INVOLVEMENT OF THE EXTRACELLULAR POLYSACCHARIDE ALGINATE IN THE VIRULENCE AND EPIPHYTIC FITNESS OF *PSEUDOMONAS SYRINGAE*

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CHAPTER 1

INTRODUCTION

P. syringae is a necrogenic bacterial plant pathogen that causes economically important diseases of plants in many parts of the world. The typical disease symptoms induced by *P. syringae* include leaf spots, blights, and galls (Alfano and Collmer, 1996). The species *P. syringae* is subdivided into pathovars (pathogenic variants, pvs.), which vary in host specificity. The pathogenesis of *P. syringae* in susceptible host plants involves prolonged bacterial multiplication, dissemination to surrounding tissues, and production of macroscopic symptoms characteristic of the disease. During the incompatible interaction with resistant host plants, *P. syringae* elicits a plant defense reaction known as the hypersensitive response (HR). The plant cells in contact with the pathogen become rapidly necrotic, which restricts the multiplication and spread of the pathogen and results in host resistance. Although phytopathogenic bacteria are diverse in their taxonomy and pathology, they all contain hypersensitive response and pathogenicity (*hrp*) genes that enable them to elicit the HR in resistant plants or to cause disease in susceptible host plants (Lindgren, 1997). In addition to the *hrp* gene cluster, a variety of virulence factors including extracellular polysaccharides (EPS), phytotoxins and cell wall degrading enzymes contribute

to the full expression of symptoms (Alfano and Collmer, 1996). EPS are carbohydrate polymers produced by a large variety of bacteria, including many plant pathogens. EPS is an important virulence factor in *Ralstonia* (*Pseudomonas*) *solanacearum*, *Erwinia amylovora*, and *Pantoea* (*Erwinia*) *stewartii* (Denny, 1995).

Prior to infection, *P. syringae* commonly exists as an epiphyte and resides on the surface of healthy plants (Beattie and Lindow, 1995). A major role of epiphytic bacterial populations is to serve as a reservoir for the potentially phytopathogenic bacteria to gain entrance into plants. The bacteria are then able to initiate interactions with the plant that result in either disease or the HR. However, the leaf surface is a harsh environment subject to desiccation stress, temperature extremes, and both UV and visible light irradiation. The ability of bacteria to establish or maintain populations on leaf surfaces is critical for a subsequent successful infection. Several traits developed by phytopathogens have been proposed to improve epiphytic fitness of phytopathogenic bacteria, including EPS production, UV tolerance, and osmotolerance (Beattie and Lindow, 1995).

P. syringae pathovars generally produce two well-characterized EPS molecules: levan (a polymer of fructofuranoses) and alginate, a copolymer of *O*-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Gross and Rudolph, 1987). Previous studies implied that alginate may contribute to virulence in *P. syringae* (Fett et al., 1986; Osman et al., 1986); however, the role of alginate in pathogenesis has not been critically assessed using a genetic

approach. In the present study, an alginate-defective (Alg^-) mutant was generated in *P. syringae* pv. syringae strain 3525, which causes bacterial brown spot on beans. The involvement of alginate in the virulence and epiphytic fitness of this pathogen was investigated.

CHAPTER 2

LITERATURE REVIEW

Pseudomonas syringae: biology and pathogenesis

Pseudomonas syringae is a necrogenic, Gram-negative plant pathogen. *P. syringae* shows an excellent ability to survive harsh environmental conditions on leaf surfaces and develop or maintain a high population size on plants prior to infection. The survival of epiphytic bacteria under adverse environmental conditions on the leaf surface may be achieved either by avoidance or tolerance of environmental stress (Beattie and Lindow, 1994; 1995). Epiphytic bacteria may occupy sites such as the depressions between cells, substomatal cavities, or the base of trichomes, which provide protection against direct exposure to UV radiation or desiccation. Epiphytic bacteria have also developed adaptive mechanisms, including the production of extracellular polysaccharides (EPS), tolerance to UV irradiation, motility, and plant hormone production, which are traits that increase tolerance to environmental stress (Andersen et al., 1998; Beattie and Lindow, 1994; 1995; Brandl and Lindow, 1998; Poplawsky and Chun, 1998).

When conditions are favorable, *P. syringae* invades plant hosts through wounds, stomata, and natural openings, colonizes the intercellular spaces and causes typical disease symptoms including leaf spots and blights (Alfano and Collmer, 1996). Strains of *P. syringae* are subdivided into pathovars (pathogenic variants, pvs.) based on host specificity. When *P. syringae* encounters nonhost or resistant host plants, the hypersensitive response (HR) is elicited. The HR is a plant defense reaction characterized by rapid, programmed death of plant cells in contact with the pathogen, which localizes the pathogen in the initial infection site. In contrast, the pathogenesis of *P. syringae* in compatible host plants involves prolonged bacterial multiplication, dissemination to surrounding tissues, and production of macroscopic symptoms characteristic of the disease. The ability of phytopathogenic bacteria to elicit the HR in resistant plants or to cause disease in susceptible host plants depends upon the *hrp* (hypersensitive response and pathogenicity) genes (Collmer, 1998; Lindgren, 1997; Willis et al., 1991). In addition to *hrp* gene products, a variety of virulence factors including EPS, phytotoxins and cell wall degrading enzymes contribute to the full expression of symptoms (Alfano and Collmer, 1996).

Biological role of extracellular polysaccharides

Extracellular polysaccharides are carbohydrate polymers that are produced by a large variety of bacteria, including many plant pathogens. EPS may be secreted into the extracellular milieu of the bacterial cell to form a loose extracellular slime or remain closely associated with the cell wall as a capsular

layer (Whitfield, 1988). EPS can consist of homopolymeric sugars, or heteropolymers composed of a complex mixture of sugars in precise, repeating subunits, which can carry a variety of noncarbohydrate substituents such as acetate, pyruvate, hydroxybutyrate and succinate (Leigh, 1992). Among the most common bacterial homopolysaccharides are alginate (2 and/or 3 O-acetylated, β-1,4-linked polymannuronic acid) and levan (β-2,6-D-fructofuranan). One of the most important commercial heteropolysaccharides is xanthan gum, which consists of a β-1,4-linked D-glucose backbone with trisaccharide side chains (Jansson et al., 1975).

EPS plays multiple roles in protecting free-living bacteria from a variety of environmental stresses and also functions in the pathogenesis of human, animal and plant pathogens (Whitfield, 1988; Leigh, 1992). Because of their hydrophilic and anionic properties, EPS polymers may modify the physical and chemical environment around the bacterial cells to a more favorable environment for growth and survival. Furthermore, EPS polymers help bacteria to absorb water (preventing desiccation), accumulate minerals and nutrients, and shield the bacteria against hydrophobic and toxic macromolecules (Denny, 1995; Rudolph, 1994).

Characterization of extracellular polysaccharides in several important phytopathogens

EPS production, particularly its role in pathogenesis as shown through transposon mutagenesis, has been extensively studied in *Ralstonia* (*Pseudomonas*) *solanacearum* (Cook and Sequeira, 1991; Denny et al., 1988; Denny and Baek 1991; Kao et al., 1992), *Erwinia amylovora* (Bernhard et al., 1993; Geier and Geider, 1993; Geider et al., 1993; Gross et al., 1992), *Pantoea* (*Erwinia*) *stewartii* (Bernhard et al., 1996; Coplin et al., 1990, 1992a,b; Dolph et al., 1988) and *Xanthomonas campestris* (Katzen et al., 1998; Pierce et al., 1993; Ramirez et al., 1988). EPS is generally considered to be an important virulence factor in pathogenesis by aiding bacterial adhesion to inert and living surfaces, which minimizes morphological contact of the bacteria with plant cells and reduces the host defense reaction. EPS may also enhance colonization of plant surfaces, prolong water-soaked symptoms in susceptible plant tissue, and inhibit bacterial agglutination in plant hosts (Denny, 1995).

Ralstonia solanacearum

R. solanacearum is a wilt-inducing pathogen that colonizes the xylem vessels of host plants. Tn*5* inactivation of the *eps* operon to create EPS deficient mutants indicated that the acidic EPS is a necessary wilt-inducing factor of *R. solanacearum.* Despite their ability to multiply in the inoculated site, *eps* mutants produce little if any acidic EPS *in planta* and are greatly reduced in virulence on

tomato, tobacco and eggplant (Denny and Baek 1991; Denny et al., 1988; Kao et al., 1992).

Erwinia amylovora and *Pantoea stewartii*

Both *E. amylovora* and *P. stewartii* are necrogenic, wilt-inducing pathogens. *E. amylovora* produces at least three types of EPS: (1) levan, (2) a poorly characterized low molecular weight glucan, and (3) an acidic EPS called amylovoran. Levan-deficient mutants of *E. amylovora* were created by Tn*5* mutagenesis of the levansucrase gene and showed delayed development of necrotic symptoms, indicating that levan synthesis may play an important role for multiplication of the pathogen *in planta* (Geier and Geider, 1993). Amylovoran-defective mutants, created by transposon mutagenesis of the *ams* genes in *E. amylovora*, were nonpathogenic, and both necrosis and bacterial multiplication were inhibited *in vivo* (Geider et al., 1993; Gross et al., 1992).

P. stewartii synthesizes an EPS called stewartan. *P. stewartii* multiplies in the intercellular spaces of corn leaves, producing both water-soaked lesions and systemic wilting. Transposon mutagenesis of the *cps* genes showed that stewartan is involved in pathogenicity probably by aiding bacterial movement within the xylem, although the initial multiplication of the pathogen within corn leaves was not affected (Coplin et al., 1990, 1992a,b; Dolph et al., 1988). Interestingly, amylovoran production restored virulence to *P. stewartii cps* mutants, but *E. amylovora ams* mutants that produced stewartan remained avirulent or weakly virulent (Bernhard et al., 1993; 1996).

Xanthomonas campestris

X. campestris is a necrogenic pathogen that causes leaf spot and blight diseases in many different plant species. All *X. campestris* pathovars make an EPS called xanthan gum. Infrared spectral analysis showed that the xanthan gum produced by *X. c.* pv. campestris may have a role in virulence (Ramirez et al., 1988). However, the cotton pathogen *X. c.* pv. malvacearum produced equal amounts of xanthan per bacterium during the first 48 h in isogenic susceptible and resistant cultivars, suggesting that host resistance does not interfere with EPS production and that EPS production does not interfere with host resistance (Pierce et al, 1993). Recently, Katzen et al. (1998) assigned biochemical functions to xanthan gum gene products. Although xanthan gum was not essential for plant virulence, alterations in the later stages of xanthan biosynthesis reduced the aggressiveness of *X. campestris*.

Alginate biosynthesis and regulation in *Pseudomonas aeruginosa*

Alginic acids (alginate) constitute a group of structurally related homopolysaccharides composed of a linear backbone of β-1,4-linked, partially acetylated D-mannuronic acid and its C5-epimer, L-guluronic acid (Fig. 1). Brown algae are the major source of alginate for a wide variety of commercial products such as gelling, stabilizing, and thickening agents. The pharmaceutical and biotechnology industries also use alginate in the administration of drugs, in tissue transplantation, and as a material for dental impressions (Rehm and Valla,

1997). The major difference between bacterial and algal alginate is the presence of O-acetyl groups in bacterial alginate (Gacesa, 1998).

P. aeruginosa is a well-studied human pathogen that infects the lungs of cystic fibrosis patients and produces an alginate capsule that protects the pathogen from the host immune system, antibiotic treatment, and desiccation (Govan and Deretic, 1996; Shankar et al., 1995). Alginate is an important virulence factor of *P. aeruginosa*, and its biosynthesis and regulation have been extensively explored in this species (Chitnis and Ohman, 1993; Darzins and Chakrabarty, 1984; Deretic et al., 1993; Shankar et al., 1995).

The alginate biosynthetic pathway is shown in Fig. 2 (Govan and Deretic, 1996; Shankar et al., 1995). The starting point for alginate biosynthesis is fructose 6-phosphate. Fructose 6-phosphate is converted into mannose 6-phosphate via phosphomannose isomerase (PMI; encoded by *algA*) (Darzins et al., 1991). Mannose-6-phosphate is then converted to mannose-1-phosphate via phosphomannomutase (PMM; encoded by *algC*) (Coyne et al., 1994). Conversion of mannose-1-phosphate to GDP-mannose is catalyzed by GDP-mannose pyrophosphorylase (GMP; also encoded by *algA*). The conversion of GDP-mannose into the GDP-mannuronate monomer via GDP-mannose dehydrogenase (GMD; encoded by *algD*) is a dedicated step in the biosynthesis of alginate (Deretic et al., 1987; May and Chakrabarty, 1994). GDP-mannuronate residues are then polymerized, epimerized and acetylated to form mature alginate. Two putative membrane proteins, Alg8 and Alg44 are thought to be involved in the polymerization of GDP-mannuronate monomers to a

polymannuronate polymer (Maharaj et al., 1993). AlgX(Alg60) is also thought to have a role in alginate polymerization by facilitating the transfer of an acetyl group from acetyl-CoA to a transacetylase, the product of the *algF* gene (Franklin and Ohman, 1993; Monday and Schiller, 1996). The *algI* and *algJ* gene products are also required for alginate acetylation (Franklin and Ohman, 1996). Another alginate modifying enzyme, the epimerase encoded by *algG*, catalyzes the epimerization of the mannuronate residues at the C5 position, thus introducing guluronate residues into polymannuronate (Franklin et al., 1994). The ratio of mannuronate to guluronate residues may control the rheological properties of alginate. A newly identified protein, AlgK, may also be involved in the polymerization of mannuronate to alginate (Jain and Ohman, 1998). Secretion of alginate may be facilitated by a pore-forming protein encoded by *algE* (Rehm et al., 1994). Alginate degradation is catalyzed by alginate lyase, which is encoded by *algL*, a gene located in the same operon as the alginate biosynthetic genes (Schiller et al., 1993).

The alginate biosynthetic and regulatory genes are clustered at four locations in the *P. aeruginosa* chromosome (Fig. 3) (Gacesa, 1998; May and Chakrabarty, 1994). Most of the alginate structural genes, *algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF* and *algA* (listed in the order of transcription) are located at 34 min. The major promoter in this cluster is the *algD* promoter, which is thought to transcribe the entire alginate biosynthetic gene cluster as an operon (Chitnis and Ohman, 1993). The only structural gene located outside of this gene cluster is *algC*, which is located at 10 min (Fig. 3).

The expression of the alginate biosynthesis genes in most pseudomonads, including *P. aeruginosa*, is normally silent, but can be expressed under certain environmental conditions, including high osmolarity, nutrient starvation, or exposure to oxidative stress (Berry et al., 1989; Mathee et al., 1999). The genetic conversion from the nonmucoid to mucoid form of *P. aeruginosa* is controlled by five genes, including *algT* (*algU*), *mucA* (*algS*), *mucB* (*algN*), *mucC* (*algM*), and *mucD* (*algW*), which are located at 68 min on the chromosome. The alternative sigma factor encoded by *algT* plays a key role in activating alginate biosynthesis by initiating transcription at the *algD*, *algT* and *algR1* promoters (Hershberger, et al., 1995; Schurr et al., 1995; Wozniak and Ohman 1994). The *mucA*, *mucB*, *mucC*, and *mucD* gene products are regulators of AlgT, and *muc* mutations result in the overproduction of alginate (Boucher et al., 1996 and 1997; Schurr et al., 1996; Xie et al., 1996). Four additional regulatory genes *algB*, *algR1* (*algR*), *algR2* (*algQ*), and *algR3* (*algP*), which are located at 13 and 10 min in the chromosome, function as auxiliary regulators of mucoidy and modulate the production of alginate (Govan and Deretic, 1996; Gacesa, 1998). AlgR1 functions as a response regulator member of the two-component signal transduction system and activates *algD* transcription in conjunction with *algT*. Additional auxiliary genes are also required for *algD* expression and have been described elsewhere (Gacesa, 1998; Ohman et al., 1996).

Alginate and pathogenesis in *P. syringae*

Pathovars of *P. syringae* generally produce loose slime layers of alginate and levan. When grown on media with excess sucrose, many *P. syringae* pathovars produce levan *in vivo*. However, studies on EPS polymers produced by several phytopathogenic *P. syringae* pathovars (pv. phaseolicola, syringae, tomato and lachrymans) showed that only alginate was produced by bacterial pathogens in water-soaked lesions, whereas no levan was produced *in planta* (Fett and Dunn, 1989). Furthermore, Osman et al. (1986) demonstrated that *P. syringae* pv. glycinea produced alginate in susceptible soybean cultivars, but did not produce significant levels of alginate in resistant cultivars. In a similar study, Gross and Rudolph (1987) reported that the more virulent race 2 isolates of *P. syringae* pv. phaseolicola produced higher amounts of alginate than race 1 isolates both *in planta* and *in vitro*. These results imply that alginate may have an important role in both pathogenesis and the development of water-soaked lesions *in planta*. However, it is important to note that the role of alginate in pathogenesis has not been critically evaluated using a genetic approach.

