AN ABSTRACT OF THE THESIS OF

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Title: Effect of Iron on Biological Control of Fire Blight by *Pseudomonas fluorescens* A506.

Abstract approved:

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Competitive exclusion has been the mechanism hypothesized to account for the biological control of fire blight disease of pear and apple by the bacterium *Pseudomonas fluorescens* A506 (A506). Recent laboratory assays demonstrated, however, that A506 produces an antibiotic that is toxic to the fire blight pathogen, *Erwinia amylovora*, when cultured on media amended with iron (Fe^{+2} or Fe^{+3}). This study investigated this iron-dependent antibiosis by A506 by: 1) examining bioavailability of iron to A506 on blossom surfaces, 2) mutagenizing A506 to disrupt genes involved in antibiotic production, and 3) evaluating suppression of fire blight by A506 when co-treated with an iron chelate (FeEDDHA). Bioavailability of iron on blossoms was investigated with an iron biosensor [iron-regulated promoter (*pvd*) fused to an ice nucleation reporter gene (*inaZ*)] in A506. A506 (*pvd-inaZ*) expressed high ice nucleation activity (INA) on blossoms indicating a low-iron environment unlikely to induce antibiosis by A506.

Spraying blossoms with FeEDDHA at concentrations $\geq$ 0.1 mM significantly suppressed INA by A506 (*pvd-inaZ*).

Transposon mutagenesis was used to generate and select mutants of A506 exhibiting altered antibiotic production profiles. One antibiotic-deficient mutant, A506 Ant^-, was recovered; this mutant showed reduced epiphytic fitness on blossoms of apple and pear trees compared to the parent stain, A506. Another mutant, A506 Ant^+, lost the characteristic fluorescent phenotype and exhibited iron-independent antibiotic production in defined culture media. A506 Ant^+ established high populations on blossoms of apple and pear trees, similar to populations attained by A506, and reduced incidence of fire blight between 20 to 40%, levels comparable to A506 in orchard trials.

In orchard trials, A506 was co-treated with FeEDDHA and fire blight suppression was evaluated. Bacterial strains established high populations on blossoms when co-treated with 0.1 mM FeEDDHA or in water. Significantly enhanced suppression of fire blight incidence by antibiotic producing strains of A506 amended with 0.1 mM FeEDDHA was observed in 2 of 5 trials, providing some evidence that iron-induced antibiosis can be a contributing mechanism in disease control. Lack of disease control by the antibiotic deficient strain, A506 $GacS^-$, and by 0.1 mM FeEDDHA alone added support to this hypothesis.

Effect of Iron on Biological Control of Fire Blight by *Pseudomonas fluorescens* A506

By
Todd N. Temple

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CONTRIBUTION OF AUTHORS

Drs. Kenneth Johnson and Virginia Stockwell provided ideas for experiments, and were instrumental in planning the designs, and aided in data analysis, the writing and editing of the thesis. Dr. David Sugar assisted in fire blight orchard experiments in Medford, OR. Dr. Joyce Loper provided ideas, advice and laboratory supplies and space for this project.

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Chapter 1

Intoduction

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Not for publication

FIRE BLIGHT

***Biology*:** The bacterium *Erwinia amylovora* [(Burr.) Winslow et al.] causes fire blight, a serious disease of pome fruits in many regions of the world including the Pacific Northwest of the United States (van der Zwet and Kiel, 1979). The name, "fire blight," describes the foliar symptoms of the disease on pear (*Pyrus*) and apple (*Malus*), that include a rapid dieback and blackening of twigs, flowers, and foliage "as though burnt by fire" (van der Zwet and Kiel, 1979). The dieback of host tissues can be extensive, resulting in devastating economic losses. In southern Oregon, a severe outbreak of fire blight in 2002 resulted in removal of hundreds of pear trees (D. Sugar, *personal communication*).

Fire blight is considered an "annual" disease, meaning that the disease cycle is initiated as new infections each spring as opposed to re-initiation of disease from expansion of infections formed in the preceding season. The disease cycle is initiated when cells of *E. amylovora* are transferred from holdover cankers (infections from the preceding season) to a small proportion of blossoms where the pathogen grows as an epiphyte. Epiphytic growth is defined as reproduction on a plant or plant organ without causing disease. For *E. amylovora*, this epiphytic growth occurs principally on the floral stigmas on the tips of the styles (Thomson, 1986). On the stigmas, the pathogen multiplies to attain populations of 10^5 to 10^7 viable cells or 'colony forming units' (CFU) per flower (Thomson, 1986). Significantly, once established on some blossoms, epiphytic populations of *E. amylovora* can be redistributed to additional flowers by

insects, such as honey bees (Johnson et al., 1993a; Johnson et al., 1993b; Nuclo et al., 1998; Thomson et al., 1992), or through rain splash or wind (McManus and Jones, 1994a; Pusey, 2000; Stockwell et al., 1999; Thomson, 1986). The most severe epidemics of fire blight are usually preceded by a series of warm days that accelerate the growth rate of *E. amylovora* (Thomson et al., 1982) and increase the number of insect visitations to blossoms (Johnson, 1993a; Kiett and Ivanoff, 1941; van der Zwet and Keil, 1979; de Wael et al., 1990).

Infection of a blossom occurs shortly after a rain or dew event washes epiphytic cells of *E. amylovora* from stigmas onto the hypanthium (Thomson, 1986). The rain or dew also dilutes nectar sugars to concentrations that permit growth of *E. amylovora* on the hypanthial surface (Keitt and Ivanoff, 1941; Pusey, 1999). *E. amylovora* enters the plant through secretory openings termed 'nectarthodes' that are on the hypanthium (Wilson et al., 1989). Inside the plant, *E. amylovora* reproduces rapidly and spreads through host tissue intercellullarly and through vascular elements (Bogs et al., 1998). Wilting, necrosis, and ooze formation are symptoms associated with infection of blossoms by *E. amylovora* (van der Zwet and Kiel, 1979). Once symptoms are visible, removing the advancing necrotic infection is the only effective means of limiting disease progression within the plant. Consequently, the suppression of epiphytic growth and prevention of floral infection is a focal point in fire blight management for two reasons: first, the epiphytic phase occurs before any host infection or damage is initiated, and second, the activity of the pathogen during the bloom period has proven

amenable (sensitive) to cost-effective control technologies (van der Zwet and Kiel, 1979).

Fire blight suppression: Suppression of floral infection by *E. amylovora* has been attained by topical application of chemical agents, notably the antibiotic streptomycin which came into use in 1950 (Ark, 1953). Over the years, however, effectiveness of streptomycin to control fire blight in many pear and apple production regions has been compromised by the development of strains of *E. amylovora* resistant to this antibiotic (Loper et al., 1991; McManus and Jones, 1994b; McManus et al., 2002; Moller et al., 1981; Stockwell et al., 1996a). Oxytetracycline and fixed copper compounds are other chemicals that have been used for fire blight suppression but are considered to be only partially effective (McManus and Jones, 1994b; McManus, 2002, Stockwell et al., 2002a).

The lack of effective chemical products for fire blight suppression has stimulated research into the use of biological methods for control of this disease. Biological control is defined as "the reduction in the amount of inoculum or disease producing activity of a pathogen by or through one or more organisms other than man" (Cook and Baker, 1983). The first report of biological control of fire blight is in 1930, when an isolated yellow bacterium controlled the disease (Beer and Rundle, 1984). Subsequently, fluorescent pseudomonads, *Pantoea agglomerans*, *Bacillus* spp., and yeasts have been isolated from stigmatic surfaces of pear and apple trees (Isenbeck and Schulz, 1985; Kearns and Hale, 1993; Lindow, 1985; Manceau et al., 1990; Stockwell et al., 1999) and investigated for their ability to suppress epiphytic growth of *E.*

amylovora. Remarkable to negligible levels of control of fire blight have been observed (Wilson and Lindow, 1993; Lindow, 1985: Lindow et al., 1996), which has led to investigations of the properties and mechanisms utilized by the effective strains to achieve disease suppression. Mechanisms hypothesized to contribute to effective suppression of *E. amylovora* include 'competitive exclusion' and 'antibiosis' (Cook and Baker, 1983).

BIOLOGICAL CONTROL

***Competitive exclusion*:** When two organisms compete with one another within the same ecological niche, exclusion of one organism can occur. Critical requirements for 'competitive exclusion' are shared nutrient resources and shared habitats for growth and survival (Beattie and Lindow, 1995; Cook and Baker, 1983; Kinkel and Lindow, 1993; Lindow, 1985; Wilson and Lindow, 1994). In scanning electron micrographs, Hattingh et al. (1986) and Wilson et al. (1992) have shown that some non-pathogenic, bacterial epiphytes (e.g. *P. agglomerans*) colonize the same microsites on the surface of the stigma that are colonized by *E. amylovora*. Hattingh et al. (1986) proposed that *P. agglomerans* suppresses growth of *E. amylovora* by competition for sites on stigmas. From the perspective of biological control, the apparently high degree of overlap of colonization sites for *E. amylovora* and non-pathogenic epiphytes suggests that large populations of introduced antagonists can exclude the fire blight pathogen via the consumption of shared and limiting resources (Johnson et al., 1993b; Lindow et al., 1996; Wilson and Lindow, 1994).

***Antibiosis*:** Antibiosis is proposed commonly as a mechanism of biological control among many bacterial antagonists evaluated for fire blight suppression (Ishimaru et al., 1988; Johnson and Stockwell, 2000; Johnson et al., 1993b; Kearns and Hale, 1996; Lindow, 1985; Stockwell et al., 2001; Stockwell et al., 2002b; Vanneste et al., 1992; Wright et al., 2001). Antibiosis has been defined as the microbial secretion of small, organic molecules that are detrimental to growth or metabolic activity of another microorganism (Thomashow and Weller, 1995). For example, strains of *P. agglomerans* produce antibiotics that inhibit growth of *E. amylovora* in defined culture media (Ishimaru et al., 1988; Kearns and Hale, 1996; Stockwell et al., 2002b; Vanneste et al., 1992; Wright et al., 2001). A recent study (Stockwell et al., 2002b) evaluated the importance of antibiosis by *P. agglomerans* strain Eh252 in suppression of fire blight in orchard environments. Using an antibiotic-deficient derivative of Eh252, they found that antibiosis significantly contributed to the control of fire blight, but competition and other factors also were important in the activity of Eh252 (Stockwell et al., 2002b). The authors concluded that the antibiotic-producing bacterium *P. agglomerans* Eh252 was an effective antagonist for fire blight and that use of similar strains could be a valuable control strategy, especially in areas where streptomycin sensitivity was lost.

***Biological control of fire blight by Pseudomonas fluorescens strain A506*:** *Pseudomonas fluorescens* strain A506 (A506) (BlightBan A506, Nufarm Americas, Inc., Houston TX) was commercialized in 1996 as a biological control agent for suppression of fire blight and frost injury. A506 was isolated from a pear leaf in California and has demonstrated partial control of fire blight under greenhouse and field

experiments (Johnson et al., 1993b; Lindow, 1985; Wilson and Lindow, 1993; Lindow et al., 1996). Biological control of fire blight requires that A506 establish large populations on blossoms prior to colonization by *E. amylovora* (Lindow, 1985; Wilson et al., 1992). For example, in controlled studies, if inoculation of *E. amylovora* was delayed twenty-four hours after the application of A506 on flowers, then A506 suppressed growth of the pathogen (Wilson et al., 1992). In contrast, co-inoculation of A506 and *E. amylovora* resulted in little to no suppression of growth of *E. amylovora* on pear flowers (Wilson et al., 1992; Wilson and Lindow, 1993). In practice, application of A506 at 30% bloom followed by another application at 80% bloom leads to populations of A506 on blossoms of sufficient size to suppress disease (Johnson and Stockwell, 1998; Wilson and Lindow, 1993).

When first investigated, the mechanism of control of fire blight by A506 was considered to be competition for nutrients and protective sites on the stigmatic surface, thereby excluding *E. amylovora* from these niches (Thomas, 1986; Wilson and Lindow, 1993). Stockwell (2001) observed that the addition of iron to defined culture media induces A506 to produce a previously unreported, diffusible compound that is toxic to *E. amylovora* (Fig. 1.1A) (Stockwell et al., 2001; Temple et al., 2001). When A506 is cultured on minimal media not amended with iron, no apparent inhibition of *E. amylovora* can be observed (Fig. 1.1B) (Stockwell et al., 2001; Temple et al., 2001).

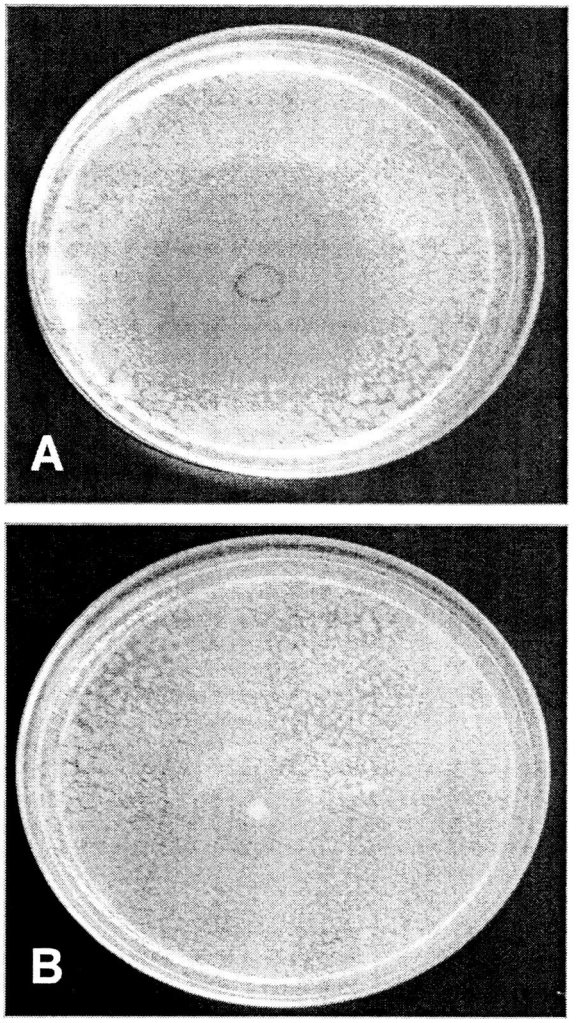

Figure 1.1. Antibiotic production bioassay for inhibition of *Erwinia amylovora* strain 153N by *Pseudomonas fluorescens* A506. A506 was cultured for 2 days at 27°C on 925 minimal medium amended with 0.1mM $FeCl_3$ (panel A) or without exogenous iron added (panel B). After 2 days, A506 was removed with a cotton swab, plates were exposed to chloroform vapors, and then oversprayed with 1 x 10^6 CFU/ml of Ea153N suspended in water. A distinct zone of inhibition of Ea153N is visible on iron amended media (A), whereas growth of Ea153N was not inhibited by A506 on media that was not amended with iron (B).

The zone of inhibition is observed in media amended with either the ferrous and ferric forms of iron. Intriguingly, a chemically–induced mutant of A506 named Fl-1

that no longer produces the fluorescent, iron-scavenging siderophore (see below) also inhibited growth of E. *amylovora* when cultured on defined media regardless of iron content (Lindow, 1985; V. Stockwell, *unpublished data*).

Iron is the fourth most abundant element on earth, but the scarcity of biologically available iron in some terrestrial habitats has led to evolution of genes that encode for production of molecules designed to scavenge this element from the environment (Neilands, 1984). For example, to overcome iron deficiency, A506 and *E. amylovora* secrete iron (Fe^{+3}) scavenging molecules called "siderophores" (Expert et al., 2000; Neilands, 1984). The fluorescent siderophore produced by A506 is a pyoverdine and the siderophores produced by *E. amylovora* are termed desferrioxamines (Expert et al., 2000; Neilands, 1984). Genes for siderophore production are commonly regulated by iron bioavailability in the environment: when iron is scarce, siderophore production is induced, and when abundant, siderophore production is suppressed (Neilands, 1984). On stigmas, bioavailable iron is considered a limiting factor to *E. amylovora* and is a requirement to initiate necrotic lesions (Dellagi et al., 1998; Expert et al., 2000).

While high bioavailability of iron may repress genes related to siderophore synthesis, many genes are positively regulated by iron. For example, iron stimulates the production of the phytotoxins syringomycin and syringopeptide by the plant pathogenic bacterium *P. syringae* (Gross, 1985). These cyclic depsipeptides also inhibit the growth of several fungi and bacteria, in addition to disrupting plant cell membranes (Gross et

al., 1977). The iron-induced antibiotic produced by A506 is inhibitory only to bacteria and not fungi sensitive to depsipeptides (V. Stockwell, *personal communication*).

POTENTIAL FOR IRON-MEDIATED ENHANCEMENT OF BIOCONTROL BY A506

The overall objective of this study was to investigate the significance of iron-induced antibiosis by A506 in the biological control of fire blight of pear and apple. Induction of the antibiotic on blossoms treated with A506 has the potential to enhance suppression of fire blight in commercial pear and apple orchards in the Pacific Northwest. Below is a review of the scientific tools available to address questions related to the overall objective.

Tools to address the significance of antibiotic production in biocontrol.

To determine if antibiosis by A506 plays a role in suppression of *E. amylovora* on stigmas, a derivative of A506 deficient in the production of the antibiotic is desired. One available strain is the *gacS* mutant of A506 (A506 GacS$^-$) (V. Stockwell, *unpublished data*), which does not produce the antibiotic in culture media regardless of iron content (V. Stockwell, *personal communication*). A506 GacS$^-$ has been used as an antibiotic-deficient control strain in laboratory assays (Temple et al., 2001). The gene product, GacS, is a sensor kinase component of a two-part regulatory system (i.e., GacS/GacA) in Gram-negative bacteria (Cui et al., 2001; Heeb and Dieter, 2001; Kitten et al., 1998; Saleh and Glick, 2001). This system functions through signal recognition by GacS (signal unknown), which activates GacA, which in turn triggers expression of target genes (Heeb and Dieter, 2001). The two-component system regulates expression

of secondary metabolites and extracellular enzymes and a mutation in *gacS* or *gacA* often results in a loss of expression of many of these target genes (Cui et al., 2001; Heeb and Dieter, 2001; Saleh and Glick, 2001; Whistler et al., 1998). For example, mutations in the *gacS* gene of *P. syringae* B728a are associated with a loss of virulence due to lack of syringomycin production (Willis et al., 2001). The mutation in the *gacS* gene of *P. syringae* B728a was not, however, associated with a loss of epiphytic fitness when inoculated on leaves (Willis et al., 2001). Similarly, growth of A506 GacS$^-$ in broth culture has a similar growth rate as the wild type A506. A506 GacS$^-$ is useful as a non-antibiotic producing control strain, however, a mutation in *gacS* will have pleiotropic effects, many of which are uncharacterized (Cui et al., 2001; Heeb and Dieter, 2001; Saleh and Glick, 2001). Thus, a portion of the research in this thesis will be focused on the selection of a mutant of A506 with a mutation in gene directly related to antibiotic production. In this regard, the only expected difference between an antibiotic biosynthesis mutant and the parental strain A506 would be due solely to antibiosis.

Tools to measure iron bioavailability in the environment.

The amount of bioavailable iron to A506 on pear and apple blossoms may influence antibiotic production on floral surfaces, but little is known about the biological availability of iron in these habitats. Bacterial strains equipped with an 'iron biosensor' have been used to detect iron available to bacteria on plant leaves, roots, and in soil (Loper and Lindow, 1994; Loper and Henkels, 1997; Loper and Henkels, 1999; Marschner and Crowley, 1997a). An iron biosensor consists of the iron regulated

promoter (e.g., the promoter for pyoverdin (*pvd*)) with a transcriptional fusion to a reporter gene (e.g., the gene *inaZ*) that transcribes for the ice nucleation protein, InaZ. Strains that harbor an iron biosensor "report" or express the biosensor gene when the concentration of iron in the environment is low (Lindow, 1987; Loper and Lindow, 1994; Wolber, 1986). For ice nucleation specifically, the InaZ protein is aggregated in the outer membrane of the bacterium and orients water into a crystalline structure; thus, limiting the ability of water to supercool (Kajava and Lindow, 1993; Lindow, 1987). Thus, water containing cells of the iron biosensor strain sampled from a low iron environment will freeze at -5°C, but water containing cells of the same strain grown in a high iron environment will supercool and not freeze (Lindow, 1987).

Tools to enhance iron bioavailability in the environment .

Biologically available iron can come in many forms, but commonly, the element is associated with a co-molecule, termed a 'chelate'. Chelate is derived from the Greek word for "claw" where the co-molecule physically surrounds and forms several bonds to a single metal ion. For example, two iron chelators are ethylenediamine-*N*-*N*'-bis (2-(hydroxyphenyl) acetic acid (EDDHA) and ethylenediaminetetraacetic acid (EDTA). In addition, soluble iron-containing compounds such as ferrous sulfate ($FeSO_4$) can be a source of bioavailable iron. Many chelates have practical applications, for example as components of fertilizers designed to provide iron to plants to enhance growth and prevent iron deficiency. Chelates can be characterized by the relative stability (strength) at which they hold the metal ion. For example, the stability order of the chelates mentioned above, in aerobic environments at pH 7.0, is EDDHA > EDTA.

More specifically, the synthetic chelate EDDHA (binding ferric (Fe^{+3}) iron) has a stability constant of 33.9 (Chaney, 1988) and holds the ferric ion more tightly than EDTA, which has a stability constant of 25.0 (Lindsay, 1979). The binding constants for the classes of the iron chelating siderophores of *P. fluorescens* spp. and *E. amylovora* have been estimated. Pyoverdine siderophores produced by *P. fluorescens* spp have a binding stability constant of 32.0 (Meyer and Abdallah, 1978), meaning it is a better iron scavenger (it holds iron more tightly) than the desferrioxamines produced by *E. amylovora* that have a stability constant of approximately 20.0 (Expert, 1999; Expert et al., 2000).

From a microbial perspective, the chelate stability constants are important because the introduction of an exogenous chelate to the environment could allow iron to become available to one organism but not to another. FeEDDHA, for example, with a high stability constant, should provide iron to A506 (pyoverdine stability constant is 32) but should not increase iron availability to *E. amylovora.* FeEDDHA is a registered agricultural chemical on pear and apple and marketed for treating iron chlorosis (Sequestrene 138, Becker-Underwood, Ames IA).

RESEARCH OBJECTIVES:

The principal hypothesis to be addressed in this thesis is: Does iron, when added to the floral environment of pear or apple, influence the degree to which *P. fluorescens* strain A506 suppresses infection of host tissues by *E. amylovora*? If so, is the biological control activity in iron-amended environments due to the antibiotic produced by *P. fluorescens* strain A506? A biosensor, reporter gene system will be used in

experiments to evaluate bioavailability of iron on pear and apple flowers. Several mutants of *P. fluorescens* strain A506 will be generated and utilized to examine the significance of antibiosis to suppression of fire blight. Finally, field experiments will be conducted to evaluate biological control activity of A506 with and without exogenous iron added to the floral environment.

Chapter 2

Iron Bioavailability to *Pseudomonas fluorescens* strain A506 on Pear and Apple Flowers

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Applied and Environmental Microbiology

Abstract:

Pseudomonas fluorescens strain A506 (A506) produces an antibiotic toxic to *Erwinia amylovora* in defined culture media containing at least 0.1mM $FeCl_3$. To estimate the relative availability of iron on blossoms, A506 was transformed with an 'iron biosensor', which consisted of an iron-regulated promoter (*pvd*) fused to an ice nucleation reporter gene (*inaZ*). A506 (*pvd-inaZ*) established high populations on blossoms, ranging from 10^4 to 10^7 colony forming units on pear and apple. In four trials on pear and apple trees, A506 (*pvd-inaZ*) expressed high ice nucleation activity on blossoms indicating limited iron bioavailability, or a low-iron environment unlikely to induce antibiosis by A506. A506 (*pvd-inaZ*) also colonized blossoms when mixed with $FeSO_4$ or the iron chelate, ferric ethylenediaminedi-(o-hydroxyphenylacetic) acid (FeEDDHA). Co-treatment of blossoms with a mixture of A506 (*pvd-inaZ*) and 0.1mM FeEDDHA significantly decreased ice nucleation activity compared to blossoms treated with A506 (*pvd-inaZ*) in water. Lower concentrations (0.01mM) of FeEDDHA did not measurably increase iron available to A506 on blossoms. These results indicate that apple and pear blossoms represent an iron-limited environment to A506 and that co-treatment with at least 0.1mM FeEDDHA is required to significantly increase the level of iron biologically available to this bacterium.

Introduction:

The bacterium *Pseudomonas fluorescens* strain A506 (A506) is a commercially available biological control agent (BlightBan A506, Nufarm Americas, Houston, TX) used for the suppression of fire blight on pear and apple trees. Effective biocontrol with A506 requires that the bacterium preemptively colonizes and establish large populations on blossom surfaces prior to colonization by the fire blight pathogen, *E. amylovora* (Johnson et al., 1993a; Johnson et al., 1993b; Johnson and Stockwell, 1998; Lindow, 1985; Lindow and Wilson, 1993; Lindow et al., 1996; Nuclo et al., 1998; Stockwell et al., 2002; Wilson et al., 1992; Wilson and Lindow, 1993). Disease suppression by A506 was proposed to occur by competitive exclusion, whereby A506 out-competes *E. amylovora* for sites and nutrients essential for epiphytic growth and subsequent infection of the host plant (Lindow, 1987; Thomson, 1986; Wilson and Lindow, 1993; Wilson and Lindow, 1994; Hattingh et al., 1986; Manceau et al., 1990; Wilson et al., 1989; Wilson et al., 1992).

