AN ABSTRACT OF THE DISSERTATION OF


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Joyce E. Loper

Tn5 mutagenesis of apdA (for antibiotic production) and deletion of gacA (for global antibiotic and cyanide) resulted in the same pleiotropic phenotype in Pseudomonas fluorescens (i.e. production of an array of secondary metabolites including the antibiotics pyrrolnitrin, pyoluteorin, and 2,4-diacyethylphloroglucinol as well as a tryptophan-side-chain oxidase, hydrogen cyanide, and an extracellular protease was abolished). The apdA and gacA loci were identified and cloned from the genome of Pf-5. Nucleotide sequencing of the apdA and gacA loci was used to identify the open reading frames for these genes. The deduced amino acid sequences for apdA and gacA exhibited similarity to sensor kinase (ApdA) and response regulator (GacA) proteins that comprise two-component regulatory systems. The C-terminal domain of GacA containing the putative helix-turn-helix DNA-binding motif was fused to the glutathione S-transferase protein. The glutathione S-transferase GacA C-terminal fusion protein was used in a cycle selection procedure that was designed to identify GacA binding sites from a complex pool of DNA fragments. Although a putative binding site for GacA was identified using the cycle selection procedure, the results were inconclusive due to several inconsistencies in the DNA-binding assay. The upstream region of one gene, which codes for a putative porin, was identified as a putative binding site for GacA by the cycle selection procedure. Studies initiated to determine whether gacA regulates transcription of this putative porin gene have been unsuccessful, so it remains unclear whether this gene is regulated by GacA. Also, asymptotic limits to biological control of Rhizoctonia damping-off of cotton were observed with the biological control agent P. fluorescens Pf-5.
A Two-Component Regulatory System Controlling Antibiotic Production by *Pseudomonas fluorescens* Pf-5.

by

Nathan Corbell

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.
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Chapter 1. Introduction

The control of soilborne pathogens responsible for plant disease is an ongoing challenge in agriculture. Crop rotation or fallowing reduces the number of propagules of certain soilborne pathogens and thus the amount of disease, but these practices may not be employed in some cases for economic reasons. In many instances, crop varieties with resistance to soilborne pathogens either do not exist or are available only for high value or high acreage crops. Few chemical treatments are effective for the control of soilborne pathogens and an increasing number of those that are effective are under review for removal from the market due to environmental concerns. For example, methyl bromide, an effective soil fumigant used extensively for the control of numerous soilborne plant pathogens has been slated for removal from the marketplace because it damages the ozone layer (methyl bromide update at www.ars.usda.gov/is/np/mbrweb). However, since there are currently no other economically viable methods for disease control in many of the crop systems that utilize this fumigant, the removal of methyl bromide from the market has been delayed. Other pathogens, such as *Rhizoctonia solani*, have never been adequately controlled by chemical means. Thus, there is an urgent need for additional methods for the control of soilborne plant pathogens. Biological control may provide an alternative to conventional means of plant disease control that is also less harmful to the environment.

Biological control, as defined by Cook and Baker (1983), is 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. More recently, the definition of biological control has been expanded to “the use of natural or modified organisms,
genes, or gene products to reduce the effects of undesirable organisms (pests), and to favor desirable organisms such as crops, trees, animals, beneficial insects, and microorganisms (Fravel, 1988). Historically, biological control has been achieved through agricultural practices such as tillage, crop rotation, and the addition of organic amendments or composts (Hoitink et al, 1986). Typically, such practices result in conditions that favor beneficial microorganisms and are less favorable for pathogenic organisms (Cook and Baker, 1983).

Biological control may also occur naturally in disease suppressive soils due to abiotic or biotic factors or a combination of both. For example, a naturally suppressive soil from North Carolina has been reported that suppresses black root rot of tobacco caused by *Thielaviopsis basicola* (Meyer and Shew, 1991). The factor(s) responsible for disease suppression in this soil is apparently abiotic because autoclaving of the soil has no effect on the suppressiveness of the soil. In contrast, soils suppressive to take-all of wheat become conducive to the disease when autoclaved indicating that the factor(s) responsible for suppressiveness in this soil is biotic in nature. Take-all disease of wheat is caused by *Gaeumannomyces graminis* var. *tritici* and can cause severe losses when a field is newly sown with wheat. The disease typically continues to increase for about the first four years of wheat monoculture. Thereafter, disease levels decline to economically unimportant levels with continued wheat monoculture. This decline in disease severity is attributed to the proliferation of fluorescent pseudomonads in the wheat rhizosphere (Raaijmakers and Weller, 1998). The examples mentioned above serve to illustrate the point that biological control in suppressive soils may be due to either abiotic or biotic factors. In most instances, biological control results from complex interactions among the biological control agent, the pathogen, the host plant, and other members of the microbial community as well as the physical environment.

Cook and Baker stated that biological control organisms may be found where disease is expected but is absent (1983). Indeed, many of the microorganisms that are now used, or are being developed as potential biological control agents, were originally isolated from naturally suppressive soils. A recent review lists the many
biological bacteria and fungi that have shown promise for the biological control of soilborne plant pathogenic fungi (Whipps, 1997).

1.1 Pseudomonads as biological control bacteria.

Among the bacterial biological control agents, *Pseudomonas* spp. have been one of the most thoroughly characterized groups, due to their abundance in the rhizosphere (Weller, 1988), their ability to utilize compounds found in root exudates (Loper and Schroth, 1986), and their production of numerous secondary metabolites that inhibit bacterial and fungal plant pathogens (Defago and Keel, 1995; Loper et al., 1993; Loper et al., 1996; Loper et al., 1997; Thomashow and Weller, 1995; Thomashow et al., 1996). Other plant-beneficial effects that have been attributed to rhizosphere pseudomonads are: 1) an increase in availability of mineral nutrients, 2) enhanced mycorrhizal establishment, and 3) direct growth promotion. These plant-beneficial attributes may also ameliorate the effects of disease by enabling plants to quickly pass through periods in their life cycles, when they are particularly susceptible to infection by pathogens (Bakker and Schippers, 1988).

Despite the proven ability of certain fluorescent pseudomonads to act as crop protectants, there remain several obstacles to their widespread exploitation as disease control agents in agricultural systems. Chief among these is the variable performance of fluorescent pseudomonads under field conditions (Weller, 1988). Perhaps the most important reason for this inconsistency is that the mechanisms by which fluorescent pseudomonads inhibit disease are only partially understood, as are the environmental factors that induce expression of genes involved in the inhibition of pathogens. It is likely that an understanding of the regulation of these genes will be prerequisite for predicting the environmental conditions under which these bacteria will perform optimally. Towards this end, many research groups are currently investigating the genetic mechanisms underlying the traits of fluorescent pseudomonads that are responsible for biological control.
1.2 Mechanisms of biological control by rhizosphere bacteria.

Biological control bacteria suppress plant diseases through a number of mechanisms including: 1) nutrient competition, 2) induced resistance, and 3) antibiosis (van Loon et al., 1998; Thomashow and Weller, 1995). Hypovirulence and parasitism are other mechanisms by which biological control may be achieved but these are typically associated with biocontrol fungi. Therefore, the present discussion will be limited to those mechanisms that are applicable to biological control bacteria with an emphasis on Pseudomonas spp. that utilize each mechanism.

Nutrient competition. Nutrient competition may occur when the ecological niches of two or more organisms overlap and a particular substance is limiting. The success of biological control organisms that utilize nutrient competition for the control of soilborne diseases, depends on their abilities to become established in the spermosphere or rhizosphere prior to the pathogen, and to utilize the same resources as the pathogen. Because propagules of certain plant pathogens such as Pythium spp. require an exogenous nutrient source for germination, it has long been speculated that the utilization of carbon compounds present in the seed and root exudates by biocontrol bacteria play a role in disease suppression. An example of this is seen in the biological control of Pythium by Pseudomonas putida N1R (Paulitz, 1991). Application of P. putida to seeds of pea and soybean reduced the concentration of available carbon for use by the pathogen and correspondingly reduced the amount of disease that was observed in Pythium-infested soil. Recently, van Dijk and Nelson convincingly demonstrated that several biocontrol bacteria, including Pseudomonas spp., reduced germination of Pythium sporangia by catabolizing the long chain fatty acids that are the primary germination stimulants of Pythium ultimum in cotton seed extracts (van Dijk and Nelson, 1997). These results demonstrate that biological control bacteria can suppress pathogens either by the general reduction of limiting nutrients or by scavenging molecules that act as specific signals for the development of plant pathogens.
Competition for iron is another mechanism by which pseudomonads inhibit plant pathogens. Although iron is abundant in most soils, it is largely present in the oxidized form (Fe$^{3+}$), which is extremely insoluble at neutral or alkaline pH. Most microorganisms use siderophores for iron acquisition. Siderophores are small molecular weight molecules that are produced under iron-limiting conditions, chelate Fe$^{3+}$ with high specificity, and transport iron into the cell via specific outer membrane receptors (Bakker et al., 1990). Fluorescent pseudomonads produce siderophores that fluoresce under UV light, which are termed pyoverdines or pseudobactins. Evidence that competition for iron played a role in biological control originally came from the observation that fluorescent pseudomonads exhibited greater disease suppression than their corresponding nonfluorescent mutants (Becker and Cook, 1988; Loper, 1988). Pyoverdine siderophores produced by fluorescent pseudomonads have been implicated in the biological control of Pythium spp. (Buysens et al., 1996; Loper, 1988) and *Fusarium oxysporum* (Lemenceau and Alabouvette, 1993). Historically, it has been difficult to assess the bioavailability of nutrients, such as iron, in soil or in the microhabitats that are occupied by biocontrol bacteria and plant pathogens. Therefore, it has been difficult to establish a role for nutrient competition in many cases. The recent development of biological sensors for assessing the levels of biologically-available nutrients will be useful in addressing the role of nutrient competition in biological control. For example, Loper and Henkels recently used an ice nucleation reporter gene fused to an iron promoter to show that iron availability to a biological control organism is greater in the rhizosphere compared to that of bulk soil (Loper and Henkels, 1997). Therefore biological control organisms that effectively sequester this nutrient should make it less available to pathogens. The development of other nutrient sensors should make it possible to define other resources that are targets for competition-mediated biological control.

**Induced resistance.** Induced resistance is defined as a state of enhanced disease resistance developed by a plant in response to a stimulus (Sticher et al., 1997; van Loon et al., 1998). Induced resistance occurs naturally when plants have limited
exposure to a plant pathogen or are infected by avirulent pathogens that elicit the
hypersensitive response (HR). HR is characterized by rapid and localized cell death in
response to infection, which limits the spread of the pathogen into neighboring tissues.
Induced resistance also can be triggered by chemicals or by nonpathogens. Induced
resistance may be localized (local acquired resistance; LAR), but is usually systemic
(systemic acquired resistance; SAR). SAR is characterized by an accumulation of
salicylic acid and pathogenesis related proteins (PRs). Salicylic acid appears to be a
signalling molecule that mediates SAR and application of salicylic acid to plants
induces SAR that is virtually indistinguishable from that induced by pathogens. The
functions of only a few of the PRs are known. These include chitinases and B-1,3-
glucanases, which are capable of hydrolyzing fungal cell walls.

As mentioned previously, induced resistance can be mediated by
nonpathogens. The term ISR (for induced systemic resistance) (Kloepper et al., 1992)
has been coined to differentiate this type of induced resistance from that mediated by
chemicals or pathogens, but it is unclear whether the underlying mechanisms of differ
from those of SAR. The fact that ISR may result from treating plants with purified
siderophores (Leeman et al., 1996) or purified outer membrane lipopolysaccharides
(van Peer et al., 1991) indicate that both ISR and SAR can be induced by chemical
compounds. To date, most described nonpathogens that mediate ISR are
Pseudomonas spp. that are used as biological control bacteria (van Loon et al., 1998).
Because these bacteria also inhibit pathogens directly, the contribution of induced
resistance to disease suppression has been difficult to prove. Recently, however,
researchers used split root systems to demonstrate that ISR is a mechanism by which
biocontrol bacteria mediate disease control (Liu et al., 1995; Zhou and Paulitz, 1994).
In general, split-root assays are performed by: 1) inoculating only some roots of a
plant with the inducing bacteria, 2) planting the exposed roots in one pot and the
unexposed roots in an adjacent pot with the plant supported between the two pots, 3)
challenging the unexposed roots with a pathogen at a later time, and 4) observing a
decrease in disease compared to similar plants that were not treated with the inducing
bacteria. Because the roots that are challenged with the pathogen are spatially
separated from those that are treated with the inducing bacteria, such systems provide strong evidence that certain biocontrol bacteria mediate disease suppression by induced resistance. Spatial separation of the biocontrol agent from the infection site is a criterion used to differentiate induced resistance from direct inhibition of a pathogen, but it is also possible that metabolites produced by the biological control organism are absorbed and translocated through the plant to provide systemic disease protection. Herbicolin A, an antifungal antibiotic produced by *Erwinia herbicola* in the rhizosphere of wheat, for example, is detectable in the crowns as well as the roots of wheat seedlings (Kempf et al., 1993). Thus, because many biocontrol bacteria produce compounds that inhibit plant pathogens, it may be difficult to ascertain whether or not induced resistance is a mechanism for disease suppression in many cases.

**Antibiosis.** Antibiotics are small organic molecules produced by microorganisms that are deleterious to the growth or metabolic processes of other microorganisms. Usually antibiotics are considered secondary metabolites because they are not strictly required for growth or survival of the producing microorganism. Many biological control bacteria produce secondary metabolites that inhibit plant pathogenic fungi and bacteria (Thomashow and Weller, 1995; Loper et al., 1996; Pierson et al., 1996; Ligon et al., 1996; Thomashow et al., 1996; Stohl et al., 1996). Two approaches have been used to demonstrate that antibiotics produced by biological control bacteria are involved in disease suppression. One approach has been to apply the purified antifungal compound to seed. For example, when the antifungal compounds pyoluteorin and pyrrolnitrin, products of *Pseudomonas fluorescens* Pf-5, were applied to cotton seeds, disease suppression was similar to that observed when Pf-5 was applied to seed (Howell and Stipanovic, 1979; Howell and Stipanovic, 1980). A second approach has been to obtain mutants that no longer produce an antibiotic and then compare the disease suppression obtained with this mutant to its wild-type counterpart. The contributions of antifungal compounds such as pyoluteorin (Maurhofer et al., 1994), phenazines (Thomashow and Weller, 1988), 2,4-
diacetylphloroglucinol (Keel et al., 1992), and hydrogen cyanide (Voisard et al., 1989) towards biological control were all demonstrated in this manner. However, mutants deficient in the production of specific antibiotic compounds generally provide some degree of disease suppression. These results indicate that antibiotics contribute to, rather than account for, the biological control activity of a microorganism. Certain biocontrol organism are likely to mediate biological control through a combination of antibiotics, rather than solely through one antibiotic.

1.3 Antifungal compounds produced by fluorescent pseudomonads.

Fluorescent pseudomonads produce numerous secondary metabolites, each having a unique spectrum of activity against plant pathogenic fungi (Thomashow and Weller 1995). Some of the more studied compounds are discussed below.

Pyoluteorin. The example of pyoluteorin is interesting because it demonstrates that an antifungal compound isolated from a biological control organism is not, by virtue of its antifungal activity, necessarily involved in disease suppression. Pyoluteorin, a compound produced by *P. fluorescens* Pf-5 (Howell and Stipanovic 1980), suppresses disease caused by *Pythium ultimum* when the purified compound is applied to cotton seeds before planting. Kraus and Loper (1992) showed that a Tn5 mutant of Pf-5 that did not produce pyoluteorin was just as effective as the wild type for the biological control of *Pythium* damping-off of cucumber. Pyoluteorin produced by *P. fluorescens* CHA0, a strain closely related to Pf-5, also does not contribute to the biocontrol of *Pythium* damping-off of cucumber, but does contribute to the suppression of this disease on cress (Maurhofer et al., 1994). In a later study, transcriptional activity of a pyoluteorin promoter fused to an ice nucleation reporter gene was measured in the spermospheres of cotton and cucumber (Kraus and Loper, 1995). Although the genes required for pyoluteorin production were expressed by Pf-5 in the spermospheres of both cotton and cucumber, transcription in the cucumber
spermosphere was delayed for over 12 h after planting, whereas transcription occurred within the first few hours after cotton seeds were planted (Kraus and Loper, 1995). Since *P. ultimum* infects cucumber seed within 12 h after planting (Nelson et al., 1986), the most probable reason for the differential contribution of pyoluteorin to disease suppression on these two hosts is that insufficient amounts of pyoluteorin were produced in the spermosphere of cucumber prior to infection by *Pythium*. These results illustrate the profound effect that the host can have on the efficacy of biological control. Recently, the cluster of biosynthetic genes for the production of pyoluteorin were sequenced (Nowak-Thompson et al. 1997) and possible regulatory genes were identified (Nowak-Thompson et al., 1999). Analysis of these regulatory genes should provide additional insights into the factors that influence the production of pyoluteorin.

**2,4-Diacetylphloroglucinol.** The antifungal compound 2,4-diacetylphloroglucinol (Phl) is produced by many *Pseudomonas* spp. of worldwide origin (Keel et al., 1996). *Pseudomonas* strains with transposon insertions in the Phl biosynthetic locus are less effective than the wild-type strain for the suppression of *P. ultimum* (Fenton et al., 1992); *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all of wheat (Keel et al., 1990); and *Thielaviopsis basicola*, the causal agent of black root rot (Keel et al., 1992). The genetic locus conferring Phl production has been cloned from two strains of *Pseudomonas* spp. and expressed in heterologous hosts (Fenton et al., 1992; Bangera and Thomashow, 1996). Furthermore, when the cloned *phl* genes were introduced into a *Pseudomonas* strain that did not suppress Pythium damping-off of sugar beet, they conferred a level of suppression of this disease that was equivalent to the biocontrol strain of *Pseudomonas* from which the *phl* genes originated. These results indicate that Phl is a major determinant of biological control against Pythium damping-off of sugar beet.

**Pyrrolnitrin.** Pyrrolnitrin was first isolated from *Pseudomonas pyrrocinia* (Arima et al., 1964). The first report of pyrrolnitrin as an inhibitor of plant
pathogenic fungi was by Howell and Stipanovic (1979). Pyrrolnitrin is produced by several Pseudomonas spp. (Leisinger and Margraff, 1979) and, in bioassays, is highly inhibitory to Thielaviopsis basicola, Alternaria spp. and Verticillium dahliae (Howell and Stipanovic, 1979). The isolated compound provides protection from Rhizoctonia damping-off of cotton seedlings (Howell and Stipanovic, 1979). Production of pyrrolnitrin by Pseudomonas spp. has also been correlated with disease suppression of Pyrenophora tritici-repentis (Pfender et al., 1993) and Rhizoctonia solani (Howell and Stipanovic, 1979; Hill et al., 1994). The inhibitory activity of pyrrolnitrin against R. solani has received a great deal of attention because effective control of this pathogen has not been achieved by the use of conventional fungicides. In addition, this compound has long been marketed as a treatment for dermatological infections in Japan and has a safe toxicological profile (Tawara et al., 1989). Recently, the biosynthetic genes for pyrrolnitrin have been identified and cloned (Ligon et al., 1996). Overexpression of the cloned pyrrolnitrin genes in a Pseudomonas biocontrol strain yielded increased biocontrol, providing compelling evidence that this compound is a major determinant of biological control. The recent sequencing of the pyrrolnitrin biosynthetic genes (Hammer et al., 1997) should facilitate investigations into the regulation of this antifungal compound.

Hydrogen cyanide. Hydrogen cyanide (HCN) is a secondary metabolite produced by numerous Pseudomonas spp. under low oxygen levels (Castric, 1983) and during the transition from exponential to stationary phase in culture (Castric et al., 1979). The HCN biosynthetic region has been identified in P. fluorescens CHA0 (Voisard et al., 1989). Mutants in the HCN biosynthetic genes are less suppressive than CHA0 of black root rot caused by T. basicola. Complementation of the mutants with the cloned HCN biosynthetic genes restores hydrogen cyanide and suppression of T. basicola to wild-type levels (Voisard et al., 1989). In addition, the cloned HCN biosynthetic genes have been expressed in other Pseudomonas spp., resulting in improved biocontrol activity (Flaishman et al., 1996). These results indicate that HCN contributes to the biocontrol activity of this strain. The recent sequencing of the HCN
biosynthetic locus (Laville et al., 1998) should provide more information on the role of HCN in biocontrol.

**Phenazines.** Phenazines are produced by certain fluorescent pseudomonads (Leisinger and Margraff, 1979) and play an important role in the suppression of take-all of wheat caused by *Gaeumannomyces graminis var. tritici* (Thomashow and Weller, 1988). Phenazines are thought to affect target organisms by undergoing oxidation-reduction reactions within the cell which lead to the toxic accumulation of reactive superoxide radicals, as has been shown for the human pathogen *Pseudomonas aeruginosa* (Hasset et al., 1993). The genes for phenazine biosynthesis appear to be highly conserved among certain pseudomonads (Mavrodi et al., 1998). Studies involving phenazine deficient mutants have shown that the ability to produce this compound is associated with the long-term survival of these *Pseudomonas* spp. in their natural environment (Mazzola et al., 1992). Numerous naturally-occurring phenazines have been identified, and certain strains have shown the ability to produce several phenazines at the same time (Turner and Messenger, 1986).

1.4 **Regulation of secondary metabolites in *Pseudomonas* spp.**

Many biosynthetic genes are controlled by regulatory genes that are closely associated with these regions and act as repressors or transcriptional activators (Prag et al., 1997). Increasingly, global regulators are being identified that control numerous and often seemingly unrelated biosynthetic loci. Many of these are transcriptional activators that fall into two main categories: 1) the LysR family of autoregulatory transcriptional activators (Schell, 1993), and 2) the two-component regulatory systems (Albright et al., 1989; Parkinson, 1993; Parkinson, 1995; Stock et al., 1995; Volz, 1995). Often combinations of these and other proteins form complex regulatory networks for gene expression in procaryotes. Investigations into the regulation of antifungal secondary metabolite production in *Pseudomonas* spp. have revealed that
expression of these metabolites is globally controlled, in part, by a two-component regulatory system (Laville et al., 1992; Gaffney et al., 1994; Corbell and Loper, 1995).

Two-component regulatory systems are a major mechanism by which bacteria sense and respond to their environment. Two-component systems regulate such diverse cellular processes as sugar, nitrogen and phosphate uptake and assimilation (Kadner et al., 1995; Porter et al., 1995; Stewart and Rabin, 1995; Wanner, 1995), cellular differentiation (Msadek, 1995), pathogenesis (Dziejman and Mekalanos), and, as mentioned above, biological control via antibiotic production. New two-component systems are continually being discovered and a two-component system has even been identified in Arabidopsis, the model organism used in a great deal of plant research (Chang and Meyerowitz, 1995).

The canonical two-component regulatory system is composed of a histidine kinase and a response regulator (Albright, 1989; Parkinson, 1993). Typically, the sensor kinase contains a transmembrane region that acts as a receptor to detect signal molecules outside the cell. When the appropriate ligand is bound, the intracellular kinase domain autophosphorylates at a conserved histidine residue using ATP. The phosphoryl group is then transferred to a conserved aspartate residue in the response regulator, typically resulting in its activation as a positive transcriptional factor (Parkinson, 1993). The system is modular by design (Figure 1-1) with sensor kinases usually being composed of an input domain and a transmitter domain. Response regulators are usually composed of a receiver domain and an output domain. Input domains contain ligand binding domains; transmitters, the conserved histidine domain; receivers, the conserved aspartate residue; and output domains, the conserved helix-turn-helix DNA-binding motif (Figure 1-1). As mentioned previously, the typical sensor kinase contains a transmembrane region, but there are examples of sensor kinases that detect intercellular signals and consequently do not contain transmembrane regions (Ronson et al., 1987). Other sensor kinases contain additional receiver and transmitter modules, which may provide an additional level of regulation
input signal results in autophosphorylation of sensor kinase (transmitter)

ATP  ADP

sensor

phosphorylation of receiver by transmitter

P

receiver  output

response regulator

phosphorylated receiver results in activation of the output domain (typically DNA binding as an activator or repressor of transcription).

Figure 1-1. Schematic representation of a two-component regulatory system.
(Ishige et al., 1994; Uhl and Miller, 1995). There are also examples of response regulators for which the corresponding output domain is provided by a separate protein (Amsler and Matsumura, 1995).

1.5 Statement of research objectives.

The research described within this dissertation was initiated for the purpose of elucidating the mechanisms regulating secondary metabolite production by the biological control bacterium Pseudomonas fluorescens Pf-5, under the hypothesis, that by understanding the regulatory mechanisms, we will be able to more fully utilize the biological control potential of this microorganism. Pf-5 was originally isolated from the rhizosphere of cotton as a microorganism that suppresses Rhizoctonia damping-off of cotton (Howell and Stipanovic, 1979). Subsequently, Pf-5 was shown to suppress Pythium damping-off of cotton (Howell and Stipanovic, 1980) and of cucumber (Kraus and Loper, 1992), and ascocarp formation by Pyrenophora tritici-repentis on wheat straw (Pfender et al., 1993). Pf-5 produces the antifungal secondary metabolites pyoluteorin (Howell and Stipanovic, 1979), 2,4-diacetylphloroglucinol (Nowak-Thompson et al., 1994), pyrrolnitrin (Howell and Stipanovic, 1980), hydrogen cyanide (Kraus and Loper, 1992), and a pyoverdine siderophore (Kraus and Loper, 1992).

In a previous study, 6286 mutants of P. fluorescens Pf-5, each with a single transposon insertion in the genome, were screened for pyoluteorin and pyrrolnitrin production by thin layer chromatography (Kraus and Loper, 1992). Five of these mutants produced neither pyoluteorin nor pyrrolnitrin and had lost the ability to inhibit Pythium ultimum and Rhizoctonia solani in vitro. The goals of this research were: 1) the identification of the regulatory mechanism causing the pleiotropic loss of production of pyoluteorin and pyrrolnitrin, and 2) the identification of the specific genes regulated by this putative regulatory mechanism. The third goal was to use the
pleiotropic antibiotic deficient mutant in a disease assay to assess a dose-response model for biological control (Johnson, 1994)(see Chapter 4).

2.1 Introduction

The biological control agent Pseudomonas fluorescens strain Pf-5 inhabits the spermosphere and rhizosphere, where it suppresses the soilborne fungi Rhizoctonia solani (Howell and Stipanovic, 1979) and Pythium ultimum (Howell and Stipanovic, 1980). These fungi infect seeds and roots thereby reducing seedling emergence and survival. Strain Pf-5 also colonizes wheat straw residue, where it suppresses ascocarp formation by Pyrenophora tritici-repentis (Pfender et al., 1992), the tan spot pathogen of wheat. Pf-5 produces the antifungal antibiotics pyrrolnitrin (Howell and Stipanovic, 1979), pyoluteorin (Howell and Stipanovic, 1980), and 2,4-diacetylphloroglucinol (Nowak-Thompson et al., 1994; hydrogen cyanide (Kraus and Loper, 1992); and a non-characterized pyoverdine siderophore. Each antibiotic has a unique spectrum of activity against fungal pathogens suppressed by Pf-5: pyrrolnitrin inhibits R. solani (Howell and Stipanovic, 1979) and P. tritici-repentis (Pfender et al., 1992), pyoluteorin inhibits P. ultimum (Howell and Stipanovic, 1980), and 2,4-diacetylphloroglucinol inhibits all three fungi (Keel et al., 1992; Nowak-Thompson et al., 1994). The profile of secondary metabolites produced by Pf-5 is remarkably similar to that of P. fluorescens CHAO, a biological control organism in which secondary metabolite production is required for effective suppression of black root rot of tobacco (Laville et al., 1992; Voisard et al., 1989), Pythium damping-off of cress (Maurhofer et al., 1994) and take-all of wheat (Keel et al., 1992). In strain CHAO, the gacA gene (for global antibiotic and cyanide control), encodes a regulatory protein (GacA) that is required for antibiotic and hydrogen cyanide production (Laville et al., 1992), and tryptophan-side-chain oxidase (Oberhansli et al., 1991), and protease activities (Sacherer, 1994). Similarly, gacA mutants of P. fluorescens BL915 are pleiotropic; they fail to synthesize several antifungal factors including hydrogen.
cyanide and pyrroline (Gaffney et al., 1994). GacA is a response regulatory protein in the FixJ/DegU family of two-component regulatory systems (Laville et al., 1992; Gaffney et al., 1994). Prokaryotic two-component regulatory systems typically are composed of a transmembrane protein that functions as an environmental sensor and a cytoplasmic response regulatory protein that mediates changes in gene expression in response to sensor signals (Parkinson et al., 1992).

In a previous study, five mutants, each containing a single genomic Tn5 insertion, were identified that were deficient for the production of pyoluteorin and pyrroline and had lost the capacity to inhibit Pythium ultimum and Rhizoctonia solani in vitro (Kraus and Loper, 1992). The pleiotropic phenotype of these mutants is designated here as Apd' (for antibiotic production). Pf-5 mutants that display the Apd' phenotype are similar to gacA mutants for other strains of P. fluorescens (i.e., they do not produce pyrroline, pyoluteorin, 2,4-diacetylphloroglucinol or hydrogen cyanide). In this report, we describe further functional characterization of the Apd' phenotype and the sequence of the apdA gene. Evidence is presented that the apdA gene 1) is distinct from gacA, 2) encodes a protein that contains domains conserved among the sensor kinases of two-component regulatory proteins, 3) is responsible for the global regulation of secondary metabolites in strain Pf-5, and 4) is the functional equivalent of lemA, which encodes a putative sensor kinase of a two-component regulatory system required for the pathogenicity of Pseudomonas syringae pv. syringae (Hrabak and Willis, 1992; Willis et al., 1994).

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are described in Table 2-1. Escherichia coli strains were cultured in Luria Bertani (LB) broth (Sambrook et al., 1989) or on LB agar plates incubated at 37°C. Pseudomonas strains were routinely
Table 2-1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Relevant Characteristics (^a)</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pf-5</td>
<td>Field isolate</td>
<td>Apd(^+)</td>
<td>Howell and Stipanovic, 1979</td>
</tr>
<tr>
<td>JL4097</td>
<td>Derivative of Pf-5, apdA::Tn5</td>
<td>Apd(^-) Km(^r)</td>
<td>Kraus and Loper, 1992</td>
</tr>
<tr>
<td>JL4106</td>
<td>Derivative of Pf-5, apdA::Tn5</td>
<td>Apd(^-) Km(^r)</td>
<td>Kraus and Loper, 1992</td>
</tr>
<tr>
<td>JL4135</td>
<td>Derivative of Pf-5, apdA::Tn5</td>
<td>Apd(^-) Km(^r)</td>
<td>Kraus and Loper, 1992</td>
</tr>
<tr>
<td>JL4209</td>
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<td>Apd(^-) Km(^r)</td>
<td>Kraus and Loper, 1992</td>
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<tr>
<td>JL4210</td>
<td>Derivative of Pf-5, apdA::Tn5</td>
<td>Apd(^-) Km(^r)</td>
<td>Kraus and Loper, 1992</td>
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<tr>
<td><strong>P. syringae</strong></td>
<td></td>
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<tr>
<td>B728a</td>
<td>Field isolate causing brown spot of bean</td>
<td>Lem(^+)</td>
<td>Hrabak and Willis, 1992</td>
</tr>
<tr>
<td>NPS3136</td>
<td>Derivative of B728a, lemA::Tn5</td>
<td>Lem(^-) Km(^r)</td>
<td>Hrabak and Willis, 1992</td>
</tr>
<tr>
<td><strong>E. coli DH5</strong></td>
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<tr>
<td></td>
<td>F(^-) endA1 hsdR17(r(^K)m(^K)) supE44 thi-1 recA1 gyrA96 relA1 80dlacZ M15 -</td>
<td></td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC18</td>
<td>ColE1 replicon</td>
<td>Ap(^r)</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Mobilizing plasmid</td>
<td>Tra(^+) Km(^r)</td>
<td>Figurski and Helinski, 1979</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Traits</td>
<td>Reference</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
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<tr>
<td>pRK415</td>
<td>$incP1$ replicon, polylinker of pUC19</td>
<td>Mob$^+$ Tc$^r$</td>
<td>Keen et al., 1988</td>
</tr>
<tr>
<td>pBR322</td>
<td>ColE1 replicon</td>
<td>Ap$^r$ Tc$^r$</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td>pME3066</td>
<td>1.65-kb BamHI-BglII fragment containing the $gacA$ gene of <em>P. fluorescens</em> CHAO cloned into pLAFR3</td>
<td>Tc$^r$</td>
<td>Laville et al., 1992</td>
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<tr>
<td>pJEL1657</td>
<td>13.5-kb Tn5 containing $EcoRI$ fragment from JL4106 cloned into pBR322</td>
<td>Ap$^r$ Km$^r$ Tc$^r$</td>
<td>This study</td>
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<td>pJEL5591</td>
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<td>Tc$^r$ Apd$^+$</td>
<td>This study</td>
</tr>
<tr>
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<td>Tc$^r$ Apd$^+$</td>
<td>This study</td>
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<tr>
<td>pJEL5680</td>
<td>6.5-kb KpnI fragment from Pf-5 cloned into pUC18</td>
<td>Ap$^r$</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$Ap$^r$, Km$^r$, and Tc$^r$ indicate resistance to ampicillin, kanamycin, and tetracycline, respectively.
cultured at 27°C in King's medium B (KMB) (King et al., 1954) broth or on KMB agar plates. Antibiotic concentrations were as follows unless otherwise specified: 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 20 μg/ml tetracycline.

