STRAIN TYPING AND CHARACTERIZATION OF σ^{54}-DEPENDENT TRANSCRIPTIONAL ACTIVATOR MUTANTS IN *PSEUDOMONAS SYRINGAE* PV. TOMATO

By

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CHAPTER I

INTRODUCTION

Pseudomonas syringae is a gram negative plant pathogenic bacterium divided into pathogenic variants (pathovars, pv.), which are named according to their host range. *P. syringae* produces a wide variety of symptoms in plants including blights (extensive tissue death), wilting, and leaf spots. *P. syringae* strains are diverse and interact with their host plants in specific interactions. *P. syringae* pv. tomato DC3000 (*Pst* DC3000) is pathogenic on tomato (Cuppels, 1986), edible *Brassica* spp., (collard, turnip) (Elizabeth and Bender, 2007; Zhao *et al.*, 2000) and Arabidopsis (Whalen *et al.*, 1991), and is a model organism for studying plant-microbe interactions (Buell *et al.*, 2003). The infection cycle of *P. syringae* involves epiphytic (surface) colonization, and entry (typically by stomata) into the intercellular space (apoplast). Multiplication of the bacteria to high levels often results in visible symptoms, which are typically necrotic lesions, often surrounded by chlorotic halos (Buell *et al.*, 2003). In incompatible interactions with non-host plants, *P. syringae* elicits a plant defense mechanism known as the hypersensitive response (HR).

Pst DC3000 is related to several bacterial species that are on the USDA APHIS select agent list. However, unlike the select agents, *P. syringae* is an endemic pathogen

that can be easily utilized as a model for biosecurity research as it does not require strict containment procedures. *Pst* DC3000 is the causal agent of bacterial speck disease, an economically important disease of tomato. On edible, leafy *Brassica spp*., *Pst* DC3000 produces disease symptoms that are indistinguishable from the very closely related *P. syringae* pv. maculicola (*Psm*) (Zhao *et al.*, 2000), which is also able to infect tomato (Cuppels and Ainsworth, 1995; Hendson *et al.*, 1992). Both *P. syringae* pv. tomato and *P. syringae* pv. maculicola belong to genomospecies III (Gardan *et al.*, 1999), and taxonomic studies have shown that *Pseudomonas* spp. and pathovars of *P. syringae* can be identified with metabolic assays, phenotypic tests, and molecular diagnostic assays. The ability to distinguish among bacterial strains is important for epidemiological tracing of pathogen outbreaks and monitoring spread of pathogens. Although numerous assays have been utilized for typing *Pst*, these were unable to distinguish strains of the pathogen (Clerc *et al.*, 1998, Gardan *et al.*, 1999, Zhao et al., 2000). Studies with other bacterial species demonstrated that variable number tandem repeat (VNTR) loci, which are found throughout bacterial genomes, are useful for rapid and reliable strain typing by multiple-locus VNTR analysis (MLVA). Hence, as part of this work, a more discriminatory strain typing method was developed utilizing VNTR loci, and this was used to type a large collection of *P. syringae* pv. tomato strains of diverse origin by MLVA analysis.

Pst DC3000 is a model organism for studying bacterial pathogenesis and plant-microbe interactions (Preston, 2000). In bacteria, the alternative sigma factor σ^{54} (encoded by *rpoN*) interacts with RNA polymerase and associated activator proteins to regulate the transcription of target genes involved in metabolic and physiological processes. Previous research suggested that σ^{54} is involved in regulating many

pathogenicity and virulence factors in *P. syringae*, as well as factors important for bacteria survival including carbon, nitrogen and amino acid assimilation. *rpoN* mutants of *P. syringae* pv. maculicola ES4326 (Hendrickson *et al.*, 2000a, Hendrickson *et al.*, 2000b) and *P. syringae* pv. glycinea PG4180 (Alarcon-Chaidez *et al.*, 2003) were unable to induce disease symptoms and multiply in host plants, and were also deficient in the ability to utilize various carbon, nitrogen and amino acid sources. While much is known about pathogenicity and virulence factors in *P. syringae*, virtually nothing is known about regulatory loci controlled by σ^{54}. The genome sequence of *Pst* DC3000 contains over 70 predicted recognition sites for σ^{54} (http://www.promscan.uklinux.net/), and there are 14 σ^{54}-dependent activators annotated in the *Pst* DC3000 genome (http://www.tigr.org/), suggesting that these activators play a critical role in gene regulation in this organism. In order to better understand regulatory networks in *P. syringae*, mutations were generated in several σ^{54}-dependent activators, and these mutants were characterized as part of this work. The characterization of these activators will yield insights into the regulatory networks in *P. syringae*, which will be highly significant and relevant to the regulation of survival and pathogenesis in gram-negative bacteria.

CHAPTER II

LITERATURE REVIEW

***Pseudomonas syringae* disease symptoms**

Pseudomonas syringae pv. tomato (formerly known as *P. tomato*) causes a disease known as bacterial speck of tomato. Lesions on tomato leaves, fruit and stems appear dark brown to black as tissues become necrotic (Bashan *et al.*, 1978). Foliar lesions are circular and irregular in shape, range from 2-5 mm in diameter, and are often surrounded by chlorotic halos. Fruit lesions are primarily superficial and range in size from tiny flecks to up to 1 mm in diameter and are either raised or sunken (Getz *et al.*, 1983a). On edible *Brassica* species, *P. syringae* pv. tomato strain DC3000 (*Pst* DC3000) produces disease symptoms similar to *P. syringae* pv. maculicola (*Psm*), which include brown, irregular shaped interveinal lesions (Zhao *et al.*, 2000). Foliar lesions on *Brassica* spp. can be classified into three groups, chlorotic, water-soaked and necrotic (Wiebe and Campbell, 1993; Zhao *et al.*, 2000). Disease symptoms on the model Brassica species, *Arabidopsis thaliana* include water-soaked spreading grey-brown lesions, which are sometimes surrounded by a chlorotic margin (Preston, 2000; Whalen *et al.*, 1991).

***Pst* transmission**

Pst occurs world-wide wherever tomatoes are grown (Colin & Chafik, 1986, Goode & Sasser, 1980, Lawton & MacNeill, 1986) and is disseminated by multiple means including animals, people, insects, mites, agricultural tools, aircraft, soil particles, and water sources (Bashan, 1986). Airborne dispersal of *Pst* has also been demonstrated via plant canopies harboring epiphytic bacteria, which serve as a source of aerosolized bacteria (McInnes *et al.*, 1988). Sources of inoculum for *Pst* include infested weeds, crop and plant debris, seeds, volunteer tomato plants, agricultural tools and water (Bashan *et al.*, 1982; Jardine *et al.*, 1988; McCarter *et al.*, 1983; Schneider and Grogan, 1977b; Zhao *et al.*, 2002). In greenhouses, *Pst* can be disseminated through water and infected seedlings, which are then a source of inoculum in the field.

Pst can survive in the soil (Bashan *et al.*, 1978; Devash *et al.*, 1980; Goode and Sasser, 1980; Schneider and Grogan, 1977a) and on weeds (McCarter *et al.*, 1983; Schneider and Grogan, 1977a) and seeds; the latter is important as *Pst* has been recovered from commercial seed lots (Bashan *et al.*, 1978, 1982; McCarter *et al.*, 1983). Seeds are thought to be surface-contaminated when infected fruit is smashed during seed extraction (Devash *et al.*, 1980). However, there is some controversy about whether the bacteria actually survive on the seed during various processes. *Pst* has been isolated from seed obtained from infected fruit (Chambers & Merriman, 1975), but was not isolated from seed extracted by the fermentation process (Chambers & Merriman, 1975) or seed subjected to heat treatment (Devash *et al.*, 1980).

Epidemiology and life cycle

Bacterial speck disease is affected by weather and other environmental factors; e.g. rain increases disease severity, and blowing sand or hail can induce wounding (Goode & Sasser, 1980). Bacterial speck is favored and enhanced by 'cooler' temperatures ranging from 13-28^{O}C and high relative humidity (Bonn, 1980; McCarter *et al.*, 1983; Smitley and Mccarter, 1982; Yunis *et al.*, 1980a); furthermore, *Pst* can survive and overwinter in plant debris (Bonn, 1980; Chambers and Merriman, 1975; Jardine *et al.*, 1988).

As mentioned above, inoculum sources for *Pst* include infected seeds, transplants, and host and nonhost plant debris (Fig.1). *Pst* can survive as an epiphyte on host and nonhost plants, and during favorable environmental conditions, the resident population multiplies, infects the host, and causes disease (Schneider & Grogan, 1977a). *Pst* can survive as a epiphyte on symptomless tomato transplants in both the growth chamber and the field (Smitley & McCarter, 1982). The presence of *Pst* on seeds used for transplant production is problematic for the transplant industry since *Pst* spreads rapidly (McCarter *et al.*, 1983, Smitley & McCarter, 1982). In one study, seedlings grown from infested seeds developed disease symptoms, and asymptomatic plants developed symptoms when grown under high relative humidity (McCarter *et al.*, 1983).

On tomatoes, *Pst* survives and multiplies on the leaf surface, specifically in areas such as stomata and trichome bases (Bashan *et al.*, 1981). Trichomes and stomata serve as entry sites for foliar infection by *Pst* (Bashan *et al.*, 1978, Bashan *et al.*, 1981, Schneider & Grogan, 1977b). Disease outbreaks occur more frequently after adverse weather conditions, creating wounds and environmental conditions favorable to disease

development, indicating that these natural openings are limited (Melotto *et al.*, 2008). In the period following anthesis, green fruit less than 3 cm in diameter are most susceptible to *Pst* infection (Getz *et al.*, 1983b). Infection of fruit occurs via open trichome bases that remain after trichomes are lost and before the cuticle is fully developed (Getz *et al.*, 1983a). Fruit infection results in infestation of seeds and seedlings.

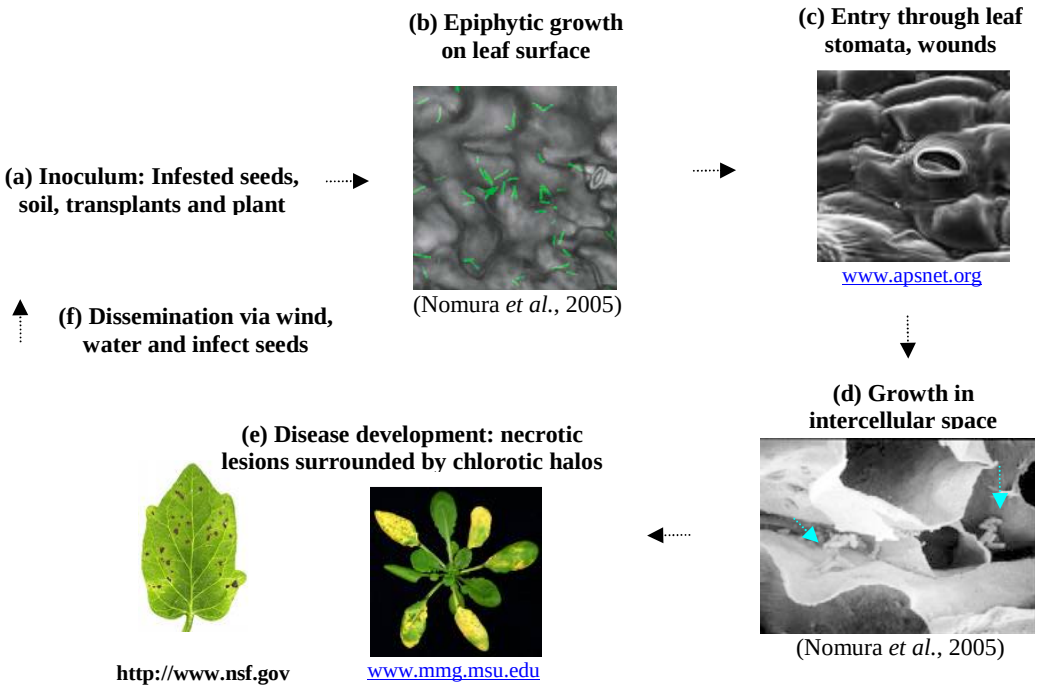


Fig. 1. The infection process of *Pst*. (a) Infested seeds, soil and plant debris provide inoculum. (b) *Pst* DC3000 labeled with GFP (green rods) growing epiphytically on an Arabidopsis leaf (Hirano & Upper, 2000). (c) Tomato leaf stomata. (d) SEM image of *Pst* DC3000 (arrows pointing to rod-shaped bacteria) growing in the intercellular space of an Arabidopsis leaf. (e) Tomato (left) and Arabidopsis (right) plants showing symptoms of *Pst* DC3000 infection. (f) Dissemination of bacteria. Model taken from http://pseudomonas-syringae.org/pst_ab_DC3000.htm and (Nomura *et al.*, 2005)

Economic impact

In the field, *Pst* spreads rapidly by natural means (Smitley & McCarter, 1982) and causes a reduction in both fruit quality and yield (McCarter *et al.*, 1983). Yield losses can be significant when plants are infected at early growth stages (Bashan *et al.*, 1978, Getz *et al.*, 1983b, Yunis *et al.*, 1980b). Fruits that develop after severe disease defoliation are

small, sunscalded, and of poor quality (Goode & Sasser, 1980). *Pst* infection reduces the market quality of tomato fruit (Smitley & McCarter, 1982), and lesions may make the fruit unfit for the fresh market (Goode & Sasser, 1980). In some cases the lesions can be deep enough to reduce quality after removal of the skin (Goode & Sasser, 1980).

Control of *Pst*

Cultural practices and copper bactericides alone or in combination with other treatments are primary mechanisms for control of *Pst* (Colin & Chafik, 1986, Goode & Sasser, 1980); however, survival of *Pst* on symptomless hosts, weeds, and nonhost plants makes it difficult to control by cultural practices (Lawton & MacNeill, 1986). One effective control technique is to obtain pathogen-free seed and treat with hot water (Goode & Sasser, 1980); in one study, surface-sterilized seeds were free of the pathogen and no disease developed on seedlings or plants (Bashan *et al.*, 1978).

Control with compounds such as streptomycin and cupric hydroxide (copper compound) can reduce fruit infection and disease spread, and increase yield, even in conditions optimal for disease development (Smitley & McCarter, 1982, Yunis *et al.*, 1980b); however, there is resistance to copper bactericides (Bender & Cooksey, 1986, Bender & Cooksey, 1987). Resistant tomato varieties have also been developed (Yunis *et al.*, 1980a), but sufficient levels of resistance to *Pst* do not exist in commercial cultivars (Goode & Sasser, 1980), and *Pst* isolates capable of overcoming the resistance have been detected and described (Lawton & MacNeill, 1986).

In one study, Actigard, which induces defense response pathways, was used for disease control and was similar or better than standard copper bactericides in efficacy

(Louws *et al.*, 2001). In other studies, biological control using various nonpathogenic *Pseudomonas* spp. was used to partially control and protect tomato plants from *Pst* (Colin & Chafik, 1986); however, efforts to integrate this approach with copper bactericides failed due to copper sensitivity of the *Pseudomonas* spp. used for biocontrol (Wilson *et al.*, 2002).

***Pst* persistence and dispersal**

Bacterial speck was first demonstrated as a disease in the United States in the 1930s (Bryan, 1933, Smitley & McCarter, 1982). It has been suggested that the widespread dissemination of *Pst* increased during the 1970s (Goode & Sasser, 1980, Pohronezny *et al.*, 1979, Wilkie & Dye, 1974, Yunis *et al.*, 1980b). The disease was first observed in Israel in 1970 (Bashan *et al.*, 1978), and it was prevalent in Australia in the 1970s (Chambers & Merriman, 1975). An intensive study of the physiological and biochemical characteristics of *Pst* isolates in New Zealand was conducted in the early 1970s (Wilkie & Dye, 1974).

Despite regulatory efforts, infestations of transplants can remain undetected due to a lack of symptoms or difficulties in distinguishing *Pst* symptoms from those of similar diseases such as bacterial spot, which is caused by *Xanthomonas campestris* pv. vesicatoria (Cuppels & Elmhirst, 1999, Goode & Sasser, 1980). Seed and seedling infestations provide a mechanism for long-distance dispersal. *Pst* is able to survive on seed for 20 years (Bashan *et al.*, 1982), and one study suggested that the spread of *Pst* throughout Israel occurred due to contaminated commercial seeds (Bashan *et al.*, 1978). Evidence also suggests that *Pst* was introduced into transplant fields in Georgia in the

1970s via commercial seed (McCarter *et al.*, 1983). Contaminated seeds (Denny *et al.*, 1988, McCarter et al., 1983) and/or infected plant tissue (Bonn *et al.*, 1985) facilitate long- distance transmission and help explain the worldwide distribution of the pathogen (Gitaitis & Walcott, 2007).

Agriculture and select agent biosecurity

Pst is related to two pathogens that are included on the APHIS select agent list *Ralstonia solanacearum* and *Xanthomonas oryzae* pv. oryzae. All three pathogens can be spread through infested seed, transplants, and water and can overwinter on diseased plants, plant debris and seed. All three of these bacteria enter plants via natural openings and wounds and have shared virulence mechanisms such as a type III secretion system (T3SS) and exopolysaccharide production (Grant *et al.*, 2006). *R. solanacearum, X. oryzae* pv. oryzae and *Pst* are not insect-transmitted. Unlike some pathogens (e.g. *Ralstonia*, *X. oryzae*, and *Xylella*), *Pst* is not thought to move in the vasculature (Bashan *et al.*, 1978). *Pst* fits many of the criteria suggested by Norm Schaad as indicative of potential risk and probability of causing harm (see Table 1, which was modified from the following web site: http://www.apsnet.org/online/feature/BioSecurity/Top.html).

Plant pathogen microbial forensics

While crop producers and agricultural scientists have traditionally focused on the prevention and management of plant diseases, the new area of plant pathogen microbial forensics is also concerned with criminal attribution of agricultural bioterrorism (Fletcher *et al.*, 2006). Some plant pathogens could be chosen as biological weapons, plant

pathologists have compiled the select agent list, expanded diagnostic laboratories, and networked these into a national system, improving disease control and diagnostic methods (Madden & Wheelis, 2003).

Table 1: Possible Bioterrorist Pathogen Rating Criteria

Pathogen rating criteria	***P. syringae* pv. tomato**
Produces toxin	√ √ √ Coronatine
Easy to obtain, handle and deliver	√ √ √ Can be transported easily
Easy to grow in large amounts	√ √ √ Large scale fermentations
Highly infectious under many conditions	√ Some weather dependence
Results in the establishment of a quarantine	√ √ √ Infected transplants = quarantine
No chemical control or host resistance available	√ Chemical controls often ineffective
No method for rapid and reliable detection	Some probes, PCR primers available
Infects systemically by natural means	√ *fruit* → *seed* → *transplant*
Spreads quickly by natural means	√ √ √
Causes severe crop losses	√ √
Survives long periods and is persistent	√ √ √ Can survive for many years on seed

The attributes listed in the left column make these pathogens highly infectious, and features listed on the right illustrate how *P. syringae* pv. tomato compares and why it makes a good model. See text sections above for more detailed information on the attributes of *P. syringae* pv. tomato.

Application to forensic investigation

Pathogen introductions occur frequently in the United States; although most of these are accidental, some introductions could be intentional. Thus, it would be ideal if the introduction could be traced back to a source, to either attribute an individual to the event, or to exclude them from possible involvement. While select agent organisms are regulated, hindering research studies, *Pst* DC3000 is endemic and not regulated, making it an ideal model for biosecurity research. The ability to discriminate an organism to the strain level is important in epidemiological tracking and the potential identification of the inoculum source, thus ensuring the safety and security of our food supply.

Pseudomonad genomes

Related strains of *P. syringae* that have been sequenced include *P. syringae* pv. phaseolicola 1448A, which causes halo blight of bean (Joardar *et al.*, 2005), and *P. syringae* pv. syringae (Feil *et al.*, 2005), which causes brown spot of bean. Other Pseudomonads have been sequenced including *P. aeruginosa* PA01 (Stover *et al.*, 2000), *P. aeruginosa* PA14 (Lee *et al.*, 2006), *P. putida* KT2440 (Nelson *et al.*, 2002), *P. entomophila* L48 (Vodovar *et al.*, 2006), *P. fluorescens* Pf-5 (Paulsen *et al.*, 2005) and *P. fluorescens* SBW25 (http://www.sanger.ac.uk/Projects/P_fluorescens/). These sequences provide a wealth of genome information that can be drawn on.

Pst and *P. syringae* pv. maculicola (*Psm*)

On edible, leafy *Brassica spp.*, *Pst* DC3000 can produce disease symptoms that are indistinguishable from the very closely related pathovar, *P. syringae* pv. maculicola (*Psm*) (Zhao *et al.*, 2000). *Psm* causes bacterial leaf spot of crucifers, and is also able to infect tomato (Cuppels & Ainsworth, 1995, Hendson *et al.*, 1992); furthermore, *Psm* and *Pst* are phenotypically similar and grouped into genomospecies III (Gardan *et al.*, 1999). However, molecular diagnostic assays for subtyping are often unable to distinguish among strains of *P. syringae* pv. tomato and *P. syringae* pv. maculicola (Clerc et al., 1998, Gardan et al., 1999, Zhao et al., 2000).

Overview of bacterial typing methods

For a typing method to be successful, it must meet certain criteria. The method must be able to type all organisms within a species, it must be able to differentiate clearly

between unrelated strains, and it must yield reproducible results when a particular strain is tested repeatedly. A few current technologies for typing include PFGE of whole chromosomal DNA, Southern blotting and restriction fragment length polymorphism (RFLP) methods, PCR-based locus-specific RFLP, random amplification of polymorphic DNA (RAPD) assays, rep-PCR, cleavase fragment length polymorphism (CFLP), amplified fragment length polymorphism (AFLP) assays and DNA sequencing (Olive & Bean, 1999).

Specific regions of DNA must also meet certain criteria before they can be used in typing. The region of DNA chosen must be variable, but flanked by conserved regions, to allow amplification and typing of all organisms in the species. The variability in the DNA region selected for typing must be sufficient to differentiate between strains, and preferably the region must not be transmissible horizontally to other strains of the species (Olive & Bean, 1999)

Methods for typing and detection of *P. syringae*

Several methods have been used for typing *P. syringae*, including DNA-DNA hybridization and RFLP (Denny *et al.*, 1988), PCR techniques (Bereswill *et al.*, 1994), RFLP and DNA fingerprinting (Zhao *et al.*, 2000), and Biolog (metabolic profiling) (Ji & Wilson, 2002). PCR and restriction analysis of the coronatine (COR) biosynthetic gene cluster were effective at detecting and showing relatedness between COR- producing strains and pathovars of *P. syringae*. Pathogenic strains of *P. syringae* from pathovars glycinea, maculicola, morsprunorum, and tomato that do not produce COR have been

isolated (Bender *et al.*, 1999), making coronatine probes and primer sets ineffective in detection of these isolates (Bereswill *et al.*, 1994).

Few methods exist for rapid and reliable detection of *P. syringae*. Cuppels and Elmhirst (Cuppels & Elmhirst, 1999) described a detection method involving colony lifts and hybridization with TPRI (a probe containing a fragment from the COR biosynthetic gene cluster). Although they detected low numbers of the pathogen on plant material, this method is not rapid and is specific only for COR-producing strains of *Pst*. Diagnosis of *P. syringae* pv. papulans and *P. syringae* pv. phaseolicola has been successful also with primers specific for these pathovars (Kerkoud *et al.*, 2002, Rico *et al.*, 2003). Recently, the *hrpZ* gene, which maps to the *hrp* T3SS pathogenicity island (PAI; discussed below), was amplified in *Pst* for identification and detection of *Pst in planta* (Zaccardelli et al., 2005).

Strains belonging to *P. syringae* pv. tomato are homogeneous (Denny *et al.*, 1988, Sawada *et al.*, 1999), and to date the techniques cited above are unable to distinguish strains of *Pst* (Clerc *et al.*, 1998). In the current study, a recent typing technique utilizing tandem repeat loci was investigated for its ability to discriminate strains of *P. syringae* pv. tomato.

Introduction to bacterial tandem repeats

Polymorphic bacterial tandem repeats, which are also referred to as variable number tandem repeats (VNTRs), were first identified in humans for use in fingerprinting (Lindstedt, 2005). These polymorphic markers have now been identified in bacteria as well and are classified by their size. Minisatellites are defined as repeat units in the range

of 6-100 bp that span hundreds of nucleotides (Le Fleche *et al.*, 2001). Microsatellites are short DNA sequence motifs (e.g. 1-6 nucleotides) that are tandemly repeated from as few as two times up to a dozen or more times at a specific locus; these span only a few tens of nucleotides (Gur-Arie *et al.*, 2000). There is no clear definition of the border between microsatellites and minisatellites, but a repeat unit size of about 6-9 nucleotides is often used to distinguish them (Lindstedt, 2005). Microsatellites are sometimes referred to as simple sequence repeats (SSRs) (Danin-Poleg *et al.*, 2006b, Diamant *et al.*, 2004, Gur-Arie *et al.*, 2000). Other bacterial repeat elements are mononucleotide repeats (MNRs), which are a subgroup of SSRs (Danin-Poleg *et al.*, 2006b)

It is important to note that bacterial tandem repeats can be found in genes, intergenic regions (DNA sequences between genes) and transposable elements (Rocha *et al.*, 1999). Tandem repeat loci vary among strains with respect to the number of repeat units and/or their primary structure and are often referred to as variable number tandem repeat or VNTR loci (van Belkum *et al.*, 1998). These DNA loci mutate as a result of the repair of a double strand break initiated within, or very close to, the tandem repeat (Le Fleche *et al.*, 2001).

Tandem repeat loci are unstable and play a role in generating and maintaining bacterial diversity. Genomic changes such as recombination, slipped strand mispairing, and mutation occur frequently in the tandem repeat region. Thus these hypermutable TR loci allow for bacterial adaptation, while simultaneously maintaining a stable genome with a low overall genomic mutation rate (Legendre *et al.*, 2007, Moxon *et al.*, 2006, U'Ren *et al.*, 2007).

Tandem repeat loci can be used to study strain relatedness and serve as targets for epidemiological typing (van Belkum, 1999). The variable number of repeat units at each locus is characterized by using locus-specific primers to PCR-amplify the DNA region. Multiple-locus variable number tandem repeat analysis (MLVA) is a type of VNTR analysis based on several to dozens of polymorphic tandem repeat loci (Lindstedt, 2005, Onteniente *et al.*, 2003, Sabat *et al.*, 2006, van Belkum, 2007, van Belkum *et al.*, 1998).

In bacteria, loci consisting of a microsatellite repeat (1-8 bp) have also been designated 'simple sequence contingency loci' (SSRs), a term that refers to a region of hypermutable DNA that mediates high-frequency, stochastic, heritable, genotypic switching (Moxon *et al.,* 2006). The altered number of repeats allows for reversible on/off states of expression of the corresponding gene. If the mutation rate is extreme (such as in *H. influenza*) the microsatellite repeat is of limited value for strain identification, epidemiological and phylogenetic studies (Le Fleche *et al.*, 2001).

Overview of MLVA techniques used for human pathogens

Since the discovery of tandem repeats in bacteria, numerous studies have reported the use of tandem repeats for the identification and typing of bacteria pathogenic to humans. The genetic diversity of homogeneous/monomorphic species such as *Bacillus anthracis* and *Yersinia* has been studied using MLVA (Le Fleche *et al.*, 2001). MLVA also has been used for *Brucella* (Al Dahouk *et al.*, 2007), *Staphylococcus aureus* (Sabat *et al.*, 2003, Sabat *et al.*, 2006), *Francisella tularensis* (Farlow *et al.*, 2001, Farlow *et al.*, 2005, Johansson *et al.*, 2004), *Salmonella enterica* (Liu *et al.*, 2003), and *Vibrio cholerae* (Danin-Poleg *et al.*, 2006a).

Overview of tandem repeats for study of plant pathogens

There is little known about the utility of tandem repeat analysis for typing plant pathogens. SSRs have been used to study strains of *X. fastidiosa* strains (Lin *et al.*, 2005), *Erwinia amylovora* (Ruppitsch *et al.*, 2004), and *R. solanacearun* (Coenye & Vandamme, 2003); however, few tandem repeat typing schemes have been developed for plant pathogens. To develop a typing scheme, polymorphic loci must be identified, and each locus must be checked for variation among strains. As reported in Chapter III, tandem repeat loci were identified within the *Pst* DC3000 genome and characterized for their ability to discriminate among strains of *P. syringae* pv. tomato. Chapter III describes the first effort to identify and characterize tandem repeat loci within a *P. syringae* genome.

P. syringae recombination, phylogeny and evolution

Bacterial genomes are sometimes distinguished by two parts: the core genome which contains essential genes, and the flexible genome, which contains genes associated with virulence (Hacker & Carniel, 2001). One way pathogens adapt to different niches is by gene adaptation via the flexible genome. These flexible genomes evolve largely through horizontal genetic exchange or transfer (HGT). Many of the genes encoding virulence and pathogenicity products occur as gene clusters, which are often referred to as “pathogenicity islands” or PAIs (Gal-Mor & Finlay, 2006, Hacker & Kaper, 2000). Mobile elements often make up the flexible genome. These elements are sometimes associated with genomic islands (GEIs) such as fitness or PAIs that are often associated with HGT and help bacteria adapt to a niche (Chen, 2006). The process of acquiring new

virulence factors or host specificities may be largely mediated by HGT (Preston *et al.*, 1998b).

One study which used PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of rRNA to study diversity of *P. syringae* strains, showed that *Pst* and related pathovars form a homogeneous and distinct group (Manceau & Horvais, 1997). RAPD and AFLP were used to compare and analyze 23 *P. syringae* strains. The results correlated well with other methods and techniques and was used to distinguish *P. syringae* pv. maculicola from *P. syringae* pv. tomato. However, neither RAPD nor AFLP distinguished *P. syringae* pv. tomato at the level of race specificity (Clerc *et al.*, 1998).

DNA-DNA hybridization also has been used to study DNA relatedness among 48 pathovars of *P. syringae*. This resulted in the grouping of strains into nine genomospecies, and certain strains were recommended as 'type' strains. Additionally, ribotyping was performed and the results of this method partially correlated to the genomospecies groupings (Gardan *et al.*, 1999).

A phylogenetic study (Sawada *et al.*, 1999) used 56 strains belonging to 19 pathovars of *P. syringae.* Using the housekeeping genes *gyrB* and *rpoD,* as well as the T3SS genes *hrpS* and *hrpL,* they constructed 12 phylogenetic trees, in three phylogenetic groups. Strains of the same pathovar were closely located within the genome trees, suggesting homogeneity. Results also showed that the *hrp* gene cluster has been stable and evolved with the *P. syringae* genome (Sawada *et al.*, 1999).

The population structure and dynamics of 60 *P. syringae* strains were recently studied by multi-locus sequence tagging (MLST). This study which included 21 pathovars and used *gyrB, rpoD, hrpS* and *hrpL* as index genes, showed that the core

genome of *P. syringae* is quite stable (Sarkar & Guttman, 2004). In a subsequent study, 95 *P. syringae* pathovars were characterized phylogenetically by MLST and assayed for toxin production and antibiotic or copper resistance (Hwang *et al.*, 2005). More than 50% of the strains assayed did not produce toxins (e.g. COR, phaseolotoxin, syringomycin, and tabtoxin), and the authors concluded that toxin production is driven by HGT.

The genomes of *P. syringae* pv. syringae B728a and *Pst* DC3000 have been compared in great detail. *Pst* DC3000 has 28 unique T3SS effectors, and the plasmids of *Pst* DC3000 contain genes that are largely missing from the *Pss* B728a genome. The B728a genome has fewer IS elements than *Pst* DC3000 and contains 976 genes with no counterparts in *Pst* DC3000. *Pss* B728a is a better epiphyte than *Pst* DC3000 and this is reflected in its genome. For example, B728a is more resistant to UV irradiation, presumably because it has more enzymes for quenching reaction oxygen species (ROS), and it contains two copies of the *rulAB* DNA repair operon. The G+C ratios of these organisms are skewed, and it is thought that these two organisms diverged from each other long ago (Feil *et al.*, 2005).

OBJECTIVES

Objective I: Select VNTR loci for study of strains of *P. syringae* pv. tomato

Objective II: Design primer sets for PCR amplification of VNTR loci

Objective III: Identify primer sets useful for strain typing

Objective IV: Type a collection of *P. syringae* pv. tomato strains using VNTR and primer sets selected in Objectives I-III.

CHAPTER III

Identification and Characterization of Variable-Number Tandem Repeat (VNTR) Loci within the *Pseudomonas syringae* pv. tomato Genome and Application for Strain Typing by Multiple-Locus VNTR Analysis (MLVA)

A similar version of the following chapter will be submitted to a peer reviewed journal for publication with Oklahoma State University Regents Professors Carol Bender, Ulrich Melcher and Jacqueline Fletcher as co-authors. The first author (C. Baker) was responsible for the complete intellectual concept for the project as well as the plan, design and execution of all project experiments and writing of the publication. The co-authors were responsible for project funding as well as the editing of the publication.

SUMMARY

Pseudomonas syringae pv. tomato strain DC3000 (*Pst* DC3000) is a gram-negative plant pathogenic bacterium that causes bacterial speck, an economically important disease of tomato. The ability to distinguish among bacterial strains is important for epidemiological tracing of pathogen outbreaks, detecting the source of an outbreak, monitoring spread of pathogens and for forensic identification. Many different typing methods have been investigated for classification of *P. syringae* pv. tomato; however these fail to distinguish *P. syringae* pv. tomato at the strain level. Studies with

other bacterial species demonstrated that variable number tandem repeat (VNTR) loci, which are found throughout bacterial genomes, are useful for rapid and reliable strain typing by multiple-locus VNTR analysis (MLVA). In the current study, the genome of *Pst* DC3000 was analyzed, and oligonucleotide primers were designed to amplify 34 VNTR loci. The primers were screened using seven representative strains of *P. syringae* pv. tomato, and a subset of five primer sets yielding polymorphic products were identified for use in strain typing. These five primer sets were used to type a collection of 58 *P. syringae* pv. tomato strains, and approximately 23 different MLVA sequence types were identified. In this study, strains that clustered together in the same MLVA sequence type originated from different geographic locations, thus no obvious relationship was observed between MLVA sequence type and geographic origin of strains. 47 *P. syringae* pv. tomato strains grouped into twelve MLVA sequence types, while eleven *P. syringae* pv. tomato strains produced a unique MLVA sequence type. Two *P. syringae* pv. tomato strains with unique MLVA sequence types were originally isolated over 60 years ago in the early 1940's, while an additional three *P. syringae* pv. tomato strains with unique MLVA sequence types were originally isolated from host plants other than tomato. The latter finding supports the hypothesis that genetic diversity occurs in relation to the host plant from which bacteria are isolated. The stability and reproducibility of the MLVA sequence types was investigated by culturing all 58 *Pst* strains for 20 generations *in vitro* and re-typing, and in all cases the sequence types defined by the MLVA primers were reproducible. This is the first report of using MLVA to type a large collection of *P. syringae* pv. tomato strains of diverse origin. As rapid detection is the key to protecting

crops from accidental or deliberate of pathogens (Schaad *et al.*, 2003), MLVA will help to facilitate rapid strain discrimination in diagnostic or forensic situations.

INTRODUCTION

Pseudomonas syringae is a gram-negative plant pathogenic bacterium that is divided into approximately 50 pathovars based on host range (Hirano and Upper, 2000). Several strains of *P. syringae* have been sequenced (Feil et al., 2005, Joardar et al., 2005), including *P. syringae* pv. tomato DC3000 (Buell *et al.*, 2003), which causes bacterial speck of tomato. Bacterial speck disease occurs worldwide wherever tomatoes are grown (Colin & Chafik, 1986, Lawton & MacNeill, 1986, Pernezny *et al.*, 1995), and *P. syringae* pv. tomato can survive epiphytically on both host and nonhost plants for extended periods of time (Voloudakis *et al.*, 1991). Inoculum sources for the pathogen include infested seeds, tomato transplants, and plant debris (Goode and Sasser, 1980). Infested seeds and tomato transplants facilitate long distance transmission and help explain the worldwide distribution of the pathogen (Gitaitis & Walcott, 2007).

Subtyping (e.g. strain characterization) is an important epidemiological tool for recognizing pathogen outbreaks, detecting the source of the outbreak, monitoring spread of pathogens and for forensic identification. Many different typing methods have been investigated for classification of *P. syringae* pv. tomato including serological assays, fatty acid analysis, metabolic profiling, phage typing, and carbohydrate utilization (Denny et al., 1988, Fackrell & Sinha, 1983, Ji & Wilson, 2002, Saunier *et al.*, 1996). Various nucleic-acid based methods have also been used for typing *P. syringae*, including DNA-

DNA hybridization, restriction fragment length polymorphism (RFLP), DNA fingerprinting, PCR techniques, and ribotyping (Bereswill *et al.*, 1994, Clerc *et al.*, 1998, Cuppels *et al.*, 2006, Denny, 1988, Gardan *et al.*, 1999, Manceau & Horvais, 1997, Zhao *et al.*, 2000). According to Olive and Bean (1999), typing methods must meet several criteria to be broadly useful (Olive & Bean, 1999). These criteria include applicability of the method to all organisms within the species or subspecies, the ability to clearly differentiate unrelated strains, and reproducibility (Olive & Bean, 1999). Unfortunately, the techniques cited above for subtyping *P. syringae* pv. tomato are of limited use since they fail to distinguish among *P. syringae* pv. tomato at the strain level (Clerc et al., 1998, Gardan et al., 1999, Zhao et al., 2000).

Bioinformatic analysis of sequenced genomes has revealed a high percentage of DNA repeats that vary in size, location and complexity (Lindstedt, 2005). Tandem DNA repeats are usually classified as satellites, minisatellites, or microsatellites, which span megabases of DNA, tens to hundreds of nucleotides, and tens of nucleotides, respectively (Le Fleche *et al.*, 2001). Microsatellites are often polymorphic and have been used in fingerprinting human DNA for forensic studies (Lindstedt, 2005). Polymorphic minisatellite markers also have been identified in bacteria and consist of 6-100 bp repeat units spanning hundreds of nucleotides (Le Fleche *et al.*, 2001). These polymorphic minisatellites may occur in bacterial genes or intergenic regions and vary among strains with respect to the number of repeat units and/or their primary structure; hence they have been termed ‘variable number tandem repeat’ (VNTR) regions (van Belkum *et al.*, 1998)

Numerous studies have reported the utility of tandem repeats for bacterial strain typing, and the availability of whole-genome sequence data has made this a powerful

approach for strain discrimination (Lindstedt, 2005, van Belkum, 2007, van Belkum et al., 1998). Multiple-locus variable number tandem repeat analysis (MLVA) is a type of VNTR analysis based on several to dozens of polymorphic tandem repeat loci. The variable number of repeat units at each locus is characterized by using locus-specific primers to amplify each locus by PCR. MLVA is a PCR-based technique, it is rapid, easy to perform and analyze, cost effective, and reproducible (Onteniente *et al.*, 2003, Sabat *et al.*, 2006). The discrimination of strains that are members of relatively homogeneous species such as *Bacillus anthracis* and *Yersinia pestis* has been successfully studied using MLVA (Le Fleche *et al.*, 2001).

