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VIRULENCE OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* STRAIN DC3000 ON *ARABIDOPSIS THALIANA*

BY

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ABSTRACT

VIRULENCE OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO*

STRAIN DC3000 ON *ARABIDOPSIS THALIANA*

By

Julie Zwiesler-Vollick

Bacterial diseases are an important cause of economic loss to the agricultural community. The Gram negative plant pathogenic bacterium, *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) has emerged as a model for the study of plant-microbe interactions because it infects the model plant *Arabidopsis thaliana*. Disease symptoms caused by *Pst* DC3000 include water-soaking, followed by the development of necrotic spots surrounded by diffuse chlorotic halo. The *Pst* DC3000-*A. thaliana* interaction is being used to understand the processes that underlie the complex pathogen-host interactions. My thesis work has focused on how *Pst* DC3000 causes disease.

One virulence system essential for *Pst* DC3000 pathogenesis is the type III protein secretion system. The type III protein secretion system is found in a variety of Gram negative bacterial pathogens, and is thought to deliver bacterial proteins, termed effectors, directly into the host cell cytoplasm. However, the identities and functions of the type III effector proteins of bacterial plant pathogens remain mysterious. My research provides new information on type III effector proteins in *Pst* DC3000, *Pst* DC3000 mutants with reduced virulence on *A. thaliana*, and the effect of transgenic expression of a *Pst* DC3000 type III effector, AvrE, on *A. thaliana*.

In collaboration with other members of the lab, I analyzed the *Pst* DC3000 genome for the presence of a *cis* promoter element, the *hrp* box, which is found in all

known *Pst* DC3000 type III effector genes. The expression of these *hrp* box-containing genes was then assessed with both microarray and northern blot analyses. *hrp* box-containing genes which showed expression only in minimal medium were further characterized with an *in planta* translocation assay. This study revealed six orthologues of effectors known in other *P. syringae* pathovars and eight novel candidate effectors, one of which was shown to be secreted via the type III protein secretion system. This work has contributed to the knowledge of the effector inventory in *Pst* DC3000.

I also analyzed *Pst* DC3000 mutants which showed reduced growth in *A. thaliana*. Reduced virulence mutants with insertions in the *PtsP*, *UvrD*, and *OprF* genes were isolated. The *PtsP* gene encodes a phosphoenolpyruvate protein phosphotransferase, which is involved in sugar uptake and catabolite repression. *UvrD* encodes a DNA helicase II involved in DNA replication and repair. The *OprF* gene encodes an outer membrane protein F precursor. This protein has been implicated in adaptation to low-osmolarity environments and host cell adhesion. These genes had previously not been implicated in *Pst* DC3000 virulence.

AvrE is a type III effector in *Pst* DC3000. In order to study the affect of AvrE on the host plant, I expressed the *avrE* gene in *A. thaliana* under the control of a DEX-inducible promoter. After induction with DEX, *avrE* transgenic plants developed symptoms which mimicked *Pst* DC3000 infection and stomatal aperture was affected. In addition, expression of the *avrE* transgene promotes the growth of the non-pathogenic *hrpH* mutant, which cannot deliver any type III effectors. These data suggest that AvrE may promote *Pst* DC3000 pathogenesis in *A. thaliana*.

To my family

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A.M.D.G.

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Introduction

Plant pathogens are a significant cause of crop loss worldwide. These losses of major crop species for the years 1988-1990 are estimated at $76.9 billion (US), accounting for approximately 13.3% of the estimated production. A significant proportion of this loss occurs in Asia ($43.8 billion US) where the majority of the world's population resides and thus the need for a reliable food supply is great (Orke et al., 1994). In addition to concern about naturally occurring plant pathogens, there is now concern in the wake of September 11, 2001, that plant pathogens could be used as agents of bioterror.

Among the variety of organisms which can cause disease are bacterial plant pathogens. These pathogens can be classified into two groups on the basis of their Gram-staining reactions. Gram-staining can be either positive or negative and is based on the properties of the bacterial cell wall. The Gram-positive bacterial plant pathogen genera include *Streptomyces* and *Clavibacter*. The Gram-negative genera include *Ralstonia, Xanthomonas, Erwinia, Agrobacterium, Xylella,* and *Pseudomonas*. The bacterial plant pathogens significantly impact agricultural economics around the world. For example, *Xylella fastidiosa* is a pathogen of grape vines (Hopkins, 1981), and it has recently become a problem for citrus fruits (Simpson et al., 2000). The vineyards of California will have increasing difficulty with the control of *Xylella* due to importation of the glassy-winged sharpshooter, the preferred insect vector for *Xylella* (Purcell and Saunders, 1999). In addition, *Erwinia amylovora* devastates orchards in the Eastern United States to such an extent that it has virtually eliminated the commercial pear industry from these locations (Jones and Sutton, 1996). It is also important to note that several bacterial

pathogens have been used as model systems to further our knowledge of the molecular basis of plant-pathogen interactions.

Virulence mechanisms of plant pathogenic bacteria

Plant bacterial pathogens utilize a variety of virulence mechanisms to infect their plant hosts. Unlike many intracellular animal bacterial pathogens, most bacterial phytopathogens remain outside the plant cell. This extracellular lifestyle probably requires the bacteria to find ways to release water and nutrients from the host cell into the apoplastic space. In addition, the extracellular space is also thought to contain anti-microbial defense compounds. Plant cells, in addition to the cell membrane of animal cells, contain an additional barrier, the plant cell wall. Plant bacterial pathogens have devised a number of methods to overcome these barriers to modulate plant cell functions: i) injection of bacterial DNA, ii) production of extracellular polysaccharides, iii) degradation of the host cell wall, iv) production of toxins which harm the plant cells, and v) transfer of bacterial effector proteins directly into the plant cell. There may be additional virulence mechanisms which have not yet been discovered. Interestingly, many of the virulence factors described rely upon specific bacterial secretion systems. These bacterial secretion systems have recently reviewed thoroughly (Lee and Schneewind, 2001). I will discuss only those secretion systems shown to pertain to plant-bacterial interactions.

The sequencing of a number of plant bacterial pathogen genomes has also begun to aid in our understanding of phytobacterial virulence. These genomes include *Xylella fastidiosa*, *Ralstonia solanacearum*, *Agrobacterium tumefasciens*, *Xanthomonas*

campestris, Xanthomonas axonopodis, and *Pseudomonas syringae* pv. *tomato.* Functional genomic analyses of sequence data is providing new insight into the mechanisms of bacterial pathogenesis. In this review, I will discuss the variety of virulence mechanisms employed by bacterial plant pathogens, focusing on bacteria of the genus *Pseudomonas.* This genus includes the model pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000. *Pst* DC3000 is providing a great deal of new information about bacterial-plant interactions. Many of the new virulence mechanisms discovered in this bacterium may have broader implications in the study of bacterial pathogenesis of plants.

T-DNA transfer

One unique virulence mechanism utilized by the bacteria of the genus *Agrobacterium* is T-DNA transfer (Zupan et al., 2000). The symptoms of *Agrobacterium* infection include cell proliferation at the site of bacterial infection resulting in galls at the crown of the plant or hairy roots. These bacteria possess the ability to metabolize the plant compounds opines (Dessaux et al., 1993). However, plants do not usually produce large quantities of these compounds. *Agrobacteria* are able to form a type IV secretion system which delivers a strand of bacterial DNA into plant cells (Winans et al., 1996). This bacterial DNA is encoded on the Ti plasmid and is bordered by two 25-base pair direct repeats, the right and left border T-DNA sequences. The *Agrobacterium* first identifies the presence of a plant wound via the detection of exudates such as acetosyringone. These exudates activate the transcription of the *vir* genes which are responsible for the generation of a single strand copy of the T-DNA and the assembly of

the T-complex transporter, a type IV secretion system. Accompanying the T-DNA are the VirD2 and VirE2 proteins. The VirE2 protein is a single-stranded DNA binding protein thought to protect the T-DNA from nucleolytic degradation (Christie et al., 1988; Citovsky et al., 1988). In addition, the VirE2 protein contains nuclear localization sequences (NLSs) which promote localization of these proteins to eukaryotic nuclei (Citovsky et al., 1992). The VirD2 protein also contains an NLS (Ballas and Citovsky, 1997). Thus, the NLSs present in both the VirD2 and VirE2 proteins are thought to direct the T-DNA-protein complex to the plant nucleus, where the bacterial T-DNA is then integrated into the genomic DNA of the plant host (Gheysen et al., 1991). The inserted genes include those required for the biosynthesis of opines. In addition, genes for the production of the plant hormones cytokinin and auxin, which promote cell division, are transferred (Akiyoshi et al., 1984; Inze et al., 1984). Thus, the targeted expression of bacterial genes in the plant cell provides an *Agrobacterium*-specific carbon source and niche.

Extracellular polysaccharides

Many plant bacterial pathogens produce extracellular polysaccharides (EPSs) both *in vitro* and *in planta* (Denny, 1995). These polysaccharides can consist of polymers of simple sugar moieties or can have more complex branching structures. The EPSs can be secreted yet anchored to the bacterial membrane via a lipid A moiety forming a polysaccharide capsule around the bacterium. Alternatively, the EPS is secreted and diffuses away from the bacterium as extracellular slime. EPS production has been observed in several plant pathogenic bacteria, including *Ralstonia solanacearum,*

Clavibacter michiganensis, Agrobacterium tumefasciens, Xanthomonas campestris, and *Erwinia amylovora* (Denny, 1995). Early studies with naturally occurring *R. solanacearum* (formerly *Pseudomonas solanacearum*) isolates noted a correlation between virulence and EPS production (Buddenhagen and Kelman, 1964). In these bacteria, the major EPS is acidic and the genes responsible for its biosynthesis comprise the *eps* operon. Mutants which disrupt the *eps* operon are unable to trigger the wilt symptoms typical of a successful infection of tomato (Denny and Baek, 1991; Kao et al., 1992). A correlation between EPS production and pathogen virulence has also been observed in *Erwinia stewartii* (Poetter and Coplin, 1991), *Erwinia amylovora* (Geier and Geider, 1993), *Xanthomonas campestris* (Denny, 1995), and *Xanthomonas oryzae* (Rajeshwari and Sonti, 2000). While *Clavibacter michiganensis* has been shown to make EPS, it is not clear whether these molecules contribute to the virulence of this pathogen (Bermpohl et al., 1996; Meletzus et al., 1993; Van Alfen et al., 1987).

There is an ongoing debate about the mechanism by which EPS production promotes virulence in plant pathogenic bacteria. EPS-producing bacteria often cause similar symptoms in their host plants. These symptoms include wilt and water-soaking (Denny, 1995). One contribution of the EPSs to vascular pathogen virulence is the blockage of the host xylem and disruption of host water relations. If the tissue blocked is leaf vasculature, then the result is water-soaking. If the tissue affected is the stem or root vasculature, the result is wilt. Exogenous application of an EPS from *R. solanacearum* to tomato shoot cuttings can cause wilt (Buddenhagen and Kelman, 1964). In *E. amylovora*, infected apple and pear fruits develop an ooze. This ooze is composed of free EPS molecules in which bacteria are suspended and is thought to aid in the spread of the

bacteria (Jones and Sutton, 1996). In addition, the EPS capsule is thought to protect the bacteria from dessication (Denny, 1995). EPSs may even play a role in the attachment of *R. solanacearum* to its host (Sequeira, 1985). Secreted EPS may form a protective layer around the bacteria isolating them from anti-microbial compounds produced by the plant host or prevent contact with the plant cells and thus impede recognition (Braun, 1990). EPSs have also been shown to be important for the initiation of symbiotic interactions between *Rhizobioum meliloti* and *Medicago sativa* (Gonzalez et al., 1996). It is thought that the EPSs are acting as signaling molecules which promote nodule invasion. While EPSs have not yet been implicated in signaling between pathogen and host, the signaling role that EPSs play in symbiotic interactions may indicate that this role should be investigated.

Cell wall-degrading enzymes

Several of the bacteria within the genus *Erwinia*, e.g. *E. chrysanthemi* and *E. carotovora*, are collectively referred to as soft-rot bacteria. These bacteria are able to infect many different vegetative tissues from a broad variety of host plants (Beaulieu and Vangijsegem, 1992). These bacteria macerate plant tissues via the destruction of pectins and cellulose. The degradation products are then utilized as a carbon source by the bacterium (Hugouvieux-Cotte-Pattat and Reverchon, 2001). Pectins are important components of the middle lamella which help to maintain the position of individual plant cells with respect to each other and the cuticle. In addition, pectins and celluloses are important components of the plant cell wall. The destruction of these structural plant compounds results in tissue rot. The enzymes responsible for this maceration are the

pectate lyases, pectin methylesterases, and the polygalacturonases, collectively called cell wall-degrading enzymes (Barras et al., 1994). These enzymes are secreted from the bacterium via a type II secretion system (general secretory pathway) called the Out pathway (Bouley et al., 2001; He et al., 1991). In *E. chrysanthemi*, an initial set of five pectate lysases were identified and encoded by the *pelABCDE* genes. These five enzymes accounted for the majority of pectate lyase activity *in vitro*. However, deletion of the *pelABCDE* genes does not cause a complete loss of the ability to elicit soft-rot symptoms on plants (Ried and Collmer, 1987). This prompted researchers to look for *pel* genes which are induced *in planta*. Three additional *pel* genes, *pelL, pelI and pelZ*, were identified as genes which were induced *in planta* (Jafra et al., 1999). Mutations in these genes showed that each contributes to the soft-rot ability of *E. chrysanthemi* on potato tubers. However, it is likely that there are more soft-rot enzymes which all contribute to pathogenesis in a quantitative manner.

While the role of pectate lyases has been described most extensively in the soft-rot *Erwinia* species where they are required for pathogenicity, evidence suggests that cell wall-degrading enzymes may contribute to the virulence of other plant pathogenic bacteria which do not cause soft-rot. The burgeoning genomic data available for other bacterial plant pathogens is revealing many pectate lyases in a broad variety of pathogens (da Silva et al., 2002; Wood et al., 2001). The *R. solanacearum* genome is predicted to contain five genes which are predicted to encode proteins with similarity to known cell wall-degrading enzymes (Salanoubat et al., 2002). Although the complete *Pst* DC3000 genome sequence has not yet been published, there is evidence for the presence of cell wall-degrading enzymes in this pathogen as well. At least one pectate lyase gene may be

co-regulated with other important pathogencity factors in this system (Fouts et al., 2002). Early studies with *P. syringae* indicated that pectate lyase activity is present during the infection process (Bashan et al., 1985). Indeed, the plant cell wall is thought to present a formidable barrier for all bacterial plant pathogens. In bacteria which do not cause soft-rotting, these enzymes may be present in lower quantities or produced and applied to the plant cell wall at specific sites, resulting in small patches of degraded plant cell wall. This would then allow other virulence factors unfettered access to the plant cell membrane without causing total loss of structural integrity of the plant tissues.

Toxins

Toxins are chemicals produced by pathogens which cause tissue damage. All currently known bacterial toxins are non-specific toxins, having effects on plants that are not hosts for the toxin-producing bacterium. Thaxtomin A is a phytotoxin produced by *Streptomyces scabies*. Exogenous application of thaxtomin A to potatoes causes necrotic lesions (Lawrence et al., 1990). Mutants of *S. scabies* which lack thaxtomin A production show reduced virulence on potatoes (King et al., 1991). Thaxtomin A is a 2,5-dioxopiperazine which acts to trigger necrosis by an unknown mechanism. Glycosylated thaxtomin A is less toxic than unmodified thaxtomin A, and plant hosts may glycosylate thaxtomin A during infection as a method of detoxification (Acuna et al., 2001). The majority of the phytobacterial toxins have been described in the genus *Pseudomonas*.

Phytotoxins of the phytopathogenic *Pseudomonads*

Tabtoxin

Tabtoxin is a phytotoxin produced by *P. syringae* pv. *tabaci*, *P. syringae* pv. *coronafasciens*, and *P. syringae.* pv. *garcae.* Spontaneous mutants of these pathogens which lack tabtoxin production remain pathogenic, but do not trigger chlorosis (Kinscherf et al., 1991; Willis et al., 1991). Thus, tabtoxin is considered to be a virulence factor. Tabtoxin is a monocyclic β-lactam which elicits chlorosis after hydrolytic release of the active moiety, the tabtoxinine-β-lactam (Levi and Durbin, 1986). Tabtoxinine-β-lactam inhibits the plant host enzyme glutamine synthetase, resulting in accumulation of toxic ammonia, which is thought to lead to chlorosis and have other deleterious effects on the plant cell (Thomas et al., 1983).

Phaseolotoxin

Phaseolotoxin is a phytotoxin produced by *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *actinidiae.* Mutants of *P. s.* pv. *phaseolicola* which do not produce phaseolotoxin are reduced in virulence (Patil, 1974). This toxin has a tripeptide moiety, consisting of ornithine, alanine, and homoarginine, and an inorganic moiety, an *N'*-sulfodiaminophosphinyl (Moore et al., 1984). Phaseolotoxin inhibits the host plant enzyme ornithine carbamoyltransferase which is involved in arginine biosynthesis (Mitchell and Bieleski, 1977). This inhibition results in lower levels of arginine which has been implicated in the inhibition of plant growth and development of chlorosis (Patil, 1974).

Syringomycin and syringopeptin

Syringomycin and syringopeptin are phytotoxins produced by *P. syringae* pv. *syringae*. Biosynthetic *Ps* pv. *syringae* mutants which produce neither syringomycin or syringopeptin are reduced in virulence (Scholz-Schroeder et al., 2001). Syringomycin and syringopeptin are lipopeptide toxins which can trigger necrosis after exogenous application to plants (Hutchison and Gross, 1997). Syringomycin and syringopeptin cause pore formation in plant plasma membranes (Hutchison and Gross, 1997). Pore formation then leads to electrolyte leakage, cell death, and tissue necrosis. Application of syringopeptin SP22A was also shown to induce stomatal closure (Di Giorgio et al., 1994).

Coronatine

Coronatine is a phytotoxin produced by *P. syringae* pvs. *morsprunorum, atropurpurea, maculicola, glycinea* and *tomato* (including *Pst* DC3000). Coronatine is the only known toxin produced by *Pst* DC3000 (Bender et al., 1999). Coronatine is a polyketide toxin which has two main moieties, coronafacic acid and coronamic acid (Ichihara et al., 1977). The enzymes required for the biosynthesis of coronafacic acid are encoded by the *cfa* genes. The enzymes required for the biosynthesis of coronamic acid are encoded by the *cma* genes. The *corRS* operon encodes a two-component regulatory system which regulates expression of the *cfa* and *cma* genes (Bender et al., 1999). The overall structure of coronatine shares some similarities with the phytohormone, jasmonic acid (JA). This similarity may be of importance in its role in promoting the virulence of

Pst DC3000 (Wasternack and Parthier, 1997). Coronatine-insensitive *A. thaliana* mutants have been isolated due to the ability of their roots to grow normally in the presence of coronatine (Feys et al., 1994). These *coi1* mutants are also insensitive to the plant hormone JA. They are male sterile due to a defect in pollen development. In addition, they are more resistant to bacterial infection, but less resistant to some fungal pathogens (Thomma et al., 1998).

Coronatine causes chlorotic symptoms when applied to tomato leaves, as well as other physiological changes in a broad variety of plants (Gnanamanickam et al., 1982). When genes involved in the biosynthesis of coronatine were mutated, *Pst* DC3000 became less virulent on tomato (Bender et al., 1987). Bacterial multiplication of these coronatine-deficient mutants was decreased relative to wildtype *Pst* DC3000. In addition, leaves infected with coronatine-deficient mutants of *Pst* DC3000 showed smaller, less chlorotic lesions. However, the role that coronatine plays in the virulence of *Pst* DC3000 on *A. thaliana* is less clear. Work with another coronatine-deficient mutant of *Pst* DC3000 (DC3661) on *A. thaliana* showed that it grew to lower levels than wildtype, but only when the plants were infected by dipping the plants in a bacterial suspension and this difference was not seen when plants were infiltrated with bacteria using a needleless syringe (Mittal and Davis, 1995). In addition, this coronatine-deficient mutant induced an increase in the levels of some defense-related mRNAs, relative to wildtype *Pst* DC3000. This study did not include the standard genetic proof of restoration of virulence by complementation with the wildtype version of the disrupted gene. Subsequent analysis indicated that multiple mutations may have produced the reduction of virulence seen in *Pst* DC3661 (unpublished data from the He lab). This

result, therefore, does not indicate whether or not coronatine plays a role in the virulence of *Pst* DC3000 on *A. thaliana.*

Recent work focused on the discovery of new effector proteins secreted via the type III secretion system has yielded some interesting results with respect to the regulation of coronatine production in *Pst* DC3000. A Hidden Markov Model (HMM) search for hrp box-containing promoters revealed that the *corRS* operon is a candidate *hrp*-regulated operon (Fouts et al., 2002). This could indicate that the expression of many of the genes required for *Pst* DC3000 virulence are coordinately regulated. While the *hrp*-dependent regulation of *corRS* could not be shown experimentally (by either microarray or northern analysis), two genes required for the biosynthesis of the coronofacic acid moiety of coronatine (*cfa1* and *cfa6*) were shown to be expressed in a *hrp*-dependent manner (Fouts et al., 2002). Our laboratory also found that the *cfa2* gene is expressed in a *hrp*-dependent manner. However, a gene required for biosynthesis of the coronamic acid moiety *cmaU* was not shown to be coordinately regulated with the *hrp* genes. This lack of regulation implies that the mechanism by which coronatine biosynthesis and the type III secretion system are coordinated is more complicated than *hrp*-dependent expression of *corRS.*

The type III protein secretion system

The type III protein secretion system is important for the pathogenicity of many Gram-negative bacterial pathogens (Galan and Collmer, 1999). Many of the animal bacterial pathogens, such as enteropathic *Escheria coli*, *Salmonella typhimurium*, and *Yersinia pestis*, utilize the type III protein secretion system during pathogenesis. All

known Gram-negative plant bacterial pathogens, with the exception of *A. tumefaciens*, also rely upon the type III protein secretion system to cause disease. The type III secretion system is a protein secretion system which is thought to have the ability to transport bacterial proteins, called type III effectors, directly into the eukaryotic host cell.

Type III protein secretion in the phytopathogenic pseudomonads

The type III secretion system was first described in the plant bacterial pathogen *P. s.* pv. *phaseolicola*. Mutants of *P. s.* pv. *phaseolicola* were isolated which were unable to elicit the hypersensitive response in the non-host tobacco or disease in the normal host bean. These mutants were classified as *hrp* mutants (for hypersensitive response and pathogenicity) (Lindgren et al., 1986). Further analysis revealed that other Gram-negative bacterial plant pathogens, including *Pst* DC3000, also carry the *hrp* genes. In the genome of a given pseudomonad, the *hrp* genes are found in a cluster. In *Pst* DC3000, the *hrp* gene cluster consists of 27 *hrp* genes organized into six operons. The majority of these *hrp* genes were later found to encode a type III protein secretion system, suggesting that the elicitation of both the HR and pathogenicity rely upon the bacterial translocation of effector proteins into the plant cell.

Type III secretion system appendages

Type III secretion systems of mammalian and plant pathogenic bacteria produce surface appendages. One of the differences between the appendages of plant and animal

pathogens are the physical dimensions. Animal bacterial pathogens produce a very short appendage called the needle (Kubori et al., 1998). This appendage has a diameter of 8 nm and has a length which is less than 100 nm. Plant bacterial pathogens, on the other hand, produce a surface appendage termed the Hrp pilus (Roine et al., 1997). This pilus is 8 nm in diameter, like the needle, but is significantly longer, up to several μm in length. It has been suggested that this increase in length is necessary for plant pathogens to penetrate the plant cell wall (Roine et al., 1997). The HrpA protein is the major structural component of the Hrp pilus. *Pst* DC3000 *hrpA* mutants do not produce Hrp pili and are unable to cause disease on hosts or trigger the HR on non-hosts (Roine et al., 1997). The *hrpA* mutant also does not secrete effector proteins *in vitro* (Wei et al., 2000). This suggests that the Hrp pilus plays an important role in the type III protein secretion process. Recent work has shown that some effector proteins are actually secreted from the tip of the Hrp pilus *in vitro* (Jin and He, 2001; Li et al., 2002). This indicates that the Hrp pilus is serving as a conduit for the delivery of type III effector proteins to the host cell.

Regulation of the type III protein secretion system

The type III protein secretion system is regulated tightly at the transcriptional level. As mentioned previously, there is now evidence which suggests that, in *Pst* DC3000 at least, other virulence factors such as coronatine may also be regulated with the type III protein secretion system. The type III protein secretion system is governed by the *hrpRS* regulatory system. *hrpR* and *hrpS* show sequence similarity to the NtrC

class of response regulators which are σ^{54}-dependent enhancer-binding proteins (Grimm et al., 1995; Xiao et al., 1994). These proteins are members of two-component regulatory systems which control transcription in bacterial systems (Stock et al., 2000). *hrpR* and *hrpS* are required for the transcription of the *hrpL* gene. *hrpL* encodes an alternative sigma factor of the extracytoplasmic function (ECF) family (Xiao et al., 1994). HrpL is thought to bind a cis-element present in the promoter sequence of *hrpRS* regulated genes. This cis-element is called the *hrp* box and is defined as KGGAACY-N14/15-CCACNNA (K is T or G, Y is C or T) (Innes et al., 1993; Shen and Keen, 1993; Xiao and Hutcheson, 1994). These *hrp* boxes are found in the promoters of *hrp* genes as well as genes that encode type III effector proteins.

This regulatory system prevents the expression of the genes that encode components of the type III secretion system and the effectors except under specific circumstances, such as *in planta* and also in *hrp*-inducing medium, which is deficient in complex nitrogen and is acidic. It is thought to mimic the conditions which are present in the apoplastic space. The sensor for this regulatory system has not yet been identified and it is not yet understood how the bacteria perceive that they are *in planta* or in *hrp*-inducing minimal media.

While it is not currently known how *hrpR* and *hrpS* are regulated at the transcriptional level, the Lon protease has been shown to influence the *hrpRS* regulatory system at the post-translational level (Bretz et al., 2002). In wildtype *Pst* DC3000, the HrpR protein has a shorter half life when in rich media than when in *hrp*-inducing minimal media. However, if the stability of the HrpR protein is measured in a *lon* mutant, the half life remains the same in both types of media. Thus, the Lon protease is

required for the differential stability of the HrpR protein under different growth conditions. Increased stability of HrpR under *hrp*-inducing conditions would favor the expression of the *hrp* genes and genes which encode effectors. The mechanism by which the Lon protease is able to regulate the stability of HrpR is not yet known. HrpS appears to have a constant stability.

There are additional factors that influence *hrp* gene regulation. The HrpV protein is thought to act as a suppressor of *hrp* gene regulation. Overexpression of the *hrpV* gene significantly reduces the mRNA levels of the *hrp* genes (Preston et al., 1998). However, overexpression of both *hrpV* and *hrpRS* results in normal levels of *hrp* gene transcripts. In addition to encoding the major structural component of the Hrp pilus, the *hrpA* gene is also thought to play a role in *hrp* gene regulation. A *Pst* DC3000 *hrpA* mutant not only lacks Hrp pili, but also shows reduced levels of *hrp* gene transcripts (Wei et al., 2000). Again, the overexpression of *hrpRS* can compensate for the lack of *hrpA*. A full understanding of how all these components contribute to the regulation of the *hrp* genes will require further study.

Genome-wide inventory of effector proteins

Despite the importance of type III effector proteins in bacterial infection, a complete effector inventory has thus far not been established for any bacterial pathogen. Forward genetic screens for *P. syringae* mutants that show reduced virulence phenotypes have yielded few effector proteins probably because of apparent functional redundancy. Recently, a multitude of alternative approaches, coupled with the availability of the *P. s.*

pv. *tomato* DC3000 genome, have proven fruitful for the identification of putative effector proteins and increasing our knowledge of the complete arsenal of effector proteins in *P. syringae*.

One approach that has provided a large number of candidate effectors is the identification of avirulence (*avr*) genes. Plants have developed a defense mechanism that enables them to recognize specific pathogen strains. If a pathogen harboring an *avr* gene attempts to infect a plant possessing the corresponding resistance (*R*) gene, the so-called gene(*avr*)-for-gene(*R*) resistance will prevent disease from developing. *avr* genes have been identified through heterologous expression. In this approach, the genomic library from strain A (which contains one or more *avr* genes and is therefore avirulent on plant A that contains one or more cognate *R* genes) was introduced into strain B (which lacks *avr* genes and is therefore virulent on plant A); the response elicited by the recombinant strain B is then monitored. Any genes from strain A that cause strain B to become avirulent are considered *avr* genes. The avirulence function of several examined *P. syringae avr* genes is dependent on a functional type III secretion system (Gopalan et al., 1996; Keen et al., 1990; Pirhonen et al., 1996). This observation, coupled with the fact that the action site of many Avr proteins is inside the plant cell (He, 1997; Kjemtrup et al., 2000), suggests that Avr proteins are effector proteins. It is widely believed now that the original function of the Avr proteins was to promote disease; the avirulence function of these proteins results from the ability of some plants to have evolved recognition capability. Indeed, the virulence function of several Avr proteins has been demonstrated (Kjemtrup et al., 2000; White et al., 2000) and will be discussed later.

Because effector proteins are secreted via the type III secretion system, another approach used is identification based on protein secretion. This method has been used to identify many effectors of animal pathogens. Yuan and He (1996) used this method to identify the HrpW, HrpZ, and HrpA proteins of *Pst* DC3000. However, this approach has a limitation; only proteins that are present in abundant quantities can be visualized and then sequenced using this method. Unfortunately, *P. syringae* and other plant pathogens appear to produce the majority of their effector proteins in quantities too small to be detected by this method.

A third approach is based on the fact that all known effector genes are coordinately regulated with *hrp* genes, which are induced in planta. Therefore, *in vivo* expression technology (IVET) can be employed to search for effector genes. For example, Boch *et al.* (2002) fused a *Pst* DC3000 genomic library to a promoterless *hrcC-uidA* reporter fusion. These constructs were then introduced into the type III secretion-deficient mutant, Δ*hrcC*, of *Pst* DC3000, and the candidate gene-*hrcC-uidA* fusion was allowed to recombine into the genome. In principle, any genes that contain the hrp box in the promoter region will be able to trigger the expression of the *hrcC-uidA* fusion, which would restore the *hrcC* mutant to grow *in planta* and elevate the reporter gene activity in *hrp*-inducing medium, but not in nutrient-rich medium. Several known and suspected type III effector genes were identified in this study (Boch et al., 2002).

Finally, the recent release of the *P. s.* pv. *tomato* DC3000 genome sequence has facilitated several genome-wide surveys for type III effector genes based on the presence of the *hrp* box motif in the promoter, induction of gene expression in *hrp*-inducing minimal medium, and secretion and translocation assays (Fouts et al., 2002; Guttman et

al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002). According to these studies, *Pst* DC3000 alone contains more than 30 putative effector genes (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). Additional effector genes will likely be found in other *P. syringae* strains in the future.

The large number of effectors revealed by these recent studies has allowed further study of the structure of type III effectors. It is not known how effectors are targeted to the type III protein secretion system within the bacterial cell. Previous studies have supported conflicting theories about the identity of the secretion signal. Anderson *et al* (1997, 1999), suggested that the secretion signal resided in the mRNA structure of the effectors of *Yersinia enterocolitica*. However, Lloyd *et al* (2001) in work with *Yersinia pseudotuberculosis* provided evidence that the secretion signal resides in the N-terminal amino acids of the effector proteins. While no specific amino acid motifs are seen in the *P. syringae* type III effectors, certain biophysical properties do seem to be important. These properties can be summarized as follows: i) the first five amino acids are solvent exposed, ii) acidic amino acids are absent in the first twelve residues, and iii) the subsequent 6 to 50 or more residues are amphipathic. Two recent studies utilized these biophysical properties to search the *Pst* DC3000 genome. These studies yielded 38 and 28 candidate effectors, respectively (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). While all of these candidates may not prove to be true effectors, at least one candidate effector from each study was verified to be a secreted effector protein. The observation of these biophysical properties should not only aid in the identification of new effector proteins but should provide more information about the mechanism of type III protein secretion.