2.2.2 Recombinant DNA techniques.

Transformation of E. coli, restriction digests, ligations, electrophoresis in Tris-phosphate-EDTA, and plasmid DNA isolation were performed by standard methods (Sambrook et al., 1989). Enzymes were from Gibco BRL Life Technologies (Gaithersburg, MD). Southern blots of plasmid and genomic DNA were prepared with nylon membranes (Nytran; Schleicher and Schuell, Keene, NH) according to the supplier's directions. DNA probes were apdA::Tn5 (the 13.5-kb Tn5-containing EcoRI fragment of pJEL1657) and the gacA gene of P. fluorescens CHA0 (the 1.65-kb BamHI-BglII fragment of pME3066) (Laville et al., 1992) labelled with biotinylated dATP or 32P-dCTP using a nick translation kit (Gibco BRL Life Technologies), and purified with a D50 column (International Biotechnologies Inc., New Haven, CT). Plasmids pLAFR3, pRK415 and derivatives were mobilized from E. coli DH5α donors into P. fluorescens and P. syringae in triparental matings using helper plasmid pRK2013 (Figurski and Helinski, 1979). Due to the high degree of natural tetracycline resistance exhibited by P. fluorescens Pf-5, transconjugants were selected on KMB containing 200μg/ml tetracycline and 100μg/ml ampicillin.

2.2.3 Cloning of the apdA gene of Pf-5.

An existing genomic library of Pf-5 (Pfender et al., 1993), constructed in cosmid vector pLAFR3, was screened by colony hybridization to identify clones that hybridized to the apdA::Tn5 probe or the gacA probe. Filters for colony hybridization were prepared with Whatman 541 paper (Whatman International Ltd., Maidstone,
England) and hybridized with the $^{32}$P-dCTP-labeled probe as described by (Gergen et al., 1979).

### 2.2.4 DNA sequencing and 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 (Alvarado-Urbina et al., 1986). Initial sequencing of the apdA locus was performed on regions flanking the Tn5 inserts of three apdA mutants (JL4106, JL4135, and JL4210). EcoRI fragments containing the Tn5 and flanking genomic DNA from each of the three mutants were cloned into pUC18 in both orientations. Derivatives of these plasmids, constructed by deleting a fragment bordered by the BamHI site internal to Tn5 and the BamHI site of the pUC18 polylinker, contained a single inverted repeat of Tn5 and contiguous genomic DNA. These plasmids were sequenced using an oligonucleotide primer complementary to bases 37 through 18 in the inverted repeat of Tn5 (5'-GGTTCCGTTCAGGACGCTAC-3') (Rich and Willis, 1990). Further sequencing of the apdA gene cloned in pJEL5680 was directed by oligonucleotide primers designed from previous sequence determinations of the apdA region. DNA sequence analysis and comparisons with sequences contained in the GenBank and EMBL databases were accomplished with software from the Genetics Computer Group, Inc., Madison, WI (Devereux et al., 1984).

The GenBank accession number for the DNA sequence of the apdA gene of *P. fluorescens* Pf-5 is U30858.
2.2.5 Characterization of the Apd' phenotype.

The Apd' phenotype of Pf-5 and derivatives was characterized for protease and tryptophan-side-chain oxidase (TSO) activities, hydrogen cyanide and antibiotic production, and inhibition of *R. solani* in culture. Protease production was determined qualitatively by streaking strains on Bacto litmus milk agar (Difco Laboratories, Detroit, MI). After cultures were incubated for 36 hours at 27°C, the presence of a cleared zone surrounding bacterial growth indicated protease production. TSO activity was determined as previously described (Takai et al., 1987). Production of cyanide was assessed by the method originally described by Castric and Castric (Castric and Castric, 1983) as modified by Sarniguet et al. (Sarniguet et al., 1992).

**Antibiotic quantification.** The antibiotics pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol were quantified by high pressure liquid chromatography (HPLC). *P. fluorescens* was grown at 20°C either for 48 hours in 5 ml nutrient broth (NB) (Difco) supplemented with 1% (wt/vol) glycerol, a medium in which pyoluteorin and pyrrolnitrin are produced preferentially, or for 96 hours in 5 ml NB supplemented with 2% (wt/vol) glucose, a medium in which 2,4-diacetylphloroglucinol is produced preferentially (Nowak-Thompson et al., 1994). Cells and supernatants of 5 ml cultures were separated by centrifugation. Cells were extracted once in 1 ml of acetone. Cell extracts were taken to dryness, resuspended in MeOH, and analyzed by HPLC as described below. Culture supernatants were adjusted to pH 2 with 1 M HCl and extracted three times with 1 ml ethyl acetate. Organic phases were combined and back extracted once with 1 ml H2O. The organic phase extracts were dried, dissolved in MeOH, and analyzed on a Waters Nova-pak C18 reverse phase column (0.8 x 10 cm, 4 μm) eluted isocratically (45% H2O, 30% acetonitrile, 25% MeOH) at a flow rate of 1.5 ml per minute. Antibiotics were detected quantitatively with a UV-photo-diode array detector at 225 nm (pyrrolnitrin), 310 nm (pyoluteorin), and 278 nm (2,4-
diacetylphloroglucinol), and compared to authentic samples of the compounds, as described previously (Nowak-Thompson et al., 1994).

**Inhibition of Rhizoctonia solani.** *P. fluorescens* was grown overnight in KMB broth. Cells were collected by centrifugation, washed, and adjusted to an optical density of 0.2 at 600 nm. Ten microliters of the cell suspension was applied to the centers of petri plates containing nutrient agar (Difco) supplemented with 1% (wt/vol) glycerol or 2% (wt/vol) glucose. Following three days of incubation at 27°C, bacterial cells were killed by exposure to chloroform vapors for 30 minutes. Plates were vented for 30 minutes, and the medium was overlaid with 5 ml molten potato dextrose agar (Difco) containing hyphal fragments of *R. solani* isolate J1 (obtained from C. R. Howell, USDA, ARS, Cotton Research Laboratory, College Station, TX). Hyphal suspensions were prepared by growing isolate J1 in 20 ml potato dextrose broth (Difco) for 3 days at 27°C with shaking and then homogenizing the hyphal mat and spent broth. Inhibition zone diameters from duplicate plates were measured after incubation for 2 days at 27°C. The experiment was repeated.

**Protein analysis.** Cells from cultures grown overnight in KMB were pelleted, and suspended in ice-cold phosphate-buffered saline (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, pH 7.5) to an optical density of 0.2 at 600 nM. Cells were concentrated 10-fold by centrifugation, resuspended in ice-cold saline, mixed with 2X Laemmli sample buffer (Sambrook et al., 1989), and boiled for 5 min. Lysates were separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Proteins were stained with Coomassie blue or transferred to reinforced nitrocellulose membranes for Western blot analysis (Sambrook et al., 1989). Immunoblotting was performed using polyclonal anti-Lema antiserum (raised in New Zealand White rabbits) (Rich et al., 1994), which was generously supplied by T. Kitten and D. K. Willis (University of Wisconsin, Madison). The secondary antibody, a goat anti-rabbit IgG (Fc) conjugated to alkaline phosphatase, was detected with 5-bromo-4-
chloro-3-indolyl-1-phosphate and nitro blue tetrazolium (Promega Corp., Madison, WI).

2.3 Results

2.3.1 Identification and cloning of the \textit{apdA} gene.

 Localization of Tn5 insertions in the genome of Apd\textsuperscript{-} mutants. Five Apd\textsuperscript{-} mutants, each of which has a single Tn5 insertion in the genome, were described previously (Kraus and Loper, 1995). Southern analysis of genomic DNA of the five mutants probed with the Tn5-containing EcoRI fragment isolated from Apd\textsuperscript{-} mutant JL4106 (ie. the \textit{apdA}::Tn5 probe) revealed that four of the mutants (JL4097, JL4106, JL4135, and JL4210) contained a single Tn5 insertion within the same 13.5-kb EcoRI fragment of genomic DNA. The 13.5-kb EcoRI fragment, comprised of 7.8 kb of genomic DNA and Tn5, was cloned from each of these mutants and the Tn5 insertions were mapped to a 1.5-kb region by restriction analysis (Figure 2-1). The single Tn5 insertion in the genome of the Apd\textsuperscript{-} mutant JL4209 was localized to the adjacent EcoRI fragment by Southern analysis. A 1.6-kb \textit{BamHI-BglII} fragment that hybridized to the \textit{gacA} gene of \textit{P. fluorescens} CHA0 (Laville et al., 1992) was present in genomic DNA of Pf-5 and the five Apd\textsuperscript{-} mutants, indicating that the Apd\textsuperscript{-} mutants did not have a Tn5 insertion within \textit{gacA}.

 Cloning of the \textit{apdA} gene. Three cosmids, each containing the wild type \textit{apdA} gene, were identified from a genomic library of \textit{P. fluorescens} Pf-5 by hybridization to the \textit{apdA}::Tn5 probe. Southern hybridization indicated that each of the three cosmids contained a 7.8-kb EcoRI fragment and a 6.5-kb KpnI fragment that hybridized to the \textit{apdA}::Tn5 probe. By probing the Pf-5 cosmid library with the \textit{gacA} gene, we identified two other cosmids, each of which contained a \textit{gacA} homolog. In Southern blots, the \textit{gacA} probe hybridized to a 1.6-kb \textit{BamHI-BglII} fragment in both
Figure 2-1. Schematic representation of the apdA locus. Numbered triangles indicate Tn5 insertion sites in Apd' mutants. pJEL5591 and pJEL5771 contain the functional apdA gene; Overhead arrow, direction of apdA transcription. Restriction sites: E, EcoRI; K, KpnI; St, SstI; S, SunI.
gacA-containing cosmids but it did not hybridize to any of the apdA-containing cosmids. The two cosmids that hybridized to the gacA probe did not hybridize to the apdA::Tn5 probe. Together, these data presented convincing evidence that the apdA and gacA loci of *P. fluorescens* Pf-5 were distinct and unlinked loci.

2.3.2 Sequence analysis of the *apdA* gene.

A 3188-bp region of DNA flanking the Tn5 insertions of *apdA* mutants and present within the 6.5-kb *KpnI* fragment of pJEL5771 was sequenced. The Tn5 insertions of JL4210, JL4106, and JL4135 were localized to within a 2751-bp open reading frame identified as the *apdA* locus (Figure 2-2). The open reading frame, beginning at bp 209 with a GTG initiation codon and terminating at bp 2960 with a TAG stop codon, was predicted to encode a protein of 917 amino acids with a molecular weight of 101 kDa. Upstream from the GTG codon, there was an AG-rich region from bp 194 to bp 203 containing two overlapping sequences similar (five of six nucleotides) to Shine-Dalgarno sites for ribosome binding (Shine and Dalgarno, 1974). Several methionine codons downstream from the putative translational start site (bp 209) could also serve as translational start sites. However, only one of these alternate start sites (the two consecutive methionine codons from bp 548 to bp 553) was preceded by a possible ribosome binding site.

The sequence of the *apdA* gene is similar to those of a class of regulatory proteins. The *apdA* nucleotide and predicted amino acid sequences were similar to those for sensor kinase components of two-component regulatory systems (Parkinson and Kofoid, 1992). All of the conserved domains associated with the kinase portion of “orthodox” sensor kinase genes (Albright et al., 1989; Parkinson and Kofoid, 1992) were identified in the *apdA* gene (Figure 2-2). The N-terminal region of ApdA contains two long hydrophobic regions (Figure 2-2), which could constitute transmembrane regions typical of sensor kinases. The C-terminal region of the
Figure 2-2. Complete nucleotide and predicted amino acid sequences of *apdA*. Restriction sites are underlined. The putative translational start codon is indicated by an arrow; the most probable ribosome binding site is indicated by a series of asterisks. Numbered triangles are sites of Tn5 insertion. Bold underlined regions are hydrophobic regions. Conserved sensor kinase domains are indicated by open boxes. Conserved residues of the receiver module (Stock et al., 1995) are circled. A region in the C terminus that is conserved among certain sensor kinases (Uhl and Miller, 1995) is indicated by a shaded box. A stop codon is indicated by a single asterisk at the end of the amino acid sequence.
Figure 2-2.
Figure 2-2. continued
Figure 2-2. continued
predicted ApdA amino acid sequence contained conserved aspartate and lysine residues (Figure 2-2) that are common to response regulators and also are found in some sensor kinases of two-component regulatory systems (Parkinson and Kofoid, 1992).

The most striking sequence similarities were found to lemA (Hrabak and Willis, 1992) and repA (Liao et al., 1994), genes encoding sensor kinases in phytopathogenic species of Pseudomonas. ApdA, LemA, and RepA are 77% identical in a 917 amino acid overlap (Figure 2-3). Like lemA and repA, the apdA gene contains no recognizable consensus sequences for σ70, σ54, σ32 type pseudomonad promoters (Deretic et al., 1989).

2.3.3 Phenotype analysis of apdA.

Complementation of apdA and lemA mutants with the cloned apdA gene. apdA mutants of Pf-5 are deficient in pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, and hydrogen cyanide production and fail to inhibit R. solani in culture (Kraus and Loper, 1992). apdA mutants also failed to produce an extracellular protease and tryptophan-side-chain oxidase in culture. Plasmid pJEL5591, which contains a 6.5-kb KpnI fragment encompassing the apdA gene (Figure 2-1), restored cyanide synthesis, extracellular protease production, and tryptophan-side-chain oxidase activity to apdA mutants JL4097, JL4106, JL4135, JL4209, and JL4210. Function of the apdA region was further localized to a 3.5-kb KpnI-SalI fragment containing the 2751 bp apdA locus (Figure 2-1), which restored the Apd+ phenotype to JL4135 (Table 2-2). We established that the products of the apdA and lemA loci were cross-functional by restoring extracellular protease production to a lemA mutant of Pseudomonas syringae pv. syringae upon introduction of pJEL5771 (data not shown) or pJEL5591 (Figure 2-4).
<table>
<thead>
<tr>
<th></th>
<th>Lemma</th>
<th>Repa</th>
<th>ApdA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MLLLTILPAS LMAAMLGGGF TWQLSSELQS QLQLQGEMIA</td>
<td>MLLLTILPAS LMAAMLGGGF TWQLSSELQS QLQLQGEMIA</td>
<td>MLLLTILPAS LMAAMLGGGF TWQLSSELQS QLQLQGEMIA</td>
</tr>
<tr>
<td>2</td>
<td>QDLAPLANA LMRKDKVLLS RIAQTQLEQT DRYAVSFPLDT DRTVLADLQ</td>
<td>QDLAPLANA LMRKDKVLLS RIAQTQLEQT DRYAVSFPLDT DRTVLADLQ</td>
<td>QDLAPLANA LMRKDKVLLS RIAQTQLEQT DRYAVSFPLDT DRTVLADLQ</td>
</tr>
<tr>
<td>3</td>
<td>TMISPSPIGS GSQLLSTSTT DTRYLLLPVF GSRQHLTISP IPABADTLLG</td>
<td>TMISPSPIGS GSQLLSTSTT DTRYLLLPVF GSRQHLTISP IPABADTLLG</td>
<td>TMISPSPIGS GSQLLSTSTT DTRYLLLPVF GSRQHLTISP IPABADTLLG</td>
</tr>
<tr>
<td>4</td>
<td>WVELEISHNG TLLRGYRFSLF ASLLLILTGL AFTATLAVRM SRTINGPSQ</td>
<td>WVELEISHNG TLLRGYRFSLF ASLLLILTGL AFTATLAVRM SRTINGPSQ</td>
<td>WVELEISHNG TLLRGYRFSLF ASLLLILTGL AFTATLAVRM SRTINGPSQ</td>
</tr>
<tr>
<td>5</td>
<td>IKQAVSQLKD GNLETRLPPL GSRELDELAS GINRMAATLQ NAQEELQLSI</td>
<td>IKQAVSQLKD GNLETRLPPL GSRELDELAS GINRMAATLQ NAQEELQLSI</td>
<td>IKQAVSQLKD GNLETRLPPL GSRELDELAS GINRMAATLQ NAQEELQLSI</td>
</tr>
<tr>
<td>6</td>
<td>EQATEDVRQN QELTIEIQNIE LDLARKEALE ASRIKS LA NMSHEIRTPL</td>
<td>EQATEDVRQN QELTIEIQNIE LDLARKEALE ASRIKS LA NMSHEIRTPL</td>
<td>EQATEDVRQN QELTIEIQNIE LDLARKEALE ASRIKS LA NMSHEIRTPL</td>
</tr>
<tr>
<td>7</td>
<td>NVILGFTHLL QKSELTPRQF DYLGTIEKSA DNLLSIINEI LDFSKIEAGK</td>
<td>NVILGFTHLL QKSELTPRQF DYLGTIEKSA DNLLSIINEI LDFSKIEAGK</td>
<td>NVILGFTHLL QKSELTPRQF DYLGTIEKSA DNLLSIINEI LDFSKIEAGK</td>
</tr>
<tr>
<td>8</td>
<td>LRQLTNLVS NAIKFRFQGT IVARAMLEDE TEEHAQCRIS VQDTGIGLSS</td>
<td>LRQLTNLVS NAIKFRFQGT IVARAMLEDE TEEHAQCRIS VQDTGIGLSS</td>
<td>LRQLTNLVS NAIKFRFQGT IVARAMLEDE TEEHAQCRIS VQDTGIGLSS</td>
</tr>
<tr>
<td>9</td>
<td>GQVRALFQAF SQAINLSLSRQ PGSTGLGLVI SKRLIEQMGG EIGV DSTEGP</td>
<td>GQVRALFQAF SQAINLSLSRQ PGSTGLGLVI SKRLIEQMGG EIGV DSTEGP</td>
<td>GQVRALFQAF SQAINLSLSRQ PGSTGLGLVI SKRLIEQMGG EIGV DSTEGP</td>
</tr>
<tr>
<td>10</td>
<td>GSEFWISLKL PKAREDKES LSNIPLGGLRA AVLHDDQQL QALEHQOLED</td>
<td>GSEFWISLKL PKAREDKES LSNIPLGGLRA AVLHDDQQL QALEHQOLED</td>
<td>GSEFWISLKL PKAREDKES LSNIPLGGLRA AVLHDDQQL QALEHQOLED</td>
</tr>
</tbody>
</table>

Figure 2-3 continued.
Figure 2-3. Amino acid sequence alignments of LemA, RepA, and ApdA. Residues that are identical in all three sequences are shaded. Sequence comparison begins at the predicted first amino acid of RepA. Boxed residues indicate the following conserved domains which are named according to their signature amino acids: H box; a.a. 288-305, N box; 394-415, D/F box; 437-464, G box; 473-492 (Stock et al., 1995). A conserved C-terminal domain (a.a. 858-869 [Uhl and Miller, 1995]) is also boxed. A black dot indicates the autophosphorylated histidine residue. Conserved residues typical of a receiver domains are circled.
Table 2-2. Phenotypic characterization and complementation of an Apd<sup>-</sup> mutant of *P. fluorescens* Pf-5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Secondary Metabolite Production&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inhibition of <em>R. solani</em>&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plt µg/ml Phl µg/ml Prn µg/ml HCN Protease TSO</td>
<td></td>
</tr>
<tr>
<td>Pf-5 (pRK415)</td>
<td>17.1±0.5 37.7±1.8 0.7±0.1 + + + 2.6 cm</td>
<td></td>
</tr>
<tr>
<td>JL4135 (pRK415)</td>
<td>- - - - - - -</td>
<td></td>
</tr>
<tr>
<td>JL4135 (pJEL5771)</td>
<td>9.9±0.5 21.5±10. 1.2±0.1 + + + 2.6 cm</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are the average of two replications ± SD. Abbreviations: Prn, pyrrolnitrin; Plt, pyoluteorin; Phl, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide; TSO, tryptophan-side-chain oxidase; +, metabolite detected; -, no metabolite or inhibition detected.

<sup>b</sup>Values are diameters of the inhibition zone minus the diameter of the bacterial colony grown on nutrient agar supplemented with 1% glycerol.
Figure 2-4. Protease production on Bacto Litmus Milk agar showing complementation of JL4135, an Apd⁻ mutant of *P. fluorescens* Pf-5, and NPS3136, a *lemA* mutant of *P. syringae* pv. *syringae* by a plasmid containing the *apdA*⁺ gene of Pf-5. Clear zones around bacterial colonies indicate protease production.
Plasmid pME3066, which contained the gacA gene of *P. fluorescens* CHAO (Laville et al., 1992) did not restore protease, cyanide, or TSO activity to any of the five apdA mutants.

**Western Analysis.** Western analysis of total protein extracted from bacterial cells identified a 101-kDa protein that reacted with the anti-LemA antiserum in strain Pf-5 but not in the apdA mutant strain JL4135 (Figure 2-5). When the wild-type apdA gene cloned in pJEL5771 or pJEL5591 was introduced into JL4135, production of the 101-kDa protein was restored. The anti-LemA antisera also reacted with a 75-kDa protein in mutant JL4135 and the complemented mutants JL4135(pJEL5771) and JL4135(pJEL5991). Based on the location of the Tn5 insertion, the predicted size of the truncated ApdA protein in JL4135 was 75 kDa.

### 2.4 Discussion

A mutation in a single locus that we have termed apdA abolished biosynthesis of an array of secondary metabolites produced by the biological control bacterium *P. fluorescens* Pf-5. Although the phenotype of Apd- mutants of Pf-5 was remarkably similar to that of gacA mutants of the biological control bacterium *P. fluorescens* CHA0 (Laville et al., 1992), apdA was distinct from the gacA homolog of Pf-5. Sequence analysis of the apdA gene showed that it was closely related to genes from two plant pathogens: 1) the lemA gene of *P. syringae*, which is a regulatory gene controlling protease and syringomycin production (Hrabak and Willis, 1993) and lesion formation (Hrabak and Willis, 1992), and 2) the repA gene of *P. viridiflava*, which is required for the production of pectate lyase and other metabolites (Liao et al., 1994). The lemA and repA genes have been proposed to be the sensor kinase components of two-component regulatory systems controlling pathogenicity genes in these strains (Hrabak and Willis, 1992; Liao et al., 1994) Anti-LemA antiserum cross-reacted with the 101-kDa ApdA protein of Pf-5. When taken together, the sequence
Figure 2-5. Western blot analysis of protein isolated from strains of *P. fluorescens* Pf-5 using anti-LemA antisera. Lanes: 1) Pf-5(pRK415), 2) JL4135(pRK415), 3) JL4135(pJEL5771), 4) JL4135(pJEL5991). The 101-kDa ApdA protein and 75-kDa truncated ApdA protein are indicated.
data, results from western analysis, and capacity of apdA to restore protease activity to a lemA mutant of P. syringae provide convincing evidence that apdA is a homolog of lemA. Because apdA and gacA mutants share common phenotypes and because the sequences of apdA and gacA are similar to the sequences of genes encoding known sensor kinases and response regulators, respectively, we infer that these two loci compose a two-component system controlling antibiotic production by P. fluorescens. This conclusion is in agreement with reports that lemA interacts with gacA to regulate antibiotic biosynthesis by the biocontrol agent P. fluorescens strain BL915 (Lam et al., 1993) and the expression of pathogenicity genes in Pseudomonas syringae (Rich et al., 1994; Willis et al., 1994).

Sensor components of two-component regulatory systems are defined by the presence of a transmitter domain, which contains an ATP-binding site and a conserved histidine residue (Parkinson and Kofoid, 1992; Stock et al., 1995). Following autophosphorylation at the conserved histidine residue, the sensor protein typically serves as a kinase, transferring a phosphoryl group to the receiver domain of the cognate response regulatory protein; the phosphorylated response regulator then functions as a transcriptional activator of target genes (Parkinson and Kofoid, 1992). By this model, sensor kinases control gene expression by controlling the concentration of the phosphorylated response regulator, which can be achieved through regulation of the autophosphorylation rate or kinase versus phosphatase activities of the sensor (Stock et al., 1995). Certain sensor proteins, including ApdA, LemA, ArcB, and BvgS, contain both transmitter and receiver domains (Parkinson and Kofoid, 1992) but lack a helix-turn-helix DNA binding domain (Brennan and Mathews, 1989), which is characteristic of response regulators. For at least two of these sensor proteins, ArcB and BvgS, conserved amino acids in both the transmitter and receiver domains are essential for efficient phosphorylation of the cognate response regulators (ArcA and BvgA) (Iuchi and Lin, 1995; Uhl and Miller, 1995). Intramolecular transphosphorylation, in which a phosphate group is transferred from the histidine in the transmitter domain to an aspartic acid residue in the receiver domain, is thought to cause conformational changes in the sensor protein that facilitate phosphorylation of
the cognate response regulator (Iuchi and Lin, 1995; Uhl and Miller, 1995). A conserved histidine residue in the C-terminal region of ApdA, LemA, ArcB, and BvgS may serve as an additional site of phosphorylation; deletion of the C-terminal region destroys function of BvgS (Uhl and Miller, 1995). Thus, ApdA is a member of a family of complex sensor proteins, which contain multiple cytoplasmic domains that participate in a phosphorylation cascade involved in signal transduction.

The transmembrane sensor kinases of two-component regulatory systems are thought to be autophosphorylated in response to a signal molecule(s), thereby mediating changes in gene expression in response to environmental signals (O'Sullivan and O'Gara, 1992). Although extracellular signals may trigger autophosphorylation of these proteins directly, it may be just as common that sensor kinases perform an intermediate role in signal transduction rather than acting as the receptor for an environmental signal. For example, at least some of the signals triggering autophosphorylation of the sensor protein ArcB are in the cytosol; these may be metabolites produced by E. coli under anaerobic conditions, which trigger the phosphorylation cascade regulating an array of genes responsive to O₂ tension (Iuchi and Lin, 1995). The nature of environmental or physiological signals to which ApdA responds has not been established. Because phenotypes under the control of apdA are expressed by Pf-5 in culture media (Nowak-Thompson et al., 1994) and in the rhizosphere (Kraus and Loper, 1995) autophosphorylation of ApdA is likely to be prompted by multiple signals or a single signal molecule that is commonly produced or encountered by Pseudomonas spp. occupying these diverse habitats. The identification of ApdA as a sensor kinase serving as a global regulator of antibiotic biosynthesis genes provides an opportunity to identify the environmental cues to which P. fluorescens responds. Such information may have predictive value, allowing identification of those environmental conditions conducive to antibiotic production, and may also lead to the genetic improvement of biological control agents. For example, as more two-component systems are identified, it may be possible to exchange an input domain from a sensor with a known signal for the wild-type input
domain of ApdA, thereby providing greater control over the expression of genes required for antifungal metabolite production by \textit{P. fluorescens}.

It must be recognized, however, that bacterial signalling systems may rarely be as simple as a system composed of two proteins. In \textit{Pseudomonas} spp., several regulatory cascades in which two-component systems play a role have been partially characterized. For example, alginate biosynthesis by \textit{Pseudomonas aeruginosa} is coordinated by a complex regulatory cascade consisting of at least two response regulators, the alternate sigma factor AlgU, and regulators of AlgU; sensor kinases for the system have yet to be identified (Deretic et al., 1994). In \textit{P. syringae}, the regulation of \textit{hrp} (for hypersensitive response and pathogenicity) and avirulence (\textit{avr}) genes is controlled by a multicomponent cascade involving HrpR, HrpS, and HrpL (Xiao et al., 1994). HrpR and HrpS are believed to be response regulators that interact with $\sigma^{54}$ to promote transcription of \textit{hrpL}, which codes for an alternate sigma factor similar to AlgU of \textit{P. aeruginosa}. The RNA polymerase-HrpL holoenzyme then activates transcription of \textit{hrp} and \textit{avr} genes (Xiao et al., 1994).

Regulation of secondary metabolite biosynthesis by \textit{P. fluorescens} may be equally complicated as those systems mentioned above. For example, a regulatory locus coding for the stationary phase sigma factor \textit{rpoS} has been identified in \textit{P. fluorescens} Pf-5 (Sarniguet et al., 1995). A mutation in this locus abolished production of pyrrolnitrin and enhanced production of pyoluteorin and 2,4-diacyltphloroglucinol. Such information leads us to speculate that antibiotic production by Pf-5 is controlled by a complex cascade of genetic regulation in which the \textit{apdA-gacA} regulatory system represents a single layer.

3.1 Introduction.

In prokaryotes, the major mechanism for signal transduction is the two-component regulatory system. The model two-component system is composed of two proteins; a sensor kinase, which autophosphorylates in response to environmental signals, and a response regulator, which is activated via transfer of a phosphoryl group from the sensor kinase. Once activated, the phosphorylated response regulator typically serves as a transcriptional activator of target genes (Parkinson and Kofoid, 1992; Parkinson, 1995; Stock et al., 1995). In fluorescent pseudomonads, the production of secondary metabolites is globally regulated by a two-component system composed of ApdA (the sensor kinase) (Corbell and Loper, 1995) and GacA (the response regulator) (Laville et al., 1992; Gaffney et al., 1994). Mutations in either of these two loci pleiotropically abolish production of antifungal secondary metabolites by fluorescent pseudomonads and can render these bacteria ineffective as biological control agents. Mutants with nucleotide substitutions in *apdA* and *gacA* accumulate in late-stationary-phase cultures of *P. fluorescens* (Duffy and Defago, 1995; Corbell et al., 1997). The functional *gacA* allele *gacA*(Y49), which specifies a tyrosine at amino acid 49 (Laville et al., 1992), apparently was isolated from such a mutant of *P. fluorescens* CHAO. The wild-type gene specifies an aspartate at amino acid 49 (Reimmann et al., 1997; Bull, 1998). Previously this allele was referred to as *gacA* (Chapter 2). Hereafter *gacA* refers to the wild-type allele and *gacA*(Y49) to the functional mutant allele.

Previously, the *apdA* gene of *P. fluorescens* Pf-5 was identified (Corbell and Loper, 1995; Chapter 1). Mutants in *apdA* are pleiotropic; they fail to synthesize the antifungal compounds pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol; as well as extracellular protease(s), hydrogen cyanide, and tryptophan side chain oxidase.
This study reports 1) the identification of the gene for the response regulator (gacA) in this two-component system in Pf-5, 2) the affect of apdA/gacA mutations on the transcription of a gene in the pyoluteorin biosynthetic cluster, and 3) the characterization of a spontaneous mutant having a partial apdA/gacA phenotype.

3.2 Materials and Methods.

3.2.1 Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are described in Table 3-1. *Escherichia coli* strains were cultured in Luria Bertani (LB) broth (Sambrook et al., 1989) or on LB agar plates incubated at 37°C. *Pseudomonas* strains were routinely cultured at 27°C in King's medium B (KMB)(King et al., 1954) broth or on KMB agar plates. Antibiotic concentrations were as follows unless otherwise specified: 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 20 µg/ml tetracycline.

