REGULATION OF ALGINATE BIOSYNTHESIS

IN *PSEUDOMONAS SYRINGAE*

BY

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CHAPTER I

Introduction

The exopolysaccharide alginate is an important virulence factor in cystic fibrosis (CF) patients infected with the opportunistic pathogen, *Pseudomonas aeruginosa.* Alginate is also produced by phytopathogenic bacteria such as *Pseudomonas syringae*, and the production of alginate is correlated with the appearance of water-soaked lesions *in planta.* The identification of factors that regulate alginate synthesis could lead to the discovery of nontoxic inhibitors of alginate synthesis that control the pulmonary infections in humans or the foliar symptoms *in planta.* Virulence mechanisms that function in clinical pathogens of animals, such as the type III secretion systems in *Salmonella*, *Shigella*, and *Yersinia*, are also conserved in phytopathogenic bacteria (Pettersson et al., 1996; Roine et al., 1997). The biosynthesis of alginate by *P. aeruginosa* and *P. syringae* presents another unique opportunity to study a virulence factor which functions in both human and plant pathogenic bacteria.

Alginate is commercially produced from marine algae and has important commercial uses as a gelling agent and as a viscosity modifier. The production and isolation of alginate from a bacterial source could provide a consistent product throughout the year and would avoid the seasonal variations in the yield and quality of algal alginate. Furthermore, *P. syringae* might be considered a more appropriate source of alginate since *P. aeruginosa* is an opportunistic human pathogen.

The unique physical properties of the different forms of alginate have been exploited for a wide variety of uses (Gacesa, 1988; Sanford and Baird, 1983). For

example, alginate is the most versatile immobilization matrix for living cells with potential use in transplantation (Skjåk-Bræk and Espevik, 1996). Alginate has also been used as a viscosifier, stabilizer, and gelling agent in biomaterials, food and beverage, paper and printing, and in the pharmaceutical industries. Furthermore, the control of alginate modifying enzymes (lyase, acetylase, and epimerase) can result in alginates of specific sizes, water-binding capacities and fluidity. Perhaps *P. syringae* alginate can be used to create EPS polymers with specific properties, i.e. designer polysaccharides of commercial importance.

While several reports indicate that the biosynthetic route to alginate in *P. syringae* is similar to that established for *P. aeruginosa*, complementation analysis indicated that the structural gene cluster in *P. aeruginosa* and *P. syringae* were not functionally interchangeable when expressed from their native promoters (Peñaloza-Vázquez et al., 1997). This result suggests that the regulation and transcriptional activation of alginate biosynthesis is different in the two species. Thus, investigating the regulation of alginate biosynthesis in *P. syringae* will shed light on how the regulation of this virulence factor in plant pathogens differs from regulation in the human pathogen, *P. aeruginosa*.

In the present study, *algR1*, a positive regulator of alginate biosynthesis, was cloned from *P. syringae* pv. syringae FF5 and overexpressed in *Escherichia coli*. The role of *algR1* in the transcriptional regulation of *algD* and *algC* in *P. syringae* was investigated. An alginate-defective mutant of *P. syringae* was created by mutagenizing an indigenous plasmid that confers constitutive alginate production, and efforts to clone and identify the plasmid-encoded genes were undertaken. Lastly, this study describes the

cloning, sequencing and overexpression of a novel extracellular epimerase from *P. syringae* pv. glycinea.

CHAPTER II

Literature Review

Biology and pathogenicity of Pseudomonas syringae. *Pseudomonas syringae* is a plant pathogen that causes a variety of symptoms on plants including galls, blights, leaf spots and cankers (Alfano and Collmer, 1996). Many *P. syringae* strains are ice-nucleation active, i.e., they serve as nuclei for ice formation and cause frost injury to plants at relatively high freezing temperatures (Agrios, 1988). Pseudomonads also encode gene products that significantly enhance pathogen virulence including extracellular polysaccharides, plant hormones, and cell wall degrading enzymes (Alfano and Collmer, 1996; Boucher et al., 1992; Denny, 1995).

The species *P. syringae* is subdivided into pathogenic variants (pathovars; pvs.), which vary in host range. When *P. syringae* cells are infiltrated into plant tissues, two distinct reactions are possible; a compatible, susceptible interaction or a hypersensitive response (HR). The susceptible reaction is characterized by a symptom called "water soaking" and is followed by pathogen proliferation and advanced symptom development. The HR is characterized by a rapid necrosis that occurs 12-24 hrs after inoculation and severely restricts multiplication of the pathogen, which results in host resistance (Willis et al., 1991).

Gram-negative bacteria have developed a variety of secretion pathways to export toxins and enzymes into the extracellular medium. Type I secretion system involves ABC transporters that function in a one-step transfer across the inner and outer

membrane with no periplasmic intermediate. Non-proteinaceous compounds can be secreted via type I systems since no recognition sequence is involved (Mishima et al., 2001). In type II secretion pathways, proteins to be secreted are produced with N-terminal signal peptides, which allows for Sec-dependent translocation across the cytoplasmic membrane. This is followed by removal of the signal peptide, folding, and release of the mature proteins into the periplasmic space (Sandkvist, 2001). Type III secretion systems are host-cell-contact-dependent,and function to deliver bacterial virulence effectors to the interior of host cells (Galan and Collmer, 1999). Proteins secreted by the type III system have no obvious amino acid motif suggestive of a secretion signal; however, the tertiary 5' mRNA structure may be involved in the secretory (Anderson and Schneewind, 1997). Type IV secretion includes cell-to-cell transfer of DNA or protein-DNA complexes during conjugation or toxin transport. The *Agrobacterium tumefaciens* T-DNA transfer system is an excellent example of the type IV secretion process (Christie and Vogel, 2000).

The genetic basis of pathogenicity and virulence in *P. syringae* is complex and include global regulators (Hrabak and Willis, 1992), a type III secretion system (the *hrp* cluster), and virulence factors such as phytotoxins and alginate (Bender et al., 1999; Yu et al., 1999). The *hrp* region (for *h*ypersensitive *r*esponse and *p*athogenicity) is conserved in phytopathogenic prokaryotes and affects the ability of a bacterium to induce a hypersensitive response (HR) in nonhost plants, pathogenicity on host plants, and the ability to grow within or on the surface of plants (He, 1998; Hirano et al., 1999). The *hrp* genes encode for the regulation and biosynthesis of a type III secretion pathway that is used by both plant and animal pathogens to secrete virulence proteins (Salmond, 1994).

A subset of the *hrp* genes was renamed *hrc* (HR and *c*onserved) because of their conservation in the type III secretion apparatus used by *Yersinia*, *Shigella*, and *Salmonella* (Bogdanove et al., 1996; Galán and Collmer, 1999). The *hrp* genes have been extensively characterized in *P. syringae* where they are clustered in the chromosome and encode regulatory, secretory, or effector proteins (Galán and Collmer, 1999). Genome wide identification of ORFs in the Hrp regulon of *P. syringae* pv. tomato DC3000 yielded an inventory of candidate effector proteins that control host range and virulence (Fouts et al., 2002; Petnicki-Ocwieja et al., 2002).

Biological roles of exopolysaccharides (EPS). Exopolysaccharides (EPSs) are produced by most bacteria, including many plant pathogens, and are secreted as loose slime layers or as capsular material. EPSs are thought to protect free-living bacteria from a variety of environmental stresses and may aid pathogenesis by sustaining water-soaking of intercellular spaces, altering the uptake of antimicrobial compounds or defense-activating signals, and blocking the xylem and thereby producing wilt symptoms (Alfano and Collmer, 1996). Most of the functions ascribed to EPS are of a protective nature. The ability of a microorganism to surround itself in a highly hydrated EPS layer may provide it with protection against desiccation and predation by protozoans. EPS can also help microorganisms adhere to and colonize solid surfaces (Leigh and Coplin, 1992). EPS production, particularly its role in plant pathogenesis as determined through transposon mutagenesis, has been most extensively explored in *Ralstonia solanacearum*, *Erwinia amylovora*, and *E. stewartii* (Denny, 1995). EPS is generally a virulence factor in these bacteria and contributes to both wilt and water-soaking symptoms.

The capacity of pseudomonads and xanthomonads to induce persistent water-soaking in leaves plays a crucial role during pathogenesis that seems to be accomplished by a synergistic interaction between bacterial EPS and plant polymers. These bacteria are not able to cause typical water-soaked disease symptoms under conditions of low EPS production. The main EPS components in these bacteria are alginate and levan (*Pseudomonas*), xanthan *(Xanthomonas*), and heteropolysaccharides in *Erwinia* spp. When bacteria are embedded in a gel-like matrix *in planta*, they are not easily recognized by the plant. Therefore, EPS molecules may protect phytopathogenic bacteria from toxic molecules produced during the plant defense response.

Biological roles of the exopolysaccharide alginate. The exopolysaccharide alginate is a co-polymer of O-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Figure 1) (Evans and Linker, 1973). The uronic acid units can be arranged randomly or contiguously within the linear molecule. Heteropolymeric block structure (polyMG) and/or the homopolymeric block structure(s) (polyM or polyG) can be found within a single alginate molecule (Figure 1). In bacteria, the mannuronate residue is usually acetylated on C2 or C3 (Davidson et al., 1977). Acetylation increases the viscosity of the polymer and decreases its ability to bind calcium ions (Skjåk-Bræk et al., 1989).

Alginate is a major structural polysaccharide of brown seaweeds, which are the primary source of alginate for commercial applications. The most common form of alginate used commercially is sodium alginate. Alginate is produced by brown seaweeds

such as *Macrocystis*, *Laminaria*, and *Ascophyllum*, and several bacterial genera, including *Pseudomonas* and *Azotobacter*.

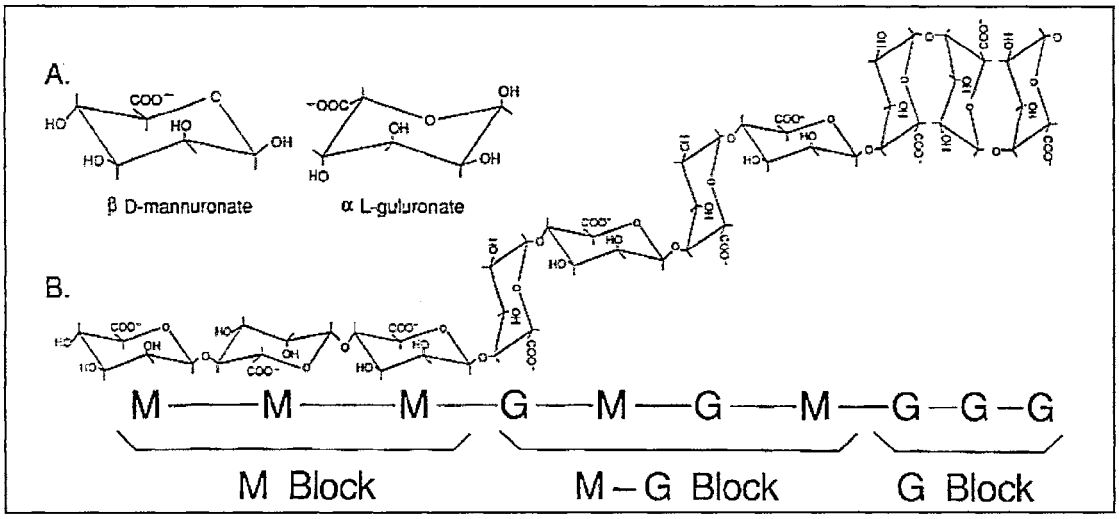


Figure 1. The structure of alginate. Individual monomers of alginate include mannuronic acid (M) and guluronic acid (G). Alginate chain conformation, include homopolymeric M blocks, heteropolymeric M-G blocks, and homopolymeric G blocks. (Franklin et al., 1994).

The biosynthesis of alginate has been extensively studied in *Pseudomonas aeruginosa* where it functions as a virulence factor in strains infecting the lungs of cystic fibrosis (CF) patients (Shankar et al., 1995). In CF patients, *P. aeruginosa* usually resists treatment, because the alginate capsule surrounding the bacterium provides a protective barrier against antibiotics and host immune defenses (Govan and Deretic, 1996; Pier et al., 1994). Alginate may also protect the bacterium from dehydration and facilitate adherence to the lung epithelium.

Other pseudomonads, including *P. fluorescens*, *P. putida*, *P. mendocina*, and *P. syringae* are known to produce alginate (Fialho et al., 1990). As in *P. aeruginosa*, the alginate biosynthetic genes in these species are normally silent and expressed only when

the bacteria undergo a genotypic transition to mucoidy. Possible roles for alginate production in the plant pathogen, *P. syringae*, are varied and include avoidance of host cell recognition, resistance of bacterial cells to desiccation, and enhancement of epiphytic fitness (Kasapis et al., 1994; Lindow, 1991). Alginate has been also implicated in a symptom known as water-soaking where the intercellular tissues of infected plants become filled with water (Fett et al., 1989; Gross and Rudolph, 1987a; 1987b). Using a genetic approach, alginate was shown to contribute to the virulence of *P. syringae* pv. syringae 3525, perhaps by facilitating colonization or dissemination of the bacterium *in planta* (Yu et al., 1999). The same study also indicated that alginate has a role in the epiphytic fitness of *P. syringae*, perhaps helping cells tolerate desiccation and osmotic changes.

Biosynthesis of alginate. The alginate biosynthetic gene cluster is organized as an operon at 34 min on the *P. aeruginosa* chromosome (Chitnis and Ohman, 1993) (Figure 2), and its organization is virtually identical in *P. syringae* (Peñaloza-Vázquez et al., 1997), *Azotobacter vinelandii* (Rehm et al., 1996; Vazquez et al., 1999), and *A. chroococcum* (Peciña et al., 1999). Transcription of these genes in *P. aeruginosa* and *P. syringae* is controlled by the promoter for *algD*, the first gene of the operon (Figure 2) (Chitnis and Ohman, 1993; Peñaloza-Vázquez et al., 1997). Interestingly, the biosynthetic gene *algC* is located away from the biosynthetic cluster at 10 min (Figure 2). The regulatory genes map at 10 and 13 min, and the loci responsible for the genotypic switch to alginate production are located at 68 min (Figure 2) (May and Chakrabarty, 1994).

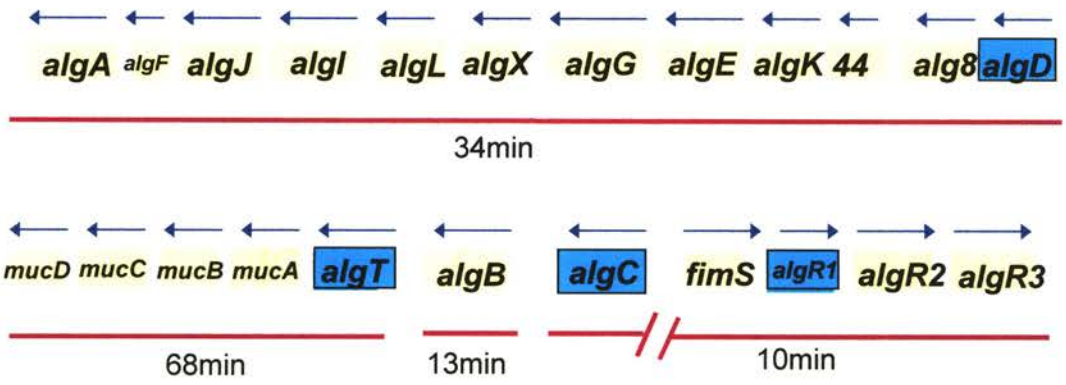


Figure 2. Organization of the alginate gene clusters in *P. aeruginosa*. The alginate genes are clustered at four locations in the *P. aeruginosa* chromosome. Except for the *algC* gene, which is located at 10 min, all of the known alginate structural genes are located at 34 min. The regulatory genes 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 the translation.

Fructose-6-phosphate is the building block for alginate synthesis (Figure 3). The first gene in the alginate pathway, *algA*, encodes a bifunctional enzyme with phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activities (Shinabarger et al., 1991). PMI converts fructose-6-phosphate to mannose-6-phosphate, and the product of *algC*, phosphomannose mutase (PMM) converts mannose-6-phosphate to mannose 1-phosphate (Zielinski et al., 1991). GMP (encoded by *algA*) then converts mannose 1-phosphate to GDP-mannose, which becomes GDP-mannuronic acid as a result of GDP-mannose dehydrogenase (encoded by *algD*) (Figure 3). Subsequent steps in the pathway (i.e. the polymerization and modification of alginate) are not fully understood. One hypothesis is that the polymerization of GDP-mannuronic acid occurs in the periplasm via the *algL*-encoded alginate lyase (Boyd et al., 1993), perhaps with a second protein, AlgX (Figure 3). Evidence exists supporting the requirement of

both AlgL and AlgX in alginate synthesis (Monday and Schiller, 1996). The *algL* gene from *P. syringae* was recently cloned and overexpressed in *E. coli* and proved to be similar to *algL* from *Halomonas marina*, *P. aeruginosa*, *A. chroococcum*, and *A. vinelandii* (Preston et al., 2000; 2001). Recently, Jain and Ohman (1998) demonstrated that an *algK* mutant secreted monomeric uronic acids, providing evidence for the role of AlgK in the polymerization of alginate. In *P. aeruginosa*, epimerization of mannuronic to guluronic acid is catalyzed by *algG*, a C5 epimerase (Chitnis and Ohman, 1990; Franklin et al., 1994) (Figure 3). Recently, *algG* was also characterized in *P. fluorescens* (Morea et al., 2001). The acetylation of mannuronic acid residues is catalyzed by the products of *algF*, *algJ* and *algI* (Franklin and Ohman, 1993; Franklin and Ohman, 1996; Shinabarger et al., 1993). Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins in *P. aeruginosa* was recently investigated and the study suggested that AlgI-AlgJ-AlgF complex may form in the membrane and constitute a reaction center for O-acetylation of alginate (Franklin and Ohman, 2002). Transport of alginate to the extracellular milieu is thought to occur via an outer membrane porin encoded by *algE* (Rehm et al., 1994).

Regulation of alginate biosynthesis in P. aeruginosa. Normally, alginate biosynthetic genes in *P. aeruginosa* are silent, but are activated in the CF lung. The transition to mucoidy is accompanied by transcriptional activation of the *algC* and *algD* promoters (*PalgC* and *PalgD*, respectively). Both *PalgC* and *PalgD* are activated by high osmolarity (Berry et al., 1989; Zielinski et al., 1992) and when cells of *P. aeruginosa* are grown on solid surfaces (Davies et al., 1993; Hoyle et al., 1993). The

latter phenomenon may be relevant to biofilm formation by *P. aeruginosa* in the CF lung and the resistance of mucoid cells to antibiotic treatment and the host immune system (Nichols et al., 1989; Jensen et al., 1990). Other signals for alginate gene activation in *P. aeruginosa* include nutritional starvation and exposure to membrane-damaging agents such as ethanol (DeVault et al., 1989; 1990).

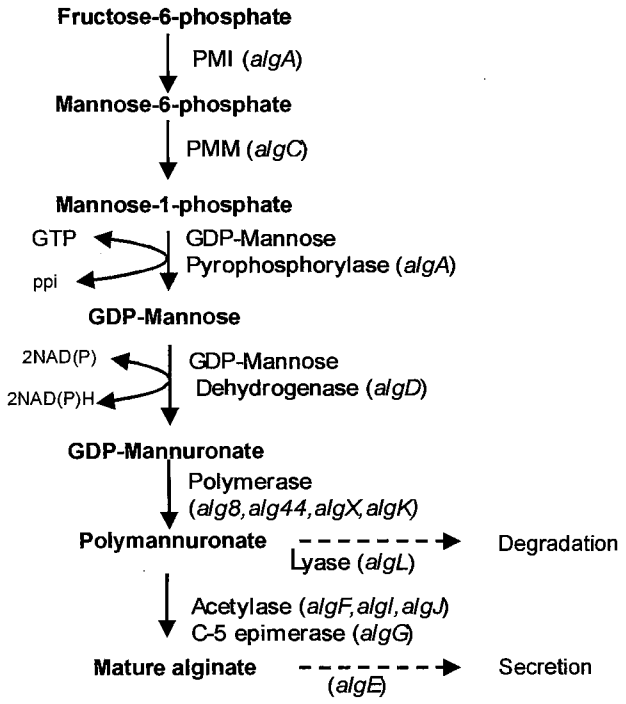


Figure 3. Hypothetical scheme for the biosynthesis of alginate by *P. aeruginosa*. Fructose-6-phosphate is converted to GDP-mannuronic acid, which provides mannuronate residues for polymerization. Guluronate residues may be incorporated into the polymer via the epimerization of mannuronate by the AlgG protein. Mannuronic residues of bacterial alginates are partially O-acetylated by the gene products of *algF*, *algI* and *algJ*. Secretion of mature alginate is catalyzed by the AlgE protein.

The cluster of genes located at 68 min on the *P. aeruginosa* chromosome is responsible for the conversion to mucoidy (Figure 4) and includes *algT* (*algU*), *mucA* (*algS*), *mucB* (*algN*), *mucC* (*algM*), and *mucD* (*algY*) (DeVries and Ohman, 1994; Schurr et al., 1994). These genes are arranged similarly in *A. vinelandii* (Fialho et al., 1990; Martinez-Salazar et al., 1996; Núñez et al., 2000); however, *P. syringae* lacks a *mucC* homologue (Keith and Bender, 1999; Keith and Bender, 2001). The product of *algT* is an

alternate sigma factor, σ^{22}, which is functionally equivalent to σ^{E}, the heat shock sigma factor in *Escherichia coli* and *Salmonella typhimurium* (Yu et al., 1995). The transcription of *algD, algR1* and *algT* are dependent on AlgT (Hershberger et al., 1995; Schurr et al., 1995; Wozniak et al., 1994) (Figure 4). Recently, a global genomic analysis of AlgT (AlgU)-dependent promoters was done in *P. aeruginosa* and AlgT promoters were found upstream of predicted lipoprotein genes suggesting a connection between conversion to mucoidy and expression of lipoproteins (Firoved et al., 2002).

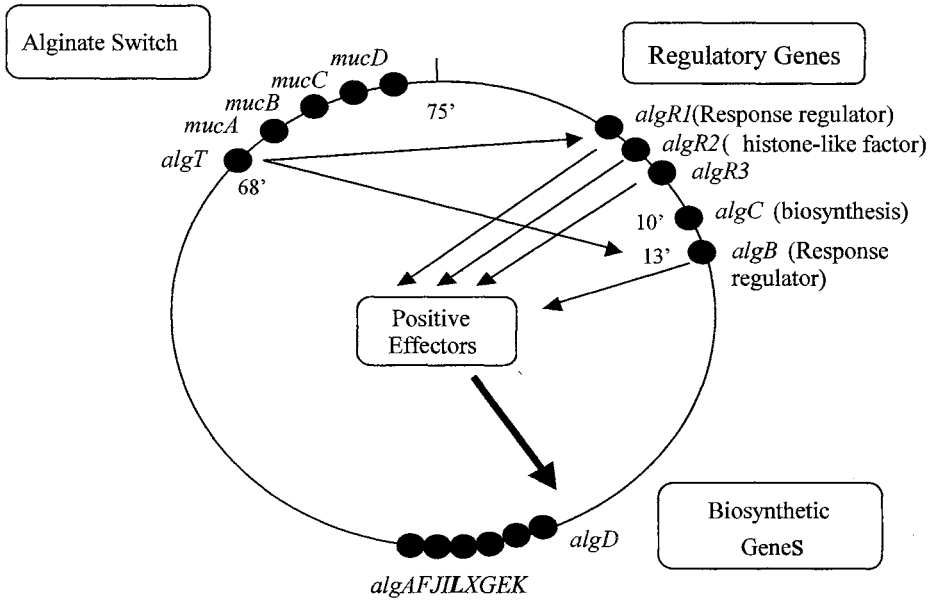


Figure 4. Chromosomal map of *P. aeruginosa* depicting genes involved in alginate biosynthesis.

MucA is a negative regulator of *algT* transcription and encodes an anti-σ factor with affinity for σ^{22} (Schurr et al., 1996; Xie et al., 1996). MucB is a second negative regulator and is thought to interact with the periplasmic domain of MucA, thereby altering its conformation so that it binds σ^{22} and targets it for degradation (Mathee et al.,

1997). In *P. aeruginosa,* mutations in MucA or MucB alleviate the negative regulation they impart on *algT* resulting in the mucoid phenotype. Using real-time PCR, Edwards and Saunders (2001) suggested that *mucB* may have a role in recognition of stress conditions and a disrupted *mucA* gene does not always result in a mucoid phenotype in *P. aeruginosa.* The most common mutations that occur in CF patients are nonsense or frameshift mutations in *mucA*. Reversion from mucoidy to nonmucoidy results from spontaneous mutations in *algT*, which suppresses *mucA* mutations (Schurr et al., 1994). Alternatively, alginate production and expression of *algD* may depend on a second sigma factor, σ^{54} (RpoN), which acts as a negative regulator when *algD* is overexpressed by σ^{22} (Boucher et al., 2000); this phenomenon is referred to as σ factor antagonism.

The gene *mucC* maps downstream of *mucB* in *P. aeruginosa* and was speculated to have a positive regulatory role in alginate production under certain environmental conditions including high salt and elevated temperatures (Boucher et al., 1997). The next gene in the *algT-muc* operon is the negative regulator *mucD,* which is similar to HtrA, a periplasmic serine protease (Boucher et al., 1996). In both *P. aeruginosa* and *P. syringae*, *mucB* and *mucD* are translated in different open reading frames with respect to *algT* and *mucA* (Keith and Bender, 2001; Schurr et al., 1994). The translation of negative regulatory genes (*mucB*, *mucD*) in different reading frames assures that *algT* is not overexpressed in the absence of a negative regulator, since an abundance of σ^{22} is toxic to the cell (Schurr et al., 1994).

Other regulators of the mucoid phenotype include *algR* (*algR1*), *algQ* (*algR2*), *algP* (*algR3*), and *algB*, which are located at 9 min and 13 min, respectively (Goldberg and Dahnke, 1992; Wozniak and Ohman, 1991) (Figure 4). AlgR and AlgB are response

regulators in two-component regulatory systems that activate gene expression through transduction of environmental signals (Parkinson and Kofoid, 1992). AlgR binds to three sites upstream of the *algD* promoter and is required for transcription of *algD* and *algC* (Kato and Chakrabarty, 1991; Mohr and Deretic, 1992; Zielinski et al., 1992). Upstream of *algR* in *P. aeruginosa* is *fimS* (or *algZ*), which has been postulated to function as the cognate sensor kinase for *algR* (Whitchurch et al., 1996; Yu et al., 1997). AlgB belongs to the NtrC subfamily of response regulators (Wozniak and Ohman, 1991) and is required for overproduction of alginate. Ma et al. (1998) identified *kinB* as the cognate sensor kinase for *algB*. In contrast to many other response regulators, phosphorylation of AlgR and AlgB is not required for alginate production (Ma et al., 1998); consequently, phosphorylation-dependent signal transduction is unlikely to function in the regulation of alginate via AlgR and AlgB. AlgP is a histone-like factor presumably involved in DNA folding and the subsequent interaction(s) of AlgR with *alg* promoters (Kato et al., 1990; Konyecsni and Deretic, 1990). AlgQ was originally proposed to be the kinase for AlgR but more recent studies have proven this hypothesis to be incorrect (Deretic et al., 1992).

Regulation of alginate production in P. syringae. Alginate production has been reported in *P. syringae* pv. glycinea, a soybean pathogen, and the molecular weight of *P. syringae* alginate was lower than that produced by *P. aeruginosa* (Osman et al. 1986). Gross and Rudolph (1987b) showed that alginate was produced by seven different *P. syringae* pathovars including: phaseolicola, pisi, lachrymans, aptata, tomato, syringae, and glycinea. Strains that were analyzed soon after isolation from plant material produced more alginate than strains subcultured for an extended time *in vitro*. EPS that

was extracted from diseased leaves exhibiting water-soaked lesions, suggesting that alginate was produced in the water-soaked tissue (Fett and Dunn, 1989).

Although many of the alginate regulatory loci are arranged similarly in *P. aeruginosa* and *P. syringae*, there are significant differences in the regulation of alginate production. As mentioned above, the alternate sigma factor encoded by *algT* (σ^{22}) and the response regulator AlgR1 are required for transcription of *algD* in *P. aeruginosa* (Figure 5). Furthermore, AlgR1-binding sites (ABS; represented by green boxes in Figure 5) are located upstream of the *algD* promoter and are required for full activation of *algD* transcription in *P. aeruginosa*. AlgR1 binds to multiple sites upstream of *algC* and *algD* (Kato and Chakrabarty, 1991; Mohr et al., 1992; Zielinski et al., 1992). Both the *algD* and *algR1* promoters show a consensus sequence at the -35/10 region which is consistent with recognition by σ^{22}, suggesting that a RNAP-σ^{22} complex binds to both promoters and positively regulates transcription (Schurr et al., 1995).

