EXPRESSION OF ALGINATE IN RESPONSE TO ENVIRONMENTAL STRESS AND PLANT SIGNALS

BY

RONALD CECIL KEITH

Bachelor of Science

Queens University

Belfast, Northern Ireland

1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2002

EXPRESSION OF ALGINATE IN RESPONSE TO

ENVIRONMENTAL STRESS

AND PLANT SIGNALS

Thesis Approved:

Carol Bender

Thesis Advisor

Jacqueline Fletcher

Jeanmarie Verchot-Lubicz

Timothy A. Peltonen

Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Carol Bender, for her intelligent supervision, constructive guidance, inspiration, and friendship. My sincere appreciation extends to my other committee members, Dr. Jacqueline Fletcher, and Dr. Jeanmarie Verchot-Lubicz, whose guidance and assistance are also invaluable.

My undying love, appreciation and respect to my wife Dr. Lisa Keith, who was my mentor, my sounding board, my support when things looked their darkest. Her hope and inspiration helped me through all the ups and downs in my research, writing and health.

Special thanks are due to my colleagues and friends, Dr. Alejandro Peñaloza-Vázquez, Dr. Mohamed Fakhr, Dr Rao Uppalapati. and Dr. Gustavo Hernández-Guzmán for their suggestions, constructive comments, and friendship.

I would also like to give my special appreciation to my parents for their love and support throughout this whole process. I give all the praise and the glory to the Lord Jesus Christ for all He has done for me and all He continues to do.

Finally, I would like to thank Dr. Carol Bender and the Department of Entomology and Plant Pathology for providing me with this research opportunity and their generous financial support.

I. TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Although phytopathogenic bacteria are taxonomically and pathologically diverse, their ability to elicit the hypersensitive response (HR) in resistant plants or to cause disease in susceptible host plants depends upon the *hrp* (hypersensitive response and pathogenicity) gene cluster (He, 1998). In addition to the *hrp* genes, a variety of virulence factors including extracellular polysaccharides (EPS), phytotoxins and cell wall degrading enzymes contribute to the full expression of symptoms in *Pseudomonas* spp. infections (Alfano and Collmer, 1996). EPS's are carbohydrate polymers produced by a large variety of bacteria, including many phytopathogens. EPS is an important virulence factor in *Ralstonia* (*Pseudomonas*) *solanacearum*, *Erwinia amylovora*, and *Pantoea* (*Erwinia*) *stewartii* (Denny, 1995). My research focused on the production of the EPS alginate by *P. syringae*.

Pseudomonas syringae is a necrogenic bacterial phytopathogen that causes economically important diseases of plants in many parts of the world. The typical disease symptoms induced by *P. syringae* include leaf spots, blights, and galls (Alfano and Collmer, 1996). The species *P. syringae* is subdivided into pathovars (pathogenic variants, pvs.), which vary in host specificity. The pathogenesis of *P. syringae* in susceptible host plants involves prolonged bacterial multiplication, dissemination to surrounding tissues, and production of macroscopic symptoms characteristic of these diseases. During the incompatible interaction with resistant host plants, *P. syringae* elicits

a plant defense reaction known as the hypersensitive response (HR), which is a necrotic reaction that restricts the multiplication and spread of the pathogen.

Prior to infection, *P. syringae* commonly exists as an epiphyte and resides on the surface of healthy plants (Beattie and Lindow, 1995). A major role of epiphytic bacterial populations is to serve as a reservoir for potentially phytopathogenic bacteria to gain entry into plants. The bacteria are then able to initiate interactions within the plant that result in either disease or the HR. Several traits developed by phytopathogens have been proposed to improve epiphytic fitness of phytopathogenic bacteria, including EPS production, UV tolerance, and osmotolerance (Beattie and Lindow, 1995).

Once bacteria have gained entry into the leaf, plants try to protect themselves by various mechanisms including the production of antimicrobial compounds, structural barriers, and the oxidative burst. The oxidative burst is an early, localized defense response that involves the production of potentially cytotoxic quantities of reactive oxygen species (ROS), such as H_2O_2 and $O_2^{\cdot}$ (Legendre *et al.*, 1993). The production of ROS commonly occurs in two distinct phases (Levine *et al.*, 1994). The initial, rapid phase ends within an hour of initiation and is followed by a second slower burst that may last 3-6 h (Baker and Orlandi, 1995). The second burst was initially shown only in incompatible reactions (Levine *et al.*, 1994). Chandra *et al.* (1996) have shown that the second phase of oxidative burst in tomato is dependent on co-expression of the R gene *Pto* in the host and *avrPto* in the pathogen, *P. syringae* pv. tomato.

It has been suggested that *E. amylovora* uses the production of ROS by the plant as a tool to provoke host cell death during pathogenesis for subsequent invasion of plant tissues (Venisse *et al.*, 2001). Bacterial EPS was hypothesized to protect *E. amylovora*

from the toxic effects of ROS since a non-capsular mutant of *E. amylovora* induced the same responses as the wild-type, but was unable to further colonize the plant (Venisse *et al.*, 2001).

P. syringae pathovars generally produce two well-characterized EPS molecules: levan (a polymer of fructofuranan) and alginate, a copolymer of *O*-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Gross and Rudolph, 1987). Previous studies implied that alginate may contribute to virulence in *P. syringae* (Fett *et al.*, 1986; Osman *et al.*, 1986); however, the role of alginate in pathogenesis has not been critically assessed using a genetic approach. In the present study, the *algD* gene and promoter of *P. syringae* pv. tomato strain DC3000, which causes bacterial speck on tomato and peppery spot on crucifers, were sequenced and phylogenetic trees showing evolutionary relatedness to other bacteria were constructed. Furthermore, a transcriptional fusion to *algD* was constructed by fusing the *algD* promoter to a promoterless glucuronidase gene (*uidA*). The *algD*::*uidA* transcriptional fusion was used to monitor alginate gene expression in both host and nonhost plants inoculated with *P. syringae* pv. tomato DC3000.

CHAPTER II

Literature Review

Pseudomonas syringae: biology and pathogenesis.

Pseudomonas syringae is a necrogenic, gram-negative phytopathogen. The bacteria enter susceptible host plants through wounds, stomates and other natural openings. Once inside the plant, the bacteria colonize the intracellular space (the apoplast). The typical disease symptoms associated with *P. syringae* include leaf spots and blights.

Strains of *P. syringae* are subdivided into pathovars (pathogenic variants, pvs.) based on their host specificity. Pseudomonads infect a wide variety of plants including tobacco (pv. tabaci), cucumber (pv. lachrymans), beans (pv. phaseolicola), oats (pv. coronafaciens), peas (pv. pisi), soybeans (pv. glycinea), pear (pv. syringae) and tomato (pv. tomato) (Agrios, 1989). Their ability to elicit the hypersensitive response (HR) in resistant plants or to cause disease in susceptible host plants depends upon the *hrp* (hypersensitive response and pathogenicity) genes (He, 1998).

The *hrp* cluster

The HR is an important defense response in plants and generally occurs only in incompatible combinations of host plants and phytopathogens (Klement, 1963). In resistant plants, a number of physiological changes occur in infected cells and in the plant cells close to the infection site. In susceptible varieties, such changes either do not occur or occur more slowly. The changes that occur during the HR include loss of permeability

in cell membranes, production of reactive oxygen species, increased respiration, accumulation and oxidation of phenolic compounds, and production of phytoalexins (He, 1998). The outcome of these reactions is collapse and death of the infected adjacent cells.

The *hrp* genes encode a type III protein secretory system, which is shared among plant and animal pathogens (Galán and Collmer, 1999). The elicitation of the HR requires contact between plant and bacterial cells that are both metabolically active and synthesizing new proteins (Holliday *et al.*, 1981). The *hrp* genes were first identified in *P. syringae* pv. phaseolicola and *P. syringae* pv. syringae (Niepold *et. al.*, 1985). The *hrp* genes are encoded by chromosomally-borne pathogenicity islands, which often contain additional virulence genes (Alfano *et al.*, 2000). Nine *hrp* genes are conserved in plant and animal pathogens and have been designated as *hrc* (hypersensitive response and conserved); the *hrc* genes encode protein products that are required for assembly of the type III secretion system (Roine *et al.*, 1997). The type III secretion pathway is essentially a bacterial pilus that can deliver virulence proteins directly into host cells (Wei *et al.*, 2000). Virulence proteins can be 'injected' into the host cell or into the intercellular milieu (Rosqvist *et al.*, 1994).

hrp genes are not expressed in rich media (Bonas, 1994), but are strongly expressed in minimal medium that mimics the plant apoplast (Huynh *et al.*, 1989). Secretion of *hrp*-encoded effectors does not occur in culture medium because it requires intimate contact between host and pathogen (Rosqvist *et al.*, 1994). The *hrp* system secretes a class of effector proteins called "harpins", which are glycine rich proteins with heat stable elicitor activity (Wei *et al.*, 1992). In *Erwinia carotovora* subsp. carotovora, the production of harpin and other proteins is regulated by RsmA, an RNA-binding

protein, and *rsmB*, a regulatory RNA (Liu *et al.*, 1998). Subsequent work by Cui *et al.* (1999), showed that these two regulators were either negatively or positively regulated by *rsmC*.

Avirulence proteins

Avirulence (*avr*) genes control host specificity in phytopathogenic bacteria at the race-cultivar level by triggering the HR in host plants carrying a corresponding resistance (*R*) gene (Flor, 1956). Over thirty different *avr* genes have been cloned from *P. syringae* and *X. campestris* (Alfano and Collmer, 1996). In general, Avr proteins have no obvious effect when infiltrated into plants and no known biochemical activity; an exception is AvrD in *P. syringae* pv. tomato, which directs the synthesis of syringolide elicitors (Keen, 1990). Substantial evidence exists indicating that *hrp* and *avr* genes are co-regulated (Leach and White, 1996). Furthermore, when the type III *hrp* system was cloned *in trans* and introduced into non-pathogens, avirulence "signals" were expressed (Gopalan *et al.*, 1996). Delivery of Avr signals is absolutely dependent on the *hrp* secretion system (Alfano and Collmer, 1996). Many characteristics of the HR elicitation process can be explained by *hrp*-mediated delivery of *avr* gene products into cells, including the one-to-one relationship between bacterial cells and HR-responding plant cells.

Virulence factors in Pseudomonads

P. syringae also produces factors that significantly enhance pathogen virulence including exopolysaccharides (discussed below), phytotoxins, and phytohormones

(Bender *et al.*, 1999; Costacurta and Vanderleyden, 1995; Denny, 1995). In general, phytotoxins are not required for *P. syringae* pathogenicity, but their production results in increased disease severity. For example, *P. syringae* phytotoxins can contribute to systemic movement of bacteria *in planta* (Patil *et al.*, 1974), lesion size (Bender *et al.*, 1987; Xu and Gross, 1988), and multiplication of the pathogen in the host (Mittal and Davis, 1995; Peñaloza-Vázquez *et al.*, 2000).

The plant growth regulator indole-3-acetic acid (IAA; auxin) is produced by many pathovars of *P. syringae* and has a role in the virulence of some strains (Mazzola and White 1994; Glickmann *et al.* 1998). *P. syringae* pv. savastanoi also produces elevated levels of cytokinins that induce the hyperplasia associated with gall production (MacDonald *et al.*, 1986). Production of another hormone, ethylene, by several pathovars of *P. syringae* has been established (Weingart and Völksch 1997). In *P. syringae*, 2-oxoglutarate is converted to ethylene by the ethylene-forming enzyme, which is encoded by *efe*, a plasmid-borne gene in *P. syringae* pvs. phaseolicola and glycinea (Watanabe *et al.* 1998). In some pathovars of *P. syringae*, the *efe* gene contributes to virulence (Weingart *et al.*, 2001).

Bacterial cell-to-cell signaling and global regulation of virulence

In order for gram negative phytopathogens to cause disease, there must be substantial development of the epiphytic population (Hirano and Upper, 1990). In *Ralstonia solanacearum*, a hierarchy of several regulatory proteins and a volatile factor, which is analogous to an autoinducer, regulates virulence, the production of exopolysaccharides (EPS), and several extracellular proteins (Clough *et al.*, 1994). In *X.*

campestris a similar subset of virulence factors is coordinately regulated by several independent regulatory genes (Dow and Daniels, 1994). *E. carotovora* and *E. sterwartii* use cell-to-cell signaling for quorum-sensing regulation of virulence gene expression (Fuqua *et al.*, 1993). *N*-acylhomoserine lactones (HSLs), known as autoinducers (AIs), are widely conserved signal molecules present in quorum-sensing systems of many gram negative bacteria. AIs are involved in the regulation of diverse biological functions, including the expression of virulence genes in *R. solanacearum*, *Erwinia* spp., and *Pseudomonas aeruginosa* (Dong *et al.*, 1999). At low cell densities, AIs are present at low concentrations; however, at high cell densities, AIs can accumulate to levels sufficient for activation of regulatory genes (Fuqua and Winans, 1996). Flavier *et al.* (1997) showed that control of HSL production in *R. solanacearum* is mediated by a novel regulatory system responsive to a 3-hydroxypalmitic acid methyl ester. This acyl-HSL system has an elaborate system of checks and balances for autoregulation and is activated with virulence genes (Flavier *et al.* 1999). Dong *et al.* (1999) cloned and sequenced *aiiA*, which encodes an auto-inducing (AI) inactivation enzyme, from *Bacillus*. Expression of *aiiA* in *Escherichia coli* and *E. carotovora* results in AI inactivation and significantly reduces AI release from *E. carotovora* (Dong *et al.*, 1999). AiiA has no significant homology to known proteins; however, a conserved HXHXDH motif was found in AiiA making it a possible zinc metalloenzyme (Dong *et al.*, 1999).

The *luxI\R* homologs in *E. carotovora*, like the *araC* homologs and the ECF (extracytoplasmic factor) sigma factors, are representatives of several global regulatory factors that have been recruited to control the expression of multiple virulence genes (Fuqua *et al.*, 1993). Similarly, in *P. syringae*, the two-component regulatory proteins

GacS (LemA) and GacA control production of syringomycin, extracellular proteases and lesion development in some *P. syringae* pathovars (Hrabak and Willis, 1992; Rich *et al.*, 1994). In *P. syringae* pv. syringae, Kinscherf and Willis (1999) reported that swarming of bacterial cells also requires *gacS* and *gacA*. A review of functions controlled by *gacA*/*gacS* has been recently published (Heeb and Haas, 2001).

Importance of epiphytic colonization

Epiphytic populations of *P. syringae* on asymptomatic leaves of susceptible and resistant host plants and non-host plants may serve as sources of inoculum for disease. However, there are few host-pathogen systems for which a quantitative relationship has been found between epiphytic population size and disease (Hirano and Upper, 2000). For example, Lindemann *et al.* (1984) reported that the epiphytic population of *P. syringae* pv. syringae was a predictive indicator of disease on beans. Population sizes of *P. syringae* are frequently small on young annual plants or on emerging leaves during plant development, but may increase to large numbers under suitable conditions (Hirano and Upper, 1994). Increases in population size occur in short bursts and can decrease rapidly, but usually the changes are relatively small for long periods of time (Hirano and Upper, 1994).

Bacteria may arrive on the leaf surface in several ways. *P. syringae* can be seedborne and may eventually proliferate on emerging cotyledons. Leaf habitats themselves are unusually open systems. The exposure to the atmosphere provides ample opportunity for aerial immigration and emigration of bacteria in the phyllosphere. The

fate of a newly arrived *P. syringae* in a leaf habitat depends on the biological, physical and chemical environments (Hirano and Upper, 1994).

There must be a substantial development of the epiphytic population for *P. syringae* to cause disease (Hirano and Upper, 1990). Prior to establishment of an epiphytic population, the pathogen must contact the leaf surface and physical attachment may occur. Epiphytic population size is significantly greater for pathogens compared to non-pathogenic bacteria or pathogens on incompatible hosts (Hirano and Upper, 1990). Ideally, the bacteria should exist on the leaf surface and multiply, but the leaf habitat is a harsh environment and bacteria are subjected to various environmental stresses. These include fluctuations in temperature, relative humidity and desiccation as well as UV and visible irradiation (Hirano and Upper, 1990). Due to these factors, bacteria have evolved mechanisms for tolerance and avoidance of various stresses in the phyllosphere. Leaf-associated populations may avoid stress through colonization of sites that are protected or buffered from the external environment (Beattie and Lindow, 1995). Following inoculation of *P. syringae* onto bean leaves, the epiphytic population initially decreases and then increases to a stable, higher level (Wilson *et al.*, 1999). This is the carrying capacity of the leaf and comprises the multiplication of bacteria that colonize protected sites. Survival and multiplication in protected sites, which include stomata, hydathodes, trichomes and wounds (Leben, 1981), is correlated with pathogenicity (Wilson *et al.*, 1999; Yu *et al.*, 1999). At high relative humidity (RH), the populations of non-pathogenic and pathogenic bacteria are not significantly different. At low RH the pathogenic bacterial populations are significantly higher than non-pathogenic strains (Yu *et al.*,

1999). Beattie and Lindow (1995) suggest that pathogens are physiologically able to occupy buffered sites more rapidly than are non-pathogenic species.

Many phytopathogenic bacteria produce exopolysaccharides (EPS) on compatible hosts. EPS may facilitate adhesion to surfaces, provide protection from environmental stresses, and contribute to both epiphytic fitness and virulence (Ophir and Gutnick, 1995; Yu *et. al.*, 1999). EPS may reduce desiccation stress by contributing to a biofilm around bacterial masses (Beattie and Lindow, 1995). Desiccation stimulates EPS synthesis in both *P. syringae* and *P. aeruginosa* (Devault *et al.*, 1990; Singh *et al.*, 1992). The correlation of pathogenicity with survival in the phyllosphere under stressful environmental conditions suggests that the epiphytic survival of *Pseudomonas* spp. on host plants may involve successful initial colonization of protected niches on the leaf surface (Wilson *et al.*, 1999).

Copper resistance in phytopathogenic bacteria

Copper sprays are frequently applied to plants for the control of bacterial diseases (Bender and Cooksey, 1986). Copper, a cofactor in a variety of enzymatic reactions, can cause extensive damage in the prokaryotic cell and result in growth inhibition and loss of cell viability (Leitão and Sá-Correia, 1997). Copper readily reacts with the superoxide anion and with hydrogen peroxide via a Fenton-like reaction to generate the highly reactive hydroxyl radical (Halliwell and Gutteridge, 1989). Bacterial defense and adaptation to oxidative stress requires the induction of the synthesis of a subset of proteins. Many of these proteins act by destroying oxidants or by repairing the resulting damage (Storz *et al.*, 1990).

The occurrence of copper-resistant strains of the phytopathogen *P. syringae* has been established (Bender and Cooksey, 1986). In *P. syringae* pv. tomato strains isolated from tomato fields in California, the copper resistance genes reside on 35 kb plasmid (Cooksey, 1987). In *P. syringae* spp. carrying the plasmid-borne *cop* operon, copper is excluded from the cytoplasm by specific proteins that bind copper in the periplasm and at the outer membrane (Cha and Cooksey, 1991). Copper resistance in *P. syringae* is specifically induced by copper ions. The *copABCD* genes are under the control of a single copper inducible promoter (Mellano and Cooksey, 1988b). Two genes are required for induction of the *cop* promoter (Mills *et al.*, 1993), *copR* and *copS*. The amino acid sequence for CopR and CopS show similarities to regulatory proteins of two-component regulatory systems, which are commonly used by bacteria to alter gene expression in response to environmental stimuli (Charles *et al.*, 1992). In a hypothetical model of copper resistance, the putative transmembrane protein CopS may 'sense' high levels of free Cu^{2+} in the periplasm and phosphorylate CopR in the cytoplasm. Phosphorylation of CopR converts it from the inactive to the active state, which results in transcriptional activation of the *copABCD* operon.

CopA and CopB are absolutely essential for copper resistance, while CopC and CopD are required for manifestation of a full level of resistance (Mellano and Cooksey, 1988). CopA, a 72 kDa protein similar to multicopper oxidases (Wackett *et al.*, 1989), and CopC (12 kDa) are located in the periplasm, while CopB is a 39 kDa outer membrane protein (Cha and Cooksey, 1991). These proteins mediate the sequestration of copper, while CopD, an inner membrane protein, balances the concentration of these proteins with respect to copper transport into the cytoplasm (Mills *et al.*, 1993).

The genes for plasmid-borne copper resistance mechanisms in *E. coli* and *Xanthomonas campestris* are related to the *cop* operon in *P. syringae* (Lee *et al.*, 1994). The predicted amino acid sequence of the *pco* operon in *E. coli* (*pcoABCDRS*) is similar to *copABCDRS*, although the former functions as an efflux system and the latter in the sequestration of copper (Mills *et al.*, 1994). Chromosomally-encoded two-component copper resistance systems have also been identified (Munson *et al.*, 2000). For example, the *cus* genes encode a copper ion efflux system homologous to known metal ion antiporters (Munson *et al.*, 2000); an equivalent system has not been identified in *P. syringae*.

Biological role of exopolysaccharides

Bacterial EPS occurs in two basic forms: (*i*) the capsular polysaccharides (CPS or K-antigen) and (*ii*) the slime polysaccharides. CPS is intimately associated with the cell surface via covalent bonding, while slime polysaccharides are loosely associated with the cell surface (Whitfield, 1988). Most bacteria produce either CPS or slime EPS. However, *Klebsiella* spp. can produce both simultaneously (Whitfield, 1988), and *Rhizobium* and *Agrobacterium* can produce more than one kind of homo- or heteropolymer. Differentiation between the groups is sometimes difficult as overproduction of CPS can cause peripheral release thus resembling slime (Whitfield, 1988).

Exopolysaccharides may be homopolymers or heteropolymers (Whitfield, 1988). Heteropolysaccharides are composed of repeating units of more than one polysaccharide and are generally anionic (Whitfield, 1988). Their structural diversity arises from a variety of monosaccharide components and is increased by non-carbohydrate substituents

and different linkages (Kenne and Lindburg, 1983). The glycan portions of the repeating unit structure are considered to be invariable, but the heteropolymers synthesized by *X. campestris* and *Rhizobium trifolii* show flexibility with respect to glycan subunits (Sutherland, 1982). Homopolysaccharides such as alginate and levan are synthesized by mechanisms that vary according to each distinct polysaccharide. However, heteropolysaccharides are generally synthesized from nucleotide diphosphate sugar precursors on a membrane-bound lipid carrier, followed by polymerization and secretion (Leigh and Coplin, 1992).

The most common EPS molecules in plant-associated bacteria are: (*i*) periplasmic β-1-2 glucans as found in Rhizobacteriaceae; (*ii*) cellulose in *Agrobacterium* and *Rhizobium*; (*iii*) levan in *Erwinia* and *Pseudomonas*; and (*iv*) alginate in fluorescent *Pseudomonas* spp. The ability to produce EPS is a direct and 'logical' response to selective pressures in the natural environment (Dudman, 1977). EPS protects bacterial cells from desiccation, phagocytosis, antibacterial compounds, toxic metals, and also facilitates the sequestering and binding of microminerals in growth-limiting conditions (Dudman, 1977). Although they are not the initial factor for cellular adhesion to surfaces, EPS are produced relatively quickly to aid stabilization and persistence of the bacterial colony (Vandevivere and Kirchman, 1993).

Exopolysaccharides produced by plant pathogenic bacteria

Enterobacteriaceae synthesize a wide range of capsular polysaccharides (CPS). *E. coli* synthesizes over 70 CPS variants, although a given strain makes only one CPS (Leigh and Coplin, 1992). CPS's are classified into two groups based on acidic

components, charge density, lipid substitution and expression at low temperatures (Leigh and Coplin, 1992). Colanic acid and K_{30} antigen are typical of Group I CPS. They are mainly composed of hexuronic sugars and are synthesized at temperatures below 30°C (Leigh and Coplin, 1992). The Group II CPS's are low molecular weight compounds, substituted with phosphatidic acid at the reducing terminus; they rarely contain hexuronic acids. Group II CPS are synthesized at temperatures above 20°C (Leigh and Coplin, 1992). *Erwinia amylovora* and *E. sterwartii* produce amylovoran and stewartan, respectively, which are structurally related CPS molecules in Group I (Goodman *et al.*, 1974; Nimtz *et al.*, 1996a, b).