3.2.2 Recombinant DNA techniques.

Transformation of *E. coli*, restriction digests, ligations, electrophoresis in Tris-phosphate-EDTA, and plasmid DNA isolation were performed by standard methods (Sambrook et al., 1989). Enzymes were from Gibco BRL Life Technologies (Gaithersburg, MD). PCR mixtures (50 µl) contained template DNA (1 µl), 5X Elongase Buffer B (Gibco BRL) (10 µl), 400 µM dNTPs, 2 units of Elongase Enzyme Mix, and 2 µM of each primer. Southern blots of plasmid and genomic DNA were prepared with nylon membranes (Nytran; Schleicher and Schuell, Keene, NH) according to the supplier's directions. DNA probes were the gacA(Y49) gene of *P. fluorescens* CHA0 (the 1.65-kb BamHI-BglII fragment of pME3066) (Laville et al.,
Table 3-1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Description</th>
<th>Relevant Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pf-5</td>
<td>Field isolate</td>
<td>Gac⁺</td>
<td>Howell and Stipanovic, 1979</td>
</tr>
<tr>
<td>JL4365</td>
<td>Derivative of Pf-5 containing a $pltE::inaZ$ transcriptional fusion</td>
<td>Plt⁻</td>
<td>Kraus and Loper, 1995</td>
</tr>
<tr>
<td>JL4477</td>
<td>Spontaneous mutant of Pf-5 containing the $gacA(V203)$ allele</td>
<td>Gac⁺</td>
<td>This study</td>
</tr>
<tr>
<td>JL4485</td>
<td>Spontaneous $gacA$ mutant of JL4365</td>
<td>Gac⁻ Plt⁻</td>
<td>This study</td>
</tr>
<tr>
<td>JL4486</td>
<td>Spontaneous $apdA$ mutant of JL4365</td>
<td>Apd⁻ Plt⁻</td>
<td>This study</td>
</tr>
<tr>
<td>JL4577</td>
<td>Derivative of Pf-5, $\Delta gacA::aphl$</td>
<td>Gac⁻ Km⁻</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli DH5α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>$Tp^r$ $Sm^r$ $recA$ $thi$ $pro$ $hsdRM^+$ $RP4:2$ $Tc:Mu:Km$ $Tn7$</td>
<td></td>
<td>Simon et al., 1983</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>ColE1 replicon</td>
<td>Ap⁺</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td>pUC4K</td>
<td>ColE1 replicon, source of kanamycin resistance gene $aphl$</td>
<td>Km⁺</td>
<td>Vieira and Messing, 1982</td>
</tr>
<tr>
<td>pRK415</td>
<td>IncP1 replicon; polylinker of pUC19</td>
<td>Mob⁺ Tc⁺</td>
<td>Keen et al., 1988</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Mobilizing plasmid</td>
<td>Tra⁺ Km⁻</td>
<td>Figurski et al., 1979</td>
</tr>
<tr>
<td>pME3066</td>
<td>1.65-kb $BamHI-BglII$ fragment containing the $gacA$ gene of P. fluorescens Pf-5</td>
<td>Tc⁺</td>
<td>Laville et al., 1992</td>
</tr>
<tr>
<td>pME6000</td>
<td>pBBR1 replicon; stable, high copy vector in <em>Pseudomonas</em> spp.</td>
<td>Mob⁺ Tc⁺</td>
<td>D. Haas</td>
</tr>
</tbody>
</table>
Table 3-1 continued

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>Selection</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJEL5520</td>
<td>pLAfR3 containing ca. 30 kb of Pf-5 genomic DNA including <em>gacA</em>.</td>
<td>Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL5680</td>
<td>6.5-kb <em>KpnI</em> fragment of Pf-5 cloned containing <em>apdA</em> cloned into pUC18</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Corbell and Loper 1995</td>
</tr>
<tr>
<td>pJEL5771</td>
<td>3.5-kb <em>KpnI-SunI</em> fragment of Pf-5 containing <em>apdA</em> cloned into pRK415</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Corbell and Loper 1995</td>
</tr>
<tr>
<td>pJEL5835</td>
<td>Derivative of pUC18 in which the <em>SmaI-HincII</em> fragment in the polycloning site has been deleted</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL5935</td>
<td>1.65-kb <em>BamHI-BglII</em> fragment of Pf-5 containing <em>gacA</em> cloned into pRK415</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL5937</td>
<td>1.65-kb <em>BamHI-BglII</em> fragment of Pf-5 containing <em>gacA</em> cloned into pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Whistler et al., 1998</td>
</tr>
<tr>
<td>pJEL5938</td>
<td>6.6-kb <em>EcoRI</em> fragment of Pf-5 containing <em>gacA</em> cloned into pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL5965</td>
<td>pME6000 containing the 1.65-kb <em>EcoRI-HindIII</em> <em>gacA</em> fragment from pJEL5937</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL5999</td>
<td>pME6000 containing the 6.7-kb <em>KpnI</em> <em>apdA</em> fragment from pJEL5680</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6053</td>
<td>960-bp <em>BamHI-EcoRI</em> fragment containing <em>gacA</em>(V203) generated by the PCR from JL4477, cloned into pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6054</td>
<td>960-bp <em>BamHI-EcoRI</em> fragment containing <em>gacA</em> generated by the PCR from pJEL5937, cloned into pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6057</td>
<td>960-bp <em>BamHI-EcoRI</em> fragment containing <em>gacA</em>(V203) from pJEL6053 cloned into pRK415</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6058</td>
<td>960-bp <em>BamHI-EcoRI</em> fragment containing <em>gacA</em> from pJEL6054 cloned into pRK415</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6060</td>
<td>1.65-kb <em>EcoRI-HindIII</em> fragment containing <em>gacA</em>(V203) cloned into pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6061</td>
<td>1.65-kb <em>EcoRI-HindIII</em> fragment of pJEL6060 cloned into pME6000</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>plasmid</td>
<td>description</td>
<td>resistance</td>
<td>source</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
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<td>--------</td>
</tr>
<tr>
<td>pJEL6064</td>
<td>2.1-kb EcoRI fragment in which gacA was deleted and replaced with an SpeI site; cloned into pUC19</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6065</td>
<td>6.6-kb EcoRI fragment of pJEL5938 cloned into pJEL5835</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6070</td>
<td>1.65-kb BamHI-BglII fragment of pJEL6065 has been replaced with the 1-kb BamHI-BglII fragment from pJEL6064</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6071</td>
<td>1.3-kb BamHI fragment containing aphI from of pUC4K cloned into the blunted SpeI site of pJEL6070</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;, Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6072</td>
<td>7.2-kb EcoRI fragment of pJEL6071 containing gacA::aphI cloned into pRK415</td>
<td>Mob+ Tc&lt;sup&gt;f&lt;/sup&gt;, Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6164</td>
<td>960-bp HindIII-EcoRI fragment containing gacA(V203) from pJEL6053, cloned into the blunted XbaI site of pRK415, lacZ and gacA(V203) promoters oppose each other</td>
<td>Mob+ Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6165</td>
<td>960-bp HindIII-EcoRI fragment containing gacA(V203) from pJEL6053, cloned into the blunted XbaI site of pRK415, lacZ and gacA(V203) promoters are in the same direction</td>
<td>Mob+ Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6167</td>
<td>960-bp EcoRI fragment containing gacA generated by the PCR from pJEL5937, cloned into pRK415, lacZ and gacA promoters oppose each other</td>
<td>Mob+ Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6168</td>
<td>960-bp EcoRI fragment containing gacA generated by the PCR from pJEL5937, cloned into pRK415, lacZ and gacA promoters are in the same orientation</td>
<td>Mob+ Tc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ap<sup>f</sup>, Km<sup>f</sup>, and Tc<sup>f</sup> indicate resistance to ampicillin, kanamycin, and tetracycline, respectively. GacA<sup>p</sup> indicates partial phenotype. The Gac<sup>-</sup> and Apd<sup>-</sup> phenotypes are the same but are given different designations since their genotypes are known.
1992) and the mutated gacA gene of Pf-5 containing the kanamycin resistance gene (the linearized plasmid pJEL6071) labelled with biotinylated dATP or $^{32}$P-dCTP using a nick translation kit (Gibco BRL Life Technologies), and purified with a D50 column (International Biotechnologies Inc., New Haven, CT). Plasmids pRK415, pME6000 and derivatives either were mobilized from E. coli DH5α donors into P. fluorescens Pf-5 in triparental matings using helper plasmid pRK2013 (Figurski and Helinski, 1979), or were mobilized from E. coli S17-1 in biparental matings. Due to the high degree of natural tetracycline resistance exhibited by P. fluorescens Pf-5, transconjugants were selected on KMB containing 200 µg/ml tetracycline and 100 µg/ml ampicillin, unless otherwise specified.

3.2.3 Cloning of gacA from Pf-5.

An extant genomic library of Pf-5 (Pfender et al., 1993) was screened by colony hybridization (Gergen et al., 1979) to identify cosmids that hybridized to gacA(Y49) of P. fluorescens CHAO (Laville et al., 1992). Southern analysis identified restriction fragments in cosmids that hybridized to the gacA(Y49) probe. A 1.65-kb BamHI-BglII fragment from cosmid pJEL5520 that hybridized to the probe was cloned into pUC19 to construct pJEL5937. A 6.6-kb EcoRI fragment from cosmid pJEL5520 that hybridized to the probe was cloned into pUC19 to construct pJEL5938.

3.2.4 Sequence analysis of gacA alleles.

DNA sequencing and oligonucleotide syntheses were done at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, OR. Sequencing of double-stranded templates was done 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 either by the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, Oregon, or DNAexpress at Colorado State University, Fort Collins, Colorado. Sequencing of the gacA gene of Pf-5 was done 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 (Devereux et al. 1984). Theoretical secondary structures of proteins encoded by alleles of gacA were predicted by PepPlot and PlotStructure programs (Genetics Computer Group, Inc.).

3.2.5 Derivation of a spontaneous gacA mutant of a 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 (Duffy and Defago, 1995). Pf-5 was grown in nutrient broth amended with 0.5% yeast extract (NBY) 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 apdA/gacA mutants (Duffy and Defago, 1995)) 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 (Kraus and Loper, 1992).
3.2.6 Separation of gacA from uvrC.

Construction of pRK415 derivatives carrying gacA alleles are shown in Figure 3-1. The PCR was used to amplify the 960-bp fragment containing wild-type gacA and its upstream noncoding sequences from pJEL5937. The primers used in the PCR are shown below.

5’-CGGGATCCGGATCCAGAGCGGCGG-3’
(GACFOR nucleotides 1-22 of gacA; Figure 3-3)

5’-CCGGAATTCTTCAGAGGCTGGCATCAACCATGC-3’
(GACREV complementary to nucleotides 934-957 of gacA; Figure 3-3)

The EcoRI and BamHI sites (underlined) contained in these respective primers were used to clone the product into the BamHI-EcoRI site of pUC19 and pRK415. The resulting plasmids (pJEL6054 and pJEL6058, respectively) contained gacA in the same orientation as the lacZ promoter in these vectors. The same method was used to clone the gacA allele of spontaneous mutant JL4477 [gacA(V203)] to create the pUC19 (pJEL6053) and pRK415 (pJEL6057) derivatives, except that the template for the PCR was chromosomal DNA of JL4477.

To reverse the orientation of gacA with respect to the lacZ promoter, the PCR was used with the pUC19 forward and GACREV primers, to amplify gacA and the adjacent vector sequences containing an EcoRI site from pJEL5937. The EcoRI sites flanking this sequence were used to clone the fragment into the EcoRI site of pRK415 in both the forward (pJEL6168), and reverse (pJEL6166) orientations with respect to the lacZ promoter.

To reverse the orientation of gacA(V203) with respect to the lacZ promoter, the HindIII/EcoRI fragment of pJEL6058 was blunted using the fill-in reaction of T4 DNA polymerase. The blunted fragment was cloned into the blunted XbaI site of pRK415 in
Figure 3-1. Recombinant plasmids carrying the wild-type gacA of Pf-5 and the mutant gacA(V203) in vector pRK415. The direction of the gacA promoter for all constructs is shown by the arrow within the gene. All constructs are shown with respect to the direction of the lacZ promoter (arrow). gacrev and pUC19 forward are primers used in the PCR. gacA is indicated by the open box. Light grey area indicates Pf-5 genomic DNA downstream of gacA. Darker grey area indicates vector DNA. DNA from Pf-5 is drawn to scale (gacA including its upstream untranslated region is 953 bp). Distance between restriction sites in the vector are not drawn to scale.

Restriction sites are abbreviated as follows: H, HindIII; B, BamHI, Bg, BglII; E, EcoRI; Xb, XbaI.
both the forward (pJEL6163), and reverse (pJEL6162) orientations with respect to the lacZ promoter.

### 3.2.7 Description of a Pf-5 derivative containing a genomic plt::inaZ fusion.

The pyoluteorin biosynthetic genes are clustered in a 20-kb region in the genome of Pf-5 (Nowak-Thompson et al. 1997). JL4365, a Pf-5 derivative containing a chromosomal plt::inaZ transcriptional fusion, was constructed by insertion of the transposon Tn3-nice into the pyoluteorin biosynthetic region such that inaZ is transcribed from the pyoluteorin promoter. JL4365 expresses ice nucleation activity (INA) that is proportional to pyoluteorin production (Kraus and Loper, 1995). INA was quantified by the droplet freezing assay as described previously (Loper and Lindow, 1994). Spontaneous apdA/gacA mutants of JL4365 were generated as described previously (Duffy and Defago, 1995). The cloned apdA (pJEL5771) and gacA (pJEL5935) genes were mobilized from E.coli DH5α donors into protease negative mutants via triparental matings with helper plasmid pRK2013 (Figurski and Helinski, 1979).

### 3.2.8 Construction of a gacA deletion mutant.

A derivative of gacA, pJEL6072, in which 626 bp of gacA were replaced with the kanamycin gene of pUC4K (Figure 3-2), was accomplished by the series of PCR and cloning steps described below. Primers used in PCR are shown below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19 forward</td>
<td>5-CCCAGTCACGACGTTGTAAAACG-3</td>
</tr>
<tr>
<td>pUC19 reverse</td>
<td>5-AGCGGATAAACAAATTTCAUCAGG-3</td>
</tr>
</tbody>
</table>
Figure 3-2. Schematic representation describing the construction of the gacA deletion mutant (JL4577). The translational start site of gacA is indicated by an arrow. Direction and corresponding sequence homology of primers DEL-F, DEL-R, pUC19 forward, and pUC19 reverse are indicated by arrows. Shaded regions indicate identical regions of sequence. Saw-toothed boxes indicate vector sequences. The kanamycin-resistance gene (aphI) is from pUC4K. Restriction sites in the vector and primer lengths are not drawn to scale.
DEL-F
5' GACCATATCGCGAGGTGTCTGCACTAGTGATGCCAGCCTCTGAAATGACC 3'
DEL-R
5' GGTCATTTCAGAGGCTGGCATCACTAGTGCAGACACCTCGCGATATGGTC 3'

DEL-F and DEL-R are complementary to each other and contain an SpeI site (underlined) that is flanked by sequences complementary to nucleotides 293-311 and 941-963 of the gacA sequence (Figure 3-3). PCRs were performed using Elongase enzyme mix (Gibco BRL) in a Stratagene Robocycler Gradient 960, with 35 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), extending (2 min at 68°C), and followed by an additional polishing of incomplete products (10 min at 68°C).

PCR 1. The 406-bp fragment, containing the upstream region of gacA and 65 bp of vector DNA, was amplified by the PCR from pJEL5937 using the pUC19 forward and DEL-F primers.

PCR 2. The 1.7-kb fragment, containing the last 16 nucleotides of gacA, the region downstream of the gene, and 45 bp of vector DNA was amplified by the PCR from pJEL5938 using the pUC19 reverse and DEL-R primers.

PCR 3. Samples (0.5 µl) of the products of PCR 1 and PCR 2 were combined. From this mixture, the 2-kb fragment, in which 626 bp of gacA were replaced by an SpeI site, was amplified by PCR using the pUC19 forward and reverse primers.

The 2-kb fragment amplified by PCR 3 was phenol chloroform extracted and ethanol precipitated to inactivate the polymerases in the Elongase Enzyme Mix (BRL). The fragment was digested with EcoRI, gel purified, and ligated into the EcoRI site in pUC19 to create pJEL6064.

The 6.6-kb EcoRI fragment of pJEL5938, which contains gacA, was cloned into the EcoRI site of pJEL5835, a pUC18 derivative in which the BamHI site had been deleted, to yield pJEL6065. pJEL6065 was digested with BamHI and BglII. The
resulting 7.7-kb fragment, composed of the pUC18 vector and the sequences flanking gacA, was gel purified and ligated to the 1-kb BamHI-BgIII fragment of pJEL6064 containing the mutated gacA. The orientation of the mutated gacA in this construct was determined by restriction digestion with BamHI and EcoRI. The construct containing the mutated gacA cloned in the same orientation as the wild-type gacA was designated pJEL6070. In order to introduce a kanamycin resistance gene, pJEL6070 was digested with SpeI and then treated with T4 DNA polymerase for the creation of blunt ends. The BamHI fragment of pUC4K containing the aphl gene for kanamycin resistance (Vieira and Messing, 1982) also was treated with T4 DNA Polymerase to create blunt ends. This 1,264-bp fragment was cloned into the blunted SpeI site of pJEL6070 to create pJEL6071. The 7-kb EcoRI fragment of pJEL6071, containing the kanamycin resistance gene within the mutated gacA, was cloned into the EcoRI site of the broad host range vector pRK415 to create pJEL6072. Plasmid pJEL6072 was introduced to Pf-5 via biparental mating. Transconjugants were selected on KB medium amended with 50 µg/ml kanamycin and 100 µg/ml ampicillin. The resulting transconjugants were grown for 24 h in KB broth in the absence of antibiotic selection to facilitate marker exchange between the chromosome and the plasmid, plated on KB amended with 50 µg/ml kanamycin, and screened for loss of tetracycline resistance conferred by the vector. Marker exchange mutants were confirmed by Southern blot analysis using linearized plasmid pJEL6071 as the probe. The marker exchange mutant created by this process was designated JL4577.

3.2.9 Assessment of secondary metabolite production.

Antibiotic quantification. Antibiotics were extracted from cells and spent media of cultures grown in duplicate as described previously (Nowak-Thompson et al., 1994). Pyoluteorin and pyrrolnitrin production were assessed from cultures grown for 3 days at 20°C in 5 ml of nutrient broth containing 1% glycerol, a medium that favors their production. The culture time was changed from 2 to 3 days because production
of pyrrolnitrin by Pf-5 strains was more reliable after 3 days of culture. 2,4-
diacetylphloroglucinol was assessed 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 and excess water was removed
with molecular sieve with a pore size of 3 Angstroms (Mallinckrodt). The bacterial
pellet was extracted with acetone. Extracts were dissolved in MeOH and the
production of antibiotics was assessed by thin layer chromatography as previously
described (Kraus and Loper, 1992).

**Enzyme production.** Extracellular protease was assessed visually as a cleared
zone around bacterial colonies on Bacto Litmus milk agar (Difco Laboratories, Inc.)
following incubation at 27°C for 48 h. TSO activity was determined qualitatively by
the method of Takai and Hayaishi (1987).

**HCN production.** Qualitative assessment of HCN production was made by
the method originally described by Castric and Castric (Castric and Castric, 1983) as
modified by Sarniguet et al. (Sarniguet, 1992).

3.3 Results.

3.3.1 Identification and sequence analysis of the gacA gene.

Two cosmids containing the gacA gene of *P. fluorescens* Pf-5 were identified
from an existing genomic library by hybridization to the gacA(Y49) probe of *P.
fluorescens* CHA0. Southern hybridization indicated that each of the two cosmids
contained a 6.6-kb *EcoRI* fragment and a 1.65-kb *BamHI-BglII* fragment that
hybridized to the gacA(Y49) probe.

A 1151-bp region of DNA within the 1.65-kb *BamHI-BglII* fragment of
pJEL5937 was sequenced and found to contain a 639-bp open reading frame (ORF)
identical to the wild-type gacA of *P. fluorescens* CHA0 (Reimmann, 1997). This 639-bp ORF was therefore identified as the gacA locus of Pf-5. The ORF, beginning at bp 315 with a TTG initiation codon and terminating at bp 954 with a TGA stop codon, was predicted to encode a protein of 213 amino acids with a molecular mass of 26 kDa. Upstream from the TTG codon, there is a putative to Shine-Dalgarno site for ribosome binding from bp 304 to bp 307 (Figure 3-3). The gacA gene contains no recognizable consensus sequences for σ^{70}, σ^{54}, or σ^{32} type pseudomonad promoters (Deretic et al., 1989).

The sequence of gacA is similar to those of a class of regulatory proteins. The gacA nucleotide and predicted amino acid sequences were similar to those for response regulator components of two-component regulatory systems (Parkinson and Kofoid, 1992). The conserved N-terminal portion of the putative protein contained the conserved aspartate and lysine residues that are commonly found within the phosphorylation domains of response regulators (Figure 3-3). A consensus LuxR-type helix-turn-helix DNA binding motif (PDOC00542 in the Prosite database) characterized by the sequence [GDC]-2X-[NSTAVY]-2X-[IV]-[GSTA]-2X-[LIVMFYWCR]-3X-[NST]-[LVIM]-5X-[NRHSA]-[LIVMSTA]-2X-[KR] was identified in the C-terminus of GacA from a.a. 163 to a.a. 184. Similarly, an overlapping region from a.a. 164 to a.a. 185 was identified as a putative helix-turn-helix using the hth program by Dodd and Egan (Dodd and Egan, 1990), although the probability of this region being a helix-turn-helix was not considered significant by that method.

The gacA gene of Pf-5 is homologous to the gacA genes of other *Pseudomonas* species. The GacA of Pf-5 was >86% identical to the deduced GacA proteins *P. fluorescens* BL915 (Gaffney et al., 1994), *P. aeruginosa* PAO1 (Reimmann et al., 1997), *P. fluorescens* CHA0 (Laville et al., 1992), *P. viridifalava* (Liao et al., 1994) and *P. syringae* B728a (Rich et al., 1994) in a 213-amino acid
Figure 3-3. Complete nucleotide and predicted amino acid sequence of gacA. The putative translational start codon is indicated by an arrow; the putative ribosome binding site is indicated by a series of asterisks. Conserved residues of the receiver module are circled. The putative LuxR-type helix-turn-helix DNA binding motif is boxed. A stop codon is indicated by a single asterisk at the end of the amino acid sequence.
overlap (Figure 3-4). The GacA of Pf-5 is identical to the wild-type GacA of *P. fluorescens* CHA0 (Reimmann et al., 1997).

### 3.3.2 Identification of a GacA*\(^{-}\) spontaneous mutant.

Forty percent of the colonies that grew from 6-day old broth cultures had a Apd'/Gac*\(^{-}\)* phenotype (i.e. colonies were large and orange in comparison to the wild type and failed to produce an extracellular protease). One such mutant, JL4477, had an allele of *gacA* with a T rather than a C at nucleotide 607. Consequently, the deduced amino acid sequence of the mutant allele designated *gacA*(V203), has a valine rather than an alanine at a.a. 203 (Figure 3-5). The 292 nucleotides immediately upstream of the *gacA* ORF in JL4477 were identical to those upstream of the wild-type *gacA* of Pf-5.

### 3.3.3 Complementation of the *gacA*(V203) mutant with cloned *gacA* gene alleles.

Mutant JL4477 of Pf-5 containing *gacA*(V203) still produced trace amounts of pyoluteorin, TSO, and HCN, but did not produce pyrrolnitrin, 2,4-diacyctylphloroglucinol, or the extracellular protease(s). Plasmid pME3066, which contained the *gacA*(Y49) allele of *P. fluorescens* CHA0 restored production of pyrrolnitrin, 2,4-diacyctylphloroglucinol, and the extracellular protease(s). TSO activity and the production of pyoluteorin and HCN were also restored to at least wild-type levels (Table 3-2). Complementation of JL4477 with the 1.65-kb *BamHI/BgIII* fragment containing the wild-type *gacA* of Pf-5 cloned in pJEL5935 yielded results similar to that seen with pME3066 (Table 3-2).

The first 600 nucleotides of the *uvrC* homolog in *Pseudomonas* species lies immediately downstream of the *gacA* alleles (data not shown) in both pME3066 and pJEL5935 so I investigated the unlikely possibility that *uvrC* rather than *gacA* was
Figure 3-4. Alignment of the amino acid sequences of GacA from Pseudomonas species. Genbank accession numbers are: P. aeruginosa PA01 (U89528), P. syringae B728a (U09767), P. viridiflava (L30102), P. fluorescens Pf-5 (AF065156), P. fluorescens BL915 (L29642), P. fluorescens CHAO (M80913).
Figure 3-5. Alignment of the amino acid sequences of GacA from *P. fluorescens* Pf-5 wild-type, GacA(V203) derivative of Pf-5, and GacA(Y49) derivative of *P. fluorescens* CHAO. Boxed amino acids indicate differences from the wild-type GacA of Pf-5.
Table 3-2. Phenotypic characterization and complementation of a spontaneous gacA mutant of *P. fluorescens* Pf-5.

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<th>Strain</th>
<th>Secondary Metabolite Production</th>
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<td>Plt</td>
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<tr>
<td>Pf-5 (pRK415)</td>
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<tr>
<td>JL4477 (pRK415)</td>
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<tr>
<td>JL4477 (pJEL5935)</td>
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<td>JL4477 (pJEL6057)</td>
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<tr>
<td>JL4477 (pJEL6164)</td>
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<td>JL4477 (pJEL6165)</td>
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<td>JL4477 (pJEL6058)</td>
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<td>JL4477 (pJEL6167)</td>
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<td>JL4477 (pJEL6168)</td>
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Abbreviations: Plt, pyoluteorin; Phl, 2,4-diacetylphloroglucinol; Prn, pyrrolnitrin; HCN, hydrogen cyanide; TSO, tryptophan-side-chain oxidase; ++++, greater than wild-type levels; ++, wild-type levels; +, very low levels; -, not detected; nt, not tested. Two replicate cultures of each strain were assayed.
responsible for the observed complementation. Both wild-type gacA and gacA(V203) were separated from the uvrC sequence as described in Figure 3-1 and used in complementation experiments. Complementation of JL4477 with wild-type gacA (pJEL6166 and pJEL6168) restored the production of all secondary metabolites, whereas complementation with the gacA(V203) allele (pJEL6162 and pJEL6163) did not fully restore production of secondary metabolites to this strain (Table 3-2). The orientation of gacA with respect to the lacZ promoter in the vector had no affect on complementation (Table 3-2). In preliminary work, the wild-type gacA allele (pJEL6058) and gacA(V203) (pJEL6057), both of which are transcribed in the same direction as the lacZ promoter in the vector, restored the production of pyoluteorin to mutant JL4477 (data not shown). The amount of pyoluteorin produced by both of these strains was more than that produced by wild type (data not shown). However, complementation with gacA(V203) did not restore pyrrolnitrin production (data not shown). Reexamination of the JL4477 strains bearing these constructs confirmed that pyoluteorin production was higher than that of the wild type (Table 3-2). However neither strain produced detectable amounts of pyrrolnitrin (Table 3-2).

### 3.3.4 Effects of gene dose of apdA and gacA on the metabolites produced by Pf-5.

**Complementation of apdA and gacA(V203) mutants with their respective genes.** Comparisons between gacA(V203) mutant JL4477 and apdA mutant JL4135 harboring gacA in a low copy plasmid (1-4 copies of pJEL5935), gacA in a high copy plasmid (16-20 copies of pJEL6001), apdA in a low copy plasmid (1-4 copies of pJEL5771), and apdA in a high copy plasmid (16-20 copies of pJEL5999) are shown in Table 3-3. Low copy plasmids pJEL5935 (gacA) and pJEL5771 (gacA) restored the production of all secondary metabolites that were assessed to their respective mutants except that 2,4-diacytethylphloroglucinol was not restored to JL4477 containing pJEL5935 (gacA). High copy plasmids pJEL6001 and pJEL5999 (gacA and apdA,
Table 3-3. Gene dose effects of *apdA* and *gacA* on complementation of an *apdA* mutant and a *gacA* null mutant.

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<td>JL4135 (pJEL5771)</td>
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<td>JL4135 (pJEL5999)</td>
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<td>JL4135 (pJEL5935)</td>
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<td>JL4477 (pJEL5965)</td>
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<td>JL4477 (pJEL5999)</td>
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Abbreviations: Plt, pyoluteorin; Phl, 2,4-diacetylphloroglucinol, Prn, pyrrolnitrin; HCN, hydrogen cyanide; TSO, tryptophan-side-chain oxidase; ++, wild-type levels; +, very low levels; -, not detected; nt, not tested. Results are from one replicate for each strain.
respectively) did not have a predictable effect when introduced into their respective mutants. Most notably, the high copy plasmids did not restore pyrrolnitrin to their respective mutants, but did restore 2,4-diacetylphloroglucinol to at least wild-type levels. A high copy number of *apdA* did not restore TSO activity to *apdA* mutant JL4135. TSO activity of *gacA*(V203) mutant JL4477 containing a high copy number of *gacA* (pJEL6001) was not assessed because JL4477 produces detectable levels of TSO activity.

**Cross complementation with *gacA* and *apdA***. A high gene dose of *gacA* (pJEL6001) restored protease and TSO activity to *apdA* mutant JL4135, but did not restore production of pyoluteorin, pyrrolnitrin, or 2,4-diacetylphloroglucinol. Neither a high (pJEL6061) nor low gene dose of *apdA* (pJEL5771) restored the production of secondary metabolites to the spontaneous *gacA* mutant JL4477 (Table 3-3).

### 3.3.5 Identification of *gacA* and *apdA* mutants of a Pf-5 derivative containing a genomic *plt::inaZ* fusion.

Following six days culturing in NBY medium, JL4485 and JL4486 were isolated as a protease deficient mutants of JL4365 and further determined to be deficient in pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, HCN, and TSO. Because these mutants had the pleiotropic Gac−/Apd− phenotype, the wild-type *gacA* (pJEL5935) and *apdA* (pJEL5771) genes were introduced into these strains to determine whether or not these mutants were *gacA* or *apdA* mutants. JL4485 was determined to be a *gacA* mutant and JL4486 an *apdA* mutant based on complementation by their respective genes, pJEL5935 and pJEL5771 (Table 3-4). Due to the *plt::inaZ* fusion, pyoluteorin was not produced by the *pltE::inaZ* parental strain, JL4365, or its derivatives.
Table 3-4. Phenotypic characterization and complementation of spontaneous *gacA* and *apdA* mutants of Pf-5 derivative containing a *pltE::inaZ* fusion.

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<th>Strain</th>
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<td>JL4485</td>
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<td>JL4486 (pJEL5935)</td>
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<td>JL4486 (pJEL5771)</td>
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Abbreviations: Plt, pyoluteorin; Phl, 2,4-diacetylphloroglucinol, Prn, pyrrolnitrin; HCN, hydrogen cyanide; TSO, tryptophan-side-chain oxidase; +, metabolite detected; -, metabolite not detected. Two replicate cultures of each strain were assayed.
3.3.6 Transcription from the plt::inaZ fusion is reduced in gacA and apdA mutants.

Compared to the plt::inaZ parental strain (JL4365), INA was reduced 1000-fold in the gacA mutant (JL4485) and 10,000-fold in the apdA mutant (JL4486) (Figure 3-6). Wild-type Pf-5 had no ice nucleation activity. Thus, all INA was attributable to the plt::inaZ fusion. The difference in INA activity for gacA and apdA mutants was reproducible. Introduction of gacA and apdA in low copy plasmids (pJEL5935 and pJEL5771, respectively) into their respective mutants enhanced ice nucleation activity beyond that of JL4365 (Figure 3-6).

3.3.7 Phenotype analysis of a gacA::aphI deletion mutant.

JL4577 (gacA::aphI) did not express TSO activity and produced no detectable antibiotics, HCN, or extracellular protease(s). TSO activity and production of pyoluteorin, pyrrolnitrin, 2,4-diacyltetracosanoyl chain, extracellular proteases, and HCN by JL4577 were restored with pJEL5935, which contains the wild-type gacA (Table 3-5). In addition, the wild-type gacA allele that was separated from uvrC (pJEL6167 and pJEL6068) restored the production of secondary metabolites to JL4577, regardless of the orientation of gacA with respect to the lacZ promoter in the vector (Table 3-5). The mutant allele, gacA(V203) (pJEL6164 and pJEL6165), restored HCN production and trace amounts of pyoluteorin, but did not restore 2,4-diacyltetracosanoyl chain and pyrrolnitrin production or protease and TSO activity (Table 3-5). When the wild-type gacA in pJEL6058 and the mutant gacA(V203) in pJEL6057 were introduced into JL4577, the results were quite different from those seen when these constructs were introduced into JL4477, the gacA(V203) mutant. The phenotype of JL4577(pJEL6057) was similar to the phenotype observed when either pJEL6164 or pJEL6165 (both contain the gacA(V203) allele) were introduced into JL4577 (i.e. trace amounts of pyoluteorin were produced and HCN production was restored, but
Figure 3-6. Ice nucleation activity expressed by Pf-5 containing a pltE::inaZ transcriptional fusion. All strains are Pf-5 derivatives that contain the pltE::inaZ fusion. Strains are: JL4365, Pf-5; JL4485, gacA mutant; JL4486, apdA mutant, JL4485 (pJEL5935), gacA restored; JL4486 (pJEL5771), apdA restored. Bars topped with the same letter are not significantly different (LSD; P=.05).
Table 3-5. Phenotypic characterization and complementation of the gacA::aphI mutant of *P. fluorescens* Pf-5.

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<td>JL4577 (pJEL6168)</td>
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Abbreviations: Plt, pyoluteorin; Pn, pyrrolnitrin; Phl, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide; TSO, tryptophan-side-chain oxidase; ++++, greater than wild-type levels; ++, wild-type levels; +, very low levels; -, not detected. Two replicate cultures of each strain were assayed.
2,4-diacetylphloroglucinol and pyrrolnitrin were not produced). However, JL4577(pJEL6057) did not have a detectable protease activity. JL4577 containing the wild-type allele in pJEL6058 produced pyoluteorin, pyrrolnitrin, and HCN, but did not have protease activity (Table 3-5). In addition TSO activity was less than wild type.

3.4 Discussion

This study shows that the global activator GacA, which has been described in other species of *Pseudomonas* (Laville et al., 1992; Gaffney et al., 1994; Rich et al., 1994; Reimmann et al., 1997), is present in *P. fluorescens* Pf-5. GacA is the response regulator in a two-component global regulatory system in *Pseudomonas* spp., in which ApdA (Hrabak and Willis, 1992; Corbell and Loper 1995; Liao et al., 1994) is the cognate sensor kinase. As expected, deletion of gacA abolished biosynthesis of the same array of secondary metabolites as affected by a mutation in apdA (Corbell and Loper, 1995; see Chapter 1). Signal transduction is often mediated by such two-component systems in prokaryotes. According to the two-component paradigm, an environmental signal triggers the ATP-dependent autophosphorylation of the sensor kinase, which then transfers the phosphoryl group to the cognate response regulator. The phosphorylated response regulator then typically acts as a transcriptional activator of target genes (Parkinson and Kofoid, 1992; Stock et al., 1995). Sequence analysis previously placed GacA in the FixJ family of DNA-binding proteins (Kahn and Ditta, 1991; Laville et al., 1992). Members of this family, for which DNA-binding sites have been established, include FixJ (Galinier et al., 1994), BvgA (Karimova et al., 1997; Sibetz, 1998), and NarL (Darwin and Stewart 1995). Analyses of the interactions of these proteins with their target sequences have revealed complex patterns of binding, not only to transcriptional activating sites, but also to repressor sites. Although the binding site(s) for GacA have not been defined, the highly fluorescent phenotype exhibited by *apdA/gacA* mutants suggests that GacA acts as a repressor of the pathway leading to production of the fluorescent siderophore in Pf-5. Alternatively, the lower
fluorescence of the wild type in comparison to the *apdA* and *gacA* mutants may be due to quenching by antibiotics. Nevertheless, GacA, like other members of the FixJ family, may serve the dual role of activator and repressor of transcription in Pf-5.

The amino acid sequence of GacA is highly conserved among biocontrol, plant pathogenic, and human pathogenic species of *Pseudomonas* (for a discussion of sequence similarity of ApdA to other species of *Pseudomonas* see Chapter 1). The most obvious role of GacA appears to be the regulation of secondary metabolites in all of these species. In fluorescent pseudomonads, for example, *gacA* is required for the production of antifungal compounds inhibitory towards phytopathogenic fungi (Laville et al., 1992; Gaffney et al., 1994). In the plant pathogenic bacteria *P. syringae* and *P. viridiflava* *gacA* is required for the production of extracellular metabolites involved in pathogenicity such as protease(s), syringomycin (Rich et al., 1994) and pectate lyase (Liao et al., 1994). In the human pathogen *P. aeruginosa*, mutations in *gacA* reduce the production of virulence factors such as HCN, lipase, and pyocyanin (Reimmann et al., 1997). Although GacA is required for the production of secondary metabolites in *Pseudomonas*, the level at which this protein exerts its control over these products is not known. A functional ApdA/GacA two-component system is required for the transcription of genes for pyoluteorin biosynthesis in Pf-5 (this work) and tabtoxin biosynthesis in the pathogen *P. syringae* pv. *coronafaciens* (Barta et al., 1992). Although the results of such studies are in agreement with the proposed role of GacA as a transcriptional activator, they do not indicate whether GacA directly interacts with the promoter for these biosynthetic genes or indirectly through a regulatory cascade involving intermediate genes. In fact, the region upstream of the gene coding for tabtoxin biosynthesis apparently does not bind GacA, but binds to another protein that requires GacA for its production (Rich et al., 1994).