The *algD* promoter region in *P. syringae* (*PsalgD*) was characterized and shown to diverge significantly from the *algD* promoter in *P. aeruginosa* (Fakhr et al., 1999). *algD* expression was stimulated by the addition of copper and also by osmotic stress (Penaloza-Vazquez et al., 1997); furthermore, Singh et al. (1992) showed that osmolarity and dehydration can stimulate alginate production in most fluorescent pseudomonads. Although the *P. syringae algD* promoter (*PsalgD*) lacked the consensus sequence recognized by AlgR1, the consensus sequence recognized by σ^{22} was present, suggesting that *algT* is required for the transcription of *algD* (Fakhr et al., 1999). *algT* was cloned and sequenced from *P. syringae* pv. syringae and showed 90% amino acid identity with its *P. aeruginosa* homologue (Keith and Bender, 1999). AlgT was essential for alginate

production in *P. syringae*, and *algT* expression was activated by heat shock, osmotic stress, and by exposure to paraquat, H_2O_2 and copper sulfate (Keith and Bender, 1999). The *algT-muc* operon was cloned and sequenced from *P. syringae* to determine whether the organization of this gene cluster was conserved in this plant pathogen. Although the *algT* flanking region in *P. syringae* was similar to *P. aeruginosa*, *P. syringae* lacked a *mucC* homologue (Keith and Bender, 2001). More research is needed to fully understand the regulation of alginate biosynthesis in *P. syringae*.

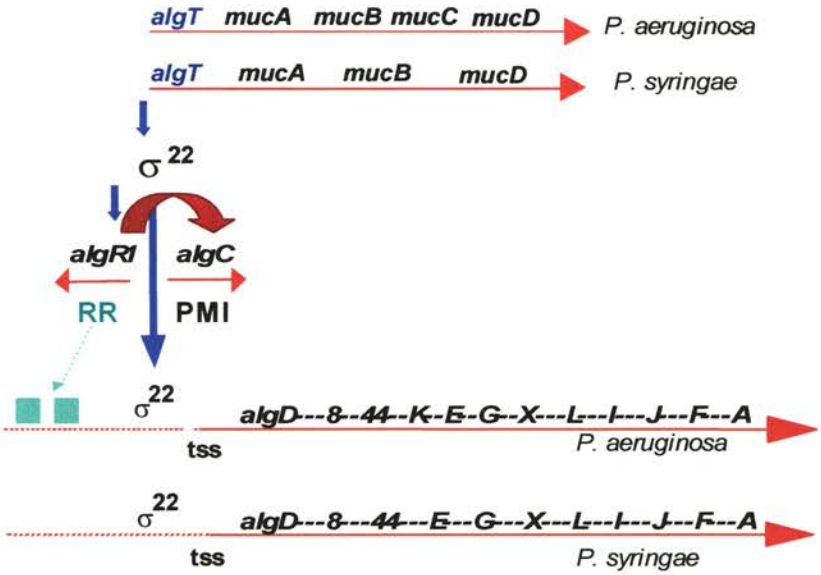


Figure 5. Regulation of the *algD* promoter in *P. aeruginosa* and *P. syringae*. AlgT encodes σ^{22}, which is required for the expression of *algR1* and *algD*. Expression of the *algD* promoter in *P. aeruginosa* also requires AlgR1, which binds upstream of the transcriptional start site (tss) in *P. aeruginosa* (the green boxes indicate AlgR1 binding sites). In *P. syringae*, a σ^{22} recognition site is located upstream of *algD*, but AlgR1 binding sites are not present.

Role of AlgR1 in the regulation of alginate biosynthesis. In *P. aeruginosa*, the transition to mucoidy starts with the activation of the *algD* and *algC* promoters. Both *PalgD* and *PalgC* are activated by specific environmental factors such as osmolarity and adherence to solid surfaces (Shankar et al., 1995). In addition to regulating its own

expression (Kimbara and Chakrabarty, 1989), AlgR1 functions as a positive transcriptional regulator of *algD*, *algC,* and the neuraminidase gene, *nanA* (Calcano et al., 1992). Two AlgR1-binding sites (ABS) were found upstream of *algD*, whereas three ABS were identified for the *algC* gene (two upstream of *algC*, and one located within the gene) (Fujiwara et al., 1993). Interestingly, no AlgR1 binding sites were found upstream of *algD* in *P. syringae* pv. syringae (Fakhr et al., 1999).

The gene *algR1* is related to the response regulator members of two component regulatory genes, including *ompR*, *phoB*, *sfrA*, *ntrC*, *spoA*, *dctD*, and *virG*; these transcriptional activators control cellular reactions to osmotic stress, phosphate limitation, and specific chemicals (Deretic et al., 1989). AlgR1 has been purified and overproduced in *E. coli*, and the purified protein bound to two separate DNA fragments upstream of *algD* (Kato and Chakrabarty, 1991). While the presence of at least one AlgR1 binding site is important for *algD* activation, the presence of both binding sites in the upstream region leads to a higher level of activation.

AlgR1, like other response regulators, contains highly conserved residues known to be critical for the phosphorylation and signal transduction processes. Deretic et al. (1992) demonstrated that AlgR1 undergoes phosphorylation *in vitro* when interacting with the histidine protein kinase, CheA, which indicates that AlgR1 is capable of undergoing phosphorylation typical of other response regulators. Roychoudhury et al. (1992) reported that AlgR2, the 18-kDa protein product of *algR2,* undergoes phosphorylation in the presence of ATP, and the phosphoryl group acquired by AlgR2 is then transferred to AlgR1. The same authors have also shown that AlgR1 can be phosphorylated by an AlgR2 homologue in *E. coli.* However, Ma et al. (1998) showed

that phosphorylation of AlgR1 is not required for alginate production, which suggests that phosphorylation-dependent signal transduction is unlikely to function in the regulation of alginate via AlgR1.

Indigenous plasmids in phytopathogenic bacteria. The presence of indigenous plasmids in phytopathogenic bacteria is thought to confer selective advantages to the host, although in most cases specific traits associated with the plasmids are unknown (Shaw, 1987; Coplin, 1989). The stable maintenance of plasmids in plant pathogens suggests a potential relevance to the host-pathogen interaction; for example, the symbiotic plasmids of *Rhizobium* and the tumor-inducing plasmids of *Agrobacterium* play critical roles in the interaction of these bacteria with their respective plant hosts (Long and Staskawicz, 1993). Plasmid-encoded genes known to be important in the interaction of *P. syringae* with host plants include avirulence genes (Kobayashi et al., 1990) and genes for biosynthesis of ethylene, indoleacetic acid, and the phytotoxin coronatine (Comai and Kosuge, 1980; Bender et al., 1991; Nagahama et al., 1994). The involvement of a 55-kb plasmid in melanin production was reported in a Turkish isolate of *Rhizobium cicer*, while a 130-kb plasmid in the same isolate is thought to be necessary for EPS production and symbiotic functions (Yildiz et al., 1999)

Other plasmid-encoded traits function primarily to enhance fitness and include resistance to bactericidal compounds and ultraviolet radiation (Bender and Cooksey, 1986; Sundin et al., 1994; 1996; Sundin and Murillo, 1999). Some plasmid-encoded traits have been transferred from plant to human pathogens, especially in environmental niches where bacteria indigenous to animals intermingle with plant-associated bacteria

(Sundin and Bender, 1996). Thus, the identification of plasmid-encoded genes and an understanding of the relationships of native plasmids and their hosts is essential in establishing the role of these elements in the evolution of bacteria.

Extracellular C5-epimerases. Alginates from *Azotobacter* contain homopolymeric blocks of mannuronic (M) and guluronic (G) residues, as well as MG blocks (Figure 1). Alginates from *Pseudomonas* spp. differ from those produced by *Azotobacter* because they are not known to contain homopolymeric G-blocks (Sherbrock-Cox et al., 1984). When alginate is first synthesized, it is comprised solely of mannuronic acid residues. G residues are introduced at the polymer level by the periplasmic enzyme, mannuronan C5-epimerase, which is a product of *algG*, a gene encoded by the alginate biosynthetic gene clusters of *P. aeruginosa* (Franklin et al., 1994), *P. syringae* (Peñaloza-Vázquez et al., 1997) and *A. vinelandii* (Rehm et al., 1996). The absence of G-blocks in *P. aeruginosa* indicates that AlgG is unable to introduce repeating G residues into alginate and suggests that this bacterium does not contain an epimerase capable of introducing G-blocks. Previous studies have demonstrated that an *algG* mutant of *P. aeruginosa* produces only polymannuronic acid, which suggests that *algG* is the sole mannuronic epimerase in this bacterium (Franklin et al., 1994).

In *A. vinelandii*, multiple mechanisms exist for the epimerization of mannuronic acid (Rehm et al., 1996). In addition to AlgG, *A. vinelandii* synthesizes a group of extracellular epimerases encoded by the *algE* gene family. These enzymes are capable of introducing alternating and/or repeating G residues into the polymer (Ertesvåg et al., 1995). It is important to note that *algE* is a multicopy gene family and not part of the

alginate biosynthetic gene cluster in *A. vinelandii* (Ertesvåg et al., 1995). The epimerase genes *algE1-4, algE6*, and *algE7* are clustered in the chromosome.

Unlike AlgG, AlgE epimerases are secreted into the growth medium, have a strict requirement for Ca^{2+}, and consist of one or two "A" modules and up to seven "R" modules. The A module consists of 385 amino acids and is presumed to catalyze the epimerization reaction (Ertesvåg and Valla, 1999). The R module(s) is located at the C-terminus of the enzyme with respect to the A module and contains ~155 amino acids (Ertesvåg et al., 1995). Each R module contains four to six repeats of a nonameric amino acid sequence characteristic of Ca^{2+}-binding motifs; furthermore, R modules are responsible for secretion of the enzyme (Ertesvåg and Valla, 1999). The A modules are highly conserved among AlgE epimerases, suggesting a common evolutionary ancestor; however, R modules exhibit more diversity than A modules.

The molecular mass of AlgE epimerases is correlated with the number of A and R modules and ranges from 57.7 kDa (AlgE4) to 191 kDa (AlgE3). Nuclear magnetic resonance (NMR) spectroscopy analysis has shown that AlgE2 and AlgE6 introduce stretches of guluronic residues (G blocks), whereas AlgE4 results in alginates with MG blocks. AlgE7 is unique because it exhibits both epimerase and lyase activity (Svanem et al., 1999). The epimerase function of AlgE7 results in alginates with both single and repeated G residues, whereas the lyase function may provide smaller oligomers needed for cyst formation or cyst germination (Svanem et al., 1999). Svanem et al. (2001) suggested that the catalytic activities of the epimerase and lyase encoded by *algE7* probably originate from the same active site and a complex interplay exists between the two enzymatic activities. AlgE1-AlgE7 and AlgY have been successfully cloned from *A.*

vinelandii and overproduced as functional epimerases in *Escherichia coli* (Ertesvåg et al., 1995; Svanem et al., 1999). To date there are no reports of any extracellular C5-epimerases in *Pseudomonas* spp.

CHAPTER III

The Response Regulator AlgR1 is Required for Alginate Production in *Pseudomonas syringae* pv. syringae, But is Not Required for Transcription of *algD*

Abstract

Both *Pseudomonas aeruginosa* and the phytopathogen *P. syringae* produce the exopolysaccharide alginate. However, the environmental signals that trigger alginate gene expression in *P. syringae* are different, with copper being a major signal. In *P. aeruginosa,* the alternate sigma factor encoded by *algT* (σ^{22}) and the response regulator AlgR1 are required for transcription of *algD*, a gene which encodes a key enzyme in the alginate biosynthetic pathway. In the present study, we cloned and characterized the gene encoding *algR1* from *P. syringae*. The deduced amino acid sequence of *algR1* from *P. syringae* showed 86% identity with its *P. aeruginosa* counterpart. Sequence analysis of the region flanking *algR1* in *P. syringae* revealed the presence of *argH*, *algZ*, and *hemC* in an arrangement virtually identical to that reported in *P. aeruginosa*. An *algR1* mutant, *P. syringae* FF5.32, was defective in alginate production but could be complemented when *algR1* was expressed *in trans*. Unlike *P. aeruginosa*, *algR1* was not required for the transcription of *algD* in *P. syringae*. However, the *algR1* upstream region in *P. syringae* contained the consensus sequence recognized by σ^{22}, suggesting that *algT* is required for transcription of *algR1*.

Introduction

The exopolysaccharide alginate is a copolymer of O-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Rehm and Valla, 1997). Alginate biosynthesis has been extensively studied in *Pseudomonas aeruginosa* where it functions as a major virulence factor in strains infecting the lungs of cystic fibrosis (CF) patients (Pier, 1998). 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 structural genes 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 synthesis, respectively (Aarons et al., 1997; Chu et al., 1991; Jain and Ohman, 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* (*alg60*) (Maharaj et al., 1993; Monday and Schiller, 1996; Wang et al., 1987); 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. A region mapping at 68 min on the *P.*

aeruginosa chromosome harbors a gene cluster consisting of *algT* (*algU*), *mucA*, *mucB* (*algN*), *mucC*, and *mucD*. These genes modulate the conversion to constitutive alginate production; at the head of this regulatory hierarchy is *algT* (*algU*). The alternative sigma factor encoded by *algT*, σ^{22}, is required for transcription of *algD*, *algT*, and *algR1* (Hershberger et al., 1995; Schurr et al., 1995). The *mucA* gene is a negative regulator of *algT* transcription and encodes an anti-sigma factor with affinity for σ^{22} (Schurr et al., 1996; Xie et al., 1996). Mutations in *mucA* inactivate the MucA protein and result in the Alg^+ phenotype; however, these mutations are unstable and spontaneous reversion to the Alg^- phenotype often occurs due to suppressor mutations in *algT* (DeVries and Ohman, 1994; Schurr et al., 1994; Schurr et al., 1996). The remaining *muc* genes also modulate the expression of *algT* and have been described elsewhere (Goldberg et al., 1993; Mathee et al., 1997; Schurr et al., 1996; Xie et al., 1996).

Other genes controlling the regulation of alginate production include *algR1* (*algR*), *algR2* (*algQ*), *algR3* (*algP*), and *algB* (Govan and Deretic, 1996; Shankar et al., 1995). AlgR1 functions as a response regulator member of the two-component signal transduction system and binds to multiple sites upstream of *algC* and *algD* (Kato and Chakrabarty, 1991; Mohr et al., 1992; Zielinski et al., 1992). Both the *algD* and *algR1* promoters show a consensus sequence at the -35/10 region which is consistent with recognition by σ^{22}, suggesting that a RNAP-σ^{22} complex binds to both promoters and positively regulates transcription (Schurr et al., 1995). Like *P. aeruginosa*, phytopathogenic strains of *P. syringae* are normally nonmucoid in vitro. Kidambi et al. (1995) previously showed that exposure to copper ions stimulated alginate production in selected strains of *P. syringae*. Furthermore, an indigenous plasmid designated pPSR12 conferred constitutive alginate

production to *P. syringae* pv. syringae strain FF5. Plasmid pPSR12 does not contain homologs of the biosynthetic or regulatory genes which control alginate production in *P. aeruginosa*; instead this plasmid presumably contains regulatory genes which remain uncharacterized (Kidambi et al., 1995). Mutagenesis of FF5(pPSR12) with Tn*5* resulted in the isolation of alginate defective (Alg^-) mutants, including FF5.31 and FF5.32 (Kidambi et al., 1995). The Tn*5* insertion in FF5.31 was located in *algL*, which encodes alginate lyase. Alginate production in FF5.31 was restored by pSK2, a cosmid clone containing homologues of *algD*, *alg8*, *alg44*, *algG*, *algX*, *algL*, *algF*, and *algA*. The order and arrangement of the alginate 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 (Peñaloza-Vázquez et al., 1997).

In the present study, the Alg^- mutant FF5.32 was shown to contain a Tn*5* insertion in *algR1*. Unlike *P. aeruginosa*, expression from the *P. syringae algD* promoter (*PsalgD*) did not require a functional copy of *algR1*. However, *algR1* was required for alginate synthesis, implying that it activates transcription of other genes required for alginate synthesis.

Materials and Methods

Bacterial strains, plasmids, and media

Table 1 lists the bacterial strains and plasmids used in the present study. *Pseudomonas* spp. were routinely maintained at 28°C on King's medium B (King et al., 1954), mannitol-glutamate medium (Keane et al., 1970), or MG supplemented with yeast

extract at 0.25 g/liter (MGY); *E. coli* strains were grown on LB medium (Miller, 1972) at 37°C. Antibiotics were added to media at the following concentrations (μg/ml): ampicillin (100), tetracycline (25), kanamycin (25), spectinomycin (25), streptomycin (25), piperacillin (250), and chloramphenicol (25).

Molecular genetic techniques

Plasmid DNA was isolated from *Pseudomonas* spp. by alkaline lysis (Sambrook et al., 1989). Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, and isolation of DNA fragments from agarose gels were performed using standard protocols (Sambrook et al., 1989). Genomic DNA was isolated from *P. syringae* using established procedures (Staskawicz, et al., 1984), and a total genomic library of FF5.32 was constructed in pRK7813 as described previously (Barta et al., 1992). Clones were mobilized into nonmucoid recipient strains using a triparental mating procedure and the mobilizer plasmid pRK2013 (Bender et al., 1991).

Table 1. Bacterial strains and plasmids used in this study.

Strains or Plasmids	Relevant characteristics*	Source or Reference
Escherichia coli		
DH5α		Sambrook et al., 1989
Pseudomonas syringae pv. syringae		
FF5	Cu^s; no detectable plasmids, nonmucoid	Kidambi et al., 1995
FF5.31	Cu^r Km^r; contains pPSR12, nonmucoid, *algL*::Tn*5*	Peñaloza-Vázquez et al.,1997
FF5.32	Cu^r Km^r; contains pPSR12, nonmucoid, *algR1*::Tn*5*	Kidambi et al., 1995
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
pCP13	Tc^r; cosmid vector	Darzins and Chakrabarty, 1984
pBluescript SK+	Ap^r; ColEI origin, cloning vehicle	Stratagene
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
pSK4	Sm^r Sp^r; contains a 1.0-kb fragment from *PsalgD* in pRG960sd in the transcriptionally inactive orientation	Peñaloza-Vázquez et al.,1997
pAP32	Tc^r Km^r; contains Tn*5*-inactivated alginate genes from FF5.32 in pRK7813	This study
pAP32.1	Ap^r Km^r; a 5.3-kb *Bam*HI fragment consisting of 2.8 kb from Tn*5* and 2.5 kb of FF5.32 in pBluescript SK+	This study
pMF4	Tc^r; cosmid clone from FF5(pPSR12) in pRK7813	This study
pMF6	Tc^r; cosmid clone from FF5(pPSR12) in pRK7813	This study
pMF6.1	Ap^r; contains a 2.7-kb *Eco*RI fragment from pMF6	This study
pMF6.2	Ap^r; contains a 2.0-kb *Pst*I fragment from pMF6	This study
pMF6.21	Tc^r; 2.0-kb *Pst*I fragment from pMF6.2 in pRK415 in the transcriptionally active orientation with respect to *lacZ* and *algR1*	This study
pMF6.22	Tc^r; 2.0-kb *Pst*I fragment from pMF6.2 in pRK415 in the transcriptionally inactive orientation with respect to *lacZ* and *algR1*	This study
pAD1039	Tc^r; contains *algR1* from *P. aeruginosa* in pCP13	V. Kapatral

DNA fragments were isolated from agarose gels by electroelution (Sambrook et al., 1989) and labelled with digoxigenin (Genius Labelling and Detection Kit; Boehringer Mannheim, Indianapolis, Ind.) or with [α-^{32}P]dCTP according to the Rad Prime DNA Labeling System (Gibco BRL, Gaithersburg, MD). Hybridizations and post-hybridization washes were conducted using high stringency conditions (Sundin and Bender, 1993).

Isolation and quantitation of alginate

Selected strains were inoculated by dilution streaking to MGY agar (three plates per strain) and incubated at 28°C for 72 h. Each plate was handled separately for quantification of alginate. Cells were washed from each plate and resuspended in 0.9% NaCl. Removal of cellular material from the mucoid growth and estimation of alginate content and total cellular

protein were performed as described previously (May and Chakrabarty, 1994). Alginic acid from seaweed (*Macrocystis pyrifera*; Sigma Chemical Co., St. Louis, MO) was used as a standard in these experiments. Mean values of three replicates were expressed as μg alginate per mg of protein.

Glucuronidase assays

Transcriptional activity was initially screened by spotting bacterial suspensions (A_{600nm} of 0.1) on MG agar medium amended with spectinomycin and 20 μg/ml X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide); plates were then incubated at 28°C for 24 to 72 h. Glucuronidase (GUS) activity was quantified by fluorometric analysis of cells grown for 18-20 h in 3 ml MG medium. Fluorescence was monitored with a Fluoroscan II Version 4.0 microplate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 96-well microtiter plates. GUS activity was expressed in U/mg protein with one unit equivalent to 1 nmol of methylumbelliferone formed per min. Values presented for GUS activity represent the average of three replicates per experiment. When significant differences in GUS activity were detected, the experiment was repeated.

DNA sequencing and analysis

Nucleotide sequencing reactions were performed by the dideoxynucleotide method and Ampli*Taq* DNA polymerase (Perkin Elmer, Foster City, Calif.). Automated DNA sequencing was accomplished using an ABI 373A apparatus and the ABI PRISM Dye Primer Cycle Sequencing Kit (Perkin Elmer, Foster City, Calif.). Automated sequencing was provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility.

The Tn*5* insertion in FF5.32 was localized by sequencing the DNA flanking the transposon using the oligonucleotide 5' GGTTCCGTTCAGGACGCTAC, which is derived from the border region of IS*50*. Sequence data were aligned and homology searches were executed using the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Package, Version 9.0. Sequences associated with σ^{22} were located using the MOTIFS program included with the UWGCG software.

Nucleotide sequence accession numbers

The nucleotide sequence shown in this study was deposited in GenBank under accession number AF131199.

Results

Location of Tn*5* insertion in FF5.32

A genomic library of FF5.32 was constructed in pRK7813, and a clone containing the Tn*5* insertion from FF5.32 was recovered and designated pAP32. The internal *Bam*HI site in Tn*5* and 2.5 kb of FF5.32 DNA were cloned from pAP32 into pBluescript SK+ resulting in a clone named pAP32.1 (Fig. 6). A primer specific for the border region of IS*50* was used to sequence approximately 300 bp of FF5.32 DNA flanking the Tn*5* insertion site. This sequence showed 76% nucleotide identity to *algR1* from *P. aeruginosa*, and the Tn*5* insertion was located at nucleotide 51 of *algR1* from *P. aeruginosa* (Deretic et al., 1989). Genomic DNA from FF5(pPSR12) and FF5.32 was digested with *Eco*RI and analyzed by Southern blotting using the 2.3-kb *Hin*dIII/*Eco*RI fragment from pAP32.1 as a probe (Fig. 6).

The probe hybridized to 2.7- and 8.4-kb *Eco*RI fragments in FF5(pPSR12) and FF5.32,

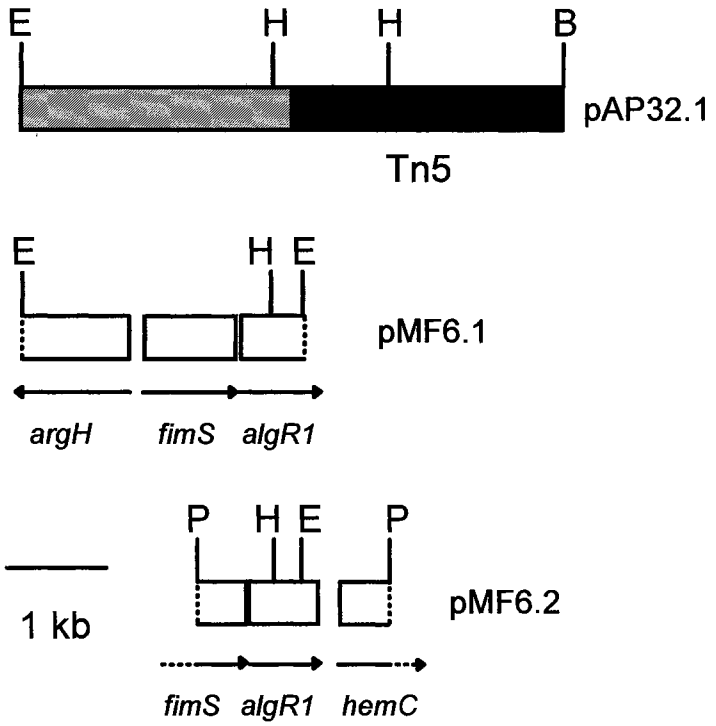


Figure 6. Constructs utilized for the cloning and sequencing of *algR1* from *P. syringae* pv. syringae FF5. pAP32.1 is a subclone containing Tn*5* (shaded region) and flanking DNA from *P. syringae* pv. syringae FF5.32 (cross-hatched region). The *Hin*dIII/*Eco*RI fragment in pAP32.1 was used as a probe for *algR1* in the current study. pMF6.1 and pMF6.2 are subclones derived from pMF6, a cosmid which complemented FF5.32 for alginate production. The 2.0-kb *Pst*I fragment in pMF6.2 was sequenced on both strands and shown to contain an intact copy of *algR1*. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

respectively (data not shown). These results indicated that the region associated with *algR1* was located in a 2.7-kb *Eco*RI fragment, and the 2.7-kb fragment was inactivated by Tn*5* (5.7 kb) in FF5.32.

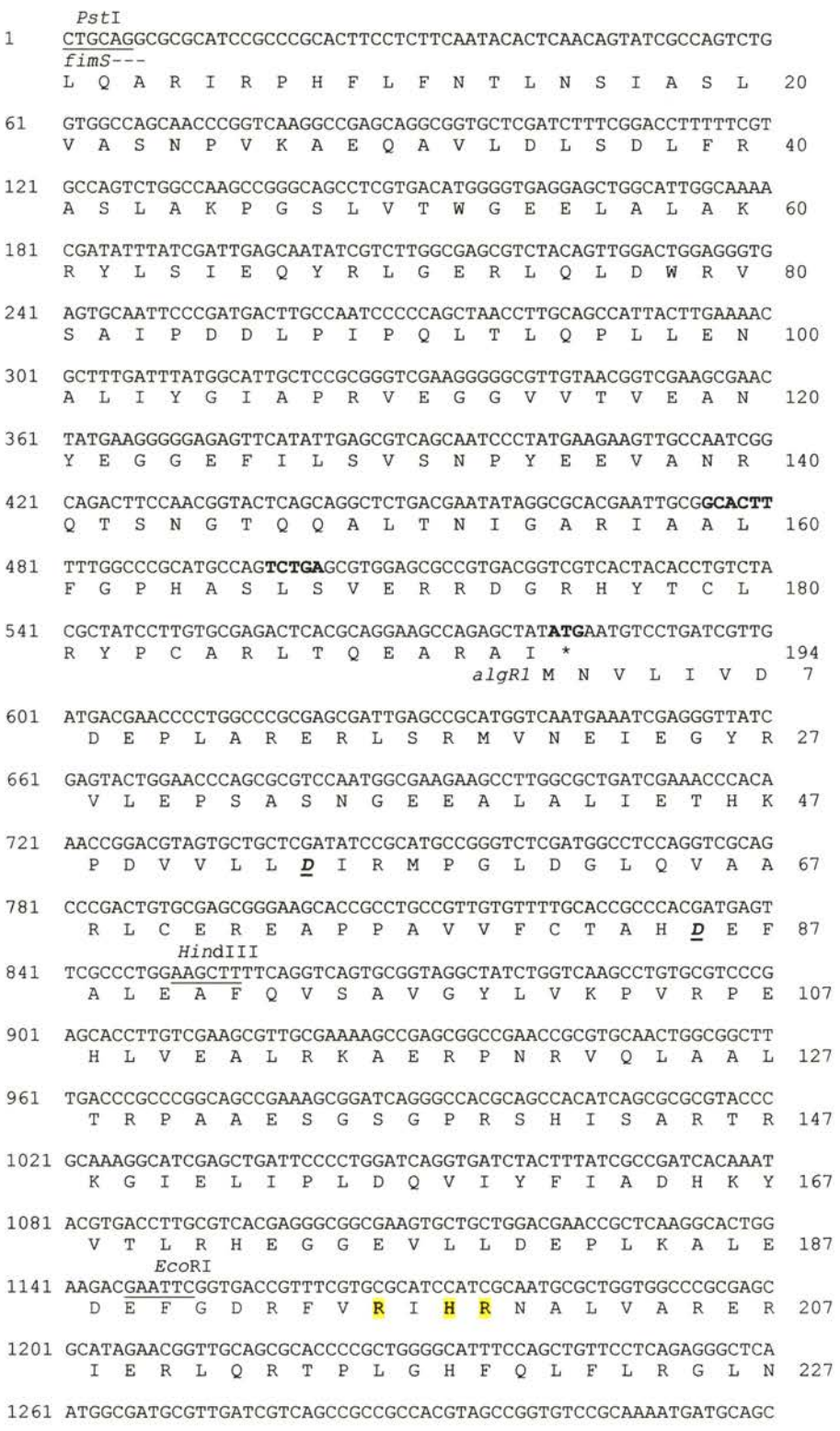
Cloning of *algR1* from *P. syringae*

A genomic library of *P. syringae* pv. syringae FF5(pPSR12) was previously constructed in pRK7813 (Peñaloza-Vázques et al., 1997). In the current study, the 2.3-kb

*Hin*dIII/*Eco*RI fragment from pAP32.1 (Fig. 6) was used to screen the library for clones containing the complete *algR1 coding* region. Seven cosmid clones hybridized with the probe; two clones designated pMF4 and pMF6 were chosen for further study and contained a 2.7-kb *Eco*RI fragment that hybridized with the probe. This fragment was subcloned from pMF6 in pBluescript SK+, resulting in pMF6.1 (Fig. 6). Sequence information for pMF6.1 was generated using the T7 and T3 primers and indicated that this fragment contained DNA homologous to *argH*, *fimS*, and *algR1*. In previous studies, the gene encoding *fimS* showed relatedness to sensor kinases of two-component systems and mapped immediately upstream of *algR1* in *P. aeruginosa* (Whitchurch et al., 1996). It is important to note that *fimS*, which was also named *algZ* (63), is distinct from the *algZ* described by Baynham and Wozniak (Baynham and Wozniak, 1996). To avoid further confusion in nomenclature, *fimS* will be used hereafter to describe the sensor kinase that maps adjacent to *algR1*.