Recently, Stockwell (2001) observed that A506 produced a large zone of inhibition to *E. amylovora* when cultured on defined media amended with 0.1 mM $FeCl_3$ (Stockwell et al., 2001). Because the zone of inhibition on iron-amended media is indicative of antibiotic production, questions arise concerning the potential significance of antibiosis in this biocontrol interaction, and concerning the relative bioavailability of iron to A506 on stigma and hypanthium surfaces. If sufficient iron is available to A506 *in situ*, production of the antibiotic may occur, suggesting that A506 utilizes an additional mechanism to attain an epiphytic advantage over *E. amylovora* on

floral surfaces. Conversely, if little iron is available on floral surfaces, competitive exclusion may be the principal mechanism of suppression, raising the question as to whether biocontrol can be enhanced by the addition of an exogenous source of iron to the blossom environment.

Iron is the fourth most abundant element on earth, but at neutral or basic pH in aerated environments, it exists principally as insoluble iron oxides (Lindsay, 1982; Neilands, 1984). Prior studies (Joyner and Lindow, 2000) have found that concentrations of bioavailable iron on plant surfaces can be limiting to the growth of microorganisms. Bacteria that reside on plant surfaces possess genes that are regulated by the environmental availability of iron (Bender et al., 1999; Blumer and Haas, 2000; Gross, 1985; Meyer and Stinzi, 1998; Sauvage and Expert, 1994). Fluorescent pseudomonads, the largest group in the genus *Pseudomonas*, produce pyoverdine: a distinct yellow-green, water-soluble, low-molecular weight compound possessing a strong affinity for ferric iron (e.g., siderophore) (Abdallah, 1991; Loper and Lindow, 1994; Meyer and Stinzi, 1998; Neilands, 1984). Typically, pyoverdine is secreted into the environment when iron bioavailability is low, and its production is suppressed when iron is abundant (Meyer and Stinzi, 1998; Mossialos, 2002; Neilands, 1984).

Iron-regulated promoters for synthesis of bacterial siderophores are an integral component of 'iron biosensors' which are used to investigate iron bioavailability on plants or in soils (Joyner and Lindow, 2000; Lindow, 1990; Loper and Lindow, 1994; Loper and Henkels, 1997; Loper and Lindow, 1997; Loper and Henkels, 1999; Marschner and Crowley, 1997a; Marschner and Crowley, 1997b; Marschner and

Crowley, 1998). The iron biosensor often used for *Pseudomonas* spp. consists of a transcriptional fusion between the iron-regulated promoter for pyoverdine biosynthesis (*pvd,* from *P. syringae*) and a promoterless ice nucleation gene (*inaZ,* also from *P. syringae*) (Lindow, 1990; Loper and Lindow, 1994; Loper and Lindow, 1997). The *inaZ* gene product is an outer-membrane protein (InaZ) that catalyzes the formation of ice by orienting water into a crystalline structure, a process called ice nucleation (Lindow et al., 1982; Wolber et al., 1986). Bacteria that express *inaZ* catalyze ice formation when suspended in water at temperatures ranging from -2 to -10°C, whereas, in the absence of ice nuclei, water may supercool to temperatures approaching -40°C before freezing (Lindow, 1990; Loper and Lindow, 1994; Loper and Lindow, 1997). In experiments with bacteria containing the *pvd-inaZ* fusion, the ice nucleation activity (INA) is quantified by first measuring the population size of bacteria in the environment of interest. The frequency of ice nucleation in these samples is then estimated in a droplet-freezing assay (Lindow et al., 1982; Loper and Lindow, 1997). Bacterial strains carrying the *pvd-inaZ* fusion show INA related to the iron concentration in the environment: abundant biologically available iron results in low INA, whereas limited bioavailable iron results in high INA (Loper and Lindow, 1994; Loper and Henkels, 1997; Loper and Lindow, 1997; Loper and Henkels, 1999; Marschner and Crowley, 1997).

The purpose of this study was to estimate the relative biological availability of iron to A506 on surfaces of pear and apple blossoms by using iron biosensor constructs of *P. fluorescens* strain A506. Moreover, exogenous sources of iron were co-applied

with A506 (*pvd-inaZ*) to blossoms to evaluate if a deficiency in bioavailable iron could be alleviated by this treatment.

Materials and Methods:

Bacterial strains: The bacterium used in this study was *P. fluorescens* strain A506. This strain was isolated from a pear leaf in California by S. Lindow (University of California, Berkeley), and is resistant to streptomycin and rifampicin. Constructs of A506 included: (1) A506 (*pvd-inaZ*), which contained the iron biosensor (an iron regulated promoter for pyoverdine production (*pvd*) fused to promoterless *inaZ*, (2) A506 Ice$^-$ which is a control strain consisting of promoterless *inaZ* cloned in opposite orientation to the *lac* promoter in pVSP61a, so that *inaZ* is not transcribed, and (3) A506 *iceC* (referred to as A506 IceC), which is a control strain where *inaZ* is transcribed constitutively from its native iron-independent promoter. The plasmid pVSP61a was used as the cloning vector for the iron biosensor and INA controls; this plasmid is considered stable in *Pseudomonas spp.* and confers resistance to kanamycin. This iron biosensor and INA controls have been used successfully in strains of *P. putida* N1R and *P. fluorescens* Pf-5 (Loper and Henkels, 1996; Loper and Henkels, 1999).

Inoculum Preparation: Constructs of A506 were cultured for 4 days at 27°C on nutrient agar (Difco Laboratories, Detroit, MI) containing 0.4% w/v glycerol, 0.5% w/v sucrose and 0.1mM ferric citrate. Ferric citrate was added to suppress INA in the initial inoculum. Bacteria were scraped from surface of the medium, suspended in 10mM potassium phosphate buffer (pH 7.0), and bacterial suspensions were adjusted to concentrations of approximately 1 x 10^8 CFU per mL (OD_{600} absorbance of 0.1) with the aid of a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY).

Screenhouse Experiment: Experiments to measure INA in constructs of A506 applied to pear and apple blossoms were conducted during the springs of 2001 and 2002 in a screenhouse facility located at the Botany and Plant Pathology Field Laboratory near Corvallis, OR. Pear and apple trees in the screenhouse ranged from 5 to 10 years old, and were protected from rain and ultraviolet radiation by a translucent, fiberglass roof, and from insect visitations by 2 x 2 mm steel screen walls. Newly-opened blossoms of pear (*Pyrus calleryana* cv. 'Aristocrat' in 2002 experiments or *Pyrus communis* cv. 'Bartlett' in 2001 and 2002 experiments) or apple (*Malus* X *domestica* cv. 'Golden Delicious' in 2001 experiments) were spray-inoculated with the suspensions of strains A506 (*pvd-inaZ*), A506 Ice^- and A506 IceC in water. All bacterial suspensions were sprayed to near runoff with hand-held trigger sprayers (800 ml capacity). In all experiments, individual branches of a tree served as the experimental units (marked with colored flagging), with individual trees serving as experimental blocks. Each A506 construct was replicated on three trees.

Iron Treatments: Several additional treatments involved inoculum of A506 (*pvd-inaZ*) mixed with the iron chelate, ferric ethylenediaminedi-(o-hydroxyphenylacetic) acid (FeEDDHA) (Sequestrene 138, Becker Underwood, Ames, IA), or with $FeSO_4$ (Sigma Chemicals, St. Louis, MO). For Bartlett pear and Golden Delicious apple in 2001, concentrations of FeEDDHA in the inoculum mixture were 0.1 and 0.01 mM; two other treatments involved these same concentrations of FeEDDHA applied as an overspray at 48 h after inoculation with A506 (*pvd-inaZ*). In 2002, Aristocrat and Bartlett pear blossoms were treated with A506 (*pvd-inaZ*) mixed with FeEDDHA at concentrations

of 0.001, 0.01, 0.1, and 1.0 mM. Mixtures of A506 (*pvd-inaZ*) with $FeSO_4$ were included as treatments also in the experiment on Golden Delicious apple in 2001 at rates of 0.1 and 18 mM; an additional treatment represented a 1:1 mixture of 0.1 mM $FeSO_4$ plus 0.1 mM FeEDDHA. Lastly, for Bartlett pear in 2002, a treatment A506 (*pvd-inaZ*) plus 0.1 mM EDDHA (chelate without iron) was included as an experimental control. All mixed treatments of A506 (*pvd-inaZ*) with FeEDDHA, $FeSO_4$, or EDDHA were also replicated three times.

Estimation of Population Size of A506 Constructs on Blossoms: For each of the experimental treatments, blossoms were sampled three to four times during bloom to estimate population size of the inoculated construct of A506. In 2001, blossoms were sampled at 0, 48 and either 96 (Bartlett pear) or 120 h (Golden Delicious apple) after inoculation. In 2002, blossom were sampled at 0, 24 (Bartlett pear only), 48, 96 (Aristocrat pear only), and 120 h after inoculation. Sample sizes were 10 blossoms per treatment except for Golden Delicious in 2001 apple where only 6 blossoms were sampled at each specified interval after inoculation. Sampled blossoms were transported to the laboratory in individual wells of sterile, 24-well microtiter plates, and then processed individually for dilution plating. The pistil and hypanthium from pear blossoms or pistils only from apple blossoms were dissected using sterile forceps and sonicated (Sonix IV, Inglewood, CA) in 1 mL of sterile 10 mM potassium phosphate buffer (pH 7.0) for 3 min. After sonication, 10 µl drops of the wash and two 100-fold serial dilutions were spread onto Pseudomonas Agar F (PAF) medium (Difco laboratories, Detroit, MI) amended with 50 µg/ml rifampicin, 50 µg/ml kanamycin, and

50 µg/ml cycloheximide. Detection limit for enumeration of bacteria for each blossom was 1 x 10^2 CFU/ml. Colonies were counted after three days.

Ice Nucleation Assay: INA was determined using the same samples used for estimation of bacterial population size in a droplet-freezing assay (Lindow, 1990). Forty 10 µl drops from the blossom wash or an appropriate sample in a 10-fold dilution series were dispensed on aluminum foil boats covered with a thin layer of Johnson's paste wax (SC Johnson, Racine, WI). The foil boats were floated on 100% ethanol bath at a temperature of –5°C (Lindow, 1990; Loper and Lindow, 1997). Numbers of drops that froze at each dilution were recorded after visual inspection and were used for estimating ice nucleation frequency.

Data Analysis: The number of colony forming units (CFU) of each applied A506 construct per blossom and the INA per blossom were estimated from the count data obtained in the dilution plating and freezing-droplet assays. Population size data were transformed to $\log_{10}$ (CFU per blossom). Calculation of INA was completed for each blossom as follows (Vali,1989):

$$INA = V_t \bullet \ln(1/(1-P_f) / V_d \bullet D$$

where V_t and V_d, represent volumes of the dilution tube and the droplet, respectively, D is the serial dilution (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} or 10^{-5}), and P_f is the proportion of frozen drops at the selected dilution. The INA per blossom was divided by the CFU per blossom and transformed to $\log_{10}$ (INA per CFU).

For each construct, the mean $\log_{10}$ (CFU per blossom) and mean $\log_{10}$ (INA per CFU per blossom) were plotted as a function of hours after inoculation. For individual

sampling dates, analysis of variance (ANOVA) was conducted to test equality of means among constructs of A506 inoculated in water, and separately, among rates of iron co-treatments applied with A506 (*pvd-inaZ*) (PROC GLM, Statistical Analysis System, SAS Institute, Cary, NC). Similarly, values for relative area under the curve for $\log_{10}$ (CFU per blossom) (RA_{CFU}) and for $\log_{10}$ (INA per CFU per blossom) (RA_{INA}) were also subjected to ANOVA. RA_{CFU} and RA_{INA} were computed as:

$$RA = \sum_{i=1}^{n} \{[(y_i + y_{i-1})/2] \bullet (t_i - t_{i-1})\} / t_{total}$$

where y and t are the values of the response variable and hours after inoculation, respectively, for the ith sample date, and n is the total number of sample dates during the experiment. Because initial inocula of A506 constructs were cultured on an iron-amended medium, calculation of RA_{INA} values excluded samples taken at 0 h after inoculation. Fischer's protected least significant difference ($P \leq 0.05$) was used to evaluate differences among means.

Results:

Population size of P. fluorescens strain A506 (pvd-inaZ), A506 Ice⁻ and A506 IceC applied to blossoms in water. A506 (*pvd-inaZ*), A506 Ice⁻ and A506 IceC established on pear and apple blossom surfaces and were recoverable from blossoms sampled over the time course of the experiment (Fig. 2.1). In general, measured population sizes of the A506 constructs were in the range of 10^4 to 10^7 CFU per blossom, except for A506 Ice⁻ on Golden Delicious apple in 2001, which ranged from 10^2 to 10^5 CFU per blossom (Fig. 2.1C). Analysis of variance (ANOVA) for mean $\log_{10}$ CFU populations at individual sampling times and for relative area under the population curve (RA_{CFU}) indicated no significant differences ($P > 0.05$) among the A506 *inaZ* constructs in any of the pear experiments (data not shown). For Golden Delicious apple in 2001, the population sizes of A506 Ice⁻ at 48 and 120 h after inoculation and the RA_{CFU} for this strain were significantly smaller ($P < 0.05$) than measured for A506 IceC and A506 (*pvd-inaZ*) (data not shown).

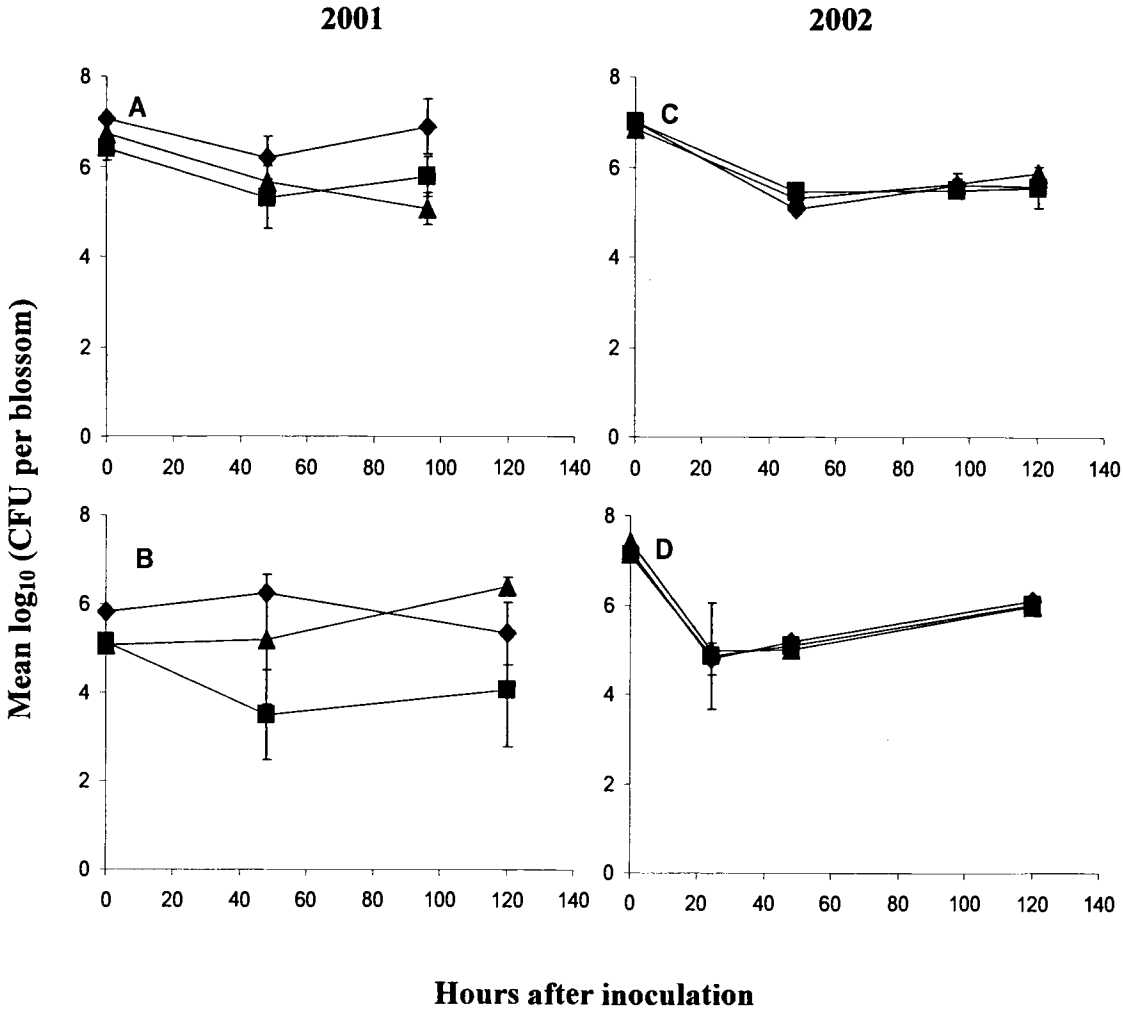

Fig. 2.1 Population size (log_{10}) of *P. fluorescens* strain A506 constructs A506 (*pvd-inaZ*) (▲), A506 IceC (◆),and A506 Ice$^-$ (■) on water treated blossoms. Panels represent experiments conducted on screenhouse-grown trees of **A**) Bartlett pear, 2001, **B**) Golden Delicious apple, 2001, **C**) Aristocrat pear, 2002, or **D**) Bartlett pear, 2002. Vertical bars represent one standard error of the mean population size measured on individual blossoms.

Ice nucleation activity of P. fluorescens constructs A506 (pvd-inaZ), A506 IceC and A506 Ice⁻ applied to blossoms in water. As expected, INA for blossoms treated with A506 Ice⁻ was low, with values for $\log_{10}$ (INA per CFU) after inoculation on pear trees ranging from -4.4 to -6.3, and -5.1 to -4.1 on apple (Fig. 2.2). In contrast, and also as expected, A506 IceC yielded high INA after inoculation with values for $\log_{10}$ (INA per CFU) ranging from -0.3 to -4.0 over the four experiments (Fig. 2.2) In general, relative to each other, the INA of A506 Ice⁻ and A506 IceC remained low and high, respectively, over the four experiments.

Compared to A506 Ice⁻, the initial inoculum and the first sample of blossoms treated with A506 (*pvd-inaZ*) expressed low INA, with values for $\log_{10}$ (INA per CFU) ranging from -7.0 to -4.3 over the four experiments. By 48 h after inoculation, $\log_{10}$ (INA per CFU) for blossoms treated with A506 (*pvd-inaZ*) had increased from an average of -5.3 at 0 h to -2.7 (Fig. 2.2), and this increase in expression was maintained over the course of the experiment (Fig 2.2). ANOVA for RA_{INA} based on the second, third and fourth samples resulted in significant differences ($P < 0.05$) among constructs. In three of four experiments, means of RA_{INA} for A506 IceC and A506 (*pvd-inaZ*) were significantly larger than means obtained for A506 Ice⁻ (Table 2.1). For Bartlett pear in 2001, RA_{INA} for A506 (*pvd-inaZ*) was intermediate to and significantly different ($P < 0.05$) from the responses measured for both A506 IceC and A506 Ice⁻ (Table 2.1).

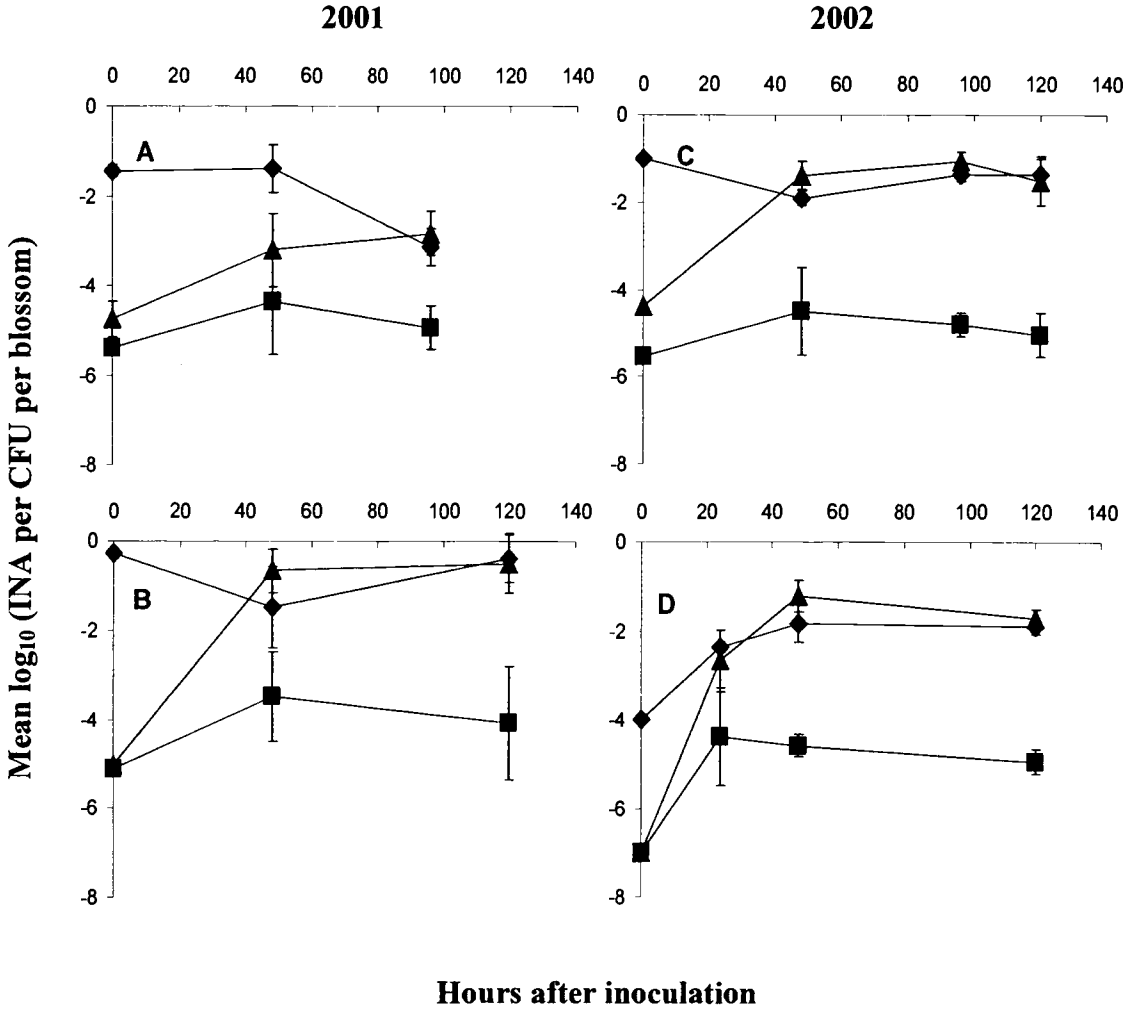

Fig. 2.2 Ice nucleation activity per colony forming unit per blossom (transformed $\log_{10}$) for *P. fluorescens* strain A506 constructs A506 (*pvd-inaZ*) (▲), A506 IceC (◆), or A506 Ice$^-$ (■) on water treated blossoms. Panels represent experiments conducted on screenhouse-grown trees of **A)** Bartlett pear, 2001, **B)** Golden Delicious apple, 2001, **C)** Aristocrat pear, 2002, or **D)** Bartlett pear, 2002. Vertical bars represent one standard error of the mean ice nucleation activity per CFU measured on individual blossoms.

Table 2.1. Relative area under the curve for $\log_{10}$ (ice nucleation activity per colony forming unit per blossom) measured on blossoms of pear and apple treated with constructs of *Pseudomonas fluorescens* A506[x] mixed with water.

Construct of A506	Relative Area Under the Curve[y]			
	2001		2002	
	Bartlett	Golden Delicious	Aristocrat	Bartlett
IceC	-2.8 a[z]	-0.9 a	-1.5 a	-2.2 a
pvd-inaZ	-3.3 b	-0.3 a	-2.1 a	-2.2 a
Ice⁻	-4.6 c	-3.6 b	-4.7 b	-4.7 b

[x] Constructs were applied to blossoms by spraying a 1 x 10^8 CFU/mL suspension to near runoff.

[y] The relative area under the ice nucleation curve (RA_{INA}) was calculated from 24 h or 48h to 120 h after inoculation by the equation:

$$RA_{INA} = \sum_{i=1}^{n} \{[(y_i + y_{i-1})/2] \bullet (t_i - t_{i-1})\}/t \text{ total}$$

where y is the mean ice nucleation activity of bacterial strains at the ith sample date and t is the corresponding sample time.

[z] Means within a column followed by the same letter are not significantly different according to Fisher's protected least significant difference at $p = 0.05$.

Effect of FeEDDHA on population size of A506 (pvd-inaZ) on blossoms.

Similar to the measured population sizes of A506 (*pvd-inaZ*) applied to blossoms in water, populations of A506 (*pvd-inaZ*) co-treated with FeEDDHA ranged from 10^4 to 10^7 CFU per blossom among the experiments (Fig. 2.3). ANOVA of RA_{CFU} for A506 (*pvd-inaZ*) revealed no significant effects ($P > 0.05$) of co-treatment with FeEDDHA in any of the experiments (data not shown).

