | 10.073 | 0.058 | | |
| Total (corrected) | 221 | 12.167 | | | |

**Analysis of variance, survival**

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | *F* Ratio | *P*- Value |
|---|---|---|---|---|---|
| Block | 41 | 1.794 | 0.042 | 0.82 | 0.772 |
| Treatment | 5 | 0.064 | 0.128 | 2.40 | 0.039 |
| Residual | 175 | 9.299 | 0.053 | | |
| Total (corrected) | 221 | 11.736 | | | |

**Table 4.7. Biological control of Pythium damping-off of cucumber by Pf-5 and derivatives**

| Strain | Treatment | Percent emergence[1] | Percent survival[1] |
|---|---|---|---|
| | No Pathogen | 83 | 83 |
| | No Treatment | 30 a | 10 a |
| | Pf-5 | 30 a | 27 ab |
| JL4292 | *lon*::Tn*5* | 26 a | 23 ab |
| JL4297 | *ptsP*::Tn*5* | 30 a | 17 ab |
| JL3985 | *rpoS*::Tn*5* | 40 a | 40 b |
| JL4296 | *pltB*::Tn*5* | 30 a | 20 a |

[1] values followed by a common letter designate that the square transformed means did not differ significantly at the 5% level as determined by Duncan's multiple range test

**Analysis of variance, emergence**

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | *F* Ratio | *P*-value |
|---|---|---|---|---|---|
| Block | 5 | 0.463 | 0.0927 | 0.64 | 0.670 |
| Treatment | 5 | 0.186 | 0.0372 | 0.26 | 0.932 |
| Residual | 25 | 3.607 | 0.1442 | | |
| Total (corrected) | 35 | 4.257 | | | |

**Analysis of variance, survival**

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | *F* Ratio | *P*- Value |
|---|---|---|---|---|---|
| Block | 5 | 1.134 | 0.227 | 1.75 | 0.161 |
| Treatment | 5 | 1.209 | 0.242 | 1.86 | 0.137 |
| Residual | 25 | 3.244 | 0.130 | | |
| Total (corrected) | 35 | 5.587 | | | |

influence of a *ptsP* mutation on Plt production and *plt* biosynthetic gene transcription was modest compared to the two previously characterized negative regulators of Plt, $\sigma^S$ and Lon protease. Whereas *ptsP* mutants produced two times the amount of Plt produced by Pf-5 (Table 4.2), *lon* and *rpoS* mutants produce ten-fold the amount produced by the *ptsP* mutants (Table 3.2). Second, both Lon and $\sigma^S$ are important regulators of stress response in Pf-5, and a mutation in either gene compromises the strain's ability to tolerate certain stresses. In contrast, the sensitivity of Pf-5 to a number of environmental stresses was not compromised substantially by a *ptsP* mutation. The *ptsP* mutant was as tolerant or more tolerant than Pf-5 to oxidative stress. The *ptsP* mutant was as tolerant as Pf-5 to freezing at –80°C. When exposed to starvation, the *ptsP* mutant was not as sensitive as an *rpoS* mutant, although in one experiment, the population decline of the *ptsP* mutant differed significantly from Pf-5. The temporal pattern of the decline in viability differed between *rpoS* and *ptsP* mutants exposed to starvation. Specifically, the population of the *rpoS* mutant declined significantly between days 5-10, whereas a *ptsP* mutant and Pf-5 did not decline during this time. Different temporal patterns of decline suggest that the mechanism of starvation sensitivity in *rpoS* and *ptsP* mutants differs. Only one stress tolerance characterized herein was notably compromised in one experiment in the *ptsP* mutant: freezing at –20°C. The lower tolerance of the *ptsP* mutant to freezing at –20°C that was not also observed at –80°C could reflect differences in ice crystal formation since rapid freezing at –80°C likely favors the formation of small and potentially less harmful ice crystals within cell membranes whereas slower freezing at –20°C likely favors the formation of larger and potentially more disruptive ice crystals in cell membranes.

PtsP (also called Enzyme $I^{Ntr}$) is presumed to function in a sugar phosphotransferase system called $PTS^{Ntr}$. The most characterized analogous PTS is that of *E. coli*, which transports across the cell membrane classic PTS sugars such as glucose, fructose, mannose, N-acetylglucosamine, mannitol, sorbitol, and lactose (117, 133). PTS also influences utilization of sugars not transported directly by the system, presumably by regulating other proteins involved in utilization of those sugars. Four proteins participate in the PTS group translocation of sugars (Fig, 4.5). First, the cytoplasmic enzyme I harvests phosphate from phosphoenolpyruvate and transfers it to a specific histidine residue of HPr. HPr transfers the phosphate to Enzyme III, a peripheral cytoplasmic membrane associated protein. Enzyme II forms the transmembrane channel, determines the sugar specificity, and catalyzes phosphoryl transfer from enzyme III to the sugar substrate, which is concomitantly translocated across the membrane.

In bacteria where PtsP has been characterized, including Pf-5, it is not required for the utilization of classic PTS sugars (140). In *A. vinelandii*, *ptsP* is required for accumulation of the carbon storage compound poly-β-hydroxybutyric acid, which can serve as an electron acceptor protecting nitrogenase from oxidative damage (140). In the human pathogen *P. aeruginosa*, a *ptsP* mutant was impaired in virulence on two artificial hosts including the plant *Arabidopsis thaliana* and the nematode *Caenorhabditis elegans* (148). Interestingly, other regulators identified in the screen for factors that influence virulence by *P. aeruginosa* include GacA/S (148) that, like PtsP, influence Plt production by Pf-5. The involvement of common global regulators in both species of *Pseudomonas* demonstrates a intriguing link between antibiotic and virulence factor production. These findings suggest that in *Pseudomonas* spp., the expression of factors important to