To develop a tandem repeat typing scheme, polymorphic minisatellite loci must be identified. As mentioned above, repeat loci can be found in genes, within intergenic regions and in transposable elements (Rocha *et al.*, 1999). Each locus must be checked for variations in the repeat number among strains. In the current study five repeat loci were identified and validated for strain typing of *P. syringae* pv. tomato. These five loci allow for discrimination among some strains of *P. syringae* pv. tomato with a high degree of stability and reproducibility. This is the first report of using MLVA to type a large collection of *P. syringae* pv. tomato strains of diverse origin.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The *P. syringae* strains used in the present study, and their geographical origin and year of isolation, are indicated in Table 2. *P. syringae* strains were stored at -70^{O}C in 20% glycerol and cultured on King's medium B (KMB) (King *et al.*, 1954) at 28^{O}C.

Table 2. *Pseudomonas syringae* strains used in this study.

Pathovar	Strain	Place of isolation	Year of isolation	Host	Source[c]	Reference
Tomato	DC3000[d]	Guernsey, Channel Islands, UK	1960	Tomato	A. Collmer	(Buell et al., 2003, Cuppels, 1986)
Tomato	PT23	USA	1983	Tomato	C. Bender	(Bender & Cooksey, 1986)
Tomato	4325	Canada	1944	Tomato	NCPPB, ICMP	(Bereswill *et al.*, 1994, Mitchell *et al.*, 1983)
Tomato	Pto 119		1997[a]	Tomato	B. Volksch	(Weingart & Volksch, 1997a)
Tomato	487	Greece	1979	Tomato	GSPB	(Cuppels & Ainsworth, 1995, Cuppels & Elmhirst, 1999, Cuppels *et al.*, 2006, Sarkar *et al.*, 2006, Volksch & Weingart, 1998, Yan *et al.*, 2008, Zhao *et al.*, 2000)
Tomato	1318	Switzerland	1971[b]	Tomato	CFPB	(Cuppels & Ainsworth, 1995, Cuppels et al., 2006, Cuppels *et al.*, 1990, Sarkar et al., 2006, Yan et al., 2008)
Tomato	3435	New Zealand	1972	Tomato, Peach, Nightshade	ICMP	(Hendson *et al.*, 1992, Innes *et al.*, 1993, Whalen *et al.*, 1991, Yan *et al.*, 2008)
Tomato	Pst26L	South Africa	1995[a]	Tomato	D. Cuppels	(Cuppels & Ainsworth, 1995,

						Cuppels *et al.*, 2006, Yan *et al.*, 2008)
Tomato	Pto 483		1988[a]	Tomato	GSPB	(Knirel *et al.*, 1998, Volksch & Weingart, 1998, Weingart & Volksch, 1997b)
Tomato	Pto 2811		1998[a]	Tomato	V. Catara	
Tomato	3357	New Zealand	1972	Tomato	ICMP	(Charity *et al.*, 2003, Cuppels & Ainsworth, 1995, Cuppels & Elmhirst, 1999, Cuppels et al., 2006, Cuppels et al., 1990, Denny, 1988, Zhao et al., 2000)
Tomato	3455	New Zealand	1972	Nightshade, Tomato	ICMP	(Hendson *et al.*, 1992, Innes *et al.*, 1993, Whalen *et al.*, 1991, Yan *et al.*, 2008)
Tomato	2844	Guernsey, Channel Islands, UK	1960	Tomato	ICMP, NCPPB, CFBP	(Charity *et al.*, 2003, Hendson *et al.*, 1992, Mitchell *et al.*, 1983)
Tomato	RG4	Venezuela	1985	Tomato	S. Hutcheson	(Charity et al., 2003, Denny, 1988, Denny et al., 1988)
Tomato	880	Yugoslavia	1953	Tomato	NCPPB, ICMP	(Charity *et al.*, 2003, Cuppels *et al.*, 1990, Denny, 1988, Denny *et al.*, 1988, Hendson *et al.*, 1992,

						Whalen *et al.*, 1991)
Tomato	2424	Switzerland	1969	Tomato	NCPPB, ICMP	(Charity *et al.*, 2003, Cuppels *et al.*, 1990, Denny, 1988, Denny *et al.*, 1988, Whalen *et al.*, 1991)
Tomato	1323	France	1971	Tomato	CFBP	(Charity *et al.*, 2003, Cuppels & Elmhirst, 1999, Cuppels *et al.*, 1990, Denny, 1988, Denny *et al.*, 1988)
Tomato	1108	UK	1960	Tomato	NCPPB	(Cuppels & Ainsworth, 1995, Cuppels et al., 2006, Cuppels et al., 1990, Yan et al., 2008)
Tomato	864	Canada	1941	Tomato	ICMP, NCPPB	(Whalen *et al.*, 1991)
Tomato	2846	Canada	1956	Tomato	ICMP, NCPPB	(Mitchell *et al.*, 1983, Whalen *et al.*, 1991)
Tomato	3358	New Zealand	1971	Tomato	ICMP	(Whalen *et al.*, 1991)
Tomato	9501	New Zealand	1987	Pepino, Tomato	ICMP	(Whalen *et al.*, 1991)
Tomato	DCT6D1	Ontario, Canada	1981	Tomato	D. Cuppels	(Cuppels & Ainsworth, 1995, Cuppels & Elmhirst, 1999, Cuppels *et al.*, 1990, Sarkar *et al.*, 2006)
Tomato	DAR 31861	Australia	1975	Tomato	DAR	(Charity et al., 2003, Denny, 1988)
Tomato	30555	Tasmania Australia	1978	Tomato	DAR	(Charity et al., 2003,

						Denny, 1988)
Tomato	CPST147	Moravia, Czech Republic	1993	Tomato	K. Pernezny	(Pernezny *et al.*, 1995)
Tomato	CPST236	Slovakia	1993	Tomato	K. Pernezny	(Pernezny *et al.*, 1995)
Tomato	Pto 479		1987[a]	Tomato	B. Volksch	(Bereswill *et al.*, 1994, Volksch & Weingart, 1998, Weingart & Volksch, 1997a)
Tomato	Pto 34	Germany	1980	Tomato	B. Volksch	(Bereswill *et al.*, 1994)
Tomato	Pto 55	Germany	1979	Tomato	B. Volksch	(Naumann *et al.*, 1986, Volksch & Weingart, 1998, Weingart & Volksch, 1997a)
Tomato	Pto 2851			Tomato	V. Catara	
Tomato	DC84-1	Ontario, Canada	1984	Tomato	D. Cuppels	(Cuppels & Ainsworth, 1995, Cuppels *et al.*, 1990, Sarkar *et al.*, 2006)
Tomato	T1	Canada	1983[a]	Tomato, Brassica	B. Kunkel	(Charity et al., 2003, Hendson et al., 1992, Manceau & Horvais, 1997, Whalen et al., 1991, Yan et al., 2008)
Tomato	JL1035	California, USA	1983	Tomato	B. Kunkel	(Cuppels & Ainsworth, 1995, Cuppels & Elmhirst, 1999, Cuppels et al., 1990)
Tomato	B125	Canada	1981	Tomato	S. Hutcheson	(Charity *et al.*, 2003, Denny *et al.*, 1988)

Tomato	SM78-1	Georgia, USA	1983[a]	Tomato	S. McCarter	(Cuppels & Ainsworth, 1995, Cuppels et al., 2006, Cuppels et al., 1990)
Tomato	AV80	Nebraska, USA	1983[a]	Tomato	A. Vidaver	(Cuppels & Ainsworth, 1995, Cuppels et al., 2006, Cuppels et al., 1990, Denny, 1988, Denny et al., 1988)
Tomato	OH314	Ohio, USA	1978	Nettle, Tomato, Crucifers	D. Cuppels	(Cuppels & Ainsworth, 1995, Cuppels *et al.*, 2006, Cuppels *et al.*, 1990, Yan *et al.*, 2008, Zhao *et al.*, 2000)
Tomato	KN10		1981	Tomato	D. Guttman	(Hwang *et al.*, 2005, Sarkar *et al.*, 2006, Sarkar & Guttman, 2004)
Tomato	TF1	USA	1997	Tomato	D. Guttman	(Hwang et al., 2005, Sarkar et al., 2006)
Tomato	PT17	USA	1983[a]			(Bender & Cooksey, 1986, Sesma *et al.*, 1998)
Tomato	DC89-4H	Ontario, Canada	1989	Tomato	D. Cuppels	(Cuppels & Ainsworth, 1995, Cuppels & Elmhirst, 1999, Sarkar et al., 2006)
Tomato	IPV-BO 2973	Italy		Tomato	M. Zaccardelli	(Zaccardelli *et al.*, 2005)
Tomato	224	Italy		Tomato	M. Zaccardelli	(Zaccardelli *et al.*, 2005)
Tomato	407	Italy		Tomato	M. Zaccardelli	(Zaccardelli *et al.*, 2005)
Tomato	443.1/96	Italy		Tomato	M.	(Zaccardelli

					Zaccardelli	*et al.*, 2005)
Tomato	171	Italy		Tomato	M. Zaccardelli	(Zaccardelli *et al.*, 2005)
Tomato	PT12	California, USA	1982	Tomato	C. Bender	(Bender & Cooksey, 1986, Cooksey, 1988)
Tomato	T4B1	Canada	1981	Tomato	S. Hutcheson	(Charity *et al.*, 2003, Denny, 1988)
Tomato	188B	Ontario, Canada		Tomato	B. MacNeill	(Cuppels & Ainsworth, 1995, Cuppels *et al.*, 1990)
Tomato	CPST116	Moravia, Czech Republic	1993	Tomato	K.L. Pernezny	(Pernezny *et al.*, 1995)
Tomato	CPST145	Moravia, Czech Republic	1993	Tomato	K.L. Pernezny	(Pernezny *et al.*, 1995)
Tomato	CPST211	Slovakia	1993	Tomato	K.L. Pernezny	(Pernezny *et al.*, 1995)
Tomato	CPST232	Slovakia	1993	Tomato	K.L. Pernezny	(Pernezny *et al.*, 1995)
Tomato	9S	South Africa		Tomato	D. Cuppels	(Cuppels *et al.*, 2006)
Tomato	519	Greece		Tomato	D. Cuppels	(Cuppels *et al.*, 2006)
Tomato	3647	Australia	1973	Tomato	ICMP	(Cuppels et al., 2006, Whalen et al., 1991)
Tomato	1008	USA	1942	Tomato	NCPPB, ICMP	(Cuppels & Elmhirst, 1999, Cuppels et al., 1990)
Syringae	4355	New Zealand	1975	Tomato	ICMP	(Charity *et al.*, 2003)
Atropurpurea	1304	Japan	1982[a]	Ryegrass	D. Kobayashi	
Coronafaciens	PC27			Oat	P. Shaw	(Bender *et al.*, 1991, Bereswill *et al.*, 1994, Sato *et al.*, 1983)
Glycinea	Race 4			Soybean	A. Collmer	(Piwowarski & Shaw, 1982, von Bodman & Shaw, 1987)
Maculicola	2744	UK	1968	Mustard,	ICMP	(Fett &

				Cabbage		Sequeira, 1981)
Maculicola	921	New Zealand	1963	Cauliflower	ICMP	(Cuppels & Ainsworth, 1995, Hendson *et al.*, 1992, Mitchell, 1982, Whalen *et al.*, 1991)
Maculicola	4326	USA	1965	Radish	ICMP, NCPPB	(Bereswill *et al.*, 1994, Mitchell, 1982, Volksch & Weingart, 1998)
Maculicola	4981	Zimbabwe	1970	Cabbage, Cauliflower	ICMP, NCPPB, CFBP	(Cuppels & Ainsworth, 1995, Hendson et al., 1992, Whalen et al., 1991)
Maculicola	438	USA		Crucifer	D. Cuppels	(Cuppels & Ainsworth, 1995, Hendson *et al.*, 1992, Sarkar *et al.*, 2006, Whalen *et al.*, 1991)
Mori	1413	Hungary	1958	White Mulberry	NCPPB, ICMP	(Bereswill *et al.*, 1994, Cuppels & Ainsworth, 1995, Cuppels *et al.*, 1990, Volksch & Weingart, 1998)
Morsprunorum	567	UK	1958	Cherry	ICMP, NCPPB ATCC	(Sesma *et al.*, 1998)
Phaseolicola	1448A			Bean	A. Vivian	(Bender *et al.*, 1991, Mitchell, 1982)
Syringae	B728a	USA		Snap Bean	D. K. Willis	(Joardar *et al.*, 2005)
Tabaci	1086-1	USA			D. Kobayashi	(Feil *et al.*, 2005, Loper & Lindow,

					1987, Rich *et al.*, 1994)
Tagetis	53534			Marigold	ATCC; J. Lydon

[a] The exact year of isolation was not available for some strains. When this occurred, the year of isolation was based on the first citation in previously published literature.
[b] Year the strain was transmitted to the CFPB collection.
[c] GSPB, Göttingen Collection of Phytopathogenic Bacteria, Göttingen Germany; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; CFPB, Collection Francaise des Bacteries Phytopathogenes, INRA Angers, France; NCPPB, National Collection Plant Pathogenic Bacteria, UK; ATCC, American Type Culture Collection, USA; DAR, Plant Pathology Herbarium, Orange Agricultural Institute, Australia.
[d] DC3000 is a rifampicin-resistant derivative of ICMP 2844 (NCPPB 2844, CFPB 2212), which is the pathotype strain of *P. syringae* pv. tomato.
Blank entries indicate that the year or place of isolation is unknown.

Preparation of genomic DNA

Genomic DNA was isolated from *P. syringae* strains using a rapid cell lysis protocol that has been described previously (Chen & Kuo, 1993). Briefly, bacteria were suspended in lysis buffer (40 mM Tris-acetate, pH 7.8; 20 mM sodium acetate; 1 mM EDTA; 1% SDS) and mixed with vigorous pipetting. A volume of 5 M NaCl was added, and the mixture was centrifuged at 16,110 g for 20 min. The supernatant was transferred to a clean microfuge tube, an equal volume of chloroform was added, and the tube contents were mixed by inversion briefly until a milky solution formed. This liquid was centrifuged at 16,110 g for 10 min., the aqueous phase was transferred to a clean microfuge tube, and the DNA was precipitated with one volume 100% EtOH. Following centrifugation the DNA was washed in 70% EtOH and the DNA pellet was dissolved in TE buffer (Sambrook *et al.*, 1989) (pH 8.0) and stored at 4°C.

Tandem repeat locus identification and primer design

Pst DC3000 tandem repeat loci were selected from the Variable Number Tandem Repeat Locus Database (VNTRDB) web site (http://vntr.csie.ntu.edu.tw/) (Chang *et al.*, 2007a). Briefly, this involved selecting the unique intra-genus tandem repeat (TR) option on the VNTRDB home page and submitting a query for the complete genome of *Pst* DC3000. This process generated a tabulated list of TRs unique to *Pst* DC3000 showing the positions of the TRs in the genome, TR length, and number of copies (Table 3). The tabulated list generated by VNTRDB also provided access to 1,000 bp nucleotide sequences flanking each TR. These sequences and BLASTN analysis were used to design forward and reverse primer pairs (Table 4) for PCR amplification of TR loci with a match percent score of 90% or above as calculated by the VNTRDB.

Table 3: Characteristics of *Pst* DC3000 VNTR loci evaluated in the present study. The TR index number was obtained from the VNTRDB website.

TR Index Name	Genome Placement	Raw Length	Copies	Size
104	23314..23262	53	1.5	35
105/106	26664..26704	---	---	---
	26671..26704			
107	30875..30909	35	5.8	6
108	47218..47272	55	13.8	4
112	66902..66956	55	6.9	8
116	81588..81648	61	2.0	31
166	221197..221938	742	2.0	371
250	377372..377415	44	1.7	26
337	617007..617366	360	2.9	125
479/480	906563..906595	---	---	---
	906565..906597			
638	1159090..1159129	40	1.9	22
643	1176753..1176784	32	1.5	21
715	1409170..1409220	51	7.3	7
803	1531725..1532074	350	3.1	111
830	1591825..1591863	39	1.9	21
919/920	1807887..1808588	---	---	---

	1807989..1808588			
1038	2051164..2051193	30	2.7	11
1142	2257099..2257159	61	1.9	31
1309	2681647..2681684	38	1.9	20
1570	3110236..3110316	81	13.5	6
1649	3184210..3184254	45	1.6	28
1651	3191757..3191871	115	2.5	46
1661	3225593..3225622	30	4.3	7
1929	3678033..3678311	279	1.9	144
2334	4524836..4524873	38	1.9	20
2522	4949827..4949856	30	1.0	29
2629	5294028..5294057	30	1.4	21
2658	5365287..5366095	809	1.9	425
2662	5408861..5408896	36	3	12
2663	5408861..5408896	36	6	6
2759	5635437..5635494	58	9.7	6
2885	5907215..5907264	50	1.9	26
2955	6080438..6080474	37	2.8	14
3053	6361293..6361342	50	3.1	16

Table 4. Characteristics of *Pst* DC3000 VNTR primers used in the present study.

Repeat Locus Primers[a]	**Associated ORFs in *Pst* DC3000**	**T_m (^OC)** [b]	**Primer Length (bp)**	**Primer Sequence**	**Product Size (bp) in *Pst* DC3000**
715-F	PSPTO_1280	60.4	24	TGTGCGATGACACGCTTACCCATA	314
715-R	PSPTO_1281	59.6	24	TATTCGCGGACATTCGTGACAAGC	
1570-F	PSPTO_2791	60.2	24	AGTCTCTGCTCTTTGGTTGGCGTA	216
1570-R	PSPTO_2792	59.6	24	GTCTGATGTACATTGTGCGCTGGT	
1929-F	PSPTO_3252	59.9	24	CGAACAGAACGCGGCCTTCAAATA	511
1929-R	PSPTO-3254	60.1	24	ACAGCGACTGAGCTGATTCAGGAT	
919/920-F	PSPTO_1648	60.4	24	AAACATCAGCCAGCAAATCACCCG	829
919/920-R	PSPTO_1649	60.2	24	AACTGTTATGCCTTGTCGCACAGC	
337-F	PSPTO_0560	60.2	24	TGGAGCACAAACTGCTCTGAGTCT	440
337-R	PSPTO_0561	60.6	23	TACAGAGATGGCGCGATTGAGCA	

[a] F, forward primer, R, reverse primer
[b] Tm, melting temperature

PCR amplification and genotyping

The polymorphisms of candidate VNTR loci were evaluated initially for strain discrimination by using a subset of seven *P. syringae* pv. tomato strains (PT23, 4325, Pto 119, 487, 1318, 3435, and Pst 26L) of diverse geographical origin. PCR reactions were

conducted in 96-well microtiter plates and consisted of 5 µl of 5X GoTaq Flexi buffer (Promega, Madison, WI), 2.25-7 mM $MgSO_4$, 0.5 µl 10 mM dNTPs, 1 µl genomic template DNA, 0.5 µl of each primer (50 mM), 0.25 µl GoTaq polymerase (Promega, Madison, WI) and sterile distilled water to a total volume of 25 µl. PCR reaction conditions consisted of denaturation at 95^{O}C (step 1, 1-2 min), incubation at 94^{O}C (step 2, 1 min), annealing at 55^{O}C (step 3, 1 min), and extension at 72^{O}C (step 4, 1 min). Steps 2-4 were repeated for 30 cycles, followed by 7 min of final extension at 72^{O}C and a final incubation at 4^{O}C.

The five primer pairs representing the five polymorphic loci (Table 4) were used to amplify VNTRs and type the strains in Table 2. In these experiments, PCR products were separated by electrophoresis in ultrapure agarose-1000 (Invitrogen, Carlsbad, CA) gels. Agarose concentrations were 2% for larger loci designated 919/920 and 1929, and 4% for the smaller loci named 337, 715 and 1570. Electrophoresis was in 0.5X TBE (Tris-borate-EDTA) buffer (Sambrook *et al.*, 1989) with a 1-kb sizing ladder (Invitrogen, Carlsbad, CA). PCR products were excised from gels and prepared for sequencing using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Sequencing was provided by the OSU Recombinant DNA/Protein Resource Facility using locus specific primers (Table 4).

Validation of repeat loci

P. syringae strains used in strain typing were stored at –70°C in 20% glycerol. To investigate the stability of the repeat loci, *P. syringae* strains were cultured for 20

generations on KMB agar (King *et al.*, 1954) at 28^{O}C and re-typed using the polymorphic repeat primers.

VARscore analysis

VARscore, which predicts TR variability and assigns a numeric value, is available on the Sequenced-based Estimation of Repeat Variability (SERV) website http://hulsweb1.cgr.harvard.edu/SERV/ (Legendre *et al.*, 2007). The VARscore ranges for two loci (1570 and 715) in the 58 *P. syringae* pv. tomato strains were determined by submitting the sequenced alleles into the publicly available SERV website, which then produced a tabulated list showing the nucleotide sequence for that particular TR locus and the VARscore (Table 5). TR repeat loci with VARscores between 1 and 3 are most useful for genotyping (Legendre *et al.*, 2007).

Table 5: Characteristics of *Pst* DC3000 tandem repeat loci used to study *P. syrinage* pv. tomato isolates.

Locus	**Locus length (bp) in *Pst* DC3000 genome**	**Motif length (bp)**	**# units in *Pst* DC3000**	**% G+C content**	**Size Range (bp)**	**No of alleles**	**VARscore range[a]**
1570	81	6	13.5	49	51-105	10	0.37-1.15
715	51	7	7.3	45	16-58	6	0.016-0.67
1929	279	144	1.9	64	135-279	2	---
919	702	359	2.0	59	573-702	3	---
920	600	120	5.0	59			---
337	360	125	2.9	63	108-608	4	---

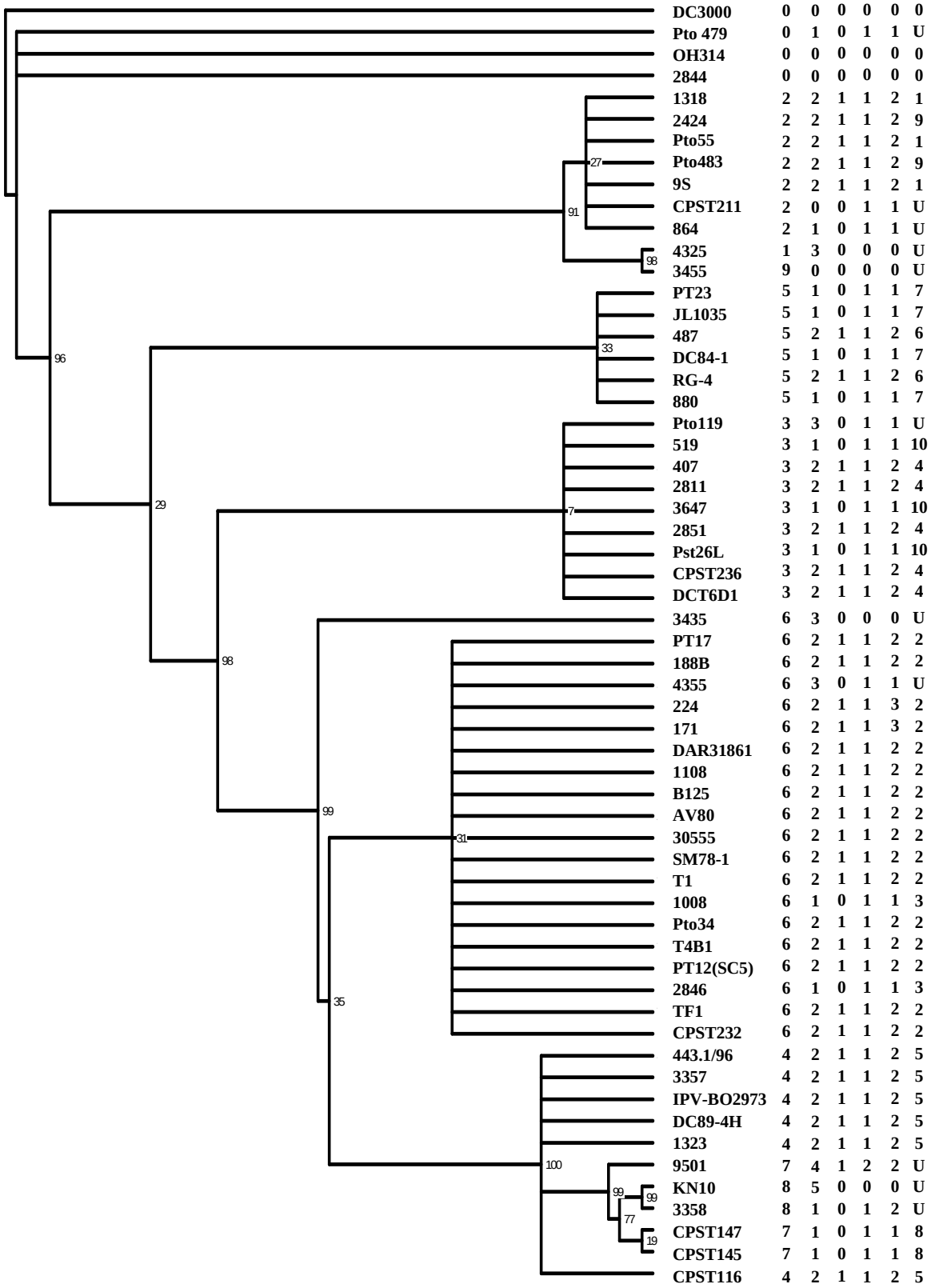
[a] For the two most polymorphic loci (in **bold**) the VARscore range was determined using the publicly available SERV website http://hulsweb1.cgr.harvard.edu/SERV/ (Legendre *et al.*, 2007). Tandem repeat loci with VARscores between 1 and 3 are most useful for genotyping.

Phylogenetic analysis

ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) (Thompson *et al.*, 1994) was used to align the DNA sequences. Phylogenetic analysis used programs in the Phylip (version3.68) package (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 2005). For each of the five loci, 100 bootstrapped data sets were created and combined into one file using SEQBOOT with default settings. The combined data sets were analyzed by DNAPARS with 25 jumbles including gaps in the analysis, and *P. syringae* pv. tomato DC3000 was assigned as the outgroup. A consensus tree for the 500 data sets was generated using CONSENSE and viewed using Treeview (Page, 1996).

Fig 2: Phylogenetic tree (see below) for the *Pst* strains utilized in this study (Table 2). The columns from left to right indicate the sequence types at loci 1570, 715, 1929, 919/920 and 337 respectively. The final column indicates the MLVA sequence type shown in Table 6 below, with U indicating a unique MLVA sequence type.

Fig. 2 Parsimony tree



RESULTS

Screening for VNTR loci in the *Pst* DC3000 genome

Analysis of *Pst* DC3000 using the VNTRDB revealed 90 unique tandem repeat loci in the DC3000 genome. Due to the large number of repeat loci, a subset of 34 loci with a match percent score of 90% or above was selected for the ability to discriminate strains of *P. syringae* pv. tomato. The nucleotide sequences flanking each TR that were provided by the VNTRDB were used to design primer sets for each TR locus (Table 3), which were screened in PCR reactions using seven *Pst* strains (PT23, 4325, Pto 119, 487, 1318, 3435, and Pst 26L). Twenty nine of these TR primer sets resulted in either a weak amplification signal or a monomorphic PCR signal. These were considered of limited value for *Pst* strain typing and were removed from further consideration. In the initial analysis of the seven strains, five of the 34 loci were polymorphic and consisted of two or more alleles, which are defined as PCR products of different size as resolved by agarose gel electrophoresis. Table 4 shows the characteristics of the five VNTR primer sets, and Table 5 shows the characteristics of the tandem repeat loci used to study *P. syringae* pv. tomato isolates. One of these loci (919/920) actually encompasses two TR sequences listed in the TR database, indexed as 919 and 920. These loci overlapped, thus one primer set was designed to amply the entire region of the genome as one locus. The number of alleles for the five TR loci ranged from two to ten. Three of the loci (1929, 919/920 and 337) contained 2-4 alleles, whereas the other two loci (1570 and 715) were more polymorphic and contained ten and six alleles, which varied by multiples of 6 and 7 nucleotides, respectively.

MLVA of *P. syringae* isolates

MLVA analysis was performed by using the five loci identified above (1570, 715, 1929, 919/920 and 337) to PCR amplify products from an additional collection of 51 *Pst* strains. The five loci resulted in detectable amplicons in all strains when used in PCR reactions. Each amplicon was excised from the agarose gel and sequenced, and a MLVA sequence type was generated for each *P. syringae* strain.

Isolates with identical or similar repeat lengths at each locus were grouped into MLVA sequence types. Based on the pattern of sequenced alleles at the five loci, twelve MLVA sequence types were identified for 47 strains and arbitrarily designated 0-11 (Table 6). Eleven *P. syringae* pv. tomato strains (4325, Pto119, 3435, 3455, 864, 3358, 9501, 4355, Pto 479, KN10, and CPST211) had a unique MLVA genotype and are not shown in Table 6. In summary, MLVA discriminated the 58 strains of *P. syringae* pv. tomato into 23 MLV sequence types.

Table 6. Isolates belonging to each MLVA sequence type.

MLVA Sequence Type	Isolates
0	DC3000, 2844 OH314,
1	**1318**, Pto 55, 9s
2	1108, DAR31861, 30555, Pto 34, T1, B125, Sm78-1, AV80, TF1, PT17, PT12 (SC5), T4B1, 188B, CPST232
3	2846, 1008
4	Pto 2811, DCT6D1, CPST236, Pto 2851, 407
5	3357, 1323, DC89-4H, IPV-BO 2973, 443.1/96, CPST116
6	**487**, RG-4
7	**PT23**, 880, DC84-1, JL1035
8	CPST147, CPST145
9	Pto 483, 2424
10	**Pst 26L**, 519, 3647
11	171, 224

Strains in **bold** were part of the initial screening collection.

The stability and reproducibility of the MLVA sequence types was investigated by culturing all strains for 20 generations *in vitro* and re-typing. The PCR products for loci 1570 and 715 in the 58 strains of *P. syringae* pv. tomato were re-sequenced, while the PCR products from the remaining loci (919/920, 1929 and 337) were evaluated by visually examining each agarose gel. In all cases, the genotypes defined by the MLVA primers were reproducible, and the number of repeat units for each PCR product did not change.

VARscore analysis was used to predict TR variability from the DNA sequences generated in this study and to assign a numeric value. The VARscore range for the most polymorphic loci, 1570 and 715, was determined by submitting the sequence data obtained at each locus in the 58 *P. syringae* pv. tomato strains into the SERV website. The VARscores ranged from 0.37-1.15 and 0.016-0.67 for loci 1570 and 715 (Table 5), respectively. According to Legendre et al. (Legendre *et al.*, 2007), tandem repeat loci with VARscores between 1 and 3 are most useful for genotyping, indicating that locus 1570 might have some value for typing.

The five VNTR primer sets were used to amplify products from each strain, which were then sequenced and used to place a particular strain into one of the twelve MLVA sequence types described above. When the five VNTR primer sets were utilized to amplify products from *P. syringae* pathovars atropurpurea, coronafaciens, mori, morsprunorum, tabaci, tagetis, syringae, phaseolicola, and glycinea (Table 2), either PCR products were not obtained or the sequenced product did not correspond to the correct repeat locus. Due to the absence of a sequence for one or more loci using the primer sets, these strains could not be assigned to one of the MLVA sequence types, confirming that

the primer sets designed and utilized in this study are very specific for typing strains of *P. syringae* pv. tomato.

Figure 2 shows the results of the PHYLIP clustering analysis generated using parsimony for the 58 *Pst* strains analyzed in this study. This clustering agrees with the assignment of the MLVA sequence types shown in Table 6. In some cases one or more of the MLVA sequence groups differed in sequence at only one locus, and these cluster together in the tree.

DISCUSSION

The present study evaluated tandem repeats unique to *Pst* DC3000. Although the *Pst* DC3000 genome is rich in TRs, only five of those identified using the VNTRDB were polymorphic. This result is reminiscent of that obtained with *P. aeruginosa* in which seven polymorphic loci were identified out of 201 primer sets (Onteniente *et al.*, 2003). In the present study, the most highly polymorphic loci, 1570 and 715, consisted of 10 and 6 alleles, respectively. The least polymorphic loci (1929, 919/920 and 337) had motif lengths larger than 100 nucleotides and contained 2-4 alleles per locus. It is unlikely that the low allele frequency at VNTR loci is due to the strains selected for screening, since many originated from different countries at different points in time. Three of the seven test strains (4325, Pto119, and 3435) gave unique MLVA genotypes, while the remaining four (1318, 487, PT23, Pst26L) were typed into MLVA sequence type groups 1, 6, 7 and 10. The most polymorphic repeats, 1570 and 715, vary by multiples of 6 and 7 nucleotides, indicating that the smaller repeat units are more

polymorphic in *Pst*. This finding is consistent with those obtained for *P. aeruginosa*, in which the smallest repeat units were also the most polymorphic (Onteniente *et al.*, 2003).

Unlike simple sequence contingency loci, which may be of limited value as epidemiological markers (Le Fleche *et al.*, 2001), MLVA has been used to trace bacterial dissemination and to determine genetic relatedness of isolates (Al Dahouk et al., 2007, Johansson et al., 2004, van Belkum, 2007). Thus MLVA typing methodology could potentially yield insights into the worldwide dispersal of *Pst* and the geographic origin of strains. The strains utilized in the present study were selected based on their diverse geographic origin to determine if MLVA could be used to determine the genetic relationship of diverse isolates. In the present study, MLVA sequence group 2 was 2-3 times larger than the other groups and contained 16 strains of *P. syringae* pv. tomato, nine of which were originally isolated in North America. However, other strains in this group were isolated elsewhere, including strain 1108 (United Kingdom), DAR31861 (Australia), 30555 (Tasmania), Pto 34 (Germany), and CPST232 (Slovakia). Thus, the results of the present study show no obvious relationship between MLVA sequence type and geographic origin of strains, since strains that clustered together in the same MLVA sequence type originated from different geographic locations. Only 11 strains (4325, Pto119, 3435, 3455, 864, 3358, 9501, 4355, Pto 479, KN10, and CPST211) produced unique MLVA patterns, while the remaining strains clustered into one of twelve MLVA groups. Three of the strains having unique MLVA genotypes, e.g. 3435, 3455 and 9501, were isolated from host plants other than tomato (the first two from woolly nightshade, and the third from pepino; Table 2). Two of the strains having unique MLVA genotypes, 4325 and 864, were isolated originally in 1944 and 1941, respectively. According to the

ICMP, strain 4355 is designated as pv. syringae, yet it is pathogenic on tomato and was typed with the primer sets used in this study. It is important to note that *P. syringae* pv. syringae often occurs with *P. syringae* pv. tomato and does cause minor leaf spotting (Gitaitis *et al.*, 1985, Jones *et al.*, 1981, Voloudakis *et al.*, 1991), although physiological and biochemical tests are used to distinguish between these two pathovars. In summary, the strains that produced unique MLVA patterns had attributes such as wide host range, or were isolated originally over 60 years ago, factors that could account for the some of the unique MLVA sequence types seen.

In an earlier RFLP analysis, all strains of *P. syringae* pv. tomato clustered into one RFLP group (Manceau & Horvais, 1997). However, MLVA separated two of these strains (1318 and DC3000) into different types (Table 6), suggesting that MLVA may be more discriminatory than RFLP. Furthermore, Zhao et al. (Zhao *et al.*, 2000) assigned *P. syringae* pv. tomato strains DC84-1, 487 and 3357 to the same rep-PCR group, but MLVA discriminated these strains to different groups, suggesting that MLVA may be more discriminatory than rep-PCR.

P. syringae pv. tomato and the related pathogen, *P. syringae* pv. maculicola, are phenotypically very similar, having overlapping host ranges (Cuppels & Ainsworth, 1995) and belonging to genomospecies III (Gardan *et al.*, 1999). In a previous study, *P. syringae* pv. tomato strains DC3000 and OH314 showed rep-PCR fingerprints identical to those of ten strains of *P. syringae* pv. maculicola (Zhao *et al.*, 2000); furthermore, DC3000 and OH314 infect both tomato and crucifers (Cuppels & Ainsworth, 1995). More recently, analysis by MLST revealed that DC3000 and OH314 were in the same

sequence type group (Yan *et al.*, 2008), a finding that is in agreement with the results presented here.

P. syringae pv. tomato strains have been reported to comprise a relatively homogeneous group (Denny et al., 1988, Manceau & Horvais, 1997), and it has been suggested that strain homogeneity could reflect worldwide dispersal by seed (Denny et al., 1988, McCarter et al., 1983) and/or infected plant tissue (Bonn *et al.*, 1985). An alternative explanation is that strains have evolved host specificity, as a high degree of host specificity would result in low genetic diversity. This idea is supported by a previous study, in which strains of *P. syringae* pvs. tomato and maculicola that were pathogenic on tomato and crucifers, respectively, separated into two distinct groups using AFLP and RAPD (Clerc *et al.*, 1998). In another study, four strains of *P. syringae* pv. maculicola isolated from Chinese cabbage were genetically identical when analyzed by MLST, even when isolated at distinctly different times (Sarkar & Guttman, 2004). More recently, MLST typing indicated that *P. syringae* pv. tomato strains that were pathogenic solely to tomato clustered separately from pv. tomato strains capable of infecting multiple host plants (Yan *et al.*, 2008). In the current study, three *P. syringae* pv. tomato strains with unique MLVA genotypes (3435, 3455, and 9501) were isolated originally as pathogens on host plant species other than tomato (woolly nightshade, woolly nightshade and pepino respectively). Thus, our results support the hypothesis that genetic diversity occurs in relation to the host plant from which bacteria are isolated (Clerc *et al.*, 1998).

P. syringae pv. tomato DC3000 is a rifampcin-resistant derivative of ICMP 2844 (CFPB 2212; NCPPB 1106), which is the pathotype strain of *P. syringae* pv. tomato (Cuppels & Ainsworth, 1995, Yan *et al.*, 2008). Previously this strain clustered separately

from other *P. syringae* pv. tomato strains when analyzed by AFLP and RAPD (Clerc *et al.*, 1998). Pathogenicity tests, biochemical assays, and rep-PCR have suggested that DC3000 resembles strains of *P. syringae* pv. maculicola more closely than other pv. tomato strains (Cuppels & Ainsworth, 1995, Zhao et al., 2000). Recently, DC3000 was shown to be phylogenetically distinct from strains pathogenic solely to tomato (Yan *et al.*, 2008). In the present study, DC3000 was assigned to MLVA sequence type 0, which contains only three of the 58 *P. syringae* pv. tomato strains analyzed by MLVA. Thus, our results agree with previous reports showing that DC3000 is an atypical *P. syringae* pv. tomato strain (Clerc et al., 1998, Yan et al., 2008, Zhao et al., 2000).