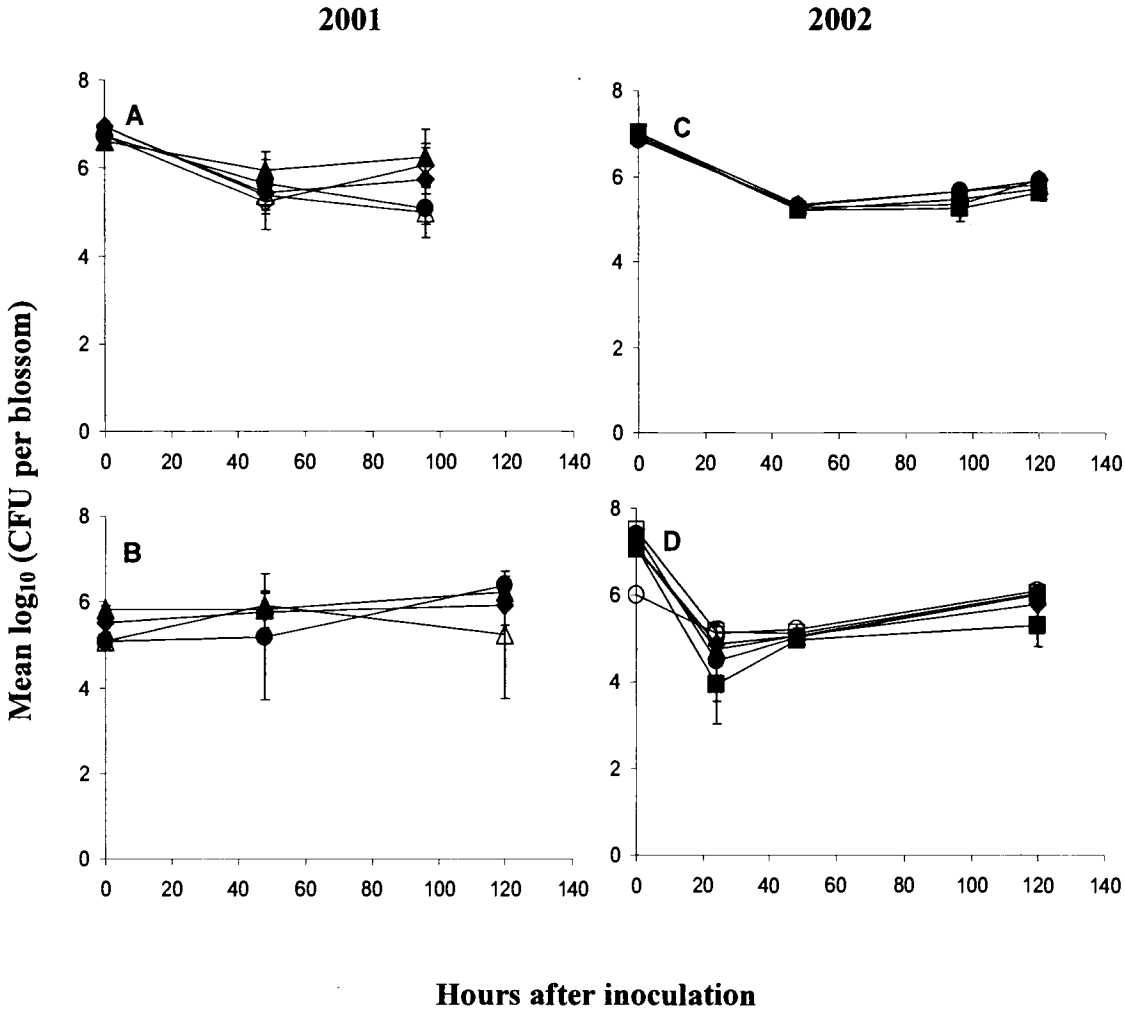

Fig. 2.3 Population size (log_{10}) of *P. fluorescens* strain A506 (*pvd-inaZ*) on blossoms treated with water (•), 0.001 (□), 0.01 (◆), 0.1 (▲), 1.0mM FeEDDHA (■), or 0.1mM EDDHA(○) or after treatment with 0.01 (◇) or 0.1mM FeEDDHA (△) at 48 h after inoculation with A506 (*pvd-inaZ*). Panels represent experiments conducted on screenhouse-grown trees of **A)** Bartlett pear, 2001, **B)** Golden Delicious apple, 2001, **C)** Aristocrat pear, 2002, or **D)** Bartlett pear, 2002. Vertical bars represent one standard error of the mean population size measured on individual blossoms.

Effect of FeEDDHA on ice nucleation activity of A506 (pvd-inaZ) on blossoms. INA of A506 (*pvd-inaZ*) was affected significantly ($P < 0.05$) by the concentration of FeEDDHA in the bacterial inoculum. Blossoms treated with A506 (*pvd-inaZ*) in water or co-treated with 0.01 or 0.001 mM FeEDDHA showed increases in mean INA from an initial value for $\log_{10}$ (frequency of ice nuclei per CFU) averaging approximately -5.3 to values that averaged -1.4 for sampling dates at or beyond 24-h after inoculation (Fig. 2.4). Conversely, in both 2001 and 2002, treatment with a mixture of A506 (*pvd-inaZ*) and either 1.0 or 0.1 mM FeEDDHA resulted in a significant ($P < 0.05$) suppression of INA compared to the blossoms that received A506 (*pvd-inaZ*) in water (Fig. 2.4, Table 2.2). Similarly, RA_{INA} for A506 (*pvd-inaZ*) mixed with FeEDDHA at concentrations of 1.0 or 0.1 mM were significantly different ($P < 0.05$) from the RA_{INA} values of A506 (*pvd-inaZ*) mixed with either 0.01 or 0.001 mM FeEDDHA (Table 2.2).

In 2001, delayed oversprays of FeEDDHA at 48 h after inoculation with A506 (*pvd-inaZ*) tended to reduce INA in comparison to blossoms treated with A506 (*pvd-inaZ*) in water (Fig 2.4A and C). For Golden Delicious apple , means values of $\log_{10}$ (INA per CFU) for the final blossom sample of the 0.1 mM FeEDDHA delay treatment (96 to 120 h after inoculation) were significantly smaller ($P < 0.05$) than values obtained for the water treated control; in Bartlett pear, however, the corresponding reductions in $\log_{10}$ (INA per CFU) observed with either delay treatment (0.01 or 0.1 mM FeEDDHA) were not significant ($P > 0.05$).

For Bartlett pear in 2002, the additional control treatment A506 (*pvd-inaZ*) mixed with 0.1 mM EDDHA (chelate without iron) yielded responses similar to those observed for A506 (*pvd-inaZ*) in water. (Fig. 2.3, 2.4; Table 2.2).

Table 2.2. Relative area under the curve for $\log_{10}$ (ice nucleation activity per colony forming unit per blossom) measured on blossoms of pear and apple treated with *Pseudomonas fluorescens* A506 (*pvd-inaZ*) mixed with water or co-treatments of iron[w]

	Relative Area Under the Curve[x]			
	2001		2002	
Iron Treatment	Bartlett	Golden Delicious	Aristocrat	Bartlett
water control	-2.8 a[y]	-0.3 a	-1.5 a	-2.2 a
0.1mM EDDHA	.....[z]	.....	.....	-2.0 a
0.001mM FeEDDHA	.....	.....	-2.1 a	-2.7 a
0.01mM FeEDDHA	-3.3 a	-1.2 b	-2.1 a	-2.7 a
0.1mM FeEDDHA	-4.5 b	-2.8 c	-4.6 b	-4.8 b
1.0mM FeEDDHA	.....	.....	-4.6 b	-4.4 b
0.1mM $FeSO_4$	.....	-2.4 c	.....	.....
18mM $FeSO_4$	.....	-1.9 b,c	.....	.....
1:1 mix of 0.1mM FeEDDHA & 0.1mM $FeSO_4$	.....	-2.4 c	.....	.....

[w] Iron treatments were sprayed onto blossoms to near run off in co-suspension with A506:*pvd-inaZ* at 1 x 10^8 CFU/mL

[x] The relative area under the ice nucleation curve (RA_{INA}) was calculated from 24 h or 48 h to 120 h after inoculation by the equation:

$$RA_{INA} = \sum_{i=1}^{n} \{[(y_i + y_{i-1})/2] \bullet (t_i - t_{i-1})\}/t \text{ total}$$

where y is the mean population size of bacterial strains at the ith sample date and t is the corresponding sample time.

[y] Means within a column followed by the same letter are not significantly different according to Fisher's protected least significant difference at $p = 0.05$.

[z] Not tested.

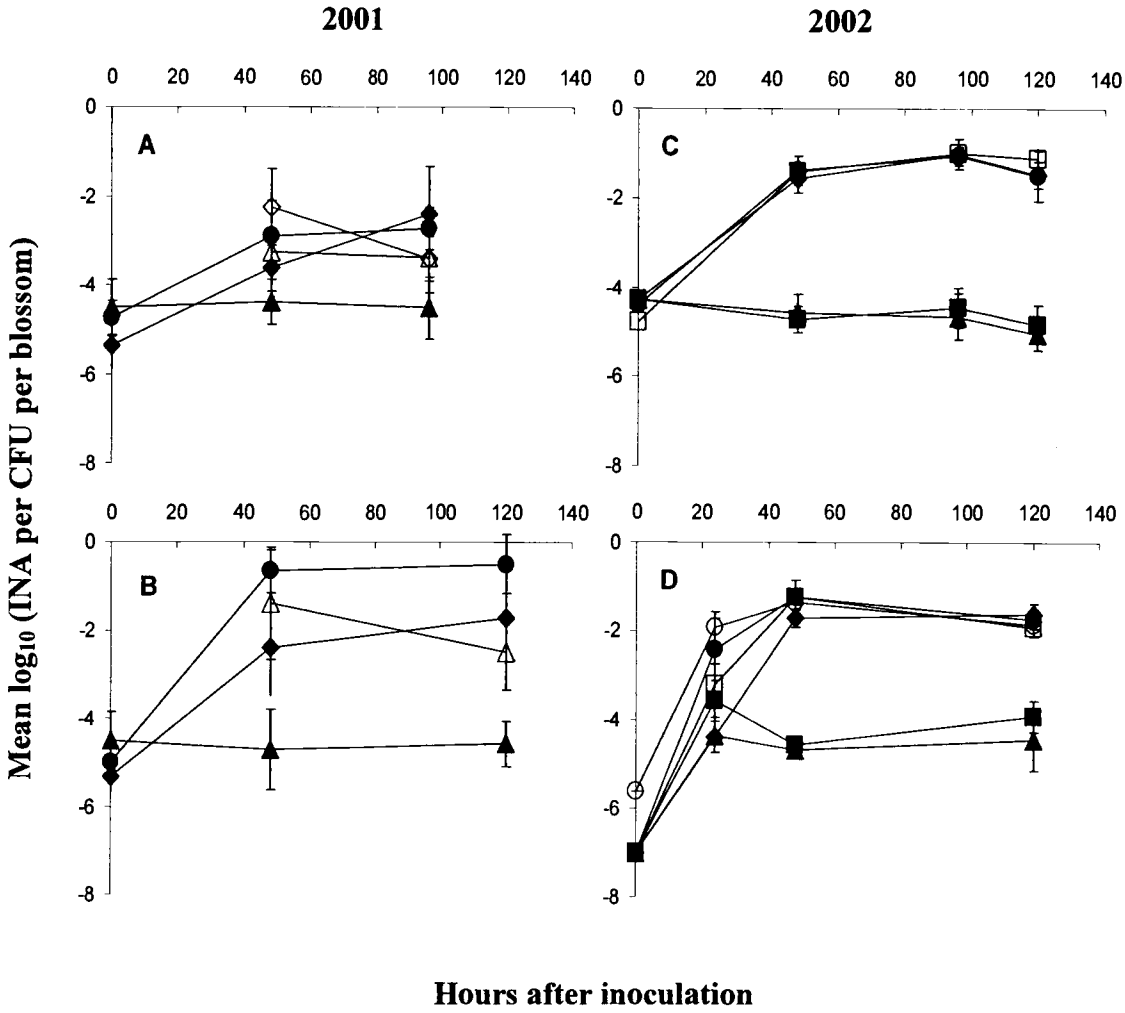

Fig. 2.4 Ice nucleation activity per colony forming unit per blossom (transformed $\log_{10}$) for *P. fluorescens* strain A506 (*pvd-inaZ*) on blossoms treated with water (•), 0.001 (□), 0.01 (◆), 0.1 (▲), 1.0mM FeEDDHA (■), or 0.1mM EDDHA(○) or after treatment with 0.01 (◇) or 0.1mM FeEDDHA(△) at 48 h after inoculation with A506:*pvd-inaZ*. Panels represent experiments conducted on screenhouse-grown trees of **A)** Bartlett pear, 2001, **B)** Golden Delicious apple, 2001, **C)** Aristocrat pear, 2002, or **D)** Bartlett pear, 2002. Vertical bars represent one standard error of the mean ice nucleation activity per CFU measured on individual blossoms.

Effects of $FeSO_4$. In 2001, on blossoms of Golden Delicious apple, mean population sizes of A506 (*pvd-inaZ*) after co-treatment with 0.1 mM $FeSO_4$ were not significantly different ($P > 0.05$) from those of A506 (*pvd-inaZ*) inoculated in water (data not shown). The higher, 18 mM co-treatment of $FeSO_4$, however, resulted in significantly smaller ($P < 0.05$) population sizes of A506 (*pvd-inaZ*) when compared to the water treated control (data not shown). Mean population sizes of A506 (*pvd-inaZ*) mixed with FeEDDHA and $FeSO_4$ (1:1) were similar to those of A506 (*pvd-inaZ*) inoculated in water up to 48 h after inoculation, but significantly smaller ($P < 0.05$) at the last sample time of 120 h after inoculation (data not shown).

From 48 to 120 h after inoculation, the INA detected from blossom washes of A506 (*pvd-inaZ*) co-treated with $FeSO_4$ ranged from -3.3 to -5.2 (frequency of ice nuclei per CFU), which was significantly ($P > 0.05$) suppressed relative to the activity measured on blossoms treated with A506 (*pvd-inaZ*) in water (Table 2.2). The relative degree of suppression of INA with co-treatments of $FeSO_4$ was statistically similar to the degree of suppressions obtained with 0.1 mM FeEDDHA (Table 2.2).

Discussion:

This study was designed to examine the relative biological availability of iron to A506 (*pvd-inaZ*) on surfaces of pear and apple blossoms through the suppression or expression of an iron-regulated promoter at the transcriptional level. This question is of interest because A506, a biological control agent for fire blight disease of pear and apple, produces an antibiotic inhibitory to the target pathogen when cultured on defined media that contains iron at concentrations ≥ 0.1 mM. Thus, the working hypothesis is that iron bioavailability on blossoms surfaces may directly influence the effectiveness of the biocontrol interaction.

After inoculation, population size determinations were made to compare relative reproductive fitness among constructs of A506 and to express ice nucleation frequency in terms of INA per CFU. With specific consideration to fitness, after 24 h, all three constructs (A506 (*pvd-inaZ*), A506 Ice$^-$ and A506 IceC) attained populations sizes of 10^4 to 10^6 CFU per blossom and maintained these population sizes over the time course of the experiment (Fig. 2.1), with the exception of A506 Ice$^-$ on Golden Delicious apple in 2001, mean values of RA_{CFU} (i.e., the mean population size weighted for length of time between samples) revealed no statistical differences ($P > 0.05$) among the constructs of A506 (data not shown). Thus, evidence that a specific construct (A506 (*pvd-inaZ*), A506 Ice$^-$ and A506 IceC) could be detrimental to the reproductive fitness of A506 was not obtained. Similarly, constructs of the biocontrol strains *P. syringae* 31R1, *P. putida* N1R, and *P. fluorescens* Pf-5 containing *pvd-inaZ,* IceC, or Ice$^-$ showed no apparent differences in epiphytic fitness in rhizosphere or phyllosphere of

bean, rhizosphere of cucumber, and rhizosphere of white lupine, respectively (Loper and Lindow, 1994; Loper and Henkels,1997; Loper and Henkels, 1999; Marschner and Crowley, 1997).

The initial INA of inoculated cells was an important consideration in the experimental design. All three constructs were grown on a medium amended with 0.1 mM ferric citrate. Thus, the expectation was that the INA of A506 (*pvd-inaZ*) cells in the initial inoculum would be at a minimal levels and similar to A506 Ice$^-$ as the cells were sprayed on to the flowers; in contrast, A506 IceC was expected to show high INA regardless of the amount of iron in the culture media. Accordingly, for inoculum and blossom samples taken at zero hours after inoculation, the INA of A506 (*pvd-inaZ*) was low and similar to A506 Ice$^-$ (Figs. 2.2 & 2.4). A506 Ice$^-$ also served as a check to evaluate background INA on blossoms maintained in the screenhouse.

A conclusion of this study is that bioavailable iron is in limited supply on the surfaces of pear and apple blossoms. This conclusion is supported principally by the observed shift in INA of A506 (*pvd-inaZ*) from initially low levels (i.e., similar to A506:Ice$^-$) to higher activities by 48 h of incubation. In most of the experiments, these higher INAs approached those of A506 IceC. In a related study, Loper and Lindow (1994), using *P. syringae* containing the *pvd-inaZ* iron biosensor, also found low iron bioavailability in the phyllosphere of bean leaves. In 2000, Joyner and Lindow (2000), using a whole cell GFP-based biosensor, also found a patchy distribution of low iron bioavailability on the surface of bean leaves. Loper and Henkels (1997), with a *pvd-inaZ* construct of *P. fluorescens* Pf-5, found that the rhizosphere of cucumber was

initially iron limiting, but became less limiting as time progressed. Marschner and Crowley (1997a) concluded that the rhizosphere of white lupine was only partially iron limited, with iron availability decreasing with nearness to the root tip (Marschner and Crowley,1997b).

In one experiment only (Bartlett pear in 2001), the INA for A506 IceC, but not population size, decreased significantly over the period from 48 to 96 h after inoculation (Fig. 2.2A). This decrease in ice nucleation expression coincided with very warm temperatures in the screenhouse (daily maximum temperatures of 34°C). Optimal temperatures for INA of gram-negative bacteria is 18 to 24°C (Gurian-Sherman and Lindow, 1995). For *P. syringae* in culture at 30°C, INA can decrease 1000-fold (Loper and Lindow, 1997). INA, but not population size, in *P. syringae* is known to be reduced on snap bean leaflets by increases in ambient temperatures (Hirano and Upper, 1995) and this may explain both the drop in INA observed in A506 IceC and the INA response of A506 (*pvd-inaZ*) that was weaker than in the other three trials.

The partially ferrated chelate, FeEDDHA, was introduced to blossoms as the source of exogenous iron. As in the comparison of constructs, the population sizes of A506 (*pvd-inaZ*) applied with FeEDDHA were measured to evaluate if different concentrations of this chemical affected the relative growth capacity of this strain on blossoms. Overall, the addition of FeEDDHA at different concentration levels to the inoculum of A506 (*pvd-inaZ*) had no observable effect of the measured population size of A506 (*pvd-inaZ*) in blossom washings (Fig. 2.3).

Nonetheless, FeEDDHA, significantly suppressed the shift from initially low to high frequencies of ice nucleation when applied at concentrations $\geq$ 0.1 mM (Fig. 2.4). Conversely, INAs of A506 (*pvd-inaZ*) amended with 0.001 and 0.01 mM FeEDDHA showed high INAs that were similar to those observed with A506 (*pvd-inaZ*) applied in water (Table 2.2). Similarly, the treatment of A506 (*pvd-inaZ*) amended with 0.1 mM EDDHA (chelate with no iron) on Bartlett pear in 2002, had no effect on the relative population size and observed frequency of INA when compared to the A506 (*pvd-inaZ*) in water control. These data indicate the 0.1 mM FeEDDHA treatment was at or slightly above the minimal concentration of iron required to suppress the transcriptional activity of *pvd-inaZ*.

In 2001, the FeEDDHA treatments delayed until 48 h after inoculation with A506 (*pvd-inaZ*) also resulted in a significant effect on frequency of INA relative to the blossoms treated with A506 (pvd-inaZ) cells in water (Fig 2.4). This effect was observable as a decrease in INA in blossoms oversprayed with either 1.0 or 0.1 mM FeEDDHA and sampled 48 to 72 h after the time of the iron overspray (96 to 120 h after inoculation). The suggestion from this result is that iron is not required to be mixed with A506 (pvd-inaZ) to have an effect on iron bioavailability on the surface of a pear or apple blossom.

Ferrous sulfate ($FeSO_4$) was included as a treatment to determine if the form of iron (FeEDDHA versus $FeSO_4$) could suppress INA of A506 (*pvd-inaZ*) cells on blossoms. In 2001, both 0.1 and 18 mM $FeSO_4$ suppressed the INA of A506 (*pvd-inaZ*) to a degree that was similar to that observed in the treatment amended with 0.1 mM

FeEDDHA (Table 2.2). The high rate of $FeSO_4$ (18 mM), however, caused a significant reduction in the recoverable population of the A506 (*pvd-inaZ*) cells when compared to treatments of FeEDDHA, EDDHA (chelate only) or water, and severely blackened treated blossoms, especially on petals and the tips of pistils (stigmas). This apparent phytotoxicity and reduction in measured populations of A506 (*pvd-inaZ*), however, were not observed with the 0.1 mM concentration of this chemical.

FeEDDHA shows more promise than $FeSO_4$ as a co-treatment with A506 in field applications for fire blight suppression. Both compounds are used to treat iron chlorosis on fruit trees. Structurally, FeEDDHA, a chelate, has a high affinity for ferric iron and has a stability constant of 33.9 (Chaney, 1988), which means that it holds the ferric ion relatively tightly. Conversely, in water, the ferrous iron dissociates from the sulfate compound readily. From a microbial perspective, in iron-poor environments, siderophores are produced and secreted to dissolve ferric iron for microbial growth (Matzanke, 2000). The tightness with which iron is held in a chelate could lead to availability to one organism but not to another. For example, this study indicates that co-treatment with FeEDDHA can enhance iron availability to A506, for which its siderophore, pyoverdine, has a high affinity for ferric iron and an iron binding coefficient of 32.0 (Meyer and Abdallah, 1978). The siderophore of *E. amylovora*, desferrioxamine, which is a hydroxamate, has a stability constant of around 20.0 (Expert, 1999; Expert et al., 2000) and grows poorly in culture media amended with the ferrated chelate, which suggests that it will be more difficult for this pathogen to acquire iron in an environment where the element is held by EDDHA

In conclusion, the findings of this study provide evidence that a construct of *P. fluorescens* strain A506 containing the iron biosensor, *pvd-inaZ,* showed levels of INA consistent with the hypothesis that iron has limited bioavailability on surfaces of pear and apple blossoms. Moreover, amendments of the iron chelate, FeEDDHA, at concentrations $\geq$ 0.1mM suppressed this INA, indicating that A506 (*pvd-inaZ*) acquired sufficient iron to suppress this iron-regulated promoter. The implications of these results are that production of the iron-induced antibiotic of A506 is unlikely to occur in field applications without the addition of exogenous iron to blossom surfaces. Applications of FeEDDHA, however, may modify the habitat on pear and apple blossoms and thus, may positively influence antibiotic production by A506 and fire blight suppression.

Chapter 3

Selection of mutants of *Pseudomonas fluorescens* strain A506 with altered antibiotic production

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Not for publication

Abstract:

Pseudomonas fluorescens strain A506 (A506) produces an antibiotic on iron-amended, defined media. A506 was mutagenized using Tn*5-km* and Tn*5gfp-km*, and mutants were selected that showed altered antibiotic production. One antibiotic deficient mutant, named A506 Ant$^-$, was mutagenized with Tn*5gfp-km* promoter fusion. The epiphytic fitness of A506 Ant$^-$ on blossoms of apple and pear trees was reduced compared to the parent strain A506. Results of Southern analysis and cloning indicated that Tn*5gfp-km* had inserted into genomic and also the plasmid DNA of A506 Ant$^-$. A Tn*5* mutant, A506 Ant$^+$, was found to be a non-fluorescent, iron-independent antibiotic producer. A506 Ant$^+$ established high populations on blossoms of apple and pear and reduced fire blight incidence in three field trials.

Introduction:

The biocontrol bacterium *Pseudomonas fluorescens* strain A506 (A506), when cultured on iron-amended defined media, produces an antibiotic toxic to the fire blight pathogen, *Erwinia amylovora* (Stockwell et al., 2001). Iron is required by living organisms for metabolic growth and enzymatic reactions and regulates expression of numerous genes. Iron-regulated secondary metabolite production occurs in bacteria, for example, siderophore production in iron-poor environments, iron-induced toxin production, or iron-stimulated biosynthesis of hydrogen cyanide (Askeland and Morrison, 1983; Blumer and Haas, 2000; Gross, 1985; Neilands, 1984). When iron is limited, A506 produces a yellow-green pigmented siderophore used by the bacterium for iron acquisition (Neilands, 1984).

Transposon mutagenesis (Tn*5*) is a tool used to disrupt biosynthetic or regulatory genes and has been used to elucidate the importance of genes involved in biological control (Stockwell et al., 2002b; Wright et al., 2001). Tn*5* mutagenesis disrupts genes by random insertion of its DNA within the DNA of a recipient organisms, thereby inhibiting transcription of gene products (de Lorenzo et al., 1990). Loss of gene transcription, i.e., detected as loss of phenotype expression, allows identification of disrupted genes. Promoter probing using Tn*5* mutagenesis relies on transcription of a promoterless antibiotic resistance gene (a reporter gene) to identify genes of interest (Tang et al., 1999). For example, Tn*5gfp-km* mutants of *Agrobacterium tumefaciens* were selected based on GFP expression and resistance to kanamycin, transcribed from a promoter (Tang et al., 1999). Promoter probes direct the

selection of disrupted genes to a subset of transcribed genes associated with reporter gene activity (Tang et al., 1999). Using Tn*5*, mutations in genes for regulation or secondary metabolite biosynthesis have been shown to positively or negatively impact biocontrol (Kraus and Loper, 1992; Kraus and Loper, 1995; Saleh and Glick, 2001; Whistler et al., 1998). Therefore, elucidation of genes involved in biosynthesis or regulation of antibiosis by A506 may potentially increase biocontrol efficacy.