In *P. syringae*, *argH*, which encodes arginosuccinate lyase, mapped adjacent to *fimS*; in *P. aeruginosa*, *argH* was divergently transcribed with respect to both *fimS* and *algR1* (Mohr and Deretic, 1990; Yu et al., 1997). Sequence analysis of pMF6.1 indicated that the same arrangement is conserved in *P. syringae* (Fig. 6). Sequence analysis indicated that pMF6.1 contained 560 bp of *algR1* but lacked approximately 180 bp located at the 3' end. Southern blot analysis of pMF6 and pMF6.1 suggested that the intact *algR1* was likely contained in a 2.0-kb *Pst*I fragment; this was subcloned in pBluescript SK+ and designated pMF6.2 (Fig. 6). The pMF6.2 plasmid was completely sequenced on both strands (Fig. 7) and shown to contain DNA homologous to the 3' end of *fimS* (585 bp), an intact copy of *algR1* (747 bp), and the 5' end of *hemC* (432 bp). In *P. aeruginosa*, *hemC* encodes porphobilonogen deaminase and maps adjacent to *algR1* (Mohr et al., 1994). The *P.*

syringae homologues showed a high degree of relatedness to the corresponding *P. aeruginosa* genes;



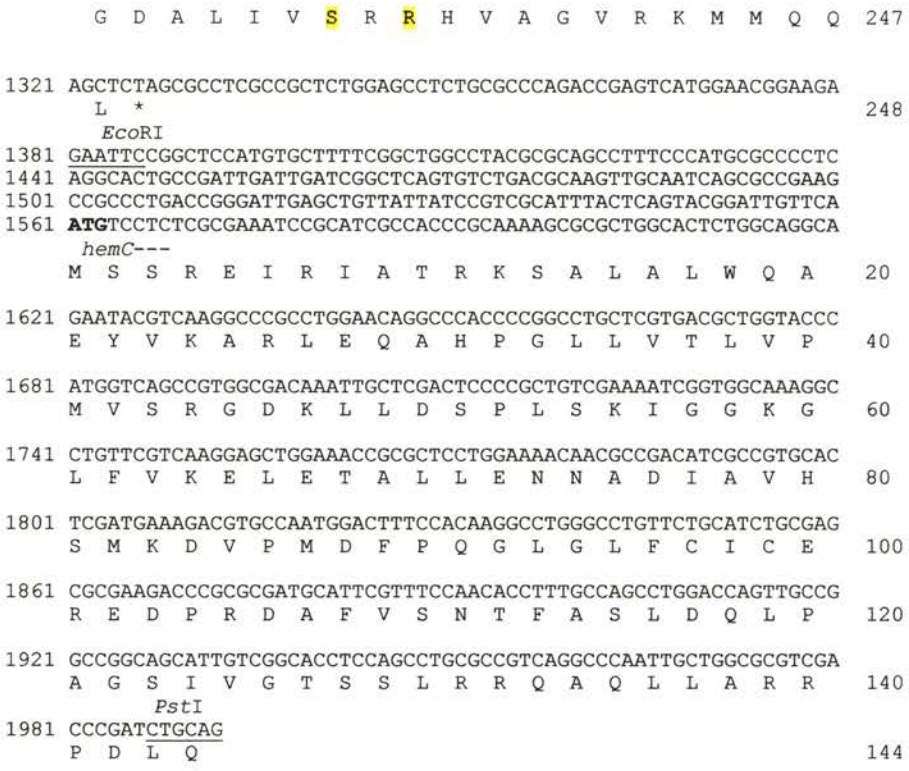


Figure 7. Nucleotide sequence of the 1,992-bp *Pst*I fragment in pMF6.2 from *P. syringae* pv. syringae FF5 containing the 3' end of *fimS*, an intact copy of *algR1*, and the 5' end of *hemC*. Numbering of nucleotide and amino acid residues is shown on the left and right, respectively. Restriction sites are underlined and indicated above the nucleotides. Translational start sequences are indicated in bold and translational stop codons are indicated by asterisks (*). The consensus sequence upstream of *algR1* recognized by σ^{22} is shown in bold, and the aspartate residues presumably involved in the phosphorylation of AlgR1 are italicized and underlined. Conserved amino acid residues of the LytTR DNA binding domain that could be involved in DNA binding are highlighted in yellow.

for example, nucleotide identity between *fimS*, *algR1*, and *hemC* in the two species was 88, 84, and 80%, respectively. Furthermore, the *algR1* homologue in *P. syringae* showed extensive relatedness (86-88% nucleotide identity) to *algR* from *Azotobacter vinelandii* (Núñez et al., 1998) and *pprA*, an *algR1* homologue in *P. putida* (Venturi et al., 1995). In *P. aeruginosa*, AlgR1 contains two aspartate residues (D54 and D85), which have been suggested to function as phosphorylation sites (Ma et al., 1998; Whitchurch et al., 1996); both aspartate residues were present in the predicted translation product of *algR1* from *P.*

syringae (Fig. 7). A DNA-binding domain was found in the C-terminus of the predicted translation product of *P. syringae algR1* and showed relatedness to the newly reported LytTR DNA-binding domain of transcriptional response regulators (Nikolskaya and Galperin, 2002). The conserved amino acid residue potentially involved in DNA binding were identical to those of *P. aeruginosa* AlgR1 (Fig. 7). A consensus sequence for σ^{22} was located 108 bp upstream of the *algR1* translational start site (Fig. 7 and Fig. 8), a location that is also conserved in *P. aeruginosa* (Yu et al., 1997).

σ^{22} Consensus GCACTT ----------17 bp---------- TCTCA

Pa algR1 GG**GCACTT**TTCGGGCCTAAAGCGAG**TCTCA**GCGTCG
Ps algR1 CG**GCACTT**TTTGGCCCGCATGCCAG**TCTGA**GCGTGG

Figure 8. Alignment of the *algR1* promoter sequences from *P. syringae* pv. syringae FF5 (Ps algR1) and *P. aeruginosa* (Pa algR1). The σ^{22} recognition sequence in both species is indicated in **bold** and underscored.

Complementation experiments

pMF4 and pMF6, the cosmid clones containing *argH*, *fimS*, *algR1*, and *hemC*, were evaluated for their ability to complement *P. syringae* pv. syringae FF5.32 for alginate production. Transconjugants of FF5.32 containing pMF4 or pMF6 were visibly mucoid and produced significantly more alginate than the mutant FF5.32 (Table 2). Since Tn*5* frequently causes polar mutations on downstream genes, the 2.0-kb *Pst*I fragment in pMF6.2 was used to investigate whether the Alg$^-$ phenotype in FF5.32 was caused by the mutation in *algR1*. pMF6.2 contains an intact copy of *algR1* with the cognate σ^{22} recognition site and truncated

copies of *fimS* and *hemC* (Fig. 6). The 2.0-kb *Pst*I fragment in pMF6.2 was subcloned in pRK415 to form pMF6.21 and pMF6.22 which contain *algR1* in the transcriptionally active

TABLE 2. Alginate production by derivatives of *P. syringae* pv. syringae FF5.

Strain	Alginate production (μg/mg of protein)[a]
FF5(pPSR12)	3,791 a
FF5.32	401 b
FF5.32(pMF4)	2,635 a
FF5.32(pMF6)	2,619 a
FF5.32(pMF6.21)	3,450 a
FF5.32(pMF6.22)	3,804 a

[a] Mean values followed by the same letter are not significantly different at $P = 0.05$ using Duncan's multiple range test. Values are the means from one experiment containing three replicates.

and inactive orientations with respect to the *lac* promoter (Table 1). Both pMF6.21 and pMF6.22 restored alginate production to FF5.32 (Table 2), indicating that the Alg$^-$ phenotype of FF5.32 was caused by the Tn*5* insertion in *algR1*. FF5.32 was complemented with both clones irrespective of the orientation of the *lac* promoter and without the addition of IPTG, indicating that a functional promoter for *algR1* was present on the 2.0-kb *Pst*I fragment. To further confirm that FF5.32 was indeed an *algR1* mutant, we investigated whether this mutant could be complemented by *algR1* from *P. aeruginosa*. Plasmid pAD1039, which contains *algR1* from *P. aeruginosa* (Table 1), complemented FF5.32 and restored alginate production in the mutant to a level equivalent to FF5(pPSR12) (data not shown).

Expression of the *PsalgD* promoter does not require AlgR1

In *P. aeruginosa*, AlgR1 is required for expression of the *algD* promoter (*PalgD*) and has been shown to bind *PalgD* at several conserved sites (Kato and Chakrabarty, 1991; Mohr et al., 1992). A portion of *PsalgD* was previously cloned as a 1-kb fragment in the promoter

probe vector, pRG960sd, creating pSK3 (*PsalgD::uidA*; transcriptionally active orientation) and pSK4 (*uidA::PsalgD*; transcriptionally inactive) (Peñaloza-Vázquez et al., 1997). In the present study, we investigated whether *PsalgD* was transcriptionally active in FF5.32, the *algR1* mutant. GUS activity in FF5(pPSR12) and FF5.32 containing pSK3 was not significantly different (Table 3), indicating that a functional copy of *algR1* was not required for transcription of *algD* in *P. syringae*.

TABLE 3. Glucuronidase activity (U GUS/mg protein)[a] for *P. syringae* pv. syringae FF5 and FF5.32 containing various promoter constructs with the *algD* upstream region.

Strain[b]	pSK3[c]	pSK4	pRG960sd
FF5(pPSR12)	537 a	88 b	66 b
FF5.32	398 a	82 b	64 b

[a] Mean values followed by the same letter are not significantly different at $P = 0.05$ using the Student-Newman Keuls Test. Values are the means from one experiment containing three replicates.
[b] FF5(pPSR12) is the wild-type and FF5.32 is an *algR1* mutant derived from the former strain.
[c] pSK3 contains the *algD* promoter in the transcriptionally active orientation (*algD::uidA*), pSK4 contains *algD* in the transcriptionally inactive orientation (*uidA::algD*), and pRG960sd is the vector used for construction of pSK3 and pSK4. FF5(pPSR12, pSK3) and FF5.32(pRG960sd) were regarded as positive and negative controls for the GUS assay, respectively.

Discussion

The AlgR1 mutant characterized in the present study, FF5.32, was previously shown to be completely defective in alginate synthesis (Kidambi et al., 1995), thereby demonstrating that AlgR1 is absolutely required for alginate production in *P. syringae*. Yet the role of AlgR1 in *P. syringae* is unclear since this protein is not required for *algD* expression; however, it remains possible that AlgR1 is required for trancriptional activation of *algC* in *P. syringae*, which is true in *P. aeruginosa* (Zielinski et al., 1992). Alternatively, AlgR1 may

function differently in *P. syringae*, perhaps as part of a signal transduction cascade which controls alginate production. A complex regulatory network for alginate synthesis in *P. syringae* seems plausible since plasmid-encoded regulatory genes are known to mediate the constitutive production of alginate in the *P. syringae* strains which harbor them (Kidambi et al., 1995).

The organization of the region flanking AlgR1 is conserved in both *P. aeruginosa* and *P. syringae* (*argH-fimS-algR1-hemC*). In both species, the σ^{22} recognition site preceding *algR1* is located within the 3' end of *fimS* (Yu et al., 1997). FimS shows relatedness to the histidine protein kinases which function as environmental sensors, and both AlgR1 and FimS are required for twitching motility in *P. aeruginosa*, a process mediated by Type IV pili. Although Type IV pili have been identified in *P. syringae* (Roine et al., 1998), our efforts to demonstrate twitching motility in *P. syringae* pv. syringae FF5 were completely unsuccessful; therefore, the involvement of AlgR1 in twitching motility in *P. syringae* remains unclear. It has also been proposed that FimS may function as the cognate sensor kinase for AlgR1, but the exact role of FimS in alginate production remains unclear (Whitchurch et al., 1996; Yu et al., 1997). Interestingly, phosphorylation of AlgR1 was not required for alginate production in *P. aeruginosa* (Ma et al., 1998). Sequence analysis of the *algR1* upstream regions in *P. syringae* revealed the presence of σ^{22} recognition sites (Fig. 8). The σ^{22} recognition site identified in the *algR1* upstream region was identical to that identified in *P. aeruginosa.* Although the transcriptional start site for *algR1* was not identified in *P. syringae*, the position of the σ^{22} recognition site relative to the translational start site are conserved in both species. The conservation of a σ^{22} recognition sequence upstream of *algR1* strongly suggests that transcriptional activation of these genes

requires a functional copy of *algT*. An *algT* homologue in *P. syringae* has recently been identified, and the role of *algT* in the transcriptional activation of *algD* and *algR1* in *P. syringae* has been investigated (Keith and Bender, 1999).

The % nucleotide identity in the *algD* coding region of *P. syringae* pv. syringae and *P. aeruginosa* ranged from 80-90%; however, upstream of the translational start site, relatedness between the two species diverged and nucleotide identity decreased to approximately 20% (Fakhr et al., 1999). This divergence is consistent with the absence of specific sequences in *PsalgD* that are involved in transcriptional activation of *algD* in *P. aeruginosa*. These include the consensus sequences for binding AlgR1 (Kato and Chakrabarty, 1991), integration host factor (Mohr and Deretic, 1992), and cyclic AMP receptor protein (DeVault et al., 1991). The absence of the conserved motifs for AlgR1 binding could explain why the *P. syringae algD* promoter does not require a functional copy of *algR1* for transcriptional activity.

Although some signals for activation of the *algD* promoter are conserved in *P. aeruginosa* and *P. syringae* (Berry et al., 1989; Leitáo et al., 1992; Peñaloza-Vázquez et al., 1997), the *algD* promoter in *P. syringae* is stimulated by exposure to copper ions (Peñaloza-Vázquez et al., 1997) and does not require a functional copy of AlgR1 for transcriptional activation. Recently, Yu et al. (Yu et al., 1999) provided the first genetic evidence for the role of alginate in the virulence and epiphytic fitness of *P. syringae*. Consequently, the differential regulation of *algD* expression in *P. syringae* and CF isolates of *P. aeruginosa* and the marked divergence in their *algD* promoter regions likely reflects their adaptation to plant and human hosts, respectively.

CHAPTER IV

Role of AlgR1 in the Activation of *algC* in *Pseudomonas syringae* pv. syringae

Abstract

In *Pseudomonas aeruginosa*, the *algC* and *algD* genes, which encode phosphomannomutase and GDP-mannose dehydrogenase, respectively, are under positive control of the response regulator, AlgR1. Although AlgR1 was not required for the activation of *algD* in the related bacterium *Pseudomonas syringae*, an *algR1* mutant of *P. syringae* was nonmucoid, indicating an undefined role of *algR1* in alginate biosynthesis (Chapter III). In this study, AlgR1 was investigated as to whether it is a positive activator of *algC* expression in *P. syringae* and if AlgR1 specifically binds the *algC* promoter region. A 2.6-kb *Hin*dIII–*Sst*I probe containing *algC* from *P. aeruginosa* was used to screen a genomic library of *P. syringae* pv. syringae strain FF5(pPSR12). A cosmid clone designated pMF8 hybridized to the probe and was shown to contain the *algC* homologue from *P. syringae* as a 3.9-kb *Xho*I fragment. The 3.9-kb fragment was cloned in pBluescript, and sequence analysis of the *algC* promoter region indicated the presence of four putative AlgR1 binding sites, which are similar to those previously reported in *P. aeruginosa*. Using PCR, an 810-bp fragment was amplified that contains 456 bp of the *algC* upstream region plus 354 bp of the 5' coding region of *algC*. This 0.8-kb fragment was cloned in the transcriptionally active orientation in pBBR.Gus, which contains a promoterless glucuronidase gene (*uidA*). The *PsalgC-uidA* transcriptional

fusion was used to monitor *algC* transcription in strain FF5.7, an *algR1* mutant of *P. syringae* pv. syringae FF5. Expression of the *P. syringae PsalgC-uidA fusion* was reduced ~two-fold in FF5.7 with respect to the wild-type strain, FF5. This indicates that AlgR1 is required for full activation of *algC* transcription in *P. syringae* pv. syringae. *P. syringae* AlgR1 was successfully overproduced in *E. coli* as a C-terminal translational fusion to the maltose binding protein (MBP). Gel shift experiments indicated MBP-AlgR1 strongly binds to the *algC* promoter. These results, along with the gene expression studies, indicate that AlgR1 has a positive role in the activation of *algC* in *P. syringae*.

Introduction

Alginate, a co-polymer of O-acetylated β-1,4-linked D-mannuronic acid and L-guluronic acid, has been reported to function in the virulence of *Pseudomonas syringae* by facilitating dissemination of the bacterium *in planta* and by enhancing epiphytic fitness (Yu et al., 1999). Alginate biosynthesis has been extensively studied in *Pseudomonas aeruginosa*, where it functions as a major virulence factor in strains infecting the lungs of cystic fibrosis (CF) patients (Pier, 1998). The alginate genes are normally silent in *P. aeruginosa* but are specifically activated during growth of this organism in the lungs of CF patients. Activation involves two critical promoters, *PalgC* (Zielinski et al., 1991; 1992) and *PalgD* (Deretic et al., 1987). The *algC* and *algD* promoters, while mapping at two separate locations on the *P. aeruginosa* chromosome, are nevertheless both responsive to environmental signals such as high osmolarity (Berry et al., 1989; Zielinski et al., 1992).

The *algD* gene, which encodes GDP-mannose dehydrogenase (Deretic et al., 1987), is the first gene to be transcribed in the alginate biosynthetic cluster of both *P. aeruginosa* and *P. syringae* (Chitnis and Ohman, 1993; Peñaloza-Vázquez et al., 1997). *algC*, which does not map with *algD* and the other alginate structural genes, encodes phosphomannomutase (PMM), an enzyme that catalyzes the second step in alginate biosynthesis by converting mannose-6-phosphate to mannose-1-phosphate. AlgC is also involved in lipopolysaccharide (LPS) biosynthesis through its phosphoglucomutase activity, which is required for the synthesis of the complete LPS core (Coyne et al., 1994). AlgC also participates in rhamnolipid production, presumably by catalyzing the conversion of glucose-6-phosphate to glucose-1-phosphate, the first step in the deoxy-thymidine-diphospho-L-rhamnose (dTDP-L-rhamnose) pathway (Olvera et al., 1999).

In *P. aeruginosa*, both *algD* and *algC* are under positive control of AlgR1, which functions as a response regulator member of the two-component signal transduction system and binds to multiple sites upstream of *algC* and *algD* (Deretic et al., 1989; Kato and Chakrabarty, 1991; Mohr et al., 1992; Zielinski et al., 1992). There are two AlgR1-binding sites (ABS) upstream of *algC* (Zielinski et al., 1992) and one binding site inside the coding region of *algC* in *P. aeruginosa* (Fujiwara et al., 1993). The high affinity ABSs, such as *algC*-ABS1, *algD*-ABS1, and *algD*-ABS2, contain a core sequence ($CCGTTCGTCN_5$), whereas the two weakly binding ABSs, *algC*-ABS2 and *algD*-ABS3, which occur near or within the coding region contain, 1-2 bp deviations from the core sequence (Mohr et al., 1992; and Zielinski et al., 1992). The high affinity ABS, *algC*-ABS3, also contains a single nucleotide derivation from the consensus (Fujiwara et al., 1993).

Expression of *P. aeruginosa algC* was up-regulated in biofilm cells compared with planktonic cells in liquid medium (Davies et al., 1993). Furthermore, *algC* was activated shortly after attachment of *P. aeruginosa* to Teflon or glass, suggesting that adherence to solid surfaces may be a signal for alginate production (Davies et al. 1993). Fujiwara and Chakrabarty (1994) demonstrated that *algC* contains a long (244 bp) 5' untranslated leader region (5' UTR), which was hypothesized to enhance the translational efficiency of the *algC* transcript. Like other genes in the alginate regulon, transcriptional activity of *algC-lacZ* fusions increased with osmotic stress (Zielinski et al., 1992).

P. aeruginosa AlgR1 was previously overproduced, purified, and shown to be required for transcriptional activation of both the *algD* and *algC* promoter regions (Kato and Chakrabarty, 1991; Mohr et al., 1992; Zielinski et al., 1992). The *algR1* gene was also cloned from *P. syringae* pv. syringae; however, a functional copy of *algR1* was not required for *algD* transcription in *P. syringae* (Chapter III). Interestingly, *algR1* was not required for *algD* transcription in *Azotobacter vinelandii* (Núñez et al., 1999), suggesting that its role in *P. syringae* and *A. vinelandii* is not identical to *P. aeruginosa*.

The objective of this study was to determine whether AlgR1 is involved in the regulation of alginate production in *P. syringae* by functioning as a positive activator of *algC*. Transcriptional fusions and gel retardation studies were utilized to convincingly demonstrate that AlgR1 functions in the transcriptional activation of *algC* by binding to its promoter region.

Materials and Methods

Bacterial strains, plasmids, and media

Table 4 lists the bacterial strains and plasmids used in the present study. *Pseudomonas* spp. were routinely maintained at 28°C on King's medium B (King et al., 1954), mannitol-glutamate medium (Keane et al., 1970), or MG supplemented with yeast extract at 0.25 g/liter (MGY). *E. coli* strains were grown on LB medium (Miller, 1972) at 37°C. Antibiotics were added to media at the following concentrations (μg/ml): ampicillin (100), tetracycline (25), kanamycin (25), streptomycin (25), and chloramphenicol (25).

Molecular genetic techniques

Plasmid DNA was isolated from *Pseudomonas* spp. by alkaline lysis (Sambrook et al., 1989). Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, colony hybridization, and isolation of DNA fragments were performed using standard protocols (Sambrook et al., 1989). Plasmid DNA was prepared for DNA sequencing using the Plasmid DNA Midi Kit from Qiagen (Qiagen, Valencia, Calif.), and genomic DNA was isolated from *P. syringae* using established procedures (Staskawicz et al., 1984). Clones were mobilized into nonmucoid recipient strains using a triparental mating procedure and the mobilizer plasmid pRK2013 (Bender et al., 1991). The cosmid pAP32, which contains *algR1*::Tn*5*, was mobilized into *P. syringae* pv. syringae FF5, and the *algR1* homologue was inactivated by homologous recombination (Bender et al., 1991), resulting in the *algR1* mutant, FF5.7.

DNA fragments were isolated from agarose gels by electroelution and labeled with digoxigenin (Genius Labeling and Detection Kit; Boeringer Mannheim, Indianapolis, Ind.) or with [α-^{32}P]dCTP using the Rad Prime DNA Labeling System (Gibco BRL, Gaithersburg, Md.). Hybridization and post-hybridization washes were conducted under high-stringency conditions. The *P. syringae* pv. syringae FF5(pPSR12) genomic library was screened for an *algC* homologue by hybridization with pNZ15 (containing *algC* from *P. aeruginosa*); hybridization was conducted for two days at 42°C (Fett et al., 1992).

Table 4. Bacterial strains and plasmids used in this study.

Strains or Plasmids	Relevant characteristics*	Source or Reference
Escherichia coli		
DH5α		Sambrook et al., 1989
P. syringae pv. syringae		
FF5	Cu^s; no detectable plasmids, nonmucoid	Kidambi et al., 1995
FF5.7	Cu^s Km^r; nonmucoid, *algR1* mutant of FF5, *algR1*::Tn*5*	This study
FF5.31	Cu^r Km^r; contains pPSR12, nonmucoid, *algL*::Tn*5*	Peñaloza-Vázquez et al., 1997
FF5.32	Cu^r Km^r; contains pPSR12, nonmucoid, *algR1*::Tn*5*	Kidambi et al., 1995
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
pRK7813	Tc^r; cosmid vector	Jones and Gutterson, 1987
pBluescript SK+	Ap^r; ColEI origin, cloning vehicle	Stratagene
pBBR.Gus	Cm^r; 6.6-kb promoter probe broad-host-range vector containing the *uidA* gene	Peñaloza-Vázquez and Bender, 1998
pNZ15	Km^r; contains 2.6-kb *Hin*dIII/*Sst*I fragment with *algC* from *P. aeruginosa* in pJRD215	Zielinski et al., 1991
pMAL-c2	Ap^r; ColEI origin, *tac* promoter, encodes *lacI*^{q} *malE* LacZα, contains factor X_a cleavage site	New England Biolabs
pAP32	Tc^r Km^r; contains Tn*5*-inactivated alginate genes from FF5.32 in pRK7813	Chapter III
pMF6	Tc^r; cosmid clone from FF5(pPSR12) in pRK7813	Chapter III
pMF6.2	Ap^r; contains a 2.0-kb *Pst*I fragment from pMF6	Chapter III
pMF6.4	Ap^r; contains *algR1* on a 0.747-kb *Bam*HI/*Pst*I	This study

	fragment derived from pMF6.2 by PCR cloning in pMAL-c2	
pMF8	Tc^r; cosmid clone from FF5(pPSR12) in pRK7813	This study
pMF8.1	Ap^r; contains a 3.9-kb *Xho*I fragment from pMF8 in pBluescript SK+	This study
pMF8.2	Ap^r; contains a 0.8-kb *Xho*I/*Hin*dIII-fragment amplified by PCR from pMF8.1 and cloned in pBluescript SK+	This study
pMF8.3	Cm^r; contains a 0.8-kb *Xho*I/*Hin*dIII-fragment of the *algC* promoter from pMF8.1 in pBBR.Gus in the transcriptionally active orientation (*algC-uidA*)	This study

The 0.8-kb *Xho*I/*Hin*dIII DNA fragment that contains the *algC* promoter region of *P. syringae* pv. syringae FF5(pPSR12) was cloned by PCR amplification using plasmid pMF8.1 as a template. Two primers were synthesized by the OSU Recombinant DNA/Protein Resource Facility: forward primer, 5'-CCCAAGCTTCTCGAGTTCACGCCC (*Xho*I site is underscored); and reverse primer, 5'-CCCAAGCTTGCCGTTGTAGTCCTT (*Hin*dIII site is underscored).

The 0.747-kb *Bam*HI/*Pst*I DNA fragment containing *algR1* gene from *P. syringae* pv. syringae FF5(pPSR12) was constructed by PCR amplification using plasmid pMF6.2 as a template. Two oligonucleotide primers were synthesized by the OSU Recombinant DNA/Protein Resource Facility: forward primer, 5' TGCGGATCCATGAATGTCCTGATCGT (*Bam*HI site is underscored); and reverse primer, 5'-TACCTGCAGCTAGAGCTGCTGCATCAT (*Pst*I site is underscored).

Glucuronidase (GUS) activity was quantified by fluorometric analysis of cells grown for 12-72 h in 100 ml MG medium supplemented with 0.2 M NaCl. The initial inoculum was adjusted to OD_{600}=0.1. Fluorescence was monitored with a Fluoroscan II Version 4.0 microplate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 96-well microtiter plates. GUS activity was expressed in U/mg protein with one unit equivalent

to 1 nmol of methylumbelliferone formed per min. Values presented for GUS activity represent the average of three replicates per experiment. When significant differences in GUS activity were detected, the experiment was repeated.

DNA sequencing and analysis

Automated DNA sequencing was provided by the OSU Recombinant DNA/Protein Resource Facility and was performed with an ABI 373A apparatus and the ABI PRISM Dye Primer Cycle Sequencing Kit (Perkin-Elmer, Foster City, Calif.). Oligonucleotide primers used for sequencing were also synthesized by the OSU Recombinant DNA/Protein Resource Facility. Sequence manipulations, amino acid alignments, and restriction maps were constructed using the Vector NTI Suite, Version 6.0 (Informax, San Francisco, CA). Database searches were performed with the BLAST service of the National Center for Biotechnology Information.