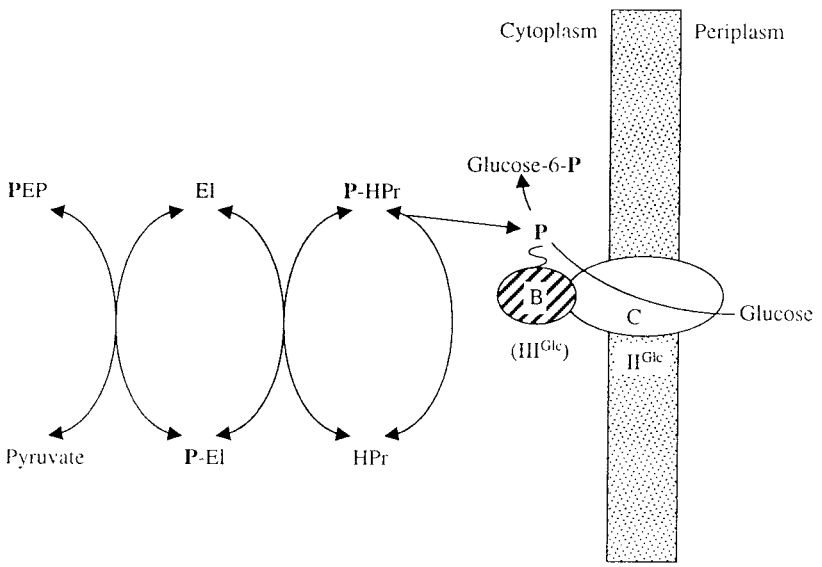


**Figure 4.5**: PTS transporters of bacteria. PEP = phosphoenol pyruvate, **P** = Phosphoryl group, EI = Enzyme I, EII = Enzyme II, EIII = enzyme III, HPr=(Heat stable) Histidine protein.

colonization or survival in association with eukaryotic hosts is governed by common regulatory and signal transduction systems whether the interaction is benign or harmful.

Although *ptsP* and *ptsI* share sequence identity, there is an important distinction between the paralogs: the presence in PtsP of an N-terminal domain similar to nitrogen responsive regulators and to regulators of polyketide antibiotic production (32, 42). The function of this domain remains unexplored, but based on its deduced amino acid sequence, it is likely to be involved in sensory transduction. The linkage of certain components of $PTS^{Ntr}$ to the *rpoN* operon (118) and the similarity of the domain to nitrogen responsive regulators led Reizer and colleagues (125) to propose that PtsP may provide a link between nitrogen and carbon utilization. Current research in *E. coli* with PtsP undoubtedly will test this hypothesis. No additional information on SrmR, a regulator of polyketide antibiotic production by *Streptomyces* spp. similar to the N-terminal domain of PtsP, is currently available. That both PtsP and SrmR regulate polyketide antibiotic production is particularly striking. Future analysis of the role of source and quality of nitrogen on Plt production by Pf-5 should elucidate the possible role of PtsP in linking nitrogen metabolism and antibiotic production.

Plt production by Pf-5 is subject to regulation in response to growth substrates for which PtsP may play a role. Plt production by Pf-5 in culture is repressed by glucose and enhanced by glycerol (75). The impact of growth substrate was also implied *in situ* where *plt* biosynthetic gene transcription by Pf-5 was enhanced on cotton but not on cucumber in the first 12 hours after inoculated seeds were planted in soil (76). Because PtsP is similar to proteins involved in uptake and regulation of carbon utilization, further characterization of PtsP may shed light on how growth substrates influence Plt

production. However, the *ptsP* mutation did not relieve glucose repression of Plt production. Previously, researchers suggested that pyrroloquinoline quinone (PQQ) was involved in glucose and ethanol dehytrogenase regulation of Plt production by a similar biological control strain *P. fluorescens* CHA0, (139). In strain CHA0, *pqqF* mutants overproduce Plt and, because of the role of PQQ as a cofactor in alcohol dehydrogenase, the mutants also could not utilize ethanol as a sole carbon source (139). PtsP influenced *plt* biosynthetic gene transcription; however, both PqqF and PtsP could also influence the flow of Plt biosynthetic substrates such as acetyl-CoA, or alternatively may regulate transcription of necessary components of Plt production.

In addition to the preliminary phenotypic characterization of the *ptsP* mutant in culture, we evaluated the impact of the mutation on biological control of Pythium damping-off of cucumber and cotton. For Pf-5, the contribution of Plt to suppression of disease on cotton may be more important than on cucumber because *plt* biosynthetic genes are transcribed on cotton seeds during early stages of disease (76). Furthermore, an *rpoS* mutant that overproduces Plt, Phl, and HCN is better than Pf-5 at biological control of post-emergence damping-off on cucumber caused by *P. ultimum*, suggesting that enhanced antibiotic production could improve biological control (136). Therefore, the *ptsP*, *lon*, and *rpoS* mutants were tested in parallel with Pf-5 and a Plt non-producing derivative to evaluate the contribution of Plt production to disease suppression. In these preliminary experiments, Pf-5 did not suppress disease significantly on cotton or cucumber. This result is surprising in contrast to previous reports demonstrating that Pf-5 suppresses Pythium damping-off on these plant hosts (75,136). Several reasons for the discrepancy are possible. In particular, the age of inoculum for *P. ultimum* differed in

these experiments from previous reports. Inoculum used in these experiments was two years old whereas inoculum used in previous experiments was six months old. Additionally, differences in soil type could account for poor biological control. Specifically, due to the sandy nature of the soil used for these experiments, the percentage of water added to reach field capacity was lower than in previous experiments (11% and 17% respectively), and likely did not favor disease. For this reason, the number of propagules necessary to achieve disease could be greater for these experiments than in previous experiments. On both cotton and cucumber there was no difference between Pf-5 and its derivatives (Tables 4.6 and 4.7), but trends in these studies are consistent with previous reports (75,136). Cucumber seeds treated with the *rpoS* mutant had the greatest seedling survival, whereas the *ptsP* and *lon* mutants were more similar to Pf-5. These findings may allude to the importance of overproduction of multiple antibiotics by the *rpoS* mutant to enhanced suppression of disease on cucumber. For cotton, seeds treated with the *ptsP* and *lon* mutants had greater seedling survival than seeds treated with Pf-5 or the *rpoS* mutant. The findings on cotton and cucumber, taken together, could suggest 1) that the contribution of Plt over-production alone is more important on cotton than cucumber or 2) that overproduction of HCN and Phl by the *rpoS* mutant was harmful to cotton. Indeed, these preliminary experiments only serve to demonstrate the utility of the mutants for evaluating the contribution of Plt to biological control, but no conclusions can be made without more thorough analysis.