Based on similarity to the better-characterized response regulators within the FixJ family of response regulators, 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 (Kahn and Ditta, 1991). Amino acid substitutions within these functional domains typically destroy
GacA function, manifested in the loss of multiple phenotypes controlled by the apdA/gacA two-component regulatory system (Black et al., 1996). 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. The fact that the gacA(V203) allele introduced into the gacA::aphI mutant JL4577 in trans resulted in a phenotype similar to that of mutant JLA477, the gacA(V203) mutant strain, further indicates that this mutation is responsible for the differential phenotype that is observed in mutant JLA4477. 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 (Stibitz, 1994). 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 (Chou and Fasman, 1978; Garnier et al., 1978). Stibitz (1994) 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. In addition, deletions of two or three amino acids from the C-terminus of BvgA markedly inhibit growth of B. pertussis. This growth inhibition is thought to be the result of an inappropriate interaction of the mutant BvgA protein with the the α subunit of RNA polymerase since a second site mutation in rpoA, the gene coding for α subunit of RNA polymerase, relieves this growth inhibition. (Stibitz, 1998). Thus, the differential effect of the gacA(V203) mutation on phenotypes regulated by apdA/gacA in Pf-5 is not unprecedented, and may reflect the importance of the α-helical region either as a specificity determinant for recognition of various promoters or as a region that interacts with other proteins of the transcriptional apparatus. 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.

Mutants in the apdA/gacA regulatory system regularly arose and proliferated in late-stationary phase cultures of P. fluorescens. Colonies of apdA/gacA mutants were
easily identified because they are large, orange, and highly fluorescent compared to the wild type (Duffy and Defago, 1995). Although a high proportion (40%) of colonies isolated from 6-day old cultures of Pf-5 exhibited this mutant phenotype, it is unclear whether this is due to a high spontaneous mutation frequency or greater fitness conferred by this phenotype in late stationary phase cultures. Because spontaneous mutations in apdA/gacA frequently have been isolated from aged cultures of other Pseudomonas species, it has been suggested that these loci are particularly susceptible to mutation when grown into late stationary phase. Although this may be the case, it is also just as plausible that the entire genome is susceptible to spontaneous mutations under these conditions, and that apdA/gacA mutants are commonly isolated due to the distinct change in colony morphology conferred by a mutation in these loci. Mutations that are lethal or do not result in obvious phenotypic changes may simply go unnoticed. Whatever the mechanism by which these mutants arise, it is clear that such mutants flourish amidst the antibiotic producing wild-type Pf-5. Because the production of antibiotics is associated with biocontrol activity by P. fluorescens (Thomashow and Weller, 1990), the occurrence of such a high proportion of spontaneous antibiotic mutants in laboratory cultures has the potential to compromise the level of disease control achieved by these bacteria. There are numerous examples of genetic instability in pathogenic bacteria leading to loss of pathogenicity or altered virulence. For example, nonpathogenic mutant colonies commonly occur in stock cultures of Ralstonia solanacearum as a result of spontaneous mutation in a transcriptional regulator of virulence genes in this organism (Brumbly et al., 1993). Relatively few studies have investigated the frequency of spontaneous mutation in biocontrol bacteria, however, and so it is not known how common or how important this phenomenon is for the efficacy of biocontrol.

Complementation studies of gacA mutants with the wild-type gacA (pJEL6167 and pJEL6168) and mutant gacA(V203) (pJEL6164 and pJEL6165) alleles confirmed the hypothesis that the point mutation resulting in the gacA(V203) allele of JL4477 was responsible for the differential loss of certain phenotypes associated with gacA mutants. The most convincing evidence for this hypothesis was that complementation
of the \textit{gacA} null mutation JL4577 with the mutant allele, \textit{gacA(V203)}, resulted in a phenotype similar to the phenotype of the \textit{gacA(V203)} mutant JL4477. Furthermore, the wild-type allele restored production of all secondary metabolites to the \textit{gacA(V203)} mutant JL4477 except for production. The failure of the wild-type \textit{gacA} allele to restore 2,4-diacetylphloroglucinol production to \textit{gacA(V203)} mutant JL4477 was not surprising, however, because production of this metabolite by Pf-5, and especially by Pf-5 derivatives containing genes cloned into the pRK415 vector, has been very mercurial in my experience. It should also be noted that wild-type Pf-5 sometimes fails to produce 2,4-diacetylphloroglucinol.

The overproduction of pyoluteorin by JL4477 strains containing the wild-type (pJEL6058) and mutant alleles (pJEL6057) of \textit{gacA} remains unexplained, but may be due to second site mutations either within the \textit{gacA(V203)} allele or elsewhere in the genome of this strain. The fact that the wild-type allele in pJEL6058 did not confer overproduction of pyoluteorin on the \textit{gacA} null mutant JL4577 supports this hypothesis. Likewise, the fact that the \textit{gacA(V203)} allele in pJEL6057 did not confer overproduction of pyoluteorin on the \textit{gacA} null mutant also supports this hypothesis. However, neither pJEL6058 nor pJEL6057 were as effective at restoring secondary metabolite production to the \textit{gacA} null mutant as the constructs created later that contained the wild-type allele (pJEL6164 and pJEL6165) or the \textit{gacA(V203)} mutant allele. This suggests that constructs pJEL6057 and pJEL6058 are defective as well.

Complementation studies comparing the effects \textit{apdA} and \textit{gacA} in low (pRK415) and high copy (pME6000) vectors revealed two interesting trends. First, the overexpression of either gene apparently inhibits the production of pyrrolnitrin while 2,4-diacetylphloroglucinol is more reliably produced. The lack of pyrrolnitrin production by the \textit{gacA(V203)} mutant of Pf-5 that contains the wild-type allele in a high copy vector is in contrast to the effect that overexpression of \textit{gacA} has in the biocontrol strain BL915. In that strain, \textit{gacA} in a high copy vector led to an increase in pyrrolnitrin production (Ligon et al., 1996). The reason that overexpression of \textit{gacA} in Pf-5 had the opposite effect is unclear. One explanation is that the function of GacA may rely on its ability to form dimers or oligomers. If this is the case, then it
may be that heterodimers or multimers of GacA and GacA(V203) have different properties than heterodimers of GacA. This could also explain the lack of protease activity, the lowered TSO activity, and the lowered production of HCN by the gacA(V203) strain containing gacA in a high copy vector. Also, GacA may function as a repressor of a repressor for 2,4-diacetylphloroglucinol production (discussed more fully in Chapter 4 section 4.4.3). In accordance with its putative role as a repressor, heterodimers of GacA and GacA(V203) may be just as functional as homodimers of GacA if the mutation in GacA(V203) does not interfere with DNA binding.

The relative amounts and pattern of secondary metabolites produced by the apdA mutant strain JL4135 containing apdA in a high copy plasmid relative to the apdA mutant containing apdA in a low copy vector were similar to the pattern observed for complementation of the gacA(V203) mutant with high and low copy vectors containing gacA. Because ApdA may act as both a kinase and a phosphatase, increasing the cellular concentration may lead to either a greater or lesser proportion of GacA that is phosphorylated. If overexpression of ApdA leads to an increased level of phosphorylated GacA, then the observed pattern of secondary metabolite production might be expected to be similar to a strain in which GacA was overexpressed, although the mechanism in the former may be due to a greater proportion of the available GacA being phosphorylated, whereas the latter may be due to the same proportion of GacA being phosphorylated but a higher overall concentration of GacA, as compared to wild-type levels.

The second interesting result of complementation studies of apdA and gacA mutants with high and low copy plasmids containing the apdA and gacA genes was that a high copy number plasmid containing gacA was able to restore the production of a subset of secondary metabolites to the apdA mutant strain. The ability of a high copy number of the response regulator to provide some degree of complementation to a sensor kinase mutant may be a common feature of FixJ-like response regulators since overexpression of another member of this family of response regulators, BvgA, also results in complementation of a mutant in its cognate sensor kinase (Boucher et al., 1994). It must be stressed, however, that these are the results of one replication each
in a screening process and, as such, may not accurately portray the production of secondary metabolites by these strains. A more rigorous evaluation of the quantities of secondary metabolites and an analysis of the relative amounts of ApdA and GacA produced by these strains should help to clarify the role of overexpression of ApdA and GacA in the production of secondary metabolites by Pf-5. Also, it would also be interesting to determine whether or not the same pattern of secondary metabolite production occurs in the gacA null mutant JL4577 harboring high and low copy plasmids that contain gacA.
Chapter 4. GacA Binding Studies

4.1 Introduction

GacA is a member of the FixJ family of response regulators (Laville et al., 1992; Kahn and Ditta, 1991). Some of the better-characterized members of this large family of response regulators are FixJ from *Rhizobium meliloti* (reviewed by Agron and Helinski, 1995), BvgA from *Bordetella pertussis* (reviewed by Uhl and Miller, 1995), and NarL and NarP from *E. coli* (reviewed by Stewart and Rabin, 1995). Target sites that bind these proteins have been identified in the promoters of genes directly regulated by these response regulators (Galinier et al., 1994; Scarlato et al., 1990; Roy and Falkow, 1991; Tyson et al., 1993). In addition, DNA binding sites for the response regulator NtrC, a member of another family of response regulators, have been well defined (Porter et al., 1993). In contrast, a DNA binding site for GacA has not been reported, although the biosynthetic genes for many of the secondary metabolites that it regulates have been identified. For example, the biosynthetic genes for pyoluteorin (Kraus and Loper, 1995; Nowak-Thompson et al., 1997), 2,4-diacetylphloroglucinol (Fenton et al., 1992; Bangera and Thomashow, 1996), pyrrolnitrin (Lam et al., 1995; Hammer et al., 1997), and hydrogen cyanide (Laville et al., 1998) from biological control species of *Pseudomonas* have been identified. Biosynthetic genes for pathogenicity factors that are regulated by GacA have been identified from pathogenic species of *Pseudomonas* (Kinscherf et al., 1991; Zhang et al., 1995). In addition, regulatory genes for the production of quorum sensing compounds that are controlled by GacA have been identified in both biocontrol (Pierson et al., 1996) and pathogenic species of *Pseudomonas* (Passador et al., 1993; Ochsner and Reiser, 1995; Dumenyo et al., 1998). Because of the global regulatory nature of GacA, it is uncertain whether this protein directly interacts with the promoter regions for the above-mentioned biosynthetic genes, for regulatory genes controlling these biosynthetic pathways, or both. Furthermore, since GacA is nearly identical
among the *Pseudomonas* spp. from which it has been reported (Reimmann et al., 1997; see Chapter 2), it is likely that the DNA-binding sites are very similar among these species, possibly regulating genes that are common to all *Pseudomonas* species.

The traditional approach for identifying genes regulated by GacA would be to use transposon mutagenesis with a reporter gene in the transposon. However, because GacA regulates so many genes, it may be near the top of a hierarchical regulatory cascade. Therefore, such an approach would invariably yield numerous biosynthetic genes that have already been identified, possibly without identifying a gene directly controlled by GacA. The mutants identified by such a study might have transposon insertions located several kb from a GacA-regulated promoter. For example, seven open reading frames (ORFs) in the pyoluteorin biosynthetic cluster, representing approximately 20 kb, are thought to be transcribed from only two promoters (Nowak-Thompson et al., 1999). Since GacA regulates pyoluteorin production, a transposon anywhere within this region would show that the promoter was transcriptionally regulated by GacA. Obviously a pyoluteorin mutant could quickly be eliminated from such a screening process since its sequence is known, but many genes regulated by GacA may yet be unknown. In addition, GacA might act as both an activator and a repressor of transcription. If this is the case, then transposon mutagenesis would have to be performed in both the wild-type and mutant backgrounds in order to detect genes activated and/or repressed by GacA.

Historically, the genes regulated by GacA have been identified by first observing a differential phenotype between the wild type and an *apdA* or a *gacA* mutant. This has been a reasonably fruitful endeavor, leading to the discovery of numerous genes (mentioned above) that are controlled by GacA, but nevertheless suffers from the fundamental flaw that a phenotype must first be observed. Many of the phenotypes regulated by *apdA/gacA* are only detectable by using specific media or growth conditions. Thus, genes controlled by GacA may remain unknown simply because no phenotype is readily apparent. Therefore, an alternative approach is needed for identifying genes directly controlled by GacA.
The goal of the present study was to identify genes regulated by GacA through direct interaction of the protein with its target DNA binding sites (i.e. DNA-binding). The approach presented herein for identifying target sequences for GacA binding relies heavily on the cycle selection process used by Ochsner and Vasil (Ochsner and Vasil, 1996) for the identification of genomic sequences of \textit{Pseudomonas aeruginosa} that bind the FUR protein, although several modifications were employed. This cycle selection process is a form of SELEX (for systematic evolution of ligands by exponential enrichment) (Tuerk and Gold, 1990), in which specific target sites for a DNA-binding protein are selected and amplified from a pool of DNA fragments. The original SELEX procedure used a pool of random DNA fragments created from synthetic oligonucleotides, and as such may identify sites that bind to the protein but are not biologically relevant. The use of genomic DNA has the potential advantage of identifying actual DNA target sites for a DNA-binding protein and the associated genes that it regulates. Since DNA binding by GacA has not been demonstrated, this study drew on the information gained by studies of similar response regulators, under the hypothesis that the conditions that were required for DNA binding of similar proteins would also provide the necessary conditions for GacA binding.

Here, I report the development of a system for selecting putative genomic target sites for GacA, the identification of a putative binding site for GacA in the genome of \textit{P. fluorescens} Pf-5, and the isolation of putative GacA binding sites from a random pool of synthetic DNA fragments. Although many of the results are as yet inconclusive, the methodology provides a framework for future studies evaluating the interactions between GacA and its DNA target sites.
4.2 Materials and methods.

4.2.1 Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are described in Table 4-1. *Escherichia coli* strains were cultured in Luria Bertani (LB) broth (Sambrook et al., 1989) or on LB agar plates incubated at 37°C. *Pseudomonas* strains were routinely cultured at 27°C in King's medium B (KMB) (King et al., 1954) broth or on KMB agar plates. Antibiotic concentrations were as follows unless otherwise specified: 100 μg/ml ampicillin, 20 μg/ml tetracycline, 34 μg/ml chloramphenicol, 50 μg/ml kanamycin.

4.2.2 Recombinant DNA techniques.

Transformation of *Escherichia coli* strains, restriction digests, ligations, electrophoresis in Tris-phosphate-EDTA, and plasmid DNA isolation were performed by standard methods (Sambrook et al., 1989). Restriction endonucleases, T4 DNA ligase, and Elongase Enzyme Mix were from Gibco BRL Life Technologies (Gaithersburg, MD). *Taq* polymerase and glutathione sepharose 4-B were from Promega (Madison, WI). *Vent* polymerase was from New England Biolabs (Beverly, MA). Nonradioactive PCR mixtures (50 μl) contained template DNA (1 μl), 5X Elongase Buffer B (10 μl), 400 μM dNTPs, 2 units of Elongase Enzyme Mix, and 2 μM of each primer unless otherwise specified. For radiolabeling probes, the PCR was performed in an Amplitron thermacycler and the PCR mixtures (50 μl) contained 10X Taq buffer (5 μl), 1.5 mM MgCl₂, 400 μM 3dNTPs (dATP, dGTP, dTTP), 2 μM dCTP, 5 μl of [α-³²P] dCTP (NEN, Boston, MA), 2 units of *Taq* DNA polymerase (Promega), and 2 μM of each primer unless specified otherwise. Radiolabeled probes were purified away from unincorporated nucleotides using a Bio-Spin P-6 column.
Table 4-1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Relevant Characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source or Reference</th>
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<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
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<tr>
<td>Pf-5</td>
<td>Field isolate</td>
<td>Gac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Howell and Stipanovic, 1979</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>JLA4577</td>
<td>Derivative of Pf-5, ΔgacA</td>
<td>Gac&lt;sup&gt;−&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
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<tr>
<td><strong>E. coli DH5</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; endA1 hsdR17(ryk&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt; r) supE44 thi-1 recA1 gyrA96 relA1 80dlacZ M15-</td>
<td></td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td><strong>E. coli S17-1</strong></td>
<td>Tp&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; recA thi pro hsdR&lt;sup&gt;+&lt;/sup&gt; RP4:2-Tc:Mu:Km Tn7</td>
<td></td>
<td>Simon et al., 1983</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>B F&lt;sup&gt;−&lt;/sup&gt; dcm ompT hsdS&lt;sup&gt;(rB&lt;sup&gt;−&lt;/sup&gt; mB&lt;sup&gt;−&lt;/sup&gt;)&lt;/sup&gt; gal (DE3) [pLysS CmR]</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
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<tr>
<td>BL21(DE3) pLysS</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC19</td>
<td>ColE1 replicon</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Sambrook</td>
</tr>
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<td>pGEX-2T</td>
<td>Glutathione S-transferase gene fusion vector</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Pharmacia</td>
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<td>pME6000</td>
<td>pBBR1 replicon</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>D. Haas</td>
</tr>
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<td>pPROBE-TI</td>
<td>pBBR1 replicon, promoter probe vector containing a promoterless inaZ</td>
<td>Mob&lt;sup&gt;−&lt;/sup&gt; Tc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>B. Miller</td>
</tr>
<tr>
<td>pPROBE-TI'</td>
<td>pBBR1 replicon, promoter probe vector containing a promoterless inaZ</td>
<td>Mob&lt;sup&gt;−&lt;/sup&gt; Tc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>B. Miller</td>
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<tr>
<td>Construction</td>
<td>Description</td>
<td>Notes</td>
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<td>pMP220</td>
<td>IncP1 replicon, Promoter probe vector containing a promoterless lacZ</td>
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<td>Spaink et al., 1987</td>
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<td>pJEL5937</td>
<td>1.65-kb BamHI-BgII fragment of Pf-5 containing gacA cloned into pUC19</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Whistler et al., 1998</td>
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<td>pJEL6169</td>
<td>642-bp BamHI-EcoRI fragment containing gacA ORF cloned into pUC19</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6170</td>
<td>228-bp BamHI-EcoRI fragment containing the gacA region coding for the helix-turn-helix DNA binding motif cloned into pUC19</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6171</td>
<td>642-bp BamHI-EcoRI fragment containing gacA ORF cloned into pGEX-2T</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6172</td>
<td>228-bp BamHI-EcoRI fragment containing the gacA region coding for the helix-turn-helix DNA binding motif cloned into pGEX-2T</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6173</td>
<td>58-bp blunted fragment containing a putative GacA binding site isolated from the dsR26 library cloned into the HincII site of pUC19</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6174</td>
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<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6175</td>
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<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6176</td>
<td>58-bp blunted fragment containing a putative GacA binding site isolated from the dsR26 library cloned into the <em>Hinc</em> II site of pUC19</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6177</td>
<td>58-bp blunted fragment containing a putative GacA binding site isolated from the dsR26 library cloned into the <em>Hinc</em> II site of pUC19</td>
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<td>This study</td>
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<td>This study</td>
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<td>pJEL6179</td>
<td>58-bp blunted fragment containing a putative GacA binding site isolated from the dsR26 library cloned into the <em>Sma</em>I site of pUC19</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6180</td>
<td>58-bp blunted fragment containing a putative GacA binding site isolated from the dsR26 library cloned into the <em>Sma</em>I site of pUC19</td>
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<td>pJEL6181</td>
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<td>This study</td>
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<td>Plasmid</td>
<td>Description</td>
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<td>pJEL6185</td>
<td>85-bp blunted fragment containing a putative Pf-5 genomic DNA binding site for GacA cloned into the HincII site of pUC19</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td>pJEL6186</td>
<td>223-bp blunted fragment containing a putative Pf-5 genomic DNA binding site for GacA cloned into the HincII site of pUC19</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6187</td>
<td>663-bp BamHI-EcoRI fragment containing a putative Pf-5 genomic DNA binding site for GacA cloned into pPROBE-TI. Orientation of the putative promoter is opposite of the promotersless inaZ</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pJEL6188</td>
<td>663-bp BamHI-EcoRI fragment containing a putative Pf-5 genomic DNA binding site for GacA cloned into pPROBE-TI'. Orientation of the putative promoter is in the same direction the promotersless inaZ</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJEL6189</td>
<td>1.3-kb KpnI-EcoRI fragment generated by PCR containing the gst::gacA fusion from pJEL6171 cloned into pME6000</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6190</td>
<td>900-bp KpnI-EcoRI fragment containing the gst::gacAC fusion from pJEL6172 cloned into pME6000</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL6191</td>
<td>1.3-kb KpnI-EcoRI fragment generated by PCR containing a point mutation in the gst::gacA fusion from pJEL6171 cloned into pME6000</td>
<td>Mob&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ap<sup>r</sup>, Cm<sup>r</sup>, Km<sup>r</sup>, and Te<sup>r</sup> indicate resistance to ampicillin, chloramphenicol, kanamycin, and tetracycline, respectively.
(BioRad, Hercules California) in which the SSC buffer had been exchanged previously for 10 mM Tris EDTA [pH 7.5] unless otherwise specified.

pPROBE-TI, pPROBE-TI', pMP220, pME6000, and derivatives were mobilized from *E. coli* S17-1 in biparental matings. Due to the high degree of natural tetracycline resistance exhibited by *P. fluorescens* Pf-5, transconjugants were selected on KMB containing 200 µg/ml tetracycline and 100 µg/ml ampicillin.

4.2.3 **Construction of GST translational fusions.**

**GST-GacAC.** The C-terminal portions of FixJ and BvgA, which contain the helix-turn-helix DNA binding motifs for these proteins, retain binding activity when severed from the N-terminal phosphorylation domain. An alignment of the amino acid sequences of these truncated proteins with the deduced amino acid sequence of GacA (Figure 4-1) was used to design DNA primers for amplification of the region of gacA that encoded the corresponding C-terminal portion of GacA. The 228-bp fragment, containing the gacA sequence coding for the C-terminal helix-turn-helix DNA-binding motif, was amplified from pJEL5937 by the PCR with 35 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), and extending (1 min at 68°C) on a Stratagene RoboCycler Gradient 960 thermacylcer (Stratagene) using the oligonucleotides shown below.

5'-GTGGATCCAGCCTTCCAGTGATTCACCGTFCG-3'
(nucleotides 729-753 of gacA; see Chapter 3 Figure 3-2)
5-CCGGAATTCTTCAGAGGCTGGCATCAACCATGC-3
(complementary to nucleotides 934-957 of gacA; see Chapter 3 Figure 3-2)

The BamHI and EcoRI sites (underlined) in the oligonucleotides were used to clone the 228-bp fragment into the BamHI-EcoRI site in pUC19 to create pJEL6170. DNA sequencing was used to confirm that the 228-bp sequence was identical to the last 228
| A) | 1 | 50 |
| GacA | --LIRLVLVD | HHLVRLGTVT RMLADIDGLOQ VVGQAESGEE SLLLKARELP |
| BvgA | --MYNKLIIID | DHPVLRLFAV RVLMEKEGFGE VIGETDNGID GLKIAKELKP |
| FixJ | MTDYTVHIVD | DEEVPVRKSLA FMLT.MMGF AVKMQQAEDA FLAFAPDVRN |
| Consensus | ------LIVD | E--VR--- RML---GF-- VVG---SGE-- L--ARE--P |
| 51 | 100 |
| GacA | DVVLNMVRMP GIGGLEATRK LLRSHPDIKV VATYCEDEDP FPTRLLQAGA |
| BvgA | NLVKLHTGIP KDGLQVLRA LQSLGQLLRV LVLIRQGPGSL FARCRNLNGA |
| FixJ | GVLVTLRMP DMGVELLRN LGDKINPIS IVITGHGDPV MAVEAMKAGA |
| Consensus | --VVV-MP | --GLE-R-- L--L--I-V --V-TG--P FA-R-L-AGA |
| 101 | 150 |
| GacA | AGYLTIAGAGL NEMVQAIRLW FAGQRYISPQ IAQQQLFKSF QPSSSLFPDA |
| BvgA | AGFVDKHENL HEVINAAPA KAGYTYFFST TLSMAMGDKN AKSDTLISV |
| FixJ | VDFIKQFPEF TVIIAEIARA ..........SEH LVAASEADVDD ANDIRARKQT |
| Consensus | AGF--V--L --E-I-AI-- V--G--Y-S -- -- -- -- -- -- -- |
| 151 | 200 |
| GacA | LSEREIQIAL MIVGGQKVQI ISDKLCLSPK TVNTYQRIFIP EKLSISSDVE |
| BvgA | LSNRLETVQI LQAOGQNKD IDASMFLNQ TVSVYFRQRL KQMNLISLVE |
| FixJ | LSERERQVLIS AVQAPNLNKS IAYDLDISPR TVPVRANKVM AMMVAKSLPH |
| Consensus | LSERE-QVL-- --V--O--NK--IAD-L-LPK TV--TIR--R-- KL-A-SLVE |
| 201 | 250 |
| GacA | LTLAVRHGM VDASL |
| BvgA | LLIDKARNNL A----- |
| FixJ | LVRMAGGDF GPS-- |
| Consensus | L--LA-R-G-- --- |

| B) | 121 | 171 |
| GacAC | ---------------------QP SSDSPFDALS EREIQIALMI VGOQKVQIIS |
| BvgAC | GYTFQPSSTL SEMAGNDAK SDSLTVQSL AQMNKNDIA |
| FixJC | ---------------------MTDAN DIRARQQLS ERERQVLINAV VAGFLNSIA |
| Consensus | ------------------------A S---LS ERE-QVL-- V-G--NK--IA |
| 172 | 215 |
| GacAC | DKLCLSPRTV NTYTRIFEK LSISSDVELT LLLAVRHGMV DLALVLDSL |
| BvgAC | DSMPLEKRVT SYTKRLQQK LNATSLVLEI DLAKRNNLA-- ---- |
| FixJC | YDLDISPRTV EHVHMRVMAK KAKSLPHLV RMLAGGFSP S-- |
| Consensus | D-L-LSPRTV --TYR-R--K L-A-SLVEL-- LA-R-G-- --- |

Figure 4-1. Amino acid sequence alignments of a) GacA, BvgA, and FixJ and b) their respective N-terminal deletions designated GacAC, BvgAC, and FixJC. Overhead numbers refer to the consensus sequence for the full-length proteins. Highly conserved residues in response regulator receiver domains are circled. The conserved helix-turn-helix DNA-binding motif is boxed. b) The entire FixJC protein is shown. The first amino acid shown for BvgAC is fused to the maltose-binding protein (designated MBP-dN BvgA by Bouche et al., 1994). The first amino acid of GacAC is fused to glutathione S-transferase (designated as GST-GacAC in the text).
bp of gacA. The 228-bp fragment of pJEL6170 was cloned into the BamHI-EcoRI site in pGEX-2T to create pJEL6172. Expression of the resulting construct yields a chimeric protein consisting of the GST protein fused to the C-terminal 75 a.a. of GacA (GST-GacAC; Figure 4-2).

**GST-GacA.** The gacA coding sequence was amplified from pJEL5937 by the PCR using the parameters described above for the GST-GacAC construct, and the oligonucleotides shown below.

5-GTGGATCCTTGATAAGGGTGCTAGTAGTCGATG-3
(nucleotides 315-339 of gacA; see Chapter 3 Figure 3-2)

5-CCGGAATTCTTCAGAGGCTGGCATCAACCATGC-3
(complementary to nucleotides 934-957 of gacA; see Chapter 3 Figure 3-2).

Amplification was followed by a final extension of 5 min at 68°C. The 5' terminal ends of the oligonucleotides contained BamHI and EcoRI sites (underlined), respectively. The 642-bp BamHI-EcoRI fragment containing the gacA open reading frame was cloned into the BamHI-EcoRI site of pUC19 to create pJEL6169. DNA sequencing was used to confirm that the gacA ORF in pJEL6169 was identical to wild-type gacA. The 642-bp BamHI-EcoRI fragment from pJEL6169 was cloned into the BamHI-EcoRI site in pGEX-2T to create pJEL6171. Expression of the resulting construct yields a chimeric protein consisting of the glutathione S-transferase protein (GST) fused to the N-terminus of GacA (GST-GacA; Figure 4-2).

**4.2.4 Expression and purification of GST-GacA and GST-GacAC.**

GST and GST fusion proteins were expressed in E. coli BL21 (DE3) pLysS cells (Stratagene, La Jolla, CA) transformed with pGEX-2T (GST), pJEL6171 (GST-
Figure 4-2. Schematic representation of *gst* gene fusions. Constructs are drawn to scale. *gst* (dark grey box) is the glutathione S-transferase gene in pGEX-2T. *gacA* (open box) indicates the *gacA* gene of Pf-5 and the light grey region within the gene represents the location coding for helix-turn-helix DNA-binding motif. Arrows indicate primers. Sequences of primers are given in section 3.2.3. Letters at the end of primers and above the constructs indicate the following restriction sites: B, *BamHI* and E, *EcoRI*. The plasmid bearing each construct is given to the right. The corresponding fusion protein is given in parentheses.
Cultures of these strains were grown overnight in LB amended with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Overnight cultures were diluted ten-fold into fresh media and incubated until the cultures reached mid-exponential growth (O.D. 600nm of 0.6-0.8). Then, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce production of the fusion proteins. Incubation was continued from 1 to 3 h. Cells were harvested by centrifugation in a Beckman Model J2-21 centrifuge at 4°C for 10 minutes at 7000 rpm in a JA-14 rotor. Cells were washed once with 1 ml of ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4 [pH 7.6]) per 50 ml of original culture, centrifuged at 4°C for 10 minutes at 7000 rpm in a Beckman JA-17 rotor to pellet the cells, and frozen at -20°C. Frozen cells were lysed by resuspension in one volume of ice-cold PBS amended with 1% Triton X-100, 10 mM MgCl2, and 1 mM DNase (Sigma, St. Louis, MO). Protease inhibitor cocktail for bacterial cell extracts (Sigma cat. P8465) was added at a rate of 100 µl of inhibitor per 100 ml of original culture. The cell suspension was mixed by pipetting until the suspension lost viscosity (i.e. DNA released from the lysed cells was cleaved by the DNase) and then incubated another 20 minutes on ice. Intact cells and cellular debris were removed by centrifugation at 4°C for 10 minutes at 10,500 rpm in a Beckman JA-17 rotor. When necessary, the pH of the cleared lysate was adjusted to pH 7.0-7.5, by the addition of 1 N NaOH. Glutathione sepharose 4B (Pharmacia), equilibrated in cold PBS, was added to the cleared lysate at a rate of 1 µl of 50% glutathione sepharose slurry per 1 ml of original culture and the mixture was incubated for 20-30 min with gentle agitation at 4°C. Proteins bound to glutathione sepharose beads were sedimented by centrifugation at 2000 rpm for 10 min at 4°C in a JA-17 rotor. Sedimented glutathione sepharose beads were washed with 10 ml of cold PBS amended with 1% Triton X-100 and centrifuged at 2000 rpm for 2 min at 4°C. The wash was repeated a total of three times. Following the last wash, the glutathione sepharose was transferred to microcentrifuge tubes and centrifuged at 14,000 rpm for 10 sec at 4°C, in an Eppendorf Model 5415C microcentrifuge. The supernatant was carefully removed with a pipette. Fusion proteins were eluted from the glutathione
sepharose by adding 1 ml of protein elution buffer [10 mM reduced glutathione (Pharmacia), 50 mM Tris-HCl, pH-adjusted after addition of glutathione to pH 8] per 500 ml of original culture and incubating at room temperature for 10-20 min. The eluted proteins were separated from the beads by centrifugation at 14,000 rpm for 30 sec in a microcentrifuge. Additional elutions were performed in the same manner and the yield of GST fusion proteins in the fractions was estimated by measuring the absorbance at 280 nm (1 A_{280} = 0.5 mg/ml) in a Beckman DU-70 Spectrophotometer. Fractions containing protein were pooled and either stored at -20°C in 50% glycerol or frozen in liquid nitrogen and stored at -80°C in 10% glycerol. Protein purity was assessed by SDS-PAGE. Lysates were mixed with 2X Laemmli sample buffer (Sambrook et al., 1989), boiled for 5 min and separated on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Proteins were stained with Coomassie blue or transferred to reinforced nitrocellulose membranes for western blot analysis (Sambrook et al., 1989). Immunoblotting was performed using polyclonal anti-GST antiserum (raised in New Zealand White rabbits), which was generously supplied by Walt Ream. The secondary antibody, an anti-rabbit IgG conjugated to horseradish peroxidase, was detected by chemiluminescence with an ECL kit according to the protocol supplied by the manufacturer (Amersham Life Sciences).

4.2.5 DNA probes for binding assays.

4.2.5.1 DNA libraries.

Random 26-nucleotide DNA library. The random 26 nucleotide DNA library was generated as described previously (Ausubel et al., 1994). The oligonucleotide R26 is 58 nucleotides in length, contains a core sequence of 26 random nucleotides, and is flanked by two 16-nucleotide sequences for primer binding (Figure 4-3). A library of radiolabeled 58-bp fragments was created by annealing primer FOR to oligonucleotide R26 and synthesizing the second strand using T4 DNA
Figure 4-3. Nucleotide sequences of the oligonucleotide R26 and FHA and the complementary primers used in constructing the dsR26 fragment library and for subsequent PCR amplification of cycle selected fragments. Names of the oligonucleotides are given above each sequence. Underlined sequences indicate BamHI (left) and XbaI (right) sites. The target DNA-binding site for BvgA is boxed on the FHA oligonucleotide. FHA refers to a site upstream of the fha gene of Bordetella pertussis which is a strong BvgA binding site (Roy and Falkow, 1991).
polymerase according to the manufacturer's protocol. For annealing, primer FOR (80 ng) and oligonucleotide R26 (190 ng) were heated to 80°C in 15 µl of 1X T4 DNA polymerase buffer and gradually cooled to 25°C. The synthesis reaction (30 µl) contained the primer template duplex (15 µl), additional 5X T4 DNA polymerase buffer (3 µl), 67 µM 3dNTP (dATP, dGTP, and dTTP), 3 µM dCTP, 5 µl of [α-32P] dCTP, and 10 units of T4 DNA polymerase. The reaction was incubated 1 h at 15°C. Specific activity of the probe was determined using a scintillation counter. As a control, an oligonucleotide containing the DNA binding site of BvgA, a protein in the same family of response regulators as GacA, was prepared using the same protocol (Figure 4-3)

**Genomic DNA fragment library.** Preparation of a genomic library of Pf-5 fragments was prepared essentially as described by Ochsner and Vasil (1996) with the exception that genomic DNA of Pf-5 was completely digested with HpaII instead of the partial digestion that was used by Ochsner. Genomic DNA of P. fluorescens Pf-5 was isolated by standard methods (Sambrook et al., 1989), digested with HpaII, phenol/chloroform extracted, and ethanol precipitated. DNA linkers were made by annealing the complementary oligonucleotides shown below.