Overproduction of fusion proteins

Overproduction of fusion proteins was evaluated in *E. coli* DH5α. Cells were grown at 18°C in Terrific Broth to an OD_{600} of 0.4 to 0.5, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubated an additional 6 h. Aliquots of cells (1 ml) were removed before and after induction, pelleted by centrifugation, resuspended in lysis buffer (Sambrook et al., 1989), and incubated on ice for 30 min. The cell suspension was then sonicated as described previously (Riggs, 1994) and centrifuged at 14,000 X *g* for 20 min at 4°C. The pellet was discarded, and the supernatant (which contains the soluble fraction of the crude extract) was analyzed by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel (Sambrook et al., 1989).

Maltose binding protein (MBP)-AlgR1 fusion proteins used in gel retardation analysis were isolated from *E. coli* DH5α cells grown as described above except the cells were grown for 15 hrs after induction with 1 mM IPTG. Subsequent steps were performed at 0 to 4°C in TEDG buffer (50 mM Tris [pH 7.5], 0.5 mM EDTA, 2 mM dithiothreitol, 10% [wt/vol] glycerol). Cells were harvested by centrifugation (500 X *g*, 1 min), supernatants were discarded, and cells were washed in TEDG buffer and collected by centrifugation. Cells were then resuspended in TEDG and lysed by sonication. Lysates were centrifuged at 23,000 X g for 10 min at 4°C; supernatants were then collected and used in gel shift assays.

Gel shift assays

To facilitate end labeling with [α-^{32}P]dCTP, DNA fragments used for gel retardation were excised with enzymes that generate 5' overhanging ends. DNA fragments were then separated on 5% polyacrylamide gels and end-labeled with [α-^{32}P]dCTP (Sambrook et al., 1989). Gel retardation assays were performed by incubating 100 ng total cellular protein with 2,000 cpm of end-labeled DNA in binding buffer [10mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 10% glycerol, and 1 μg of poly(dI-dC)]. After 60 min on ice, 2 μl of loading buffer (binding buffer supplemented with 0.4% bromophenol blue and 1% glycerol) was added, and the samples were loaded onto a 5% polyacrylamide gel. After electrophoresis, the gels were dried and autoradiographed.

Results

Cloning of the *algC* promoter from *P. syringae*

In the current study, a 2.6-kb *Hin*dIII/*Sst*I fragment from pNZ15 (containing *algC* and promoter region from *P. aeruginosa*) was used to screen a genomic library of *P. syringae* pv. syringae FF5(pPSR12) for clones containing the *P. syringae algC* homologue. A cosmid clone designated pMF8 hybridized with the probe and was chosen for further study. Restriction digestion of pMF8 and Southern blot analysis revealed a 3.9-kb *Xho*I fragment that hybridized to the probe; this fragment was isolated by electroelution, and ligated to pBluescript II SK+, forming pMF8.1 (Table 4 and Fig. 9). Sequence analysis of pMF8.1 showed that the 3.9-kb insert contains a homologue of *algC* with 456 bp of its upstream region. Two PCR primers were designed (Figure 10, highlighted in yellow) to amplify the *P. syringae algC* promoter region from pMF8.1. The amplified fragment was 810 bp and contained 456 bp upstream of the translational start site of *algC* and 354 bp of the coding region. This 0.8-kb fragment was cloned in pBluescript SK+ and pBBR.Gus as a *Xho*I/*Hin*dIII fragment, resulting in pMF8.2 and pMF8.3, respectively (Table 4 and Fig. 9).

Sequence analysis of the *algC* promoter region

$CCGTTCGTCN_5$ was previously reported as a consensus sequence recognized and bound by AlgR1, and AlgR1-binding sites (ABS) were identified in the promoter regions of *algD* and *algC* from *P. aeruginosa* (Kato and Chakrabarty, 1991; Zielinski et al., 1992). Nucleotide sequencing (Figure 10) of the region upstream of the translational

start site of *P. syringae algC* contained two putative ABS. The two binding sites, located at 420 and 116 bp upstream of the predicted ATG, resemble *algC*-ABS1 and *algC*-ABS2 in *P. aeruginosa* (Zielinski et al., 1992) with two and three mismatches in the core sequence, respectively. Interestingly, within the *P. syringae algC* coding region, two putative ABSs were located 45 and 306 bp downstream from the ATG (Fujiwara et al., 1993). The putative ABS at 306 bp contains only one divergent base from the core sequence identified in *algC*-ABS3 of *P. aeruginosa* (Fujiwara et al., 1993). The location of the three *algC*-ABSs in *P. aeruginosa* and the four putative *algC*-ABSs in *P. syringae* are shown in Figure 12.

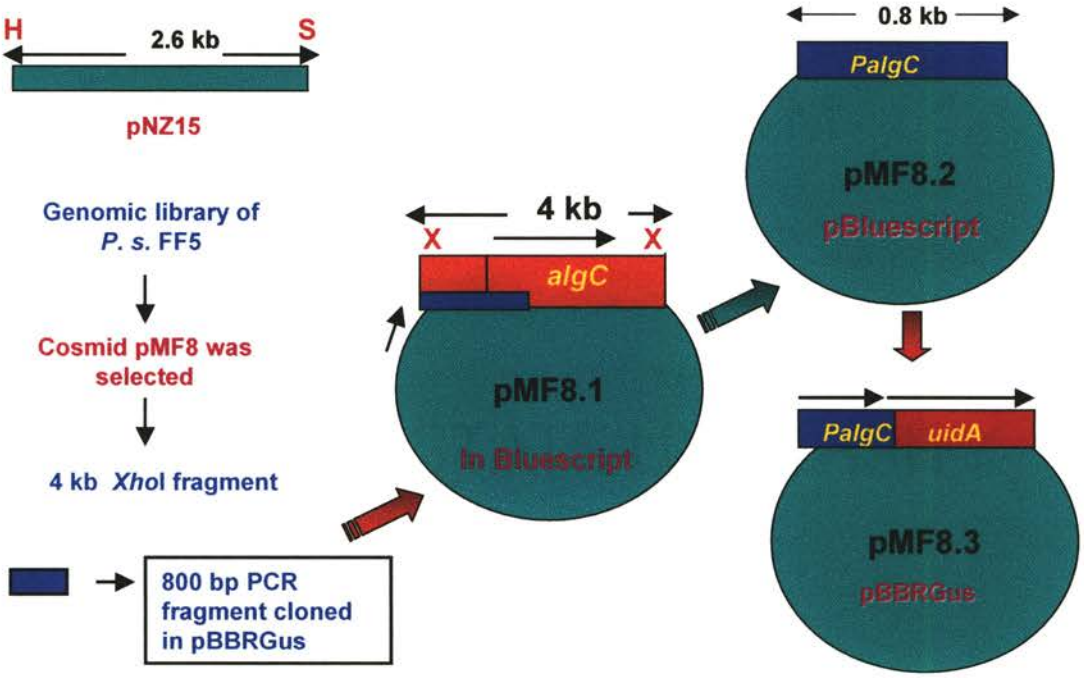


Figure 9. Cloning of the *algC* promoter region from *Pseudomonas syringae* pv. syringae.

The sequence of the *P. syringae algC* promoter contained three putative sequences resembling σ^{54} recognition motifs with the consensus GG-N_{10}-GC (Figure 10). This suggests that transcription of *algC* requires *rpoN*, which encodes the alternate sigma factor, σ^{54}. In *P. aeruginosa*, two σ^{54} recognition motifs were reported upstream of *algC*, and transcription of *algC* was significantly reduced in a *rpoN* mutant of *P. aeruginosa* (Zielinski et al., 1992).

BLAST analysis

BLAST analysis of the 1-kb region shown in Figure 9 showed 66% nucleotide identity to *algC* and its upstream region in *P. aeruginosa*. The 544 nucleotides of the

```
  1 CTCGAGTTCACGCCCGGTCCCGGGCTGAAAACCTCTCCGTCCGTGATGCTTATGCTGCTGGCATT
 66 GATTATCGCTGCAGCGGGTGTGCTGCTTGGCTTGCACCGCAATGAACGGGCCCTGCAGAAGCGTA
131 TTTGCGCCGACGCACGGCAACTGGATCAGCTGCTCCAAGAACTCTCACGCGGAAAAGCCGTCAAA
196 CCCTTCGGTTTGAGCCTCCCGGCCCTTAGTGGTCTGGCTCAGGCACTTGCTCGTGTCTCGCTTCG
261 TAGTCAGCCTCAGGCAGCGCCTGTCCTGGCGGCGGGCGAGCAGCGCGATGCAGATATTGCCAAAG
326 GCAGTGCTTCGTCCACCGTGGCTGCATCACGCACCGACTGGACCGATCCGCTGTTTCAAGATACC
391 GATATCCTTGATATCGACCTTCTCGACGAAAACCAGGACTTCCTGAGATCGGAGCATAACCTCGC
456 TATGAACAGCCCAGCATCCGTGGCACCCAACCTTCCCGAGACTATTTTCCGTGCCTACGACATTC
521 GTGGCGTGGTCGGGGACACCCTCAACGCTGAAACGGCTTACTGGATCGGCCGCGCCATTGGCTCC
586 GAAAGCCTGGCGCAGAACGAACCCAACGTCAGCGTTGGCCGCGACGGTCGCCTTTCCGGCCCTGA
651 ACTGGTAGAGCAACTGATTCAAGGTCTGCACGACAGCGGCTGCCACGTCAGCGATGTGGGCCTGG
716 TGCCAACGCCTGCGCTGTATTACGCAGCCAACGTGCTGGCCGGCAAGACCGGCGTCATGCTGACC
781 GGCAGCCACAACCCCAAGGACTACAACGGCTTCAAGATCGTCATCGCCGGCGACACCCTCGCCAA
846 CGAGCAGATCCAGGCACTGCACGAGCGCATCAAGACCAACAACCTGACGTCGCAGAAAGGCAGCA
911 TCACCAAAGTCGACATCCTTGATCGCTACTTCCAGCAGATCAAGAATGACATCGTCATGGCGCGC
976 AAGCTGAAGGTCGTGGTCGACTGCG
```

Figure 10. Nucleotide sequence of the DNA region upstream and downstream of the *algC* translational start site of *P. syringae* pv. *syringae* FF5. Nucleotides are numbered on the left side of the sequence and only the coding strand is shown. The translational start site (ATG) is highlighted in green. Sequences that are homologous to the consensus *rpoN* recognition sequence, **GG**-N10-**GC** are shown in violet and underlined. The four putative AlgR1 binding sites are shown in **blue** with the bases that diverge from those in *P. aeruginosa* indicated in **red**. The PCR primers used to amplify the 0.8-kb promoter fragment are highlighted in yellow.

algC coding region in *P. syringae* pv. syringae showed 87% amino acid similarity with *P. aeruginosa* phosphomannomutase (AlgC). It also showed 70 and 66% amino acid similarity with phosphoglucomutase of *Ralstonia solanacearum* and *Neisseria meningitidis*, respectively. Sequence analysis of ~2 kb downstream of *algC* in *P. syringae* pv. syringae showed 92% amino acid similarity to acetylglutamate kinase (ArgB) from *P. aeruginosa* (GenBank accession no. AE004945). The *argB* homologue in *P. syringae* pv. syringae that follows *algC* is oriented in the same direction with respect to transcription as previously described for *P. aeruginosa*. The DNA downstream of the *argB* homologue in *P. syringae* pv. syringae shows 77% amino acid similarity to a hypothetical protein in *P. aeruginosa* (PA5330; GenBank accession no. AE004945). Interestingly, the *P. aeruginosa* genome contains six additional genes between *argB* and the hypothetical protein (PA5330) that are absent in this region of the *P. syringae* pv. syringae genome (Figure 11).

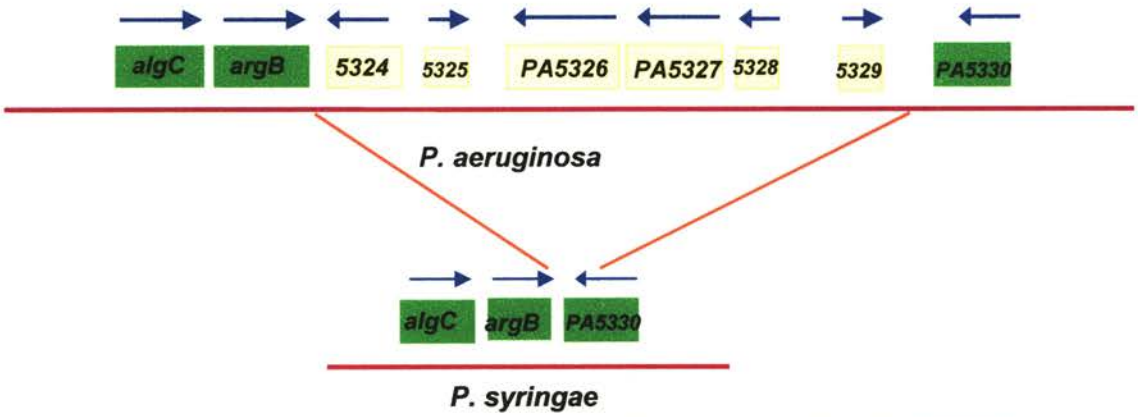


Figure 11. Comparison between the region downstream of *algC* in *P. syringae* and *P. aeruginosa*. *P. syringae* lacks six open reading frames (ORFs) that are present in *P. aeruginosa* between *argB* and the hypothetical protein PA5330. These six ORFs (shown in yellow) starting from *PA5324* to *PA5329* are speculated to encode the following functions; a probable transcriptional regulator (5324), a hypothetical protein (5325), a probable oxidoreductase (5326), a probable cytochrome c that functions in energy metabolism (5327), and a hypothetical unclassified protein (5328).

An alignment of the *algC* promoter and the 5' end of the coding regions of *P. syringae* pv. syringae (Ps algC) and *P. aeruginosa* (Pa algC) is shown in Figure 12. The *algC* coding region sequenced from *P. syringae* pv. syringae and that reported for *P. aeruginosa* were 76% identical at the nucleotide level; however, upstream of the translational start site, the relatedness between the two species diverged and nucleotide identity decreased to approximately 54%.

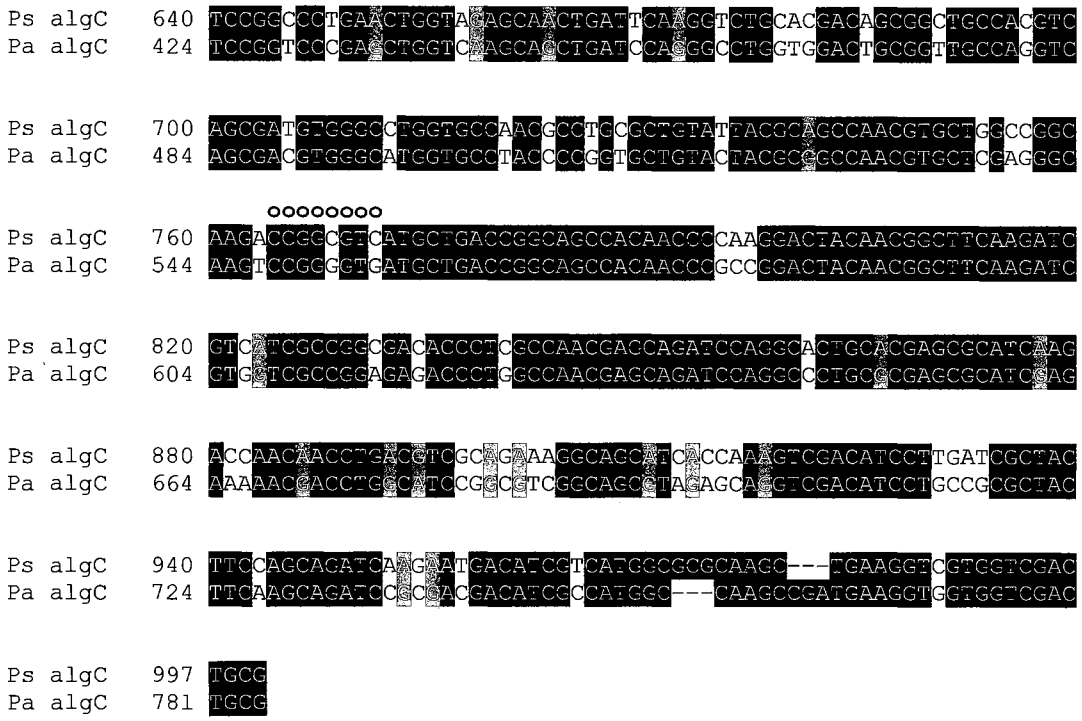


Figure 12. Alignment of the *algC* promoter sequences from *P. syringae* pv. syringae FF5 (Ps algC) and *P. aeruginosa* (Pa algC). The *P. aeruginosa* sequence was previously reported (Zielinski et al., 1992; Fujiwara et al., 1993); the nucleotides for this sequence are shown on the left with +1 (asterisk) corresponding to the transcriptional start site. Nucleotides for the *P. syringae* pv. *syringae algC* promoter are also shown on the left. Gaps (--) were used to maximize the alignment. Identical bases are shown in white font with black background, whereas similar bases are shaded. The three reported AlgR1 binding sites in *P. aeruginosa* are indicated by **red** open circles beneath the specific bases. The four putative AlgR1 binding sites in *P. syringae* pv. *syringae* are shown with **blue** open circles above the specific bases. The *algC* translational start site is indicated by an arrow (*algC*—→).

Full expression of the *PsalgC* promoter requires AlgR1

In *P. aeruginosa*, AlgR1 is required for expression of the *algC* promoter (*PalgC*) (Zielinski et al., 1991) and binds to *PalgC* at multiple sites (Zielinski et al., 1992; Fujiwara et al., 1993). To investigate whether *algC* expression requires *algR1* in *P. syringae*, an *algR1*::Tn*5* mutant was constructed in *P. syringae* pv. syringae FF5.

Homologous recombination of Tn*5* into *algR1* was confirmed by Southern blot analysis and resulted in strain FF5.7. The two transconjugants, FF5(pMF8.3) and FF5.7(pMF8.3) were grown on minimal media (MG) supplemented with 0.2 M NaCl. FF5(pBBRGus) and FF5.7(pBBRGus) were included as negative controls. The media was supplemented with 0.2 M NaCl because FF5 is normally nonmucoid and elevated osmolarity is known to stimulate alginate gene expression in this strain (Peñaloza-Vázquez et al., 1997). Table 5 shows that *algC* expression (GUS activity) was reduced two-fold in FF5.7 as compared to the wild-type FF5, indicating that a functional copy of *algR1* is required for full activation of *algC* expression.

TABLE 5. Glucuronidase activity (U GUS/mg protein)[a] for *P. syringae* pv. syringae FF5 and FF5.7 containing pMF8.3 (*algC-uidA*) and pBBR.Gus (promoterless *uidA*).

Strain[b]	pMF8.3	pBBR.Gus
FF5	354.66 a	21.57 c
FF5.7	181.29 b	18.79 c

[a] Mean values followed by the same letter are not significantly different at $P = 0.05$ using the Student-Newman Keuls Test. Values are the means from one experiment containing three replicates.
[b] FF5 is the wild-type and FF5.7 is an *algR1* mutant derived from the former strain.

Overproduction of *P. syringae* AlgR1

algR1 was first amplified using PCR and pMF6.2 as template DNA (Table 4 and Fig. 13); restriction sites were incorporated into the oligonucleotide primers to facilitate cloning of a 747-bp *Bam*HI/*Pst*I fragment that contains *algR1* with its stop codon. This fragment was subcloned into pMAL-c2, a construct designed for making C-translational fusions to the maltose-binding protein (MBP; product of *malE* gene). The construct

resulting from this experiment, pMF6.4, was then introduced into *Escherichia coli* DH5α (Figure 13).

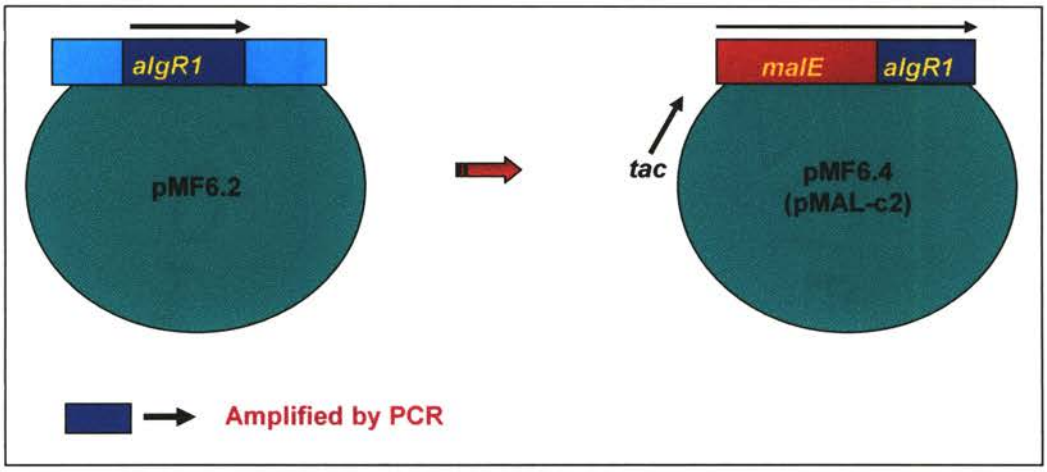


Figure 13. Construction of the MBP-AlgR1 translational fusion protein.

When *E. coli* DH5α(pMF6.4) cells were induced with IPTG, a 70-kDa protein (Figure 14, lane 3) was observed, which corresponds to the predicted size of the fusion protein, MBP-AlgR1. This band was absent from uninduced cells of DH5α(pMF6.4) (Figure 14, lane 2) and from uninduced and induced DH5α(pMAL-c2) cells (data not shown). It is important to note that the MBP-AlgR1 fusion protein could not be overproduced when *E. coli*(pMF6.4) cells were induced and incubated at 37°C; it was necessary to grow cells at 18°C, a temperature suboptimal for growth, in order to achieve overproduction. This suggests that a high concentration of AlgR1 is toxic to *E. coli* cells, which is consistent with previous results showing that AlgR1 from *P. aeruginosa* could not be overproduced using high-copy number plasmids (Kato and Chakrabarty, 1991).

All efforts to purify MBP-AlgR1 using affinity chromatography on amylose resin were unsuccessful.

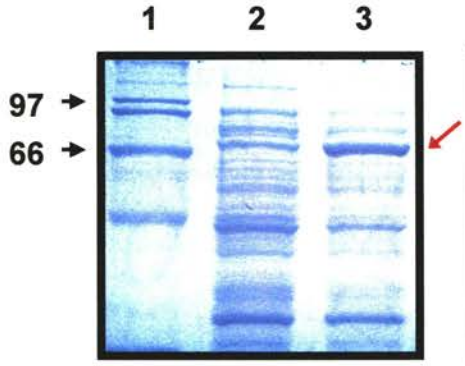


Figure 14. SDS-PAGE analysis of *E. coli* DH5α cells containing pMF6.4. Lane **1** shows the migration of molecular mass markers in kilodaltons (kDa). Lanes **2-3** show total cellular proteins from the following: lane **2**, DH5α(pMF6.4), uninduced; lane **3**, DH5α(pMF6.4), induced with IPTG. The 70 kDa MBP-AlgR1 fusion product of pMF6.4 is indicated (red arrow).

Gel retardation assays

The ability of *P. syringae* AlgR1 to bind the *PsalgC* promoter region was investigated. The 0.8-kb *Xho*I-*Hin*dIII fragment in pMF8.2 containing the *P. syringae algC* promoter was used in gel shift assays. When the 0.8-kb *algC* promoter fragment was incubated with 200 ng of MBP-AlgR1, migration of the labeled fragment was markedly reduced (Figure 15A, lane 3) as compared to labeled fragment alone (Figure 15A, lane 1) or labeled fragment incubated with 200 ng of MBP (Figure 15A, lane 2). These results show that AlgR1 binds to the *algC* promoter in *P. syringae*, possibly to the ABS sites that are conserved in *P. syringae* and *P. aeruginosa*. The MBP-AlgR1 fusion also retarded the migration of a 0.9-kb *Xho*I-*Bss*HII fragment from pNZ15, which contains the *algC* promoter of *P. aeruginosa* (Figure 15B, lane 3). This is not unexpected given the conservation between *P. syringae* and *P. aeruginosa* in putative and known ABSs; furthermore, *algR1* homologues in the two species are highly related (84% nucleotide identity; see Chapter III).

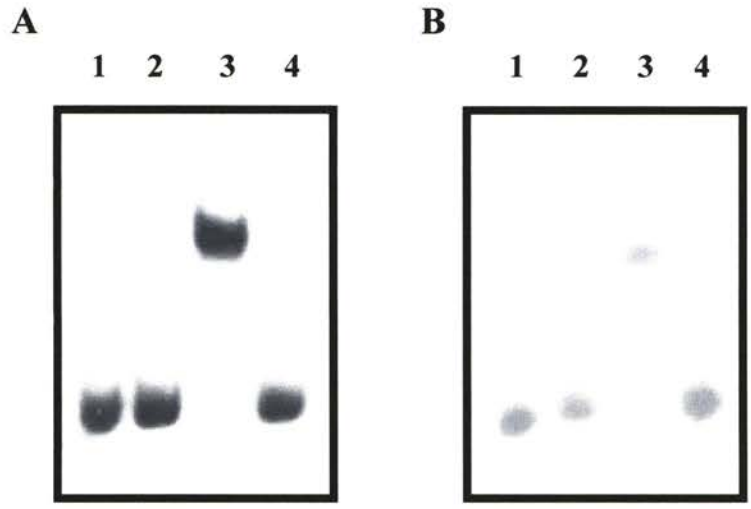


Figure 15. Gel shift assays. (**A**) Gel shift assays of the MBP-AlgR1 fusion and a 0.8-kb *Xho*I-*Hin*dIII fragment containing the *algC* promoter of *P. syringae*. Lanes 1 and 4 contain 20 ng of end-labeled target DNA and 0 ng of MBP-AlgR1. Lane 2 contains the target DNA fragment and approximately 200 ng of MBP. Lane 3 contains the target DNA fragment and approximately 200 ng of MBP-AlgR1. (**B**) Gel shift assay of the MBP-AlgR1 fusion and a 0.9-kb *Xho*I-*Bss*HII fragment containing the *algC* promoter of *P. aeruginosa*. Lanes 1 and 4 show approximately 20 ng of end-labeled target DNA and 0 ng of MBP-AlgR1. Lane 2 contains the target DNA fragment and 200 ng of MBP. Lane 3 contains the target DNA fragment and 200 ng of MBP-AlgR1.

The specificity of complex formation between MBP-AlgR1 and the fragment containing the *P. syringae algC* promoter was investigated by adding increasing amounts of the unlabeled *Xho*I-*Hin*dIII fragment to the reaction mixture. When cold fragment was added as a competitor in amounts of 200 ng or higher, binding was either significantly reduced or completely abolished (Figure 16, lanes 6 and 7). However, when poly(dI-dC) was added to the reaction mixture, binding was not altered (Figure 16, lanes 3 and 4). These results indicate that the MBP-AlgR1 specifically binds the 0.8-kb *Xho*I-*Hin*dIII fragment. This result is consistent the presence of the putative AlgR1 binding sites in this fragment (Figure 10).

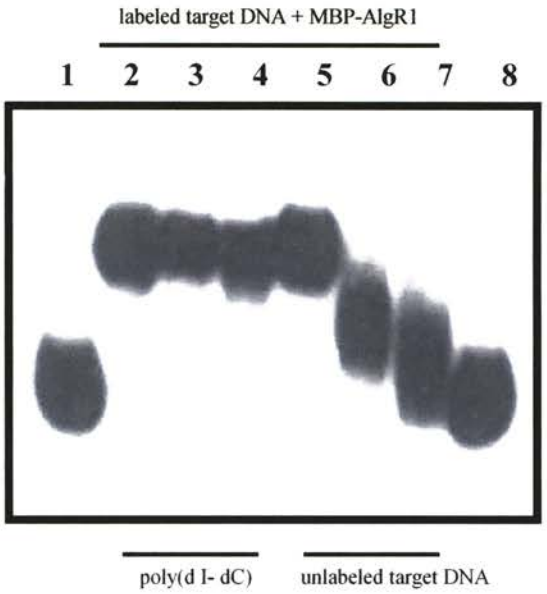


Figure 16. Competition assays using the MBP-AlgR1 fusion and the 0.8-kb *Xho*I-*Hin*dIII fragment containing the *algC* promoter from *P. syringae*. Lanes 1 and 8 show approximately 20 ng of end-labeled target DNA and 0 ng of MBP-AlgR1. Lanes 2 to 7 contain the target DNA fragment and approximately 200 ng of MBP-AlgR1. The addition of the nonspecific competitor poly(dI-dC) is shown in lanes 2 to 4, which contain 0, 200, and 800 ng of poly(dI-dC), respectively. The specific inhibition of binding is shown in lanes 5, 6, and 7, which contain 0, 200, and 600 ng of unlabeled target fragment:

Discussion

The protein product of *algC* is phosphomannomutase, which catalyzes the second biosynthetic step in the pathway to alginate. In *P. aeruginosa*, AlgC functions in LPS biosynthesis via its phosphoglucomutase activity and in rhamnolipid synthesis (Coyne et al., 1994; Olvera et al., 1999). Although these roles for *algC* in *P. syringae* have not been demonstrated, it is quite likely that AlgC has multiple roles in this bacterium. This hypothesis is supported by the fact that *algC* transcriptional activity in the *algR*1 mutant,

FF5.7, was only two-fold lower than transcriptional activity in the wild-type strain, FF5. Thus it is likely that *algC* transcription is necessary for other processes in *P. syringae*, since complete abrogation of *algC* expression was not observed in the *algR1* mutant.