# Chapter 5

## Two Mutants of *Pseudomonas fluorescens* Pf-5 Deficient in Linoleic Acid Utilization and Impaired in Biological Control of Pythium Post-emergence Damping-off

Cheryl A. Whistler and Joyce Loper

## 5.1 Abstract

Long-chain fatty acids (LCFAs) such as linoleic acid present in seed exudates are known to trigger sporangial germination of *Pythium ultimum* (103). Utilization of linoleic acid by the biocontrol organism *Enterobacter cloacae* suppresses the stimulatory activity of seed exudates on *P. ultimum* and reduces Pythium damping-off disease of cotton (153,154). Many soil bacteria utilize LCFAs including the biological control agent *Pseudomonas fluorescens* Pf-5 (153). One mechanism by which *Pseudomonas fluorescens* Pf-5 could suppress Pythium damping-off is by degrading seed exudates that function as germination signals for sporangia of *P. ultimum*. Tn*5* mutants of Pf-5 were screened for utilization of linoleic acid, a long-chain fatty acid (LCFA), as a sole carbon source on minimal agar plates. Two mutants were selected and characterized. Sequence analysis of the DNA flanking the transposon in one isolate identified the disrupted gene as a homolog of *aceB*. The product of *aceB*, malate synthase, is required for growth of bacteria on fatty acids as a sole carbon source, which is consistent with a linoleic utilization deficiency. In a second mutant, the sequence flanking the transposon had identity to portions of two genes: *cti* and *cysI*, but the disrupted DNA was not homologous to the complete coding sequence of either gene thus preventing assignment of identity. Both linoleic utilization deficient derivatives were tested for suppression of a seedling disease of cucumber caused by *Pythium ultimum*. Reduction in the biocontrol ability of the linoleic utilization mutants suggests that Pf-5 could protect seedlings from disease by signal interference.

## 5.2 Introduction

*Pseudomonas fluorescens* Pf-5 is a biological control agent that suppresses seedling diseases of cotton (59) and cucumber (75). Pf-5 produces at least three secondary metabolites that inhibit growth of the pathogen *P. ultimum*, including pyoluteorin (Plt) (59), 2,4-diacetylphloroglucinol (Phl) (105), and hydrogen cyanide (23). Mutants of Pf-5 deficient in Plt production or in global control of antibiotic production and cyanide still suppress Pythium damping-off of cucumber (75). Therefore, Pf-5 must suppress disease by mechanisms other than antibiosis.

*P. ultimum* is a common seed-rotting pathogen that survives in the soil as dormant sporangia. Within the first six to twelve hours after seeds are sown and presumably in response to seed exudates, sporangia germinate, infect seeds, and prevent seedling emergence (103). After an initial period of susceptibility, the seedlings become more resistant to infection; therefore, for infection to succeed, *Pythium* sporangia must perceive early signals from planted seeds. Long-chain fatty acids (LCFA) present in cotton seed exudates trigger sporangial germination (131) and can serve as sources of carbon for bacterial inoculants (103,153). The biological control bacterium *Enterobacter cloacae* can utilize LCFAs, thereby reducing sporangial germination by *P. ultimum* in response to seed exudates (153,154). Mutations in one of two genes, *fadB* involved in β-oxidation and *fadL* required for fatty acid uptake, impair utilization of the LCFA linoleic acid by *E. cloacae* (154). The *fadL* and *fadB* derivatives were unable to inhibit sporangial germination and did not suppress *Pythium* seed rot in cotton seedling bioassays (154).

These properties suggest that interference with certain sporangial germination signals is an important mechanism by which *E. cloacae* suppresses Pythium seed rot (103).

Strains of seed-associated *P. fluorescens* can utilize LCFAs but the possibility that signal interference is a mechanism of biocontrol in *P. fluorescens* Pf-5 has not been investigated. To evaluate the role of LCFA signal interference as a mechanism for biological control by *P. fluorescens* Pf-5, we isolated two Tn*5* mutants deficient in utilization of linoleic acid as a sole carbon source. Sequence analysis determined that the disrupted gene in one mutant was a homolog of *aceB,* which encodes malate synthase (124), an enzyme necessary for the use of fatty acids and acetate as sole carbon sources (107). A second mutant was also characterized but the identity and function of the mutated gene could not be deduced due to limited nucleotide sequence identity with known genes. Both linoleic acid utilization mutants produced the full complement of antifungal compounds of Pf-5. Biocontrol assays demonstrated that the linoleic acid utilization mutants were less effective thatn Pf-5 had lower seedling survival in biocontrol of Pythium damping-off of cucumber, implying that signal interference may contribute to the biocontrol capacity of Pf-5.

## 5.3 Materials and Methods

### 5.3.1 Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table 5.1. *P. fluorescens* strains were grown routinely on King's medium B (KB) (70) at 27°C. *Escherichia coli* strains were cultured routinely on Luria-Bertani agar (LB) (135) at 37°C. Auxotrophy and linoleic acid utilization were determined from replica-plated bacterial colonies on M9 minimal medium (135) containing 0.5 mg/mL glucose (M9Glc) or 0.5 mg/mL linoleic acid (M9Lin), respectively. Antibiotics were at 20 µg/mL tetracycline ($Tc_{20}$) for cultures of *E. coli*, 200 µg/mL tetracycline ($Tc_{200}$) for cultures of *P. fluorescens*,100 µg/mL streptomycin (Sm), 50 µg/mL chloramphenicol (Cm) or 50 µg/mL kanamycin (Km). Growth on acetate was determined with acetate differential agar (AD) (130). Ability of bacteria to utilize acetate as the sole carbon source on this medium was assessed by the change of the agar from green to blue. Antibiotics were assessed from cultures grown in nutrient broth (NB) (Difco Laboratories) supplemented with 2% glucose or 1% glycerol.

### 5.3.2 Storage and handling of linoleic acid

A 10 mg/mL stock solution of linoleic acid sodium salt (Sigma Chemical Co., St Louis, MO) was prepared in 1% (wt/vol) Brij-58 (Sigma), filter sterilized, and 10 mL aliquots were placed in sterile ampules. Rubber septa sealed ampules were sparged with

**Table 5.1: Bacterial strains and plasmids used in this study**

| Strain or Plasmid | Description | Reference |
|---|---|---|
| ***Psedomonas fluorescens*** | | |
| Pf-5 | Rhizosphere isolate | (58) |
| JL4400 | Derivative of Pf-5 impaired in linoleic acid utilization; *aceB*::Tn*5* | This study |
| JL4425 | Tn*5* derivative of Pf-5 impaired in linoleic acid utilization | This study |
| JL4432 | Marker-exchanged derivative of Pf-5; *aceB*::Tn*5* | This study |
| JL4636 | Marker-exchanged derivative of Pf-5 | This study |
| ***Eschericia coli*** | | |
| DH5α | F$^-$, *endA1*, *hsdR17*, ($r_K^-$ $m_K^+$), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*,ϕ80*dlacZ*, ΔM15, λ- | (135) |
| C600 | | (10) |
| HB101 | | (135) |
| **Plasmids** | | |
| pBR322 | ColE1 replicon, $Ap^r$, $Tc^r$ | (135) |
| pUC19 | ColE1 replicon, $Ap^r$ | (135) |
| pRK2013 | Mobilizing plasmid, $Tra^+$, $Km^r$ | (37) |
| pLG221 | Tn*5* delivery plasmid | (10) |