L1: 5’-CGGAGTGACTGTTGACCTCGTCTAGAGCA-3’
L2: 5’-TGCTCTAGACGAGGTCAACAGTCACTC-3’

The L1 oligonucleotide was chemically phosphorylated at the 5’ end (DNAexpress, Colorado State University, Fort Collins, CO). When annealed, the linkers contain an internal XbaI site (underlined) and a protruding HpaII site. Linkers (1 µg) were ligated to the HpaII-digested chromosomal DNA (4 µg) using T4 DNA ligase. This pool of chromosomal DNA was amplified and radiolabeled by the PCR, with 30 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), and extending (1 min at 72°C). The PCR mixture (50 µl) contained approximately 10 ng of template DNA,
10X Taq buffer (Promega) (5 µl), 1.5 mM MgCl₂, 400 µM 3dNTPs (dATP, dGTP, dTTP), 2 µM dCTP, 5 µl of [α-3²P] dCTP, 4 µM of linker specific primer (L3: 5'-TGCTCTAGACGAGGTCAAGAGTCACTCC-3'), and 2 units of Taq DNA polymerase (Promega).

**Modified construction of the genomic library.** A second genomic library of Pf-5 fragments was prepared, as described above, with the following modifications. After ligation of genomic DNA fragments to the linkers, the pool of fragments was amplified by a nonradioactive PCR using the same cycling parameters previously mentioned. The amplified pool of fragments was phenol/chloroform extracted and ethanol precipitated. Then, the pool of fragments was digested with XbaI (XbaI sites are contained within the linkers). After digestion with XbaI, fragments less than 500 bp in length were purified on a 5% non-denaturing acrylamide gel (0.5X TBE). The smear of bands was sectioned into the three following size categories: 500 to 300-bp fragments (Large), 300 to 100-bp fragments (Medium) and fragments less than 100-bp (Small). Fragments were electroeluted using Little Blue Tank electroelution apparatus (Isco, Lincoln, NE) according to the manufacturer's instructions. Fragments were labeled with [α-3²P] dCTP at the XbaI sites by using the fill-in reaction of T4 DNA polymerase.

**4.2.5.2 Specific DNA probes.**

*gacA.* The 300-bp BamHI-SpeI fragment containing the region upstream from the gacA ORF was amplified from pJEL5937 by the PCR using the DEL-F and GAC-F primers (see Chapter 3). Following digestion with BamHI and SpeI, the fragment was gel isolated in a 2% agarose gel. The fragment was labeled with [α-3²P] dCTP using the fill-in reaction of T4 DNA polymerase.

*pltR-pltA intergenic region.* Binding of GST-GacAC to the pltR-pltA intergenic region was assessed because transcription of the pyoluteorin genes requires
GacA (see Chapter 2). Overlapping DNA fragments (approximately 200 bp each) spanning the intergenic region between pltR and pltA were labeled with \([\alpha-^{32}\text{P}]\) dCTP and amplified by the PCR using the primers listed in Figure 4-4. Fragments were purified on 5% polyacrylamide (0.5X TBE) non-denaturing gels. DNA was eluted from the gel by soaking the gel slice containing the fragment in 250 µl of elution buffer [0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS] overnight at 37°C. DNA was precipitated at -20°C by adding 1-2 µl of glycogen and 1.25 ml of ethanol.

**dsFHA.** The BvgA binding site (sequence shown in Figure 4-3) in the upstream region of fha was used as a test binding site for GacA. The rationale for using this sequence was that GacA might have a low affinity for the target site of another protein (BvgA) in the FixJ family of response regulators. If so, then the GacA-dsFHA complexes might be used in electrophoretic mobility shift analysis (EMSA) to locate the approximate region in a gel which corresponded protein-DNA complexes composed of GacA and DNA sequences from the synthetic DNA library (Figure 4-3). The dsFHA fragment was labeled as described for the random 26-nucleotide DNA library.

**Putative GacA binding sites identified from the R26 library and the Pf-5 genomic library.** Putative GacA binding sites from the double-stranded R26 (dsR26) library and the Pf-5 genomic DNA libraries were labeled with \([\alpha-^{32}\text{P}]\) dCTP and amplified, from plasmids containing these sequences using the PCR. Primers FOR and REV (Figure 4-3) were used for amplification of dsR26 fragments. The L3 primer (shown on p.84) was used for the genomic fragments. DNA fragments were gel purified as described above (under pltR-pltA intergenic region).
Figure 4-4. Schematic representation of the intergenic region between *pltR* (left) and *pltA* (right) showing the overlapping fragments used in EMSA analysis. *pltR* and *pltA* are indicated by the hatched boxes. Arrows above genes indicate direction of transcription. Intergenic region is indicated by the labeled open box. Primers are indicated by arrows and the name of each is given above the arrow. PCR products are shown immediately below primers. Each PCR product was approximately 200 bp. Primer sequences are also listed.
### 4.2.6 Determining Binding Conditions for GST-GacAC.

**Buffers.** DNA binding by GST-GacAC was assessed in three DNA binding buffers (Table 4-2). These buffers were based on those used in DNA binding experiments with response regulatory proteins similar to GacA (Roy and Falkow, 1991; Ames and Nikaido, 1985; Galinier et al., 1994).

<table>
<thead>
<tr>
<th>Buffer A</th>
<th>Buffer C</th>
<th>Buffer J</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris HCl pH 7.5</td>
<td>50 mM Tris HCl pH 7.4</td>
<td>40 mM Tris acetate pH 8</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td>50 mM KCl</td>
<td>50 mM KCl</td>
</tr>
<tr>
<td>30 mM potassium acetate</td>
<td>10 mM MgCl₂</td>
<td>8 mM MgCl₂</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>.1 mM EDTA</td>
<td>27 mM ammonium acetate</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>1 mM DTT</td>
<td>1 mM DTT</td>
</tr>
<tr>
<td>15% glycerol</td>
<td>10% glycerol</td>
<td>10% glycerol</td>
</tr>
<tr>
<td>100 µg/ml BSA</td>
<td>100 µg/ml BSA</td>
<td>100 µg/ml BSA</td>
</tr>
<tr>
<td>50 µg/ml poly dI-dC</td>
<td>50 µg/ml poly dI-dC</td>
<td>50 µg/ml poly dI-dC</td>
</tr>
</tbody>
</table>

Buffer A = BvgA binding buffer (Roy and Falkow, 1991)
Buffer C = NtrC binding buffer (Ames and Nikaido, 1985)
Buffer J = FixJ binding buffer (Galinier et al., 1994)

**DNA pull-down assays.** Binding of GST-GacAC to the dsR26 library was assessed in DNA pull-down assays. GST-GacAC was purified as described above but was not eluted from the Glutathione Sepharose 4B. Approximately 50 µl of packed protein-glutathione sepharose bead complexes were put into a microcentrifuge tube.
To exchange the PBS buffer used in the purification process for the DNA binding buffer, the beads were washed with 1 ml of 1X binding buffer and pulse centrifuged to pellet the beads. The wash was repeated once. It was previously determined that the proteins remained bound to the glutathione sepharose beads when buffers were exchanged. The glutathione sepharose bound proteins were resuspended in 200 μl of 1X binding buffer and combined with radiolabeled R26 DNA (circa 10^6 cpm). The suspension was incubated with gentle agitation for 30 min at 4°C. Protein bead complexes were pelleted by pulse centrifugation in a microcentrifuge. The supernatant was removed and saved for scintillation counting. The pellet was washed quickly with 500 μl of 1X binding buffer. The wash was repeated one to three times. To isolate bound DNA, the beads were incubated in 350 μl of protein elution buffer for 30 min at room temperature and then heated to 94°C for 15 min. DNA was precipitated with four volumes of ethanol, 1-2 μl glycogen, and 0.25 volumes of ammonium acetate at -20°C overnight. DNA was pelleted by centrifugation at 13,000 rpm in a microcentrifuge and resuspended in 10 μl of water. The purified DNA was amplified and labeled by the PCR using primers FOR and REV (Figure 4-3) with 30 cycles of denaturing (1 min at 94°C), annealing (1 min at 45°C), and extending (1 min at 72°C). Scintillation counting was used to determine the specific activity of the probe. The probe was mixed with glutathione sepharose bound protein and the cycle repeated three times. Binding assays were conducted in all three buffers (Table 4-2) in parallel. The amount of protein-bound DNA, as measured by scintillation counting, was assessed at each step of the binding assay.

**Electrophoretic Mobility Shift Assays (EMSAs).** EMSAs were performed as previously described (Ausubel et al., 1994). Protein concentrations of GST, GST-GacAC, or GST-GacA varied from 0 to 7 μg per 20 μl reaction. Salt concentration was varied from 50 to 400 mM KCl by adding salt from a stock of 2 M KCl. DNA probes labeled with [α^{32}P] dCTP ranged from 100,000 cpm to 800,000 cpm per 20 μl reaction. Each 20 μl reaction consisted of buffer, protein, labeled probe, and 1 μl Bromphenol Blue as a tracking dye. Reactions were gently mixed and incubated at
with gentle agitation for 30 to 40 min. The samples were electrophoresed on a 5% polyacrylamide (0.5X TBE) non-denaturing gel at a constant current of 25 mA. After electrophoresis, gels were transferred to Whatman 3MM paper for support and dried under vacuum at 85°C. The dried gel was exposed to X-ray film for 24 h to 2 weeks.

4.2.7 Cycle selection procedures for identifying GacA binding sites.

Cycle selection procedure for the dsR26 DNA library using EMSAs. EMSAs were carried out as described above with the random library of 26-bp sequences. Autoradiographs of the gel were used as templates for locating the shifted bands within the dried gel. Templates were made by cutting the bands out of the film. Templates then were placed over dried gels and the positions of bands on the gel were marked with a pencil. Bands were excised from the gel with a clean razor blade. The dried gel was exposed to X-ray film a second time to ensure that the band of interest had been removed from the gel. DNA was eluted from the gel by soaking the excised gel slice in 250-400 µl of water for 10 min, boiling 15 min, and precipitating the DNA at -20°C for 30 min or longer with 4 volumes of ethanol, 1-2 µl glycogen, and 0.25 volumes of ammonium acetate. DNA was pelleted by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. DNA was resuspended in 20 µl of sterile water and stored at -20°C until the next cycle of selection.

For subsequent rounds of selection, DNA fragments isolated from the previous EMSA were labeled and amplified by the PCR using primers FOR and REV (Figure 4-3). DNA fragments were amplified with 30 cycles of denaturing (1 min at 94°C), annealing (1 min at 45°C), and extending (1 min at 72°C). Specific activity of the probe was determined using a Scintillation counter. The cycle selection procedure was repeated a total of three times.

Cycle selection procedure for the Pf-5 genomic DNA library. The cycle selection procedure for the Pf-5 genomic DNA library of HpaII-fragments was
performed as described for the dsR26 library with the following exceptions. The concentration of the linker specific primer (L3 shown on p.84) was 4 μM and the annealing temperature was 55°C in the PCR.

4.2.8 Cloning and sequence analysis of putative GacA DNA binding sites.

Cloning of cycle selected DNA fragments. Following the last round of selection, DNA fragments from the R26 library and genomic library were amplified by the PCR as described above but in the absence of [α-32P] dNTPs. Vent polymerase was used in these reactions to create blunted DNA fragments. DNA fragments were purified by phenol/chloroform extraction followed by ethanol precipitation (4 volumes of ethanol, 1-2 μl glycogen, and 0.25 volumes of ammonium acetate). The DNA fragments were cloned into either the SmaI or HincII site of pUC19 and screened for loss of β-galactosidase activity conferred by lacZ in the vector. Cloned fragments were confirmed by the PCR using the pUC19 forward (5'-CCCAGTCACGACGTTGTAAAACG-3') and pUC19 reverse primers (5'-AGCGGATAACAATTTCACACAGG-3').

DNA sequencing and sequence analyses. DNA sequencing was done at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, OR. Sequencing of double-stranded templates was done on an ABI model 373A or 377 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 either by the Center for Gene Research and Biotechnology at Oregon State University, Corvallis, Oregon, or DNAexpress at Colorado State University, Fort Collins, Colorado. DNA and deduced protein sequence analyses, and comparisons with sequences in the GenBank database were accomplished with software from the Genetics Computer Group (GCG), Inc., Madison, WI (Devereux et al. 1984) or the basic local alignment
tool (Blast; (Karlin et al., 1993; Altschul et al., 1997)) via the National Center for Biotechnology Information (NCBI at www.ncbi.nlm.nih.gov). GCG version 9 programs fasta, pileup, and pretty were used for making multiple alignments of DNA fragments. The blastX program was used for making comparisons to the deduced amino acid sequences of genes in the non-redundant GenBank database and the blastn program was used for making comparisons to the unfinished microbial genome of *P. aeruginosa* (*Pseudomonas Genome Project* [PGP] is available via NCBI or www.pseudomonas.com).

**Obtaining sequences flanking putative genomic GacA binding sites.**
Inverse PCR (Ochman et al., 1989) was used to obtain sequences flanking the putative genomic GacA binding sites that were identified above. Southern analysis was used to identify useful restriction sites for the inverse PCR. Southern blots of plasmid and genomic DNA were prepared with nylon membranes (Nytran; Schleicher and Schuell, Keene, NH) according to the supplier's directions. DNA probes were the 85-bp *EcoRI-HindIII* fragment of pJEL6185 and the 223-bp *EcoRI-HindIII* fragment of pJEL6186, both of which contain putative binding sites for GacA. Biotinylated probes for each fragment were prepared using biotinylated dATP with a nick translation kit (Gibco BRL Life Technologies, Gaithersburg, MD), and purified with a D50 column (International Plasmids Biotechnologies Inc., New Haven, CT). Detection was by chemiluminescence using an ECL kit (Amersham Life Science, Arlington Heights, IL). A radiolabeled probe comprised of the 85-bp fragment of was made by the PCR (reaction conditions described in section 4.2.5) using pJEL6185 as the template and the pUC19 forward and pUC19 reverse primers. This probe was purified with a Bio-Spin P-6 column.

Genomic DNA of Pf-5 was prepared for inverse PCR by digestion with *BclII*, phenol/chloroform extraction, ethanol precipitation, and religation using T4 DNA ligase. The concentration of genomic DNA in the ligation (15 μl) was adjusted to 10 ng/μl to favor the formation of monomeric circles over concatamerization (Ochman et al., 1989). The sequences flanking the putative binding site were amplified from 1 μl
of the ligation mixture by the inverse PCR with 35 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), and extending (5 min at 68°C). The primers used in this PCR and the corresponding plasmids from which they were designed are given below. EcoRI restriction sites are underlined.

pJEL6185 (85-bp fragment)
5'-GGAATTCTGTTGTCTGCATCACGAAACAATC-3'
5'-GGAATTCGCTGGAGTATGTGCAGCATTGGG-3'

pJEL6186 (223-bp fragment)
5'-GAAGGTTGGGATTGCCTCAGG-3'
5'-TTGCCACGGCAATGGGTCAG-3'

PCR products were cloned into pGEM-T Easy and sequenced using the T7 primer.

4.2.9 Functional analysis of a genomic DNA fragment containing a putative GacA binding site.

inaZ promoter probe. The promoter probe vectors pPROBE-TI and pPROBE-TI' were obtained from Bill Miller (University of California at Berkeley). pPROBE-TI contains a promoterless inaZ, the pBBR1 replicon, and a tetracycline resistance gene. pPROBE-TI' is essentially the same as pPROBE-TI, but the polycloning site is in the opposite orientation. Stop codons are present in all three reading frames on both sides of the polycloning site to prevent translational readthrough either from the vector or from fragments inserted in the polycloning site. Primers derived from the sequences flanking the 223-bp putative GacA binding site were used in the PCR to amplify a 663-bp fragment from genomic DNA of Pf-5. EcoRI and BamHI sites (underlined) were incorporated into the primers as shown below.
The PCR was performed with 35 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), and extending (1 min at 68°C). The EcoRI and BamHI sites in the primers were used to clone this fragment into the promoter probe vectors pPROBE-TI to create pJEL6187 and pPROBE-TI’ to create pJEL6188. Plasmids were mobilized from S17-1 donors into Pf-5 and the GacA deletion derivative (JL4577) by biparental mating. Promoter activity was assessed by ice nucleation activity (INA). INA was quantified by the droplet freezing assay as described previously (Loper and Lindow 1994).

**lacZ promoter probe.** pMP220 is a promoter probe vector (Spaink 1987) containing a promoterless lacZ, an incP replicon, and a tetracycline resistance gene. Primers derived from the sequences flanking the 223-bp putative GacA binding site were used in the PCR to amplify a 663-bp fragment from genomic DNA of Pf-5. EcoRI restriction sites (underlined) were incorporated into both primers (shown below) to facilitate cloning of the fragment in both orientations.

5'-CGGAATTCTGATGCTCCTTCTATGCAAGCC-3'
5'-CGGGATCCCTTGCGGCCATTCTTCACGGGTAGG-3'

These EcoRI sites were used to clone the 663-bp fragment into pMP220 in both orientations.

**4.2.10 Expression and function of GST-GacA and GST-GacAC in Pf-5.**

The DNA sequences coding for GST-GacAC (pJEL6172) and GST-GacA (pJEL6171) were amplified by the PCR using a primer upstream of gst in pGEX-2T
(5'-CGGGGTACCTCTGGCGTCAGGCAGCCATCGG-3'; nts 31-52, accession U13850 in Genbank) and the primer complementary to nucleotides 934-957 of the gacA sequence (see section 3.2.3). The primers contained KpnI and EcoRI sites (underlined). The PCR was carried out with 35 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), and extending (3 min at 68°C). DNA fragments were cloned into the KpnI-EcoRI site of pME6000 to create pJEL6189 (GST-GacA) and pJEL6190 (GST-GacAC). Three clones each of pJEL6189 and pJEL6190 were partially sequenced from the 3' end using the T7 primer. Expression of these constructs was assessed in E. coli S17-1 and in the gacA mutant of Pf-5 (JL4577). Antibiotic production by JL4577 strains harboring these plasmids was assessed by thin layer chromatography, as described previously (Kraus and Loper, 1992).

4.3 Results

4.3.1 Expression and purification of GST fusion proteins.

Lysates of cells containing GST fusion proteins. E. coli BL21(DE3) pLysS cells containing GST produced a transparent lysate when frozen cells were resuspended in PBS + 1% Triton X-100. Cells of this strain containing either GST-GacAC or GST-GacA produced a cloudy lysate when frozen cells were resuspended in PBS + 1% Triton X-100. This cloudy lysate did not differ visually from unlysed cells, except for the increase in viscosity of the lysate due to the release of chromosomal DNA from the lysed cells. Sonication of lysates containing GST-GacAC and GST-GacA did not result in a cleared lysate. Sonication of lysates also did not appear to increase the yield of protein (data not shown). Since over-sonication can destroy protein function, the sonication step was dropped from the protein isolation protocol.
Influence of cell density on purification of GST fusion proteins. The density of cells in PBS + Triton X-100 did not appear to affect lysis but did appear to influence the pH of the lysate. Since binding of GST fusion proteins to glutathione sepharose is pH-dependent, it was necessary to obtain a lysate of the appropriate pH (pH 7.0 to pH 7.5). Resuspension of cells in PBS + Triton X-100 at less than 1/100th of the original culture volume typically resulted in a low pH (<pH 6.0 on one occasion) indicating that release of cellular contents had exceeded the buffering capacity of PBS. No attempt was made to determine the actual cell density at which the buffering capacity of PBS was exceeded. Instead, it was empirically determined that the lysate remained buffered between pH 7.0 and pH 7.5 when a ratio of one volume of lysate per 50 volumes of original culture was used. The effect of pH on binding of the fusion proteins to glutathione sepharose was more pronounced as the size of the fusion protein increased (data not shown). For example, GST bound to glutathione sepharose under low pH conditions, whereas GST-GacAC appeared to bind with less affinity, and no binding of GST-GacA was detected. Typically, yields of purified fusion proteins decreased with increasing size of the fusion proteins (Figure 4-5).

4.3.2 Establishing binding conditions for GST-GacAC.

Preliminary EMSAs. Initial EMSAs were performed using the FHA probe (Figure 4-3) with the GST-GacAC protein and the three DNA binding buffers listed in Table 4-2, under the hypothesis that the target site of BvgA, a protein closely related to GacA, would bind to GST-GacAC with some affinity. No binding of GST-GacAC to the FHA probe was observed in these experiments (data not shown). These initial results were inconclusive and could indicate that the DNA target site for GacA was not similar to the BvgA target site, that the conditions for binding were not correct, or that the GST fusions altered the binding capacity of the protein. GST-GacAC,
Figure 4-5. Coomassie stained gels of GST fusion proteins. a) Representative gel of purified GST-GacAC (lane 2) and GST (lane 3) that were used in EMSA analysis. b) Representative gel of the relative amounts of purified GST (lane 3), GST-GacAC (lane 4), and GST-GacA (lane 5). Corresponding lysates for the purified proteins after incubation with glutathione sepharose are shown in lanes 6,7, and 8 (GST, GST-GacAC and, GST-GacA, respectively). Proteins shown in (a) were isolated as described in the text. Proteins shown in (b) were isolated without Triton X-100. Numbers indicate the molecular weight of standards in kD (lane 1 for both gels).
likewise, showed no apparent affinity for the 300-bp *BamHI-SpeI* fragment containing the region upstream of the *gacA* ORF (data not shown).

**DNA pull-down assays with GST-GacAC indicated that binding occurred in buffer A and buffer C.** The amount of double stranded R26 (dsR26) DNA bound to GST-GacAC in DNA-binding buffer C (as assessed by scintillation counts of $^{32}$P) increased approximately 13-fold over the last three rounds of selection in DNA pull-down assays (Table 4-3). The amount of dsR26 bound to GST-GacAC in DNA-binding buffer A increased approximately 5-fold over these last rounds of selection, whereas no increase in binding was seen in buffer J. Because binding and enrichment of dsR26 fragments appeared to occur in both buffer A and buffer C, gel shift assays were performed using the pools of dsR26 fragments isolated from each selection cycle performed in these buffers. The results of these EMSAs indicated that, in buffer C, GST-GacAC bound to sequences in the random pool of DNA fragments that were isolated from each selection cycle, whereas no binding was observed in buffer A (Figure 4-6). After the shifted bands (Figure 4-6a) were isolated and amplified, EMSAs were performed on these pools of fragments and on the unselected dsR26 DNA library using buffer C. The shifted bands for selection rounds one, two, and three were of similar intensity to that of the original pool of dsR26 (Figure 4-7). No shifted band was observed for DNA isolated from selection cycle four, but this was most likely due to the fact that this DNA did not amplify well in the preceding PCR reaction.

**Optimization of protein and salt concentrations in binding reactions.**
Optimum protein concentration and potassium chloride concentration were determined empirically using unselected dsR26 DNA and two different preparations of purified GST-GacAC protein. A GST-GacAC concentration between 5 µM (Figure 4-8) and 8 µM (Figure 4-9) (approximately 2.6 µg to 4 µg per 20 µl binding reaction) resulted in the highest intensity of the shifted dsR26 band. In one EMSA (Figure 4-8), a shifted band appeared in the lane containing the GST protein. No such shifted band appeared
Table 4-3. Influence of buffer on Cycle selection of dsR26 fragments in DNA pull-down assays with GST-GacAC.

<table>
<thead>
<tr>
<th>Round of selection</th>
<th>Buffer A</th>
<th>Buffer C</th>
<th>Buffer J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PCR 1</td>
<td>450,640</td>
<td>481,680</td>
<td>788,800</td>
</tr>
<tr>
<td>Selection 2</td>
<td>6,395</td>
<td>7,266</td>
<td>6,265</td>
</tr>
<tr>
<td>PCR 2</td>
<td>1,205,800</td>
<td>1,127,320</td>
<td>1,170,880</td>
</tr>
<tr>
<td>Selection 3</td>
<td>11,673</td>
<td>30,654</td>
<td>758</td>
</tr>
<tr>
<td>PCR 3</td>
<td>1,404,820</td>
<td>1,360,200</td>
<td>1,258,720</td>
</tr>
<tr>
<td>Selection 4</td>
<td>34,374</td>
<td>96,223</td>
<td>1,852</td>
</tr>
</tbody>
</table>

Values for each round of selection are the amounts of DNA eluted in counts per minute of $^{32}$P, as determined by scintillation counting, eluted after washes with the respective 1X binding buffers. The number of washes for each round of selection were: Rd. 2, 2 washes, Rd 3, 3 washes; Rd 4, 4 washes. ND = no data. DNA for the first round of binding was the labeled R26 library DNA. The same amount of DNA was used for the first round of binding; specific activity undetermined.
Figure 4-6. Binding of GST-GacAC to pools of dsR26 fragments isolated in DNA pull-down experiments (section 4.3.2). The autoradiogram shows the result of an EMSA in which approximately $10^5$ cpm of radioactively labeled dsR26 DNA from different rounds of selection in the DNA pull-down assay were incubated without protein (F) or with approximately 1.5 μg of GST-GacAC (+). DNA-binding buffer was either buffer C (a) or buffer A (b). Numbers above pairs of lanes indicate selection rounds from which each pool of fragments originated. GST-GacAC was from protein preparation 1. P-D indicates protein-DNA complex; W, wells of the gel; F, free probe.
Figure 4-7. Binding of GST-GacAC to dsR26 fragments isolated from shifted bands in EMSA analysis conducted with buffer C (Figure 4-6a). The autoradiogram shows the result of an EMSA in which approximately $2 \times 10^5$ cpm of radioactively labeled dsR26 that appeared as shifted bands in a previous EMSA was incubated without protein (F) or with approximately 1.5 μg of GST-GacAC (+). Numbers above pairs of lanes indicate lanes in Figure 4-6a from which fragments were isolated. The pair of lanes labeled zero contain unselected dsR26 DNA. P-D indicates protein-DNA complex; W, wells of the gel. The free probe has been run off the gel. GST-GacAC was from preparation 1.
Figure 4-8. Effect of protein concentration on binding of GST-GacAC to unselected dsR26. The autoradiogram shows the result of an EMSA in which $4 \times 10^6$ cpm of dsR26 DNA was incubated without protein (lane 1), with increasing concentration of GST-GacAC (lanes 3-6), or with 2.5 µg of GST (lane 8). GST-GacAC concentrations were 0.01 µg (lane 3), 0.1 µg (lane 4), 1 µg (lane 5), and 2.6 µg (lane 6). P-D indicates protein-DNA complex; W, wells of the gel; F, free probe. GST and GST-GacAC were from preparation 1.
Figure 4-9. Effect of protein concentration on binding of GST-GacAC to unselected dsR26 fragments. The autoradiogram shows the result of an EMSA in which 6 x 10^6 cpm of dsR26 DNA was incubated without protein (lane 1), with increasing concentrations of GST (lanes 2-4), or with increasing concentrations of GST-GacAC (lanes 5-7). GST concentrations were 1 µg (lane 2), 4 µg (lane 3), and 7 µg (lane 4). GST-GacAC concentrations were 1 µg (lane 5), 4 µg (lane 6), and 7 µg (lane 7). GST and GST-GacAC were from preparation 2. Lane 9 contains labeled dsR26 DNA and 1 µg of GST-GacAC from preparation 1. P-D indicates protein-DNA complex; W, wells of the gel; F, free probe.
in the GST lane in Figure 4-9, in which a new preparation of GST was used, nor did a shifted band appear in the GST lane in further gel shift assays. A potassium chloride concentration between 150 mM and 200 mM resulted in the highest intensity of the shifted dsR26 band (Figure 4-10). In further experiments, a protein concentration of 2 μg to 4 μg and a salt concentration of 150 mM KCl were used.

4.3.3 Cycle selection of dsR26 DNA fragments bound to the GST-GacAC protein.

DNA fragments that contained putative target sequences for GacA binding were isolated using the cycle selection procedure shown in Figure 4-11. A representative gel shift assay is shown in Figure 4-12. After three cycles of selection, fragments containing putative GacA binding sites were cloned into either the HincII site or SmaI site of pUC19. Comparison of the DNA sequences of 12 such cloned sequences indicated that all were A+T-rich (Figure 4-13). Four of the 12 fragments (pJEL6173, pJEL6174, pJEL6179, and pJEL6180) were used in EMSAs. All four fragments were shifted, but the patterns of shifted bands were very different with respect to each other and with respect to the unselected dsR26 fragment pool (Figure 4-14). Relative affinity of GST-GacAC for these fragments was pJEL6173 > pJEL6179 ≈ pJEL6180 > pJEL6174.

A new preparation of GST-GacAC was used to assess binding to a consensus sequence 5'-GATCAACAAACAGCTAG-3' that was derived from alignment of the 12 cloned sequences and the cloned genomic sequence (Figure 4-13). The fragment containing this consensus sequence was not shifted in EMSAs with the new preparation of GST-GacAC protein (data not shown). The previously identified fragments (pJEL6173, pJEL6174, pJEL6179, and pJEL6180) also were not shifted by the new preparation of GST-GacAC (data not shown).

No site for GacA binding was revealed by EMSA analysis of the pltR-pltA intergenic region. None of the overlapping DNA fragments spanning the intergenic
Figure 4-10. Effect of salt concentration [KCl] on binding of GST-GacAC to unselected dsR26 fragments. The autoradiogram shows the result of an EMSA in which 6 x 10^6 cpm of dsR26 DNA was incubated with 4 µg of GST-GacAC and increasing concentrations of potassium chloride. KCl concentrations were 50 mM (lane 1), 75 mM (lane 2), 100 mM (lane 3), 150 mM (lane 4), 200 mM (lane 5), and 400 mM (lane 6). Lane 7 contains the probe, 4 µg of GST, and 50 mM KCl. GST and GST-GacAC were from preparation 2. P-D indicates protein-DNA complex; W, wells of the gel; F, free probe.
synthesize 2nd strand of R26 oligonucleotide using T4 DNA polymerase, $^{32}$P dCTP, and primer FOR to create radiolabeled pool of dsR26

amplify and radiolabel using PCR

Electrophoretic mobility shift assay with GST fusion proteins

repeat cycle 3 times

excise shifted band and elute DNA

Amplify shifted band by the PCR and clone putative GacA binding sites

Figure 4-11. Cycle selection procedure for dsR26 DNA fragments. Putative GacA binding sites are enriched in successive EMSAs and amplified by the PCR. The starting pool of random DNA sequences was generated by synthesizing the second strand of the R26 oligonucleotide, which contains a 26 nucleotide random core (open box) flanked by two 16 base sequences (grey boxes) for binding of primers FOR and REV (indicated by the labeled arrows).
Figure 4-12. EMSA from the 2nd cycle of selection of dsR26 fragments with GST-GacAC. The autoradiogram shows the result of an EMSA in which $5 \times 10^6$ cpm of dsR26 was incubated without protein (lane 1), with 4 µg of GST (lanes 2 and 3), and 4 µg of GST-GacAC (lanes 4 and 5). KCl concentration was 50 mM (lanes 1 and 2) and 150 mM (lanes 3, 4, and 5). P-D indicates protein-DNA complex; W, wells of gel; F, free probe. GST and GST-GacAC were from preparation 2. dsR26 DNA was from the pool of shifted bands isolated from the previous EMSA (selection cycle 1). Blank area in lane 5 is where the band was excised from the autoradiogram so that the film could be used as a template for excision of the shifted band from the gel.
| pJEL6184 | +++ | pJEL6173 | +++ | pJEL6175 | +++ | pJEL6178 | + | pJEL6174 | ++ | pJEL6180 | +++ | pJEL6183 | + | pJEL6177 | ++ | pJEL6181 | ++ | pJEL6179 | + | pJEL6176 | + | pJEL6182 |
|-----------|-----|----------|-----|-----------|-----|-----------|---|----------|----|-----------|-----|-----------|---|-----------|----|-----------|----|-----------|---|-----------|
| ~~~ATGATAA AGAACCAAGC ACGCCACA~~ | ~~~ATGATACA CCACACAACA CCACCACA~~ | ~~~AGGTACAC CCAACTACC CACCTACA~~ | ~~~AATGTTA AACAAGCAGC CCACCC~~ | ~~~AATAGCAA AACAAGCACC ACCTACCA~~ | ~~~ATAAAA AACAAGGAAGA CGACCCACAT | ~~~ATAAAAAC CAACACGAC GCACCCACCA |
| AC GCACACCAAGC -AAAA AA AA -AAAAATAC | ATAAAAAC GATAACCATT ACACACACCC | TAAAATAC AAACACAGAC GCACCCACCA | TAAAATAC AAACACAGAC GCACCCACCA |
| AT AAAATAAT AA | GATAACCATT ACACACCAAA | ATAAAATAAT AA | ACAGTAGCAC GACATCATAAA ACTCCA |
| GCCACACAA | GCCACACAAGC -AAAA AA AC | CCCACCAAA | CAAA ACTCC |

Consensus: **---AAAAA-AA AA-AACAATC CCACCACAAAT**

Figure 4-13. Sequence alignment of the A+T-rich dsR26 sequences cloned after three cycles of selection. Sequence comparisons were made with the GCG programs pileup and pretty. pJEL numbers refer to plasmids containing each sequence. Only the 26-nucleotide core sequence is shown for each fragment. The consensus sequence was used to design oligonucleotides to test this sequence for binding. Sequences pJEL6173, pJEL6174, pJEL6179, and pJEL6180 were used in EMSAs. Relative binding affinities of GST-GacAC for these fragments are indicated by plus signs. +++ is high affinity; ++, moderate; +, weak.
Figure 4-14. Binding of GST-GacAC to specific dsR26 fragments cloned after three selection cycles. The autoradiogram shows the result of an EMSA in which four of the cloned dsR26 fragments were incubated with either 4 μg of GST (-) or 4 μg of GST-GacAC (+). dsR26 sequences were unselected dsR26 (lanes 1 and 2), pJEL6173 (lanes 4 and 5), pJEL6174 (lanes 6 and 7), pJEL6179 (lanes 8 and 9), pJEL6180 (lanes 10 and 11). Specific probes were approximately $2 \times 10^5$ cpm of DNA per lane. Unselected dsR26 DNA was $10^6$ cpm per lane. GST and GST-GacAC were from preparation 2. P-D indicates protein-DNA complexes; W, wells of gel; F, free probe.
region between \textit{pltR} and \textit{pltA} depicted in Figure 4-4 were shifted in EMSAs with GST-GacAC (data not shown).