Cells of *Pseudomonas* and *Xanthomonas* spp. produce loosely associated EPS molecules ("slime layers"). *X. campestris* pathovars produce xanthan gum, which consists of a cellulose backbone with trisaccharide side chains (Whitfield, 1988). *Pseudomonas* spp. produce alginate or the homopolysaccharide levan. Levan, a β, 2-6-D fructan, is a homopolymer of fructose and is synthesized when *P. syringae* is maintained on sucrose as a sole carbon source (Whitfield, 1988). Alginate is a co-polymer of O-acetylated β, 1-4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Rehm and Valla, 1997).

Role of EPS in the pathogenesis of *P. syringae*

Evidence for EPS production in plants infected with *P. syringae* initially came from ultrastructural studies. Fibrillar material, which was assumed to be bacterial EPS, was evident around cells of *P. syringae* pv. coronafaciens (Ebrahim-Nesbat and Slusarenko, 1983), pv. phaseolicola (Harper *et al.*, 1987) and pv. tabaci (Smith and

Mansfield, 1982) in infected oat, bean and tobacco plants, respectively. The addition of the cationic dye ruthenium red to the fixative enhanced visualization of the fibrillar material and indicated that it was anionic in composition (Luft, 1971).

Gross and Rudolph (1984) were the first to report that *P. syringae* pv. phaseolicola, a bean pathogen, produces the EPS alginate. Osman *et al.* (1986) reported that *P. syringae* pv. glycinea also produces alginate. Alginate biosynthesis is a common feature for most pseudomonads in rRNA-DNA homology group I, which includes all fluorescent and a few non-fluorescent species (Fett *et al.*, 1992). Within the superfamily B, the alginate genes are distributed throughout the *Pseudomonas* group 1-*Azotobacter-Azomonas* lineage. Only a few alginate genes are retained in the *Pseudomonas* group V, which contains *Xanthomonas* and enteric lineages (Fialho *et al.*, 1989; Fett *et al.*, 1992).

The main hindrance to bacterial multiplication in the intercellular space is the scarcity of water (Rudolph, 1980). It is assumed that EPS accumulates around the bacterial cells *in planta*, so that moisture is retained and micro-colonies are protected from sudden desiccation. The bacterial slime prevents close contact between the phytopathogen and plant cell walls and may block recognition and development of the HR. EPS may also protect bacterial cells from agglutination by plant polymers or from phytoalexins and reactive oxygen species (Rudolph, 1980).

Alginate is presumably responsible for the water-soaking observed in leaves infected by *P. syringae* (Rudolph *et al.*, 1994). Gross and Rudolph (1987) studied the kinetics of alginate production by *P. syringae* pv. phaseolicola in bean leaves and showed that alginate production was correlated with the appearance of water-soaked lesions. In

healthy plants from the same study, alginate was not detected, and there was no evidence of water-soaked lesions.

Fett and Dunn (1989) showed that alginate-producing strains of *P. syringae* induce water-soaked lesions on leaves of host plants, while alginate non-producing strains do not. Therefore production of alginate *in vivo* is important for the induction and maintenance of water-soaked leaf tissue. These same strains produced alginate *in vitro* on solid media, but were incapable of alginate production in liquid media. Darzins and Chakrabarty (1984) found that *P. aeruginosa* 8830 produced alginate on solid media, but remained incapable of synthesizing alginate in liquid media regardless of the carbon source. Elevated EPS production on solid media reflects the stimulation of alginate biosynthesis via cell-to-cell contact, and suggests that this is a density-dependent phenomenon controlled by quorum sensing.

Biosynthesis and regulation of alginate in *P. aeruginosa*

The alginate biosynthetic pathway in shown in **Figure 1** (Govan and Deretic, 1996). Fructose 6-phosphate is converted into mannose 6-phosphate via phosphomannose isomerase (PMI; encoded by *algA*). Mannose 6-phosphate is then converted to mannose 1-phosphate by phosphomannomutase (PMM; encoded by *algC*). Conversion of mannose 1-phosphate to GDP-mannose is catalyzed by GDP-mannose pyrophosphorylase (GMP; also encoded by *algA*). The conversion of GDP-mannose into the GDP-mannuronate monomer via GDP-mannose dehydrogenase (GMD; encoded by *algD*) is a rate-limiting step in the biosynthesis of alginate. GDP-mannuronate residues are then polymerized, epimerized and acetylated to form mature alginate (**Figure 1**). 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. AlgX is also required for alginate synthesis and may have a role in polymerization (Monday and Schiller, 1996) by facilitating the transfer of an acetyl group from acetyl-CoA to a transacetylase, the product of gene *algF*. Two other genes, *algI* and *algJ* (Franklin and Ohman, 1993; Franklin and Ohman, 1996) are required for the acetylation of alginate. Another alginate modifying enzyme, AlgG, catalyzes the epimerization of the mannuronate residues at the C-5 position, thus introducing guluronate residues into polymannuronate. In addition to acetylation and epimerization, a major modification of the *P. aeruginosa* alginate is catalyzed by alginate lyase, which is encoded by *algL*. AlgL physically degrades alginate and may have a role in detachment of bacterial cells. Transport of alginate to the extracellular milieu may occur via an outer membrane porin encoded by *algE* (Rehm *et al.*, 1994).

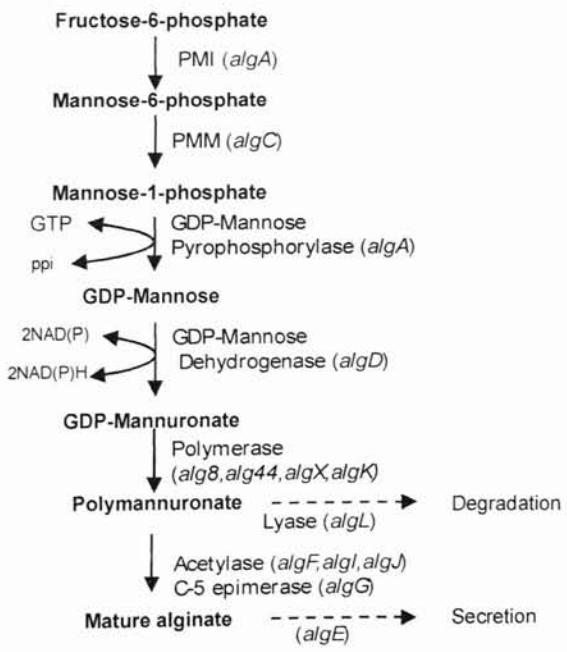

Figure 1. The biosynthesis of alginate by *P. aeruginosa*. Fructose-6-phosphate obtained from the metabolic pool is converted to GDP-mannuronic acid, which provides mannuronate residues for polymerization. Guluronate residues may be incorporated into the polymer via the epimerization of mannuronate by 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 aided by the AlgE protein.

Alginate in cystic fibrosis

Pseudomonas aeruginosa can cause severe and life-threatening infections in immunosuppressed hosts, such as patients with respiratory diseases, burns, cancer, and cystic fibrosis (CF) (Ohman *et al.*, 1996). The overproduction of alginate enables *P. aeruginosa* to persist in the lungs of CF patients and cause bronchopulmonary infection (Govan and Harris, 1986). Alginate also provides an adherence mechanism, confers increased resistance to the patient's immune response, and interferes with antibiotic therapy (Govan and Harris, 1986).

CF arises from mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), a plasma membrane protein involved in chloride ion secretion (Kubesch *et al.*, 1993). Patients with CF have salty lungs that are usually colonized by nonmucoid (alginate-nonproducing) *P. aeruginosa* strains typical of those found in the environment. The factor responsible for long-term persistence of *P. aeruginosa* in the lungs is the genetic switch to the mucoid (alginate-producing) phenotype (Deretic *et al.*, 1994). When *P. aeruginosa* cells become mucoid, they no longer produce long O-side chains (Pier, 1998). The production of alginate protects *P. aeruginosa* from both complement-mediated and non-opsonic phagocytosis by host cells. Govan (1988) proposed that alginate contributes to a 'frustrated phagocytosis', which allows the organism to remain in the lungs. Alginate also allows *P. aeruginosa* to grow as a biofilm, which protects the organism from phagocytosis and reduces complement activation (Lam *et al.*, 1980). Anwar *et al.* (1992) stated that alginate biofilms present a polyanionic barrier to antimicrobial agents. Due to the slow, iron-limited growth of *P. aeruginosa* within the biofilm, susceptibility to antibiotics is reduced and they are

removed from the biofilm before they can be effective in combating the bacterial infection.

Alginate interferes with non-opsonic (Goldberg and Ohman, 1987) and opsonic (Baltimore and Mitchell, 1980) phagocytosis. This property is further enhanced by the ability of alginate to quench reactive oxygen species and scavenge hypochlorite generated by phagocytic cells (Learn *et al.*, 1987). Opsonins, such as complement deposited onto the bacterial surface and antibiotics, are masked by the alginate coating on bacterial cells, which become inaccessible for interactions with cognate receptors on phagocytic cells (Baltimore and Mitchell, 1980). Alginate inhibits complement-driven (Stiver *et al.*, 1988) and N-fMLP induced chemotaxis (Pedersen *et al.*, 1986), while decreasing the effect of the oxidative burst from phagocytic cells (Bender *et al.*, 1987a). The mitogenic activity of alginate and lipopolysaccharide contributes to the hypergammaglobulinemia associated with the clinical deterioration in CF patients (Pier *et al.*, 1984). Alginate elicits the production of proinflammatory cytokine IL-1 and tumor necrosis factor α (Daley *et al.*, 1985). It also stimulates the release of IL-8 from epithelial cells and contributes to the recruitment of neutrophils (Anwar *et al.*, 1983). The emergence of the mucoid phenotype in the lungs of CF patients correlates with a grim prognosis (Høiby, 1982).

Alginate as a food additive

All alginates used for commercial purposes are produced from the harvesting of brown seaweed (King, 1983). Commercial grade alginate is generally the sodium salt of a linear polyuronic alginate harvested from the alga, *Macrocystis pyrifera*. However,

harvesting seaweed is environmentally unsound and the overall quality of the alginate is low (Rehm and Valla, 1997). The rheological properties of pure alginates are determined by the degree of polymerization, which controls viscosity, and by their monomeric composition and sequence of mannuronic and glucuronic residues (Moe *et al*, 1995). The sequence of alginate monomers can be predicted from statistical models (Stokke *et al.*, 1991).

In food products, polypropylene glycol alginates (PGA) are used. PGAs are insensitive to low pH and polyvalent cations, which makes solutions containing them quite stable (Clare, 1993). For example, solutions containing PGA are insensitive to calcium ions and can be used in the dairy industry. The hydrophobic polypropylene glycol moiety imparts to alginate properties that facilitate foaming, emulsification and emulsion-stabilizing properties. The addition of low concentrations of calcium ions increases the viscosity of PGA, thus allowing it to be used as a thickener (Clare, 1993). Alginate gels are reasonably heat-stable and show little synergesis. Gels containing fruit can be used for pie fillings and remain stable through pasteurization and cooking. These gels are not thermoreversible and can be used as dessert gels that do not require refrigeration (Clare, 1993).

Research on alginate in the C. Bender laboratory

Role of alginate in lesion development and epiphytic fitness. Yu *et al.* (1999) studied the role of alginate in the bean spot pathogen, *P. syringae* pv. *syringae* strain 3525. Comparisons were made between *P. syringae* pv. syringae 3525, an alginate-producing strain, and *P. syringae* pv. syringae 3525.L, an alginate-defective mutant.

Although the Alg$^-$ mutant 3525.L remained pathogenic on bean, symptom severity and bacterial multiplication were reduced in comparison to 3525 (**Figure 2**). When brown spot lesions first became apparent 48 h after inoculation, the population of 3525 increased dramatically and was significantly higher than the initial inoculum density (Yu *et al.*, 1999). In contrast, the population of 3525.L remained low and was approximately 100-fold lower than strain 3525 throughout most of the sampling period. These results correlated with a reduction in the symptoms induced by the Alg$^-$ mutant. Lesions induced by 3525 initially had a water-soaked appearance with a necrotic lesion in the center, and the necrosis gradually intensified with time. Cells surrounding the necrotic lesion collapsed, possibly because water was withdrawn from the adjacent tissue, and some lesions coalesced (**Figure 2A**). These symptoms are typical of bacterial brown spot on bean (Fahy and Lloyd, 1983). In contrast, 3525.L induced fewer lesions, and these did not coalesce (**Figure 2B**).

Our results indicate that alginate production facilitates the dissemination of *P. syringae* pv. syringae 3525 in bean leaves. When the Alg$^-$ mutant 3525.L was infiltrated into bean leaves, lesions were restricted to the inoculation site (**Figure 2C**, left arrow). However, numerous secondary lesions developed around the site where 3525 was infiltrated (**Figure 2C**, right arrow), which indicates that the wild-type bacterium had successfully colonized neighboring plant cells. The hydrostatic pressure created by EPS production in the intercellular spaces of plant hosts may cause plant cells to rupture and assist in the dissemination of bacteria into areas surrounding the initial infection site (Leigh and Coplin, 1992).

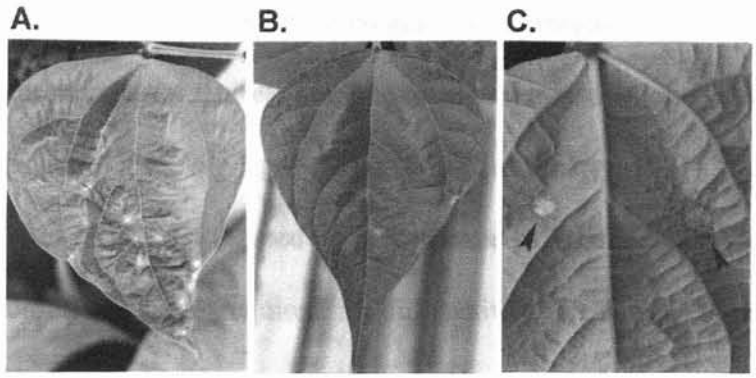

Figure 2. Lesion development on bean leaves spray-inoculated with (**A**) *P. syringae* pv. syringae 3525 and (**B**) the Alg⁻ mutant 3525.L. (**C**) Lesion development (see arrows) on a bean leaf infiltrated with *P. syringae* pv. syringae 3525.L (left) and the wild-type 3525 (right) (from Yu *et al.*, 1999).

The alginate biosynthetic genes in P. syringae pv. syringae. As in *P. aeruginosa*, the alginate biosynthetic genes in other pseudomonads are normally silent (Goldberg *et al.*, 1993). Interestingly, an indigenous plasmid designated pPSR12 conferred constitutive alginate production to *P. syringae* pv. syringae strain FF5 (Kidambi *et al.*, 1995). 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, which have not been characterized (Kidambi *et al.*, 1995). Mutagenesis of FF5(pPSR12) with Tn*5* resulted in the isolation of several alginate defective (Alg⁻) mutants, including FF5.31, which contains a Tn5 insertion in *algL* (Peñaloza-Vázquez *et al.*, 1997). The plasmid pSK2 restored alginate production to *P. syringae* pv. syringae FF5.31; this plasmid was shown to contain the alginate structural gene cluster in an arrangement virtually identical to that in *P. aeruginosa*. However, complementation analyses 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).

***Regulation of alginate in P. syringae pv.* syringae.** AlgR1 functions as a response regulator member of the two-component signal transduction system and binds to multiple sites upstream of the *algD* gene (Kato and Chakrabarty, 1991). In *P. aeruginosa*, AlgR1-binding sites (ABS; represented by green boxes in **Figure 3**) are located upstream of the *algD* promoter and are required for full activation of *algD* transcription in *P. aeruginosa*. Fakhr *et al.* (1999) generated an *algR1* mutant of *P. syringae* pv. syringae, which was designated FF5.32. This mutant was defective in alginate production but could be complemented when *algR1* from *P. aeruginosa* was expressed *in trans.* Unlike the situation in *P. aeruginosa*, *algR1* was not required for the transcription of *algD* in *P. syringae,* and *PsalgD* lacked the consensus sequence recognized by AlgR1 (**Figure 3**). The relatedness of the two species upstream of the *algD* translational start site was less than 40% at the nucleotide level (Fakhr *et al.*, 1999). This divergence in the *algD* promoter of *P. aeruginosa* and *P. syringae* is consistent with the fact that signals for gene induction differ in the two species (Kidambi *et al.*, 1995; Peñaloza-Vázquez *et al.*, 1997).

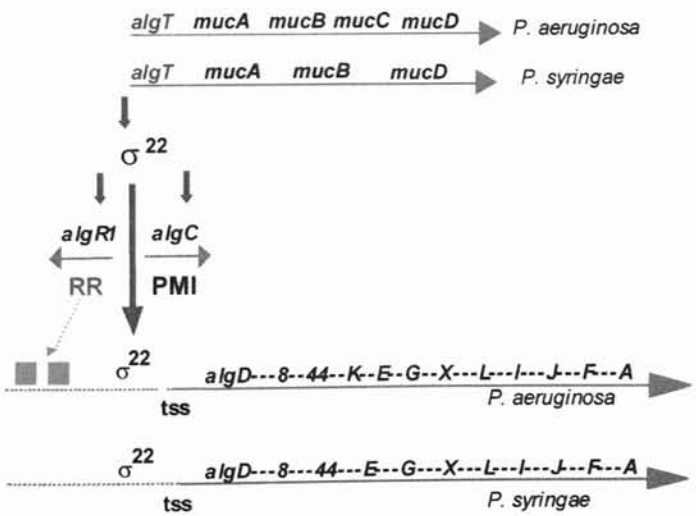

Figure 3. Regulation of the *algD* operon. AlgT encodes an alternate sigma factor, σ^{22}, which is required for the expression of *algR1*, *algC*, and the *algD* promoters. In addition to σ^{22}, expression of the *algD* promoter in *P. aeruginosa* also requires AlgR1, a response regulator protein that 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 this region.

AlgT (σ^{22}) controls alginate production in P. syringae. In *P. aeruginosa*, a cluster of genes located at 68 min is responsible for the conversion to mucoidy and includes *algT*, *mucA*, *mucB*, *mucC*, and *mucD* (DeVries and Ohman, 1994; Schurr *et al.*, 1994). These genes are arranged similarly in *P. syringae* (Keith and Bender, 1999; Keith and Bender, 2001); however, *P. syringae* does not have a *mucC* gene. 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). Keith and Bender (1999) demonstrated that the *algT* gene product, σ^{22}, from *P. syringae* pv. *syringae* shows 90% amino acid identity with σ^{22} from *P. aeruginosa*. Regions flanking *algT* in *P. syringae* contain *mucA*, *mucB* and *mucD* in an arrangement similar to that in *P. aeruginosa* (**Figure 3**) (Keith and Bender, 2001). An *algT* mutant of *P. syringae* was nonmucoid and could be complemented with *algT* from *P. aeruginosa* (Keith and Bender, 1999). The *algT* mutant of *P. syringae* was hypersensitive to heat, paraquat and reactive oxygen intermediates (ROIs); the increased sensitivity to ROIs was especially significant since these are generated during the oxidative burst, a defense response that occurs *in planta*. Furthermore, these stress-related signals also stimulated *algT* expression at the transcriptional level (Keith and Bender, 1999).

***Signals for alginate production in P. syringae pv.* syringae**. Peñaloza-Vázquez *et al.* (1997) showed that some environmental signals for alginate gene expression in *P. syringae* differ from those for *P. aeruginosa*; for example, exposure to copper ions triggers alginate production in *P. syringae* but not in *P. aeruginosa*. Copper was shown to dramatically increase alginate production in certain strains of *P. syringae* (Kidambi *et al.*, 1995), and many copper-resistant *P. syringae* strains are heavily mucoid on media

containing $CuSO_4$. Copper also activates transcription of *algD* and *algT* in *P. syringae* (Keith and Bender, 1999; Peñaloza-Vázquez *et al.*, 1997). Exposure to Cu^{2+} ions can generate free radicals and expose bacterial cells to oxidative stress (Keith and Bender, 1999). During colonization of plant tissue, *P. syringae* may be exposed to hydrogen peroxide (H_2O_2), another source of oxidative stress, which also increased the transcription of *algT* (Keith and Bender, 1999). As in *P. aeruginosa*, increased levels of NaCl and sorbitol activate the transcription of alginate promoters in *P. syringae* (Keith and Bender 1999), indicating that elevated osmolarity is a general signal for alginate production.

***algD* and its promoter**.

The *P. aeruginosa algD* gene is the first gene to be transcribed in the alginate operon, which encodes most of the proteins necessary for the biosynthesis of alginate (Chitnis *et al.*, 1993). In *P. aeruginosa,* AlgR1 is required for activation of the *algD* operon (Kato and Chakrabarty, 1991) and binds at several conserved sites in the promoter (**Figure 3**). Auxiliary proteins that are needed for the activation of *algD* include integration host factor (Mohr and Deretic, 1992), cyclic AMP receptor protein (DeVault *et al.*, 1991), AlgZ (a DNA-binding protein) (Baynham *et al.*, 1999), CysB (Delic-Attree *et al.*, 1997), and AlgB (Wozniak and Ohman, 1991). In *P. syringae*, the *algD* promoter region contains a putative recognition site for σ^{22} (Fakhr *et al.*, 1999), but the requirement of σ^{22} for *algD* transcription has not yet been demonstrated. However, the transcriptional activation of *algT* and *algD* in response to heat, osmotic stress, and copper

sulfate supports the hypothesis that *algT* may control activation of *algD* transcription in *P. syringae* (Keith and Bender, 1999; Peñaloza-Vázquez *et al.* 1997).

The reporter gene encoding β-glucuronidase

The enzyme β-glucuronidase (GUS) (E.C. 3.2.1.31) catalyses the hydrolysis of a wide variety of glucuronides. Its substrate consists of D-glucuronic acid conjugated through a β-O-glucosidic linkage to virtually any aglycone (non-sugar residue attached to a carbohydrate by an acetal linkage). The gene encoding β-glucuronidase (GUS) from *E. coli* K12, *gusA* (*uidA*), has been cloned and sequenced; the predicted protein product is a stable homotetramer of 68 kDa (Jefferson, 1986). The GUS reporter gene is very robust, assays are relatively simple, and expression of the gene is not deleterious to the plant host (Jefferson, 1989). An important advantage of GUS is the absence of glucuronidase activity in most organisms (Jefferson, 1989). Furthermore, minute quantities of GUS can be accurately measured, even down to single cells (Jefferson, 1989). GUS fusions are widely used to study plant-pathogen and plant-symbiont interactions. These fusions can be used to study both expression of particular genes and to mark and monitor populations of plant-associated microorganisms. *gusA,* unlike the β-galactosidase reporter, *lacZ,* can readily transverse membranes when fused to a transit peptide. Xiao *et al.* (1992) constructed *hrp-gusA* fusions in *P. syringae* pv. syringae 61 by Tn5-*gusA*1 mutagenesis to elucidate the organization and environmental regulation of the *hrp* cluster. Similar work was carried out by Schulte and Bonas (1991) with *gus* fusions of *hrp* genes in *X. campestris* pv. vesicatoria. Work conducted by Cook and Sequeira (1991) with *hrp-gusA* fusions demonstrated that *hrp* gene expression is constitutive in *R. solanacearum*. In the

Bender lab, *gusA* fusions have been used to monitor gene expression of both coronatine and alginate in *P. syringae* (Fakhr *et al.*, 1999; Keith and Bender, 1999; Palmer *et al.*, 1997; Peñaloza-Vázquez *et al.*, 1997; 2000; Rangaswamy *et al.*, 1997; 1998).

Objectives

As mentioned above, *algD* encodes GDP-mannose dehydrogenase, the committed step in the alginate biosynthetic pathway. *algD* is also the first gene to be transcribed in the alginate biosynthetic gene cluster of *P. syringae*. In the study reported in Chapter III, the *algD* promoter, gene and protein from multiple bacteria species were compared to achieve a better understanding of the evolutionary relatedness of *algD* homologues and their regulation.