The goal of this study was to select and characterize several Tn*5* mutants of A506 with altered antibiotic production. The research describes the selection and phenotype characterization of one antibiotic deficient mutant of A506, A506 Ant$^-$. In addition, two fluorescent-deficient, iron-independent (constitutive antibiosis) mutants of A506 (A506 Ant$^+$ and A506 Fl-1) were evaluated for epiphytic fitness and enhanced disease suppression relative to the parental strain.

Materials and Methods:

Bacterial strains and plasmids:

The bacteria and plasmids used in this study are listed in Table 3.1.

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

Strain	Relevant characteristics[a]	Source
Pseudomonas fluorescens		
JL4654 A506	*Pseudomonas fluorescens* A506; phyllosphere inhabitant and biological control agent for fire blight; iron-dependent antibiotic production; Sm^r, Rf^r.	Lindow, 1985
JL4639 A506 GacS$^-$	Mini-Tn*5km* insertion in regulatory gene *gacS*; antibiotic deficient; Km^r, Sm^r, Rf^r.	Stockwell
JL4633 Fl-1	Non-fluorescent mutant of A506 selected after treatment with ethyl methane sulfonate; iron-independent antibiotic production; Sm^r, Rf^r	Lindow, 1985
JL4658 A506 Ant$^-$	Antibiotic-deficient mutant with two insertions of mini-Tn*5gfp-km*; Gm^r, Km^r, Sm^r, Rf^r; GFP positive.	This study
JL4657 A506 Ant$^+$	Non-fluorescent, putative mini-*Tn5km* mutant; iron-independent antibiotic production; Km^r, Sm^r, Rf^r.	This study
JL4716 to JL4718	Three putative mini-Tn*5km* mutants exhibiting delayed iron-dependent antibiotic production,; Km^r, Sm^r, Rf^r.	This study
JL4689 to JL4698	Ten non-fluorescent, putative mini-Tn*5km* mutants; iron-independent antibiotic production; Km^r, Sm^r, Rf^r.	This study
JL4699 to JL4714	Sixteen non-fluorescent, putative mini-Tn*5km* mutants; iron-dependent antibiotic production; Km^r, Sm^r, Rf^r.	This study

Table 3.1 continued on page 47.

Strain	Relevant characteristics[a]	Source
JL4720	A506 (pJEL4658); antibiotic deficient; Gm^r, Km^r, Sm^r, Rf^r; GFP positive.	This study
JL4722	A506 (pJEL6310); antibiotic deficient; Gm^r, Km^r, Sm^r, Rf^r; GFP positive.	This study
Erwinia amylovora		
LA391 Ea153N	Bacterial pathogen that causes fire blight of pome fruit trees; Nx^r.	Johnson et al., 1993a
Escherichia coli		
JL1931	S17 (pUT); mini-Tn*5km*; Ap^r, Km^r.	Loper
L6256	S17.1 (pTGN); mini-Tn*5gfp-km*; Gm^r, Km^r.	Loper
DH5α	Competent cells	Loper
DH10B	Electrocompetent cells	Life tech
L6310	DH5α (pUC18 containing 6 to 9 kb (*spe*I) genomic DNA region of JL4658 with insertion); Ap^r, Gm^r, Km^r; GFP negative.	This study
JL6311	DH10B (pJEL4658); Gm^r, Km^r, GFP-negative.	This study

[a] Ap^r, Gm^r, Km^r Nx^r, Rf^r, Sm^r, indicates resistance to ampicillin, gentamicin, kanamycin, nalidixic acid, rifampicin, and streptomycin, respectively. GFP indicates green fluorescent protein.

All strains were stored at -80°C in nutrient broth (Difco laboratories, Detroit, MI) amended with 15% v/v glycerol and were recovered from storage on solid King's medium B (King et al., 1954) or Luria-Bertani medium (Sambrook et al., 2001) amended with appropriate antibiotics. Bacteria were cultured routinely in Luria Broth (LB, Sambrook et al., 2001) unless otherwise noted. Bacteria were screened on 925-TNG medium which is a modification of 925 minimal medium consisting of 20 mM $K_2HPO_4 \cdot 3H_2O$, 10 mM $Na_2H_2PO_4$, 20 mM NH_4Cl, 3 mM $MgSO_4 \cdot 7H_2O$, 0.3 μM

thiamine, 0.1 μM nicotinic acid, 1% w/v potassium gluconate, and 1.5% w/v agar (Bacto-agar, Difco laboratories, Detroit, MI). All ingredients were prepared as filter-sterilized stocks and added to agar after autoclaving. Medium 925-TNG Fe indicates that 0.1 mM $FeCl_3$ was added to 925-TNG. Incubation temperatures for *P. fluorescens* and *E. amylovora* were 27°C, unless otherwise noted. Strains of *E. coli* were incubated at 37°C. Antibiotics were obtained from Sigma Chemicals (Sigma Chemicals, St Louis, MO) and added to media at the following concentrations: 100 μg/ml ampicillin, 50 μg/ml cycloheximide, 12.5 μg/ml gentamicin (*E. coli)*, 40 μg/ml gentamicin (*P. fluorescens)*, 50 μg/ml kanamycin, 50 μg/ml nalidixic acid, 50 μg/ml rifampicin, and 50 μg/ml streptomycin unless otherwise noted.

Transposon mutagenesis:

Two transposon constructs were used for mutagenesis of A506. One was a 2.0 kb transposon Tn*5* (mini-Tn*5km*) on plasmid pUT. The transposon Tn*5* encodes for neomycin phosphotransferase (*npt*II) driven off its own promoter, and this gene confers resistance to the antibiotic kanamycin (de Lorenzo et al., 1990). A second transposon construct used is a bi-functional mutagen and promoter probe called mini-Tn*5gfp-km* (Fig. 3.1) on plasmid pTGN (Tang et al., 1999). The promoter probe portion of this 2.1 kb construct consists of a promoterless artificial operon containing a gene encoding for the UV-shifted, green fluorescent protein (*gfp-uv*) as well as *npt*II (Tang et al., 1999). The gene encoding for gentamicin resistance (*aacCi*) of this construct is expressed constitutively (Tang et al. 1999).

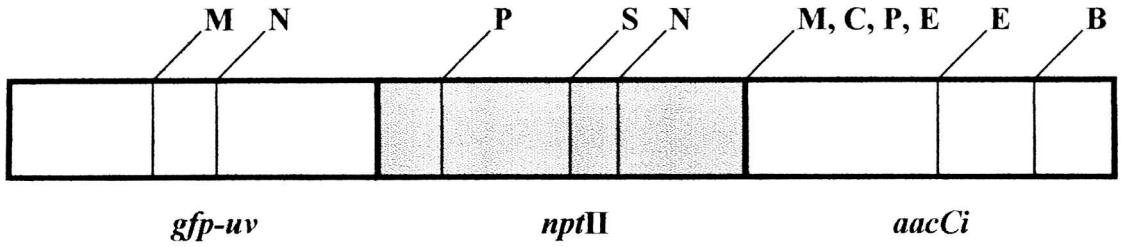

Fig. 3.1. Tn*5gfp-km* transposon restriction map. Restriction enzymes are M) *Mlu*I, C) *Cla*I, B) *Bgl*II, S) *Sph*I, N) *Nco*I, E) *Eco*RV, and P) *Pst*I.

Plasmids carrying transposon constructs were introduced into A506 from strains of *E. coli* by standard methods for conjugal transfer (Sambrook et al, 2001). Putative mini-Tn*5km* transconjugants were selected on King's medium B amended with rifampicin and kanamycin. Putative mini-Tn*5gfp-km* transconjugants were selected on 925-TNG Fe amended with rifampicin and kanamycin. Putative Tn*5gfp-km* mutants of A506 that grew on the defined medium and expressed GFP were selected for further evaluation. All putative transconjugants were transferred to individual wells of 96-well plates containing nutrient broth amended with 15% v/v glycerol and stored at -80°C.

Selection assay:

Putative transposon mutants of A506 were screened for loss of antibiotic production in a preliminary broth selection assay. This assay consisted of transferring mutants of A506 from solid King's medium B amended with rifampicin and kanamycin with a 48-pin replicator into 96-well plates containing 925-TNG Fe broth. In each plate, A506 and A506 GacS$^-$ were included in two wells as antibiotic-positive and antibiotic-deficient controls, respectively. After incubation for 48 h at 20°C, a 50 µl suspension of Ea153N at 10^6 CFU/ml was introduced into each well with a multi-channel pipettor (Titertek Octapippetter, Eflab, Finland). After an additional 48 h incubation at 20°C, a 5 µl sample from each well was transferred to solid CCT (Ishimaru and Klos, 1984) amended with 100 µg/ml nalidixic acid (Ea153N selective media) and solid King's medium B amended with rifampicin and kanamycin. Putative mutants from wells that had both culturable populations of Ea153N and a derivative of

A506 were evaluated on solidified 925-TNG Fe in 90 mm petri dishes for lack of inhibition of Ea153N.

Selection of non-fluorescent mutants of A506.

The chemically-induced non-fluorescent mutant of A506, called A506 Fl-1 (Table 3.1), does not require excess iron to produce the antibiotic (Stockwell, personal communication). Non-fluorescent mini-Tn*5km* mutants of A506 were selected by culturing cells in King's media B broth amended with 1.0 mM of the iron chelator HBED (N,N'-di(2-hydroxy benzyl) ethylene diamine-N,N'-diacetic acid monohydrochloride hydrate (Sigma Chemicals)) in 96-well plates for 48 h at 20°C. Colonies in individual wells were examined with long UV radiation (404 nm) for lack of fluorescence. Non-fluorescent culturable mutants from solidified King's medium B, that corresponded to wells of the broth plus HBED were selected.

Antibiotic production assay:

Antibiotic production was assayed on solidified 925-TNG Fe and on 925-TNG. Producer test strains were precultured on solidified King's medium B amended with rifampicin for A506 or King's medium B amended with rifampicin and kanamycin for mutants of A506. Bacterial suspensions at an optical density of 0.1 OD_{600nm} (Spectronic 20, Bausch and Lomb, Rochester, NY) were spotted in 12µl samples onto the center of 90 mm petri plates containing 925-TNG Fe or 925-TNG. After growth at 27°C for 72 h, colonies were scraped from the surface of the media and residual cells were killed by a 5 min exposure to UV radiation or 10 min exposure to chloroform vapors. A 10^6 CFU/ml suspension of Ea153N was atomized over the surface of the medium or 0.5 ml

of a 10^8 CFU/ml suspension of Ea153N was added to 4.5 ml molten 925-TNG and overlaid on the medium. The plates were incubated at 27°C for 48 h before examination for a zone of inhibition.

Characterization of mutants:

All mutants that exhibited altered patterns of antibiotic production were subjected to standard tests, such as resistance to rifampicin and streptomycin, production of a fluorescent pigment on King's medium B, and oxidase-positive to ensure that they were *Pseudomonas fluorescens* strain A506 (King et al., 1954; Schaad, 1980; Lindow, 1985). Additionally, all antibiotic-deficient mutants were tested for their capacity to produce extracellular protease on Litmus milk agar (LMA, Difco) to select against putative GacS$^-$ deficient strains of A506 (Corbell and Loper, 1995). Mutants satisfying all of these criteria were selected for genetic characterization.

DNA isolation: Genomic DNA was extracted and purified with the CTAB (hexadecyl-trimethyl ammonium bromide) method described by Ausubel et al. (1991). Plasmid DNA of A506 was extracted by the alkaline lysis method described by Sambrook et al. (2001). Plasmids of *E. coli* were isolated with a plasmid mini-preparation kit (Wizard mini-prep, Promega).

Southern blot analysis: Southern blots of genomic DNA of A506 Ant$^+$ and genomic and plasmid DNA of A506 Ant$^-$ followed standard procedures (Sambrook et al., 2001). A506 Ant$^+$ genomic DNA were digested with *Eco*RV (no restriction sites within the Tn*5-km*) with expected insert size of 2.0 kb. Probe was prepared from Tn*5-km Eco*RI insert fragment with biotynylated dNTPs (BioPrme DNA Labeling System,

Gibco BRL Life Technologies). A506 Ant⁻ genomic and plasmid DNA were digested with enzymes that have no restriction sites within the Tn*5-gfp-km* (*Spe*I & *Mun*I; 2.1 kb expected size), one site (*Eco*RI; 1.5 and 0.5 kb fragment size, *Bgl*II; 1.8 and 0.2 kb, & *Bam*HI; 2.0 and 0.02 kb), or two sites (*Nco*I; 0.6, 0.7, and 0.8 kb, *Mlu*I; 1.1, 0.7 and 0.3 kb, *Eco*RV; 1.5, 0.5 and 0.1 kb, & *Pst*I; 0.9, 0.7, and 0.8 kb). Southern blots were prepared using nylon membranes (Nytran; Schleicher & Schuell). Probe was Tn*5gfp-km Hind*III-*Mlu*I insert fragment with biotynylated dNTPs (BioPrime DNA Labeling System, Gibco BRL Life Technologies). Hybridizations were visualized using the Blue Gene kit (Gibco BRL Life Technologies).

Inverse PCR: Inverse PCR of A506 Ant⁻ genomic DNA followed standard procedures (Sambrook et al., 2001) using the primers listed in Table 3.5. The inverse PCR reaction mixture consisted of 1X PCR buffer (Invitrogen Corporation, Carlsbad, CA), 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ (Invitrogen), 0.15 μM of each primer, and 1.0 Units of Taq DNA polymerase (Invitrogen). Inverse PCR reaction conditions were 1 cycle at 94°C (2 min), 35 cycles at 94°C (45 sec); 58°C (1 min); 72°C (2 min), and 1 cycle at 72°C (10 min), and amplification was carried out on a Power block II™ system easycycler series (Ericomp, Inc. San Diego, CA).

Table 3.2. Primers used for inverse PCR reactions.

Primer	Sequence 5' to 3'	Length	Tm[z]
EcoRVGm-R	TGTTAGGTGGCGGTACTTGGG	21	64
EcoRVnpt-F	GGCTGACCGCTTCCTCGTG	19	66
PstInpt-F	GTGGAGAGGCTATTCGGCT	19	62
PstIGm-R	GATGAATGTCTTACTACGG	19	56
mTn5O-end	TTAACCCATGACATATACC	19	54
mTn5I-end	GATGGCGCAGGGGATCAAG	19	64

[z] Tm = Melting temperature

Thermal asymmetric interlaced (TAIL) PCR: Genomic DNA of A506 Ant$^-$ was used as a template for TAIL PCR procedures as described by Liu and Whittier (1995) using the primers listed in Table 3.6. The TAIL PCR primary reaction mixture consisted of 1X PCR buffer (Promega), 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ (Promega), 0.15 µM specific primer, 5.0 µM degenerate primer, and 2.5 Units of Taq DNA polymerase (Invitrogen), the secondary reaction mixture consisted of 1X PCR buffer (Promega), 0.025 mM of each dNTPs, 1.5 mM $MgCl_2$ (Promega), 0.2 µM specific primer, 3.0 µM degenerate primer, and 2.5 Units of Taq DNA polymerase (Invitrogen), and the tertiary reaction mixture consisted of 1X PCR buffer (Promega), 0.025 mM of each dNTPs, 1.5 mM $MgCl_2$ (Promega), 0.2 µM specific primer, 3.0 µM degenerate primer, and 3.5 Units of Taq DNA polymerase (Invitrogen). The TAIL PCR secondary reaction mixture template (5 µl) was a dilution (1:100) of primary reaction PCR products, and tertiary reaction mixture template (5 µl) was a dilution (1:100) of secondary reaction PCR products. TAIL PCR reaction conditions for the primary reaction were 1 cycle at 92°C (2 min); 95°C (1 min), 5 cycles at 94°C (15 sec); 63°C (1 min); 72°C (2 min), 1 cycle at 94°C (15 sec); 30°C (3 min); 30-72°C (0.2 deg/sec) (3 min); 72°C (2 min), 10 cycles at 94°C (5 sec); 44°C (1 min); 72°C (2 min), 12 cycles at 94°C (5 sec); 63°C (1 min); 72°C (2 min); 94°C (5 sec); 44°C (1 min); 72°C (2 min), and 1 cycle at 72°C (5 min). Reaction conditions for the secondary reaction were 10 cycles at 94°C (5 sec); 63°C (1 min); 72°C (2 min); 94°C (5 sec); 44°C (1 min); 72°C (2 min) and 1 cycle at 72°C (5 min). Reaction conditions for the tertiary reaction were 20 cycles

at 94°C (10 sec); 44°C (1 min); 72°C (2 min) and 1 cycle at 72°C (5 min). Amplification was carried out in a Minicycler (MJ Research Inc., Waltham, MA).

Table 3.3. Primers used for TAIL PCR reactions.

Primer	Sequence 5' to 3'	Length	Tm
tpg_R1[x]	GGAGAGGGTGAAGGTGATGC	20	64
tpg_R2	ACAAGACGCGTGCTGAAGTCAA	22	66
tpg_R3	GCTAACTTCAAAATTCGCCACAAC	24	68
AD1[y]	TGWGNAGWANCASAGA[z]	16	46
AD2	AGWGNAGWANCAWAGG	16	44
AD3	GTNCGASWCANAWGTT	16	46
AD4	WGTGNAGWANCANAGA	16	45

[x] AD = arbitrary degenerate primer
[y] tpg_R = TAIL PCR GFP reverse primer
[z] W = random A or T, N = random A, T, G, or C , and S = random G or C.

PCR of gfp region: Amplification of the *gfp* region of Tn*5gfp-km* followed standard procedures (Sambrook et al., 2001) using the primers gfpUV-F 5' to 3' (ATGGTCTGCTAGTTGAACGG) and gfpUV-R (AGAGGGTGAAGGTGATGCAA). The reaction mixture, conditions and amplification were similar to inverse PCR procedures.

DNA Sequence: DNA sequencing was done at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis. Sequencing of inverse PCR and TAIL PCR products were done with primers complementary to the Tn*5* DNA. Analysis of DNA and comparisons with sequences in the GenBank database used the Blast software program.

Subcloning: Genomic DNA of A506 Ant$^-$ was digested with *Spe*I and subjected to agarose gel electrophoresis. Agarose containing fragments approximately 6.0 kb to 9.0kb was removed and DNA was extracted using a QIAEX II agarose gel extraction kit (Qiagen, Valencia CA). The plasmid vector pUC18 (Gibco-BRL, Life Technologies)

was digested with *Xba*I and the ends were dephosphorylated by standard methods. Isolated *spe*I-genomic fragments from A506 Ant$^-$ were ligated into *Xba*I-digested pUC18 using T4 DNA ligase (Gibco-BRL Life Technologies). *E. coli* DH5α was transformed with the ligation products by standard procedures (Sambrook et al., 2001) and spread on Luria-Bertani media amended with ampicillin, gentamicin, and Xgal to select for transformants harboring pUC18 with genomic DNA of A506 Ant$^-$ containing Tn*5gfp-km* (Sambrook et al., 2001).

Electroporation: Isolated plasmid DNA of A506 Ant$^-$ was transformed into *E. coli* DH10B (Gibco-BRL, Life Technologies) electrocompetent cells following the protocol of Sambrook (2001) using a Cell-porator voltage booster, pulse control and power supply, and chamber safe as recommended by the manufacturer (Gibco-BRL, Life Technologies). Transformants were selected on Luria-Bertani media amended with gentamicin or kanamycin.

Characterization of selected mutants:

Two mutants A506 Ant$^-$ and A506 Ant$^+$ were evaluated for epiphytic growth on blossoms in orchards by methods described below. A506 Ant$^-$ also was subjected to additional phenotypic tests in the laboratory. Strains A506 and A506 GacS$^-$ served as control strains in each assay. A506 Ant$^+$ also was evaluated for its capacity to suppress fire blight in three orchard trials.

Growth rates: Bacteria were cultured in 25 ml of King's medium B or 925-TNG Fe broth at 27°C and 200 rpm in a 125 ml side-arm flask with two replicates flasks per strain per medium. Initial cell densities were adjusted to values between 0.01

to 0.05 OD_{600nm} using a Spectronic 20 (Bausch and Lomb). Optical density was measured over time until cultures reached saturation.

Desiccation tolerance: Assays were performed in sealed plastic chambers containing distilled water or a saturated solution of NaCl to maintain relative humidity at 100% or 75%, respectively. 10 µl samples of bacterial suspensions (OD_{600nm} 0.1) were spotted to 1-cm^2 pieces of sterile filter paper (Whatman No. 1; Whatman International, Ltd., Maidstone, England) and incubated at room temperature (~20°C). Periodically five replicate filter papers per treatment were removed, placed in 1 ml sterile 10 mM phosphate buffer, sonicated (Sonix IV, Inglewood, CA) for 3 min, and the sample and two 100-fold serial dilutions were spread on King's medium B plates. Populations of bacteria were counted after the plates were incubated at 27°C for three days. This experiment was repeated 3 times.

UV radiation tolerance: 10 µl samples of four 100-fold serial dilutions of OD_{600nm} 0.1 (ca. 1 x 10^8 CFU per ml) bacterial suspensions were spread on King's medium B. Five replicate dilution series plates were spread per strain. Bacteria on the surface of agar were treated with timed exposures of UV radiation (254 nm) and incubated in darkness at room temperature for three days before colonies were enumerated.

Motility assay: Motility was assayed on two media, semi-solid King's medium B and semi-solid 925-TNG plus 0.1 mM ferric citrate. Each medium contained 0.4% (w/v) Difco-Bacto agar and was poured into 90 mm polystyrene petri plates (Sambrook et al., 2001). Bacterial strains were cultured on solid media and transferred to semi-

solid media with either a toothpick or 5 µl of an aqueous suspension (1 x 10^8 CFU/ml) was pipetted onto the test media Petri plates were incubated on a level surface at 27°C for 48 hours and measurements of colony diameter (mm) were recorded over time.

Epiphytic growth of A506 Ant⁻ on blossoms: A506 and A506 Ant⁻ were applied to four replicate pear trees (*Pyrus communis* L. cv. 'Bartlett') twice between 30% to 100% bloom at the southern Oregon Research and Extension Center near Medford, OR, and at 100% bloom on apple trees (*Malus* X *domestica* Borrkh. cv. 'Rome Beauty') at the Oregon State University, Botany and Plant Pathology Field Laboratory near Corvallis, OR.

Preparation, treatment, and recovery of A506 Ant⁻: A506 and A506 Ant⁻ were cultured for 4 d at 27°C on nutrient agar (Difco) amended with 0.4% w/v glycerol and 0.5% w/v sucrose followed by scraping bacterial lawns into 10 mM potassium phosphate buffer (pH 7.0) to 0.1 OD_{600nm} (Spectronic 20, Bausch and Lomb) or approximately 1 x 10^8 CFU/ml. A506 and A506 Ant⁻ bacterial suspensions were applied to blossom clusters using a hand-held sprayer (Corvallis, Rome Beauty) or applied with a 12 L backpack sprayer fitted with a hand-held wand (Medford, Bartlett) till run-off. On Bartlett pear and Rome Beauty apple, an iron chelate was added to A506 Ant⁻ bacterial suspensions applied at a concentration of 0.1 mM FeEDDHA (Sequestrene 138, 6% a.i.; Becker Underwood, Ames, IA).

Ten to 20 blossoms were sampled per treatment per sample date. The pistil and hypanthia for pear blossoms and the stigmas and styles for apple blossoms were excised and placed in 1 ml sterile 10 mM potassium phosphate buffer (pH 7.0) and sonicated

(Sonix IV, Inglewood, CA) for three min. A 10 µl sample of wash and two 100-fold serial dilutions were spread on Pseudomonas Agar F (PAF, Difco) amended with 50 µg/ml of rifampicin and cycloheximide for selective recovery of A506 or PAF amended with 50 µg/ml of kanamycin and cycloheximide for selective recovery of A506 Ant$^-$. Bacteria were incubated at 27°C for three days and bacterial colonies were enumerated.

Epiphytic growth and relative disease suppression by non-fluorescent mutants of A506: Field experiments were conducted at the southern Oregon Research and Extension Center near Medford, OR on Bartlett pear trees (*Pyrus communis* L.) and at the Oregon State University, Botany and Plant Pathology Field Laboratory located near Corvallis, OR on Golden Delicious and Rome Beauty apple trees (*Malus* X *domestica* Borrkh). For each treatment, there were 5 replicate trees in Corvallis or 4 replicate trees in Medford at 30 and 80% bloom.

Treatment of blossoms with non-fluorescent mutants: A506, A506 Ant$^+$, and A506 Fl-1 were cultured for 4 d at 27°C on nutrient agar (Difco) amended with 0.4% w/v glycerol and 0.5% w/v sucrose followed by scraping bacterial lawns into 10 mM potassium phosphate buffer (pH 7.0) to 0.1 OD_{600nm} (Spectronic 20, Bausch and Lomb) or approximately 1 x 10^8 CFU/ml. A506, A506 Ant$^+$ and A506 Fl-1 bacterial suspensions were applied to blossoms at 30 and 80% bloom with a 12 L backpack sprayer fitted with a hand-held wand till near run-off. Additional control trees in each field plot were treated with water or streptomycin sulfate (Agristrep 17%, 100 µg/ml, Novartis Crop Protection, Greensboro, NC). Water was applied to trees at 30% and

80% bloom (negative control), and streptomycin was applied at 70% and at full bloom, immediately following pathogen inoculation.