**Table 5.1** (Continued)

| | | |
|---|---|---|
| pJEL5784, pJEL5785 | 22.1-kb *Eco*RI fragment containing *aceB*::Tn*5* cloned in pBR322,$Tc^r$, $Ap^r$, $Km^r$. | This study |
| pJEL5803 | A *Hin*dIII deletion derivative of pJEL5784 containing a single 20.1- kb *Eco*RI-*Hind*III fragment, $Ap^r$. | This study |
| pJEL5804 | A *Hin*dIII deletion derivative of pJEL5785 containing a single 10.1- kb *Eco*RI-*Hind*III fragment, $Ap^r$. | This study |
| pJEL6223 | 20.7-kb *Eco*RI fragment from JL4425 containing Tn*5* cloned in pUC19, $Ap^r$. | This study |
| pJEL6224 | 13-kb *Bam*HI fragment from pJEL6223 cloned in pUC19, $Ap^r$, $Km^r$. | This study |
| pJEL6225 | A *Bam*HI deletion derivative of pJEL6223 containing a 4.8-kb *Eco*RI-*Bam*HI genomic fragment in pUC19, $Ap^r$. | This study |
| pJEL6226 | 2.9-kb *Bam*HI fragment from pJEL6223 cloned in pUC19, $Ap^r$. | This study |
| pJEL6227 | 20.7-kb *Eco*RI fragment from JL4425 containing Tn*5* cloned in pBR322, $Tc^r$, $Ap^r$, $Km^r$. | This study |

Abbreviations: $Ap^r$, $Km^r$, and $Tc^r$; resistance to ampicillin, kanamycin, and tetracycline, respectively.

nitrogen gas to displace the air and were stored at 4°C in the dark. Agar plates were prepared frequently and stored in the dark at 4°C.

### 5.3.3 Nucleic acid methods

Plasmids were isolated from *E. coli* with a Promega Magic Mini-prep Kit (Madison, WI). Genomic DNA was isolated by a cetyltrimethyl ammonium bromide method and precipitated with isopropanol (4). Electrophoresis in agarose gels in Tris-phosphate EDTA buffer, restriction digests with enzymes from GibcoBRL (Bethesda, MD), and ligation procedures were standard (135).

### 5.3.4 Derivation of linoleic acid utilization mutants

Transposon Tn*5* was randomly inserted into the genome of Pf-5 as previously described (75). The Tn*5* delivery plasmid pLG221 (10) was mobilized from *E. coli* C600 donors into Pf-5 in triparental matings with *E. coli* DH5α containing helper plasmid pRK2013 (37). 3528 putative transposon mutants were selected on KB Km Sm. Following two days incubation, putative Tn*5* mutants of Pf-5 were replica plated onto KB Km and M9Lin Km. Colonies unable to grow after three days on M9Lin Km were re-streaked from KB Km plates to obtain individual colonies, which were re-tested on M9Lin Km Sm to confirm lack of growth, and M9Glc Km Sm to determine auxotrophy. For a later mutagenesis experiment, 700 transconjugants were selected on M9Glc Km Sm, to eliminate bacteria auxotrophic for growth on glucose minimal medium and to

facilitate more rapid screening. Two mutants unable to utilize linoleic acid, strains JL4400 and JL4425, and 20 mutants that could not grow on glucose minimal medium, strains JL4401 through JL4420, were identified by these methods.

### 5.3.5 Cloning of Tn*5* and flanking DNA

The genomic DNA from JL4400 was digested with *Eco*RI and ligated to *Eco*RI-digested pBR322. The recombinant plasmids pJEL5784 and pJEL5785 were selected as transformants of *E. coli* HB101 on LB Km $Tc_{20}$. Both plasmids contained the same Tn*5* containing 22.1-kb genomic fragment, each in a different orientation. The location of a single Tn*5* insertion within the *Eco*RI fragment was determined by restriction mapping. Two additional sub-clones were constructed by deleting most of the transposon but maintaining one terminal repeat to provide template for sequencing. From pJEL5784, 1.1-kb of the genomic fragment was deleted following digestion with *Hin*dIII and ligation. The resultant plasmid, pJEL5802, contained a 21-kb *Eco*RI-*Hin*dIII fragment. From pJEL5785, 10.1-kb of the genomic fragment was deleted following digestion with *Hin*dIII and ligation. The resultant plasmid, pJEL5803, contained a 12-kb *Eco*RI-*Hin*dIII fragment.

The genomic DNA from JL4425 was digested with *Eco*RI and a 20.7-kb fragment was ligated to *Eco*RI-digested pBR322 and pUC19 to create pJEL6227 and pJEL6223 respectively. Transformants of *E. coli* DH5α containing these plasmids were selected on LB Km Ap. The location of a single Tn5 insertion within the *Eco*RI fragment was determined by restriction mapping. Three additional sub-clones were constructed by

digesting pJEL6223 with *Bam*HI, which cuts within the transposon, at another site within the flanking genomic DNA, and in the multiple cloning site of pUC19, resulting in three *Bam*HI fragments. The 7.5-kb *Bam*HI fragment containing pUC19 and 4.8-kb of genomic DNA was self-ligated creating pJEL6225. Two additional *Bam*HI fragments, each containing half of the transposon and flanking genomic DNA, were ligated to pUC19: a 13-kb *Bam*HI fragment containing the neomycin resistance cassette of Tn*5* was cloned into pUC19 to create pJEL6224, whereas a 2.9-kb *Bam*HI fragment was cloned into pUC19 to create pJEL6226.

### 5.3.6 Marker-exchange mutagenesis

To determine if the Tn*5* insertion was associated with inability of strains JL4400 and JL4425 to utilize linoleic acid, plasmids pJEL5784 and pJEL6227 were used to replace the wild-type copy of the mutated gene in the genome of Pf-5 by taking advantage of the inability of pBR322 to replicate in *Pseudomonas* spp.. Plasmids were moved into Pf-5 for marker exchange mutagenesis in tri-parental matings as previously described (75). Putative mutants were purified on KB KmCm, and then replica-plated onto M9Lin to confirm inability to utilize linoleic acid (for JL4400), AD to evaluate growth on acetate (JL4425), onto KB Km to confirm presence of Tn*5* transposon, and onto KB Km $Tc_{200}$ to confirm loss of plasmid.