Several reports indicate that the biosynthetic route to alginate in *P. syringae* is similar to that established for *P. aeruginosa*. DNA homologous to several essential alginate genes of *P. aeruginosa* has been found in several pathovars of *P. syringae* (Fialho et al., 1990; Fett et al. 1992). Penaloza-Vazquez et al. (1997) cloned the alginate structural gene cluster from *P. syringae* pv. syringae FF5. The arrangement of the structural gene cluster was virtually

identical to that previously described for *P. aeruginosa*. Complementation analyses, however, indicated that the structural gene clusters in *P. aeruginosa* and *P. syringae* were not functionally interchangeable when expressed from their native promoters. This result suggests that the regulation and transcriptional activation of alginate biosynthesis is different in the two species. Singh et al. (1992) found that sodium chloride and ethanol stimulated alginate production in most fluorescent pseudomonads, indicating that osmolarity and dehydration are general signals for alginate production. However, Kidambi et al. (1995) showed that copper was a signal for alginate production in *P. syringae* but not in *P. aeruginosa*. It is possible that some of the signals for alginate production in *P. syringae* are plant-induced since the bacteria are only slightly mucoid *in vitro* but are known to produce alginate *in planta*. More recently, Fakhr et al. (1999) cloned and characterized the gene encoding AlgR1 from *P. syringae*. Although AlgR1 and the flanking region were highly homologous to *P. aeruginosa*, AlgR1 was not required for transcription of *algD* in *P. syringae*. Further analysis revealed that the *algD* promoter region in *P. syringae* (*PsalgD*) diverged significantly from the *algD* promoter in *P. aeruginosa*, and *PsalgD* lacked the consensus sequence recognized by AlgR1. Recognition sites for integration host factor and the cyclic AMP receptor binding protein, which are known to be involved in the transcriptional activation of *algD* in *P. aeruginosa*, were also missing in *PsalgD*. The differential regulation of alginate gene expression in *P. aeruginosa* and *P. syringe* and the marked divergence in their *algD* promoter regions probably reflects their adaptation to plant and human hosts, respectively.

Similar results were also observed in the transcriptional regulation of *algT*, which encodes the alternate sigma factor, σ^{22} (Keith and Bender, 1999). Heat shock and osmotic stress were common stimuli for *algT* activation in both *P. syringae* and *P. aeruginosa.* But H_2O_2 and copper sulfate, compounds frequently encountered by the pathogen during colonization of plant tissue, may serve as unique signals for *algT* activation in *P. syringae*.

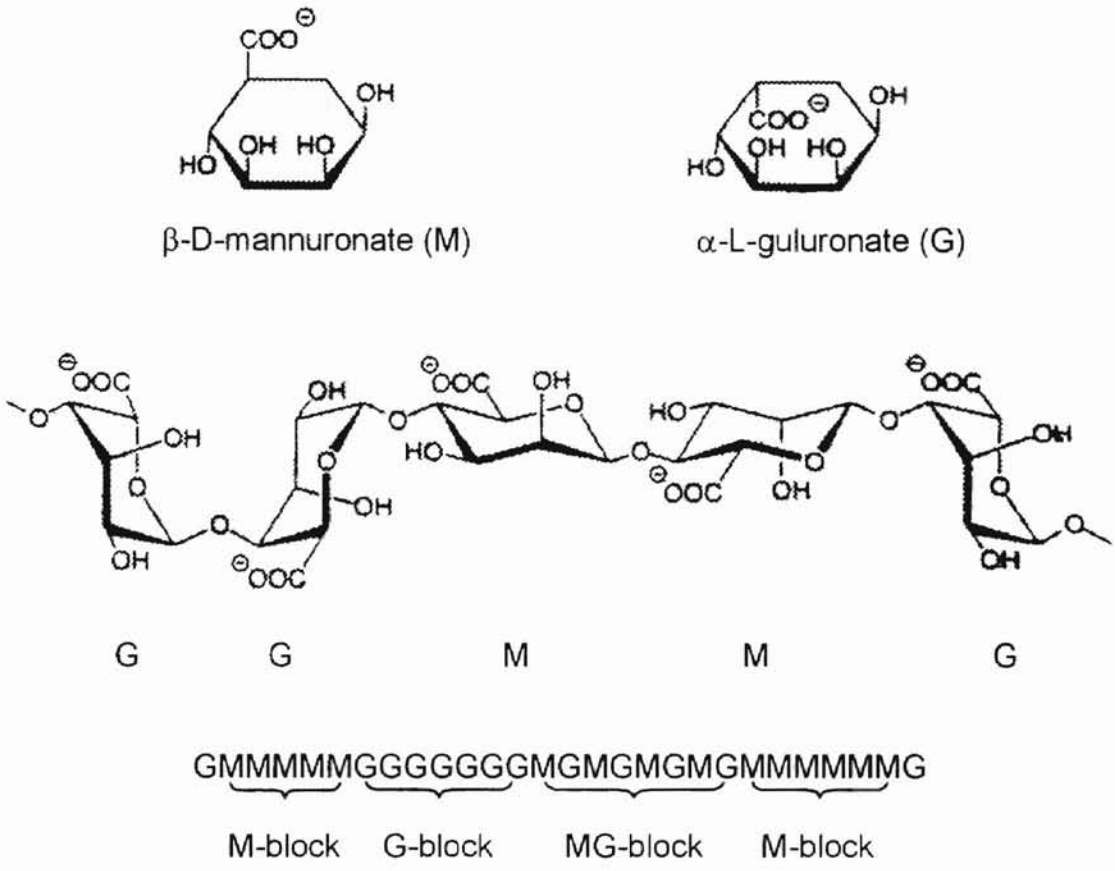


Fig. 1. Structure of alginate. The alginate monomers β-D-mannuronate (M) and α-L-guluronate (G) are assembled into (1-4)-linked blocks of continuous mannuronate residues (M-blocks), guluronate residues (G-blocks), or alternating residues (MG-blocks). Normally, bacterial alginates are *O*-acetylated at the 2 and/or 3 positions of the mannuronate residues.

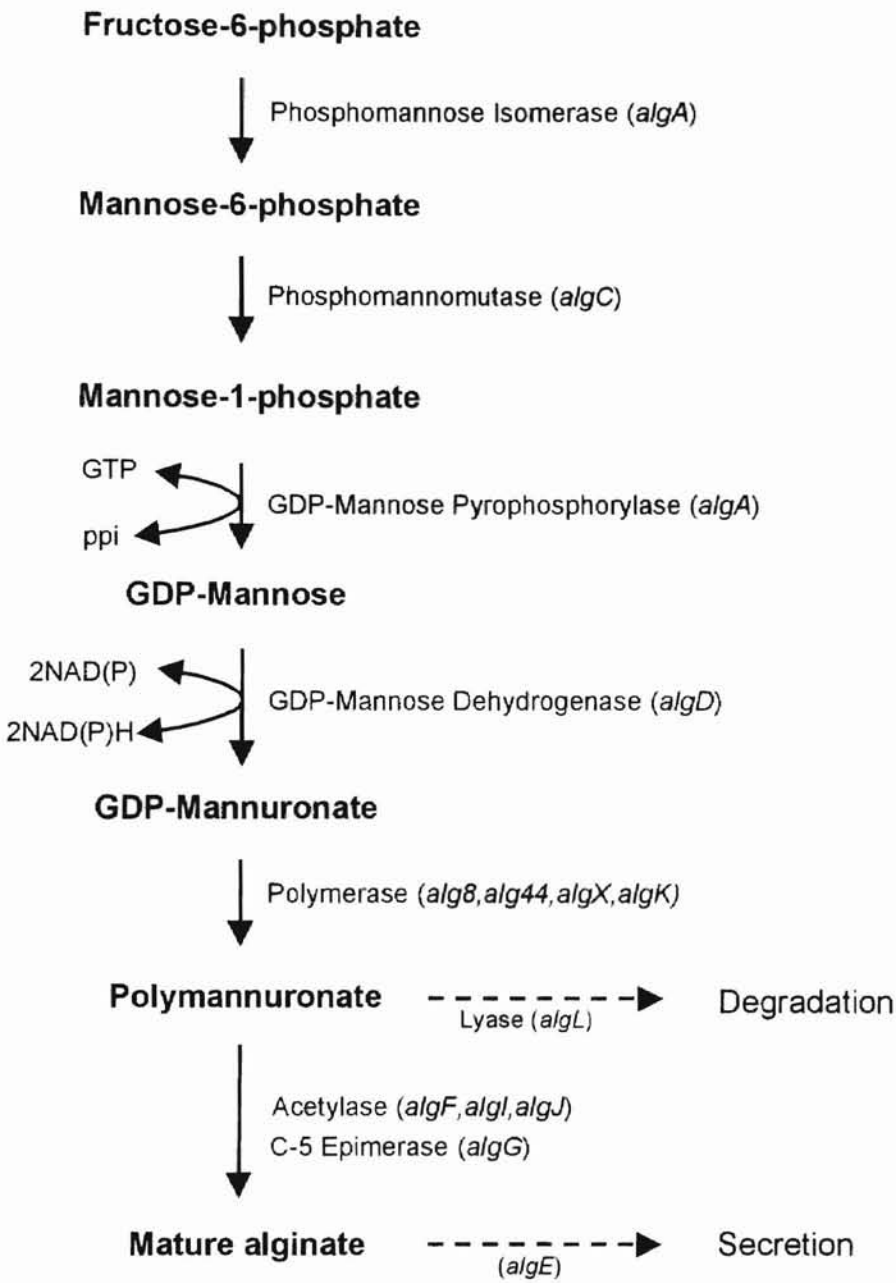


Fig. 2. The biosynthesis of alginate by *Pseudomonas aeruginosa*. Fructose 6-phosphate obtained from the metabolic pool is converted to GDP-mannuronic acid, which provides mannuronate residues for polymerization. Occasionally guluronate residues are incorporated into alginate chain via epimerization of mannuronate residues by the AlgG protein. Mannuronic acid residues of bacterial alginates are partially O-acetylated by the gene products of *algF*, *algI*, and *algJ*. Secretion of mature alginate is aided by the AlgE protein.

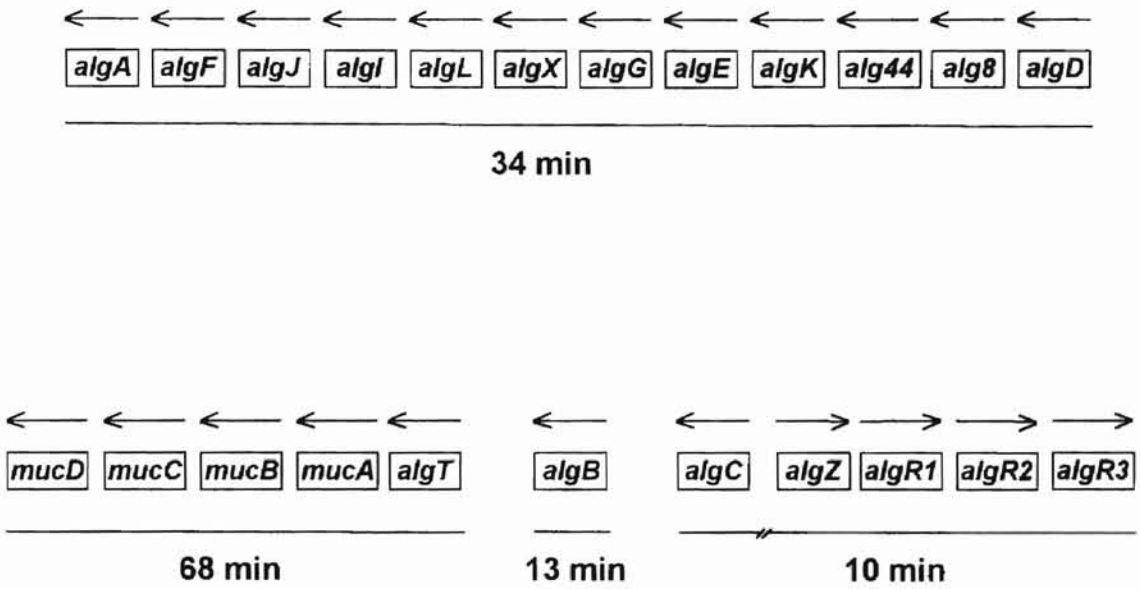


Fig. 3. Organization of the alginate gene clusters in *Pseudomonas aeruginosa*. The alginate genes are clustered at four locations in the *P. aeruginosa* chromosome. Except for the *algC* gene, which is located at 10 min, all of the known alginate structural genes are located at 34 min. The regulatory genes map at 10 min and 13 min, and the genes responsible for the genotypic switch to alginate production are located at 68 min. The arrows above the genes represent the direction of translation.

CHAPTER 3

INVOLVEMENT OF THE EXTRACELLULAR POLYSACCHARIDE ALGINATE IN THE VIRULENCE AND EPIPHYTIC FITNESS OF *PSEUDOMONAS SYRINGAE*

Abstract

Alginate, a copolymer of *O*-acetylated β-1,4 linked D-mannuronic acid and L-guluronic acid, has been reported to function in the virulence of *P. syringae*, although genetic studies to test this hypothesis have not been previously undertaken. In the present study, we used a genetic approach to evaluate the role of alginate in the pathogenicity of *Pseudomonas syringae* pv. syringae 3525, which causes bacterial brown spot on beans. Alginate biosynthesis in strain 3525 was disrupted by recombining Tn*5* into *algL*, which encodes alginate lyase, resulting in 3525.L. Alginate production in 3525.L was restored by the introduction of pSK2 or pAD4033 which contains the alginate biosynthetic gene cluster from *P. syringae* pv. syringae FF5 or the *algA* gene from *P. aeruginosa*, respectively. The role of alginate in the epiphytic fitness of strain 3525 was assessed by monitoring the populations of 3525 and 3525.L on tomato, which is not a host for this pathogen.

The mutant 3525.L was significantly impaired in its ability to colonize tomato leaves as compared to 3525, indicating that alginate functions in the survival of strain 3525 on leaf surfaces. The contribution of alginate to the virulence of strain 3525 was evaluated by comparing the population dynamics and symptom development of 3525 and 3525.L in bean leaves. Although 3525.L retained the ability to form lesions on bean leaves, symptoms were less severe and the population was significantly reduced in comparison to 3525. These results indicate that alginate contributes to the virulence of *P. syringae* pv. syringae 3525, perhaps by facilitating colonization or dissemination of the bacterium *in planta*.

Introduction

Exopolysaccharide (EPS) molecules are carbohydrate polymers produced by bacteria, which are either secreted into the growth media, form a loosely associated extracellular slime, or remain closely attached to cells as a capsule layer (Whitfield, 1988). EPSs provide a selective advantage to bacteria and have multiple functions including the absorption of water, the accumulation of minerals and nutrients, and protection from hydrophobic and toxic macromolecules (Denny, 1995; Rudolph et al., 1994). The virulence of numerous phytopathogenic bacteria including *Ralstonia (Pseudomonas) solanacearum*, *Erwinia amylovora*, *Pantoea stewartii* and *Xanthomonas campestris* has been correlated with their ability to produce EPS polymers *in planta* (Dolph et al., 1988; Geier and Geider, 1993; Kao et al., 1992; Katzen et al., 1998; Saile et al., 1997). However, the role of EPS in the virulence

of *Pseudomonas syringae* has not been assessed using a genetic approach. *P. syringae* pathovars generally produce two EPS molecules: levan (a fructofuranan) and alginate, a copolymer of *O*-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Fett et al., 1986; Gross and Rudolph, 1987). When grown on media with excess sucrose, many *P. syringae* pathovars produce levan (Hettwer et al., 1998). However, studies on the EPS molecules produced by *P. syringae in planta* indicated that alginate was the major exopolysaccharide produced in water-soaked lesions (Fett and Dunn, 1989; Rudolph et al., 1989). Furthermore, a positive correlation between the virulence of *P. syringae* and the quantity of alginate produced *in planta* has been demonstrated (Gross and Rudolph, 1987; Osman et al., 1986).

Several reports indicate that the biosynthetic route to alginate in *P. syringae* is similar to that established for *Pseudomonas aeruginosa* (Fialho et al., 1990; Fett et al., 1992; Peñaloza-Vázquez et al., 1997). Alginate biosynthesis has been extensively studied in *P. aeruginosa* where it functions as a major virulence factor in strains infecting the lungs of cystic fibrosis (CF) patients (Pier, 1998). Alginate protects *P. aeruginosa* from the host immune system, antibiotic treatment, and desiccation (Shankar et al., 1995). In *P. aeruginosa*, genes that encode the biosynthesis and regulation of alginate map to four chromosomal locations. With the exception of *algC*, which is located at 10 min, the genes encoding alginate biosynthesis are clustered within an 18-kb region located at 34 min (Gacesa, 1998; Rehm and Valla, 1997). Structural genes that have been characterized in this region include: *algA*, encoding a bifunctional enzyme which functions as a

phosphomannose isomerase and a GDP-mannose pyrophosphorylase (PMI-GMP) (Shinabarger et al., 1991); *algG*, which encodes a C-5 epimerase (Chitnis and Ohman, 1990); *algF*, *algI*, and *algJ*, genes involved in acetylation of the alginate polymer (Franklin and Ohman, 1993; 1996; Shinabarger et al., 1993); and *algD*, which encodes GDP-mannose dehydrogenase (Deretic et al., 1987). This region also contains *algE* and *algK*, which encode proteins with putative roles in polymer export and polymerization, respectively (Aarons et al., 1997; Chu et al., 1991; Jain et al., 1998), and *algL*, a gene encoding alginate lyase (Boyd et al., 1993; Schiller et al., 1993). Other genes which map within this region include *alg44*, *alg8*, and *algX* (Maharaj et al., 1993; Monday and Schiller, 1996); however, the functional role of the proteins encoded by these genes remains unclear. Chitnis and Ohman (1993) postulated that the alginate biosynthetic gene cluster in *P. aeruginosa* is organized as an operon with transcription initiating at the *algD* promoter.

Peñaloza-Vázquez et al. (1997) cloned and characterized the alginate biosynthetic gene cluster from *P. syringae* pv. syringae FF5. The arrangement of the alginate biosynthetic gene cluster in *P. syringae* FF5 was virtually identical to that described for *P. aeruginosa*. However, the regulation and signals for transcriptional activation of alginate biosynthesis differed in the two species, presumably because of their adaptation to plant and animal hosts, respectively (Kidambi et al., 1995; Peñaloza-Vázquez et al., 1997).