In the work described in Chapter IV, an *algD-uidA* transcriptional fusion was constructed from the *algD* promoter in *P. syringae* pv. tomato DC3000. The activity of the *algD-uidA* fusion was evaluated in host plants (tomato and collard) and nonhost plants (tobacco and tomato containing the *Pto* resistance gene). The purpose of this study was to determine when *algD* was expressed in susceptible and resistant hosts, and to evaluate the role of alginate in compatible and incompatible host-pathogen-interactions.

CHAPTER III

Comparative Analysis of the *algD* Promoter, Gene and Protein from *Pseudomonas syringae*, *Pseudomonas aeruginosa*, and *Azotobacter vinelandii*

ABSTRACT

The *algD* gene encodes GDP-mannose dehydrogenase, which catalyzes the committed step in alginate biosynthesis. The *algD* promoter is a major controlling element in the biosynthesis of the exopolysaccharide alginate because it is the first gene transcribed in the structural gene cluster. The *algD* promoter from *P. syringae* pv. tomato DC3000 was cloned and sequenced in the current study, and the nucleotide sequence of the *algD* gene from *P. syringae* pv. syringae FF5 and pv. tomato DC3000 was derived. These sequences were compared to *algD* homologues in *Pseudomonas aeruginosa* and *Azotobacter vinelandii* by constructing multiple sequence alignments and phylogenetic trees. The results indicate that the *algD* promoter, genes, and proteins from *P. syringae* pv. tomato, phaseolicola, and syringae are closely related. Molecular modeling of the *P. syringae* DC3000 AlgD protein was accomplished using the HasB protein from *Streptococcus pyrogenes* as a template. The model reveals several conserved motifs that are candidates for site-directed mutagenesis and structure/function studies.

INTRODUCTION

The phytopathogenic bacterium *Pseudomonas syringae* produces two well-characterized EPS molecules: levan, a polymer of fructofuranan, and alginate, a

copolymer of *O*-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Fett *et al.*, 1986; Gross and Rudolph, 1987). Possible roles for the EPS molecules produced by *P. syringae* are varied and include avoidance of host plant cell recognition, resistance of bacterial cells to desiccation, and enhancement of epiphytic fitness (Kasapis *et al.*, 1994; Yu *et al.*, 1999). Furthermore, alginate has been implicated in the symptom water-soaking, in which the intercellular spaces of infected plants become filled with water (Gross and Rudolph, 1987; 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 (Shankar *et al.*, 1995). In *P. aeruginosa*, genes that encode the biosynthesis and regulation of alginate map to four chromosomal locations. With the exception of *algC*, which is located at 10 min, most of the structural genes are located at 34 min. The conversion from the nonmucoid to the mucoid phenotype is governed by a cluster of genes located at 68 min (Schurr *et al.*, 1996) and includes *algT* (*algU*), *mucA* (*algS*), *mucB* (*algN*), *mucC* (*algM*), and *mucD* (*algY*) (Boucher *et al.*, 1996; 1997). Other genes involved in the regulation of alginate synthesis include *algR1*, *algR2*, and *algR3* at 10 min and *algB* at 13 min (Wozniak and Ohman, 1994).

Most of the structural genes for alginate biosynthesis are clustered within an 18-kb operon in the *P. aeruginosa* chromosome (Chitnis and Ohman, 1993). The first gene to be transcribed in this operon is *algD*, which encodes a GDP-mannose dehydrogenase (Deretic *et al.*, 1987). In *P. aeruginosa,* the transcriptional activator encoded by *algR1* is required for activation of the *algD* operon (Kato and Chakrabarty, 1991) and binds to several conserved sites in the promoter. Auxiliary proteins that are needed for the

activation of *algD* include integration host factor (Mohr and Deretic, 1992), cyclic AMP receptor protein (DeVault *et al.*, 1991), AlgZ (a DNA-binding protein) (Baynham *et al.*, 1999), CysB (Delic-Attree *et al.*, 1997), and AlgB (Wozniak and Ohman, 1991).

In *P. syringae*, the arrangement of the structural and regulatory gene clusters are similar to that in *P. aeruginosa* (Fakhr *et al.*, 1999; Keith and Bender, 1999; 2001; Peñaloza-Vázquez *et al.*, 1997). The *P. syringae algD* promoter contains a putative recognition site for σ^{22} (Fakhr *et al.*, 1999), but the requirement of σ^{22} for *algD* transcription has not yet been demonstrated. However, the transcriptional activation of *algT* and *algD* in response to heat, osmotic stress, and copper sulfate supports the hypothesis that *algT* may control activation of *algD* transcription in *P. syringae* (Keith and Bender, 1999; Peñaloza-Vázquez *et al.* 1997).

In this study, the *algD* promoter, gene and protein from multiple bacteria species were compared to achieve a better understanding of the evolutionary relatedness of *algD* homologues and their regulation.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Table 1 lists the bacterial strains and plasmids used in this study. *P. syringae* was routinely maintained at 28°C on mannitol-glutamate (MG) agar (Keane et al., 1970). *Escherichia coli* strains were grown on Luria-Bertani (LB) medium (Miller, 1972) at 37°C. Antibiotics were added to the media at the following concentrations (μg/ml): ampicillin, 100; tetracycline, 12.5.

Molecular genetic techniques

Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, and isolation of DNA fragments from agarose gels were performed by standard protocols (Sambrook *et al.*, 1989). A genomic library of *P. syringae* pv. tomato DC3000 (Boch *et al.*, 2002) was probed with [α-^{32}P] dCTP labeled pSKK3.9 (**Table 1**) using the Rad Prime DNA labeling system (Gibco BRL, Gaithersburg, MD). Hybridizations and post-hybridization washes were conducted under high stringency conditions as described previously (Sundin and Bender, 1993).

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

STRAIN OR PLASMID	RELEVANT CHARACTERISTICS	REFERENCE/SOURCE
Escherichia coli DH5α	Δ(*lacZYA-argF*)$_{u169}$	Sambrook *et al.*, 1989
Pseudomonas syringae		
pv. tomato DC3000	Alginate producer; pathogenic on tomatoes, crucifers, Arabidopsis	Moore *et al.*, 1989
pv. syringae FF5	Alginate producer; pathogenic on ornamental pear	Kidambi *et al.*, 1995
pv. phaseolicola	Alginate producer; pathogenic on bean	Koopman *et al.*, 2001
Pseudomonas aeruginosa	Alginate producer; CF isolate	Deretic et al., 1987
Azotobacter vinelandii	Alginate producer; soil isolate	Campos et al., 1996
Streptococcus pyrogenes	Hyaluronic acid producer; clinical isolate	Dougherty and van de Rijn, 1993
Plasmids		
pBluescript SK+	Ap^R, ColE1 origin, cloning vehicle	Stratagene
pRCK1	Tc^R; cosmid clone from *P. syringae* DC3000 library	This study
pBRCK1	Ap^R; pBluescript SK(+) containing 8-kb *Bam*HI fragment	This study
pSKK3.9	Ap^r, contains 3.9-kb *Kpn*I fragment from pSK2 in pBluescript SK(+)	Peñaloza-Vázquez *et al.*, 1997

DNA sequencing and analysis

Nucleotide sequencing reactions were performed by the dideoxynucleotide method (Sambrook *et al.*, 1989) with AmpliTaq DNA Polymerase (Perkin-Elmer, Foster City, CA). Automated DNA sequencing was accomplished using an ABI 373A apparatus and the ABI PRISM Dye Primer Cycle Sequencing Kit (Perkin-Elmer). Automated sequencing of DNA was provided by the OSU Recombinant DNA-Protein Resource Facility. Sequence data was aligned and homology searches were executed using the National Center for Biotechnology Information Blast Network server. CLUSTALX (Thompson *et al.*, 1997) was used to construct multiple sequence alignments.

Multiple sequence alignments and construction of phylogenetic trees

A multiple sequence alignment of selected dehydrogenases (*algD* homologues) was constructed using CLUSTALX (Thompson *et al.*, 1997) and BOXSHADE 3.21 (Hoffman and Baron; www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed using the Vector NTI Suite, Version 6.0 (Informax, San Francisco, CA). Values obtained using this program are based on alignments created with CLUSTALW (Thompson *et al.*, 1994) and on the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987).

Nucleotide sequence accession numbers

The nucleotide sequence for *algD* and promoter region from *P. syringae* pv. tomato DC3000 was deposited in Genbank under accession no. AY095346. The

nucleotide sequence for *algD* in *P. syringae* pv. syringae FF5 was deposited in Genbank under the accession no. AY095347.

RESULTS AND DISCUSSISON

Cloning of *algD* from *P. syringae* pv. tomato DC3000

A genomic library of *P. syringae* pv. tomato DC3000 was previously constructed in pRK7813 (Boch *et al.*, 2002). In this study, a 3.9-kb *Kpn*I fragment from pSKK3.9, which contains *algD* from *P. syringae* pv. syringae FF5, was used to screen the DC3000 genomic library for clones containing *algD*. A cosmid clone designated pRCK1 contained an 8-kb *Bam*HI fragment that hybridized with the probe. This fragment was subcloned into pBluescript SK(+), resulting in pBRCK1, which was partially sequenced using the T7 and T3 primers. Sequence analysis indicated that the right border of this fragment contained DNA homologous to *alg8*. Since *algD* is the first gene in the alginate operon and located near *alg8*, we suspected that pBRCK1 contained a functional copy of *algD*.

Sequence analysis of *algD* from *P. syringae* DC3000

Sequence data for *algD* from *P. syringae* pv. tomato DC3000 was initially derived using pBRCK1 as template and the T7 and T3 primers, which anneal to the vector polylinker. Additional sequence data was obtained by generating internal oligonucleotide primers, and both DNA strands were sequenced for verification. The *P. syringae* pv. tomato DC3000 *algD* homologue was 1,317 bp (**Figure 4**) **(Table 2)** and was highly

related to *algD* from *P. syringae* pv. phaseolicola (91.8 and 98.4% nucleotide/ amino acid identity)

```
1    ATGCGTATCAGCATATTTGGTTTGGGTTATGTCGGTGCAGTCTGTGCCGGTTGCCTCTCT
     M  R  I  S  I  F  G  L  G  Y  V  G  A  V  C  A  G  C  L  S    20
61   GCCCGCGGCCACGACGTAGTGGGTGTGGACATCTCCAGCACCAAGATCGACCTGATCAAC
     A  R  G  H  D  V  V  G  V  D  I  S  S  T  K  I  D  L  I  N    40
121  AATGGCAAGTCGCCTATCGTTGAACCGGGCCTGGAAGAGCTGCTGCAAAAAGGTCTGGCC
     N  G  K  S  P  I  V  E  P  G  L  E  E  L  L  Q  K  G  L  A    60
181  ACCGGCAAACTGCGCGGCACTACCGACTTCGCCGAAGCCATTCGCGCCACTGACCTGTCG
     T  G  K  L  R  G  T  T  D  F  A  E  A  I  R  A  T  D  L  S    80
241  ATGATCTGCGTGGGTACGCCAAGCAAGAAGAACGGCGACCTGGAACTGGACTACATCGAA
     M  I  C  V  G  T  P  S  K  K  N  G  D  L  E  L  D  Y  I  E   100
301  TCAGTGTGCCGTGAAATCGGTTATGTCCTGCGTGACAAGAACACTCGCCACACTATCGTG
     S  V  C  R  E  I  G  Y  V  L  R  D  K  N  T  R  H  T  I  V   120
361  GTTCGCAGCACCGTACTGCCTGGCACCGTCGCCAACGTGGTTATCCCGATCCTGGAAGAC
     V  R  S  T  V  L  P  G  T  V  A  N  V  V  I  P  I  L  E  D   140
421  TGCTCCGGCAAGAAAGCCGGCGTTGACTTCGGCGTTGCCGTAAACCCAGAGTTCCTGCGT
     C  S  G  K  K  A  G  V  D  F  G  V  A  V  N  P  E  F  L  R   160
481  GAAAGTACCGCAATCAAAGACTACGACCTGCCGCCAATGACCGTGATCGGCGAGTTCGAC
     E  S  T  A  I  K  D  Y  D  L  P  P  M  T  V  I  G  E  F  D   180
541  AAGGCCTCGGGTGACGTTCTGCAATCGCTGTACGAAGAACTTGACGCACCGATCATCCGC
     K  A  S  G  D  V  L  Q  S  L  Y  E  E  L  D  A  P  I  I  R   200
601  AAGGACATCGCCGTTGCCGAAATGATCAAGTACACCTGCAACGTGTGGCACGCCACCAAA
     K  D  I  A  V  A  E  M  I  K  Y  T  C  N  V  W  H  A  T  K   220
661  GTGACCTTCGCCAACGAAATCGGCAACATTGCCAAAGCAGTCGGCGTTGATGGCCGTGAA
     V  T  F  A  N  E  I  G  N  I  A  K  A  V  G  V  D  G  R  E   240
721  GTGATGGACGTGGTCTGCCAGGACAAGGCATTGAACCTGTCCCAGTACTACATGCGCCCA
     V  M  D  V  V  C  Q  D  K  A  L  N  L  S  Q  Y  Y  M  R  P   260
781  GGCTTCGCCTTCGGCGGTTCGTGCCTGCCTAAAGACGTCCGCGCCCTGACCTACCGCGCC
     G  F  A  F  G  G  S  C  L  P  K  D  V  R  A  L  T  Y  R  A   280
841  GGTAGCCTGGATGTCGACGCACCGCTGCTCAACTCGCTGATGCGCAGCAACACCTCGCAA
     G  S  L  D  V  D  A  P  L  L  N  S  L  M  R  S  N  T  S  Q   300
901  GTGCAGAACGCTTTCGACATGGTTGCCAGCTACGACACCCGCAAGGTTGCACTGCTGGGT
     V  Q  N  A  F  D  M  V  A  S  Y  D  T  R  K  V  A  L  L  G   320
961  CTGAGCTTCAAGGCCGGCACCGACGATCTGCGTGAAAGCCCGCTGGTAGAGCTGGCTGAA
     L  S  F  K  A  G  T  D  D  L  R  E  S  P  L  V  E  L  A  E   340
1021 ATGCTGATCGGCAAGGGCTTCGACCTGAGCATTTTCGACAGCAACGTCGAATACGCCCGT
     M  L  I  G  K  G  F  D  L  S  I  F  D  S  N  V  E  Y  A  R   360
1081 GTTCACGGTGCCAACAAGGACTACATCGAATCGAAGATTCCGCACGTTTCCTCGCTGCTC
     V  H  G  A  N  K  D  Y  I  E  S  K  I  P  H  V  S  S  L  L   380
1141 AATTCCGACTTCGATCAGGTCATCAACGACTCCGACGTGATCATCCTTGGCAACCGCGAC
     N  S  D  F  D  Q  V  I  N  D  S  D  V  I  I  L  G  N  R  D   400
1201 GAGCGTTTCCGCTCCCTCGCCAACAAGACGCCTGAAGGCAAGCGCGTTATCGACCTGGTG
     E  R  F  R  S  L  A  N  K  T  P  E  G  K  R  V  I  D  L  V   420
1314 GGCTTCATGACCAATGCCACCACCGAAGACGGTCGTGCCGAAGGTATCTGCTGGTAA
     G  F  M  T  N  A  T  T  E  D  G  R  A  E  G  I  C  W  -      439
```

Figure 4. Nucleotide and deduced amino acid sequence of *algD* from *Pseudomonas syringae* pv. tomato DC3000. Translational start codon is highlighted in white font and surrounded by a black box. The putative NAD^+ binding site is shown in **blue** font, and the predicted active site is shown in **red** font.

AlgD consists of 438 amino acids with a predicted mass of 47.6 kDa. A potential ribosome-binding site was identified –6 to –12 bp upstream of the predicted translational start site, and a potential σ^{22} recognition site was present 501 and 478 bp upstream of the translational start site (**Figure 5**).

```
  1   GATTGACGCTGTAATCGTCGGTGTAACGCACCGCTGTTACACGACGCCCC
 51   CTCCGATCCGCCCCCAAATGACAACTTAGTGACGTCAGATAAACGTCAAC
101   TTGCCGACGACTCGTTCACGTCGATTTTTTTCATGAAAAAAAATCCATCT
151   TTTTAAGTAGTGGCCTTTTGGCTATTTGCTGTAGTAGCACTTTCATATCC
201   ACCCGGCTTTTATCCCCGCACACTAAGGCTTTTAAGAAACAGGCCATTAT
251   TTTGCAACTGCCATTGTCACAAACTGGATACACCGATTTTCTCAAATCAG
301   GACGAGCGGCCCGGAACTAAATGCGAATTTGTACGCTCCTACATCCCGTA
351   ACAATTCTGGGCGAATGTGTAAGCGATCACTTACACGCAGAACAGGAAAC
401   GGGAAGTTACCAACCGCTAAGCCGGGTCCACGGGATTCGGAGGTCTGGTA
451   CAGGATTAAACTCACATCACTAGTTGATGTTGTAGACAGACGAAGCACCG
501   GCCAGGTGCGACGTGCAGGACTCGAGCAAAGGCAGCAATGGCTCTGGCCA
551   GAGGCTTTCCGACCGAGGTCCGCCGTCAATCGTCAGACAGGAGTAGTTTA
601   CTGCATCCAAGGAGAGCAGGGCCCTGCCCACCGGACAGGTCACTTGCCGC
651   TTGCACTTAGTGTCATTTGAGTTCGCCTCGCTTGTTTGGGCACTCACTCA
701   TTCAATATGCCTGCGTTAGTTCTACGGTTCTGTTAGCCGTGACAACTGAC
751   GCGTGACATCTGCTGGCCAAAATTAATATCCAGCCACTTTATGGCGTGAA
820   AATCTAGGAGAATGATGATG
```

Figure 5. Nucleotide sequence of *algD* promoter from *Pseudomonas syringae* pv. tomato DC3000. The translational start codon is highlighted in white font and surrounded by a black box. A potential σ^{22} recognition sequence is indicated in bold and underlined. A possible ribosome-binding site is indicated in bold and double-underscored.

Table 2. Results of BlastP search for proteins that show a high degree of relatedness to the predicted amino acid sequence of AlgD from *Pseudomonas syringae* pv. tomato DC3000.

Organism	Enzyme	Score	E-Value
Pseudomonas phaseolicola	GDP-mannose 6-dehydrogenase	866	0.0
Pseudomonas aeruginosa	GDP-mannose 6-dehydrogenase	722	0.0
Pseudomonas aeruginosa	GDP-mannose 6-dehydrogenase	721	0.0
Azotobacter vinelandii	GDP-mannose 6-dehydrogenase	691	0.0
Streptomyces coelicolor	UDP-glucose/GDP-mannose dehydrogenase	437	e-122
Pyrococcus furiosus	NDP-sugar dehydrogenase	294	1e-78
Pyrococcus abyssi	UDP-glucose dehydrogenase	288	8e-77
Archaeoglobus fulgidus	UDP-glucose dehydrogenase	284	2e-75
Pyrococcus furiosus	NDP-sugar dehydrogenase	260	2e-68
Archaeoglobus fulgidus	UDP-glucose dehydrogenase	253	3e-66
Halobacterium sp. NRC-1	UDP-glucose dehydrogenase	247	2e-64
Mesorhizobium loti	UDP-glucose dehydrogenase	228	8e-59
Aquifex aeolicus	Nucleotide sugar dehydrogenase	223	4e-57
Bacillus subtilis	Similar to NDP-sugar dehydrogenase	223	4e-57
Xylella fastidiosa 9a5c	UDP-glucose dehydrogenase	221	9e-57
Bacillus anthracis	pXO1-95 Bacillus dehydrogenase	220	2e-56
Clostridium perfringens	probable NDP-sugar dehydrogenase	216	3e-55
Bacillus subtilis	UDP-glucose 6-dehydrogenase	213	4e-54
Synechocystis sp. PCC 6803	UDP-glucose dehydrogenase	204	2e-51
Nostoc sp. PCC 7120	UDP-glucose dehydrogenase	201	1e-50
Bacteroides fragilis	putative UDP-dehydrogenase	200	2e-50
Rickettsia conorii	UDP-glucose 6-dehyrogenase	199	5e-50

The presence of a potential σ^{22} recognition sequence in the *algD* promoter of DC3000 suggests that *algT* is involved in the regulation of alginate in this strain. The alternative sigma factor encoded by *algT*, σ^{22}, is required for transcription of *algD*, *algR1*, and *algT* in *P. aeruginosa* (Hershberger *et al.*, 1995; Schurr *et al.*, 1995; Wozniak and Ohman, 1994). Both the *algD* and *algR1* promoters in *P. aeruginosa* show a consensus sequence that 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). In *P. syringae* pv. syringae FF5, potential σ^{22} recognition sites are located upstream of *algD*, *algR1*, and *algT* (Fakhr *et al.*, 1999; Keith and Bender, 1999).

No AlgR1 binding sites were apparent in the DC3000 *algD* promoter region, which suggests that DC3000, like *P. syringae* pv. syringae FF5, does not require AlgR1 for expression of *algD* (Fakhr *et al.*, 1999). This contrasts with *P. aeruginosa*, where AlgR1 is required for *algD* expression and physically binds to the *algD* promoter region at conserved AlgR1-binding sites (Kato and Chakrabarty, 1991; Mohr *et al.*, 1992).