The presence of three putative sequences resembling σ^{54} recognition motifs (GG-N_{10}-GC) in the *P. syringae* pv. syringae *algC* promoter suggests that σ^{54} is necessary for *algC* transcription. This is true in *P. aeruginosa* where two σ^{54} recognition motifs were reported in the *algC* promoter region; furthermore, *algC* expression was greatly reduced in a *rpoN* mutant, which encodes σ^{54} (Zielinski et al., 1992). Although a *rpoN* mutant of *P. syringae* FF5 is not available, *rpoN* mutants of *P. syringae* pv. glycinea (Alarcón-Chaidez et al., submitted) and *P. syringae* pv. maculicola (Hendrickson et al., 2000) have been constructed and could be used to assess whether *algC* transcription is *rpoN*-dependent.

An MBP-AlgR1 translational fusion was used in this study for the DNA binding assays, and MBP was used as a negative control. MBP translational fusions have been used in numerous studies to investigate the DNA binding function of regulatory proteins (Boucher et al., 1994; Grob and Guiney, 1996; Lee et al., 1993; Peñaloza-Vázquez and Bender, 1998). Overproduction of MBP-AlgR1 was successful when IPTG-induced *E. coli* cells were incubated at 18°C, a temperature suboptimal for growth. This suggests that a high concentration of AlgR1 is toxic to *E. coli*, which was also suggested for AlgR1 from *P. aeruginosa* (Kato and Chakrabarty, 1991). In the current study, trials to purify AlgR1 using an amylose affinity column were unsuccessful, possibly because AlgR1 was insoluble after cleavage from MBP. Furthermore, low solubility was a problem in the purification of AlgR1 from *P. aeruginosa* since the protein was shown to

form insoluble aggregates at low salt concentrations and ~85% of AlgR1 precipitated during dialysis following column chromatography (Kato and Chakrabarty, 1991).

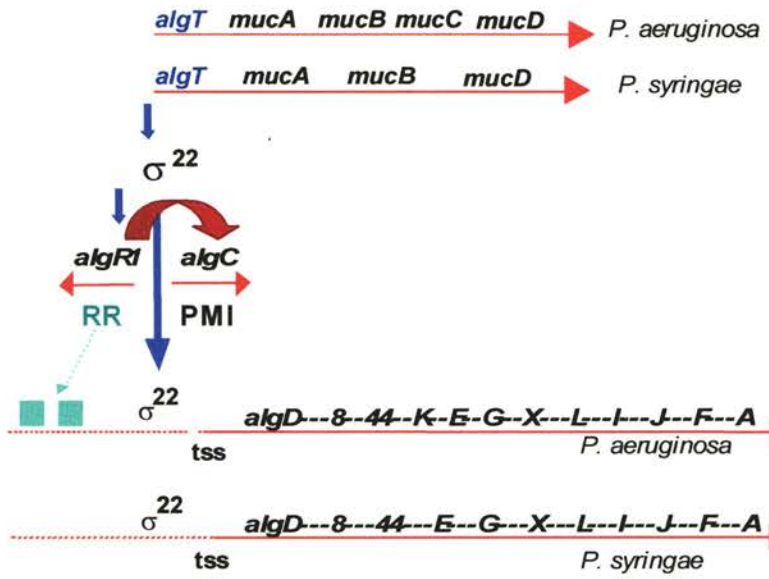


Figure 17. Comparison of the roles of AlgR1 in *P. aeruginosa* and *P. syringae*. AlgT encodes σ^{22}, which is required for the expression of *algR1* and *algD*. Expression of the *algD* promoter in *P. aeruginosa* also requires AlgR1, which binds upstream of the transcriptional start site (tss) in *P. aeruginosa* (the green boxes indicate AlgR1 binding sites). In *P. syringae*, a σ^{22} recognition site is located upstream of *algD*, but AlgR1 binding sites are not present.

In *P. aeruginosa*, AlgR1 is required for transcriptional activation of both *algD* and *algC* and binds to multiple ABSs in the *PalgD* and *PalgC* promoter regions (Fujiwara et al., 1993; Kato and Chakrabarty, 1991; Mohr et al., 1992; Zielinski et al., 1992). In *P. syringae*, *algD* expression does not require *algR1* (Chapter III), and the *PsalgD* promoter region lacks AlgR1 binding sites (Fakhr et al., 1999). However, it is important to note that an *algR1* mutant is still nonmucoid, indicating a role for this gene in alginate biosynthesis (Chapter III). In this study, *algR1* was required for full activation of *algC* transcriptional activity in *P. syringae*. Furthermore, binding of AlgR1 to the *algC*

promoter was demonstrated. These results indicate that *algR1* mediates alginate biosynthesis via transcriptional activation of *algC*. The roles of AlgR1 in *P. aeruginosa* and *P. syringae* are compared in Figure 17.

Fujiwara et al. (1993) demonstrated that activation of the *algC* promoter by AlgR1 was independent of the relative location (upstream or downstream of the transcriptional start), the number of copies, or the orientation of *algC*-ABS1. Consequently, the ABS may function in a manner similar to eukaryotic enhancer elements and could also facilitate the formation of a DNA loop when AlgR1 is bound (Fujiwara et al., 1993). In *P. syringae*, four putative ABS were identified; two upstream of the translational start site and two within the *algC* coding region. The gel retardation experiments clearly demonstrated that *P. syringae* AlgR1 bound strongly and specifically to DNA fragments containing the ABS from both *P. syringae* and *P. aeruginosa*. Therefore, it is highly likely that AlgR1 from *P. syringae* recognizes the ABS in *P. aeruginosa*, and these binding sequences are conserved in *P. syringae*. The absence of ABS in the *algD* promoter of *P. syringae*, may explain why *P. syringae*, unlike *P. aeruginosa*, does not require a functional copy of *algR1* for *algD* transcriptional activity (Figure 17).

CHAPTER V

Mutagenesis of a Plasmid that Confers Constitutive Alginate Production to *Pseudomonas syringae*

Abstract

Pseudomonas syringae pv. syringae FF5 is normally nonmucoid *in vitro*; however, a large 200-kb plasmid designated pPSR12 confers constitutive alginate production to FF5. Plasmid pPSR12 is not known to contain homologs of the biosynthetic or regulatory genes that control alginate production; instead, this plasmid presumably contains regulatory genes that remain uncharacterized. The aim of the present study was to clone and identify the gene(s) on pPSR12 that confers mucoidy to FF5. Mutagenesis of FF5(pPSR12) with a mini-Tn*5* transposon resulted in the isolation of an alginate-defective mutant named FF5.MF1. The Tn5 insertion in FF5.MF1 was localized to a 8-kb *Sst*I fragment in pPSR12. The mutant FF5.MF1 showed an 80-fold decrease in alginate production when compared to the mucoid parent strain, FF5(pPSR12). pSM51, a clone isolated from a cosmid library of pPSR12, partially complemented the nonmucoid mutant FF5.MF1 and caused a 12-fold increase in alginate production. When pSM51 was mobilized to the mucoid parent strain FF5(pPSR12), a 60-fold decrease in alginate production was observed, possibly because the cosmid contains both positive and negative regulatory genes or due to a copy number effect. A 5-kb *Eco*R1 fragment from pSM51 was cloned in pBluescript that contains the wild-type

region of the disrupted gene in FF5.MF1. DNA sequence analysis of the 5-kb *Eco*RI fragment indicated the presence of a 1026 bp open reading frame, which lacked DNA relatedness to genes deposited in various databases. However, the predicted amino acid sequence of this ORF showed a low amount of relatedness (36% similarity) to the plasmid-borne *kfrA* gene in *Enterobacter aerogenesis*. The 5-kb *Eco*R1 fragment was subcloned in pRK415 resulting in pRKE5.1; however, transconjugants of FF5.MF1(pRKE5.1) were not complemented for alginate production. To further characterize the mutant FF5.MF1, the expression of the alginate regulatory genes *algT* and *algR1* was compared in the mutant FF5.MF1 and the parent strain FF5(pPSR12). The transcription of both genes was significantly reduced in FF5.MF1 as compared to FF5(pPSR12), which agrees with the phenotypic reduction of alginate synthesis in the mutant FF5.MF1.

Introduction

The exopolysaccharide alginate is a copolymer of O-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Rehm and Valla, 1997). Alginate biosynthesis has been extensively studied in *Pseudomonas aeruginosa* where it functions as a major virulence factor in strains infecting the lungs of cystic fibrosis (CF) patients (Pier, 1998). Alginate also contributes to both the virulence and epiphytic fitness of *Pseudomonas syringae* (Yu et al., 1999).

The alginate biosynthesis genes in pseudomonads are normally silent (Goldberg et al., 1993). Interestingly, an indigenous plasmid designated pPSR12 conferred constitutive

alginate production to *P. syringae* pv. syringae FF5 (Kidambi et al., 1995). Plasmid pPSR12 was first identified in *P. syringae* pv. syringae 7A36, a strain isolated from ornamental pears grown in eastern Oklahoma (Sundin et al., 1994). Plasmid pPSR12 does not contain homologs of the biosynthetic or regulatory genes that control alginate production in *P. aeruginosa*; instead, this plasmid presumably contains regulatory genes that have not been characterized (Kidambi et al., 1995). Although pPSR12 encodes resistance to copper (Cu) and streptomycin (Sm), the plasmid is stably maintained in the absence of Cu or Sm selection (Kidambi et al., 1995). The stable maintenance of pPSR12 suggests that the plasmid might encode additional factors that may improve the fitness of the host bacterium. Traits known to increase the fitness of phytopathogenic bacteria include motility, tolerance to desiccation, and epiphytic fitness (the ability to colonize the surface of both host and nonhost plants) (Lindow, 1991; Lindow et al., 1993).

The presence of indigenous plasmids in phytopathogenic bacteria is thought to confer selective advantages to the host bacterium, although in many cases specific traits associated with the plasmids are unknown (Shaw, 1987; Coplin, 1989). The stable maintenance of plasmids in plant pathogens suggests a potential relevance to the host-pathogen interaction; for example, the symbiotic plasmids of *Rhizobium* and the tumor-inducing plasmids of *Agrobacterium* play critical roles in the interaction of these bacteria with their respective plant hosts (Long and Staskawicz, 1993). Plasmid-encoded genes known to be important in the interaction of *P. syringae* with host plants include avirulence genes (Kobayashi et al., 1990) and genes for biosynthesis of ethylene, indoleacetic acid, and the phytotoxin coronatine (Comai and Kosuge, 1980; Bender et al.,

1991; Nagahama et al., 1994). Other plasmid-encoded traits that enhance fitness include those that confer resistance to bactericidal compounds and ultraviolet radiation (Bender and Cooksey, 1986; Sundin et al., 1994; 1996; 1999). Some of these traits have been transferred to human pathogens, especially in environmental niches where bacteria indigenous to animals intermingle with bacteria indigenous to plants (Sundin and Bender, 1996). Thus the identification of plasmid-encoded genes and an understanding of the relationships of native plasmids and their hosts is essential in establishing the role of these elements in the evolution of bacteria.

The purpose of the present study was to use a genetic approach to isolate the genes encoded by pPSR12 that confer constitutive alginate production to *P. syringae* pv. syringae strain FF5.

Materials and Methods

Bacterial strains, plasmids, and media

Table 6 lists the bacterial strains and plasmids used in the present study. *Pseudomonas* spp. were routinely maintained at 28°C on King's medium B (King et al., 1954), mannitol-glutamate medium (Keane et al., 1970), or MG supplemented with yeast extract at 0.25 g/liter (MGY); *E. coli* strains were grown on LB medium (Miller, 1972) at 37°C. Antibiotics were added to media at the following concentrations (μg/ml): ampicillin (100), tetracycline (25), kanamycin (25), spectinomycin (50), streptomycin (25), chloramphenicol (25) and copper (200).

Molecular genetic techniques

Plasmid DNA was isolated from *Pseudomonas* spp. by alkaline lysis (Sambrook et al., 1989). The large plasmids pPSR12 and pPSR12.1 were isolated using the Portnoy-White method of plasmid isolation (Crosa and Falkow, 1981). Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, colony hybridization, and isolation of DNA fragments from agarose gels were performed using standard protocols (Sambrook et al., 1989). Plasmid DNA was prepared for DNA sequencing using the plasmid DNA Midi Kit from Qiagen (Qiagen, Valencia, Calif.), and genomic DNA was isolated from *P. syringae* using established procedures (Staskawicz, et al., 1984). A total genomic library of FF5.MF1 was constructed in pRK7813 as described previously (Barta et al., 1992). Clones were mobilized into *P. syringae* recipient strains using a triparental mating procedure and the mobilizer plasmid pRK2013 (Bender et al., 1991).

Table 6. Bacterial strains and plasmids used in this study.

Strains or Plasmids	Relevant characteristics*	Source or Reference
Escherichia coli		
DH5α		Sambrook et al., 1989
S17-1 λ-*pir*	λ-*pir* lysogen of S17-1	Wilson et al., 1995
Pseudomonas syringae pv. syringae		
FF5	Cu^s; no detectable plasmids, nonmucoid	Kidambi et al., 1995
FF5. MF1	Cu^r Sm^r Sp^r; nonmucoid, mini-Tn*5* mutant of FF5(pPSR12), mutation is believed to disrupt one of the *muc* genes on plasmid pPSR12	This study
Plasmids		
pPSR12	Cu^r Sm^r; 200 kb, confers constitutive alginate production to *P. syringae* pv. syringae FF5	Kidambi et al., 1995
pPSR12.1	Cu^r Sm^r Sp^r; mutated plasmid pPSR12 with a mini-Tn5 insertion in '*muc*' gene	This study
pRK2013	Km^r; helper plasmid	Figurski and Helinski, 1979
pRK7813	Tc^r; cosmid vector	Jones and Gutterson, 1987
pRK415	Tc^r; RK2-derived cloning vector	Keen et al., 1988
pBluescript SK+	Ap^r; ColEI origin, cloning vehicle	Stratagene
pBBR.Gus	Cm^r; 6.6-kb promoter probe broad-host-range vector containing the *uidA* gene	Peñaloza-Vázquez and Bender, 1998
pCAM140	Sm^r Sp^r Ap^r; contains mini-Tn*5*-uidA (promoterless *uidA* for transcriptional fusions) suicide plasmid	Wilson et al., 1995
pMF6	Tc^r; cosmid clone from FF5(pPSR12) in pRK7813	Chapter III

pMF6.2	Ap^r; contains a 2.0-kb *Pst*I fragment from pMF6	Chapter III
pMF6.3	Ap^r; contains *algR1* promoter region as a 0.7-kb *Pst*I fragment; derived from pMF6.2 by PCR cloning in pBluescript SK+	This study
pMF6.31	Cm^r; contains a 0.7-kb *Pst*I fragment of the *algR1* promoter from pMF6.3 in pBBR.Gus in a transcriptionally active orientation with regard to the *uidA* gene	This study
pAlgTA	Cm^r; 1-kb *Hin*dIII/*Pst*I fragment containing the *algT* promoter region in pBBR.Gus in transcriptionally active orientation with regard to the *uidA* gene	Keith and Bender, 1999
pMF10	Tc^r Sm^r Sp^r; cosmid clone from the mutated plasmid pPSR12.1 in pRK7813	This study
pSM51	Tc^r; cosmid clone from pPSR12 library in pRK7813	This study
pBSS8	Ap^r Sm^r Sp^r; contains 8kb fragment from pMF10 with the mini-Tn*5* insertion	This study
pBSE5	Ap^r; contains a 5-kb EcoRI fragment from pSM51	This study
pRKE5.1	Tc^r; contains a 5-kb *Eco*RI fragment from pBSE5 in pRK415 in the transcriptionally-active orientation with respect to *lacZ* and the '*muc*' gene	This study
pRKE5.2	Tc^r; contains a 5-kb *Eco*RI fragment from pBSE5 in pRK415 in the transcriptionally inactive orientation with respect to *lacZ* and the *muc* gene	This study

DNA fragments were isolated from agarose gels by electroelution (Sambrook et al., 1989) and labeled with digoxigenin (Genius Labeling and Detection Kit; Boehringer Mannheim, Indianapolis, Ind.) or with [α-^{32}P]dCTP using the Rad Prime DNA Labeling System (Gibco BRL, Gaithersburg, Md.). Hybridizations and post-hybridization washes were conducted using high stringency conditions (Sundin and Bender, 1993).

The 0.7-kb *Pst*I fragment that contains the *algR1* promoter region of *P. syringae* pv. syringae FF5(pPSR12) was cloned by PCR amplification using plasmid pMF6.2 as a template. Two primers were synthesized by the OSU Recombinant DNA/Protein Resource Facility: forward primer, 5'-AGTCTGCAGCCGCACTTCCTCTTCAATAC; and reverse primer, 5'-TGACTGCAGTTCTTCGCCATTGGACG (*Pst*I sites are underscored).

FF5(pPSR12) was mutagenized with the suicide plasmid pCAM140, which contains a mini-Tn*5-uidA* transposon (Table 6). pCAM140 was introduced into the recipient using the triparental mating technique. The donor strain *E. coli* S17-1 λ-*pir*

(pCAM140) and the helper strain *E. coli* DH5α (pRK2013) were grown on LB agar supplemented with the appropriate antibiotics for 36 h at 37°C. The recipient strain *P. syringae* FF5(pPSR12) was cultured for 36 h on KMB medium supplemented with the appropriate antibiotics at 28°C. Equal amounts of bacteria were removed from the agar media and then suspended in 500 µL of 10% glycerol. The three suspensions were then mixed together vigorously and spotted on the surface of KMB agar in 100 µL aliquots, air-dried and incubated for 12 h at 28°C. Each mating 'spot' was then removed from the agar surface, suspended in 1 ml of 10% glycerol, vortexed and transferred to selective media containing MG medium supplemented with Sp and Cu. Plates were then incubated at 28°C for 3-4 days, and individual colonies were then transferred to microtiter dishes containing 15% glycerol and stored at -80°C. A 1.8-kb *Pst*I/SstI fragment from plasmid pBBR.Gus containing the *gusA* gene was used as a probe to screen for mini-Tn*5* insertions.

Isolation and quantitation of alginate

Selected strains were inoculated by dilution streaking to MGY agar (three plates per strain) and incubated at 28°C for 72 h. Each plate was handled separately for quantification of alginate. Cells were washed from each plate and resuspended in 0.9% NaCl. Removal of cellular material from the mucoid growth and estimation of alginate content and total cellular protein were performed as described previously (May and Chakrabarty, 1994). Alginic acid from seaweed (*Macrocystis pyrifera*; Sigma Chemical Co., St. Louis, MO) was used as a standard in these experiments. Mean values of three replicates were expressed as µg alginate per mg of protein.

Glucuronidase assays

Glucuronidase (GUS) activity was quantified by fluorometric analysis of cells grown for 36 h in 5 ml MGY medium supplemented with the appropriate antibiotics. The initial inoculum was adjusted to OD_{600}=0.1. Fluorescence was monitored with a Fluoroscan II Version 4.0 microplate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 96-well microtiter plates. GUS activity was expressed in U/mg protein with one unit equivalent to 1 nmol of methylumbelliferone formed per min. Values presented for GUS activity represent the average of three replicates per experiment.

DNA sequencing and analysis

Automated DNA sequencing was provided by the OSU Recombinant DNA/Protein Resource Facility and was performed with an ABI 373A apparatus and the ABI PRISM Dye Primer Cycle Sequencing Kit (Perkin-Elmer, Foster City, Calif.). Oligonucleotide primers used for sequencing were also synthesized by the OSU Recombinant DNA/Protein Resource Facility. DNA flanking the mini-Tn*5* transposon in plasmid pBSS8 was sequenced using the oligonucleotide primer 5' AGATCTGATCAAGAGACAG, which is derived from the 'I' border of IS*50*.

Sequence manipulations, amino acid alignments, and restriction maps were constructed using the Vector NTI Suite, Version 6.0 (Informax, San Francisco, CA). Database searches were performed with the BLAST service of the National Center for Biotechnology Information.

Results

Creation of the alginate-defective mutant FF5.MF1

The 200-kb plasmid pPSR12 presumably contains regulatory genes that confer constitutive alginate production to *Pseudomonas syringae* pv. syringae FF5. Therefore, generating an alginate-defective mutant by mutating this plasmid was a crucial step towards the identification of these genes. Random mutagenesis of *P. syringae* FF5(pPSR12) was accomplished using the suicide plasmid pCAM140. Approximately 2000 Sp^r Cu^r colonies of FF5(pPSR12) were isolated and maintained at -80°C. Genomic DNA was isolated from ten mutants, digested with *Sst*I (which does not cut within the mini-Tn*5* transposon), blotted, and probed with a 1.8-kb *Pst*I/*Sst*I fragment derived from the *uidA* gene. Southern blot analysis indicated that the mini-Tn*5* had inserted at random sites in the FF5 genome.

The mutant bank was then screened visually for mucoid, and nonmucoid mutants were selected. Since the nonmucoid phenotype could be due to insertional activation of either chromosomal or plasmid DNA, both genomic and plasmid DNA were isolated from each mutant and probed with the 1.8-kb *uidA* gene. Only one mutant contained a mini-Tn*5* insertion in pPSR12, and this mutant was designated FF5.MF1. Phenotypically, FF5.MF1 is nonmucoid on MGY agar and resembles the nonmucoid, plasmid-free strain, FF5 (Figure 18).

Figure 18. Morphology of *P. syringae* FF5 and derivatives on MGY agar. **A**, FF5; **B**, FF5(pPSR12); and **C**, FF5.MF1. When introduced into FF5, pPSR12 results in the constitutive production of alginate and a mucoid phenotype (**B**). The mutant FF5.MF1 contains a transposon insertion in pPSR12 that results in a nonmucoid phenotype (**C**).

Mapping the mini-Tn*5* insertion and library screening

Plasmid DNA was isolated from the nonmucoid mutant FF5.MF1, digested with *Sst*I, blotted and probed with the 1.8-kb *uidA* gene. The probe hybridized to a 8-kb *Sst*I fragment in the digested preparation of the mutant plasmid, which was designated pPSR12.1 (Figure 19, lane 3).

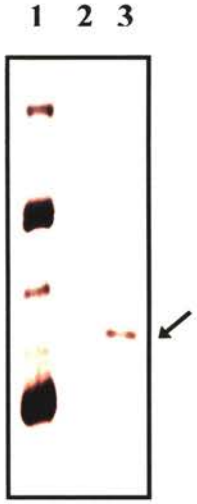


Figure 19. Southern blot analysis of *Sst*I-digested wild-type and mutated pPSR12. Lanes: **1**, plasmid pCAM140 containing mini-Tn5-*uidA*; **2**, *Sst*I digest of the wild-type plasmid, pPSR12; and **3,** *Sst*I digest of the mutated plasmid, pPSR12.1 (from the nonmucoid mutant FF5.MF1). A 1.8-kb digoxigenin-labeled *uidA* fragment was used as a probe in this experiment. Red arrow shows hybridization of 8-kb *Sst*I fragment to the *uidA* probe.

Efforts to clone the 8.0-kb *Sst*I fragment from pPSR12.1 directly into pBluescript II SK+ were unsuccessful. A second strategy involved constructing a cosmid library of FF5.MF1 in pRK7813. The FF5.MF1 library was then screened for cosmids containing the mini-Tn*5* insertion by selecting for resistance to spectinomycin (selective marker for mini-Tn5 construct). Five Sp^r clones were obtained from the library, and a cosmid designated pMF10 (Table 6) was chosen for further studies. Southern blot analysis indicated that an 8.0-kb *Sst*I fragment in pMF10 hybridized to the 1.8-kb *uidA* probe. The 8.0-kb *Sst*I fragment was then subcloned from pMF10 into pBluescript II SK+, resulting in pBSS8 (Table 6). pBSS8 was digested with several enzymes to identify potential fragments that could be used to obtain the wild-type gene(s). A 2.8-kb *Eco*RI fragment from pBSS8 was shown to map adjacent to the mini-Tn*5* insertion (data not shown). This fragment contains an *Eco*RI site in the 'I' border of the transposon; the second *Eco*RI site is located in the DNA flanking the insertion site. This 2.8-kb *Eco*RI fragment was labeled with ^{32}P and used to screen a wild-type library of pPSR12 (Kidambi et al., 1995). Forty-nine clones from the pPSR12 library hybridized to the probe and were used in complementation studies.

Alginate production and complementation analysis

Alginate production by mutant FF5.MF1 was compared to the parent strain FF5(pPSR12) and FF5 lacking pPSR12. Mutant FF5.MF1 produced 80-fold less alginate than the mucoid parent strain, FF5(pPSR12) (Figure 20A). Alginate production by the mutant FF5.MF1 was not significantly different than FF5, which supports (but does not prove) that the Tn*5* insertion in FF5.MF1 abolished constitutive production of alginate.

Attempts to complement mutant FF5.MF1 involved mobilizing the 49 clones from the pPSR12 library (described above) into this mutant and visually assessing the transconjugants for restoration of mucoidy. Only a few clones partially restored alginate production to FF5.MF1, and a clone designated pSM51 was chosen for further study since FF5.MF1(pSM51) was slightly mucoid in appearance. Quantitative analysis of alginate production showed that FF5.MF1(pSM51) produced ~12-fold more alginate than the mutant FF5.MF1 (Figure 20B). Interestingly, when pSM51 was mobilized to the parent strain FF5(pPSR12), alginate production was 60-fold lower than the wild-type (Figure 20C), possibly because of copy-number or regulatory effects. It also remains possible that pSM51 contains both positive and negative regulatory genes, which might explain the partial, rather than full complementation of the mutant.

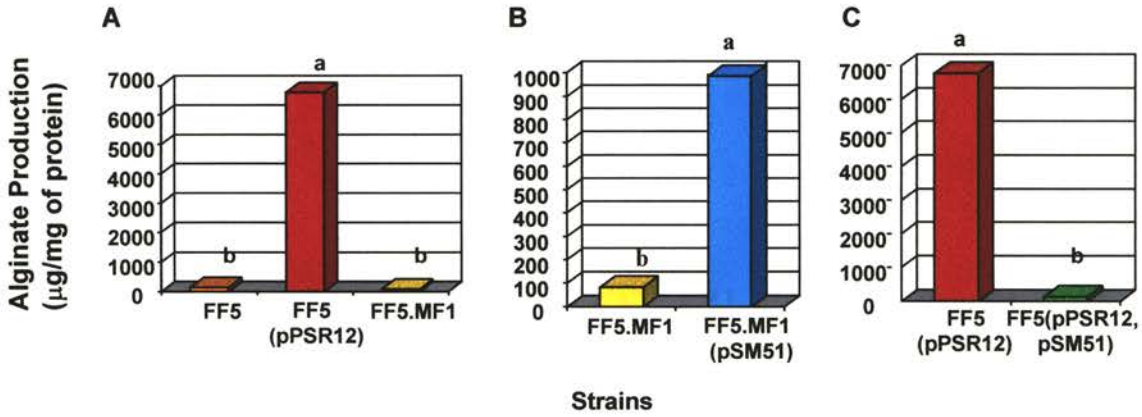


Figure 20. Alginate production by derivatives of *P. syringae* pv. syringae FF5. Mean values of three replicates with the same letter were not significantly different at P=0.05 (Student-Newman Keuls test). **A**, Alginate production by FF5, FF5(pPSR12), and FF5.MF1. The mutant FF5.MF1 produced 80-fold less alginate than the parent strain, FF5(pPSR12). **B**, Alginate production by FF5.MF1 and FF5.MF1(pSM51). The introduction of pSM51 into FF5.MF1 resulted in a 12-fold increase in alginate production. **C**, Alginate production by FF5(pPSR12) and FF5(pPSR12, pSM51). pSM51 caused 60-fold reduction in alginate production when introduced into FF5(pPSR12).

pSM51 was selected for further subcloning of the putative *muc* gene. Restriction digestion of pSM51 and Southern blot analysis revealed a 5-kb *Eco*RI fragment that hybridized to the EcoR*I* fragment derived from pBSS8; this fragment was isolated by electroelution and cloned in pBluescript II SK+, resulting in pBSE5 (Table 6 and Figure 21).