Pathogen inoculation onto blossoms: At full bloom, suspensions of Ea153N (2.5×10^5 for pear and 1×10^6 CFU per mL for apple) were misted onto trees in the evening under still wind conditions. Applied volumes ranged from 2 to 3 L per tree depending on tree size. Streptomycin antibiotic treatment was applied to trees at 80% bloom and the day following inoculation with Ea153N.

Recovery of bacterial treatments from blossoms: One day after pathogen inoculation, eight to 12 blossoms were sampled per treatment every 5 to 10 days for a total of 4 sample dates per experiment. Bacterial populations were recovered and enumerated as described above. Strains A506 and A506 Fl-1 were enumerated on PAF amended with rifampicin and cycloheximide and A506 Ant^+ was enumerated on PAF amended with kanamycin and cycloheximide.

Disease incidence: In each plot, blossom clusters with fire blight symptoms (necrosis, wilting, and/or bacterial ooze) were counted over a five week period. The sum of fire blight strikes was calculated for each treatment in each block.

Data Analysis: For the experiments comparing epiphytic fitness of A506 to derivatives of A506, the mean and standard error of population size recovered from blossoms were calculated by averaging $\log_{10}$ values obtained from individual trees. Prior to $\log_{10}$ transformations, bacterial counts of zero were set to values of 99 (i.e., below the detection limit of 1×10^2 CFU per ml). Analysis of variance (ANOVA) and

Fischer's t-test protected least significant difference was used to determine significance between the means (GLM, Statistical Analysis System, SAS Institute, Cary, NC).

Percent survival values of A506, A506 Ant$^-$ or A506 GacS$^-$ for UV radiation tolerance were determined from colony counts ($\log_{10}$ transformed) of recovered cells of UV irradiated plates compared to non-irradiated plates. Non-transformed ratios were subjected to ANOVA and Fischer's protected least significant difference t-test (GLM, Statistical Analysis System, SAS Institute, Cary, NC).

For the desiccation tolerance experiment comparing the population responses of A506, A506 Ant$^-$ and A506 GacS$^-$, the mean and standard error of population size recovered from blossoms were calculated by averaging $\log_{10}$ values obtained from the dilution-plated filter papers. Log transformed mean populations were subjected to ANOVA and Fischer's protected least significant difference t-test (GLM, Statistical Analysis System, SAS Institute, Cary, NC).

For the motility experiment, the diffusion distance (mm per 24 h) was calculated for A506, A506 Ant$^-$ or A506 GacS$^-$ and non-transformed rates were subjected to ANOVA and Fischer's protected least significant difference t-test to separate rate values (GLM, Statistical Analysis System, SAS Institute, Cary, NC).

Diseased blossom cluster numbers per tree were converted to a relative disease incidence by dividing the incidence of blossom blight strikes on treated trees by the incidence on water-only treated trees within each respective block. Arcsine square root-transformation was calculated before ANOVA of the relative disease incidence data.

Fisher's protected least significant difference (LSD) test at $P = 0.05$ was used to separate the mean relative values calculated for each treatment in each trial.

Results:

Selection of antibiotic-deficient mutants: A total of 8736 putative mini-Tn*5km* mutants of A506 were selected and screened in a broth inhibition assay for loss of antibiosis. Of these mutants, 8186 inhibited Ea153N in the preliminary 925-TNG Fe selection assay and 550 did not. Of the 550 mutants, 40 (7%) exhibited auxotrophy and were unable to grow on 925-TNG. Nineteen (4%) antibiotic-deficient mutants also were extracellular protease-deficient, two phenotypes exhibited by A506 GacS$^-$, and these mutants were not characterized further. Three mutants did not inhibit Ea153N on solid 925-TNG Fe when precultured for 24 to 48 h (Table 3.1), but after 5 d these mutants inhibited growth of Ea153N on solid 925-TNG Fe medium (Fig. 3.2). These mutants were not characterized further.

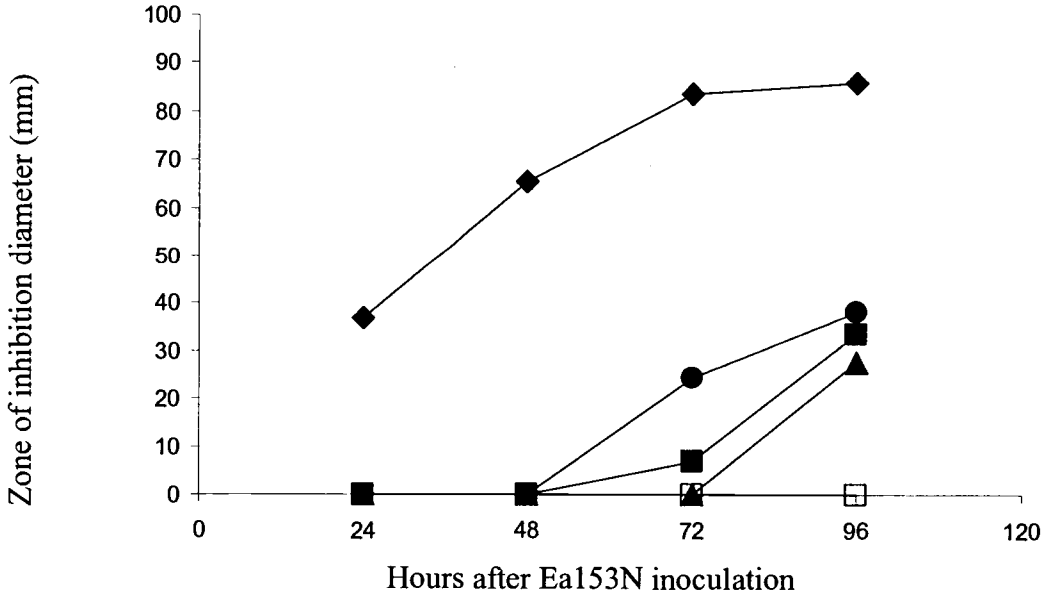

Fig 3.2. Average diameter of the zone of inhibition to *E. amylovora* 153N produced by *P. fluorescens* A506 (◆), A506 GacS$^-$ (□), JL4716 (▲), JL4717 (●), or JL4718 (■) when cultured up to five days on solidified 925-TNG plus 0.1mM $FeCl_3$.

Promoter probe to detect antibiotic deficient mutant: In another mutagenesis procedure using a gfp promoter probe, 1504 putative mini-Tn*5gfp-km* mutants of A506 expressing GFP were selected on a solidified 925-G Fe medium. Of these mutants, 1288 inhibited Ea153N in the initial broth 925-TNG Fe screening assay and 216 (14%) did not. The 216 mutants were subjected to repeated inhibition assays on solidified 925-TNG Fe and only 11 mutants did not inhibit Ea153N. Ten of these mutants were extracellular protease deficient, presumed to be regulatory gene mutants, and were not characterized further. One mutant named A506 Ant$^-$ or JL4658 did not inhibit growth of Ea153N in repeated assays and was extracellular protease positive (Table 3.1). GFP expression was not iron-regulated in A506 Ant$^-$ as this mutant was GFP$^+$ on iron-amended and non-amended media.

Genetic characterization of A506 Ant$^-$: Southern analysis of genomic DNA preparations of A506 Ant$^-$ digested with restriction enzymes that cut outside the miniTn*5gfp-km* cassette showed a single insertion (Fig. 3.3). Southern analysis of plasmid DNA preparations of A506 Ant$^-$ indicated that the cassette also inserted into a plasmid (Fig. 3.4).

The procedures inverse PCR and TAIL PCR were used to amplify genomic DNA flanking the miniTn*5gfp-km* cassette for sequencing. PCR products ranging in size from 1.0 to 2.0 kb were obtained. Sequence analysis showed that *gfp*, *npt*II or *aacCi* in the Tn*5* insert were consistently amplified from genomic DNA of A506 Ant$^-$. Unfortunately, sequence data could not be obtained from genomic DNA flanking the

insert because of long regions of unreadable sequence where nucleotides could not be identified.

Because direct amplification of genomic DNA flanking the insert in A506 Ant$^-$ was inconclusive, an 8.0 kb region of *Spe*I cut genomic DNA containing the insert was subcloned into *Xba*I cut pUC18. *E. coli* DH5α transformed with pUC18::Ant$^-$ (L6310) was resistant to gentamicin but GFP$^-$, although the presence of *gfp* was confirmed by PCR. A506 transformed with pUC18::Ant$^-$ (L6310) was resistant to kanamycin and gentamicin, GFP$^+$, and did not inhibit growth of Ea153N on solid 925-TNG Fe medium (Table 3.1).

From Southern analysis, it appeared that the plasmid of A506 Ant$^-$ (pJEL4658 or pAnt$^-$) also contained the promoter probe cassette. *E. coli* DH10B transformed with pAnt$^-$ (L6311) by electroporation was resistant to gentamicin. Like DH5α (L6310), GFP was not expressed by DH10B (L6311), but *gfp* was confirmed by PCR. A506 transformed with pAnt$^-$ was resistant to kanamycin and gentamicin, GFP$^+$. Significantly, A506 (pAnt$^-$) did not inhibit growth of Ea153N on solid 925-TNG Fe medium (Table 3.1). No further genetic characterization was completed.

Epiphytic fitness of A506 Ant$^-$: In two trials on apple and pear trees when applied suspended in 0.1 mM FeEDDHA, the population size of A506 Ant$^-$ from blossom washings was significantly lower ($P < 0.05$) than the populations of the parental strain A506 (Fig. 3.5). The population size of A506 Ant$^-$ applied to trees suspended in water also was significantly lower ($P < 0.05$) than A506 nine days after inoculation onto Rome Beauty apple blossoms (Fig. 3.5).

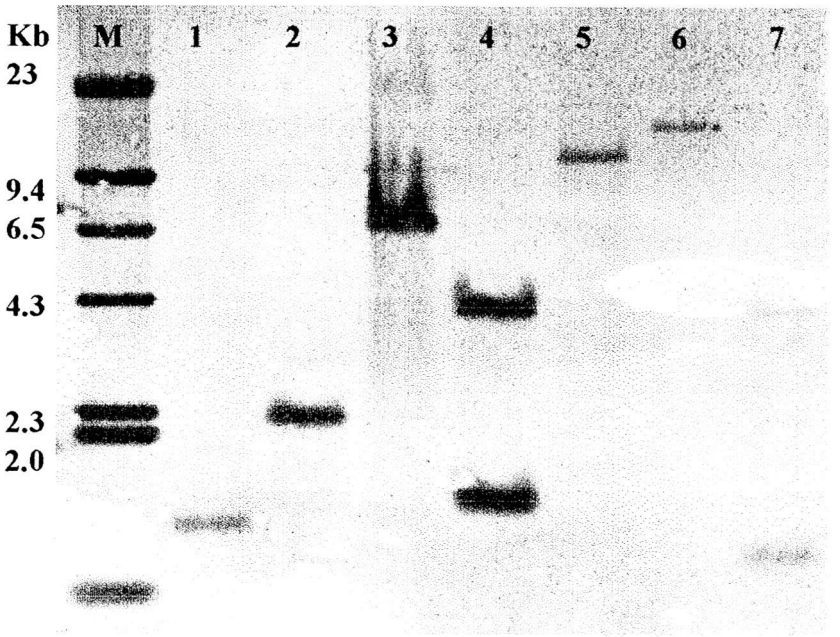

Fig 3.3. Southern blot of genomic DNA of A506 Ant$^-$. Lanes include M) biotin labeled lambda *Hind*III molecular weight marker and 1) *Eco*RI, 2) *Mun*I, 3) *Spe*I, 4) *Nco*I, 5) *Bam*HI, 6) *Bgl*II and 7) *Pst*I restriction enzyme digested DNA. Bands visualized using biotin labeled *npt*II region of pTGN. Enzymes in lanes 1, 2, 3 & 5 do not cut within *npt*II region of insert and show single bands. Enzymes in lanes 4, 6, & 7 cut once in *npt*II region of insert and shows two bands.

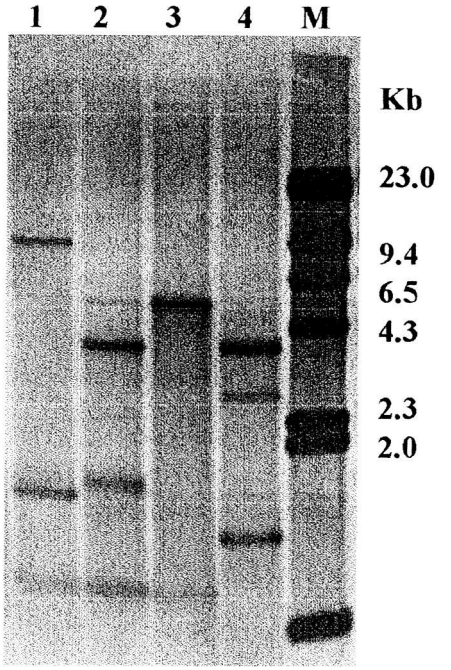

Fig. 3.4. Southern Blot of plasmid DNA of pAnt$^-$. Lanes include M) biotin labeled lambda *Hind*III molecular marker and 1) *Mlu*I, 2) *Pst*I, 3) *Eco*RV, and 4) *Nco*I restriction enzyme digested DNA. Bands visualized using biotin labeled *gfp-km-aacCi* region of pTGN. Enzymes in lanes 1, 2, 3, & 4 cut twice within the insert and show three expected bands, with the exception of lane 3 where a 0.1 kb DNA fragment was not detected.

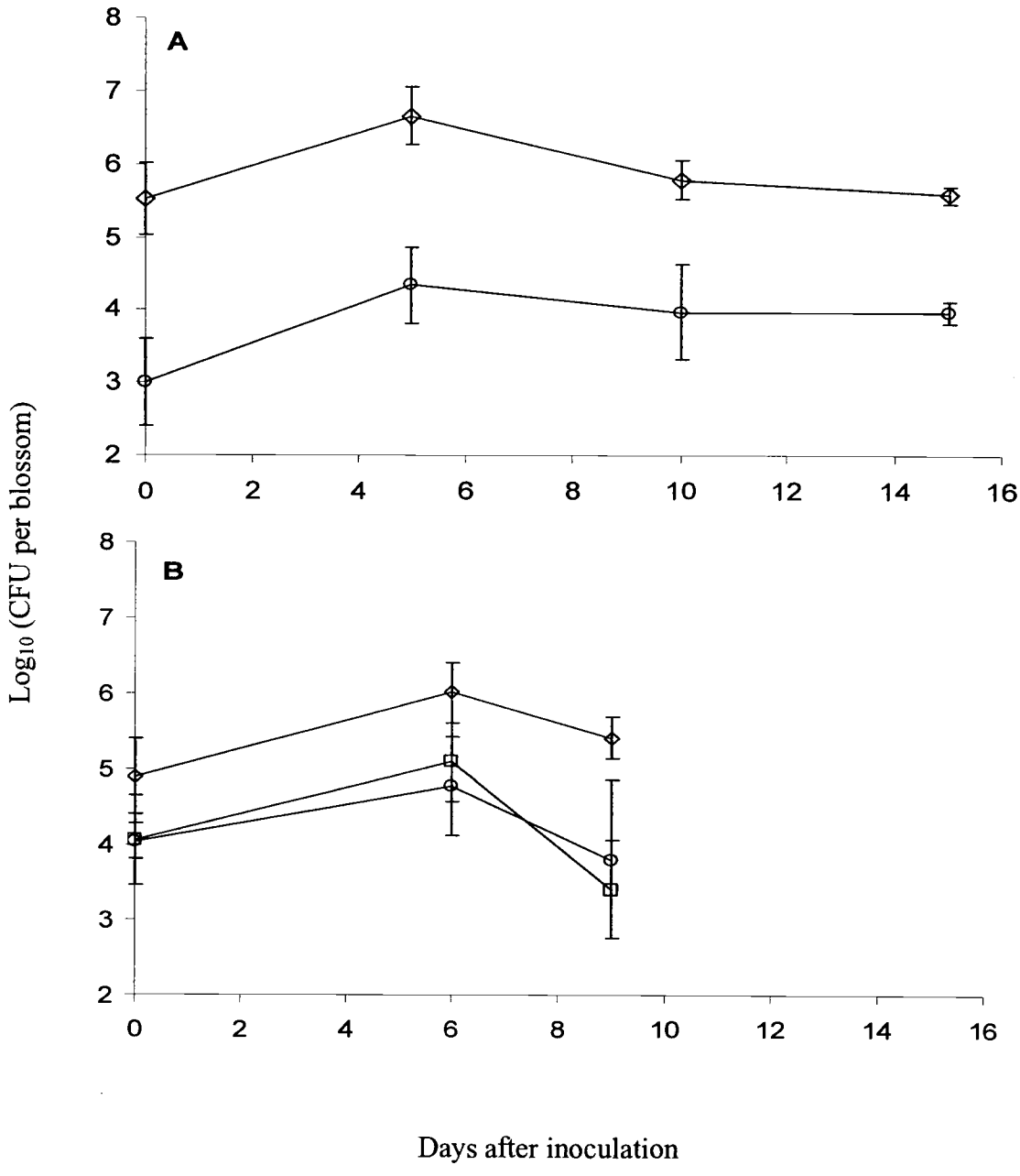

Fig 3.5. Population size (log_{10}) of *P. fluorescens* A506 plus water (◇), A506 Ant$^-$ plus 0.1 mM FeEDDHA (○), or A506 Ant$^-$ plus water (□) on blossoms of **A)** Bartlett pear and **B)** Rome Beauty apple. Vertical bars represent one standard error of the mean population size on individual blossoms.

Growth rates and relative stress tolerance of A506 Ant⁻ in culture: A506 and A506 Ant⁻ exhibited similar growth rates at 27°C with a doubling time of 90 min in 925 minimal broth medium amended with potassium gluconate and ferric citrate and 30 min in King's medium B broth (data not shown). A506 and A506 Ant⁻ also demonstrated similar tolerance to UV radiation, whereby doses of ca. 165 Erg/mm^2/sec were required before significant decreases in population sizes were measured (data not shown). A506 and A506 Ant⁻ populations were maintained (10^6 CFU per sample) when incubated at 100% relative humidity (data not shown). A506 Ant⁻ demonstrated decreased tolerance to desiccation tolerance compared to A506 when incubated at 75% relative humidity and had significantly smaller ($P < 0.05$) populations after 2 h incubation (Fig. 3.6). A506 GacS⁻ behaved similarly to A506 Ant⁻ in response to desiccation stress (Fig. 3.6).

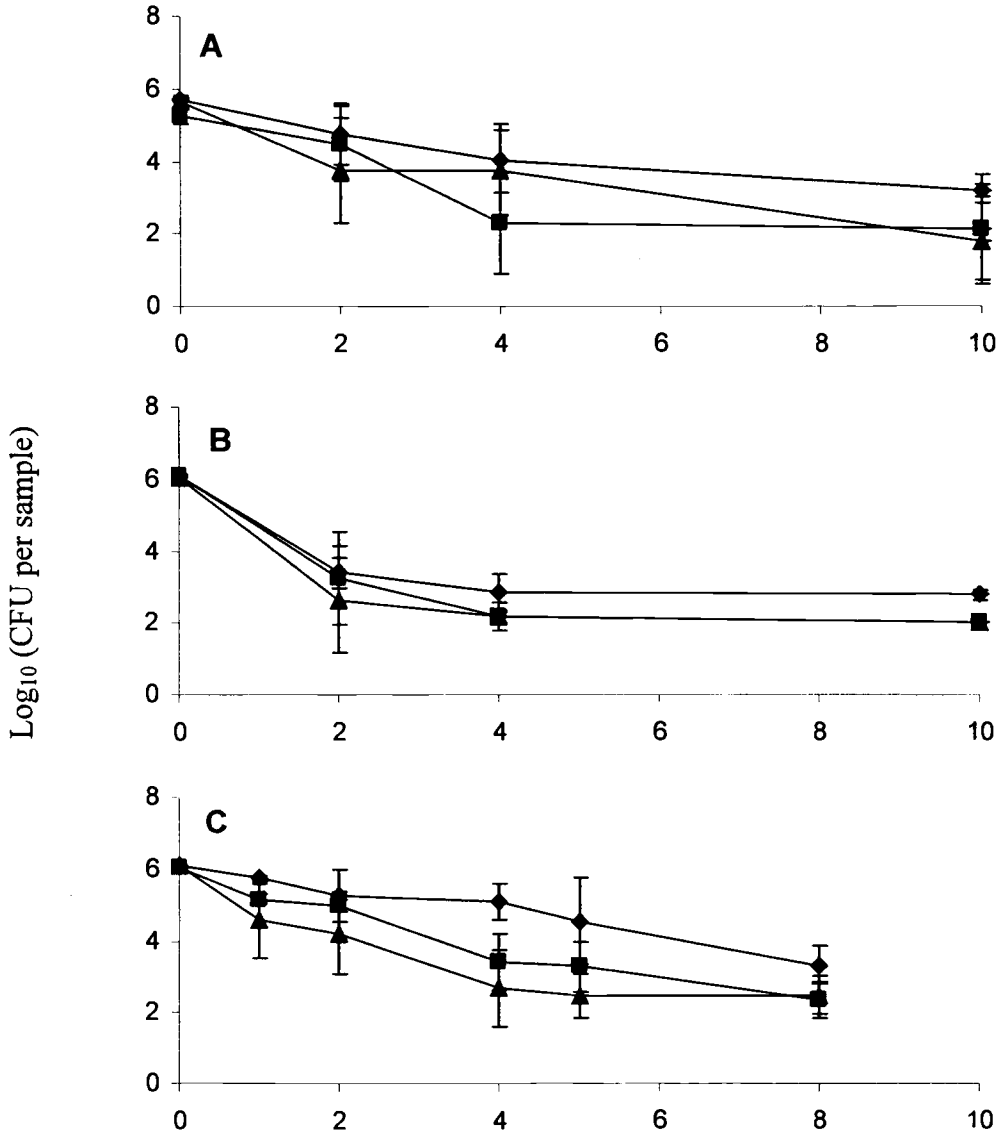

Fig 3.6. Population size (log_{10}) of *P. fluorescens* A506 (◆), A506 Ant$^-$ (■), or A506 GacS$^-$ (▲) applied to filter paper at 10^8 CFU per mL and incubated at 75% relative humidity in three trials for 24 h (**A** & **B**) (data for 24 h time point not shown but similar to 10 h) or 8 h (**C**). Sample size consisted of five replicate filter papers per strain per sample. Detection limit was 1 x 10^2 CFU per sample and bacterial counts of zero were set to values of 99. Vertical bars represent one standard error of the mean population size on individual filter papers.

A506 Ant$^-$ exhibited a rate of motility of 24 mm per 24 h, which was significantly decreased ($P < 0.05$) compared to A506, which had an average rate of 50 mm per 24 h on semi-solid King's medium B amended with ferric citrate. On an iron-amended defined medium, motility of A506 Ant$^-$ was 12 mm per 24 h and not significantly different ($P > 0.05$) to the parental strain. As a comparative control, A506 GacS$^-$ exhibited no significant difference ($P > 0.05$) in motility on defined media (12 mm per 24 h or complex media (24 mm per 24 h)) compared to A506 Ant$^-$.

Non-fluorescent mutants of A506: Of the 8736 putative miniTn*5km* mutants of A506, 23 (0.3%) non-fluorescent mutants were selected from an iron-depleted medium. Ten (44%) of the 23 non-fluorescent mutants were iron-independent in antibiosis, that is, an antibiotic was produced on solidified 925-TNG with and without iron amendment (Table 3.1). This iron-independent-antibiosis phenotype of these 10 mutants is similar to the phenotype of a non-fluorescent mutant of A506 (A506 Fl-1) selected previously by Lindow (1985).

Southern analysis of four of these mutants indicated a single insertion of mini-Tn*5km* in genomic DNA (data not shown). No additional genetic characterization was conducted.

Epiphytic fitness and relative disease suppression by A506 Ant$^+$: One of the non-fluorescent iron-independent-antibiosis mutants named A506 Ant$^+$ (JL4657) was selected for evaluation of blossom blight disease suppression in apple and pear orchards in comparison to the parental strain A506 and a chemically-induced non-fluorescent mutant named A506 Fl-1 (Lindow, 1985). The non-fluorescent mutant A506 Ant$^+$

colonized blossoms similar to the parent strain A506 in each of three field trials as did A506 Fl-1 (Fig. 3.7). In each field trial, A506 and A506 Fl-1 reduced the incidence of fire blight by similar levels (Table 3.11). The non-fluorescent mutant A506 Ant$^+$ reduced the incidence of fire blight to levels similar to A506 on Bartlett pear and Golden Delicious apple (Table 3.11). On Rome Beauty apple, the non-fluorescent mutant A506 Ant$^+$ provided significantly greater ($P > 0.05$) suppression of fire blight than the parental strain A506, but less disease suppression than the antibiotic streptomycin (Table 3.11).

Table 3.4. Relative incidence of fire blight on trees treated with water, streptomycin, *P. fluorescens* A506, A506 Ant$^+$, or A506 Fl-1

	Experimental location and cultivar		
	Medford	Corvallis	
Treatment[a]	Bartlett	Golden Delicious	Rome Beauty
Water	1.00 a[c]	1.00 a	1.00 a
A506	0.68 b	0.54 b	0.59 b
A506 Ant$^+$	0.84 a,b	0.63 b	0.43 c
A506 Fl-1	0.59 b	0.58 b	0.60 b
Streptomycin	0.15 c	0.23 c	0.12 d

[a] Trees were arranged in a complete randomized block design with four or five replications per treatment of water, A506, A506 Ant$^+$ (JL4657), or A506 FL-1which were applied at 30% and 80% bloom. At full bloom, the pathogen *E. amylovora* strain 153N was applied at 2.5 x 10^5 CFU/ml for pear and 1x 10^6CFU/ml for apple. All trials had a post-pathogen application of streptomycin within 36 hours after inoculation with the streptomycin-sensitive pathogen strain.