**Supershift and competitive binding analysis.** No supershift occurred in either of two experiments, when anti-GST antibody was added to the binding reaction containing the fragment from pJEL6180. However, a decrease in intensity and smearing of the shifted band was observed (Figure 4-15 and Figure 4-16). Addition of unlabelled competitor DNA slightly reduced the intensity of the shifted band in one experiment (Figure 4-15) but not in another (Figure 4-16). Labeled and unlabeled dsFHA were also used in these experiments. The appearance of a shifted dsFHA band (Figure 4-16) contradicted previous results obtained in section 4.3.2. However, previous EMSAs with the FHA probe were conducted under suboptimal binding conditions. Unlabelled dsFHA appeared to have no influence on binding of the pJEL6180 fragment to GST-GacAC (Figure 4-16).

### 4.3.4 Cycle selection of genomic DNA fragments bound to GacAC.

Genomic DNA fragments that contained putative target sequences for GacA binding were isolated using the cycle selection procedure shown in Figure 4-17. Because the \textit{HpaII} fragments in the genomic DNA fragment library were of variable size, the shifted band in the first selection cycle was within a smear of background DNA fragments (data not shown). In subsequent cycles of selection, a band of unshifted DNA persisted in EMSAs (Figures 4-18 and 4-19). In an attempt to remove this contaminating band from the cycle selection process, the fragments isolated from the third selection cycle were gel isolated in the absence of GST-GacAC (Figure 4-20). The smear corresponding to the DNA below the contaminating band was sectioned into 3 regions according to size as shown in Figure 4-20. The amplified fragments from each section were subjected to electrophoretic mobility shift assays. The shifted bands that were isolated from this fourth round of selection (Figure 4-21)
Figure 4-15. Binding of GST-GacAC to the radioactively labeled fragment from pJEL6180 in the presence of the unlabeled fragment from pJEL6180 and a GST antibody. Labeled DNA (4 x 10^6 cpm) was incubated with 1 μg GST (lane 1), 1 μg GST-GacAC (lane 2), 1 μg GST-GacAC plus 500 ng of unlabeled DNA from pJEL6180 (lane 3), and 1 μg GST-GacAC plus 1 μl of anti-GST antibody (lane 4). GST and GST-GacAC were from preparation 3. P-D indicates protein-DNA complex; W, wells of gel; F, free probe.
Figure 4-16. Binding of GST-GacAC to the radioactively labeled fragment from pJEL6180 in the presence of the unlabeled fragment from pJEL6180, unlabeled dsFHA, and a GST antibody. Labeled DNA (4 x 10^6 cpm) was incubated without protein (lane 1), with 1 µg GST (lane 2), 1 µg GST-GacAC (lane 3), 1 µg GST-GacAC plus 500 ng of unlabeled FHA (lane 4), 1 µg GST-GacAC plus 500 ng of unlabeled DNA from pJEL6180 (lane 5), and 1 µg GST-GacAC plus 1 µl of anti-GST antibody (lane 6). Radioactively-labeled dsFHA DNA was incubated without protein (lane 7), with 1 µg GST (lane 8), and 1 µg GST-GacAC (lane 9). GST-GacAC were from preparation 3. P-D indicates protein-DNA complex; W, wells of gel; F, free probe.
Figure 4-17. Cycle selection of procedure for genomic DNA of Pf-5. Putative GacA binding sites are enriched in successive EMSAs and amplified by the PCR. The starting pool of DNA fragments was prepared by digesting genomic DNA with HpaII (open boxes) and ligating linkers (grey boxes) to these fragments. Fragments were radiolabeled and used in EMSAs. Following isolation and amplification of shifted bands, the PCR was used to amplify and radioactively label the pool of fragments enriched for GacA binding sites that was used in the next cycle of selection.
Figure 4-18. Binding of GST-GacAC to genomic DNA fragments from Pf-5. The autoradiogram shows the EMSA from the second cycle of selection. Radioactively-labeled genomic DNA was incubated without protein (lane 1), with 4 µg of GST (lanes 3 and 4), and with 4 µg of GST-GacAC (lanes 5 and 6). GST and GST-GacAC were from preparation 2. P-D indicates protein-DNA complexes; W, wells of gel; F, free probe. The arrows indicate unbound DNA that has the same relative mobility as protein-DNA complexes.
Figure 4-19. Selection cycle three of binding of GST-GacAC to genomic DNA fragments from Pf-5. Radioactively-labeled genomic DNA isolated from the EMSA in selection cycle 2 and amplified by the PCR was incubated with 4 µg of GST (lane 1), with 4 µg of GST-GacAC (lane 2), and without protein (lane 4). GST and GST-GacAC were from preparation 2. P-D indicates protein-DNA complex; W, wells of gel; F, free probe. The arrows (lanes 1 and 4) indicate unbound DNA that have the same relative mobility as protein-DNA complexes.
Figure 4-20. Gel isolation of the GST-GacAC bound genomic DNA from cycle selection 3. The starting pool of radioactively-labeled DNA for selection cycle 3 is in lane 1. The total DNA recovered after selection cycle 3 is in lane 2. The arrow indicates the contaminating band that migrates at the same position as the shifted DNA. Letters indicate the three size categories isolated from the gel: large (L), medium (M), small (S). The DNA isolated from each section was amplified and labeled using the PCR and used in selection cycle 4.
Figure 4-21. Selection cycle four of binding of GST-GacAC to genomic DNA fragments from Pf-5. Three size classifications of genomic DNA fragments from selection cycle 3 were radioactively amplified by the PCR. Lanes 1-3 contain large DNA fragments (L). Lanes 3-6 contain medium fragments (M). Lanes 7-9 contain small fragments (S). Probes were incubated without protein (F), with 4 μg of GST (-), or with 4 μg of GST-GacAC (+). GST and GST-GacAC were from preparation 2. Arrows indicate shifted bands of DNA that were isolated and cloned. W indicates wells of gel. F indicates free probe.
were cloned into either the HincII site or Smal site of pUC19. Sequence analysis of four such clones revealed that two fragments (an 85-bp fragment in pJEL6185 and a 223-bp fragment in pJEL6186) were unique; the other two fragments were duplicates of these fragments. Both the 85-bp fragment and the 223-bp fragment were shifted in EMSAs with GST-GacAC (Figure 4-22). Sequence analysis revealed that both fragments were A+T-rich. Sequence similarity between these two fragments was 58% over 53 bp. Neither the 85-bp fragment nor the 223-bp fragment were similar to any sequence in the GenBank or the unfinished microbial genome of *P. aeruginosa*.

One round of cycle selection of genomic DNA using the modified selection protocol described in section 4.2.5 (under Modified construction of the genomic library) demonstrated that binding to genomic fragments was reproducible (Figure 4-23). Attempts to clone additional fragments, which might contain GacA binding sites, from this selection process failed.

The deduced amino acid sequence of the ORF adjacent to a putative GacA DNA-binding site is similar to pore-forming proteins. Southern hybridization indicated that the Pf-5 genome contained a 2-kb *BclI* fragment that hybridized to the 223-bp putative DNA-binding site for GacA (data not shown). Inverse PCR, as depicted in Figure 4-24, was used to obtain the 2 kb of DNA in the *BclI* fragment that flanked this 223-bp sequence. Sequence analysis of this 2-kb region indicated that the 223-bp putative GacA binding site was immediately upstream of an open reading frame (hereafter referred to as ORF P). ORF P begins at bp 570 with an ATG start codon (Figure 4-25). Upstream from the ATG codon, there is an AG-rich region from bp 559 to 564 similar (five of six nucleotides) to Shine-Dalgarno sites for ribosome binding (Shine and Dalgarno, 1974). The region upstream of ORF P contains no recognizable σ70, σ54, or σ32-type pseudomonad promoters (Deretic et al., 1989). The deduced amino acid sequence of ORF P is similar to certain porin proteins of *Pseudomonas* spp. The partial N-terminal sequence of the deduced protein coded for by ORF P (hereafter referred to as porin P) is most similar to the PhaK protein of *Pseudomonas putida*, which is a putative pore forming protein in the recently
Figure 4-22. Binding of GST-GacAC to cloned fragments of genomic DNA of Pf-5. The radioactively labeled 85-bp putative GacA binding fragment from pJEL6185 was incubated with 4 µg of GST (lane 1) and 4 µg of GST-GacAC (lane 2). The radioactively labeled 223-bp putative GacA binding fragment from pJEL6186 was incubated with 4 µg of GST (lane 3) and 4 µg of GST-GacAC (lane 4). GST and GST-GacAC were from preparation 2. Shifted bands are indicated by the arrows. W indicates wells of gel. F indicates free probe.
Figure 4-23. Binding of GST-GacAC to a second library of genomic Pf-5 fragments that was prepared by a modification of the original method (see section 3.2.5). Radioactively labeled genomic DNA of 3 size classes were incubated with 1 μg of GST (-) or 1 μg of GST-GacAC (+). Lanes 1 and 2 contain fragments between 500 and 300 bp; lanes 4 and 5, fragments between 300 and 100 bp; lanes 7 and 8, fragments less than 100 bp. GST and GST-GacAC were from preparation 3. P-D indicates protein-DNA complex; W, wells of gel; F, free probe.
putative GacA binding site in the genome of Pf-5 (223 bp) labeled with biotin and used as a probe

Southern analysis probe identifies a 2-kb BclI fragment

Pf-5 genomic DNA digested with BclI

ligation under conditions favoring formation of monomeric circles

anneal primers

PCR

design new primers

crossing lines are used to indicate that the orientation of the new primers in the inverse PCR product and the Pf-5 genome

Pf-5 genome

PCR larger fragment from the genome of Pf-5

Figure 4-24. Schematic representation of the inverse PCR process used to obtain sequences flanking the 223-bp fragment contained in pJEL6186. Primers used to amplify the 663-bp fragment contain EcoRI sites to facilitate cloning of the PCR product.
Figure 4-25. Comparison of sequences isolated from the dsR26 library to the region upstream of a putative porin gene of Pf-5 (ORF P). Sequence is of the 5' end of ORF P and its upstream untranslated sequence. The 223-bp sequence between the HpaII sites at positions 420 and 643 was cycle selected from the genomic library. The 663-bp fragment amplified by PCR and cloned into promoter probe vectors is boxed and shaded. The numbers refer to plasmid numbers containing putative GacA binding sites that were cycle selected from the dsR26 library (i.e. 6173 is pJEL6173) and are above the ORF P sequences to which they are similar. Italics, bold, and underlined text is used to differentiate overlapping regions of similarity. Sequence that is outside the shaded box is unconfirmed sequence generated by inverse PCR. The overhead arrow indicates the putative start codon for porin P. The putative second half of an FNR binding site is boxed (dark grey). A possible Shine-Dalgarno ribosome binding site is boxed.
discovered phenylacetyl catabolic pathway (Olivera et al., 1998). However, \textit{phaK} is part of an operon in which the preceding gene terminates 19 bp upstream of the \textit{phaK} ORF. The region immediately upstream of the putative Pf-5 porin gene does not contain any identifiable ORF for at least 500 bp (data not shown). The putative Pf-5 porin is also similar to the anaerobically-induced porin E1 (OprE) of \textit{P. aeruginosa} PAO1 (Yamano et al., 1993) and the oprD2-like porin (OprD3) of \textit{P. aeruginosa} PAO1 (GenBank accession AF033849). The N-terminus of the putative Pf-5 protein is more than 41\% identical to PhaK, OprE, and OprD3 over a 127 amino acid overlap beginning at amino acid 32 in Figure 4-26. The first 31 amino acids of these proteins show little similarity.

The 85-bp fragment containing the putative GacA binding site could not be identified in the genome of Pf-5 by Southern hybridization with either a biotinylated probe or a \textsuperscript{32}P-radiolabelled probe of this fragment. Attempts to amplify DNA flanking this region by the inverse PCR also failed (data not shown).

\textbf{Sequence similarities between dsR26 and the genomic putative GacA binding sites.} The putative GacA binding sites isolated from the dsR26 library were compared to the upstream region and coding sequence of ORF P. The vast majority of the dsR26 sequences (21 out of 24) were similar to regions upstream of the putative start codon for ORF P, and many (11 out of 24) were similar to regions within the 223-bp \textit{HpaII} fragment originally isolated by the cycle selection procedure (Figure 4-25). Sequence analysis of the first 536 bp of the putative porin ORF indicated only three regions with similarity to the dsR26 fragments (Figure 4-25).

\textbf{4.3.5 Promoter activity of the putative GacA DNA-binding fragment.}

\textbf{Ice nucleation activity as conferred by an inaZ promoter probe was not a reliable measure of promoter activity.} The DNA sequence of the inverse PCR product was used to design primers that could then be used to amplify a region of
Figure 4-26. Alignment of the deduced amino acid sequence of hypothetical porin P of Pf-5 with the PhaK, OprE, and OprD3 porins from Pseudomonas species. Residues that are identical are shaded. The boxed residues contain the alanine-X-alanine sequence that is a potential cleavage site for a procaryotic signal peptidase. The N-terminal amino acid of the mature OprE protein is indicated by an asterisk.
DNA flanking the 223-bp *Hpall* fragment containing a putative GacA binding site. Using these primers, I obtained the 663-bp PCR product (Figure 4-25). This 663-bp fragment was cloned into the promoter probe vectors pPROBE-TI and pPROBE-TI', each of which contain a promoterless *inaZ*. Unfortunately, these vectors were not useful for assessing promoter activity of the putative GacA DNA-binding site because INAs conferred by these vectors were very high (10^{-3} to 10^{-1} ice nuclei per cfu) in the *gacA* mutant (JL4577) background (Figure 4-27). An approximate 100-fold decrease in INA occurred in the *gacA* mutant background (Figure 4-27) when the putative GacA DNA-binding site (the 663-bp fragment depicted in Figure 4-27) was placed opposite the orientation of *inaZ* (pJEL6187). INA was no different than the vector alone when the putative DNA-binding site was placed in the same orientation as *inaZ* (pJEL6188) (Figure 4-27). There was little difference in INA between exponential phase cultures (12 h) and stationary phase cultures (24 h) of the *gacA* mutant carrying these promoter probe constructs.

In the wild-type background, INA was conferred only by pJEL6187, which contains the putative GacA DNA-binding site placed opposite the orientation of *inaZ*. There was an approximate 1000-fold reduction in INA in stationary phase cultures of this strain compared to exponential phase cultures (Figure 4-27).

Isolation of plasmids pPROBE-TI, pPROBE-TI', pJEL6187, and pJEL6188 from *gacA::aphI* (JL4577) and wild-type backgrounds indicated that these plasmids were stable in the mutant but not in the wild-type. Restriction endonuclease digestion of plasmids isolated from the mutant produced fragments of expected size, whereas digestion of the plasmids isolated from the wild type did not (Figure 4-28).

**Promoter activity of the putative binding site in pMP220.** Promoter activity of the 663-bp fragment containing the ORF P promoter region was not assessed because the 663-bp fragment was never successfully cloned into this vector.
Figure 4-27. Ice nucleation activity of promoter probes pPROBE-TI, pPROBE-TI', and derivatives containing the porin P promoter in the opposite orientation as inaZ gene in the vector (pJEL6187) or the same orientation as inaZ in the vector (pJEL6188). JL4577 is the gacA::aphI mutant and Pf-5 is the wild type. Values are the mean of two replications.
Figure 4-28. Plasmid DNA of promoter probe vectors pPROBE-TI, pPROBE-TI’, and their derivatives containing the 663-bp porin P promoter region. Plasmids were isolated either from the gacA::aphI mutant JL4577 (lanes 2-7) or the wild-type strain Pf-5 (lanes 9-14). Equal volumes of culture were used to purify plasmids. Lanes 2 and 9 contain pPROBE-TI; lanes 3 and 10, pPROBE-TI’; lanes 4,5,11, and 12, pJEL6187; lanes 6,7,13, and 14, pJEL6188. E indicates that the plasmid was digested with EcoRI. B indicates that the plasmid was digested with EcoRI and BamHI. Lanes 1 and 8 contain a 1 kb ladder (GibcoBRL) for a size standard.
4.3.6 Difficulties in obtaining reproducible results in EMSAs.

After obtaining putative GacA binding sites from dsR26 and genomic libraries with two preparations of GST-GacAC, several further preparations of this protein did not result in shifted bands in EMSAs (data not shown). Furthermore, western analysis of purified GST, GST-GacAC, and GST-GacA indicated that proteins co-purified with GST-GacAC and GST-GacA that did not co-purify with GST (data not shown). Together with the band observed in the GST lane in Figure 4-8, these results suggested that an *E. coli* protein that co-purified with the GST-GacAC protein might be responsible for the shifted bands that were observed in EMSAs. To test this hypothesis, total protein extracts and purified protein from *E. coli* containing either GST, GST-GacAC, or GST-GacA were used in EMSA analysis with the 223-bp genomic fragment. Unfortunately, the probe was degraded completely in the lanes containing crude protein extracts (Figure 4-29). Degradation of the probe was most likely due to a combination of the magnesium in the buffer and DNases within the protein extract since no such degradation occurred in the lanes that contained purified protein. No shifted bands were observed in the lanes containing purified protein that was prepared in parallel with these total protein extracts. It is noteworthy that two other protein preparations were used in this EMSA, both of which had previously resulted in shifted bands in EMSA analysis. These two protein preparations gave opposite results; one resulted in at least two shifted bands, whereas the other resulted in no shifted bands (Figure 4-29).

4.3.7 Expression and function of GST-GacA and GST-GacAC in Pf-5.

Partial sequences of two of the three clones of pJEL6189 (GST-GacA) were obtained. One of the clones had a point mutation in *gacA* that changes the glycine to a serine at position 97 in the amino acid sequence of GacA. This plasmid was designated pJEL6191 and the deduced protein is hereafter referred to as GST-GacA1.
Figure 4-29. EMSA of crude extracts containing GST, GST-GacAC and GST-GacA. The 223-bp putative GacA binding site from pJEL6186 was radioactively-labeled and incubated without protein (lane 1); with 4 µg of GST-GacAC (lanes 2 and 3); with crude lysates from E. coli containing GST (lane 4), GST-GacAC (lane 5), GST-GacA (lane 6); or with purified GST (lane 7), GST-GacAC (lane 8), or GST-GacA (lane 9). Shifted bands are indicated by the arrows (lane 2). GST-GacAC in lanes 2 and 3 are from different protein preparations. W indicates wells of the gel. F indicates free probe.
The partial sequences of all three clones of pJEL6190 were identical to the 3' end of gacA, which codes for the GacAC portion of the fusion protein. The molecular weight of GST-GacA and GST-GacAC are predicted to be 49 kD and 34 kD, respectively. Coomassie stained gels of protein extracts from the gacA::aphl mutant (JLA577), which contained plasmid-borne genes coding for GST-GacA (pJEL6189), GST-GacAC (pJEL6190), and GST-GacA1 (pJEL6191) were inconclusive for determining expression of these proteins. Expression of GST-GacA and GST-GacA1 in E. coli S17-1 was also inconclusive because an E. coli protein similar in size to GST-GacA was induced by the addition of IPTG. In addition, a protein of approximately 23 kD was expressed in E. coli S17-1 containing GST-GacA, and a protein of approximately 42 kD was expressed in E. coli S17-1 containing GST-GacA1. A protein corresponding to the predicted size of GST-GacAC (34 kD) was expressed in E. coli S17-1.

When in vivo activities of GST-GacA and GST-GacAC were assessed, complementation of the gacA::aphl mutant was incomplete with both constructs, indicating that the fusion proteins were not fully functional. However, the full-length fusion (GST-GacA) restored the production of low levels of pyoluteorin and Phl to the gacA::aphl mutant of Pf-5. The C-terminal fusion (GST-GacAC) restored production of a low level of PHL to the gacA::aphl mutant, which was comparable to complementation with GST-GacA (data not shown). GST-GacA1 also restored production of pyoluteorin in when the gacA::aphl mutant containing this construct was cultured in NB amended with 1% glycerol. Interestingly, GST-GacA1 restored production of pyoluteorin to wild-type levels in KB, a medium in which the wild type GST-GacA protein did not (data not shown).
4.4 Discussion

4.4.1 Identification of a putative GacA binding site.

This study reports the identification of an A+T-rich region in the Pf-5 genome that is a putative DNA binding site for GacA. Several A+T-rich DNA sequences that formed complexes with a GacA fusion protein were isolated from a synthetic pool of DNA fragments, supporting the hypothesis that GacA specifically binds to an A+T-rich DNA sequence. Furthermore, the synthetic A+T-rich sequences were similar to several different regions within the genomic A+T-rich sequence, indicating that GacA may bind to multiple sites in the promoter region of this gene. Other response regulators in the FixJ family recognize sequences that either are A+T rich or are surrounded by A+T rich regions of DNA. These regulatory proteins also bind to numerous sites in the promoter regions of the genes that they regulate. For example, DNase I footprint analysis of the promoter region recognized by NarL, which regulates genes required for nitrate respiration in *E. coli*, indicates that several A+T-rich sequences are protected from DNase I digestion by the NarL protein (Kaiser and Sawyers, 1995). Likewise, BvgA, a response regulator necessary for the expression of virulence factors by *Bordetella pertussis*, binds to A+T-rich regions upstream of the promoters that it regulates (Roy et al., 1991). FixJ, a response regulator that controls the expression of nitrogen fixation genes by *Rhizobium meliloti*, also protects an A+T-rich sequence from DNase I digestion (Galinier et al., 1994). Consensus heptamers within these A+T-rich sequences are the proposed binding sites for NarL and BvgA (Kaiser and Sawyers, 1995; Roy et al., 1991). However, these consensus sequences are rather loosely conserved for both the NarL and BvgA binding sites. Although a binding site has been identified for FixJ, no consensus sequence has been reported for this protein.

The response regulators OmpR, VirG, and NtrC, which belong to other families of response regulators based on their consensus helix-turn-helix domains, also bind within A+T-rich regions in the promoter regions of the genes that they regulate.
OmpR, a response regulator involved in the osmo-regulated expression of two porins by *E. coli*, binds to several 18-bp A+T-rich regions upstream of the *ompC* and *ompF* porin genes. The consensus OmpR binding sequence is ratherly loosely conserved with only one G and one C near the center of the A+T-rich region being highly conserved (Huang et. al, 1996). Interestingly, it is the relative affinity of OmpR for these sites in the *ompC* and *ompF* promoters that dictate the expression these two genes (Huang et al., 1996). VirG is required for the expression of the *vir* genes of *Agrobacterium tumefaciens* in response to plant exudates (reviewed by Heath et al., 1995). The consensus VirG binding site is a 12-bp A+T-rich sequence (Jin et al., 1990). The consensus binding site for NtrC, a response regulator involved in nitrogen regulation by enteric bacteria, differs from the other binding sites mentioned because it consists of two half sites for NtrC binding that are separated by a 4-bp A+T sequence (reviewed by Porter et al., 1995).

The A+T-rich region isolated from the genome of Pf-5 was immediately upstream of an open reading frame (ORF P). The deduced amino acid sequence of ORF P has similarity to certain bacterial porins. The porins of gram-negative bacteria form water-filled channels that permit the diffusion of hydrophilic solutes across the outer membrane (Hancock, 1987). Generally, they are divided into the following two classes: nonspecific porins that allow diffusion into the cell of molecules below a certain size, and specific porins that facilitate the entry of specific substrates based on stereospecific binding sites (Hancock, 1987).

The putative Pf-5 porin (porin P) probably belongs to the class of specific porins based on its similarity to members of that class, such as PhaK, OprD, and OprE. Porin P was most similar to the PhaK porin of *P. aeruginosa*, but it is probably not the Pf-5 homolog of this protein. *phaK* encodes a putative porin involved in the phenylacetic acid degradation pathway and, as part of a larger operon, does not appear to have its own promoter (Olivera et al., 1998). The ORF upstream of *phaK* ends only 19 bp from the putative translational start site of *phaK*. In contrast, no ORF could be identified within 500 bp upstream of the putative translational start site of the Pf-5 porin gene. OprD is a selective porin for the uptake of basic amino acids and small
peptides containing these amino acids (Trias and Nikaido, 1990). Like ORF P, the upstream region of oprD is A+T-rich. OprE is an anaerobically-induced porin of P. aeruginosa (Yamano et al., 1993). Anaerobically-induced genes in Pseudomonas spp. typically contain an FNR binding site (consensus TTGAA-N4-ATCAA) approximately 40 bp upstream of the transcriptional start site (Galimand et al., 1991; Cuypers et al., 1995). The upstream region of oprE contains a sequence similar to the second half of the FNR binding site (TTCAA) that is located 34-38 bp upstream of the transcriptional start site. This sequence is also located upstream of ORF P and, although its position relative to the transcriptional start site of ORF P has not been determined, it is located at the same position as that of oprE relative to the translational start site of ORF P. The presence of this sequence suggests that ORF P may be anaerobically induced in Pf-5. There are also a few other notable differences between ORF-P and these other porin genes. For example, oprD and oprE both contain the σ54 binding consensus sequence (GG-N10-GC) (Deretic et al., 1989), whereas this sequence is not present upstream of ORF P. In addition, the upstream region of oprE is not A+T-rich like that of ORF P. Furthermore, PhaK, OprE, and OprD contain sequences similar to procaryotic signal sequences (a hydrophobic core followed by the sequence alanine-X-alanine) (Perlman and Halvorson, 1983) that are removed from the mature protein (Figure 4-25), whereas no such sequence was found in porin P.

As the levels of OprE increase in P. aeruginosa under anaerobic conditions, the levels of another putative porin (OprC) also increase, whereas levels of another putative porin (protein E2) decrease (Yamano et al., 1993). The expression of these porins by P. aeruginosa is similar to the alternate expression of porins OmpF and OmpC by E. coli. The expression of OmpF and OmpC are controlled by a two-component regulatory system in response to the osmolarity of the growth medium (reviewed by Pratt and Silhavy, 1995). As mentioned earlier, the regions upstream of ompF and ompC contain several A+T-rich binding sites for the two-component response regulator OmpR. Both ompF (GenBank accession M74489) and ompC (GenBank accession E00506) have extensive 5' untranslated regions (>500 bp)
containing OmpR binding sites for both transcriptional activation and repression (Head et al., 1998). Because the 5’ untranslated region of ORF P contains numerous A+T-rich sequences and because no ORFs could be identified within this region, it is tempting to speculate that the production of porin P might be regulated by GacA in a similar fashion to that observed for the OmpR-mediated regulation of OmpF and OmpC. Unfortunately, efforts to clarify the role of GacA in regulating porin P were inconclusive.

GacA regulation of the promoter for porin P could not be determined using the inaZ promoter probe vectors for two reasons. First, the vector alone conferred a very high level of background ice nucleation activity in the gacA background. This was unexpected since these vectors confer very low levels of background activity in other Pseudomonas spp. (Steve Lindow and Bill Miller, personal communication). Second, the inaZ promoter probe vectors were unstable in the wild-type Pf-5 background, but stable in the gacA mutant background. This result suggests that GacA regulates factors controlling plasmid stability. Several researchers have noticed that it is easier to introduce plasmids into gacA or apdA mutants, but, to my knowledge, none have reported an effect of gacA on plasmid stability. Efforts to use the alternative promoter probe vector pMP220, which carries a promoterless lacZ cassette, to assess the promoter activity for porin P in wild-type and gacA mutant backgrounds have been unsuccessful. Therefore, the role of GacA in the regulation of porin P remains speculative.

4.4.2 Requirements for the cycle selection procedure.

The cycle selection procedure that was used to identify a putative GacA binding sites was derived from the SELEX-like strategy used to identify genes specifically regulated by the iron uptake regulatory protein FUR of P. aeruginosa (Ochsner and Vasil, 1996). In that system, a consensus binding sequence for the FUR protein and the conditions for FUR binding had previously been determined.
Consequently, the researchers were able to use this information to identify numerous other genes that were regulated by FUR. In contrast, nothing was previously known about the DNA sequence recognized by GacA, although the biosynthetic genes for many of the secondary metabolites regulated by GacA have been identified and their characterization was underway when this study was initiated. Evidence that GacA was a DNA-binding protein was based on its amino acid sequence similarity to the response regulator proteins of well characterized two-component signal transduction systems (Laville et al., 1992). Therefore, the successful identification of DNA target sites for GacA binding required 1) a purified active form of GacA (see section 4.4.3), 2) identification of buffer conditions required for DNA binding (determined empirically), 3) a representative starting pool of DNA fragments (see section 4.4.5), and 4) a method for isolating and identifying target DNA fragments from the starting pool (electrophoretic mobility shift assay, see Figures 4-11 and 4-17).

4.4.3 Rationale behind the creation of GST-GacA and GST-GacAC.

Using sequence similarities to related response regulators, GST fusion proteins linking the GST protein to the proposed full length GacA protein (GST-GacA) and to the C-terminal end of GacA (GST-GacAC), which contains the helix-turn-helix DNA-binding motif, were created. The hypothesis was that removal of the receiver domain would result in a protein that would retain the ability to bind specific DNA sequences with high affinity but would not require phosphorylation. The fusion of the full-length GacA to GST was prepared as an alternative should the C-terminal fusion fail.

Response regulators of two-component systems are composed of an N-terminal receiver domain, which controls the activity of the C-terminal output domain in response to phosphorylation of a conserved aspartate residue (Albright et al., 1989; Parkinson, 1993; Stock et al., 1995). In general, response regulators can be placed in two main classes (Fiedler and Weiss, 1995). In class I, exemplified by NtrC, phosphorylation of the receiver works positively to allow dimerization of these
response regulators, which is a prerequisite for high affinity binding (Mettke et al., 1995). For a class I response regulator, deletion of the receiver domain does not activate the protein (Porter et al., 1995). In class II, exemplified by FixJ, phosphorylation of the receiver is thought to relieve inhibition of the output domain imposed by the receiver (Kahn and Ditta, 1991). In contrast to class I, the region involved in dimerization appears to be in the output domain of class II response regulators (Kahn and Ditta, 1991; Re et al., 1994). Consequently, deletion of the receiver domain of a class II response regulator may result in a constitutively active protein. Further evidence that the main role of the receiver domain of class II response regulators is inhibition of the output domain is provided by the crystal structure of NarL, a member of the FixJ family, which shows that the DNA-binding domain is blocked by the receiver domain (Baikalov et al., 1996). However, as discussed further below, it may be hazardous to assume that the role of the receiver of class II response regulators is simply to inhibit the output domain since the consequences of deleting this domain have been studied for only a few proteins.

Sequence similarity previously placed GacA in the large FixJ family (i.e. class II) of response regulators (Laville et al., 1992). Also included in this family are the well-characterized response regulators BvgA and NarL. The C-terminal domain of FixJ-like response regulators, which contains the helix-turn-helix DNA binding motif, have significant homology to a class of autoregulatory DNA-binding proteins, such as LuxR from Vibrio fischeri (Choi and Greenberg, 1991) and GerE from Bacillus subtilis (Zheng et al., 1992). Indeed, the prosite database groups the FixJ-like proteins with the LuxR-like proteins based on the consensus sequence of their helix-turn-helix motifs (PDOC00542 available through the ExPASy molecular biology server at expasy.hcuge.ch). Because LuxR and GerE function in the absence of an N-terminal receiver, this further suggests that the non-phosphorylated N-terminal domain of the FixJ family of response regulators inhibits the function of the C-terminal domain. A flexible stretch of amino acids linking the N-terminal domain to the C-terminal domain has been postulated, which allows the non-phosphorylated N-terminal domain to interact with and inhibit the DNA binding activity of the C-
terminal domain (Parkinson, 1993). Evidence that a flexible linker tethers the two domains has come from experiments in which the two domains are expressed as separate proteins. For example, when the N-terminal domain was removed from FixJ, the derivative (FixJC) was not only constitutively active, but more active than FixJ in vivo (Kahn and Ditta, 1991). In addition, FixJC formed more stable complexes with its target DNA than phosphorylated FixJ (Kahn and Ditta, 1991). These results suggested that removal of the N-terminal domain of GacA might result in a protein that retained sequence-specific binding and was constitutively active in vivo.

Like FixJ, derivatives of BvgA bind their specific DNA targets when the N-terminal receiver domain is removed (Boucher et al., 1994). Unlike FixJ, however, the C-terminal of BvgA was not functional in vivo (Boucher et al., 1994). There are at least three explanations for the lack of activity of the BvgA C-terminus. First, the BvgA C-terminal proteins were created as fusion proteins to the 6X Histidine-tag (His-tag) or the 45 kD maltose-binding protein (MBP) (Boucher et al., 1994), whereas the FixJ derivative was created by inframe deletion of the nucleotides coding for the receiver domain (Kahn and Ditta, 1991). The large MBP moiety could interfere with activity, but it seems less likely that the much smaller His-tag would also interfere with activity. Second, the BvgA C-terminal proteins were created by deletion of the first 120 amino acid (i.e. the conserved receiver domain). As such, this construct contains not only the C-terminal domain, but also the flexible linker region. FixJC is shorter and may not contain any of the linker residues. It should be stressed that the positions at which this linker begins and ends are not well characterized. Therefore, an inhibitory role for this stretch of amino acids cannot be ruled out. Third, the N-terminal domain may have an additional function in BvgA that is not contained within the N-terminal domain of FixJ. Thus, because of the numerous differences in the construction of the BvgA and FixJ derivatives, it is not possible to ascertain the reason that one is inactive while the other is active.

Both His-tag and MBP fusions to full-length BvgA were functional *in vivo* as assessed by reporter genes, although expression of the reporter gene by the MBP fusion was lower than that of the His-tag fusion (Boucher et al., 1994). Similarly,
fusions of the maltose-binding protein to NarL and NarP were also functional in vivo (Darwin et al., 1998). Together with the FixJ results, these results suggested that a GST fusion to the C-terminal domain of GacA might bind specific DNA targets and possibly activate transcription in vivo, whereas a GST fusion to the full-length GacA might result in a protein that would bind specific DNA targets and also be able to activate transcription in vivo.