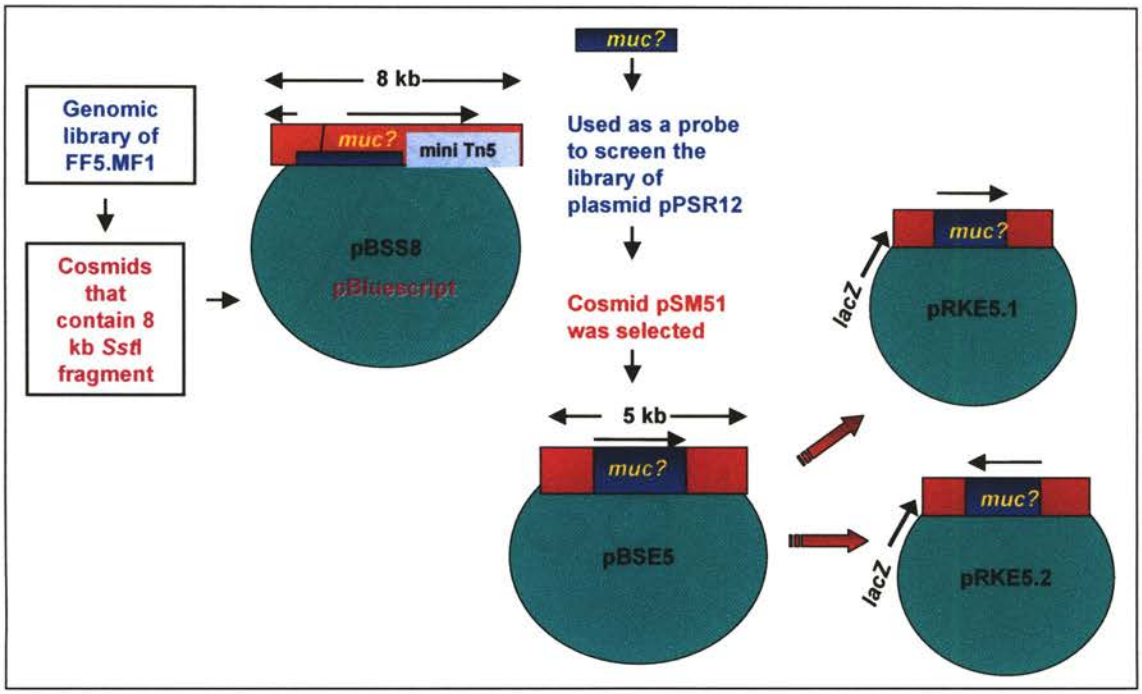


Figure 21. Cloning of the *muc* gene from plasmid pPSR12 of *P. syringae* pv. syringae.

Sequence analysis

The sequence of the region flanking the transposon insertion site in FF5.MF1 was derived using an oligonucleotide primer homologous to the 'I' end of the mini-Tn*5* construct. Sequence analysis indicated that the mini-Tn*5* had inserted into a gene with no

DNA homology to any gene in public databases; however, the predicted amino acid sequence showed similarity (38%) to *kfrA* from *Enterobacter aerogenes*.

Sequence analysis of the 5-kb *Eco*RI fragment in pBSE5 revealed that it contains the wild-type counterpart of the disrupted *muc* gene with ~2 kb of flanking DNA on each side (Figure 22).

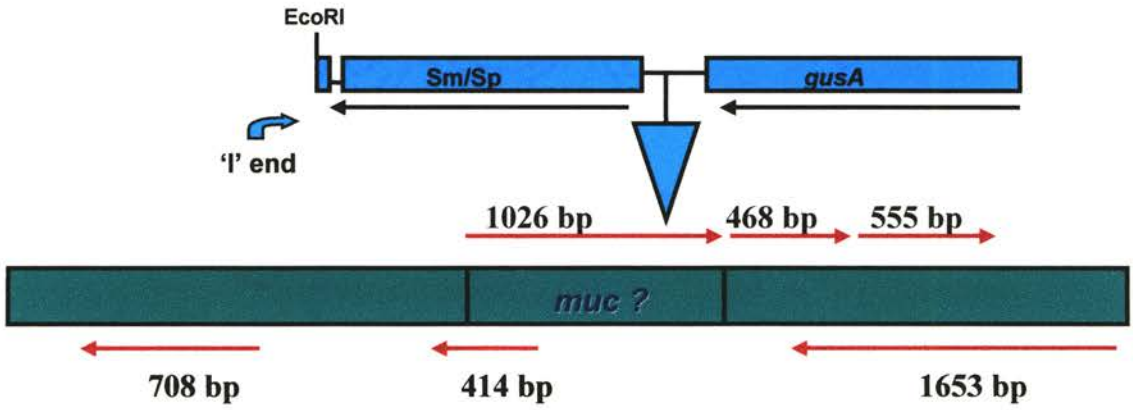


Figure 22. Open reading frames (ORFs) within the 5-kb *Eco*R1 fragment in pBSE5 (**green** rectangles). The **red** arrows represent the ORFs and the direction of transcription. The location and orientation of the **mini-Tn5** insertion in the 1026-bp ORF (putative *muc* gene) is shown.

The DNA sequence of the 1,026-bp ORF (putative *muc* gene) and ~800 bp of the upstream region is shown (Figure 23). The predicted amino acid sequence of this ORF (shown in **red**) and the putative translational start site (highlighted in yellow) are indicated.

```
   1 TCGGGGTCCGTACATCCCAGTGGTCGCCGCGGCTTATGAGCAAGG
  46 TACCGTGGGGCTCTGTCCTCGGAAGTAATACTGAGGACGTTTCCG
  91 GAACTGATTCCTGGACCGTTGCGTTCATCCTCCAAGTTCACGAAT
 136 TACTTCCCGCCGTTGGCAGCCATGCCTCAGACGTCTGGATCACCG
 181 CCGGCGCACAGACTGCTCAACAAGACGGTTTTGCCTGGGCGTTTG
 226 ACCCAGGAGCATCAGTGAGGCTTTGCCCATACAGCAGGTTTTCTG
 271 CAGCTGATCGGGCAATGATCGGGTTGCGGCCCGCTCTGTGCTAGG
 316 GTTTGAAAGTGTCCATGGTTCGTTTCAGCTATGGTGCCCCGCAGA
 361 TCCGGTGCTGCCTTGCTTTAGCATTTTTGGCCCGTGGCTTCGCAT
 406 TTATGGCTGACGTTGATACTTGATCTCCAGCGCAAGCAAATTTGC
 451 CAGAACCCCCTTGTTCATAGGGGTTAACGAGTAATCAAAAACGCC
 496 TCTTTGAAAAGCCAGCTACTGATAGCGATGCGCTTTTGGAGGGGC
 541 GTTTTTTCTTGCTTGAAAAAACTCGTGATAATCGGAGATATTCAC
 586 GAATTTCCCAATTTTTTAATAAGAATCGTATGATAATCAATAAGA
 631 AATTGATTTTAACCCGGCGTCGCACTATGCTGCGATTTCCTCTGC
 676 AGCACGCTGTGCGCCGGCGCGTTGCGCTACAAATGATGCCGCCGT
 721 GGCCGGGGTTATCCCGCCACACCCATCACAGATTAAGGATTCGCC
 766 ATGACGACCGCCGACGGAGTTCAACCTGACAAGCGCCTGGGCACA
     M  T  T  A  D  G  V  Q  P  D  K  R  L  G  T   15
 811 CGTGAGCTGGTCCACCGTTATGCGAAAGAGTTGCTCGATGCTGGG
     R  E  L  V  H  R  Y  A  K  E  L  L  D  A  G   30
 856 CGTGAGGTTCGCCAGGCAGATATCCGTGAGTGCATCTTCATCCAC
     R  E  V  R  Q  A  D  I  R  E  C  I  F  I  H   45
 901 CACGACCTCAAGGCCTCGCCGAACCTGGTCAACGAGGAGATCAAG
     H  D  L  K  A  S  P  N  L  V  N  E  E  I  K   60
 946 AAATTCTGGAGCGAAATTGGCCCCGTGCTGAGTGCCAGATTGCGC
     K  F  W  S  E  I  G  P  V  L  S  A  R  L  R   75
 991 AGACCTGGCATTCCTGACGCCGTGTGCGAAAAGCTCGATGAGATC
     R  P  G  I  P  D  A  V  C  E  K  L  D  E  I   90
1036 TGGGATGTAGCCCTGAGCGCAGCCACCAGCTCTCACGAGGTTGAG
     W  D  V  A  L  S  A  A  T  S  S  H  E  V  E  105
1081 CGCAAGGCGTTTGAGGCCGCCACTGCTGCAGCTGAAGCGACAGCC
     R  K  A  F  E  A  A  T  A  A  A  E  A  T  A  120
1126 AAGGAATCGCGGGAGAATGAACTGACTGCTGTGTCAGCCCTTGAA
     K  E  S  R  E  N  E  L  T  A  V  S  A  L  E  135
1171 GCTCAGCGCCGAGAGCTGGCCGGCCTCATCGCAGACAAGGAGCGC
     A  Q  R  R  E  L  A  G  L  I  A  D  K  E  R  150
1216 TTGACCGATCAGCTTGAACAGTCGAGTGCTGATATTCGGCAGCTT
     L  T  D  Q  L  E  Q  S  S  A  D  I  R  Q  L  165
1261 CGAGCTGAGCTAGCAGATCTGAACAAGAAACTAACTGCCTCAAGC
     R  A  E  L  A  D  L  N  K  K  L  T  A  S  S  180
1306 CAGGCCCATGGCGAGGAGATCAAGCGCCTGCAGGACGTGCACAAC
     Q  A  H  G  E  E  I  K  R  L  Q  D  V  H  N  195
1351 GGCGTCATTGAGCGTGCCCAGGACATGCATCGTGGGGAACTTGAG
     G  V  I  E  R  A  Q  D  M  H  R  G  E  L  E  210
1396 CGCCTACAGAAGCAGGTTCAAGCAGCAAACGATGCGACTGAATCC
     R  L  Q  K  Q  V  Q  A  A  N  D  A  T  E  S  225
1441 GCACGTGTGAAGGCCGAGTCTGCACGGATTGCCGCAGAGGAGCAT
     A  R  V  K  A  E  S  A  R  I  A  A  E  E  H  240
1486 CTTGAGAGAACCGAAAATCACCTGATGATGGAGACCGCCAGGGTT
     L  E  R  T  E  N  H  L  M  M  E  T  A  R  V  255
1531 CGGGATGAAGAGAGAGGCAAAACAGAGAAGGTCAGCAAGGAGCTG
     R  D  E  E  R  G  K  T  E  K  V  S  K  E  L  270
1576 CAGCATGCGTTGACGCTCGCTGATCAATTAAGAGTACAGCGCTCC
     Q  H  A  L  T  L  A  D  Q  L  R  V  Q  R  S  285
1621 AAGGCCAATGACGATGCTGCAGAGACCCGCGGTCGGTTGGAGGTT
     K  A  N  D  D  A  A  E  T  R  G  R  L  E  V  300
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1666 ACCCAGCAGAATTTGAGCGCCCTAGAGGCGCAGAACAAGGAGCTG
     T  Q  Q  N  L  S  A  L  E  A  Q  N  K  E  L   315
1711 CGTGAACTGAACAGCACCCTGCAGGCTGCGCTGCTAGAAGGCTTT
     R  E  L  N  S  T  L  Q  A  A  L  L  E  G  F   330
1756 AGAGGTGGTAAGGCAGGAGCGACTGGCGAGGGTAAATAG
     R  G  G  K  A  G  A  T  G  E  G  K  *         342
```

Figure 23. Nucleotide sequence of the 1026-bp ORF (putative *muc* gene) and upstream region. The deduced amino acid sequence of the ORF is shown in red and the putative translational start site is highlighted in yellow.

Blast analysis of the 1026-bp putative *muc* gene and associated ORFs (Figure 22) did not result in DNA homology to any genes deposited with public databases (including both finished and unfinished microbial genomes). However, the predicted protein product of the putative *muc* gene showed relatedness (30-31% identity, 36% similarity) to *kfrA* homologues in *Enterobacter aerogenes* and *Pseudomonas* sp. *kfrA* encodes a protein consisting of 308 amino acids and was originally isolated from plasmid RK2, where it presumably functions in plasmid stability and partitioning (Jagura-Burdzy and Tomas, 1992). The putative *muc* gene showed relatedness to a SMC1-family ATPase involved in DNA repair in *Methanopyrus kandleri* (27% identity/46% similarity), a cell wall binding protein in *Bacillus halodurans*, (24% identity/46% similarity) (19% identity/41% similarity), a probable chemoreceptor (methyl accepting chemotaxis transmenbrane protein) in *Sinorhizobium meliloti* (19% identity/41% similarity), and an intracellular protein transport protein in *Methanothermobacter thermautotrophicus* (23% identity/42% similarity). The putative *muc* gene showed a low amount of relatedness to several eucaryotic myocin-like proteins, but a protein motif search was not successful in identifying any domains or motifs that would suggest a particular function.

The 1653 bp ORF (Figure 22) showed relatedness (45% identity/63% similarity) to an unknown protein in *Ralstonia metallidurans* and (16% identity/27% similarity) to a cell wall surface 'anchor' protein in *Streptococcus pneumoniae*. The 414 bp ORF (Figure 22) showed limited relatedness (60% similarity over 35 amino acids) to the LysR family in *Vibrio cholerae*. The other ORFs (708, 468, and 555 bp) showed no significant relatedness to other genes in the database.

Complementation experiments using the putative *muc* gene

The 5-kb *Eco*R1 fragment in pBSE5 (Figure 22) was assayed for its ability to restore alginate production to mutant FF5.MF1. pBSE5 contains an intact copy of the putative *muc* gene with ~2 kb of additional DNA flanking each side of the *muc* gene (Figure 22). The 5-kb *Eco*RI fragment in pBSE5 was subcloned in pRK415 to form pRKE5.1 and pRKE5.2, which contain the *muc* gene in the transcriptionally active and inactive orientation with respect to the *lac* promoter (Table 6 and Figure 21). When mobilized to FF5.MF1, neither pRKE5.1 nor pRKE5.2 restored alginate production to FF5.MF1. The two constructs did not reduce alginate production when mobilized to the heavily mucoid strain, FF5(pPSR12). Neither pRKE5.1 nor pRKE5.2 conferred alginate production to the nonmucoid strain FF5. It remains possible that the Tn*5* insertion may have impacted the transcription of additional genes that might work solely or in cooperation with the disrupted gene to confer constitutive alginate production to strain FF5.

Expression of *algT* and *algR1* in the mutant FF5.MF1

To further characterize mutant FF5.MF1, the expression of two alginate regulatory genes, *algT* and *algR1*, were compared in FF5.MF1, FF5, and FF5(pPSR12). The *P. syringae algT* promoter region was previously cloned in pBBR.Gus in the transcriptionally active orientation relative to a promoterless *uidA* gene, thus forming palgTA (Keith and Bender, 1999). The *P. syringae algR1* promoter region was cloned as a 677 bp PCR fragment in pBluescript, resulting in pMF6.3 (Table 6). The 0.7-kb *Pst*I fragment containing the *algR1* promoter was then excised from pMF6.3 and subcloned in pBBR.Gus in the transcriptionally active orientation, resulting in pMF6.31 (Figure 25).

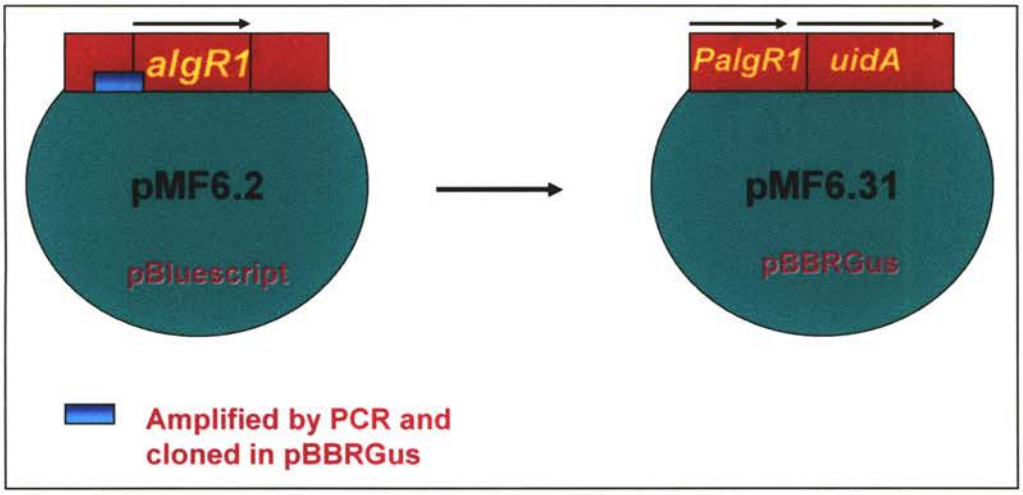

Figure 24. Cloning of *algR1* from *P. syringae algR1* and construction of the *algR1*::*uidA* transcriptional fusion.

Both palgTA (*algT-uidA*), pMF6.31 (*algR1-uidA*), and pBBR.Gus (promoterless *uidA*) were introduced into FF5 (nonmucoid), FF5(pPSR12) (heavily mucoid), and FF5.MF1 (nonmucoid mutant). Expression of both *algT* (Figure 25A) and *algR1* (Figure

25B) was significantly lower in FF5.MF1 as compared to the parent strain FF5(pPSR12), which is consistent with the nonmucoid phenotype of the mutant. Furthermore, transcription of both *algT* and *algR1* in FF5.MF1 (harboring the mutated plasmid pPSR12.1) was significantly lower than strain FF5 (totally lacking the plasmid), which suggests (but does not prove) that pPSR12 contains both positive and negative regulators of alginate production.

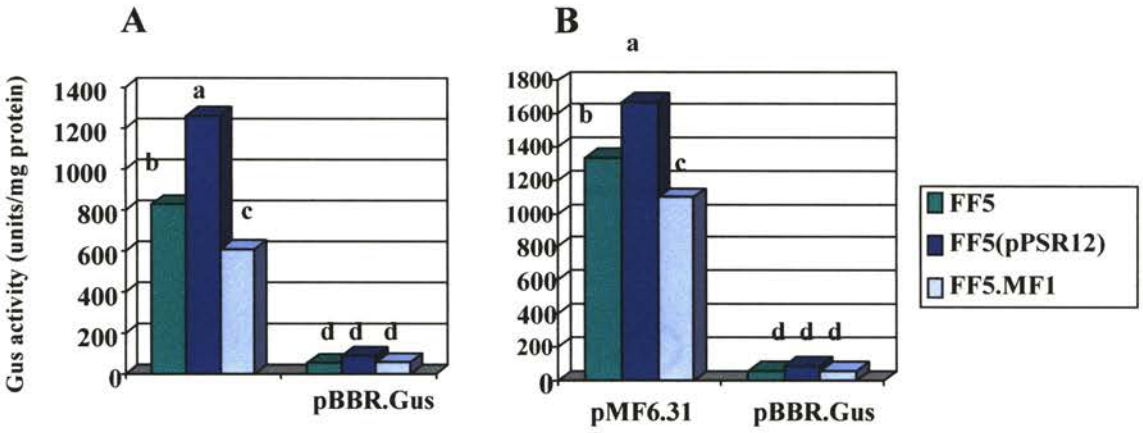

Figure 25. GUS activity of *P. syringae* FF5 (nonmucoid), FF5(pPSR12) (heavily mucoid), and FF5.MF1 (nonmucoid mutant) containing *algT-uidA* (panel **A**) and *algR1-uidA* (panel **B**). Values are the means from one experiment containing three replicates. Treatments accompanied by the same lowercase letter were not significantly different at P=0.05 (Student-Newman Keuls test.) pAlgTA contains the *algT* promoter in the transcriptionally active orientation, pMF6.31 contains *algR1* in the transcriptionally active orientation, and pBBR.Gus is the vector used to construct pAlgTA and pMF6.31.

Discussion

P. syringae pv. syringae FF5 is normally nonmucoid *in vitro*; however, the introduction of the 200-kb plasmid pPSR12 confers constitutive alginate production to

FF5 (Figure 18). In the present study, mutagenesis of FF5(pPSR12) resulted in the isolation of an alginate-defective mutant named FF5.MF1 containing a mini-Tn*5* insertion in pPSR12. Since plasmid pPSR12 does not contain homologs of any known alginate biosynthetic or regulatory genes (Kidambi et al., 1995), the inactivated gene(s) on the mutated plasmid pPSR12.1 may be a new regulator of alginate production.

FF5.MF1 was only partially complemented for alginate production by cosmid pSM51, possibly because this clone contains both positive and negative regulators. This hypothesis was supported by the 60-fold reduction in alginate production that occurred when pSM51 was mobilized to FF5(pPSR12) (Figure 20C) and by the gene expression data shown in Figure 25. Failure to complement FF5.MF1 with pRKE5.1, which contains the putative *muc* gene, may indicate that the Tn*5* mutation has polar effects on other genes required for constitutive mucoidy. Another possibility is that pRKE5.1 encodes a negative regulator in addition to the proposed positive regulator (*muc*) (discussed below).

The similarity of the *muc* gene to *kfrA*, which has a proposed role in plasmid stability and partitioning, may suggest a similar role for *muc*. However, the relatedness between *muc* and *kfrA* is low (36% similarity), and the ORFs surrounding *muc* (Figure 22) have no homology to ORFs associated with *kfrA* (Jagura-Burdzy and Tomas, 1992). The putative *muc* gene showed some relatedness to a SMC1-family ATPase involved in DNA repair. SMC proteins, which are involved in *s*tructural *m*aintenance of the *c*hromosome, constitute a protein family (110 to 170 kDa) that function in a range of chromosomal transactions, including chromosome condensation, sister-chromatid cohesion, recombination, DNA repair and epigenetic silencing of gene expression (Harvey et al., 2002). Since *recA* was shown to be involved in switching between rough

and smooth phenotypes in *Pseudomonas tolaasii* (Sinha et al., 2000), a possible role for a SMC homologue (e.g. *muc*) cannot be discounted.

Perhaps the most interesting relatedness found in database searches was the similarity between *muc* and *exoF* of *Sinorhizobium meliloti* (37% similarity). Interestingly, *exoF* is encoded by a megaplasmid and is organized within an operon of other *exo* genes that are essential for biosynthesis of the exopolysaccharide (EPS) succinoglycan. Furthermore, *exoX*, which maps near *exoF* in *S. meliloti*, is a negative regulator of succinoglycan and inhibits completely EPS synthesis production if expressed at high levels (Müller et al, 1993). A similar phenomenon might explain the failure of pRKE5.1 to complement mutant FF5.MF1; for example, pRKE5.1 might contain an additional ORF that functions as a negative regulator. Efforts to complement FF5.MF1 with the *muc* gene and individual ORFs in the region disrupted by mini-Tn*5* might clarify whether a negative regulator is present.

It is also important to note that the putative *muc* gene shared 41% similarity with ClpB, a heat shock protein in *E. coli*. ClpB functions as a molecular chaperone, whereas other Clp family members function both as chaperones and as components of ATP-dependent proteases (Schirmer et al., 1996). The *muc* gene product also showed a low level of relatedness (21%) to DegS from *E. coli*. DegS, a putative serine protease, regulates the basal and induced activity of the essential *E. coli* sigma factor, σ^E, which is involved in the cellular response to extracytoplasmic stress. DegS promotes the destabilization of the σ^E-specific anti-sigma factor RseA, thereby releasing σ^E to direct gene expression (Alba et al., 2001). It is tempting to speculate that the *muc* gene product could function in an analogous manner; for example, it could destabilize the anti-sigma

factor MucA, thereby releasing AlgT (σ^E homologue), which then initiates alginate biosynthesis. Although these ideas remain highly speculative, further work is needed to confirm the identity and function of the pPSR12-encoded gene conferring constitutive mucoidy to *P. syringae*.

CHAPTER VI

Sequence Analysis and Expression of a Putative Extracellular Epimerase from *Pseudomonas syringae* pv. glycinea

Abstract

Pseudomonas syringae pv. glycinea PG4180, a pathogen of soybean plants, produces the exopolysaccharide alginate. Previously, a clone from PG4180 named p561 was shown to be related (57% amino acid identity) to the extracellular epimerase, AlgE4, from *Azotobacter vinelandii*. This enzyme catalyzes the extracellular formation of α-L-guluronic acid (G) from its C5 epimer β-D-mannuronic acid (M). Since a complete open reading frame (ORF) was not found in p561, a genomic library of *P. syringae* pv. glycinea PG4180 was screened for an intact ORF using a probe constructed from p561. A cosmid clone designated pMF9 hybridized to the probe, and a 4.4-kb *Afl*III fragment of pMF9 with homology to the probe was subcloned into the expression vector pET21a, creating pMF9.2. *Escherichia coli* BL21(DE3) cells containing pMF9.2 were induced with IPTG and produced a protein band of ~165 kDa that was absent from non-induced cells. Several regions within the putative *P. syringae* epimerase (PAlgE) showed significant relatedness to the A and R structural modules present in the extracellular epimerases of *A. vinelandii*. PAlgE contains one A module and six putative R modules; the latter each contain two to six imperfect, nonameric repeats indicative of Ca^{2+} binding. While the N-terminus of the putative epimerase showed relatedness to the AlgE

epimerases, the C-terminus showed relatedness to adenylate cyclase. The presence of extracellular mannuronan C5-epimerases in *Pseudomonas* spp. has not been previously reported.

Introduction

Plant pathogenic bacteria are able to sense changes in temperature and can adapt accordingly by altering the expression of genes specifically required during pathogenesis or epiphytic growth. For example, *P. syringae* pv. glycinea PG4180 causes typical bacterial blight symptoms on soybean plants when the bacteria are grown at 18°C prior to inoculation but not when bacteria are grown at 28°C (Budde and Ullrich, 2000). Furthermore, PG4180 produces optimal levels of the chlorosis-inducing phytotoxin coronatine at 18°C, but negligible quantities at 28°C (Palmer and Bender, 1993). Although PG4180 has the capacity to produce the exopolysaccharide alginate (Keith and Bender, unpubl.), temperature-dependent production of alginate has not been reported for this strain. It remains possible that alginate has specific role(s) during the different temperature shifts that occur during colonization of host plants.

Alginate is a copolymer of β-D-mannuronic acid (M) and its C5 epimer, α-L-guluronic acid (G). These residues may be continuous (M blocks or G blocks) or mixed (MG blocks). The M/G ratio and the amount of M and G blocks vary with the organism and the conditions used for isolating the polymer (Haug et al., 1974). *Azotobacter vinelandii* produces alginate as part of a vegetative capsule and as a component of a metabolically dormant cyst (Sadoff, 1975). Alginates from *Azotobacter* contain

homopolymeric blocks of M and guluronic G residues, as well as MG blocks. Other alginate-producing bacteria include *Pseudomonas aeruginosa*, which overproduces alginate in the lungs of cystic fibrosis patients, and *P. syringae*, which produces alginate as a loosely attached capsule that contributes to virulence (Whitfield, 1988; Yu et al., 1999). Alginates from *Pseudomonas* spp. differ from those produced by *Azotobacter* because they are not known to contain homopolymeric G-blocks (Sherbrock-Cox et al. 1984).

The structural variability of the alginate polymer gives rise to a corresponding variation in its physical properties. Randomly alternating M and G monomers may form "kinks" and "disrupted" ribbons. Poly-M blocks form an extended "ribbon" conformation and produce elastic gels. Poly-G blocks form a "buckled" chain conformation and may produce firm but brittle gels by binding to divalent cations such as Ca^{2+}, Ba^{2+}, and Sr^{2+} (Rees, 1972; Rehm, 1998; Stokke et al., 1991). The diverse properties of alginate make it useful in a variety of biotechnological applications. For example, G-rich alginates have been used as gel-forming agents for the encapsulation of cells for transplantation into humans (Skjåk-Bræk and Espevik, 1996).

When alginate is first synthesized, it is comprised solely of mannuronic acid residues. G residues are introduced at the polymer level by the periplasmic enzyme, mannuronan C5-epimerase (Figure 26), which is a product of *algG*, a gene encoded by the alginate biosynthetic gene clusters of *P. aeruginosa* (Franklin et al., 1994), *P. syringae* (Penaloza-Vazquez et al., 1997) and *A. vinelandii* (Rehm et al., 1996). The absence of G-blocks in *P. aeruginosa* indicates that AlgG is unable to introduce repeating G residues into alginate and suggests that this bacterium does not contain an epimerase

capable of introducing G-blocks. Previous studies have demonstrated that an *algG* mutant of *P. aeruginosa* produces only polymannuronic acid, which suggests that *algG* is the sole mannuronic epimerase in this bacterium (Franklin et al., 1994).