[b] Experiments were conducted on pear cultivar Bartlett near Medford, OR and apple cultivars Golden Delicious and Rome Beauty in experimental orchards near Corvallis, OR.

[c] Values of relative incidence of fire blight followed by the same letter within a column indicate no significant difference relative to the water-treated trees. Relative incidence values were arcsine square root-transformed and means separated using Fisher's protected least significant difference at $P = 0.05$.

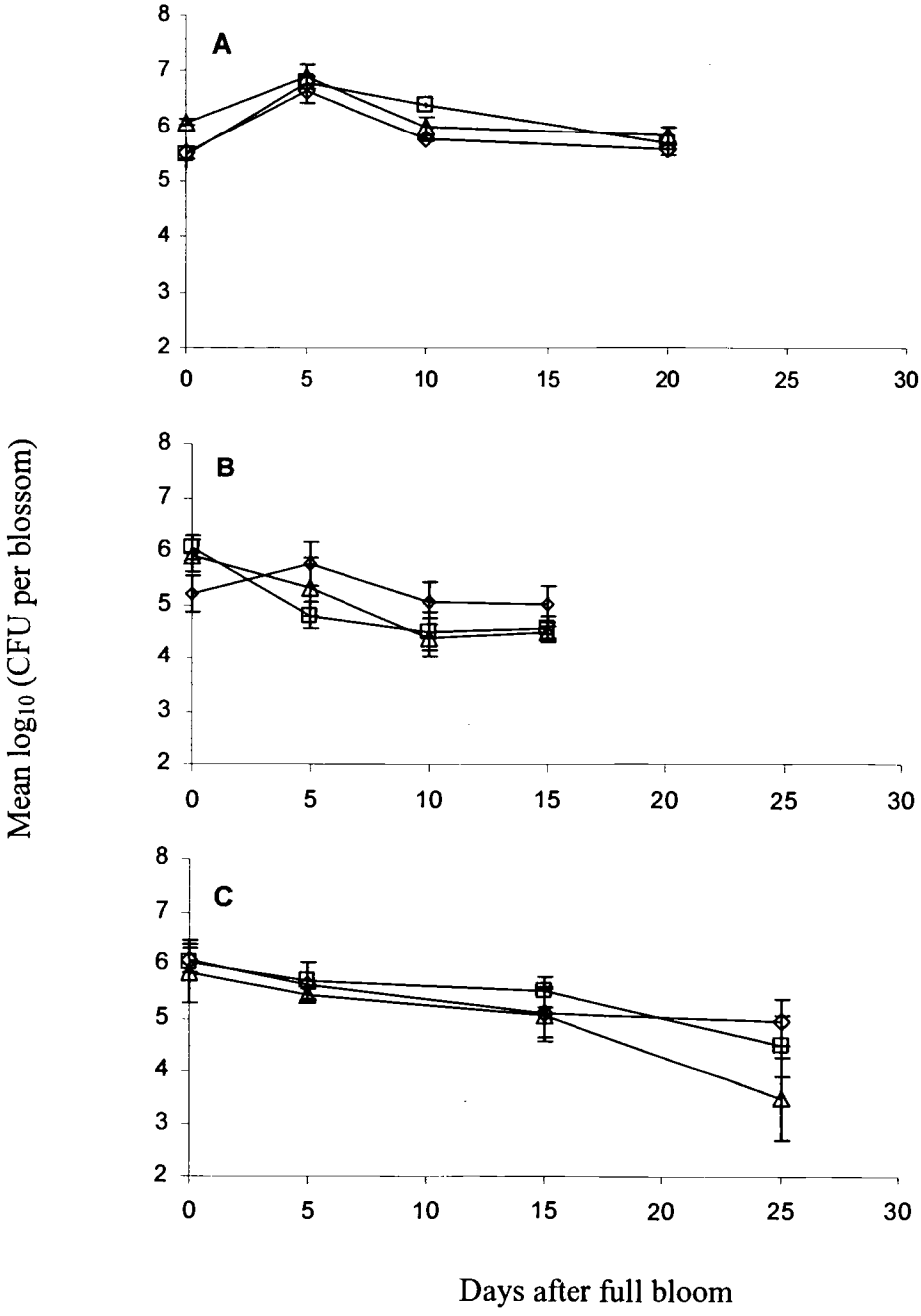

Fig 3.7. Population size (log_{10}) of *P. fluorescens* A506 (◇), A506 Ant$^+$ (□), or A506 Fl-1 (△) on blossoms of **A)** Bartlett pear, **B)** Golden Delicious apple, and **C)** Rome Beauty apple. Vertical bars represent one standard error of the mean population size on individual blossoms.

Discussion:

In this study, the transposon, Tn*5* was used to mutageneize *P. fluorescens* strain A506 to generate mutants altered in antibiotic production. The selection assay was a rapid screen of Tn*5* mutants for loss of antibiotic production. The strains A506 (antibiotic producer) and A506 GacS$^-$ (non-antibiotic producer) were used as controls in the selection assay, demonstrating the assay had potential for selecting an antibiotic deficient mutant.

Out of the 8736 putative mutants generated using Tn*5km*, no antibiotic deficient isolates were detected. Random distribution of the Tn*5* insert into DNA was determined by the 7% frequency of auxotrophy that was detected, similar to random insertion in other mutagenesis experiments (Lindow et al., 1993b). Nevertheless, distribution of the Tn*5* insert into all regions of DNA (e.g., supercoiled DNA) in A506 may not have occurred. Lack of detecting antibiotic deficient mutants could be due to the selection assay conditions, or an insufficient number of mutants analyzed.

Of the 1504 putative mutants from the promoter probe Tn*5gfp-km* mutagenesis, one antibiotic deficient mutant was isolated, named A506 Ant$^-$. The Tn*5* insert was detected in A506 Ant$^-$ in preparations of genomic and plasmid DNA. Insertion in genomic and plasmid DNA was identified through Southern blot analysis. The parent strain A506 contains an indigenous plasmid which could potentially be isolated in a genomic DNA preparation. Tests were not performed to determine conclusively if there are indeed two separate and unique inserts in genomic and plasmid DNA. Additional

analyses to identify genes disrupted in plasmid and genomic DNA of A506 Ant$^-$ should clarify the number and the location of the insertion.

The conjugal transfer of pAnt$^-$ into the parent strain A506 resulted in the loss of antibiotic production and expression of GFP in transconjugates. Transfer of pUC18 containing a ~8.0 kb genomic DNA fragment into the parent strain A506 (JL4722) also resulted in the loss of antibiotic production and expression of GFP. These results confirm that both plasmid DNA and subcloned genomic DNA of A506 Ant$^-$ contained and expressed the insert. The disruption in antibiosis by both sources of the insert when reintroduced into the parent A506 is curious. Sequencing DNA from pAnt$^-$ and the subcloned genomic DNA flanking the Tn*5* will help define if the disrupted gene is similar for genomic and plasmid DNA of A506 Ant$^-$.

Epiphytic fitness of A506 Ant$^-$ was compromised on blossoms of pear and apple trees. The Tn*5* cassette by itself should not compromise epiphytic fitness of the recipient (Tang et al., 1999). To evaluate if containing the insert was detrimental to epiphytic fitness, one trial on Rome Beauty apple was conducted using a derivative of A506, as a control strain, that contained the Tn*5gfp-km* insert. Epiphytic fitness in this control strain was not compromised (data not shown). Therefore, it is not clear why epiphytic fitness of A506 Ant$^-$ was reduced on orchard blossoms. Bacterial survival in the phyllosphere can be decreased by harsh environmental stresses, two of which are UV radiation and drying (Jacobs and Sundin; 2001; Lindow et al., 1993a). Motile epiphytes are able to survive these stresses by escaping exposed sites to protected niches (Beattie and Lindow, 1994a; Beattie and Lindow, 1999; Haefele and Lindow,

1987). UV tolerance, growth rates in culture, and motility in defined media of A506 Ant$^-$ were similar to the parent strain A506. A506 Ant$^-$ and A506 GacS$^-$ were similar in growth or stress tolerance. Although in laboratory assays, A506 GacS$^-$ was similar to A506 Ant$^-$, in orchard trials A506 GacS$^-$ colonized blossoms with high populations (chapter 4 orchard experiments). Beattie and Lindow (1994b) observed that epiphytic fitness of some *P. syringae* Tn*5* mutants were reduced under field conditions but laboratory assays failed to detect defects. When A506 Ant$^-$ DNA flanking the insert is characterized, it may give greater understanding to genes influencing growth and survival on blossoms.

The mutant A506 Fl-1 is constitutive for antibiotic production (iron-independent) and exhibits loss of fluorescence on iron-poor media. The correlation between non-fluorescence and iron-independent antibiotic production, both of which are observed in iron-rich media by parental A506, were of interest. Several iron associated biosynthetic genes could be responsible for loss of fluorescence, such as biosynthesis genes *pvd*D, E, F, or A regulated by *pvd*S, or *pvc*A, B, C, or D regulated by *ptx*R (Visca et al., 2002). Of the 23 non-fluorescent Tn*5* mutants selected, 13 were iron-dependent antibiotic producers. A mutation in a biosynthetic gene for pyoverdine may explain this phenotype (Visca et al., 2002). The remaining 10 non-fluorescent mutants shared the iron-independent antibiotic production phenotype associated with A506 Fl-1. The high number of mutants expressing this phenotype suggests several regulatory genes may be involved, based on the effect one regulatory gene has on many genes i.e., a link between antibiotic production and siderophore production. Characterization of genes associated

with the iron-independent phenotype may give greater understanding to regulation of antibiotic production.

A506 Ant$^+$ is a non-fluorescent Tn*5km* mutant that is iron-independent in antibiotic production. A506 Ant$^+$ was evaluated for epiphytic fitness and suppression of fire blight. A506 Ant$^+$ established well and was recovered in high populations on apple and pear blossoms (Fig 3.7). Suppression of blossom blight symptoms proved to be variable, with 16%, 37%, and 57% reduction in relative incidence on Bartlett pear, Golden Delicious apple and Rome Beauty apple, respectively.

In conclusion, we developed several derivatives of A506 that may serve as useful tools in the understanding of the synthesis and regulation of antibiosis. A506 Ant$^-$ was the only antibiotic-deficient mutant detected out of thousands of mutants screened. A506 Ant$^-$ may provide essential information on antibiotic production through sequencing DNA flanking the insertions. In addition, we selected several non-fluorescent iron-independent mutants that once characterized, may reveal genes involved in regulation of iron-induced antibiosis by A506. The potential of increased disease control through elucidation of the genes involved in constitutive antibiotic production could enhance biological control.

Chapter 4

Effect of an Iron Chelate on Suppression of Fire Blight with *Pseudomonas fluorescens* strain A506

T. N. Temple, Stockwell, V. O., and Johnson, K. B.

Plant Disease

Abstract:

Competitive exclusion has been the mechanism postulated to account for the biological control of fire blight disease of pear and apple by the bacterium *Pseudomonas fluorescens* A506 (A506). Recent laboratory assays demonstrate that A506 cultured on media amended with iron (Fe^{+2} or Fe^{+3}) produces a compound that is toxic to *Erwinia amylovora*, the fire blight pathogen. Four field trials in 2001 and 2002 on Bartlett and Bosc pear and Golden Delicious and Rome Beauty apple were conducted to evaluate if iron oversprays onto pear or apple trees could improve the degree of biocontrol obtained after co-treatment with A506 by induction of antibiosis. The bacterial treatments, A506 Ecp^- (an extracellular protease deficient mutant) in 2001, A506 in 2002, and A506 $GacS^-$ (an antibiotic deficient mutant) in both 2001 and 2002 were combined in a factorial arrangement with 0.1 mM FeEDDHA. All bacterial treatments achieved high populations on blossoms throughout the field trials. Over both years, the incidence of blighted blossom clusters on trees treated with 0.1 mM FeEDDHA averaged 88%. In 2001, A506 Ecp^- applied to blossoms in water suppressed disease development on average by 26%, whereas A506 Ecp^- applied to blossoms in 0.1 mM FeEDDHA significantly enhanced disease suppression to an average of 47%. In 2002, A506 applied to blossoms in water suppressed disease development on average by 40%, whereas A506 applied to blossoms in 0.1 mM FeEDDHA suppressed disease development on average by 38%. A506 $GacS^-$ applied to blossoms in water suppressed disease on average by 17% and applied in 0.1 mM FeEDDHA suppressed disease on

average by 14%. The lack of significant control by the antibiotic deficient strain A506 GacS$^-$ or by 0.1 mM FeEDDHA alone also adds support to this hypothesis.

Introduction:

Fire blight, caused by *Erwinia amylovora* [(Burr.) Winslow et. al.], is an insidious bacterial disease in pear and apple production regions of the Pacific Northwest of North America (van der Zwet, 1979; Vanneste, 2000). Historically, control of fire blight has been achieved through application of the antibiotic, streptomycin sulfate, during bloom to prevent epiphytic colonization by the pathogen and subsequent floral infections (Ark, 1953). Over the last decade, however, strains of *E. amylovora* resistant to streptomycin have been isolated from fields with a history of streptomycin applications (Burr et al., 1993; Loper et al.,1991; McManus and Jones, 1994a; McManus et al., 2002; Stockwell et al., 1996a). Emergence of these resistant strains has prompted research into the development of biological control agents for fire blight suppression.

Introduction of bacterial antagonists to floral surfaces of pear and apple trees is directed at limiting colonization of stigmatic surfaces by *E. amylovora* (Beer and Rundle, 1984; Isenbeck and Schulz, 1985; Johnson et al., 1993a; Johnson et al., 1993b; Johnson and Stockwell, 1998; Johnson and Stockwell, 2000; Kearns and Hale, 1996; Lindow, 1985; Lindow et al., 1996; Nuclo et al., 1998; Stockwell et al., 1996b; Stockwell et al., 1999; Stockwell et al., 2002b; Thomson, 1986; van der Zwet and Keil, 1979; Vanneste et al., 1992; Wilson et al., 1992; Wilson and Lindow, 1993). Suppression occurs not only through preemptive exclusion and/or direct competition with *E. amylovora* for shared nutrients and habitat, but through antibiosis, whereby these two mechanisms can operate together to exclude the pathogen from colonizing

blossom stigma surfaces (Beer and Rundle, 1984; Lindow, 1985; Nuclo et al., 1998; Johnson et al., 1993a; Johnson et al., 1993b; Stockwell et al., 2002b; Vanneste et al., 1992; Wilson and Lindow, 1993). Prior experiments with bacterial antagonists have successfully reduced the incidence of blossom blight (Johnson et al., 1993a; Johnson and Stockwell, 1998; Lindow et al., 1996; Stockwell et al., 2002b; Wilson and Lindow, 1993). Yet, variation in suppression, i.e. excellent suppression one year followed by less suppression the following year, has been observed (Lindow et al., 1996; Nuclo et al., 1998). Reducing the variation in the degree of disease control attained with biological antagonists is essential for further adoption of this control strategy.

On average, the antagonist *Pseudomonas fluorescens* strain A506 (A506) attains 30 to 50% fire blight suppression in field trials, whereas streptomycin sulfate averages up to 90% control on streptomycin-sensitive isolates of *E. amylovora* (Johnson and Stockwell, 1998; Wilson and Lindow, 1993). Recently, Stockwell et al. (2001) observed inhibition of *E. amylovora* by A506 through *in vitro* antibiosis on a culture medium amended with at least 0.1 mM ferric chloride. Pear and apple blossoms are limited in the bioavailability of iron to A506 as determined using an iron-biosensor (Chapter 2). The addition of 0.1 mM ferric-ethylenediaminedi (o-hydroxyphenlacetic) acid (FeEDDHA) to blossoms significantly increased bioavailable iron to A506. It is hypothesized that addition of iron to blossoms co-treated with A506 will induce antibiosis by modifying the blossom environment. The iron source, FeEDDHA, is a registered product (Sequestrene 138, Becker Underhill, Ames, IA) for alleviation of iron chlorosis of pear trees. Addition of 0.1 mM FeEDDHA plus A506 in a field trial in the

spring of 2000 reduced blossom blight incidence dramatically compared to A506 applied without FeEDDHA (V. O. Stockwell, personal communication).

The importance of antibiosis by A506 in disease suppression was investigated using an antibiosis deficient mini-Tn*5* mutant strain called A506 GacS$^-$. The A506 GacS$^-$ mutant contains a single insertion in *gacS*, the sensor kinase portion of a two-component regulatory system (Cui et al., 2001; Heeb and Dieter, 2001; Saleh and Glick, 2001; V. O. Stockwell, personal communication). The *gacS* gene regulates several biosynthetic pathways, and often GacS mutants do not produce antibiotics (Cui et al., 2001; Heeb and Dieter, 2001; Saleh and Glick, 2001). For another patho-system, a GacS mutant in *P. syringae* pv. *syringae* strain B728a was used to investigate toxin production and its importance in lesions produced on plants (Hrabak and Willis, 1992; Kitten et al., 1998; Willis et al., 1990). Previous experiments using insertional mutants to disrupt gene expression have successfully demonstrated the importance or lack of importance of the target gene (Stockwell et al., 2002b; Vanneste et al., 1992; Wright and Beer, 1996; Wright et al., 2001; Steinberger and Beer, 1987).

The primary purpose of this study was to investigate the effect of treating pear and apple blossoms with A506 or A506 GacS$^-$ co-treated with 0.1 mM FeEDDHA and its results on the development of blossom blight in orchard trials. The establishment and incidence of A506 and A506 GacS$^-$ on pear and apple blossoms co-treated in water or 0.1 mM FeEDDHA also was examined. Subsequent incidence and epiphytic growth by *E. amylovora* on blossoms inoculated with water-only, 0.1 mM FeEDDHA-only, A506 and A506 GacS$^-$ co-treated with water or 0.1 mM FeEDDHA was evaluated.

Materials and Methods:

Bacterial Strains: The bacterial strains used in this study were *P. fluorescens* strain A506 (A506), A506 Ecp$^-$ (A506 Ecp$^-$), and A506 GacS$^-$ (A506 GacS$^-$). A506 was isolated from a pear leaf in California by S Lindow (University of California, Berkley) and is resistant to streptomycin and rifampicin. A506 Ecp$^-$ and A506 GacS$^-$ were provided by VO Stockwell (Oregon State University, Corvallis) and are resistant to kanamycin, streptomycin, and rifampicin. A506 GacS$^-$ is antibiotic deficient. A506 Ecp$^-$ contains a single mini-Tn*5* insertion in a metalloprotease gene and lacks production of an extracellular protease. The insertion of Tn*5* does not effect the growth of A506 GacS$^-$ or A506 Ecp$^-$ in culture broth or on detached orchard blossoms as compared to the parent strain A506.

The pathogen, *E. amylovora* 153N (Ea153N), is a spontaneous nalidixic acid-resistant (100 μg per ml) mutant of strain 153 isolated in Milton-Freewater, OR from a fire blight canker on Gala apple (Johnson et al., 1993a). Ea153N is similar to the parent strain Ea153 in growth rate and colony morphology and is streptomycin sensitive. Each bacterial strain was stored in nutrient broth with 15% glycerol at –80°C.

Experimental Design: Field experiments were conducted to evaluate population dynamics and relative disease suppression of fire blight by the biocontrol agent A506, A506 Ecp$^-$, and A506 GacS$^-$ (Table 4.1). Field experiments were conducted at the Oregon State University, Botany and Plant Pathology Field Laboratory located near Corvallis, OR in 2001 and 2002 in 0.5-ha blocks of pear (*Pyrus communis* L. cvs. 'Bartlett' and 'Bosc') and 0.4-ha plots of apple (*Malus* X *domestica* Borrkh. cvs. 'Rome

Beauty' and 'Golden Delicious'). In 2002, at the Southern Oregon Research and Extension Center near Medford, OR, two trials were conducted on cv Bartlett pear (spray block and north block). Bacterial and chemical treatments were assigned to individual trees in field plots in randomized complete block designs. Trees were grouped into blocks based on relative bloom density and location in the orchard. In 2001 on Bartlett pear, treatments were applied to 5 trees, and on Bosc pear, treatments were applied to 3 trees. In 2002, all field trials had treatments applied to 5 replicate trees, with the exception of the Medford North plot that had 4 replicate trees per treatment.

Table 4.1. Location, cultivar, treatment, and disease assessment dates.

Year	Location	Cultivar (block)	Treatment dates			Disease assessment
			First [x]	Second [y]	Pathogen [z]	
2001	Corvallis	Bartlett	5 April	11 April	13 April	20 April to 30 June
	Corvallis	Bosc	17 April	19 April	20 April	26 April to 30 June
2002	Medford	Bartlett (spray block)	3 April	5 April	7 April	29 Apr to 7 May
	Medford	Bartlett (north block)	3 April	5 April	7 April	22 Apr to 7 May
	Corvallis	Golden Delicious	23 April	25 April	27 April	22 May to 30 June
	Corvallis	Rome Beauty	30 April	3 May	5 May	30 May to 6 July

[x] Date of first application of *Pseudomonas fluorescens* strains, water, FeEDDHA or streptomycin to trees at 30% bloom.
[y] Date of second application of *Pseudomonas fluorescens* strains, water, FeEDDHA or streptomycin to trees at 80% bloom.
[z] Date of application of *Erwinia amylovora* strain 153N to trees at full bloom.

Treatment preparation and application: A506, A506 Ecp$^-$ and A506 GacS$^-$ were cultured for 4 days at 22°C, or room temperature, on nutrient agar (Difco Laboratories, Detroit, MI) amended with 0.5% w/v sucrose and 0.4% w/v glycerol. Bacteria were scraped from the agar surface and suspended in sterile 10mM potassium phosphate buffer (pH 7.0) and bacterial suspensions were at a cell concentration of approximately 1×10^{10} colony forming units (CFU) per ml based on spectrophotometric readings (Spectronic 20, Bausch & Lomb, Rochester, NY). The concentrated stock solution of bacteria was transported to the field in 1 liter Nalgene bottles in a Styrofoam cooler. Once in field plots, the concentrated bacterial suspensions were diluted to approximately 10^8 CFU per ml using local tap water at the field station. Bacterial treatments that included iron amendments were tank mixed with the iron chelate, FeEDDHA (Sequestrene 138 6% a.i.; Becker Underwood, Ames, IA) at a concentration of 0.1 mM. In 2001, A506 GacS$^-$ or A506 Ecp$^-$ were applied to trees with the surfactant 0.002% polyether-polymethylsiloxane-copolymer (Break-Thru™, Goldschmidt Chemical Corp, Essen, Germany).

At 30% and again at 80% bloom, trees were treated with A506, A506 Ecp$^-$, or A506 GacS$^-$ at daybreak in still air conditions to prevent drift. Treatments were applied to near runoff with a 12-liter backpack sprayer fitted with a hand-held spray wand. Individual trees received approximately 2 L of the bacterial suspensions. As controls for disease development and suppression, additional trees in each field plot were treated at 30 and 80% bloom with water-only, 0.1 mM FeEDDHA-only, and at 80% and post-pathogen application oxytetracycline (2002 only) (Mycosheild 17% a.i., 200 μg/ml,

Syngenta Crop Protection, Greensboro, NC) or streptomycin sulfate (2001 and 2002) (Agristrep 17%, 100 μg/ml, Novartis Crop Protection, Greensboro, NC).

Inoculation with pathogen, Ea153N, occurred at full bloom. One hour prior to inoculation, a lyophilized cell preparation of Ea153N was suspended in water in a 1 L Nalgene bottle. After re-hydration of the lyophilized cells, the stock solution was added to local tap water in a 100-liter tank connected to a motorized sprayer fitted with an agitator and handgun. The pathogen suspension (2.5×10^5 CFU per ml for pear trees and $1x\ 10^6$CFU per ml for apple trees) was misted onto trees in the evening in 2001 and 2002 (around 8:00pm to 9:00pm) under still wind conditions; spray volumes were 2 liters per tree depending on tree size. In 2001 and 2002, a second streptomycin and in 2002 an oxytetracylcine treatment was applied to trees the day following inoculation with Ea153N.

Recovery of bacteria from blossoms: After inoculation with the pathogen, mature blossoms (dark anthers) were sampled periodically until detection of fire blight symptoms. In 2001, 6 and 10 blossoms were sampled from each replicate tree in Bartlett and Bosc pear trials, respectively. In 2002, 8 blossoms were sampled for the first two dates and 12 blossoms sampled for the last two sampling dates in the north plot pear trials in Medford. In Corvallis for 2002, the first two samples contained 6 blossoms of each replicate tree followed by 10 blossoms for the second set of samples. After blossoms were collected, they were transported to the laboratory in sterile individual wells of 12-well plastic plates (Corning Inc., Corning, NY). In the laboratory, the pistil and nectary of each pear blossom (or pistil alone for apple

blossoms) were excised and placed into test tubes containing 1 ml sterile 10 mM potassium phosphate buffer (pH 7.0) and sonicated (Sonix IV, Inglewood, CA) for 3 min. After sonication, a 10µl sample of the wash and two 100-fold serial dilutions were spread onto Pseudomonas Agar F (PAF, Difco Laboratories, Detroit, MI) amended with 50 µg/ml of rifampicin and cycloheximide for selective recovery of A506 or PAF amended with 50 µg/ml of kanamycin and cycloheximide for selective recovery of A506 Ecp^- or A506 $GacS^-$, and CCT medium (Ishimaru and Klos, 1984) containing 50 µg/ml nalidixic acid and cycloheximide for selective recovery of the pathogen Ea153N. The detection limit was

1×10^2 CFU per blossom.