P. syringae pv. syringae FF5 was originally isolated from ornamental pear trees showing the extensive necrosis associated with bacterial blight symptoms (Sundin and Bender, 1993). *algL*, which encodes alginate lyase, was previously

inactivated by Tn*5* in strain FF5 to produce the Alg⁻ mutant, FF5.31 (Peñaloza-Vázquez et al., 1997). Because the assays required to reproduce bacterial blight symptoms on pear seedlings were difficult and time-consuming, we searched for a host plant more amenable to virulence testing. Therefore, the present study describes experiments with a different strain of *P. syringae* pv. syringae; strain 3525 produces bacterial brown spot of beans and is much more amenable to *in planta* studies. In the present study, we constructed an alginate-defective (Alg⁻) mutant of *P. syringae* pv. syringae strain 3525, which causes bacterial brown spot on beans. The involvement of alginate in the pathogenicity of 3525 was evaluated by comparing the population dynamics and symptom development of the wild-type and Alg⁻ mutant on bean leaves. The role of alginate in the epiphytic fitness of 3525 was assessed by comparing populations of the wild-type and Alg⁻ mutant on tomato, which is not a host of strain 3525. Our results provide the first genetic evidence that alginate functions in the virulence and epiphytic fitness of *P. syringae*.

Materials and Methods

Bacterial strains and plasmids

The plasmids and bacterial strains used in this study are described in Table 1. *E. coli* cells were grown in Luria-Bertani medium (Miller, 1972) at 37°C, and *P. syringae* strains were routinely cultured at 28°C on mannitol-glutamate medium (MG) (Keane et al., 1970), MG supplemented with yeast extract at 0.25 g/liter, or King's medium B (KMB) (King et al., 1954). Antibiotics were used at the following

concentrations (μg/ml): ampicillin, 100 for *E. coli* and 20 for *P. syringae;* kanamycin, 25; tetracycline, 12.5; streptomycin, 25; and piperacillin, 250.

Recombinant DNA methods

Isolation of plasmid DNA, agarose gel electrophoresis, restriction digests, Southern blots, PCR, and other DNA manipulations were performed using standard protocols (Sambrook et al., 1989). Genomic and plasmid DNA were isolated from *P. syringae* as described previously (Crosa and Falkow, 1981; Staskawicz et al., 1984). DNA fragments were labeled with digoxigenin with the Genius Labeling and Detection Kit (Boehringer Mannheim). DNA was prepared for sequencing with the Plasmid Midi Kit (Qiagen). Oligonucleotide synthesis and automated DNA sequencing were provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility.

Construction of an Alg$^-$ mutant of *P. syringae* 3525

The *algL*::Tn*5* insertion in pSK31 was subcloned as a 12-kb *Eco*RI fragment in pRK415 to create pJYE12 (Fig. 4B). pJYE12 was mobilized into *P. syringae* pv. syringae 3525, and the *algL* homologue in 3525 was inactivated by homologous recombination (Bender et al., 1991), resulting in *P. syringae* pv. syringae 3525.L. A 1-kb probe specific for *algL* was constructed by PCR amplification using pAPE6.2 (Fig. 4C) as template. Primers for the PCR reaction were: 5' CCGGAATTCATCGCCAGATGATGTCGCTG (forward primer) and 5' CCGGAATTCCCCAGTACGAGTGGTTGTTG (reverse primer); the underscored

bases represent an *Eco*RI recognition site which facilitated cloning of the PCR product into pBluescript SK+, resulting in pJYLE1 (Fig. 4D). The 1-kb insert in pJYLE1 was sequenced and shown to contain DNA homologous to *algL* from *P. aeruginosa* (nucleotides 41-1078 as described in Boyd et al., 1993).

PCR was also used to confirm the insertion of Tn*5* into *algL* in *P. syringae* pv. syringae 3525.L. A 1.1-kb DNA fragment was amplified from genomic DNA of 3525.L with the following oligonucleotide primers: 5' CCGGAATTCATCGCCAGATGATGTCGCTG and 5' GGTTCCGTTCAGGACGCTAC, which are derived from *algL* of *P. syringae* FF5 and the IS*50* portion of Tn*5*, respectively. The amplified fragment was cloned into pCR2.1, and the 1.l-kb insert was sequenced to confirm the insertion of Tn*5* into *algL*.

Alginate assay

Selected strains were grown on MG agar (three plates per strain) supplemented with appropriate antibiotics at 28°C for 36 h. Cells were washed from each plate and resuspended in 0.9% NaCl. Alginate isolation and quantitation were performed as described previously (May and Chakrabarty, 1994), and alginic acid from seaweed (*Macrocystis pyrifera*; Sigma Chemical Co., St. Louis, Mo.) was used as a standard. The experiment was repeated twice, and mean values were expressed as the quantity of alginate produced per milligram of protein.

Complementation of the Alg$^-$ mutant 3525.L

pPSR3, a 220-kb conjugative plasmid which confers constitutive production of alginate to *P. syringae* recipients, was used to facilitate the visualization and quantitation of alginate *in vitro*. *P. syringae* pv. syringae strain H12, which harbors pPSR3, was used as a donor in matings with 3525 and 3525.L. The transfer of pPSR3 to the two recipient strains was detected by selection for copper resistance (Cu^r), a natural marker on pPSR3. In subsequent experiments, pSK2 and pAD4033 were mobilized into the nonmucoid transconjugant 3525.L(pPSR3) using pRK2013 as a helper plasmid.

Evaluation of epiphytic fitness

The epiphytic fitness of 3525 and 3525.L was evaluated on tomato (*Lycopersicon esculentum* cv. Glamour). Tomato seedlings were grown for 3 weeks in the greenhouse, and care was taken to minimize the wetting of leaves during daily watering. Bacteria were grown for 36 h on MG agar supplemented with the appropriate antibiotics and suspended in sterile distilled water. Bacterial inoculum (10^6 cfu/ml) was applied to plants with an airbrush (~ 8 psi) until leaf surfaces were uniformly wet. Plants were incubated in a growth chamber at 20-26°C with 40-60% relative humidity. Random leaf samples (three leaves per time point) were taken daily from 0 to 9 days after inoculation. Leaves were weighed individually, transferred to 10 ml 0.01 M potassium phosphate buffer (pH 7.0) and washed for 2 h at 250 rpm. Serial dilutions were plated on MG or MG containing kanamycin (MGKm) to enumerate colonies of 3525 and 3525.L, respectively. The experiment

was performed three times.

Virulence tests

The virulence of *P. syringae* pv. syringae 3525 and the Alg$^-$ mutant 3525.L was evaluated on bean (*Phaseolus vulgaris* cv. Bush Blue Lake 274). Plants were grown in the greenhouse for about 2 weeks or until the first trifoliate leaf was fully expanded. Bacteria were cultured for 36 h on MG agar supplemented with the appropriate antibiotics and then suspended in sterile distilled water. Bacterial inoculum of 10^6 cfu/ml was applied by spraying individual plants with an airbrush as described above. Plants were either inoculated directly and placed in a growth chamber or incubated in a dew chamber for 24 h before and after inoculation and subsequently moved to a growth chamber. Inoculated plants were then incubated for 14 days at 20-26°C with a 12-h photoperiod and 60-80% relative humidity.

In the experiments designed to follow the population dynamics in bean, random leaf samples were taken at 0 to 14 days after inoculation (three replicates per time point). Leaves were weighed separately and ground in 10 ml sterile distilled water using a mortar and pestle. Bacterial counts of 3525 and 3525.L were determined by plating dilutions of the leaf homogenate onto MG or MGKm, respectively. Fluorescent colonies were counted after incubating plates for 48 h, and the experiment was performed three times. Symptoms were evaluated by determining lesion size and number on individual leaves daily for 7 days after inoculation. Each strain was tested on a minimum of nine seedlings.

Virulence was also evaluated by infiltrating bean leaves with 3525 or 3525.L.

Bean plants (approximately 2 weeks old) were incubated in a dew chamber for 24 h before and after inoculation to induce the opening of stomata. Bacterial inoculum was prepared as described above, and a 1 ml syringe (without a needle) was used to inject inoculum (10^6 cfu/ml) into the leaf tissue. Plants were then returned to the growth chamber and incubated as described above.

Results

Inactivation of *algL* in *P. syringae* pv. syringae 3525

Although *algL* was not required for alginate biosynthesis in *P. syringae* pv. syringae FF5, the *algL*::Tn*5* mutant FF5.31 was nonmucoid because of polar effects on *algA*, an essential gene for alginate synthesis which maps downstream of *algL* (Fig. 4A) (Peñaloza-Vázquez et al., 1997). In this study, the *algL*::Tn*5* mutation from FF5.31 was introduced into *P. syringae* pv. syringae 3525 on pJYE12 (Fig. 4B) and exchanged into the 3525 genome by homologous recombination (Bender et al., 1991). Genomic DNA was isolated from the wild-type 3525 and several Tc^s Km^r derivatives of 3525, digested with *Eco*RI, and analyzed by Southern blotting using the 1.0-kb insert in pJYLE1 (Fig. 4D). *Eco*RI was chosen as a restriction enzyme for Southern blot analysis because Tn*5* is not digested by this enzyme. The probe hybridized to 4.4- and 10.1-kb *Eco*RI fragments in 3525 and the Tc^s Km^r derivatives, respectively (data not shown). These results indicated that the region associated with *algL* was located in a 4.4-kb *Eco*RI fragment in 3525, and this 4.4-kb fragment was inactivated by Tn*5* (5.7 kb) in the Tc^s Km^r derivatives. The *algL*::Tn*5* mutation

in 3525.L was further confirmed by PCR analysis using primers complimentary to *algL* and the IS*50* portion of Tn*5*; sequence analysis of the PCR product confirmed that Tn*5* was located in the coding region of *algL* in 3525.L.

Complementation studies

Like many *P. syringae* strains, 3525 is only slightly mucoid when cultured on MG or KMB medium (Kidambi et al., 1995). Plasmid pPSR3 is a Cu^r plasmid that confers constitutive alginate production to *P. syringae*, thereby converting recipient strains to a visibly mucoid phenotype (Kidambi et al., 1995). When plasmid pPSR3 (Table 2) was introduced into 3525, the transconjugant 3525(pPSR3) was stably mucoid and produced alginate at levels equivalent to the mucoid donor, H12, from which pPSR3 was originally isolated (Sundin and Bender, 1993) (Table 2). As expected, 3525.L remained nonmucoid and produced very little alginate when pPSR3 was introduced (Table 2); however, 3525.L(pPSR3, pSK2) and 3525.L(pPSR3, pAD4033) were visibly mucoid and produced approximately 50-fold more alginate than 3525.L(pPSR3) (Table 2). pSK2 and pAD4033 only partially restored alginate production to 3525.L (Table 2), and similar results were obtained in complementation experiments with FF5.31, the *algL* mutant of strain FF5 (Peñaloza-Vázquez et al., 1997). The lack of full complementation by pSK2 and pAD4033 could be caused by the low copy number of pRK7813 (the cosmid vector present in pSK2) or the use of the P*tac* promoter in pAD4033. Although P*tac* is recognized by *P. syringae* (Peñaloza-Vázquez *et al.*, 1996; 1997), expression of downstream genes is substantially lower than that obtained with *E. coli* and might

explain the lack of full complementation with pAD4033. Alternatively, *algL* may have a role in the biosynthesis of alginate by *P. syringae*, and the Tn*5* insertion in this gene may prevent full complementation. Furthermore, it is important to note that *algL* was required for alginate production in *P. aeruginosa* FRD (Schiller et al., 1993), but not in *P. aeruginosa* 8830 (Boyd et al., 1993).

Contribution of alginate to epiphytic fitness

The involvement of alginate in epiphytic fitness was evaluated by monitoring the epiphytic populations of 3525 and 3525.L on tomato, which is not a host for strain 3525. Prior to these experiments, both 3525 and 3525.L were shown to grow similarly in liquid media, indicating that the lack of alginate production in the mutant did not impair its growth *in vitro* (data not shown). At 24 h after inoculation, the population of both 3525 and 3525.L decreased approximately 15-fold to 10^3 cfu/g leaf tissue. Thereafter, the epiphytic population of 3525 increased significantly and was 10 to 15-fold higher than 3525.L throughout the remainder of the sampling period (Fig. 5A). These results clearly indicate that alginate contributes to the epiphytic survival of 3525. Interestingly, the population of both the mutant and wild-type strains followed the same trend for the duration of this experiment, perhaps because factors other than alginate production contributed to epiphytic survival in both strains.

Involvement of alginate in virulence

The colonization of 3525 and 3525.L were compared on bean, the natural

host of this organism. As in the epiphytic studies, the population of both 3525 and 3525.L decreased significantly 24 h after inoculation (Fig. 5B). When brown spot lesions first became apparent 48 h after inoculation, the population of 3525 increased dramatically and was significantly higher than the initial inoculum density (Fig. 5B). In contrast, the population of 3525.L remained low and was approximately 100-fold lower than strain 3525 throughout most of the sampling period. These results correlated with a reduction in the symptoms induced by the Alg$^-$ mutant. Lesions induced by 3525 initially had a water-soaked appearance with a necrotic lesion in the center; the necrosis gradually intensified, and water-soaking was replaced by chlorotic leaf tissue. Cells surrounding the necrotic lesion collapsed, possibly because water was withdrawn from the adjacent tissue, and some lesions coalesced (Fig. 6A). These symptoms are typical of bacterial brown spot on bean (Fahy and Lloyd, 1983). In contrast, 3525.L induced fewer lesions that did not coalesce (Fig. 6B). When they remained separated, lesions were 1-6 mm in diameter and lesion sizes were not significantly different for 3525 and 3525.L (data not shown). However, in bean leaves inoculated with 3525, individual lesions often coalesced (Fig. 6A), resulting in a larger amount of diseased tissue.

Several inoculation methods were used to further evaluate the role of alginate in symptom development. In one series of experiments, beans were incubated in a moist chamber for 24 h before and after inoculation and then placed in a growth chamber. A second group of beans was inoculated without incubation in the moist chamber and immediately placed in the growth chamber. Plants in the first group developed more lesions than those in the latter group, regardless of the strain

inoculated. When plants were incubated in the dew chamber, the average number of lesions observed per leaf was 124±18 and 52±11 for 3525 and 3525.L, respectively. However, when plants were not incubated in the dew chamber, lesion numbers were 11.2±1.6 and 2±0.6 for 3525 and 3525.L, respectively. Regardless of the inoculation method, the number of lesions induced by 3525.L was lower than 3525, which agrees with the population studies (Fig. 5B).

Bean leaves were also inoculated with 3525 or 3525.L by infiltration. When 3525.L was used as inoculum, the necrotic lesion which developed was limited to the area selected for infiltration and resembled a localized hypersensitive reaction (Fig. 6C, left arrow). In contrast, numerous secondary lesions developed around the area infiltrated with strain 3525 (Fig. 6C, right arrow). Another brown spot pathogen, *P. syringae* pv. syringae B728a, produced symptoms similar to strain 3525 when infiltrated into bean leaves (Willis et al., 1990).

Discussion

Our results suggest that the organization of the alginate biosynthetic gene clusters in *P. syringae* strains FF5 and 3525 may be similar. Attempts to mutate the 3525 *algL* gene using *algL*::Tn*5* from FF5.31 were successful, indicating that the nucleotides flanking *algL* in FF5 were conserved in 3525, thereby allowing homologous recombination. In FF5.31, the *algL*::Tn*5* mutation abolished alginate production because of the polarity of Tn*5* on *algA*, a gene essential for alginate production which maps downstream of *algL* (Peñaloza-Vázquez et al., 1997) (Fig.

4A). Alginate production was partially restored to FF5.31 by pAD4033, which contains the *P. aeruginosa algA* gene under control of the P*tac* promoter (Peñaloza-Vázquez et al., 1997). In the current study, pAD4033 also partially complemented 3525.L for alginate production, thereby indicating that the transcriptional organization of the alginate gene cluster may be similar in strains FF5 and 3525.

Although the Alg$^-$ mutant 3525.L remained pathogenic on bean, symptom severity and bacterial multiplication were reduced in comparison to 3525. Several studies suggest that EPS synthesis can significantly enhance the colonization of phytopathogenic bacteria. For example, EPS-defective strains of *P. stewartii* were reduced in virulence partly because they spread more slowly than the wild-type in the vascular system of maize plants (Braun, 1990). In a similar study, Saile et al. (1997) used mutants to show that EPS production increased the dissemination of *R. solanacearum* in tomato stem tissue. Our results indicate that alginate production facilitates the dissemination of *P. syringae* pv. syringae 3525 in bean leaves. When the Alg$^-$ mutant 3525.L was infiltrated into bean leaves, lesions were restricted to the inoculation site (Fig. 6C, left arrow). However, numerous secondary lesions developed around the site where 3525 was infiltrated (Fig. 6C, right arrow), which indicates that the wild-type bacterium had successfully colonized the intercellular space of neighboring plant cells. The hydrostatic pressure created by EPS production in the intercellular spaces of plant hosts may cause plant cells to rupture and assist in the dissemination of bacteria into areas surrounding the initial infection site (Leigh and Coplin, 1992).