A previous study showed that the *P. syringae* core genome is highly clonal and stable, and that mutation was more likely than recombination to induce single nucleotide changes (Sarkar & Guttman, 2004). More recently, homologous recombination was shown to contribute significantly to the variation observed among closely related *P. syringae* strains, whereas more distantly related strains differed from each other primarily due to mutation (Yan *et al.*, 2008). It is important to note that TR loci play a role in generating and maintaining bacterial diversity, because genomic changes (recombination, slippage, mutation) occur frequently in TRs. These hypermutable loci allow for bacterial adaptation while maintaining low genomic mutation rates (Legendre *et al.*, 2007, Moxon *et al.*, 2006, U'Ren *et al.*, 2007). In the current study, the 58 *P. syringae* pv. tomato strains were passed for 20 generations *in vitro* and re-typed by re-sequencing the PCR products derived from loci 1570 and 715 or evaluating PCR products visually by agarose gel electrophoresis (loci 919/920, 1929 and 337). In all cases, the number of repeat units

for each PCR product did not change, indicating that our typing results were reproducible.

The publicly available predictive model SERV (http://hulsweb1.cgr.harvard.edu/SERV) was used to estimate repeat variability. This tool uses the characteristics of the tandem repeat (unit length and number of repeated units) to accurately predict repeat variability. The numeric "VARscore" produced by the SERV web tool correlates well with experimental repeat mutation rates, and TRs with VAR scores between one and three have been recommended for genotyping (Legendre *et al.*, 2007). This web tool is able to accurately predict the variability of repeats with units under 75 nucleotides, thus, this tool was used to estimate the repeat variability of loci 1570 and 715 (Table 5). VARscore analysis indicated that for locus 1570 some of the scores were in the ideal range (0.37-1.15), while all the scores for locus 715 make it less than ideal for genotyping (0.016-0.67). In the *Pst* DC3000 genome, loci 1570 and 715 contained similar unit lengths (6 and 7, respectively), while they differ in the number of repeated units (13.5 and 7.3, respectively), thus contributing to the lower VARscore for locus 715. These low VARscores provide further evidence that the processes that induce genomic changes, such as mutation, occur at low rates in *Pst*.

In the current study, products from *Pst* DC3000 were amplified using all five VNTR primer pairs, and were used as outgroups for generating phylogenetic trees. The MLVA sequence types assigned in Table 6 are congruent with the clustering seen in the parsimony tree, which is supported by branches with high bootstrap values. In some cases, strains were grouped into different MLVA sequence types yet clustered together in the phylogenetic tree. This can be explained by the fact that these strains contained

identical sequences at several loci, and differed in sequences at only one locus. Thus, the tree clustering analysis is more conservative than the MLVA sequence typing.

In summary, MLVA has the potential to discriminate strains of *P. syringae* pv. tomato that are indistinguishable by other techniques. It has been suggested that the current strains assigned to *P. syringae* pv. tomato be separated into two distinct pathovars; e.g. one group including typical strains that are pathogenic solely on tomato, and a second group containing DC3000 and strains that are pathogenic to tomato and other hosts (Yan *et al.*, 2008). Since DC3000 is an atypical pv. tomato strain, it may not be the best choice for predicting unique TR loci for discriminating more typical *P. syringae* pv. tomato strains. For example, comparison of a more typical *P. syringae* pv. tomato strain such as T1 with a pv. maculicola strain would greatly facilitate the bioinformatic search for unique TR loci in *P. syringae.* As more *P. syringae* genomes are sequenced, additional VNTR loci could be identified and evaluated for their ability to monitor the dissemination of *P. syringae* pv. tomato, explore its diversity and better define its relationship to pv. maculicola. The ability to discriminate strains is important in a forensics investigation, and this study demonstrates that MLVA is useful for discrimination of some *Pst* strains.

CHAPTER IV

LITERATURE REVIEW II

Pseudomonas syringae **biology and pathogenicity**

Pseudomonas syringae is a gram-negative plant pathogenic bacterium related to other important Pseudomonads including the human pathogen *P. aeruginosa* and the environmentally important *P. fluorescens* and *P. putida* (Buell *et al.*, 2003). Strains of *P. syringae* are divided into pathogenic variants, which are named according to their host range. This species incites a wide variety of diseases including bacterial blight of soybean (*P. syringae* pv. glycinea), halo blight of beans (*P. syringae* pv. phaseolicola), bacterial canker of stone fruits (*P. syringae* pv. syringae), angular leaf spot of cucumber (*P. syringae* pv. lachrymans), wildfire of tobacco (*P. syringae* pv. tabaci, leaf spot of *Brassica* spp (*P. syringae* pv. maculicola) and bacterial speck of tomato (*P. syringae* pv. *tomato*).

P. syringae pv. tomato DC3000 (*Pst* DC3000) is pathogenic on multiple host plant species including tomato (Cuppels, 1986), edible *Brassica* spp., (collard, turnip) (Elizabeth & Bender, 2007, Zhao et al., 2000), and the model plant *Arabidopsis thaliana* (Whalen *et al.*, 1991). In the field *P. syringae* is persistent and can survive as an epiphyte on the surface of host and nonhost plants until environmental conditions are favorable for growth (Schneider & Grogan, 1977a, Smitley & McCarter, 1982). After *P. syringae* is

established on the leaf surface, it gains entry into hosts via wounds, stomata and other natural openings. Typically, *P. syringae* uses leaf stomata to gain access to intercellular spaces (the apoplast), where it then multiplies and colonizes host tissues. Multiplication to high levels is necessary for visible symptom development, which are typically necrotic lesions that are often surrounded by chlorotic halos (Buell *et al.*, 2003).

In nonhost or resistant plants, phytopathogens elicit a plant defense mechanism known as the hypersensitive response (HR). During the HR, physiological changes within plant cells at the infection site include ion fluxes, membrane depolarization, and generation of reactive oxygen species (ROS). These events result in the collapse and death of cells at or near the infection site, observeable 12-24 h post-inoculation. The HR shares similarities to programmed cell death, and this reaction prevents the spread of the pathogen (Heath, 2000).

***P. syringae* pv. tomato DC300: a model for studying host-pathogen interactions**

Pst DC3000 is considered a model organism for studying molecular-plant microbe interactions, largely because of its genetic tractability, its pathogenicity on the model plant Arabidopsis, and the availability of its genomic sequence (http://pseudomonas-syringae.org/pst_DC3000_gen.htm) (Buell *et al.*, 2003). The *Pst* DC3000 genome consists of a circular chromosome (6,397,126 bp) and two plasmids, pDC3000A (73,661 bp) and pDC3000B (67,473 bp), which encode a total of 5,763 open reading frames (ORFs) (Buell *et al.*, 2003). Additionally, several related pathovars have been sequenced including *P. syringae* pv. phaseolicola 1448A and *P. syringae* pv.

syringae B728a, which causes halo blight and brown spot of bean, respectively (Feil *et al.*, 2005, Joardar *et al.*, 2005)

The type III secretion system (T3SS)

Highly conserved genes encoding the type III secretion system (T3SS) in bacteria are called the *hrc* genes (for *h*ypersensitive *r*esponse and *c*onserved) (Bogdanove *et al.*, 1996). The *hrc*/*hrp* (for "*h*ypersensitive *r*esponse and *p*athogenicity") gene cluster is required for the pathogenicity of *P. syringae* on host plants. Genes that encode the T3SS apparatus were first identified by random Tn*5* mutagenesis and were termed *hrp* genes because they were required for a HR on nonhost plants and for pathogenicity on host plants (Lindgren *et al.*, 1986).

The T3SS of *P. syringae* resembles those of other bacterial pathogens and is used to transport effector proteins into host cells, where they modulate host processes to promote virulence or defense (Grant et al., 2006, He & Jin, 2003, Jin & He, 2001, Li *et al.*, 2002). Like other bacterial and fungal pathogens, the pathogenicity of DC3000 is controlled by "gene-for-gene" interactions, in which dominant alleles in the host and pathogen interact (Buell *et al.*, 2003). More than 53 potential *hop*/*avr* genes have been identified in *Pst* DC3000, although the function of many of these genes is unclear (Schechter *et al.*, 2006). In some cases, Avr effector proteins suppress the plant host defense response, ultimately leading to bacterial multiplication and disease (Jin *et al.*, 2003).

The T3SS of gram negative bacteria is relatated evolutionarily to the flagellar apparatus (Desvaux *et al.*, 2006, McCann & Guttman, 2008). Distinguishing features of

the T3SS include the absence of a signal peptide in the secreted protein and a requirement for chaperones and intimate contact with a host cell for delivery of secreted proteins (Buttner & Bonas, 2006). The components of the T3SS in plant pathogenic bacteria are more closely related to flagellar components than to the T3SS in animal pathogens. It has been theorized that T3SS in phytopathogenic bacteria may be an evolutionary adaptation of flagella to secrete proteins other than flagellin, a capability that would allow plant pathogenic bacteria to associate closely with host plant cells (Galan & Collmer, 1999).

Regulation of virulence factors in *Pst* DC3000

Pathogenicity and virulence factors in *P. syringae* include the production of phytotoxins (Bender *et al.*, 1999, Bender & Scholz-Schroeder, 2004, Bender *et al.*, 1987) and exopolysaccharides (Keith *et al.*, 2003, Penaloza-Vazquez *et al.*, 1997, Yu *et al.*, 1999), both of which function to enhance disease severity. A two-component regulatory system consisting of the response regulator GacA and its cognate sensor kinase, GacS, also is involved in disease (Heeb & Haas, 2001, Hrabak & Willis, 1992, Rich *et al.*, 1994). The GacS/GacA system has been found to control diverse phenotypes and processes including survival, pathogenicity, production of toxin and antibiotics, secretion systems, biofilm formation, quorum sensing, motility, synthesis of secondary metabolites, and production of extracellular polysaccharides (Chatterjee *et al.*, 2003, Heeb & Haas, 2001, Kitten *et al.*, 1998, Tang *et al.*, 2006). The GacS/GacA two-component system of *P. syringae* pv. tabaci 6605 was shown to be indispensable for virulence on host tobacco, and important for the full expression for various virulence factors including *N*-acyl homoserine lactones, motility, pigment production, adhesion, *hrp* genes and the *algT*

gene (Marutani *et al.*, 2008). *P. syringae* pv. tabaci 6605 mutants that were defective in *gacA, gacS* or both genes were still able to induce the HR in nonhost tomato plants (Marutani *et al.*, 2008). The *gacA* gene was speculated to play a central role in the virulence of *P. syringae* pv. tomato DC3000 by controlling various sigma factors, quorum sensing, and regulatory RNA (Chatterjee *et al.*, 2003).

Production of the phytotoxin coronatine by *P. syringae*

The phytotoxin coronatine (COR) is produced and secreted as a virulence factor by several pathovars of *P. syringae* including pvs. tomato, glycinea, maculicola, morsprunorum, atropurpurea and alisalensis (Bender et al., 1987, Brooks *et al.*, 2004, Mitchell, 1982, Tamura *et al.*, 1998). In *Pst* DC3000, COR functions as a virulence factor in tomato, Arabidopsis, and *Brassica* spp. (Brooks *et al.*, 2004, Elizabeth & Bender, 2007, Penaloza-Vazquez *et al.*, 2000) and was required for lesion formation and bacterial multiplication in tomato and Arabidopsis (Brooks *et al.*, 2004, Mittal & Davis, 1995, Penaloza-Vazquez *et al.*, 2000). Mutants defective in COR production had lower populations in tomato compared with wild-type and caused smaller lesions as compared to the wild-type *Pst* DC3000 (Brooks et al., 2004, Penaloza-Vazquez et al., 2000).

COR also promotes virulence in Arabidopsis. A COR insensitive (*coi1*) mutant of Arabidopsis was inoculated with *Pst* DC3000 and while the growth of *Pst* DC3000 was at a high level, pathogen growth was not restricted and these plants did not exhibit disease symptoms (Kloek *et al.*, 2001). Studies conducted using Arabidopsis inoculated with the wild-type DC3000 strain and a COR^- mutant showed that COR production may suppress host defense genes, and may be critical during the early infection stages (Mittal

& Davis, 1995). The nature of COR in promoting disease during the early stages of infection remained elusive until very recently. On the leaf surface, the wild-type *Pst* DC3000 was observed to migrate toward stomata to gain access to the apoplast (Melotto *et al.*, 2006). Although stomata are generally viewed as passive openings, stomata on Arabidopsis leaves were shown to respond to both human and plant pathogenic bacteria by closing. After three hours of incubation with wild-type *Pst* DC3000, stomata re-opened to the pre-inoculation state, whereas a COR^- mutant was unable to re-open closed stomata. These results indicated that COR was responsible for suppressing early stomatal defense in Arabidopsis (Melotto *et al.*, 2006).

Studies focusing on the host side of the plant-microbe interaction have provided insights into additional functions for COR in plant tissues. COR has a variety of effects and is known to induce chlorosis *in planta* (Bender *et al.*, 1999), inhibit root elongation, stimulate anthocyanin accumulation (Feys *et al.*, 1994), cause stunting, induce hypertrophy and stimulate ethylene production (Bender *et al.*, 1999, Kenyon & Turner, 1992). COR is also known to mimic the endogenous plant signaling molecules collectively known as the jasmonates (Lauchli & Boland, 2003). In tomatoes, COR impacts host signaling pathways by modulating the jasmonic acid, ethylene, and auxin pathways (Uppalapati *et al.*, 2005). Furthermore, COR inhibits host defense by suppressing salicylic acid (SA) dependent plant defense responses, which leads to activation of the JA pathway (Nomura *et al.*, 2005). Thus, COR actively inhibits host defense mechanisms to promote *P. syringae* virulence.

Regulation of coronatine production

COR is a nonhost specific phytotoxin, its structure consists of two distinct moieties that function as intermediates in the biosynthetic pathway: (a) the polyketide coronafacic acid (CFA), and (b) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977, Parry *et al.*, 1994). CMA and CFA are synthesized by separate pathways and joined by an amide bond to form COR (Bender *et al.*, 1993). In *Pst* DC3000, the CMA and CFA biosynthetic gene clusters are physically separated by ~26,000 bp (Brooks et al., 2004, Buell et al., 2003). In *Pst* DC3000 and in the related pathogen, *P. syringae* pv. glycinea PG4180, the regulatory genes for COR include *corR, corS* and *corP* (Sreedharan *et al.*, 2006, Ullrich *et al.*, 1995). COR regulation in PG4180 is via a two-component regulatory system, where the protein products of *corP* and *corR* show relatedness to transcriptional regulators in the two-component family, and *corS* shows similarity to genes encoding histidine protein kinases (HPKs) (Bender & Scholz-Schroeder, 2004, Braun *et al.*, 2008, Rangaswamy & Bender, 2000, Ullrich *et al.*, 1995). Cross-talk between the T3SS and COR regulation is discussed in more detail below.

Regulation of the T3SS

Induction of the T3SS is mediated by *hrpR*, *hrpS* (encoding response regulator members of a two-component regulatory system), *rpoN* (encoding sigma factor σ^{54}), and *hrpL* (encoding sigma factor σ^{L}). HrpR and HrpS are encoded by the *hrpRS* operon, and these two enhancer binding proteins form a stable heterodimeric complex that interacts with σ^{54} to positively regulate the *hrpL* promoter (Hendrickson et al., 2000a, Hutcheson

et al., 2001, Xiao *et al.*, 1994). σ^L then activates the expression of *hrp* and *avr* genes (Bretz *et al.*, 2002, Hutcheson *et al.*, 2001, Xiao *et al.*, 1994) (see Fig 3). When the *hrpL* promoter was inactivated, *hrpR* and *hrpS* were expressed constitutively (Bretz *et al.*, 2002). *hrpA*, which encodes the major structural protein of the Hrp pilus (Roine *et al.*, 1997), also has a regulatory role in the expression of *hrpR/hrpS* in *Pst* DC3000 (Wei *et al.*, 2000).

Lon protease functions in the degradation of HrpR and negatively regulates the T3SS (Bretz et al., 2002, Lan *et al.*, 2007). When the gene encoding Lon protease was mutagenized in *P. syringae* pv. syringae 61 and *Pst* DC3000, the HrpR protein showed increased stability, and expression of *hrpL* was enhanced (Bretz *et al.*, 2002). Furthermore, mutation of *hrpV* elevated *hrp/hrc* expression, while overexpression of *hrpV* reduced *hrp/hrc* gene expression, a phenotype that was restored by constitutive expression of *hrpRS*. Collectively, these results indicate that HrpV functions as a negative regulator upstream of *hrpRS* in the regulatory cascade shown in Fig. 3 (Preston *et al.*, 1998a)

Analysis of *P. syringae* pv. syringae 61 *hrpG* (encoding a component of the T3SS) showed that it is conserved in several *P. syringae* pathovars including *Pst* DC3000 but is not found in other bacteria (Deng *et al.*, 1998, Wei et al., 2005). A *hrpG* mutant of *P. syringae* pv. syringae 61 showed reduced virulence and was impaired in the HR (Deng *et al.*, 1998). In a subsequent study HrpV was shown to interact with HrpG and the positive regulator HrpS (Wei *et al.*, 2005), and a model was proposed in which HrpG acts as a suppressor of the negative regulator HrpV. The interaction of HrpV and HrpG

suppresses HrpV activity, leading to the dissociation of HrpS and HrpV and freeing HrpS to activate the *hrp* regulon (Wei *et al.*, 2005).

More recently, microarray analysis was conducted to identify genes in *Pst* DC3000 that were regulated by *hrpL* and *hrpRS* (Lan *et al.*, 2006). HrpL recognizes a conserved promoter sequence referred to as the "hrp box" upstream of HrpL-dependent genes (Fouts *et al.*, 2002, Lan *et al.*, 2006, Schechter *et al.*, 2004, Xiao & Hutcheson, 1994, Zwiesler-Vollick *et al.*, 2002). Genes regulated by *hrpL* were mostly regulated by *hrpRS*, but a large number of genes regulated by the *hrpRS* two-component system were *hrpL*-independent. This finding indicates that *hrpL* is just one branch of the regulatory pathway downstream of *hrpRS* (Lan *et al.*, 2006).

A *Pst* DC3000 *gacA* mutant showed reduced expression of *hrpR*, *hrpS*, *rpoN* and *hrpL* transcripts, suggesting a role for GacA in the positive regulation of the T3SS (Chatterjee *et al.*, 2003). More recently, *rhpS* and *rhpR* were reported to function upstream of *hrpR* and to regulate T3SS genes in *P. syringae* pv. phaseolicola NPS3121 (Xiao *et al.*, 2007). *rhpR* and *rhpS* were suggested to form a two-component system in which phosphorylated RhpR represses the T3SS, and RhpS reverses this phosphorylation under T3SS-inducing conditions (Xiao *et al.*, 2007).

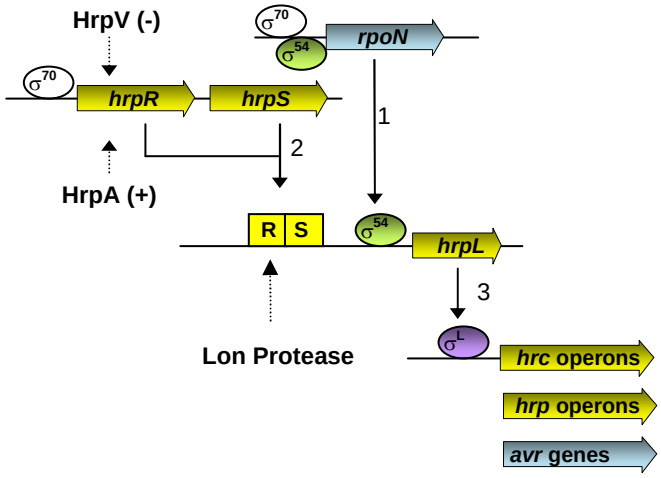


Fig. 3. σ^{54}-mediated activation of the *hrp* regulon. Arrow 1, binding of the *rpoN* gene product (σ^{54}) to the *hrpL* promoter. Transcriptional initiation of *hrpL* is activated by HrpR [R] and HrpS [S] gene products (arrow 2), which are related to proteins in the NtrC family. Arrow 3 depicts HrpL (σ^{L}) activation of the *hrp/hrc* operons and the *avr* genes, which modulate host range and fitness. HrpA and HrpV also control *hrp* gene expression presumably upstream of *hrpR* and *hrpS* (Wei *et al.*, 2005). Lon protease has been shown to negatively regulate the T3SS by degrading HrpR (Bretz *et al.*, 2002). Aspects of this model are based on previous reports (Bretz *et al.*, 2002; Preston *et al.*, 1998a; Tang *et al.*, 2006; Wei *et al.*, 2000; Xiao *et al.*, 1994)

Cross-talk between the *hrp* and *cor* systems

Cross-talk exists between the T3SS and COR regulation systems in *P. syringae* (Boch *et al.*, 2002, Fouts et al., 2002, Penaloza-Vazquez et al., 2000, Sreedharan et al., 2006). *hrcC* encodes an outer membrane protein required for secretion via the T3SS (Preston *et al.*, 1998a). A *hrcC* mutant of *Pst* DC3000 overproduced COR (Penaloza-Vazquez *et al.*, 2000), demonstrating that a functional T3SS is not required for COR production *in vitro* and that mutations in the T3SS have a direct effect on COR production in *Pst* DC3000. When *hrpV* was expressed *in trans* in the *hrcC* mutant, COR production was reduced to wild-type levels, suggesting that the overproduction of COR in the *hrcC* mutant was due to polar effects on *hrpV*, a regulatory gene in the *hrp* cluster (Penaloza-Vazquez *et al.*, 2000).

Another study using *in vivo* expression technology (IVET) was aimed at identifying the *Pst* DC3000 genes that are induced upon infection of Arabidopsis (Boch *et al.*, 2002). Several CFA structural genes such as *cfl*, *cfa1* and *cfa7* were induced *in planta*. Additional support for regulatory cross-talk in *Pst* DC3000 was reported when a

promoter trapping screen was used to identify genes controlled by *hrpL* (Fouts *et al.*, 2002). *hrpL*-regulated insertions were identified in two COR biosynthesis genes, *cfa1* and *cfa6*. This was an intriguing result, because neither COR gene contains a recognizable '*hrp*' box. A Hidden Markov Model was utilized to look for variations in functional Hrp boxes, and this analysis indicated the potential existence of a '*hrp*' box upstream of *corR*, the response regulator known to control expression of both the CFA and CMA operons (Fouts *et al.*, 2002). This result suggested that the COR regulatory genes (*corRS*) might be modulated by σ^L, the alternate sigma factor encoded by *hrpL*. Using reverse-transcription PCR, Sreedharan *et al*. (2006) showed that the effector gene *holPtoAA*, which was associated with the *hrp* box and located immediately upstream of *corR*, was co-transcribed with *corRS*, thus constituting an operon (*holPtoAA-corR-corS*). The mutation in *corR* also had effects on the expression of *hrpL* (Sreedharan, 2005, Sreedharan *et al.*, 2006); thus the *cor* regulatory system directly impacts the expression of the *hrp* regulon in *P. syringae* and mutations in the T3SS can impact COR expression and production.

σ^{54}-mediated gene regulation

Global virulence regulators are important for the pathogenicity of *P. syringae* (Alarcon-Chaidez et al., 2003, Chatterjee et al., 2003). *rpoN* encodes σ^{54} and is required for many metabolic functions including the utilization of nitrogen and carbon sources, the expression of virulence determinants and nitrogen fixation (Alarcon-Chaidez et al., 2003, Kustu *et al.*, 1989, Merrick, 1993, Studholme & Buck, 2000, Wosten, 1998). Processes such as dicarboxylic acid transport, pilin synthesis, hydrogen uptake, flagellar assembly,

degradation of xylene and toluene, acetoin catabolism, rhamnolipid production, arginine catabolism and alginate production also are controlled by σ^{54} (Alarcon-Chaidez et al., 2003, Barrios *et al.*, 1999). Furthermore, σ^{54} functions in many cellular and environmental responses including flagellation, chemotaxis, biodegradation and expression of other sigma factors. Bacteria have different mechanisms to control σ^{54}; for example, some bacteria contain more than one *rpoN* gene, but *Pst* DC3000 contains a single copy of *rpoN* (Alarcon-Chaidez et al., 2003, Hendrickson et al., 2000a, Hendrickson et al., 2000b).

σ^{54} and the RNA polymerase holoenzyme initiate transcription at σ^{54}-dependent promoters (Fig. 4 below). These promoter sequences are located between 24 and 12 bp upstream of the transcriptional start site and have the consensus sequence TGGCACG-N4-TTGC (Barrios et al., 1999, Cases *et al.*, 2003, Morett & Segovia, 1993). These elements are highly conserved, and deletion of one or more of these nucleotides abolishes the function of the promoter (Barrios *et al.*, 1999).

σ^{54} interacts with enhancer binding proteins to modulate transcription

σ^{54} binds to RNA polymerase to form a closed complex (Fig. 4B) that requires further activation by members of the NtrC class of bacterial enhancer binding proteins (EBPs) (Cases & de Lorenzo, 2001, Studholme & Buck, 2000, Studholme & Dixon, 2003). These EBPs or transcriptional activator proteins are regulatory proteins that bind to DNA sequences referred to as enhancer-like elements (ELE) approximately 100-200 bp upstream of the transcriptional start site (Cases *et al.*, 2003, Morett & Segovia, 1993, Studholme & Dixon, 2003). The interaction between σ^{54} and the EBP has been difficult to

study biochemically due to its transient nature (Grande *et al.*, 1999). σ^{54} controls many physiological processes, and each involves different EBPs interacting with unique ELE sequences (Fig. 4A), thus activating transcription of σ^{54}-dependent genes from a distance (Barrios et al., 1999, Cases et al., 2003, Morett & Segovia, 1993).

The closed holoenzyme complex formed by σ^{54} and RNA polymerase requires further activation by members of the NtrC class of enhancer binding proteins (EBPs) (Studholme & Buck, 2000, Studholme & Dixon, 2003). The DNA region between the binding sites for σ^{54} and the activator bend to position the two protein complexes in such a way that they can physically interact (Carmona *et al.*, 1997, Cases *et al.*, 2003) (Fig. 4). The closed promoter complex remains stable until an activator protein binds to the ELE, which results in an open promoter complex and allows transcription initiation (Fig. 4C). This final step requires energy, which is obtained by the ATP hydrolysis catalyzed by the EBP (Cases et al., 2003, Morett & Segovia, 1993) (Fig. 4B). A phosphate-binding loop is present in σ^{54}-dependent activators, and the amino acids that make up the loop are conserved and present in other ATP and GTP-binding proteins. It is this loop motif that binds the phosphate group of the nucleoside triphosphate (Gao *et al.*, 1998).

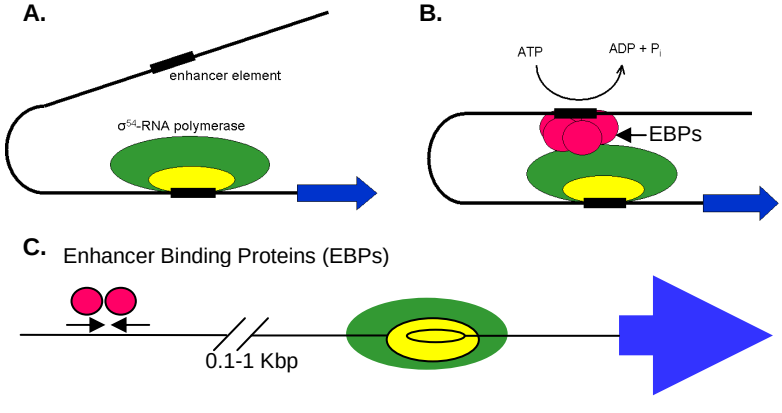


Figure 4: σ^{54} interacts with enhancer binding proteins to modulate transcription. The enhancer binding proteins (EBPs; represented by the red circles) bind to the enhancer-like element (represented as a black bar in Figure 2A & 2B). These then interact with the RNA polymerase complex (green oval) and σ^{54} (yellow oval) to modulate transcription. Reference: Studholme: http://www.promscan.uklinux.net/faq.html

Classification of σ^{54}-dependent activators

Some σ^{54}-dependent activators are associated with histidine protein kinases (HPKs) in two component regulatory systems; whereas others are not. A typical two-component system consists of an HPK and a response regulator (RR), which may be a σ^{54}-dependent activator. The HPKs are often transmembrane proteins that sense environmental changes, but also contain cytosolic domains that transduce the signal. In the signal transduction event, the HPK is autophosphorylated at a conserved histidine residue, which then transphosphorylates the receiver domain of the response regulator, which is often a transcription factor. Once this transphosphorylation is complete the response regulator is activated (West & Stock, 2001).

It is important to note that σ^{54}-dependent activators may occur in the proteome in the absence of HPKs. This group of activators contains response regulator EBPs without sensor partners. The HrpR/HrpS regulators of *P. syringae* are examples of this group. These EBPs lack the regulatory input domains seen in two-component systems, but are negatively regulated by HrpV protein (Preston *et al.*, 1998a, Studholme & Dixon, 2003).

σ^{54} is required for virulence and elicitation of the HR in *P. syringae*

Global regulation of COR in selected *P. syringae* pathovars requires *hrpL* and σ^{54} (encoded by *rpoN*). The *rpoN* mutants of *P. syringae* pv. *maculicola* ES4326 and *P. syringae* pv. *glycinea* PG4180 were nonpathogenic and unable to elicit the HR when infiltrated into tobacco (Alarcon-Chaidez et al., 2003, Hendrickson et al., 2000b). When *hrpL* was introduced into the ES4326 *rpoN* mutant, the HR was restored and the *rpoN* mutant was partially complemented for disease symptoms in Arabidopsis; however, the

partially complemented mutant was unable to grow *in planta* (Hendrickson et al., 2000a, Hendrickson et al., 2000b). This finding suggests that the absence of the T3SS system is not the sole explanation for the nonpathogenic phenotype of the *rpoN* mutant in ES4326. The results of both of these studies are consistent with the signal transduction cascade in Fig. 3, in which σ^{54} activates *hrpL*, which then activates various *hrp* and *avr* genes.

σ^{54} is required for *hrp* and *cor* gene expression

Several reports implicate *rpoN* in the regulation of the *hrp* gene cluster in *P. syringae*. The HrpR and HrpS regulatory proteins encoded by the *hrp* cluster (Deng et al., 1998, Hutcheson et al., 2001, Lan et al., 2006, Xiao et al., 1994) show relatedness to NtrC, an activator protein that interacts with σ^{54} and RNA polymerase (RNAP) to initiate transcription (Morett & Segovia, 1993, North *et al.*, 1993). Furthermore, σ^{L} (encoded by *hrpL*) is required for the expression of several transcripts in the *hrp* gene cluster and contains a promoter region with strong homology to the consensus recognized by σ^{54} (Xiao *et al.*, 1994).

In *P. syringae* pv. maculicola ES4326 and in *P. syringae* pv. glycinea PG4180, σ^{54} was required for the transcription of *hrpL* (Alarcon-Chaidez *et al.*, 2003, Hendrickson *et al.*, 2000b). Additionally, *rpoN* mutants of ES4326 and PG4180 did not produce COR *in vitro* and were defective in *cor* gene expression (Alarcon-Chaidez *et al.*, 2003, Hendrickson *et al.*, 2000b). The complementation of the ES4326 *rpoN* mutant with *hrpL* eliminated the requirement for σ^{54}-dependent activation of *hrpL*, and resulted in expression of the *hrc* and *hrp* transcripts and selected *avr* genes, but did not fully restore the mutant for COR production and *cor* gene expression (Hendrickson *et al.*, 2000b).

These results suggest that additional *rpoN*-dependent genes are required for COR expression in ES4326, and that *hrpL* did not restore the functional activity of these genes. The *rpoN* mutant of PG4180 was also impaired in *hrpL* transcription, and while complementation with *rpoN* restored *cor* gene expression and COR biosynthesis, the mutant was not fully complemented for *hrpL* expression (Alarcon-Chaidez *et al.*, 2003). The authors concluded that *rpoN* is required for *cor* gene expression and COR production in PG4180, although it is not clear how this occurs.

It is important to note that both the *cfl*/CFA and *cmaABT* promoter regions of the COR gene cluster of PG4180 lack the conserved–24(GG)/-12(GC) motif that is bound by σ^{54} (Alarcon-Chaidez *et al.*, 2003, Barrios *et al.*, 1999, Liyanage *et al.*, 1995, Ullrich & Bender, 1994), which suggests that σ^{54} does not bind directly to these promoters. Thus, σ^{54} control of *cor* gene expression is likely to be mediated through another regulatory gene whose expression is controlled directly by σ^{54}. It is important to mention that the *cfl*/CFA and *cmaABT* promoter regions contain motifs similar to sequences recognized by σ^{54}-dependent activators, NifA and NtrC, respectively (Liyanage *et al.*, 1995, Ullrich & Bender, 1994).

The transcriptional start sites for the COR regulatory genes *corR*, *corP*, and *corS* are unmapped in PG4180, but several motifs for σ^{54} were found ~500 bp upstream of the *corR* translational start site (Alarcon-Chaidez *et al.*, 2003). σ^{54} also modulates σ^{L} expression which activates transcription of *corRS*. Although the role of σ^{54} *in P. syringae* remains unclear, it is tempting to speculate that σ^{54} may coordinately regulate *hrp* and *cor* gene expression in *P. syringae* (Preston, 2000).

Aspects of virulence modulated by σ^{54}-dependent regulators

FleQ and role in flagellar synthesis.

σ^{54} interacts with the FleQ enhancer binding protein to modulate flagellar synthesis. σ^{54} is known to control other traits that may be involved in virulence including motility (Wosten, 1998). Bacterial flagella are required for motility and are broadly conserved in gram-negative bacteria (McCarter, 2006, Moens & Vanderleyden, 1996). Mutants defective in motility and formation of flagella have been previously isolated and some were impaired in virulence (Chatterjee *et al.*, 2003, Haefele & Lindow, 1987, Hattermann & Ries, 1989, Kinscherf & Willis, 1999).

The *fleQ* gene of *Pst* DC3000 shows homology to *fleQ* genes characterized in other organisms, including *P. fluorescens* (Capdevila *et al.*, 2004) and *P. aeruginosa* (Arora et al., 1997). *fleQ,* together with *rpoN,* is located at the top of the flagellar regulatory hierarchy and regulates the *fleSR* two-component regulatory system (Fig. 5) to control motility and adhesion in *P. aeruginosa* (Arora *et al.*, 1997, Dasgupta *et al.*, 2003). It is important to note that some aspects of flagellar biosynthesis are regulated differentially in *Pseudomonas* spp. (Redondo-Nieto *et al.*, 2008). A mutant defective in *fleQ*, which belongs to the NtrC family of activators, was characterized as part of this work.

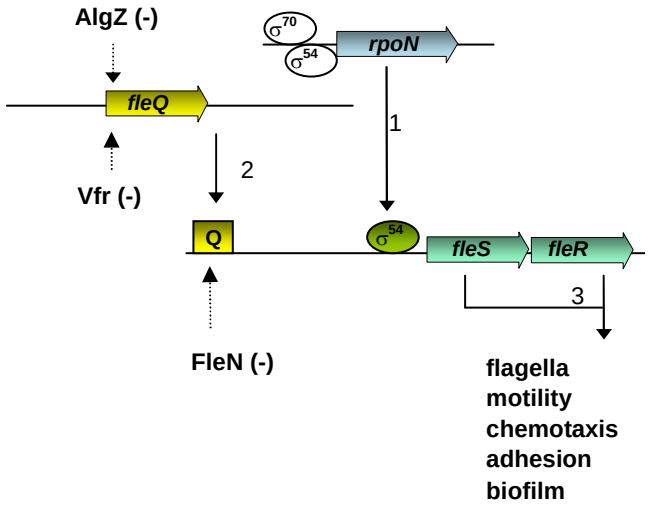


Fig. 5. σ^{54}-mediated activation of the flagellar regulon. Arrow 1, binding of the *rpoN* gene product (σ^{54}) to the consensus σ^{54}-dependent promoter. Transcriptional activation of the *fleSR* two-component system by the enhancer binding protein FleQ [Q] gene product (arrow 2) is from a distance. FleQ binds upstream of the *fleSR* transcriptional start site and interacts with σ^{54} via looping (Jyot *et al.*, 2002). Arrow 3 depicts FleSR transcriptional activation, which modulates flagella, chemotaxis and adhesion traits (Arora *et al.*, 1997; Capdevila *et al.*, 2004; Robleto *et al.*, 2003). The alternative sigma factor AlgT (algU; σ^{22}) represses flagellum biosynthesis indirectly by promoting expression of AmrZ (AlgZ), which represses *fleQ* in *P. aeruginosa* (Tart *et al.*, 2005; Tart *et al.*, 2006). Vfr represses *fleQ* transcription in *P. aeruginosa* by binding to the Vfr binding site in the *fleQ* promoter (Dasgupta *et al.*, 2002). FleN interacts with FleQ to regulate flagellar number in *P. aeruginosa* (Dasgupta *et al.*, 2000; Dasgupta and Ramphal, 2001). Aspects of this model are based on previous reports (Dasgupta *et al.*, 2003).

Regulatory links between the flagellar system and other bacterial processes.

Flagella sense the external environment and regulate physiological processes in addition to flagellar biogenesis (Wang *et al.*, 2005). Results obtained with *P. fluorescens* indicated that FleQ (AdnA) impacts the expression of at least 23 ORFs either directly or by polar effects (Robleto et al., 2003). These results support the concept that FleQ regulates transcription at multiple promoters and also regulates cell processes other than flagellar synthesis (Robleto *et al.*, 2003).

There are regulatory links between the flagellar system and the T3SS in several bacterial species. In *Erwinia amylovora* the *hrp* and flagellar systems are inversely regulated (Cesbron et al., 2006). In *P. aeruginosa*, an inverse relationship between the T3SS and flagellar assembly was described. For example, a strain lacking flagella sustained higher T3SS gene expression, effector secretion and cytotoxicity (Soscia et al.,

2007). Correspondingly, in a *P. aeruginosa* strain overproducing the T3SS master regulator ExsA, both motility and flagellar gene expression were lower than in the wild-type (Soscia *et al.*, 2007).

Regulatory interactions between the flagellar and the chemotaxis and quorum sensing systems also have been reported. Mutants of *Salmonella typhimurium* defective in the chemotaxis signaling pathway exhibit fewer and shorter flagella than the wild-type strain (Wang *et al.*, 2005). In *P. syringae* pv. syringae, quorum sensing defective mutants were hypermotile, invaded leaves more rapidly, and caused more lesions than did the wild-type (Quiniones *et al.*, 2005).