Disease incidence: Over a five-week period, blossom clusters with fire blight symptoms of necrosis, wilting and/or bacterial ooze, were counted and pruned from pear and apple trees. The sum of fire blight strikes was then calculated for each treated tree in each block.

Data analysis: Mean population size and standard error of treatments on individual blossoms were calculated by transforming colony forming units (CFU) (obtained from serial dilution plates) to $\log_{10}$ (CFU per blossom) for each sampling date. For statistical analysis, blossom populations of zero were set below the detection limit at 99 CFU per blossom. The incidence of detectable populations from blossom washing was calculated for each bacterial strain for each sample time in all trials.

Comparison of growth differences for bacterial strains on blossoms and to evaluate treatment effectiveness on growth of the pathogen, the relative area under the population curve (RAUPC) was calculated for each bacterial treatment in each trial. The following formula was used to calculate RAUPC:

$$\text{RAUPC} = \sum_{i=1}^{n} \{[(y_i + y_{i-1})/2] \bullet (t_i - t_{i-1})\}/t_{total}$$

Where y is the mean population size of a bacterial strain in the ith sample date and t is the corresponding sample time. Mean separation using the Fisher's protected least significant difference (LSD) test at $P = 0.05$ was used for RAUPC values calculated for each bacterial treatment of each trial (PROC GLM, Statistical Analysis System, SAS Institute, Cary, NC).

Diseased blossom cluster numbers per treated tree were converted to a relative disease incidence by dividing the incidence of fire blight strikes on treated trees by the incidence on water-only treated trees within each respective block. Arcsine square root-transformed relative disease incidence data were subjected to analysis of variance. Fisher's protected least significant difference (LSD) test at $P = 0.05$ was used to separate the mean relative values calculated for each bacterial treatment of each trial.

Results

***Establishment of A506, A506 Ecp⁻ and A506 GacS⁻ applied in water to pear and apple blossoms in water*:** For all field trials, A506, A506 Ecp⁻ and A506 GacS⁻ inoculated in water established on pear and apple blossoms and were recovered from blossoms sampled over the time course of the experiment (Fig. 4.1). Over the sampling periods, the incidence of recovery of a strain from the tree to which it was applied ranged from 79 to100% of sampled blossoms (data not shown). The mean population size of A506 or a derivative strain averaged between 1 x 10^3 and 1 x 10^7 CFU per blossom (Fig. 4.1). For four of the five experiments, analysis of variance (ANOVA) of $\log_{10}$ (colony forming unit) per blossom at individual sample times and for the summary statistic, relative area under the population curve (RAUPC), revealed no significant differences ($P > 0.05$) among treatments of A506 Ecp⁻ and A506 GacS⁻ (2001) or among A506 and A506 GacS⁻ (2002). For the Golden Delicious experiment in 2002, however, the measured population size of A506 GacS⁻ was significantly smaller ($P \leq 0.05$) than that of A506 on two of the four sampling dates (i.e., at 5 and 10 days after first bloom).

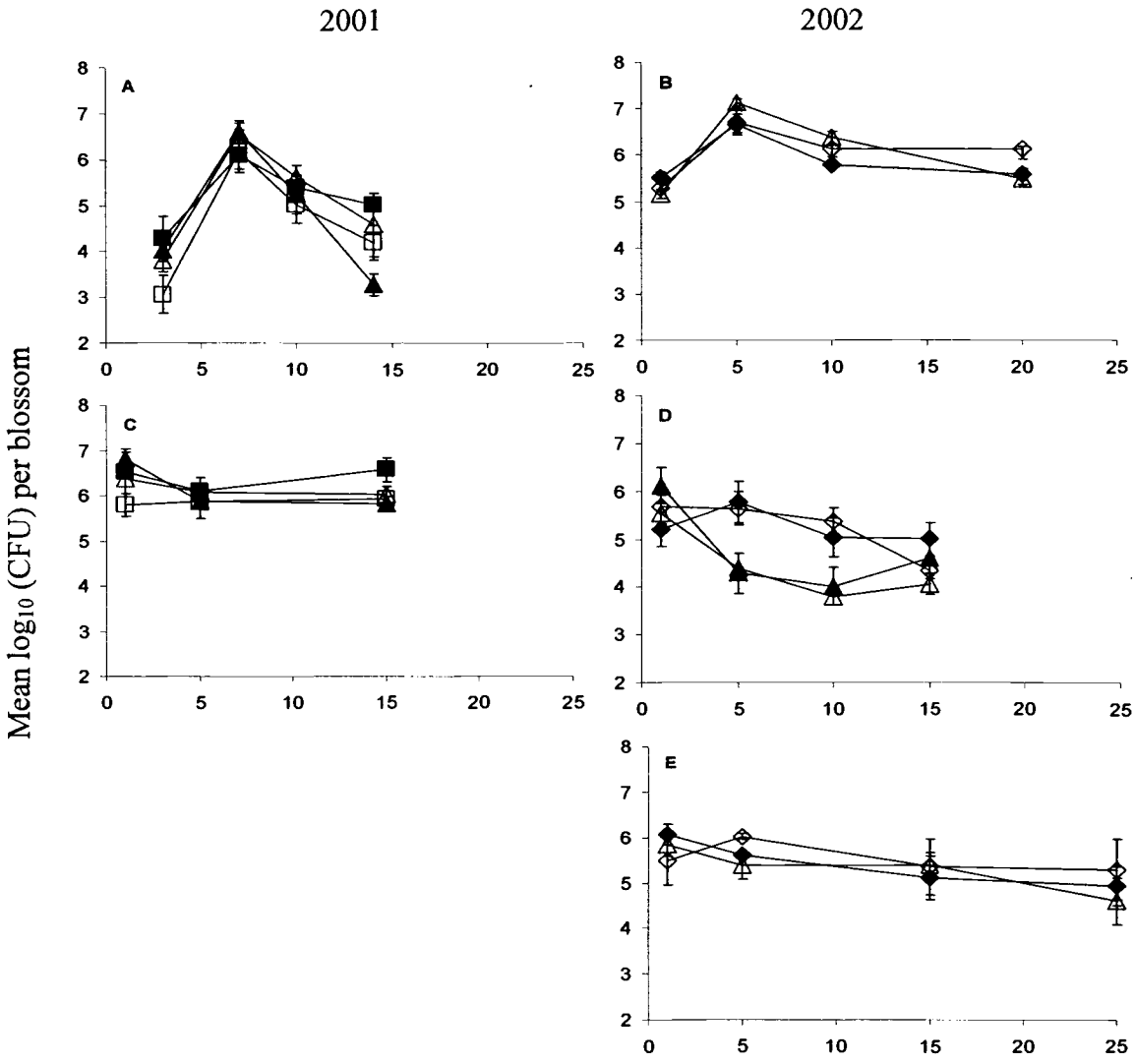

Fig 4.1. Population size (log_{10}) of *P. fluorescens* strain A506 (2002; ◆,◇), A506 Ecp$^-$ (2001; ■, □), or A506 GacS$^-$ (2001 & 2002; ▲, △) for experiments conducted in orchards of **A)** Bartlett pear (Corvallis) **B)** Bartlett pear (Medford) **C)** Bosc pear (Corvallis) **D)** Golden Delicious apple (Corvallis) and **E)** Rome Beauty apple (Corvallis). Closed symbols represent the bacterial strain applied to trees in water and open symbols represent co-treatment of the bacterial strain with 0.1 mM FeEDDHA. Vertical bars represent one standard error of the mean population size on individual blossoms.

Establishment of A506, A506 Ecp⁻, and GacS⁻ applied to pear and apple blossoms with 0.1mM FeEDDHA: Similar to the recovery of A506, A506 Ecp⁻, and A506 GacS⁻ applied to blossoms in water, the incidence of recovery of these strains on blossoms co-treated with 0.1mM FeEDDHA ranged from 91 to 100% (data not shown). Mean populations of A506, A506 Ecp⁻ and A506 GacS⁻ inoculated with 0.1 mM FeEDDHA also ranged from 1 x10^3 to 1 x 10^7 CFU per blossom (Fig. 4.1). Comparison of RAUPC values for strains A506, A506 Ecp⁻, and A506 GacS⁻ by analysis of variance indicated no significant differences ($P > 0.05$) among water and the 0.1 mM FeEDDHA co-treatment on recovery of the strains from blossoms .

Establishment of E. amylovora 153N on pear and apple blossoms treated with A506, A506 Ecp⁻ or A506 GacS⁻: In 2001, after inoculation with the pathogen, the recovery of *E. amylovora* strain Ea153N from water-treated Bartlett and Bosc pear blossoms increased over time from 62% to 92%, and mean population size ranged from 1 x 10^4 and 1 x 10^6 CFU per blossom (data not shown). In general, the pathogen population sizes measured on trees that received a treatment of A506 or one of its derivatives were statistically similar to pathogen population sizes measured on the water-treated control (Table 4.2). Similarly, the incidence of Ea153N populations $\geq$ 1 x 10^5 CFU per blossom on trees that received a biological treatment was either significantly greater (Bartlett pear) or of similar magnitude (Bosc pear) to trees that received treatments water or of FeEDDHA (Table 4.3). For both 2001 experiments, when compared to the bacterial strain applied in water, the co-treatment of 0.1 mM

FeEDDHA with a bacterial strain had no apparent effect on the population size or the incidence of high populations of Ea153N (Tables 4.2 and 4.3).

For the three experiments conducted in 2002, average incidence of recovery of Ea153N from blossoms on trees treated with water ranged from 53 to 91 %, and mean population size ranged from 1 x 10^2 to 2 x 10^5 CFU per blossom (data not shown). Population size of Ea153N increased on water-treated trees during the sampling period from an average of 1 x 10^2 to 7 x 10^3 immediately after inoculation to 8 x 10^3 to 2 x 10^5 CFU per blossom for samples taken near petal fall. Compared to water treated trees, population sizes of Ea153N on blossoms treated with A506 and A506 GacS$^-$ were significantly reduced ($P < 0.05$) (Table 4.2); the, incidence of recovery of Ea153N populations $\geq$ 1 x 10^5 CFU also was significantly reduced ($P < 0.05$) by most of the biological treatments (Table 4.3). Compared to the water-treated control, biological treatments mixed with 0.1 mM FeEDDHA also significantly suppressed ($P < 0.05$) the population size and the incidence of high populations of Ea153N (Tables 4.2 and 4.3).

Table 4.2. Relative area under the population curve for *Erwinia amylovora* as influenced by treatments[w] of *Pseudomonas fluorescens* A506, A506 Ecp⁻, A506 GacS⁻, or FeEDDHA.

	Experimental season, location and cultivar				
	2001		2002		
Treatments	Corvallis Bartlett pear	Corvallis Bosc pear	Medford Bartlett (North)	Corvallis Golden Delicious	Corvallis Rome Beauty
Water	5.0 a,b[x,y]	6.0 a	4.3 a	4.7 a	3.5 a
FeEDDHA	4.0 b	6.3 a	....[z]	....	....
A506 Ecp⁻	5.8 a	6.2 a	....	....	....
A506 Ecp⁻ plus FeEDDHA	6.2 a	6.4 a	....	....	....
A506	....	....	1.6 c	3.2 b	2.6 b
A506 plus FeEDDHA	....	....	1.7 c	3.6 b	2.6 b
A506 GacS⁻	5.6 a,b	6.1 a	....	3.9 a,b	....
A506 GacS⁻ plus FeEDDHA	5.6 a,b	6.3 a	2.9 b	4.0 a,b	2.4 b

[w] Treatments were applied to four or five, individual tree replicates at 30% and 80% bloom to near runoff at concentrations of 0.1 mM FeEDDHA and 1 x 10^8 CFU/ml for *P. fluorescens* A506 or its derivatives.

[x] The Relative Area Under the Population Curve (RAUPC) was calculated over all sample dates by the equation:

$$\text{RAUPC} = \sum_{i=1}^{n} \{[(y_i + y_{i\text{-}1})/2] \bullet (t_i - t_{i\text{-}1})\}/t \text{ total}$$

where y is the mean $\log_{10}$ (colony forming units) per blossom at the ith sample date and t is the corresponding sample time.

[y] Means within a column followed by the same letter are not significantly different according to Fisher's protected least significant difference at $P \geq 0.05$.

[z] Not tested.

Table 4.3. Incidence of recovery of *Erwinia amylovora* population ≥1 x 10^5 CFU per blossom[v] as influenced by treatments[w] of *Pseudomonas fluorescens* A506, A506 Ecp$^-$, or A506 GacS$^-$, and FeEDDHA.

	Experimental season, location and cultivar				
	2001		2002		
Treatments	Corvallis Bartlett pear	Corvallis Bosc pear	Medford Bartlett (North)	Corvallis Golden Delicious	Corvallis Rome Beauty
Water	0.42 b [x,y]	0.85 a,b	0.48 a	0.62 a	0.34 a
FeEDDHA	0.45 b	0.82 b	....[z]	....	....
A506 Ecp$^-$	0.77 a	0.97 a	....	....	....
A506 Ecp$^-$ plus FeEDDHA	0.88 a	0.98 a	....	....	....
A506	....	....	0.13 b	0.31 b	0.18 b
A506 plus FeEDDHA	....	....	0.10 b	0.48 a,b	0.13 b
A506 GacS$^-$	0.77 a	0.90 a,b	....	0.34 b	....
A506 GacS$^-$ plus FeEDDHA	0.77 a	0.90 a,b	0.23 b	0.56 a	0.11 b

[v] Blossom samples were taken from full bloom to approximately 7 days post petal fall.

[w] Treatments were applied to four or five, individual tree replicates at 30% and 80% bloom to near runoff at concentrations of 0.1mM FeEDDHA and 1 x 10^8 CFU/ml for *P. fluorescens* A506 and its derivatives.

[x] Incidence values were acrsine square root transformed for analysis.

[y] Means within a column followed by the same letter are not significantly different according to Fisher's protected least significant difference at $P \geq 0.05$.

[z] Not tested.

Fire blight development: Symptoms of fire blight were observed in all experiments. In 2001, blossom blight development was very high on water-treated blossoms averaging 451 and 825 strikes (infections) per tree in the Bartlett and Bosc pear experiments, respectively. In 2002, blossom blight on water-treated trees averaged 168, 255, 358 and 229 strikes per tree on Bartlett pear (Medford north and spray blocks), Golden Delicious and Rome Beauty apple, respectively. In each experiment, the most effective treatment was streptomycin sulfate, which averaged 89% suppression of infection relative to the water-treated control (Table 4.4).

Except for the Bartlett pear experiment in 2001, biological treatments of A506 or A506 Ecp$^-$ resulted in a significant reduction in the observed incidence of fire blight symptoms (Table 4.4), the mean reduction for either of these treatments was 44%. Similarly, trees treated with either A506 or A506 Ecp$^-$ plus 0.1 mM FeEDDHA showed reduced blossom blight incidence relative to the water-treated control, but on average, the mixture of A506 or A506 Ecp$^-$ plus 0.1mM FeEDDHA did not enhance disease suppression relative to the degree of suppression obtained by the strains applied in water (Table 4.4.). The one possible exception was the Golden Delicious experiment in 2002 where A506 plus FeEDDHA resulted in a degree of fire blight suppression that was similar to that observed with the antibiotic, streptomycin. Overall, the control treatments, 0.1 mM FeEDDHA (no direct effect on *E. amylovora* expected) and A506 GacS$^-$ (no iron induced antibiosis expected), and A506 GacS$^-$ plus 0.1 mM FeEDDHA, provided inconsistent levels of fire blight suppression (Table 4.4.); results from these

treatments tended to be intermediate to treatment with water and to the results obtained with A506 or A506 Ecp$^-$ with or without FeEDDHA.

Table 4.4. Relative incidence of fire blight on pear and apple trees as influenced by treatments[w] of *Pseudomonas fluorescens* A506, A506 Ecp⁻, A506 GacS⁻, or FeEDDHA.

	Experimental season, location and cultivar					
	2001		2002			
Treatments	Corvallis Bartlett pear	Corvallis Bosc pear	Medford Bartlett (North)	Medford Bartlett (Spray)	Corvallis Golden Delicious	Corvallis Rome Beauty
Water	1.00 a[x,y]	1.00 a	1.00 a	1.00 a	1.00 a	1.00 a
FeEDDHA	0.81 b	0.31 d	0.79 b,c	2.28 a	0.82 b,c	0.85 b,c
A506 Ecp⁻	1.03 a	0.44 c,d	....[z]	.....	.....	.....
A506 Ecp⁻ plus FeEDDHA	0.71 b	0.34 d	.....	.....	.....	.....
A506	.....	.....	0.68 b,c	0.54 b	0.54 c,d	0.59 c
A506 plus FeEDDHA	.....	.....	0.55 c	0.69 b	0.23 e	0.61 b,c
A506 GacS⁻	1.06 a	0.54 b,c	.....	.....	0.86 a,b	.....
A506 GacS⁻ plus FeEDDHA	0.97 b	0.65 b	0.97 a,b	0.82 b	0.98 a	0.82 b
Oxytetracycline	....	....	0.83 b,c	0.73 b	.....	.....
Streptomycin	0.01 c	0.04 e	0.13 d	0.13 c	0.23 e	0.12 d

[w] Treatments 0.1mM FeEDDHA, streptomycin (100 µg/ml), oxytetracycline (200 µg/ml), or 1 x 10^8 CFU/ml for *P. fluorescens* A506 or its derivatives were applied to near runoff to four or five individual tree replicates at 30% and 80% bloom.

[x] Counts of infections per tree, within a replication, were divided by the count obtained on the water treated tree and the resulting incidence values were acrsine square root transformed for analysis.

[y] Means within a column followed by the same letter are not significantly different according to Fisher's protected least significant difference at $P \geq 0.05$.

[z] Not tested.

Discussion:

This study was designed to examine the suppressive effect of co-treatments of *Pseudomonas fluorescens* strain A506 and 0.1 mM FeEDDHA on the development of fire blight disease in pear and apple blossoms. This question is of interest because the biological control agent, A506, has been observed to produce an antibiotic inhibitory to the fire blight pathogen, *E. amylovora*, when cultured on media that contains iron at concentrations ≥ 0.1 mM. Thus, the working hypothesis is that the bioavailability of iron on blossom surfaces is limited and that combining this element with inoculations of A506 may directly enhance fire blight suppression.

Results from Chapter 2 of this thesis provided evidence that bioavailable iron is indeed limiting on surfaces of pear and apple blossoms, and that augmenting the blossom environment with an iron chelate, such as FeEDDHA, can alleviate this limitation. Also as in Chapter 2, the experiments conducted in this chapter confirmed that the co-treatment of FeEDDHA with A506, A506 Ecp^- or A506 $GacS^-$ did not enhance or decrease the ability of the bacterial strains to establish and colonize blossom surfaces. Observed population sizes of A506 and its derivatives (Fig 4.1) were typical of those reported previously (Johnson et al., 1993a; Johnson et al., 1993b; Nuclo et al., 1998; Wilson and Lindow, 1993; Lindow et al., 1996; Stockwell et al., 1998) regardless of whether the bacterial strains were in water or mixed with 0.1 mM FeEDDHA.

Nonetheless, the second aspect of the working hypothesis, that combing iron with inoculations of A506 may directly enhance fire blight suppression, was not confirmed by the disease incidence data collected in the orchard experiments. Fire

blight developed in all experiments, and significant disease suppression by at least one treatment also was observed. The experiments reconfirmed that the antibiotic, streptomycin sulfate, is a very effective agent for fire blight suppression when used against a streptomycin-sensitive strain such as Ea153N. Moreover, A506 or its extracellular protease deficient mutant, A506 Ecp$^-$, provided significant disease suppression in five of six experiments, and the levels of suppression were generally similar to those reported previously (Johnson et al., 1993a; Johnson et al., 1993b; Lindow et al., 1996; Nuclo et al., 1998; Wilson and Lindow, 1993). Disease suppression with co-treatments of FeEDDHA mixed with either A506 or A506 Ecp$^-$, however, were generally of similar magnitude to the bacterial strains applied to trees in water. This data was in contrast to a field trial conducted in 2000, where Stockwell observed enhanced suppression of blossom blight on trees co-treated with A506 and 0.1 mM FeEDDHA (V.O. Stockwell, personal communication).

One field trial, Golden Delicious apple in 2002, was an apparent exception, where blossom blight was reduced by approximately 75% by the mixture of A506 and FeEDDHA (Table 4.4), and this reduction was significantly greater than the reduction obtained by A506 alone. In 2002, all orchards exhibited a high incidence of secondary bloom (i.e., flowers that open after the primary bloom period). In the Golden Delicious apple trial, this late bloom was physically pruned from orchard trees, which limited the number of blossoms that opened late in the experiment. Consequently, a reason for lack of enhanced disease suppression by A506 when mixed with FeEDDHA could be due in part to the timing of the iron application. Trees were co-treated with A506 and

FeEDDHA at 30% and 70% bloom. In general, after inoculation, A506 continues to spread to blossoms that open after the time of inoculation (Nuclo et al., 1998). For iron, however, blossoms that open after the time of treatment likely remain untreated. As a result, variation in disease suppression would be expected due to the non-iron amended. environment of late opening flowers. Similarly, the iron treatments also may become diluted by rains that occur after the time of treatment. Consequently, an iron overspray at either full bloom or after a heavy rain may be worth further investigation with regard to enhanced fire blight suppression by A506.

Iron has been shown to be a virulence factor for *E. amylovora* (Dellagi et al., 1998; Expert et al., 2000). Because of this, there was some concern that additional iron bioavailability on blossoms surfaces could result in a higher incidence of diseased blossom clusters. As discussed in Chapter 2, this concern was addressed by choosing a chelate, FeEDDHA, which has a stronger affinity for iron than the iron chelating siderophore of Ea153N. Thus, the expectation was that the iron in the applied chelate would be available to A506 (see results of Chapter 2) but unavailable to the pathogen. In five of six trials, blossoms treated with 0.1 mM FeEDDHA had slightly but significantly less disease than the water treated controls (Table 4.4), suggesting that the chelated iron was not enhancing disease on pear and apple trees.

Commonly, investigations of antibiosis and its importance to suppression of plant disease has been accomplished through the use insertion mutants in biosynthesis genes generated from the parental bacterial strain (Bangera and Thomashow, 1999; Kraus and Loper, 1995; Pfender et al., 1993; Steinberger and Beer, 1987; Stockwell et

al., 2002b; Vanneste et al., 1992; Wright and Beer, 1996; Wright et al., 2001). For our interests, the treatment A506 GacS$^-$ was employed due to its lack of antibiosis to Ea153N. A506 GacS$^-$ contains a disruption in the global regulatory gene, *gacS* (V.O. Stockwell, unpublished data). While this strain served a useful purpose in the field trials, it was not the ideal choice for this study as *gacS* regulates the expression of several genes, only one of which is antibiosis (Cui et al., 2001; Duffy, 2000; Heeb and Dieter, 2001; Saleh and Glick, 2001; Whistler et al., 1998). In most of our field trials, we observed A506 GacS$^-$ to be variable in disease suppression. Duffy and Défago (2000) also described reduced efficacy in biocontrol by *P. fluorescens* CHA0 containing a mutation in *gacS*. Conversely, A506 GacS$^-$ was not compromised in its ability to colonize blossoms and attained similar population sizes as the parent strain A506 in our field trails. Prior to the discovery of the iron-dependent antibiotic produced by A506, researchers hypothesized that A506 suppressed *E. amylovora* by colonizing blossoms rapidly and out-competing the pathogen for space and nutrients (Wilson and Lindow, 1993). That A506 GacS$^-$ was a good colonist of blossoms and reduced disease in four of six trials compared to water treated trees is suggestive that competition for habitat and resources is important but may not be the only mechanism employed by A506 in suppression of pathogen populations on pear and apple blossoms.

An interesting contrast between the two field seasons was the differing effects of the treatments on the measured population sizes of Ea153N. In 2001, populations of Ea153N were very high across all treatments and in the Bartlett pear experiment, the incidence of Ea153N populations $\geq 1 \times 10^5$ were significantly higher on trees that

received biologicals than those that water or FeEDDHA treatments (Table 4.3). In 2002, Ea153N populations were lower, and the biological treatments reduced the proportion of blossoms with high pathogen populations. Two possible causes for the differences among season are differences in environmental conditions or differences in the amount of pathogen inoculum that established on blossom in each orchard. A third cause may relate to a silicone surfactant that was used in conjunction with biological treatments in 2001 but not in 2002. In 2001, the inocula of A506 Ecp$^-$ and A506 GacS$^-$ applied to pear were amended with the surfactant, polyether-polymethylsiloxane-copolymer (Break-Thru™, Goldschmidt Chemical Corp, Essen, Germany), to aid surface wetting and bacterial establishment (Holtz et al., 2002). Potentially, the residue of this chemical on the tree may have also aided the establishment of the pathogen, Ea153N.

In conclusion, we found that the iron chelate FeEDDHA plus A506 added to blossoms of pear and apple trees had no influential effect on suppression of blossom blight symptoms compared to A506 inoculated alone, with the exception of one trial on Golden Delicious apple. In future research, to understand the role of the antibiotic and its importance in disease suppression *in situ*, the biosynthetic genes of antibiosis must be characterized. Once identified, a near-isogenic mutant of A506, lacking only the biosynthesis genes, may elucidate the impact of antibiosis on fire blight suppression.