An interesting parallel between the reaction of humans and plants to microbial

pathogens is the production of reactive oxygen intermediates (ROI) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Van Camp et al., 1998). There is a striking correlation between the production of ROI in plant cell cultures and the outcome of resistance (the HR) or susceptibility. The formation of ROI is known to be critical for establishing plant disease resistance during the HR. Consequently, pathogen products which interfere with the toxicity of ROI, such as alginate, could interfere with plant defense. The proposed functions for alginate in the lungs of CF patients include: (1) suppression of the oxidative burst in neutrophils (Jensen et al., 1990); and (2) a direct role in scavenging ROI species which are produced by phagocytic cells (Simpson et al., 1989). Furthermore, the *algT* gene product, which encodes an alternate sigma factor required for alginate biosynthesis, is involved in the expression of genes that determine resistance to ROI. AlgT is required for the full resistance of *P. aeruginosa* and *P. syringae* to toxic oxygen species (Keith and Bender, 1999; Martin et al., 1994; Yu et al., 1995); consequently, alginate production *in planta* may help *P. syringae* evade the host defense response.

The epiphytic fitness of phytopathogenic bacteria can also contribute to disease severity when a host plant is colonized. Mechanisms which reduce desiccation stress, such as the formation of microbial biofilms or EPS matrices (Beattie and Lindow, 1995; Morris et al., 1998), can significantly increase epiphytic fitness. In the present study, it was difficult to discriminate between epiphytic and intercellular colonization in bean plants, the natural host of strain 3525. Consequently, we evaluated epiphytic survival on tomato, which is not a host for *P.*

syringae pv. syringae 3525. The epiphytic population of 3525 on tomato leaves was ten to fifteen-fold higher than 3525.L, which suggests a role for alginate in epiphytic fitness. Although the mechanisms which explain how alginate contributes to epiphytic colonization remain unclear, alginate has been shown to reduce desiccation stress and assist in the formation of microbial biofilms. Desiccation stress stimulated alginate synthesis in both *P. syringae* and *P. aeruginosa* (DeVault et al., 1990; Singh et al., 1992), thereby increasing resistance to dehydration (Ophir and Gutnick, 1994). Furthermore, alginate was important in the formation of biofilms by *P. aeruginosa* (Boyd and Chakrabarty, 1994); biofilms often play an important role in the attachment of microbial populations to solid surfaces (Cammarota and Sant'Anna, 1998). Although it is not clear whether alginate contributes to biofilm formation on plant surfaces, a role for alginate in attachment to leaf surfaces remains a viable possibility.

The lesion numbers produced by the wild-type and Alg$^-$ mutant were higher when beans were incubated in the dew chamber as compared to direct inoculation and incubation in the growth chamber. The high relative humidity in the dew chamber would facilitate bacterial entry into the plant because stomates would be induced to open under these conditions. Under more stressful conditions (direct inoculation and incubation in the growth chamber), fewer bacteria survived desiccation and were able to enter the plant through natural openings or wounds. Consequently, the lesion numbers induced by the wild-type and Alg$^-$ mutant were significantly less than those observed on plants incubated in the dew chamber.

The production of EPS polymers by phytopathogenic bacteria has been

implicated in several symptoms including the wilting induced by vascular pathogens and the water-soaking associated with foliar pathogens (Denny, 1995). Bacterial brown spot is a foliar pathogen of bean and water-soaked lesions were apparent during the onset of disease in leaves inoculated with both 3525 and 3525.L. Since alginate was not responsible for water-soaking in 3525, we believe alginate has a more subtle role in brown spot of bean, perhaps by facilitating the dissemination of the bacterium as noted above. It is important to note that alginate may have a more obvious role in symptom development in other *P. syringae* pathovars.

TABLE 1

BACTERIAL STRAINS AND PLASMIDS

Strain/plasmid	Relevant characteristics [a]	Reference [b]
Escherichia coli		
DH5α	Δ(*lacZYA-argF*)$_{U169}$	Sambrook et al., 1989
Top10F′	*lacI*^{q} Tc^{r} *mcr*A Δ*lacZ* ΔM15	Invitrogen
Pseudomonas syringae pv. syringae		
FF5	Cu^{s}	Sundin and Bender, 1993
FF5.31	Cu^{r} Km^{r}, contains pPSR12, *algL*::Tn*5*	Peñaloza-Vázquez et al., 1997
H12	Cu^{r}, contains pPSR3, stably mucoid	Sundin and Bender, 1993
3525	Cu^{s}, nonmucoid, causal agent of brown spot of bean	ICMP
3525.L	Cu^{s} Km^{r}, *algL*::Tn*5*	This study
Plasmids		
pPSR12	Cu^{r} Sm^{r}, 200 kb, confers constitutive alginate production to *P. syringae* pv. syringae FF5	Kidambi et al., 1995
pSK2	Tc^{r}, contains alginate biosynthetic cluster from *P. syringae* pv. syringae FF5 in pRK7813	Peñaloza-Vázquez et al., 1997
pRK2013	Km^{r}, helper plasmid	Figurski and Helinski, 1979
pRK415	Tc^{r}, RK2-derived cloning vector	Keen et al., 1988
pRK7813	Tc^{r}, cosmid vector	Jones and Gutterson, 1987
pBluescript SK+	Ap^{r}, ColEI origin, cloning vehicle	Stratagene
pCR2.1	Ap^{r} Km^{r}, 3.9-kb cloning vector	Invitrogen
pPSR3	Cu^{r} Sm^{r}, 220 kb, confers constitutive alginate production to *P. syringae* pv. syringae	Sundin and Bender, 1993
pAD4033	Ap^{r}, contains *algA* from *P. aeruginosa* under P*tac* control in pMMB22	Boyd et al., 1993
pAPE6.2	Ap^{r}, contains 6.2 *Eco*RI fragment from pSK2 in pBluescript SK+	Peñaloza-Vázquez et al., 1997
pJYLE1	Ap^{r}, contains 1.0-kb PCR fragment (including part of *algL*) amplified from pAPE6.2 in pBluescript SK+	This study
pSK31	Tc^{r} Km^{r}, contains *algL*::Tn*5* from FF5.31 in pRK7813	Peñaloza-Vázquez et al., 1997
pJYE12	Tc^{r} Km^{r}, contains a 12-kb fragment with *algL*::Tn*5* from pSK31 in pRK415	This study

a. Ap, ampicillin; Cu, copper; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; r, resistant; s, sensitive.

b. ICMP is the International Collection of Microorganisms from Plants, Auckland, New Zealand.

TABLE 2

ALGINATE PRODUCTION BY DERIVATIVES OF *PSEUDOMONAS SYRINGAE* PV. SYRINGAE

Strain	Alginate production [a] (μg/mg of total cellular protein)
H12(pPSR3)	10,177 a
3525(pPSR3)	12,364 a
3525.L(pPSR3)	128 c
3525.L(pPSR3, pSK2)	6,149 b
3525.L(pPSR3, pAD4033)	5,359 b

a. Mean values followed by the same letter are not significantly different at P=0.05 by the Student-Newman-Keuls test.

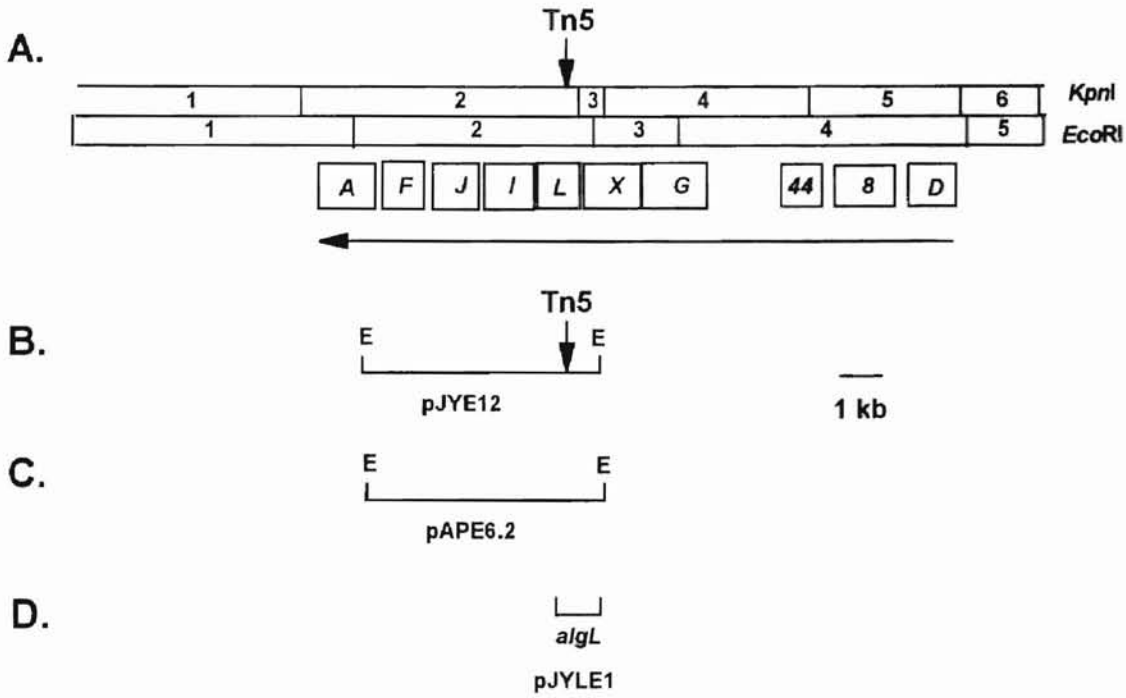


Fig. 4. A, Organization of the alginate structural genes in *P. syringae* pv. syringae FF5. Abbreviations: A. *algA*; F, *algF*; J, *algJ*; I, *algI*; X, *algX*; G, *algG*; 44, *alg44*; 8, *alg8*; D, *algD*. The horizontal arrow beneath the gene cluster indicates the direction of transcription; the Tn*5* insertion in *algL* abolished alginate production in FF5(pPSR12) due to polar effects on *algA* (Peñaloza-Vázquez *et al.*, 1997). **B,** pJYE12 was constructed by subcloning the *algL*::Tn*5* insertion in *Eco*RI fragment #2 into pRK415. Plasmid pJYE12 was then introduced into *P. syringae* pv. syringae 3525, and *algL* was inactivated by homologous recombination, creating 3525.L. **C,** pAPE6.2 contains *Eco*RI fragment #2 from pSK2 in pBluescript SK+. This fragment was used as template in a PCR reaction to amplify a 1.0-kb fragment containing *algL*. **D,** pJYLE1 contains an internal portion (1.0-kb) of *algL* in pBluescript SK+.

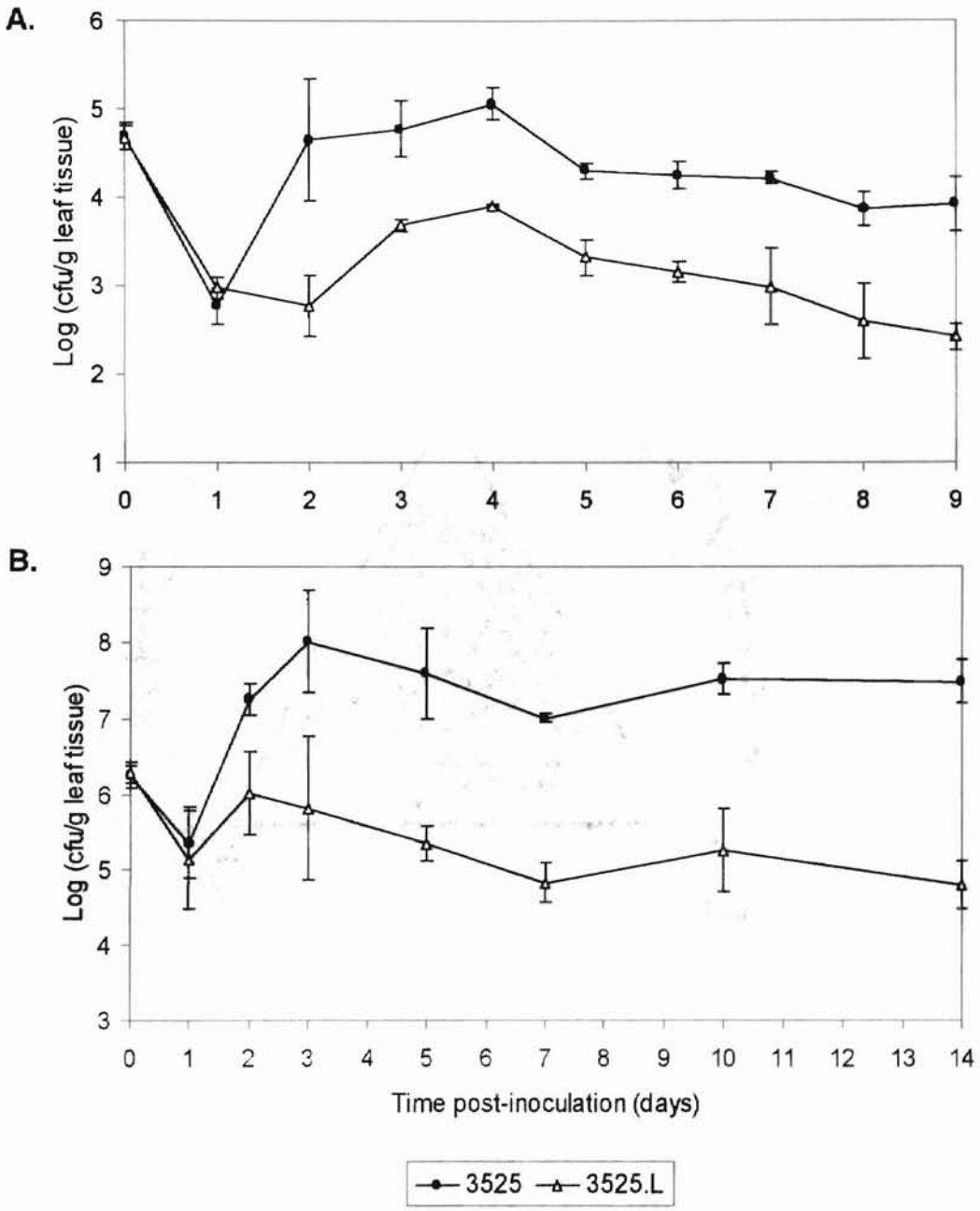


Fig. 5. Population of *P. syringae* pv. syringae 3525 and the Alg⁻ mutant 3525.L on nonhost and host plants. **A**, Epiphytic population of *P. syringae* pv. syringae 3525 and the Alg⁻ mutant 3525.L on leaves of tomato, which is not a host for strain 3525. Bacterial populations were determined by washing leaves in 0.01 **M** potassium phosphate buffer (pH 7.0) followed by dilution plating. **B**, Population dynamics of *P. syringae* pv. syringae 3525 and the Alg⁻ mutant 3525.L on bean, which is a host for strain 3525. Bacterial populations were determined by homogenizing the leaves in sterile distilled water followed by dilution plating. All experiments were performed three times with similar results.

Fig. 6. Lesion development on bean leaves inoculated with suspensions of (**A**) *P. syringae* pv. syringae 3525 and (**B**) the Alg⁻ mutant 3525.L. Bacterial suspensions of 10^6 cfu/ml were applied to the first trifoliate leaves until leaf tissue was uniformly wet. (**C**) Lesion development (see arrows) on a bean leaf infiltrated with *P. syringae* pv. syringae 3525.L (left) and the wild-type 3525 (right). Bacterial inoculum (10^6 cfu/ml) was infiltrated into leaves using a 1 ml syringe without a needle. After inoculation, all plants were incubated in the growth chamber at 20-26°C with 60-80% relative humidity and a 12-h photoperiod. Photographs were taken five days after inoculation.

BIBLIOGRAPHY

Aarons, S.J., Sutherland, I.W., Chakrabarty, A.M., and Gallagher, M.P. 1997. A novel gene, *algK*, from the alginate biosynthetic cluster of *Pseudomonas aeruginosa*. Microbiology 143: 641-652.

Alfano, J.B., and Collmer, A. 1996. Bacterial pathogens in plants: life up against the wall. The Plant Cell 8: 1683-1698.

Andersen, G.L., Beattie, G.A., and Lindow, S.E. 1998. Molecular characterization and sequence of a methionine biosynthetic locus from *Pseudomonas syringae*. J. Bacteriol. 180: 4497-4507.

Baynham, P.J., Brown, A.L., Hall, L.L., and Wozniak, D.J. 1999. *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. Mol Microbiol. 33: 1069-1080.

Beattie, G.A., and Lindow, S.E. 1994. Epiphytic fitness of phytopathogenic bacteria: physiological adaptations for growth and survival. Curr. Top. Microbiol. Immunol. 192: 1-27.

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

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

Bernhard, F., Coplin, D.L., and Geider, K. 1993. A gene cluster for amylovoran synthesis in *Erwinia amylovora*: characterization and relationship to *cps* genes in *Erwinia stewartii*. Mol. Gen. Genet. 239: 158-168.

Bernhard, F., Schullerus, D., Bellemann, P., Nimtz, M., Coplin, D.L., and Geider, K. 1996. Genetic transfer of amylovoran and stewartan synthesis between *Erwinia amylovora* and *Erwinia stewartii*. Microbiology 142: 1087-1096.

Berry, A., Devault, J.D., and Chakrabarty, A.M. 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. J. Bacteriol. 171: 2312-2317.

Boucher, J.C., Martinez-Salazer, J.M., Schurr, M.J., Mudd, M.H., Yu, H., and Deretic, V. 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine-protease HtrA. J. Bacteriol. 178: 511-523.

Boucher, J.C., Schurr, M.J., Yu, H., Rowen, D.W., and Deretic V. 1997. *Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity. Microbiology 143: 3473-3480.

Boyd, A., and Chakrabarty, A.M. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 60: 2355-2359.

Boyd, A., Ghosh, M., May, T.B., Shinabarger, D., Keogh, R., and Chakrabarty, A.M. 1993. Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. Gene 131: 1-8.

Brandl, M.T., and Lindow, S.E. 1998. Contribution of indole-3-acetic acid production to the epiphytic fitness of *Erwinia herbicola*. Appl. Environ. Microbiol. 64: 3256-3263.