Sequence analysis of *algD* from *P. syringae* FF5

The nucleotide sequence for *algD* from *P. syringae* pv. syringae FF5 was initially derived using a primer constructed from -2 to +14 of *algD* (5' CGATGCGTATCAGCAT, Fakhr *et al.*, 1999). Plasmid pSKK3.9 was used as template and additional sequence was generated using internal oligonucleotide primers. Both DNA strands were sequenced for verification. The *P. syringae* pv. syringae FF5 *algD* homologue was 1,317 bp and was highly related to *P. syringae* pv. tomato DC3000 (92.1 and 97.9% nucleotide and amino

acid identity, respectively). The predicted translational product of *algD* consists of 438 amino acids with a mass of 47.5 kDa (**Figure 6**).

```
   1  ATGCGTATCAGCATATTTGGTTTGGGTTACGTCGGTGCAGTCTGTGCCGGTTGCCTGTCT
      M  R  I  S  I  F  G  L  G  Y  V  G  A  V  C  A  G  C  L  S    20
  61  GCCCGCGGTCACGAAGTAGTTGGTGTGGATATTTCCAGCACCAAGATCGACCTGATCAAC
      A  R  G  H  E  V  V  G  V  D  I  S  S  T  K  I  D  L  I  N    40
 121  AACGGCAAATCACCTATCGTTGAACCGGGTCTGGAAGAGCTGTTGCAGAAAGGTATCAGT
      N  G  K  S  P  I  V  E  P  G  L  E  E  L  L  Q  K  G  I  S    60
 181  ACCGGAAAACTGCGCGGCACTACAGACTTCGCCGAAGCCATTCGCGCCACCGACCTGTCG
      T  G  K  L  R  G  T  T  D  F  A  E  A  I  R  A  T  D  L  S    80
 241  ATGATCTGCGTCGGTACGCCGAGCAAGAAGAACGGCGATCTGGAACTGGATTACATTGAA
      M  I  C  V  G  T  P  S  K  K  N  G  D  L  E  L  D  Y  I  E   100
 301  TCGGTGTGCCGCGAAATCGGTTATGTCCTGCGTGACAAAGCCACTCGCCACACTATCGTG
      S  V  C  R  E  I  G  Y  V  L  R  D  K  A  T  R  H  T  I  V   120
 361  GTTCGCAGTACCGTACTGCCGGGCACTGTTGCCAACGTTGTTATCCCGATTCTGGAAGAC
      V  R  S  T  V  L  P  G  T  V  A  N  V  V  I  P  I  L  E  D   140
 421  TGCTCCGGCAAGAAAGCCGGTGTTGACTTCGGCGTCGCCGTCAACCCTGAGTTCCTGCGC
      C  S  G  K  K  A  G  V  D  F  G  V  A  V  N  P  E  F  L  R   160
 481  GAAAGTACTGCGATCAAGGACTACGACCTGCCGCCAATGACCGTGATCGGCGAGTTCGAC
      E  S  T  A  I  K  D  Y  D  L  P  P  M  T  V  I  G  E  F  D   180
 541  AAAGCGTCGGGCGACGTTCTGCAATCGCTGTACGAAGAACTCGATGCACCGATCATCCGC
      K  A  S  G  D  V  L  Q  S  L  Y  E  E  L  D  A  P  I  I  R   200
 601  AAGGACATCGCTGTTGCCGAGATGATCAAGTACACCTGCAATGTGTGGCACGCCACCAAG
      K  D  I  A  V  A  E  M  I  K  Y  T  C  N  V  W  H  A  T  K   220
 661  GTTACTTTCGCCAACGAAATCGGCAACATTGCCAAGGCCGTCGGCGTCGATGGCCGTGAA
      V  T  F  A  N  E  I  G  N  I  A  K  A  V  G  V  D  G  R  E   240
 721  GTGATGGACGTGGTCTGCCAGGACAAGGCGCTGAACCTGTCCCAGTACTACATGCGCCCT
      V  M  D  V  V  C  Q  D  K  A  L  N  L  S  Q  Y  Y  M  R  P   260
 781  GGCTTCGCATTCGGCGGCTCCTGCCTGCCGAAAGACGTTCGTGCCCTGACCTACCGCGCC
      G  F  A  F  G  G  S  C  L  P  K  D  V  R  A  L  T  Y  R  A   280
 841  GGCAGCCTGGACGTTGAAGCTCCCCTGCTCAACTCGCTGATGCGCAGTAACACGTCGCAA
      G  S  L  D  V  E  A  P  L  L  N  S  L  M  R  S  N  T  S  Q   300
 901  GTACAGAACGCTTTCGACATGGTTGCCAGCTACGACGCCCGCAAGGTTGCACTGCTGGGC
      V  Q  N  A  F  D  M  V  A  S  Y  D  A  R  K  V  A  L  L  G   320
 961  CTGAGCTTCAAGGCCGGTACTGACGATCTGCGTGAAAGCCCGCTGGTAGAGCTGGCCGAA
      L  S  F  K  A  G  T  D  D  L  R  E  S  P  L  V  E  L  A  E   340
1021  ATGCTGATCGGCAAGGGCTTCGACCTGAGCATCTTCGACAGCAACGTTGAATATGCACGC
      M  L  I  G  K  G  F  D  L  S  I  F  D  S  N  V  E  Y  A  R   360
1081  GTTCACGGTGCCAACAAGGACTACATCGAATCCAAGATTCCGCACGTTTCCTCGCTGCTG
      V  H  G  A  N  K  D  Y  I  E  S  K  I  P  H  V  S  S  L  L   380
1141  AACTCGGACTTCGACCAAGTCATCAATGACTCCGACGTCATCATCCTCGGCAACCGCGAT
      N  S  D  F  D  Q  V  I  N  D  S  D  V  I  I  L  G  N  R  D   400
1201  GAGCGTTTCCGCGCCCTCGCCAACAAGACCCCTGAAGGCAAGCGCGTCATCGATCTGGTG
      E  R  F  R  A  L  A  N  K  T  P  E  G  K  R  V  I  D  L  V   420
1314  GGCTTCATGGCCAACGCCACCAGCGAAGACGGTCGTGCCGAAGGTATCTGCTGGTAA
      G  F  M  A  N  A  T  S  E  D  G  R  A  E  G  I  C  W  -      439
```

Figure 6. Nucleotide and deduced amino acid sequence of *algD* from *Pseudomonas syringae* pv. syringae FF5. Translational start codon is highlighted in white font and surrounded by a black box. The putative NAD^+ binding site is shown in **blue** font, and the predicted active site is shown in **red** font.

Multiple sequence alignments

The *algD* promoter, gene and protein of *P. syringae* pv. tomato DC3000 was compared to other *algD* homologues. These included *P. syringae* pv. syringae FF5 (*algD* promoter deposited as accession no. AF131068), *P. syringae* pv. phaseolicola (accession no. AF001555), *P. aeruginosa* (accession no. Y00337), *Azotobacter vinelandii* (accession no. U11240) and *Streptococcus pyrogenes* (accession no. L08444). A multiple sequence alignment of the dehydrogenases was constructed using CLUSTALX, and several regions were highly conserved in the promoter and the gene products (**Table 3** and **Figures 7 and 8**).

Table 3. Percent identity and similarity for nucleotide residues in the protein, gene, and promoter regions of selected *algD* homologues compared with the respective sequences in *P. syringae* pv. tomato DC3000.

Organism	Protein/ Gene/ Promoter % Identity	Protein % Similarity
P. syringae pv. tomato DC3000	100/ 100/ 100	100
P. syringae pv. syringae FF5	97.9/ 92.1/ 81.6	99.3
P. syringae pv. phaseolicola	98.4/ 91.8/ 77.2	99.5
Pseudomonas aeruginosa	78.5/ 77.4/ 45.7	87.2
Azotobacter vinelandii	77.2/ 77.4/ 45.6	84.9
Streptococcus pyogenes	20.4/ 39.1/ ----	20.4

Phylogenetic trees

The construction of phylogenetic trees provided some clues regarding the evolutionary relatedness of *algD*. Phylogenetic analysis of the *algD* promoter and coding regions (**Figure 9A, B**) indicated that *P. syringae* pv. syringae FF5 and *P. syringae* pv. phaseolicola cluster together. *P. syringae* pv. tomato DC3000 was in an evolutionarily

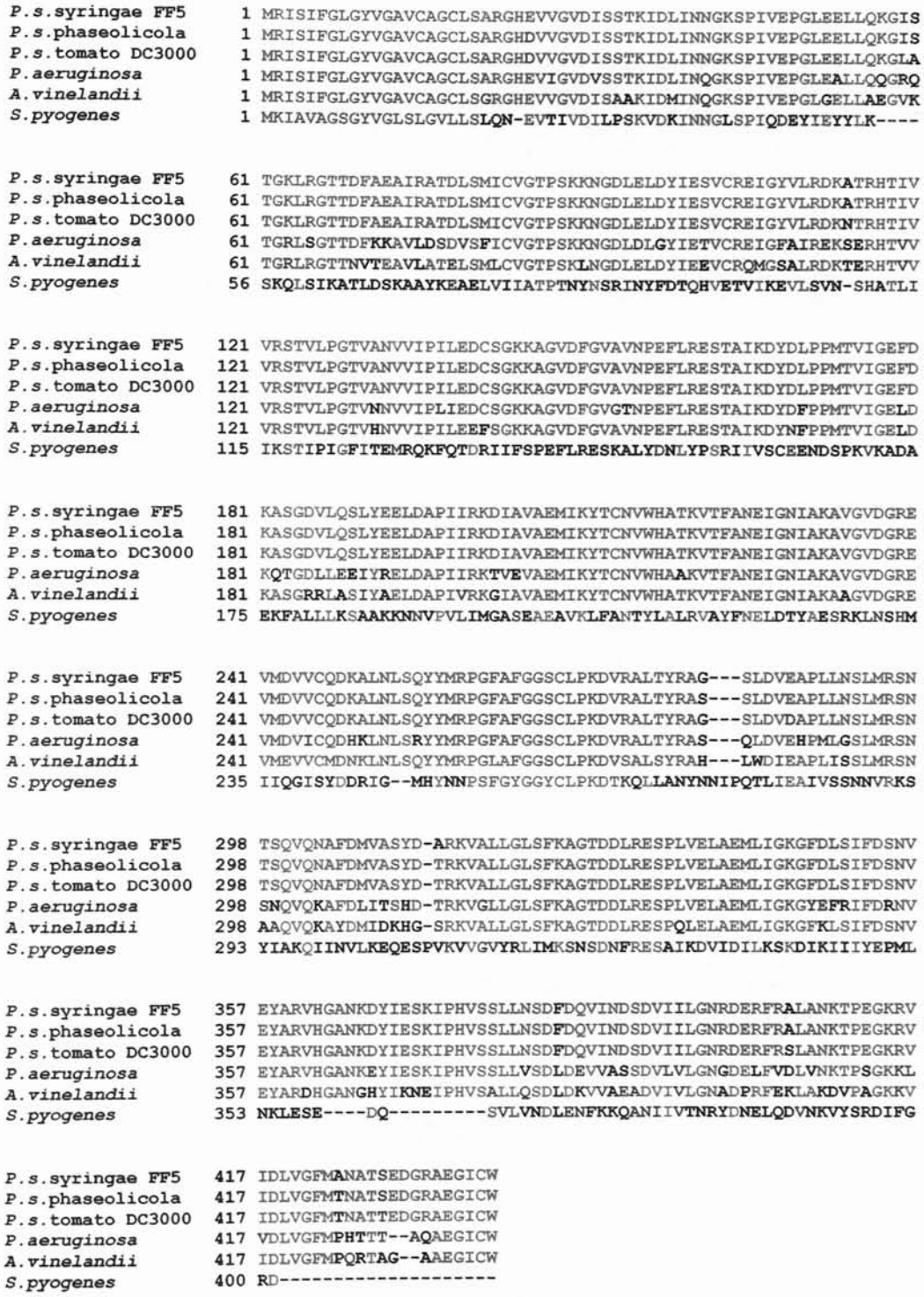

Figure 7. Multiple sequence alignment of selected GDP-mannose dehydrogenases. Conserved residues are shown in **red** font, similar residues in **black** and non-related residues in **blue**. NAD^+ binding site residues are highlighted in **green** and the active site residues are **pink**.

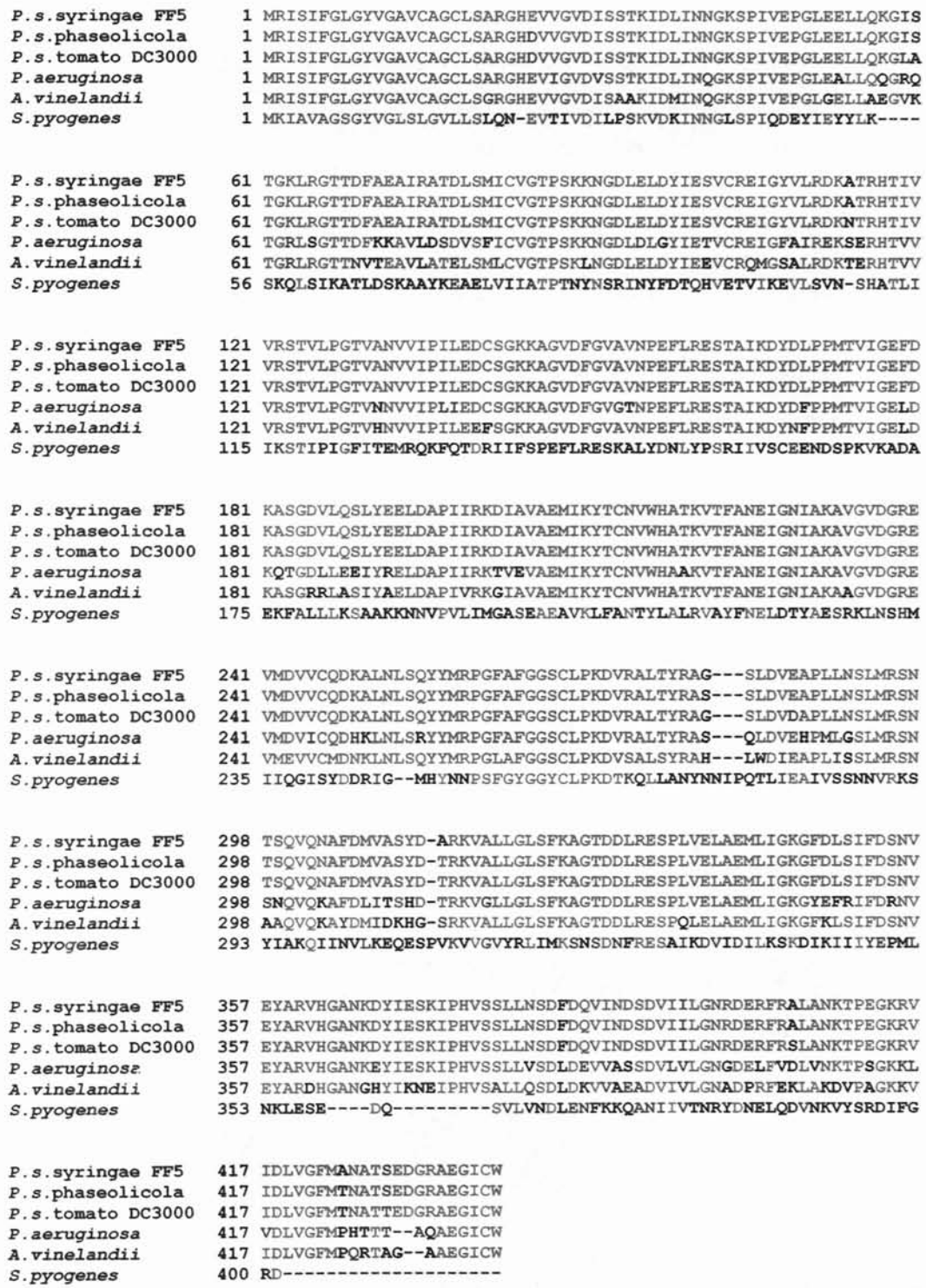

Figure 7. Multiple sequence alignment of selected GDP-mannose dehydrogenases. Conserved residues are shown in red font, similar residues in **black** and non-related residues in **blue**. NAD^+ binding site residues are highlighted in green and the active site residues are pink.

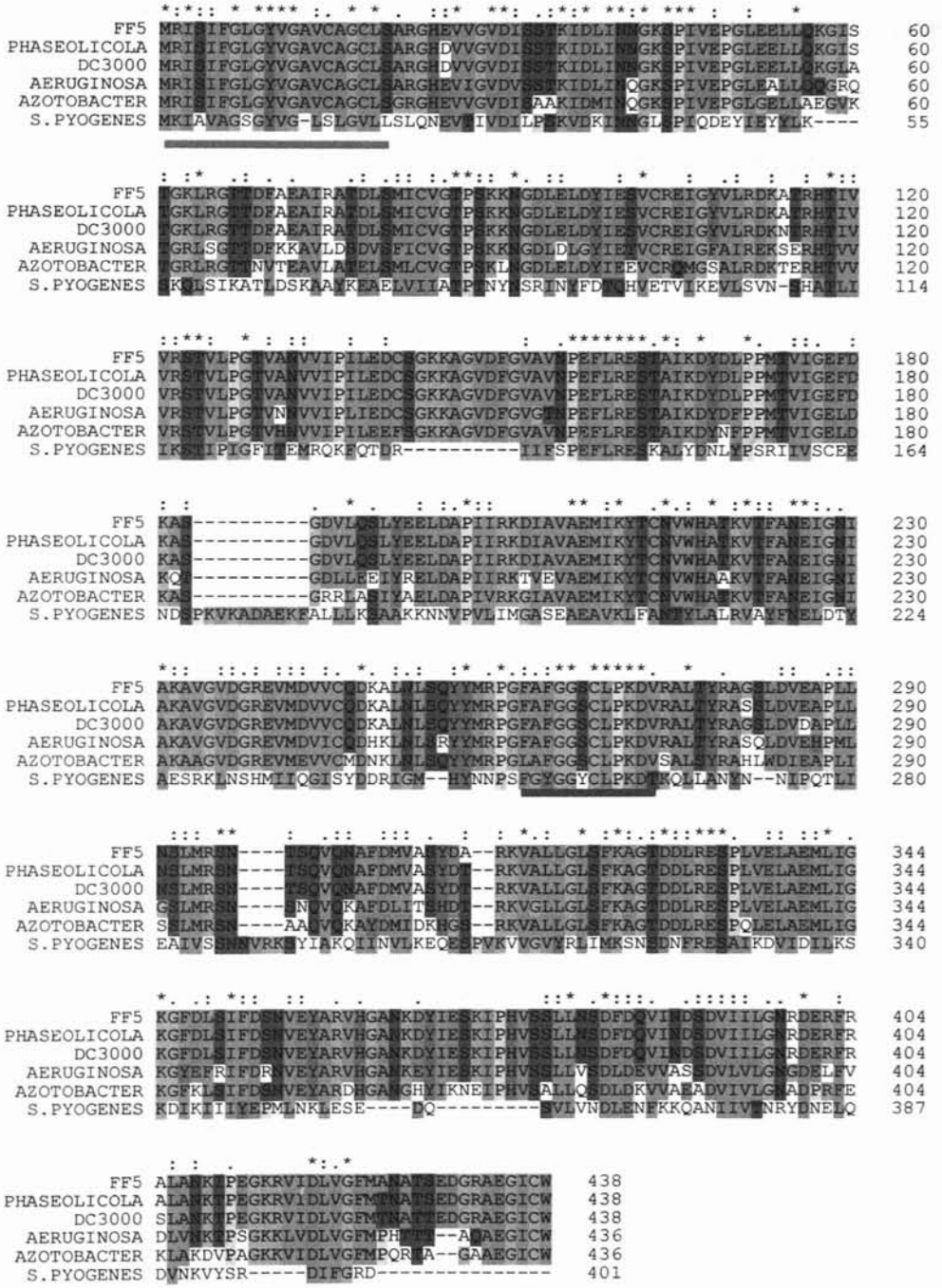

Figure 8. Multiple sequence alignment of selected dehydrogenases. Conserved residues are highlighted in color: orange, glycine; dark orange, positively-charged residues; purple, negatively charged residues; yellow, proline; dark blue, hydrophobic residues; light blue, slightly hydrophobic residues; and green, polar residues. Asterisks (*) represent identical amino acids, whereas colons (:) and dots (.) represent similar residues. Red underscore shows NAD^+ binding site and blue underscore shows active site.

close, but separate group. The reason for the separate groupings is not clear, but we have previously found that the *algD* promoter from FF5 does not function properly in DC3000, and this may be reflected in the phylogenetic tree. Although the *algD* promoter from *P. aeruginosa* and *Azotobacter vinelandii* form a separate clade, the length of the branches indicate that the promoter regions of the *algD* homologues are not closely related to each other and may have diverged in the past from a common ancestor. The groupings shown in Figure 9A are important indicators of divergence in the regulation of *algD*, which has been well established for *P. syringae* and *P. aeruginosa* (Fakhr *et al.*, 1999; Peñaloza-Vázquez *et al.*, 1997).

In the phylogenetic trees constructed for the *algD* gene (**Figure 9B**) and protein (**Figure 9C**), we see a marked increase in the degree of similarity between the *P. syringae* pathovars and the group comprised of *P. aeruginosa* and *A. vinelandii*. The high degree of similarity among the three *P. syringae* pathovars reflects their evolutionary relatedness. At the protein level, this similarity indicates a conservation of function and the specific amino acids located at conserved sites within the secondary structure.

The *algD* gene and protein trees were constructed using the *hasB* gene from *Streptococcus pyogenes* as an "outlier". The *hasB* gene encodes UDP-glucose dehydrogenase (Dougherty and van de Rijn, 1993), an enzyme that is functionally similar to GDP-mannose dehydrogenase (the activity catalyzed by AlgD).

AlgD modeling studies

HasB from *Streptococcus pyogenes* was used as a template for constructing a 3-D structure for AlgD from *P. syringae* pv. tomato DC3000 using the Swiss-Pdb Viewer

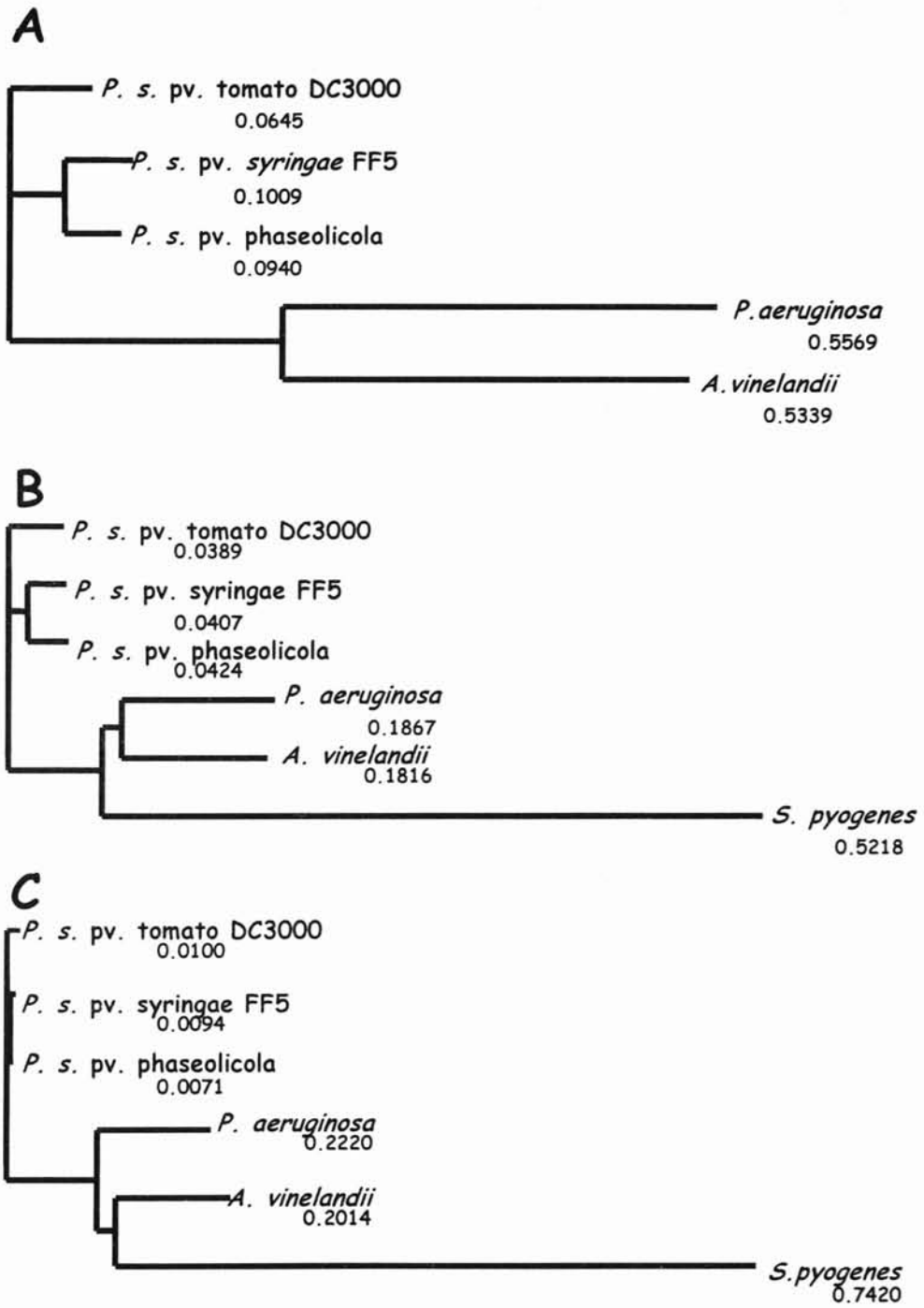

Figure 9. Phylogenetic analysis of (**A**) promoter regions, (**B**) genes, and (**C**) proteins of *algD* homologues from three *P. syringae* pathovars, *P. aeruginosa*, *A. vinelandii*, and *S. pyrogenes*. Tree analysis based on the Neighbor Joining (NJ) algorithm.