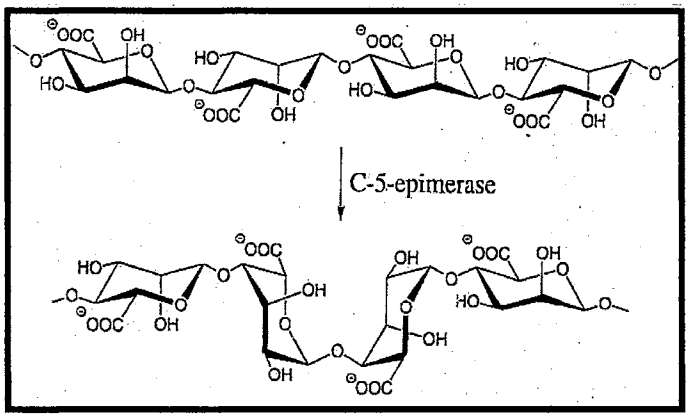

Figure 26. Epimerization of mannuronic to guluronic acid, a reaction catalyzed by mannuronic C5-epimerase.

In *A. vinelandii*, multiple mechanisms exist for the epimerization of mannuronic acid (Rehm et al., 1996). In addition to AlgG, *A. vinelandii* synthesizes a group of extracellular epimerases encoded by the *algE* gene family. These enzymes are capable of introducing alternating and/or repeating G residues into the polymer (Ertesvåg et al. 1995). It is important to note that *algE* is a multicopy gene family and not part of the alginate biosynthetic gene cluster in *A. vinelandii* (Ertesvåg et al. 1995). The epimerase genes *algE1-4, algE6*, and *algE7* are clustered in the chromosome (Figure 27). The 3' end of *algE6* is located 276 bp upstream of *algE4*, and the 3' end of *algE7* is located 5 kb upstream of *algE6* (Svanem et al., 1999). Interestingly, *algE5* is located in a separate part of the *Azotobacter* genome. Unlike AlgG, AlgE epimerases are secreted into the growth medium, have a strict requirement for Ca^{2+}, and consist of one or two "A" modules and up to seven "R" modules. The A module consists of 385 amino acids and is presumed to catalyze the epimerization reaction (Ertesvåg and Valla, 1999). The R module(s) is

located at the C-terminus of the enzyme with respect to the A module and contains ~155 amino acids (Ertesvåg et al., 1995). Each R module contains four to six repeats of a nonameric amino acid sequence characteristic of Ca^{2+}-binding motifs; furthermore, R modules are responsible for secretion of the enzyme (Ertesvåg and Valla, 1999). The A modules are highly conserved among AlgE epimerases, suggesting a common evolutionary ancestor; however, R modules exhibit more diversity than A modules.

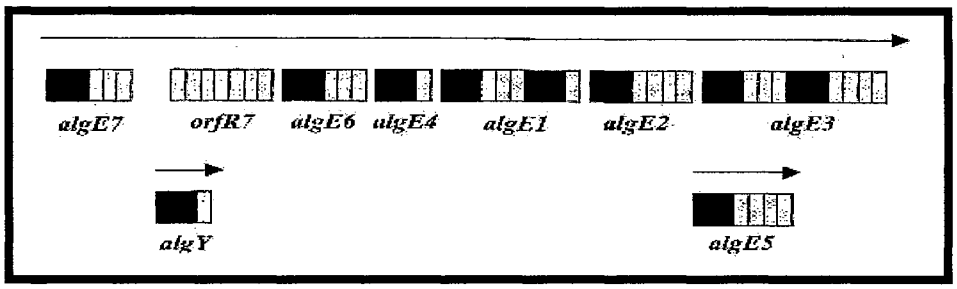

Figure 27. Physical map of the DNA region encoding *algE* epimerases in *A. vinelandii*. Black and white boxes represent the regions encoding the A- and R-modules, respectively. The physical location of *algY* and *algE5* relative to the main cluster is unknown (after Valla et al., 2001).

The molecular mass of AlgE epimerases is correlated with the number of A and R modules and ranges from 57.7 kDa (AlgE4) to 191 kDa (AlgE3). Nuclear magnetic resonance (NMR) spectroscopy analysis has shown that AlgE2 and AlgE6 introduce stretches of guluronic residues (G blocks), whereas AlgE4 results in alginates with MG blocks. AlgE7 is unique because it exhibits both epimerase and lyase activity (Svanem et al., 1999). The epimerase function of AlgE7 results in alginates with both single and repeated G residues, whereas the lyase function may provide smaller oligomers needed for *A. vinelandii* cyst formation or cyst germination (Svanem et al., 1999). Svanem et al. (2001) suggested that the catalytic activities of the epimerase and lyase encoded by *algE7*

probably originate from the same active site and a complex interplay exists between the two enzymatic activities. AlgE1-AlgE7 and AlgY have been successfully cloned from *A. vinelandii* and overproduced as functional epimerases in *Escherichia coli* (Ertesvåg et al., 1995; Svanem et al., 1999).

In addition to the C5 epimerization of mannuronic to guluronic acid, many other carbohydrate epimerization reactions occur in microorganisms. The conversion of D-glucuronic acid to L-iduronic acid by glucuronyl C5-epimerase, a key enzyme in the biosynthesis of heparin, is carbohydrate modification that cannot be achieved by chemical methods (Li et al., 2001). Allard et al. (2001) grouped epimerases according to their mechanism of action and concluded that each position on the carbohydrate can be epimerized by a microbial enzyme.

Alginates are used for a variety of purposes and are generally isolated from brown seaweeds. The use of alginate for immobilization of cells has resulted in efforts to create artificial alginate-based organs for patients suffering from diabetes (Darzins and Chakrabarty, 1984). The cost of polymers for this type of application is high because product quality and reproducibility is absolutely critical. Consequently, the use of bacterial C5-epimerases for improving the quality of alginates from seaweeds or for the direct production of high-quality polymers in bacteria may be cost-effective for certain applications (Valla et al., 2001).

The purpose of this study was to clone, sequence and overexpress a gene encoding a putative extracellular epimerase from *P. syringae* pv. glycinea PG4180. This epimerase was originally identified in a screen for genes specifically induced at 18°C, a

feature that may have biological significance in protecting *P. syringae* from the desiccation stress that occurs at reduced temperatures.

Materials and Methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 7. *E. coli* strains were maintained at 37°C on Luria-Bertani (LB) medium supplemented with ampicillin or tetracycline at 100 or 25 μg/ml, respectively.

Molecular genetic techniques

Plasmid p561 was provided by Dr. Matthias Ullrich at the Max Planck Institute for Microbiology in Marburg, Germany, and was isolated and purified from *E. coli* DH5α using the Plasmid DNA Midi Kit from Qiagen (Qiagen, Valencia, Calif.). Restriction enzyme digests, plasmid DNA isolations, agarose gel electrophoresis, Southern transfers and colony hybridizations were performed by standard methods (Sambrook et al., 1989). The 835-bp DNA probe used to screen the *P. syringae* pv. glycinea PG4180 genomic library was constructed by PCR amplification using plasmid p561 as a template. Two oligonucleotide primers were synthesized by the OSU Recombinant DNA/Protein Resource Facility: forward primer, 5'-ATACAGCAGCCATTCAGGCCACTA; and reverse primer, 5'-TGCTCAGGGTGTTATCAAAGACATCCAC. DNA fragments were isolated from agarose gels by electroelution and labeled with digoxigenin (Genius Labeling and Detection Kit; Boeringer Mannheim, Indianapolis, Ind.) or with [α-

^{32}P]dCTP using the Rad Prime DNA Labeling System (Gibco BRL, Gaithersburg, Md.). Hybridization and post-hybridization washes were conducted under high-stringency conditions.

Table 7. Bacterial strains and plasmids.

Strains or Plasmids	Relevant characteristics	Source or Reference
Escherichia coli		
DH5α	Contains T7 RNA polymerase	Sambrook et al., 1989
BL21(DE3)		Novagen
P. syringae pv. glycinea		
PG4180	Pathogenic on soybeans	Bender et al., 1993
Plasmids		
p561	Ap^r; contains 2.0 kb of PG4180 DNA in pBluescript II SK+	M. Ullrich
pRK7813	Tc^r; cosmid vector	Jones and Gutterson, 1987
pMF9	Tc^r ; cosmid clone from PG4180 library containing ~40-kb insert in pRK7813	This study
pBluescript SK+	Ap^r; ColEI origin, cloning vehicle	Stratagene
pET21a	Ap^r; contains T7 promoter and His-tag peptide	Novagen
pMF9.1	Ap^r; contains 4.4 -kb *Afl*III fragment of pMF9 in pBluescript II SK+	This study
pMF9.2	Ap^r; contains 4.4-kb *Eco*RI-*Sst*I fragment from pMF9.1 in pET21a	This study
pMT9.2	Ap^r; contains 11-kb *Hin*dIII fragment of pMF9 in pET21a	This study

DNA sequencing and analysis

Automated DNA sequencing was provided by the OSU Recombinant DNA/Protein Resource Facility and was performed with an ABI 373A apparatus and the ABI PRISM Dye Primer cycle-sequencing kit (Perkin-Elmer, Foster City, Calif.). Oligonucleotide primers used for sequencing were also synthesized by the OSU Recombinant DNA/Protein Resource Facility. Sequence manipulations, amino acid alignments, and restriction maps were constructed using the Vector NTI Suite, Version 6.0 (Informax, San Francisco, CA). Database searches were performed with the BLAST

service of the National Center for Biotechnology Information. Preliminary genomic sequence data was obtained for *P. syringae* pv. tomato DC3000 from The Institute for Genomic Research (*http://www.tigr.org*).

Overproduction of proteins

E. coli BL21(DE3) cells containing selected constructs were grown at 37°C in LB broth to an OD_{600}=0.5-0.6, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubated an additional 3 h. Aliquots of cells (1 ml) were collected from induced and uninduced treatments and pelleted by centrifugation. The total cellular protein was prepared and analyzed on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing 5% polyacrylamide (Sambrook et al., 1989).

Results

Cloning of the putative epimerase from *P. syringae*

Previously, *P. syringae* pv. glycinea PG4180 was randomly mutagenized with a mini-Tn*5* construct containing a promoterless glucuronidase gene (*uidA*). Mutants were screened for elevated gene expression at 18°C to identify thermoregulated genes (Ullrich et al., 2000). A transposon mutant designated PG4180.561 showed increased transcriptional activation of glucuronidase at 18°C as compared to 28°C. The region disrupted by the transposon insertion in PG4180.561 was cloned into *Sal*I-digested pBluescript II SK+ and designated p561 (Ullrich et al., 2000). Sequence analysis of p561

revealed a 2.0-kb fragment with 57% amino acid identity to the extracellular epimerase, AlgE4, from *A. vinelandii* (Preston, 2001). In the current study, a 835-bp PCR fragment from p561 (with relatedness to the A modules of *Azotobacter* epimerases) was used to screen a genomic library of *P. syringae* PG4180 for clones containing the intact epimerase (PAlgE) coding region (Figure 28). Several cosmids hybridized with the probe, and a clone designated pMF9 was chosen for further study. Restriction digestion of pMF9 and Southern blot analysis revealed a 4.4-kb *Afl*III fragment that hybridized to the probe; this fragment was isolated by electroelution, end-filled with Klenow, and ligated to *Sma*I-digested pBluescript II SK+, forming pMF9.1 (Table 7 and Figure 28). Sequence analysis of pMF9.1 showed that the 4.4-kb insert contained a large ORF but lacked a stop codon. Therefore, an 11-kb *Hin*dIII fragment of pMF9 that also hybridized to the probe was cloned in pET21a, forming pMT9.2 (Table 7). Sequence analysis of pMT9.2 showed a definite stop codon that was absent in pMF9.1.

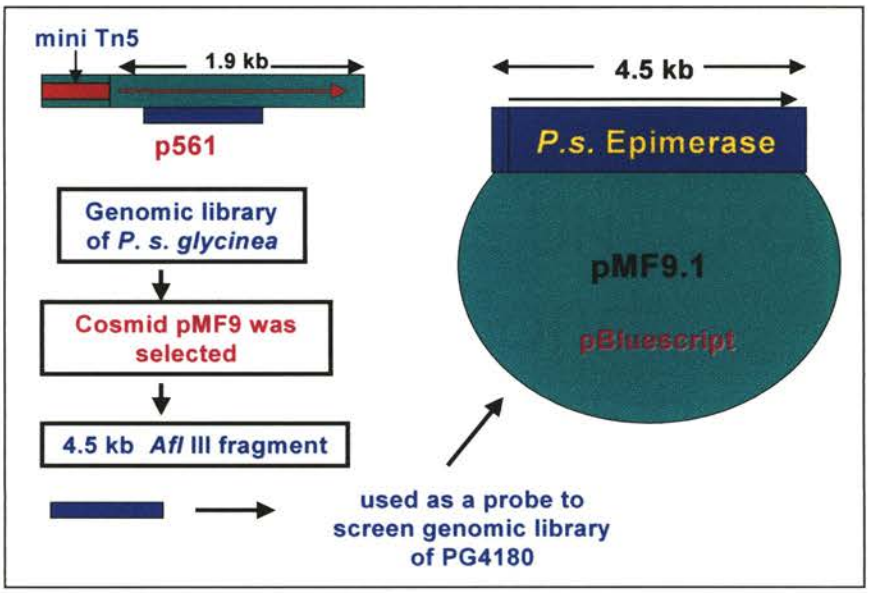

Figure 28. Cloning of the putative extracellular epimerase from *P. syringae* pv. glycinea.

Sequence analysis

DNA sequencing (Figure 29) of ~5 kb in pMF9.1 and pMT9.2 revealed a 4,830 bp ORF that encodes *pAlgE*, the putative epimerase of *P. syringae* PG4180. PAlgE contains 1,610 amino acid residues, with a single A module and six putative R modules. The A module (Figure 29; residues in red font) comprises 387 amino acids and terminates with the sequence VTVQGT. Five amino acids (residues 388-392) separate the A module from the first R module (Figure 29). PAlgE contains six putative R modules (Figure 29, blue font), each with two to six imperfect, nonameric Ca^{2+}-binding motifs (Figure 29; residues underscored in blue) that are characteristic of R modules in *A. vinelandii*.

```
  1 ATGATATTAAACACAAAAGACTTTGGTGCAGTGGGCGACAGCGTC
    M  I  L  N  T  K  D  F  G  A  V  G  D  S  V  15
 46 ACGGACGATACAGCAGCCATTCAGGCCACTATTGATGCAGCTGCA
    T  D  D  T  A  A  I  Q  A  T  I  D  A  A  A  30
 91 GCGGCGGGCGGTGGAGAAGTTGTGCTGGCAGCCGGGACCTACATT
    A  A  G  G  G  E  V  V  L  A  A  G  T  Y  I  45
136 GTGTCCGGTGGCGAGGAGCCTTCCGACGGCTGCCTGATGCTCAAA
    V  S  G  G  E  E  P  S  D  G  C  L  M  L  K  60
181 AGCAACGTGACCCTATCGGGCGCGGGCATGGGCGAGACAGTCATC
    S  N  V  T  L  S  G  A  G  M  G  E  T  V  I  75
226 AAGCTGGCGGATGGCTCGGACACCAAGGTCACCGGCATCGTGCGT
    K  L  A  D  G  S  D  T  K  V  T  G  I  V  R  90
271 TCCGCCTACGGCGAAGAAACCCACGACTTCGGCATGAAAAATCTG
    S  A  Y  G  E  E  T  H  D  F  G  M  K  N  L  105
316 ACATTGGACGGTAACCGGGACGCCACCACCGGCAAGGTGGACGGC
    T  L  D  G  N  R  D  A  T  T  G  K  V  D  G  120
361 TGGTTCAACGGCTACATTCCAGGTTCTGACGGCAAGGATTCGAAC
    W  F  N  G  Y  I  P  G  S  D  G  K  D  S  N  135
406 GTTACGCTCGACAGCGTCGAGATCAAGGACTGCTCGGGCTATGGC
    V  T  L  D  S  V  E  I  K  D  C  S  G  Y  G  150
451 TTCGACCCGCACGAGCAGACCGTCAACATGGTGATCAAAAACAGC
    F  D  P  H  E  Q  T  V  N  M  V  I  K  N  S  165
496 GTATCCCATGGCAACGGCCTGGACGGTTTCGTGGCCGATTACCTG
    V  S  H  G  N  G  L  D  G  F  V  A  D  Y  L  180
541 AGCGACAGCGTGTTTGAAAATAACATTGCTTATAACAACGACCGT
    S  D  S  V  F  E  N  N  I  A  Y  N  N  D  R  195
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586 CACGGCTTCAACGTGGTGACCAGCACTCACGATTTCACCCTGAGC
    H  G  F  N  V  V  T  S  T  H  D  F  T  L  S  210
631 AACAACGTCGCCTACGGCAATGGCAGCACCGGCATCGTGGTGCAG
    N  N  V  A  Y  G  N  G  S  T  G  I  V  V  Q  225
676 CGCGGCAGTGAAAACATCCCCTCGCCTGCCAATATCACCATCACC
    R  G  S  E  N  I  P  S  P  A  N  I  T  I  T  240
721 GGTGGCGCGGTCTATGGCAACGGTGCTGAAGGCGTGCTGATCAAG
    G  G  A  V  Y  G  N  G  A  E  G  V  L  I  K  255
766 CTGTCCAGTCAGGTCTCTGTCAGTGGCCTGGACATTCACGACAAC
    L  S  S  Q  V  S  V  S  G  L  D  I  H  D  N  270
811 GGCAGTGCCGGTGTGCGCATCTACGGCAGTTCCGGGGTGGATGTC
    G  S  A  G  V  R  I  Y  G  S  S  G  V  D  V  285
856 TTTGATAACACCCTGAGCAACAACTCCCTCGGCGCCCCGGTGCCG
    F  D  N  T  L  S  N  N  S  L  G  A  P  V  P  300
901 GAGATTATCATCCAGTCTTACGACGACACTTCAGGCGTTTCCGGC
    E  I  I  I  Q  S  Y  D  D  T  S  G  V  S  G  315
946 AAGTATTTCAACGGCAGCGACAACCTGATCCGCGGCAACCTGATC
    K  Y  F  N  G  S  D  N  L  I  R  G  N  L  I  330
991 ACCGGCAGCGACAACTCCACCTACGGCGTTGCCGAGCGCAACGAA
    T  G  S  D  N  S  T  Y  G  V  A  E  R  N  E  345
1036 GATGGCACCGATCGCAACAGCATCGTGGGCAACACCATCAGCCAC
    D  G  T  D  R  N  S  I  V  G  N  T  I  S  H  360
1081 ACCAGCAAAGGCCTGACGCTGGTGTATGGCGACGGCAGCTTTGCA
    T  S  K  G  L  T  L  V  Y  G  D  G  S  F  A  375
1126 GGCGATTCGTTTCCGCTGGTCACCGTACAGGGTACCGACGCCAAC
    G  D  S  F  P  L  V  T  V  Q  G  T..D  A  N  390
1171 GATGCGATCACCGGTGGCGCAGCCAATGAAATGATTTTCGGTCTG
    D  A..I  T  G  G  A  A  N  E  M  I  F  G  L  405
1216 GCTGGCAAGGACACGCTCAACGGCGCTGCGGGCGACGATATTCTG
    A  G  K  D  T  L  N  G  A  A  G  D  D  I  L  420
1261 GTCGGCGGTGCGGGAGCCGACAAGCTCACCGGTGGCGCGGGTGCC
    V  G  G  A  G  A  D  K  L  T  G  G  A  G  A  435
1306 GATACTTTTCGCTTCGACCAGCTGACCGATAGCTATCGCACGGCG
    D  T  F  R  F  D  Q  L  T  D  S  Y  R  T  A  450
1351 ACCACCAGCGCCACCGATCTGGTCACCGACTTCGACGTCAGCCAG
    T  T  S  A  T  D  L  V  T  D  F  D  V  S  Q  465
1396 GACCGCATCGACCTGTCAAACCTTGGTTTCAGCGGGCTGGGCAGC
    D  R  I  D  L  S  N  L  G  F  S  G  L  G  S  480
1441 GGCAAGGGCGGTACTCTGAACATCAGCTACAACGCCACACTTGAT
    G  K  G  G  T  L  N  I  S  Y  N  A  T  L  D  495
1486 CGCACTTACGTCAAATCACTCGACGCCGATGCGAGCGGCAACCGA
    R  T  Y  V  K  S  L  D  A  D  A  S  G  N  R  510
1531 TTCGAGCTGGGCCTGAGCGGCAACCTGAAAGACACCCTCAATGCC
    F  E  L  G  L  S  G  N  L  K  D  T  L  N  A  525
1576 AGCCATTTCATCTTCCAGCGGGTCATAGAAGGCACTGCGGGCGGC
    S  H  F  I  F  Q  R  V  I  E  G  T  A  G  G  540
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1621 GATACCCTGACCGGCACCGACGGCAACGACGTGATGAACGGCAAC
     D  T..L  T  G  T  D  G  N  D  V  M  N  G  N  555
1666 GCCGGTACAGACCGCATCAATGGCGGTGCCGGTGCAGATCTCATC
     A  G  T  D  R  I  N  G  G  A  G  A  D  L  I  570
1711 AATGGCGGGGCTGATGCCGATATTCTGACCGGTGGCGCGGGCGCG
     N  G  G  A  D  A  D  I  L  T  G  G  A  G  A  585
1756 GACCTGTTCATCTACAACTCGCGCCTGGACAGCTACCGCAATTAC
     D  L  F  I  Y  N  S  R  L  D  S  Y  R  N  Y  600
1801 ACCGCCAGCGGCACCAAGCAGAGCGACACCATCACCGACTTCAAC
     T  A  S  G  T  K  Q  S  D  T  I  T  D  F  N  615
1846 CCGGCCGAAGACCGGATCGACCTGTCCAGCATCGGCCTGCGAGGC
     P  A  E  D  R  I  D  L  S  S  I  G  L  R  G  630
1891 CTGGGCGATGGCAGTGCCAACACTATCTATCTGTCCGTCAATGCC
     L  G  D  G  S  A  N  T  I  Y  L  S  V  N  A  645
1936 GACGGCAGCAAGACCTATATCAAGACCAATGCGGTCGACACCACC
     D  G  S  K  T  Y  I  K  T  N  A  V  D  T  T  660
1981 GGCAATCGCTTCGAGATTGCACTGGAAGGCAACCTGCTCGACAAA
     G  N  R  F  E  I  A  L  E  G  N  L  L  D  K  675
2026 CTGAGCGCGTCCAGCTTCATCTTCTCTACAGCCTCAGCCGCCAAT
     L  S  A  S  S  F  I  F  S  T  A  S  A  A  N  690
2071 CAGGCCCCGGTACTCAATACACCGCTGATGGATCAGAACGTTACC
     Q  A  P  V  L  N  T  P  L  M  D  Q  N  V  T  705
2116 GAGTTGAAGGCGTTTTCCTACGCGGTACAATCGGGCAGTTTCAGT
     E  L  K  A  F  S  Y  A  V  Q  S  G  S  F  S  720
2161 GATCCGGACAGCAACACGCTGACCTACAGCGCGACCCTGGCCGAT
     D  P  D  S  N  T  L  T  Y  S  A  T  L  A  D  735
2206 AACAGCGCCCTGCCCGACTGGTTGAAGTTCGACAGCAAGACCCTG
     N  S  A  L  P  D  W  L  K  F  D  S  K  T  L  750
2251 ACCTTCAGCGGCACACCGGGCGGCAAGGCATCCGGGCTTTACTCG
     T  F  S  G  T  P  G  G  K  A  S  G  L  Y  S  765
2296 GTATTGCTGACCGCAAGCGATGCCACCGGCGCGTCGGTGGCCGAC
     V  L  L  T  A  S  D  A  T  G  A  S  V  A  D  780
2341 AGTTTTGCCATCACGGTAGGCAACGTTACACCGGGCGTTCTGACC
     S  F  A  I  T  V  G  N  V  T  P  G  V  L  T  795
2386 GGCACCGAAAATGCGGAAGCGCTGTATGGCACCGAAGGTGACGAC
     G  T  E  N  A  E  A..L  Y  G  T  E  G  D  D  810
2431 ACCATTCTCGGGCTGGGTGGCGACGATACGCTGCGTGGCGATACC
     T  I  L  G  L  G  G  D  D  T  L  R  G  D  T  825
2476 GGTGCCGACATCATCAACGGTGGCGCTGGCCGCGACGCATTGTAC
     G  A  D  I  I  N  G  G  A  G  R  D  A  L  Y  840
2521 GGTGGAGACGGTGCCGATACGTTCGTCTACAGCGCACTCACCGAC
     G  G  D  G  A  D  T  F  V  Y  S  A  L  T  D  855
2566 AGCTACCGCGATTACGATGCTGGCGGGCTGACAGCCACCGACACA
     S  Y  R  D  Y  D  A  G  G  L  T  A  T  D  T  870
2611 ATCTATGACTTCACCCCCGGCCAGGACAAAATAGACGTCTCTGCT
     I  Y  D  F  T  P  G  Q  D  K  I  D  V  S  A  885
2656 CTGGGTTTCCTGGGGCTGGGCAACGGTGAAGATCACACCTTGTAC
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     L  G  F  L  G  L  G  N  G  E  D  H  T  L  Y  900
2701 ATGACCCTCAACGAAGCCGGCGACAAGACCTATGTCAAATCCGCC
     M  T  L  N  E  A  G  D  K  T  Y  V  K  S  A  915
2746 ACGCCGGATGCCGACGGCAATCGCTTCGAAATCGCCCTGAGCGGC
     T  P  D  A  D  G  N  R  F  E  I  A  L  S  G  930
2791 AACCTGATCGACACCCTGACCGATGCGGACTTCGTGTTCGGCCAG
     N  L  I  D  T  L  T  D  A  D  F  V  F  G  Q  945
2836 CGCGAGGCTCAGGAGATTCTCTATCTGCCGACGCTTGGCCAGTCC
     R  E  A  Q  E  I  L  Y  L  P  T  L  G  Q  S  960
2881 AACGCACGCCTGCTGCGCATGACCGAAGACGACAATCAGTCCGGC
     N  A  R  L  L  R  M  T  E  D  D  N  Q  S  G  975
2926 ACCTCGGAAATGGTCAAGGACCTGGCCCGTTACACCGATTATGAC
     T  S  E  M  V  K  D  L  A  R  Y  T  D  Y  D  990
2971 GTGCGCAGCCAGTTCACCGACGCCAACGGTGATGGCATCGACCTT
     V  R  S  Q  F  T  D  A  N  G  D  G  I  D  L  1005
3016 GCCGTAGGCGGCAGCACCGTGGTCGGCTATTCGACCGGCACGCAG
     A  V  G  G  S  T  V  V  G  Y  S  T  G  T  Q  1020
3061 GAAGAACAGCGTGTTTCGTGGTGGCTGGTGGATACCGATCAGCCA
     E  E  Q  R  V  S  W  W  L  V  D  T  D  Q  P  1035
3106 GGCCCTGCGCTGCTGCGTGCGACCGAACTTCTCAAGTCGCAACTG
     G  P  A  L  L  R  A  T  E  L  L  K  S  Q  L  1050
3151 GCGTCGCTGACGGCTATCGATAAAGTGACCACCGGGATTATCTGG
     A  S  L  T  A  I  D  K  V  T  T  G  I  I  W  1065
3196 GGTCAGGGTGAAGAAGCCGCGCAGGAAATAGCCCGCGCCACGGAC
     G  Q  G  E  E  A  A  Q  E  I  A  R  A  T  D  1080
3241 AAGCAGGCTGCAGCCGACCTCTACAAAGCCTCGACCCTGAAGGTG
     K  Q  A  A  A  D  L  Y  K  A  S  T  L  K  V  1095
3286 TTCGATTACCTGCACGCGCAGATAGGCGACTTCACCGTGTACATG
     F  D  Y  L  H  A  Q  I  G  D  F  T  V  Y  M  1110
3331 GCCGAAACCGGCCACTATCAGACCGAGGCGGCCAAGGCACGTGGC
     A  E  T  G  H  Y  Q  T  E  A  A  K  A  R  G  1125
3376 TACACCGAAGAAAAGATCAACGCTATTGTCGAGGGTGCCGGATAC
     Y  T  E  E  K  I  N  A  I  V  E  G  A  G  Y  1140
3421 GTCAGAAACGCTCAGGAAGCCATCGCCAATGAGCGTGCCGACGTC
     V  R  N  A  Q  E  A  I  A  N  E  R  A  D  V  1155
3466 AAGCTGGCAGTTGACTACACCGACCTGCCATTGCGTTACGAGGTC
     K  L  A  V  D  Y  T  D  L  P  L  R  Y  E  V  1170
3511 AACCCGCTGGTCTACCCCGATGACGTCTGGCACCTGCACGAAGAG
     N  P  L  V  Y  P  D  D  V  W  H  L  H  E  E  1185
3556 TCCGCCGAAATCGTCGGTCAGCGCCTAGCCGACTTCATTGCCGAT
     S  A  E  I  V  G  Q  R  L  A  D  F  I  A  D  1200
3601 GACCTGGGTTTTCGTGGCGATGCCAGCGACAACAACGACCCGGCC
     D  L  G  F  R  G  D  A  S  D  N  N  D  P  A  1215
3646 GCCATTTTCGAGAGTGGCCAGAATGAAGGCGGCAACATCTTCGGC
     A  I  F  E  S  G  Q  N  E  G  G  N..I  F  G  1230
3691 ACCAGTGACGACGACACTTTGGTGGGCAGCGCCGGCAATGACGTG
     T  S  D  D  D  T  L  V  G  S  A  G  N  D  V  1245
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3736 CTGGATGGCGATCAGGGCGCAGATGACATGACCGGCGGGGACGGC
     L  D  G  D  Q  G  A  D  D  M  T  G  G  D  G  1260
3781 AACGACATCTATGTAGTCGATAACGCACTCGATACCGTCACCGAG
     N  D  I  Y  V  V  D  N  A  L  D  T  V  T  E  1275
3826 AGCAATGATTCGCCATCGCAGGTTGATACCGTGGTGTCTTCCGTC
     S  N  D  S  P  S  Q  V  D  T  V  V  S  S  V  1290
3871 AGTTGGCAGTTGGGGGCGAACGTGGAAAACCTGCTGCTTACCGGC
     S  W  Q  L  G  A  N  V  E  N  L  L  L  T  G  1305
3916 GTATCTGCCATCAACAGCACCGGCAATGCGCTGAAAAACGTCATC
     V  S  A  I  N  S  T  G  N  A  L  K  N  V  I  1320
3961 ACCGGCAATGCCAGCAACAATGTTCTCGACGGCGCAGCGGGTGCC
     T  G  N  A  S  N  N  V..L  D  G  A  A  G  A  1335
4006 GACCTGCTGACCGGCGGCGACGGATCGGACAGTTATTACGTTGAC
     D  L  L  T  G  G  D  G  S  D  S  Y  Y  V  D  1350
4051 GATGCAGCCGACCGAGTGGTCGAGACCAACGCAGATCAGCAGGTA
     D  A  A  D  R  V  V  E  T  N  A  D  Q  Q  V  1365
4096 GGCGGCATCGACACGGTGCTCAGTTCGCTGGCCAGCTACACGCTG
     G  G  I  D  T  V  L  S  S  L  A  S  Y  T  L  1380
4141 GGGGCCAACCTGGAAAACATCGTCATCACCGGCACAGGGGCCGCG
     G  A  N  L  E  N  I  V  I  T  G  T  G  A  A  1395
4186 AACGCAACCGGCAATACCCTTGATAACCTGATCTACGCAGGCGCT
     N  A  T  G  N  T  L  D  N  L  I  Y  A  G  A  1410
4231 GGCGACAACGTCATGGACGGTCGTGACGGCAATGACACGGTGTCT
     G  D  N  V  M  D  G  R  D  G  N  D  T  V  S  1425
4276 TATCTGTTTGCCACGGCAGGCGTTACGGTTGCGCTGAACACCAGC
     Y  L  F  A  T  A  G  V  T  V  A  L  N  T  S  1440
4321 GCTCAACAGGCCACCGGCGGCTCCGGGTTGGACACGCTCAAGGGT
     A  Q  Q  A  T  G  G  S  G  L  D  T  L  K  G  1455
4366 ACAGAGAACCTGACTGGCAGCCAGTTTGCCGACACGTTGACCGGC
     T  E  N..L  T  G  S  Q  F  A  D  T  L  T  G  1470
4411 AACAAAAACGCCAACGTCCTGAACGGCGGCAGTGGCAATGACACC
     N  K  N  A  N  V  L  N  G  G  S  G  N  D  T  1485
4456 TTGTCCGGCGGCGTGGGTGACGACGTACTGATCGGCGGGTCAGGC
     L  S  G  G  V  G  D  D  V  L  I  G  G  S  G  1500
4501 GCTGATACGCTGATTGGCGGCACCGGTGCAGACCGTTACGTGTTC
     A  D  T  L  I  G  G  T  G  A  D  R  Y  V  F  1515
4546 AACAACAGCAACGAGACAGGCCTGGGCGGCCTGCGCGACATCATC
     N  N  S  N  E  T  G  L  G  G  L  R  D  I  I  1530
4591 AACGGCTTCAAGGCAGCCGAAGGCGACAAGCTGGACTTCACAGGC
     N  G  F  K  A  A  E  G  D  K  L  D  F  T  G  1545
4636 TTCGATGCCCGACCGCTGACCGATGCCCACGATGCGTTCGTGTTC
     F  D  A  R  P  L  T  D  A  H  D  A  F  V  F  1560
4681 ATAGGCAACGCGGCGTTCAGCGCCAACAACACCGGCGAGCTTCGC
     I  G  N  A  A  F  S  A  N  N  T  G  E  L  R  1575
4726 TTTGCCGATGGGGTGCTGTACGGCAATCTCGATGACAACATCGGC
     F  A  D  G  V  L  Y  G  N  L  D  D  N  I  G  1590
4771 GCGGACTTTGAAATCCAGCTGACTGGCGTGCAGAGTCTGCAAGCG
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     A  D  F  E  I  Q  L  T  G  V  Q  S  L  Q  A  1605
4816 GCCGATATCATCGTCTGA
     A  D  I  I  V  *                          1610
```