The progress made toward the identification of type III effector proteins in *P. syringae* is summarized in Table 1-1. Here, we have chosen to use the first published gene name. We have also chosen to maintain the *avr* name for a gene even if the protein encoded was later directly proven to be secreted. Proposed *hop* (hrp outer protein) names or names of orthologues in other species are shown in the alternative name column.

Table 1-1 A list of confirmed and putative type III effector proteins in *Pseudomonas syringae.*

Effector name	**First found in pathovar**	**Reference or accession**	**Alternative name**	**Alternative reference**	**Secreted/ translocated**	**Secretion reference**
HopPsyV	*syringae*	AF232005	Orf 5	[1]	Unpublished	[1]
HopPtoA1	*tomato*	AF232004	CEL Orf5	[1]	Yes	[2]
			HopPtoA1pma	[3]	Yes	[3]
HopPtoB	*tomato*	AF232004	EEL Orf1	[1]	Unpublished	[4]
AvrA	*glycinea*	M15194				
AvrB	*glycinea*	M21965				
AvrC	*glycinea*	M22219				
AvrPphB	*phaseolicola*	M86401				
AvrPphF	*phaseolicola*	AF231453	Chp6	[5]		
AvrPpiB1	*pisi*	X84843				
AvrPpiG1	*pisi*	AJ277495				
HrpA		L41863			Yes	[6, 7, 8]
HrpW	*syringae*	U25812			Yes	[9]
			HrpWpma	[3]	Yes	[3]
HrpZ	*syringae*	L14775			Yes	[7, 8, 10, 11]
HrmA	*syringae*	L14926	HopPsyA	[11]	Yes	[11]
AvrE	*tomato*	U97505	AvrE pma	[3]	Yes	[3]
AvrPphD	*phaseolicola*	AJ277494	HopPtoD	[4]	Yes	[4]
AvrPphE	*phaseolicola*	U16817	AvrPphEPma	[3]	Yes	[3]
AvrPpiC2	*pisi*	AJ277496	HopPtoC	[4]	Yes	[4]
AvrPto	*tomato*	L20425			Yes	[12, 5, 11]
VirPphA	*phaseolicola*	AF141883	HopPmaL	[3]	Yes	[3]
			AvrPtoB	[13]	Yes	[13]
			VirPtoA	[5]	Yes	[5]
AvrRpm1	*maculicola*	X67808	AvrPmaA1.RPM1	[3]	Yes	[3]
AvrRpt2	*tomato*	L11355			Yes	[14]
AvrXv3	*tomato*	[15]	HopPtoJ	[4]	Yes	[4]
AvrRps4	*pisi*	L43559	HopPtoK/avrPpiE	[4]	Yes	[4]

Table 1-1 cont.

Effector name	First found in pathovar	Reference or accession	Alternative name	Alternative reference	Secreted/ translocated	Secretion reference
HopPtoI	*tomato*	[4]	HolPtoO	[3]	Yes	[4]
			Chp3b	[5]		
HopPtoH	*tomato*	[4]			Yes	[4]
HopPtoE	*tomato*	[4]	HolPtoV	[3]	Yes	[4]
			Chp1	[5]		[5]
HopPtoG	*tomato*	[4]			Yes	[4]
HopPtoL	*tomato*	[4]			Yes	[4]
HopPtoO	*tomato*	[3]	HopPtoS1	[4]	Yes	[3]
			HopPtoS1	[4]	Yes	[4]
HopPtoS2	*tomato*	[4]			Yes	[4]
HopPmaI	*maculicola*	[3]	Ipx67	[16]	Yes	[3]
			Chp2	[5]		
HopPmaJ	*maculicola*	[3]			Yes	[3]
HopPmaK	*maculicola*	[3]			Yes	[3]
HopPmaG	*maculicola*	[3]			Yes	[3]
HopPmaH	*maculicola*	[3]			Yes	[3]
HopPmaA	*maculicola*	[3]			Yes	[3]
HopPtoP	*tomato*	[3]	HopPtoPpma	[3]	Yes	[3]
			Chp5	[5]		
HopPmaB	*maculicola*	[3]			Yes	[3]
HopPmaD	*maculicola*	[3]			Yes	[3]

[1] Alfano *et al,* 2000
[2] Badel *et al,* 2002
[3] Guttman *et al,* 2002
[4] Petnicki-Ocweija *et al,* 2002
[5] Zwiesler-Vollick *et al,* 2002
[6] Roine *et al,* 1997
[7] Li *et al,* 2002
[8] Yuan and He, 1996
[9] Wei *et al,* 2000
[10] He *et al,* 1993
[11] van Dijk *et al,* 1999
[12] Jin and He, 2001
[13] Kim *et al,* 2002
[14] Mudgett and Staskawicz, 1999
[15] Fouts *et al,* 2002
[16] Boch *et al,* 2002

Overall, the number of predicted effector proteins in *P. syringae* appears to be greater than the effectors known in any mammalian pathogen, possibly reflecting a requirement for *P. syringae*, as a species, to infect a broad range of plant hosts. Alternatively, the presence of a large number of effector genes in the *P. syringae* genome may reflect the presumably aggressive co-evolution which is taking place between the pathogen and the plant host. It is apparent that plants use type III effectors as a main source of recognition to activate innate defense and turn virulence-intended effector genes into *avr* genes. To survive, *P. syringae* must either mutate these *avr* genes to evade recognition or evolve or acquire additional effector genes to mask the presence of the *avr* genes. This evolution may have resulted in rapid proliferation of effector genes in the *P. syringae* genome.

Virulence role of type III effector proteins

While type III effector proteins have been implicated as having a role in virulence because most are delivered into the host cell, research is only now beginning to elucidate the virulence contributions made by individual effector proteins. Effector proteins can generally be classified into two classes. While most type III effector proteins are thought to be delivered into the host cell, a few are merely secreted outside the bacterial cell. Those that are not thought to enter the host cell are called harpins and include HrpW and HrpZ. The harpins also have the ability to trigger a hypersensitive response (HR) when infiltrated into leaves. Because they are secreted by the type III secretion system, harpins are thought to play a role in virulence. HrpZ has also been shown to be required for the

delivery of AvrB into the host cell which then triggers an HR (Gopalan et al., 1996). HrpZ has been shown to associate with plant protoplasts (Lee et al., 2001a) and can trigger pore formation in lipid bilayers *in vitro* (Lee et al., 2001b). The terminal portion of HrpW shows similarity to pectate lyases and can bind to pectate *in vitro,* although no enzymatic activity has been shown (Charkowski et al., 1998). Genetic analysis of *hrpZ, hrpW,* and *hrpZhrpW* double mutants showed that loss of these harpins had no effect on virulence. However, the *hrpZhrpW* double mutant has a reduced ability to trigger the HR (Charkowski et al., 1998). Taken together, these results suggest that the harpins may aid bacterial growth by interacting with the plant cell wall or membrane and allowing nutrient/water leakage, or by aiding the penetration of the Hrp pilus and the delivery of type III effectors into the host cell.

The role that translocated effector proteins play in virulence has also been the subject of intense research. The *avr* genes are conserved among many bacterial plant pathogens and are coordinately regulated with the *hrp* genes (Leach and White, 1996). The maintenance in the bacterial genome of these *avr* genes has been an enigma in the field (Dangl, 1994). Selection should have eliminated *avr* genes from pathogens, unless selection is also favoring the retention of these genes. It has been suggested that the *avr* genes, named for their role in the incompatible interaction, may function as virulence factors in the compatible interaction. From the perspective of the plant, the most efficient way to detect a pathogen is to recognize those factors which make the bacterium virulent. There have been several demonstrations that *avr* genes are virulence factors. The *avrRpm1* gene of *P. s.* pv. *maculicola* was shown to be contribute to virulence on *A. thaliana*, in the absence of the corresponding *R* gene, *RPM1* (Ritter and Dangl, 1995). In

Xanthomonas campestris, the *avrBs2* gene is needed for full virulence on its host (Kearney and Staskawicz, 1990). As mentioned previously, research in this area has been slow due to the apparent redundancy among effector proteins. However, several studies have been able to provide clues about the role that effector proteins play in virulence.

Many genes involved in virulence are encoded on plasmids. The bean pathogen *P. s.* pv. *phaseolicola* contains such a plasmid. A *P. s.* pv. *phaseolicola* strain which had been cured of this plasmid was shown to have an altered host range (Jackson *et al,* 1999). This prompted an analysis of the genes encoded on this plasmid. One gene, *virPphA,* was shown to be required for virulence on some cultivars of bean, and for triggering an HR in others. Another gene encoded on the plasmid, *avrPphC,* was shown to act as an *avr* gene in certain bean cultivars. In addition, the presence of *avrPphC* or *virPphA* was shown to block the recognition of *avrPphF* or an unidentified *avr* gene, respectively. This mechanism would allow virulence determinants which had become targets of the host gene-for-gene resistance system to be maintained without losing the ability to infect these hosts (Tsiamis *et al,* 2000).

While most type III effectors show no similarity to genes which code for proteins of known function, new motif searches are revealing enzymatic motifs in some effectors. The effector AvrPphB was found to be similar to the *Yersinia* effector YopT. YopT is a cysteine protease which acts to promote virulence by disrupting the host actin cytoskeleton. AvrPphB was tested and also has cysteine protease activity (Shao et al., 2002). The investigation of the enzymatic activity of type III effector proteins should reveal more about their role in pathogenesis.

Transgenic expression of effector proteins in host plants is also proving to be a method which can shed light on the virulence contribution of these proteins. The effector AvrRpt2 has been investigated via transgenic expression in *A. thaliana* plants which lack the corresponding *R* gene, *RPS2* (Chen et al., 2000). These plants show enhanced growth of *Pst* DC3000. The expression of AvrRpt2 in these plants also interferes with *avrRpm1-Rpm1* mediated resistance. Ectopic expression of *avrRpt2* in *Pst* DC3000 also allows this pathogen to grow in the *cpr5* and *coi1 A. thaliana* mutants which are resistant to *Pst* DC3000. The expression of *avrPto* from *Pst* DC3000 in *A. thaliana* promotes the growth of the non-pathogenic *Pst* DC3000 *hrpH* mutant. These transgenic plants also fail to respond to the *hrpH* mutant with the formation of callose-containing papillae as is seen with wildtype *A. thaliana* (P. Hauck, R. Thilmony and S.Y. He, unpublished data). Thus, transgenic expression of effectors *in planta* is helping to reveal the role that effectors play in pathogenesis.

Project Summary

Great strides have been made in understanding virulence mechanisms utilized by bacterial plant pathogens. The identification and characterization of the Hrp type III protein secretion system has proven to be essential for our understanding of the interaction between bacterial plant pathogens and their hosts. However, research into the modes of action of the type III effectors has been impeded by the presumed functional redundancy among the effectors. Unlike animal bacterial pathogens, the number of effectors present within any given plant bacterial pathogen strain there are estimated to be

at least 35, and may be even more (Collmer et al., 2002; Genin and Boucher, 2002; Petnicki-Ocwieja et al., 2002). The proposed redundant functions of these effectors has made forward genetic analysis difficult. Identification of more effector proteins is an essential first step before reverse genetic or other approaches can be undertaken to determine the role of any given effector in virulence. Also, unlike in the study of animal bacterial pathogens, we currently lack a multitude of markers to monitor the process of pathogenesis. The initial work done with a handful of effectors should provide testable hypotheses for further characterization of all effector proteins. The advent of new technologies such as microarray host expression analysis and *in planta* visualization of bacterial infection should also help to elucidate mechanisms by which effectors contribute to pathogenesis.

At the time this project was started the only type III effectors known in *Pst* DC3000 were AvrPto and AvrE. My goal was to further characterize virulence in *Pst* DC3000. *Pst* DC3000 is an especially attractive pathogen for study. The type III secretion system has been studied extensively and is amenable to genetic manipulation. In addition, it is a pathogen of the economically important crop plant tomato as well as the model plant *A. thaliana.* Recent sequencing initiatives have made the *Pst* DC3000-*A. thaliana* pathosystem the first where genome sequence for both the host and the bacterial pathogen are available. This interaction can be investigated via the study of the pathogen as well as the host.

Chapter 2 reports my research on the identification of novel type III effector proteins of *Pst* DC3000. A preclosure sequence of the *Pst* DC3000 genome was used to search for the *hrp* box motif. The expression patterns of the *hrp* box-containing open

reading frames were then determined by both microarray and northern blot analyses. Six orthologues of effectors known in other systems, but not previously known in *Pst* DC3000, were identified. In addition, eight new candidate effectors which showed no similarity to any previously-described effectors were identified. An AvrRpt2 based translocation system was used to show that at least one of the effectors is secreted by the type III protein secretion system.

Chapter 3 describes my research on the identification of *Pst* DC3000 mutants which show reduced virulence. *Pst* DC3000 was mutagenized with a *uidA*-containing transposon. Mutants were initially screened for minimal medium-induced *uidA* reporter expression, followed by determination of virulence in *A. thaliana*. Six mutants were found which showed reduced virulence. These mutations were localized to three genes: The *oprF* gene which encodes the outer membrane protein F precursor, the *pstP* gene which encodes the enzyme I subunit of the phosphoenolpyruvate phosphotransferase, and *uvrD* which encodes a helicase involved in DNA replication and repair.

Chapter 4 communicates my work on the *Pst* DC3000 effector AvrE. Although AvrE was shown to be important for virulence in *Pst* PT23, *avrE* mutants of *Pst* DC3000 do not show a reduction in virulence. In order to investigate the role that AvrE may play in virulence, I expressed the *Pst* DC3000 *avrE* gene transgenically in *A. thaliana* under the control of a dexamethasone-(DEX-)inducible promoter. Transgenic plants which express the signal peptide fused to AvrE collapse 24-48 hours after induction of expression under dry conditions. If however, the plants are maintained under high humidity, they develop water-soaking followed by chlorosis and necrosis. These symptoms mirror those seen with *Pst* DC3000 infection. Concurrent with water-soaking,

stomatal closure was observed. In addition, these plants promoted the growth of the *hrpH Pst* DC3000 mutant to near wildtype levels. These experiments have provided the basis for further investigation of the role of AvrE in *Pst* DC3000 pathogenesis.

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Chapter 2

Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome

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Abstract

Pseudomonas syringae pv. *tomato* (*Pst*) strain DC3000 infects the model plants *Arabidopsis thaliana* and tomato, causing disease symptoms characterized by necrotic lesions surrounded by chlorosis. One mechanism employed by *Pst* DC3000 to infect host plants is the type III protein secretion system, which is thought to deliver multiple effector proteins to the plant cell. The exact number of type III effectors in *Pst* DC3000 or any other plant pathogenic bacterium is not known. All known type III effector genes of *P. syringae* are regulated by HrpS, an NtrC-family protein, and the HrpL alternate sigma factor, which presumably binds to a conserved *cis* element (called the 'hrp box') in the promoters of type III secretion-associated genes. In this study, we designed a search motif based on the promoter sequences conserved in 12 published *hrp* operons and putative effector genes in *Pst* DC3000. Seventy-three predicted genes were retrieved from the January 2001 release of the *Pst* DC3000 genome sequence, which had a 95% genome coverage. The expression of the 73 genes was analyzed by microarray and northern blot, revealing 24 genes/operons (including 8 novel genes) whose expression was consistently higher in *hrp*-inducing minimal medium than in nutrient-rich Luria-Bertani broth. Expression of all 8 genes was dependent on the *hrpS* gene. Most were also dependent on the *hrpL* gene, but at least one was dependent on the *hrpS* gene, but not on the *hrpL* gene. An AvrRpt2-based type III translocation assay provides evidence that some of the *hrpS*-regulated novel genes encode putative effector proteins.

Introduction

Pseudomonas syringae infects a wide range of susceptible plants and causes mainly localized necrosis in infected tissues. A given strain of *P. syringae* may infect only a few cultivars of a host plant, exhibiting a high degree of specificity. Host specificity is the basis for classifying various *P. syringae* strains into more than 40 pathovars (Gardan *et al.*, 1999). *P. syringae* pathovar (pv). *tomato* strain DC3000 (*Pst* DC3000 hereafter) infects tomato and Arabidopsis and causes necrotic spots surrounded by diffuse chlorotic haloes (Cuppels, 1986; Whalen *et al.*, 1991; Katagiri *et al.*, 2002). The molecular basis of pathogenicity of *Pst* DC3000, like that of the majority of plant pathogenic bacteria, is not well understood. An essential weapon in the virulence arsenal of *Pst* DC3000 is the type III protein secretion system, which is conserved in many Gram-negative plant and mammalian pathogenic bacteria (He, 1998; Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000; Staskawicz *et al.*, 2001). In plant pathogenic bacteria, the type III secretion system is encoded by *hrp* (for hypersensitive reaction and pathogenicity) and *hrc* (hrp gene conserved) genes (Van Gijsegem *et al.*, 1993; Bonas, 1994; Alfano and Collmer, 1997; Lindgren, 1997; He, 1998; Hutcheson, 1999; Mudgett and Staskawicz, 1998). This secretion system is responsible for the assembly of the Hrp pilus (Roine *et al.*, 1997; Jin and He, 2001) and is thought to deliver virulence effector proteins directly into the host cell. Once there, the effector proteins are believed to modulate the physiology of the host cell to favor pathogenesis. However, different plant cultivars may evolve specific resistance genes to recognize individual bacterial effectors and convert them into elicitors of host defense responses. In such situations, these virulence effector proteins have been named avirulence (Avr) proteins (Leach and White, 1996).

The expression of *hrc, hrp,* and effector genes is tightly controlled in *P. syringae*. All known *hrp, hrc,* and type III effector genes are expressed at a low level in standard nutrient-rich media, such as Kings B (King *et al.*, 1954) or Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). The expression of *hrc/hrp* genes is induced in plant tissues or in *hrp*-inducing minimal medium (Huynh *et al.*, 1989; Rahme *et al.*, 1992; Xiao *et al.*, 1992; Wei *et al.*, 2000) that mimic the *in planta* conditions. Three regulatory proteins are required for the expression of type III secretion-associated genes in *P. syringae*: HrpR and HrpS, which belong to the NtrC family of two-component regulatory proteins (Grimm *et al.*, 1995; Xiao *et al.*, 1994; Hutcheson *et al.*, 2001), and HrpL, a member of the ECF family of alternate sigma factors (Xiao *et al.*, 1994). The HrpS, HrpR, and HrpL proteins function as a regulatory cascade in which HrpS and HrpR activate the expression of the *hrpL* gene in response to an undefined signal in host tissue or in *hrp*-inducing minimal medium (Xiao *et al.*, 1994; Grimm *et al.*, 1995). HrpL activates the *hrp*, *hrc*, and *avr* genes presumably by binding to a consensus bipartite *cis* element ('hrp box') present in the promoter region of *hrp*, *hrc*, and type III effector genes (Innes *et al.*, 1993; Shen and Keen, 1993; Xiao and Hutcheson, 1994).

Identification of genes that encode type III effectors is a key step toward a comprehensive understanding of the function of the type III secretion system in plant pathogenesis. In *Yersinia pseudotuberculosis*, a pathogenic bacterium of mammalian hosts, there are 13 known virulence effectors determined by secretion analysis (Hueck, 1998). Less is known about the number of effectors in plant pathogenic bacteria that employ type III secretion as a virulence system. A recent cDNA-amplified fragment length polymorphism (AFLP) study of *Xanthomonas campestris* pv *vesicatoria hrpG-*

regulated genes revealed several known *hrp* gene operons, effector proteins, and unknown genes (Noel *et al.*, 2001). In *Pst* DC3000, three approaches have been used in the past to discover type III effectors: Cloning based on avirulence phenotype (Ronald *et al.*, 1992; Lorang and Keen, 1995), sequence analysis of extracellular proteins secreted via the Hrp secretion system (Yuan and He, 1996), and characterization of genes located in the so-called Hrp pathogenicity island (Alfano *et al.*, 2000). These approaches have resulted in the identification of four type III-secreted proteins, HrpA, HrpZ, HrpW, and AvrPto, and several putative effector genes (including *avrE*) located in the Hrp pathogenicity island (Alfano *et al.*, 2000). In this study, we used functional genomics approaches to discover new putative type III effector genes in *Pst* DC3000. We utilized the available *Pst* DC3000 genome sequence to search for genes that contain the 'hrp box'-like sequence in their promoters, which is indicative of possible HrpL regulation. The expression of these genes was compared between *hrp*-repressing and *hrp*-inducing conditions by microarray and northern blot analyses. Further comparisons of gene expression were made between wild-type bacteria and *hrpS* and *hrpL* mutants using northern blot analysis. Then a selected group of Hrp-regulated genes were subjected to an AvrRpt2-based type III translocation assay in *RPS2*$^{+}$ *Arabidopsis* plants to identify putative type III effectors.

Materials and methods

Computer analysis

The *Pseudomonas syringae* DC3000 genome of approximately 6.2 megabases was sequenced by The Institute for Genomic Research (TIGR; http://www.tigr.org/tdb/mdb/mdbinprogress.html). The 2nd release (January 2001, 95% coverage) was imported into BioNavigator (http://www.bionavigator.com), a web-based genome analysis tool from Entigen Corporation of Sunnyvale, California. The genome was searched with the FindPatterns(GCG) program using the 'hrp box' motif (KGGARCY(N){13,20}CCACNNA) derived from the alignment of 12 known 'hrp-box'-containing genes in *Pst* DC3000. The first base of the bipartite 'hrp box,' is either a T or a G in the 12 hrp-regulated genes. The following three bases, GGA, are invariably conserved in all 12 genes. For the fifth base of our search motif, we chose to use R, representing either A or G, although all 12 genes contain an A at this position. This is because the *hrpW* gene has a second 'hrp-box'-like sequence at the –18 nt position. In this 'hrp-box'-like sequence, there is a G, instead of an A, in the 5^{th} position. The 6^{th} position is a C in all 12 genes, whereas the 7^{th} position is a C in all genes, except for *hrpJ* and *hrpP*, which have a T. We therefore designated a Y to represent either C or T at this position. The two motifs in the 'hrp boxes' of the 12 genes were separated by 15 to 16 nts. However, to investigate the importance of the spacing between the two motifs and to identify a maximum number of candidate genes, we purposely expanded this spacing range to 13 to 20 nts.

Construction of an *hrpL* mutant of *Pst* DC3000 (by Vinita Joardar and Barbara Kunkel)

The strain VJ202 (*hrpL*::Ω) was constructed using a marker replacement strategy. A 3.2-kb *Bsa*BI fragment containing *hrpL* and flanking sequences (1.5 kb upstream and 1.2 kb downstream) was cloned into pBluescript II KS+ (Amp^r, Stratagene). This construct was moved into *Escherichia coli* strain GM2163 (*dam*$^-$; from NEB) to facilitate digestion of internal *Bsa*BI sites in the insert that are sensitive to dam methylation. The *hrpL* coding region (0.5 kb) in this construct was replaced with the Sp^r-Ω-cassette from pUC4 (Prentki and Krisch, 1984) using the internal *Bsa*BI sites. This insertion/deletion construct was moved into the suicide plasmid pJP5603 (Km^r; Penfold and Pemberton, 1992). The pJP5603 construct was conjugated into *Pst* DC3000 to generate strain VJ201 (Sp^r, Km^r) in which the construct was integrated into the *Pst* DC3000 genome via a single recombination event. The mutant strain VJ202 (Sp^r, Km^s) was identified by screening for derivatives of VJ201 in which a second recombination event resulted in loss of Km^r. Strain VJ202 was confirmed by PCR and by assaying for disease and HR production. Strain VJ202 was unable to cause disease on *A. thaliana* (Col-0) or elicit an HR on tobacco (*Nicotiana tabacum*). Complementation of strain VJ202 with the *hrpL* gene from *Pst* DC3000 restored both phenotypes.

Bacterial DNA isolation and polymerase chain reaction (PCR) amplification

Genomic DNA from DC3000 was prepared as described in Chen and Kuo (Chen and Kuo, 1993). The isolated genomic DNA was used as template for PCR amplification

of HCOs. Primers (22mers; see Appendix A) were designed using the Prime3 program from BioNavigator to amplify HCOs (hrp box-containing ORFs). Primers were manufactured by Integrated DNA Technologies (IDT) of Coralville, Iowa. The PCR fragments were verified for correct sizes, purified by ethanol precipitation, and resuspended in 40 µl of 3X SSC.

Microarray slide preparation and analysis

PCR products for a total of 79 genes ('hrp-box'-containing genes and 10 spiking controls) were spotted from microtiter plates onto SMA-25 superaldehyde-coated glass slides (Telechem International, Inc). Spotting was done at high density using an Omnigridder robot (Gene Machines, San Carlos, CA) and 16Arraylt chipmaker 1 pin (Telechem). Slides were washed and blocked according to the Telechem protocol. Microarray slides were prepared at the Michigan State University Arabidopsis Functional Genomics Consortium (AFGC) microarray facility. Spiking controls are human cDNA clones: B-cell receptor protein (AF126021), myosin heavy chain (X13988), myosin reg. light chain 2 (M21812), insulin-like growth factor (X07868), FLJ10917fis (AK001779), HSPC120 (AF161469), beta2 microglobulin (NM_004048), phosphoglycerate kinase (pgk1) (NM_000291), tyrosine phosphatase (pac-1) (L11329), and G10 homolog (edg-2) (U11861).

Isolation of bacterial RNA

RNA was isolated from *Pst* DC3000, and the *hrpL* and *hrpS* (Yuan and He, 1996) mutants of DC3000 following a protocol described by Tao *et al.* (Tao *et al.*, 1999).

Bacteria were grown in Luria-Bertani (LB; Sambrook *et al.*, 1989) medium till OD_{600} reached 0.6, when stop buffer (1.25 ml; 5% acid phenol in EtOH) was added to 10 ml culture and the mixture was centrifuged in Sorvall RC-5B at 7,000 rpm for 10 min. The resulting pellet was frozen at -80°C until being subjected to microarray or northern blot analysis.

For RNA isolation from bacteria grown in *hrp*-inducing medium (Wei *et al.*, 2000), a 10-ml LB culture of OD_{600}=0.6 was centrifuged in a clinical centrifuge at 3,000 rpm for 10 min. The LB medium was removed and bacteria resuspended in 10 ml of *hrp*-inducing medium. Bacteria were incubated at 20°C for 6 h with shaking. Again, the phenol/ethanol stop buffer was added, samples were centrifuged as above, and RNA pellets frozen at -80°C.

Aminoallyl labeling of bacterial RNA

RNA samples were labeled by Cy3 or Cy5 fluorescent dye, following a protocol by Ben Schroeder, which was modified from Chris Seidel's protocol (http://www.pangloss.com/seidel/Protocols/amino-allylRT.html). Three mg/ml Gibco random hexamers were used in the reverse transcription reaction. The labeled probes were hybridized to a microarray in a total volume of 30 µl of hybridization buffer (3.4 x SSC, 0.32% SDS, and 5 µg yeast tRNA) for 16 h at 60°C. The microarray slide was then washed at room temperature sequentially in 2 x SSC/0.1% SDS for 5 min, 1 x SSC/0.1% SDS for 5 min, and 0.1 x SSC for 15 s. The slide was centrifuged, dried, and scanned with a Scanarray 4000 (GSI Lumonics, Billerica, MA). Each microarray experiment was

repeated three times (two technical replicates with the same RNA samples and one biological replicate using RNA isolated from a different culture).

Due to the small size of the array, normalization to spiking controls was used. Spike mRNA (0.5 ng each) was added to both RNA samples before labeling. Once the data were obtained, the ratios of the spikes were set to one and from this a normalization constant was determined.

Northern hybridization

Twenty μg of total bacterial RNA was run on each lane of a denaturing formaldehyde gel and transferred to Millipore Immobilon N membrane. ^{32}P probes were made using purified PCR products of 43 'hrp-box'-containing genes for which expression was detectable in microarray experiments.

AvrRpt2 fusion translocation analysis

The truncated *avrRpt2*$_{80\text{-}255}$ gene, which encodes type III secretion/translocation-incompetent, but biologically active, AvrRpt2 (Mudgett *et al.*, 2000) was cloned into the *Xba*I-*Hin*dIII sites of pUCP19 (Schweizer, 1991). Candidate type III effector genes were amplified using appropriate primers (see below) and cloned into the *Eco*RI- *Xba*I sites in pUCP19::*avrRpt2*$_{80\text{-}255}$ to create in-frame fusions (5'-effector gene-*avrRpt2*$_{80\text{-}255}$-3'). All gene fusions contained the full-length putative effector genes. The recombinant plasmids were then electroporated into *Pst* DC3000. The tansformants were grown in LB to OD_{600}=0.6. Bacteria were collected by centrifugation and resuspended in sterile water to an OD_{600}=0.2. The bacterial suspensions were infiltrated into leaves of 6-week-old

RPS2^{+} or *rps2*$^{-}$ Arabidopsis plants (ecotype Col-0). Tissue collapse was monitored over a 48-h period at room temperature.

Primers used to amplify chp genes for making *avrRpt2* fusions:

hrpA: sense primer 5'TGAATTCTTGCAAAGACGCTGGAACCG3'

antisense primer 5'AATCTAGAGTAACTGATACCTTTAGCG3'

avrPto sense 5'CGAATTCAAGTCAGTGACGCTTTGATG3'

antisense 5'TGTCTAGATTGCCAGTTACGGTACGGG3'

virPtoA: sense 5'GCGAATTCGGGCATGGAAAAATCCTCTTC3'

antisense 5' GCT CTA GAG GGG ACT ATT CTA AAA GC 3'

chp1: sense primer 5'CGAATTCTGCCGTGGCGCCGCAACCTG3'

antisense primer 5'AGTCTAGAGTCAATCACATGCGCTTGG3'

chp5: sense primer 5'CGAATTCAGCTACATCTCTGGTTCGCG3'

antisense primer 5'AGTCTAGAAGCGGGTAAATTGCCCTGC3'

chp6: sense primer 5'GGAATTCGAACCGGGAGACGGATAGA3'

antisense primer 5'CATTAAACTACGCGCTCCAGTCTAGACG3'

chp7: sense primer 5'CGAATTCCTATCACTTAACAGACGCTT3'

antisense primer 5'AATCTAGACTGCGACGACCTCACAGCC3'

chp8: sense primer 5'CGAATTCTTCGAACTGTCCGACATGCC3'

antisense primer 5'CCTCTAGAGATACGGCACGCGGCTGCA3'

Further genomics searches

Search for potential myristoylation signals was done with ExPasy's scan Prosite (http://ca.expasy.org/tools/scnpsite.html). Analysis for protein domains of Chp protein sequences was done by searching Pfam HMMs (http://pfam.wustl.edu/hmmsearch.shtml). Analysis of plant organelle targeting sequences was done using PSORT (http://psort.nibb.ac.jp).