(Guex and Peitsch, 1997; www.expasy.ch/spdbv). AlgD from DC3000 (438 amino acids) and HasB (401 amino acids; no. 1DLIA in the Brookhaven Protein Databank) belong to the UDP-glucose/GDP-mannose dehydrogenase family, which is a small group of enzymes possessing the ability to catalyze the NAD-dependent two-fold oxidation of an alcohol to an acid without releasing an aldehyde intermediate. AlgD is a homohexamer (Roychoudhury *et al.*, 1989), and the structural analysis conducted in this study indicates that AlgD consists of two domains with 16 α-helices and 11 β-strands in 3 β-sheets **(Figure 10A and 10B)**. The N-terminal domain of AlgD contains a highly conserved NAD^+ binding site consisting of residues R2 to L19 (**Figure 11A**). The proposed active site of the protein is C268 (Roychoudhury *et al.*, 1989), which is situated at the top of the cleft formed by the two domains (**Figure 11B**). These conserved sites are also reflected in the deduced amino acid sequence of *P. syringae* pv. tomato DC3000 (**Figure 4**) and *P. syringae* pv. syringae FF5 (**Figure 6**). Comparison of the three dimensional *Pseudomonas syringae* pv. tomato DC3000 model **(Figure 12A)**, with the *S. pyogenes* template **(Figure 12B)**, shows the highly conserved nature of the dehydrogenases, even though the amino acid identity is only 20%. Here we see the conservation of the three dimensional structure especially at the NAD^+-binding and active sites. This suggests that there has been an evolutionary divergence of the two structures from an ancestral dehydrogenase. This ancestral dehydrogenase was probably more closely related to the UDP-glucose dehydrogenase than the mannose dehydrogenase because production of alginate is not essential for bacterial survival, but instead serves an adaptation to provide bacteria with a selective advantage in survival and colonization of susceptible hosts.

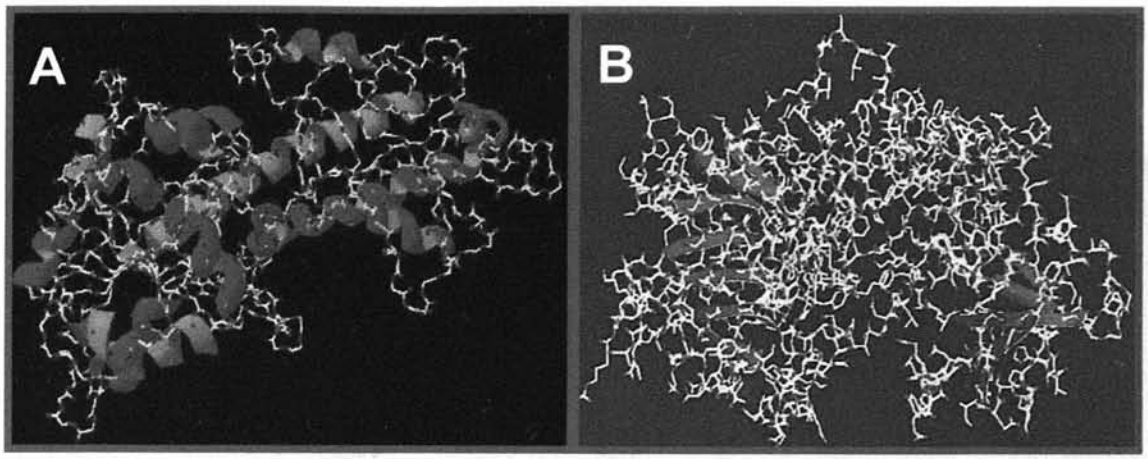

Figure 10. Molecular modeling of AlgD from *P. syringae* pv. tomato DC3000. **A,** α-helices imposed over wireframe. **B,** β sheets imposed over wireframe.

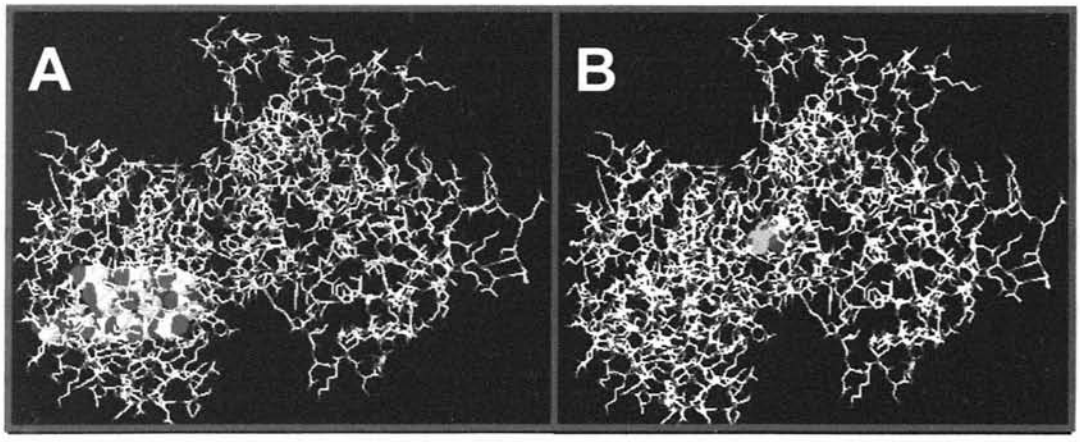

Figure 11. Molecular modeling of AlgD from *P. syringae* pv. tomato DC3000. Wireframe diagrams show the two discrete domains of the enzyme. **A,** Space-filling portion of model shows a potential NAD^+-binding site. **B,** The catalytic cysteine residue (C268) is shown in yellow.

The motif GFAFGGSCLPKD is related to the catalytic sites of other NAD^+-linked, four-electron transfer dehydrogenases, including L-histidine dehydrogenase from *Escherichia coli* (Ordman and Kirkwood, 1977). Secondary structural analysis of amino acid composition using nnpredict (www.cmpharm.uscf.edu/cgi-bin/nnpredict.pl) indicated a

>99% probability of AlgD having a cytoplasmic location with no membrane spanning domains or motifs.

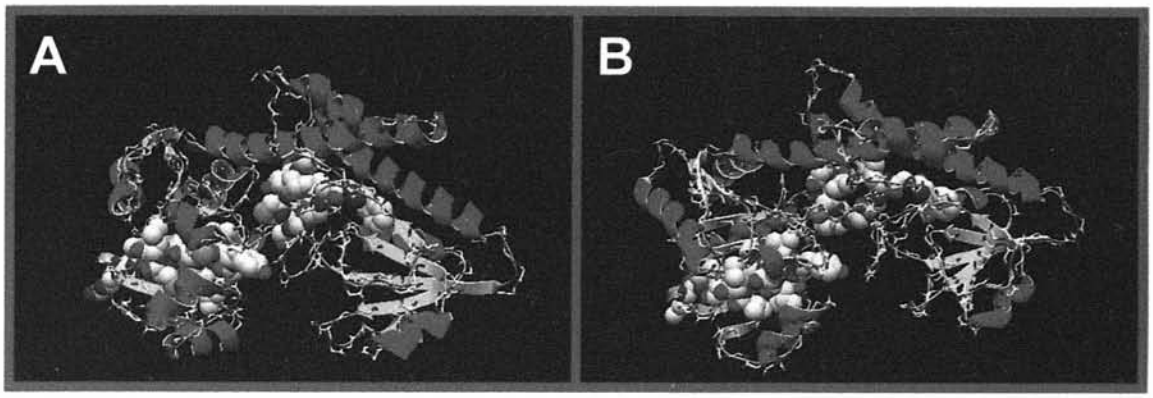

Figure 12. Molecular modeling of AlgD and template (HasB). **A,** AlgD from *P. syringae* pv. tomato DC3000 showing α-helices and β sheets along with putative NAD^+-binding site and active site. **B,** UDP-glucose dehydrogenase (HasB) from *S. pyogenes*, which was used as a modeling template and showing structural similarity to AlgD.

In this study, the *algD* promoter, nucleotide sequence and translational product were shown to be very similar in *P. syringae* pvs. tomato, syringae and phaseolicola. Phylogenetic trees constructed using *algD* from *P. aeruginosa* and *A. vinelandii* reflected the adaptation of these bacteria to different niches (CF lung vs. soil). HasB from *S. pyrogenes* was a useful template for molecular modeling of AlgD, and a number of residues in AlgD were identified that would be useful for site-directed mutagenesis and structure/function studies.

CHAPTER IV

Expression of the *algD* Promoter of *P. syringae* pv. tomato DC3000 in Host and Nonhost Plants

ABSTRACT

Pseudomonas syringae produces the exopolysaccharide alginate, a copolymer of mannuronic acid and guluronic acid. Although alginate is produced by *P. syringae in planta*, the signals and timing of alginate gene expression have not been described. In this study, an *algD*::*uidA* transcriptional fusion designated pDCalgDP was constructed and used to monitor alginate gene expression in host and nonhost plants infected with *P. syringae* pv. tomato DC3000. When leaves of susceptible collard plants were spray-inoculated with DC3000(pDCalgDP), *algD* was activated within 48-72 hpi and was associated with the development of water-soaked lesions. In susceptible tomato leaves, *algD* activity was generally lower than in collard and was not associated with water-soaking. *algD* expression was also monitored in leaves of the tomato cv. Rio Grande containing the *Pto* transgene. A hypersensitive response (HR) could be observed in single cells of Rio Grande *Pto* tomato leaf tissue; furthermore, *algD* expression was activated quickly, and glucuronidase activity was visualized in single bacterial cells. *algD* expression was also monitored in tobacco leaves infiltrated with DC3000(pDCalgDP); *algD* expression was induced during the HR in tobacco, and the timing closely followed expression of a *hrpA*::*uidA* transcriptional fusion. In tobacco inoculated with DC3000-*hrcC*(pDCalgDP), an HR did not occur and expression of *algD* remained low. In

summary, these results suggest that events occurring during the oxidative burst are signals for alginate gene expression *in planta*.

INTRODUCTION

Both *Pseudomonas aeruginosa* and *Pseudomonas syringae* produce the exopolysaccharide alginate, a copolymer of *O*-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer, L-guluronic acid. Several studies have shown that alginate functions as a virulence factor in *P. syringae* (Osman *et al.*, 1986; Gross and Rudolph, 1987; Yu *et al.*, 1999). For example, alginate enhances epiphytic fitness, increases resistance to desiccation and toxic molecules, and has been associated with the induction of water-soaked lesions on infected leaves (Fett and Dunn, 1989; Rudolph *et al.*, 1994; Yu *et al.*, 1999). In plants inoculated with alginate-defective mutants of *P. syringae* pv. syringae, symptoms were less severe and bacterial multiplication was reduced (Yu *et al.*, 1999). It has been speculated that alginate may interfere with the oxidative burst and the formation of reactive oxygen intermediates, responses that are associated with the plant defense response (Keith and Bender, 1999; Yu *et al.*, 1999).

Strains of *P. syringae*, like *P. aeruginosa*, are normally nonmucoid; however, some of the signals for conversion to the mucoid phenotype differ in these two species. Copper has been shown to dramatically increase alginate production in certain strains of *P. syringae* (Kidambi *et al.*, 1995), possibly because Cu^{2+} ions can generate free radicals and expose bacterial cells to oxidative stress (Keith and Bender, 1999). During colonization of plant tissue, *P. syringae* may be exposed to hydrogen peroxide (H_2O_2), another source of oxidative stress and one that increased the transcription of *algT*, which

encodes the alternative sigma factor, σ^{22} (Keith and Bender, 1999). In *P. syringae*, the *algD* promoter region contains a putative recognition site for σ^{22} (Fakhr *et al.*, 1999), but the requirement of σ^{22} for *algD* transcription has not yet been demonstrated. However, the transcriptional activation of *algT* and *algD* in response to heat, osmotic stress, and copper sulfate supports the hypothesis that *algT* may control activation of *algD* transcription in *P. syringae* (Keith and Bender, 1999; Peñaloza-Vázquez *et al.* 1997). As in *P. aeruginosa*, increased levels of NaCl and sorbitol activate the transcription of alginate promoters in *P. syringae* (Keith and Bender, 1999), indicating that elevated osmolarity is a general signal for alginate production.

In *P. syringae*, the *hrp/hrc* genes encode a type III secretory system, which controls the ability to induce a hypersensitive response (HR) in nonhost plants, pathogenicity on host plants, and growth in the apoplast and phylloplane (Galán and Collmer, 1999; Hirano *et al.*, 1999; Wei *et al.*, 2000). In *P. syringae*, the *hrp/hrc* genes are encoded by chromosomally-borne pathogenicity islands and encode regulatory, secretory, or effector proteins (Alfano *et al.*, 2000; Collmer *et al.*, 2000). Activities in the type III secretion apparatus have been assigned to some of the Hrp and Hrc proteins. For example, *hrcC* encodes an outer membrane protein that is essential for type III protein secretion and has a primary role in protein translocation across the outer membrane (Charkowski *et al.*, 1997). The *hrpA* gene encodes the major subunit of the Hrp pilus and is required for secretion of putative virulence proteins (Wei *et al.*, 2000). The *hrpA* gene also has regulatory functions and is known to impact expression of *hrpR* and *hrpS*, which encode response regulators (Wei *et al.*, 2000).

P. syringae pv. *tomato* DC3000, a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower), and *Arabidopsis thaliana* (Moore *et al.*, 1989; Whalen *et al.*, 1991; Zhao *et al.*, 2000), is a model strain for investigating plant-microbe interactions. This is largely because of its genetic tractability, pathogenicity on *A. thaliana*, and the availability of genomic sequence (http://www.tigr.org). When DC3000 is inoculated to tomato leaves, it induces necrotic lesions that are surrounded by a yellow chlorotic halo; the latter symptom is caused by production of coronatine (COR), a chlorosis-inducing phytotoxin (Moore *et al.*, 1989). DC3000 induces a typical HR when infiltrated into leaves of tobacco, which is not a host for this pathogen. A *hrcC* mutant of DC3000 (DC3000-*hrcC*) failed to induce an HR when infiltrated into leaves of the nonhost tobacco; however, when DC3000-*hrcC* was inoculated to tomato leaves, chlorotic haloes were visible (Preston, 1997). Subsequent experiments indicated that DC3000-*hrcC* overproduced COR relative to the wild-type strain and indicated that the chlorosis observed *in planta* was due to COR production (Peñaloza-Vázquez *et al.*, 2000). These results indicated that a functional type III secretion system was not required for COR production and further suggested that mutations in the *hrp/hrc* genes can have regulatory effects on other virulence factors (Peñaloza-Vázquez *et al.*, 2000). The term 'HR' was first used by Stakman (1915) to describe the rapid and localized plant cell death induced when races of rust fungi were inoculated to resistant cereal cultivars. Goodman and Novacky (1994) defined HR as 'the rapid death of plant cells in association with the restriction of pathogen growth'. The HR of plants resistant to microbial pathogens is a complex form of programmed cell death (PCD) that involves the induction of local and systemic defense responses (Heath, 2000). The HR is generally recognized by the presence of

brown, dead cells at the infection site and may involve one or many plant cells (Heath, 2000). The HR may involve a single plant resistance gene (*R* gene) (Heath, 1996), which interacts with an avirulence (*avr*) gene product in a gene-for-gene relationship (Flor, 1956). In bacteria, this can be more complex with single *R* genes interacting with more than one *avr* gene (Hammond-Kosack and Jones, 1997).

R genes share common motifs, and plant genomes contain large numbers of *R* genes with similar sequences (Michelmore and Meyers, 1998). The predominant class of *R* gene products has a nucleotide binding site, leucine rich repeats, and a leucine zipper or transcriptional initiation region (TIR). Some *R* genes have a predicted transmembrane sequence, but others have an intracellular location (Boyes *et al.*, 1998). The *Pto* resistance gene in tomato encodes a serine-threonine protein kinase and confers resistance to isolates of *P. syringae* pv. tomato that express the avirulence gene *avrPto* (Martin *et al.*, 1993). The AvrPto protein is secreted from *P. syringae* into the plant cell through the *hrp* pilus where its interaction with the *Pto* kinase results in disease resistance (Jin *et al.*, 2001). The *Pto* resistance gene was originally isolated from *Lycopersium pimpinellifolium* (Miller and Tanksley, 1990) and was shown to confer resistance to *P. syringae* pv. tomato when inserted into the sensitive, domesticated tomato, *L. esculentum* (Tanksley *et al.*, 1996).

Plants commonly respond to external stimuli, including microbial elicitors of cell death, by calcium influx into the cell (Ebel and Scheel, 1997). For bacterial pathogens, one of the earliest signs of the HR is membrane dysfunction (Pike *et al.*, 1998). Incompatible bacteria cause membrane depolarization, potassium efflux, and alkalization of the external medium (Pike *et al.*, 1998). The generation of inter- or intracellular

reactive oxygen species (ROS; e.g. O_2^-, H_2O_2) has also been demonstrated in response to incompatible bacteria (Baker and Orlandi. 1995). *In planta*, the initial stimulation of extracellular ROS is followed by intracellular generation (Heath, 1998), probably due to cellular decompartmentalization. Plants can generate extracellular ROS in several ways (Bolwell, 1997), including wall-bound peroxidases (Bestwick *et al.*, 1998) and plasma membrane-bound NADPH oxidase (Higgins *et al.*, 1998). Hydrogen peroxide (H_2O_2) may play a more important physiological role than O_2^-, but H_2O_2 does not induce cell death in tomato (Delledonne, 1998, 2001). In tomato, the effect of H_2O_2 is potentiated by the accompanying generation of nitric oxide (NO) (Delledonne, 1998, 2001), and evidence has emerged for the involvement of NO in the plant defense response (Durner and Klessig, 1998). NO is a well-recognized signal in animal defense systems and involves redox changes where coupled cGMP-dependent and independent signaling pathways exist. Clarke *et al.* (2000) demonstrated that NO induces cGMP-dependent PCD in *Arabidopsis* (including chromatin condensation and capase-like activity) in an incompatible interaction. A direct role for NO in plant disease resistance has been hypothesized in which NO reacts with ROS to produce $ONOO^-$ (peroxynitrite), which is known to kill mammalian pathogens during phagocytosis (Delledonne *et al.*, 2001).

In *P. syringae*, the expression of genes encoding the exopolysaccharide alginate has not been demonstrated *in planta*. Although alginate is known to be produced by *P. syringae* in host plants, the signals and timing of alginate gene expression have not been described. Previous studies indicated that alginate was the major EPS produced in water-soaked lesions (Fett and Dunn, 1989; Rudolph *et al.*, 1989). Furthermore, alginate contributed significantly to the virulence and epiphytic survival of *P. syringae* pv.

syringae 3525, presumably by facilitating colonization and/or dissemination of the bacterium *in planta* (Yu *et al.*, 1999).

The primary objectives of the current study were to construct an *algD*::*uidA* (*algD*::*gus*) transcriptional fusion and to use this construct to monitor alginate gene expression in host and nonhost plants infected with *P. syringae* pv. tomato DC3000. *algD* expression was monitored in susceptible hosts (tomato and collard), a resistant host (tomato carrying the *Pto* resistance gene), and a nonhost plant (tobacco), which undergoes the HR in response to infection with *P. syringae* pv. tomato.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in **Table 4**. *P. syringae* strains were grown at 28°C on mannitol-glutamate medium (MG) (Keane *et al.*, 1970). *Escherichia coli* strains were grown on Luria-Bertani medium (Miller, 1972) at 37°C. Antibiotics were used at the following concentrations (μg ml^{-1}): kanamycin (Km), 25; ampicillin (Ap), 100; chloramphenicol (Cm), 25: spectinomycin (Sp), 25; and tetracycline, (Tc), 12.5.

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

Strain or plasmids	Relevant characteristics	Reference or source
P. syringae pv. tomato		
DC3000	Alginate producer; pathogenic on tomatoes, crucifers, Arabidopsis	Moore *et al.*, 1989
DC3000-*hrc*	Cm^r; *hrcC*::Tn*5*-Cm	Yuan and He, 1996
Escherichia coli DH5α	$\Delta(lacZYA\text{-}argF)_{u169}$	Sambrook *et al.*, 1989
Plasmids		
pDCalgDP	Cm^r; 1-kb *Hin*dIII/*Pst*I fragment containing the *algD* promoter region in pBBR.GUS	This study
pDCalgDP.Km	Cm^r, Km^r; 1-kb *Hin*dIII/*Pst*I fragment	This study

	containing the *algD* promoter region in pBBR.GUS.Km	
pBBR.Gus	Cm^r; 6.6-kb promoter probe vector containing *uidA* in pBBR1MCS	Peñaloza-Vázquez and Bender, 1998
pBBR.Gus.Km	Cm^r, Km^r: 6.6-kb promoter probe vector containing *uidA* in pBBR1MCS	L. Keith
pCR2.1	Ap^r, Km^r; 3.9-kb cloning vector	Invitrogen
pRK2013	Km^r: helper plasmid	Figurski and Helinski, 1979
pCRalgDP	Ap^r, Km^r, contains *algD* promoter as a 1-kb *Pst*I-*Hin*dIII fragment in pCR2.1	This study
pBRCK1	Ap^r, 8-kb *Bam*HI fragment cloned in pBluescript SK+	This study
pCPP2438	Sp^r, Tc^r; *hrpA::uidA* fusion	Alan Collmer

Molecular genetic techniques

Restriction enzyme digests, agarose gel electrophoresis, PCR, and other routine molecular methods were performed using standard protocols (Sambrook *et al.*, 1989). Plasmids were isolated from *P. syringae* as described by Kado and Liu (1981). Clones were mobilized into recipient strains by using a triparental mating procedure and mobilizer plasmid pRK2013 (Bender *et al.*, 1991). Nucleotide sequencing reactions were performed by the dideoxynucleotide method using Ampli*Taq* DNA polymerase and the ABI PRISM Dye Primer Cycle Sequencing Kit (Perkin Elmer, Foster City, Calif.). The synthesis of oligonucleotide primers and automated DNA sequencing were provided by the Recombinant DNA/Protein Resource Facility at Oklahoma State University. Sequence data was aligned and homology searches were executed using the National Center for Biotechnology Information Blast Network server.

Construction of an *algD*::*uidA* transcriptional fusion

The *algD* promoter in *P. syringae* pv. syringae FF5 was previously defined by deletion analysis (Peñaloza-Vázquez *et al.* 1997). The corresponding region in *P.*

syringae pv. tomato DC3000 showed 81.6% nucleotide identity with the *algD* promoter of FF5 (Chapter III). Therefore, the strategy used for cloning the DC3000 *algD* promoter was based on prior results with FF5, since these two strains were highly homologous in their *algD* upstream regions.

pBBR.Gus, which contains a promoterless glucuronidase gene (*uidA*) downstream of the polylinker in pBBR1MCS, was used to create an *algD::uidA* transcriptional fusion. To obtain the *algD* promoter region in the transcriptionally active orientation, a 1053 bp PCR product was cloned into the *Hin*dIII/*Pst*I sites of pBBR.Gus. The promoter region was amplified from pBRCK1 by using the forward primer 5'-CGGAAAGCTTTAAACCAGTTCGATG (the *Hin*dIII site is underscored) and the reverse primer 5'-GGCGACGTCCCATTGTTGATCAAGTC (*Pst*I site is underscored). After amplification of the 1053 bp PCR product, cloning into pCR2.1, and transformation into *E. coli* DH5α, plasmid pCRalgDP was recovered. The presence of the *algD* promoter region in pCRalgDP was confirmed by sequence analysis using the T7 and M13 oligonucleotide primers. pCRalgDP was then digested with *Hin*dIII and *Pst*I and ligated into pBBR.Gus, resulting in pDCalgDP.

Quantitative GUS assays

Liquid cultures of DC3000(pBBR.Gus) and DC3000(pDCalgDP) were incubated at 28°C in MG broth supplemented with 25 μg ml^{-1} chloramphenicol. Each strain carrying an individual construct was inoculated to triplicate aliquots of medium (100 ml of MG broth) and incubated at 28°C. At different time points (0, 2, 4, 6, 8, 12, 24 and 48 h), a 1-ml aliquot of bacterial cells was removed from each tube and assayed for GUS

activity as described previously (Peñaloza-Vázquez and Bender, 1998). An additional 3 ml of culture was removed at the same time and the OD_{600} was determined. The protein concentration of cell lysates was determined using the Bio-Rad (Richmond, CA) protein assay kit, following the manufacturers protocol. GUS activity was expressed in units (U) per mg of protein, with 1 U equivalent to 1 nmol of methylumbelliferone formed min^{-1}.