Braun, E. J. 1990. Colonization of resistant and susceptible maize plants by *Erwinia stewartii* strains differing in exopolysaccharide production. Physiol. Mol. Plant Pathol. 36: 363-379.

Cammarota, M.C., and Sant'Anna, G.L. 1998. Metabolic blocking of exopolysaccharide synthesis: effects on microbial adhesion and biofilm accumulation. Biotechnol. Lett. 20: 1-4.

Chitnis, C.E., and Ohman, D.E. 1990. Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J. Bacteriol. 172: 2894-2900.

Chitnis, C.E, and Ohman, D.E. 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol. Microbiol. 8: 583-590.

Chu, L., May, T.B., Chakrabarty, A.M., and Misra, T.K. 1991. Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. Gene 107: 1-10.

Collmer, A. 1998. Determinants of pathogenicity and avirulence in plant pathogenic bacteria. Curr. Opin. Plant Biol. 1: 329-335.

Cook, D., and Sequeira, L. 1991. Genetic and biochemical characterization of a *Pseudomonas solanacearum* gene cluster required for extracellular polysaccharide production and for virulence. J. Bacteriol. 173: 1654-1662.

Coplin, D.L., and Majerczak, D.R. 1990. Extracellular polysaccharide genes in *Erwinia stewartii*: directed mutagenesis and complementation analysis. Mol. Plant-Microbe Interact. 3: 286-292.

Coplin, D.L., Frederick, R.D., and Majerczak, D.R. 1992. New pathogenicity loci in *Erwinia stewartii* identified by random Tn5 mutagenesis and molecular cloning. Mol. Plant-Microbe Interact. 5: 266-268.

Coplin, D.L., Frederick, R.D., Majerczak, D.R., and Tuttle, L.D. 1992. Characterization of a gene cluster that specifies pathogenicity in *Erwinia stewartii.* Mol. Plant-Microbe Interact. 5: 81-88.

Coyne, M.J., Russell, K.S., Coyle, C.L., and Goldberg, J.B. 1994. The *Pseudomonas aeruginosa algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. J. Bacteriol. 176: 3500-3507.

Crosa, J.H., and Falkow, S. 1981. Plasmids. pp. 266-282. In Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R., and Phillips, G.B. (eds), Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC.

Darzins, A., and Chakrabarty, A.M. 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. 159: 9-18.

Darzins, A., Frantz, B., Vanags, R.I., and Chakrabarty, A.M. 1986. Nucleotide sequence analysis of the phosphomannose isomerase gene (*pmi*) of *Pseudomonas aeruginosa* and comparison with the corresponding *Escherichia coli* gene *manA*. Gene 42: 293-302.

Denny, T.P., Makini, F.W., and Brumbley, S.M. 1988. Characterization of *Pseudomonas solanacearum* Tn*5* mutants deficient in extracellular polysaccharide. Mol. Plant-Microbe Interact. 1: 215-223.

Denny, T.P., and Baek, S.R. 1991. Genetic evidence that extracellular polysaccharide is a virulence factor of *Pseudomonas solanacearum.* Mol. Plant-Microbe Interact. 4: 198-206.

Denny, T.P. 1995. Involvement of bacterial polysaccharide in plant pathogenesis. Annu. Rev. Phytopathol. 33: 173-197.

Deretic, V., Gill, J.F., and Chakrabarty, A.M. 1987. Gene *algD* encoding GDP-mannose dehyhrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. 169: 351-358.

Deretic, V., Gill, J.F., and Chakrabarty, A.M. 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucl. Acids Res. 15: 4567-4581.

Deretic, V., Martin, D.W., Schurr, M.J., Mudd, M.H., Hibler, N.S., Curcic, R., and Boucher, J.C. 1993. Conversion to mucoidy in *Pseudomonas aeruginosa.* Biotechnology. 11: 1133-1136.

DeVault, J.D., Kimbara, K., and Chakrabarty, A.M. 1990. Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates alginate gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. Mol. Microbiol. 4: 737-745.

Dolph, P.J., Majerczak, D.R., and Coplin, D.L. 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. J. Bacteriol. 170: 865-871.

Fahy, P.C., and Lloyd, A.B. 1983. *Pseudomonas*: The fluorescent pseudomonads. pp. 141-188. In Fahy, P.C., and Persley, G.J. (eds.) Plant Bacterial Diseases: A Diagnostic Guide. Academic Press, Sydney.

Fakhr, M.K., Peñaloza-Vázquez, A., Chakrabarty, A.M., and Bender, C.L. 1999. Regulation of alginate biosynthesis in *Pseudomonas syringae* pv. syringae. J. Bacteriol. 181: 3478-3485.

Fett, W.F., Osman, S.F., Fishman, M.L., and Siebles, T.S. III. 1986. Alginate production by plant-pathogenic pseudomonads. Appl. Environ. Microbiol. 52: 466-473.

Fett, W.F., and Dunn, M.F. 1989. Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. Plant Physiol. 89: 5-9.

Fett, W.F., Wijey, C., and Lifson, E.R. 1992. Occurrence of alginate gene sequences among members of the pseudomonad rRNA homology groups I-IV . FEMS Microbiol. Lett. 99: 151-158.

Fialho, A.M., Zielinski, N.A., Fett, W.F., Chakrabarty, A.M., and Berry, A. 1990. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-*Azomonas-Azotobacter* lineage of superfamily B procaryotes. Appl. Environ. Microbiol. 56: 436-443.

Figurski, D., and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76: 1648-1652.

Franklin, M.J. and Ohman, D.E. 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J. Bacteriol. 175: 5057-5065.

Franklin, M.J., Chitnis, C.E., Gacesa, P., Sonesson, A., White, D.C., and Ohman, D.E. 1994. *Pseudomonas aeruginosa algG* is a polymer level alginate C5-mannuronan epimerase. J. Bacteriol. 176: 1821-1830.

Franklin, M.J. and Ohman, D.E. 1996. Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O-acetylation. J. Bacteriol. 178: 2186-2195.

Gacesa, P. 1998. Bacterial alginate biosynthesis – recent progress and future prospects. Microbiology 144: 1133-1143.

Geier, G., and Geider, K. 1993. Characterization and influence on virulence of the levansucrase gene from the fireblight pathogen *Erwinia amylovora*. Physiol. Mol. Plant Pathol. 42: 387-404.

Geider, K., Geier, G., Bellemann, P., Bernhard, F., Bugert, P., and Metzger, M. 1993. Exopolysaccharides in pathogenicity of *Erwinia amylovora*. Acta Horticulturae 338: 255-262.

Govan, J.R.W., and Deretic, V. 1996. Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60: 539-574.

Gross, M., Geier, G., Rudolph, K., and Geider, K. 1992. Levan and levansucrase synthesized by the fireblight pathogen *Erwinia amylovora*. Physiol. Mol. Plant Pathol. 40: 371-381.

Gross, M., and Rudolph, K. 1987. Demonstration of levan and alginate in bean plants *(Phaseolus vulgaris*) infected by *Pseudomonas syringae* pv. phaseolicola. J. Phytopathol. 120: 9-19.

Hershberger, C.D., Ye, R.W., Parsek, M.R., Xie, Z.D., and Chakrabarty, A.M. 1995. The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative sigma factor (σ^E). Proc. Natl. Acad. Sci. USA 92: 7941-7945.

Hettwer, U., Jaeckel, F.R., Boch, J., Meyer, M., Rudolph, K., and Ullrich, M.S. 1998. Cloning, nucleotide sequence, and expression in *Escherichia coli* of

levansucrase genes from the plant pathogens *Pseudomonas syringae* pv. glycinea and *P. syringae* pv. phaseolicola. Appl. Environ. Microbiol. 64: 3180-3187.

Jain, S., and Ohman, D.E. 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J. Bacteriol. 180: 634-641.

Jansson, P.E., Kenne, L., and Lindberg, B. 1975. Structure of extracellular polysaccharide from *Xanthomonas campestris.* Carbohydr. Res. 45: 275-282.

Jensen, E.T., Charisma, A., Lam, K., Costerton, J.W., and Høiby, N. 1990. Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. Infect. Immun. 58: 2383-2385.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. 1987. GUS fusions, β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Jones, J.D.G., and Gutterson, N. 1987. An efficient mobilizable cosmid vector, pRK7813, and its use in a rapid method for marker exchange in *Pseudomonas fluorescens* strain HV37a. Gene 61: 299-306.

Kao, C.C., Barlow, E., and Sequeira, L. 1992. Extracellular polysaccharide is required for wild-type virulence of *Pseudomonas solanacearum.* J. Bacteriol. 174: 1068-1071.

Katzen, F., Ferreiro, D.U., Oddo, C.G., Ielmini, M.V., Becker, A., Puhler, A., and Ielpi, L. 1998. *Xanthomonas campestris* pv. campestris gum mutants: effects on xanthan biosynthesis and plant virulence. J. Bacteriol. 180: 1607-1617.

Keane, P.J., Kerr, A., and New, P.B. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23: 585-595.

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene 70: 191-197.

Keith, L.M.W., and Bender, C.L. 1999. AlgT (σ^{22}) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae.* J. Bacteriol. 181: 7176-7184.

Kidambi, S.P., Sundin, G.W., Palmer, D.A., Chakrabarty, A.M., and Bender, C.L. 1995. Copper as a signal for alginate synthesis in *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. 61: 2172-2179.

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.

Leigh, J.A., and Coplin D.L. 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. 46: 307-346.

Lindgren, P.B. 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. 35: 129-152.

Maharaj, R., May, T.B., Wang, S.K., and Chakrabarty, A.M. 1993. Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene 136: 267-269.

Martin, D.W., Schurr, M.J., Yu, H., and Deretic, V. 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship of σ^E and stress response. J. Bacteriol. 176: 6688-6696.

Mathee, K., Ciofu, O., Sternberg C., Lindum, P.W., Campbell, J.I., Jensen, P., Johnsen, A.H., Givskov, M., Ohman, D.E., Molin, S., Hoiby, N., and Kharazmi, A. 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiology 145: 1349-1357.

May, T.B., and Chakrabarty, A.M. 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. Trends Microbiol. 2: 151-157.

May, T.B., and Chakrabarty, A.M. 1994. Isolation and assay of *Pseudomonas aeruginosa* alginate. Meth. Enzymol. 235: 295-304.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Monday, S.R., and Schiller, N.L. 1996. Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX. J. Bacteriol. 178: 625-632.

Morris, C.E., Monier, J.-M., and Jacques, M.-A. 1998. A technique to quantify the population size and composition of the biofilm component in communities of bacteria in the phyllosphere. Appl. Environ. Microbiol. 64: 4789-4795.

Ohman, D.E., Mathee, K., McPherson, C.J., DeVries, C.A., Ma, S., Wozniak, D.J., and Franklin, M.J. 1996. Regulation of the alginate (*algD*) operon in *Pseudomonas aeruginosa*. p. 472-483. In T. Nakazawa et al. (eds), Molecular biology of pseudomonads. ASM Press, Washington, DC.

Ophir, T., and Gutnick, D.L. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. Appl. Environ. Microbiol. 60: 740-745.

Osman, S.F., Fett, W.F., and Fishman, M.L. 1986. Exopolysaccharides of the phytopathogen *Pseudomonas syringae* pv. glycinea. J. Bacteriol. 166: 66-71.

Palmer, D.A., Bender, C.L., and Sharma, S.B. 1997. Use of Tn*5*-gus*A5* to investigate environmental and nutritional effects on gene expression in the coronatine biosynthetic gene cluster of *Pseudomonas syringae* pv. glycinea. Can. J. Microbiol. 43: 517-525.

Peñaloza-Vázquez, A., Rangaswamy, V., Ullrich, M., Bailey, A. M., and Bender, C. L. 1996. Use of translational fusions to the maltose binding protein to produce and purify proteins in *Pseudomonas syringae* and assess their activity in vivo. Mol. Plant-Microbe Interact. 9: 637-641.

Peñaloza-Vázquez, A., Kidambi, S.P., Chakrabarty, A.M., and Bender, C.L. 1997. Characterization of the alginate biosynthetic gene cluster in *Pseudomonas syringae* pv. syringae. J. Bacteriol. 179: 4464-4472.

Peñaloza-Vázquez, A., and Bender, C.L. 1998. Characterization of CorR, a transcriptional activator which is required for biosynthesis of the phytotoxin coronatine. J. Bacteriol. 180: 6252-6259.

Pier, G.B. 1998. *Pseudomonas aeruginosa*: a key problem in cystic fibrosis. ASM News 64: 339-347.

Pierce, M.L., Essenberg, M., and Mort, A.J. 1993. A comparison of the quantities of exopolysaccharide produced by *Xanthomonas campestris* pv. malvacearum in susceptible and resistant cotton cotyledons during early stages of infection. Phytopathology 83: 344-349.

Poplawsky, A.R., and Chun, W. 1998. *Xanthomonas campestris* pv. campestris requires a functional *pigB* for epiphytic survival and host infection. Mol. Plant-Microbe Interact. 11: 466-475.

Ramirez, M.E., Fucikovsky, L., Garcia-Jimenez, F., Quintero, R., and Galindo, E. 1988. Xanthan gum production by altered pathogenicity variants of *Xanthomonas campestris*. Appl. Microbiol. Biotechnol. 29: 5-10.

Rehm, B.H.A., Boheim, G., Tommassen, J., and Winkler, U.K. 1994. Overexpression of AlgE in *Escherichia coli* – subcellular localization, purification, and ion channel properties. J. Bacteriol. 176: 5639-5647.

Rehm, B.H.A., and Valla, S. 1997. Bacterial alginate: biosynthesis and applications. Appl. Microbiol. Biotechnol. 48: 281-288.

Rudolph, K.W.E., Gross, M., Neugebauer, M., Hokawat, S., Zachowski, A., Wydra, K., and Klement, Z. 1989. Extracellular polysaccharides as determinants of leaf spot diseases caused by pseudomonads and xanthomonads. p. 177-218. In Graniti, A., Durbin, R.D., and Ballio, A. (eds), Phytotoxins and plant pathogenesis. Springer-Verlag. Berlin.

Rudolph, K.W.E., Gross, M., Ebrahim-Nesbat, F., Nollenburg, M., Zomorodian, A., Wydra, K., Neugebauer, M., Hettwer, U., Wagihel-Shouny, Sonnenberg B., and Klement, Z. 1994. The role of extracellular polysaccharide as virulence factors for phytopathogenic pseudomonads and xanthomonads. p.357-348. In C. I. Kado and J. H. Crosa (eds), Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers. Netherlands.

Saile, E., McGarvey, J.A., Schell, M.A., and Denny, T.P. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology 87: 1264-1271.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schiller, N.L., Monday, S.R., Boyd, C.M., Keen, N.T., and Ohman, D.E. 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*) – cloning, sequencing, and expression in *Escherichia coli*. J. Bacteriol. 175: 4780-4789.

Schurr, M.J., Yu, H., Boucher, J.C., Hibler, N.S., and Deretic, V. 1995. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (σ^E) which controls mucoidy in cystic fibrosis isolates of *Pseudomonas aeruginosa*. J. Bacteriol. 177: 5670-5679.

Schurr, M.J., Yu, H., Martinez-Salazer, J.M., Boucher, J.C., and Deretic, V. 1996. Control of *algU*, a member of the σ^E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. 178: 4997-5004.

Shankar, S., Ye, R.W., Schlictman, D., and Chakrabarty, A.M. 1995. Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. p. 221-255. In A. Meister (ed.), Advances in enzymology and related areas of molecular biology. John Wiley & Sons Inc.

Shankar, S., Ye, R., Schlictman, D., and Chakrabarty, A.M. 1995. Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. Adv. Enzymol. 70: 221-255.

Shinabarger, D., Berry, A., May, T.B., Rothmel, R., Fialho, A., and Chakrabarty, A.M. 1991. Purification and characterization of phosphomannose isomerase – guanosine diphospho-D-mannose pyrophosphorylase. J. Biol. Chem. 266: 2080-2088.

Shinabarger, D., May, T.B., Boyd, A., Ghosh, M., and Chakrabarty, A.M. 1993. Nucleotide sequence and expression of the *Pseudomonas aeruginosa algF* gene controlling acetylation of alginate. Mol. Microbiol. 9: 1027-1035.

Simpson, J.A., Smith, S.E. and Dean, R.T. 1989. Scavenging by alginate of free radicals released by macrophages. Free Radical Biol. Med. 6: 347-353.

Singh, S., Koehler, B., and Fett, W.F. 1992. Effect of osmolarity and dehydration on alginate production by fluorescent pseudomonads. Curr. Microbiol. 25: 335-339.

Staskawicz, B.J., Dahlbeck, D., and Keen, N.T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. glycinea determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81: 6024-6028.

Sundin, G.W., and Bender, C.L. 1993. Ecological and genetic analysis of copper and streptomycin resistance in *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. 59: 1018-1024.

Terry, J.M., Pina, S.E., and Mattingly, S.J. 1992. Role of energy metabolism in conversion of nonmucoid *Pseudomonas aeruginosa* to the mucoid phenotype. Infect. Immun. 60: 1329-1335.

Van Camp, W., Van Montagu, M., and Inzé, D. 1998. H_2O_2 and NO: redox signals in disease resistance. Trends Plant Sci. 3: 330-334.

Van den Eede, G., Deblaere, R., Goethals, K., Montagu, M.V., and Holsters, M. 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol. Plant-Microbe Interact. 5: 228-234.

Whitfield, C. 1988. Bacterial extracellular polysaccharide. Can. J. Microbiol. 34: 415-420.

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

Willis, D.K., Rich, I.J., and Hrabak, E.M. 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact 4: 132-138.