Figure 29. Nucleotide sequence of pAlgE, which encodes a putative extracellular epimerase in *P. syringae* pv. glycinea PG4180. The amino acid sequence of the A module is indicated in red font. The putative R modules are shown in blue font and separated by two periods (. .). Nonameric Ca^{2+}-binding motifs are underscored.

BLAST analysis

When compared to amino acid sequences in the database, PAlgE is related (45% identity/57% similarity) to the extracellular mannuronan C5-epimerase, AlgE4, from *A. vinelandii*. PAlgE is also related to other extracellular epimerases in the AlgE family, including AlgE1, AlgE2, AlgE4, AlgE5, AlgE6, and AlgE7. The A module of PAlgE is 63% and 62% identical to the A modules of AlgE4 and AlgE5 (Figure 30), respectively. Furthermore, the R modules of PAlgE and the the AlgE family of epimerases are related; for example the first two R modules in PAlgE are 52% identical to the R modules in AlgE3 and AlgE5 and 48% identical to the R module in AlgE4. It is interesting to note that the A modules of AlgE1-7 and PAlgE are more highly conserved while the corresponding R modules display greater sequence divergence.

```
PAlgE: 1   MDYNVKDFGALGDGVSDDTAAIQAAIDAAYAAGGGTVYLPAGEYRVSGGEEPSDGCLTIK 60
           M  N KDFGA+GD V+DDTAAIQA IDAA AAGGG V L AG Y VSGGEEPSDGCL +K
AlgE5: 1   MILNTKDFGAVGDSVTDDTAAIQATIDAAAAAGGGEVVLAAGTYIVSGGEEPSDGCLMLK 60

PAlgE: 61  SNVYIVGAGMGETVIKLVDGWDQDVTGIVRSAYGEETSNFGMSDLTLDGNRDNTSGKVDG 120
           SNV + GAGMGETVIKL DG D  VTGIVRSAYGEET +FGM +LTLDGNRD T+GKVDG
AlgE5: 61  SNVTLSGAGMGETVIKLADGSDTKVTGIVRSAYGEETHDFGMKNLTLDGNRDATTGKVDG 120

PAlgE: 121 WFNGYIPGEDGADRDVTLERVEIREMSGYGFDPHEQTINLTIRDSVAHDNGLDGFVADFQ 180
           WFNGYIPG DG D +VTL+ VEI++ SGYGFDPHEQT+N+ I++SV+H NGLDGFVAD+
AlgE5: 121 WFNGYIPGSDGKDSNVTLDSVEIKDCSGYGFDPHEQTVNMVIKNSVSHGNGLDGFVADYL 180

PAlgE: 181 IGGVFENNVSYNNDRHGFNIVTSTNDFVLSNNVAYGNGGAGLVIQRGSYDVAHPYGILID 240
              VFENN++YNNDRHGFN+VTST+DF LSNNVAYGNG  G+V+QRGS ++  P  I I
AlgE5: 181 SDSVFENNIAYNNDRHGFNVVTSTHDFTLSNNVAYGNGSTGIVVQRGSENIPSPANITIT 240
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PAlgE: 241 GGAYYDNGLEGVQIKMAHDVTLQNAEIYGNGLYGVRVYGAEDVQILDNYIHDNSQSGSYA 300
           GGA Y NG EGV IK++  V++   +I+ NG  GVR+YG+  V + DN + +NS
AlgE5: 241 GGAVYGNGAEGVLIKLSSQVSVSGLDIHDNGSAGVRIYGSSGVDVFDNTLSNNSLGAPVP 300

PAlgE: 301 EILLQSYDDTAGVSGNFYTTTGTWIEGNTIVGSANSTYGIQER-ADGTDYSSLYANSVSN 359
           EI++QSYDDT+GVSG ++  +   I GN I GS NSTYG+ ER  DGTD +S+  N++S+
AlgE5: 301 EIIIQSYDDTSGVSGKYFNGSDNLIRGNLITGSDNSTYGVAERNEDGTDRNSIVGNTISH 360

PAlgE: 360 VQSGSVRLYGTNSVVSD 376
              G   +YG  S   D
AlgE5: 361 TSKGLTLVYGDGSFAGD 377
```

Figure 30. Pairwise comparison of the **A** modules of PAlgE from *P. syringae* (pink font) and AlgE5 from *A. vinelandii* (green font). The two **A** modules show 62% amino acid identity.

Since pseudomonads are not known to produce extracellular epimerases, the nucleotide sequence of *PalgE* was compared to sequences in the unfinished genome of *P. syringae* pv. tomato DC3000. Interestingly, PAlgE showed 90% amino acid identity (94% similarity) and 84% nucleotide identity to an ORF of 8,330 bp in the DC3000 genome. Interestingly, no similar ORF was found in the *P. aeruginosa* genome, although limited relatedness was noted between PAlgE and the periplasmic mannuronan C5-epimerase (AlgG) of *P. aeruginosa*. It is also important to mention that the 3' end of *palgE* (~1 kb) shares relatedness (39% similarity) with the amino acid sequence of the adenylate cyclase hemolysin of *Bordetella bronchiseptica*.

Overproduction of the putative epimerase

The 4.4-kb fragment in pMF9.1 contains *palgE* under control of the T7 promoter of pBluescript. However, efforts to overproduce a protein from *E. coli* BL21(pMF9.1) cells were unsuccessful. In a subsequent experiment, the 4.4-kb insert in pMF9.1 was excised as an *Eco*RI-*Sst*I fragment and subcloned in the active orientation with respect to the T7 promoter in pET21a, generating a new construct named pMF9.2. When *E. coli*

BL21(pMF9.2) cells were induced with IPTG, a 165-kDa protein (Figure 31, lane 5) was observed, which corresponds to the predicted size of the truncated PAlgE epimerase. This band was absent from uninduced and induced BL21(pET21a) cells and from uninduced cells of BL21(pMF9.2) (Figure 31, lanes 2-4). Efforts to overproduce the 177 kDa predicted PAlgE protein using BL21(DE3) cells harboring pMT9.2 were not successful.

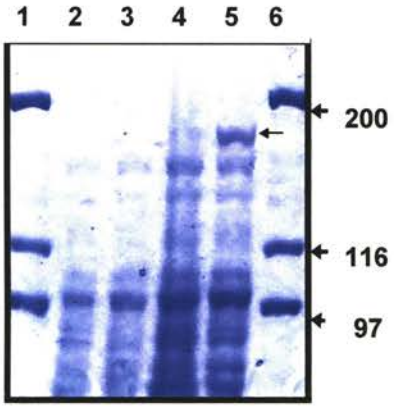

Figure 31. SDS-PAGE analysis of *E. coli* BL21(DE3) cells containing pET21a and pMF9.2. Lanes **1** and **6** show the migration of the molecular mass markers in kilodaltons (kDa). Lanes **2-5** show total cellular proteins from the following: lane **2**, BL21(pET21a), uninduced; lane **3**, BL21(pET21a), induced with IPTG; lane **4**, BL21(pMF9.2), uninduced; and lane **5**, BL21(pMF9.2), induced with IPTG. The 165 kDa protein product of pMF9.2 in lane **5** is indicated (arrow).

Discussion

We conclude from this study that *P. syringae* pv. glycinea PG4180 has a putative extracellular epimerase of ~177 kDa. The AlgE family epimerases have been studied in *A. vinelandii*, but have not been previously reported in *Pseudomonas* spp. (Valla et al., 2001). Sequence analysis of PAlgE revealed that it has one A module and six putative R modules. The AlgE epimerases in *Azotobacter* contain one or two A modules of 385 amino acids and generally terminate with the sequence TGQQAT; an exception is AlgE4 where the A module ends with TPQQPS. The A module in PAlgE terminates with VTVQGT, and five amino acid residues precede the first R module (Figure 29). This is

consistent with previous data showing that the A and R modules in AlgE from *A. vinelandii* are separated by four to nine amino acids, which are not part of either module (Ertesväg et al., 1995). The six R modules of PAlgE contain two to six imperfect nonameric Ca^{2+}-binding motifs (Figure 29), which is similar to the number found in the AlgE epimerase family. Although PAlgE is related to most members of the AlgE epimerase family, its structure is most similar to AlgE5, which contains one A module and four R modules (Figure 27).

It was previously reported that the A modules alone are sufficient for epimerization and that the patterns of epimerization are largely determined by the A-modules (Ertesväg and Valla, 1999). It seems reasonable to assume that the AlgE-type epimerases originally had only A modules; fusion to the R modules and extensive gene duplication events presumably took place later (Valla et al., 2001). The question then arises why the DNA sequences encoding the R modules were recruited and why the duplication events survived throughout evolution. The presence of Ca^{2+}-binding motifs in the R modules might increase amount of Ca^{2+} in the extracellular milleau, which is needed for enzymatic activity. Since alginate is known to have the ability to bind Ca^{2+}, the existence of multiple R modules with Ca^{2+}-binding motifs might increase the chance for the AlgE epimerases to stay in the vicinity of the cell capsule instead of diffusing away from the bacterial cell. In addition to Ca^{2+} binding, it has been shown that R modules stimulate reaction rates (Ertesväg and Valla, 1999), and promote translocation of the enzyme to its target on the cell surface or in the extracellular environment (Ertesväg et al., 1994). Although these theories may partly explain the extensive amplification of the R modules, more work is needed to fully explain this phenomenon.

The very high amino acid relatedness (94% similarity) of PAlgE to a single ORF in *P. syringae* pv. tomato DC3000 may indicate that this gene is conserved in *P. syringae*. Furthermore, since only a single ORF in the DC3000 genome showed relatedness to pAlgE, it is unlikely that *P. syringae* contains a family of related extracellular epimerases, which is true for *A. vinelandii*. It is interesting that this gene is not present in the genome of *P. aeruginosa*, another alginate producer.

The relatedness of the C-terminus of PAlgE to adenylate cyclase hemolysins may indicate a bifunctional role for PAlgE. Alternatively, this relatedness might be attributed to the fact that AlgE epimerases share some sequence similarity to proteins secreted via the hemolysin export pathway, which does not involve N-terminal cleavage (Ertesväg et al., 1994). The relatedness of the C-terminus of PAlgE to various extracellular proteases might be caused by the presence of the Ca^{+2}-binding motifs in the R modules.

In a previous study, the *palgE* promoter was thermoregulated and exhibited increased transcriptional activity at 18°C as compared to 28°C (Ullrich et al., 2000). The biological significance of a thermoregulated epimerase in *P. syringae* is unclear; however, one function may be the modification of alginate to form a firm, gel-like protective coat around the bacterium analogous to alginate-containing cysts of *A. vinelandii*. A correlation between cyst coat organization and the amount and appearance of mannuronan C-5 epimerases in the extracellular environment has been reported for *A. vinelandii* (Høidal et al., 2000). It is possible that the incorporation of the G residues into alginate via an extracellular epimerase provides the bacterium with tensile strength.

Although the PAlgE overproduced in this study is truncated at the C-terminus, the protein may still be functional with respect to epimerase activity since it contains an

intact A module. The overproduction of A modules was previously shown to be sufficient for epimerization, and the specificity of the epimerase reaction is largely determined by the A modules (Ertesväg and Valla, 1999). Preliminary results in Dr. Svein Valla's laboratory indicate that pAlgE is functional with respect to epimerase activity.

CHAPTER VII

General Conclusions

Role of AlgR1 in P. syringae. Both *Pseudomonas aeruginosa* and the phytopathogen *P. syringae* produce the exopolysaccharide alginate. However, the environmental signals that trigger alginate gene expression in *P. syringae* are different, with copper being a major signal. Recent studies in the Bender laboratory have indicated that signals produced as a result of the oxidative burst in plant hosts (O_2^-, H_2O_2) are also signals for alginate gene induction (Keith, 2002; Keith and Bender, 1999). This is consistent with the stimulation of alginate synthesis in response to environmental stress.

In *P. aeruginosa,* the alternate sigma factor encoded by *algT* (σ^{22}) and the response regulator AlgR1 are required for transcription of *algD*, which encodes GDP-mannose dehydrogenase, the committed step in alginate biosynthesis. In the present study, we cloned and characterized the gene encoding *algR1* from *P. syringae.* The deduced amino acid sequence of *algR1* from *P. syringae* showed 86% identity with its *P. aeruginosa* counterpart. An *algR1* mutant, *P. syringae* FF5.32, was defective in alginate production but could be complemented when *algR1* was expressed *in trans,* thereby demonstrating that AlgR1 is absolutely required for alginate production in *P. syringae.* The *algR1* upstream region in *P. syringae* contained the consensus sequence recognized by σ^{22}, suggesting that *algT* is required for transcription of *algR1.* The conservation of a σ^{22} recognition sequence upstream of *algR1* strongly suggests that transcriptional activation of these genes requires a functional copy of *algT.* Although an *algT*

homologue in *P. syringae* was identified (Keith and Bender, 1999), the lack of an *algT* mutant curtailed studies designed to determine the role of *algT* in the transcriptional activation of *algR1*.

The role of AlgR1 as a positive activator of *algC* expression in *P. syringae* was investigated. The *algC* homologue was cloned from *P. syringae*, and sequence analysis of the *algC* promoter region indicated the presence of four putative AlgR1 binding sites (ABS), which are similar to those reported in *P. aeruginosa*. The *algC* promoter region (*PsalgC*) was cloned from *P. syringae* pv. syringae FF5, and a *PsalgC-uidA* transcriptional fusion was used to monitor *algC* transcription in strain FF5.7, an *algR1* mutant of *P. syringae* pv. syringae FF5. The expression of the *PsalgC-uidA* fusion was reduced approximately twofold in FF5.7 with respect to the parent strain, FF5, suggesting a positive role in the activation of *algC* in *P. syringae*. *P. syringae* AlgR1 was successfully overproduced in *E. coli* as a C-terminal translational fusion to the maltose binding protein (MBP). Gel shift experiments indicated MBP-AlgR1 strongly binds to the *algC* promoter. These results indicate that *algR1* mediates alginate biosynthesis via transcriptional activation of *algC*. In *P. syringae*, four putative ABS were identified; two upstream of the translational start site and two within the *algC* coding region. The gel retardation experiments clearly demonstrated that *P. syringae* AlgR1 bound to DNA fragments containing the ABS from both *P. syringae* and *P. aeruginosa*. Therefore, it is likely that AlgR1 from *P. syringae* recognizes the ABS in *P. aeruginosa*, and these binding sequences are conserved in *P. syringae*.

It is likely that *algC* transcription is necessary for other processes in *P. syringae*, since complete abrogation of *algC* expression was not observed in the *algR1* mutant. In

addition to its role as a phosphomannomutase in alginate biosynthesis, *algC* also functions in LPS biosynthesis via its phosphoglucomutase activity and in rhamnolipid synthesis (Coyne et al., 1994; Olvera et al., 1999). Although these roles for *algC* in *P. syringae* have not been demonstrated, it is quite likely that AlgC has multiple roles in this bacterium.

Three putative sequences resembling σ^{54} recognition motifs (GG-N_{10}-GC) were found in the *P. syringae* pv. syringae *algC* promoter suggesting that σ^{54} is necessary for *algC* transcription, which is the case for *P. aeruginosa*. Although a *rpoN* mutant of *P. syringae* FF5 is not available, *rpoN* mutants of *P. syringae* pv. glycinea (Alarcón-Chaidez et al., submitted) and *P. syringae* pv. maculicola (Hendrickson et al., 2000) have been constructed and could be used to assess whether *algC* transcription is *rpoN*-dependent.

Plasmid-encoded genes and alginate production. A complex regulatory network for alginate biosynthesis in *P. syringae* seems plausible since plasmid-encoded regulatory genes are known to mediate the constitutive production of alginate in this pathogen. For example, *P. syringae* pv. syringae FF5 is normally nonmucoid *in vitro*; however, the introduction of the 200-kb plasmid pPSR12 confers constitutive alginate production to FF5. In the present study, mutagenesis of FF5(pPSR12) resulted in the isolation of an alginate-defective mutant named FF5.MF1, which contains a mini-Tn*5* insertion in pPSR12. Since plasmid pPSR12 does not contain homologs of any known alginate biosynthetic or regulatory genes (Kidambi et al., 1995), the inactivated gene(s) on the mutated plasmid (pPSR12.1) may be a new regulator of alginate production. The mutant strain FF5.MF1 was only partially complemented for alginate production by cosmid pSM51, possibly because this clone contains both positive and negative regulators. This

hypothesis was supported by the 60-fold reduction in alginate production that occurred when pSM51 was mobilized to FF5(pPSR12). A 5-kb *Eco*R1 containing the disrupted gene from FF5.MF1 was subcloned in pRK415 and named pRKE5.1; however, transconjugants of FF5.MF1(pRKE5.1) were not complemented for alginate production. To further characterize the mutant FF5.MF1, the expression of the alginate regulatory genes *algT* and *algR1* was compared in the mutant FF5.MF1 and the parent strain FF5(pPSR12). The transcription of both genes was significantly reduced in FF5.MF1 as compared to FF5(pPSR12), which agrees with the phenotypic reduction of alginate synthesis in the mutant FF5.MF1. Failure to complement the FF5.MF1 with pRKE5.1, which contains the putative *muc* gene, may indicate that the Tn*5* mutation has polar effects on other genes required for constitutive alginate production. Another possibility is that pRKE5.1 encodes a negative regulator in addition to the proposed positive regulator (*muc*).

DNA sequence analysis of the 5-kb *Eco*RI fragment indicated the presence of a 1026 bp open reading frame, which lacked DNA relatedness to genes deposited in various databases. However, the predicted amino acid sequence of this ORF showed a low amount of relatedness to the plasmid-borne *kfrA* gene in *Enterobacter aerogenesis* and several proteins in the microbial genomes database. Perhaps the most interesting relatedness found in database searches was the similarity between *muc* and *exoF* of *Sinorhizobium meliloti* (37% similarity). Interestingly, *exoF* is encoded by a megaplasmid and is organized within an operon of other *exo* genes that are essential for biosynthesis of the exopolysaccharide (EPS) succinoglycan. Furthermore, *exoX*, which maps near *exoF* in *S. meliloti*, is a negative regulator of succinoglycan and completely

inhibits EPS synthesis production if expressed at high levels (Müller et al, 1993). A similar phenomenon might explain the failure of pRKE5.1 to complement mutant FF5.MF1; for example, pRKE5.1 might contain an additional ORF that functions as a negative regulator. Efforts to complement FF5.MF1 with the *muc* gene and individual ORFs in the region disrupted by mini-Tn*5* might clarify whether a negative regulator is present.

The *muc* gene product also showed a low level of relatedness (21%) to DegS from *E. coli*. DegS, a putative serine protease, regulates the basal and induced activity of the essential *E. coli* sigma factor, σ^E, which is involved in the cellular response to extracytoplasmic stress. DegS promotes the destabilization of the σ^E-specific anti-sigma factor RseA, thereby releasing σ^E to direct gene expression (Alba et al., 2001). It is tempting to speculate that the *muc* gene product could function in an analogous manner; for example, it could destabilize the anti-sigma factor MucA, thereby releasing AlgT (σ^E homologue), which then initiates alginate biosynthesis (Figure 32, Tier 1). Although these ideas remain highly speculative, further work is needed to confirm the identity and function of the pPSR12-encoded gene conferring constitutive mucoidy to *P. syringae*.

A model summarizing the findings of this study with respect to alginate regulation in *P. syringae* is presented in Figure 32.

Extracellular C5 epimerases. C5-epimerases catalyze the formation of α-L-guluronic acid (G) from its C5 epimer β-D-mannuronic acid (M). The AlgE family of extracellular epimerases has been studied in *A. vinelandii*, but has not been previously reported in *Pseudomonas* spp. (Valla et al., 2001). A putative extracellular epimerase of ~177 kDa was cloned and sequenced from *P. syringae* pv. glycinea PG4180. Sequence

analysis of the putative epimerase (PAlgE) revealed that it has one A module, which is presumed to catalyze the epimerization reaction, and six putative R modules (R modules are thought to catalyze secretion of the epimerase). The six R modules of PAlgE contain two to six imperfect nonameric Ca^{2+}-binding motifs, which is similar to the number found in the AlgE epimerases in *Azotobacter* spp. Although PAlgE is related to most members of the AlgE epimerase family, its structure is most similar to AlgE5, which contains one A module and four R modules.

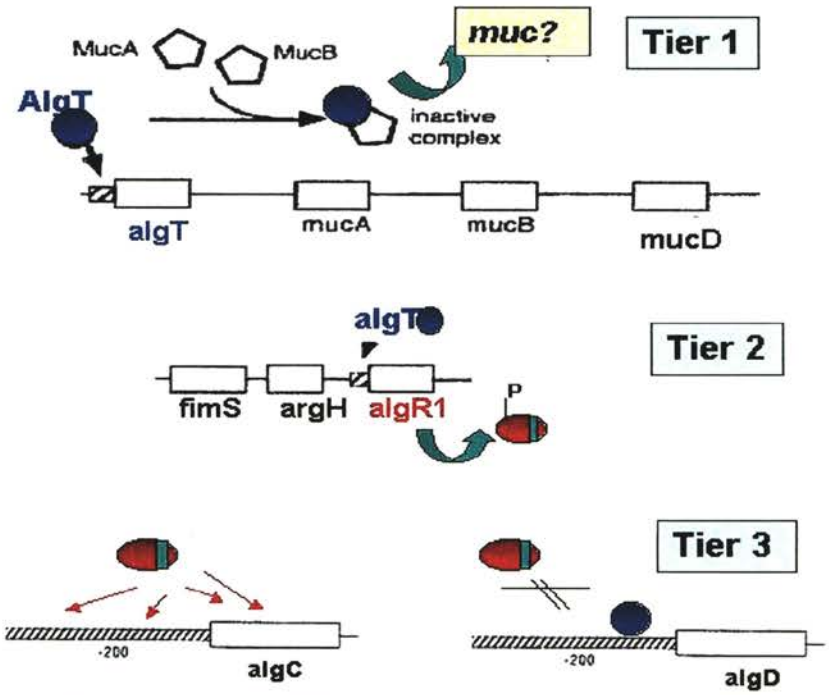

Figure 32. A model summarizing the findings of this study with respect to alginate regulation in *P. syringae*. **Tier 1:** The *muc* gene product in the plasmid pPSR12 is proposed to be an anti-anti-sigma factor that might destabilize the anti-sigma factor MucA, thereby releasing AlgT (σ^{22}) (blue circle), which then initiates alginate biosynthesis through biding to its promoter and also to the *algD* promoter. **Tier 2:** AlgT is believed to bind upstream of *algR1* and positively regulate its transcription. The protein product AlgR1 (red oval) has a DNA-binding motif in its C-terminus (green rectangle) and two aspartate residues that are phosphorylated. **Tier 3:** AlgR1 (phosphorylation not required) may bind to four putative sites in the *algC* promoter and positively regulate its transcription. AlgR1 is not required for *algD* expression and the *algD* promoter lacks AlgR1 binding sites.

The very high relatedness (94% amino acid similarity) of PAlgE to a single ORF in *P. syringae* pv. tomato DC3000 may indicate that this gene is conserved in *P. syringae*. Furthermore, since only a single ORF in the DC3000 genome showed relatedness to *pAlgE*, it is unlikely that *P. syringae* contains a family of related extracellular epimerases, which is true for *A. vinelandii*. Furthermore, it is interesting that a *pAlgE* homologue is not present in the genome of *P. aeruginosa*.

Although the PAlgE construct overproduced in this study was truncated at the C-terminus, the protein may still be functional with respect to epimerase activity since it contains an intact A module. The overproduction of A modules was previously shown to be sufficient for epimerization, and the specificity of the epimerase reaction is largely determined by the A modules (Ertesväg and Valla, 1999). Consequently, efforts to assay the truncated version of PAlgE for epimerase activity are currently underway in collaboration with Dr. Svein Valla's laboratory in Norway and preliminary results indicate that pAlgE is functional with respect to epimerase activity.

CHAPTER VIII

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