Results

Computer-assisted identification of putative 'hrp box'-containing genes

Although several 'hrp box' *cis* elements have been described based on promoter regions of *hrp* and/or *avr* genes from various *P. syringae* strains, none were based on strain DC3000 (Innes *et al.*, 1993; Shen and Keen, 1993; Xiao and Hutcheson, 1994). We aligned the promoter regions of 12 known *hrp*, *hrc*, and *avr* genes in *Pst* DC3000 (Figure 2-1). This alignment was used to generate an initial DC3000-specific consensus bipartite 'hrp box' (KGGARCY[N_{15-16}]CCACNNA). Although the 5th base of the first motif was an A in all 12 genes, we designated an R in this position because *hrpW* has a second hrp-box-like sequence at -18 nts (TGGAGCT[N_{17}]CCACTTA; not shown in Table 2-1), in which the 5th base is a G. In the KGGARCY[N_{15-16}]CCACNNA sequence, the two highly conserved motifs were separated by 15 to 16 nucleotides (nts). However, to investigate the importance of the spacing between the two motifs and to identify a maximum number of candidate genes, we purposely expanded this spacing range to 13 to 20 nts. We searched the January 2001, release of the *Pst* DC3000 genome sequence (95% coverage) from The Institute for Genomic Research (TIGR; http://www.tigr.org/tdb/mdb/mdbinprogress.html) for 'hrp box'-containing sequences using FindPatterns(GCG) available through BioNavigator (http://www.bionavigator.com). This search produced a total of 73 hits. The region downstream of each 'hrp box'-like sequence was analyzed for the presence of an open reading frame (ORF) using the BioNavigator ORF finder program. In the majority of DC3000 HrpL-regulated genes, the space between the 'hrp box' and the putative ORF start site varied from 31 bases (*hrpJ*) to 306 bases (*hrpP*). The only exception is the

putative type III effector gene *orf2* of the EEL, which is located 837 nts downstream of the 'hrp box' (Table 2-1). However, upstream of *orf2* we found another small, predicted ORF (encoding an 82-aa polypeptide) 55 nts downstream of the 'hrp box.' We allowed up to 600 bases between the 'hrp box'-like sequence and the putative ORF start site to maximize the number of candidate genes. The identified ORFs were then used to search for sequence similarity in GenBank. Of the 73 putative hrp-box-containing ORFs (HCOs hereafter), we found 11 of the 12 known type III secretion-associated genes/operons shown in Figure 2-1. The 12th gene, *orf2* of EEL, was not present in the January, 2001, sequence release. We found 6 putative orthologues of type III effectors, 26 HCOs with significant similarity to known bacterial proteins, 25 with similarity to hypothetical proteins in other bacteria, and 5 with no homology to any known protein (Appendix A).

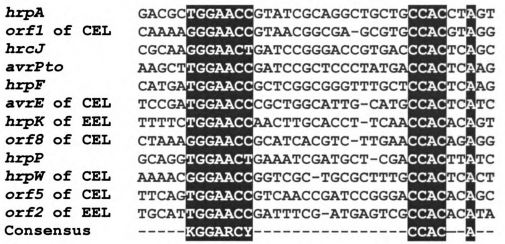

Figure. 2-1. Consensus 'hrp box' sequence in *P. syringae* pv. *tomato* DC3000. The 'hrp box' regions of 12 *Pst* DC3000 genes/operons were aligned using the ClustalW program. A consensus sequence is indicated at the bottom. CEL: Conserved effector locus (Alfano *et al.*, 2000). EEL: Exchangeable effector locus (Alfano *et al.*, 2000). K: T or G. R: A or G. Y: C or T.

Gene expression analysis of putative 'hrp box'-containing genes

Primers (see Appendix A) were designed to amplify all 73 HCOs. Of the 73 HCOs, 69 were amplified successfully. The amplified DNA was arrayed onto glass slides for microarray analysis (see Experimental Procedures), or was labeled with ^{32}P for northern blot analysis. In microarray experiments, we also included 10 human cDNAs (see Experimental Procedures) for the purpose of data normalization. Results of microarray expression and northern analysis are summarized in Table 1 and Figure 2-2.

Microarray analysis showed that the expression of most of the positive control group (*hrp* gene operons, *avrPto*, *orf2* of EEL, and *orf1*, *avrE*, *hrpW*, *orf5*, and *orf7* of CEL) were induced in *hrp*-inducing medium, with induction ratios close to 2-fold or higher (Table 2-1). The only two exceptions were the *hrpP* and *hrpK* operons, which did not show consistent differential expression in LB vs. *hrp*-inducing medium in the microarray experiments. The expression of the *hrpK* gene was at background level, giving rise to great variations in microarray experiments (Table 2-1). Also induced were 5 HCOs that show sequence homologies to known *avr* or *vir* genes (*virPphA*, *avrPpiB*, *avrPpiC*, *avrPphD*, and *hopPsyA/hrmA*) of other pathovars of *P. syringae* (Appendix A). Finally, six additional HCO genes (HCO1, 2, 3, 4, 5 and 8) were also differentially expressed (>2 fold) (Table 2-1). The remaining 49 HCOs either were not expressed at a detectable level, were not expressed at a significantly higher level in *hrp*-inducing medium compared with that in LB, or were expressed at a higher level in LB compared with that in *hrp*-inducing medium (data not shown).

The *hrpP* operon was previously shown to be induced in *hrp*-inducing medium in northern blot experiments (Wei *et al.*, 2000). Our inability to detect its induction by microarray assay in this study prompted us to verify expression of the HCOs by northern blotting. Of the 49 HCOs that did not show enhanced expression in *hrp*-inducing medium in the microarray experiments, 43 were subjected to northern blot analysis. The other 6 HCOs were not analyzed because their expression was not detectable in the microarray assay. As positive controls, we included *hrpA*, *hrcU* (*hrpP* operon), *avrE*, *avrPpiC2*, *avrPphD*, and *hopPsyA/hrmA* in northern blot analysis (see Figure 2-2 for *hrpA* and *hrcU* expression). The northern blot results were consistent with the microarray results, except for two HCOs (HCO6 and HCO7; (Table 2-1). These two HCOs were induced in *hrp*-inducing medium in northern blot assay (Figure 2-2).

Thus, with our combined microarray and northern blot analyses, we identified a total of 24 genes/operons that were expressed at higher levels in *hrp*-inducing medium than in LB (Table 2-1). Of the 24 genes/operons, 11 are known 'hrp box'-containing genes located in the *hrp* pathogenicity island, 5 show significant homology to known *avr* or *vir* genes of other *P. syringae* pathovars, and 8 are uncharacterized genes. Because the primary goal of this study was to discover new type III effector genes in *Pst* DC3000, our subsequent analyses were focused on the eight uncharacterized genes. We determined the dependence of these novel genes on the *hrpL* and/or *hrpRS* regulatory genes for expression by northern blot analysis. Of the 8 HCOs, most showed dependence on *hrpL* and *hrpRS* for high expression with no detectable level of basal expression (Figure 2-2). The expression of HCO8 was clearly dependent on the *hrpRS* operon, but a high level of expression was observed in the *hrpL* mutant background, suggesting an alternative

regulatory pathway downstream of the *hrpRS* regulatory step (Figure 2-2). Based on the dependence of their expression on the *hrpS* and/or *hrpL* gene, we named the 8 HCO genes *chp,* for co-regulated with the *hrp* genes.

Although we allowed a 13- to 20-nt gap between the two conserved motifs in the initial 'hrp-box' search sequence to identify putative HrpL-dependent genes in our bioinformatic analysis, the genes that were confirmed to be HrpL-dependent by northern blot analysis had either a 15- or 16-nt space between the two motifs of the bipartite 'hrp box' sequence. Thus, the 15- to 16-nt space appears to be crucial for HrpS/HrpL regulation in *Pst* DC3000. Also, in our genomic search we designated a maximum of 600 bases between the 'hrp box' and the putative ORF start site. However, the genes that showed HrpL-dependent regulation in this study had spacing that varied from only 28 bases to 125 bases. In comparison, spacing between the 'hrp box' and the translational start site in the previously characterized *Pst* DC3000 *hrp*-regulated genes varied from 31 (*hrpJ*) to 306 bases (*hrpP*) (Table 2-1).

Table 2-1. Features of 'hrp box' sequences and expression analysis of 'hrp box'-containing genes[a]

Gene name	Features of 'hrp box'[b]				Microarray analysis[c]		Cy5 signal[d]	Northern blot;
	Position	Motif 1	Spacing	Motif 2	MM/LB ratio	SD		induced in MM[e]
Genes previously described *(Alfano et al.*, 2000; *Wei et al.*, 2000)								
hrpRS	none	-	-	-	2.64	0.44	medium	yes
hrpL	none	-	-	-	21.55	10.84	low	yes
hrcC (hrpF operon*)*	-35	TGGAACC	16	CCACTCA	1.98	0.38	medium	yes
hrpA	-50	TGGAACC	16	CCACCTA	13.28	4.58	high	yes
hrpJ	-31	GGGAACT	16	CCACTCA	9.21	4.13	low	yes
hrcU (*hrpP* operon)	-306	GTGGAAC	15	CCACTTA	1.25	0.25	medium	yes
hrpW	-123	GGGAACC	15	CCACTCA	5.84	1.13	high	yes
avrPto	-41	TGGAACC	16	CCACTCA	7.94	4.96	high	yes
avrE	-72	TGGAACC	15	CCACTCA	4.15	0.42	medium	yes
hrpK	-37	TGGAACC	15	CCACACA	9.59	9.75	low	nd
orf2 of EEL	-873[f]	TGGAACC	15	CCACACA	1.85	0.22	high	nd
orf1 of CEL	-32	GGGAACC	15	CCACGTA	2.81	0.89	high	nd
orf5 of CEL	-167	TGGAACC	16	CCACACA	3.54	0.87	high	nd
orf7 of CEL (*orf8* operon)	-36	GGGAACC	15	CCACAGA	2.14	0.69	high	nd
Putative orthologues of known type III effectors								
VirPphA	-59	TGGAACT	16	CCACACA	27.55	5.81	high	nd
avrPpiB	-39	GGGAACC	15	CCACGAA	13.30	4.84	high	nd
AvrPpiC2	-35	GGGAACT	15	CCACTCA	5.98	2.64	high	yes
avrPphD	-42	TGGAACC	15	CCACACA	5.40	1.71	high	yes
hrmA/hopPsyA	-80	TGGAACC	16	CCACTCA	4.16	0.66	medium	yes
avrPphE[g]	-40	GGGAACT	15	CGACATA	3.16	0.76	high	nd

Table 2-1 cont.

New *hrp*-regulated genes

HCO1 (*chp1*)	-43	TGGAACC	16	CCACACA	2.25	0.38	medium	yes
HCO2 (*chp2*)	-29	TGGAACC	15	CCACCAA	8.83	2.43	medium	yes
HCO3 (*chp3*)	-34	TGGAACC	15	CCACCAA	3.60	0.72	medium	yes
HCO4 (*chp4*)	-35	TGGAACC	15	CCACTCA	9.22	3.96	medium	yes
HCO5 (*chp5*)	-94	GGGAACC	15	CCACTCA	28.21	14.95	medium	yes
HCO6 (*chp6*)	-38	GGGAACC	15	CCACTCA	1.13	0.31	medium	yes
HCO7 (*chp7*)	-125	GGGAACC	16	CCACTCA	1.44	0.20	medium	yes
HCO8 (*chp8*)	-28	GGGAACT	16	CCAGCCA	10.74	5.48	medium	yes

a. Only those genes whose mRNA levels in *hrp*-inducing minimal medium (MM) were >2 fold higher than those in Luria-Bertani (LB) medium in microarray analysis and/or qualitatively higher than in LB medium in northern blot analysis are shown.

b. Position indicates the position of the last nucleotide (A) of an 'hrp box' relative to the start codon of an ORF. Motifs 1 and 2 are the two subsequences of an 'hrp box' (Fig. 1). Spacing is the distance between the two motifs in an 'hrp box.'

c. The MM/LB ratio indicates the relative ratio of gene expression in MM vs. LB. SD indicates standard deviation.

d. The Cy5 signal indicates gene expression level. "High" indicates a reading of >7,500, "medium" indicates a reading of 1,000-7,500, and "low" indicates a reading of <1,000.

e. "Yes" indicates a qualitative increase in the transcript level (see Fig. 2 and Wei *et al.*, 2000 for example). "nd" indicates not determined.

f: *orf2* is 873 nts away from the 'hrp box.' However, we found another ORF (encoding an 82-aa polypeptide) 55-nt downstream of the 'hrp box.'

g: *avrPphE* was not found in this search. It represents a gene that is expressed at a higher level in MM than in LB, but that its 'hrp box' motif deviates from the one we used.

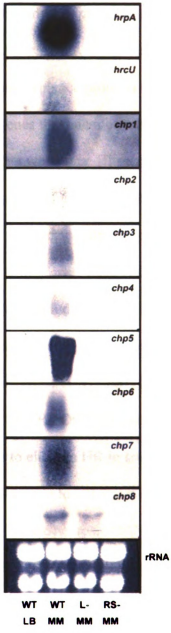

Figure 2-2. Northern blot analysis of eight novel 'hrp-box'-containing genes in *Pst* DC3000 (WT) and *hrpL* (L-) and *hrpS* (S-) mutants. The name of each gene is indicated on the right. The rRNA visualized after ethidium bromide staining was used as loading control. LB: Luria-Bertani medium (Sambrook *et al.*, 1989). MM: *hrp*-inducing medium (Wei *et al.*, 2000).

Type III translocation analysis of selected 'hrp-box'-containing genes

The *hrpS*- and/or *hrpL*-dependent expression of the eight *chp* genes suggests that these genes encode type III effector proteins, components of the secretion machinery (e.g., chaperone proteins), or other virulence proteins that function independently of type III secretion. To identify those genes that encode putative type III effectors, we took advantage of the recent finding that when type III secretion/translocation-incompetent, but biologically active, AvrRpt2$_{80\text{-}255}$ is fused to a protein carrying a type III secretion signal, the fusion protein can be translocated into the plant cell and elicits a hypersensitive response (HR) in *Arabidopsis* plants carrying a functional copy of the corresponding resistance gene, *RPS2* (Mudgett *et al.*, 2000; Guttman and Greenberg, 2001). We attempted to fuse each of the eight *hrpS/hrpL*-regulated genes ('hrp box' + ORF) to *avrRpt2*$_{80\text{-}255}$. Five fusion constructs (Chp1, 5, 6, 7 and 8) were made successfully and were mobilized into *Pst* DC3000. We examined the ability of DC3000 carrying the recombinant fusions to elicit an HR in leaves of *RPS2*$^+$ and *rps2*$^-$ *Arabidopsis* plants (ecotype Col-0). We found that the negative control strain DC3000 caused *RPS2*-independent, slow disease necrosis visible only after 24 h post-inoculation (Figure 2-3). As positive controls, DC3000 expressing the wild-type AvrRpt2 elicited an *RPS2*-dependent HR at about 9 h post-inoculation and DC3000 expressing the *virPtoA* (the homologue of *P. s.* pv. *phaseolicola virPphA*)-AvrRpt2$_{80\text{-}255}$ fusion or AvrPto-AvrRpt2$_{80\text{-}255}$ fusion elicited an *RPS2*-dependent HR at about 16 h after inoculation (Figure 2-3). Chp1-AvrRpt2$_{80\text{-}255}$ fusion also caused an *RPS2*-dependent HR at about 16 h post-inoculation (Figure 2-3). In contrast, the Chp6 fusion did not elicit an HR (Figure

2-3). Chp5, 7, and 8- AvrRpt2$_{80-255}$ fusions did not consistently elicit an *RPS2*-dependent HR (data not shown).

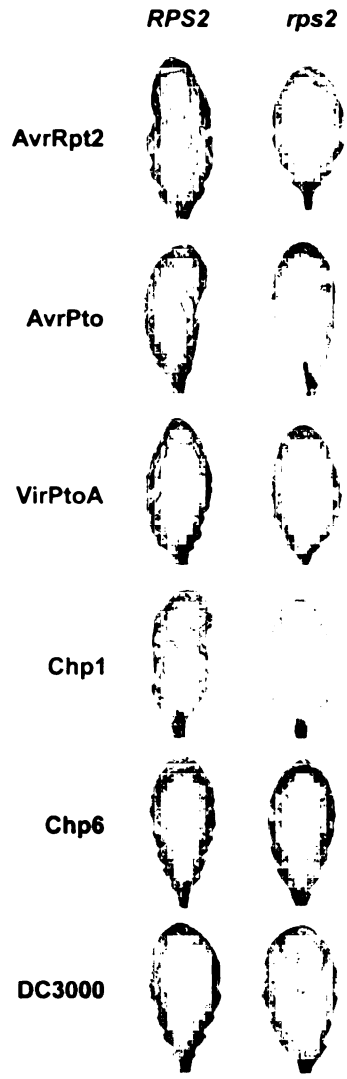

Figure 2-3. Symptoms on *RPS2* and *rps2* Arabidopsis leaves infiltrated with DC3000, DC3000 (pUCP19::AvrRpt2), or DC3000 (pUCP19::effector-AvrRpt2$_{80-255}$ fusion). Pictures were taken at 24 h after bacterial infiltration. Note that all *rps2* leaves as well as *RPS2* leaves infiltrated with DC3000 or DC3000(pUCP19::Chp6-AvrRpt2$_{80-255}$) did not show HR necrosis (leaves remained green and smooth), whereas *RPS2* leaves infiltrated with DC3000(pUCP19::AvrRpt2), DC3000(pUCP19::VirPtoA-AvrRpt2$_{80-255}$), DC3000(pUCP19::AvrPto-AvrRpt2$_{80-255}$), DC3000(pUCP19::Chp1 -AvrRpt2$_{80-255}$) showed grey HR necrosis and the infiltrated areas appeared wrinkled.

Further computer analysis of Chp proteins

We performed further analysis on the eight *chp* genes (Table 2-2). All eight Chp proteins are hydrophilic. The G+C contents of *chp6* (48%) and *chp8* (43%) are significantly lower than the G+C content of the *Pst* DC3000 genome (about 60%; Alfano *et al.*, 2000), indicating the possibility of recent introduction of these genes by horizontal gene transfer. Chp1, Chp3, and Chp4 have no similarity to known proteins, whereas the C-terminus of Chp2 has significant similarity to the DnaJ family of proteins (BLAST score 72; E value 2.0E-12; 72% similar over 63 aa) (Table 2-2). Chp5 has a putative transglycosylase SLT domain (PF01464) and shares 25% identity with the N-terminus HrpW (BLAST score 64; E value 3.0E-9; 36% similar over 281 aa) (Charkowski *et al.*, 1998). Chp6 shows sequence similarity to Orf1 of the *avrPphF* locus in *P. s.* pv. *phaseolicola* (BLAST score 155; E value 6.0E-38; 75% similar over 130 aa) (Tsiamis *et al.*, 2000). Chp7 shows similarity to proteins of the ApbE family (PF02424) (Pfam score 228; E value 6.0E-61; 93% aligned over 314 aa), some of which are involved in thiamine biosynthesis (Gralnick *et al.*, 2000). Chp8 shows sequence similarity to several hypothetical proteins with the GGDEF domain. The most significant similarity (BLAST score 266; E value 1e-70; 68% similar over 246 aa) is to a hypothetical 91.8-kD protein, AGR_L_1027p, of *Agrobacterium tumefaciens*. Several GGDEF domain (PF00990) proteins may possess diguanylate cyclase activity, modulating cyclic diguanylic acid levels in the cell (Ausmees *et al.*, 2001). Chp8 also contains the Pfam EAL domain (PF00563), which has no known function. No specific plant organelle-targeting sequences are present in these proteins.

Table 2-2. Properties of Chp proteins

Gene name	% G +C	Predicted length (aa)	Predicted mass (kDa)	Predicted pI	Sequence similarity[a] Protein, organism [accession number]	E value
chp1	50.6	211	24.0	8.4	none	-
chp2	63.1	488	52.6	11.1	heat shock protein DnaJ homologue Pfj4, *Plasmodium falciparum* [BAB17689.1]	2.0E-12
chp3	54.8	142	15.6	5.5	none	-
chp4	57.6	157	17.2	5.2	none	-
chp5	59.5	324	32.4	4.2	HrpW, *Pst* DC3000 [AAC62526.1]	3.0E-09
chp6	47.9	131	15.3	6.5	Orf1, *P. s.* pv. *phaseolicola*, [AF231452_1]	6.0E-38
chp7	58.6	340	37.3	7.2	hypothetical ApbE protein, *Pseudomonas aeruginosa* [NP_251683.1]	6.0E-84
chp8	43.2	262	28.6	4.6	AGR_L_1027p, *Agrobacterium tumefaciens* [NP_356302.1]	1.0E-70

a. The protein sequence with the lowest E value is shown.

Discussion

The availability of the *Pst* DC3000 genome makes it possible to conduct global searches for virulence-related genes. Our 'hrp box' motif search of the January, 2001, genomic sequence release revealed a large number of genes, including known *hrp, hrc,* and effector (*avr* and *vir*) genes, novel genes, and genes that show sequence similarity to known genes, but that had not been implicated in type III secretion. We took a functional genomics approach to analyze these genes. From a total of more than 73 candidate genes, which resulted from our 'hrp box' motif search, we identified eight new *hrp*-regulated genes (*chp1*, *2*, *3*, *4*, *5*, *6*, *7*, and *8*).

Our search for 'hrp box'-containing genes has served two purposes. First, it provided a pool of candidate genes for identification of novel putative type III effectors. The efficacy of this method was verified with the identification of *hrp/hrc* gene operons, known *hrp*-regulated genes located in the Hrp pathogenicity island, and putative orthologues of several known type III effector (*avr* and *vir*) genes identified previously in other *P. syringae* pathovars. There were a total of 20 known *hrp*, *avr*, and putative effector genes/operons in the January, 2002, release of the DC3000 genome (95% genome coverage). The 'hrp box' search motif used in our study enabled us to find 17 of these 20 genes/operons, giving a search efficiency of 85%. The three missing genes are *orf4* of CEL, and putative orthologues of *avrPphE* (Mansfield *et al.*, 1994) and *avrRps4* (Hinsch and Staskawicz, 1996). The promoter regions of the *avrPphE* orthologue and *orf4* contain a G, instead of a C, at the 2nd position of the 2nd motif (Figure 2-1). In retrospect, we could have designed the 2nd base of the 2nd motif wobble (G or C) to accommodate genes like *orf4* and *avrPphE*. However, this putative 'hrp box' was not

annotated in the literature nor the expression precisely defined in *Pst* DC3000 at the time we initiated our search. Also, the 5' end of the *avrPphE* orthologue was not available when we conducted our search. Interestingly, when we used a revised 'hrp box' sequence, which allows a G or C at the 2nd base of the 2nd motif, to search the January, 2001, release, more than twice as many genes (151 compared to 73) were recovered. That is, even though *orf4* and *avrPphE* represent a small minority of *hrp*-regulated genes, one has to screen twice as many candidate genes to find them. We could not find a sequence closely resembling an 'hrp box' in the promoter region of the putative *avrRps4* orthologue presumably because of a lack of a genuine 'hrp box' in this gene. Second, we were able to further elucidate the structure/function relationship of the 'hrp box' in *Pst* DC3000. Our gene expression analyses indicate that the spacing of 15 to 16 nucleotides between the two conserved motifs is crucial. Incorporation of this knowledge will aid future analysis of HrpL-regulated genes and the *hrpR-hrpS-hrpL* signal transduction cascade in *Pst* DC3000.

An important finding of our study is that genes containing the same 'hrp box' motif (with the 15- to 16-nt spacing) had very different steady-state transcript levels (Table 1) and many were not even induced in *hrp*-inducing medium in both microarray and northern blot assays (data not shown). This observation raises the possibility that, at least in *hrp*-inducing medium, either the 'hrp box' is not the only determinant for HrpL-mediated regulation, or different 'hrp-box'-containing genes produce transcripts with different stabilities that affect an accurate measurement by microarray or northern blot assay. Additionally, there was no correlation between the level of a transcript and the distance of the 'hrp box' relative to the start site of the first ORF. Therefore, a major

conclusion from this study is that a simple deduction of *hrpL*-dependent mRNA accumulation based solely on the presence of an 'hrp-box'-like sequence in the promoter region is not valid. The biological significance of the different expression levels of type III effector genes is not clear. The different transcript abundances could, however, influence the amounts of effector proteins produced and delivered into the host cells, which may be biologically optimized to alter specific host metabolic and signaling pathways in favor of pathogenesis.

The regulation of Chp8 by HrpS, but not by HrpL (Figure 2-2), was not consistent with the current model in which DC3000 senses environmental signals through the HrpR-HrpS-HrpL cascade in a single linear fashion. However, our reproducible demonstration of the HrpS-dependent and HrpL-independent expression of *chp8* in *hrp*-inducing medium strongly suggests that in addition to the HrpL pathway, there is an alternative regulatory circuit downstream of HrpS. This is a very intriguing finding. Future whole genome microarray analysis is needed to determine whether this phenomenon is more prevalent than is revealed in this study.

Our analysis has resulted in the identification of a subset of putative type III effector genes that are clearly dependent on *hrpS* and/or *hrpL* genes for mRNA accumulation. The actual number of type III effectors in *Pst* DC3000 is likely larger than that revealed in this study, for two reasons. First, a minority of *hrp*-regulated genes/operons (e.g., *orf4* of CEL, and the putative homologues of *avrPphE*, and *avrRps4*) do not appear to have a typical 'hrp box' motif as defined in Figure 2-1. It is not known whether these genes/operons are expressed in a HrpL-dependent manner in *Pst* DC3000, although the *orf4* operon of CEL is induced in *hrp*-inducing medium (Lorang and Keen,

1995) and we showed in this study that the *avrPphE* orthologue was induced in *hrp*-inducing medium (Table 2-1). It is likely these putative orthologues are additional type III effectors in *Pst* DC3000. Second, our microarray, northern blot, and AvrRpt2-based type III secretion/translocation analyses were performed with the first ORF downstream of the 'hrp box' motif. We purposely limited our analysis to the first ORFs to avoid misleading conclusions due to the incomplete annotation of the *Pst* DC3000 genome used for this study. Some type III effectors could be encoded by additional genes downstream of the first ORFs analyzed in this study (e.g., genes that are part of an operon). Indeed, we found that the second ORF (a putative orthologue of *avrPphF* of *P. s.* pv. *phaseolicola*) downstream of the *chp6* gene, when its was fused with the $avrRpt2_{80\text{-}255}$, elicited an RPS2-dependent HR (data not shown).

It is important to point out that not all HrpL-dependent genes would encode type III effectors; some may encode accessory secretion functions (e.g., chaperone) or play an important role in pathogenesis other than as type III effector proteins. As the Chp6-AvrRpt2 fusion consistently did not elicit an *RPS2*-dependent HR, *chp6* is a candidate for this class of HrpL-regulated genes.

AvrRpt2 fusions with Chp5, 7, and 8 did not consistently elicit an *RPS2*-dependent HR (data not shown). It is possible that Chp5, 7, and 8 are normally secreted via the type III secretion system, but are not translocated into the host cell. Alternatively, these Chp-$AvrRpt2_{80\text{-}255}$ fusion proteins are translocated into Arabidopsis cells, but the Chp portion may interfere with the HR-eliciting function of the $AvrRpt2_{80\text{-}255}$, preventing elicitation of an HR in *RPS2* plants. It is also possible that fusion of these

full-length Chp proteins to $AvrRpt2_{80-255}$ interferes with the ability of the Chp and/or $AvrRpt2_{80-255}$ portion to be secreted.

Previously, the AvrBs3 family of type III effectors in *Xanthomonas* was shown to carry eukaryotic nuclear localization signals (NLSs) and to function inside the plant nucleus (Yang and Gabriel, 1995; Van den Ackerveken *et al.*, 1996; Yang *et al.*, 2000). Furthermore, several *P. syringae* type III effectors (AvrRpm1, AvrB, AvrPphB, and AvrPto) carry fatty acid modification signals that target these effector proteins to the host membrane (Nimchuk *et al.*, 2000; Shan *et al.*, 2000). None of the four putative type III effectors (Chp5, 6, 7, and 8) identified in this study have recognizable NLSs, transmembrane helices, or fatty acid modification signals. However, all four new putative type III effectors share sequence similarity to other proteins. Chp5 has limited similarity to the N-terminal region of another type III effector, HrpW (Charkowski *et al.*, 1998), and contains a putative transglycosylase SLT (soluble lytic transglycosylase) domain. No transglycosylase SLT domain was found in HrpW, but HrpW contains a putative pectate lyase domain at its C-terminus (amino acids 187 to 425), which is involved in plant cell wall binding (Charkowski *et al.*, 1998). Purified HrpW elicits an HR-like necrosis in tobacco (Charkowski *et al.*, 1998). We do not know whether Chp5 also elicits an HR in tobacco. Chp6 shares sequence similarity with Orf1 of the AvrPphF locus (Tsiamis *et al.*, 2000). Finally, Chp8 shares sequence similarity to GGDEF-containing proteins, some of which may be involved in the diguanylate cyclase activity (Ausmees *et al.*, 2001). These homologies will guide future investigation of the virulence functions of Chp proteins in the host.

In summary, this study illustrates the usefulness of functional genomics approaches, used concomitantly with biological assays, in the successful identification of novel *hrp*-regulated genes and putative type III effector genes from a large number of candidate genes in *Pst* DC3000. We are currently investigating biological activities of the products of the *chp* genes. This and other genome-based studies (Boch *et al.*, 2002; Fouts *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002) mark the beginning of a new stage of research aimed at a complete inventory of *hrp*-regulated genes and type III effectors in *Pst* DC3000. Identification of all *hrp*-regulated genes and type III effectors is an essential step toward comprehension of bacterial pathogenesis in higher plants.

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Chapter 3

A bacterial mutagenesis approach for the discovery of genes required for *Pseudomonas syringae* pv. *tomato* strain DC3000 virulence in *Arabidopsis*

The initial mutagenesis and *uidA* screening described in this chapter was done by Wensheng Wei and Xin Rong.

Abstract

Pseudomonas syringae pv. *tomato* strain DC3000 (*Pst* DC3000) is the causal agent of bacterial speck disease in tomato and *Arabidopsis thaliana*. The phytotoxin coronatine and the effector proteins secreted by the type III secretion system are thought to be important for virulence. The genes which are important for production of coronatine and the effectors are transcriptionally regulated such that they are not expressed in rich media. In order to increase our chances of finding reduced-virulence mutants, a *Pst* DC3000 mutant population was generated with a mini-Tn5 *uidA* transposon. Mutants were first examined for *uidA* expression in minimal, but not rich, media. Those mutants which satisfied these criteria were then assessed for virulence in *A. thaliana*. Six mutants which demonstrated an apparent reduction in virulence were isolated. Three mutants contained disrupted *oprF* genes which encode the outer membrane porin F precursor protein. These mutants also had a reduced growth rate in minimal media. One of the mutants contained a disrupted *ptsP* gene which encodes the Enzyme I subunit of the phosphoenolpyruvate protein phosphotransferase system. The final mutant was disrupted in the *uvrD* gene which encodes a type II DNA helicase involved in DNA replication and repair. Isolation of these mutants provides new tools for the study of virulence of *Pst* DC3000.

Introduction

Pseudomonas syringae pv. *tomato* strain DC3000 (*Pst* DC3000) is a bacterial plant pathogen. It can infect both the economically important crop plant tomato as well as the model plant species *Arabidopsis thaliana. Pst* DC3000 causes disease symptoms typified by water soaking followed by the development of necrotic spots surrounded by diffuse chlorosis. Although there has been a great deal of research, especially in the last 10 years, on the interaction between *Pst* DC3000 and its model host *A. thaliana,* there is still little known about how this bacterium invades plant leaves, colonizes the apoplastic space, and causes disease. Although other factors may be involved, it is known that *Pst* DC3000 virulence is dependent upon both the phytotoxin coronatine (Bender *et al.*, 1999) and the proteins secreted by the type III secretion system (He, 1998).

The phytotoxin coronatine has been shown to induce chlorosis in tomato (Palmer and Bender, 1995) but its role in the *Pst* DC3000 interaction with *A. thaliana* has not been clearly defined. Coronatine is structurally similar to forms of jasmonic acid (JA), a plant hormone (Wasternack and Parthier, 1997). An *A. thaliana* mutant, *coi1*, which is insensitive to coronatine is also impaired in JA signaling. These results suggest that coronatine may act in *A. thaliana* via perturbation of the JA signaling pathway. Neither the actual mechanism of action of coronatine nor the benefits conferred to *Pst* DC3000 by coronatine production are known.