Regulatory links between the flagellar system and alginate production also have been established. The alternative sigma factor AlgT (σ^{22}) is required for the transcription of many alginate genes. AlgT negatively regulates flagellar expression (Tart *et al.*, 2006, Tart *et al.*, 2005). In *P. aeruginosa* AlgT represses flagellum biosynthesis indirectly by promoting expression of AmrZ (AlgZ). AmrZ then interacts directly with the *fleQ* promoter, which represses expression of *fleQ* while inducing transcription of the alginate gene cluster (Tart *et al.*, 2006, Tart *et al.*, 2005) (Fig. 5). Interestingly, *algB* and *algR*, which also are activators of alginate synthesis, do not directly interact with *fleQ* (Tart et al., 2006); however, *algR* is negatively regulated by FleQ (Giddens et al., 2007).

Recently, a suppressor IVET (SPyVET) screen was used to identify *P. fluorescens* SBW25 plant environment-induced loci (EIL). Previously, the cellulose biosynthetic gene *wssE* was identified as a plant environment-induced locus (Gal *et al.*, 2003), and a strain carrying a fusion to this operon was used to identify regulators. In the SPyVET screen, FleQ was implicated in the negative transcriptional control of the *wss*

operon (Giddens *et al.*, 2007). These studies suggested that SBW25 coordinates the expression of flagella and the *wss* operon via FleQ. The authors hypothesize that FleQ may function as a repressor by interacting with σ^{54} at the *amrZ* promoter, promoting the transcriptional activation of the *wss* repressor AmrZ (Giddens et al., 2007).

Most recently, FleQ from *P. aeruginosa* was identified as a c-di-GMP-responsive transcription factor (Hickman & Harwood, 2008), adding an additional layer of complexity to the regulation of the flagellar system. High levels of the intracellular signaling molecule cyclic diguanylate (c-di-GMP) suppress motility and activate EPS production in a variety of bacterial species. This most recent model for flagellar regulation in *P. aeruginosa* indicates that when c-di-GMP levels are low, FleQ binds and represses transcription of genes in the *pel* operon involved in EPS biosynthesis. When c-di-GMP levels are high, FleQ binds to c-di-GMP, allowing transcription of the *pel* operon (Hickman & Harwood, 2008).

Flagellin protein is a PAMP implicated in host specificity. In both animal and plant pathogens, innate immunity in the host plant and the basal defense response are triggered by host detection of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) (He *et al.*, 2007, Nurnberger & Brunner, 2002, Zipfel & Felix, 2005). Recently, flagella and flagellin have been implicated as PAMPs in both plant and animal systems (Asai *et al.*, 2002, Verma *et al.*, 2005). Plants have a receptor that perceives flagellin, triggering expression of defense genes and restricting bacterial invasion (Zipfel *et al.*, 2004). In a recent study, stomata of Arabidopsis plants treated with flagellin closed

(Melotto *et al.*, 2006); this is consistent with stomatal closure functioning as a basal defense against bacterial invasion.

It is also important to note that flagella and flagellin, which are potentially regulated in a σ^{54}-dependent manner, have been implicated in host specificity. The ability of flagella or flagellins to elicit the plant defense response varies in different host-pathogen interactions (He et al., 2007); however, in *P. syringae* pathovars, the flagellin protein elicits both host and nonhost plant defense responses (Li *et al.*, 2005, Shimizu *et al.*, 2003). The flagellins of *P. syringae* pv. tabaci and *P. syringae* pv. glycinea are identical, but are modified post-translationally and affect both compatible and incompatible plant-pathogen interactions. Mutants defective in flagellin glycosylation genes were able to induce HR on nonhost tobacco leaves, but were unable to infect host soybean leaves (Takeuchi *et al.*, 2003).

AlgB and role in alginate synthesis

Role of EPS *in planta.* Exopolysaccharides (EPS) are involved in the invasion, multiplication and survival of bacteria in host plant tissues and can help the bacterium avoid plant defense responses (Gacesa, 1998, Leigh & Coplin, 1992, Quiniones *et al.*, 2005, Rahme *et al.*, 1995, Von Bodman *et al.*, 2003). It is assumed that EPS remain closely associated with the bacterial cell as a capsule or slime layer (Denny, 1995). Water is scarce in the apoplast of plant tissue, and EPS retain moisture and protect bacterial cells from desiccation, promoting bacterial multiplication (Denny, 1995). During pathogenesis, EPS are also thought to benefit the pathogen by minimizing their contact with plant cells, promoting colonization (Denny, 1995). On the surface of plant

leaves, EPS production increases the dissemination and epiphytic fitness of bacteria, and protects bacteria from desiccation stress and ROS (Keith *et al.*, 2003, Yu *et al.*, 1999).

Alginate is the major EPS produced in water-soaked lesions of susceptible plant leaves inoculated with *P. syringae* pathovars (Fett & Dunn, 1989, Gross & Rudolph, 1987). *P. syringae* and the human opportunistic pathogen *P. aeruginosa* both produce alginate EPS, a copolymer of O-acetylated β-1,4 linked D-mannuronic acid and its C-5 epimer L-guluronic acid. Alginate has been shown to function in the virulence of some plant pathogenic *P. syringae* strains and is involved in lesion formation and increased epiphytic fitness (Keith *et al.*, 2003, Penaloza-Vazquez *et al.*, 2004, Yu *et al.*, 1999). Alginate-defective mutants of *P. syringae* showed reduced epiphytic fitness and lower disease severity on host plants when compared to wild-type strains (Allen & Bender, Yu *et al.*, 1999), which suggests that alginate contributes to *P. syringae* virulence *in planta*. Boch *et al*. used *in vivo* expression technology (IVET) to show that the alginate biosynthetic gene, *algA*, was induced during infection of Arabidopsis with *Pst* DC3000 (Boch *et al.*, 2002). Furthermore, the *algD* gene (another alginate biosynthesis gene) was induced when tomatoes were inoculated with *Pst* DC3000 (Keith *et al.*, 2003).

Regulation of alginate production. The genes involved in alginate regulation and production in *P. aeruginosa* are grouped into three main clusters. The biosynthetic gene cluster consists of *algD* to *algA* (Fig. 6), and the order and arrangement is identical in *P. syringae* (Buell *et al.*, 2003, Penaloza-Vazquez *et al.*, 1997). The regulatory gene cluster consists of *algB, algC, algR3, algR* (*algR1*), genes that have a positive regulatory effect on transcription of the *algD* promoter (Ramsey & Wozniak, 2005) (Fig. 6).

Another gene cluster is involved in the switch from the nonmucoid to the mucoid mode of growth, and consists of *algT* and the *mucABCD* operon. The *muc* genes have a negative regulatory role in alginate production in *P. aeruginosa* and *P. syringae*, and inhibit the *algT* (*algU*) gene product, σ^{22} (Keith & Bender, 1999, Schenk *et al.*, 2008).

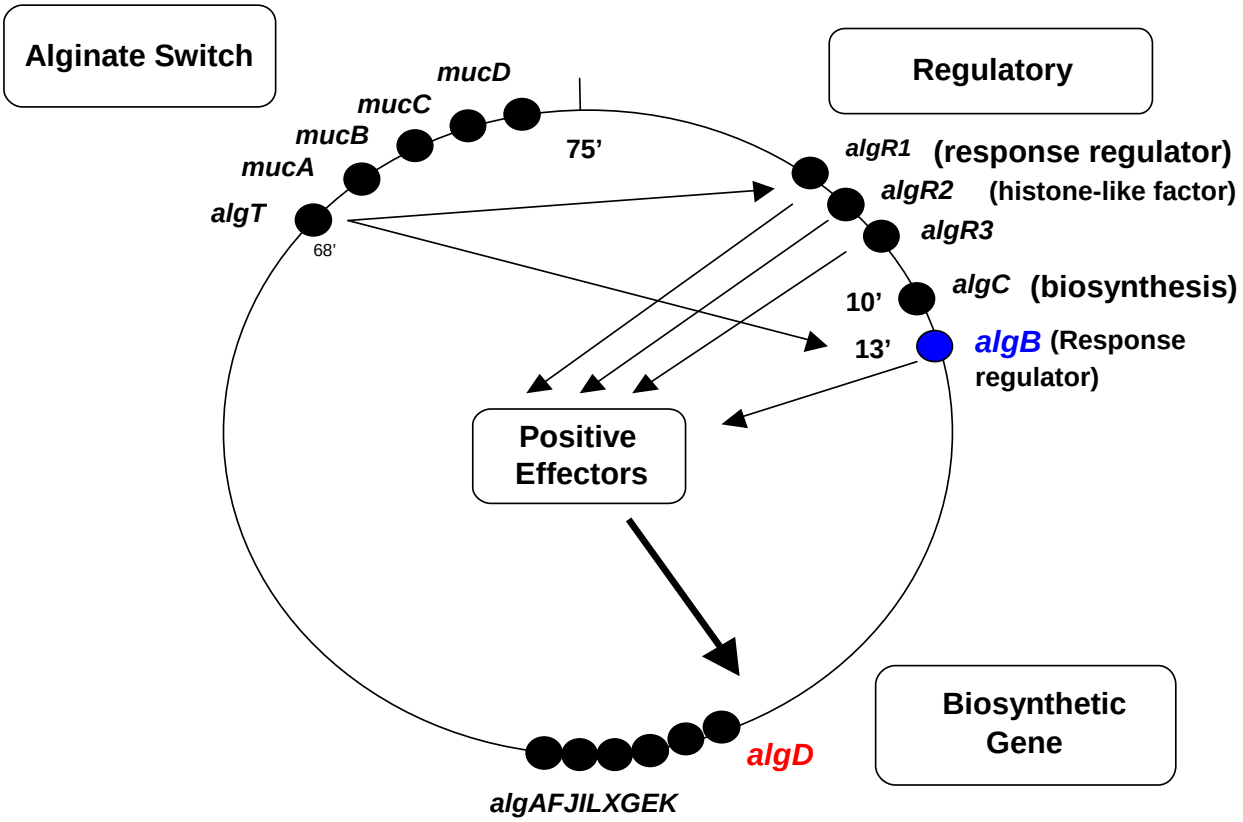


Fig. 6: Chromosomal map of *P. aeruginosa* showing genes involved in alginate biosynthesis. The *muc* cluster consists of *algT* and the *mucABCD* operon (Wood & Ohman, 2006) *mucA*, *mucB* (*algN*) and *mucD* negatively impact the *algD* promoter by inhibiting the activity of σ^{22}, as mutations in these genes lead to conversion to mucoidy (Boucher *et al.*, 1997, Goldberg *et al.*, 1993a, Martin *et al.*, 1993, Schurr *et al.*, 1994, Wood & Ohman, 2006, Xie *et al.*, 1996). *algT* encodes the alternate sigma factor, σ^{22} (Hershberger *et al.*, 1995), which is required for the transcription of *algD*, *algR*, and *algT* in *P. aeruginosa* (Wozniak & Ohman, 1994). The *algB* gene encodes an NtrC activator that positively regulates *algD* transcription.

The alginate biosynthetic genes are normally silent (not expressed) (Goldberg *et al.*, 1993), and the switch from a nonmucoid to a mucoid phenotype depends on the presence of environmental factors, which activate expression of the alginate regulatory genes, leading to alginate production. Factors such as copper sulfate (Kidambi et al., 1995), paraquat, and hydrogen peroxide (H_2O_2) (Keith & Bender, 1999) have been shown to induce alginate production by *P. syringae*.

Activation of alginate production in response to environmental signals involves two promoters, P*algC* and P*algD*, which map at two distinct locations in *P. aeruginosa* and *P. syringae* (Chitnis & Ohman, 1993, Penaloza-Vazquez *et al.*, 1997). The *algD* biosynthetic gene encodes GDP-mannose dehydrogenase and is the first gene to be transcribed in the alginate biosynthetic cluster in *P. aeruginosa* (Deretic *et al.*, 1987a) and *P. syringae* (Penaloza-Vazquez et al., 1997). The *algC* biosynthetic gene (which encodes a phosphomannomutase) (Zielinski *et al.*, 1991, Zielinski *et al.*, 1992) is also involved in lipopolysaccharide (LPS) biosynthesis (Coyne *et al.*, 1994).

Several regulators are required for transcription of *algD*, one of which is AlgR (AlgR1). In *P. aeruginosa*, the response regulator AlgR positively controls transcription at the *algD* promoter (Deretic *et al.*, 1987b, Kato & Chakrabarty, 1991, Mohr *et al.*, 1992). However, in *P. syringae* pv. syringae FF5, AlgR is not required for the activation of *algD* (Fakhr *et al.*, 1999). In contrast, *algC* is transcriptionally activated by AlgR in both *P. aeruginosa* (Zielinski *et al.*, 1991, Zielinski *et al.*, 1992) and *P. syringae* (Penaloza-Vazquez et al., 2004). In *P. aeruginosa*, AlgT is required for transcriptional activation of *algR* and *algD* (Hershberger *et al.*, 1995, Schurr *et al.*, 1995, Wozniak & Ohman, 1994), and σ^{22} recognition sequences were found upstream of *algR1* and *algD* in

P. syringae, suggesting that *algT* is required for transcription of these genes in *P. syringae* as well (Fakhr *et al.*, 1999). As in *P. aeruginosa*, an *algT* mutant of *P. syringae* was defective in alginate production (Keith & Bender, 1999), and analysis of an *algT* mutant of *P. syringae* pv. glycinea revealed that σ^{22} (AlgT) activates *algD* (Schenk *et al.*, 2006).

The transcriptional regulator *algB* also modulates alginate production in *P. aeruginosa* and is a member of the NtrC family of response regulators, which interact with *rpoN* (Goldberg & Dahnke, 1992, Wozniak & Ohman, 1991). AlgB is known to positively regulate *algD*, the first gene in the alginate biosynthetic pathway that encodes GDP-mannose dehydrogenase (Chitnis & Ohman, 1993, Deretic *et al.*, 1987a, Wozniak & Ohman, 1991). AlgB is necessary for overproduction of alginate (Wozniak & Ohman, 1991), and *algB* mutants of *P. aeruginosa* are impaired in their ability to produce high levels of alginate (Goldberg & Ohman, 1987). Transcriptional analysis in *P. aeruginosa* showed that both the AlgB and AlgR pathways require σ^{22} (AlgT) for transcriptional activation (Wozniak & Ohman, 1993, Wozniak & Ohman, 1994), placing *algT* at the top of the alginate gene expression hierarchy (Muhammadi & Ahmed, 2007, Ohman *et al.*, 1996, Wozniak & Ohman, 1994). Although *P. syringae* contains *algB* (Buell *et al.*, 2003), it is not known whether *algB* functions similarly in *Pst* DC3000 and *P. aeruginosa*. A mutant defective in *algB* was studied as part of the current work.

DctD and role in nutrient assimilation

The *dct* system. Although virulence factors such as the T3SS, COR, flagella and alginate are important, the ability of *P. syringae* and persist and multiply in the plant

apoplast is dependent upon its ability to import and metabolize the available nutrients. Boch *et al*. used IVET to identify *Pst* genes that were induced upon infection of Arabidopsis (Boch *et al.*, 2002). As expected many plant-inducible genes included those involved in virulence. In addition, metabolic genes that are presumably important for *Pst* adaptation and growth in plant tissue such as those involved in amino acid biosynthesis and nutrient acquisition were also identified (Boch *et al.*, 2002).

In bacteria, the transport and catabolism of dicarboxylic acids involves the *dct* (dicarboxylic acid transport) gene system. Since dicarboxylic acids such as succinic acid fuel nitrogen fixation, this system is well-characterized in symbiotic, nitrogen-fixing *Rhizobium* spp. (Labes *et al.*, 1993, Lodwig & Poole, 2003, Rastogi *et al.*, 1992, Yurgel & Kahn, 2005). In *R. meliloti* the sensor *dctB* and the transcriptional regulator *dctD* form a two-component system (Wang *et al.*, 1993). *dctD*, which encodes C_4 dicarboxylic acid transport protein D, then interacts with σ^{54} in the presence of dicarboxylates to activate transcription of the *dctA*-encoded transport protein (Engelke *et al.*, 1989, Janausch *et al.*, 2002, Jiang *et al.*, 1989, Labes & Finan, 1993, Lee & Hoover, 1995, Lodwig & Poole, 2003, Ronson *et al.*, 1987, Watson, 1990).

Mutations in the *dct* genes are known to result in impaired transport and growth on medium containing C_4 dicarboxylic acids (Engelke *et al.*, 1989, Lodwig & Poole, 2003, Ronson *et al.*, 1987). *dctBD* was required for the expression of *dctA* in *Rhizobium meliloti* (Wang *et al.*, 1989), while mutations in *dctD* in resulted in reduced taxis to aspartate (Robinson & Bauer, 1993). In *R. leguminosarum*, the absence of the DctA protein caused the DctB-DctD pair to improperly signal and cross-talk with other operons (Reid & Poole, 1998). There is also evidence of the *dct* system in other bacterial species,

including *Mesorhizobium loti*, *Sinorhizobium meliloti*, and *Agrobacterium tumefaciens* (Dombrecht *et al.*, 2002). In *P. putida*, there is evidence of σ^{54}-dependent transcriptional control of C_4 dicarboxylic acid transport via *dctA* and *dctB* (Studholme & Buck, 2000).

Virulence and nutrient assimilation in *P. syringae.* An *rpoN* mutant of *P. syringae* pv. glycinea PG4180 was unable to use proline, histidine, and methionine as nitrogen sources (Alarcon-Chaidez *et al.*, 2003). Additionally, an *rpoN* mutant of *P. syringae* pv. maculicola ES4326 was unable to utilize nitrate, urea, C_4-dicarboxylic acids, several amino acids, or concentrations of ammonia below 2 mM as nitrogen sources (Hendrickson *et al.*, 2000b). Both mutants failed to induce disease symptoms and multiply in host plants. It is important to mention that the T3SS and its effector proteins (T3SEs) play a major role in the invasion and extraction of nutrients by bacteria from their plant hosts (McCann & Guttman, 2008). The T3SS is induced by nutritional conditions including plant sugars such as sucrose, fructose and mannitol (Rico & Preston, 2008). These findings indicate that nutrient assimilation is important for the survival and pathogenicity of *P. syringae*, which is significant because *P. syringae* resides in the plant apoplast where nutrients (sugars and amino acids) are limiting (Hancock & Huisman, 1981).

Virtually nothing is known about how the *dct* system functions in a plant pathogenic bacterium such as *P. syringae*, and the regulatory interactions this system has with other bacterial survival and virulence systems. In other organisms, the σ^{54}-dependent transcriptional activator *dctD* together with σ^{54} is known to activate transcription of *dctA* (Reid & Poole, 1998, Ronson *et al.*, 1987, Wang *et al.*, 1989, Yurgel & Kahn, 2004).

Curiously, there are two *dctA* genes (designated *dctA1* and *dctA2*) annotated in the *Pst* DC3000 genome, as well as three *dctD* genes (*dctD1*, *dctD2*, and *dctD3*). Mutants defective in *dctD1, 2*, and 3 which belong to the NtrC family of activators were studied as part of the current work.

OBJECTIVES

In this study I characterized σ^{54}-dependent transcriptional activator mutants of *Pst* DC3000. The main objectives of the study were:

Objective I: Validate transcriptional activator mutants in *Pst* DC3000 using Southern hybridization

Objective II: Determine the phenotype of the σ^{54}-dependent transcriptional activator mutants on host and nonhost plants

Objective III: Analyze σ^{54}-dependent transcriptional activator mutants for traits relevant to specific activator (motility, nutrient utilization, alginate production)

Objective IV: Complementation analysis for selected σ^{54}-dependent transcriptional activator mutants by restoring the defective gene on a stable plasmid

CHAPTER V

Characterization of σ^{54}-Dependent Transcriptional Activator Mutants in *Pseudomonas syringae* pv. *tomato* DC3000

SUMMARY

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000), is a gram-negative plant pathogenic bacterium that is pathogenic on tomato, Arabidopsis and *Brassica* spp., and is a model organism for studying plant-microbe interactions. In bacteria, the alternative sigma factor σ^{54}, which is encoded by the *rpoN* gene, interacts with RNA polymerase and associated activator proteins to regulate the transcription of target genes involved in various metabolic and physiological processes. *P. syrinage rpoN* mutants are unable to induce disease symptoms and multiply in host plants; however, the role of individual σ^{54}-dependent activators in *P. syringae* is not clear, and the majority of target genes have yet to be identified in this pathogen. To better understand gene regulation in *Pst* DC3000, mutants were generated in eight of the associated σ^{54}-dependent activator genes. In this work these were characterized for traits associated with each activator and evaluated with respect to virulence and ability to elicit a hypersensitive response (HR) on non-host tobacco plants. The *DC3000-fleQ* mutant was non-motile, non-flagellated, and defective in biofilm formation, but was able to induce disease symptoms on hosts tomato

and Arabidopsis, suggesting that *fleQ* is not required for *Pst* DC3000 virulence in host plants. Complementation studies demonstrated that motility, flagella, and biofilm formation can be restored with the *fleQ* gene. DC3000-*algB* showed a reduction in symptom production on Arabidopsis, suggesting a role for alginate in *Pst* DC3000 virulence. DC3000-*dctD2* (dicarboxylic acid transport protein D) mutant was defective in the ability to transport and/or utilize succinic acid as a carbon source, but *dctD2* was not required for pathogenicity or the HR. To our knowledge, these results provide the first example of a mutation in the *dctD* gene system in a plant pathogenic bacterium. In summary, this study shows that σ^{54}-dependent activator genes in *Pst* DC3000 are important for pathogenicity, nutrient assimilation, and various physiological processes.

INTRODUCTION

Pseudomonas syringae is a gram-negative bacterium widely used for studying bacterial pathogenesis and plant-microbe interactions (Preston, 2000), largely because of its genetic tractability and pathogenicity on Arabidopsis, and the availability of its genomic sequence (Buell *et al.*, 2003). *P. syringae* is related to other important Pseudomonads including the human pathogen *P. aeruginosa,* and the environmentally important *P. fluorescens* and *P. putida.* Pseudomonads have been shown to use similar traits to infect both plants and animals.

In bacteria, the alternative sigma factor σ^{54} (RpoN), which is encoded by the *rpoN* gene, interacts with RNA polymerase and associated activator proteins to regulate the transcription of target genes. These activators are required for various functions including nutrient assimilation, pilin synthesis, alginate production, hydrogen uptake, and flagellar

assembly (Alarcon-Chaidez *et al.*, 2003, Barrios *et al.*, 1999, Kustu *et al.*, 1989, Merrick, 1993, Studholme & Buck, 2000, Wosten, 1998). Furthermore, σ^{54} functions in many cellular and environmental responses including flagellation, chemotaxis, biodegradation and expression of other sigma factors.

rpoN was shown to be important during pathogenesis in *P. syringae* pv. maculicola ES4326 and *P. syringae* pv. glycinea PG4180. *rpoN* mutants of ES4326 and PG4180 were unable to induce disease symptoms and multiply in host plants (Arabidopsis and soybean, respectively) and failed to elicit the hypersensitive response (HR) when infiltrated into non-host tobacco leaves (Alarcon-Chaidez et al., 2003, Hendrickson *et al.*, 2000b). Furthermore, these mutants were unable to utilize various carbon, nitrogen and amino acid sources. While much is known about pathogenicity and virulence factors in *P. syringae*, the role of individual σ^{54}-dependent activators is not clear, and the majority of target genes have yet to be identified in this pathogen.

Previous research has suggested that σ^{54} (and hence activator proteins) are involved in regulating many factors in *P. syringae* (Alarcon-Chaidez et al., 2003, Hendrickson *et al.*, 2000a, Hendrickson et al., 2000b) and *P. fluorescens* (Jones et al., 2007). There are 14 annotated σ^{54}-dependent activators in the *Pst* DC3000 genome (http://www.tigr.org/) (Table 7), and the DC3000 genome contains over 70 predicted recognition sites for σ^{54} (http://www.promscan.uklinux.net/). Since transcriptional activation by σ^{54} requires a positive activator protein (Merrick, 1993, Morett & Segovia, 1993), this suggests that these activators play a critical role in gene regulation within this organism.

Table 7. Genes in *Pst* DC3000 having relatedness to known σ^{54}-dependent activators in other organisms, particularly *Pseudomonas* spp.

Activator name	Position in DC3000 genome (bp)	DC3000 Annotation Name	Probable targets	Potential function/relevant literature/notes
dctD1	4835207..4836541	4292	PSPTO4296 metabolite-proton symporter, putative	Glycerol, fructose, or arginine transport; (Llamas *et al.*, 2003)
dctD2	6132127..6133512	5399	Homologous to activators in *P. syringae* pv. syringae, *P. fluorescens*	Defective in utilization of succinate acid (C. Baker, this study)
algB	363589..365935	0334	algD	(Wozniak & Ohman, 1991) (also see text)
ssuD	3913277..3914383	3467	PSPTO3471-monovalent cation:proton antiporter, putative PSPTO3451 *(ssuE*) (FMN reductase, NADH-dependent)	sulfur metabolism (Endoh et al., 2003, Kahnert et al., 2000)
dctD3	4704777..4706105	4176	PSPTO4171-4176 (4171-4174 amino acid ABC transporters; periplasmic amino acid binding protein) (4176 indicates autoregulation)	May have transport defective phenotype
fleQ (adnA)	2139870..2141345	1954	regulates *fleSR* system in *P. aeruginosa*; biofilm formation and adhesion in *P. fluorescens*	(Arora *et al.*, 1997, Casaz *et al.*, 2001, Robleto *et al.*, 2003)
nifA	Complement 1047150..1048586	0964	PSPTO0963 polyA polymerase has sig 54 recognitation	DNA polymerase I, lethal mutation
TR	Complement (3531672..3532976)	3142	(no strong clues on function); no strong defects in pathogenesis	Homologous to activators in *P. syringae* pv. syringae, *P. fluorescens*, *P. putida*
ISPsy12, orfB	Complement (3425886..3427889)	3044	transposase	According to recent re-annotation this is not an activator
	130125..131444		in *Agrobacterium* acetoin catabolism regulatory protein	Homologous to activators
fleR	2142682..2144097		regulation of mucin adhesion and flagellar expression	(Arora *et al.*, 1997)
	2501881..2504172		Not present in *P. syringae* B728a	Homologous to activators-but not in Pseudomonas spp.
	2815564..2817093		PSPTO2550-PSPTO2554 (conserved hypothetical proteins)	Homologous to activator in *P. fluorescens* & to *Pst* DC3000 orf #5424
	3316244..3317929		PSPTO2950: benzaldehyde dehydrogenase (encoded by *xylC* in *P. putida*; involved in degradation of toluene and xylene	(Inoue *et al.*, 1995)
	4225753..4227606		PSPTO3752 (*acnB*) aconitate hydratase 2; PSPTO3755 membrane protein	*P. syringae* pv. syringae, *P. fluorescens*, & many others acetoin/glycerol metabolism
	Complement (6165047..6166564)		lacks W in conserved motif	Homologous to activators in *P. fluorescens*, *P.*

			aeruginosa, *Pst* DC3000 orf # 2549
ntrC	Complement (384711..386147)	Two-component system w/*ntrB*; probably regulating entire operon; *glnA* & downstream genes (including *ntrB*)	
pilR	Complement (889587..890927)	*pilA* in *P. aeruginosa*; *Pst* orf #0927 and other *pil* genes in *Pst* including orf # 0810 (putative pilus biogenesis protein)	(Roine *et al.*, 1998)
hrpR, hrpS	1522289..1523233 1523279..1524187	PSPTO1404 (*hrpL*)	(Deng et al., 1998, Hutcheson et al., 2001, Preston et al., 1998a, Wei et al., 2000)

The σ^{54}-dependent activator *fleQ* is required for motility, which may be important for invasion of plant tissues. However, loss of *fleQ* may confer benefits *in planta* if motility is not required, as flagellin is an elicitor of innate immunity. In *Pst* DC3000, *fleQ* may also regulate traits such as alginate biosynthesis, the T3SS, stress tolerance and nutrient assimilation, which are important for pathogenesis. These regulatory networks are highly significant and relevant to the regulation of survival and pathogenesis in gram-negative bacteria.

AlgB has a role in the regulation of alginate biosynthesis (Wozniak & Ohman, 1991), and *algB* mutants of *P. aeruginosa* are impaired in their ability to produce high levels of alginate (Goldberg & Ohman, 1987). Although *P. syringae* contains *algB* (Buell *et al.*, 2003), it is not known whether *algB* functions similarly in *Pst* DC3000 and *P. aeruginosa*. This is one of the first studies to assess the role of an alginate regulatory gene *in planta*.

P. syringae resides in the plant apoplast, where nutrients (sugars and amino acids) are limiting (Hancock & Huisman, 1981). Ultimately, the ability of *P. syringae* to persist and multiply in the plant apoplast is dependent upon its ability to import and metabolize the available nutrients. In bacteria, the transport and catabolism of dicarboxylic acids

involves the *dct* (dicarboxylic acid transport) gene system. *rpoN* mutants of *P. syringae* pv. glycinea PG4180 (Alarcon-Chaidez *et al.*, 2003) and *P. syringae* pv. maculicola ES4326 (Hendrickson et al., 2000b) were deficient in the assimilation of various nutrients including carbon, nitrogen, C_4-dicarboxylic acids, and amino acids. Virtually nothing is known about how the *dct* system functions in a plant pathogenic bacterium such as *P. syringae*. Three annotated *dctD* genes (*dctD1*, *dctD2*, and *dctD3*) annotated in the *Pst* DC3000 genome were studied as part of the current work (Table 8).

Table 8. Presence and homology of the *dctD* genes in *Pst* DC3000 to *Pseudomonas* using BLASTx megablast max identity.

Pseudomonas strains	*dctD1*	*dctD2*	*dctD3*
P. syringae pv. phaseolicola 1448a	84%	93%	100%
P. syringae pv. syringae B728a	84%	90%	100%
P. putida GB-1	75%	79%	81%
P. putida Pf-1	---	---	---
P. fluorescens Pf0-1	---	88%	73%
P. fluorescens Pf-5	---	80%	81%
P. aeruginosa PA7	---	77%	78%
P. aeruginosa UCBPP-PA14	76%	---	---
P. aeruginosa PAO1	76%	---	---

Blanks (---) indicate that a *dctD* homolog was not found in that strain.

Previously, eight σ^{54}-dependent activator genes were mutated in *Pst* DC3000 (Hernandez-Guzman and Bender, unpublished; described in Materials & Methods). However, the mutant strains were not analyzed for pathogenicity, characterized for their metabolic phenotypes, or genetically complemented, which are objectives of the present study.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 9**.** *E. coli* DH5α was cultured on Luria Bertani (LB) medium or in Terrific Broth (TB) (Sambrook *et al.*, 1989) at 37^{O}C. *P. syringae* strains were cultured on King's medium B (KMB) (King *et al.*, 1954) or mannitol-glutamate (MG) medium (Keane *et al.*, 1970) at 28^{O}C. Antibiotics were used at the following concentrations (µg/ml): kanamycin (Km), 25; chloramphenicol (Cm), 25; and rifampicin (Rif), 50.

Table 9. Bacterial strains and plasmids used in activator study

Strain or plasmid	Relevant characteristics[1]	Reference or source
P. syringae pv. tomato DC3000	derivative of NCPPB 1106; Rif^{R}	(Moore *et al.*, 1989)
DC3000-*dctD1*	Rif^{R}, Km^{R}; contains pCR2.1 in *dctD1*	This study
DC3000-*dctD2*	Rif^{R}, Km^{R}; contains pCR2.1 in *dctD2*	This study
DC3000-*algB*	Rif^{R}, Km^{R}; contains pCR2.1 in *algB*, derivative of *Pst* DC3000	This study
DC3000-*ssuD*	Rif^{R}, Km^{R}; contains pCR2.1 in *ssuD*	This study
DC3000-*dctD3*	Rif^{R}, Km^{R}; contains pCR2.1 in *dctD3*	This study
DC3000-*fleQ*	Rif^{R}, Km^{R}; contains pCR2.1 in *fleQ*	This study
DC3000-A10	Rif^{R}, Km^{R}; contains pCR2.1 in PSPTO3142	This study
DC3000-A11	Rif^{R}, Km^{R}; contains pCR2.1 in PSOTO3044	This study
PG4180-*rpoN* (*Psg-rpoN*)	Km^{R}, *rpoN*::*nptII*; non-pathogenic in soybean; defective in COR production	(Alarcon-Chaidez *et al.*, 2003)
DC3000-DB4G3	Rif^{R},Km^{R}; *cfa6*::Tn*5*	(Brooks *et al.*, 2004)
E. coli DH5α	*recA lacZ*_M15	Bethesda Research Labs
Plasmids		
pCR2.1	Ap^{R} Km^{R}; cloning vector for PCR products; replicated in *E.*	Invitrogen, Carlsbad, CA

	coli but not *P. syringae*	
pBBR1MCS	Cm^R; broad-host range cloning vector, replicates well in *Pseudomonas* without selection	(Kovach *et al.*, 1994)
pBBR-*fleQ*	Rif^R, Km^R; Cm^R; contains *fleQ in trans* as a 1.8 kb *Bam*HI fragment in pBBR1MCS	This study
pBBR-5399	Rif^R, Km^R; Cm^R; contains *dctD2 in trans* as a 1.5 kb *Sal*I-*Xba*I fragment in pBBR1MCS	This study
pCR2.1-*dctD1*	Km^R; contains a 404-bp internal fragment of the *dctD1* gene in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-*dctD2*	Km^R; contains a 579-bp internal fragment of the *dctD2* gene in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-*algB*	Km^R; contains a 780-bp internal fragment of the *algB* gene in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-*ssuD*	Km^R; contains a 397-bp internal fragment of the *ssuD* gene in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-*dctD3*	Km^R; contains a 375-bp internal fragment of the *dctD3* gene in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-*fleQ*	Km^R; contains a 359-bp internal fragment of the *fleQ* gene in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-A10	Km^R; contains a 554-bp internal fragment of the 3142 ORF in pCR2.1	Hernandez-Guzman, unpublished
pCR2.1-A11	Km^R; contains a 266-bp internal fragment of the 3044 ORF in pCR2.1	Hernandez-Guzman, unpublished

[1]**Abbreviations: Rif^R (rifampicin), Km^R (kanamycin), and Cm^R (chloramphenicol).**

Recombinant DNA methods

Agarose gel electrophoresis, restriction enzyme digestions, and DNA isolations were performed using standard techniques (Sambrook *et al.*, 1989). All enzymes used in genetic manipulations were purchased from Promega (Madison, WI) or Invitrogen

(Carlsbad, CA). Plasmid DNA isolated for sequencing was prepared using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Sequence data and homology searches were done using BLAST (http://www.ncbi.nih.gov).

Construction of activator mutants

Dr. Gustavo Hernandez-Guzman, a former postdoctoral researcher in the Bender lab, constructed the activator mutants used in this study. His work is summarized below for clarity. The *fleQ* mutant of *Pst* DC3000 was created using primers fleQ forward 5' ACC GCG CTT TTC GTG TTC CAT A 3') and fleQ reverse (5' CGT TTA CCG GGG CAA TCA CCA G 3'). The resulting PCR product and pCR2.1 were then ligated, resulting in plasmid pCR2.1-*fleQ*. This construct was introduced by electroporation into the *Pst* DC3000 genome, and the *fleQ* gene was disrupted by a single crossover event. The resulting mutant was designated DC3000-*fleQ*, and the disruption of *fleQ* was confirmed by PCR and Southern blot analysis (see results). Additional activator defective mutants were created in the same manner using the primers summarized in Table 10.

Table 10. Characteristics of primers used in mutant study.

Target Gene/ Primer	Primer Sequence	Tm ^{O}C	Size (bp)
dctD1 forward	5' CAG TTG CGC AAT GTC CTC A 3'	52.8	
dctD1 reverse	5' GCG CCG GCA AAC CCT TCG TG 3'	66.1	404
dctD2 forward	5' CGC GCC GGG GCT TAT GAC TTT C 3'	65.2	
dctD2 reverse	5' TCC TTG GTG GCG GCG ATG ATG C 3'	67.5	579
algB forward	5' CCG GGC GGG CGT ACT CTT TGA 3'	64.7	
algB reverse	5' TGC GCG TGG TGA TCG TGA CTG C 3'	65.8	789

ssuD forward	5’ TGC CCG GCC AAC TGT AGG TC 3’	59.4	
ssuD reverse	5’ AAG GCC GGC TGG TTT GAA GAG G 3’	62.3	397
dctD3 forward	5’ GCG CAA GGA CAG CCA GTT CGT G 3’	63.6	
dctD3 reverse	5’ AGC GGC GGC AAT TCC AGA GTG A 3’	64.5	375
fleQ forward	5’ ACC GCG CTT TTC GTG TTC CAT A 3’	60.3	
fleQ reverse	5’ CGT TTA CCG GGG CAA TCA CCA G 3’	62.0	359
A10 forward	5’ ACA TTG CCC GGC CAG TCG TG 3’	62.4	
A10 reverse	5’ CGC GGT GCC GGC AAA GAG TT 3’	63.9	554
A11 forward	5’ AAG CGG CAC CGG CAA GGA GTT A 3’	63.5	
A11 reverse	5’ GCA AGA CGC GCA AAA GGC TGA C 3’	62.7	266

Southern blot analysis

Genomic DNA used in Southern hybridizations was isolated from *Pst* DC3000 and the activator mutants using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI)). Extracted DNAs were digested with *Cla*I (mutants DC3000-*fleQ* and DC3000-A10) or *Sal*I (mutants DC3000-*dctD1*, DC3000-*dctD2*, DC3000-*algB*, DC3000-*ssuD*, DC3000-*dctD3*, DC3000-A11) (Invitrogen, Carlsbad CA) for 24 h at 37^{O}C. The corresponding activator gene fragment and pCR2.1 were labeled with digoxigenin-11-dUTP (DIG) (Roche Applied Science) by the random primed DNA labeling method according to the manufacturer’s instructions. Southern blotting was performed using Hybond-N nylon membranes (Amersham Biosciences) as described previously (Sambrook *et al.*, 1989). Hybridization was detected using an anti-DIG antibody and chemiluminescence using the DIG Luminescent Detection Kit (Roche Applied Science).

Mutant complementation

For complementation analysis, the 1.8-kb *fleQ* gene was amplified by PCR and cloned into pBBR1MCS. Successful cloning of *fleQ* in pBBR1MCS was confirmed by sequence analysis. The resulting construct, pBBR-*fleQ*, was introduced by electroporation into DC3000-*fleQ* for complementation studies. Two restriction sites were added to the primer sequences (Table 11; primers were synthesized by Integrated DNA Technologies) to facilitate cloning into pBBR1MCS. The PCR product and pBBR1MCS were then digested with *Bam*HI, purified from agarose gels, and ligated, resulting in plasmid pBBR-*fleQ*. The DC3000-*dctD2* mutant was complemented in the same manner as described using the *dctD2* forward and reverse primer sets shown in Table 11.