Plant growth and inoculation procedures

Tomato (*Lycopersium esculentum* cv. 'Glamour') and collard (*Brassica oleracea* var. *viridis* L. cv. 'Vates') seedlings were maintained in a growth chamber at 24-25°C, 30-40% relative humidity (RH), with a photoperiod of 12 h. Plants were maintained at ≥ 90% RH for 48 h before inoculation. *P. syringae* pv. tomato DC3000(pBBR.Gus) and *P. syringae* pv. tomato DC3000(pDCalgDP) were grown for 48 h on MG agar supplemented with 25 μg ml^{-1} chloramphenicol at 28°C, and cells were resuspended to an OD_{600}=0.3 (~5 x 10^8 cfu ml^{-1}) in sterile distilled water. Silwet L77 (Osi Specialties Inc., Danbury, CT) was added to bacterial inoculum at a concentration of 0.2 μg ml^{-1}. Six-week old plants were spray-inoculated with an airbrush (~8 psi) until leaf surfaces were uniformly wet. After inoculation, tomato and collard plants were incubated at 24°C, 60% RH with a 12 h photoperiod for the duration of the experiment.

Tomato (*Lycopersium esculentum* cv. 'Rio Grande' and Rio Grande with the *Pto* transgene) and tobacco *(Nicotiana tabacum* cv. 'Petite Havana') were either spray-inoculated (OD_{600}=0.3) or infiltrated (OD_{600}=1.0) with selected bacterial strains using established methods (Schaad, 1988).

Histochemical detection of glucuronidase activity

Tomato and collard leaves were sampled at 0, 24, 48, 72, 120 and 168 h post-inoculation and vacuum-infiltrated with a substrate surfactant solution (5-bromo-4-chloro-3-indoyl β-D-glucuronide, 0.5 mg ml^{-1}, and L77 at 0.2 µl ml^{-1} in 50 mM sodium phosphate buffer, pH 7.0). Infiltrated leaves were incubated at 37°C overnight and fixed and decolorized in 80% ethanol at 37°C (Hugouvieux *et al.*, 1988).

Samples from infiltrated tomato and tobacco were collected hourly by removing leaf samples (1 cm in diameter) with a sterile cork borer. Samples were vacuum infiltrated, incubated and destained as described above.

Determination of bacterial growth *in planta*

In the experiments designed to follow the total population of the DC3000 strains, random leaf samples were taken at 0, 24, 48, 72, 120 and 168 h after inoculation. Each leaf was weighed separately (3 replicates per time point) and macerated in 2 ml (tomato) or 5 ml (collard) sterile distilled H_2O. 500 µl of leaf homogenate was removed and stored at –80°C for protein determination and GUS activity. Bacterial counts were determined by plating dilutions of the leaf homogenate onto MG agar supplemented with 25 µg ml^{-1} chloramphenicol. Colonies were counted after incubating the plates 48-96 h, and the experiment was then repeated.

RESULTS

GUS assays *in vitro*

GUS activity of DC3000 containing DC3000(pBBR.Gus) and DC3000

(pDCalgDP) was measured at 0, 12, 24, 36, and 48 hours post inoculation (hpi) to MG broth. GUS activity increased approximately two-fold in DC3000(pDCalgDP) during the 48 h sampling period, whereas GUS activity in DC3000(pBBR.Gus) remained negligible. These results confirmed that the *algD* promoter was active in DC3000(pDCalgDP), and the level of expression *in vitro* was consistent with values obtained for other *algD*::*uidA* fusions (Fakhr *et al.*, 1999; Peñaloza-Vázquez *et al.* 1997).

algD expression in tomato cv. 'Glamour'

Histochemical staining was used to study expression of the *algD* promoter *in planta*. Conceptually, if the *algD* promoter is activated, β-glucuronidase will be produced, and leaves incubated with the chromogenic substance X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) will stain blue where the bacteria are located. When DC3000(pBBR.Gus) and DC3000(pDCalgDP) were inoculated to tomato plants, necrotic lesions were visible using 5X magnification at 48 hpi and to the unaided eye at 96 hpi (data not shown). On tomato leaves infected with DC3000(pDCalgDP) and stained for glucuronidase activity, small blue spots were visible 48 hpi using 5X magnification (**Figure 13A**). The intensity of staining was more pronounced at 96 hpi, indicating that the *algD* promoter was active at the onset of visible lesion development (**Figure 13B**).

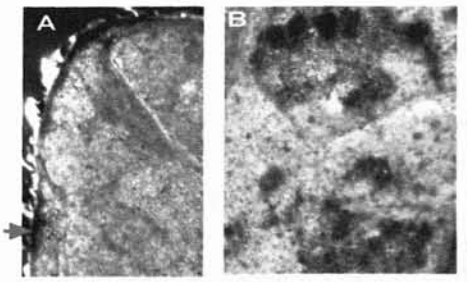

Figure 13. *P. syringae* pv. tomato DC3000 (pDCalgDP) lesions on tomato cv. 'Glamour'. Tissue was stained for glucuronidase activity as described in methods. (**A**) X-gluc stained lesions first appear at 48 hpi near the hydathodes (red arrow, 5X magnification), and (**B**) subsequently appear throughout the leaf (96 hpi, 31.5X magnification).

Both DC3000(pBBR.Gus) and DC3000(pDCalgDP) grew equally well in tomato var. Glamour and obtained a population of 5 x 10^9 cfu per gram in 72 h (**Figure 17**). This indicates that neither pBBR.Gus nor pDCalgDP negatively impacted the growth of DC3000 *in planta*.

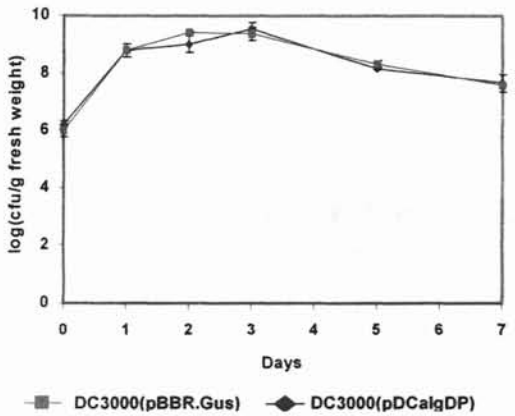

Figure 17. Growth of *P. syringae* pv. tomato DC3000(pDCalgDP) and *P. syringae* pv. tomato DC3000(pBBR.Gus) on tomato cv. Glamour.

algD expression in collard cv. Vates

In collard leaves inoculated with DC3000(pBBR.Gus) and DC3000(pDCalgDP), lesions were visible at 72 hpi and chlorosis and necrosis were evident beginning at 120 hpi (**Figure 18A**). When collards inoculated with DC3000(pDCalgDP) were stained for GUS activity, maximal activity of the *algD* promoter was coincided with the appearance of spreading necrotic lesions (**Figure 18A, B**).

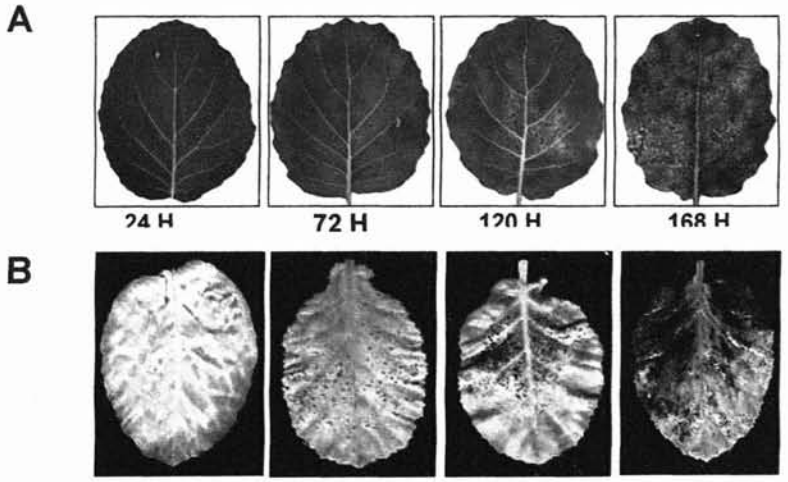

Figure 18. Symptom development (**A**) and detection of GUS activity (**B**) in collard leaves inoculated with *P. syringae* pv. tomato DC3000 pDCalgDP). GUS activity coincided with the onset of visible lesions (72 hpi).

At 72 hpi, collard leaves inoculated with DC3000(pDCalgDP) and DC3000(pBBR.Gus) developed water-soaked lesions (**Figure 19A, E**). When collard leaves inoculated with DC3000(pDCalgDP) were stained for GUS activity, the water-soaked lesions stained blue (**Figure 19D**), indicating that *algD* expression occurred within the water-soaked lesion. A closer inspection of the leaves incubated with X-gluc indicated that the zone of histochemical staining was most intense in the center of water-soaked lesions and more diffuse in the perimeter (**Figure 19D**).

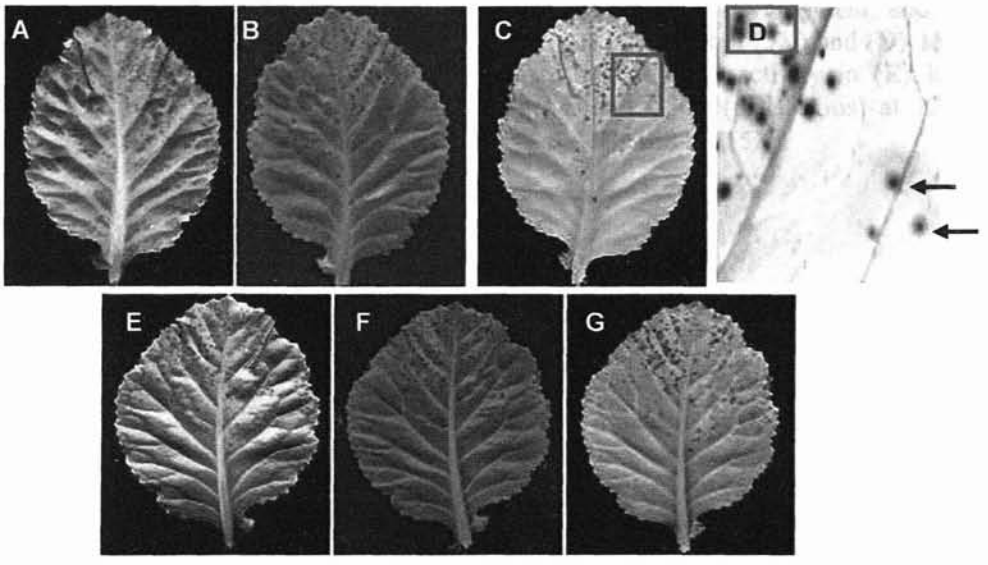

Figure 19. Collard leaves inoculated with DC3000(pDCalgDP) and photographed 72 hpi. Panels **A-D** show the lower surface of a single leaf, whereas panels **E-G** show the upper surface. Panels **A** and **E** show symptoms, panels **B** and **F** show X-gluc staining prior to clearing, and panels **C** and **G** show the same leaf after X-gluc treatment and clearing. In panel **D**, the red rectangle shown in panel **C** is enlarged. Arrows indicate individual water-soaked lesions that stain intensively with X-gluc.

Microscopic examination of collard leaves inoculated with DC3000(pDCalgDP) indicated that lesion development and staining became more intense 72-96 hpi (**Figure 20A, B**). Collard leaves inoculated with DC3000(pBBR.Gus) did not stain when treated with X-gluc, regardless of the time following inoculation (**Figure 20E, F**). Both

DC3000(pBBR.Gus) and DC3000(pDCalgDP) grew equally well in collard reaching a population of 5 x 10^9 cfu per gram in 120 h (**Figure 21**); this indicates that the constructs pBBR.Gus and pDCalgDP did not negatively impact growth of DC3000 *in planta*.

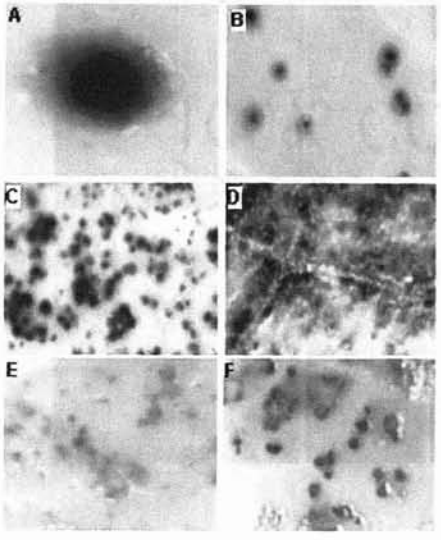

Figure 20. Histochemical staining for GUS activity in collard leaves inoculated with *P. syringae* pv. tomato DC3000(pDCalgDP) (**A-D**) and DC3000(pBBR.Gus) (**E-F**). GUS activity in water-soaked lesions at (**A**) 72 hpi (31.5X magnification) and (**B**) 96 hpi (5X). Widespread lesion development and GUS activity at (**C**) 120 hpi (5X) and (**D**) 168 hpi (5X). Lack of GUS activity in (**E**) lesions induced by DC3000(pBBR.Gus) at 120 hpi (5X) and (**F**) 168 hpi (5X).

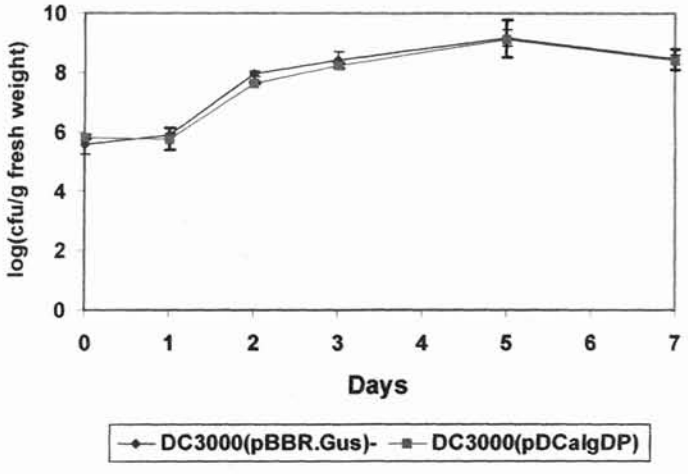

Figure 21. Growth of *P. syringae* pv. tomato DC3000(pDCalgDP) and *P. syringae* pv. tomato(pBBR. Gus) in collard leaves.

Compatible and incompatible reactions on tomato cv. Rio Grande

Tomato cv. Rio Grande was inoculated with DC3000(pBBR.Gus) and DC3000(pDCalgDP) as described for cv. Glamour. The results obtained with the susceptible cv. Rio Grande were similar to those obtained with Glamour. Necrotic lesions

were first visible at 96 hpi (**Figure 22A**), and those induced by DC3000(pDCalgDP) exhibited dark blue necrotic centers when stained with X-gluc (**Figure 22B**). Lesions induced by DC3000(pBBR.Gus) showed no evidence of GUS activity (data not shown).

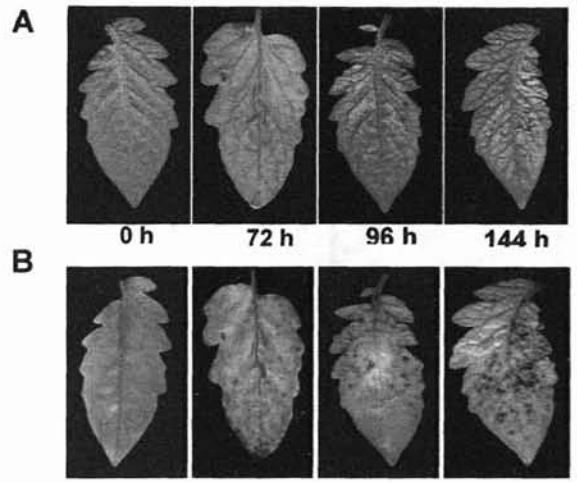

Figure 22. Compatible response of tomato cv. Rio Grande inoculated with *P. syringae* pv. tomato DC3000 (pDCalgDP). (**A**), Symptom development at various times post inoculation. (**B**), Histochemical staining of leaves shown in panel A for GUS activity.

When the resistant cv. Rio Grande containing *Pto* was spray-inoculated with DC3000(pDCalgDP) and DC3000(pBBR.Gus), no visible symptoms were apparent from 0-168 hpi (**Figure 23A**). However, upon removal of chlorophyll, the leaf tissue became increasingly brown, indicating some cellular damage (**Figure 23B**).

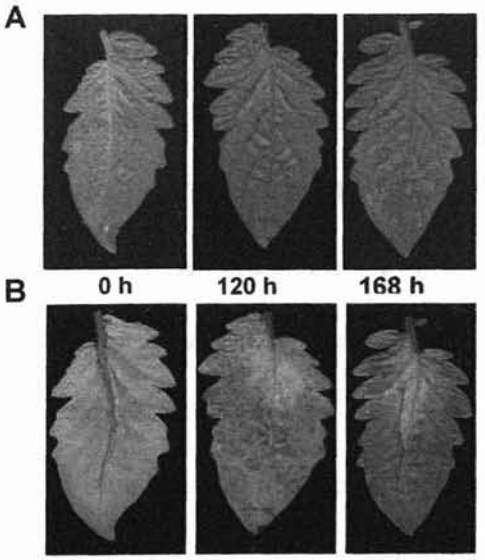

Figure 23. Incompatible response of tomato cv. Rio Grande *Pto* inoculated with *P. syringae* pv. tomato DC3000(pDCalgDP). (**A**), Symptom development at various times post inoculation. (**B**), Histochemical staining of leaves shown in panel A for GUS activity.

The formation of a 'micro HR' during the incompatible reaction between Rio Grande *Pto* and *P. syringae* pv. tomato DC3000(pDCalgDP) was evident on leaves stained for GUS activity beginning 24 hpi (**Figure 24A**). Small, microscopic blue lesions increased in number at 48 hpi and became most obvious 72 hpi (**Figure 24B, C**).

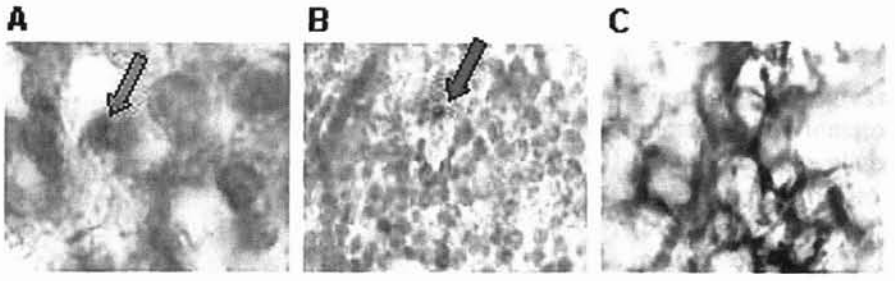

Figure 24. Infection of tomato cv. Rio Grande *Pto* with *P. syringae* pv. tomato DC3000(pDCalgDP). Tissue was stained for GUS activity as described in methods. GUS activity was visible by light microscopy beginning at 24 hpi (panel **A**, blue arrow, 20X magnification), became more obvious at 48 hpi (panel **B**, 10X, red arrow), and reached a maximum intensity at 72 hpi when the HR developed in adjoining cells and limited spread of the bacteria (panel **C**, 20X).

When investigated at higher magnification (500X), the development of the HR and induction of *algD* activity could be visualized near individual stomates. For example, on Rio Grande *Pto* inoculated with DC3000(pDCalgDP), the development of an HR near an invaded stomate was apparent 12 hpi (**Figure 25A**). At 24 hpi, individual bacteria associated with the guard cells stained blue, indicating that the *algD* promoter was active (**Figure 25B**). The HR was more pronounced at 48 hpi, and cells adjacent to the HR stained blue, indicating the presence of bacteria (**Figure 25C**). Interestingly, epiphytic bacteria associated with stomata continued to stain blue at 72 hpi, suggesting that *algD* expression continued during the HR (**Figure 25D**). At 96 and 120 hpi, the intensity of blue staining decreased, while cells undergoing the HR increased in size and could be visualized as small circular lesions at 10X magnification (data not shown). In tomato leaves infected with DC3000(pBBR.Gus), cells undergoing 'micro-HR' were

evident, but did not stain blue due to the absence of an active *uidA* gene. Interestingly, leaves of Rio Grande *Pto* did not show any obvious signs of the HR when examined without the aid of a microscope and remained green and asymptomatic throughout the experiment (**Figure 22A**). The results of this experiment are summarized in **Table 5**.

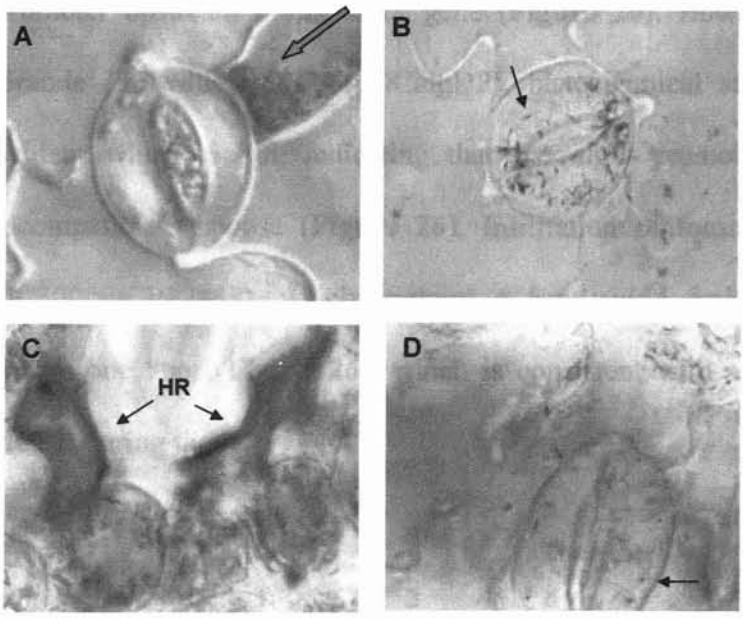

Figure 25. Progression of a micro-HR in tomato cv. Rio Grande *Pto* inoculated with *P. syringae* pv. tomato DC3000 (pDCalgDP), (500X magnification). (**A**) T=12 hpi; cell adjacent to stomate undergoes an HR (blue arrow). (**B**) T=24 hpi; bacteria on guard cells induce *algD* promoter and turn blue (black arrow). (**C**) T=48 hpi; cells undergo HR while the number of cells staining blue increase in number. (**D**) T=72 hpi; cells close to the stomate undergo HR; epiphytic bacteria continue to stain blue (see arrow).

Table 5. Resistant and susceptible reactions on Rio Grande and Rio Grande *Pto* spray-inoculated with *P. syringae* pv. tomato DC3000.

Bacterial strain	Resistant tomato (Rio Grande Pto)	Sensitive tomato (Rio Grande)
DC3000(pDCalgDP)	Micro-HR evident within 24-48 hpi; 'blue' bacteria visualized when tissue is stained with X-gluc; algD promoter 'on' within 24 hpi.	No micro-HR; bacteria initiate disease symptoms; lesions stain blue when tissue is treated with X-gluc; algD promoter 'on' at late stages of infection 96-144 hpi.
DC3000(pBBR.Gus)	Micro-HR evident within 24-48 hpi; bacteria colorless throughout duration of experiment.	No micro-HR; bacteria initiate disease symptoms; necrotic lesions observed.

Infiltration studies

When tomato cv. Rio Grande *Pto* was infiltrated with DC3000(pBBR.Gus) (OD_{600}=1.0), an HR developed within 12 hpi (data not shown). After vacuum infiltration with X-gluc, incubation and destaining, no GUS activity was observed with tomato tissue inoculated with DC3000(pBBR.Gus), which is consistent with the absence of a functional promoter upstream of the *uidA* gene (**Figure 26**). However, after infiltration of Rio Grande *Pto* with DC3000(pDCalgDP), histochemical staining of tomato tissue was evident within 6 hpi, indicating that the *algD* promoter was activated during the incompatible response (**Figure 26**). Infiltration of tomato cv. Rio Grande *Pto* with DC3000(pCPP2438), which contains a *hrpA::uidA* fusion, resulted in GUS activity within one hour (**Figure 26**), which is consistent with activation of the *hrp/hrc* gene cluster during the HR.