Virulence in *Pst* DC3000 is also known to require the type III secretion system. The type III protein secretion system is responsible for the delivery of effector proteins into the host cell cytoplasm. Mutations which disrupt the type III secretion system render the mutant bacteria non-pathogenic (Lindgren, 1997; Lindgren *et al.*, 1986). This implies

that some effector proteins delivered by a functional type III secretion system are involved in virulence. In several cases, type III effector proteins have been shown to contribute to virulence. The presence of either *avrRpm1* or *avrRpt2* allows increased pathogen growth on susceptible hosts (Chen *et al.*, 2000; Ritter and Dangl, 1995). In a few cases, evidence is beginning to suggest the mode of action of these type III effector proteins. These mechanisms include proteolytic activity, suppression of general host defense responses, and interference with host recognition of another *avr* gene and development of the hypersensitive response (Chen *et al.*, 2000; Guttman and Greenberg, 2001; Shao *et al.*, 2002; Tsiamis *et al.*, 2000).

Recent work has revealed that coronatine biosynthesis and type III secretion may be coordinately regulated. Expression of the genes required for coronatine biosynthesis is controlled by a modified two component regulatory system encoded by *corRS* (Ullrich *et al.*, 1995), whereas expression of the type III secretion system is regulated by the *hrpRS* regulatory system (Xiao *et al.*, 1994). However, recent studies revealed that the *corRS* operon might be controlled by the *hrpRS* regulatory system. The transcription of the *cfa1, cfa2,* and *cfa6* genes required for coronatine biosynthesis is dependent on *hrpRS* (Fouts *et al.*, 2002)(Zwiesler-Vollick, Plovanich-Jones and He, unpublished data). In addition, the promoter of the *corS* gene in *Pst* DC3000 has a *hrp* box-like sequence (Fouts *et al.*, 2002).

Although the initial characterization of coronatine and the effectors of the type III protein secretion system has begun, many questions remain regarding the role they play in disease development. In addition, other factors may also be required which have not yet been discovered. For example, while we are beginning to understand how pathogens

suppress defense responses, we do not yet understand how bacterial pathogens metamorphose from epiphytes to pathogens, enter the apoplastic space, adjust to the environmental conditions in the apoplastic space, attain and maintain their close physical proximity to the plant cells within the apoplastic space, and control the release of water and/or nutrients from the host cell.

Coronatine biosynthesis and type III protein secretion appear to be coordinately regulated and both processes occur in minimal media. We mutagenized *Pst* DC3000 with a mini-Tn5 transposon containing a promoterless *uidA* gene and screened for insertions into genes which were induced in minimal media. This may enrich for insertions in pathogenesis related genes. We then further screened for mutants which showed reduced virulence when infiltrated into plants. The genes which had been insertionally-inactivated were identified and the mutants were complemented.

Material and methods

Bacterial culture conditions

Bacterial cultures were grown at 30°C in Luria-Bertani (LB) (Katagiri *et al.*, 2002) medium supplemented with appropriate antibiotics unless otherwise specified. Rifampicin (Rif) was used at the concentration of 100 mg/L. Kanamycin (Km) was used at the concentration of 50 mg/L. 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) was used at the concentration of 50 mg/L.

Transposon mutagenesis

A population of bacterial mutants was generated by transposon mutagenesis with the mini-Tn5 *uidA* transposon in Sm10λ_{pir} on the suicide plasmid pGP704 (Taylor *et al.*, 1989). Sm10λ_{pir}(mini-Tn5*uidA*) and *Pst* DC3000 were grown to log phase in LB medium (Sambrook *et al.*, 1989). Bacteria were then centrifuged in a tabletop centrifuge at 3000 rpm (approx. 2000xg) for 10 min. The supernatant was decanted and the bacterial pellet was resuspended in sterile water to an OD_{600}=0.002 (10^6 cfu(colony forming units)/ml). Sm10λ_{pir}(mini-Tn5*uidA*) was then mixed with *Pst* DC3000 in a ratio of 1:5 (V/V). The mixture was then plated onto non-selective LB plates, allowed to grow for three days and then transferred onto LB plates supplemented with Rif and Km for selection.

Screening of mutants for *uidA* expression

Mutants were cultured in liquid LB media with Rif and Km to OD_{600}=0.2 to 0.8. They were then centrifuged in a tabletop centrifuge at 3000 rpm (approx. 2000xg) for 10 min. The supernatant was decanted and the bacterial pellet was resuspended in water to an OD_{600}=2.0 100 µL of the bacterial suspension were plated onto King's B (KB) agar media (King *et al.*, 1954) supplemented with Km and X-gluc and then replica-plated onto *hrp*-inducing minimal media (MM) (Wei *et al.*, 2000)supplemented with Km and X-gluc. Colony color on both KB X-gluc and MM X-gluc plates was monitored. Those which were blue, indicating *uidA* expression, on MM but not KB plates were selected. A second round of differential *uidA* expression screening was performed to confirm the *uidA* expression phenotype.

Pathogenesis assays

A. thaliana (Col-0 *gl*) plants were grown in growth chambers at 20°C with 70% humidity, a light intensity of 100 µEinsteins, and a 12-hour photoperiod. Bacteria were grown in liquid LB medium with Rif to an OD_{600}=0.4 to 0.8. Bacteria were then centrifuged in a tabletop centrifuge at 3,000 rpm (approx. 2,000xg) for 10 min. The supernatant was decanted and the bacterial pellet was resuspended in water to an OD_{600}=0.002 (10^6 cfu/ml). Leaves of 6-week-old *A. thaliana* (Col-0 *gl*) plants were hand-infiltrated using a needleless syringe. The plants were kept under high humidity at approximately 25°C. After three days the symptoms were recorded. Typical symptoms are characterized by water-soaking at approximately two days post infiltration which then

develops into necrotic spots surrounded by chlorotic haloes at about 3 days after infiltration. Quantification of bacterial growth was performed (Katagiri *et al.*, 2002).

Growth assays in liquid media

Mutants and wildtype *Pst* DC3000 were grown in LB broth supplemented with Rif overnight to an OD_{600} of approximately 1.0. For growth assessment in LB broth, the overnight culture was diluted to 10 ml of OD_{600}=0.05 in LB supplemented with Rif. The bacteria were then allowed to grow until OD_{600}=0.8. Cell density was assessed at regular intervals. For growth assessment in liquid minimal media (MM), the overnight LB culture was centrifuged, the supernatant decanted, and the pelleted cells were resuspended in MM to an OD_{600}=0.1. No antibiotic selection was used. The cell density was monitored at various intervals. Two previously characterized *Pst* DC3000 mutants, *hrpH* and *hrpS*, which do not form the functional type III secretion system and are therefore not pathogenic, were used as controls (Yuan and He, 1996).

Southern blot analysis of bacterial mutants

Bacterial DNA was extracted (Chen and Kuo, 1993). The DNA was digested overnight at 37°C with the *Pst*I restriction enzyme (NEB cat #R0140). Ten μg of digested DNA were separated on a 1.0% agarose gel run at 60 mV for approximately 2 hours. The bands of the DNA ladder were identified in the gel under UV light and a Pasteur pipet was used to remove part of each band. The gel was treated with 0.25 M HCl for 10 minutes, and rinsed in water. The DNA was transferred to nitrocellulose membrane via capillary action in 0.4 M NaOH (Sambrook *et al.*, 1989). The lanes of the

gel as well as the positions of the bands of the DNA ladder were marked on the membrane. ^{32}P-labelled *uidA* probe was prepared using approximately 100 ng of polymerase chain reaction (PCR) product (Sambrook *et al.*, 1989) and purified with Bio-Rad columns (CAT #732-6223) according to the manufacturer's instructions. The approximate size of the fragment which hybridized to the probe was estimated by comparison with the marked bands of the ladder.

Cloning and sequencing of transposon-containing fragments

Bacterial genomic DNA was extracted from the mutants, digested with *Pst*I and separated with a 0.7% agarose gel as above. DNA fragments of the approximate size of the transposon-containing *Pst*I fragments (revealed by Southern blot analysis) were cut out of the gel . The DNA was extracted from the gel using the BioRad Prep-A-Gene kit (Cat #732-6012) according to manufacturer's instructions, except that incubation with the DNA-binding matrix was lengthened to 6 hours. The purified DNA was ligated into the *Pst*I site of pBluescript SK+ treated with calf alkaline phosphatase (NEB Cat #M0290) (Sambrook *et al.*, 1989). *E. coli* DH5α was transformed with ligation mix and plated on LB Km agar plates, selecting for plasmids containing miniTn5*uidA*. Km-resistant colonies were selected and cultured in LB Km broth. Plasmid was obtained using the Promega Wizard mini-prep kit (CAT #A7100) and sequenced using the *uidA* reverse primer 5' CAGACTGAATGCCCACAGGCC 3'. The DNA sequence obtained was compared to existing sequence available in GenBank using Blastx (Altschul *et al.*, 1990).

UV tolerance assays

Wildtype *Pst* DC3000 and mutant W56 were grown in liquid LB broth supplemented with Rif to OD_{600}=0.4 to 0.8. Bacteria were centrifuged at room temperature (2000 x g) for 10 min, the supernatant was decanted, and the bacterial pellet was resuspended in sterile water to OD_{600}=0.002 (10^6 cfu/ml). Fifty µL of this suspension were plated onto LB Rif plates. The plates were then placed (agar surface down with the lids removed) onto a UV light (UVP dual intensity transilluminator). One half of the plate was protected by aluminum foil while the other half was exposed to UV light on the high setting (365 nm) for 5 seconds. The plates were then incubated at 30°C for two days.

Complementation of bacterial mutations

The sequence flanking the *uidA* gene in each insertion was used to search the *Pst* DC3000 genome sequence available through the TIGR unfinished microbial genome website (http://tigrblast.tigr.org/ufmg/) for the full-length genes and/or operons by using the Blastn algorhythm (Altschul *et al.*, 1990). The Primer3 software (available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design primers to amplify the identified genes (Rozen and Skaletsky, 2000). The primers are listed below.

(*PtsP* Forward 5' ATGCTGGTCAGGTCCTATGG 3',

PtsP Reverse 5' GGTCGGTACAGCCAATGAAG 3',

PtsP + orfs Forward 5' GCTCTAGAAAGAAGTCGGGCTTGAACG 3',

PtsP + orfs Reverse 5' AGAGAGCGAATCTAGCGCTGGAGCTCGC 3'

OprF Forward 5' CACGATATCTGGGGAACGAT 3'

OprF Reverse 5' CCGCATTCGAGCTGAAAA 3'

UvrD Forward 5' CGAAGCTTGCAGGGTTGTGTCGAAAATC 3'

UvrD Reverse 5' GCTCTAGACCACGCAACGAGGTTATACA 3'). For PCR amplification, *Pst* DC3000 genomic DNA (Chen and Kuo, 1993) was used as the template. HiFi Platinum Taq (Gibco Cat # 11304-011) was used according to manufacturer's instructions, and PCR products were purified using Qiagen QIAquick columns (cat # 28104) according to manufacturer's instructions. The *PtsP* and *OprF* genes were cloned into pGEM-T easy and transformed into *E coli* DH5α. They were subsequently sub-cloned into the *Eco*RI site of pUCP18 (Schweizer, 1991). All other PCR products were digested with restriction enzymes (*PtsP* + orfs *Xba*I (NEB # R0145) and *Sac*I (NEB #R0156), and *uvrD Hind*III (NEB # R0104) and *Xba*I (NEB # R0145)) ligated directly into pUCP18 directly and transformed into *E. coli* DH5α. Plasmids were extracted as above and the inserts were sequenced using JFL7 and M13 Forward primers. pUCP18 derivatives were electroporated into the corresponding *Pst* DC3000 mutants (Keen *et al.*, 1990). Transformants were then assessed virulence and bacterial growth on *A. thaliana* Col-0 *gl* plants.

Results

Isolation of *Pst* DC3000 mutants with differential *uidA* expression in LB vs. MM

The two previously described virulence factors of *Pst* DC3000, coronatine and the effectors of the type III protein secretion system, are transcriptionally regulated and expressed *in planta* and in minimal medium (MM). In order to enrich the pool of mutants for those genes which are induced in minimal medium, differential *uidA* expression screening was used. *Pst* DC3000 was mutagenized with miniTn5-*uidA* Km. The use of this construct permits the identification of the expression pattern of the insertionally-inactivated genes. Mutants which showed more *uidA* expression on minimal media than on rich (KB) media were selected. A summary of this scheme is shown in Figure 3-1.

Approximately 12,000 mutants were screened for *uidA* expression. One hundred and thirty eight mutants were isolated from the first round of selection. This number was reduced to 96 after a second round of screening. Figure 3-2 shows the *uidA* expression phenotypes observed for bacterial mutants that were chosen for further study. The criteria by which these mutants were selected are described in the next section.

Mutant Isolation Strategy

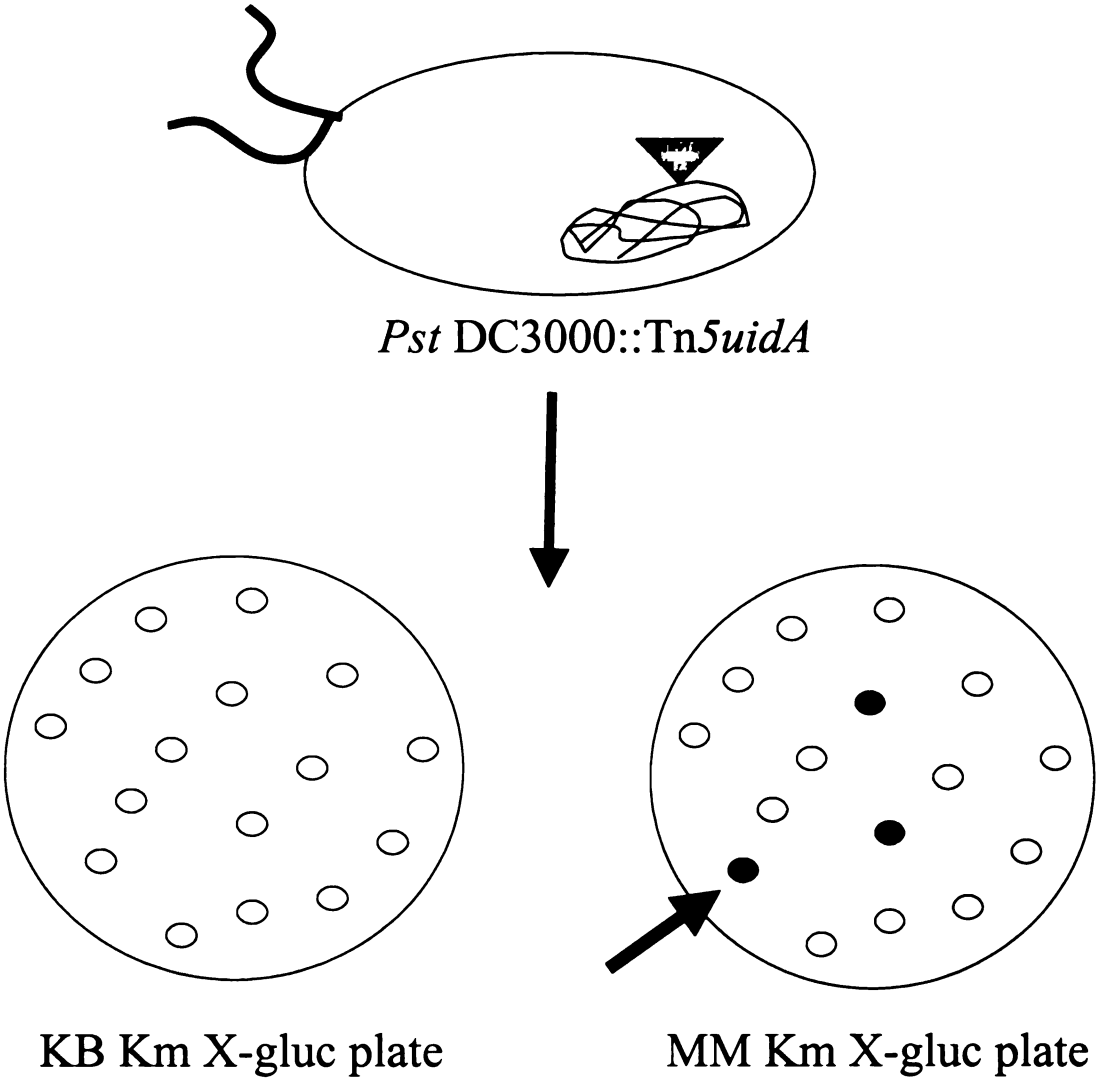

Figure 3-1. The *Pst* DC3000 mutant isolation strategy. *Pst* DC3000 was mutagenized with mini-Tn*5uidA* which confers Km resistance. Mutants were screened on rich King's B (KB) medium with X-gluc which confers blue color to colonies which express *uidA*. Screening was then done on minimal media (MM) plates with X-gluc. Colonies which were not blue on KB but were blue on MM were selected for further study.

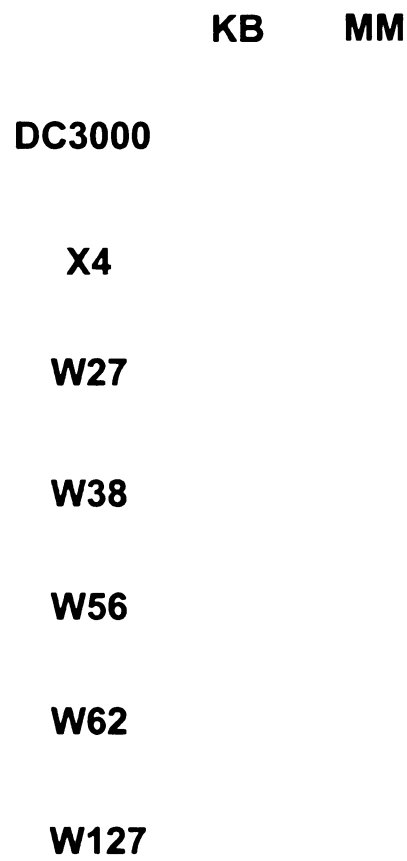

Figure 3-2. *uidA* expression of selected mutants. The colonies were allowed to grow for 3 days at 22° C. The pictures on the left are colonies which were plated onto rich King's B (KB) medium with X-gluc, the substrate for the enzyme β-glucuronidase encoded by the *uidA* gene. The pictures on the right are colonies which were plated onto minimal medium (MM) with X-gluc. The selected mutants show higher *uidA* expression in minimal medium than in rich medium.

Screening for mutants with reduced virulence

Mutants showing reproducible, differential *uidA* expression in rich vs. MM medium were tested for reduced virulence in *A. thaliana* (Col-*gl*). Six mutants were repeatedly shown to cause reduced necrosis and chlorosis compared to wildtype *Pst* DC3000. These six mutants are X4, W23, W38, W56, W62 and W127. *A. thaliana* plants infected with the six mutants are shown in Figure 3-3. *A. thaliana* plants infected with the W56 mutant showed one to two leaves leaves with prominent chlorosis and necrosis while the majority of the sixteen to eighteen leaves showed a significant reduction in chlorosis and no necrosis. *A. thaliana* infected with the other mutants showed a significant reduction in symptoms, one to three leaves with small chlorotic patches while the majority of the sixteen to eighteen leaves showed no disease symptoms. Multiplication of these mutants within *A. thaliana* leaves was also assessed quantitatively. Bacterial growth levels after three days of infection are shown in Figure 3-4. The X4 mutant, which caused a significant reduction in symptoms, grew to near wildtype levels in leaves (approximately a 10-fold reduction in growth). The W56 mutant, which showed a moderate reduction in symptoms, showed an approximately 1000-fold reduction in growth when compared to the growth of *Pst* DC3000. The W23, W38, W62 and W127 mutants, which also showed significant reduction in symptoms in *A. thaliana,* also showed an approximately 1000-fold reduction in growth relative to that of *Pst* DC3000.

Reduction in growth *in planta* may indicate a more generalized lack of bacterial fitness. To examine this possibility, growth in both MM and LB media was analyzed.

All mutants grew at rates and to levels similar to those of *Pst* DC3000 in rich media (Figure 3-5). However, while mutants X4 and W56 grew to near wildtype levels, mutants W23, W38, W62 and W127 showed a significant reduction in growth in MM (Figure 3-6). This result indicates that these mutants are affected in growth in MM. Therefore, reduced growth observed in MM could cause the reduced growth which occurs *in planta* as MM is thought to mimic conditions within the apoplastic space.

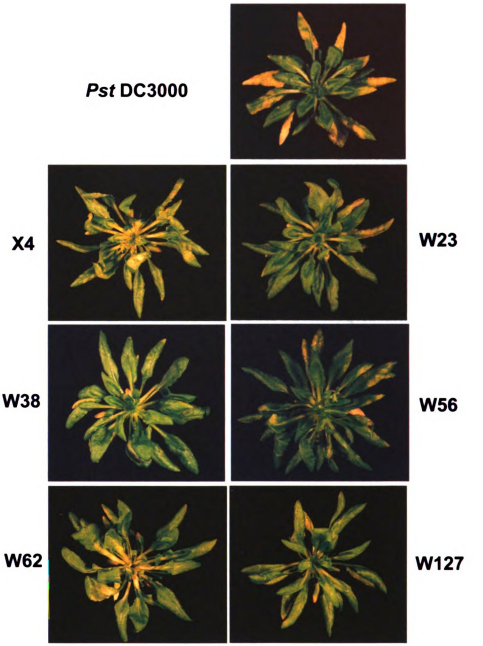

Figure 3-3. Symptoms of *Pst* DC3000 mutants in *A. thaliana* Col-0 *gl* plants. Bacteria were syringe-infiltrated into Col-0 *gl* leaves at 10^6 cfu/ml. Pictures were taken three days after infection.

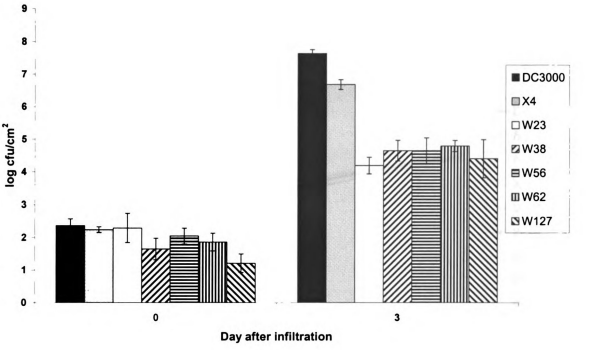

Figure 3-4. Bacterial proliferation in *A. thaliana* Col-0 *gl* plants. Bacteria were syringe-infiltrated into Col-0 *gl* plants at 10^6 cfu/ml. Bacterial growth was monitored after three days. Each bar represents the mean titer of 12 leaf discs from three individual leaves. Error bars were created using standard deviation.

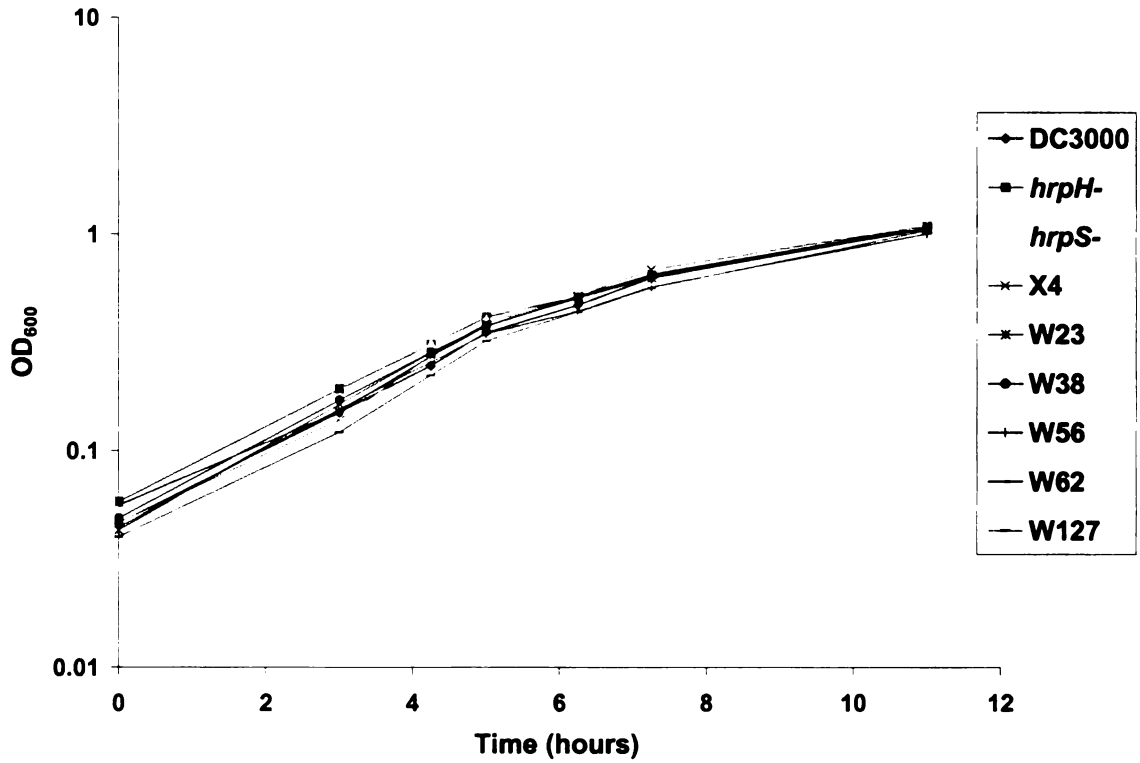

Figure 3-5. Growth of *Pst* DC3000 mutants in LB medium. Bacteria were grown in LB medium at 30°C for 11 hours. The OD_{600} was measured at various time points. The data points represent the mean OD_{600} values from three readings at each time point. Error bars were created using the standard deviation. *Pst* DC3000 mutants *hrpH* and *hrpS* were included as controls.

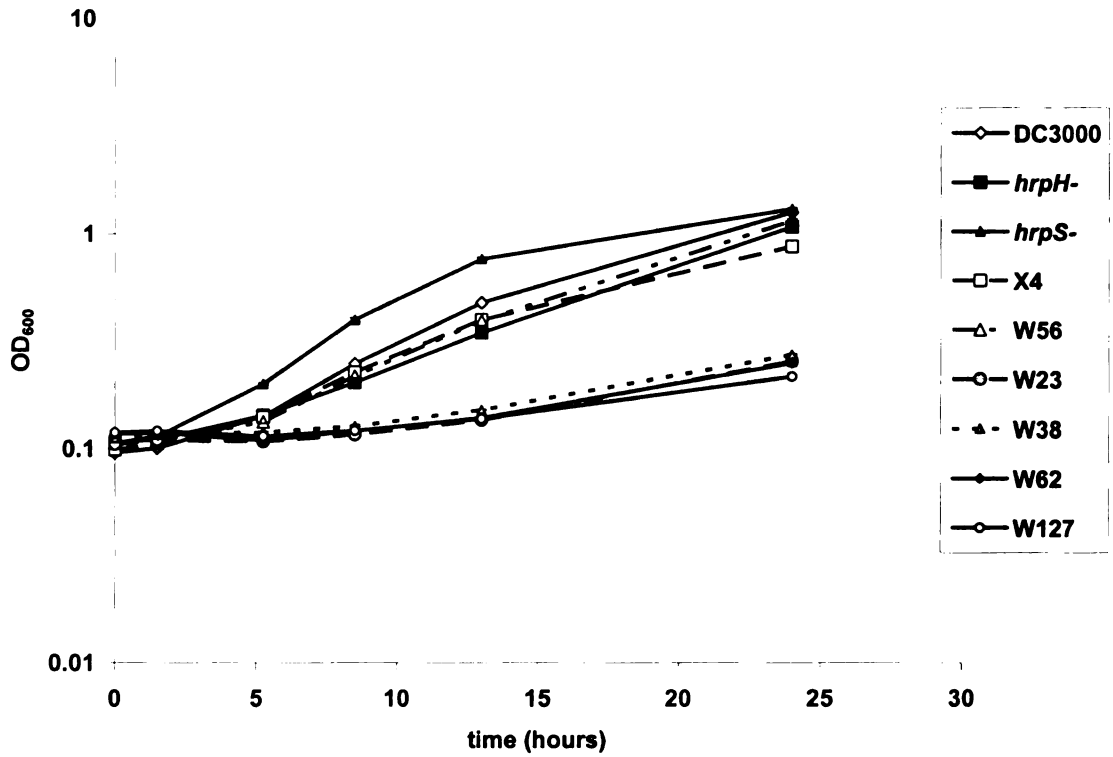

Figure 3-6. Growth of *Pst* DC3000 mutants in MM. Bacteria were grown in MM at 22°C for 24 hours. The OD_{600} values were determined at various time points. The data points represent the mean OD_{600} values from three readings at each time point. Error bars were created using the standard deviation. *Pst* DC3000 mutants *hrpH* and *hrpS* were included as controls.

Identification of the insertionally-inactivated genes

To identify genes mutated in the six mutants, genomic DNA was isolated from each mutant and digested with *Pst*I which does not cut within the transposon. Southern blot analysis with the *uidA* gene as a probe was performed to determine the number of transposon insertions and to estimate the size of the *Pst*I fragments which contain the transposon insertion. The result of the Southern blot analysis, suggesting a single insertion in each mutant, is shown in Figure 3-7. *Pst*I fragments of estimated size transposon were extracted from an agarose gel, purified, and ligated into pBluescript KS+. In order to isolate these fragments which contained the transposon, the transformants were selected on LB Km plates. Resistance to Km is encoded within the transposon. This method prevents the cloning and sequencing of non-transposon-containing *Pst*I fragments of a similar size. The recombinant plasmids containing the transposon-containing fragments were isolated from Km-resistant transformants, the DNA inserts were sequenced with a reverse *uidA* prime to obtain the sequence directly upstream of the transposon insertion site. The sequence similarities are shown in Table 3-1. Four of the six mutants (W23, W38, W62, W127) isolated contained transposon insertions in the *oprF* gene. Two of the mutants (W23 and W38) contained insertions at the same site within the *oprF* gene. Thus, three independent insertion events in the *oprF* gene were found. Predicted annotation for these *Pst* DC3000 genes can be found in Appendix B.

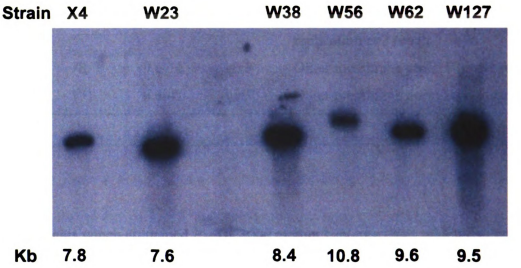

Figure 3-7. Southern blot analysis of the genomic DNA from *Pst* DC3000 mutants. Ten μg of genomic DNA was digested with *Pst*I, which does not cut within the transposon. ^{32}P-labelled *uidA* probe was used in the hybridization. The *Pst* DC3000 mutant strain from which the genomic DNA was isolated is listed above each lane. The estimated size of the transposon-containing *Pst*I fragments is shown below each lane.

Table 3-1. Summary of the identification of the insertionally-inactivated genes.

Strain	**BlastX Score**	**Blast E-value**	**Gene**	**Predicted Protein**
X4	163	2 e-39	*ptsP*	Enzyme I of the phosphoenolpyruvate protein phosphotransferase system
W23	160	2 e-38	*oprF*	Outer membrane porin F precursor
W38	109	5 e-22	*oprF*	Outer membrane porin F precursor
W56	180	3 e-64	*uvrD*	Type II helicase involved in DNA replication and repair
W62	78	9 e-14	*oprF*	Outer membrane porin F precursor
W127	171	6 e-42	*oprF*	Outer membrane porin F precursor

Transformants which contained *Pst* DC3000 mutant genomic DNA which conferred Km resistance were sequenced using a reverse *uidA* primer. BlastX was then used to determine the identity of the sequences. The BlastX score, expectation (E-value), gene name and putative protein identity are given above.