Table 11. Primers used for mutant complementation

Target Gene/ Primer	Primer Sequence	Restriction Site	Tm	Size (kb)
FleQ forward	5' GGG GGA TCC CGC CAT AAA TTT GAC TGT GTT C -3'	*Bam*HI	66.3	
FleQ reverse	5' AAA GGA TCC GGA CAT CTG ATT GAA CAG CGA G -3'	*Bam*HI	65.4	1800
dctD2-5399 forward	5' ATC GTC GAC GCT TCA GGG GCC AAT CTT CAA C 3'	*Sal*I	66.2	
dctD2-5400 reverse	5' CTT TCT AGA TTA ACG CAG CGT GGA AAC AAG 3'	*Xba*I	62.4	1588

Motility assays

Bacteria were inoculated onto motility agar plates (KMB medium containing 0.5% agar) with an inoculating needle and were incubated for 24 and 48 h at 28^{O}C. Movement of the bacterial colony from the inoculation point (colony diameter) was

measured in millimeters and compared to that of the wild-type *Pst* DC3000 and an *rpoN* mutant of *P. syringae* pv. glycinea PG4180.

Electron microscopy

Bacterial strains were grown overnight on KMB medium solidified with agarose and supplemented with the appropriate antibiotics. Cells were then suspended in 10 mM $MgSO_4$ buffer or water and placed on formvar-coated nickel grids (150 mesh) for 1-2 min and allowed to adhere to the grids. Unabsorbed cell suspensions were removed by blotting the edge of grids with Whatman #2 filter paper. Grids were then stained with phosphotungstic acid (PTA) or uranyl acetate (UA) and examined with a JEOL JEM-100CX II transmission electron microscope at 80 KV.

Plant material and inoculation procedures

Tomato (*Lycopersicum esculentum* cv. 'Glamour') plants were grown from seed and maintained at 24-25^{O}C at 30-40% relative humidity (RH), with a photoperiod of 12 h. Plants were maintained in growth chambers at 90% RH for 48 h before inoculation. All tomato plants used for pathogenicity assays were four to five weeks old. *Pst* DC3000 and derivatives were grown at 28^{O}C for 48 h on MG agar supplemented with the appropriate antibiotics, and cells were suspended to an optical density (OD_{600}) of 0.1 (~10^6 cfu/ml) in sterile distilled water. Tomato plants were spray-inoculated with an airbrush (55.2 kPa) until leaf surfaces were uniformly wet. After inoculation, tomato plants were incubated in growth chambers where the RH was 90% for the initial 48 h and

70% for the remainder of the experiment. The photoperiod was 12 h day/night for the duration of the experiment.

Arabidopsis ecotype Colombia (Col-0) seeds were placed on 0.1% water agar which was sealed with parafilm to hold moisture, and placed at 4^{O}C for 3-5 days to break dormancy. The seeds were then transferred to moistened peat discs (3 seeds per disc) with a small glass Pasteur pipette. The tray containing the discs was covered with a clear dome for five days. After germination the dome was removed and the seedlings thinned to one plant per disc. Plants used in pathogenicity studies were grown at 24-25^{O}C with a 12 h photoperiod and were four to five weeks old at the time of inoculation. Dip inoculation of Arabidopsis was conducted by immersing plant rosettes in bacterial suspensions (~10^{6} cfu/ml).

Symptoms on tomato and Arabidopsis were evaluated 7-10 days post inoculation The leaves were graded on a visual scale of 0-100 depending on the percentage of infected leaf area exhibiting symptoms, with 100 being the maximum disease severity. It is important to note that necrotic, water-soaked and chlorotic lesions were taken into consideration when evaluating virulence.

Determination of bacterial growth *in planta*

After spray inoculation growth of *Pst* DC3000, DC3000-*fleQ*, and DC3000-*fleQ*(pBBR-fleQ) was monitored in host tomato plants according to standard methods (Keith *et al.*, 2003, Penaloza-Vazquez *et al.*, 2000). Experiments were designed to monitor the total population of *Pst* DC3000 and derivatives over a 10 day time period. Random leaf samples were taken at 0, 1, 3, 5, 7 and 10 days after inoculation. At each

sampling, three replicate leaves were removed and dissected along the midrib. One half of the leaf was macerated using a mortar and pestle in sterile distilled H_20 to obtain the total population of bacteria. The remaining half was surface-sterilized in 15% hydrogen peroxide for 5 min, rinsed twice prior to rinsing in sterile distilled H_20 (to remove epiphytic bacteria) and then macerated using a mortar and pestle. Bacterial counts were determined by plating serial dilutions of the leaf homogenate on MG medium supplemented with the appropriate antibiotics, and colonies were counted 24-48 h after incubation at $28^{O}C$. To obtain epiphytic bacterial populations, the internal population counts were subtracted from the total population counts.

Assay for the hypersensitive response (HR)

In infiltration studies, four-week old tobacco leaves (*Nicotiana tabacum*) were infiltrated with bacterial suspensions (OD_{600}=0.1, approximately 10^6cfu/ml) of wild-type *Pst* DC3000, and mutants DC3000-*dctD2*, DC3000-*algB* and DC3000-*fleQ* using a needle-less syringe (Schaad, 2001). After infiltration, tobacco plants were maintained at $25^{O}C$ under constant light and monitored for a HR at the site of infiltration 24 h post-inoculation.

Metabolic phenoarray assay

P. syringae rpoN mutants were analyzed for growth on minimal medium supplemented with various carbon, nitrogen and amino acid sources using established protocols (Alarcon-Chaidez et al., 2003, Hendrickson et al., 2000b, Michiels *et al.*, 1998). In brief, the metabolic profiles of *P. syringae Pst* DC3000 and derivatives were evaluated

by growing strains overnight in KMB medium supplemented with appropriate antibiotics. Cells were then suspended in 10 mM $MgSO_4$ solution, washed twice and adjusted to an OD_{600}=0.05 in M9 medium (Ausubel, 1997). Strains were then inoculated into 96-well microtiter plates containing M9 medium supplemented with various carbon, nitrogen, or amino acid sources (carbon, 25 mM; nitrogen, 100 mM; amino acids, 100mM). Microtiter plates were incubated at 28^{O}C on an aerobic rotary shaker, and bacterial growth (OD_{600}) was monitored for 48 h using a microtiter plate reader [Bio-Tek Microplate Reader (www.bio-tek.com)].

Assay for coronatine production

Bacterial strains were grown overnight on MG medium supplemented with appropriate antibiotics. Cells were then adjusted to an OD_{600}=0.1 in Hoitink-Sinden sucrose medium (HSS) (Palmer & Bender, 1993, Penaloza-Vazquez *et al.*, 2000). Each strain was grown as a 10 ml culture (3-4 replicates per strain) and incubated at 18^{O}C for 7 days. Cultures were then centrifuged, and pellets were stored at -20°C for total protein quantification. For assessing COR production, culture supernatants were extracted with ethyl acetate. The organic phase was saved, the ethyl acetate was removed by evaporation, and the organic acid extracts were suspended in 200-750 µl of trifluoroacetic acid (TFA) and acetonitrile solution. Organic acid extracts were analyzed by HLPC with an Ultrasphere C-8 reverse phase column (Beckman Coulter, Fullerton, CA) at 208 nm using Gold Noveaux Chromatography Software (Brooks *et al.*, 2004). COR production was expressed as a function of soluble protein concentration (Penaloza-Vazquez & Bender, 1998). Total protein content from the bacterial cell lysates was determined using

the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc; Hercules, CA) as recommended by the manufacturer. Experiments were repeated two or more times.

Biofilm assay

A bioassay was used to indicate the potential of *Pst* DC3000 and derivatives to for biofilms. Bacterial strains were suspended to an OD_{600}=0.1 in Hoitink-Sinden medium (Palmer & Bender, 1993, Penaloza-Vazquez *et al.*, 2000) amended with 0.25 M citric acid, casamino acids (CAA, 0.5%), and 10 ml of 2mM $FeCl_3$, pH=6.5. Bacterial cells were then transferred to a 96-well flexible polyvinylchloride (PVC) microtiter plate and incubated overnight at 28^{O}C to allow cells to adhere. Crystal violet (CV) was used to stain the attached cells, but not the PVC. Excess stain was removed, the CV was solubilized using ethanol, and biofilm formation was evaluated by measuring the OD at 600 nm (O'Toole & Kolter, 1998b)

Assay for alginate production

For induction of alginate, *Pst* DC3000 and derivatives were grown in Bruegger and Keen (BK) medium (Bruegger & Keen, 1979, Fett *et al.*, 1986) supplemented with sodium gluconate (BKG; 25g/L). BK medium (1 L) contains the following reagents: $(NH_4)_2SO_4$ (1.32 g), $MgSO_4$ (0.08 g), $ZnSO_4$ (0.008 g), potassium phosphate (8.709 g) and casamino acids (2.3 g). Bacterial strains were grown overnight on MG medium supplemented with appropriate antibiotics. Bacterial strains were suspended in sterile water (OD_{600}=0.3), and inoculated onto BKG medium (six plates per strain). Plates were incubated at 28^{O}C for 7 days; at this time, bacteria were recovered from plates and re-

suspended in 10 ml of 0.9% NaCl. 500 µl of each bacterial suspension was centrifuged at 13,200 rpm for 15 min, and the bacterial pellets were stored at -20°C for total protein quantification. To precipitate uronic acid polymers the supernatant was transferred to a fresh microfuge tube, and mixed with 1 ml of 95% ethanol and 50 µl of 3 M sodium acetate. This was placed at -70^{O}C briefly and centrifuged at 13,200 rpm for 20 min. The alginate was air-dried and re-suspended in 250 µl of water. Alginate was quantified by mixing 70 µl of the suspension with 600 µl borate-sulfuric acid solution (100 mM H_3BO_3 in concentrated H_2SO_4) and 20 µl of a carbazole solution (0.1% in ethanol) (May & Chakrabarty, 1994). Samples were incubated for 30 min at 55°C to facilitate color development. One hundred and fifty microliters of the reactions were loaded into a 96-well microtiter plate and read at 540 nm using a Bio-Tek Microplate Reader. Alginate concentrations were determined from a standard plot of seaweed alginate (Sigma).

RESULTS

Confirmation of mutagenesis

To create the activator mutants, Dr. Hernandez-Guzman cloned PCR fragments containing selected activator genes into vector pCR2.1, and the corresponding constructs were electroporated into *Pst* DC3000. After electroporation, potential DC3000 mutants were selected on MG medium containing kanamycin; this screen facilitated selection for potential mutants containing activators disrupted by a single crossover event (Hernandez-Guzman, unpublished). To confirm the disruption of the activator genes by a single cross-over event, genomic DNAs of *Pst* DC3000 and the activator mutants were digested with *Cla*I (mutants DC3000-*fleQ* and DC3000-A10) or *Sal*I (mutants DC3000-*dctD1*,

DC3000-*dctD2*, DC3000-*algB*, DC3000-*ssuD*, DC3000-*dctD3*, DC3000-A11) (Invitrogen, Carlsbad CA), the products electrophoresed on 0.6% agarose gels at 60 V (BioRad Sub-Cell GT System with 15 x 20 cm tray) overnight, and transferred to nylon membranes as described in Methods. Hybridization probes (corresponding activator gene fragment and pCR2.1) were DIG labeled as described in Methods. After simultaneous hybridization with the labeled probes, bands were observed in the digested genomic DNAs of the wild-type and activator mutants for all hybridizations (Fig 7). For the activator mutants, band sizes were consistent with insertion of the corresponding pCR2.1 activator constructs (Table 9). These results confirmed that the constructs had inserted into the corresponding activator gene in the activator mutants (Fig 7). For example, Fig. 7F shows hybridization to the 1.6 kb band of the DNA ladder marker (left lane), both probes (pCR2.1 and fragment of *fleQ*) hybridizing to a 6,180 bp band of DC3000-*fleQ* (middle lane), and the *fleQ* fragment hybridizing to a 1,892 bp band in *Pst* DC3000 (right lane).

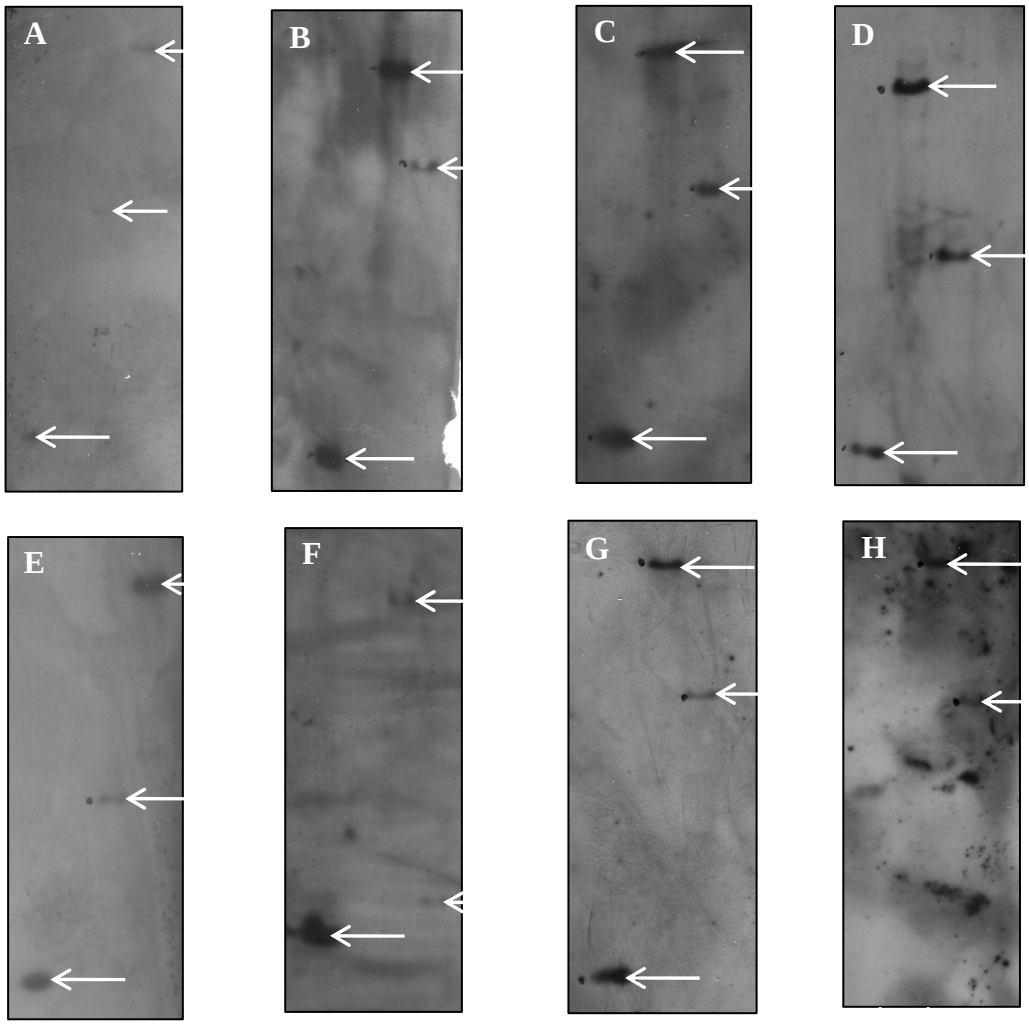


Figure 7. Southern blot analysis of digested genomic DNA from *Pst* DC3000 and the activator mutants. Extracted DNAs were digested with *Cla*I (wild-type *Pst* DC3000 and mutants DC3000-*fleQ* and DC3000-A10 in panels F and G) or *Sal*I (wild-type *Pst* DC3000 and mutants DC3000-*dctD1*, DC3000-*dctD2*, DC3000-*algB*, DC3000-*ssuD*, DC3000-*dctD3*, DC3000-A11 in panels A, B, C, D, E, and H) as stated in methods. All blots were hybridized simultaneously with the corresponding activator gene fragment and pCR2.1. All mutant band sizes correspond to the pCR2.1 plasmid (3929 bp) plus the corresponding activator amplicon (Table 9). Each panel shows the results of a Southern hybridization for one mutant with three signals, each corresponding to the kb ladder, the wild-type *Pst* DC3000 and an activator mutant. Southern blot signals from lanes in order from left to right, shown by arrows in panels: (A) 1.6 kb ladder, 4,050 bp *Pst* DC3000, and 8,383 bp DC3000-*dctD1*; (B) 1.6 kb ladder, 10,426 bp DC3000-*dctD*, and 5,918 *Pst* DC3000; (C) 1.6 kb ladder, 9,139 bp DC3000-*algB*, and 4,421 bp *Pst* DC3000; (D) 1.6 kb ladder, 8,051 bp DC3000-*ssuD*, and 3,725 bp *Pst* DC3000; (E) 1.6 kb ladder, 3,278 *Pst* DC3000, and 7,582 DC3000-*dctD3*; (F) 1.6 kb ladder, 6,180 bp DC3000-*fleQ*, and 1,892 bp *Pst* DC3000; (G) 1.6 kb ladder, 9,324 bp DC3000-A10, and 4,841 bp *Pst* DC3000; and (H) 8,107 bp DC3000-A11; 3,912 bp *Pst* DC3000

Motility of *Pst* DC3000 and derivatives

The motility of *Pst* DC3000 and derivatives was evaluated using KMB motility medium containing 0.5% agar; the low percentage of agar facilitated the evaluation of swarming motility (Robleto *et al.*, 2003). After incubation for 48 h, motility was visualized as bacteria surrounding the site of inoculation (Fig. 8A). Large bacterial colonies (15-20 mm diameter) were evident around the inoculation points for *Pst* DC3000 and pBBR-*fleQ* (DC3000-*fleQ* complemented mutant), whereas small colonies (~5 mm) were evident around the inoculation points for DC3000-*fleQ* and *Psg-rpoN* (Fig. 8, panels A and B). These results suggest that the *fleQ* mutant was significantly impaired in motility, whereas the other activator mutants were not. When *fleQ* was re-introduced into DC3000-*fleQ* on plasmid pBBR-*fleQ*, motility was restored (Fig. 8 panels A and B). Large bacterial colonies comparable to *Pst* DC3000 were evident around the inoculation points for DC3000-*dctD1*, DC3000-*dctD2*, DC3000-*algB*, DC3000-*ssuD*, DC3000-*dctD3*, DC3000-A10, and DC3000-A11 (data not shown), suggesting that motility was not impaired in these mutants.

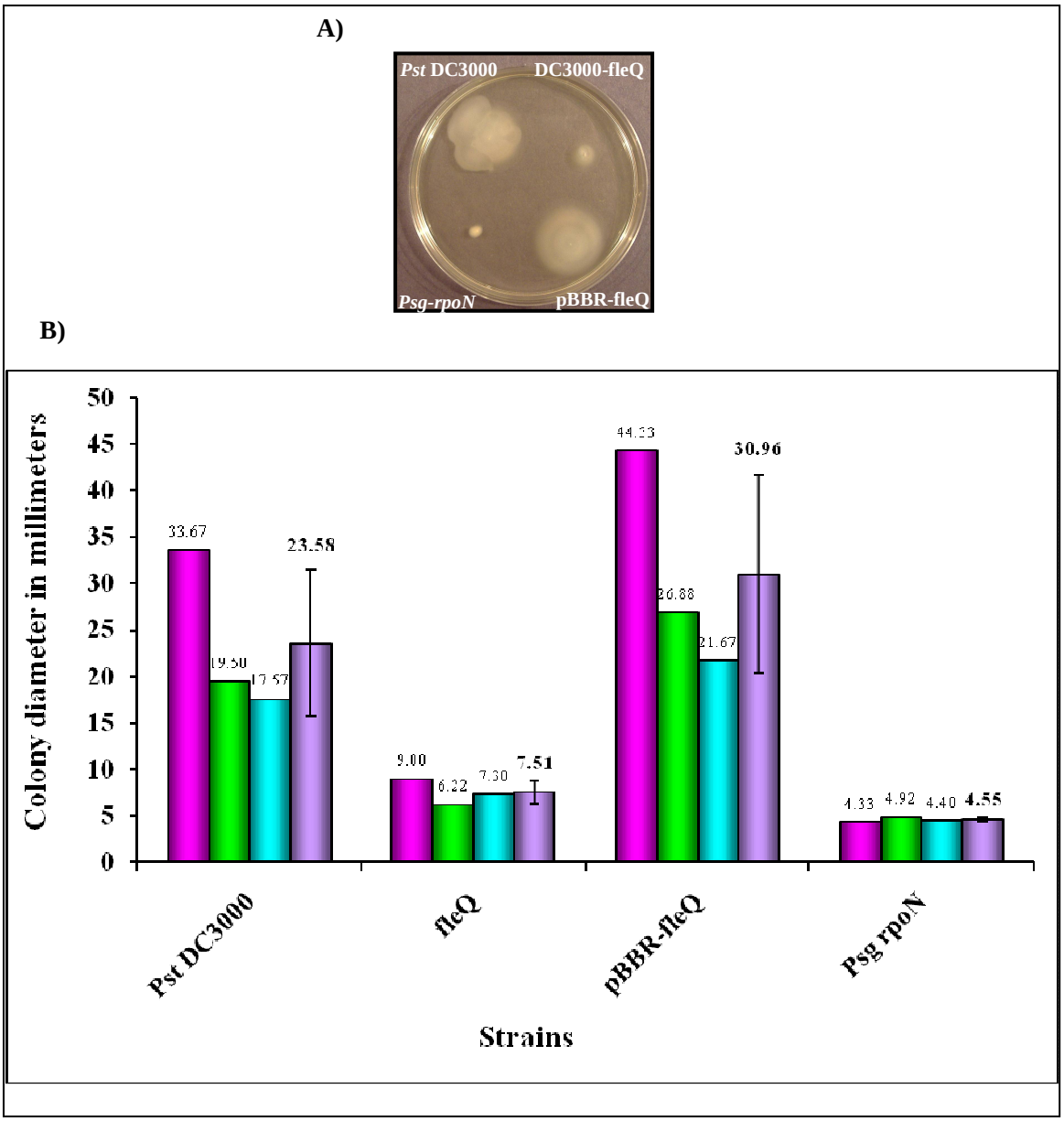


Figure 8. Motility of *Pst* DC3000 and derivatives on KMB medium containing 0.5% agar; bacteria of each strain was inoculated at a single point and incubated for 48 h at 28^{O}C. Colony formation of *Pst* DC3000, DC3000-fleQ, DC3000-fleQ (pBBR-fleQ) and *Psg-rpoN* shown (panel **A**), and colony diameter was measured in millimeters (panel **B**). The graph shows the mean from experiment 1 (pink bars), experiment 2 (green bars), experiment 3 (teal bars), and the average of the three independent experiments (purple bars), with error bars representing the standard deviation. Each experiment contained a minimum of three plates inoculated with each strain at a single point per experiment.

Visualization of flagella

To evaluate whether or not the mutation in *fleQ* impaired this mutant's ability to produce flagella, *Pst* DC3000, DC3000-*fleQ*, DC3000-*fleQ* (pBBR-fleQ) and *Psg-rpoN* were examined by electron microscopy. Polar flagella were visible on *Pst* DC3000 cells in several independent observations, whereas no flagella were visualized on the DC3000-*fleQ* mutant or on *Psg-rpoN* (Fig. 9). Multiple flagella were observed on cells of the DC3000-*fleQ* complemented mutant (Fig. 9D), and these results were reproducible in several independent examinations. These results and the absence of motility (Fig. 8) suggest that the *fleQ* gene is required for the formation of flagella in *Pst* DC3000. Furthermore, both motility and formation of flagella were restored when the *fleQ* gene was present *in trans* in DC3000-*fleQ* (see pBBR-*fleQ* in Figures 8 and 9).

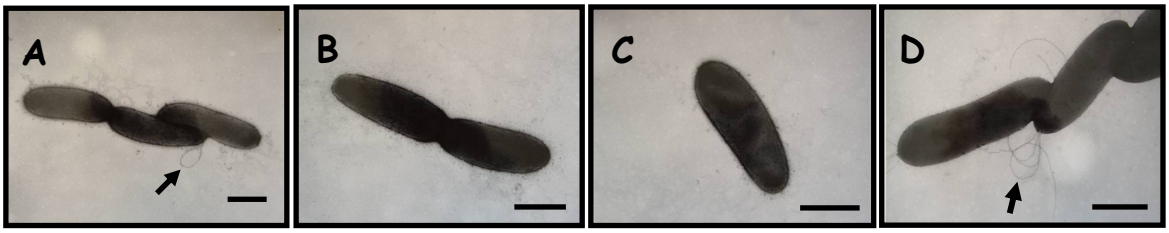


Figure 9. Electron micrographs of *Pst* DC3000 and derivatives. Panels represent the following strains: (A) flagellated wild-type *Pst* DC3000, (B) non-flagellated DC3000-*fleQ*, (C) *Psg-rpoN*, and (D) DC3000-*fleQ* containing pBBR-fleQ. Photographs were taken at taken 14,000 or 10,000 X, and results were reproducible on several separate occasions. Black bar represents 1 µm and arrows (panels A and D) show flagella.

Symptom development

The ability of *Pst* DC3000, and of activator mutants derived from it to cause disease on host plants was evaluated by dip (Arabidopsis) and spray (tomato) inoculation experiments. Symptoms were assessed visually 7-10 days post-inoculation. Tomato

plants inoculated with *Pst* DC3000 and the activator mutants developed necrotic lesions surrounded by chlorotic halos. Tomato plants inoculated with the wild-type *Pst* DC3000 plants inoculated with the activator mutants showed no significant difference with respect to overall symptom production (Fig. 10).

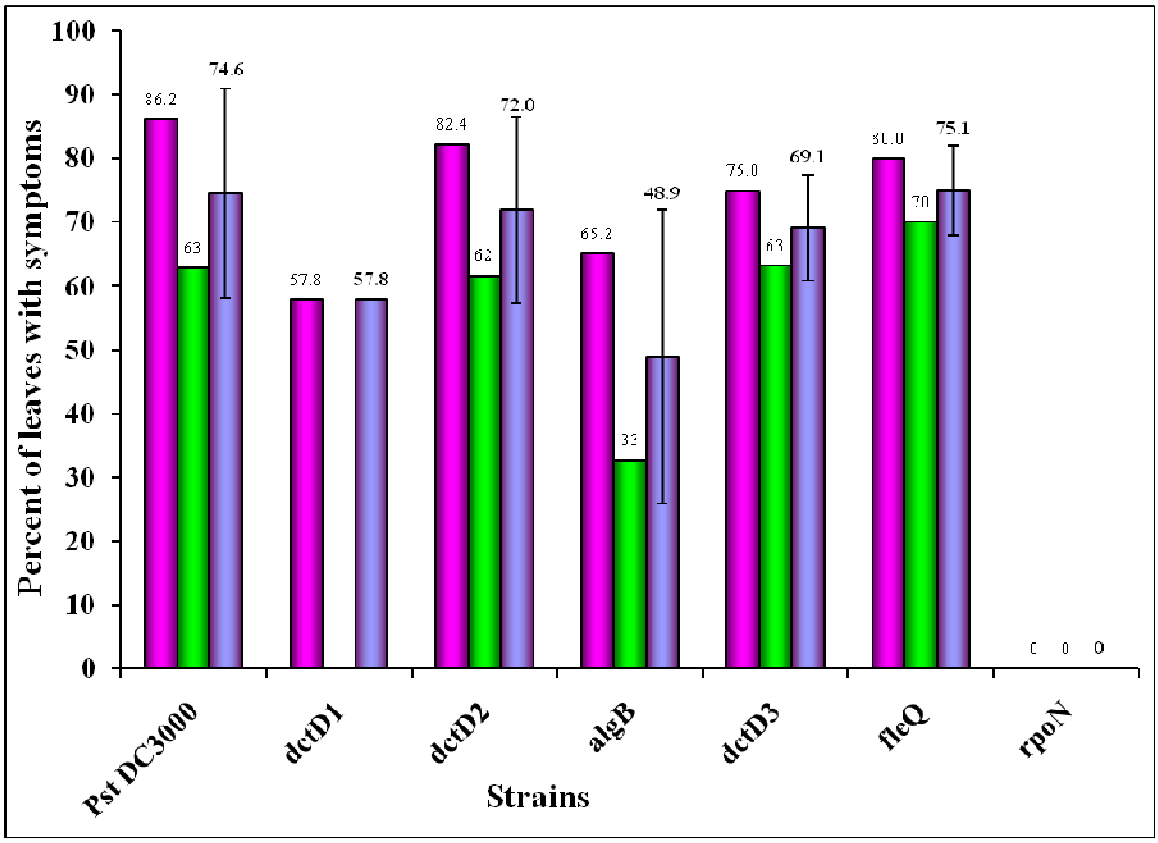


Figure 10: Symptom production by *Pst* DC3000 and activator mutants on tomato. The graph shows results from experiment 1 (pink bars), experiment 2 (green bars), and the average of the two independent experiments (purple bars), with three plants inoculated in each experiment. For *dctD1* the experiment was performed once, and graph shows only the results for experiment 1.

Arabidopsis leaves inoculated with *Pst* DC3000 developed both water-soaked and chlorotic lesions. Arabidopsis plants inoculated with DC3000-*algB* showed a reduction in symptom production (fig 11). These results suggest that mutations in *algB* interfere with the ability of *Pst* DC3000 to cause symptoms on Arabidopsis.

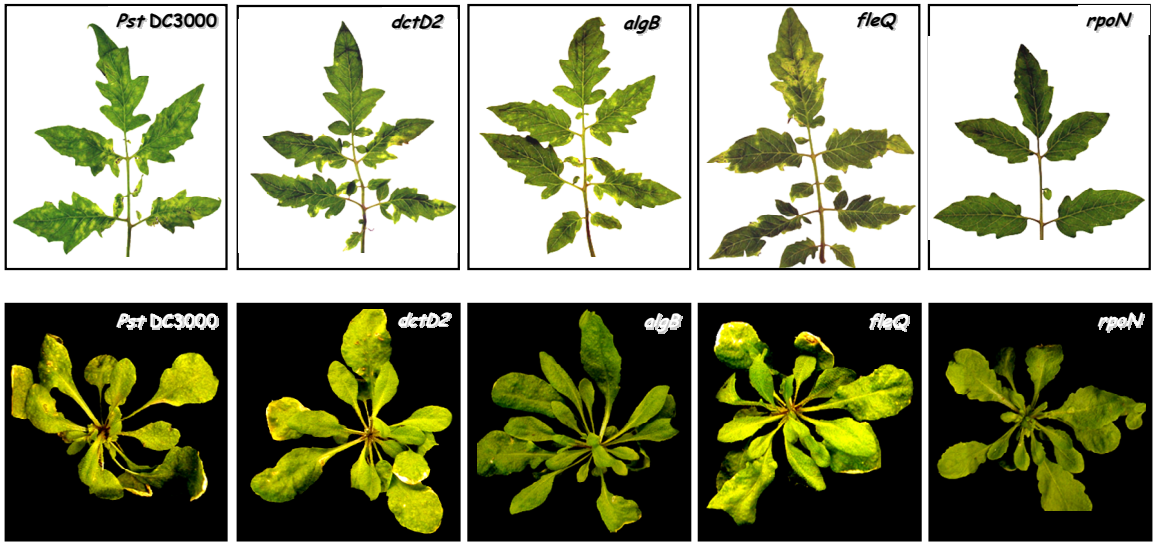


Figure 11. Symptom of *Pst* DC3000 and activator mutants on host tomato and Arabidopsis. Plants were inoculated as described in Methods, and photos were taken 7-10 days post-inoculation.

Bacterial population *in planta*

The populations of *Pst* DC3000, DC3000-*fleQ*, and DC3000-*fleQ* (pBBR-*fleQ*) were monitored in tomato after spray inoculation. The populations of *Pst* DC3000, DC3000-*fleQ* and DC3000-*fleQ* (pBBR-fleQ) increased until day five; after day seven all populations declined (data not shown). The overall population trends of all strains were similar, with no significant differences observed (data not shown). These results and the presence of symptoms in both tomato (Figs. 10, 11) and Arabidopsis (Fig. 11) suggest that the mutation in *fleQ* does not interfere with the ability of the bacterium to multiply and cause symptoms in tomato leaves.

Visualization of the hypersensitive response

The pathogenicity of *Pst* DC3000 is multifactorial, and one of the major factors involved in disease development and symptom production is the T3SS. Mutants defective

in the T3SS show reduced virulence in host plants and are unable to induce the HR in non-host plants. Since *rpoN* mutants of *P. syringae* pv. maculicola (Hendrickson et al., 2000b) and *P. syringae* pv. glycinea (Alarcon-Chaidez *et al.*, 2003) are defective in the HR, the ability of the activator mutants to elicit an HR was evaluated on tobacco plants (Fig. 12). Within 24 h after infiltration, tobacco leaves inoculated with *Pst* DC3000, DC3000-*dctD2*, DC3000-*fleQ* and DC3000-*algB* produced a necrotic region typical of the HR at the site of infiltration (Fig. 12). These results suggest that mutations in these individual activator genes in *Pst* DC3000 do not impair the T3SS. These results in particular also indicate that the reduction in virulence on Arabidopsis leaves dip-inoculated with DC3000-*algB* can be attributed to the mutation in the *algB* gene, and not to defects in the T3SS.

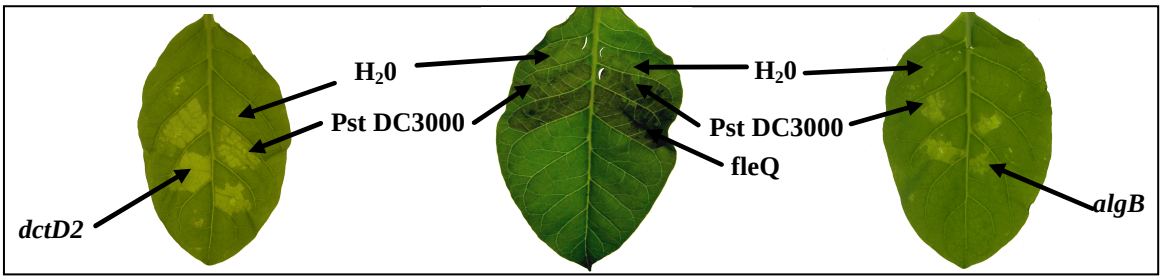


Figure 12. Assay of *Pst* DC3000 and derivatives for the hypersensitive response on tobacco leaves. Bacteria (OD_{600}=0.1) were infiltrated into tobacco leaves with a needle-less syringe, and infiltrations on left panel of each leaf were repeated on right. The photographs were taken 24 h post-infiltration.

Metabolic studies

Several of the activator mutations are in *dctD* genes, and these are presumably involved in dicarboxylic acid transport. To evaluate if the activator mutants were impaired in their ability to grow *in vitro*, *Pst* DC3000 and derivatives were grown in M9 broth supplemented with various carbon, nitrogen and amino acids (Table 12) using a 96-

well microtiter plate assay. Table 12 summarizes the activator mutants that were assayed for defects on 25 carbon, nitrogen, or amino acid sources. Figure 13 shows a more detailed view of the growth defect seen with the DC3000-*dctD2* mutant grown in M9 supplemented with succinic acid, while no significant differences were noted using the other C/N sources. However, it remains possible that differences would be revealed if a more sensitive assay were utilized.

Over a 48 h period, both *Pst* DC3000 and pBBR-*fleQ* grew to a high density (~0.8 OD) in M9 medium supplemented with succinic acid (Fig. 13). Additionally, DC3000-*fleQ* and DC3000-*algB* mutants also grew to a high level in M9 medium supplemented with succinic acid (data not shown). Interestingly, DC3000-*dctD2* only grew to ~0.4 OD, approximately a two-fold reduction in growth when compared to *Pst* DC3000 (Fig. 13). The results indicate that in comparison to *Pst* DC3000, the DC3000-*dctD2* grows more slowly than the wild-type in M9 supplemented with succinic acid. This metabolic defect was restored when the *dctD2* gene was present *in trans* in DC3000-*dctD2* (see growth curve for DC3000-*dctD2* (pBBR-5399) complemented mutant, which is essentially equivalent to that of the wild-type *Pst* DC3000).

Table 12. Nutrient sources used to supplement M9 broth medium. The activator mutants that were tested on each source are listed in the columns below each category.

Carbon Sources	Nitrogen Sources	Amino Acid Sources
Succinate	L-glutamine	Alanine
Malate	Histidine	Leucine
Aspartate	Methionine	Glutamine
Tartaric acid	Urea	Threonine
Malic acid	KNO_3	Phenylalanine
Oxaloacetic acid	Proline	Isoleucine
	Glutamate	Arginine
	L-alanine	Lysine
	Aspartic acid	Valine

Mutants Tested		
DC3000-*dctD1*	DC3000-*dctD1*	DC3000-*dctD1*
DC3000-*dctD2*	DC3000-*dctD2*	DC3000-*dctD3*
DC3000-*dctD3*	DC3000-*dctD3*	
	DC3000-*ssuD*	
	DC3000-A10	
	DC3000-A11	

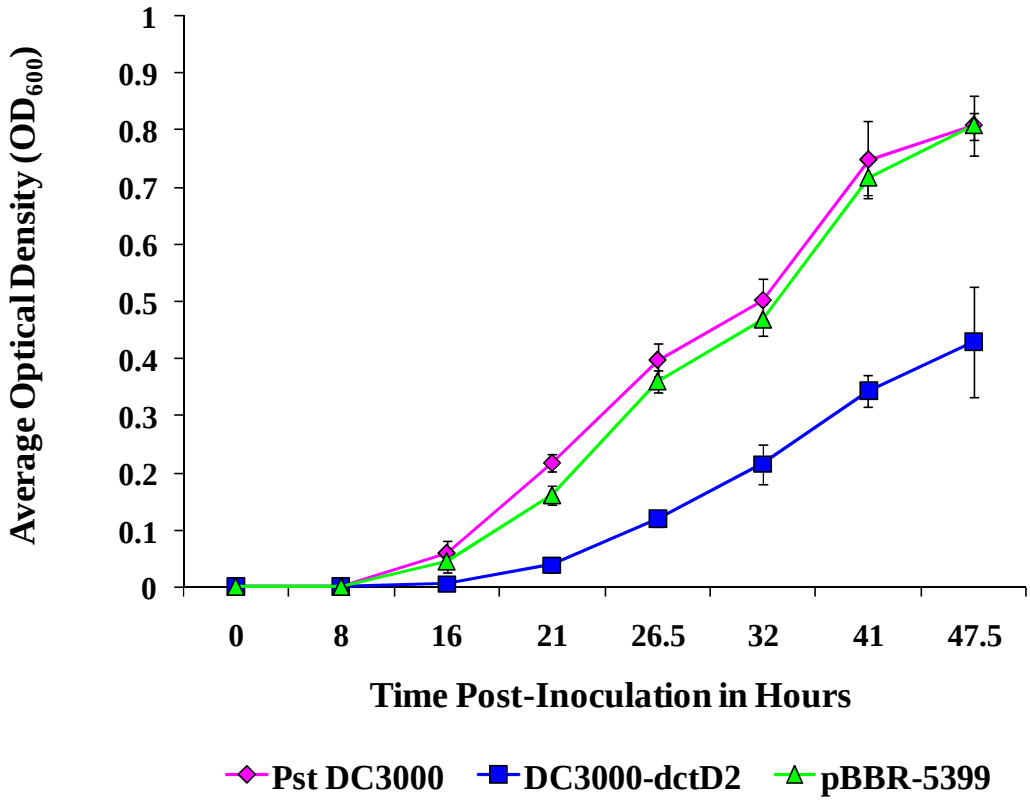


Figure 13: Growth (OD_{600}) of *Pst* DC3000, DC3000-*dctD2*, and DC3000-*dctD2* (pBBR-5399) in M9 media supplemented with succinic acid during a 48 h period. The experiment was repeated three times, and the graph shown is representative of the results obtained.