Chapter 5

Summary

Todd N. Temple

Fire blight, caused by the bacterium *Erwinia amylovora*, is a serious disease of pome fruits in many regions of the world including the Pacific Northwest of the United States. Competitive or preemptive exclusion has been the mechanism hypothesized to account for the biological control of fire blight disease of pear and apple by the bacterium *Pseudomonas fluorescens* A506 (A506). Recent laboratory assays demonstrate, however, that A506 cultured on defined media amended with iron (Fe^{+2} or Fe^{+3}) produces a compound that is toxic to the fire blight pathogen. Biological antagonism of *E. amylovora* by A506 occurs on blossom surfaces, i.e., stigma, pistillate and hypanthia. Therefore, iron availability to A506 on blossoms was investigated. Lack of biologically available iron on blossoms was determined using an iron-biosensor contained within A506 [A506 (*pvd-inaZ*)]. The iron-biosensor consisted of an iron-regulated promoter (*pvd*) fused to an ice nucleation reporter gene (*inaZ*). Overspray or co-treatment of A506 (*pvd-inaZ*) with an iron chelate, 0.1 mM FeEDDHA, increased the bioavailable iron on blossom surfaces to A506 (*pvd-inaZ*). The results of this experiment suggest that the low bioavailable iron environment on blossoms would not support iron-dependent antibiosis by A506. Adding iron as a co-treatment or as an overspray (i.e., after bacterial establishment on blossoms) increased iron bioavailability to A506 to levels that, on artificial media, induced antibiosis.

To investigate the genes involved in antibiotic production, Tn*5* mutagenesis of A506 was used to generate mutants with altered antibiotic production phenotypes. Two mutants with altered antibiotic production patterns were selected for further study. One mutant (A506 Ant^-) exhibited loss of antibiotic production and the other mutant did not

require excess iron for antibiotic production (A506 Ant^+). No genes involved in antibiotic production were identified in this experiment, but the insertion in A506 Ant^- was detected in genomic and plasmid DNA. Introduction of the cloned genomic region containing the insert or plasmid DNA of A506 Ant^- into the parent strain A506 resulted in loss of antibiotic production. Insertion in A506 Ant^- decreased epiphytic fitness on blossoms of apple and pear orchard trees compared to A506. Laboratory assays showed A506 Ant^- exhibited reduced tolerance to desiccation stress and a decreased rate of motility compared to A506. Populations of A506 Ant^- exhibited similar tolerance to A506 to UV radiation. The iron-independent antibiotic producer, A506 Ant^+, was deficient in production of the fluorescent siderophore. A506 Ant^+ was tested in field trials for suppression of fire blight. A506 Ant^+ established high populations on blossoms of apple and pear trees, similar to populations attained by A506. A506 Ant^+ reduced incidence of fire blight between 20 to 40%, levels comparable to A506 in orchard trials.

Field trials were conducted to evaluate if treatment of pear or apple trees with A506 combined with iron could improve the degree of biological control fire blight. All bacterial treatments achieved high populations on blossoms throughout the field trials co-treated with 0.1 mM FeEDDHA or in water. Over both years, the relative incidence of blighted blossom clusters on trees treated with 0.1 mM FeEDDHA averaged 87%. In both years, the A506 GacS mutant (non-antibiotic producer) applied in water or 0.1 mM FeEDDHA had a less suppressive effect than the wild type A506 with or without iron. A506 $GacS^-$ applied to blossoms combined with 0.1 mM

FeEDDHA suppressed disease on average by 14%, whereas A506 and A506 Ecp$^-$ co-treated with iron suppressed disease on average 62 and 33%, respectively. Increased suppression of fire blight incidence by A506 and A506 Ecp$^-$ amended with 0.1 mM FeEDDHA provides some evidence that iron-induced antibiosis can be a contributing mechanism in disease control. The lack of significant control by the antibiotic-deficient strain A506 GacS$^-$ or by 0.1 mM FeEDDHA alone also adds support to this hypothesis.

Bibliography

Abdallah, M. A. 1991. Pyoverdins and pseudobactins, pp 139-154 in: CRC handbook of Microbial Iron Chelates ed. G. Winkelmann. CRC Press, Boca Raton, FL.

Ark, P. A. 1953. Use of streptomycin dust to control fire blight. Plant Dis. Report. 37:404-406.

Askeland, R. A., and Morrison, S. M. 1983. Cyanide production by *Pseudomonas fluourescens* and *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 45:1802-1807.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J.G., Smith, J. A., and Struhl, K. (eds) 1991. Current Protocols in Molecular Biology. John Wiley and Sons, Interscience, NY.

Bangera, M. G., and Thomashow, L. S. 1999. Identification of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J. Bacteriol. 181:3155-3163.

Beer, S. V., and Rundle, J. R. 1984. Recent progress in the development of biological control for fire blight: A Review. Acta Hortic. 151:195-201.

Beattie, G. A. and Lindow, S. E. 1994a. Survival, growth, and localization of epiphytic fitness mutants of *Pseudomonas syringae* on leaves. Appl. Environ. Microbiol. 60:3790-3798.

Beattie, G. A. and Lindow, S. E. 1994b. Comparison of the behavior of epiphytic fitness mutants of *Pseudomonas syringae* under controlled and field conditions. Appl. Environ. Microbiol. 60:3799-3808.

Beattie, G. A., and Lindow, S. E. 1995. The secret life of foliar bacterial pathogens on leaves. Annu. Rev. Phytopathol. 33:145-172.

Beattie, G. A. and Lindow, S. E. 1999. Bacterial colonization of leaves: a spectrum of strategies. Phytopathology 89:353-359.

Bender, C. L., Malvick, D. K., and Mitchell, R. E. 1989. Plasmid mediated production of the phytotoxin coronatine in *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 171:807-812.

Bender, C. L., Young, S. A., and Mitchell, R. E. 1991. Conservation of plasmid DNA sequence in coronatine-producing pathovars of *Pseudomonas syringae*. Appl. Environ. Microbiol. 57:993-999.

Bender, C. L., Alarcon-Chaidez, F., and Gross, D. C. 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. 63:266-292.

Blumer, C. and Haas, D. 2000. Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. Microbiology 146:2417-2424.

Bogs, J., Bruchmuller, I., Erbar, C., and Geider, K. 1998. Colonization of host plants by the fire blight pathogen *Erwinia amylovora* marked with genes for bioluminescence and fluorescence. Phytopathology 88:416-421.

Budzikiewicz, H. 1993. Secondary metabolites from fluorescent pseudomonads. FEMS Microbiol. Rev. 104:209-228.

Burr, T. J., Norelli, J. L., Reid, C. L., Capron, L. K., Nelson, L. S., Aldwinckle, H. S., and Wilcox, W. F. 1993. Streptomycin-resistant bacteria associated with fire blight infections. Plant Dis. 77:63-66.

Chaney, R. L. 1988. Plants can utilize iron from Fe-*N,N'*-di-(2-hydroxybenzoyl)-ethylenediamine-*N,N'* -diacetic acid, a ferric chelate with 10^6 greater formation constant than Fe-EDDHA. J. Plant Nutr. 11:1033-1050.

Ciavatta, L., De Tommaso, G., and Luliano, M. 2002. Stability constants of iron(II) sulfate complexes. J. Analyt. Environ. Chem. (Abstr.) 92:513.

Cook, R. J., and Baker, K. F. 1983. The nature and practice of biological control of plant pathogens. American Phytopahtological Society, St. Paul.

Corbell, N., and Loper, J. E. 1995. A global regulator of secondary metabolite production in *Pseudomonas fluorescens* strain Pf-5. J. Bacteriol. 177:6230-6236.

Cui, Y., Chatterjee, A., and Chatterjee, A, K. 2001. Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and $harpin_{Ecc}$. Mol. Plant-Microbe Interact. 14:516-526.

Dellagi, A., Brisset, M., Paulin, J., and Expert, D. 1998. Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. Mol. Plant-Microbe Interact. 11:734-742.

de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. 1990. Mini-Tn*5* derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172:6568-6572.

de Wael, L., de Greef, M., and van Laere, O. 1990. The honeybee as a possible vector of *Erwinia amylovora* (Burr.) Winslow et al. Acta Hortic. 273:107-113.

Duffy, B. K., and Défago, G. 2000. Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 66:3142-3150.

Expert, D. 1999. Witholding and exchanging iron: interactions between *Erwinia spp.* and their plant hosts. Annu. Rev. Phytopathol. 37:307-334.

Expert, D., Dellagi, A., and Kachadourian, R. 2000. Iron and fire blight: Role in pathogenicity of desferrioxamine E, the main siderophore of *Erwinia amylovora.* pp 179-195 in: Fire Blight, The Disease and its Causative Agent, *Erwinia amylovora.* Vanneste, J. L. ed. CABI publishing New York, NY.

Geider, K. 2000. Exopolysaccharides of *Erwinia amylovora*: structure, biosynthesis, regulation, role in pathogenicity of amylovoran and levan. pp 117-140 in: Fire blight: The Disease and its Causitive Agent, *Erwinia amylovora.* Vanneste, J. L. ed. CABI Publishing, New York, NY.

Gurian-Sherman, D., and Lindow, S.E. 1995. Differential effects of growth temperature on ice nuclei active at different temperatures that are produced by cells of *Pseudomonas syringae.* Cryobiology 32:129-138.

Gross, D. C. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. J. Appl. Bacteriol. 58:167-174.

Gross, D. C., DeVay, J. E., and Stadtman, F. H. 1977. Chemical properties of syringomycin and syringotoxin: toxigenic peptides produced by *Pseudomonas syringae.* J. Appl. Bacteriol. 43:453-463.

Haefele, D. M. and Lindow, S. E. 1987. Flagellar motility confers epiphytic firness advantages upon *Pseudomonas syringae.* Appl. Environ. Microbiol. 53:2528-2533.

Hattingh, M. J., Beer, S. V., and Lawson, E. W. 1986. Scanning electron microscopy of apple blossoms colonized by *Erwinia amylovora* and *Erwinia herbicola.* Phytopathology 76:900-904.

Heeb, S., and Dieter, H. 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. Mol. Plant-Microbe Interact. 14:1351-1363.

Hirano. S. S. and Upper, C. D. 1995. Principles of ice nucleation, pp 41-61 in: Biological ice nucleation and its applications. R. E. Lee Jr., G. J. Warren, and L. V. Gusta eds. American Phytopathological Society, St. Paul, MN.

Holtz, B. A., Hoffman, E. W., and Lindow, S. E. 2002. Enhancing flower colonization of *Pseudomonas fluorescens* strain A506, and the efficacy of apogee and serenade, for fire blight control in the San Joaquin valley of California. Acta Hortic. 590:319-324.

Hrabak, E. M., and Willis, D. K. 1992. The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. J. Bacteriol. 174:3011-3020.

Isenbeck, M., and Schulz, F. A. 1985. Biological control of fire blight (*Erwinia amylovora* [Burr.] Winslow et al.) on ornamentals I. Control of the pathogen by antagonistic bacteria. Phytopath. Z. 113:324-333.

Ishimaru, C. A., and Klos, E. J. 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74:1342-1345.

Ishimaru, C. A., Klos, E. J., and Brubaker, R. R. 1988. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology 78:746-750.

Jacobs, J. L., and Sundin, G. W. 2001. Effect of solar UV-B radiation on a phyllosphere bacterial community. Appl. Environ. Microbiol. 67:5488-5496.

Johnson, K. B., Stockwell, V. O., Burgett, D. M., Sugar, D., and Loper, J. E. 1993a. Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees to apple and pear blossoms. Phytopathology 83:478-484.

Johnson, K. B., Stockwell, V. O., McLaughlin, R. J., Sugar, D, Loper, J. E., and Roberts, R. G. 1993b. Effect of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. Phytopathology 83:995-1002.

Johnson, K. B., and Stockwell, V. O. 1998. Management of fire blight: a case study in microbial ecology. Annu. Rev. Phytopathol. 36:227-248.

Johnson, K. B., and Stockwell, V. O. 2000. Biological control of fire blight. pp 319-337 in: Fire blight: The Disease and its Causative Agent, *Erwinia amylovora*. Vanneste, J. L. ed. CABI Publishing, New York, NY.

Joyner, D. C. and Lindow, S. E. 2000. Heterogeneity of iron bioavailability on plants assessed with a whole-cell GFP-based bacterial biosensor. Microbiology 146:2435-2445.

Kajava. A. V., and Lindow S. E. 1993. A model of the three-dimensional structure of ice nucleation proteins. J. Mol. Biol. 232:709-717.

Kearns, L. P., and Hale, C. N. 1996. Partial characterization of an inhibitory strain of *Erwinia herbicola* with potential as a biocontrol agent for *Erwinia amylovora*, the fire blight pathogen. J. Appl. Bacteriol. 81:369-374.

Kiett, G. W., and Ivanoff, S. S. 1941. Transmission of fire blight by bees and its relation to nectar concentration of apple and pear blossoms. J. Agricult. Res. 62:745-753.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.

Kinkel, L. L., and Lindow, S. E. 1993. Invasion and exclusion among coexisting *Psuedomonas syringae* strains on leaves. Appl. Environ. Microbiol. 59:3447-3454.

Kitten, T., Kinscherf, T. G., McEvoy, J. L., and Willis, D. K. 1998. A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. 28:917-930.

Kraus, J. and Loper, J. E. 1992. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biocontrol of *phythium* damping-off of cucumber. Phytopathology 82:264-271.

Kraus, J. and Loper, J. E. 1995. Characterization of a genomic region required for production of the antibiotic pyoluteorin by the biological control agent *Pseudomonas fluorescens* Pf-5. Appl. Environ. Microbiol. 61:849-854.

Lindow, S. E. 1993. Novel method for identifying bacterial mutants with reduced epiphytic fitness. Appl. Environ. Microbiol. 59:1586-1592.

Lindow, S. E., Andersen, G., and Beattie, G. A. 1993. Characteristics of insertional mutants of *Pseudomonas syringae* with reduced epiphytic fitness. Appl. Environ. Microbiol. 59:1593-1601.

Lindow, S. E. 1985. Integrated control and role of antibiosis in biological control of fire blight and frost injury. pp 83-115 in: Biological Control on the Phylloplane. C. Windels and S. E. Lindow, eds. American Phytopathological Society, St. Paul, MN.

Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by Ice^- mutants of *Pseudomonas syringae*. Appl. Environ. Microbiol. 53:2520-2527.

Lindow, S. E. and Wilson, M. 1993. Population dynamics of *Pseudomonas fluorescens* strain A506 in pear flowers following inoculation in relation to strategies of biological control of fire blight and frost injury. Acta Hortic. 338:331-332.

Lindow, S. E., McGourty, and Elkins, R. 1996. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. Phytopathology 86:841-848.

Lindsay, W. L. 1979. Chemical equilibria in soils. John Wiley & Sons, Inc., New York, NY.

Lindsay, W. L. and Schwab, A. P. 1982. The chemistry of iron in soils and its availability to plants. J. Plant Nutr. 5:821-840.

Liu, Y. and Whittier, R. F. 1995. Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25:674-681.

Loper, J. E., Henkels, M. D., Roberts, R. G., Grove, G. G., Willett, M. J., and Smith, T. J. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington state. Plant Dis. 75:287-290.

Loper, J. E. and Lindow, S. E. 1994. A biological sensor for iron available to bacteria in their habitats on plant surfaces. Appl. Environ. Microbiol. 60:1934-1941.

Loper, J. E. and Henkels, M. D. 1997. Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. Appl. Environ. Microbiol. 63:99-105.

Loper, J. E. and Lindow, S. E. 1997. Reporter gene systems useful in evaluating *in situ* gene expression by soil- and plant-associated bacteria. pp 627-637 in: Manual of Environmental Microbiology. Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D., and Walter, M. V. eds. ASM press, Washington D. C.

Loper, J. E. and Henkels, M. D. 1999. Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. Appl. Environ. Microbiol. 65:5357-5363.

Manceau, C., Lalande, J., Lachaud, G., Chartier, R., and Paulin, J. 1990. Bacterial colonization of flowers and leaf surfaces of pear trees. Acta Hortic. 273:73-81.

Marschner, P. and Crowley, D. E. 1997a. Iron stress and pyoverdine production by fluorescent pseudomonad in the rhizosphere of white lupin (*Lupinus albus* L.) and barley (*Hordeum vulgare* L.). Appl. Environ. Microbiol. 63:277-281.

Marschner, P. and Crowley, D. E. 1997b. Root colonization and iron nutritional status of *Pseudomonas fluorescens* in different plant species. Plant and Soil 196:311-316.

Marschner, P. and Crowley, D. E. 1998. Phytosiderophores decrease iron stress and pyoverdine production of *Pseudomonas fluorescens* Pf-5 (PVD-INAZ). S. Bio. Biochem. 30:1275-1280.

Matzanke, B. F. 2000. Structures, coordination chemistry and functions of microbial iron chelates, pp 15-64 in: CRC handbook of microbial iron chelates G. Winkelmann ed.. CRC Press, Boca Raton, FL.

Meyer, J. M., and Abdallah, M. A. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification, and physico-chemical properties. J. Gen. Microbiol. 107:319-328.

Meyer, J. and Stintzi, A. 1998. Iron metabolism and siderophores in *Pseudomonas* and related species. pp 201-239 in *Pseudomonas*. Montie ed. Plenum Press, New York, 1998.

McManus, P. S., and Jones, A. L. 1994a. Role of wind driven rain, aerosols, and contaminated budwood in incidence and spatial pattern of fire blight in an apple nursery. Plant Dis. 78:1059-1066.

McManus, P. S., and Jones, A. L. 1994b. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology 84:627-633.

McManus, P. S., Stockwell, V. O., Sundin, G. W., and Jones, A. L. 2002. Antibiotic use in plant agriculture. Annu. Rev. Phytopathol. 40:443-465.

Moller, W. J., Schroth, M. N., and Thomson, S. V. 1981. The scenario of fire blight and streptomycin resistance. Plant Dis. 65:563-568.

Mossialos, D., Ochsner, U., Baysse, C., Chablain, P., Pirnay, J., Koedam, N., Budzikiewicz, H., Fernandez, D. U., Schafer, M., Ravel, J. and Cornelis, P. 2002. Identification of new, conserved, non-ribosomal peptide synthetases from fluorescent pseudomonads involved in the biosynthesis of the siderophore pyoverdine. Mol. Microbiol. 45:1673-1685.

Neilands, J. B. 1984. Siderophores of bacteria and fungi. Microbiol. Sci. 1:9-14.

Nuclo, R. L., Johnson, K. B., Stockwell, V. O., and Sugar, D. 1998. Secondary colonization of pear blossoms by two bacterial antagonists of the fire blight pathogen. Plant Dis. 82:661-668.

Pfender, W. F., Kraus, J., and Loper, J. E. 1993. A genomic region from *Pseudomonas fluorescens* Pf-5 required for pyrrolnitrin production and inhibition of *Pyrenophora tritici-repentis* in wheat straw. Phytopathology 83:1223-1228.

Pusey, P. L. 1999. Effect of nectar on microbial antagonists evaluated for use in control of fire blight. Phytopathology 89:39-46.

Pusey, P. L. 2000. The role of water in epiphytic colonization and infection of pomaceous flowers by *Erwinia amylovora*. Phytopathology 90:1352-1357.

Saleh, S. S., and Glick, B. R. 2001. Involvement of *gacS* and *rpoS* in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. Can J. Microbiol. 47:698-705.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (eds.) 1989. Molecular cloning-A Laboratory Manual. 2nd Edition. Cold Spring Harbor Laboratory Press, NY.

Sauvage, C. and Expert, D. 1997. Differential regulation by iron of *Erwinia chrysanthemi* pectate lyases: pathogenicity of iron transport regulatory (*cbr*) mutants. Mol. Plant-Microbe Interact. 7:71-77.

Schaad, N. W. 1980. Laboratory guide for identification of plant pathogenic bacteria. American Phytopathological Society, St. Paul, MN.

Steinberger, E. M., and Beer, S. V. 1987. Mutants of *Erwinia amylovora* altered in pathogenicity by transposon mutagenesis. Acta Hortic. 217:167-168.

Stockwell, V. O., Sugar, D., Spotts, R., Johnson, K. B., and Loper, J. E. 1996a. Recovery of streptomycin-resistant isolates of *Erwinia amylovora* from Oregon orchards. (Abstr.) Phytopathology 86:S50.

Stockwell, V. O., Johnson, K. B., and Loper, J. E. 1996b. Compatibility of bacterial antagonists of *Erwinia amylovora* with antibiotics used to control fire blight. Phytopathology 86:834-840.

Stockwell, V. O., Johnson, K. B., and Loper, J. E. 1998. Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. Phytopathology 88:506-513.

Stockwell, V. O., McLaughlin,, R. J., Henkels, M. D., Loper, J. E., Sugar, D., and Roberts, R. G. 1999. Epiphytic colonization of pear stigmas and hypanthia by bacteria during primary bloom. Phytopathology 89:1162-1168.

Stockwell, V. O., Johnson, K. B., and Loper, J. E. 2001. Enhancement of biocontrol of fire blight with an iron chelate. (Abstr.) Phytopathology 91:S86.

Stockwell, V. O., Johnson, K. B., and Loper, J. E. 2002a. Biological control of fire blight: Understanding interactions among introduced and indigenous microbial communities. pp 225-239 in: Phyllosphere Microbiology. S. E. Lindow, E. I. Hecht-Poinar, and V. J. Elliott eds. American Phytopathological Society, St. Paul, MN.

Stockwell, V. O., Johnson, K. B., Sugar, D., and Loper, J. E. 2002b. Antibiosis contributes to biological control of fire blight by *Pantoea agglomerans* strain Eh252 in orchards. Phytopathology 92:1202-1209.

Tang, X., Lu, B. F., and Pan, S. Q. 1999. A bifunctional transposon mini-Tn*5gfp-km* which can be used to select for promoter fusions and report gene expression levels in *Agrobacterium tumefaciens*. FEMS Microbiol. Lett. 179:37-42.

Temple, T. N., Stockwell, V. O., Johnson, K. B., and Loper, J. E. 2001. Selection assay for an antibiotic deficient mutant of *Pseudomonas fluorescens* A506. (Abstr) Phytopathology 91:S87.

Thomashow, L. S., and Weller, D. M. 1995. Current concepts in the use of introduced bacteria for biological disease control: Mechanisms and antifungal metabolites. pp 187-235 in: Plant-Microbe Interactions, Vol. 1. Stacey, G, and Keen, N. eds. Chapman & Hall, New York, NY.

Thomson, S. V. 1986. The role of the stigma in fire blight infections. Phytopathology 76:476-482.

Thomson, S. V., Schroth, M. N., Moller, W. J., and Reil, W. O. 1982. A forecasting model for fire blight of pear. Plant Dis. 66:576-579.

Thomson, S. V., Hansen, D. R., Flint, K. M., and Vandenberg, J. D. 1992. Dissemination of bacteria antagonistic to *Erwinia amylovora* by honey bees. Plant Dis. 76:1052-1056.

Vali, G. 1989. Principles of ice nucleation, pp 1-28 in: Biological Ice Nucleation and its Applications. R. E. Lee Jr., G. J. Warren, and L. V. Gusta eds. American Phytopathological Society, St. Paul, MN.

van der Zwet, T., and Keil, H. L. 1979. Fire Blight: A Bacterial Disease of Rosaceous Plants. Agricultural Handbook Number 510. USDA Science and Education Administration, Washington D. C.

Vanneste, J. L., Yu, J., and Beer, S. V. 1992. Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. J. Bacteriol. 174:2785-2796.

Vanneste, J. L. 2000. What is fire blight? Who is *Erwinia amylovora*? How to control it? in Fire Blight: The Disease and its Causitive Agent, *Erwinia amylovora* J. L. Vanneste ed. CAB Publishing, New York, NY.

Visca, P., Leoni, L., Wilson, M. J., and Lamont, I. L. 2002. Iron transport and regulation, cell signaling and genomics: Lessons from *Escherichia coli* and *Pseudomonas*. Mol. Microbiol. 45:1177-1190.

Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W., and Loper, J. E. 1998. The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^S and the stress response in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 180:6635-6641.

Willis, D. K., Holmstadt, J. J., and Kinscherf, T. G. 2001. Genetic evidence that loss of virulence associated with *gacS* or *gacA* mutations in *Pseudomonas syringae*B728a does not result from effects on alginate production. Appl. Environ. Microbiol. 67:1400-1403.

Wilson, M., Epton, H. A. S., and Sigee, D. C. 1989. *Erwinia amylovora* infection of hawthorn blossom II. The stigma. J. Phytopathol. 127:15-28.

Wilson, M., Epton, H. A. S., and Sigee, D. C. 1992. Interaction between the biological control agent *Erwinia herbicola* and *Erwinia amylovora* on the stigma of hawthorn blossoms. Phytopathology 82:914-918.

Wilson, M., and Lindow, S.E. 1993. Interactions between the biological control agent *Pseudomonas fluorescens* strain A506 and *Erwinia amylovora* in pear blossoms. Phytopathology 83:117-123.

Wilson, M., and Lindow, S. E. 1994. Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl. Environ. Microbiol. 60:4468-4477.

Wolber, P., Deininger, C. A., Southworth, M. W., Vandekerckhove, J., Van Montagu, M., and Warren, G. J. 1986. Identification and purification of a bacterial ice-nucleation protein. Proc. Natl. Acad. Sci. 83:7256-7260.

Willis, D. K., Hrabak, E. M., Rich, J. J., Barta, T. M., Lindow, S. E., and Panopolous, N. J. 1990. Isolation and characterization of a *Pseudomonas syringae* pv. *syringae* mutant deficient in lesion forming ability on bean. Mol. Plant-Micro. Interact. 3:149-156.

Wright, S. A., and Beer, S. V. 1996. The role of antibiotics in biological control of fire blight by *Erwinia herbicola* strain Eh318. Acta Hortic. 411:309-311.

Wright, S., Zumoff, C., Schneider, L. and Beer, S. 2001. *Pantoea agglomerans* strain Eh318 produces two antibiotics that inhibit *Erwinia amylovora* in vitro. Appl. Environ. Microbiol. 67:284-292.