Complementation of mutations

The *Pst* DC3000 genome was used to find the genomic sequence around the putative insertionally-inactivated genes. The region downstream of the *oprF* gene contains a predicted gene in the opposite orientation to the *oprF* gene. Thus, *oprF* is probably the last gene in the operon. Analysis of the region surrounding the *uvrD* gene and the *PtsP* gene revealed that one open-reading frame (ORF) downstream of *uvrD* and two ORFs downstream of *PtsP* were in the same orientation. There was no strong evidence for either a rho-independent terminator or a Shine-Dalgarno sequence in either of these regions. Based on this information, I designed primers to clone the insertionally-inactivated genes with and without the downstream ORFs. The genes were PCR-amplified, cloned into pUCP18, and transformed into the corresponding mutants. Although the *oprF* gene was amplified, it could not be cloned despite repeated attempts. Thus, the putative *oprF* mutants W23, W38, W62 and W127 were not complemented. The genomic region containing the *uvrD* gene and the putative downstream ORFs could not be PCR-amplified. However, the W56 mutant was complemented by the *uvrD* gene alone. The presence of a functional *uvrD* gene restores the *in planta* growth of this mutant to wildtype levels (Figure 3-8). In addition, the *uvrD* gene alone could restore the UV tolerance of the W56 mutant. The *in vitro* growth of *Pst* DC3000, W56, and W56 with *uvrD* after UV exposure is shown in Figure 3-9. The X4 mutant, which shows a ten-fold reduction in growth as well as significant reduction of symptoms *in planta*, was complemented by the *ptsP* gene. The *ptsP* gene is likely present in an operon, however the *ptsP* gene alone was capable of restoring wildtype levels of *in planta* growth to the

X4 mutant, indicating that the *ptsP* gene alone is responsible for the mutant phenotype (Figure 3-10).

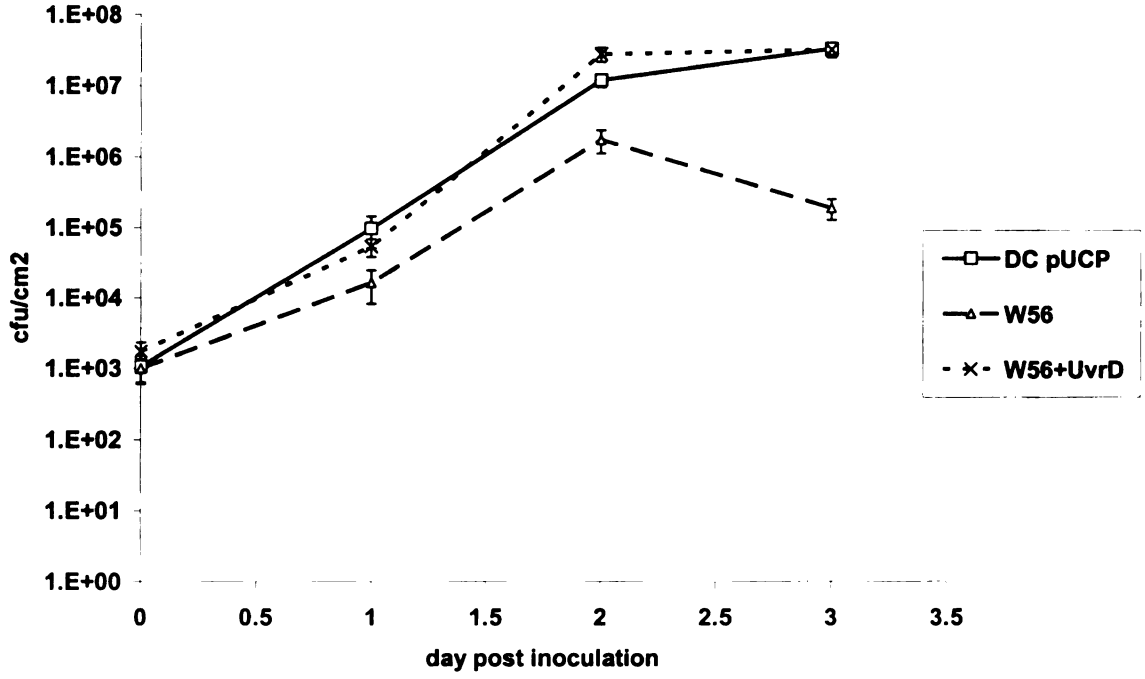

Figure 3-8. Complementation of *in planta* growth of the W56 mutant by the *uvrD* gene. Bacteria were syringe-infiltrated into Col-0 *gl* plants at 10^6 cfu/ml. Bacterial growth was monitored over three days. Each datum point represents the mean titer of 12 leaf discs from three individual leaves. Error bars were created using standard deviation. DC+pUCP represents *Pst* DC3000 with the empty vector pUCP18. W56 represents the *Pst* DC3000 mutant W56. W56+uvrD represents the *Pst* DC3000 W56 mutant with pUCP18 with the *uvrD* gene.

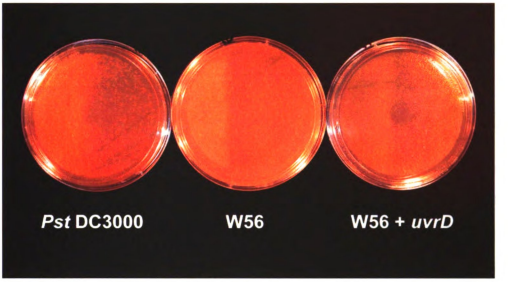

Figure 3-9. Complementation of UV tolerance of the W56 mutant by the *uvrD* gene. Bacteria were plated onto LB Rif Ap plates with 10^6 cfu/ml. One half of each plate was exposed to 5 second of UV light. The plates were then allowed to grow at 30°C for 2 days. *Pst* DC3000 represents the wildtype bacteria. W56 represents the W56 mutant. W56 + *uvrD* represents the *Pst* DC3000 W56 mutant with pUCP18 with the *uvrD* gene.

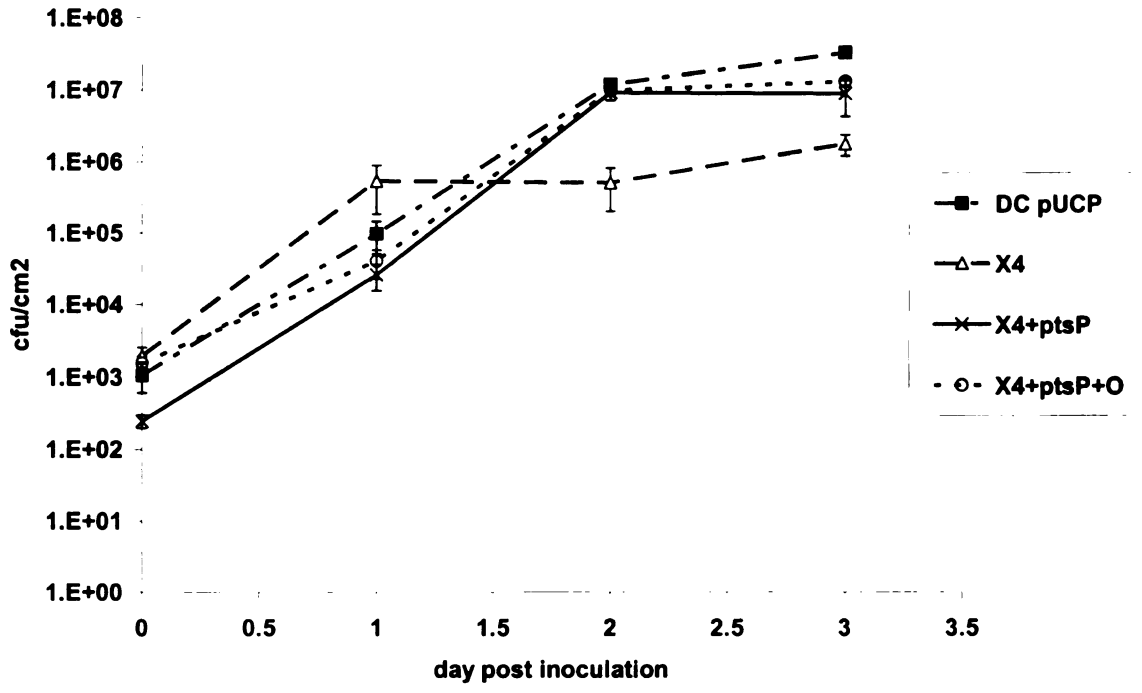

Figure 3-10. Complementation of the X4 mutant by the *ptsP* gene. Bacteria were syringe-infiltrated into Col-0 *gl* plants at 10^6 cfu/ml. Bacterial growth was monitored over three days. Each datum point represents the mean titer of 12 leaf discs from three individual leaves. Error bars were created using standard deviation. DC+pUCP represents *Pst* DC3000 with the empty vector pUCP18. X4 represents the X4 mutant. X4+EI represents the X4 mutant with pUCP18 with the *ptsP* gene. X4+EI+O represents the *Pst* DC3000 X4 mutant with pUCP18 with the *ptsP* gene and the downstream ORFs.

Discussion

The intended purpose of this study was the identification of novel virulence genes, especially type III effector genes, which would be expected to be expressed in MM. Therefore, it is surprising that no mutations in the previously described *hrp* genes were found. These genes are known to be *hrp*-regulated and should be expressed in *hrp*-inducing minimal media but not rich King's B media. In addition, mutations in many of the *hrp* genes show a loss of pathogenicity. However, the overall expression level of these genes may be low compared to the genes identified in this study. Perhaps the visual screening that we used in this study was not sensitive enough to discern the subtle *uidA* expression which would result from a mini-Tn5 *uidA* Km insertion into a *hrp* gene.

Despite the fact that no novel type III effector genes were found in this study, the mutants identified still provide insight into the process of *Pst* DC3000 pathogenesis. There were three independent insertion events into the *oprF* gene (W23/W38, W62 and W127). The *oprF* gene encodes the precursor of the outer membrane protein F. This protein has been of interest in the study of mammalian bacterial pathogens because it is an outer membrane protein and may be a target for recognition by the host immune system (Knapp *et al.*, 1999). The ability of the W23/W38, W62 and W127 mutants to be complemented by the DC3000 *oprF* gene could not be determined because positive transformants could not be obtained in *E. coli*. A literature search indicates that this is not an uncommon problem. Other studies which have complemented *oprF* mutants have had to clone the *oprF* gene with a weakened promoter or a mutated promoter to reduce the expression of the o*prF* gene (Brinkman *et al.*, 1999; Rawling *et al.*, 1998).

Overexpression of the *oprF* gene is apparently lethal. Despite the fact that the W23/W38, W62 and W127 mutants could not be complemented, the presence of three independent mutations in this gene does reinforce the idea that this gene is important for virulence. Studies of *oprF* mutants of a related bacteria, *Pseudomonas aeruginosa*, hint at the role that OprF may play in *Pst* DC3000 virulence. *P. aeruginosa* is a Gram-negative bacterial pathogen which can infect the lungs of immune-compromised individuals and cystic fibrosis patients (Oliver *et al.*, 2000). *P. aeruginosa oprF* mutants have an altered cell morphology. In addition, these mutants have a reduced ability to grow in media with low osmolarity (Rawling *et al.*, 1998). This is intriguing especially in light of the observed inability of the W23/W38, W56 and W127 mutants to grow in MM while their growth in rich media is comparable to that of *Pst* DC3000. While the environmental conditions in the apoplastic space where *Pst* DC3000 resides during infection are unknown for the most part, it has been suggested that this environment may not be rich in nutrients and/or water. The reduced virulence phenotype observed in the W23/W38, W56 and W127 mutants suggests that the apoplastic space may be a low osmolarity environment, similar to MM. There has also been a study which shows that *P. aeruginosa oprF* mutants exhibit a reduced ability to bind to human cells *in vitro* (Azghani *et al.*, 2002). The OprF protein of *P. fluorescens* has been shown to bind to plant roots (DeMot *et al.*, 1992). The mechanism by which *Pst* DC3000 binds to host plant cells within the apoplastic space is not known. The Hrp pilus of the type III protein secretion system may play a role in this process, but this has not been proven. The reduced virulence of the W23/W38, W56 and W127 mutants in *A. thaliana* leaves provides an alternative hypothesis. The development of new microscopic techniques with fluorescent-labeled bacteria now allows for the

visualization of bacteria within the apoplastic space (Badel *et al.*, 2002). These techniques could be used to observe the course of infection with the *oprF* and *hrp* mutants, compared to that of wildtype *Pst* DC3000, and determine if the *oprF* or *hrp* mutants are unable to adhere to host plant cells.

Another mutant isolated in this screen was the W56 mutant which lacks a functional *uvrD* gene. The *uvrD* gene encodes a type II DNA helicase which is required for unwinding the DNA helix during DNA replication and repair. Bacterial mutants which lack the *uvrD* gene have a higher mutation rate than wildtype bacteria, especially in response to mutagens such as UV light (Oliver *et al*, 2002). The *Pst* DC3000 W56 mutant also showed an increased sensitivity to UV light. In mammalian pathogens, the mutation rate has been shown to play an important role in pathogenesis. In *P. aeruginosa*, the loss of the *mutS, mutL* and *uvrD* genes (which enable DNA repair functions) confers an advantage in virulence over time, on a population level (Oliver *et al.*, 2002; Oliver *et al.*, 2000). The advantage accorded by a higher mutation rate is thought to be the result of co-evolution between pathogens and host. The mammalian immune system is able to recognize certain antigenic pathogen proteins. A higher mutation rate confers an advantage to a pathogen because it allows the pathogen's surface antigens to change and evade immune recognition. However, in our case the W56 mutant showed reduced virulence in a three day period and we have not followed the W56 mutant through many generations or cycles of infection.

There is evidence to suggest that UV tolerance is important for the epiphytic phase of *P. syringae* growth. As the bacteria exist on the surface of leaves, they face an onslaught of UV light (Sundin and Jacobs, 1999). *rulA* mutants of *P. syringae* showed

reduced tolerance to UV light and are not as competitive as wildtype bacteria in the phyllosphere (Sundin *et al.*, 2000). The *rul* operon is often located on plasmids of the pPT23A family which frequently encode genes involved in host-pathogen interactions (Sundin *et al.*, 2000).

The inoculation methods used in this study essentially bypassed phyllospheric growth because the bacteria were infiltrated directly into the apoplastic space. It is possible however, that some UV light is permeating the leaf and entering the apoplast. This would then impact the growth of the pathogen. Another possibility is that this mutant is less able to handle stresses such as those caused by exposure to plant defense compounds present in the apoplastic space. Preliminary evidence indicates that the W56 mutant grows to a higher level in the NahG transgenic host (data not shown), which lacks salicylic acid-mediated host defenses (Hunt *et al.*, 1996). Further experiments are needed to determine the role that *uvrD* plays in *Pst* DC3000 virulence *in planta*.

Finally, the X4 mutant contains an insertion in the *ptsP* gene which encodes a component of the phosphoenolpyruvate protein phosphotransferase system (PTS). The PTS is a sugar uptake system which has been well characterized in *E. coli* (Ginsburg and Peterkofsky, 2002; Postma *et al.*, 1993). The *ptsP* gene encodes the Enzyme I subunit of the PTS. Enzyme I is biologically active as a dimer. In the presence of Mg^{+}, it is able to self-phosphorylate. This phosphoryl moiety can then be transferred to the HPr carrier protein, then transferred to the Enzyme II subunit, and finally transferred to the sugar as it is transported into the bacterial cell. Several Enzyme II subunits have been characterized and they seem to be designed for specific sugars such as glucose, mannose, mannitol and cellobiose. The PTS has also been implicated in processes other than sugar uptake, such

as catabolite repression and chemotaxis. In two other bacterial pathogens, mutations in the *ptsP* gene have led to a reduced virulence phenotype. In a multi-host strain of *P. aeruginosa*, the loss of the *ptsP* gene leads to a loss of virulence on *Caenorhabditis elegans* and a loss of pathogenicity on burnt mice (Tan *et al.*, 1999). *Legionella pneumophila ptsP* mutants are also impaired in pathogenecity (Higa and Edelstein, 2001). In *Azotobacter vinelandii*, the *ptsP* gene has been shown to be required for the production of 8-polyhydroxy butyrate (Segura and Espin, 1998). The mechanisms by which nutrients are released from the plant host or obtained by the bacterium are not currently known. The reduced virulence of the X4 mutant may indicate that the PTS plays a role in nutrient uptake by bacteria. In addition, the reduction in virulence may stem from a loss of catabolite repression or chemotaxis which could be required for pathogen growth within the apoplastic space.

Overall, this study has identified three novel *Pst* DC3000 genes, mutations in which cause a reduction in virulence on *A. thaliana*. The role that these genes, and the proteins which they encode, play in virulence will be the focus of future studies. These novel mutants may provide a window into previously unknown aspects of *Pst* DC3000 virulence, such as the transition from epiphytic growth on the leaf surface to pathogenic growth in the leaf apoplast, uptake of nutrients, and the tolerance of plant defense compounds.

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Chapter 4

Characterization of transgenic *Arabidopsis thaliana* plants that express the AvrE effector of *Pseudomonas syringae* pv. *tomato* strain DC3000

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Abstract

Pseudomonas syringae pv. *tomato* strain DC3000 (*Pst* DC3000) is the causal agent of bacterial speck disease of tomato and *Arabidopsis thaliana*. Effector proteins secreted by the type III protein secretion system have been implicated in *Pst* DC3000 virulence. AvrE is one effector produced by *Pst* DC3000. Bacterial genetic studies have failed to determine the role that AvrE plays in *Pst* DC3000 virulence, purportedly due to functional redundancy among effector proteins. We utilized an inducible promoter system to express *avrE* with the PR1-b tobacco signal sequence in *A. thaliana*. These transgenic plants displayed symptoms similar those caused by *Pst* DC3000 infection after transgene induction. These symptoms included water-soaking followed by the development of necrotic spots surrounded by chlorotic haloes. The water-soaking phenotype was correlated with stomatal closure. These transgenic plants supported increased growth of the normally non-pathogenic *hrpH* mutant of *Pst* DC3000. This study provides evidence that AvrE does contribute to the virulence of *Pst* DC3000.

Introduction

Plant pathogens have a significant impact on agriculture. Therefore, a great deal of study regarding plant-pathogen interactions has focused on understanding plant resistance. One important resistance mechanism is gene-for-gene resistance (Flor, 1971), which occurs when a pathogen harboring a given *avr* gene attempts to infect a plant expressing the corresponding *R* gene. A hypersensitive response (HR) will then occur. The HR is a form of programmed cell death which is thought to limit the spread of the bacterial pathogen, although how this happens mechanistically is not understood.

Our lab focuses on understanding the virulence role of Avr proteins in *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000). This bacterial pathogen is the causal agent of bacterial speck in both *Arabidopsis thaliana* and tomato. In this study, we have chosen to determine the role of AvrE in virulence of *Pst* DC3000 on *A. thaliana*. A *Pst* DC3000 mutant in which the *avrE* gene has been replaced with an antibiotic marker cassette shows no reduction in virulence on *A. thaliana* (He, unpublished data). However, it has been proposed that the effectors of *Pst* DC3000 are functionally redundant. Therefore, traditional bacterial mutagenesis would not be amenable for determining the role that a given effector plays in virulence.

If the only role of an *avr* gene was to trigger host resistance, *avr* genes should not be maintained in the bacterial genome. This suggests that the *avr* genes play a role in promoting bacterial fitness. In support of this speculation, many Avr proteins have now been shown to be secreted from bacteria via the type III secretion system (Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002), which is often essential for bacterial pathogenesis (He, 1998; Galan and Collmer, 1999). Furthermore, several bacterial *avr* genes have

been shown to be required for pathogen growth in susceptible hosts (Chen *et al.*, 2000; Kearney and Staskawicz, 1990; Ritter and Dangl, 1995).

While there is no direct evidence that AvrE promotes *Pst* DC3000 virulence, there is correlative evidence. *avrE* is required for full virulence in *Pst* strain PT23 on tomato (Lorang *et al.*, 1994). Loss of DspE, an effector which shares sequence similarity with AvrE, abolishes pathogenicity of *E. amylovora* on apple and pear (Bogdanove *et al.*, 1998; Gaudriault *et al.*, 1997; Tharaud *et al.*, 1994). AvrE is a type III effector protein. The promoter of the *avrE* gene contains a classic canonical *hrp*-box motif and it is co-regulated with the *hrp* genes in a *hrpS*- and *hrpL*-dependent manner (Fouts *et al.*, 2002; Lorang and Keen, 1995; Zwiesler-Vollick *et al.*, 2002). It is secreted by the type III protein secretion system *in vitro* and is capable of promoting the translocation of secretion-incompetent but biologically-active AvrRpt2 (Guttman *et al.*, 2002). In *Pst* DC3000, *avrE* is linked to the *hrp* gene cluster, in a locus known as the conserved effector locus (CEL). A mutant which lacks four of the effectors from this region, including AvrE, shows a significant reduction in virulence on both *A. thaliana* and tomato hosts (Alfano *et al.*, 2000).

Several recent studies have focused on the identification of new phytobacterial type III effector proteins (Boch *et al.*, 2002; Fouts *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002). These studies indicate that there are many more putative effectors than previously predicted among the phytopathogenic *Pseudomonads*. Even within an individual subspecies, such as *Pst* DC3000, there are a remarkable number of putative effectors, at least 36 (Collmer *et al.*, 2002). If some of these effectors are, as previously suggested, functionally redundant,

few bacterial studies will be able to demonstrate the subtle advantage conferred by any specific effector protein. For this reason we have begun to assess the impact of production of a single effector *in planta*.

In this study, we present further evidence that AvrE contributes to *Pst* DC3000 virulence. If *Pst* DC3000 contains another virulence factor which is able to perform the same function as AvrE, bacterial mutational studies would be unlikely to yield information about the role of AvrE. Thus, we expressed the AvrE protein transgenically in *A. thaliana*. This approach investigated the effect of AvrE within the host plant cell, independent of the role that other effectors might play. We were therefore able to examine the effect of a single effector, AvrE, on *A. thaliana* health, appearance, and ability to sustain growth of a normally non-pathogenic bacteria, the *Pst* DC3000 *hrpH* mutant.

Materials and Methods

Generation of Transgenic plants

Pst DC3000 genomic DNA was extracted (Chen and Kuo, 1993). Polymerase chain reaction was used to amplify *avrE* using eLONGase polymerase (Gibco Cat # 10480028) according to manufacturer's instructions. The primers used were: *ssavrE* Forward 5' GCGGATCCCAGTCACCATCGATCCACCG 3'

avrE Forward 5' CCGCTCGAGACCATGGAGTCACCATCGATCCACCG 3'

avrE Reverse 5'GACTAGTTTCGTTATTAGCTCTTCAGTTCG 3'. The *avrE* gene of *Pst* DC3000 was cloned into the pTA7002 vector (Aoyama and Chua, 1997; McNellis *et al.*, 1998) with and without the tobacco PR1-b signal sequence (*ss*) (PR1-b Forward 5' CCGCTCGAGACCATGGGATTTTTTCTCTTTTCACAAATGCCCTCATTTTTTCTT GTCTCTACACTTCTC 3'

PR1-b Reverse 5'

CGGGATCCAGAGTTTTGGGCATGAGAAGAGTGAGATATTATTAGGAATAAGA GAAGTGTAGAGACAAG 3'). pTA7002 allows for inducible expression of transgenes after application of the animal glucocorticoid hormone, dexamethasone (DEX). The recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain GV3850 by electroporation (Keen *et al.*, 1990). Four pots of *Arabidopsis thaliana* Col-0 *gl* plants were transformed with *A. tumefaciens* carrying pTA7002-*avrE* or pTA7002-*ssavrE* via vacuum infiltration (Bechtold *et al.*, 1993). Seeds collected from the pots were kept separate to ensure that independently transformed lines could be isolated. Seeds were vapor-sterilized by incubation in a dessicator for 16 hours with 100ml of bleach mixed

with 3ml of concentrated HCl. Transformants were selected on Murashige-Skoog (MS) plates supplemented with 1x vitamins and 40 units/ml hygromycin B (hyg) (Calbiochem Cat # 400051).

Southern blot analysis

Genomic DNA from Col-0 *gl* and putative transgenic plants was extracted by grinding a single leaf in 400 µl extraction buffer (100mM Tris pH 8.0, 10 mM EDTA, and 1.0% SDS) followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). DNA was digested with *Bam*HI (NEB Cat # R0136) with overnight incubation at 37°C. Ten µg of digested DNA were separated on a 1.0% agarose gel, at 60 mV for approximately 2 hours. The bands of the DNA ladder were identified in the gel under UV light and a Pasteur pipet was used to remove part of each band. The gel was then treated with 0.25 M HCl for 10 minutes, and rinsed in water. The digested DNA was then transferred to nitrocellulose membrane via capillary action in 0.4 M NaOH (Sambrook *et al.*, 1989). The lanes of the gel, as well as the bands of the DNA ladder, were marked on the membrane. ^{32}P-labelled probe was prepared (Sambrook *et al.*, 1989) with approximately 100 ng of an internal *avrE* PCR product (Intra *avrE* Forward 5' GCCCCCGCCACCCACCGCCGC 3' and Intra *avrE* Reverse 5' CGGAGGCCTTCCCCCGGACTC 3') and purified with Bio-Rad columns (Cat #732-6223) according to manufacturer's instruction. The approximate size of the fragment which hybridized to the probe was estimated by comparison with the marked bands of the ladder.

Dexamethasone-induction of transgene expression

A 30mM stock of dexamethasone (Sigma Aldrich Cat # D1756) (DEX) was made up in 100% ethanol. This stock solution was diluted 1000-fold in water to make 30μM DEX. The transgene was induced by either infiltrating the leaves with a needleless syringe or by spraying the leaves with the DEX solution.

Northern blot analysis

A. thaliana Col-0 *gl* and transgenic plants were sprayed with 30μM DEX. Tissue was collected at selected time points between 0 hours and 36 hours and snap-frozen in liquid N_2. *A. thaliana* RNA was isolated using the Promega RNAgents kit (Cat # Z5110) according to manufacturer's instructions. Twenty μg RNA were denatured with two volumes of loading buffer (500 μl formamide, 170 μl formaldehyde, 100 μl 10X MOPS buffer, and 10 μl of 1 mg/ml ethidium bromide) for ten minutes at 65°C. The RNA was separated on a formaldehyde agarose gel and transferred to nitrocellulose membrane via capillary transfer (Sambrook *et al.*, 1989). Probe labeling and hybridization were performed as above. ^{32}P-labeled *eIF4*a (eukaryotic initiation factor 4a) probe was used to assess the amount of sample loaded.

Bacterial culture conditions

Bacterial cultures were cultured at 30°C in Luria-Bertani (LB) medium (Katagiri *et al.*, 2002) supplemented with appropriate antibiotics. Rifampicin (Rif) was used at a concentration of 100 mg/L. Ampicillin (Ap) was used at a concentration of 100 mg/L.

Pathogenesis assays

A. thaliana (Col-0 *gl* and transgenic) plants were grown in growth chambers at 20°C with 70% humidity, a light intensity of 100 μEinsteins, and a 12-hour photoperiod. Bacteria were grown to approximately OD_{600}=0.4 to 0.8. Bacteria were then centrifuged in a tabletop centrifuge at 3,000 rpm (approximately 2,000xg) for 10 min. The supernatant was decanted and the bacterial pellet was resuspended in sterile water to OD_{600}=0.002 (10^6 cfu(colony forming units)/ml). The leaves of 6-week-old *A. thaliana* (Col-0 *gl*) plants were infiltrated with the bacterial suspension using a needless syringe. Infected plants were kept under high humidity at approximately 25°C. After three days the symptoms were recorded. Typical symptoms were characterized by water-soaking, at approximately two days post infiltration followed by the development of necrotic spots surrounded by chlorotic haloes at about 3 days after infiltration. Quantification of bacterial growth was performed as in Katagiri *et al.* (2002). The bacterial strains used in this study were *Pst* DC3000, the *hprH* mutant, and *Pst* DC3000 expressing the *avrRpt2* gene. The *hrpH* mutant was previously described by Yuan and He (1996). The *Pst* DC3000 expressing the *avrRpt2* gene was previously described (Zwiesler-Vollick *et al* 2002).

Evaluation of stomatal opening

A. thaliana Col-0 *gl* and *ssavrE* plants were kept under a high humidity dome. Leaves were observed at various time points. Leaves were removed from the plants and

sliced in half with a razor blade to remove the mid-rib vein. The leaf halves were placed ventral side up on a microscope slide. A bead of water was added to each leaf and a cover slip was place on the leaf halves. Stomata were observed with a Reichert Microstar IV microscope at 40X magnification. At least 100 stomata from one leaf were examined and rated as either open or closed. Col-o *gl* stomata which were less than completely open were evaluated as closed. *ssavrE* stomata which were showed any space between the two guard cells were evaluated as open. This stringent evaluation method would underestimate the stomatal closure seen in the *ssavrE* transgenic plants. The entire process, from the removal of the leaf from the plant to the completion of the counting, took less than 15 minutes.

Results

Generation of transgenic plants and examination of expression of *ssavrE*

To ascertain the function of the AvrE protein in *Pst* DC3000 virulence, *avrE* was transformed into susceptible *A. thaliana* plants with and without the tobacco PR-1b signal sequence (*ss*). Transformants were selected on MS medium supplemented with hyg. Despite four independent transformation attempts, no transgenic plants containing *avrE* without the signal peptide could be obtained. In contrast, 21 *ssavrE* transgenic lines were obtained. Three independent *ssavrE* transgenic lines (2-9, 3-1, and 4-5) were chosen for further study. Results from studies with the representative line *ssavrE* 2-9 will be presented in this chapter. Southern blot analysis with *Bam*HI-digested DNA indicated that these plants contain *avrE* hybridizing bands (See Figure 4-1). Northern blot analysis showed that the *ssavrE* mRNA was produced after exposure of plants to DEX. A time course showed that the *ssavrE* mRNA could be detected as early as 12 hours after 30 μM DEX application and remained detectable until 36 hours after 30 μM DEX application (see Figure 4-1). The *ssavrE* mRNA levels before 12 hours and beyond 36 hours after 30 μM DEX exposure were not determined.

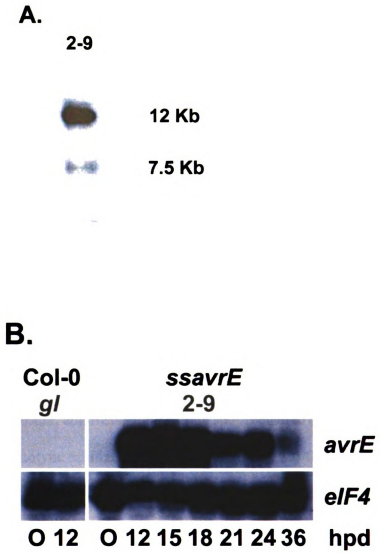

Figure 4-1. Southern blot and northern blot analyses of *ssavrE* transgenic plants. A. Southern blot analysis of *Bam*HI digested *ssavrE* 2-9 genomic DNA hybridized with ^{32}P-labeled *avrE*. The approximate size of the hybridizing bands is indicated. B. Northern blot analysis of total RNA extracted from Col-0 *gl* and *ssavrE* 2-9 transgenic plants. The plants were sprayed with 30μM DEX. hpd indicates the hour post-DEX application when the tissue was collected. *avrE* indicates that the blot was hybridized with ^{32}P-labeled *avrE*. *eIF4* indicates that the blot was hybridized with ^{32}P-labeled *eIF4*.

ssavrE transgenic plants show two distinct phenotypes

The *ssavrE* transgenic plants were slightly smaller than the parent plants but otherwise showed no obvious morphological differences from their Col-0 *gl* parents in the absence of DEX (see Figure 4-2). After treatment with 30 μM DEX, the *ssavrE* plants displayed a distinct phenotype. If the plants were treated with DEX and allowed to remain at ambient humidity, the leaves began to show signs of chlorosis by 36 hours after spraying. By 48 to 72 hours the leaves which were chlorotic had necrosed and collapsed (Figure 4-2). If, however, the plants were placed under a high humidity dome after exposure to 30 μM DEX, the phenotype was slightly altered. These plants showed water-soaking at 6 to 12 hours after DEX exposure. Then at 36 to 48 hours after DEX exposure, chlorosis with necrotic spots began to develop (see Figure 4-2). This phenotype mirrors the symptoms caused by *Pst* DC3000 infection of *A. thaliana*.