### 5.3.7 Nucleotide sequencing

DNA sequencing and oligonucleotide syntheses were performed at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, OR. Sequencing of double-stranded templates was performed on an ABI model 373A Automated DNA Sequencer using a Taq DyeDeoxy (TM) Terminator Cycle Sequencing Kit (Applied Biosystems, Inc. Foster City, CA) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI model 380B DNA synthesizer using phosphoramidite chemistry (2). Plasmids that contained only one end of the Tn*5* and flanking DNA (pJEL5803 and pJEL5804, which were subclones from JL4400, and pJEL6224 and pJEL6226, which were subclones from JL4425) were used as the templates for sequence analysis of the disrupted genomic region using an oligonucleotide primer complementary to bases 37 to 18 in the inverted repeat of Tn*5*: 5'-GGTTCCGTTCAGGACGCTAC-3' (127). Plasmid pJEL6225, a subclone from JL4425 not containing transposon DNA, also served as a template for sequencing using pUC19 forward and reverse primers. DNA sequence analysis and comparisons with sequences in the GenBank and EMBL databases were accomplished with software from the Genetics Computer Group, Inc. (Madison WI).

### 5.3.8 Antibiotic assessment

Antibiotics were extracted from cells and spent media of cultures grown in triplicate by described methods (160). Plt and Prn concentrations were quantified from

cultures grown for 2 days at 20°C in 5 mL of nutrient broth containing 1% glycerol, a medium that favors their production. The concentration of Phl was quantified from cultures grown for 4 days in 5 mL of nutrient broth containing 2% glucose, a medium that favors its production. Culture supernatants were extracted twice with ethyl acetate. The bacterial pellet was extracted with acetone. Extracts dissolved in MeOH were analyzed by TLC or HPLC and compared to authentic standards as previously described (161).

### 5.3.9 Visual assessment of HCN, protease, and tryptophan side-chain oxidase

The production of hydrogen cyanide by Pf-5 and its derivatives was determined as previously described (15)(136). Protease production was assessed as a zone of clearing on Bacto litmus milk agar (Difco Laboratories, Detroit, Mich). Tryptophan side-chain oxidase activity was assessed by the method of Takai and Hayaishi (147). Each phenotype was determined from two replicates and the experiment was repeated.

### 5.3.10 Biological control

Cucumber seeds (cv. Marketmore 76) were surface disinfested with 30% bleach for 10 min, rinsed with water, placed in 70% EtOH for 1 min, rinsed again with water and air-dried. Inoculum of *P. fluorescens* Pf-5 and derivatives was grown in KB broth, and bacterial cells were washed and suspended in 10 mM potassium phosphate buffer (pH 7.0). Seeds were soaked for 10 min in aqueous cell suspensions of *P. fluorescens* to obtain $10^8$ CFU per seed or mock treated by soaking for 10 min in 10 mM potassium

phosphate buffer (pH 7.0). Seeds were planted individually in pots containing 50 mL of pasteurized Newberg fine sandy loam soil infested with *P. ultimum* isolate N1. Water was added to a final moisture content of 17% (wt/wt), which corresponds to a matric potential of –0.01 MPa. Forty-eight pots per treatment were maintained at 25° for two weeks and the numbers of emerging and surviving seedlings were recorded daily for ten days. The number emerging (total number, recorded daily) and surviving (healthy plants at day 10) seedlings from a complete random block design with eight plants for each of six blocks were compared between treatments with a type III sum of squares multifactor analysis of variance (ANOVA). After square-root transformation of the number of emerged and surviving seedlings per block, the means were compared using Duncan's multiple range test with a 95% confidence interval (Statgraphics plus version 3 software package, Manugistics, Inc, Rockville MD).

## 5.4 Results

### 5.4.1 Isolation of Tn*5* mutants impaired in growth on linoleic acid

From 4228 putative transposon mutants of Pf-5, two derivatives, JL4400 and JL4425, were identified as having impaired growth on M9 minimal agar with linoleic acid as a sole carbon source. Strain JL4400 grew well on M9 minimal agar with glucose as a sole carbon source. Strain JL4425 grew slower than Pf-5 on M9 agar with linoleic acid as a sole carbon source, and grew on M9 glucose medium. Both JL4400 and JL4425

were also deficient in acetate utilization. Twenty derivatives of Pf-5 that did not grow on M9 minimal agar supplemented with glucose were also identified in the screen.

### 5.4.2 Identification of the Tn*5* disrupted gene in JL4400

To confirm that the Tn*5* insertion caused loss of linoleic acid utilization in JL4400, the Tn*5* was re-introduced into the corresponding location in genome of Pf-5 by marker exchange mutagenesis (76). The kanamycin-resistant colonies derived by marker exchange were unable to utilize linoleic acid or acetate as a sole carbon sources, confirming that these phenotypes were associated with the Tn*5* insertion and not due to secondary mutations at other loci.

Single stranded sequence of the disrupted DNA from *P. fluorescens* covering a total of 934 bases was obtained from each end of the transposon (Fig. 5.1). The 934-base sequence was 67% identical to a region internal to the coding sequence of the *aceB* gene encoding malate synthase B of *Corynebacterium glutamicum* (124).

### 5.4.3 Analysis of the Tn*5* disrupted gene in JL4425

The transposon and flanking DNA from JL4425 was re-introduced into the corresponding region in the genome of Pf-5 by marker exchange mutagenesis to create JL4636. JL4636 was unable to utilize acetate as a sole carbon source confirming that loss of acetate utilization is associated with the Tn*5* disruption.