Coronatine production *in vitro*

Another major factor governing the ability of *P. syringae* to cause disease is the phytotoxin coronatine. In *Pst* DC3000, COR functions as a virulence factor in tomato, Arabidopsis and *Brassica* spp. (Brooks et al., 2004, Elizabeth & Bender, 2007, Penaloza-

Vazquez et al., 2000, Uppalapati *et al.*, 2008). Since *rpoN* mutants of *P. syringae* pv. glycinea and *P. syringae* pv. maculicola do not produce COR *in vitro* (Alarcon-Chaidez *et al.*, 2003, Hendrickson *et al.*, 2000b), mutations in various activator genes were evaluated for their effect on COR production. The wild-type *Pst* DC3000 produced approximately 2 µg of COR/mg of protein, whereas the COR-defective mutant DB4G3 (Brooks et al., 2004), produced no detectable levels of COR. DC3000-*algB* and DC3000-*fleQ* produced approximately 50% less COR than the wild-type, although variability was high for DC3000-*fleQ*. DC3000-*dctD2* produced approximately four-fold less COR than *Pst* DC3000. As noted above, DC3000-*dctD2* is compromised in its ability to either utilize or transport succinic acid. This has potential implications for COR production, since succinic semialdehyde has been proposed to function as a starting unit for synthesis of coronafacic acid, the polyketide component of COR (Parry et al., 1996).

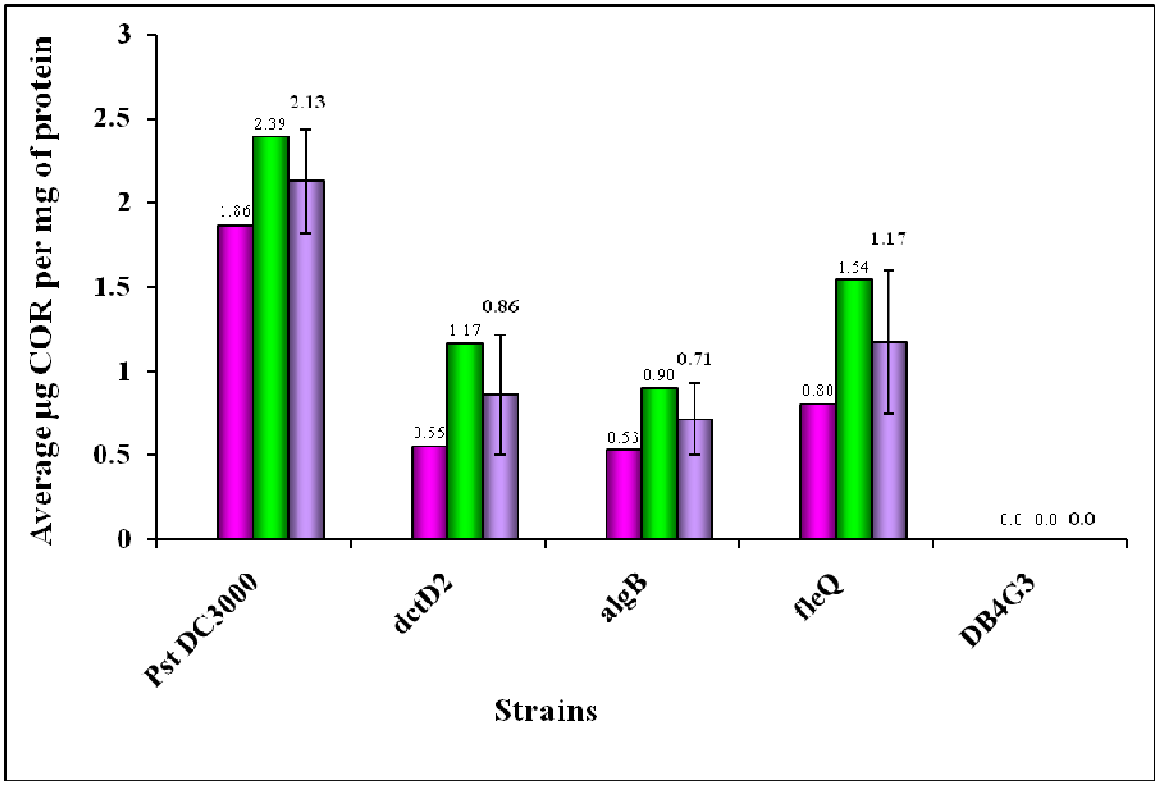


Figure 14: COR production by *Pst* DC3000 and activator mutants in HSS media. The graph shows results from experiment 1 (pink bars), experiment 2 (green bars), and the average of the two independent experiments (purple bars), with error bars representing the standard deviation. Each experiment consisted of four replicate culture tubes that were handled separately to quantify COR.

Production of biofilms *in vitro*

Pst DC3000 is known to produce EPS including alginate, and EPS molecules contribute to adhesion and biofilm formation. Since several of the activator genes are potentially involved in these traits, the activator mutants were assayed for their potential to form biofilms *in vitro* using a 96-well microtiter plate assay (Fig. 15). The relative biofilm formation of *Pst* DC3000, DC3000-*dctD2* and DC3000-*algB* was similar, whereas an over three-fold decrease in relative biofilm formation was seen with DC3000-*fleQ* (Fig. 15). This defect was restored by complementation of DC3000-*fleQ* with pBBR-*fleQ* (Fig. 15). These results indicate *fleQ* is involved in the initial formation of biofilms in *Pst* DC3000 *in vitro*.

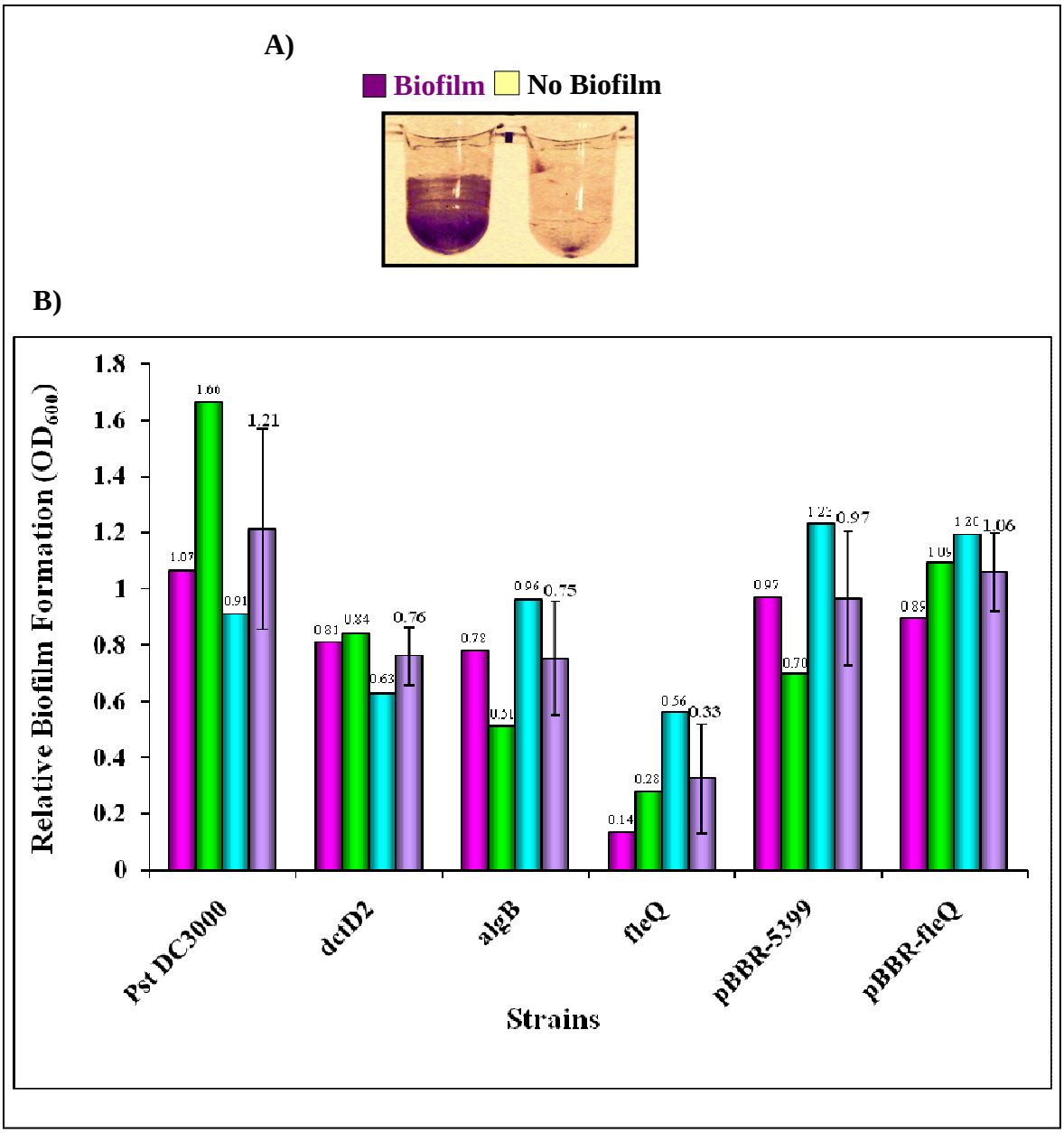


Figure 16: Biofilm formation phenotype of *Pst* DC3000 and activator mutants. Bacterial attachment was assessed on the surface of polyvinylchloride microtiter plates (a) after 24 h of growth. The data (b) shows results from experiment 1 (pink bars), experiment 2 (green bars), experiment 3 (teal bars), and the average of the three independent experiments (purple bars), with error bars representing the standard deviation.

Production of alginate *in vitro*

Several genes that regulate alginate production are known to negatively regulate flagellar expression (Tart *et al.*, 2006, Tart *et al.*, 2005). For example, the alginate regulatory gene *algR* is negatively regulated by FleQ (Giddens et al., 2007). To evaluate if the *fleQ* mutation interferes with alginate production, *Pst* DC3000, *Pst* DC3000-*fleQ*, and *Pst* DC3000-*fleQ* (pBBR-*fleQ*) were evaluated for alginate production in BKG medium. The wild-type *Pst* DC3000 produced approximately 25 µg of alginate per mg of protein, while DC3000-*fleQ* produced slightly higher levels of alginate (35 µg/mg protein) (Fig. 16). Alginate production was slightly decreased in the complemented *Pst* DC3000-*fleQ* (pBBR-*fleQ*) strain (approximately 19 µg of alginate), (Fig. 16). In summary, the results provide preliminary evidence that FleQ may have a negative regulatory effect on alginate production in *P. syringae*, as alginate production was slightly increased in the *fleQ* mutant and reduced in the complemented strain.

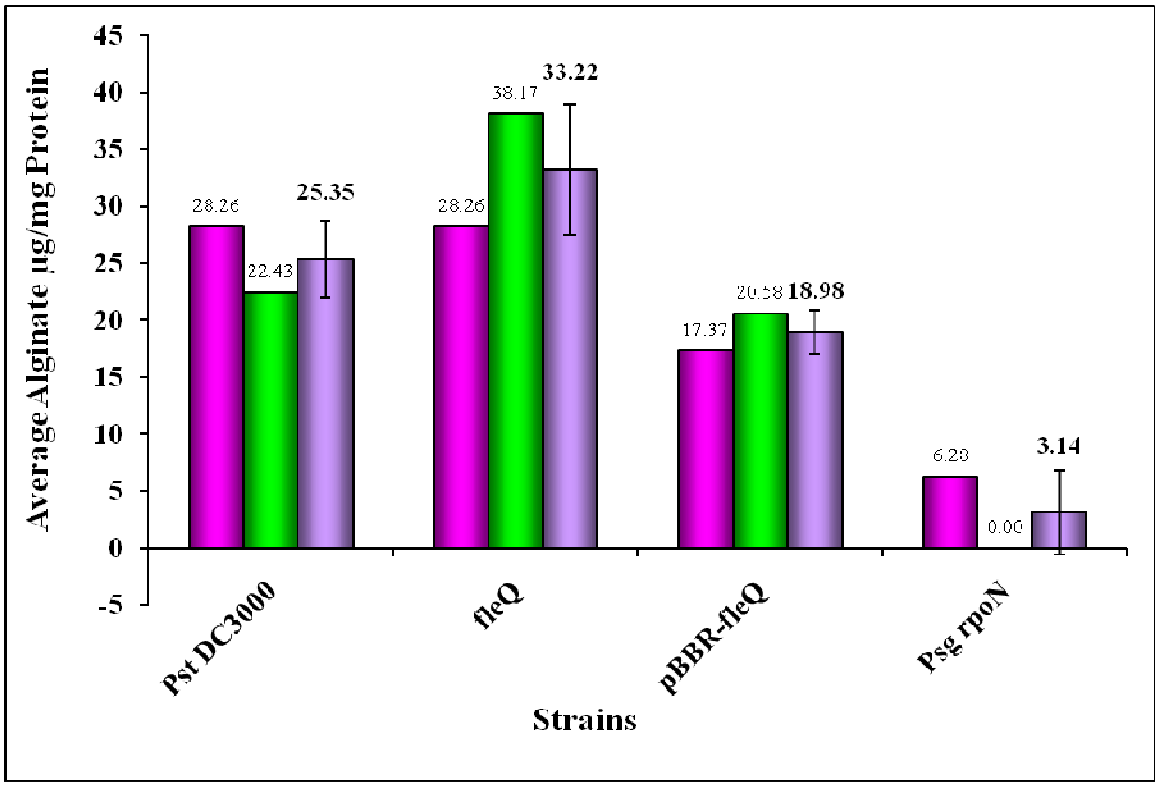


Figure 16: Alginate production by *Pst* DC3000, *Pst* DC3000-*fleQ*, pBBR-fleQ and *Psg-rpoN* on BKG medium. The graph shows results from experiment 1 (pink bars), experiment 2 (green bars), and the average of the two independent experiments (purple bars), with error bars representing the standard deviation. Each experiment consisted of six plates that were handled separately to quantify alginate as described in Methods.

DISCUSSION

Previous research suggested that σ^{54} (and hence σ^{54}-dependent activator proteins) are involved in regulating many factors in *P. syringae* (Alarcon-Chaidez et al., 2003, Hendrickson *et al.*, 2000a, Hendrickson et al., 2000b). However, the mutagenesis and characterization of multiple σ^{54}-dependent activators in *P. syringae* has not been conducted previously. In the current study, mutations in eight σ^{54}-dependent activator genes in *Pst* DC3000 were characterized for pathogenicity on tomato and Arabidopsis,

production of the HR on tobacco, and characterized for traits associated with each activator.

FleQ is a σ^{54}-dependent activator that regulates motility and flagella biogenesis (Arora et al., 1997, Dasgupta *et al.*, 2003). In the present study, an agar assay was used to demonstrate that swarming motility was abolished in the DC3000-*fleQ* mutant (Fig. 8), a result which is consistent with previous reports of *fleQ* mutants of both *P. fluorescens* (Capdevila et al., 2004) and *P. aeruginosa* ((Arora et al., 1997). After confirming that the DC3000-*fleQ* mutant was non-motile, electron microscopy confirmed the absence of flagella in the DC3000-*fleQ* mutant. Furthermore, complementation assays confirmed that *fleQ* is required for motility and formation of flagella.

The ability of σ^{54}-dependent activator mutants to elicit symptoms in tomato and Arabidopsis was visually assessed 7-10 days post-inoculation. Tomatoes inoculated with activator mutants showed no significant differences with respect to disease symptoms when compared to *Pst* DC3000 (Fig. 10; Fig 11). On Arabidopsis, a reduction in symptom production was seen with DC3000-*algB* (fig.11) (Baker & Bender, 2006). Further experiments are needed to determine whether the reduced disease symptoms on Arabidopsis are correlated with reduced bacterial multiplication. The DC3000-*algB* mutant was reduced in symptom development on host Arabidopsis, but not tomato, which is significant because this is the first report of the phenotype of an alginate regulatory gene in *Pst* DC3000 *in planta*. The results obtained in this study corroborate a previous report demonstrating that alginate is important for the virulence of *Pst* DC3000 on *Brassica* spp. such as Arabidopsis (Keith et al., 2003). It is also important to note that the DC3000-*algB* mutant was not impaired in the ability to elicit an HR in tobacco leaves

(Fig. 12). Thus the reduced symptoms on Arabidopsis inoculated with the DC3000-*algB* mutant was not due to a nonfunctional T3SS.

The evaluation of symptoms (Figs. 10, 11) and population dynamics suggest that *fleQ* does not impact the ability of *Pst* DC3000 to multiply and cause symptoms. While mutants defective in motility have been reported for other *P. syringae* pathovars (Hattermann & Ries, 1989, Kinscherf & Willis, 1999), the contribution of flagella to virulence in *Pst* DC3000 has not been previously investigated. Although motility may be important for invasion of host tissues in some pathosystems, the loss of flagella may be advantageous since flagella and flagellin function as a pathogen or microbe-associate molecular patterns (PAMP or MAMP). In both plant and animal systems, PAMPs and MAMPs are known to elicit defense responses, thus triggering innate immunity mechanisms (He *et al.*, 2007, Li *et al.*, 2005, Nurnberger *et al.*, 2004, Shimizu *et al.*, 2003, Zipfel *et al.*, 2004). Thus the loss of flagella may actually help the pathogen avoid the host recognition systems associated with innate immunity.

P. syringae is also known to form microbial aggregate communities known as biofilms under certain environmental conditions (Dulla & Lindow, 2008, Von Bodman *et al.*, 2003). Flagella and the exopolysaccharide alginate are important for biofilm formation in *Pseudomonas* spp. (Boyd & Chakrabarty, 1995, Chang *et al.*, 2007b, Daniels *et al.*, 2004, Laue *et al.*, 2006, O'Toole & Kolter, 1998a). In the current study, DC3000, DC3000-*dctD2* and DC3000-*algB* adhered in 96-well microtiter plates at similar intensities (Fig. 15), a bioassay which indicates their potential to form biofilms. However, the DC3000-*fleQ* mutant was severely impaired in biofilm formation as compared to DC3000, and this defect was restored by complementation (Fig. 15). These

results indicate that *fleQ* is involved in initial biofilm formation in *Pst* DC3000, suggesting a role for flagella in the initial cell-to-surface interactions, which was previously reported for a non-motile strain of *P. aeruginosa* (O'Toole & Kolter, 1998a).

Previous reports indicate an inverse relationship between flagella and alginate production in *Pseudomonas*. In *P. aeruginosa*, the alginate regulatory protein AlgT represses flagellum biosynthesis indirectly by promoting expression of AmrZ. AmrZ then interacts directly with the *fleQ* promoter, repressing flagellar synthesis while inducing transcription of the alginate gene cluster (Tart *et al.*, 2006, Tart *et al.*, 2005). Furthermore, *algR*, which has a positive regulatory effect on the transcription of the alginate biosynthetic gene *algD*, is negatively regulated by FleQ (Giddens et al., 2007). In the current study, a subtle effect of the *fleQ* mutation on alginate production was detected *in vitro*. Alginate production for DC3000-*fleQ* was slightly higher than in *Pst* DC3000, and somewhat reduced in the pBBR-*fleQ* complemented strain. In summary, these results suggest that FleQ in *P. syringae* may have a negative regulatory effect on alginate production as in *P. aeruginosa*.

During pathogenesis *P. syringae* gains entry into the plant apoplast, an environment where nutrients (sugars and amino acids) are limiting (Hancock & Huisman, 1981), making the survival and pathogenicity of *P. syringae* dependent upon its ability to import and metabolize the available nutrients. In support of this, there are three *dctD* genes (*dctD1*, *dctD2*, and *dctD3*) annotated in the *Pst* DC3000 genome that are presumably involved in the uptake and/or metabolism of C4-dicarboxylic acids. In the present study, *Pst* DC3000 and the three *dctD* mutants were evaluated for growth in M9 broth supplemented with various carbon, nitrogen and amino acid sources (Table 12). No

significant metabolic defects were observed for DC3000-*dctD1* or DC3000-*dctD3*. However, the DC3000-*dctD2* was impaired in growth in M9 supplemented with succinic acid. These results suggest a role for *dctD2* in either the transport and/or catabolism of succinic acid, which is one of the major organic acids found in tomato (Morris *et al.*, 1995). *dctD* is known to activate transcription of *dctA* (Reid & Poole, 1998, Ronson *et al.*, 1987, Wang *et al.*, 1989, Yurgel & Kahn, 2004). Curiously, there are two *dctA* genes (designated *dctA1* and *dctA2*) annotated in the *Pst* DC3000 genome, as well as three *dctD* genes (*dctD1*, *dctD2*, and *dctD3*; Table 8). This literature, as well as the findings in the current study, indicate an undefined role for the *dctD* gene system in nutrient assimilation in the plant pathogen *Pst* DC3000.

Many pathovars of *P. syrinage* produce the phytotoxin coronatine (COR) which functions as a virulence factor in many *P. syringae*-plant interactions (Bender *et al.*, 1987, Brooks *et al.*, 2004, Elizabeth & Bender, 2007, Mitchell, 1982, Penaloza-Vazquez *et al.*, 2000, Wiebe & Campbell, 1993). *rpoN* mutants of *P. syringae* pv. glycinea and *P. syringae* pv. maculicola do not produce coronatine *in vitro* (Alarcon-Chaidez *et al.*, 2003, Hendrickson *et al.*, 2000b). The possibility that mutations in the individual activator genes would impair COR production was evaluated *in vitro* using HSS liquid medium and ethyl acetate extraction followed by HPLC analysis. DC3000-*dctD2* produced four-fold less COR than *Pst* DC3000 (Fig 14), however it is reduced in its ability to transport or utilize succinic acid, which has been proposed to function in COR biosynthesis (Parry et al., 1996).

In order to prepare this work for publication, all of these mutants need to be complemented and evaluated for bacterial multiplication *in planta.* Furthermore, the *dctD*

and *ssuD* mutants need to be analyzed for nutritional defects using more sensitive assay methods. These additional experimental results, along with those described above, will help evaluate the role of σ^{54}-dependent activators in the invasion and multiplication of *Pst* DC3000 in host tissues.

CHAPTER VI

SUMMARY

P. syringae pv. tomato DC3000 (*Pst* DC3000) is the causal agent of bacterial speck disease, an economically important disease of tomato. *Pst* DC3000 is related to several bacterial species that are on the USDA APHIS select agent list; however, unlike the select agents, *P. syringae* is an endemic pathogen. The ability to distinguish among bacterial strains is important for epidemiological tracing of pathogen outbreaks, detecting the source of an outbreak and monitoring the spread of pathogens. Variable number tandem repeat (VNTR) loci, which are found throughout bacterial genomes, are useful for rapid and reliable strain typing by multiple-locus VNTR analysis (MLVA). In this work a MLVA typing scheme was developed for analyzing diverse strains of *P. syringae* pv. tomato. Oligonucleotide primers were designed to amplify 34 VNTR loci, the primers were screened and a subset of five primer pairs yielding polymorphic products was identified for use in strain typing. Fifty eight *P. syringae* pv. tomato strains were analyzed using the five primer sets, and 23 different MLVA sequence types were identified. Eleven *P. syringae* pv. tomato strains produced a unique MLVA sequence type, while the remaining 47 strains were assigned to one of twelve MLVA sequence types. The stability and reproducibility of the typing scheme was investigated by

culturing the *Pst* strains *in vitro* for 20 generations and re-typing, which showed that the sequence types defined by the primer sets were reproducible. Two *P. syringae* pv. tomato strains with unique MLVA sequence types were originally isolated over 60 years ago in the early 1940's. Three *P. syringae* pv. tomato strains with unique MLVA sequence types were originally isolated from host plants other than tomato, supporting the hypothesis that genetic diversity occurs in relation to the host plant from which bacteria are isolated. Additionally, strains that originated from different geographic locations clustered together in the same MLVA sequence group; thus no obvious relationship was observed between MLVA sequence type and the geographic origin of strains. This is the first report using a MLVA typing scheme to study the relatedness of a large collection of *P. syringae* pv. tomato strains of diverse origin.

Pst DC3000 has been sequenced (Buell *et al.*, 2003) and is a model organism for studying bacterial pathogenesis and plant-microbe interactions (Preston, 2000). The alternative sigma factor σ^{54} (encoded by *rpoN*) interacts with RNA polymerase and associated activator proteins to regulate the transcription of bacterial target genes involved in metabolic and physiological processes. In *P. syringae* σ^{54} is involved in regulating many survival, pathogenicity and virulence factors. *rpoN* mutants of *P. syringae* pv. maculicola ES4326 (Hendrickson *et al.*, 2000a, Hendrickson *et al.*, 2000b) and *P. syringae* pv. glycinea PG4180 (Alarcon-Chaidez *et al.*, 2003) were deficient in the ability to utilize various carbon, nitrogen and amino acid sources, and were unable to induce disease symptoms and multiply in host plants. While the genome sequence of *Pst* DC3000 contains over 70 predicted recognition sites for σ^{54} (http://www.promscan.uklinux.net/), virtually nothing is known about the regulatory loci

controlled by σ^{54}. There are 14 σ^{54}-dependent activators annotated in the *Pst* DC3000 genome (http://www.tigr.org/), suggesting that these activators play a critical role in gene regulation in this organism.

In the present study, *P. syringae* pv. tomato mutants defective in several σ^{54}-dependent activators were characterized for pathogenicity on tomato and Arabidopsis, production of the HR on tobacco, and analyzed for traits associated with each activator. A DC3000-*fleQ* mutant was defective in motility, formation of flagella, and biofilm formation, and these traits were genetically complemented by the introduction of *fleQ in trans*. Although motility may be important for invasion of host tissues in some pathosystems, the symptoms and population dynamics of DC3000-*fleQ* suggest that *fleQ* does not impact the ability of *Pst* DC3000 to multiply and cause symptoms. Tomatoes inoculated with the activator mutants showed no significant differences with respect to disease symptoms when compared to *Pst* DC3000; however, on Arabidopsis, a reduction in symptoms was seen with DC3000-*algB*. These results indicate that traits such as alginate production is important for the pathogenicity of *Pst* DC3000. None of the activator mutants were impaired in their ability to elicit an HR in tobacco leaves (Fig. 12). Thus the reduced symptoms seen on Arabidopsis were not due to a nonfunctional T3SS.

Previous reports indicate that there is an inverse relationship between flagella and alginate production in *Pseudomonas*. In the current study, alginate production for DC3000-*fleQ* was slightly higher than in *Pst* DC3000, and slightly reduced in the pBBR-*fleQ* complemented strain. These results suggest that FleQ in *P. syringae* may have a negative regulatory effect on alginate production as in *P. aeruginosa*.

During pathogenesis, *P. syringae* gains entry into the plant apoplast, an environment where nutrients (sugars and amino acids) are limiting (Hancock & Huisman, 1981). Thus, the survival and pathogenicity of *P. syringae* is dependent on its ability to import and metabolize available nutrients. In the current study, a DC3000-*dctD2* mutant was impaired in the ability to transport or catabolize succinic acid, one of the major organic acids found in tomato (Morris *et al.*, 1995). Further characterization of these activators will yield insights into the regulatory networks in *P. syringae*, and will provide new information regarding traits important for the survival and pathogenesis of gram-negative bacteria.

CHAPTER VII

BIBLIOGRAPHY

Al Dahouk, S., Fleche, P. L., Nockler, K., Jacques, I., Grayon, M., Scholz, H. C., Tomaso, H., Vergnaud, G. and Neubauer, H. (2007) Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Methods* **69**: 137-145.

Alarcon-Chaidez, F. J., Keith, L., Zhao, Y. and Bender, C. L. (2003) RpoN (sigma54) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid* **49**: 106-117.

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

Alfano, J. R. and Collmer, A. (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu Rev Phytopathol* **42**: 385-414.

Allen, C. and Bender, C. Unpublished data.

Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. and Ramphal, R. (1997) A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. *J Bacteriol* **179**: 5574-5581.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L., Gomez-Gomez, L., Boller, T., Ausubel, F. M. and Sheen, J. (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415**: 977-983.

Ausubel, F. M. (1997) *Short protocols in molecular biology : a compendium of methods* Current protocols in molecular biology F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl (eds). New York: John Wiley & Sons, Inc. , pp. xxii, 387.

Baker, C. M. and Bender, C. L. (2006) The role of sigma 54-dependent transcriptional activators FleQ, DctD2, and AlgB in *Pseudomonas syringae* pv. tomato DC3000. *Phytopathology* **96**: S8-S8.

Barrios, H., Valderrama, B. and Morett, E. (1999) Compilation and analysis of sigma54-dependent promoter sequences. *Nucleic Acids Res* **27**: 4305-4313.

Bashan, Y. (1986) Field dispersal of *Pseudomonas syringae* pv. tomato, *Xanthomonas campestris* pv. vesicatoria, and *Alternaria macrospora* by animals, people, birds, insects, mites, agricultural tools, aircraft, soil particles, and water sources. *Can J Bot* **64**: 276-281.

Bashan, Y., Okon, Y. and Henis, Y. (1978) Infection studies of *Pseudomonas tomato*, causal agent of bacterial speck of tomato. *Phytoparasitica* **6**: 135-143.

Bashan, Y., Okon, Y. and Henis, Y. (1982) Long-term survival of *Pseudomonas syringae* pv. tomato and *Xanthomonas campestris* pv. vesicatoria in tomato and pepper seeds. *Phytopathology* **72**: 1143-1144.

Bashan, Y., Sharon, E., Okon, Y. and Henis, Y. (1981) Scanning electron and light-microscopy of infection and symptom development in tomato leaves infected with *Pseudomonas tomato*. *Physiol Plant Pathol* **19**: 139-144.

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

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

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

Bender, C. L., Liyanage, H., Palmer, D., Ullrich, M., Young, S. and Mitchell, R. (1993) Characterization of the genes controlling the biosynthesis of the polyketide phytotoxin coronatine including conjugation between coronafacic and coronamic acid. *Gene* **133**: 31-38.

Bender, C. L. and Scholz-Schroeder, B. K. (2004) New insights into the biosynthesis, mode of action, and regulation of syringomycin, syringopeptin and coronatine. In: *The Pseudomonads.* J. L. Ramos. (eds). Kluwer Academic Press, Dordrecht, pp. 125-158.

Bender, C. L., Stone, H. E., Sims, J. J. and Cooksey, D. A. (1987) Reduced pathogen fitness of *Pseudomonas syringae* pv. tomato Tn*5* mutants defective in coronatine production. *Physiol Mol Plant Pathol* **30**: 273-283.

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

Bereswill, S., Bugert, P., Volksch, B., Ullrich, M., Bender, C. L. and Geider, K. (1994) Identification and relatedness of coronatine-producing *Pseudomonas syringae*

pathovars by PCR analysis and sequence determination of the amplification products. *Appl Environ Microbiol* **60**: 2924-2930.

Boch, J., Joardar, V., Gao, L., Robertson, T. L., Lim, M. and Kunkel, B. N. (2002) Identification of *Pseudomonas syringae* pv. tomato genes induced during infection of *Arabidopsis thaliana*. *Mol Microbiol* **44**: 73-88.

Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., Panopoulos, N. J. and Van Gijsegem, F. (1996) Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol Microbiol* **20**: 681-683.

Bonn, W. G., Gitaitis, R. D. and Macneill, B. H. (1985) Epiphytic survival of *Pseudomonas syringae* pv. tomato on tomato transplants shipped from Georgia. *Plant Dis* **69**: 58-60.

Boyd, A. and Chakrabarty, A. M. (1995) *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. *J Ind Microbiol* **15**: 162-168.

Braun, Y., Smirnova, A. V., Schenk, A., Weingart, H., Burau, C., Muskhelishvili, G. and Ullrich, M. S. (2008) Component and protein domain exchange analysis of a thermoresponsive, two-component regulatory system of *Pseudomonas syringae*. *Microbiology* **154**: 2700-2708.

Bretz, J., Losada, L., Lisboa, K. and Hutcheson, S. W. (2002) Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*. *Mol Microbiol* **45**: 397-409.

Brooks, D. M., Hernandez-Guzman, G., Kloek, A. P., Alarcon-Chaidez, F., Sreedharan, A., Rangaswamy, V., Penaloza-Vazquez, A., Bender, C. L. and Kunkel, B. N. (2004) Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant-Microbe Interact* **17**: 162-174.

Bruegger, B. B. and Keen, N. T. (1979) Specific elicitors of glyceollin accumulation in the *Pseudomonas glycinea*-soybean host-parasite system. *Physiol Plant Pathol* **15**: 43-51.

Bryan, M. K. (1933) Bacterial speck of tomatoes. *Phytopathology* **23**: 897-904.

Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M. J., Haft, D. H., Nelson, W. C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S. E., Feldblyum, T. V., D'Ascenzo, M., Deng, W. L., Ramos, A. R., Alfano, J. R., Cartinhour, S., Chatterjee, A. K., Delaney, T. P., Lazarowitz, S. G., Martin, G. B., Schneider, D. J., Tang, X., Bender, C. L., White, O., Fraser, C. M. and Collmer, A. (2003) The

complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc Natl Acad Sci U S A* **100**: 10181-10186.

Buttner, D. and Bonas, U. (2006) Who comes first? How plant pathogenic bacteria orchestrate type III secretion. *Curr Opin Microbiol* **9**: 193-200.

Capdevila, S., Martinez-Granero, F. M., Sanchez-Contreras, M., Rivilla, R. and Martin, M. (2004) Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiology* **150**: 3889-3897.

Carmona, M., Claverie-Martin, F. and Magasanik, B. (1997) DNA bending and the initiation of transcription at sigma54-dependent bacterial promoters. *Proc Natl Acad Sci U S A* **94**: 9568-9572.

Casaz, P., Happel, A., Keithan, J., Read, D. L., Strain, S. R. and Levy, S. B. (2001) The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. *Microbiology* **147**: 355-361.

Cases, I. and de Lorenzo, V. (2001) The limits to genomic predictions: role of sigmaN in environmental stress survival of *Pseudomonas putida*. *FEMS Microbiol Ecol* **35**: 217-221.

Cases, I., Ussery, D. W. and de Lorenzo, V. (2003) The sigma54 regulon (sigmulon) of *Pseudomonas putida*. *Environ Microbiol* **5**: 1281-1293.

Cesbron, S., Paulin, J. P., Tharaud, M., Barny, M. A. and Brisset, M. N. (2006) The alternative sigma factor HrpL negatively modulates the flagellar system in the phytopathogenic bacterium *Erwinia amylovora* under *hrp*-inducing conditions. *FEMS Microbiol Lett* **257**: 221-227.

Chambers, S. C. and Merriman, P. R. (1975) Perennation and control of *Pseudomonas tomato* in Victoria. *Aust J Agr Res* **26**: 657-663.

Chang, C. H., Chang, Y. C., Underwood, A., Chiou, C. S. and Kao, C. Y. (2007a) VNTRDB: a bacterial variable number tandem repeat locus database. *Nucleic Acids Res* **35**: D416-D421.

Chang, W. S., van de Mortel, M., Nielsen, L., Nino de Guzman, G., Li, X. and Halverson, L. J. (2007b) Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. *J Bacteriol* **189**: 8290-8299.

Charity, J. C., Pak, K., Delwiche, C. F. and Hutcheson, S. W. (2003) Novel exchangeable effector loci associated with the *Pseudomonas syringae hrp* pathogenicity island: evidence for integron-like assembly from transposed gene cassettes. *Mol Plant-Microbe Interact* **16**: 495-507.

Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J. R. and Chatterjee, A. K. (2003) GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant-Microbe Interact* **16**: 1106-1117.

Chen, L. L. (2006) Identification of genomic islands in six plant pathogens. *Gene* **374**: 134-141.

Chen, W. P. and Kuo, T. T. (1993) A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res* **21**: 2260.

Chitnis, C. E. and Ohman, D. E. (1993) Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol Microbiol* **8**: 583-593.

Clerc, A., Manceau, C. and Nesme, X. (1998) Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Appl Environ Microbiol* **64**: 1180-1187.

Coenye, T. and Vandamme, P. (2003) Simple sequence repeats and compositional bias in the bipartite *Ralstonia solanacearum* GMI1000 genome. *BMC Genomics* **4**: 10.

Colin, J. E. and Chafik, Z. (1986) Comparison of biological and chemical treatments for control of bacterial speck of tomato under field conditions in Morocco. *Plant Dis* **70**: 1048-1050.

Cooksey, D. A. (1988) Reduction of infection by *Pseudomonas syringae* pv. tomato using a nonpathogenic, copper-resistant strain combined with a copper bactericide. *Phytopathology* **78**: 601-603.

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

Cuppels, D. A. (1986) Generation and characterization of Tn*5* insertion mutations in *Pseudomonas syringae* pv. tomato. *Appl Environ Microbiol* **51**: 323-327.

Cuppels, D. A. and Ainsworth, T. (1995) Molecular and physiological characterization of *Pseudomonas syringae* pv. tomato and *Pseudomonas syringae* pv. maculicola strains that produce the phytotoxin coronatine. *Appl Environ Microbiol* **61**: 3530-3536.

Cuppels, D. A. and Elmhirst, J. (1999) Disease development and changes in the natural *Pseudomonas syringae* pv. tomato populations on field tomato plants. *Plant Dis* **83**: 759-764.

Cuppels, D. A., Louws, F. J. and Ainsworth, T. (2006) Development and evaluation of PCR-based diagnostic assays for the bacterial speck and bacterial spot pathogens of tomato. *Plant Dis* **90**: 451-458.

Cuppels, D. A., Moore, R. A. and Morris, V. L. (1990) Construction and use of a nonradioactive DNA hybridization probe for detection of *Pseudomonas syringae* pv. tomato on tomato plants. *Appl Environ Microbiol* **56**: 1743-1749.

Daniels, R., Vanderleyden, J. and Michiels, J. (2004) Quorum sensing and swarming migration in bacteria. *FEMS Microbiol Rev* **28**: 261-289.