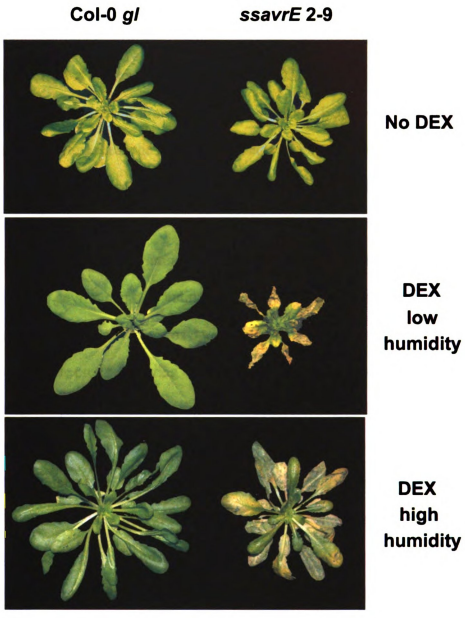

Figure 4-2. Phenotypes of *ssavrE* transgenic plants. Experiments were conducted when plants were approximately 6-weeks-old. The plants shown in the panel labeled "No DEX" were not treated with DEX. The plants shown in the panel labeled "DEX low humidity" were sprayed with 30 μM DEX and left uncovered for two days before pictures were taken. The plants shown in the panel labeled "DEX high humidity" were sprayed with 30 μM DEX and covered with a humidity dome for three days before pictures were taken.

DEX-induction of *ssavrE* causes stomatal closure

Six hours after DEX-induction, *ssavrE* transgenic plant leaves could not be vacuum-infiltrated efficiently with bacterial suspension containing surfactant L-77 nor with water and surfactant L-77. In contrast, DEX-induced Col-0 *gl* plants were able to be efficiently vacuum-infiltrated with either solution. Because vacuum-infiltration relies upon open stomata as the sole point of bacterial entry into the apoplastic space, I decided to look at the stomatal aperture after DEX-induction in Col-0 *gl* and *ssavrE* transgenic plants. Stomatal counting was completed within fifteen minutes after the leaves were removed from the high humidity environment to prevent the stomata from reacting to the change in environmental conditions. For each plant and treatment, 100 stomata per leaf were counted and evaluated as open or closed. The data collected are shown in Table 4-1. A sample picture showing the difference between DEX-induced Col-0 *gl* and *ssavrE* transgenic plants is shown in Figure 4-3. While the majority of stomata in the DEX-treated Col-0 *gl* leaves kept under high humidity were open, the majority of stomata in DEX-treated *ssavrE* leaves were closed. This phenotype is dependent on the application of DEX. Both the Col-0 *gl* and *ssavrE* transgenic plants have the majority of their stomata open if plants are kept under high humidity after spraying with water. The DEX-induced closure of stomata could only be observed in a high humidity environment. Under low humidity, most of the stomata of Col-0 *gl* plants were closed. To determine if this phenomenon is also produced by *Pst* DC3000 infection, Col-0 *gl* leaves which had been infiltrated with *Pst* DC3000 or the *hrpH* mutant were examined under high humidity. Two days after infection, water-soaking could be observed in the *Pst* DC3000

infected plants, but not in the *hrpH* infected plants. At this time point, approximately half of the stomata in the *hrpH* infected plants are closed, while approximately 75% of the stomata in *Pst* DC3000 infected plants are closed (Table 4-2). *ssavrE* expression *in planta* results in a phenotype which is reminiscent of symptoms triggered by *Pst* DC3000 infection. Because both water-soaking and stomatal closure may affect the water relations within the leaves, I checked to see if the stomatal closure preceded the appearance of visible water-soaking under high humidity. Three hours after spraying with DEX, no visible water-soaking could be seen. However, the majority of the stomata in the *ssavrE* transgenic plants were closed at this time point, in contrast to the Col-0 *gl* plants in which only half the stomata were closed (Table 4-3). Finally, I wanted to determine how stomata react to the presence of excess water in the apoplastic space. For this purpose, Col-0 *gl* leaves were infiltrated with water and kept under high humidity. The leaves were then monitored for an hour. After an hour, almost all of the stomata of these leaves were open (Table 4-4).

Table 4-1. DEX-induction of *ssavrE* causes stomatal closure

Experiment	Treatment	Plant	Open stomata	Closed Stomata
1	Mock	Col-0 *gl*	64%	36%
1	Mock	*ssavrE* 2-9	62%	38%
1	30 μM DEX	Col-0 *gl*	67%	33%
1	30 μM DEX	*ssavrE* 2-9	30%	70%
2	30 μM DEX	Col-0 *gl*	73%	27%
2	30 μM DEX	*ssavrE* 2-9	26%	74%
3	30 μM DEX	Col-0 *gl*	43%	57%
3	30 μM DEX	*ssavrE* 2-9	24%	76%

The plants were sprayed with 30 μM DEX or sprayed with water (mock) as indicated. The plants were kept under humidity domes and each row represents at least 100 stomata from one leaf observed at six hours after treatment. Three experiments were conducted with separate sets of sprayed plants on different days.

Table 4-2. *Pst* DC3000 infection of Col-0 *gl* causes stomatal closure

Experiment	Bacteria	Open Stomata	Closed Stomata
1	*hrpH*	58%	42%
1	*hrpH*	46%	54%
1	*Pst* DC3000	33%	67%
1	*Pst* DC3000	34%	66%
2	*hrpH*	49%	51%
2	*Pst* DC3000	19%	81%

The plants were syringe-infiltrated with either the non-pathogenic *hrpH* mutant or *Pst* DC3000 at 10^6 cfu/ml. The observations were made two days after bacterial inoculation when the *Pst* DC3000 plants showed water-soaking symptoms. Each row represents at least 100 stomata from one leaf. Two experiments were conducted with separate sets of infiltrated plants on different days.

Table 4-3. Time course of stomatal response.

Time (hours)	Plant	Open Stomata	Closed Stomata
0	Col-0 *gl*	25%	75%
0	*ssavrE* 2-9	34%	66%
3	Col-0 *gl*	44%	56%
3	*ssavrE* 2-9	24%	76%
6	Col-0 *gl*	54%	46%
6	*ssavrE* 2-9	30%	70%

The plants were sprayed with 30 μM DEX and stomata were observed at 0, 3, and 6 hours after DEX exposure. Each row represents at least 100 stomata from one leaf.

Table 4-4. Artificial water-soaking of Col-0 *gl* leaves causes stomatal opening.

Time (minutes)	Open Stomata	Closed Stomata
0	72%	28%
0	64%	36%
15	89%	11%
15	88%	12%
30	93%	7%
30	91%	9%
45	96%	4%
45	95%	5%

The plants were syringe-infiltrated with sterile water. The stomata were observed at 0, 15, 30, and 45 minutes after infiltration. Two leaves were used for each time point.

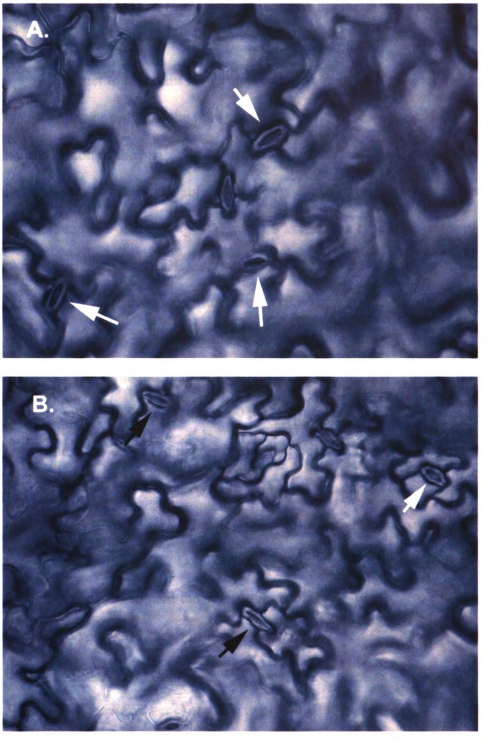

Figure 4-3. The majority of the stomata in the *ssavrE* plants are closed under high light and humidity. Pictures were taken eight hours after spraying with 30 μM DEX. Plants were kept in the light under a high humidity dome. A. Col-0 *gl* plants treated with 30 μM DEX. B. *ssavrE* 2-9 plants treated with 30 μM DEX. Black arrows indicate closed stomata. White arrows indicate open stomata.

DEX-induction of *ssavrE* promotes enhanced bacterial growth

In order to ascertain the virulence contribution of AvrE in the absence of other type III effector proteins, the transgenic *ssavrE* plants were used. I examined the multiplication of the *hrpH* mutant in Col-0 *gl* and *ssavrE* transgenic plants. The *Pst* DC3000 *hrpH* mutant is unable to form a functional type III protein secretion system and is thus incapable of secreting AvrE as well as all other type III effector proteins. The plants were treated with DEX six hours before bacterial inoculation and daily during the course of the experiment to ensure that AvrE was present in the plants. The bacterial multiplication in these plants is shown in Figure 4-4. The *hrpH* mutant was unable to multiply beyond the inoculation level in DEX-treated Col-0 *gl* plants, but was able to grow to levels similar to *Pst* DC3000 in DEX-treated *ssavrE* transgenic plants. To determine if the benefit to the *hrpH* mutant conferred by *ssavrE* expression was specific to those bacteria which lack AvrE and other type III effector proteins, the growth of *Pst* DC3000 carrying the *avrRpt2* gene was assessed. *avrRpt2* is not a naturally occurring gene in the genome of *Pst* DC3000, but is present in other *P. syringae* strains. Because the Col-0 *gl* plants contain the cognate *R* gene, *RPS2*, *Pst* DC3000 carrying a plasmid-borne copy of *avrRpt2* are avirulent and unable to infect Col-0 *gl* (Kunkel *et al.*, 1993; Yu *et al.*, 1993). However, as seen in Figure 4-5, the DEX-treated *ssavrE* plants (which also contain the *RPS2* gene) are able to promote the growth of *Pst* DC3000 carrying *avrRpt2* to levels similar to those of *Pst* DC3000. The growth of wildtype *Pst* DC3000 was unaffected in the *ssavrE* transgenic plants.

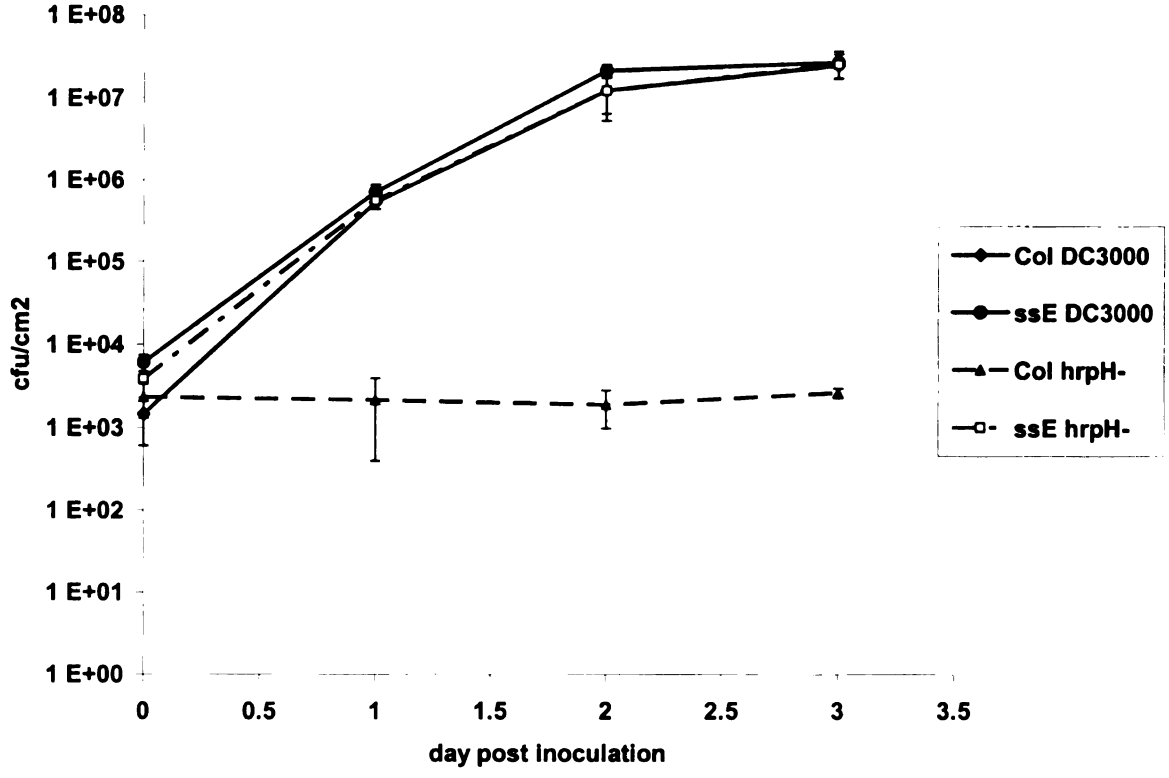

Figure 4-4. The *hrpH* mutant is able to proliferate in *ssavrE* transgenic plants. Plants were sprayed with 30 μM DEX six hours prior to bacterial infiltration and daily during the course of the experiment. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored over three days. Each datum point represents the mean titer of 12 leaf discs from three individual leaves. Error bars were created using standard deviation. Col represents Col-0 *gl* plants. ssE represents *ssavrE* transgenic plants. DC3000 represents *Pst* DC3000. hrpH- represents the *hrpH* mutant.

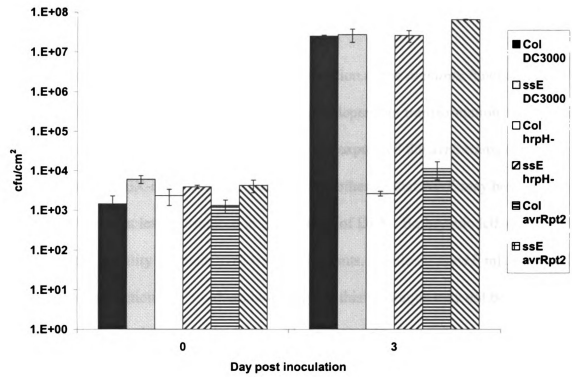

Figure 4-5. Bacterial proliferation in *A. thaliana* Col-0 *gl* and *ssavrE* transgenic plants. Plants were sprayed with 30 μM DEX six hours prior to bacterial infiltration and daily during the course of the experiment. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored after three days. Each bar represents the mean titer of 12 leaf discs from three individual leaves. Error bars were created using standard deviation. Col represents Col-0 *gl* plants. ssE represents *ssavrE* transgenic plants. DC3000 represents *Pst* DC3000. H- represents the *hrpH* mutant. avrRpt2 represents *Pst* DC3000 expressing the *avrRpt2* gene.

Discussion

The objective of this study was the identification of the *in planta* function of the *Pst* DC3000 type III effector, AvrE. Despite four independent transformation attempts, I was unable to obtain *A. thaliana* Col-0 *gl* plants that expressed the *avrE* transgene under the control of the DEX-inducible promoter. We hypothesize that AvrE may be toxic to the plant cell, even at levels produced in the absence of DEX. Embryonic lethality might account for the inability to obtain *avrE* transgenic plants. While no *ssavrE* mRNA could be seen before induction with northern blot analysis, this technique may not be sensitive enough to detect very low transcript levels. This presumed toxicity may be related to the necrosis which develops during *Pst* DC3000 infection or it may be related to overexpression of the AvrE protein in the transgenic plants.

I was able to obtain transgenic plants which express *avrE*-tobacco PR1-b signal sequence fusion. These plants could be viable due to the removal of the majority of the fusion protein from the cytoplasm of the host plant cell, as speculated for other fusion proteins (Lund and Dunsmuir, 1992; Gopalan *et al.*, 1996). Thus the tobacco PR1-b signal sequence may help to reduce toxicity due to expression of foreign proteins. Because both the *ssavrB* (Gopalan *et al.*, 1996) and *ssavrE* transgenic plants have necrosis phenotypes, this could suggest that the expression of a foreign bacterial gene fused to the signal sequence would cause a cell death phenotype. However, I have created other transgenic plants which express the *Pst* DC3000 *hrpZ* and *hrpW* genes fused to the tobacco PR1-b signal sequence under the control of the DEX-inducible promoter. These plants do not show an *ssavrE*- or *ssavrB*-like cell death phenotype after

DEX treatment (data not shown). Therefore, the necrosis is not likely due to the presence of the tobacco PR1-b signal sequence which might block the plant general secretory pathway.

Members of our lab are now developing an *Agrobacterium*-based system for the transient expression of transgenes in the leaves of *A. thaliana*. This system may allow for the transient expression of *avrE* without the tobacco PR1-b signal sequence. This assay would address whether *avrE* expression affects the plants in a manner similar to *ssavrE* expression.

The experiments described here suggest that AvrE in the plant cell is altering the plant cell physiology. The expression of *ssavrE* triggers chlorosis followed by wide-spread necrosis under low humidity. Symptoms reminiscent of *Pst* DC3000 infection, including water-soaking, chlorosis and localized necrosis, occur under high humidity. The role that symptom development plays in the *Pst* DC3000 infection process has not been determined. The symptoms may be triggered to help the bacteria reach high population levels within the leaf. Alternatively, the symptoms may be due to the high levels of bacteria present within the leaf. Our study demonstrated that the *in planta* expression of a single type III effector, AvrE, caused symptom development. Because the expression of *ssavrE* also promotes the growth of the normally non-pathogenic *Pst* DC3000 *hrpH* mutant, we hypothesize that symptoms may contribute to allowing pathogen growth.

It is tempting to conclude that because the expression of *ssavrE in planta* can restore *hrpH* mutant bacterial growth to near wildtype levels that the AvrE effector is sufficient to cause the *hrpH* mutant to regain its ability to be pathogenic. However,

several factors caution against this. First, in the transgenic plants AvrE was present at the beginning of the *hrpH* mutant growth. This would not be the case during normal pathogen growth. Second, the amount of AvrE produced *in planta* by the DEX-inducible system may be different than that produced by *Pst* DC3000 during the course of an infection. The DEX-induced AvrE may not be physiologically relevant. Finally, the promotion of growth seems to apply to *Pst* DC3000 expressing *avrRpt2* as well as the *hrpH* mutant. The modes by which these two bacteria are prevented from establishing successful infections are distinct. The *hrpH* mutant is unable to secrete any type III effectors, whereas *Pst* DC3000 with *avrRpt2* expresses an extra type III effector which confers recognition by the gene-for-gene resistance system. It is possible that AvrE is a multifunctional effector which is capable of promoting the growth of the *hrpH* mutant by one mechanism, while interfering with *RPS2*-mediated recognition of *Pst* DC3000 with *avrRpt2* by another mechanism. However, it is also possible that AvrE is preventing recognition of both of these bacterial strains by one mechanism.

One mode of action by which AvrE could accomplish the growth promotion of both the *hrpH* mutant and *Pst* DC300 expressing *avrRpt2* would be to prevent the bacteria from contacting the plant cell while also allowing nutrients to be made available for growth. It is possible that water-soaking would promote these conditions. There may be nutrients present in the apoplastic space, but these nutrients could be unavailable to the bacteria. The nutrients may be concentrated and localized until water-soaking aids in nutrient dispersal. The water could solubilize the nutrients, convert them into a form which is accessible to the bacteria, and spread the nutrients evenly throughout the apoplast. In addition, it is thought that secreted plant defense compounds are also present

in the apoplastic space (Osbourn, 1999). The release of water might dilute these compounds to a non-toxic level. Alternatively, these plant defense compounds may be water-insoluble. The plant defense compounds may be localized to specific locations within the apoplastic space, such as the cell wall. Thus, the release of water would separate the bacteria from these toxic plant defense compounds. In either case, water-soaking could act to protect the bacteria from the plant defense compounds.

The observation that *ssavrE* transgenic plants develop water soaking after transgene induction suggests that AvrE may promote the release of water during *Pst* DC3000 infection. Stomatal closure is also affected in these transgenic plants. The regulation of stomatal aperture is one way that plants control the loss of water during photosynthetically active time periods (Schroeder *et al.*, 2001). Active photosynthesis requires gas exchange in the leaf for maximum efficiency. Open stomata promote gas exchange. However, open stomata also allow for water loss via evaporation. Thus, under dry or drought conditions, fewer stomata are open. This reduces the efficiency of photosynthesis, but also reduces water loss. We have observed a correlation between *ssavrE* expression, water-soaking and stomatal closure. However, whether this relationship is causal is not currently known. One hypothesis is that the AvrE-induced stomatal closure is a cause of water-soaking. Experimental evidence suggests that stomatal closure precedes visible water-soaking. However, there may be microscopic, localized water-soaking which occurs before both stomatal closure and visual water-soaking.

However, stomatal closure may not be the cause of water-soaking. Water-soaking could be caused by loss of water from plant cells. This water loss could be perceived as

water stress and could then trigger stomatal closure. Stomata do not respond to artificially water-soaked leaves with closure. This artificial water-soaking was created by infiltration of water into the apoplast with a needleless syringe. Little is known about the microscopic characteristics of pathogen-induced water-soaking, but even visual assessment with the naked eye indicates that these two types of water-soaking are morphologically distinct. Artificially created water-soaking saturates the entire leaf apoplast with water. Pathogen-induced water-soaking is not homogeneous. There are patches of water soaking present within a leaf. Thus, stomatal closure may be a natural plant response to pathogen- or AvrE-induced water-soaking and might not play a part in the promotion of pathogen growth. It is also possible that the relationship between water-soaking and stomatal closure is merely correlative.

This study provides evidence that transgenic production of AvrE promotes pathogen infection. The observations made in this study could not have been made using bacterial studies with the *avrE* mutant. This study indicates the utility of *in planta* expression for the study of *Pst* DC3000 virulence. This study also suggests that alterations of host water relations are important for pathogenesis. The role that water-soaking and stomatal closure, possibly triggered by AvrE and other type III effectors, play in pathogenesis should be further examined.

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Chapter 5

Conclusions and Future Perspectives

The study of plant-pathogen interactions should allow researchers to help farmers reduce economic losses due to pathogen infection. While the mechanisms utilized by plants for defense are being discovered and elucidated, little is known about the processes which underlie disease. A widely studied plant-pathogen system consists of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) and its model host, *Arabidopsis thaliana*. The focus of my thesis work has been to gain a greater understanding of virulence in *Pst* DC3000. It is known that *Pst* DC3000 produces the phytotoxin coronatine, but the mechanism by which this toxin promotes disease is not yet known. *Pst* DC3000 also utilizes a type III protein secretion system during pathogenesis. This protein secretion system is thought to secrete bacterial proteins, collectively referred to as effectors, directly into the plant host cytoplasm. Once they are translocated into the plant host cell, the effectors are thought to promote disease by suppressing host defense responses and promoting the release of nutrients for utilization by the bacterial pathogen.

At the time this thesis was started, only two putative effectors were known to be present in *Pst* DC3000, AvrPto and AvrE. However, *avrPto* and *avrE* bacterial mutants did not show a reduction in virulence. This lack of phenotype was hypothesized to be due to functional redundancy with other unidentified *Pst* DC3000 effectors. In order to begin to understand the role that type III effectors play in virulence, we needed to learn more about the number and types of effectors encoded in the *Pst* DC3000 genome. A bioinformatics search was conducted followed by functional genomic characterization of putative type III effectors of *Pst* DC3000. This work is described in Chapter 2. In our search we found the eleven known and/or suggested *hrp*-regulated genes previously described and present in the available release of the *Pst* DC3000 genome sequence. We

identified six orthologues of avirulence and virulence genes which had been identified in other bacterial plant pathogens. Finally, we identified eight novel putative effectors which were shown to have *hrpS*-dependent transcription. One of these putative effectors was shown to be translocated into plant cells with an AvrRpt2-based translocation assay. This study and others have revealed that there are many putative type III effectors encoded in the *Pst* DC3000 genome. A different study has also identified a large number of putative type III effectors in another plant bacterial pathogen, *Pseudomonas syringae* pv. *maculicola* (Guttman *et al.*, 2002). Therefore, the presence of a large number of effectors is not a characteristic unique to in *Pst* DC3000, but is more broadly representative of the *P. syringae* pathovars, and possibly other plant bacterial pathogens. It is currently estimated that there are at least 36 effectors produced by *Pst* DC3000 (Collmer *et al.*, 2002). This number exceeds that of identified type III effectors in any animal bacterial pathogen. While the reason for this large number of effectors is not known, it has been suggested that it may enable the pathogenic pseudomonads to have a broad host range.

Now that many putative effectors have been identified, we must begin to characterize the role that they play in *Pst* DC3000 virulence. Efforts are currently underway to create mutations in each individual effector gene. However, due to the large number of effectors and the likelihood of functional redundancy, this approach has not yet led to any new knowledge of effector function. Information about the identity of many candidate effectors could be used to construct multi-effector knockouts which might then show a reduction in virulence. One multi-effector knockout, the delta CEL mutant, is reduced in virulence and this cannot be phenocopied by mutation of any single

effector within the CEL region (Alfano *et al.*, 2000; J. Badel, K. Nomura, S. Bandyopadhyay, A. Collmer and S.Y. He, *in prep*). However, the sheer magnitude of predicted effectors suggests that this approach would be labor intensive. The development of virulence assays which could be used to quickly test all putative effectors for a variety of functions would also help to determine the role that putative effectors may play in virulence.

One assay that is being developed is the expression of candidate effectors in non-pathogenic bacteria, such as *Pseudomonas fluorescens*, which heterologously express a *hrp* gene cluster-encoded type III protein secretion system (P. Hauck, R. Thilmony, and S.Y. He, unpublished). *P. fluorescens* which contains *hrp* cluster is unable to attain pathogenic levels of growth within plants. These bacteria could then be used to assess if the additional effector imparts greater bacterial growth *in planta*. If increased growth is observed, it could indicate that the effector is contributing to bacterial growth. Alternatively, this system could also be used to evaluate the ability of putative effectors to interfere with gene-for-gene mediated resistance. *P. fluorescens* expressing the *hrp* cluster and a known *avr* gene for the host being infiltrated should elicit a hypersensitive response (HR). If however, *P. fluorescens* expressing the *hrp* cluster, a known *avr* gene for the host being infiltrated, and an effector that interferes with the function of this *avr* gene, no HR would be observed. This approach does have a potential drawback. Guttman *et al* (2002) have indicated that putative type III effectors can be found in *P. fluorescens* even though it lacks a native functional type III secretion system and is non-pathogenic. There is evidence to suggest that there may be complex interactions among the effectors of a pathogen. The presence of unknown native putative effectors of *P.*

fluorescens could interfere with interpretation of the results of these heterologous expression studies.

Another assay being used is the expression of putative effectors *in planta*, either transiently or stably. Stable transgenic lines are currently being used to elucidate the role that individual candidate effectors play in virulence. *A. thaliana* plants which express *avrRpt2* (and lack the corresponding *R* gene *RPS2*) show increased susceptibility to *Pst* DC3000 (Chen *et al.*, 2000). Expression of *avrPto* in *A. thaliana* promotes the growth of the non-pathogenic *Pst* DC3000 *hrpH* mutant (Yuan and He, 1996) and suppresses the formation of callose-containing papillae (P. Hauck, R. Thilmony, and S.Y. He, unpublished). In addition, expression of *ssavrE* in *A. thaliana* induces stomatal closure and also promotes the growth of the *hrpH* mutant. One disadvantage of this approach is that the generation of stable transgenic *A. thaliana* plants which express putative effectors requires a great deal of labor, space, and time.

Transient-expression analysis would allow candidate effectors to be screened relatively quickly. However, this technique has not yet been perfected in *A. thaliana*. For now, transient expression is attainable in *Nicotiana benthamiana*. This system could be used to screen for phenotypes after transient expression of candidate effectors. Care must be taken because *N. benthamiana* is not a host for *Pst* DC3000. While some candidate effectors may elicit a similar response in *A. thaliana* and *N. benthamiana*, others may elicit a hypersensitive response (HR) in *N. benthamiana* which would not happen in *A. thaliana*. In addition, some putative effectors would trigger changes in the *A. thaliana* host but may not affect *N. benthamiana*.

Overall, the identification of many new effectors and candidate effectors which are thought to contribute to phytobacterial virulence will aid in future study of plant pathogen-interactions. Our field is now poised to contribute to greater understanding of pathogenesis.

Another goal of my thesis work was the identification of reduced-virulence bacterial mutants which would aid in our understanding of *Pst* DC3000 virulence. Because the previously described virulence factors in *Pst* DC3000 are transcriptionally induced in minimal medium, a transposon with a promoterless reporter gene was used to generate a population of mutagenized bacteria and subsequently determine the expression pattern of the insertionally-inactivated genes in minimal versus rich medium. Mutants disrupted in the *ptsP, uvrD,* and *oprF* genes were isolated in this way, as described in Chapter 3.

The *ptsP* gene encodes the Enzyme I subunit of the phosphoenolpyruvate protein phosphotransferase system. In concert with other subunits of this system, Enzyme I mediates the uptake of sugar into the bacterial cell. However, these systems have also been implicated in other processes such as catabolite repression and chemotaxis (Postma *et al.*, 1993). Interestingly, *ptsP* mutants of other bacteria also show reduced virulence (Higa and Edelstein, 2001; Tan *et al.*, 1999).

The specific role that the phosphoenolpyruvate protein phosphotransferase system plays in virulence has not yet been determined in any bacterial system. Loss of virulence in the *Pst* DC3000 *ptsP* mutant could be due to the inability of these mutants to acquire sugar. Little is known about the environment present in the plant apoplastic space. If indeed a defect in sugar uptake is the cause of the *ptsP* mutant phenotype, this mutant

could be used to augment our knowledge of the conditions present in the apoplastic space. Feeding experiments could be done with ^{14}C-labeled sugars to determine which sugars can/cannot be taken up by the *ptsP* mutant *in vitro*. These experiments might reveal whether certain sugars, which can be taken up by *Pst* DC3000, cannot be taken up by the *ptsP* mutant. These sugars might be present in the apoplastic space during infection, and this could account for the loss of virulence of the *ptsP* mutant. These experiments could be followed up with chemical composition analysis of *A. thaliana* apoplast wash fluid to determine if these sugars are indeed present.

An alternative hypothesis is that chemotaxis is required within the apoplastic space before the bacteria adhere to the host cells and that this process is disrupted in the *ptsP* mutant. Chemotaxis could allow the bacteria to find areas which contain nutrients or to avoid areas with plant defense compounds. Badel *et al.* (2002) have developed a green fluorescent protein (GFP)-based system for the observation of *Pst* DC3000 in the apoplastic space. This technology could be used to determine if the *ptsP* mutant bacteria fail to disperse throughout the apoplastic space to the same extent as wildtype *Pst* DC3000.