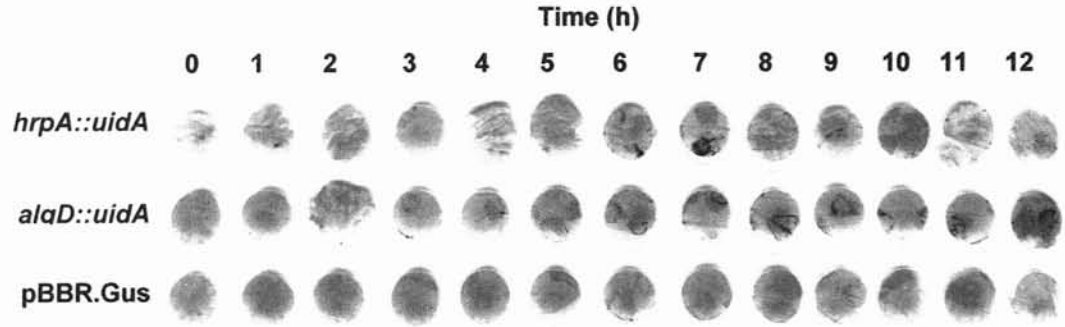

Figure 26. GUS activity in tomato cv. Rio Grande *Pto* (resistant to *P. syringae* pv. tomato DC3000) infiltrated with DC3000(pCPP2438), DC3000(pDCalgDP), and DC3000(pBBR.Gus), which contain *hrpA*::*uidA*, *algD*::*uidA*, and a promoterless *uidA* gene, respectively. Time is shown in hours (h) post-infiltration.

A similar pattern of histochemical staining was observed when the susceptible tomato cv. Rio Grande was infiltrated with DC3000(pCPP2438), DC3000(pDCalgDP),

and DC3000(pBBR.Gus). The *hrpA*::*uidA* fusion was activated within 3 hpi (**Figure 27**) and the intensity of staining decreased from 10-12 hpi. Activation of the *algD* promoter was evident at 4 hpi and continued to remain active at 12 hpi (**Figure 27**). No histochemical staining was evident when tomato tissue was inoculated with DC3000(pBBR.Gus) (**Figure 27**). These results indicate that the *hrpA*::*uidA* and *algD*::*uidA* fusions are activated in both incompatible and compatible reactions when infiltrated into tomato tissue (**Figures 26 and 27**). However, the two fusions differ in both the timing and the kinetics of induction after infiltration. For example, *hrpA*::*uidA* is activated rapidly after infiltration (3-7 hpi) and then expression decreases; whereas *algD*::*uidA* is activated after *hrpA*, and expression is more prolonged.

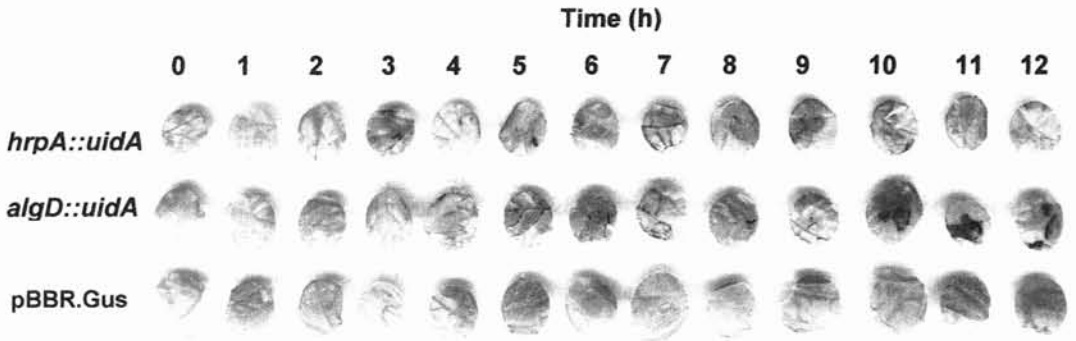

Figure 27. GUS activity in tomato cv. Rio Grande (susceptible to *P. syringae* pv. tomato DC3000) infiltrated with DC3000(pCPP2438), DC3000(pDCalgDP), and DC3000(pBBR.Gus), which contain *hrpA*::*uidA*, *algD*::*uidA*, and a promoterless *uidA* gene, respectively. Time is shown in hours (h) post-infiltration.

When tobacco cv. Petite Havana was infiltrated with DC3000(pCPP2438), DC3000(pDCalgDP), and DC3000(pBBR.Gus), an HR was visualized within 12 hpi (data not shown). After vacuum infiltration with X-gluc, incubation and destaining, a high level of GUS activity was observed with tobacco tissue inoculated with

DC3000(pCPP2438) (**Figure 28A**), which is consistent with a high level of *hrpA* activity during the HR in tobacco. Activation of the *algD*::*uidA* fusion was first observed at 4-6 hpi, and expression continued throughout the sampling period (**Figure 28A**).

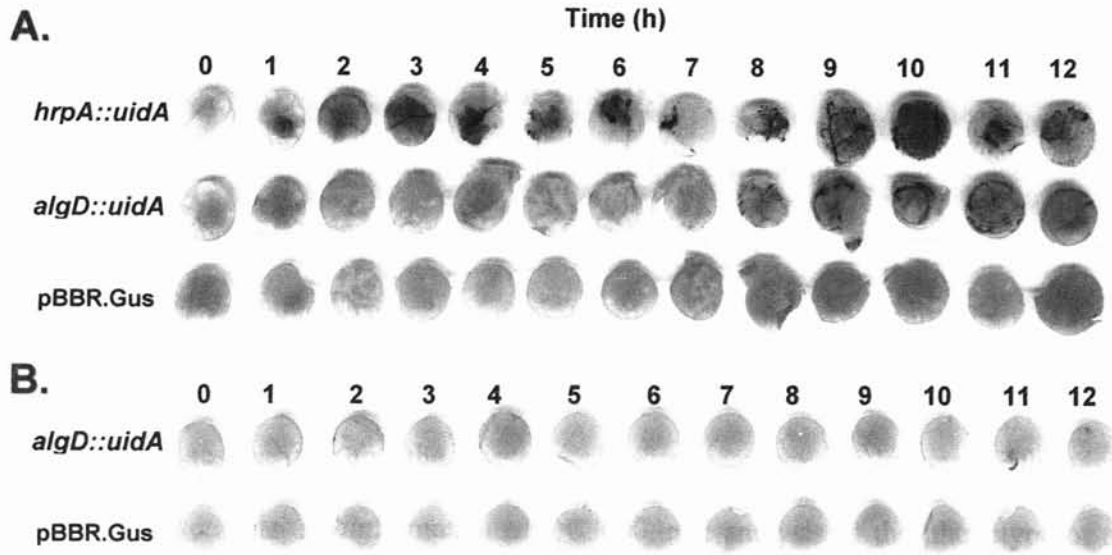

Figure 28. GUS activity in tobacco cv. Petite Havana. (**A**) Tobacco leaves infiltrated with DC3000(pCPP2438), DC3000(pDCalgDP), and DC3000(pBBR.Gus), which contain *hrpA*::*uidA*, *algD*::*uidA*, and a promoterless *uidA* gene, respectively. (**B**) Tobacco leaves infiltrated with DC3000-*hrcC*(pDCalgDP), and DC3000-*hrcC*(pBBR.Gus). Time is shown in hours (h) post-infiltration.

Tobacco leaves were also infiltrated with DC3000-*hrcC* containing the *algD*::*uidA* fusion (pDCalgDP) and the promoterless control (pBBR.Gus). Unlike the wild-type DC3000, no HR was elicited with DC3000-*hrcC* containing these constructs (data not shown). A very low level of GUS activity was visualized beginning at 2 hpi in leaves infiltrated with D3000-*hrcC*(pDCalgDP) (**Figure 28B**). When compared to DC3000(pDCalgDP), *algD* promoter activity in tobacco inoculated with DC3000-*hrcC*(pDCalgDP) remained consistently lower throughout the sampling period (**Figure**

28A, B). This suggests that the *algD* promoter is activated by signals generated during the HR, such as reactive oxygen intermediates, and these signals are absent or reduced when the HR is not elicited. No GUS activity was detected in tobacco inoculated with DC3000-*hrcC*(pBBR,Gus), regardless of the sampling period (**Figure 28B**).

DISCUSSION

The gene expression studies *in planta* clearly show that *algD* is activated in collard leaves within 48-72 hpi (**Figure 20A**). Induction of the *algD* promoter in collard was associated with the development of water-soaked lesions, which also showed GUS activity (**Figure 19D**). These results suggest that alginate is produced in collard leaves and may be responsible for the 'water-soaked' appearance of lesions. Efforts are underway to generate an *algD* mutant of DC3000, and analysis of the phenotype of an alginate-defective mutant will resolve whether alginate is responsible for the water-soaked appearance of lesions on collard.

Tomato is also a host for *P. syringae* pv. tomato DC3000, but symptom severity is generally less on tomato than on cruciferous crops (Zhou and Bender, unpublished data). In tomato, *algD* activity was evident at 48 hpi, but was localized to the hydathodes (**Figure 13A**), which serve as points of entry for the pathogen. Necrotic lesions developed on susceptible tomatoes at 96 hpi, and these lesions were smaller than those induced by DC3000 on collard and lacked water-soaking. Although histochemical staining indicated that the *algD* promoter was active in the tomato lesions, the level of activity was lower than that observed in collard leaves (compare **Figures 14B** and **18B**). In general, we have found that *Brassica* spp. are more susceptible to infection by

DC3000, and symptom severity is much more pronounced than that observed in tomato. We suspect that alginate plays a much more important role in the pathogenicity of DC3000 on collard than on tomato, and future studies will address this hypothesis using a genetic approach.

This study demonstrates that resistant tomatoes containing the *Pto* transgene exhibit a 'micro-HR' when challenged with *P. syringae* pv. tomato DC3000. *algD* expression was stimulated during the micro-HR and presumably helps protect the bacteria from host defense mechanisms, such as reactive oxygen intermediates. This is a 'last ditch' attempt at survival, which may provide a small amount of protection to bacterial cells. Repeating this experiment with an alginate-defective mutant and following viability during the HR would confirm whether alginate production provides some protection during PCD.

The oxidative burst is an early, localized defense response that involves the production of potentially cytotoxic quantities of H_2O_2 and $O_2^{\cdot}$ (Legendre *et al.*, 1993). In plants, the oxidative burst generally occurs in two distinct phases (Levine *et al.*, 1994). The initial rapid phase ends within an hour of its initiation (shortly after bacteria enter the host) and is followed by a second slower burst that may last 3-6 h (Baker and Orlandi, 1995). The second burst was initially shown to occur during incompatible reactions (Levine *et al.*, 1994). For example, Chandra *et al.* (1996) used *P. syringae* pv. tomato containing *avrPto* to demonstrate that the second phase of oxidative burst is dependent on co-expression of *Pto* in the tomato and *avrPto* in the pathogen. The induction of the *algD* promoter after infiltration of DC3000(pDCalgDP) into tobacco and resistant tomato shows that these artificial methods of inoculation remove a natural barrier and disrupt the

natural timing of disease and the HR. The infiltration of 10^9 cells directly into the apoplast induced *hrpA* and *algD* gene expression within 1-6 hpi in both compatible (Rio Grande) and incompatible (Rio Grande *Pto*) interactions. When a tomato plant is inoculated with a pathogen by spraying, bacteria are dispersed uniformly over the leaf surface. Within the first 12 hours, bacteria enter stomata and trigger the first oxidative burst. This oxidative burst, although not lethal to the bacteria, may function as a signal to trigger alginate production. Further invasion and colonization of the apoplast then occurs. In the resistant cv. Rio Grande *Pto*, a second oxidative burst may occur within 48-72 hpi in cells undergoing the HR. In the sensitive cv, Rio Grande, the second oxidative burst is either absent or occurs at sublethal levels, which do not impact multiplication of the bacteria.

In resistant tomato, alginate may function primarily as a defensive factor for *P. syringae* pv. tomato. However, in susceptible interactions, especially in those where water-soaked lesions occur (e.g. collard leaves), alginate may have an offensive role. The production of the highly hydrophilic alginate in the apoplast could facilitate dissemination of the bacteria by exerting pressure on plant cells, especially if the pressure is sufficient to propel the bacteria through the apoplast to more distant sites. A similar role has been suggested for the offensive nature of the EPS produced by *Ralstonia solanacearum* (Saile *et al.*, 1997).

In tobacco, *Erwinia amylovora* induced a sustained production of O_2^-, lipid peroxidation, electrolyte leakage, and concomitant increases of several antioxidative enzymes (Venisse *et al.*, 2001). In contrast, the compatible pathogen *P. syringae* pv. tabaci did not induce these reactions (Venisse *et al.*, 2001). In pear leaves, inoculations

with both compatible and incompatible bacteria resulted in superoxide accumulation, lipid peroxidation and electrolyte leakage (Venisse *et al.*, 2001). The ability of *E. amylovora* to generate an oxidative stress in a compatible interaction was linked to a functional *hrp* cluster because a *hrp* mutant did not induce these responses (Venisse *et al.*, 2001). It was suggested that *E. amylovora* uses the production of ROS as a tool to provoke host cell death during pathogenesis, which facilitates the invasion of plant tissue (Venisse *et al.*, 2001). Bacterial exopolysaccharide (EPS) was hypothesized to protect *E. amylovora* from the toxicity of ROS since a non-capsular mutant promoted the formation of ROS but was unable to further colonize the plant (Venisse *et al.*, 2001). These results are highly relevant to the current study since *algD* activity was substantially reduced on tobacco leaves infiltrated with the *hrp* mutant, DC3000-*hrcC* as compared to the wild-type DC3000 (**Figure 28A, B**). This suggests that the signals generated during the HR directly stimulate activity of the *algD* promoter and alginate production. Future studies designed to identify the specific reactive oxygen species that trigger *algD* expression are underway.

BIBLIOGRAPHY

Alfano, J. R., A. O. Charkowski, W.-L. Deng, J. L. Badel, T. Petnick-Ocwieja, K. van Dijk, and A. Collmer. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. USA* **97**: 4856-4861.

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

Agrios, N., 1988. Plant diseases caused by prokaryotes. *In* Plant Pathology. Vol III. Agrios. N. (ed.). Academic Press, San Diego, CA, pp. 510-609.

Anwar, H. J., L. Strap. and J. W. Costerton. 1992. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob. Agents Chemother.* **36**: 1347-1351.

Atkinson, M. M., and C. J. Baker. 1989. Role of the plasmalemma H^+-ATPase in *Pseudomonas syringae*-induced K^+/H^+ exchange in suspension-cultured tobacco cells. *Plant Physiol.* **91**: 298-303.

Baltimore, R. S., and M. Mitchell. 1980. Immunological investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. *J. Infect. Dis.* **141**: 238-247.

Baker, J. C., and E. W. Orlandi. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopath.* **33**: 299-321.

Baldi, P., S. Brunak, P. Frasconi, G. Pollastri, and G. Soda. 1999. Exploiting the past and the future in protein secondary structure prediction. *Bioinformatics* **15**: 937-946.

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

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

Bender, C. L. and D. A. Cooksey. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. tomato: conjugative transfer and role in copper resistance. *J. Bacteriol.* **165**: 534-541.

Bender, C. L., and D. A. Cooksey. 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. tomato. *J. Bacteriol.* **169**: 470-474.

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

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

Bender, J. G., A. L. Florman, and D. E. van Epps. 1987a. Correlation of serum opsonic activity in cystic fibrosis with colonization and disease state measurement of opsonins to *Pseudomonas aeruginosa* by neutrophil superoxide anion generation. *Pediatr. Res.* **22**: 383-388.

Bender, C. L., H. E. Stone, J. J. Sims, and D. A. Cooksey. 1987b . Reduced pathogen fitness of *Pseudomonas syringae* pv. *tomato* Tn*5* mutants defective in coronatine production. *Physiol. Mol. Plant Pathol.* **30**: 272-283.

Bestwick, C. S., I. R. Brown, and J. W. Mansfield. 1998. Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. *Plant Physiol.* **118**: 1067-1078.

Boch, J., V. Joardar, L. Gao, T. L. Robertson, M. Lim, and B. N. Kunkel. 2002. Identification of *Pseudomonas syringae* genes that are induced during infection of *Arabidopsis thaliana*. *Mol. Microbiol. in press.*

Bolwell, G P., and P. Wojtaszek. 1997. Mechanisms for the generation of reactive oxygen species in plant defense: a broad perspective. *Physiol. Mol. Plant Path.* **51**: 347-366.

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. *In* Current topics in microbiology and immunology, Vol. 192: Bacterial pathogenesis of plants and animals-Molecular and cellular mechanisms. J. L. Dangl, (ed.) (Berlin: Springer-Verlag), pp. 79-98.

Boucher, J. C., J. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic. 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. *J. Bacteriol.* **178:** 511-532.

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

Boyes, D. C., J. Nam, and J. L. Dangl. 1998. The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. *Proc. Natl. Acad. Sci.* USA **95**: 15849-15854.

Campos, M.-E., J. M. Martínez-Salazar, L. Lloret, S. Moreno, C. Núñez, G. Espín, and G. Soberón-Chávez. 1996. Characterization of the gene coding for GDP-mannose dehydrogenase (*algD*) from *Azotobacter vinelandii*. *J. Bacteriol.* **178**: 1793-1799.

Cha, J.-S., and D. A. Cooksey. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc. Natl. Acad. Sci. USA* **88**: 8915-8919.

Chandra, S., G. B. Martin, and P. S. Low. 1996. The Pto kinase mediates a signaling pathway leading to the oxidative burst in tomato. *Proc. Natl. Acad. Sci. USA* **93**: 13393-13397.

Charkowski, A. O., H. C. Huang, and A. Collmer. 1997. Altered localization of HrpZ in *Pseudomonas syringae* pv. syringae *hrp* mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of gram-negative bacteria. *J. Bacteriol.* **179**: 3866-3874.

Charles, T. C., S. Jin, and E. W. Nester. 1992. Two-component sensory transduction systems in phytobacteria. *Annu. Rev. Phytopathol.* **30**: 463-484.

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

Clare, K. 1993. Algin. *In* Industrial Gums. Whistler, R. L., and J. N. BeMiller, (eds.). Academic Press, San Diego, CA, pp. 105-143.

Clarke, A., R. Desikan, R. D. Hurst, J. T. Hancock, and S. J. Neill. 2000. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J.* **24**: 667-677.

Clough, S. J., M. A. Schell, and T. P. Denny. 1994. Evidence for involvement of a volatile extracellular factor in *Pseudomonas solanacearum* virulence gene expression. *Mol. Plant-Microbe Interact.* **7**: 621-630.

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

Cooksey, D. 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. tomato. *Appl. Environ. Microbiol.* **53**: 454-456.

Costacurta, A., and J. Vanderleyden. 1995. Synthesis of phytohormones by plant-associated bacteria. *Crit. Rev. Microbiol.* **21**: 1-18.

Cui, Y., Mukherjee, A., Dumenyo, C. K., Liu, Y., and A. K. Chatterjee. 1999. *rsmC* of the soft-rotting bacterium *Erwinia carotovora* subsp. carotovora negatively controls extracellular enzyme and harpin(Ecc) production and virulence by modulating levels of regulatory RNA (*rsmB*) and RNA-binding protein (RsmA). *J. Bacteriol.* **18**: 6042-52.

Daley, L., G. B. Pier, J. D. Liporace, and D. D. Eardley. 1985. Polyclonal B cell stimulation and interleukin 1 induction by the mucoid exopolysaccharide of *Pseudomonas aeruginosa* associated with cystic fibrosis. *J. Immunol.* **134**: 3089-3093.

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

Delic-Attree, I., B. Toussaint, J. Garin, and P. M. Vignais. 1997. Cloning, sequence and mutagenesis of the structural gene of *Pseudomonas aeruginosa* CysB, which can activate *algD* transcription. *Mol. Microbiol.* **24**: 1275-1284.

Delledonne, M, Y. Xia, R. A. Dixon, and C. Lamb. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**: 585-588.

Delledonne, M., J. Zeier, A. Marocco, and C. Lamb. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl. Acad. Sci. USA.* **98**: 13454-13459.

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

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

Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin. 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress regulation of bacterial virulence by alternate sigma factors. *J. Bacteriol.* **176**: 2773-2780.

Devault, J. D., W. Hendrickson, J. Kato, and A. M. Chakrabarty. 1991. Environmentally regulated *algD* promoter is responsive to the cAMP receptor protein in *Escherichia coli. Mol. Microbiol.* **5**: 2503-2509.

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

DeVries, C. A., and D. E. Ohman. 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*,

encoding a putative alternative sigma factor, and shows evidence for autoregulation. *J. Bacteriol.* **176**: 6677-6687.

Doke, N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissue to infection with an incompatible race of *Phytophthora infestans* and to the hypha wall components. *Physiol. Plant Path.* **23**: 345-357.

Dong, Y.-H., J.-L. Xu, X.-Z. Li, and L.-H. Zhang. 1999. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. USA* **97**: 3526-3531.

Dougherty, B. A., and I. Van de Rijn. 1993. Molecular characterization of *hasB* from an operon required for hyaluronic acid synthesis in group A streptococci. Demonstration of UDP-glucose dehydrogenase activity. *J. Biol. Chem.* **268**: 7118-7124.

Dow, J. M., and M. J. Daniels. 1994. Pathogenicity determinants and global regulation of pathogenicity of *Xanthomonas campestris* pv. campestris. *In* Current topics in microbiology and immunology, vol. 192: Bacterial pathogenesis of plants and animals-Molecular and cellular mechanisms. J. L. Dangl, ed. (Berlin: Springer-Verlag), pp. 29-41.

Dudman, W. F. 1997. The role of surface polysaccharides in natural environments. *In* Surface carbohydrates of the prokaryotic cell. I. W. Sutherland (ed.). Academic Press, New York. pp. 357-414.

Durner, J., D. Wendehenne, and D. F. Klessig. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. USA* **95**: 10328-10333.

Ebel, J., and D. Scheel. 1997. Signals in host-parasite interactions. In: G. C. Carroll and P. Tudzynski (Eds.) The Mycota Vol V. Plant Relationships Part A, Springer-Verlag, Berlin/Heidelberg, pp. 85-105.

Ebrahim-Nesbat, F. and A. J. Slusarenko. 1983. Ultrastructure of the interaction of cells of *Pseudomonas phaseolicola* with cell walls of a resistant and susceptible bean cultivar. *Phytopathol. Z.* **108**:148-159.

Fahy, P. C., and A. B. Lloyd. 1983. *Pseudomonas*: The fluorescent pseudomonads. *In* Plant bacterial diseases: a diagnostic guide. Fahy, P. C., and G. J. Persley (eds). Sydney-Academic Press, pp. 141-188.

Fakhr, M. K., A. Peñaloza-Vázquez, A. M. Chakrabarty, and C. L. Bender. 1999. Regulation of alginate biosynthesis in *Pseudomonas syringae* pv. syringae. *J. Bacteriol.* **181**: 2478-3485.

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

Fett, W. F., S. F. Osman, M. L. Fishman, and T. S. Siebles III. 1986. Alginate production by plant-pathogenic Pseudomonads. *J. Bacteriol.* **52**: 466-473.

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

Fialho, A. M., N. A. Zielinski, W. F. Fett, A. M. Chakrabarty, and A. Berry. 1989. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-*Azomonas-Azotobacter* lineage of superfamily b prokaryotes. *Appl. Environ. Microbiol.* **56**: 436-443.

Flavier, A. B., L. M. Ganova-Raeva, M. A. Schell, and T. P. Denny. 1997. Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J. Bacteriol.* **179**: 7089-97.

Flor, H. H. 1956. The complementary genic systems in flax and flax rust. *Adv. Genet.* **8**: 29-54.

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

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

Fuqua, C., and S. C. Winans. 1996. Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. *J. Bacteriol.* **178**: 435-40.

Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1993. Quorum sensing in bacteria. The LuxI/LuxR family of cell density responsive transcriptional regulators. *J. Bacteriol.* **176**: 269-275.

Galán, J. E., and A. C. Collmer. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**: 1322-1328.

Glickmann, E., L. Gardan, S. Jacquet, S. Hussain, M. Elasri, A. Petit, and Y. Dessaux. 1998. Auxin production is a common feature of most pathovars of *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* **11**: 156-162.