Wilson, K.J., Hughes, S.G., and Jefferson, R.A. 1992. The *Escherichia coli gus* operon, induction and expression of the *gus* operon in *E. coli* and the occurrence and use of GUS in other bacteria. p. 7-23. In S. Gallagher (ed.), GUS protocols: using the GUS gene as a reporter of gene expression. Academic Press. N. Y.

Wozniak, D.J., and Ohman, D.E. 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. 176: 6007-6014.

Xie, Z.D., Hershberger, C.D., Shankar, S., Ye, R.W., and Chakrabarty, A.M. 1996. Sigma factor - anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. J. Bacteriol. 178: 4990-4996.

Yu, H., Schurr, M.J., and Deretic, V. 1995. Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. J. Bacteriol. 177: 3529-3268.

APPENDIX A

PSEUDOMONAS SYRINGAE PATHOVARS CAPABLE OF PRODUCING WATER-SOAKING IN SUSCEPTIBLE HOSTS

Introduction

The phytopathogenic prokaryote *Pseudomonas syringae* commonly penetrates plant tissues through natural openings or wounds and induces typical water-soaking on leaves of susceptible hosts. During pathogenesis, the bacteria do not enter the host cells, but instead colonize the intercellular spaces of the plant mesophyll, absorb nutrients from the plant cells, and multiply intercellularly. This process is often accompanied by secretion of bacterial alginate, a linear copolymer of β-1,4-linked, partially acetylated D-mannuronate and L-guluronate, which is suggested to play an important role for maintenance of a compatible interaction between pathogenic *P. syringae* and susceptible hosts.

The objective of this study was to investigate the role of alginate in producing water-soaked lesions. The first step involved identifying bacterial strains that produce good water-soaking in leaves of selected host plants. The pathogenicity of thirty three strains from four *P. syringae* pathovars (syringae,

phaseolicola, tomato, and glycinea) were introduced into susceptible bean, tomato or soybean plants and the development of water-soaked lesions were evaluated visually.

Materials and Methods

Preparation of bacterial inocula

P. syringae strains were maintained on mannitol-glutamate (MG) or MG supplemented with yeast extract at 0.25 g/L (MGY) agar medium. Prior to plant inoculations, bacteria were streaked to MGY agar and incubated at 28°C for 24 to 36 h. Bacterial suspensions were made in sterile water and the OD_{600} was adjusted to an absorbance of 0.1. This value equals approximately 10^8 colony forming units per milliliter (cfu/ml). Bacterial inocula consisted of ten-fold serial dilutions of this suspension.

Preparation of host plants

Bean (*Phaseolus vulgaris* cv. Bush Blue Lake 274), tomato (*Lycopersicon esculentum* cv. Glamour), or soybean seeds (L.L Olds Seeds Company, Madison, WI) were planted in pots and maintained in a growth chamber where the temperature ranged 20-26°C with a 12-h photoperiod. Young plants with fully expanded primary leaves (two week-old bean and soybean plants or three week-old tomato plants) were used for inoculations. Bean pods were purchased from a local grocery store, and pods 10-12 cm in length were inoculated.

Pathogenicity tests

Pathogenicity tests were performed by localized infiltration or injection of bacterial suspensions into young plant leaves or pods. Ten-fold bacterial dilutions starting from $10^4 - 10^7$ cfu/ml were used to inoculate plant leaves. A 1 ml tuberculin syringe (without a needle) (Becton Dickinson & Co., Rutherford, NJ) was used to infiltrate inoculum into the lower leaf surface. Leaves infiltrated with water were used as controls. Plants were then returned to the growth chamber, where they were inspected daily for the presence or absence of water-soaked lesions daily for a 7-day period.

Inoculation of bean pods

Prior to inoculation, beans were surface-sterilized by immersion in 10% sodium hypochlorite (Clorox) for 2 min and rinsed for 10 min in sterile tap water. 20 µl aliquots of 10-fold dilutions ($10^4 - 10^7$ cfu/ml) were injected just under the epidermis of bean pods using a 1 ml tuberculin syringe and 22 gauge needle. Bean pods were then placed in separate compartments within a plastic storage box, which was surface sterilized and contained moistened sterile paper towels. Inoculated pods were incubated in a growth chamber at 24°C with a 12-h photoperiod and monitored for symptom development daily for 7 days.

Results

P. syringae pv. syringae strains capable of producing water-soaked lesions in bean pods

No water-soaked lesions were observed when bean leaves were inoculated with *P. syringae* pv. syringae strains 3525, B86-37, 4916, BBS32-5, B86-17, B728a, or NPS3136. Instead, necrotic lesions were observed with these seven strains. Therefore, we tested the ability of these strains to produce water-soaked lesions in bean pods. Water-soaked lesions were produced by all strains in bean pods with the exception of NPS3136, a *lemA* mutant of B728a; this strain produced flat, brown, dry lesions in the inoculated area.

The results of using different concentrations of inoculum indicated that 10^6 cfu/ml of *P. syringae* pv. syringae commonly induced the best water-soaked lesions in bean pods. Strains 3525, 4916, and B86-17 induced sunken, brown, water-soaked lesions in bean pods, and the symptoms were confined to the inoculated area. B728a induced sunken, dark brown, water-soaked lesions in bean pods, and the lesions spread slightly after inoculation. BBS32-5 and B86-37 induced sunken, green, water-soaked, spreading lesions in bean pods. Controls (water-inoculated pods) showed no water-soaking or necrosis.

P. syringae pv. phaseolicola strains capable of producing water-soaked lesions in bean leaves

Among the 17 strains of *P. syringae* pv. phaseolicola tested, nine strains (1448A, 501, 508, 524, 528, 461, 482, G-50, and M2/0) produced water-soaked lesions in bean leaves. The optimum inoculum level was about 10^5 cfu/ml. Dark green, translucent, water-soaked lesions were observed 5 days after inoculation, and lesions remained water-soaked for 4 d before turning necrotic. When the inoculum density was greater than 10^5 cfu/ml, water-soaked spots were confluent and became necrotic sooner. Eight other *P. syringae* pv. phaseolicola strains (6/0, F2, 106/1, 52, 539, 106/1/8, NPS3121, and HB4) produced dry, necrotic lesions without water-soaking.

P. syringae pv. tomato strains capable of producing water-soaked lesions in tomato leaves

Among five *P. syringae* pv. tomato strains tested, two (PT17 and OK-1) produced water-soaked lesions in tomato leaves, while three others (DC3000, 4325 and SC-5) produced dry, necrotic lesions. When the inoculum density was approximately 10^4 cfu/ml, both PT17 and OK-1 induced dark green, water-soaked lesions 3 days after inoculation, and the water-soaked lesions persisted for 4 d and then turned necrotic. When the inoculum density was higher than 10^5 cfu/ml, water-soaking occurred earlier (2 d after inoculation), and lesions became necrotic faster (1 d later). *P. syringae* pv. tomato DC3000, 4325 and SC-5 failed to produce water-soaked lesions in the inoculated area.

P. syringae pv. glycinea strains do not produce water-soaked lesions in soybean leaves

None of the four *P. syringae* pv. glycinea strains tested (PG4180, 4182, 5562, race 4) produced water-soaked lesions under the experimental conditions utilized in this study.

Discussion

When studying the interaction between phytopathogens and host plants, it is necessary to use a standardized amount of inoculum since inoculum levels either too high or low will lead to erroneous conclusions. This study showed that 10^4 cfu/ml (*P. syringae* pv. tomato), 10^5 cfu/ml (*P. syringae* pv. phaseolicola) and 10^6 cfu/ml (*P. syringae* pv. syringae) were the most suitable bacterial concentrations for obtaining water-soaked lesions in tomato leaves, bean leaves and bean pods, respectively. When the bacterial concentration was too high, transient water-soaking was observed in the susceptible host, and the lesions quickly became necrotic. When the bacterial concentration was too low, symptoms did not appear probably because the bacteria did not successfully colonize the plant.

The results showed that *P. syringae* pv. phaseolicola strains 1448A, 501, 508, 524, 528, 461, 482, G-50, M2/0, and *P. syringae* pv. tomato strains OK-1 and PT17 are excellent pathogens for producing water-soaked lesions in host plants. *P. syringae* pv. syringae strains 3525, B728a, B86-37, 4916, B86-17, and

BBS32-5 induced water-soaked lesions in bean pods but not in bean leaves. However, it is important to mention that the humidity of the growth chamber varied when these experiments were performed, and some might actually induce water-soaked lesions if the proper humidity was provided. For example, *P. syringae* pv. syringae 3525, which produced water-soaked lesions in bean pods but not in bean leaves in the current study, induced water-soaked lesions in bean leaves when the relative humidity was 60-80% (see Chapter 3).

APPENDIX B

EXPRESSION OF *PSALGD*::*UIDA* TRANSCRIPTIONAL FUSIONS IN *PSEUDOMONAS SYRINGAE* PV. SYRINGAE 3525

Introduction

Our previous results showed that alginate contributes significantly to the epiphytic survival and virulence of *Pseudomonas syringae* pv. syringae 3525. To further investigate alginate gene expression during epiphytic growth and pathogenesis, a system to monitor alginate gene expression *in planta* is needed. Analysis of promoter activity in plants has been extensively studied using the *Escherichia coli uidA* gene encoding β-glucuronidase (GUS). The *uidA* gene is a popular reporter gene because most plants and many bacteria do not contain endogenous β-glucuronidase activity (Jefferson et al., 1987; Wilson et al., 1992). Two *algD*::*uidA* transcriptional fusions were constructed in our lab: (1) pSK3, which contains a 1.0-kb fragment of the *algD* promoter from *P. syringae* pv. syringae FF5(pPSR12) in pRG960sd (Peñaloza-Vázquez et al., 1997), and (2) pAPDP, which contains a 2.7-kb fragment of the *algD* promoter from FF5(pPSR12) in pBBR.GUS (Fakhr et al., 1999). Both plasmids pRG960sd and

pBBR.GUS are promoter probe vectors that can be used to generate transcriptional fusions between a promoter of interest and a promoterless glucuronidase gene (Peñaloza-Vázquez et al., 1998; Van den Eede et al., 1992). Transcription in both pSK3 and pAPDP proceeds through the *algD* promoter and into the β-glucuronidase gene. Thus, *algD* gene expression can be detected by measuring GUS activity quantitatively by fluorometric analysis.

P. syringae FF5(pPSR12) was originally isolated from ornamental pear trees showing the extensive necrosis associated with bacterial blight symptoms (Sundin and Bender, 1993). Because it is very difficult and time consuming to reproduce bacterial blight symptoms on pear seedlings, the two *algD*::*uidA* promoter fusions from *P. syringae* FF5(pPSR12) were conjugated into *P. syringae* 3525, a bean pathogen that is more amenable to *in planta* studies. In the present study, the *in vitro* regulation of *PsalgD*::*uidA* transcriptional fusions in *P. syringae* 3525 was investigated.

Materials and Methods

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 3. *P. syringae* 3525 was maintained at 28°C on King's medium B, mannitol-glutamate medium (MG) or MG supplemented with yeast extract at 0.25 g/liter (MGY). *E. coli* strains were grown on Luria-Bertani (LB) medium at 37°C.

Antibiotics were added to the media at the following concentrations (μg/ml): ampicillin, 20; chloramphenicol, 25; spectinomycin 25; and streptomycin, 25.

Molecular biology techniques

Plasmid DNA was isolated by alkaline lysis, and agarose gel electrophoresis was performed using standard methods (Sambrook et al., 1989). Plasmid clones were mobilized into *P. syringae* 3525 using a triparental mating procedure with pRK2013 as a helper plasmid (Bender et al., 1991).

GUS assay

Transcriptional activity was qualitatively assayed by spotting 1 μl of bacterial suspensions (OD_{600}=0.1) onto MG agar medium amended with the appropriate antibiotics and 20 μg of X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) per ml; the plates were then incubated at 28°C for 20 to 48 h. For quantitative assays, bacterial strains were grown on MG agar supplemented with antibiotics for 36 h at 28°C. The bacteria were then suspended in MG broth to an OD_{600}=0.1 and incubated at 250 rpm for 36 h. Promoter activity was determined by fluorometric analysis of GUS as described previously (Palmer et al., 1997). Fluorescence was monitored by excitation at 365 nm and emission at 455 nm with a Fluoroscan II version 4.0 microplate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 96-well microtiter plates. The protein content in cell lysates was determined using the BioRad protein assay kit (BioRad, Hercules, CA) as recommended by the manufacturer. GUS activity was expressed in units per mg

of protein, with 1 U equivalent to 1 nmol of methylumbelliferone formed per min. Values presented for GUS activity represent the average of three replicates per experiment.

The kinetics of *algD* transcriptional activity in *P. syringae* 3525(pSK3) was evaluated by preparing fresh bacterial suspensions (OD_{600}=0.1) in MGY broth as described above. Bacteria were incubated at 28°C at 250 rpm, and aliquots of cells were removed at 1, 4, 8, 16, 24, 30, and 36 h and evaluated for transcriptional activity by glucuronidase assays.

For evaluation of *algD* expression in response to osmotic stress, fresh bacterial suspensions (OD_{600}=0.1) in MGY broth were prepared as described above and incubated at 28°C, 250 rpm for 4 h or until the OD_{600}=0.3. NaCl or sorbitol was added to the bacterial cultures at the following final concentrations: NaCl (0, 0.1, 0.15, 0.2, 0.3, 0.4 M) or sorbitol (0, 0.1, 0.3, 0.6, 0.9, 1.2 M). Cultures were returned to the incubator and sampled at different times for promoter activity by fluorometric analysis.

The effect of yeast extract on *algD* transcription was also investigated. Strains were incubated on MG agar for 36 h and suspended to OD_{600}=0.1 in MG broth amended with yeast extract (0%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%, 0.8%). Cultures were then incubated at 28°C, 250 rpm. Transcriptional activity was evaluated as described above.

Results and Discussion

The two *PsalgD*::*uidA* transcriptional fusions, pSK3 and pAPDP, and the vector controls (pRG960sd and pBBR.GUS) were introduced into *P. syringae* 3525. Colonies of 3525(pSK3) turned blue on medium containing X-Gluc, indicating that the *algD* promoter was active *in vitro*. As expected, the vector control 3525(pRG960sd) remained colorless on X-Gluc medium. Interestingly, the *PsalgD*::*uidA* transcriptional fusion in 3525(pAPDP) was inactive, while the vector control 3525(pBBR.GUS) showed very low activity. These results were consistent with quantitative analysis of *PsalgD*::*uidA* transcriptional activity in MG broth (Table 4). Therefore, 3525(pSK3) was chosen for subsequent experiments.

Time course experiments with 3525(pSK3) showed that *algD* transcriptional activity increased steadily, reaching a concentration of 810 U of GUS/mg protein at 36 h (Fig. 7). GUS activity in 3525(pRG960sd) remained low (40~105 U) throughout the sampling period (Fig. 7).

When expressed in *P. syringae* FF5(pPSR12), the *PsalgD*::*uidA* transcriptional fusion was activated by both NaCl and sorbitol, indicating that the alginate genes were activated in response to increased osmotic stress (Penaloza-Vazquez et al., 1997). Similar results were obtained in *P. aeruginosa* and several other fluorescent pseudomonads (Berry et al., 1989; Singh et al., 1992). However, when the *PsalgD*::*uidA* transcriptional fusion pSK3 was introduced into *P. syringae* 3525 and exposed to different NaCl concentrations and monitored at different times (0.3, 0.6, 1, 2, 4, 6, 9, 18 h), no stimulation of

GUS activity was observed (data not shown). Fig. 8 shows GUS activity in 3525(pSK3) at 6 h after the addition of different amounts of NaCl. Sorbitol also failed to stimulate the *PsalgD*::*uidA* fusion in *P. syringae* 3525 (Fig. 9).

Alginate production in *P. aeruginosa* increased during nutritional starvation *in vitro* (Terry et al., 1992). In the present study, the effect of nutritional supplementation (yeast extract) on *algD* transcription in 3525(pSK3) was investigated. Our results showed that the addition of yeast extract did not inhibit *PsalgD* transcription (Fig. 10).

We also investigated whether the regulation of *algD* transcriptional activity was masked by the high basal level of *algD* transcriptional activity in *P. syringae* 3525(pSK3). Therefore, GUS activity of *P. syringae* 3525 harboring pAPDP was also tested for transcriptional activity in response to NaCl and sorbitol. No significant increase in *algD* transcription occurred in response to osmotic stress in 3525(pAPDP) (Fig. 11 and Fig. 12).

In summary, when the *PsalgD*::*uidA* transcriptional fusion was expressed in *P. syringae* 3525, there was no stimulation by osmotic stress or starvation as observed in *P. syringae* FF5 (Penaloza-Vazquez et al., 1997). These results indicate that the *PsalgD*::*uidA* transcriptional fusion from FF5 is not suitable for monitoring alginate gene expression in strain 3525. Fakhr et al. (1999) showed that alginate gene expression in *P. syringae* FF5 and *P. aeruginosa* is regulated differentially, and the *algD* promoter region in *P. syringae* FF5 (*PsalgD*) diverged significantly from the *algD* promoter in *P. aeruginosa*. The present results indicate that the *algD* promoter is differentially regulated in different strains of *P.*

syringae. It would be worthwhile to compare the sequence of the *algD* promoter regions in *P. syringae* strain 3525 and FF5.