**Purification of GST fusion proteins.** The GST-GacAC protein was easily purified, whereas the GST-GacA protein proved to be difficult to purify. Because GST-GacA did not bind to glutathione sepharose when prepared in parallel with GST-GacAC, GST-GacA was thought to be insoluble. Overexpression of fusion proteins can lead to the formation of insoluble aggregates of the protein known as inclusion bodies. However, binding and recovery of GST fusions to glutathione sepharose also is dependent on the pH of the lysate (Pharmacia manual for GST fusions). Efficient binding of GST to glutathione sepharose occurs between pH 6.5 and pH 8.0. It was later discovered that the pH of the lysate containing GST-GacA was too low (pH=6.0) for efficient binding of GST to glutathione sepharose. When the pH of the lysate was increased either by the diluting it with PBS or by the addition of NaOH, it was possible to purify GST-GacA using glutathione sepharose. However, recovery of GST-GacA was not as great as that of GST or GST-GacAC. Because of the initial difficulty in purifying large amounts of GST-GacA, the cycle selection procedure for identifying target DNA sequences for GacA were performed with GST-GacAC. Considering that the C-terminal domains of FixJ and BvgA bind to their specific target sites with similar affinity to that of their phosphorylated full-length counterparts, it seemed reasonable to believe that the C-terminal of GacA (GST-GacAC) also would bind its target sequences with high affinity.

**Complementation of the gacA::aphI mutant with GST fusion proteins.** When in vivo activities of GST-GacA and GST-GacAC were assessed, complementation of a gacA::aphI mutant was incomplete with both constructs,
indicating that the fusion proteins were not fully functional. However, the full-length fusion (GST-GacA) did restore the production of low levels of pyoluteorin and 2,4-diacetylphloroglucinol (PHL) to a gacA mutant of Pf-5. The C-terminal fusion (GST-GacAC) restored production of a low level of PHL to the gacA mutant, which was comparable to complementation with GST-GacA. Regulation of PHL by *Pseudomonas* spp. is interesting in that it appears to be negatively regulated by phlF (GenBank accession U41818), a gene that is upstream of the PHL biosynthetic genes and divergently transcribed (Bangera and Thomashow, 1996). PhlF is similar to members of the family of TetR repressors (Fenton et al., 1997). Evidence that PhlF is a repressor for PHL production is two-fold. First, PHL production is conferred on nonproducing strains when a fragment containing the PHL biosynthetic genes are introduced into these strains, but not when the additional flanking sequence containing phlF is introduced (Bangera and Thomashow, 1996). Second, mutations in phlF lead to constitutive production of PHL (Fenton et al., 1997) Furthermore, mutations in phlF decouple PHL production from global regulation by GacA (Fenton et al., 1997). These results imply that GacA acts as repressor of the PhlF repressor as depicted in Figure 4-30. Because repressors typically bind to promoter regions and thereby prevent members of the transcriptional apparatus from binding, the ability of GST-GacA and GST-GacAC to restore PHL production may depend on its ability to bind specific sequences within the promoter region of phlF. Alternatively, GacA could act as positive transcriptional factor for a repressor of phlF. Generally, transcriptional activation by response regulators may be more complex than repression by the same response regulator. Transcriptional activation requires that the response regulator first bind its target site and then make the appropriate contacts with other members of the transcriptional apparatus in order to initiate transcription. In contrast, repression may be as simple as the response regulator having the ability to bind its target site. Because GST-GacAC does not restore other phenotypes to the gacA mutant, it seems unlikely that this construct acts as an activator of a repressor for the PhlF repressor. The region upstream of phlF also contains several A+T-rich regions that are possible GacA binding sites (data not shown). Therefore, the ability of GST-GacAC to restore
Figure 4-30. Proposed regulatory mechanism for 2,4-diacetylphloroglucinol production in Pf-5. \textit{phlA} is the first ORF in the biosynthetic pathway. \textit{phlF} codes for putative repressor of the biosynthetic genes. Phosphorylated GacA (GacA-P) represses transcription of \textit{phlF}. In the absence of PhlF, the biosynthetic genes for 2,4-diacetylphloroglucinol are transcribed. Arrows above \textit{phlF} and \textit{phlA} indicate direction of transcription.
PHL production to a gacA mutant is strong circumstantial evidence that GacA inhibits transcription of phlF as a consequence of directly binding to the promoter region of this gene.

The reason that GST-GacA is unable to fully complement a gacA mutant is not clear. Possible reasons are: 1) GST-GacA is not expressed well in Pf-5, 2) mutations in the PCR amplification of this fusion occurred that affect its function, or 3) the GST moiety inhibits the function of GacA. Expression of GST-GacA is under the control of a tac promoter, which is constitutive in other Pseudomonas spp (Ligon et al., 1996). Coomassie stained gels of Pf-5 crude lysates containing GST-GacA were inconclusive for determining expression of this protein. It seems likely that GST-GacAC is expressed in Pf-5, however, considering that this construct provided partial complementation of a gacA mutant. Western analysis using an anti-GST or anti-GacA antibody should clarify whether or not this protein is being expressed.

Partial sequencing of two GST-GacA constructs indicated that one of the constructs had a point mutation that changed the glycine at position 97 in the amino acid sequence of GacA to a serine (hereafter referred to as GST-GacA1). Interestingly, GST-GacA1 was more effective for complementing a gacA mutant than the presumed wild type, as indicated by its ability to restore pyoluteorin production to wild-type levels in KB media. Thus, GST-GacA1 may be a constitutively active form of the protein. Similarly, a point mutation in the gacA of P. fluorescens BL915 that changed the glycine to an arginine at position 132 results in a ApdA-independent GacA (Ligon personal communication, 1999). Because none of the three clones of GST-GacA have been fully sequenced, however, it is possible that the presumed wild-type clones have mutations that affect their function and that the mutation in GST-GacA1 actually has no affect on function. To appropriately address this question, the gst and gacA portions of these proteins will need to be sequenced completely.

It is also possible that the GST moiety interferes with the function of GST-GacA. For example, dimerization is thought to be necessary for binding and/or transcriptional activation by response regulators. Although the ability of GST-GacA to dimerize was not examined in this study, it seems unlikely that the 26 kD GST
moiety would interfere with dimerization, given that the much larger 45 kD MBP did not prevent MBP fusions to BvgA (Boucher et al., 1994), NarP, or NarL (Darwin et al., 1998) from activating transcription from their respective promoters. However, the level of transcription from BvgA regulated promoters was not as high when MBP BvgA was overexpressed as when His-tag BvgA was overexpressed (Boucher et al., 1994). Also, DNA binding by MBP BvgA was not greatly enhanced by incubating it with acetyl phosphate, whereas DNA binding by the corresponding His-tag BvgA protein was markedly increased by the addition of acetyl phosphate (Boucher et al., 1994). These results suggest that the MBP moiety may interfere 1) with the ability of this construct to dimerize, 2) with contacts between the response regulator and other components of the transcriptional apparatus, or 3) with the interaction with its cognate kinase.

A high concentration of GST-GacAC was required for DNA binding. Typically, the concentration of DNA-binding protein used in a gel shift assay is in the nanomolar range. The high concentration of GST-GacAC (6 µM) that was required for the formation of protein-DNA complexes suggests that this construct has lower affinity for its target site than the native protein (i.e. it seems unlikely that a 6 µM cellular concentration of GacA would be required for activity). It is uncertain why such a high concentration of GST-GacAC would be necessary for DNA binding but there are several plausible reasons. One is that purification and storage conditions resulted in a high proportion of the protein being inactivated. Although this possibility was considered, the conditions for maintaining a stable protein must be determined empirically and this was not done. Steric hindrance by the GST moiety or the deletion of a domain needed for high affinity binding might be another factor influencing the concentration of GST-GacAC required for effective binding. The high concentration of GST-GacAC, however, does not mean that binding is not sequence specific. For example, 5 to 50 µM concentrations of NarL (Kaiser and Sawyers, 1995), 0.8 µM concentrations of FixJC (Galinier et al., 1994), 0.54 µM concentrations of MBP-dN BvgA (Boucher et al., 1994), and 9 µM of His-tagged CitBC (Meyer et al., 1997) were
required for effective binding to their target sequences. The high concentrations of protein that were used in each of these cases did not effect the specificity of these proteins for their target sequences. Interestingly, western analysis revealed that the vast majority of MBP-dN BvgA was not complexed with DNA (Boucher et al., 1994). These results suggest that, even though a high concentration of GST-GacAC was required for the formation of protein-DNA complexes, a relatively small proportion of active protein may be responsible for the observed complexes.

**Shifted fragments may be due to a protein that co-purified with GST-GacAC.** Several lines of evidence suggest that the observed complexes represent DNA bound to a protein that co-purified with GST-GacAC rather than to GST-GacAC itself. If a co-purifying protein from *E. coli* was responsible for binding, then the high concentration of purified GST-GacAC, which was used in the binding experiments may have included enough of that co-purifying protein to account for the observed DNA binding. In support of this hypothesis is the lack of a supershift of the protein-DNA complex when GST antibody was included in the binding reaction. Also competitive binding with unlabeled DNA failed to decrease the signal from the protein-DNA complex as would be expected if GST-GacAC were responsible for binding. However, in order to appropriately assay for specific binding to a labeled fragment, it is necessary to use a molar excess of the unlabelled fragment. Because radioisotope was incorporated into the labeled fragment in a PCR reaction, it was not feasible to assess the concentration of the labeled fragment. Therefore it is not certain that a molar excess of unlabeled fragment was used in these assays. Modifications to the labeling procedure that circumvent this problem are discussed in section 4.4.5.

**4.4.4 Possible reasons that GST-GacAC did not bind to specific fragments.**

**Upstream region of gacA.** The response regulator BvgA regulates its own expression by binding to a region upstream of *bvgA* and activating transcription of that
gene (Roy and Falkow, 1991). By analogy to BvgA, GacA might regulate its own transcription. Interestingly, nucleotide sequence similarity between the upstream regions of bvgA and gacA declines near the BvgA binding site in bvgA. Binding of GST-GacAC to the upstream region of gacA was not detected. However, the binding assays that were performed using the 300-bp fragment upstream of gacA were done before the assay was optimized for salt and protein concentration. The concentrations of KCl (50 mM) and GST-GacAC (10 nM to 100 nM) that were used in these EMSAs were well below the concentrations that were eventually used. Binding of other probes was barely detectable at a GST-GacAC concentration of 1 μM. Therefore, binding may not have been detected due to the low protein concentration used in these assays.

**dsFHA fragment.** The dsFHA fragment is an A+T-rich sequence that is a strong binding site for the response regulator BvgA (Roy and Falkow, 1991). Many initial experiments were performed using this fragment under the hypothesis that GST-GacAC might bind this fragment to some degree. However, all of these experiments were performed with concentrations of GST-GacAC that were less than 100 nM. Therefore, as with the assays using the upstream region of gacA, binding may not have been detected due to the low concentration of GST-GacAC in the binding reaction. Later, when the dsFHA fragment was tested under the same conditions as a fragment containing a putative GacA binding site, GST-GacAC displayed similar affinities for both the dsFHA fragment and a putative GacA binding site isolated by the cycle selection procedure. This suggests that initial failure to detect binding of the dsFHA fragment indeed was due to the low concentration of protein used in those initial experiments.

**pltR-pltA intergenic region.** pltA is the first ORF in the pyoluteorin biosynthetic cluster. The pltR gene is located approximately 800 bp upstream of pltA, divergently transcribed, and encodes a putative LysR-type transcriptional activator (Nowak-Thompson et al., 1999). Mutagenesis of pltR reduces pyoluteorin production
by Pf-5 to undetectable levels and reduces activity from a reporter gene within the pyoluteorin biosynthesis genes, indicating that this gene is a positive transcriptional activator of the pyoluteorin biosynthesis genes (Nowak-Thompson et al., 1999). Although sequence analysis of the pltR-pltA intergenic region did not identify any significant alignments with the putative GacA binding sites isolated from either the genomic or random fragment libraries, this intergenic region contains numerous A+T-rich regions, indicating that GacA might bind somewhere within it. The lack of apparent affinity of GST-GacAC for this region may indicate that there is no GacA binding site within this region. However, numerous difficulties in obtaining binding of GST-GacAC were experienced when these assays were performed and so these experiments should be repeated.

Consensus sequence derived from putative GacA binding sites. A consensus GacA binding sequence, which was derived from the putative genomic and synthetic GacA binding sites, did not bind to GST-GacAC. As with the experiments on the pltR-pltA intergenic region, the protein used in these experiments did not bind to probes for which GST-GacAC had previously displayed affinity. Therefore, it is most likely that binding was not observed because the protein was inactive. Considering the variable affinity that GST-GacAC displayed for four different fragments isolated from the random DNA pool, it also is possible that the consensus sequence is not a very good binding site. In addition, the length (18 bp) of the consensus sequence may have been too short for efficient binding of GST-GacA. For example, the probe used to detect BvgA binding was 24 bp (Roy and Falkow, 1991) and VirG requires not only the 12-bp consensus (Jin, 1990), but also the 19 bp downstream for efficient binding (Roitsch et al., 1994). These examples suggest that GacA may require a longer fragment of DNA for efficient binding.
4.4.5 Recommendations for improving the cycle selection procedure.

The aim of this section is to discuss the numerous pitfalls in the cycle selection procedure used in this study, with respect to improvements that might be made so that the same mistakes need not be repeated in the future. Improvements unique to the cycle selection procedures for identifying sequences from random and genomic pools are discussed separately. Improvements that could be made to both procedures are then discussed together.

4.4.5.1 Improvements in the cycle selection procedure for selecting random DNA fragments.

Oligonucleotide design. Several design flaws in the oligonucleotide that was used for creating the random pool of DNA fragments caused problems in both the amplification and cloning of fragments from this pool. The main flaws were 1) the spacing between restriction sites in the sequences flanking the random core was a multiple of three, and 2) the length of the flanking sequences used as primer binding sites were too short.

Incorporation of restriction sites. As depicted in Figure 4-3, XbaI and BamHI restriction sites were incorporated into the sequences flanking the internal core of random nucleotides. The purpose of these sites was to facilitate the cloning of isolated sequences into pUC19 and then be able to screen for interruption of the lacZ gene contained in this plasmid. Because the centers of the restriction sites were placed 33 bases apart, cloning these fragments into pUC19 would have resulted in an inframe insertion that would not have inactivated lacZ, unless a stop codon was present in the fragment. Since Taq polymerase adds an extra adenine residue to the ends of DNA fragments, the PCR products could have been cloned into a T-A cloning vector such as pGEM-T (Promega) or pGEM-T Easy. Unfortunately, the total length of an individual random fragment was 58 bp and the addition of one base on each end would result in a
60 bp fragment, which also would be unlikely to inactivate lacZ in either of these two vectors. Therefore, fragments had to be cloned by the relatively inefficient method of blunt end cloning. This difficulty could be avoided by making a fragment of a length not divisible by three.

**Short length of primer binding sites.** The primer binding sites flanking the 26 base random core were 16 bases in length. Because the primer length was short, a relatively low annealing temperature (45°C) was used in the PCR. A low annealing temperature may lead to false priming within the random core sequence and may also lead to interfragment or intrafragment priming. Interfragment priming occurs, for example, when the 3' end of a PCR product (in this case a single-stranded 58 mer) acts as a primer that binds somewhere within another fragment. Intrafragment priming occurs when the single stranded DNA loops back and primes off a region within the same fragment. Elongation from such priming results in fragments that are larger than the original fragment. Several bands which ran slower than the 58-bp fragment were observed on the autoradiographs of EMSAs, indicating that there was a significant amount of such priming in the PCRs used to amplify this pool of DNA fragments. Increasing the primer length would allow specific priming at a higher annealing temperature and might decrease the amount of undesirable priming.

4.4.5.2 Improvements for the cycle selection procedure for selecting genomic fragments.

**Size fractionation of genomic fragments.** Restriction digestion of genomic DNA creates a pool of variable size fragments that appear as a smear when electrophoretically separated in a gel. This can pose a problem in EMSAs if unbound fragments have similar or less mobility than the DNA-binding protein in the gel because the signal from unbound fragments can obscure fragments shifted by the protein. This problem can be overcome by size fractionating the genomic fragments on an acrylamide gel prior to the binding assay. Size fractionating genomic fragments,
however, means that some binding sites may be lost. This problem may be overcome by creating another library of genomic fragments using a different restriction enzyme.

4.4.5.3 General improvements for the cycle selection procedure.

**PCR and radiolabeling.** Labeling fragments by incorporating a radiolabeled nucleotide in the PCR has the advantage of producing a very “hot” probe. There are, however, three major disadvantages. Those disadvantages are: 1) it is difficult to quantify the amount of probe, 2) interfragment and intrafragment priming can create larger products that may obscure shifted fragments in a gel shift assay, and 3) the unequal molar concentrations of dNTPs in the reaction may lead to bias in the fragments that are amplified. The DNA concentration of radiolabeled probes is not generally determined by direct means. Instead, the specific activity of the probe is determined by scintillation counting. The concentration of the probe can then be estimated by taking into account the half-life of the radioisotope, the proportion of the isotope incorporated, and the length of the DNA. It must be stressed that this cumbersome method can only provide a crude estimate of the DNA concentration. Not knowing the concentration of the probe made it impossible to know whether a molar excess of unlabelled probe was being used in the competitive binding experiments conducted as part of this study.

Although interfragment or intrafragment binding occurred in the PCR used to amplify the 58-bp fragments from the random library, it did not appear to cause a serious problem since the protein-DNA complex was well separated from the free probe. However, such priming occurred within the probes spanning the intergenic region between pltR and pltA and made it necessary to gel isolate the labeled probes. Another problem associated with interfragment and intrafragment priming of genomic sequences is that it creates sequences that do not exist in the genome. Another potential problem that may occur when radiolabeling fragments by PCR is the biased amplification of fragments. The reason for this is that the radiolabeled nucleotide is
generally the limiting nucleotide in the reaction. In this work, the concentrations of unlabeled nucleotides, dATP, dTTP, and dGTP, were 200-fold greater than the combined concentration of dCTP and \([\alpha-^{32}P]\) dCTP. It is possible, therefore, that the fragments amplified in this study were not representative of the overall pool of fragments.

To avoid the possible problems involved in radiolabeling fragments in the PCR, the PCR could be carried out with unlabeled dNTPs, gel isolated, quantified, and labeled by one round of PCR. The main disadvantage in this process is that not all fragments may be labeled. An alternative approach would be to amplify the pool of fragments with unlabelled dNTPs, digest the fragments with restriction enzymes that recognize sequences in either the linkers of genomic fragments or the flanking sequences surrounding a random core in a synthetic pool of DNA fragments, quantify the DNA, and fill in the restriction sites with radiolabeled nucleotides. The disadvantage in this process is that sites may be lost if the restriction enzyme cuts the fragment internally. Although these alternative approaches increase the number of steps required for labeling the probe, they avoid many of the problems that may be encountered when labeling via PCR. An added benefit of these approaches is that they eliminate any need to gel isolate radioactive probes and so reduce the user's exposure to ionizing radiation.

**Fusion proteins.** Proceeding with binding experiments using a fusion protein that was not the equivalent of the wild-type protein was possibly the greatest flaw in this study. As discussed previously, GST fusions to GacA (GST-GacA) and the C-terminal of GacA (GST-GacAC) were not fully functional *in vivo*. Therefore, it is not certain that these proteins were capable of binding GacA target sites *in vitro*. Also, it was never conclusively determined whether GST-GacAC or a co-purifying protein was responsible for the binding observed in gel shift experiments. In retrospect, preincubating GST-GacAC with thrombin prior to the DNA-binding reaction might have answered this question. The advantage of using a protein that is functional *in vivo* is that it the protein will almost certainly bind *in vitro* if the binding conditions
are correct. There are several vectors available for expressing fusion and native proteins, any one of which might have produced a functional protein. Perhaps the best alternative is to use an in vitro translation system to produce the native protein for the initial binding experiments. An in vitro translation system is advantageous because the extract, minus the plasmid carrying the gene for the protein of interest, provides a good negative control. The disadvantage of in vitro translation is that small amounts of protein are produced. However, once binding conditions were established using an in vitro translated protein, it might be possible to substitute a fusion protein in further assays.

Phosphorylation of GacA is most likely necessary for high affinity binding. Other response regulators have been successfully phosphorylated with small phosphate donors such as acetyl phosphate (Lukat et al., 1992; Boucher et al., 1994; Galinier et al., 1994; Darwin et al., 1998; Kaiser and Sawyers, 1995; Head et al., 1998; Meyer et al., 1997) but it appears that this is much less efficient than the response regulator's cognate sensor kinase (Meyer et al., 1997). Several researchers have produced constitutive sensor kinases by deleting the membrane associated domain and creating a fusion protein to the kinase domain (Huang and Igo, 1996; Meyer et al., 1997; Uhl and Miller, 1996; Jourlin et al., 1997). These constitutive kinases along with ATP are then included in the binding reaction to phosphorylate the response regulator. Such an approach might prove useful in binding experiments with GacA.

4.5 Conclusion

Although a putative binding site for GacA was identified using the cycle selection procedure, the results were inconclusive due to several inconsistencies in the DNA-binding assay. The appearance of a shifted band in the GST control lane of approximately the same mobility as the GST-GacAC fusion, the lack of a supershift when anti-GST antibody was used in EMSA analysis, and the inability of GST-GacAC to complement the gacA::aphI mutant of Pf-5 suggests that the observed
binding may have been due to a protein that was co-purified with GST-GacAC. The upstream region of only one gene, which codes for a putative porin, was identified as a putative binding site for GacA by the cycle selection procedure. Studies initiated to determine whether gacA regulates transcription of this putative porin gene have been unsuccessful, to date, so it remains unclear whether this gene is regulated by GacA. The identification of only one putative GacA-regulated gene was disappointing since the justification for the cycle selection procedure was that it should have the ability to identify numerous genes regulated by GacA. The failure to identify several potential GacA-regulated genes, however, does not indicate that the cycle selection procedure is a poor approach for identifying such sequences, but rather that the execution of the procedure was not what it could have been. Hopefully, the mistakes made in this study and knowledge gained therefrom will be useful in the design of future cycle selection schemes.

5.1 Introduction

In agricultural systems, biological control of plant disease often is achieved by the inundative introduction of the antagonistic organism to the soil, spermosphere, or phyllosphere. The requirement for a large initial dose of the antagonist implies a relationship between the amount of antagonist available to interact with the pathogen and the amount of disease suppression achieved. A model that relates pathogen dose-disease responses to the population size (dose) of an antagonist and the level of disease suppression has been developed (Johnson 1994) based on the most common type of pathogen dose-disease relationship, i.e. the negative exponential function (Van der Plank 1975). The concept of an asymptote or "refuge", which defines a proportion of the pathogen population not susceptible to influence or attack, is a central component of the proposed model (Johnson 1994). It has been hypothesized that pathogen propagules not susceptible to a biocontrol agent may be the result of several factors, including temporal (e.g., rate of antibiotic production by an antagonist vis-a-vis rate of establishment by the pathogen (Weller and Thomashow, 1990)), spatial (e.g., pathogen aggregation (Adams and Fravel, 1990)), genetic (e.g., pathogen resistance to antibiotics produced by antagonists (Cooksey and Moore, 1982; Mazzola et al., 1994; Sule and Kado, 1980)), and environmental (e.g., heterogeneity in niches or nutrient availability (Loper, 1990)) factors. The model predicts that the magnitude of such an asymptote will have a profound impact on the potential amount of disease suppression (Johnson, 1994). In terms of biological control, the existence of an asymptote indicates the point at which the introduction of additional biocontrol agent will have no further affect on disease suppression.

Typically, biological control has been assessed by applying doses of the antagonist on a logarithmic scale. Graphical depiction of the resulting disease
suppression on a logarithmic scale (Adams and Fravel, 1990; Bull et al., 1991; Hadar et al., 1979), often gives the misperception that the level of disease suppression increases linearly with increasing dose of a biological control agent. By replotting the data of previous biological control experiments on an arithmetic scale, the presence of an asymptote was indicated (Johnson 1994). Thus, to determine if an asymptote exists, it will be necessary to design experiments with both logarithmic and arithmetic scaling of the biological control doses (Johnson 1994). Empirical evidence from a selected number of biological control systems have subsequently supported the concept of an asymptote (Raaijmakers et al., 1995; Smith et al., 1997; Montesinos and Bonaterra, 1996). Further research on various biological control systems is needed to verify the model proposed by Johnson (1994) and to determine if asymptotes are commonly defined in dose-response relationships, and to evaluate the effect of asymptotes on limiting the efficacy of biological control.

In this study, the biological control model proposed by Johnson was tested on preemergence damping-off of cotton caused by *Rhizoctonia solani* AG-4. The biological control agent was *Pseudomonas fluorescens* Pf-5. *R. solani* is a major cause of seed rot, preemergence damping-off, and post emergence damping-off of cotton throughout much of the Cotton Belt in the USA. Preemergence damping-off can be caused by both *R. solani* and *Pythium ultimum* and it is thus difficult to assign losses due to *R. solani* alone. Seedling diseases, however, caused an estimated loss of 828,199 bales of cotton nationwide in 1995 (Blasingame, 1996) and 81,994 bales in California during the same period, with similar losses in previous years (Davis et al., 1994). Cotton normally escapes serious seedling infection provided that conditions are favorable for rapid seedling growth during the first 2-3 weeks after planting. Afterwards, the stem of the plant becomes more woody and more resistant to infection (Watkins, 1981). Environmental factors such as cool soil temperatures or waterlogged soil that slow the growth of seedlings therefore render the plant more susceptible to infection by *R. solani*. High nutrient levels within the pathogen propagule and the
presence of exogenous nutrients in the soil have also been shown to increase severity of the disease (Weinhold et al., 1969; Weinhold et al., 1972).

There continues to be debate over the exact mechanism by which *R. solani* infects and causes disease in cotton. Growth of the fungus towards the cotton plant is ascribed to seed and hypocotyl exudates (Armentrout et al., 1987). Most studies have focussed on infection of the hypocotyl although the pathogen infects seeds and roots as well. The developmental stages of hypocotyl infection have been defined as: 1) hyphae from the inoculum source contact the hypocotyl and grow along the surface; 2) the hyphae branch extensively; 3) infection cushions form; 4) discoloration appears beneath the infection cushion; and 5) distinct lesions form and tissue maceration occurs (Weinhold and Motta, 1973). In stage one, hyphal growth along the anticlinal walls of epidermal cells is a thigmotrophic response (Armentrout et al., 1987). Armentrout and Downer (1987) also described extensive mycelial branching that leads to the orderly formation of an infection cushion. Multiple infection pegs are produced beneath the infection cushion and these presumably are capable of penetrating the cuticle by mechanical force (Armentrout and Downer, 1987). It has been shown, however, that the cell wall is broken down prior to penetration by polygalacturonase produced by *R. solani* (Weinhold and Motta 1973; Brookhouser and Weinhold, 1979). Lesions and tissue maceration may occur in as little as 24 hrs if the pathogen is immediately adjacent to the emerging hypocotyl (Armentrout and Downer, 1987).

The efficacy of existing compounds used for the control of *Rhizoctonia* preemergence damping-off is extremely variable. Fungicides such as Captan, Thiram, PCNB, and Myclobutanil, which are typically applied as a seed coat prior to planting, vary in efficacy depending on factors such as planting date, presence of other pesticides, soil moisture (Moustafa-Mahmoud et al., 1993), cotton cultivar (Wang and Davis, 1997), and inoculum density (Davis et al., 1997). Other treatments that have shown variable control of damping-off include seed treatment with L-sorbose (Howell, 1978) and 3-O-methyl glucose applied to hypocotyls (Weinhold and Bowman, 1974).
The absence of a reliable means for the control of Rhizoctonia damping-off of cotton makes development of a biological control system for disease suppression an attractive option. Biological control of damping-off caused by R. solani has been achieved by the use of both fungal and bacterial isolates. Beneficial fungal isolates include Trichoderma spp. (Elad et al., 1982; Howell, 1994; Lewis and Papavizas, 1991; Flores et al., 1997) and Gliocladium spp. (Howell, 1987). Numerous studies have been conducted to identify rhizobacteria with antifungal activity against R. solani (Cook and Rovira, 1976; Hagedorn et al., 1989). The most abundant and demonstrably effective bacteria isolated have been Pseudomonas spp. (Hagedorn et al., 1989; Weger et al., 1995).

Pseudomonas fluorescens Pf-5, the biological control antagonist used in this study, was isolated and found effective for the suppression of damping-off caused by R. solani by Howell and Stipanovic (1979). P. fluorescens Pf-5 is known to produce at least two antifungal compounds that effectively inhibit R. solani, pyrrolnitrin (Howell and Stipanovic, 1979; Cartwright et al., 1995) and 2,4-diacetylphloroglucinol (Keel et al., 1992; Nowak-Thompson et al., 1994). Mutants of Pf-5 that do not produce these compounds (Kraus and Loper, 1992; Pfender et al., 1993; Corbell and Loper, 1995) were termed apdA mutants (for antibiotic production) but have recently been renamed gacS (for global activator sensor kinase) (Kitten et al., 1998; Whistler et al., 1998). It is anticipated that gacS mutants will prove useful for assessing the dose-response model for biological control (Johnson 1994) with respect to the affect of the antifungal compounds on the predicted asymptote.

This chapter presents the progress that was made in developing a biological control system for preemergence damping-off of cotton caused by R. solani using biological control agent P. fluorescens Pf-5 in order to test the proposed dose-response model for biological control.
5.2 Materials and Methods

5.2.1 Biocontrol strains.

Antagonist strains used in experiments were *Pseudomonas fluorescens* Pf-5 (JL4092), and its derivatives (JL3871 and JL4135). All strains were obtained from J. E. Loper (USDA Agricultural Research Service, Horticultural Research Laboratory, Corvallis, Oregon). JL4092 produces antifungal compounds including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, HCN, and a pyoverdin siderophore. JL3871 is a spontaneous rifampicin resistant mutant that produces the same antifungal compounds as JL4092. JL4135 contains the Tn5 transposon in *gacS*, is kanamycin resistant, produces the pyoverdin siderophore but does not produce the other known antifungal compounds produced by Pf-5. Pf-5 and all of its derivatives are ampicillin and streptomycin resistant.

5.2.2 Inoculum preparation of biocontrol strains.

For use in biological control assays, Pf-5 and the *gacS* mutant were cultured for five days at 20°C on nutrient agar supplemented with 1% glycerol. These are conditions conducive for the production of pyrrolnitrin, the most effective compound produced by Pf-5 for the inhibition of *R. solani*. Strains were harvested by scraping plates with a cotton swab and suspending cells in phosphate buffer [20 mM potassium phosphate, pH 7] to an O.D. of 1.0 at 600nm. Bacterial densities were assessed by dilution plating the suspension on King’s medium B (KB) (King et al., 1954). The ranges of biocontrol densities used in experiments were prepared by serial dilution of the bacterial suspension in phosphate buffer.
5.2.3 Pathogen preparation.

*Rhizoctonia solani* AG-4 isolate J1 (hereafter refered to as *R. solani*) was obtained from C. R. Howell, Cotton Research Laboratory, USDA Agricultural Research Service, College Station, Texas. Inoculum for experiments was prepared by the method described by Van Bruggen (Van Bruggen et al., 1985). Briefly, frozen green beans that had been autoclaved for 30 minutes in a 100x80 pyrex dish were inoculated with four 1 cm² plugs of *R. solani* from 4 -5 day old cultures maintained on potato dextrose agar (Difco Laboratories, Detroit, MI). Infested beans were incubated for 15 days at 27°C, then air-dried under a biohazard hood on sterile paper towels for 48 h. The dry material was ground through a 710 μm sieve and caught on a 500 μm sieve to yield large propagules (500-710 μm). Propagules were mixed with autoclaved soil at 1 g pathogen to 1 kg of autoclaved soil to produce a dilute inoculum source for use in biocontrol assays. Propagules per gram of inoculum were determined by sprinkling samples (0.5 g) of the inoculum on water agar containing streptomycin at 100 μg /ml.

5.2.4 Biological Control Assays.

Pre-emergence damping-off of cotton and post-emergence lesion formation on cotton seedlings was studied in a soil bioassay. Soil used in these experiments was Newburg sandy loam (pH 6.4). Soil was steam pasteurized for 1 h at 76°C and spread out in the greenhouse to air-dry. Air-dried soil was sieved (2 mm mesh) and stored at 20°C in 1 gallon plastic bags (1.5 kg / bag). The pathogen was incorporated into dry soil at a concentration of 2.5 or 5 propagules per 100 g soil. The pathogen was mixed thoroughly with air-dry soil by inverting the sealed bags 20-30 times. Distilled water was added to obtain a soil moisture of 20% (< -0.1 bar water potential) based on
percent water to air dry soil. Typically, soil was moistened 2 h before planting.
During this time, the soil was kept in sealed ziplock bags. Pots (4" square) were
filled with approximately 300 g of infested soil per pot and covered with a plastic bag
until planting to prevent evaporation. Acid delinted cotton seed var. Stoneville 213
(obtained from C. R. Howell, Cotton Research Laboratory, USDA Agricultural
Research Service, College Station, Texas) were neutralized to pH 6.8-7.0 by soaking
in 20% NaHCO₃ for 10 min followed by a 2-min rinse under running distilled water.
Seeds were blotted dry on paper towels and dipped for 20-40 minutes in serial
dilutions of bacterial suspensions. Cotton seeds were planted (nine seeds/pot), while
wet, on the surface of infested soil using sterile forceps and covered with another 150
g of infested soil resulting in a planting depth of approximately 1 inch. Experimental
design was a randomized block with nine, ten, or twelve replications; nine seeds per
replication. Pots were placed on cafeteria trays inside plastic garbage bags to insure
constant humidity and incubated in dark growth chambers for 6 days at 20°C. Plants
were then removed from plastic bags, transferred to the greenhouse, and grown for 5-6
more days. Plants were watered with 100 ml of distilled water per pot immediately
after transfer to the greenhouse. Greenhouse lights were set for a 14.5 h photoperiod.
Daily maximum temperature was 27°C; minimum was 15°C. Disease was evaluated
by scoring for emergence. Emergence was defined as the cotyledons having cleared
the soil surface.