Single stranded sequence of the DNA disrupted in JL4425 covering a total of

**Figure 5.1:** Nucleotide sequence of the locus disrupted by Tn*5* in JL4400

TTGAACNCCGCCNCGAAAACNCGGGGTCAATGNCGANCGGTTGAAACTNNNGNTCGTTCC 60

CTCCTGTTCGTGCCCAANCGTNGNCCACNTGATGACCATCGATGCGATCCTCGAGAAGGA 120

TGCCANCGAAGTGCNGGAAGNCATCNTCGNCGGCCTGNTCACCAGCCTGGCGTCGATCCA 180

CAACCTCAACGGCAACACGTCGCGCAAGAACAGCCGCACCGGCTCGATGTACATCGTCAA 240

GCCGAAGATGCACGGCCCTGAAGAAGCGGCGTTCACCAACGAGCTGTTCGGCCGCATCGA 300

AGAGGTGCTGAACCTGCCGCGCAACACCCTGAAAGTCGGGATCATGGACGAGGAGCNCCG 360

CACCACGGTCAACCTCAAGGCCTGCATCAAGGCGGCCAGCGAGCGCGTGGTGTTCATCAA 420

CACCGGCTTCCTCGACCGCACCGGCGACGAAATCCACACCTCCATGGATGGAGGCCGGCG 480

CCATGGTGCGCAAGGCCGACATGAAGGCCGAGAAATGGATCGGCGCCTACGAGAACTGGA 540

ACGTCGATATCGGCCTGAGCACCGGCCTGCAAGGTCGCGCGCAAAGCGGCAAGGGCATGT 600

GGGCGATGCCGGACCTGATGGCAGCGATGCTCGAACAGAAAATCGCTCACCCACTGGGCG 660

GCGCCAACACTCCTGGGTTCCATCCCCGACCGCCGCTGCGCTGCACGCGCTGCACTATCA 720

CAAGGTCGACGTATTCGCCCGCCAGGCCGAACTGGCCAAGCGTGCCCGCGCCTCGGTGGA 780

TGACATCCTGACCATCCCGCTGGTGGTCAACCCGAGCTGGACGGCGGAGCAGATCAAGAA 840

CGAACTGGTCAACAACGCCCAGGGGATTCTCGGTTACGTGGCGGGCTGGATCGACCAGGC 900

GTGGGCTTTCGAAAGTGCCGACATCATCANGCG

1200 bases was obtained from each end of the transposon and was analyzed for homology to known sequences (Fig. 5.2). Within the sequence, discrete portions of the disrupted DNA showed identity to two genes. In a portion of the sequence that flanks the transposon from nucleotide 429 to 499, the sequence was 92% identical to *cysI* which encodes sulfite reductase of *Pseudomonas aeruginosa* (62). Downstream of the transposon, from nucleotide 617 to 896, the sequence was 80% identical to *cti*, encoding a cis/trans isomerase of *Pseudomonas putida* (65). No significant matches (>25% over >30 bases) to known sequences were found in the remaining portions of the sequence.

### 5.4.4 Assessment of antibiotic production

Strains JL4400 and JL4425 were characterized quantitatively (Table 5.2) for their production of the antibiotics Plt, Prn, and Phl. JL4400 and JL4425 produce all three antibiotics. Hydrogen cyanide and siderophore, qualitatively assessed, were also produced by both derivatives.

### 5.4.5 Biological control

Pf-5, JL4400, and JL4425 were tested for their ability to suppress Pythium damping-off disease of cucumber. All three strains improved seedling emergence and survival over the no treatment control, indicating that the strains protected seedlings from disease (Table 5.3). The three strains did not differ from each other in the number of emerging seedlings but did differ in the number of surviving seedlings. Both JL4400 and

**Figure 5.2:** Nucleotide sequence of locus disrupted by Tn5 in JL4425. The two underlined regions, one flanking the transposon and one immediately downstream were >80% identical to discrete portions of two unique genes *cysI* and *cti*.

AATGGCCCAAGCATCAACGCTACTGTCACTTTTCCTTACTTTGGTTGGAT 50

GGCTCATAGCATCGCCTCTAGTAGATTTTGTCGGTGACTGCTCAAAGACT 100

TTTTGCGCATAACCGCAAACTGGTATCGTTCGGCGGCTCCGTTGGGAGTG 150

GCTCCACAGTTAAGACCAGGATTGCCCCTACCACAAATGGGGTTACTCTC 200

ACGCCCCTACCGTTAGTCGTTTTGGTTCTGTGCATATACTCAAACATCTC 250

CGCGGCCGTGCGCGCAGATTTGCTTACATGGGTGGCGTGAAGCGTGAACG 300

AAGGCAAGACGAGGTGAGACCGGCGAAAACCGCGCTGGACGCCAGTCCGA 350

GCAGCGTACCGAAGCCGTTCACCGCAGTATTTGCCAAGTGCGCTTGAGCG 400

CCGCCTGTAGCCTGAGAACTGGACCTATATGTACGTATACGACGAGTACG 450

Tn*5*

▼

ATCAGCGGATCATCGAGGACCGCGTCAAGCAGTTCCGTGATCAGACCCGA 500

CGCTATCTGGCAGGCGAGCTGAGCGAAGAAGAATTCGAGCTCGGTACCCG 550

GGGATCCGATCAACCGCTGTGACAGCGCCTACTGCTCACGCCCGAACATC 600

GACCCGGCGCTGCAGGATGCCGAACAGGCCCTGAGCCGCCTGGCATCGCG 650

CCCGGCGGCGGGCCTCAAGGTCATCGAACAGTTGCCGGAAGCGACCCTGC 700

TGCGTGTCCAGACCGCCAGCGGCAAGCGCGAGTTCTACAGCATGCTGCGC 750

AACCGCGCCCACAGTAACGTGGCCTTCATGCTCGGCGAGTCGCTGCGCTA 800

CCAGCCGGGGCTGGACACCCTGACCATATTCCCGGGGATTCTCAGCAGCT 850

ATCCGAACTTCATGTTCAACGTACCGGCCGGGCAAGTGCCGGAGTTCGTC 900

GACGCCATGCAGGCGGCCCGGGACACCGCCAGCTTCGAGAAGATCGTCGA 950

GCGCTGGGGCATCCGCCGCAGCCATCCGCAGTTCTGGCTGTACTTCCATG 1000

ACCAGACCCGCTACCTTGCAGGAACCGACCCGGTGGAAGCCGGGGTCCTG 1050

GACATGAACCGTTACGAAACCTCTTACATAGAAGTTGTCATCGCCCCCGG 1100

ACTGCGCCTTGGCGCGGCCCCGGGACGCTTCTTTCCAAAGTCTATTCCCG 1150

**Table 5.2 Secondary metabolite production by *P. fluorescens* Pf-5 and derivatives.**

| Strain | Plt (µg/ml)[1] | Prn (µg/ml)[1] | Phl (µg/ml)[1] | HCN [4] | TSO [4] | Proteases [4] |
|---|---|---|---|---|---|---|
| Pf-5 | 5.3 ± 1.4 | 1.2 ± 0.1 | 7.2 ± 0.6 | + | + | + |
| JL4400 | 5.2 ± 0.5 | 1.1 ± 0.1 | 6.9 ± 1.2 | + | + | + |
| JL4425 | 5.6 ± 0.5 | 0.8 ± 0.1 | 5.0 ± 0.3 | + | + | + |

[1] Plt and Prn were quantified from extracts of cells and spent media of cultures grown for 2 days at 20°C in 5 ml of NB containing 1% glycerol.
[2]Phl was quantified from extracts of cells and spent media of cultures grown for 4 days at 20°C in 5 ml of NB containing 2% glucose.
[3] Values are the means from three replicate cultures ± SD. Detection limit defined at 0.01 mg/ml.
[4] (+) = presence of compound assessed qualitatively.