Danin-Poleg, Y., Cohen, L. A., Gancz, H., Broza, Y. Y., Goldshmidt, H., Malul, E., Valinsky, L., Lerner, L., Broza, M. and Kashi, Y. (2006a) *Vibrio cholerae* strain typing and phylogeny study based on simple sequence repeats (SSR). *J Clin Microbiol* **45**: 736-746.

Danin-Poleg, Y., Somer, L., Cohen, L. A., Diamant, E., Palti, Y. and Kashi, Y. (2006b) Towards the definition of pathogenic microbe. *Int J Food Microbiol* **112**: 236-243.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. and Ramphal, R. (2003) A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**: 809-824.

Deng, W. L., Preston, G., Collmer, A., Chang, C. J. and Huang, H. C. (1998) Characterization of the *hrpC* and *hrpRS* operons of *Pseudomonas syringae* pathovars syringae, tomato, and glycinea and analysis of the ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants to elicit the hypersensitive response and disease in plants. *J Bacteriol* **180**: 4523-4531.

Deng, W. L., Rehm, A. H., Charkowski, A. O., Rojas, C. M. and Collmer, A. (2003) *Pseudomonas syringae* exchangeable effector loci: sequence diversity in representative pathovars and virulence function in *P. syringae* pv. syringae B728a. *J Bacteriol* **185**: 2592-2602.

Denny, T. P. (1988) Phenotypic diversity in *Pseudomonas syringae* pv. tomato. *J Gen Microbiol* **134**: 1939-1948.

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

Denny, T. P., Gilmour, M. N. and Selander, R. K. (1988) Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. *J Gen Microbiol* **134**: 1949-1960.

Deretic, V., Gill, J. F. and Chakrabarty, A. M. (1987a) Gene *algD* coding for GDP-mannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* **169**: 351-358.

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

Desvaux, M., Hebraud, M., Henderson, I. R. and Pallen, M. J. (2006) Type III secretion: what's in a name? *Trends Microbiol* **14**: 157-160.

Devash, Y., Okon, Y. and Henis, Y. (1980) Survival of *Pseudomonas tomato* in soil and seeds. *J Phytopathol* **99**: 175-185.

Diamant, E., Palti, Y., Gur-Arie, R., Cohen, H., Hallerman, E. M. and Kashi, Y. (2004) Phylogeny and strain typing of *Escherichia coli*, inferred from variation at mononucleotide repeat loci. *Appl Environ Microbiol* **70**: 2464-2473.

Dombrecht, B., Marchal, K., Vanderleyden, J. and Michiels, J. (2002) Prediction and overview of the RpoN-regulon in closely related species of the *Rhizobiales*. *Genome Biol* **3**: RESEARCH0076.

Dulla, G. and Lindow, S. E. (2008) Quorum size of *Pseudomonas syringae* is small and dictated by water availability on the leaf surface. *Proc Natl Acad Sci U S A* **105**: 3082-3087.

Elizabeth, S. V. and Bender, C. L. (2007) The phytotoxin coronatine from *Pseudomonas syringae* pv. tomato DC3000 functions as a virulence factor and influences defence pathways in edible brassicas. *Mol Plant Pathol* **8**: 83-92.

Endoh, T., Kasuga, K., Horinouchi, M., Yoshida, T., Habe, H., Nojiri, H. and Omori, T. (2003) Characterization and identification of genes essential for dimethyl sulfide utilization in *Pseudomonas putida* strain DS1. *Appl Microbiol Biotechnol* **62**: 83-91.

Engelke, T., Jording, D., Kapp, D. and Puhler, A. (1989) Identification and sequence analysis of the *Rhizobium meliloti dctA* gene encoding the C4-dicarboxylate carrier. *J Bacteriol* **171**: 5551-5560.

Fackrell, H. B. and Sinha, R. C. (1983) Serological analysis of *Pseudomonas syringae* pv. tomato. *Phytopathology* **73**: 178-181.

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

Farlow, J., Smith, K. L., Wong, J., Abrams, M., Lytle, M. and Keim, P. (2001) *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. *J Clin Microbiol* **39**: 3186-3192.

Farlow, J., Wagner, D. M., Dukerich, M., Stanley, M., Chu, M., Kubota, K., Petersen, J. and Keim, P. (2005) *Francisella tularensis* in the United States. *Emerg Infect Dis* **11**: 1835-1841.

Feil, H., Feil, W. S., Chain, P., Larimer, F., DiBartolo, G., Copeland, A., Lykidis, A., Trong, S., Nolan, M., Goltsman, E., Thiel, J., Malfatti, S., Loper, J. E., Lapidus, A., Detter, J. C., Land, M., Richardson, P. M., Kyrpides, N. C., Ivanova, N. and Lindow, S. E. (2005) Comparison of the complete genome sequences of *Pseudomonas syringae* pv. syringae B728a and pv. tomato DC3000. *Proc Natl Acad Sci U S A* **102**: 11064-11069.

Felsenstein, J. (2005) PHYLIP (Phylogeny Inference Package) version 3.6.

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

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

Fett, W. F. and Sequeira, L. (1981) Further characterization of the physiologic races of *Pseudomonas glycinea*. *Can J Bot* **59**: 283-287.

Feys, B., Benedetti, C. E., Penfold, C. N. and Turner, J. G. (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* **6**: 751-759.

Fletcher, J., Bender, C., Budowle, B., Cobb, W. T., Gold, S. E., Ishimaru, C. A., Luster, D., Melcher, U., Murch, R., Scherm, H., Seem, R. C., Sherwood, J. L., Sobral, B. W. and Tolin, S. A. (2006) Plant pathogen forensics: capabilities, needs, and recommendations. *Microbiol Mol Biol Rev* **70**: 450-471.

Fouts, D. E., Abramovitch, R. B., Alfano, J. R., Baldo, A. M., Buell, C. R., Cartinhour, S., Chatterjee, A. K., D'Ascenzo, M., Gwinn, M. L., Lazarowitz, S. G., Lin, N. C., Martin, G. B., Rehm, A. H., Schneider, D. J., van Dijk, K., Tang, X. and Collmer, A. (2002) Genomewide identification of *Pseudomonas syringae* pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc Natl Acad Sci U S A* **99**: 2275-2280.

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

Gal-Mor, O. and Finlay, B. B. (2006) Pathogenicity islands: a molecular toolbox for bacterial virulence. *Cell Microbiol* **8**: 1707-1719.

Gal, M., Preston, G. M., Massey, R. C., Spiers, A. J. and Rainey, P. B. (2003) Genes encoding a cellulosic polymer contribute toward the ecological success of *Pseudomonas fluorescens* SBW25 on plant surfaces. *Mol Ecol* **12**: 3109-3121.

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

Gao, Y., Wang, Y. K. and Hoover, T. R. (1998) Mutational analysis of the phosphate-binding loop of *Rhizobium meliloti* DctD, a sigma54-dependent activator. *J Bacteriol* **180**: 2792-2795.

Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F. and Grimont, P. A. (1999) DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *Int J Syst Bacteriol* **49** 469-478.

Getz, S., Fulbright, D. W. and Stephens, C. T. (1983a) Scanning electron-microscopy of infection sites and lesion development on tomato fruit infected with *Pseudomonas syringae* pv. tomato. *Phytopathology* **73**: 39-43.

Getz, S., Stephens, C. T. and Fulbright, D. W. (1983b) Influence of developmental stage on susceptibility of tomato fruit to *Pseudomonas syringae* pv. tomato. *Phytopathology* **73**: 36-38.

Giddens, S. R., Jackson, R. W., Moon, C. D., Jacobs, M. A., Zhang, X. X., Gehrig, S. M. and Rainey, P. B. (2007) Mutational activation of niche-specific genes provides insight into regulatory networks and bacterial function in a complex environment. *Proc Natl Acad Sci U S A* **104**: 18247-18252.

Gitaitis, R. and Walcott, R. (2007) The epidemiology and management of seedborne bacterial diseases. *Annu Rev Phytopathol* **45**: 371-397.

Gitaitis, R. D., Jones, J. B., Jaworski, C. A. and Phatak, S. C. (1985) Incidence and development of *Pseudomonas syringae* pv. syringae on tomato transplants in Georgia. *Plant Dis* **69**: 32-35.

Goldberg, J. B. and Dahnke, T. (1992) *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. *Mol Microbiol* **6**: 59-66.

Goldberg, J. B., Hatano, K. and Pier, G. B. (1993) Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. *J Bacteriol* **175**: 1605-1611.

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

Goode, M. J. and Sasser, M. (1980) Prevention - the key to controlling bacterial spot and bacterial speck of tomato. *Plant Dis* **64**: 831-834.

Grande, R. A., Valderrama, B. and Morett, E. (1999) Suppression analysis of positive control mutants of NifA reveals two overlapping promoters for *Klebsiella pneumoniae rpoN*. *J Mol Biol* **294**: 291-298.

Grant, S. R., Fisher, E. J., Chang, J. H., Mole, B. M. and Dangl, J. L. (2006) Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. *Annu Rev Microbio* **60**: 425-449.

Gross, M. and Rudolph, K. (1987) Studies on the extracellular polysaccharides (EPS) produced invitro by *Pseudomonas phaseolicola*. 1. Indications for a polysaccharide resembling alginic acid in 7 *Pseudomonas syringae* pathovars. *J Phytopathol* **118**: 276-287.

Gur-Arie, R., Cohen, C. J., Eitan, Y., Shelef, L., Hallerman, E. M. and Kashi, Y. (2000) Simple sequence repeats in *Escherichia coli*: abundance, distribution, composition, and polymorphism. *Genome Res* **10**: 62-71.

Hacker, J. and Carniel, E. (2001) Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. *EMBO Rep* **2**: 376-381.

Hacker, J. and Kaper, J. B. (2000) Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol* **54**: 641-679.

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

Hancock, J. G. and Huisman, O. C. (1981) Nutrient movement in host-pathogen systems. *Annu Rev Phytopathol* **19**: 309-331.

Hattermann, D. R. and Ries, S. M. (1989) Motility of *Pseudomonas syringae* pv glycinea and its role in infection. *Phytopathology* **79**: 284-289.

He, P., Shan, L. and Sheen, J. (2007) Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. *Cell Microbiol* **9**: 1385-1396.

He, S. Y. and Jin, Q. (2003) The Hrp pilus: learning from flagella. *Curr Opin Microbiol* **6**: 15-19.

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

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

Hendrickson, E. L., Guevera, P. and Ausubel, F. M. (2000a) The alternative sigma factor RpoN is required for *hrp* activity in *Pseudomonas syringae* pv. maculicola and acts at the level of *hrpL* transcription. *J Bacteriol* **182**: 3508-3516.

Hendrickson, E. L., Guevera, P., Penaloza-Vazquez, A., Shao, J., Bender, C. and Ausubel, F. M. (2000b) Virulence of the phytopathogen *Pseudomonas syringae* pv. maculicola is *rpoN* dependent. *J Bacteriol* **182**: 3498-3507.

Hendson, M., Hildebrand, D. C. and Schroth, M. N. (1992) Relatedness of *Pseudomonas syringae* pv. tomato, *Pseudomonas syringae* pv. maculicola and *Pseudomonas syringae* pv. antirrhini. *J Appl Bacteriol* **73**: 455-464.

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

Hickman, J. W. and Harwood, C. S. (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* **69**: 376-389.

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

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

Hutcheson, S. W., Bretz, J., Sussan, T., Jin, S. and Pak, K. (2001) Enhancer-binding proteins HrpR and HrpS interact to regulate *hrp*-encoded type III protein secretion in *Pseudomonas syringae* strains. *J Bacteriol* **183**: 5589-5598.

Hwang, M. S., Morgan, R. L., Sarkar, S. F., Wang, P. W. and Guttman, D. S. (2005) Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Appl Environ Microbiol* **71**: 5182-5191.

Ichihara, A., Shiraishi, K., Sato, H., Sakamura, S., Nishiyama, K., Sakai, R., Furusaki, A. and Matsumoto, T. (1977) Structure of coronatine. *J Am Chem Soc* **99**: 636-637.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R. and Staskawicz, B. J. (1993) Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J Bacteriol* **175**: 4859-4869.

Inoue, J., Shaw, J. P., Rekik, M. and Harayama, S. (1995) Overlapping substrate specificities of benzaldehyde dehydrogenase (the *xylC* gene product) and 2-

hydroxymuconic semialdehyde dehydrogenase (the *xylG* gene product) encoded by TOL plasmid pWW0 of *Pseudomonas putida*. *J Bacteriol* **177**: 1196-1201.

Janausch, I. G., Zientz, E., Tran, Q. H., Kroger, A. and Unden, G. (2002) C4-dicarboxylate carriers and sensors in bacteria. *Biochim Biophys Acta* **1553**: 39-56.

Ji, P. and Wilson, M. (2002) Assessment of the importance of similarity in carbon source utilization profiles between the biological control agent and the pathogen in biological control of bacterial speck of tomato. *Appl Environ Microbiol* **68**: 4383-4389.

Jiang, J., Gu, B. H., Albright, L. M. and Nixon, B. T. (1989) Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum dct* genes. *J Bacteriol* **171**: 5244-5253.

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

Jin, Q., Thilmony, R., Zwiesler-Vollick, J. and He, S. Y. (2003) Type III protein secretion in *Pseudomonas syringae*. *Microbes Infect* **5**: 301-310.

Joardar, V., Lindeberg, M., Jackson, R. W., Selengut, J., Dodson, R., Brinkac, L. M., Daugherty, S. C., Deboy, R., Durkin, A. S., Giglio, M. G., Madupu, R., Nelson, W. C., Rosovitz, M. J., Sullivan, S., Crabtree, J., Creasy, T., Davidsen, T., Haft, D. H., Zafar, N., Zhou, L., Halpin, R., Holley, T., Khouri, H., Feldblyum, T., White, O., Fraser, C. M., Chatterjee, A. K., Cartinhour, S., Schneider, D. J., Mansfield, J., Collmer, A. and Buell, C. R. (2005) Whole-genome sequence analysis of *Pseudomonas syringae* pv. phaseolicola 1448A reveals divergence among pathovars in genes involved in virulence and transposition. *J Bacteriol* **187**: 6488-6498.

Johansson, A., Farlow, J., Larsson, P., Dukerich, M., Chambers, E., Bystrom, M., Fox, J., Chu, M., Forsman, M., Sjostedt, A. and Keim, P. (2004) Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. *J Bacteriol* **186**: 5808-5818.

Jones, J., Studholme, D. J., Knight, C. G. and Preston, G. M. (2007) Integrated bioinformatic and phenotypic analysis of RpoN-dependent traits in the plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25. *Environ Microbiol* **9**: 3046-3064.

Jones, J. B., Mccarter, S. M. and Gitaitis, R. D. (1981) Association of *Pseudomonas syringae* pv. syringae with a leaf-spot disease of tomato transplants in southern Georgia. *Phytopathology* **71**: 1281-1285.

Kahnert, A., Vermeij, P., Wietek, C., James, P., Leisinger, T. and Kertesz, M. A. (2000) The *ssu* locus plays a key role in organosulfur metabolism in *Pseudomonas putida* S-313. *J Bacteriol* **182**: 2869-2878.

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

Keane, P. J., Kerr, A. and New, P. B. (1970) Crown Gall of Stone Fruit .2. Identification and Nomenclature of Agrobacterium Isolates. *Aust J Biol Sci* **23**: 585-595.

Keith, L. M. and Bender, C. L. (1999) AlgT (sigma22) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. *J Bacteriol* **181**: 7176-7184.

Keith, R. C., Keith, L. M., Hernandez-Guzman, G., Uppalapati, S. R. and Bender, C. L. (2003) Alginate gene expression by *Pseudomonas syringae* pv. tomato DC3000 in host and non-host plants. *Microbiology* **149**: 1127-1138.

Kenyon, J. S. and Turner, J. G. (1992) The stimulation of ethylene synthesis in *Nicotiana tabacum* leaves by the phytotoxin coronatine. *Plant Physiol* **100**: 219-224.

Kerkoud, M., Manceau, C. and Paulin, J. P. (2002) Rapid diagnosis of *Pseudomonas syringae* pv. papulans, the causal agent of blister spot of apple, by polymerase chain reaction using specifically designed *hrpL* gene primers. *Phytopathology* **92**: 1077-1083.

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

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

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

Kitten, T., Kinscherf, T. G., McEvoy, J. L. and Willis, D. K. (1998) A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. *Mol Microbiol* **28**: 917-929.

Kloek, A. P., Verbsky, M. L., Sharma, S. B., Schoelz, J. E., Vogel, J., Klessig, D. F. and Kunkel, B. N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. *Plant J* **26**: 509-522.

Knirel, Y. A., Ovod, V. V., Zdorovenko, G. M., Gvozdyak, R. I. and Krohn, K. J. (1998) Structure of the O polysaccharide and immunochemical relationships between the lipopolysaccharides of *Pseudomonas syringae* pathovar tomato and pathovar maculicola. *Eur J Biochem* **258**: 657-661.

Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., 2nd and Peterson, K. M. (1994) pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**: 800-802.

Kustu, S., Santero, E., Keener, J., Popham, D. and Weiss, D. (1989) Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiol Rev* **53**: 367-376.

Labes, M. and Finan, T. M. (1993) Negative regulation of sigma 54-dependent *dctA* expression by the transcriptional activator DctD. *J Bacteriol* **175**: 2674-2681.

Labes, M., Rastogi, V., Watson, R. and Finan, T. M. (1993) Symbiotic nitrogen fixation by a *nifA* deletion mutant of *Rhizobium meliloti*: the role of an unusual *ntrC* allele. *J Bacteriol* **175**: 2662-2673.

Lan, L., Deng, X., Xiao, Y., Zhou, J. M. and Tang, X. (2007) Mutation of Lon protease differentially affects the expression of *Pseudomonas syringae* type III secretion system genes in rich and minimal media and reduces pathogenicity. *Mol Plant-Microbe Interact* **20**: 682-696.

Lan, L., Deng, X., Zhou, J. and Tang, X. (2006) Genome-wide gene expression analysis of *Pseudomonas syringae* pv. tomato DC3000 reveals overlapping and distinct pathways regulated by *hrpL* and *hrpRS*. *Mol Plant-Microbe Interact* **19**: 976-987.

Lauchli, R. and Boland, W. (2003) Indanoyl amino acid conjugates: tunable elicitors of plant secondary metabolism. *Chem Rec* **3**: 12-21.

Laue, H., Schenk, A., Li, H., Lambertsen, L., Neu, T. R., Molin, S. and Ullrich, M. S. (2006) Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*. *Microbiology* **152**: 2909-2918.

Lawton, M. B. and MacNeill, B. H. (1986) Occurrence of race 1 of *Pseudomonas syringae* pv. tomato on field tomato in southwestern Ontario. *Can J Plant Path* **8**: 85-88.

Le Fleche, P., Hauck, Y., Onteniente, L., Prieur, A., Denoeud, F., Ramisse, V., Sylvestre, P., Benson, G., Ramisse, F. and Vergnaud, G. (2001) A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BMC Microbiol* **1**: 2.

Lee, D. G., Urbach, J. M., Wu, G., Liberati, N. T., Feinbaum, R. L., Miyata, S., Diggins, L. T., He, J., Saucier, M., Deziel, E., Friedman, L., Li, L., Grills, G., Montgomery, K., Kucherlapati, R., Rahme, L. G. and Ausubel, F. M. (2006) Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol* **7**: R90.

Lee, J. H. and Hoover, T. R. (1995) Protein crosslinking studies suggest that *Rhizobium meliloti* C4-dicarboxylic acid transport protein D, a sigma 54-dependent

transcriptional activator, interacts with sigma 54 and the beta subunit of RNA polymerase. *Proc Natl Acad Sci U S A* **92**: 9702-9706.

Legendre, M., Pochet, N., Pak, T. and Verstrepen, K. J. (2007) Sequence-based estimation of minisatellite and microsatellite repeat variability. *Genome Res* **17**: 1787-1796.

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

Li, C. M., Brown, I., Mansfield, J., Stevens, C., Boureau, T., Romantschuk, M. and Taira, S. (2002) The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. *EMBO J* **21**: 1909-1915.

Li, X., Lin, H., Zhang, W., Zou, Y., Zhang, J., Tang, X. and Zhou, J. M. (2005) Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc Natl Acad Sci U S A* **102**: 12990-12995.

Lin, H., Civerolo, E. L., Hu, R., Barros, S., Francis, M. and Walker, M. A. (2005) Multilocus simple sequence repeat markers for differentiating strains and evaluating genetic diversity of *Xylella fastidiosa*. *Appl Environ Microbiol* **71**: 4888-4892.

Lindgren, P. B., Peet, R. C. and Panopoulos, N. J. (1986) Gene cluster of *Pseudomonas syringae* pv. "phaseolicola" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. *J Bacteriol* **168**: 512-522.

Lindstedt, B. A. (2005) Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis* **26**: 2567-2582.

Liu, Y., Lee, M. A., Ooi, E. E., Mavis, Y., Tan, A. L. and Quek, H. H. (2003) Molecular typing of *Salmonella enterica* serovar typhi isolates from various countries in Asia by a multiplex PCR assay on variable-number tandem repeats. *J Clin Microbiol* **41**: 4388-4394.

Liyanage, H., Penfold, C., Turner, J. and Bender, C. L. (1995) Sequence, expression and transcriptional analysis of the coronafacate ligase-encoding gene required for coronatine biosynthesis by *Pseudomonas syringae*. *Gene* **153**: 17-23.

Llamas, M. A., Rodriguez-Herva, J. J., Hancock, R. E., Bitter, W., Tommassen, J. and Ramos, J. L. (2003) Role of *Pseudomonas putida tol-oprL* gene products in uptake of solutes through the cytoplasmic membrane. *J Bacteriol* **185**: 4707-4716.

Lodwig, E. and Poole, P. (2003) Metabolism of *Rhizobium* bacteroids. *Crit Rev Plant Sci* **22**: 37-78.

Loper, J. E. and Lindow, S. E. (1987) Lack of evidence for *in situ* fluorescent pigment production by *Pseudomonas syringae* pv. syringae on bean leaf surfaces. *Phytopathology* **77**: 1449-1454.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., Sahin, F. and Miller, S. A. (2001) Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Dis* **85**: 481-488.

Madden, L. V. and Wheelis, M. (2003) The threat of plant pathogens as weapons against US crops. *Annu Rev Phytopathol* **41**: 155-176.

Manceau, C. and Horvais, A. (1997) Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. tomato. *Appl Environ Microbiol* **63**: 498-505.

Marutani, M., Taguchi, F., Ogawa, Y., Hossain, M. M., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2008) Gac two-component system in *Pseudomonas syringae* pv. tabaci is required for virulence but not for hypersensitive reaction. *Mol Genet Genomics* **279**: 313-322.

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

McCann, H. C. and Guttman, D. S. (2008) Evolution of the type III secretion system and its effectors in plant-microbe interactions. *New Phytol* **177**: 33-47.

McCarter, L. L. (2006) Regulation of flagella. *Curr Opin Microbiol* **9**: 180-186.

McCarter, S. M., Jones, J. B., Gitaitis, R. D. and Smitley, D. R. (1983) Survival of *Pseudomonas syringae* pv. tomato in association with tomato seed, soil, host tissue, and epiphytic weed hosts in Georgia. *Phytopathology* **73**: 1393-1398.

McInnes, T. B., Gitaitis, R. D., McCarter, S. M. and Phatak, S. C. (1988) Airborne dispersal of bacteria in tomato and pepper transplant fields. *Plant Dis* **72**: 575-579.

Melotto, M., Underwood, W. and He, S. Y. (2008) Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu Rev Phytopathol* **46**: 101-122.

Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S. Y. (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**: 969-980.

Merrick, M. J. (1993) In a class of its own--the RNA polymerase sigma factor sigma 54 (sigma N). *Mol Microbiol* **10**: 903-909.

Michiels, J., Moris, M., Dombrecht, B., Verreth, C. and Vanderleyden, J. (1998) Differential regulation of *Rhizobium etli rpoN2* gene expression during symbiosis and free-living growth. *J Bacteriol* **180**: 3620-3628.

Mitchell, R. E. (1982) Coronatine production by some phytopathogenic pseudomonads. *Physiol Plant Pathol* **20**: 83-89.

Mitchell, R. E., Hale, C. N. and Shanks, J. C. (1983) Production of different pathogenic symptoms and different toxins by strains of *Pseudomonas syringae* pv. tomato not distinguishable by gel-immunodiffusion assay. *Physiol Plant Pathol* **23**: 315-322.

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

Moens, S. and Vanderleyden, J. (1996) Functions of bacterial flagella. *Crit Rev Microbiol* **22**: 67-100.

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

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

Morett, E. and Segovia, L. (1993) The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *J Bacteriol* **175**: 6067-6074.

Morris, V. L., Jackson, D. P., Grattan, M., Ainsworth, T. and Cuppels, D. A. (1995) Isolation and sequence analysis of the *Pseudomonas syringae* pv. tomato gene encoding a 2,3-diphosphoglycerate-independent phosphoglyceromutase. *J Bacteriol* **177**: 1727-1733.

Moxon, R., Bayliss, C. and Hood, D. (2006) Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Annu Rev Genet* **40**: 307-333.

Muhammadi and Ahmed, N. (2007) Genetics of bacterial alginate: alginate genes distribution, organization and biosynthesis in bacteria. *Curr Genomics* **8**: 191-202.

Naumann, K., Karl, H., Gierz, E. and Griesbach, E. (1986) On the detection of *Pseudomonas syringae* pv tomato (Okabe) Young, Dye and Wilkie, the causal agent of the bacterial speck disease, on tomato seeds. *Zbl Mikrobiol* **141**: 301-321.

Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M.,

DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J. A., Timmis, K. N., Dusterhoft, A., Tummler, B. and Fraser, C. M. (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**: 799-808.

Nomura, K., Melotto, M. and He, S. Y. (2005) Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. *Curr Opin Plant Biol* **8**: 361-368.

North, A. K., Klose, K. E., Stedman, K. M. and Kustu, S. (1993) Prokaryotic enhancer-binding proteins reflect eukaryote-like modularity: the puzzle of nitrogen regulatory protein C. *J Bacteriol* **175**: 4267-4273.

Nurnberger, T. and Brunner, F. (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Curr Opin Plant Biol* **5**: 318-324.

Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* **198**: 249-266.

O'Toole, G. A. and Kolter, R. (1998a) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**: 295-304.

O'Toole, G. A. and Kolter, R. (1998b) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28**: 449-461.

Ohman, D. E., Mathee, K., McPherson, C. J., deVries, C. A., Ma, S., Wozniak, D. J. and Franklin, M. J. (1996) Regulation of the alginate (*algD*) operon in *Pseudomonas aeruginosa*. In: *Molecular Biology of Pseudomonads.* T. N. e. al., (eds). Washington, DC American Society for Microbiology Press, pp. 472-483.

Olive, D. M. and Bean, P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* **37**: 1661-1669.

Onteniente, L., Brisse, S., Tassios, P. T. and Vergnaud, G. (2003) Evaluation of the polymorphisms associated with tandem repeats for *Pseudomonas aeruginosa* strain typing. *J Clin Microbiol* **41**: 4991-4997.

Page, R. D. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**: 357-358.

Palmer, D. A. and Bender, C. L. (1993) Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. glycinea. *Appl Environ Microbiol* **59**: 1619-1626.

Parry, R. J., Mhaskar, S. V., Lin, M. T., Walker, A. E. and Mafoti, R. (1994) Investigations of the biosynthesis of the phytotoxin coronatine. *Can J Chem* **72**: 86-99.

Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S., Mavrodi, D. V., DeBoy, R. T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R. J., Durkin, A. S., Brinkac, L. M., Daugherty, S. C., Sullivan, S. A., Rosovitz, M. J., Gwinn, M. L., Zhou, L., Schneider, D. J., Cartinhour, S. W., Nelson, W. C., Weidman, J., Watkins, K., Tran, K., Khouri, H., Pierson, E. A., Pierson, L. S., 3rd, Thomashow, L. S. and Loper, J. E. (2005) Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol* **23**: 873-878.

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

Penaloza-Vazquez, A., Fakhr, M. K., Bailey, A. M. and Bender, C. L. (2004) AlgR functions in *algC* expression and virulence in *Pseudomonas syringae* pv. syringae. *Microbiology* **150**: 2727-2737.

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

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

Pernezny, K., Kudela, V., Kokoskova, B. and Hladka, I. (1995) Bacterial diseases of tomato in the Czech and Slovak Republics and lack of streptomycin resistance among copper-tolerant bacterial strains. *Crop Prot* **14**: 267-270.

Petnicki-Ocwieja, T., Schneider, D. J., Tam, V. C., Chancey, S. T., Shan, L., Jamir, Y., Schechter, L. M., Janes, M. D., Buell, C. R., Tang, X., Collmer, A. and Alfano, J. R. (2002) Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. tomato DC3000. *Proc Natl Acad Sci U S A* **99**: 7652-7657.

Piwowarski, J. M. and Shaw, P. D. (1982) Characterization of plasmids from plant pathogenic *Pseudomonads*. *Plasmid* **7**: 85-94.

Pohronezny, K., Volin, R. B. and Stall, R. E. (1979) Outbreak of bacterial speck on fresh-market tomatoes in south Florida. *Plant Dis Rep* **63**: 13-17.

Preston, G., Deng, W. L., Huang, H. C. and Collmer, A. (1998a) Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J Bacteriol* **180**: 4532-4537.

Preston, G. M. (2000) *Pseudomonas syringae* pv. tomato: the right pathogen, of the right plant, at the right time. *Mol Plant Pathol* **1**: 263-275.

Preston, G. M., Haubold, B. and Rainey, P. B. (1998b) Bacterial genomics and adaptation to life on plants: implications for the evolution of pathogenicity and symbiosis. *Curr Opin Microbiol* **1**: 589-597.

Quiniones, B., Dulla, G. and Lindow, S. E. (2005) Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol Plant-Microbe Interact* **18**: 682-693.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. and Ausubel, F. M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**: 1899-1902.

Ramsey, D. M. and Wozniak, D. J. (2005) Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol Microbiol* **56**: 309-322.

Rangaswamy, V. and Bender, C. L. (2000) Phosphorylation of CorS and CorR, regulatory proteins that modulate production of the phytotoxin coronatine in *Pseudomonas syringae*. *FEMS Microbiol Lett* **193**: 13-18.

Rastogi, V., Labes, M., Finan, T. and Watson, R. (1992) Overexpression of the *dctA* gene in *Rhizobium meliloti*: effect on transport of C4 dicarboxylates and symbiotic nitrogen fixation. *Can J Microbiol* **38**: 555-562.

Redondo-Nieto, M., Lloret, J., Larenas, J., Barahona, E., Navazo, A., Martinez-Granero, F., Capdevila, S., Rivilla, R. and Martin, M. (2008) Transcriptional organization of the region encoding the synthesis of the flagellar filament in *Pseudomonas fluorescens*. *J Bacteriol* **190**: 4106-4109.

Reid, C. J. and Poole, P. S. (1998) Roles of DctA and DctB in signal detection by the dicarboxylic acid transport system of *Rhizobium leguminosarum*. *J Bacteriol* **180**: 2660-2669.

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

Rico, A., Lopez, R., Asensio, C., Aizpun, M. T., Asensio-S-Manzanera, M. C. and Murillo, J. (2003) Nontoxigenic strains of *Pseudomonas syringae* pv. phaseolicola are a main cause of halo blight of beans in Spain and escape current detection methods. *Phytopathology* **93**: 1553-1559.

Rico, A. and Preston, G. M. (2008) *Pseudomonas syringae* pv. tomato DC3000 uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. *Mol Plant-Microbe Interact* **21**: 269-282.

Robinson, J. B. and Bauer, W. D. (1993) Relationships between C4 dicarboxylic acid transport and chemotaxis in *Rhizobium meliloti*. *J Bacteriol* **175**: 2284-2291.

Robleto, E. A., Lopez-Hernandez, I., Silby, M. W. and Levy, S. B. (2003) Genetic analysis of the AdnA regulon in *Pseudomonas fluorescens*: nonessential role of flagella in adhesion to sand and biofilm formation. *J Bacteriol* **185**: 453-460.

Rocha, E. P., Danchin, A. and Viari, A. (1999) Functional and evolutionary roles of long repeats in prokaryotes. *Res Microbiol* **150**: 725-733.

Roine, E., Raineri, D. M., Romantschuk, M., Wilson, M. and Nunn, D. N. (1998) Characterization of type IV pilus genes in *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant-Microbe Interact* **11**: 1048-1056.

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

Ronson, C. W., Astwood, P. M., Nixon, B. T. and Ausubel, F. M. (1987) Deduced products of C4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. *Nucleic Acids Res* **15**: 7921-7934.

Ruppitsch, W., Stoger, A. R. and Keck, M. (2004) Stability of short sequence repeats and their application for the characterization of *Erwinia amylovora* strains. *FEMS Microbiol Lett* **234**: 1-8.

Sabat, A., Krzyszton-Russjan, J., Strzalka, W., Filipek, R., Kosowska, K., Hryniewicz, W., Travis, J. and Potempa, J. (2003) New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. *J Clin Microbiol* **41**: 1801-1804.

Sabat, A., Malachowa, N., Miedzobrodzki, J. and Hryniewicz, W. (2006) Comparison of PCR-based methods for typing *Staphylococcus aureus* isolates. *J Clin Microbiol* **44**: 3804-3807.

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

Sarkar, S. F., Gordon, J. S., Martin, G. B. and Guttman, D. S. (2006) Comparative genomics of host-specific virulence in *Pseudomonas syringae*. *Genetics* **174**: 1041-1056.

Sarkar, S. F. and Guttman, D. S. (2004) Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Appl Environ Microbiol* **70**: 1999-2012.

Sato, M., Nishiyama, K. and Shirata, A. (1983) Involvement of plasmid DNA in the productivity of coronatine by *Pseudomonas syringae* pathovar atropurpurea. *Ann Phytopathol Soc Jap* **49**: 522-528.

Saunier, M., Malandrin, L. and Samson, R. (1996) Distribution of *Pseudomonas syringae* pathovars into twenty-three O serogroups. *Appl Environ Microbiol* **62**: 2360-2374.

Sawada, H., Suzuki, F., Matsuda, I. and Saitou, N. (1999) Phylogenetic analysis of *Pseudomonas syringae* pathovars suggests the horizontal gene transfer of *argK* and the evolutionary stability of *hrp* gene cluster. *J Mol Evol* **49**: 627-644.

Schaad, N. W. (2001) *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, N. W. Schaad, J. B. Jones & W. Chun (eds). St. Paul, USA: American Phytopathological Society, pp. 398.

Schaad, N. W., Frederick, R. D., Shaw, J., Schneider, W. L., Hickson, R., Petrillo, M. D. and Luster, D. G. (2003) Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annu Rev Phytopathol* **41**: 305-324.

Schechter, L. M., Roberts, K. A., Jamir, Y., Alfano, J. R. and Collmer, A. (2004) *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter. *J Bacteriol* **186**: 543-555.

Schechter, L. M., Vencato, M., Jordan, K. L., Schneider, S. E., Schneider, D. J. and Collmer, A. (2006) Multiple approaches to a complete inventory of *Pseudomonas syringae* pv. tomato DC3000 type III secretion system effector proteins. *Mol Plant-Microbe Interact* **19**: 1180-1192.

Schenk, A., Berger, M., Keith, L. M., Bender, C. L., Muskhelishvili, G. and Ullrich, M. S. (2006) The *algT* gene of *Pseudomonas syringae* pv. glycinea and new insights into the transcriptional organization of the *algT-muc* gene cluster. *J Bacteriol* **188**: 8013-8021.

Schenk, A., Weingart, H. and Ullrich, M. S. (2008) The alternative sigma factor AlgT, but not alginate synthesis, promotes in planta multiplication of *Pseudomonas syringae* pv. glycinea. *Microbiology* **154**: 413-421.

Schneider, R. W. and Grogan, R. G. (1977a) Bacterial speck of tomato - sources of inoculum and establishment of a resident population. *Phytopathology* **67**: 388-394.

Schneider, R. W. and Grogan, R. G. (1977b) Tomato leaf trichomes, a habitat for resident populations of *Pseudomonas tomato*. *Phytopathology* **67**: 898-902.

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

Sesma, A., Sundin, G. W. and Murillo, J. (1998) Closely related plasmid replicons coexisting in the phytopathogen *Pseudomonas syringae* show a mosaic organization of the replication region and altered incompatibility behavior. *Appl Environ Microbiol* **64**: 3948-3953.

Shimizu, R., Taguchi, F., Marutani, M., Mukaihara, T., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2003) The Delta*fliD* mutant of *Pseudomonas syringae* pv. tabaci, which secretes flagellin monomers, induces a strong hypersensitive reaction (HR) in non-host tomato cells. *Mol Genet Genomics* **269**: 21-30.

Smitley, D. R. and McCarter, S. M. (1982) Spread of *Pseudomonas syringae* pv. tomato and role of epiphytic populations and environmental conditions in disease development. *Plant Dis* **66**: 713-717.

Soscia, C., Hachani, A., Bernadac, A., Filloux, A. and Bleves, S. (2007) Cross talk between type III secretion and flagellar assembly systems in *Pseudomonas aeruginosa*. *J Bacteriol* **189**: 3124-3132.

Sreedharan, A. (2005) Regulation of coronatine biosynthesis in *Pseudomonas syringae* pv. tomato DC3000. PhD Thesis. Oklahoma State University.

Sreedharan, A., Penaloza-Vazquez, A., Kunkel, B. N. and Bender, C. L. (2006) CorR regulates multiple components of virulence in *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant-Microbe Interact* **19**: 768-779.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. and Olson, M. V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959-964.

Studholme, D. J. and Buck, M. (2000) The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS Microbiol Lett* **186**: 1-9.

Studholme, D. J. and Dixon, R. (2003) Domain architectures of sigma54-dependent transcriptional activators. *J Bacteriol* **185**: 1757-1767.

Takeuchi, K., Taguchi, F., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2003) Flagellin glycosylation island in *Pseudomonas syringae* pv. glycinea and its role in host specificity. *J Bacteriol* **185**: 6658-6665.

Tamura, K., Zhu, Y., Sato, M., Teraoka, T., Hosokawa, D. and Watanabe, M. (1998) Roles of coronatine production by *Pseudomonas syringae* pv. maculicola for pathogenicity. *Ann Phytopathol Soc Jap* **64**: 299-302.

Tang, X., Xiao, Y. and Zhou, J. M. (2006) Regulation of the type III secretion system in phytopathogenic bacteria. *Mol Plant-Microbe Interact* **19**: 1159-1166.

Tart, A. H., Blanks, M. J. and Wozniak, D. J. (2006) The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. *J Bacteriol* **188**: 6483-6489.

Tart, A. H., Wolfgang, M. C. and Wozniak, D. J. (2005) The alternative sigma factor AlgT represses *Pseudomonas aeruginosa* flagellum biosynthesis by inhibiting expression of *fleQ*. *J Bacteriol* **187**: 7955-7962.