Three independent mutants which contain a disrupted *oprF* gene were identified. The *oprF* gene encodes an outer membrane porin F precursor protein. *P. fluorescens* mutants which lack the *oprF* gene are unable to grow in medium with low osmolarity (Rawling *et al.*, 1998). The *Pst* DC3000 *oprF* mutants also show a reduced growth rate in *hrp*-inducing minimal medium. Taken together, these results suggest that the *Pst* DC3000 *oprF* gene may be required for growth in low-osmolarity conditions such as *hrp*-inducing minimal medium and the apoplastic space. The protein encoded by the *oprF*

gene has also been implicated in adherence to host cells (Azghani *et al.*, 2002; DeMot *et al.*, 1992). The mechanism by which *Pst* DC3000 adheres to host cells in the apoplastic space is not currently known. The above mentioned studies (Azghani *et al.*, 2002; DeMot *et al.*, 1992) have tested the ability of bacteria to remain adhered to host cells after perturbation by washing. To examine the adherence of *Pst* DC300 to *A. thaliana* cells, leaves which had been previously infiltrated with bacteria (either *Pst* DC3000 or the *oprF* mutant) could be infiltrated again with water, followed by centrifugation. This could be used to wash away bacteria which had not adhered to the plant cells. Conditions and timing would have to be determined such that bacteria that had been infiltrated would have begun to adhere to host cells. However, water-infiltration would also have to be done before high levels of multiplication had occurred because the growth differences *in planta* between *Pst* DC3000 and the *oprF* mutants could mask the effect of differences in adhesion. If more *oprF*⁻ bacteria than wildtype *Pst* DC3000 were recovered from the apoplastic wash fluid, and assessment of the bacterial levels remaining in the washed leaves indicated higher levels of *Pst* DC3000 than the *oprF* mutant, this might indicate that OprF plays a role in the adhesion of *Pst* DC3000 to *A. thaliana* cells within the apoplastic space.

The *uvrD* mutant is also reduced in virulence. The *uvrD* gene encodes a type II DNA helicase which is important for DNA replication and repair, especially after exposure to UV light. The *Pst* DC3000 *uvrD* mutant is more sensitive to UV light. While this sensitivity has been shown to be important during the epiphytic growth of plant pathogenic bacteria (Sundin and Jacobs, 1999), the *Pst* DC3000 *uvrD* mutants never grew epiphytically during my study because they were infiltrated directly into the

apoplastic space. It is possible that UV light could impact bacterial growth within the apoplastic space, but this would require UV light to penetrate through the leaf. An alternative hypothesis is that the *uvrD* gene is required for *in planta* growth because it plays a role in tolerance to plant defense compounds present in the apoplastic space. Secreted compounds have been demonstrated to play a role in defense against plant pathogens in other systems (Osbourn, 1999). Unfortunately, there is still little known about the defense compounds produced by *A. thaliana* which are effective against bacterial pathogens. This lack of knowledge prevents *in vitro* studies which could compare the tolerance of wildtype *Pst* DC3000 and the *uvrD* mutant to known *A. thaliana* defense compounds. As indicated in Chapter 3, preliminary evidence suggests that the *uvrD* mutant is able to grow to a level similar to that of wildtype *Pst* DC3000 in NahG transgenic plants. The NahG transgenic plant expresses the bacterial napthalene hydroxylase gene (*nahG*) which converts salicylic acid to catechol (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). However, there is evidence to suggest that this transgenic plant lacks not only salicylic acid but other defense compounds derived from the phenylpropanoid pathway (Hunt *et al.*, 1996). If this phenomenon is reproducible, it may indicate that the *uvrD* mutant is less tolerant to plant secondary metabolite defense compounds which originate from the phenylpropanoid pathway.

Chapter 4 describes my efforts to characterize the role that a specific type III effector, AvrE, plays in *Pst* DC3000 virulence. Earlier studies with the *Pst* DC3000 *avrE*⁻ mutant failed to indicate a role for this effector in virulence, probably due to functional redundancy (S.Y. He, unpublished). To avoid the problems of effector redundancy, *avrE* fused to the tobacco PR1-b signal sequence (*ss)* was expressed in *A.*

thaliana under the control of a dexamethasone (DEX)-inducible promoter. After induction, the *ssavrE* transgenic plants exhibited water-soaking followed by the development of chlorosis and necrosis. The occurrence of water-soaking was correlated with stomatal closure. Expression of *ssavrE* also permitted the growth of the normally non-pathogenic *Pst* DC3000 *hrpH* mutant. In addition, *Pst* DC3000 with the *avrRpt2* gene was able to grow to higher levels in *ssavrE*-expressing plants.

One mechanism that could cause an increased level of bacterial growth would be the prevention of contact with the plant cells in the AvrE-expressing plants. Prevention of plant-bacterial contact would prevent the translocation of AvrRpt2 and thus gene-for-gene resistance would not be triggered. In addition, this might prevent the *hrpH* mutant from contacting plant defense compounds which are localized in the plant cell wall. A previous study showed that infiltration of a bacterial suspension in 0.01% agarose was able to prevent gene-for-gene resistance. The agarose was hypothesized to prevent bacterial contact with the host cells (Stall and Cook, 1979). Disruption of contact between bacterial and host cells could be accomplished by the release of water into the apoplastic space. *A. thaliana* leaves infected with *Pst* DC3000 develop water-soaking symptoms two days after bacterial infiltration. Although no specific role in virulence has yet been attributed to water-soaking, it might serve to protect the bacteria from the cell wall-localized defense compounds. This hypothesis provides a role for AvrE-induced water-soaking during the course of infection.

Water-soaking in *ssavrE* transgenic plants is correlated with the closure of stomata. This relationship may be mere correlation or it may be causal. Water-soaking may cause stomatal closure, stomatal closure may cause water-soaking, or both may

caused by AvrE independently. In leaves which were artificially water-soaked, nearly all the stomata were open. While this does suggest that water-soaking is not the cause of stomatal closure, it is important to acknowledge that this artificial water-soaking is apparently different from pathogen-induced water-soaking. The two treatments are visually different. Artificially induced water-soaking results in leaves which are thoroughly saturated with water. The pathogen-induced water soaking is more mottled in appearance. The complete saturation of the artificially water-soaked leaves probably results in an anaerobic environment within the apoplastic space. This anaerobic environment could account for the open stomata observed in the artificially water-soaked leaves. I have observed that stomatal closure occurs during *Pst* DC300 pathogenesis and is correlated with water-soaking, however, this observation still does not address the nature of the relationship.

Because I was unable to create a true reproduction of pathogen-induced water-soaking, I was unable to determine if pathogen-induced water-soaking causes the observed stomatal closure. We could, however, use chemicals which artificially alter stomatal aperture to determine if stomatal closure is sufficient to cause water-soaking. 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7), an inhibitor of protein kinase C, has been shown to inhibit stomatal opening in the light and enhance stomatal closure in the dark in *Commelina communis* (Lee and Assmann, 1991). H-7 could be used to artificially trigger stomatal closure. The leaves could then be observed to determine if water-soaking occurs. In addition we could utilize a synthetic diacylglycerol such as 1,2-dioctanoylglycerol which has been shown to inhibit dark-induced stomatal closure and enhance light-induced stomatal opening (Lee and Assmann, 1991). This diacylglycerol

could then be used to determine if artificial opening of the stomata could prevent *ssavrE*-induced water-soaking. Additional observations could determine the role that water-soaking plays in further symptom development and enhanced growth of the *hrpH* mutant. *Pst* DC3000 infected plants could also be treated with this diacylglycerol to attempt to inhibit pathogen-induced water-soaking and determine if bacterial growth is altered under these conditions. These experiments could further understanding of the relationship between stomatal aperture, water-soaking, and *Pst* DC3000 virulence.

Overall, my thesis work has provided new information about *Pst* DC3000 virulence. The effectors and bacterial mutants identified, as well as the transgenic plants generated, should be useful for further examination of the infection process.

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Appendix A

Supplementary material for Chapter 2

This appendix contains supplementary material for Chapter 2. Appendix table 1 represents the collection of hrp box containing ORFs (HCOs) found in Pst DC3000 in Chapter 2. This table provides information about the primers used to amplify the HCOs for inclusion on the microarray slide. It also contains the top blastX hit and expectation value.

Table A-1. 'Hrp box'-containing open-reading frames (HCOs)a.

HCO	Sequence similarity Protein, organism [accession number]	E value	Forward primer	Reverse primer
hrcC	AF232004	0.00E+00		
hrpA	AF232004	0.00E+00		
hrpJ	AF232004	0.00E+00		
hrcU	AF232004	0.00E+00		
hrpW	AF232004	0.00E+00		
avrPto	Avirulence protein, *Pseudomonas syringae* [AAA25728]	4.00E-91		
avrE	AF232004	0.00E+00		
hrpK	AF232004	0.00E+00		
orf1/CEL	AF232004	0.00E+00		
orf5/CEL	AF232004	0.00E+00		
orf7/CEL	AF232004	0.00E+00		

Table A-1
cont.

HCO1	None	-	GCCGACTTTTCGTACAGAG	CACACAAGCATCCGTAATAG
HCO2	DNAJ-like protein homolog, *Neurospora crassa* [BAB17689]	9.00E-11	GCCACCAAGGCGATATCAGTT	GTTCTTCATCGCGGCATCTCT
HCO3	None	-	CGCCACCAAGTGCTCATTC	TGTGGAGCCGGATATTTTCATAG
HCO4	None	-	GGCTTTTTACCACTCAATGGACAG	CGATGTGGAGCCGGATATTTT
HCO5	HrpW, *Pseudomonas syringae* [AAC62526.1]	3.00E-09	CGGCACATTCCAGGAGGTATT	CCGTTCGACACAGGCATC
HCO6	ORF1, *Pseudomonas syringae* pv. *phaseolicola* [AAF67148]	6.00E-38	TTAAATTATGAGGATATGAGGAGATG	CCAGAGTTCACAAGGCTAAAA
HCO7	Conserved hypothetical protein, *Pseudomonas aeruginosa* [NP_251683.1]	6.00E-84	AGCCACTTTGGGTGTTTTTG	GAAGGCCAATTTTTGTCTGTATG
HCO8	Hypothetical protein, *Pseudomonas aeruginosa* [NP 250999.1]	8.00E-70	CTACCTGCCTGTTCTCTGCC	TGCATCTTACCCCACGTACA
HCO9	Carboxypeptidase G2, *Bacillus halodurans* [NP 241218.1]	5.00E-31	GCTATTGATTGATCGCCAAGT	TGTAAAGAGCCCGAAAGAGC
HCO10	VirPphA, *Pseudomonas syringae* pv. *phaseolicola* [AAD47203]	1.00E-112	ATGGCGGGTATCAATAGAGCG	TCAGGGGACTATTCTAAAAGC

Table A-1
cont.

HCO11	AvrPpiB1.R3, *Pseudomonas syringae*	1.00E-160	ATGCACGCAAATCCTTTAAGC	TAGTCGCCTAGGAAATTATTTAGTTC
HCO12	AvrPpiC2 protein, *Pseudomonas syringae* pv. *pisi* [CAC16701]	1.00E-151	AACCACTCATTTCGCATCTTTT	CCACAAGGTAGAGCTTGGAGAC
HCO13	AvrPphD protein, *Pseudomonas syringae* pv. *phaseolicola* [CAC16699]	0.00E+00	CATCCGGGACAGCTGATAG	GCTGCTCGTGAAGTCCAGTA
HCO14	Putative type III chaperone, *Pseudomonas syringae* pv.*syringae* [AF232003]	1.00E-27	TCTACAAAACCCTGCTTGATGA	TCATTTAAAAAGTGCATCGAGAGT
HCO15	Hypothetical protein, *Pseudomonas aeruginosa* [NP_253648.1]	7.00E-47	AGTTATGGTGATGTGATGGG	GTGTACGAAGGCGAGCTG
HCO16	Conserved hypothetical protein, *Haemophilus influenzae* [NP_438658]	2.00E-52	TCAAAGGCGACAAGAAGC	TCTTCGTCTTCCCCAATAG
HCO17	AGR_pAT_789p, Agrobacterium tumefaciens [NP_396465.1]	4.00E-33	CCGTCGTTCATCTTCTCCAC	TCAGACAGCCCTTTGGTTTC

Table A-1
cont.

HCO18	Organic solvent tolerance protein OstA precursor, *Pseudomonas aeruginosa* [NP 249286.1]	0.00E+00	TATTGCTCGGGTGCCTACG	GCGCCGCTCAGGAGTAAC
HCO19	Hypothetical protein, 15.3 kD, *Pseudomonas putida* [AJ245436]	4.00E-61	ATCCACGAACCCCACATTT	CGTCAGGAAGGTTGTCGG
HCO20	Hypothetical protein, *Pseudomonas aeruginosa* [NP 250958.1]	4.00E-50	AGTAAGGCGTGGGGAAAAGT	GGATGGGGAGCAGGGTAT
HCO21	Probable two-component sensor, *Pseudomonas aeruginosa* [NP 252663.1]	5.00E-50	GAATTTCTTGCCACGCTCAC	GCCATAGTCGCCGAAAAAC
HCO22	Putative exported protein, *Yersinia pestis* [NP 406993.1]	1.00E-06	CATCTTAAAATCCCCATGCATAAAC	GAACCAGACAAGAGGACAAGTGC
HCO23	Hypothetical protein, *Pseudomonas aeruginosa* [NP 249035.1]	1.00E-129	GGATGCGGGTTGAGTGTT	GGAGCAACTGGCCCACTAT
HCO24	Hypothetical protein, *Pseudomonas aeruginosa* [NP 248996.1]	2.00E-43	GCTGCCACATCTTCCTCACT	GCTGTACGGCACCAATCC

Table A-1
cont.

HCO25	Peptide chain release factor 3, *Pseudomonas aeruginosa* [NP_252592.1]	1.00E-143	GTTCTTCGGGACGGCTTT	CTTATCAGTTCGTGACGCTCC
HCO26	Orf; hypothetical protein, *Escherichia coli* O157:H7 EDL933 [NP 290033.1]	1.00E-31	CTGAAACACATCGCCAACC	AGAGCAGCCCGAAATCCT
HCO27	None	-	GCTAATGACCCGCAGCACATA	GTAACACCGTGCGCAAGTACC
HCO28	Zinc transport protein ZnuC, *Pseudomonas aeruginosa* [NP 254187.1]	1.00E-104	CCAGACGGTCGAAATCGT	CTGCGAGCAAGGCATACA
HCO29	Conserved hypothetical protein, *Pseudomonas aeruginosa* [NP_252504]	1.00E-134	TGTATCAAGAAGGGAGCGTAG	CTGCATCTTCTGGCTGAACT
HCO30	PotH, *Pseudomonas fluorescens* [AAK15490]	8.00E-21	CATTTTCAGCGAACCCAGGTA	AAGCTGGTGCAGTCGTTTGTC
HCO31	Hypothetical protein, *Pseudomonas aeruginosa* [NP 251764.1]	1.00E-166	CTCAATCGTCTGCGGAGGTC	CGTAATCCTGCGCTTCGTACA
HCO32	Protease inhibitor, *Pseudomonas fluorescens* [BAA36462.1]	7.00E-19	CTGGAATGTACCCCCATC	TTATCAAATAACGCGCTCA

Table A-1
cont.

HCO33	Syringomycin synthetase, *Pseudomonas syringae* pv. *syringae* [T14593]	0.00E+00	GCCACCATTGCGCAGTTA	GAGCTTGACCTGATCGTCGT
HCO34	Probable cation-transporting P-type ATPase, *Pseudomonas aeruginosa* [NP_250240.1]	0.00E+00	CAACACCGGAACGACTGAAC	CCAGCATTGCCAGCACAA
HCO35	Unknown, *Pseudomonas putida* [U18304]	8.00E-24	CGGCCATCGATCACTTTTTC	TCATTACCTGCTCTTCGGTCAA
HCO36	ATP-dependent DNA helicase RecQ, *Pseudomonas aeruginosa* [NP_252034]	8.00E-51	GTGCCGCCTTATGTCATTTT	TTATGATCAGGCAAGCATGG
HCO37	Unknown, *Pseudomonas syringae* pv. *tomato* [U16119]	3.00E-55	GCGCTTTGCCACTCACTT	GGAAACCCCACACTACCATC
HCO38	AGR_pAT_757p, *Agrobacterium tumefaciens* [NP_396445.1]	1.00E-31	AGTGGTCGAACGCATCAAAC	TGAATAATTCGAGCGGTGCT
HCO39	Conserved hypothetical protein, *Pseudomonas aeruginosa* [NP_252504.1]	1.00E-74	GCTCTCATTCCGAACCTCTG	GCTCATTCATCTGCGTTCATT

Table A-1
cont.

HCO40	ORF4, *Pseudomonas syringae* [BAA87063.1]	3.00E-50	TTCGAGCAGCAGAGCTATTG	GACGTTTTTGTGCCGGATT
HCO41	Probable ATP dependent DNA helicase, *Mesorhizobium loti* [NP_106239.1]	2.00E-48	AGCCAGAATGATCGAGCC	CAGCGAACGGGTAAAGAAAC
HCO42	Probable hydrolase, *Pseudomonas aeruginosa* [NP_250096.1]	4.00E-05	GCGCAGCAACCCTATGTATT	CGACACCAAAAGCATCAAACT
HCO43	Similar to sorbitol dehydrogenase, *Bacillus subtilis* [NP_389115]	3.00E-22	GCGCTTTATGGCTAATGTTTTC	CTCGGCAAAAGTTCCATGTT
HCO44	Potassium-transporting ATPase, B chain, *Pseudomonas aeruginosa* [NP_250325.1]	0.00E+00	GGAACTGACTGCCATTCTGAC	GGATCAGCACGACGATGA
HCO45	Hypothetical 119.5K protein (uvrA region), *Micrococcus luteus* [JQ0405]	6.00E-03	GCAATTACAGGCAGTCACCA	ACCGAAGGTGAACACGGA
HCO46	Avirulence protein, *Xanthomonas campestris* pv. *vesicatoria* [AAG18480]	4.00E-15	CGGTGGAGAGTACACGCTTAG	GGTTATTACAGGTAGCGCTTTATTG

Table A-1
cont.

HCO47	AGR_L_3495p, *Agrobacterium tumefaciens* [NP_357538.1]	6.00E-64	CAAGAATCGCCAGCACATC	TGATCACACCCTCAAAGCAG
HCO48	Probable phosphotransferase system enzyme I, *Pseudomonas aeruginosa* [NP_252252.1]	1.00E-105	AGTCTTCGCCAACATCGG	GGGTTCATGGTCAGGGTAAG
HCO49	3-alpha-hydroxysteroid dehydrogenase, *Xylella fastidiosa* 9a5c [NP_299548]	7.00E-29	GAGCTATCAGCAGTCGCAAG	CAGCAAGCCCTCCATCTAC
HCO50	Hypothetical protein, *Pseudomonas aeruginosa* [NP_253944.1]	9.00E-45	GCACCCTCTGGCGATAAG	GCTGTCGAAGTAAAACGGAGA
HCO51	Transcriptional regulator, TetR family, *Vibrio cholerae* [NP_231051.1]	2.00E-25	CCGGTTCACTAAGGGATTGA	ACGTGAGACGCTGTTGATTG
HCO52	Orf41, *Escherichia coli* [NP_053103]	2.00E-32	CAGCTGGACTCAGGACCAAT	GTAAAACTGGCGGCTTTGAG
HCO53	Hypothetical protein, *Pseudomonas aeruginosa* [NP_252030.1]	3.00E-17	GTTTTTCCTTGGCCACGTC	CTCAATACTCGAAAACGCTGAA

Table A-1
cont.

HCO54	Probable ATP-binding component of ABC transporter, *Pseudomonas aeruginosa* [NP_250498.1]	1.00E-160	CTCAAAGCCCTGCCACAC	CGACCCTTGAGTTTGTCGTT
HCO55	Probable two-component response regulator, *Pseudomonas aeruginosa* [NP_252403.1]	2.00E-47	GTCTTGAGCGATTCCACTGA	AACGTGGTGATTCCCGTCT
HCO56	Proline dehydrogenase, *Pseudomonas putida* [AAF25000]	5.00E-36	GCGCTTTATGGCTAATGTTTTC	AGGTCTCACTCACCGTTTTTGT
HCO57	Succinate dehydrogenase (A subunit), *Pseudomonas aeruginosa* [NP_250274.1]	1.00E-114	CCGATTCCGGTAGTTCCA	TCGCGATCTTCGAAGTCTTAC
HCO58	None	-	ACCGTCATGCTGCTACACAC	GCGCCTACATCCCCTGTT
HCO59	2-DEHYDRO-3-DEOXYGLUCONOKINASE, *Pectobacterium chrysanthemi* [P45416]	2.00E-67	CGTGGATTACGTGACCGATA	TTGCGGTCAGTGCGTTAG

Table A-1
cont.

HCO60	Probable helicase, *Pseudomonas aeruginosa* [NP_250096.1]	5.00E-39	GAGAAACTGCCAGCCCAC	CTTGACCCGGTAATCGAAAC
HCO61	ABC transporter, permease protein, *Mesorhizobium loti* [NP_105920.1]	2.00E-29	CGACGAGCCACTGGAAAT	AGTTGTCCGGCTTCTTGAAA
HCO62	BH3308~unknown conserved protein in B. subtilis, *Bacillus halodurans* [NP_244174.1]	1.00E-07	CAGATACTGATGGCAACGGA	CTTTCCAGATTTACCCATACAACA

a. The entire HCO sequences can be accessed at http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=p_syringae by blasting with the listed primers.
b. The protein sequence with the lowest E value is shown.

Appendix B

Supplementary material for Chapter 3

This appendix contains supplementary material for Chapter 3. These figures represent the insertionally-inactivated genes identified in Chapter 3. These figures contain predicted operon structure, and annotation information about these *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) genes. In addition, a blast P alignment is included.

Predicted operon structure of *Pst* DC3000 *ptsP* region

Z -- Hypothetical prot
A -- Putative invasion protein or MutT/Nudix-like protein
B – *ptsP* which encodes Enzyme I of the Phosphoenolpyruvate protein phosphotransferase system
C – Prolipoprotein diacylglyceryl transferase-like protein
D – Thymidylate synthase-like protein
E – Putative terminator sequence

Figure B-1. Diagram of the predicted operon structure of the *Pst* DC3000 *ptsP* gene region.

This diagram is not to scale.

Figure B-2. Translation of the *Pst* DC3000 *ptsP* genomic region.

The putative Shine-Dalgarno sequence is highlighted in yellow. The predicted translational start site is underlined. The transposon insertion site is highlighted in green.

```
   1 H  G  V  R  P  R  A  M  L  N  T  L  R  K  I  V  Q  E  V  N
   1 CACGGAGTTCGACCCCGAGCCATGCTCAATACGCTGCGCAAGATCGTCCAGGAAGTTAAC
  21 S  A  K  D  L  K  A  A  L  G  I  I  V  L  R  V  K  E  A  M
  61 TCCGCCAAGGATCTCAAGGCGGCGTTGGGCATTATTGTGTTGCGCGTCAAGGAGGCCATG
  41 G  S  Q  V  C  S  V  Y  L  L  D  A  E  V  N  R  F  V  L  M
 121 GGCAGCCAGGTCTGCTCGGTCTACCTGCTCGATGCCGAAGTGAACCGTTTTGTGCTGATG
  61 A  S  E  G  L  N  K  R  S  I  G  K  V  S  M  A  P  N  E  G
 181 GCCTCCGAAGGCCTCAACAAGCGTTCCATCGGCAAAGTCAGCATGGCGCCGAACGAAGGC
  81 L  V  G  L  V  G  T  R  E  E  P  L  N  L  E  N  A  A  D  H
 241 CTTGTTGGTCTGGTCGGTACGCGCGAAGAACCTCTAAACCTCGAAAACGCCGCTGATCAC
 101 P  R  Y  R  Y  F  A  E  T  G  E  E  R  Y  A  S  F  L  G  A
 301 CCCCGTTATCGCTACTTCGCCGAGACCGGTGAAGAGCGTTATGCCTCGTTCCTGGGGGCG
 121 P  I  I  H  H  R  R  V  V  G  V  L  V  I  Q  Q  K  E  R  R
 361 CCGATCATCCACCATCGTCGTGTGGTTGGCGTATTGGTGATCCAGCAAAAGGAGCGCCGT
 141 Q  F  D  E  G  E  E  A  F  L  V  T  M  S  A  Q  L  A  G  V
 421 CAGTTCGACGAGGGCGAGGAAGCCTTCCTTGTCACCATGAGTGCGCAACTGGCGGGCGTT
 161 I  A  H  A  E  A  T  G  S  I  R  G  L  G  R  Q  G  K  G  I
 481 ATCGCGCATGCCGAAGCCACCGGCTCGATTCGCGGTCTGGGTCGCCAGGGCAAGGGCATT
 181 Q  E  A  K  F  V  G  V  A  G  S  P  G  A  A  V  G  V  A  V
 541 CAGGAAGCCAAGTTTGTCGGTGTGGCCGGCTCGCCGGGTGCCGCAGTCGGTGTCGCAGTG
 201 V  M  L  P  P  A  D  L  E  V  V  P  D  K  T  V  T  D  I  A
 601 GTCATGCTGCCGCCTGCCGATCTGGAAGTTGTCCCGGACAAGACCGTCACGGATATCGCT
 221 A  E  L  T  L  F  Q  N  A  L  E  G  V  R  N  D  M  R  T  L
 661 GCCGAGCTGACGCTGTTCCAGAATGCACTGGAAGGTGTGCGCAACGACATGCGCACCCTG
 241 S  A  K  L  A  T  Q  L  R  P  E  E  R  A  L  F  D  V  Y  L
 721 TCCGCCAAGCTCGCCACGCAACTGCGCCCCGAAGAGCGCGCGCTGTTCGATGTCTACCTG
 261 M  M  L  D  D  A  S  L  G  S  E  V  T  N  V  I  K  T  G  E
 781 ATGATGCTCGACGATGCTTCGCTGGGCAGCGAGGTGACCAATGTCATCAAGACCGGCGAA
 281 W  A  Q  G  A  L  R  S  V  V  S  E  H  V  K  R  F  E  L  M
 841 TGGGCACAAGGTGCCTTGCGCTCGGTGGTCAGCGAGCACGTCAAACGCTTTGAGCTGATG
 301 D  D  A  Y  L  R  E  R  A  S  D  V  K  D  L  G  R  R  L  L
 901 GACGATGCTTACCTGCGTGAGCGGGCCTCGGACGTCAAGGACCTGGGCCGCCGCCTGCTG
 321 A  Y  L  Q  E  E  R  Q  Q  A  L  V  Y  P  D  N  T  I  L  V
 961 GCGTACCTGCAGGAAGAGCGGCAACAGGCACTGGTTTACCCCGATAACACGATTCTGGTC
 341 S  E  E  L  T  P  A  M  L  G  E  V  P  E  G  K  L  V  G  L
1021 AGTGAAGAGCTGACCCCGGCCATGCTTGGCGAAGTGCCGGAAGGCAAGCTGGTCGGTCTG
 361 V  S  V  Q  G  S  G  N  S  H  V  A  I  L  A  R  A  M  G  I
1081 GTGTCGGTGCAAGGTTCCGGCAACTCCCACGTCGCGATTCTGGCGCGGGCGATGGGTATT
 381 P  T  V  M  G  L  V  D  F  P  Y  S  K  V  D  G  I  D  L  V
1141 CCCACGGTCATGGGCCTGGTGGATTTCCCGTACTCCAAGGTCGATGGCATCGATCTTGTG
 401 V  D  G  Y  H  G  E  V  F  T  N  P  S  E  I  M  R  K  Q  F
1201 GTCGACGGCTATCACGGCGAAGTCTTCACCAACCCCAGCGAAATCATGCGTAAGCAGTTC
 421 G  K  V  V  E  E  E  R  Q  L  S  Q  G  L  D  A  L  R  E  L
1261 GGCAAGGTGGTGGAGGAGGAGCGTCAGCTCTCTCAGGGCCTGGATGCCCTGCGCGAATTG
 441 P  C  V  T  L  D  G  H  R  M  P  L  W  V  N  T  G  L  L  A
1321 CCGTGCGTGACCCTCGACGGGCATCGCATGCCGCTGTGGGTCAACACCGGTCTGCTAGCC
 461 D  V  A  R  A  Q  Q  R  G  A  E  G  V  G  L  Y  R  T  E  V
1381 GACGTCGCTCGCGCCCAGCAGCGTGGCGCCGAAGGGGTTGGCCTGTATCGCACCGAAGTG
 481 P  F  M  I  N  Q  R  F  P  S  E  K  E  Q  L  A  I  Y  R  E
1441 CCGTTCATGATCAACCAGCGTTTCCCGAGTGAAAAGGAACAGCTGGCGATCTACCGCGAG
 501 Q  L  A  A  F  H  P  L  P  V  T  M  R  S  L  D  I  G  G  D
1501 CAACTGGCGGCCTTCCATCCGCTGCCGGTGACCATGCGCAGCCTGGACATCGGCGGCGAC
 521 K  S  L  S  Y  F  P  I  K  E  D  N  P  F  L  G  W  R  G  I
1561 AAGTCGCTGTCCTACTTCCCGATCAAGGAAGACAACCCGTTTCTCGGCTGGCGCGGTATT
 541 R  V  T  L  D  H  P  E  I  F  L  V  Q  T  R  A  M  L  K  A
1621 CGCGTCACCCTCGACCACCCGGAAATCTTTCTGGTTCAGACCCGCGCCATGCTCAAGGCC
 561 S  E  G  L  N  N  L  R  I  L  L  P  M  I  S  S  T  H  E  V
```

```
1681 AGCGAAGGCCTGAACAACCTGCGCATTCTGTTGCCGATGATTTCCAGCACCCATGAAGTG
 581 E  E  A  L  H  L  I  H  R  A  W  G  E  V  R  D  E  G  T  D
1741 GAAGAGGCGCTGCACCTGATCCACCGGGCCTGGGGCGAAGTGCGCGACGAAGGCACCGAT
 601 V  P  M  P  P  V  G  V  M  I  E  V  P  A  A  V  Y  Q  T  R
1801 GTGCCGATGCCGCCTGTCGGCGTGATGATCGAAGTACCCGCTGCGGTTTACCAGACTCGT
 621 D  L  A  R  Q  V  D  F  L  S  V  G  S  N  D  L  T  Q  Y  L
1861 GACCTGGCGCGGCAGGTGGACTTCCTCTCGGTGGGCTCCAACGACCTGACCCAATACCTG
 641 L  A  V  D  R  N  N  P  R  V  A  D  L  Y  D  Y  L  H  P  A
1921 CTGGCGGTCGACCGCAACAACCCGCGTGTGGCCGATCTCTACGATTACCTGCACCCGGCG
 661 V  L  Q  A  L  Q  S  V  V  R  D  A  H  A  E  G  K  P  V  S
1981 GTGCTTCAGGCGCTGCAAAGCGTGGTGCGTGATGCCCACGCCGAGGGCAAGCCGGTGAGC
 681 I  C  G  E  M  A  G  D  P  A  A  A  V  L  L  M  A  M  G  F
2041 ATCTGCGGCGAAATGGCCGGCGACCCGGCAGCCGCCGTGCTGCTCATGGCGATGGGCTTC
 701 D  S  L  S  M  N  A  T  N  L  P  K  V  K  W  M  L  R  Q  I
2101 GACAGCCTGTCGATGAACGCCACCAACCTGCCGAAAGTGAAGTGGATGCTGCGCCAGATC
 721 N  L  S  K  A  K  E  L  L  A  Q  L  M  T  N  D  N  P  Q  V
2161 AACCTCAGCAAGGCCAAGGAGTTGCTGGCGCAACTGATGACCAACGACAACCCGCAAGTC
 741 I  S  S  S  L  Q  L  A  L  K  N  L  G  L  S  R  M  I  N  P
2221 ATCAGCAGCTCCCTGCAACTGGCACTGAAAAACCTCGGTCTGTCGCGGATGATCAACCCG
 761 G  S  V  K  G  H  *  L  R  L  Q  D  R  T  Q  S  V  Q  K  G
2281 GGCTCGGTCAAAGGGCACTAGCTGCGCTTGCAGGACCGGACGCAGAGCGTCCAGAAAGGC
 781 A  T  T  R  S  V  G  A  M  T  I  V  Q  Q  I  F  I  G  C  T
2341 GCTACCACGCGGAGCGTGGGAGCGATGACCATTGTTCAGCAAATCTTCATTGGCTGTACC
 801 D
2401 GACC
```

Figure B-3. Alignment of the *Pst* DC3000 *ptsP* predicted protein with the corresponding protein in *Azotobacter vinelandii.*

Blast 2 sequences (NCBI) was used to generate this alignment.