TABLE 3

BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Strain or plasmid	Relevant characteristics	Reference
E. coli DH5α		Sambrook et al, 1989
P. syringae 3525	Amp^r, nonmucoid	ICMP[a]
3525(pRG960sd)	Amp^r Sm^r Sp^r, nonmucoid	This study
3525(pSK3)	Amp^r Sm^r Sp^r, nonmucoid	This study
3525(pBBR.GUS)	Amp^r Cm^r, nonmucoid	This study
3525(pAPDP)	Amp^r Cm^r, nonmucoid	This study
pRG960sd	Sm^r Sp^r, contains promoterless *uidA* with start codon and Shine-Dalgarno sequence	Van den Eede et al., 1992
pSK3	Sm^r Sp^r, contains a 1.0-kb fragment from *PsalgD* in pRG960sd in the transcriptionally active orientation	Peñaloza-Vázquez et al., 1997
pBBR.GUS	Cm^r, 6.6-kb promoter probe vector containing *uidA* in pBBR1MCS	Peñaloza-Vázquez et al., 1998
pAPDP	Cm^r, 7.4-kb contains *PsalgD* as a 2.7-kb *Hind*III-*Eco*RV fragment in pBBR.GUS	Peñaloza-Vázquez et al., 1997

a. ICMP is the International Collection of Microorganisms from Plants, Auckland, New Zealand.

TABLE 4

IN VITRO GUS ACTIVITY OF *P. SYRINGAE* 3525 CONTAINING DIFFERENT *PSALGD::UIDA* TRANSCRIPTIONAL FUSIONS

Strain	Mean GUS activity (U/mg protein)[a]	Plate assay[b]
3525(pSK3)	662 a	+++
3525(pRG960sd)	17 c	-
3525(pAPDP)	38 c	-
3525(pBBR.GUS)	143 b	+

a. Mean values followed by the same letter are not significantly different at P=0.05 using the Student-Newman-Keuls test.

b. +++ blue; + slightly blue; - colorless.

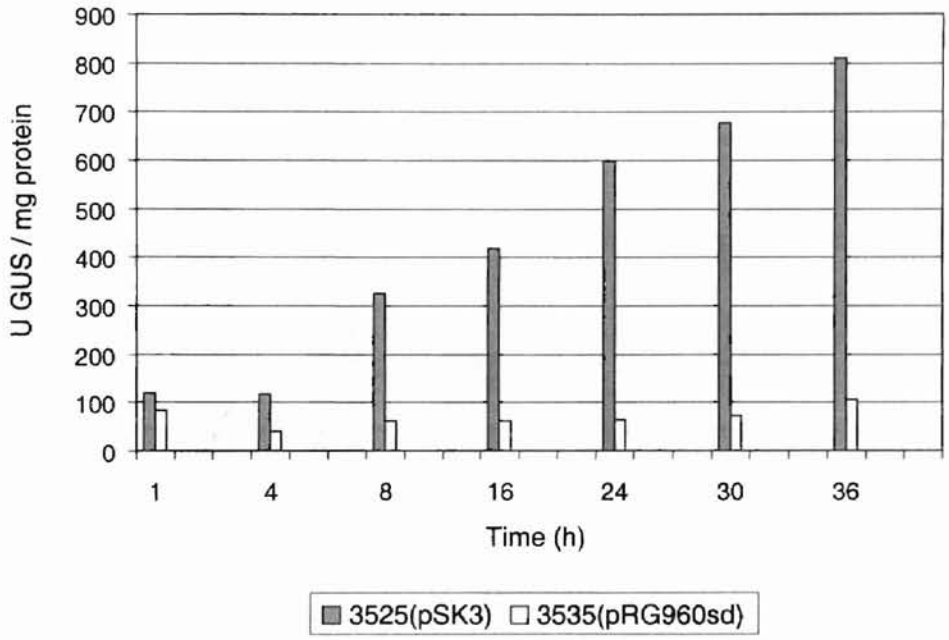


Fig. 7. *P. syringae* 3525(pSK3) transcriptional activity in MGY broth.

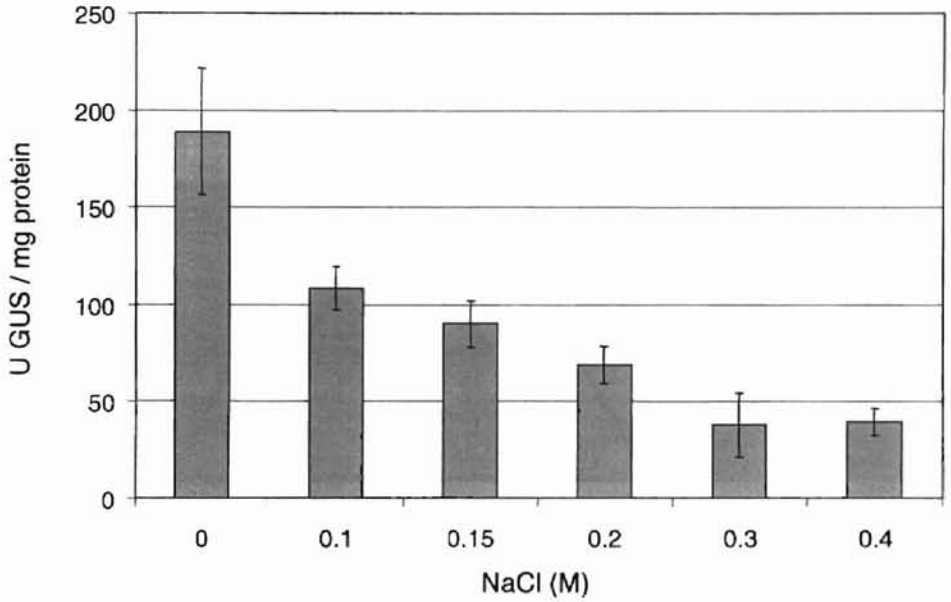


Fig. 8. *P. syringae* 3525(pSK3) transcriptional activity in response to osmotic stress (NaCl).

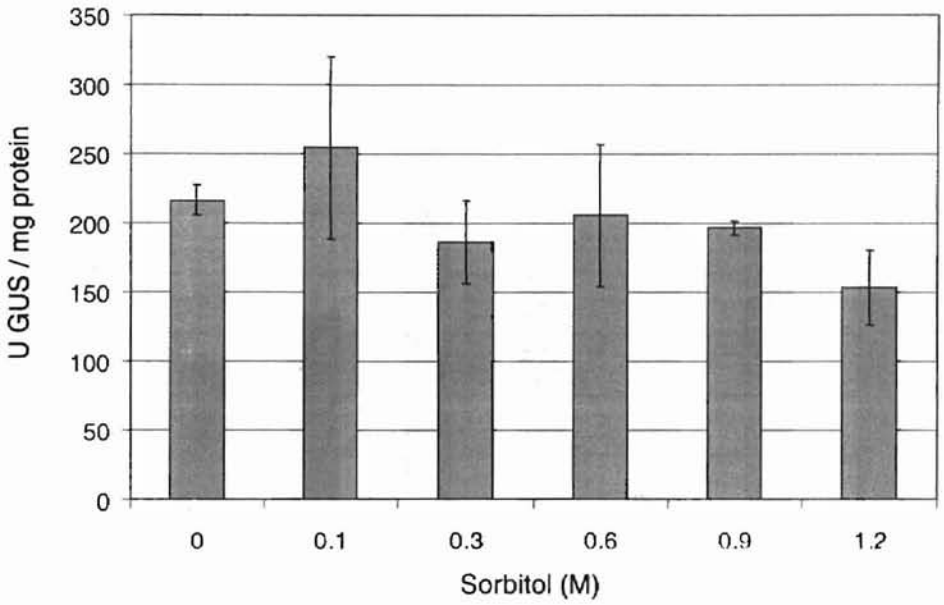


Fig. 9. *P. syringae* 3525(pSK3) transcriptional activity in response to osmotic stress (sorbitol).

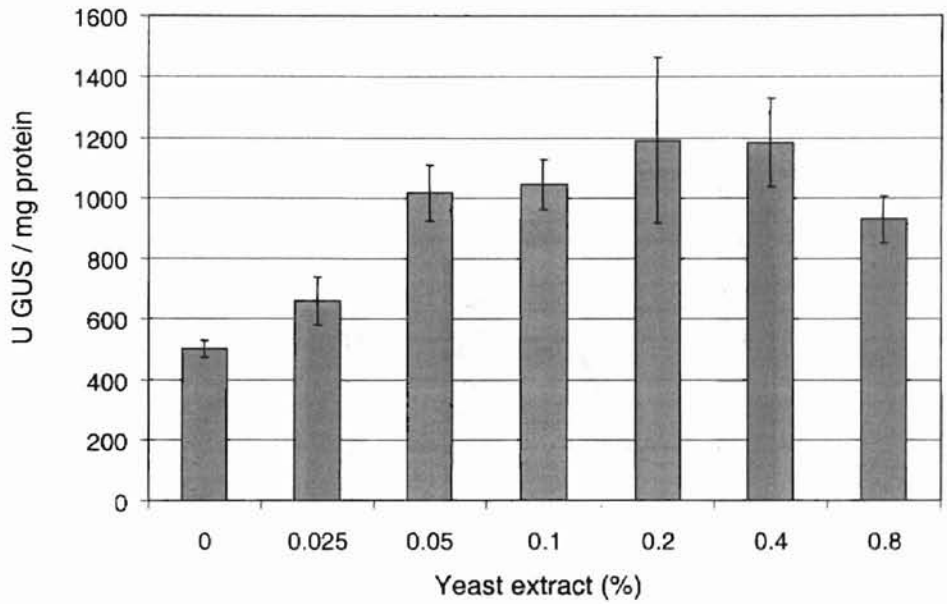


Fig. 10. *P. syringae* 3525(pSK3) transcriptional activity in response to yeast extract.

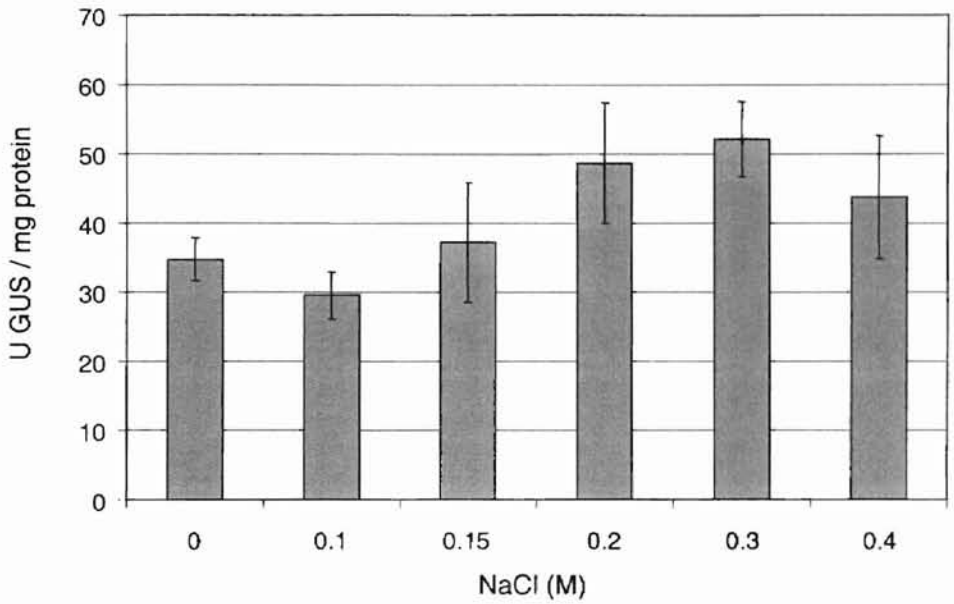


Fig. 11. *P. syringae* 3525(pAPDP) transcriptional activity in response to osmotic stress (NaCl).

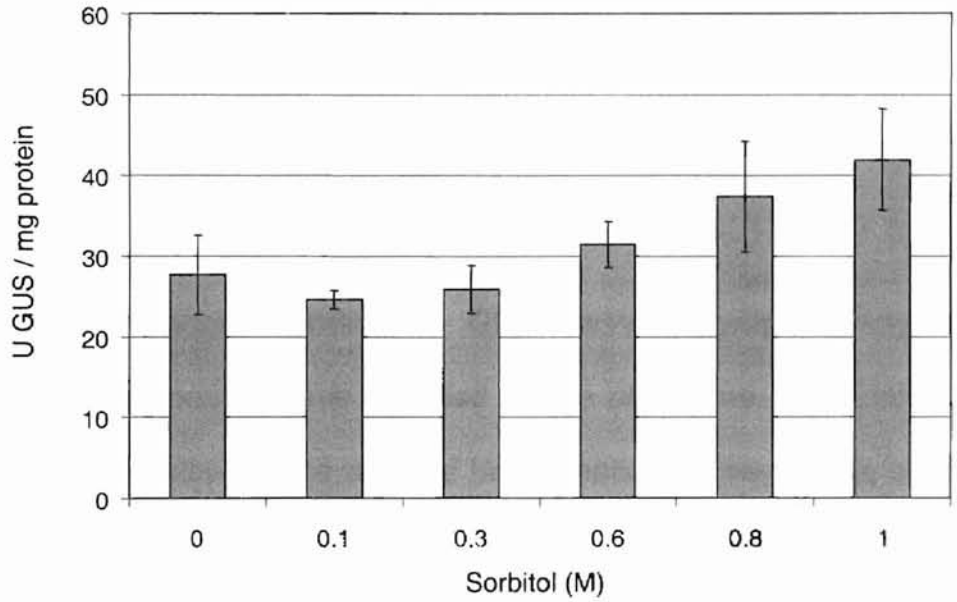


Fig. 12. *P. syringae* 3525(pAPDP) transcriptional activity in response to osmotic stress (sorbitol).

APPENDIX C

SEQUENCE ANALYSIS OF *ALGA* FROM SEVERAL DIFFERENT PATHOVARS OF *PSEUDOMONAS SYRINGAE*

Introduction

Phosphomannose isomerase – GDP-mannose pyrophosphorylase (PMI-GMP) is a bifunctional enzyme encoded by the *algA* gene. PMI-GMP catalyses the first and third steps in the alginate biosynthetic pathway and is essential for alginate biosynthesis in *P. aeruginosa* (May and Chakrabarty, 1994; Shinabarger et al., 1991) and *P. syringae* (Peñaloza-Vázquez et al., 1997). Peñaloza-Vázquez et al. showed that the *algA* homolog in *P. syringae* FF5 was 74% identical to *algA* from *P. aeruginosa*, and *algA* was functionally interchangeable between the two species when expressed from the P*tac* promoter. In the present study, a 0.87-kb *algA* fragment was cloned by PCR from several strains of *P. syringae* (pv. phaseolicola 1448A, pv. syringae B728a, and pv. tomato PT17). Sequence analysis revealed a conserved, unique *Eco*RI site within the *algA* gene that would be useful for insertion mutagenesis.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains used in this study were *Escherichia coli* Top10F' (Invitrogen), *P. syringae* pv. phaseolicola 1448A (from A. Vivian), pv. syringae B728a (from K. Willis), and pv. tomato PT17 (C. Bender). Cloning vector pCR2.1 was purchased from Invitrogen.

Molecular biology techniques

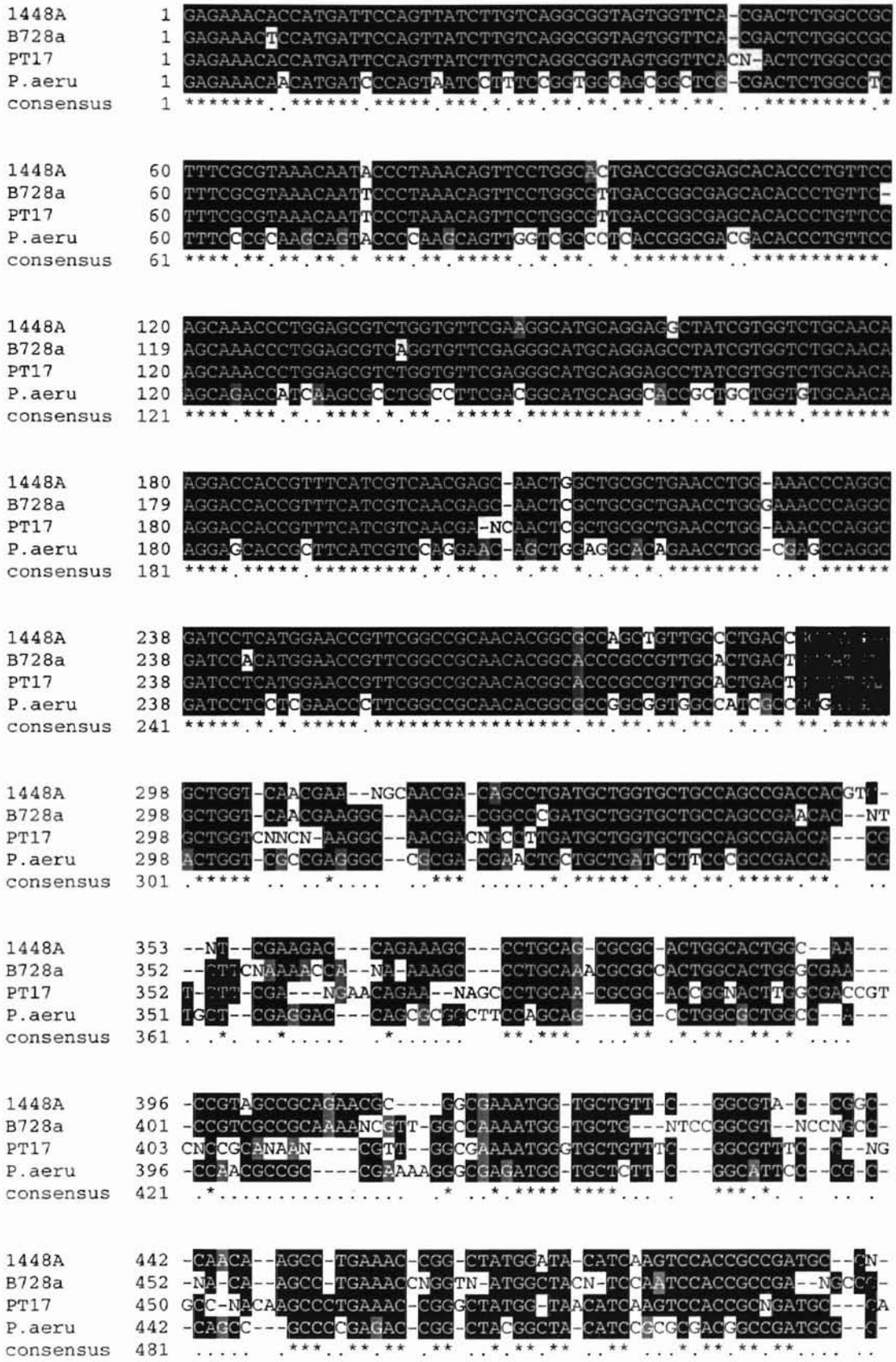
Plasmid DNA isolations, agarose gel electrophoresis, and restriction digestions were performed using standard protocols (Sambrook et al., 1989). Chromosomal DNA from *P. syringae* was extracted as described previously (Staskawicz et al., 1984). Primers for the PCR reaction were: 5' GAGAAACACCATGATTCCAG (forward primer) and 5' TTACCGTTGGCGTCCTTTG (reverse primer), which are derived from *algA* of *P. syringae* FF5. Amplification was performed in a MJ Research thermal cycler (PTC-100). Chromosomal DNA (250 ng) from *P. syringae* was used as template, and reactions contained Taq polymerase (2.5 U), 1.25 mM of each dNTP, 0.5 μM of each oligonucleotide primer, and 50 mM $MgCl_2$. PCR reactions consisted of a 30-cycle program with a 45 s denaturation at 94°C, a 30 s annealing at 50°C, and a 60 s elongation at 72°C. PCR products were cloned using the TA Cloning Kit (Invitrogen) according to the manufacturer's recommendations. DNA was prepared for sequencing using the Plasmid Midi Kit (Qiagen). Oligonucleotide