5.2.5 Population dynamics of biological control bacteria on cotton seed and
root.

In addition to biocontrol efficacy experiments, a series of experiments were
performed to assess how the populations of Pf-5 on seeds and roots change over time.
Serial dilutions of bacterial inoculum were prepared as previously described above
except that bacteria were cultured for 2 or 5 days on nutrient agar (Difco
Laboratories, Detroit, MI) supplemented with 1% glycerol. The culture time was
shortened from 5 to 2 days because I discovered that spontaneous *gacA* and *gacS* mutants arise after 4 days incubation of Pf-5 in rich media. Length of culture time for each experiment is specified in figure legends. Cotton seed and soil were prepared as described above. For assessing populations on seeds within three days of planting, either plastic cones (4 cm diameter x 20.5 cm deep) or cells in punch trays (3.5 cm diameter x 4 cm deep) were used. Plastic cones were used for all experiments in which bacterial densities on hypocotyls or roots were assessed. Seeds planted in punch trays were planted to an approximate depth of 1 cm. Seeds planted in plastic cones were planted to an approximate depth of 2 cm. Seed was planted by placing soil in the bottom of punch tray cells or cones, placing seeds on the soil surface using sterile forceps, and then covering the seed with soil to the prescribed depth. Punch trays were placed inside plastic boxes with wet paper towels on the bottom to maintain moisture. Racks containing cones were placed inside a plastic bag and sealed to maintain moisture. Treatments were arranged in a randomized block design with two to five replicates per dose; one seed per replicate. Trays or racks were held in dark growth chambers at 20°C for three to six days before plants were harvested.

For assessing bacterial densities on seeds, the seeds were recovered from the soil and placed into 10 ml culture tubes containing 1 ml of phosphate and vortexed 10 seconds to remove bacteria from the surface. After vortexing, three 100-fold serial dilutions were made. From the original suspension and dilutions, 10-μl samples were plated on KB containing 100 μg/ml cycloheximide and 50 μg/ml streptomycin for the selective recovery of Pf-5 derivatives. In population studies, in which the spontaneous rifampicin resistant derivative and *gacS* mutant were used as a mixture, KB was amended with rifampicin at 100 μg/ml or kanamycin at 50 μg/ml, respectively, for the selective recovery of these strains.

For assessing populations of Pf-5 or derivative strains at the hypocotyl-root transition zone of 6-day old seedlings, two variations on the same method were used.
When no distinction was made between populations of Pf-5 on hypocotyls and roots, populations were assessed from a 2.5-cm segment of the plant which encompassed the hypocotyl-root transition zone. For experiments in which the hypocotyl and root populations were assessed separately, a 2-cm section encompassing the same region was excised and divided at the midpoint of the hypocotyl-root transition zone, to yield a 1-cm segment of hypocotyl and a 1-cm segment of root. Bacteria were recovered from the plant segments by the same method used for recovery of bacteria from seeds.

5.2.6 Data analysis.

Data were analyzed with Statgraphics Plus Version 3.0 and SAS (Statistical Analysis System, SAS Institute Inc., Cary, North Carolina). The general linear means (GLM) procedure was used to detect significant differences (*P* ≤ .05) in emergence and experiments on population densities.

5.3 Results.

Development of an assay to evaluate a dose response model for the biological control of preemergence damping-off of cotton by *R. solani*.

5.3.1 Determining a pathogen dose.

Treatment with *P. fluorescens* Pf-5 (6 x 10^7 cfu/ml) had a significant effect on emergence of cotton seedlings from soil infested with 2.5 or 5.0 propagules of *R. solani* per 100 g of soil (*P* = .036 and *P* ≤ .0001, respectively at day 12) (Figure 5-1). In soil in which *R. solani* was absent, emergence of seeds treated only with phosphate
buffer did not differ significantly from seeds treated with Pf-5 ($P = .068$). Soil was infested with 5.0 propagules per 100 g of soil for all subsequent experiments.

5.3.2 Development of a dose response assay.

**Dose-response experiment 1.** When emergence of cotton as a function of Pf-5 inoculum density ($2.6 \times 10^8$ cfu/ml to $2.6 \times 10^{11}$ cfu/ml) was plotted on an arithmetic scale (Figure 5-2), it appeared that control of preemergence damping-off caused by *R. solani* reached a maximum near an inoculum dose of $2.6 \times 10^{10}$ cfu/ml and did not increase further with increasing dose. Emergence was not significantly different among seedlings treated with these doses of Pf-5 ($P = .055$). Emergence of cotton seedlings was higher than expected (65±7%) for the buffer treated control in comparison to previous results (39±6% in Figure 5-1).

**Dose-response experiment 2.** Pf-5 and the gacS mutant were compared for suppression of *Rhizoctonia solani* damping-off, over a 100,000-fold range of inoculum densities. The trend was a slight increase in emergence as inoculum density of Pf-5 increased, whereas emergence did not increase with increasing inoculum density of the gacS mutant. Emergence of seedlings treated with Pf-5 differed significantly ($P \leq .05$) from emergence of seedlings treated with the gacS mutant only at the two highest inoculum doses of the strains (Figure 5-3). For seeds treated with Pf-5, maximum emergence (89±5%; Figure 5-3) occurred at the highest initial inoculum ($7 \times 10^9$ cfu/ml), which was comparable to the inoculum dose previously found to give maximum emergence (85±5% at an inoculum density of $2.6 \times 10^{10}$; Figure 5-2). Mean emergence for the control without *R. solani* was 97±2%.
Figure 5-1. Emergence of cotton over time as affected by the inoculum density of *R. solani* J1 in the presence (+ Pf-5) or the absence (- Pf-5) of *P. fluorescens* Pf-5. Soil was infested with 0 (A), 2.5 (B), or 5 (C) propagules of *R. solani* per 100 g soil. Inoculum density of Pf-5 was 6 x 10^7 cfu/ml. Propagules of *R. solani* were determined by dilution plating of soil samples as described in section 5.2.3. Emergence was defined as cotyledons having cleared the soil surface. Emergence data are the means of 12 replicate pots each containing nine seeds. Error bars represent standard errors around treatment means.
Figure 5-2. Emergence of cotton from soil infested with *R. solani* (5 propagules per 100 g of soil) as affected by increasing initial inoculum doses of *P. fluorescens* Pf-5. Initial inoculum doses of Pf-5 were $2.6 \times 10^8$, $2.6 \times 10^9$, $5.2 \times 10^9$, $2.6 \times 10^{10}$, and $2.6 \times 10^{11}$ cfu/ml. Mean emergence is plotted on an arithmetic scale to display the asymptotic nature of the response (emergence) with respect to increasing dose of Pf-5. Emergence was defined as cotyledons having cleared the soil surface. Emergence data are the means of 12 replicate pots each containing nine seeds. Emergence was recorded at 11 days after planting. Error bars represent standard errors around treatment means.
Figure 5-3. Emergence of cotton from soil infested with *R. solani* (5 propagules per 100 g of soil) as affected by increasing initial inoculum doses of the wild type (JL4092) and the antibiotic deficient mutant (JL4135) of *P. fluorescens* Pf-5. Emergence data are the means of 10 replicate pots each containing nine seeds. A positive control treatment (+) pathogen (-) biocontrol was not included in the experiment. The (-) pathogen (-) biocontrol treatment is indicated by the open diamond. Emergence was recorded at 11 days after planting. Error bars represent standard errors around treatment means.
**Dose-response experiment 3.** The dose-response comparison between the Pf-5 and the gacS mutant was repeated, and a negative control (buffer-treated seed planted in infested soil) was added to the experimental design (Figure 5-4). There was no statistical difference \((P > .05)\) in seedling emergence among the four doses of Pf-5 that were applied to seeds. Two of the doses \(4 \times 10^5\) cfu/ml and \(4 \times 10^9\) cfu/ml were not statistically different \((P > .05)\) from seeds treated with the gacS mutant. Increasing the inoculum dose of the gacS mutant did not increase seedling emergence either \((P > .05)\). Because there were no differences between doses of Pf-5 or doses of the gacS mutant, the emergence data from all of the Pf-5 doses were combined and the emergence data from all of the gacS doses were combined. When the treatments were combined in this manner, there was a significant overall difference between the treatment with Pf-5 and the gacS mutant \((P = .001)\). The mean emergence was 63±3\% for the Pf-5 treatment and 44±4\% for treatment with the gacS treatment when averaged across all inoculum doses. Emergence of seedlings treated with the gacS mutant did not differ significantly from emergence of seedlings treated with buffer alone \((40±6\%)\). Mean emergence for the control without *R. solani* was 96±3\%. 
Figure 5-4. Emergence of cotton from soil infested with *R. solani* (5 propagules per 100 g of soil) as affected by increasing initial inoculum doses of the wild type (JL4092) and the antibiotic deficient mutant (JL4135) of *P. fluorescens* Pf-5. Plots for JL4092 and JL4135 have been extended to the control treatment (+) pathogen (-) biocontrol indicated by the open circle for ease of comparison. The open diamond indicates the (-) pathogen (-) biocontrol treatment. Control treatments have been offset from zero on the x axis. Emergence data are the means of nine replicate pots each containing nine seeds. Emergence was recorded at 11 days after planting. Error bars represent standard errors around treatment means.
5.3.3 Effect of initial inoculum density on spermosphere and rhizosphere colonization by Pf-5.

**Population density experiment 1.** Preliminary data suggested that Pf-5 and the gacS mutant reached similar population densities at the hypocotyl-root transition zone six days after planting although initial inoculum densities ranged from $10^4$ to $10^8$ cfu/ml for both strains (Figure 5-5b). Population densities of Pf-5 and its gacS mutant recovered from seeds, immediately after the bacteria were applied, were significantly related to density of the bacterial suspensions in which they were dipped ($P = .0016$ and $P = .015$, respectively; Figure 5-5a). Analysis using the general linear means model indicated that there were no significant differences among the populations of either Pf-5 or the gacS mutant at the hypocotyl-root transition zone 6 days after planting for any inoculum dose, despite a 10,000-fold difference in the initial inoculum doses ($P = .1586$ and $P = .3060$, respectively; Figure 5-5b).

**Population density experiment 2.** Inoculum densities of $10^5$ and $10^{10}$ cfu/ml resulted in populations of Pf-5 on cotton seeds of approximately $10^3$ and $10^8$ cfu/seed, respectively, indicating that the seed received approximately 10 µl of inoculum. Two days after planting, the density of Pf-5 on seeds treated with the highest dose ($10^{10}$ cfu/ml) had decreased to $6 \times 10^7$ cfu/seed (Figure 5-6). Population densities of Pf-5 on seeds treated with the middle dose ($10^5$ cfu/ml) increased to $2 \times 10^7$ cfu/seed; and seeds treated with the low dose ($10^2$ cfu/ml), which were not detectable on the day of planting, increased to $1 \times 10^5$ cfu/seed. Small increases in population densities for the two lower doses were observed from day 2 to day 3, after which population densities did not change. Pf-5 was recovered from all three replicate sections of hypocotyls and roots at the high and middle inoculum doses but was recovered from only one of three replicates for the low inoculum dose (data not shown). No fluorescent pseudomonads were recovered on the selective medium (KB amended with 100 µg/ml cyclohexamide and 50 µg/ml streptomycin) from control seeds treated only with buffer. The experiment was repeated once with similar results (data not shown).
Figure 5-5. Effect of increasing the initial dose of the wild type (JL4092) and the antibiotic-deficient derivative (JL4135) of *P. fluorescens* on population densities recovered from the spermosphere and rhizosphere of cotton. Population densities were enumerated by the method described in section 5.2.5. Initial populations on seeds (day0) were determined before seeds were planted in soil. Rhizosphere populations (day 6) were recovered from a 2.5-cm section centered on the root-hypocotyl transition zone at 6 days after planting. Population densities are the means of two replicates. Error bars represent standard errors around treatment means.
Figure 5-5.
Figure 5-6. Population densities of *P. fluorescens* Pf-5 (JL4092) recovered over time from the spermosphere and rhizosphere of cotton, as affected by increasing initial inoculum dose Pf-5. Spermosphere populations are the means of 5 replicates. Error bars represent standard errors around treatment means.
Population density experiment 3. When seeds were inoculated with doses of $10^2$, $10^4$, $10^6$, and $10^{10}$ cfu/ml of Pf-5, population densities in the spermosphere converged over time such that the mean densities of bacteria recovered after 3 days ranged from $1 \times 10^7$ to $5 \times 10^7$ cfu/seed (Figure 5-7). Bacterial densities recovered from seeds at day 0 were higher than expected for the two lower doses ($3 \times 10^3$ and $6 \times 10^3$ cfu/seed for doses of $10^2$ and $10^4$ cfu/ml, respectively) as compared to previous results (Figures 5-6). Populations on seeds treated with the other doses were approximately 100-fold lower than that of the inoculum and were similar to previous results. Mean population densities, on seed surfaces, peaked two days after planting (Figure 5-7). On 6-day-old seedlings, the incidence and population densities recovered from roots were higher than those from hypocotyls (data not shown).

5.3.4 Relationship between inoculum dose and population densities over time when a Rifampicin resistant derivative of Pf-5 is applied alone or in a mixture with the gacS mutant.

When seeds were inoculated, solely with increasing doses of the spontaneous rifampicin resistant derivative of Pf-5, populations peaked at day 2 and declined slightly at day 3 (Figure 5-8). At three days after planting, population densities on seeds were similar ($4 \times 10^7$ to $8 \times 10^7$ cfu/seed for lowest and highest doses, respectively) and were not statistically different ($P \geq .05$) despite a 100,000-fold difference in initial population densities. In contrast, when varying doses of the rifampicin resistant derivative of Pf-5 were applied in a mixture with the gacS mutant at $2 \times 10^9$ cfu/ml, population densities of the rifampicin resistant derivative of Pf-5, which were recovered from seeds, remained significantly different from each other ($P \geq .05$), and corresponded to initial inoculum doses. The experiment was repeated once with similar results (data not shown).
Figure 5-7. Population densities of *P. fluorescens* Pf-5 (JL4092) recovered over time from the spermosphere and of cotton, as affected by increasing initial inoculum dose Pf-5. Spermosphere populations are the means of 5 replicates. Error bars represent standard errors around treatment means.
Figure 5-8. Population densities of *P. fluorescens* Pf-5 (JL3871) recovered from the spermosphere of cotton over time in the presence (JL3871 in a mixture) or absence (JL3871 alone) of the *gacS* mutant JL4135 (10⁹ cfu/ml). Phenotypes were JL3871 (Plt⁺Prn⁺Phl⁺Rif⁺) and JL4135 (Plt Prn Phl Kan⁺). Detection limit was 10² cfu/seed. Populations below the detection limit were assigned a value of 10¹ cfu/seed for computing the mean population densities. Populations are the means of five replicates. Error bars represent standard errors around treatment means.
5.4 Discussion

Modeling of the epidemiological parameters of plant-pathogen interactions has proven useful in understanding dose-response relationships in plant disease. Extension of these principles to biological control systems will further our understanding of the complex interactions between the host, pathogen, and biological control agent. The biological control model proposed by Johnson (1994) predicts a nonlinear relationship between biocontrol dose and disease suppression. At high doses of the biocontrol agent, further increases in biocontrol inoculum are predicted to show diminishing increases in disease suppression as an asymptote is approached. The biological control system used in these studies, *Pseudomonas fluorescens* Pf-5-mediated control of *Rhizoctonia* pre-emergence damping-off of cotton, proved to be a difficult system with which to evaluate this model due to variability intrinsic to the disease assay.

5.4.1 Variations in incidence of damping-off of cotton caused by *Rhizoctonia solani*.

There was extreme variability in emergence of cotton between experiments in which soil was infested with equivalent levels of *R. solani*. In two experiments, emergence for non-treated seeds was low (<40%); while in another experiment emergence for non-treated seeds was high (>60%). This variability in emergence may have been caused by several factors, as described below.

5.4.1.1 Lack of a standard inoculum of *R. solani*.

Developing a standard inoculum source for *R. solani* was problematic because the fungus does not produce distinct propagules such as conidia or basidiospores. Instead, it produces a sclerotium-like structure composed of compact masses of
shortened hyphae called monilioid cells in the center, and looser, longer hyphae towards the margin. Because these sclerotial structures are not surrounded by a rind and are not separable from host tissue (Butler and Bracker, 1970), inoculum of *R. solani* typically is produced by growing the fungus on plant tissue or agar medium followed by drying and grinding the resultant hyphal mass (Van Bruggen et al., 1985; Lewis et al., 1990; Wang and Davis, 1997) to produce a complex inoculum consisting not only of pathogen but the medium on which it is grown. This type of complex inoculum is difficult to standardize so the inoculum, used in my experiments, was prepared using the method developed by Van Bruggen (1985) because it was reported that this method converted nearly all the bean tissue, on which the fungus was grown, into sclerotia making separation of the sclerotia from the bean tissue unnecessary. Unfortunately, the isolate of *R. solani* used in these studies did not completely convert bean tissue to sclerotia and it was impossible to separate sclerotia from the remnants of bean tissue. Thus, it was difficult to obtain reproducible levels of disease in the absence of a standard inoculum of *R. solani* obtaining reproducible levels of disease difficult.

5.4.1.2 Virulence of *R. solani* J1.

*R. solani* J1, the isolate used in these experiments, is extremely virulent on cotton, so a low pathogen density (5 propagules/100 g soil) caused 40-60% damping-off on nontreated seed. This pathogen dose-disease response is similar to that reported by Wang et al. (1997), where 9 cfu/100 g soil of a different isolate of *R. solani* AG-4 caused 57% damping-off of cotton. In those experiments, the inoculum was produced by blending cultures grown on potato dextrose agar to produce propagules of undefined size, whereas the inoculum used in my experiments was grown on green beans and the size of the propagule was defined. Also, the relative virulence of the isolate of *R. solani* used in those experiments, with respect to *R. solani* J1, is not known. Therefore, the comparison between these two systems is tenuous because virulence of *R. solani* is dependent on the size of the propagule and the quantity and quality of the medium on which this fungus is grown (McCoy and Kraft, 1984;
Weinhold et al., 1969; Weinhold et al., 1972; Van Bruggen et al., 1985). Nevertheless, the comparison between these two systems is useful because it indicates that the low density of pathogen, such as that used in my experiments, relative to the amount of disease is characteristic of the pathogen dose-disease response for preemergence damping-off caused by *R. solani*.

Another factor that may have influenced the relative aggressiveness of *R. solani* was the pasteurized soil used in my experiments. The soil, in this study, was pasteurized because of the uncharacterized nature of the soil microflora. By eliminating a portion of the indigenous microbes, the pasteurization process may increase the aggressiveness of *R. solani* in a number of ways including: 1) a release of nutrients into the soil that these microbes contain, which *R. solani* can utilize, and 2) a reduction in microbial competitors of *R. solani*. The virulence of the pathogen in raw soil was not evaluated, so the extent to which pasteurization may have affected the virulence of the inoculum is unknown.

5.4.1.3 Variations in proximity of pathogen propagules from seed and seedling.

Methods of incorporating of *R. solani* into soil vary from homogeneous mixing (Papavizas and Ayers, 1965; Ko and Hora, 1971; Wang et al., 1997) to applying a defined amount of pathogen directly to the furrow in which the cotton seed will be planted (Howell and Stipanovic, 1979). Previous studies evaluating Pf-5 for biocontrol of Rhizoctonia damping-off of cotton were done by planting cotton in furrows in flats and inoculating with the pathogen by the latter method (Howell and Stipanovic, 1979). Due to space constraints, the pathogen was homogeneously incorporated into the soil, in these experiments. In retrospect, this may have been a mistake. Because of the high virulence of *R. solani* J1 inoculum, an average of 22 propagules of the fungus per pot caused a disease incidence of 40-60%. It seems plausible, therefore, that small variations in the amount and the dispersal of pathogen in a pot could cause the differences in disease severity observed between experiments.
5.4.1.4 Genetic heterogeneity of the host.

Stoneville 213 cotton seed, a highly susceptible cotton variety used in this study (Howell personal communication), was contaminated with approximately 10% of another variety. The contaminating cultivar was identified because of the red pigmentation that developed in the hypocotyl as the seedling matured. The hypocotyls of some seedlings appeared to have a light pink pigmentation, indicating that these may have been crosses between the two varieties. The source of the seed used in this study may have been a border row between two varieties of cotton (Howell personal communication). No differences in the susceptibility of Stoneville 213 and the contaminating cultivar are known (Howell personal communication) so the effect of the genetic heterogeneity of the host on disease incidence is unknown.

5.4.2 A threshold dose of Pf-5 for the biological control of Rhizoctonia damping-off of cotton was not identified.

The hypothesis that dose-response relationships for the biological control of Rhizoctonia preemergence damping-off of cotton would show diminishing increases in disease supression with increasing concentrations of the bacterial antagonist was apparent when biological control doses were plotted on an arithmetic scale. When the data from two of the dose-response experiments were plotted on a log scale no such limit to biological control was apparent (data not shown). In the third dose-response experiment a possible asymptote was evident even when biological control doses were plotted on a log scale. Although a possible asymptote for biological control was indicated in all three dose-response experiments, the doses of Pf-5 at which this occurred varied by seven orders of magnitude.

The degree of biological control achieved with Pf-5 in these experiments was similar to the level previously reported by Howell and Stipanovic (1979), who observed an increase in emergence from approximately 40% to 80% when seeds were treated with a relatively high dose of Pf-5. Although this is a substantial increase in
emergence compared to the control, the variability within the system made it difficult to obtain informative data points without prohibitively large numbers of replicates for each dose. This difficulty was encountered in other systems in which dose-response relationships for biological control have been investigated. For example, in modeling the relationship between the dose of *Bacillus cereus* UW85 and suppression of Pythium damping-off of tomato, more than 600 seeds were required to quantify pathogen and biocontrol parameters for one dose of the pathogen (Smith et al., 1997). In another set of studies evaluating biological control of crown gall of tomato and cherry with *Agrobacterium radiobacter* K84, a biological control system in which control of pathogen strains sensitive to the antibiotic produced by *A. radiobacter* K84 is nearly complete, 800 tomato and 1440 cherry seedlings, respectively were required for assessing the biological control dose-response relationship for a single density of the pathogen (Johnson and DiLeone unpublished). From the data obtained in this study, it is estimated that, given the facilities available, a prohibitively large number of replicates, would be required to determine parameters for the biological control agent and pathogen necessary for an evaluation of the dose-response model.

5.4.3 A *gacS* mutant of Pf-5 did not suppress damping-off of cotton caused by *R. solani*.

Emergence of cotton from soil infested with *R. solani* was not improved by inoculating seed with the *gacS* mutant at any inoculum dose. These data are consistent with a role for the antifungal compounds produced by Pf-5 in disease suppression. The role of these metabolites cannot be concluded from the results presented in this work, however, due to the pleiotropic nature of the *gacS* mutation. As described previously (Chapter 1; Corbell and Loper, 1995), *gacS* mutants do not produce a number of compounds that may affect biological control, including pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, hydrogen cyanide, and an extracellular protease. In addition, *gacS* mutants are compromised in other activities, such as stress response (Whistler et al., 1998), quorum sensing (Wood et al., 1997), and uncharacterized
activities, that could affect their efficacy as biological control agents. Of the compounds produced by Pf-5, pyrrolnitrin (Howell and Stipanovic, 1979) and 2,4-diacetylphloroglucinol (Keel et al., 1992) are known to inhibit \textit{R. solani}. Mutants specific for either compound were not available at the time these studies were conducted. The biosynthetic genes for pyrrolnitrin (Hammer et al., 1997) and 2,4-diacetylphloroglucinol have recently been identified (Bangera and Thomashow, 1996) so it would be possible now to create the specific mutants that would unequivocally define the role of these compounds for biological control activity. Population size of Pf-5 and the \textit{gacS} mutant in the rhizosphere were not correlated to the initial inoculum.

5.4.4 Population size of Pf-5 and the \textit{gacS} mutant in the rhizosphere were not correlated to the initial inoculum.

All parts of the cotton seedling are susceptible to infection by \textit{R. solani}, but seeds and the root-hypocotyl transition zone are particularly susceptible (Hayman, 1969; this study). Therefore, seeds and the root-hypocotyl transition zone, were selected to monitor bacterial densities because disease suppression is likely dependant on colonization of these sites by Pf-5. These studies showed that Pf-5 and its \textit{gacS} mutant established similar population densities at the root-hypocotyl transition zone even when initial inoculum densities varied 10,000 fold. Incidence of recovery of the introduced bacteria from the seedlings indicated that roots were more likely to be colonized than hypocotyls, and that Pf-5 was more likely to become established when inoculated at higher rather than at lower initial doses. For example, an initial inoculum density of $10^2$ cfu/ml typically resulted in an initial population density of Pf-5 on seeds that was below the threshold for detection and the incidence of recovery of Pf-5 from hypocotyls and roots was only one of three, whereas the incidence of recovery for the higher doses $10^5$ and $10^{10}$ was three of three. However, since a dose of $10^2$ cfu/ml would result in an average of one bacterial cell per seed, it is not difficult to imagine that there was an insufficient density of Pf-5 in this low inoculum dose to
assure establishment on the seeds. The ability the biocontrol bacteria, applied at a low inoculum density, to multiply and reach similar doses at the infection court as that bacteria applied at higher initial densities implies that the seed and seedling have a carrying capacity for Pf-5. Thus, the carrying capacity of a seed or seedling for a biological control agent may be one of the factors contributing to an asymptote in biological control.

There was no evidence that the gacS mutant differed from Pf-5 in colonization of seeds or the root-hypocotyl transition zone. This lack of difference in colonization was expected, since a mutation in gacA, the second component in the two-component system, of which gacS is the first component, has no effect on rhizosphere competence in a closely related strain of Pseudomonas fluorescens (Natsch et al., 1994).

5.4.5 Population size of Pf-5 on the spermosphere increases over time when initial inoculum doses are below the carrying capacity of the seed.

Population densities of Pf-5 generally increased when the initial inoculum resulted in spermosphere densities below $10^8$ cfu/seed, and decreased when the initial spermosphere density was greater than $10^8$ cfu/seed, indicating that $10^8$ cfu/seed may represent a carrying capacity for Pf-5 on cotton seed. It may seem rather trivial that population densities increased over time when low initial doses were applied, since bacteria typically reach equivalent populations over time in culture, irrespective of the initial population. The findings in these studies are similar to the results obtained by other researchers in which populations of rhizobacteria were independent of initial inoculum density. In those studies either a gnotobiotic sand system (Bennett and Lynch, 1981) or a sand soil mixture (Scher et al., 1984) was used. These results contrast investigations evaluating rhizosphere populations in field soil in which rhizosphere populations were dependent on the initial inoculum level of the bacteria (Loper et al., 1985; Dupler and Baker, 1984). It must be stressed, however, that in field soil other microbes competing for the available nutrients limit the ability of the introduced microbe to multiply and fill the available niche. For example, the
population density of *Pseudomonas putida* N-1R was significantly lower when soil was moistened 1 week prior to the experiment as opposed to soil that was stored dry and moistened the day of the experiment (Dupler and Baker 1984). It was proposed that this reduction in rhizosphere colonization might be due the nutritional status of the soil, competitors, or predators detrimental to *Pseudomonas species*. Presumably, the time between moistening the soil and planting allowed the indigenous microflora to proliferate and sequester nutrients that otherwise would have been available to the introduced species. The soil used in the studies presented herein was pasteurized and stored dry until the day of the experiment. The effect of this was not determined but it is interesting to speculate that the increases in population densities of Pf-5 that were observed in this study, may not have occurred in a natural soil environment where the indigenous microflora might be expected to limit the population growth of Pf-5.

5.4.6 Coinoculation with the *gacS* mutant resulted in a correlation between initial inoculum dose and population size of Pf-5 that was stable over time.

A method for maintaining a separation of Pf-5 populations established on the plant from various doses is needed if a dose-response relationship is to be observed in this system. This was accomplished by coinoculating seeds with a spontaneous rifampicin resistant (Rif') derivative of Pf-5 and a *gacS* mutant, a kanamycin resistant derivative of Pf-5 that is as effective as Pf-5 at colonizing the seeds and rhizospheres of cotton plants but has no effect on disease suppression (this study). The kanamycin and rifampicin resistance markers made it possible to differentiate the populations of these two strains at any given time.

Coinoculation of varying doses of the Rif' derivative of Pf-5 with a high dose of the *gacS* mutant, proved that separation of initial inoculum doses for the spontaneous mutant could be maintained in the spermosphere over time. Spontaneous rifampicin-resistant mutants, such as the one used in place of the wild type in the coinoculation experiments, provide a useful marker for tracking specific bacterial
strains in natural habitats. Before using such mutants in a biological control assay it is
wise to determine that the mutant is not also a mutant in any of the properties thought
to be important for its activity as a biocontrol agent. This is especially important in
light of the recent discovery that a significant proportion of such spontaneous mutants
are deficient in DNA mismatch repair systems (Mao et al., 1997) and can therefore
exhibit a high frequency of mutation. The problem with using such mutator strains is
that the phenotype can be unstable and consequent effects on activities important for
biological control are unknown. The spontaneous Rif derivative, used in place of the
wild type in this study exhibited wild-type growth rates and antibiotic profiles.

5.4.7 **Recommendations for future research.**

The evidence presented here clearly demonstrates that the dose of the
antagonist population in the infection court at the time when infection occurs must be
varied to evaluate a biocontrol dose-plant disease response relationship.

The use of indigenous or introduced microflora to maintain proportionality
between the initial dose of a biological control strain and populations in the infection
court may prove useful in determining dose-response relationships in model systems.
Several studies, in which biocontrol strains have been paired, have demonstrated the
effect on the population of the antagonist of interest is related to the species with
which the antagonist is paired (Fukui et al., 1994a; Stockwell et al., 1996). Therefore,
it is likely that closely related species will be required to suppress population growth
of the wild-type biological control agent since these strains are more likely to occupy
the same niches as the parental strain.

The neccessity of using another microorganism to maintain separation of doses
of a biocontrol agent in the infection court will likely be dependent on the time
between planting and infection by the pathogen. For example, infection of seeds by
*Pythium* species is known to occur within 24 hours of planting in infested soils
(Nelson et al., 1986). Because the population of Pf-5 on cotton seeds 24 hrs after
planting was still proportional to the initial inoculum dose, coinoculation with another strain would be unnecessary in a dose-response assay in which *Pythium* was the pathogen. Indeed, it has been shown for several *Pseudomonas* species that a high initial inoculum density of the antagonist resulted in significantly fewer pericarp invasions of sugar beet seed over that of a low initial dose even though the population densities on the spermosphere reached similar densities by 24 hours (Fukui et al., 1994b). The experiments performed in this study were not designed to determine the time of infection by *R. solani* and so the importance of maintaining a separation in doses over time is not known.

5.5 Conclusion

Asymptotic limits to biological control of Rhizoctonia damping-off of cotton were observed with the biological control agent *P. fluorescens* Pf-5. Antibiotic production by Pf-5 appeared to influence the maximum degree of disease control, although these results should be interpreted with caution due to the pleiotropic nature of the *gacS* mutant. Populations of Pf-5 tended to converge at a possible carrying capacity of the seed when the bacteria was applied at a range of initial doses. Therefore, favorability of the environment for antagonist growth is likely to be one reason for the lack of increasing disease control with increasing dose of the biological control organism.
Chapter 6. Conclusion

The results presented in this dissertation have demonstrated that two genes, \textit{apdA} (= gacS) and \textit{gacA}, in the genome of the biological control bacterium \textit{P. fluorescens} Pf-5 comprise a two-component signal transduction system for the regulation of secondary metabolites produced by this organism. ApdA (GacS) is the sensor kinase and GacA is the response regulator in this two-component system. The ApdA/GacA system also has been identified in pathogenic and biological control species of \textit{Pseudomonas}. As in other \textit{Pseudomonas} spp., mutations in these genes typically abolished secondary metabolite production by \textit{P. fluorescens} Pf-5. However, a point mutation was identified in the extreme C-terminus of GacA that affected production of only a subset of secondary metabolites. This mutation was downstream of the helix-turn-helix DNA-binding domain and may constitute a domain that interacts with the \( \alpha \) subunit of RNA polymerase.

Because GacA is presumed to regulate transcription of target genes via binding of the helix-turn-helix DNA-binding domain, a fusion protein containing the DNA-binding domain of GacA was used in an assay in an attempt to identify the target genes directly controlled by GacA. To create a pool of possible GacA target sites random fragments of either synthetic DNA or chromosomal DNA were prepared. Following preparation of these DNA pools, a cycle selection procedure was developed for amplification and identification of putative GacA target sites from these pools of DNA fragments. Although a putative binding site for GacA was identified using the cycle selection procedure, the results were inconclusive due to several inconsistencies in the DNA-binding assay. The appearance of a shifted band in the GST control lane of approximately the same mobility as the GST-GacAC fusion, the lack of a supershift when anti-GST antibody was used in EMSA analysis, and the inability of GST-GacAC to complement the \textit{gacA::aphI} mutant of Pf-5 suggests that the observed binding may have been due to a protein that was co-purified with GST-GacAC. The upstream region of only one gene, which codes for a putative porin, was identified as a
putative binding site for GacA by the cycle selection procedure. Studies initiated to
determine whether gacA regulates transcription of this putative porin gene have been
unsuccessful, to date, so it remains unclear whether GacA regulates this gene. The
identification of only one putative GacA-regulated gene was disappointing since the
justification for the cycle selection procedure was that it should have the ability to
identify numerous genes regulated by GacA. The failure to identify several potential
GacA-regulated genes, however, does not indicate that the cycle selection procedure is
a poor approach for identifying such sequences, but rather that the execution of the
procedure was not what it could have been. Hopefully, the mistakes made in this
study and knowledge gained therefrom will be useful in the design of future.

An apdA (gacS) mutant was also used to evaluate a dose-response model for
biological control of soilborne plant pathogens. Asymptotic limits to biological
control of Rhizoctonia damping-off of cotton were observed with the biological
control agent P. fluorescens Pf-5. Antibiotic production by Pf-5 appeared to influence
the maximum degree of disease control, although these results should be interpreted
with caution due to the pleiotropic nature of the gacS mutant. Populations of Pf-5
tended to converge at a possible carrying capacity of the seed when the bacteria were
applied at a range of initial doses. Therefore, favorability of the environment for
antagonist growth is likely to be one reason for the lack of increasing disease control
with increasing dose of the biological control organism. Recommendations for further
research are given in the discussion sections of Chapters 4 and 5 (sections 4.4.5 and
5.4.7)
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