Sequence 1 gi 3641832enzyme I [*Azotobacter vinelandii*] **Length**759 (1 .. 759)
Sequence 2 lcl|PstDC3000 PtsP (X4) **Length**759 (1 .. 759)

Score = 1233 bits (3191), Expect = 0.0
Identities = 628/753 (83%), Positives = 673/753 (88%)

```
Query: 1   MLNTLRKIVQEVNSAKDLKTALGIIVRRVKEAMGSQVCSVYLLDPETNRFVLMATDGLNK 60
           MLNTLRKIVQEVNSAKDLK ALGIIV RVKEAMGSQVCSVYLLD E NRFVLMA++GLNK
Sbjct: 1   MLNTLRKIVQEVNSAKDLKAALGIIVLRVKEAMGSQVCSVYLLDAEVNRFVLMASEGLNK 60

Query: 61  RSIGKVSMASNEGLVGLVGTREEPLNLENAAAHPRYRYFAETGEERYASFLGAPIIHHRR 120
           RSIGKVSMA NEGLVGLVGTREEPLNLENAA HPRYRYFAETGEERYASFLGAPIIHHRR
Sbjct: 61  RSIGKVSMAPNEGLVGLVGTREEPLNLENAADHPRYRYFAETGEERYASFLGAPIIHHRR 120

Query: 121 VMGVLVVQQKERREFDEGEEAFLVTMSAQLAGVIAHAEATGSIRGLGRQGKGIQEARFXX 180
           V+GVLV+QQKERR+FDEGEEAFLVTMSAQLAGVIAHAEATGSIRGLGRQGKGIQEA+F
Sbjct: 121 VVGVLVIQQKERRQFDEGEEAFLVTMSAQLAGVIAHAEATGSIRGLGRQGKGIQEAKFVG 180

Query: 181 XXXXXXXXXXXXXXXXXXXXXXXXXKPAEDLKAELELFGNALEAVRADIRALSAKLATQ 240
                                    K   D+ AEL LF NALE VR D+R LSAKLATQ
Sbjct: 181 VAGSPGAAVGVAVVMLPPADLEVVPDKTVTDIAAELTLFQNALEGVRNDMRTLSAKLATQ 240

Query: 241 LRPEERALFDVYLMMLDDASLGCEVQRVIRTGQWAQGALRQVVNEHVKRFELMDDAYLRE 300
           LRPEERALFDVYLMMLDDASLG EV  VI+TG+WAQGALR VV+EHVKRFELMDDAYLRE
Sbjct: 241 LRPEERALFDVYLMMLDDASLGSEVTNVIKTGEWAQGALRSVVSEHVKRFELMDDAYLRE 300

Query: 301 RASDVKDLGXXXXXXXXXXXXXXMVYADNTILVSEELSSAMLGELPEGKLVGLVSVQGSG 360
           RASDVKDLG               +VY DNTILVSEEL+ AMLGE+PEGKLVGLVSVQGSG
Sbjct: 301 RASDVKDLGRRLLAYLQEERQQALVYPDNTILVSEELTPAMLGEVPEGKLVGLVSVQGSG 360

Query: 361 NSHVAILARAMGIPTVMGVVDLPYSKMDGIDLIVDGYHGEVYTNPSDMLRQQFADLVEEE 420
           NSHVAILARAMGIPTVMG+VD PYSK+DGIDL+VDGYHGEV+TNPS+++R+QF  +VEEE
Sbjct: 361 NSHVAILARAMGIPTVMGLVDFPYSKVDGIDLVVDGYHGEVFTNPSEIMRKQFGKVVEEE 420

Query: 421 RQLTQGLDALRELPCETLDGHRLPLWVNTGLLADVARAQERGAEGVGLYRTEVPFMNNER 480
           RQL+QGLDALRELPC TLDGHR+PLWVNTGLLADVARAQ+RGAEGVGLYRTEVPFM N+R
Sbjct: 421 RQLSQGLDALRELPCVTLDGHRMPLWVNTGLLADVARAQQRGAEGVGLYRTEVPFMINQR 480

Query: 481 FPSEKEQLAIYRDQLSAFYPLPVTMRTLDIGGDKSLSYFPIKESNPFLGWRGIRVTLDHP 540
           FPSEKEQLAIYR+QL+AF+PLPVTMR+LDIGGDKSLSYFPIKE NPFLGWRGIRVTLDHP
Sbjct: 481 FPSEKEQLAIYREQLAAFHPLPVTMRSLDIGGDKSLSYFPIKEDNPFLGWRGIRVTLDHP 540

Query: 541 EIFLVQVRAMLKASEGLNNLRVLLPMISSIHELDEALHLIHRAWGEVRDEGTDVPMPPVG 600
           EIFLVQ RAMLKASEGLNNLR+LLPMISS HE++EALHLIHRAWGEVRDEGTDVPMPPVG
Sbjct: 541 EIFLVQTRAMLKASEGLNNLRILLPMISSTHEVEEALHLIHRAWGEVRDEGTDVPMPPVG 600

Query: 601 VMVEIPAAVYQTRELARLVDFLSVGSNDLTQYLLAVDRNNPRVADLYDYLHPAVLQALNK 660
           VM+E+PAAVYQTR+LAR VDFLSVGSNDLTQYLLAVDRNNPRVADLYDYLHPAVLQAL
Sbjct: 601 VMIEVPAAVYQTRDLARQVDFLSVGSNDLTQYLLAVDRNNPRVADLYDYLHPAVLQALQS 660

Query: 661 VVQDAHAEGKPVSICGEMAGDPSAAVLLMAMGFDSLSMNATNLPKVKWLLRQITLSKARE 720
           VV+DAHAEGKPVSICGEMAGDP+AAVLLMAMGFDSLSMNATNLPKVKW+LRQI LSKA+E
Sbjct: 661 VVRDAHAEGKPVSICGEMAGDPAAAVLLMAMGFDSLSMNATNLPKVKWMLRQINLSKAKE 720

Query: 721 LLDRLMAIDNPQVIHSTLQLALRNLGLGRVINP 753
             LL +LM  DNPQVI S+LQLAL+NLGL R+INP
Sbjct: 721 LLAQLMTNDNPQVISSSLQLALKNLGLSRMINP 753
```

Predicted operon structure of *Pst* DC3000 *uvrD* region

Z – putative transcriptional regulator
A – *uvrD* which encodes a type II DNA helicase
B – Hypothetical protein
Y – Hypothetical protein

Figure B-4. Diagram of the predicted operon structure of the *Pst* DC3000 *uvrD* gene region.

This diagram is not to scale.

Figure B-5. Translation of the *Pst* DC3000 *uvrD* genomic region.

The putative Shine-Dalgarno sequence is highlighted in yellow. The predicted translational start site is underlined. The transposon insertion site is highlighted in green.

```
  1 A  *  A  *  G  P  E  T  R  C  N  Q  W  R  D  R  N  A  R  W
  1 GCTTGAGCTTGAGGTCCTGAAACCCGGTGCAACCAATGGCGCGACAGAAACGCACGATGG
 21 S  A  R  *  C  R  R  C  A  P  D  R  P  W  N  C  A  S  Q  P
 61 TCGGCTCGCTGATGCCGACGCTGTGCGCCAGATCGGCCATGGAACTGTGCATCACAGCCG
 41 Q  G  Q  A  A  H  G  R  L  L  *  A  P  I  C  A  A  G  D  V
121 CAGGGTCAAGCAGCACATGGTCGGCTACTTTGAGCTCCGATTTGCGCAGCAGGTGACGTG
 61 T  E  R  C  V  A  A  D  S  R  G  R  L  N  K  G  K  M  G  W
181 ACTGAGCGATGTGTTGCAGCAGATTCAAGGGGCAGACTCAACAAAGGGAAGATGGGCTGG
 81 V  V  A  F  L  *  L  Y  Y  M  T  C  K  P  P  S  *  T  T  L
241 GTTGTAGCTTTCTTGTAGTTATACTACATGACCTGCAAACCGCCCAGTTAAACGACGCTG
101 E  R  P  A  R  A  *  K  G  K  F  K  K  I  C  G  N  N  P  T
301 GAACGTCCTGCGCGAGCCTGAAAAGGAAAATTCAAAAAAATATGTGGGAATAATCCTACA
121 L  R  R  A  S  H  Q  T  R  A  R  S  S  G  N  D  R  L  T  L
361 TTACGTCGAGCCTCACACCAGACCCGCGCACGCTCATCGGGCAACGATAGACTGACCTTA
141 F  K  P  G  S  M  A  T  T  G  R  Q  H  L  L  I  F  Y  A  *
421 TTCAAGCCAGGCAGCATGGCGACGACCGGTCGTCAGCACCTGCTCATTTTTTATGCCTGA
161 R  K  A  W  F  A  R  H  R  A  A  P  *  N  A  L  M  R  D  D
481 CGCAAAGCATGGTTTGCCCGTCATCGCGCGGCACCCTAGAATGCGCTGATGCGCGATGAT
181 L  S  V  L  L  N  S  L  N  D  A  Q  R  Q  A  V  A  A  S  L
541 CTCTCTGTTCTTCTTAATTCCCTCAACGATGCCCAACGTCAGGCCGTAGCCGCCTCATTG
201 G  R  Q  L  V  L  A  G  A  G  S  G  K  T  R  V  L  V  H  R
601 GGTCGTCAGTTGGTCCTGGCCGGTGCTGGCTCCGGCAAAACCCGAGTGCTGGTGCATCGC
221 I  A  W  L  M  Q  V  E  Q  A  S  P  H  S  V  L  S  V  T  F
661 ATCGCCTGGCTGATGCAGGTCGAGCAAGCCTCCCCGCATTCGGTCCTGTCGGTGACGTTC
241 T  N  K  A  A  A  E  M  R  H  R  I  E  Q  L  M  G  I  S  P
721 ACCAACAAGGCAGCTGCCGAGATGCGCCACCGCATCGAGCAGCTCATGGGCATCAGCCCT
261 A  G  M  W  V  G  T  F  H  G  L  A  H  R  L  L  R  A  H  W
781 GCTGGCATGTGGGTAGGCACTTTCCACGGCCTGGCGCACCGCCTGTTGCGGGCGCACTGG
281 Q  E  A  G  L  V  Q  T  F  Q  I  L  D  S  D  D  Q  Q  R  L
841 CAGGAAGCCGGGCTGGTGCAGACCTTCCAGATTCTCGACAGCGATGACCAGCAACGTCTG
301 V  K  R  V  M  R  E  L  G  L  D  E  Q  L  W  P  A  R  Q  A
901 GTCAAGCGCGTGATGCGCGAGCTGGGTCTGGACGAGCAACTCTGGCCTGCGCGTCAGGCT
321 Q  W  F  I  N  G  Q  K  D  E  G  L  R  P  K  H  I  Q  A  S
961 CAGTGGTTCATCAATGGTCAGAAAGACGAAGGCCTGCGCCCGAAACATATTCAGGCCAGC
341 G  D  L  F  L  T  T  M  K  S  V  Y  E  A  Y  E  A  A  C  Q
1021 GGCGATCTGTTCCTGACCACCATGAAAAGCGTTTACGAAGCCTACGAGGCTGCCTGCCAG
361 R  A  G  V  I  D  F  S  E  L  L  L  R  A  L  D  L  W  R  D
1081 CGTGCAGGCGTCATCGACTTCTCTGAGTTGCTGCTGCGCGCGCTGGACCTGTGGCGTGAC
381 N  P  G  L  L  A  H  Y  Q  R  R  F  R  H  V  L  V  D  E  F
1141 AACCCTGGCTTGCTGGCGCACTACCAGCGCCGCTTCCGCCACGTACTGGTGGACGAGTTT
401 Q  D  T  N  A  V  Q  Y  A  W  L  R  L  L  A  Q  G  G  D  S
1201 CAAGATACCAACGCCGTGCAATATGCCTGGCTGCGCCTGCTTGCTCAGGGTGGCGACAGC
421 L  M  V  V  G  D  D  D  Q  S  I  Y  G  W  R  G  A  K  I  E
1261 CTGATGGTGGTCGGCGACGACGATCAGTCCATCTACGGCTGGCGTGGCGCGAAGATCGAG
441 N  I  H  Q  Y  S  D  D  F  P  D  T  E  V  I  R  L  E  Q  N
1321 AACATCCATCAGTATTCCGATGATTTCCCGGACACCGAAGTGATCCGCCTGGAGCAGAAC
461 Y  R  S  T  A  S  I  L  K  A  A  N  G  L  I  I  N  N  S  G
1381 TACCGCTCCACGGCGAGCATCCTCAAAGCCGCCAACGGTTTGATCATCAATAACAGCGGG
481 R  L  G  K  E  L  W  T  D  V  G  D  G  E  L  I  N  L  Y  A
1441 CGTCTGGGCAAAGAGTTGTGGACCGACGTCGGCGATGGCGAGCTGATCAATCTGTATGCC
501 A  F  N  E  H  D  E  A  R  Y  V  V  E  T  I  E  S  A  L  K
1501 GCCTTCAACGAACACGACGAAGCGCGTTACGTGGTCGAGACCATCGAGAGCGCCCTGAAA
521 T  G  I  S  R  N  D  I  A  I  L  Y  R  S  N  A  Q  S  R  V
1561 ACCGGCATCTCACGCAACGACATCGCCATTCTGTACCGTTCCAACGCCCAGTCTAGGGTG
541 L  E  E  A  L  L  R  E  R  I  P  Y  R  I  Y  G  G  Q  R  F
1621 CTGGAAGAAGCCCTGCTGCGCGAGCGTATTCCGTACCGCATCTATGGCGGGCAGCGCTTT
561 F  E  R  A  E  I  K  N  A  M  A  Y  M  R  L  L  E  G  R  G
```

```
1681 TTCGAGCGCGCCGAGATCAAGAACGCGATGGCCTATATGCGCCTGCTGGAAGGTCGCGGC
 581 N  D  A  A  L  E  R  V  I  N  V  P  A  R  G  I  G  E  K  T
1741 AACGATGCCGCGCTGGAGCGGGTGATCAACGTTCCGGCACGCGGCATCGGCGAGAAAACC
 601 V  E  A  I  R  E  H  A  R  H  A  D  V  S  M  W  E  A  M  R
1801 GTCGAAGCGATTCGCGAGCACGCGCGGCATGCCGACGTGTCGATGTGGGAAGCCATGCGC
 621 L  L  V  A  N  K  G  L  T  G  R  A  A  G  A  L  G  G  F  I
1861 CTGCTGGTCGCCAACAAGGGCCTGACTGGCCGCGCTGCCGGCGCGCTGGGCGGGTTTATC
 641 E  L  I  E  N  L  S  A  K  V  M  E  M  P  L  H  L  M  T  Q
1921 GAGCTGATCGAGAACCTGTCGGCCAAGGTCATGGAAATGCCGCTGCACCTGATGACTCAG
 661 T  V  I  E  Q  S  G  L  I  T  Y  H  E  Q  E  K  G  E  K  G
1981 ACAGTCATCGAGCAGTCCGGGCTGATTACCTATCACGAACAGGAAAAAGGCGAGAAAGGC
 681 Q  A  R  V  E  N  L  E  E  L  V  S  A  A  R  A
2041 CAGGCCCGGGTAGAAAACCTTGAGGAACTGGTCAGCGCCGCTCGGGCT
```

Figure B-6. Alignment of the *Pst* DC3000 *uvrD* predicted protein with the corresponding protein in *Pseudomonas aeruginosa.*

Blast 2 sequences (NCBI) was used to generate this alignment.

Sequence 1 gi 15600636DNA helicase II [Pseudomonas aeruginosa]**Length**728 (1 .. 728)
Sequence 2 lcl|PstDC3000 UvrD (W56)**Length**727 (1 .. 727)

```
Score = 1170 bits (3027), Expect = 0.0
Identities = 584/713 (81%), Positives = 642/713 (89%), Gaps = 1/713
(0%)

Query: 16  QRQAVAAPLGRQLVLAGAGSGKTRVLVHRIAWLIQVEHASPYSILSVTFTNKAAAEMRHR 75
           QRQAVAA LGRQLVLAGAGSGKTRVLVHRIAWL+QVE ASP+S+LSVTFTNKAAAEMRHR
Sbjct: 16  QRQAVAASLGRQLVLAGAGSGKTRVLVHRIAWLMQVEQASPHSVLSVTFTNKAAAEMRHR 75

Query: 76  IEQLLGINPAGMWVGTFHGLAHRLLRAHWREAGLSENFQILDSDDQQRLVKRVIRELGLD 135
           IEQL+GI+PAGMWVGTFHGLAHRLLRAHW+EAGL + FQILDSDDQQRLVKRV+RELGLD
Sbjct: 76  IEQLMGISPAGMWVGTFHGLAHRLLRAHWQEAGLVQTFQILDSDDQQRLVKRVMRELGLD 135

Query: 136 EQRWPARQAQWFINGQKDEGLRPQHIQPGGDLFLATMLKIYEAYEAACARAGVIDFSELL 195
           EQ WPARQAQWFINGQKDEGLRP+HIQ  GDLFL TM  +YEAYEAAC RAGVIDFSELL
Sbjct: 136 EQLWPARQAQWFINGQKDEGLRPKHIQASGDLFLTTMKSVYEAYEAACQRAGVIDFSELL 195

Query: 196 LRALDLWRDHPGVLEHYQRRFRHILVDEFQDTNAVQYAWLRILAKGGDSLMVVGDDDQSI 255
           LRALDLWRD+PG+L HYQRRFRH+LVDEFQDTNAVQYAWLR+LA+GGDSLMVVGDDDQSI
Sbjct: 196 LRALDLWRDNPGLLAHYQRRFRHVLVDEFQDTNAVQYAWLRLLAQGGDSLMVVGDDDQSI 255

Query: 256 YGWRGARIENIQQFSDDFADAEVIRLEQNYRSTXXXXXXXXXXXXXXQGRLGKELWTDGE 315
           YGWRGA+IENI Q+SDDF D EVIRLEQNYRST                GRLGKELWTD
Sbjct: 256 YGWRGAKIENIHQYSDDFPDTEVIRLEQNYRSTASILKAANGLIINNSGRLGKELWTDVG 315

Query: 316 DGESLSLYAAFNEHDEARYVVESIESALKGGLARSEIAILYRSNAQSRVLEEALLREKIP 375
           DGE ++LYAAFNEHDEARYVVE+IESALK G++R++IAILYRSNAQSRVLEEALLRE+IP
Sbjct: 316 DGELINLYAAFNEHDEARYVVETIESALKTGISRNDIAILYRSNAQSRVLEEALLRERIP 375

Query: 376 YRIYGGQRFFERAEIKNAMAYLRLLDGRGNDAALERVVNVPARGIGEKTVESIREFARGN 435
           YRIYGGQRFFERAEIKNAMAY+RLL+GRGNDAALERV+NVPARGIGEKTVE+IRE AR
Sbjct: 376 YRIYGGQRFFERAEIKNAMAYMRLLEGRGNDAALERVINVPARGIGEKTVEAIREHARHA 435

Query: 436 DVSMWEAIRLMIANKVLPGRAASALTGFVELIENLSAKVMDMPLHLMTQTVIEQSGLISY 495
           DVSMWEA+RL++ANK L GRAA AL GF+ELIENLSAKVM+MPLHLMTQTVIEQSGLI+Y
Sbjct: 436 DVSMWEAMRLLVANKGLTGRAAGALGGFIELIENLSAKVMEMPLHLMTQTVIEQSGLITY 495

Query: 496 HKEEKGEKGQARVENLEELVSAARAFENSEEEEDLTPLQAFLSHASLEAGETQADAHEDS 555
           H++EKGEKGQARVENLEELVSAARAFEN E +E+LTPL AFL HASLEAG+TQA  HEDS
Sbjct: 496 HEQEKGEKGQARVENLEELVSAARAFENHESDEELTPLAAFLGHASLEAGDTQAQEHEDS 555

Query: 556 VQLMTLHSAKGLEFPLVFLVGMEEGLFPHKMXXXXXXXXXXXXXXAYVGVTRAMQRLVLT 615
           +QLMTLHSAKGLEFP VFLVGMEEGLFPHKM               AYVG+TRAM++LV+T
Sbjct: 556 IQLMTLHSAKGLEFPHVFLVGMEEGLFPHKMSLEEPGRLEEERRLAYVGITRAMKQLVMT 615

Query: 616 YAETRRLYGSETYNKVSRFIREIPPALIQEVRLSNTVSRPYGGTSRSAGGNLFSGAGVPE 675
           YAETRRLYGSETYNKVSRF+REIPPALIQEVRLSN+VSRP+GGT +   G+LF+G G+PE
Sbjct: 616 YAETRRLYGSETYNKVSRFVREIPPALIQEVRLSNSVSRPFGGTPKFNSGSLFNGTGIPE 675

Query: 676 TPFSLGQRVRHALFGEGTILNFEGAGAQARVQVNFESEGSKWLMLGYAKLEAL 728
           T F++GQRV+HA+FGEG ILNFEGAGAQARVQVNF +EGSKWLM+GYAKL AL
Sbjct: 676 TEFAMGQRVQHAVFGEGVILNFEGAGAQARVQVNF-AEGSKWLMMGYAKLVAL 727
```

Predicted operon structure of *Pst* DC3000 *oprF* region

C – *cmaX*
D – *crfX*
E -- *cmpX*
F – *sigX* which encodes an ECF sigma factor like protein
G – *oprF* which encodes the outer membrane porin F precursor protein
Z – Uroporphyrin methyltransferase like protein

Figure B-7. Diagram of the predicted operon structure of the *Pst* DC3000 *oprF* gene region.

This diagram is not to scale. The operon may continue beyond the *cmaX* gene.

Figure B-8. Translation of the *Pst* DC3000 *oprF* genomic region.

The putative Shine-Dalgarno sequence is highlighted in yellow. The predicted translational start site is underlined. The transposon insertion site for W23 and W38 is highlighted in green. The transposon insertion site for W62 is highlighted in blue. The transposon insertion site for W127 is highlighted in pink.

```
   1 H  D  I  W  G  T  I  A  M  L  T  M  S  V  R  R  *  C  L  K
   1 CACGATATCTGGGGAACGATCGCGATGCTGACGATGTCTGTCAGGAGGTGATGCTTAAAG
  21 C  C  T  G  *  K  I  L  K  V  N  L  N  L  R  P  G  C  T  A
  61 TGTTGTACGGGTTGAAAAATTTTGAAGGTAAATCTAAATTTAAGACCTGGCTGTACAGCA
  41 S  P  T  M  N  A  S  R  S  T  G  R  N  G  V  S  V  A  *  W
 121 TCACCTACAATGAATGCATCACGCAGTACAGGAAGGAACGGCGTAAGCGTCGCTTGATGG
  61 T  R  *  V  W  I  H  L  R  K  R  R  K  K  R  R  P  N  L  K
 181 ACGCGTTGAGTCTGGATCCACTTGAGGAAGCGTCGGAAGAAAAGACGCCCAAACCTGAAG
  81 S  G  A  D  L  I  A  G  L  C  M  *  T  R  S  I  G  K  F  W
 241 AGCGGGGCGGACTTGATCGCTGGCTTGTGTATGTGAACCCGATCGATCGGGAAATTCTGG
 101 Y  C  V  L  L  Q  N  L  N  S  R  R  S  Q  T  S  C  T  W  A
 301 TACTGCGTTTTGTTGCAGAACTTGAATTCCAGGAGATCGCAGACATCATGCACATGGGCT
 121 *  A  Q  R  K  C  V  T  S  G  L  W  I  N  Y  V  R  N  L  R
 361 TGAGCGCAACGAAAATGCGTTACAAGCGGGCTTTGGATAAATTACGTGAGAAATTTGCGG
 141 E  S  P  K  L  N  T  V  Q  I  S  L  T  N  W  Q  G  L  V  H
 421 GAATCGCCGAAACTTAATACGGTGCAAATATCTCTAACGAACTGGCAAGGTCTGGTACAC
 161 L  P  T  S  C  P  H  L  V  G  L  L  Y  N  H  Q  M  G  I  *
 481 TTGCCGACGAGTTGTCCCCACCTTGTGGGACTGCTTTATAATCATCAGATGGGGATTTAA
 181 R  M  K  L  K  N  T  L  G  L  A  I  G  T  I  V  A  A  T  S
 541 CGGATGAAACTGAAAAACACCTTGGGCTTGGCCATTGGTACTATTGTTGCCGCAACTTCG
 201 F  G  A  L  A  Q  G  Q  G  A  V  E  I  E  G  F  A  K  K  E
 601 TTCGGCGCGCTGGCTCAAGGCCAAGGCGCAGTCGAAATCGAAGGCTTCGCCAAGAAAGAA
 221 M  Y  D  S  A  R  D  F  K  N  N  G  N  L  F  G  G  S  I  G
 661 ATGTACGACAGCGCCCGTGACTTCAAAAACAACGGCAACCTGTTCGGCGGCTCGATTGGC
 241 Y  F  L  T  D  D  V  E  L  R  L  G  Y  D  E  V  H  N  V  R
 721 TACTTCCTGACCGACGACGTTGAATTGCGTCTGGGCTACGACGAAGTTCACAACGTTCGT
 261 S  D  D  G  K  N  I  K  G  A  N  T  A  L  D  A  L  Y  H  F
 781 AGCGATGATGGCAAGAACATCAAGGGCGCAAACACCGCTCTGGACGCTCTGTACCACTTC
 281 N  N  P  G  D  M  L  R  P  Y  V  S  A  G  F  S  D  Q  S  I
 841 AACAACCCAGGCGACATGCTCGTCCATACGTTTCTGCCGGTTTCTCCGATCAGAGCATT
 301 G  Q  N  G  R  N  G  R  N  G  S  T  F  A  N  I  G  G  G  A
 901 GGCCAGAACGGTCGCAACGGTCGTAACGGTTCCACCTTCGCCAACATCGGCGGCGGTGCC
 321 K  L  Y  F  T  D  N  F  Y  A  R  A  G  V  E  A  Q  Y  N  I
 961 AAGCTGTACTTCACCGACAATTTCTATGCTCGTGCTGGCGTTGAAGCTCAGTACAACATC
 341 D  Q  G  D  T  E  W  A  P  S  V  G  I  G  V  N  F  G  G  G
1021 GACCAAGGCGACACCGAGTGGGCTCCAAGCGTCGGTATCGGCGTAACCTTCGGTGGCGGC
 361 S  K  K  V  E  A  A  P  A  P  V  A  E  V  C  S  D  S  D  N
1081 AGCAAGAAAGTTGAAGCAGCACCAGCTGCAGTAGCTGAAGTGTGCTCCGACAGCGACAAC
 381 D  G  V  C  D  N  V  D  K  C  P  D  T  P  A  N  V  T  V  D
1141 GACGGCGTGTGCGACAACGTCGACAAGTGCCCAGACACCCAGCCAACGTAACTGTTGAC
 401 A  D  G  C  P  A  V  A  E  V  V  R  V  E  L  D  V  K  F  D
1201 GCTGATGGCTGCCCGGCAGTTGCTGAAGTGGTTCGTCTTGAGCTGGACGTGAAGTTCGAC
 421 F  N  K  S  V  V  K  P  N  S  M  G  D  I  K  N  L  A  D  F
1261 TTCAACAAGTCTGTTGTCAAGCCTAACAGCATGGGCGACATCAAGAACCTCGCTGACTTC
 441 M  Q  Q  Y  P  Q  T  T  T  V  E  G  H  T  D  S  V  G  P
1321 ATGCAGCAGTACCCACAGACCACCACCACTGTTGAAGGTCACACTGACTCCGTCGGTCCT
 461 D  A  Y  N  Q  K  L  S  E  R  R  A  N  A  V  K  Q  V  L  V
1381 GACGCTTACAACCAAAAACTGTCCGAGCGTCGTGCAAACGCCGTTAAACAGGTTCTGGTT
 481 N  Q  Y  G  V  G  A  S  R  V  N  S  V  G  Y  G  E  S  R  P
1441 AACCAGTACGGTGTTGGCGCTAGCCGCGTAAACTCGGTTGGTTACGGCGAATCCCGCCCA
 501 V  A  D  N  A  T  D  A  G  R  A  V  N  R  R  V  E  A  E  V
1501 GTTGCTGACAACGCAACTGACGCTGGCCGTGCAGTAAACCGTCGCGTAGAAGCAGAAGTA
 521 E  A  Q  A  K  *
1561 GAAGCTCAAGCTAAGTAA
```

Figure B-9. Alignment of the *Pst* DC3000 *oprF* predicted protein with the corresponding protein in *Pseudomonas syringae pv. syringae.*

Blast 2 sequences (NCBI) was used to generate this alignment.

Sequence 1 gi 130681OUTER MEMBRANE PORIN F PRECURSOR *Pseudomonas syringae pv. syringae*
Length344 (1 .. 344)
Sequence 2 lcl|PstDC3000 OprF (W23/W38, W62, W127)**Length**344 (1 .. 344)

```
Score =  660 bits (1702), Expect = 0.0
Identities = 327/344 (95%), Positives = 331/344 (96%)

Query: 1   MKLKNTLGLAIGTIVAATSFGALAQGQGAVEIEGFAKKEMYDSARDFKNNGNLFGGSIGY 60
           MKLKNTLGLAIGTIVAATSFGALAQGQGAVEIEGFAKKEMYDSARDFKNNGNLFGGSIGY
Sbjct: 1   MKLKNTLGLAIGTIVAATSFGALAQGQGAVEIEGFAKKEMYDSARDFKNNGNLFGGSIGY 60

Query: 61  FLTDDVELRLGYDEVHNVRSDDGKNIKGADTALDALYHFNNPGDMLRPYVSAGFSDQSIG 120
           FLTDDVELRLGYDEVHNVRSDDGKNIKGA+TALDALYHFNNPGDMLRPYVSAGFSDQSIG
Sbjct: 61  FLTDDVELRLGYDEVHNVRSDDGKNIKGANTALDALYHFNNPGDMLRPYVSAGFSDQSIG 120

Query: 121 QNGRNGRNGSTFANIGGGPKLYFTDNFYARAGVEAQYNIDQGDTEWAPSVGIGVNFGGGS 180
           QNGRNGRNGSTFANIGGG KLYFTDNFYARAGVEAQYNIDQGDTEWAPSVGIGVNFGGGS
Sbjct: 121 QNGRNGRNGSTFANIGGGAKLYFTDNFYARAGVEAQYNIDQGDTEWAPSVGIGVNFGGGS 180

Query: 181 KKXXXXXXXXXXXCSDSDNDGVCDNVDKCPDTPANVTVDADGCPAVAEVVRVELDVKFDF 240
           KK           CSDSDNDGVCDNVDKCPDTPANVTVDADGCPAVAEVVRVELDVKFDF
Sbjct: 181 KKVEAAPAPVAEVCSDSDNDGVCDNVDKCPDTPANVTVDADGCPAVAEVVRVELDVKFDF 240

Query: 241 DKSVVKPNSYGDIKNLADFMQQYPQTTTTVEGHTDSVGPDAYNQKLSERRANAVKQVLVN 300
           +KSVVKPNS GDIKNLADFMQQYPQTTTTVEGHTDSVGPDAYNQKLSERRANAVKQVLVN
Sbjct: 241 NKSVVKPNSMGDIKNLADFMQQYPQTTTTVEGHTDSVGPDAYNQKLSERRANAVKQVLVN 300

Query: 301 QYGVGASRVNSVGYGESKPVADNATEAGRAVNRRVEAEVEAQAK 344
           QYGVGASRVNSVGYGES+PVADNAT+AGRAVNRRVEAEVEAQAK
Sbjct: 301 QYGVGASRVNSVGYGESRPVADNATDAGRAVNRRVEAEVEAQAK 344
```