**Table 5.3: Biological control of Pythium damping-off of cucumber by Pf-5 and derivatives**

| Treatment | Percent emergence[1] | Percent survival[1] |
|---|---|---|
| No Treatment | 21 a | 21 a |
| Pf-5 | 71 b | 63 b |
| JL4400 | 69 b | 58 bc |
| JL4425 | 65 b | 46 c |

[1] values followed by a common letter designate that the square-root transformed means did not differ significantly at the 5% level as determined by Duncan's multiple range test

**Analysis of variance, emergence**

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | *F* Ratio | *P*- Value |
|---|---|---|---|---|---|
| Block | 5 | 0.373 | 0.075 | 0.92 | 0.4926 |
| Treatment | 3 | 3.893 | 1.212 | 16.06 | 0.0001 |
| Residual | 15 | 1.212 | 0.081 | | |
| Total (corrected) | 23 | 5.480 | | | |

**Analysis of Variance, survival**

| Source of variation | Degrees of freedom | Sum of squares | Mean of squares | *F* Ratio | *P*- Value |
|---|---|---|---|---|---|
| Block | 5 | 0.634 | 0.127 | 2.40 | 0.0869 |
| Treatment | 3 | 3.361 | 1.120 | 21.19 | 0.0000 |
| Residual | 15 | 0.793 | 0.053 | | |
| Total (corrected) | 23 | 4.788 | | | |

JL4425 had lower seedling survival than Pf-5, although only JL4425 differed significantly from Pf-5 in seedling survival.

## 5.5 Discussion

Growth on fatty acids by bacteria requires a number of genes involved both in transport and utilization (6). In *E. coli*, transport of fatty acids requires *fadL*, encoding the membrane transporter for fatty acids. Upon transport, the LCFAs are converted to fatty acyl-CoA thioesters by the product of *fadD* (Fig. 5.3). Catabolism proceeds by the β-oxidation pathway encoded by *fadE*, *fadFG*, *fadH* and *fadBA* leading to the production of acetyl-CoA. The two-carbon acetyl-CoA units may be incorporated into fatty acids or phospholipids by *fabA* or further catabolized by the TCA cycle providing electron transport potential. If acetyl-CoA is metabolized by the TCA cycle to form energy, there is no net gain of carbon, due to the loss of two carbon molecules in the form of $CO_2$ for every acetyl-CoA metabolized. Therefore, in order to supply necessary intermediates for cellular biosynthesis in the absence of carbon sources other than fatty acids, the glyoxylate shunt is required, where the enzymes isocitrate lyase encoded by *aceA*, and malate synthase, encoded by *aceB*, catalyze the conversion of isocitrate to malate producing one mole of dicarboxylic acid for every 2 moles of acetyl-CoA (107).

Following random transposon mutagenesis of the genome of *P. fluorescens* Pf-5, we isolated two derivatives impaired linoleic acid and acetate utilization. Both linoleic acid utilization deficient derivatives were similar to Pf-5 in their production of antibiotics

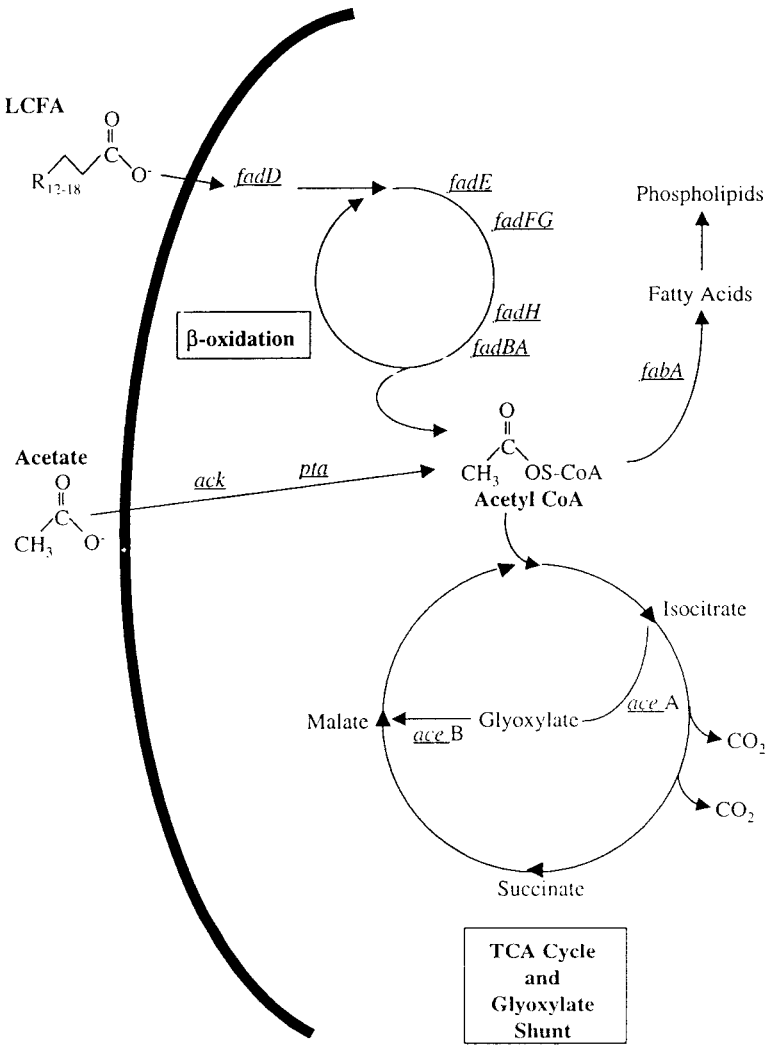


**Figure 5.2:** Long-chain fatty acid and acetate utilization by bacteria.

and growth on glucose minimal medium. Malate synthase, the product of the *aceB* gene disrupted in JL4400, may function in the glyoxylate shunt similarly to that described for *E. coli*. A second mutant of Pf-5 that grew very slowly on linoleic acid, JL4425, also was identified in this screen. We did not determine the identity of the disrupted DNA, because only two small segments of the DNA had identity to discrete portion of two unrelated genes, *cti* and *cysI*. It is possible that the mutated region is a previously uncharacterized gene, or that chromosome rearrangements or deletions in the region resulted from the transposon insertion. Neither *cti* nor *cysI* are predicted to be involved in growth on acetate or linoleic acid; although the product of *cti*, cis/trans isomerase, is involved in isomerization of unsaturated fatty acids from the cis to the trans conformation. This conversion leads to changes in cell membranes that confer tolerance to certain stresses including exposure to phenol and elevated temperature (56).