Goldberg, J. B., W. L. Gorman, J. L. Flynn, and D. E. Ohman. 1993. A mutation in *algN* permits *trans* activation of alginate production by *algT* in *Pseudomonas* species. *J. Bacteriol.* **175**: 1303-1308.

Goldberg, J. H. and D. E. Ohman. 1987. Construction and characterization of *Pseudomonas aeruginosa algB* mutants: role of *algB* in high-level production of alginate. *J. Bacteriol.* **169**: 1593-1602.

Goodman, R. N., J. S. Huang, and P. Y. Huang. 1974. Host specific phytotoxic polysaccharide from apple tissue infected by *Erwinia amylovora*. *Science* **183**: 1081-1082.

Goodman, R. N., and A. J. Novacky. 1994. The Hypersensitive Reaction in Plants to Pathogens. APS Press, St. Paul, MN.

Gopalan, S., D. W. Bauer, J. R. Alfano, A. O. Loniello, S. Y. He, and A. Collmer. 1996. Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**: 1095-1105.

Govan, J. R. W. 1988. Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis. pp. 67-96. *In* E. Griffiths, W. Donachie, and J. Stephen (eds.), Bacterial infections of respiratory and gastrointestinal mucosae. IRL Press. Oxford.

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

Govan, J. R. and G. S. Harris. 1986. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiol. Sci.* **3**: 302-8.

Gross, M., and K. Rudolph. 1987. Studies on the extracellular polysaccharides (EPS) produced in vitro by *Pseudomonas phaseolicola*. I. Indications for a polysaccharide resembling alginic acid in seven *P. syringae* pathovars. *J. Phytopathol.* **118**: 276-287.

Guex, N and M. C. Peitsch 1997. SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis* **18**: 2714-2723.

Halliwell, B., and J. M. Gutteridge. 1989. Free Radicals in Biology and Medicine, 2nd ed. Oxford: Oxford University Press.

Hammond-Kosack, K. E., and J. D. G. Jones. 1997. Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**: 575-607.

Harper, S., N. Zewdie, I. R. Brown, and J. W. Mansfield. 1987. Histological, physiological and genetical studies of the responses of leaves and pods of *Phaseolus vulgaris* to three races of *Pseudomonas syringae* pv. *phaseolicola* and to *Pseudomonas syringae* pv. *coronafaciens*. *Physiol. Plant Pathol.* **31**: 153-172.

He, S. Y. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu. Rev. Phytopathol.* **36**: 363-392.

He, S. Y., H.-C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. syringae $harpin_{pss}$: A protein that is secreted via the *hrp* pathway and elicits the hypersensitive response in plants. *Cell* **73**: 1255-1266.

Heath, M. C. 1996. Plant resistance to fungi. *Can. J. Plant Path.* **18**: 469-475.

Heath, M. C. 1997. Evolution of plant resistance and susceptibility to fungal parasites. In: G. C. Carroll and P. Tudzynski (Eds.) The Mycota Vol V. Plant Relationships Part B, Springer-Verlag, Berlin/Heidelberg, pp. 257-276.

Heath, M. C. 1998. Involvement of reactive oxygen species in the response of resistant (hypersensitive) or susceptible cowpeas to the cowpea rust fungus. *New Phytol.* **135**: 689-700.

Heath, M. C. 2000. Hypersensitive response-related death. *Plant Mol. Biol.* **44**: 321-334.

Heeb, S., and D. Haas. 2001. Regulatory roles of the GacS/GacA two-component system in plant associated and other gram-negative bacteria. *Mol. Plant Microbe Interact.* **15**: 1351-1362.

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

Higgins, V. J., H. Lu., T. Xing, A. Gellie, and E. Blumwald. 1998. The gene-for-gene concept and beyond: interactions and signals. *Can. J. Plant Path.* **20**: 150-157.

Hirano, S. S. and C. D. Upper. 1990. Population biology and epidemiology of *Pseudomonas syringae*. *Annu. Rev. Phytopathol.* **28**: 155-177.

Hirano, S. S., and C. D. Upper. 1994. Autoecology of foliar pseudomonads, pp. 227-243. *In* J. P. Blakeman and B. Williamson (eds.), Ecology of plant pathogens. CAB International, Wallingford, U.K.

Hirano, S. S., and C. D. Upper. 2000. Bacteria in the Leaf ecosystem with emphasis on *Pseudomonas syringae*---a pathogen, ice nucleus and epiphyte. *Microbiol. Mol. Biol. Rev.* **64**: 624-653.

Høiby, N. 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. A survey. *Acta Pathol. Microbiol. Scand. Sect. C. Suppl.* **262**: 3-96.

Holliday, M. J., N. T. Keen, and M. Long. 1981. Cell death patterns and accumulation of fluorescent material in the hypersensitive response to soybean leaves to *Pseudomonas syringae* pv. glycinea. *Physiol. Plant Pathol.* **18**: 279-287.

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

Huynh, T. V., D. Dahlbeck, and B. J. Staskawicz. 1989. Bacterial blight of soybean. Regulation of a pathogen gene determining host cultivar specificity. *Science* **245**:1374-1377.

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

Jefferson, R. A., S. M. Burgess and D. Hirsh. 1986. β-glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* **83**: 8447-8451.

Jefferson, R. A. 1989. The GUS reporter gene system. *Nature* **342**: 837-838.

Jin, Q. and He, S. Y. 2001. Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. *Science* **294**: 2556-2558.

Kado, C. I., and S. T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145**: 1365-1373.

Kasapis, S., E. R. Morris, M. Gross, and K. Rudolph. 1994. Solution properties of levan polysaccharide from *Pseudomonas syringae* pv. phaseolicola, and its possible role as a blocker of recognition during pathogenesis. *Carbohydr. Polym.* **23**: 55-64.

Kato, J., and A. M. Chakrabarty. 1991. Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the *algD* promoter in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **88**: 1760-1764.

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

Keen,. N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* **24**: 447-463.

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

Keith, L. M. W., and C. L. Bender. 2001. Genetic divergence in the *algT-muc* operon controlling alginate biosynthesis and response to environmental stress in *Pseudomonas syringae*. *DNA Seq.* **12**: 125-129.

Kenne, L., and B. Lindberg. 1983. Bacterial Polysaccharides. *In* The polysaccharides. Vol. 2. *Edited by* G.O. Aspinall. Academic Press, New York. pp. 287-363.

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

King, A. H. 1983. Brown seaweed extracts (alginates). *In* Food Hydrocolloids. Vol II. Glicksman, M. (ed.). CRC Press, Boca Raton, Fl. pp. 115-188.

Kinscherf, T. G. and D. K. Willis. 1999 Swarming by *Pseudomonas syringae* B728a requires *gacS* (lemA) and *gacA*, but not the acyl-homoserine lactone biosynthetic gene *ahlI*. *J. Bacteriol.* **181**: 4133-4136.

Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. *Nature* **199**: 299-300.

Koopmann, B., H. Rollwage, M. Noellenburg, and K. Rudolph 2001. Isolation and characterization of the *algD* gene of *Pseudomonas syringae* pv. phaseolicola and its distribution among other pseudomonads and related organisms. *J. Phytopathol.* **149**: 511-519.

Kubesch, P., T. Dörk, U. Wulbrand, N. Kälin, T. Neumann, B. Wulf, H. Geerlings, H. Weissbrodt, H. von der Hardt, and B. Tümmler. 1993. Genetic determinants of airways' colonization with *Pseudomonas aeruginosa* in cystic fibrosis. *Lancet* **341**: 189-193.

Lam, J., R. Chan, K. Lam, and J. W. Costerton. 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect. Immun.* **28**: 546-556.

Leach, J. E., and F. F. White. 1996. Bacterial avirulence genes. *Annu. Rev. Phytopathol.* **34**: 153-179.

Learn, D. B., E. P. Brestel, and S. Seetharama. 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. *Infect. Immun.* **55**: 1813-1818.

Leben, C. 1981. How plant-pathogenic bacteria survive. *Plant Dis.* **65**: 633-637.

Lee, Y.-A., M. Hendson, N. J. Panopolous, and M. N. Schroth. 1994. Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. juglandis: homology with small blue copper proteins and multicopper oxidase. *J. Bacteriol.* **176**: 173-188.

Legendre, L., S. Rueter, P. F. Heinstein, and P. S. Low. 1993. Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. *Plant Physiol.* **102**: 233-240.

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

Leitäo, J. H. and I. Sá-Correia. 1997. Effects of growth-inhibitory concentrations of copper on alginate biosynthesis in highly mucoid *Pseudomonas aeruginosa*. *Microbiology* **143**: 481-488.

Levine, A., R. Tenhaken, R. Dixon, and C. Lamb. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**: 583-593.

Lindemann, J., D. C. Arny, and C. D. Upper. 1984. Use of an apparent infection threshold population of *Pseudomonas syringae* to predict incidence and severity of brown spot of bean. *Phytopathology* **74**: 1334-1339.

Liu, Y., G.-Q. Jiang, Y. Cui, A. Mukherjee, W.-L. Ma, and A. K. Chatterjee. 1999. *kdgREcc* negatively regulates genes for pectinases, cellulase, protease, harpin Ecc, and a global RNA regulator in *Erwinia carotovora* subsp. *carotovora*. *J. Bacteriol.* **181**: 2411–2422.

Luft, J. H. 1971. Ruthenium red and violet I. Chemistry, purification, methods for use for electron microscopy and mechanism of action. *Anatom. Rec.* **171**: 347-368.

MacDonald, E. M. S., G. K. Powell, D. A. Regier, N. L. Glass, F. Roberto, T. Kosuge, and R. O. Morris. 1986. Secretion of zeatin, ribosylzeatin, and ribosyl-1-methylzeatin by *Pseudomonas savastanoi*. *Plant Physiol.* **82**: 742-747.

Martin, G. B., S. H. Brommonschenkel, J. Chunwongse, A. Frary, M. W. Ganal, R. Spivey, T. Wu, E. D. Earle, and S. D. Tanksley. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*. **262**: 1432-1436.

Mazzola, M. and F. F. White. 1994. A mutation in the indole-3-acetic acid biosynthesis pathway of *Pseudomonas syringae* pv. syringae affects growth in *Phaseolus vulgaris* and syringomycin production. *J. Bacteriol.* **176**: 1374-1382.

Mellano, M. A., and D. A. Cooksey. 1988a. Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. tomato. *J. Bacteriol.* **170**: 2879-2883.

Mellano, M. A., and D. A. Cooksey. 1988b. Induction of the copper resistance operon from *Pseudomonas syringae* pv. tomato. *J. Bacteriol.* **170**: 4399-4401.

Michelmore, R. W., and B. C. Meyers. 1998. Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. *Genome Res.* **8**: 1113-1130.

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

Miller, J. C., and S. D. Tanksley. 1990. RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. *Theor. Appl. Genet.* **80**: 437-448.

Mills, S. D., C. A., Jasalavich and D. A. Cooksey. 1993. A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J. Bacteriol.* **175**: 1656-1664.

Mills, S. D., C.-K. Lim, and D. A. Cooksey. 1994. Purification and characterization of CopR, a transcriptional activator protein that binds to a conserved domain (*cop* box) in copper-inducible promoters of *Pseudomonas syringae*. *Mol. Gen. Genet.* **244**: 341-351.

Mittal, S., and K. R. Davis. 1995. Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. *Mol. Plant-Microbe Interact.* **8**: 165-171.

Moe, S. T., K. I. Draget, G. Skjåk-Bræk, O. Smidsrød. 1995. Alginates. *In* Stephen, A.M. (ed), Food polysaccharides and applications. M. Decker. New York, pp. 245-286.

Mohr, C. D., and V. Deretic. 1992. In vitro interactions of the histone-like protein IHF with the *algD* promoter, a critical site for control of mucoidy in *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Comm.* **189**: 837-844.

Mohr, C. D., J. H. J. Leveau, D. P. Krieg, N. S. Hibler, and V. Deretic. 1992. AlgR-binding sites within the *algD* promoter make up a set of inverted repeats separated by a large intervening segment of DNA. *J. Bacteriol.* **174**: 6624-6633.

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

Moore, R. A., A. N. Starratt, S.-W. Ma., V. L. Morris, and D. A. Cuppels. 1989. Identification of a chromosomal region required for biosynthesis of the phytotoxin coronatine by *Pseudomonas syringae* pv. tomato. *Can. J. Microbiol.* **35**: 910-917.

Munson, G. P., D. L. Lam, F. W. Outten, and T. V. O'Halloran. 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J. Bacteriol.* **182**: 5864-5871.

Niepold, F,. D. Anderson, and D. Mills. 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syrinage*. *Proc. Natl. Acad. Sci. USA* **82**: 406-410.

Nimtz, M., A. Mort, T. Domke, V. Wray, Y. Zhang, F. Qiu, D. Coplin, and K. Geider. 1996a. Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. *Carbohydr. Res.* **287**: 59-76.

Nimtz, M., A. Mort, V. Wray, T. Domke, Y. Zhang, D. L. Coplin, and K. Geider. 1996b. Structure of stewartan, the capsular exopolysaccharide from the corn pathogen *Erwinia stewartii*. *Carbohydr. Res.* **288**: 189-201.

Ohman, D. E., K. Mathee, C. J. McPherson, C. A. DeVries, S. Ma, D. J. Wozniak, and M. J. Franklin, 1996. Regulation of the (*algD*) operon in *Pseudomonas aeruginosa*. *In* Molecular Biology of Pseudomonads. T. Nakazawa (ed.)., ASM Press, Washington, D.C.

Ophir, T., and D. L. Gutnick. 1995. A role for exopolysaccharides in the protection of microorganisms from desiccation. *Appl. Environ. Microbiol.* **60**: 740-745.

Ordman, A. B., and, S. Kirkwood. 1977. Mechanism of action of uridine diphoglucose dehydrogenase. Evidence for an essential lysine residue at the active site. *J. Biol. Chem.* **252**: 1320-1326.

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

Palmer, D. A., C. L. Bender, and S. Sharma. 1997. Use of Tn*5-gusA5* to investigate environmental and nutritional effects on gene expression in the coronatine biosynthetic gene cluster of *Pseudomonas syringae* pv. glycinea. *Can. J. Microbiol.* **43**: 517-525.

Patil, S. S., A. C. Hayward, and R. Emmons. 1974. An ultraviolet-induced nontoxigenic mutant of *Pseudomonas phaseolicola* of altered pathogenicity. *Phytopathology* **64**: 590-595.

Pedersen, S. S., T. Jensen, T. Pressler, N. Hoiby, and K. Rosendal. 1986. Does centralized treatment of cystic fibrosis increase the risk of *Pseudomonas aeruginosa* infection? *Acta Paediatr. Scand.* **75**: 840-5.

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

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

Peñaloza-Vázquez, A., G. M. Preston, A. Collmer, and C. L. Bender. 2000. Regulatory interactions between the Hrp type III protein secretion system and coronatine biosynthesis in *Pseudomonas syringae* pv. tomato DC3000. *Microbiology* **146**: 2447-2456.

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

Pier, G. B., and M. E. Elcock. 1984. Nonspecific immunoglobulin synthesis and elevated IgG levels in rabbits immunized with mucoid exopolysaccharide from cystic fibrosis isolates of *Pseudomonas aeruginosa*. *J. Immunol.* **133**: 734-739.

Pike, S. M., A. L. Ádám, X.-A. Pu, M. E. Hoyes, R. Laby S. V. Beer and A. Novacky. 1998. Effects of *Erwinia amylovora* harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from pertubations caused by inocululated *E. amylovora*. *Physiol. Mol. Plant Path.* **53**: 39-60.

Preston, G. 1997. *HrpZ and hrp expression in Pseudomonas syringae and emerging paradigms of pathogenesis and host specificity*. PhD thesis, Cornell University, USA.

Rangaswamy, V., R. Mitchell, M. Ullrich, and C. Bender. 1998. Analysis of genes involved in the biosynthesis of coronafacic acid, the polyketide component of the phytotoxin coronatine. *J. Bacteriol.* **180**: 3330-3338.

Rangaswamy, V., M. Ullrich, W. Jones, R. Mitchell, R. Parry, P. Reynolds, and C. L. Bender. 1997. Expression and analysis of *c*oron*a*facate *l*igase, a thermoregulated gene required for production of the phytotoxin coronatine in *P. syringae*. *FEMS Microbiol. Lett.* **154**: 65-72.

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

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

Rich, J. J., T. G. Kinscherf, T. Kitten, and D. K. Willis. 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J. Bacteriol.* **176**: 7468-7475.

Roine, E. W., J. Wei, E.-L. Nurmiaho-Lassila, N. Kalkkinen, M. Romantschuk, and S. Y. He. 1997. Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. USA* **94**: 3459-3464.

Rosqvist, R., K. E. Magnusson, and H. Wolf-Watz. 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J.* **13**: 964-972.

Roychoudhury, S, K. Chakrabarty, Y. K. Ho, A. M. Chakrabarty. 1992. Characterization of guanosine diphospho-D-mannose dehydrogenase from *Pseudomonas aeruginosa*. Structural analysis by limited proteolysis. *J. Biol. Chem.* **267**: 990-6.

Roychoudhury, S., T. B. May, J. F. Gill, S. K. Singh, D. S. Feingold, and A. M. Chakrabarty. 1989. Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. A key enzyme in the biosynthesis of alginate by *Pseudomonas aeruginosa*. *J. Biol. Chem.* **264**: 9380-9385.

Rudolph, K. 1980. Multiplication of bacteria in leaf tissue. *Angew. Botanik* **54**: 1-9.

Rudolph, K. W. E., M. Gross, Ebrahim-Nesbat, F. N Ilenberg, A. Zomorodian, K. Wydra, M. Neugebauer, U. Hettwer, W. El-Shouny, B. Sonnenberg and Z. Klement. 1994. The role of extracellular polysaccharides as virulence factors for phytopathogenic pseudomonads and xanthomonads. *In* C. I. Kado and J. H. Cross (eds.), Molecular mechanisms for bacterial virulence. Kluwer Academic Publishers, Netherlands, pp. 357-378.

Saitou, N and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.

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

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

Schaad, N. W. 1988. *Laboratory Guide for Identification of Plant Pathogenic Bacteria.* St. Paul, MN: American Phytopathological Society Press.

Schulte, R. and U. Bonas. 1992. Expression of the *Xanthomonas campestris* pv. vesicatoria *hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* **174**: 815-823.

Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic. 1994. Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. *J. Bacteriol.* **176**: 3375-3382.

Schurr, M. J., H. Yu, J. C. Boucher, N. S. Hibler, and V. Deretic. 1995. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor

AlgU (σ^E), which controls mucoidy in cystic fibrosis isolates of *Pseudomonas aeruginosa*. *J. Bacteriol.* **177**: 5670-5679.

Shankar, S., R. Ye, D. Schlictman, and A. M. Chakrabarty. 1995. Exopolysacharride alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. *Adv. Enzymol.* **70**: 221-225.

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

Smith, J. J., and J. W. Mansfield. 1982. Ultrastructure of interactions between pseudomonads and oat leaves. *Physiol. Plant Pathol.* **21**: 259-266.

Stakman, E. C. 1915. Relationship between *Puccinia graminis* and plants highly resistant to attack. *J. Agric. Res.* **4**: 193-200.

Stiver, H. G., K. Zachidniak, and D. P. Speert. 1988. Inhibition of polymorphonuclear leukocyte chemotaxis by the mucoid exopolysaccharide of *Pseudomonas aeruginosa*. *Clin. Invest. Med.* **11**: 247-252.

Stokke, B. F., O. Smidsrød, P. Bruheim, and G. Skjåk-Bræk. 1991. Distribution of uronate residues in alginate chains in relation to alginate gelling properties. *Macromolecules* **24**: 4637-4645.

Storz, G., L. A. Tartaglia, S. B. Farr, and B. N. Ames. 1990. Bacterial defenses against oxidative stress. *Trends Genet.* **6**: 363-368.

Sutherland, I. W. 1982. Biosynthesis of microbial exopolysaccharides. *Adv. Microbiol. Physiol.* **23**: 79-150.

Tanksley, S. D., S. Grandillo, T. M. Fulton, D. Zamir, Y. Eshed, V. Petiard, J. Lopez, and T. Beck-Bunn. 1996. Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium*. *Theor. Appl. Genet.* **92**: 213-224.

Thompson, J. D., T .J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876-4882.

Thompson, J. D., D. G. Higgins, and T .J. Gibson. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Vandevivere, P., and D. L. Kirchman. 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. *Appl. Environ. Microbiol.* **59**: 3280-3286.

Venisse, J.-S., G. Gullner, and M.-N. Brisset. 2001. Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol.* **125**: 2164-2172.

Watanabe, K., K. Nagahama, and M. Sato. 1998. A conjugative plasmid carrying the *efe* gene for the ethylene-forming enzyme isolated from *Pseudomonas syringae* pv. *glycinea*. *Phytopathology* **88**: 1205-1209.

Wackett, L. P., W. H. Orme-Johnson and C. T. Walsh. 1989. Transition metal enzymes in bacterial metabolism. In: T. J. Beveridge and R. J. Doyle (eds). Metal ions and bacteria. Wiley & Sons, New York, pp. 165-206.

Wei, Z.-M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**: 85-88.

Wei, W., A. Plovanich-Jones, W.-L. Deng, Q.-L. Jin, A. Collmer, H.-C. Huang, and S. Y. He. 2000. The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato*. *Proc. Natl. Acad. Sci. USA* **97**: 2247-2252.

Weingart, H., H. Ullrich, K. Geider, and B. Völksch. 2001. The role of ethylene production in virulence of *Pseudomonas syringae* pvs. *glycinea* and *phaseolicola*. *Phytopathology* **91**: 511-518.

Weingart, H., and B. Völksch.. 1997. Ethylene production by *Pseudomonas syringae* pathovars *in vivo* and *in planta*. *Appl. Environ. Microbiol.* **63**: 156-161.

Whitfield, C. 1986. Bacterial exopolysaccharides. *Can. J. Microbiol.* **34**: 415-420.

Wilson, M., S. S. Hirano, and S. E. Lindow. 1999. Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth with the leaf. *Appl. Environ. Microbiol.* **65**: 1435-1443.

Wozniak, D. J., and D. E. Ohman. 1991. *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. *J. Bacteriol.* **173**: 1406-1413.

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

Xiao, Y., Y. Lu., S. Heu. and S. W. Hutcheson. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. syringae 61 *hrp* cluster. *J. Bacteriol.* **174**: 1734-1741.

Xu, G.-W., and D. C. Gross. 1988. Physical and functional analyses of the *syrA* and *syrB* genes involved in syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.* **170**: 5680-5688.

Yu, J., A. Peñaloza-Vázquez, A. M. Chakrabarty, and C. L. Bender. 1999. Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. syringae. *Mol. Microbiol.* **33**: 712-720.

Yu, H., M. J. Schurr, and V. Deretic. 1995. Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. *J. Bacteriol.* **177**: 3259-3268.

Zielinski, N. A., A. Chakrabarty, and A. Berry. 1991. Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. *J. Biol. Chem.* **266**: 9754-9763.

VITA

Ronald Keith

Candidate for the Degree of

Master of Science

Thesis: EXPRESSION OF ALGINATE IN RESPONSE TO ENVIRONMENTAL STRESS AND PLANT SIGNALS

Major Field: Plant Pathology

Biographical:

Personal Data: Born in Lisburn, Northern Ireland on November 16, 1968, the son of Cecil and Kathleen Keith. Married on August 14, 1993 to Dr. Lisa Keith (nee. Wolfson).

Education: Graduated from Wallace High School, Lisburn, Northern Ireland, in June 1987; received Bachelor of Science Degree in Biology from Queens University, Belfast, Northern Ireland, in July 1991; completed requirements for the Master of Science Degree with a major in Plant Pathology at Oklahoma State University in May 2002.

Professional Experience: Research Assistant, Food Science and Technology Department, University of Nebraska-Lincoln August 1991 to December 1994. Research Technician, Department of Animal Science, University of Nebraska-Lincoln, December 1994 to August 1997. Research Technician, Department of Microbiology, Oklahoma State University, October 1997 to December 1999. Graduate Research Assistant, Department of Entomology and Plant Pathology, Oklahoma State University, January 2000 to present.

Professional Membership: American Phytopathology Society.