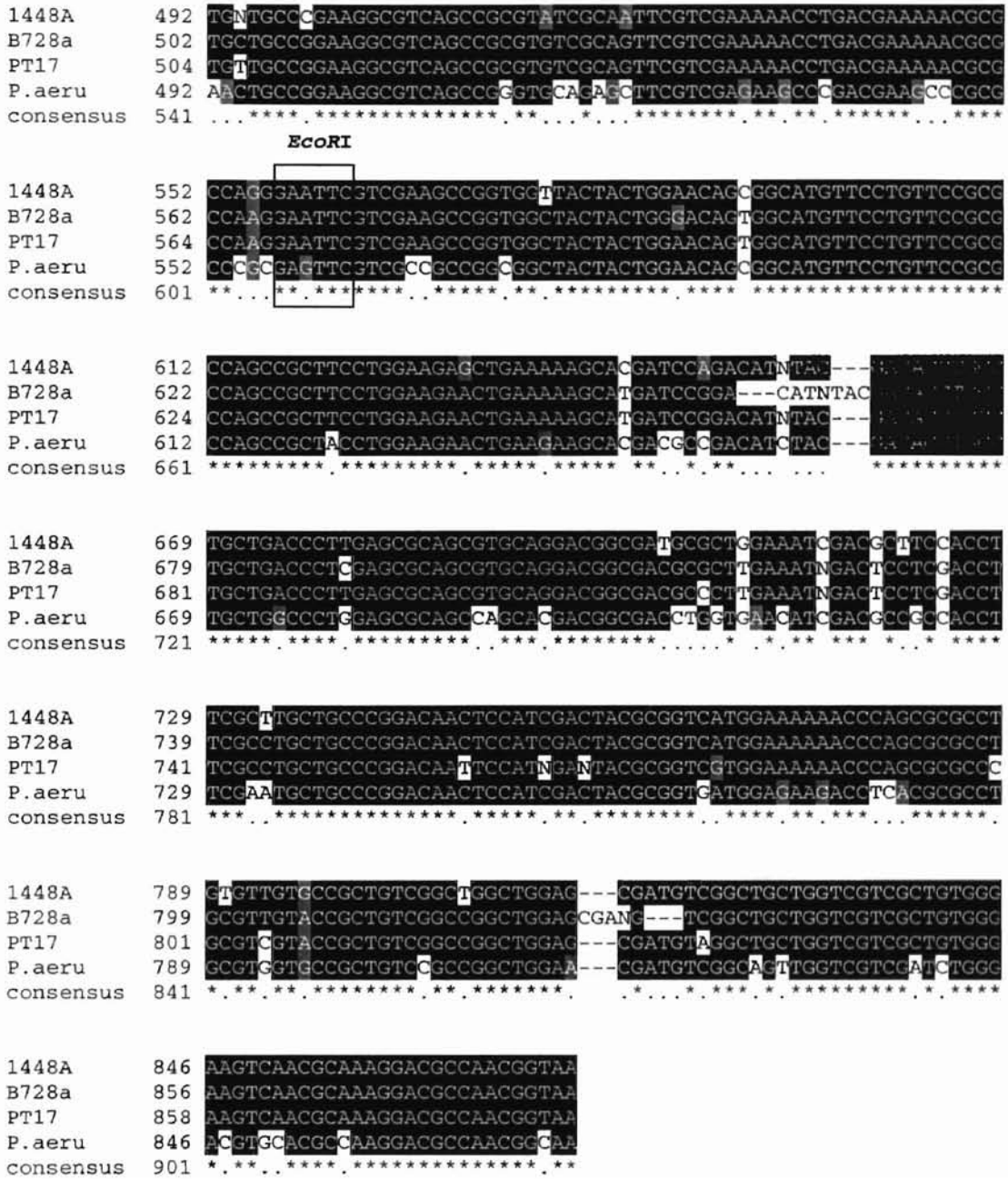
synthesis and automated DNA sequencing were provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Sequence data was assembled and homology searches were executed using MacVector Version 4.5 (IBI) and the BLAST network service at NCBI. Multiple sequence alignments were performed using the BCM Search Launcher network service at Baylor College of Medicine.

Results and Discussion

PCR products of the expected size (0.87 kb) were cloned into pCR2.1 and sequenced with the M13 forward and reverse primers. The *algA* sequence from *P. syringae* pv. phaseolicola 1448A, pv. syringae B728a, and pv. tomato PT17 were highly homologous to each other (91-93% nucleotide identity) and shared about 78% nucleotide identity to *algA* from *P. aeruginosa* (nucleotides 30-902 as described in Darzins et al., 1986). A conserved *Eco*RI site was found in all three *algA* sequences from *P. syringae*, and was located at nucleotides 586-591, with respect to the *P. aeruginosa algA* coding region (Fig. 13). This site could be used for insertion of an antibiotic cassette and the development of an *algA* mutant of *P. syringae*.

Fig. 13. Multiple sequence alignment of *algA* genes from the following *Pseudomonas* strains: *P. syringae* pv. phaseolicola 1448A, *P. syringae* pv. syringae B728A, *P. syringae* pv. tomato PT17, and *P. aeruginosa*. Conserved nucleotides are shaded in black while conserved substitutions are shaded in gray. "-" is created for the purpose of alignment. The conserved *Eco*RI site is marked with a rectangle.


1448A 1 GAGAAACACCATGATTCCAGTTATCTTGTCAGGCGGTAGTGGTTCA-CGACTCTGGCCGC
B728a 1 GAGAAACTCCATGATTCCAGTTATCTTGTCAGGCGGTAGTGGTTCA-CGACTCTGGCCGC
PT17 1 GAGAAACACCATGATTCCAGTTATCTTGTCAGGCGGTAGTGGTTCACN-ACTCTGGCCGC
P.aeru 1 GAGAAACAACATGATCCCAGTAATCCTTTCCGGTGGCAGCGGCTCG-CGACTCTGGCGTC
consensus 1 *******..******.*****.***.*.**.**.**.**.**.**. ..*********.*
1448A 60 TTTCGCGTAAACAATACCCTAAACAGTTCCTGGCACTGACCGGCGAGCACACCCTGTTCC
B728a 60 TTTCGCGTAAACAATTCCCTAAACAGTTCCTGGCGTTGACCGGCGAGCACACCCTGTTC-
PT17 60 TTTCGCGTAAACAATTCCCTAAACAGTTCCTGGCGTTGACCGGCGAGCACACCCTGTTCC
P.aeru 60 TTTCCCGCAAGCAGTACCCCAAGCAGTTGGTCGCCTCACCGGCGACGACACCCTGTTCC
consensus 61 ****.**.**.**.* ***.**.*****..*.**. *.********..**********.
1448A 120 AGCAAACCCTGGAGCGTCTGGTGTTCGAAGGCATGCAGGAGGCTATCGTGGTCTGCAACA
B728a 119 AGCAAACCCTGGAGCGTCAGGTGTTCGAGGGCATGCAGGAGCCTATCGTGGTCTGCAACA
PT17 120 AGCAAACCCTGGAGCGTCTGGTGTTCGAGGGCATGCAGGAGCCTATCGTGGTCTGCAACA
P.aeru 120 AGCAGACCATCAAGCGCCTGGCCTTCGACGGCATGCAGGCACCGCTGCTGGTGTGCAACA
consensus 121 ****.***.*..****.*.**..*****.**********...*..*..****.*******
1448A 180 AGGACCACCGTTTCATCGTCAACGAGC-AACTGGCTGCGCTGAACCTGG-AAACCCAGGC
B728a 179 AGGACCACCGTTTCATCGTCAACGAGC-AACTCGCTGCGCTGAACCTGGGAAACCCAGGC
PT17 180 AGGACCACCGTTTCATCGTCAACGA-NCAACTCGCTGCGCTGAACCTGG-AAACCCAGGC
P.aeru 180 AGGAGCACCGCTTCATCGTCCAGGAAC-AGCTGGAGGCACAGAACCTGG-CGAGCCAGGC
consensus 181 ****.*****.*********.*.**.. *.** *..**.*.******** ..*.******
1448A 238 GATCCTCATGGAACCGTTCGGCCGCAACACGGCGCCAGCTGTTGCCCTGACC
B728a 238 GATCCACATGGAACCGTTCGGCCGCAACACGGCACCCGCCGTTGCACTGACT
PT17 238 GATCCTCATGGAACCGTTCGGCCGCAACACGGCACCCGCCGTTGCACTGACT
P.aeru 238 GATCCTCCTCGAACCCTTCGGCCGCAACACGGCGCCGGCGGTGGCCATCGCC
consensus 241 *****.*.*.*****.**********.**.**.**.** .*..* **.*****
1448A 298 GCTGGT-CAACGAA--NGCAACGA-CAGCCTGATGCTGGTGCTGCCAGCCGACCAGGT
B728a 298 GCTGGT-CAACGAAGGC--AACGA-CGGCCCGATGCTGGTGCTGCCAGCCGAACAG--NT
PT17 298 GCTGGTCNNCN-AAGGC--AACGACNGCCTGATGCTGGTGCTGCCAGCCGACCA---CG
P.aeru 298 ACTGGT-CGCCGAGGGC--CGCGA-CGAACTGCTGCTGATCCTTCCCGCCGACCA---CG
consensus 301 .***** .. ..*.... ..*** ......*.*****.*.**.**.*****.**. ..
1448A 353 --NT--CGAAGAC---CAGAAAGC---CCTGCAG-CGCGC-ACTGGCACTGGC--AA---
B728a 352 --CTTCNAAAACCA--NA-AAAGC---CCTGCAAACGCGCCACTGGCACTGGCGAA---
PT17 352 T-CTT-CGA---NGAACAGAA--NAGCCCTGCAA-CGCGC-ACCGGNACTTGGCGACCGT
P.aeru 351 TGCT--CGAGGAC---CAGCGCGGCTTCCAGCAG----GC-CCTGGCGCTGGCC---A---
consensus 361 . .*. ..*..... .*..... **.***. ...** .*.**..**.* ....
1448A 396 -CCGTAGCCGCAGAACGC----GGCGAAATGG-TGCTGTT-C---GGCGTA-C--CGGC-
B728a 401 -CCGTCGCCGCAAAANCGTT-GGCCAAAATGG-TGCTG---NTCCGGCGT--NCCNGCC-
PT17 403 CNCCGCANAAN----CGTT--GGCGAAAATGGGTGCTGTTTC---GGCGTTTC--C--NG
P.aeru 396 -CCAACGCCGC----CGAAAGGGCGAGATGG-TGCTCTT-C---GGCATTCC--CC-C-
consensus 421 .*.............. .* ..*.**** **** ... . ***.*. . .. .
1448A 442 -CAACA--AGCC-TGAAAC-CGG-CTATGGATA-CATCAAGTCCACCGCCGATGC--CN-
B728a 452 -NA-CA--AGCC-TGAAACCNGGTN-ATGGCTACN-TGCAATCCACCGCCGA--NGCC-
PT17 450 GCC-NACAAGCCCTGAAAC-CGGGCTATGG-TAACATCAAGTCCACCGCNGATGC---CA
P.aeru 442 -CAGCC---GCCCCGAGAC-CGG-CTACGGCTA-CATCCCCGCGACGGCCGATGCG---C-
consensus 481 ..... .***..**.** .** ..*.**.** ..** ...*.**.**.**.... ..


EcoRI
1448A 492 TGNTGCCCGAAGGCGTCAGCCGCGTATCGCAATTCGTCGAAAAACCTGACGAAAAACGCG
B728a 502 TGCTGCCGGAAGGCGTCAGCCGCGTGTCGCAGTTCGTCGAAAAACCTGACGAAAAACGCG
PT17 504 TGTTGCCGGAAGGCGTCAGCCGCGTGTCGCAGTTCGTCGAAAAACCTGACGAAAAACGCG
P.aeru 492 AACTGCCGGAAGGCGTCAGCCGGGTGCAGAGCTTCGTCGAGAAGCCCGACGAAGCCCGCG
consensus 541 ...****.**************.**...*...********.**.**.******...****
1448A 552 CCAGGGAATTCGTCGAAGCCGGTGGTTACTACTGGAACAGCGGCATGTTCCTGTTCCGCG
B728a 562 CCAAGGAATTCGTCGAAGCCGGTGGCTACTACTGGGACAGTGGCATGTTCCTGTTCCGCG
PT17 564 CCAAGGAATTCGTCGAAGCCGGTGGCTACTACTGGAACAGTGGCATGTTCCTGTTCCGCG
P.aeru 552 CCCGCGAGTTCGTCGCCGCCGGCGGCTACTACTGGAACAGCGGCATGTTCCTGTTCCGCG
consensus 601 **...**.*******..*****.**.*********.**** ********************
1448A 612 CCAGCCGCTTCCTGGAAGAGCTGAAAAAGCACGATCCAGACATNTAC---
B728a 622 CCAGCCGCTTCCTGGAAGAACTGAAAAAGCATGATCCGGA---CATNTAC
PT17 624 CCAGCCGCTTCCTGGAAGAACTGAAAAAGCATGATCCGGACATNTAC---
P.aeru 612 CCAGCCGCTACCTGGAAGAACTGAAGAAGCACGACGGCGACATCTAC---
consensus 661 *********.*********.*****.***** **..*.**... ... **********
1448A 669 TGCTGACCCTTGAGCGCAGCGTGCAGGACGGCGATGCGCTGGAAATCGACGCTTCCACCT
B728a 679 TGCTGACCCTCGAGCGCAGCGTGCAGGACGGCGACGCGCTTGAAATNGACTCCTCGACCT
PT17 681 TGCTGACCCTTGAGCGCAGCGTGCAGGACGGCGACGGCCTTGAAATNGACTCCTCGACCT
P.aeru 669 TGCTGGCCCTGGAGCGCAGCCAGCACGACGGCGACCTGGTGAACATCGACGCCGCCACCT
consensus 721 *****.****.*********..***.********.....* .*.** *** *..* ****
1448A 729 TCGCTTGCTGCCCGGACAACTCCATCGACTACGCGGTCATGGAAAAAACCCAGCGCGCCT
B728a 739 TCGCCTGCTGCCCGGACAACTCCATCGACTACGCGGTCATGGAAAAAACCCAGCGCGCCT
PT17 741 TCGCCTGCTGCCCGGACAATTCCATNGANTACGCGGTCGTGGAAAAAACCCAGCGCGCCC
P.aeru 729 TCGAATGCTGCCCGGACAACTCCATCGACTACGCGGTCATGGAGAAGACCTCACGCGCCT
consensus 781 ***..**************.*****.**.********..****.**.***...******.
1448A 789 GTGTTGTGCCGCTGTCGGCTGGCTGGAG---CGATGTCGGCTGCTGGTCGTCGCTGTGGG
B728a 799 GCGTTGTACCGCTGTCGGCCGGCTGGAGCGANG---TCGGCTGCTGGTCGTCGCTGTGGC
PT17 801 GCGTCGTACCGCTGTCGGCCGGCTGGAG---CGATGTAGGCTGCTGGTCGTCGCTGTGGC
P.aeru 789 GCGTGGTGCCGCTGTCGCCGGCTGGAA---CGATGTCGGGAGTTGGTCGTCGATCTGGG
consensus 841 *.**.**.********.**.*******. .*...*.***.*.*********.*.****
1448A 846 AAGTCAACGCAAAGGACGCCAACGGTAA
B728a 856 AAGTCAACGCAAAGGACGCCAACGGTAA
PT17 858 AAGTCAACGCAAAGGACGCCAACGGTAA
P.aeru 846 ACGTGCACGCCAAGGACGCCAACGGCAA
consensus 901 *.**..****.**************.**

VITA

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Master of Science

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