We tested the ability of Pf-5 and the linoleic utilization mutants JL4400 and JL4425 to inhibit *P. ultimum* and to protect cucumber seedlings from Pythium damping-off disease in biological control experiments. All three strains protected seedlings from disease, suggesting that they likely colonized plants and produced antibiotics. However, they differed from each other in the degree of disease protection they provided. JL4425 was reduced in its biocontrol ability, and differed significantly from Pf-5. Without a better knowledge of the function of the locus disrupted by Tn*5* in JL4425, it is difficult to predict how the mutation has influenced protection. The strain likely produced antibiotics, since production was unaffected in liquid culture; therefore, reduced protection is probably due to impairment of another biocontrol mechanism, such as signal interference. For strain JL4400, phenotypes detrimental to biological control other than

signal interference were not seen or expected, due to the specificity of malate synthase in replenishing carbon when bacteria are grown on fatty acids as a sole carbon source. However, the resulting strain is still likely to import and utilize fatty acids which serve as only one of many potential carbon sources present in seed exudates, and, therefore, could still interfere with the exudates that trigger fungal spore germination. This may explain why JL4400, although slightly reduced in biological control capacity, did not differ significantly from Pf-5.

Although these experiments are consistent with the hypothesis that signal interference is a mechanism for biological control by Pf-5, additional studies are likely to aid in distinguishing signal interference from other mechanisms. Two approaches are currently being used. First, we have moved each Tn*5* mutation from JL4400 and JL4425 into a *gacA*(V203) derivative of Pf-5. The resulting strains should be deficient in antibiotic production and linoleic acid utilization. Once the strains are compared to the *gacA*(V203) derivative for biocontrol, we can evaluate the role of signal interference in the absence of protection provided by antibiosis. Second, because of the precedence that linoleic acid derived from cotton seed exudates is a key germination signal, biological control studies on cotton are likely to provide a stronger basis for evaluating signal interference by Pf-5 and the linoleic utilization deficient derivatives.

# Chapter 6. Concluding Remarks

A primary objective of the studies described herein was to elucidate regulatory circuits that influence Plt production by the biological control bacterium *P. fluorescens* Pf-5. Previous research determined the identity of a negative regulator of Plt and Phl production as *rpoS*. The *rpoS* mutant, although superior at biological control compared to Pf-5, was impaired in tolerance to certain stresses likely to be important for survival of Pf-5 under field conditions. Little was known about the mechanism by which *rpoS* influenced antibiotic production. Therefore, I chose to focus my efforts on identifying regulators of Plt and testing their role as intermediates of the *rpoS* regulatory circuit. Mutations in regulators that function downstream of *rpoS* in the regulation of Plt may provide enhanced biological control capacity without compromising stress tolerance of the strain. There were several additional benefits of this approach. The identification and characterization of additional regulators, regardless of their role in the *rpoS* regulatory circuit, would likely expand our understanding of factors that influence antibiotic production. Since environmental conditions are important factors that influence antibiotic production, identification of these regulators should also expand our understanding of how bacteria sense and respond to their environment.

I first tested the GacA/GacS two component regulatory system to determine if it participated in a regulatory circuit with RpoS. As described in chapter 2, we found that GacA and GacS were required for normal expression of RpoS and development of stress

tolerance by Pf-5. This study also strengthened the evidence for a connection between antibiotic production and stress response.

The role of GacA/S in regulating the timing of expression of *rpoS* and accumulation of $\sigma^S$ protein has some additional implications in biological control. Researchers have for some time known that GacA/S mutants, which are deficient in antibiotic production, often arise from late stationary-phase cultures (22). N. Corbell determined that up to 40% of the colonies derived from cells incubated for 5 days in NB exhibit the colony morphology of GacA or GacS mutants (24). Researchers have also noted that *rpoS* mutants often arise in laboratory strains and are detected in frozen stock cultures of *E. coli* (64) and *P. fluorescens* (49). In light of recent reports that cells with altered or reduced $\sigma^S$ function have an apparent growth advantage during stationary-phase (164, 165), it is possible that accumulation of a large proportion of GacA/S mutants in starving cultures may result directly from the effect of *gacA* and *gacS* mutations in reducing the amount of $\sigma^S$, leading to enhanced growth. Therefore, the connection of GacA/S to $\sigma^S$ demonstrated in these studies sheds light on why *gacA* and *gacS* mutations, which may be detrimental to biological control, appear so commonly when bacterial cultures are handled or stored improperly.

A fourth regulator described in chapter 3, Lon protease, is not an intermediate in the $\sigma^S$ regulatory circuit, but negatively influenced Plt production and was required for optimal survival when exposed to certain stresses. This study demonstrates that multiple signal transduction pathways influence both stress response and antibiotic production in *P. fluorescens*. The results of these studies imply that, under conditions where Pf-5 is experiencing stress, Plt production is likely down-regulated, which could negatively

impact biocontrol on some plant hosts. I believe that this interpretation is particularly relevant to our understanding of why biocontrol may be variable under field conditions.

One regulator characterized in these studies and described in chapter 4, PtsP, did not substantially influence stress tolerance by Pf-5. Additional analyses will be necessary to evaluate the performance of the *ptsP* mutant in biological control. Because PtsP is similar to proteins involved in regulation of carbon utilization, and different carbon sources can either enhance or reduce production of Plt (76), it is tempting to propose that PtsP may mediate carbon source regulation of Plt. However, we have no experimental evidence from this research linking PtsP to the influence of carbon on Plt production. To date, we still do not understand how carbon sources differentially influence antibiotic production.

One additional goal of this research was to test how signal interference may contribute to the ability of Pf-5 to suppress disease. I identified two mutants that were unable to grow on linoleic acid as a sole carbon source to test this hypothesis. Preliminary biological control studies were promising. However, I believe that the presence of multiple mechanisms, and in particular antibiosis, is likely to confound results. Therefore, I propose that mutants deficient in both antibiotic production and linoleic acid utilization may prove more useful in testing signal interference as a mechanism of biological control.

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