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

U'Ren, J. M., Schupp, J. M., Pearson, T., Hornstra, H., Friedman, C. L., Smith, K. L., Daugherty, R. R., Rhoton, S. D., Leadem, B., Georgia, S., Cardon, M., Huynh, L. Y., DeShazer, D., Harvey, S. P., Robison, R., Gal, D., Mayo, M. J., Wagner, D., Currie, B. J. and Keim, P. (2007) Tandem repeat regions within the *Burkholderia pseudomallei* genome and their application for high resolution genotyping. *BMC Microbiol* **7**: 23.

Ullrich, M. and Bender, C. L. (1994) The biosynthetic gene cluster for coronamic acid, an ethylcyclopropyl amino acid, contains genes homologous to amino acid-activating enzymes and thioesterases. *J Bacteriol* **176**: 7574-7586.

Ullrich, M., Penaloza-Vazquez, A., Bailey, A. M. and Bender, C. L. (1995) A modified two-component regulatory system is involved in temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *J Bacteriol* **177**: 6160-6169.

Uppalapati, S. R., Ayoubi, P., Weng, H., Palmer, D. A., Mitchell, R. E., Jones, W. and Bender, C. L. (2005) The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *Plant J* **42**: 201-217.

Uppalapati, S. R., Ishiga, Y., Wangdi, T., Urbanczyk-Wochniak, E., Ishiga, T., Mysore, K. S. and Bender, C. L. (2008) Pathogenicity of *Pseudomonas syringae* pv. tomato on tomato seedlings: phenotypic and gene expression analyses of the virulence function of coronatine. *Mol Plant-Microbe Interact* **21**: 383-395.

van Belkum, A. (1999) The role of short sequence repeats in epidemiologic typing. *Curr Opin Microbiol* **2**: 306-311.

van Belkum, A. (2007) Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). *FEMS Immunol Med Microbiol* **49**: 22-27.

van Belkum, A., Scherer, S., van Alphen, L. and Verbrugh, H. (1998) Short-sequence DNA repeats in prokaryotic genomes. *Microbiol Mol Biol Rev* **62**: 275-293.

Verma, A., Arora, S. K., Kuravi, S. K. and Ramphal, R. (2005) Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response. *Infect Immun* **73**: 8237-8246.

Vodovar, N., Vallenet, D., Cruveiller, S., Rouy, Z., Barbe, V., Acosta, C., Cattolico, L., Jubin, C., Lajus, A., Segurens, B., Vacherie, B., Wincker, P., Weissenbach, J., Lemaitre, B., Medigue, C. and Boccard, F. (2006) Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. *Nat Biotechnol* **24**: 673-679.

Volksch, B. and Weingart, H. (1998) Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. *J Basic Microbiol* **38**: 135-145.

Voloudakis, A. E., Gitaitis, R. D., Westbrook, J. K., Phatak, S. C. and McCarter, S. M. (1991) Epiphytic survival of *Pseudomonas syringae* pv. syringae and *P. s.* tomato on tomato transplants in southern Georgia. *Plant Dis* **75**: 672-675.

Von Bodman, S. B., Bauer, W. D. and Coplin, D. L. (2003) Quorum sensing in plant-pathogenic bacteria. *Annu Rev Phytopathol* **41**: 455-482.

von Bodman, S. B. and Shaw, P. D. (1987) Conservation of plasmids among plant-pathogenic *Pseudomonas syringae* isolates of diverse origins. *Plasmid* **17**: 240-247.

Wang, Q., Suzuki, A., Mariconda, S., Porwollik, S. and Harshey, R. M. (2005) Sensing wetness: a new role for the bacterial flagellum. *EMBO J* **24**: 2034-2042.

Wang, Y. P., Birkenhead, K., Boesten, B., Manian, S. and O'Gara, F. (1989) Genetic analysis and regulation of the *Rhizobium meliloti* genes controlling C4-dicarboxylic acid transport. *Gene* **85**: 135-144.

Wang, Y. P., Giblin, L., Boesten, B. and O'Gara, F. (1993) The *Escherichia coli* cAMP receptor protein (CRP) represses the *Rhizobium meliloti dctA* promoter in a cAMP-dependent fashion. *Mol Microbiol* **8**: 253-259.

Watson, R. J. (1990) Analysis of the C4-dicarboxylate transport genes of *Rhizobium meliloti*: nucleotide sequence and deduced products of *dctA*, *dctB*, and *dctD*. *Mol Plant-Microbe Interact* **3**: 174-181.

Wei, C. F., Deng, W. L. and Huang, H. C. (2005) A chaperone-like HrpG protein acts as a suppressor of HrpV in regulation of the *Pseudomonas syringae* pv. syringae type III secretion system. *Mol Microbiol* **57**: 520-536.

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

Weingart, H. and Volksch, B. (1997a) Ethylene production by *Pseudomonas syringae* pathovars in vitro and in planta. *Appl Environ Microbiol* **63**: 156-161.

Weingart, H. and Volksch, B. (1997b) Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. *J Phytopathol* **145**: 339-345.

West, A. H. and Stock, A. M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* **26**: 369-376.

Whalen, M. C., Innes, R. W., Bent, A. F. and Staskawicz, B. J. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* **3**: 49-59.

Wiebe, W. L. and Campbell, R. N. (1993) Characterization of *Pseudomonas syringae* pv. maculicola and comparison with *P. s*. tomato. *Plant Dis* **77**: 414-419.

Wilkie, J. P. and Dye, D. W. (1974) *Pseudomonas tomato* in New Zealand. *New Zeal J Agr Res* **17**: 131-135.

Wilson, M., Campbell, H. L., Ji, P., Jones, J. B. and Cuppels, D. A. (2002) Biological control of bacterial speck of tomato under field conditions at several locations in north America. *Phytopathology* **92**: 1284-1292.

Wood, L. F. and Ohman, D. E. (2006) Independent regulation of MucD, an HtrA-like protease in *Pseudomonas aeruginosa*, and the role of its proteolytic motif in alginate gene regulation. *J Bacteriol* **188**: 3134-3137.

Wosten, M. M. (1998) Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**: 127-150.

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

Wozniak, D. J. and Ohman, D. E. (1993) Involvement of the alginate *algT* gene and integration host factor in the regulation of the *Pseudomonas aeruginosa algB* gene. *J Bacteriol* **175**: 4145-4153.

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

Xiao, Y., Heu, S., Yi, J., Lu, Y. and Hutcheson, S. W. (1994) Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. syringae Pss61 *hrp* and *hrmA* genes. *J Bacteriol* **176**: 1025-1036.

Xiao, Y. and Hutcheson, S. W. (1994) A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J Bacteriol* **176**: 3089-3091.

Xiao, Y., Lan, L., Yin, C., Deng, X., Baker, D., Zhou, J. M. and Tang, X. (2007) Two-component sensor RhpS promotes induction of *Pseudomonas syringae* type III secretion system by repressing negative regulator RhpR. *Mol Plant-Microbe Interact* **20**: 223-234.

Yan, S., Liu, H., Mohr, T. J., Jenrette, J., Chiodini, R., Zaccardelli, M., Setubal, J. C. and Vinatzer, B. A. (2008) The role of recombination in the evolution of the model plant pathogen *Pseudomonas syringae* pv. tomato DC3000, a very atypical tomato strain. *Appl Environ Microbiol* **74**: 3171-3181.

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

Yunis, H., Bashan, Y., Okon, Y. and Henis, Y. (1980a) Two sources of resistance to bacterial speck of tomato caused by *Pseudomonas tomato*. *Plant Dis* **64**: 851-852.

Yunis, H., Bashan, Y., Okon, Y. and Henis, Y. (1980b) Weather dependence, yield losses, and control of bacterial speck of tomato caused by *Pseudomonas tomato*. *Plant Dis* **64**: 937-939.

Yurgel, S. N. and Kahn, M. L. (2004) Dicarboxylate transport by rhizobia. *FEMS Microbiol Rev* **28**: 489-501.

Yurgel, S. N. and Kahn, M. L. (2005) *Sinorhizobium meliloti dctA* mutants with partial ability to transport dicarboxylic acids. *J Bacteriol* **187**: 1161-1172.

Zaccardelli, M., Spasiano, A., Bazzi, C. and Merighi, M. (2005) Identification and *in planta* detection of *Pseudomonas syringae* pv. tomato using PCR amplification of hrpZ(Pst). *Eur J Plant Pathol* **111**: 85-90.

Zhao, Y. F., Damicone, J. P., Demezas, D. H., Rangaswamy, V. and Bender, C. L. (2000) Bacterial leaf spot of leafy crucifers in Oklahoma caused by *Pseudomonas syringae* pv. maculicola. *Plant Dis* **84**: 1015-1020.

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

Zielinski, N. A., Maharaj, R., Roychoudhury, S., Danganan, C. E., Hendrickson, W. and Chakrabarty, A. M. (1992) Alginate synthesis in *Pseudomonas aeruginosa*: environmental regulation of the *algC* promoter. *J Bacteriol* **174**: 7680-7688.

Zipfel, C. and Felix, G. (2005) Plants and animals: a different taste for microbes? *Curr Opin Plant Biol* **8**: 353-360.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G. and Boller, T. (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* **428**: 764-767.

Zwiesler-Vollick, J., Plovanich-Jones, A. E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B. N. and He, S. Y. (2002) Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. tomato DC3000 genome. *Mol Microbiol* **45**: 1207-1218.

APPENDIX I

Evaluation of the Exchangeable Effector Locus of *P. syringae* as a Potential Signature for Microbial Forensics

SUMMARY

Agriculture is a vital part of the United States economy, and American crops are vulnerable to accidental or deliberate introduction of pathogens. *Pseudomonas syringae* pv. tomato strain DC3000 (*Pst* DC3000), which causes bacterial speck of tomato, shares survival and virulence mechanisms with pathogens that are currently on the USDA APHIS select agent list. However, *Pst* DC3000 can easily be utilized without biocontainment facilities. In *Pst* DC3000, the type III secretion system (T3SS) is encoded by the *hrp/hrc* genes and constitutes a pathogenicity island (PAI). The *hrp/hrc* regions in *P. syringae* are flanked by a conserved effector locus (CEL) and a unique exchangeable effector locus (EEL). This effector region, which is presumably involved with host specificity, represents a potential signature that could be used in strain identification. Previous studies have predicted that a correlation may exist between variability in the EEL region, and the geographic origination of strains. In this study, conserved regions of EEL genes were amplified by polymerase chain reaction (PCR), and used as probes to

analyze a collection of *P. syringae* strains isolated from diverse geographical locations. Strains were screened and evaluated for the presence of this potential signature using a hybridization technique using dot blots. When screened for the EEL signature, no obvious correlations between the EEL and the year or geographic place of strain origination were noted. The results showed that EEL probes were able to detect some strains within a pathovar, but did not yield the same result when a particular strain was tested repeatedly. The results also showed that EEL probes cross react with strains from different pathovars and were not able to clearly differentiate between unrelated strains. In summary, this study shows that EEL probes are not specific enough to be useful for strain identification of *P. syringae* by dot blot analysis.

INTRODUCTION

Agriculture is vitally important in the economic health of the United States, and US crops are vulnerable to introduction by foreign pathogens, largely because they cover vast geographic areas and are not under constant surveillance. Protection of plants from accidental or deliberate introduction of pathogens is a national security priority in the United States (Schaad *et al.*, 2003). Most pathogens on the USDA-APHIS biosecurity threat list are exotic, and strict regulations must be followed for handling these organisms. Scientists are now starting to use model pathogens, as these can be utilized without biocontainment facilities.

P. syringae is a gram-negative plant pathogenic bacterium that is a model organism for host-pathogen interactions. The *hrp/hrc* region in strains *Pst* DC3000, *P. syringae* pv. syringae 61, and *P. syringae* pv. syringae B728a consist of a conserved pathogenicity island (PAI) flanked by a conserved effector locus (CEL) and a unique exchangeable effector locus (EEL) (Alfano *et al.*, 2000) (Fig. 17).

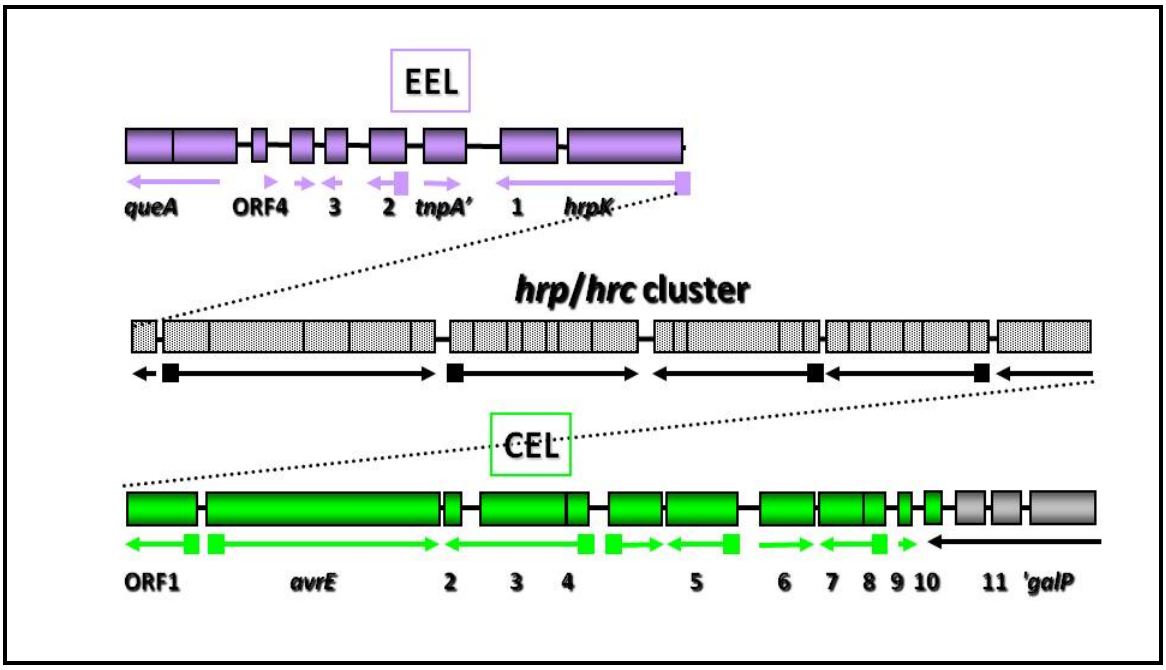


Figure 17: Diagram of the Hrp PAI of *Pst* DC3000. The *hrp/hrc* cluster is flanked by the conserved effector locus (CEL) and the exchangeable effector locus (EEL), a variable region with diagnostic potential (figure based on (Alfano *et al.*, 2000).

The CEL is required for pathogenicity, contains putative effector genes, and has a G + C content similar to the *hrp/hrc* genes which suggests that it is conserved. The EEL begins downstream of *hrpK* and is divergent among *P. syringae* strains until the conserved tRNA sequences are encountered (Fig. 18). The EEL region contains putative effector proteins, mobile genetic elements and has a lower than average G + C content, suggesting that it was acquired by horizontal transmission. This region varies in size from

2.3 to 7.3 kb between pathovars (Fig. 18). When the EEL was deleted in *Pst* DC3000, pathogen fitness was reduced (Alfano et al., 2000). The authors hypothesize that the Hrp PAI was acquired early and evolved with *P. syringae*, while the instability at the EEL suggests that it is still evolving (Alfano et al., 2000)

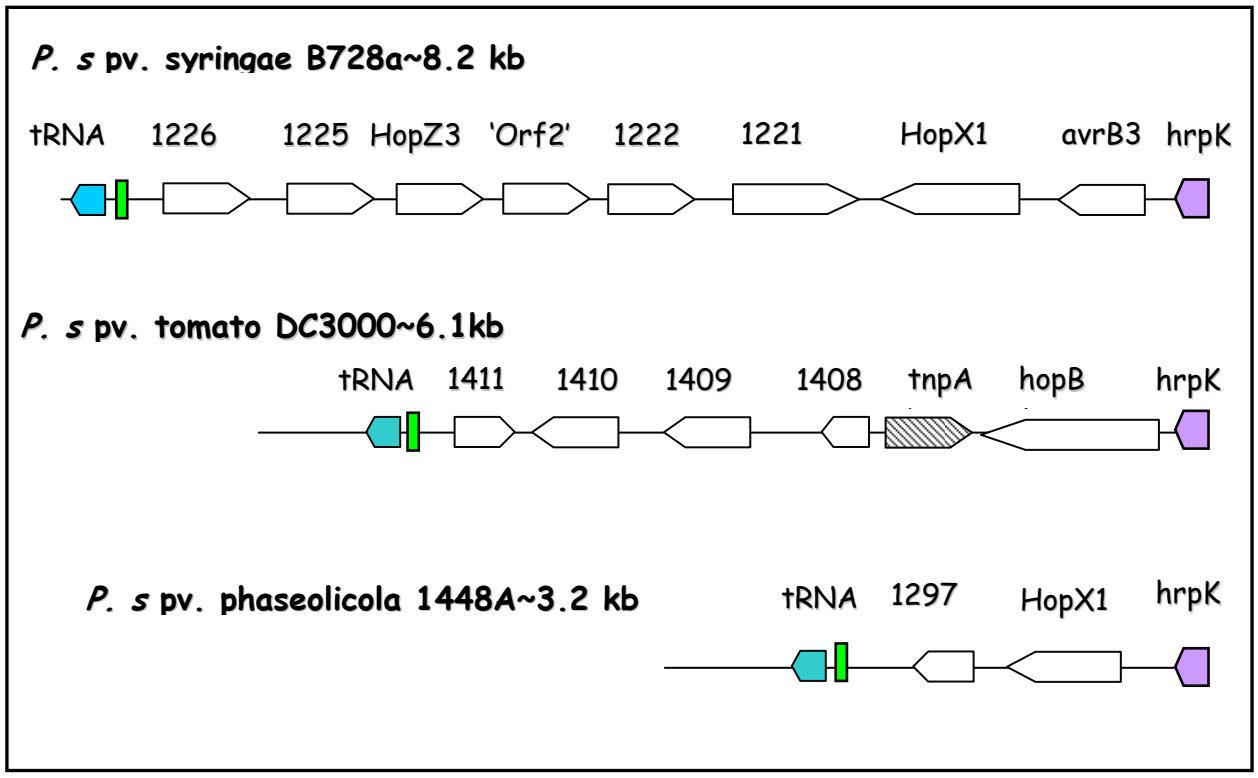


Figure 18: Diagram of the EEL of several *P. syringae* pathovars. The EEL region starts at *hrpK* and is divergent among the pathovars until conserved tRNA sequences are met. Shown above are the EEL regions from those pathovars whose genomes have been sequenced.

A study by Charity et al. used PCR techniques to amplify the EEL and identify EEL types that were classified by genetic composition (Charity *et al.*, 2003). The authors concluded that the effectors encoded by the EEL are involved in pathogenicity, and that the EEL evolved independently of the *hrp* CEL, possibly through transposed gene cassettes. The strains used in this study (Charity *et al.*, 2003) were predominantly from North America, and the authors suggested that other EEL types may be present in

geographically diverse strains. In another study, Deng et al. compared the EEL of various *P. syringae* strains and suggested that these sequences were acquired by horizontal gene transfer (HGT) (Deng *et al.*, 2003). This is thought to have occurred evolutionarily after the acquisition of the *hrp/hrc* cluster, but before the pathovars diverged from one another. The more recent divergence seen in the EEL of various *P. syringae* strains is the probable result of acquiring new effector proteins and by point mutations (Deng et al., 2003).

Charity et al. previously predicted that there may be a correlation between the EEL and place or date of strain origination (Charity et al., 2003), and the EEL has been identified as a possible polymorphic region of the *P. syringae* genome (Fletcher *et al.*, 2006). The objective of the current study was to evaluate the EEL for its potential to type strains of *P. syringae*.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Escherichia coli DH5α was routinely cultured on Luria Bertani (LB) medium or in Terrific Broth (TB) (Ausubel, 1997) at 37^{O}C. The *P. syringae* strains used in this study (Table 13) included strains isolated from distinct geographical locations and from diverse host plant species in different years. *P. syringae* strains were cultured on King's medium B (KMB) (King *et al.*, 1954) at 28^{O}C and stored at –70°C in 20% glycerol.

Table 13: *Pseudomonas syringae* strains used in the EEL study.

Pathovar	Strain	Place of isolation	Year of isolation	Host	OSUCB NO	Source/Reference[b]
Atropurpurea	1304	Japan			1086-3	D. Kobayashi
Glycinea	36a				0991-7	M. Ullrich
Glycinea	4180	New Zealand	1975		0792-1	DSIR, ICMP
Glycinea	4182	New Zealand	1975		0792-2	DSIR

Glycinea	5562	New Zealand	1978		0792-3	DSIR
Glycinea	Race 4				0596-20	A. Collmer
Maculicola	921				0493-3	ICMP
Maculicola	4326				0596-13	F. Ausubel
Maculicola	4981	Zimbabwe	1970	Cauliflower		
Mori	1413				0886-25	NCPPB
Morsprunorum	560				0886-17	NCPPB, UCPPB
Morsprunorum	567	UK		Prunus	0288-7	R. E. Mitchell; NCPPB 560
Morsprunorum	3714	Italy	1965	Sweet cherry	0288-8	NCPPB 1781
Morsprunorum	239	South Africa	1981	Prunus	0494-13	I. Roos
Phaseolicola	F2			Bush bean	1096-6	B. Volksch
Phaseolicola	**1448A**				**1096-15**	**Race 6**
Phaseolicola	482				1096-18	Race 5
Phaseolicola	501				1096-19	Race 7
Phaseolicola	524				1096-21	Race 2
Savastanoi	AT 13526				0886-21	UCRDAC 0485-9
Syringae	**B728a**				**0995-1**	**wild-type; Kyle Willis**
Syringae	B301D				0688-2	D. Gross
Syringae	FF5	Oklahoma USA	199?		0491-8	G. Sundin
Syringae	5076	France	1965	Lilac, cucumber, melon & pumpkin	0494-3	ICMP
Syringae	1391	UK	1959	Pear	0494-4	L. Gardan
Syringae	4918	Uganda	1973	*Centrosema pubescens*, lilac	0494-9	ICMP, NCPPB
Syringae	3910	Greece	1963	Lemon fruit	0494-10	ICMP
Syringae	4916	France	1965	Bean, lilac	0494-11	ICMP, NCPPB
Syringae	6006	USSR	1948	*Citrus reticulata*	0494-18	ICMP
Syringae	HS191	Australia		Millet	0494-19	
Syringae	3476	UK	1960	Lilac	0494-20	ICMP
Syringae	1680	Hungary	1958	Cherry	0494-23	L. Gardan
Syringae	847	New Zealand	1962	Cherry	0494-24	ICMP
Syringae	3525	New Zealand	1973	Bean	0494-25	ICMP
Syringae	6491	New Zealand	1978	Apricot	0494-26	ICMP
Syringae	Pss 61				1295-1	wild-type; Alan Collmer
Tabaci					1086-1	
Tagetis	53534			Marigold	0700-4	J. Lydon; ATCC
Tomato	**DC3000 TIGR**	Guernsey, Channel Islands, UK	1960	Tomato	**0700-10**	(Buell *et al.*, 2003, Cuppels, 1986)
Tomato	PT23	USA	1983	Tomato	0887-15	(Bender & Cooksey, 1986)
Tomato	4325	Canada	1944	Tomato	0288-9	NCPPB, ICMP
Tomato	Pto 483		1988[a]	Tomato	0798-5	B. Voelksch, GSPB
Tomato	Pto 2811			Tomato	0798-7	V. Catara
Tomato	Pto 119		1997[a]	Tomato	0798-8	(Weingart & Volksch, 1997a)
Tomato	DC84-1	Ontario	1984	Tomato		
Tomato	487	Greece	1979	Tomato		
Tomato	3357	New Zealand	1972	Tomato		ICMP
	Cit7			Citrus; nonpathogenic	1191-1	K. Willis; MPMI 1:80

[a] The exact year of isolation and location was not available for some strains. When this occurred, the year of isolation was based on the first citation in previously published literature.

[b] GSPB, Göttingen Collection of Phytopathogenic Bacteria, Göttingen Germany; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; CFPB, Collection Francaise des Bacteries Phytopathogenes, INRA Angers, France; NCPPB, National Collection Plant Pathogenic Bacteria, UK; ATCC, American Type Culture Collection, USA; DAR, Plant Pathology Herbarium, Orange Agricultural Institute, Australia.
Strains in **bold underline** were used for primer design.

Preparation of genomic DNA

Genomic DNA was isolated from *P. syringae* strains using a rapid cell lysis protocol that has been described previously (Chen & Kuo, 1993). Briefly, bacteria were suspended in lysis buffer (40 mM Tris-acetate, pH 7.8; 20 mM sodium acetate; 1 mM EDTA; 1% SDS), and mixed with vigorous pipetting. A volume of 5 M NaCl was added and the mixture was centrifuged at 16,110 g for 20 min. The supernatant was transferred to a clean microfuge tube, an equal volume of chloroform was added, and the contents mixed briefly by inversion, and then centrifuged at 16,110 g for 10 min. The aqueous phase was transferred to a clean microfuge tube, and DNA was precipitated with one volume 100% EtOH. Following centrifugation, DNA was washed in 70% EtOH, and the pellet was dissolved in TE buffer (Sambrook *et al.*, 1989) (pH 8.0) and stored at 4°C.

Primer design

Sequence data was obtained from NCBI (www.ncbi.nih.gov) and homology searches were done using BLAST. Table 14 includes a list of EEL genes used in screening a collection of diverse *P. syringae* strains. The table was constructed by using the National Center for Biotechnology Information (NCBI) web site to search for the conserved *hrpK* gene, and the EEL locus was then located in various *P. syringae* pathovars. The *Pst* DC3000 genome was also analyzed with Artemis

(http://monod.cornell.edu/visualization/Visualization_with_Artemis.html), which is a free genome viewer and annotation tool that allows visualization of sequence features. The EEL regions from other *P. syringae* pathovars were sequenced previously (Charity et al., 2003), and obtained from the NCBI web site by searching for *hrpK* and identifying the flanking regions. The coding sequence of individual genes was then analyzed for related sequences using BlastN, and conserved regions of 100-600 bp were identified by comparing the query alignments. Approximately 30 primer pairs were then designed using PrimerQuest on the Integrated DNA Technologies (IDT) website (http://scitools.idtdna.com/Primerquest/) to amplify conserved regions as described below.

Table 14: EEL target genes and primers used in screening *P. syringae* strains

Target Gene (Probe)	Forward and Reverse Primers	Product Size (bp)
PSPTO 1406	GCAGCCAACTGTCTGCAAATGT TACTGTCAGGTGCCCATAAACCCT	201
PSPTO 1407	GGTGGTCGATGCGACGATCATT CAGTACCCACCAGGCTATGGACTAAA	170
PSPTO 1408	ACTGAGGCATCAGGCCTTTATCAC TGGTAGTGCCCAGAGTGCAG	146
PSPTO 1409	TCGAAGATCAACATCGCAGCAGGA TACGTCATGCTACGCTCAACCACA	252
PSPTO 1410	GGCAACGACAAGGATCTGGATAACGA GCCGTTGTAGTTGTCGGTGTTGTT	255
PSPTO 1411	GGAAGTCTCGGTTAAAGTACCTACAGGC CTCATAACCGTCAGCATTGAGTTGCG	181
Psyr 1219	GCATCTGTCATTTCTTCGGACAGC GGTCCAGTTATAGGCGTAACAAACGC	404
Psyr 1220	AAGCGCAGAATGAAGCGTCTCACA GTTGCGCATCCACATCAATGTTGCC	300
Psyr 1222	TCGAAGTCATTCACGAATTGCCCG CACTGGTTTCTTCGCTGATGACATGC	84
Psyr 1223	GCACTGGTTGAGCTGGACATCAAA CAGGCGACAAATCGTGGAGTCAAA	275
Psyr 1224	GATGCTGAACTCACCGAAAGCATC	266

	GCAGATTCAAGCCGGGATTTCTTG	
Psyr 1225	CCGTTACGGATTGATGAGGATCAC CCCGCCATGGTTAGCATTAAAC	155
Psyr 1226	TGAGCATCACCAAACCTGAAACGC TAGTAGTCCGGGTCGAGAGTCG	205
Pspph 1296	CGACGTCCACTGGATGAAGACAGTAT TTAGACCACGGGTCCATGACGAT	443
Psm avrPphE	ATGAACTGGAATGTGCTCGTCGC ATCTTTCGCGAACCGGCTGTCTT	273

PCR amplification

Primers were designed to amplify the most conserved portion of selected genes from the EEL region (Fig. 18 and Table 14) of various *P. syringae* pathovar reference strains (boldface, underscored strains in Table 13). For each primer pair, 50 µl reactions consisted of 5 µl of 10x Mg free buffer, 6.5 µl $MgCl_2$, 1 µl 10 mM dNTP, 1 µl genomic template DNA, 1 µl of each primer (50mM), 1 µl Taq polymerase (Invitrogen), and 33.5 µl sterile distilled H_20. PCR cycling conditions consisted of denaturation at 95^OC (step 1, 1-2 min), incubation at 94^OC (step 2, 1 min), annealing at 55^OC (step 3, 30 sec), and extension at 72^OC (step 4, 45 sec). Steps 2-4 were repeated for 30 cycles, followed by 7 min of final extension at 72^OC and a final incubation at 4^OC. PCR products were separated by electrophoresis in ultrapure agarose (Invitrogen, Carlsbad, CA) gels. Electrophoresis was in 0.5X TBE (Tris-borate-EDTA) or 1X TAE buffer (Sambrook *et al.*, 1989) with a 1-kb sizing ladder (Invitrogen, Carlsbad, CA). PCR products were excised from gels and prepared for cloning using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Cloning and sequencing of PCR products

Products were cloned into the pCR2.1-TOPO (Invitrogen, Inc.) cloning vector using the manufacturer's protocol (Table 15). The clones were then transformed into *E. coli*, and plasmid DNA isolated for sequencing was prepared using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing of the cloned fragment in each construct was conducted by the OSU Recombinant DNA/Protein Resource Facility using T7 and M13 primers.

Table 15: Strains and constructs used in the EEL study.

Strain or plasmid	Characteristics	Reference or source
E. coli DH5α	*recA lacZ*_M15	Bethesda Research Labs
Plasmids		
pCR2.1-TOPO	Ap^R Km^R; cloning vector for PCR products	Invitrogen
pCR2.1-PSPTO 1406	Ap^R; Km^R; contains conserved portion of PSPTO 1406 in pCR2.1	This study
pCR2.1-PSPTO 1408	Ap^R; Km^R; contains conserved portion of PSPTO 1408 in pCR2.1	This study
pCR2.1-PSPTO 1409	Ap^R; Km^R; contains conserved portion of PSPTO 1409 in pCR2.1	This study
pCR2.1-Psyr1219	Ap^R; Km^R; contains conserved portion of Psyr 1219 in pCR2.1	This study
pCR2.1-Psyr1220	Ap^R; Km^R; contains conserved portion of Psyr 1220 in pCR2.1	This study
pCR2.1-Psyr1222	Ap^R; Km^R; contains conserved portion of Psyr 1222 in pCR2.1	This study
pCR2.1-Psyr1224	Ap^R; Km^R; contains conserved portion of	This study

	Psyr 1224 in pCR2.1	
pCR2.1-Psyr1225	Ap^R; Km^R; contains conserved portion of Psyr 1225 in pCR2.1	This study
pCR2.1-Psyr1226	Ap^R; Km^R; contains conserved portion of Psyr 1226 in pCR2.1	This study

DNA labeling and hybridization

After confirming the identity of the inserts by sequencing, the constructs were used as templates, and the fragments amplified and labeled with digoxigenin (DIG) by PCR (PCR DIG probe synthesis kit; Roche Applied Science). Genomic DNA from *P. syringae* strains (Table 13) was spotted onto Hybond-N nylon membrane (Amersham Biosciences), cross-linked using a UV transilluminator and denatured. Hybridizations were conducted for at least 12 h at 65^OC, and post-hybridization washes (2X SSC, 0.1% SDS and 0.1% SSC, 0.1% SDS) were used. DNA-DNA hybridization was detected using an anti-dig antibody and chemiluminesence using the DIG Luminescent Detection Kit (Roche Applied Science).

RESULTS AND DISCUSSION

Effector proteins are delivered inside host cells via the T3SS. Effectors are sometimes recognized by the host and may modify the outcome of the host-pathogen interaction (Alfano & Collmer, 2004). The EEL is a highly variable source of effectors (Alfano et al., 2000), and some of these have a proven role in plant-microbe interactions (Fouts *et al.*, 2002, Petnicki-Ocwieja *et al.*, 2002, Schechter *et al.*, 2006). The EEL is part of the *P. syringae* flexible genome, which consists of genes that vary within strains of a

species, and by definition evolves largely through HGT. The organization of this region was constructed for *Pst* DC3000, B728a, and 1448A by searching for the *hrpK* gene in the genome of these three strains (figure 18; table 14 column 1). Conserved portions of one to several hundred base pairs were identified, and approximately fifteen primer sets were designed to amplify these conserved regions in *Pst* DC3000, B728a and 1448A. Detectable amplicons of the correct size were amplified and excised for cloning, indicating that the PCR reaction and cycling conditions were appropriate and sufficient.

To characterize and confirm the identity of the PCR products obtained, these were cloned into the pCR2.1-TOPO (Invitrogen, Inc.) cloning vector to create nine individual EEL-pCR2.1 constructs (Table 15). These were transformed into *E. coli*, and sequence analysis of the cloned fragment confirmed the correct identity of each insert.

The conserved EEL fragments were used in hybridization experiments to screen and evaluate a diverse collection of *P. syringae* strains for the presence of the EEL signature by a dot blot technique. After the cloned portion was confirmed by sequencing, inserts were used as template, amplified, and labeled with DIG. These labeled probes (Table 14 column one) were then hybridized with genomic DNAs from *P. syringae* strains (Table 13) that had been spotted to nylon membranes. Hybridizations were conducted overnight at 65^{O}C, after which time DNA-DNA hybridization was detected by chemiluminesence. Table 16 summarizes the results of experiments using *Pst* DC3000 EEL probes, and Table 17 summarizes the results of experiments using B728a EEL probes.

Table 16: Results from *Pst* DC3000 EEL probes. Probes PSPTO 1406 and 1409 were used in hybridization experiments with strains from various *P. syringae* pathovars (Table 13). Check marks for DC3000 indicate the total number of times that hybridization experiments were repeated (four for PSPTO 1406 and 5 for PSPTO 1409). Check marks for *P. syringae* strains represent the number of times a signal was detected in hybridization experiments. *E. coli* was included in hybridization experiments as a negative control.

pv	strain	PSPTO 1406	PSPTO 1409
Tomato	**DC3000**	✓✓✓✓	✓✓✓✓✓
Tomato	DC84-1	✓✓	✓✓✓
Tomato	487	✓✓✓✓	✓✓✓✓
Tomato	3357	✓✓✓✓	✓✓✓✓✓
Tomato	PT23		✓✓
Tomato	4325	✓✓	✓✓
Tomato	Pto 483	✓	✓✓
Tomato	Pto 2811	✓	✓
Tomato	Pto 119	✓✓	✓✓✓
Maculicola	4981	✓✓✓✓	✓✓✓✓✓
Mori	1431		✓
Syringae	3525	✓	✓✓
Syringae	HS191		✓✓
Syringae	847		✓
	Cit7	✓	✓
E. coli			✓
Atropurpurea			
Glycinea	36a		
Glycinea	4180		
Glycinea	4182		
Glycinea	Race 4		
Glycinea	5562		
Maculicola	921		
Maculicola	4326		
Morsprunorum	567		
Morsprunorum	3714		
Morsprunorum	239		
Phaseolicola	F2		
Phaseolicola	1448A		
Phaseolicola	482		
Phaseolicola	501		
Phaseolicola	524		
Savastanoi	AT 13526		
Syringae	B728a		

Syringae	B301D
Syringae	FF5
Syringae	5076
Syringae	1391
Syringae	4918
Syringae	3910
Syringae	4916
Syringae	6006
Syringae	3476
Syringae	1680
Syringae	6491
Syringae	Pss61
Tabaci	
Tagetis	53534

Table 17: Results from *Psyr* B728a EEL probes. Probes Psyr 1219, 1222, 1224, 1225, and 1226 were used in hybridization experiments with strains from various *P. syringae* pathovars (Table 13). Hybridization experiments were performed three times (1219, 1222, 1225 and 1226), and four times (1224) respectively. Check marks for *P. syringae* strains represent the number of times a signal was detected in hybridization experiments. The absence of check marks for probes 1222 and 1225 with strain B728a indicate that a detection signal for this positive control was not observed. *E. coli* was included in hybridization experiments as a negative control.

pv	strain	Psyr 1219	Psry 1222	Psyr 1224	Psyr 1225	Psyr 1226
Syringae	**B728a**	✓✓✓		✓✓✓✓		✓
Glycinea	4180		✓	✓		
Glycinea	5562		✓✓✓			
Maculicola	4326	✓	✓✓✓	✓	✓✓	✓
Maculicola	4981	✓✓	✓✓✓	✓✓	✓✓	✓
Mori	1431	✓	✓	✓	✓	✓✓
Phaseolicola	F2			✓		
Phaseolicola	1448A	✓				✓
Phaseolicola	524	✓				
Syringae	4918	✓✓✓		✓✓✓✓✓	✓✓✓	✓✓
Syringae	4916	✓✓✓		✓✓✓✓	✓✓	✓
Syringae	HS191	✓	✓✓✓	✓	✓	✓✓
Syringae	3476			✓		
Syringae	1680		✓✓			✓✓
Syringae	847	✓		✓		✓✓✓
Syringae	3525	✓✓✓✓	✓✓✓	✓✓✓✓✓	✓✓✓✓	✓✓✓
Syringae	6491	✓	✓	✓✓✓✓	✓✓	✓
Tomato	Pto 483			✓	✓	
Tomato	Pto 119		✓✓			

Tomato	4325		✓✓✓			
Tomato	DC3000	✓✓	✓✓✓	✓✓	✓✓	✓
Tomato	DC84-1	✓✓	✓✓✓	✓✓	✓✓✓	✓✓✓
Tomato	487	✓	✓✓✓	✓✓	✓	✓
Tomato	3357	✓	✓✓✓	✓✓✓✓	✓✓	✓✓
E. coli				✓✓		✓
Atropurpurea						
Glycinea	36a					
Glycinea	4182					
Glycinea	Race 4					
Maculicola	921					
Morsprunorum	239					
Morsprunorum	567					
Morsprunorum	3714					
Phaseolicola	482					
Phaseolicola	501					
Savastanoi	AT 13526					
Syringae	B301D					
Syringae	FF5					
Syringae	5076					
Syringae	1391					
Syringae	3910					
Syringae	6006					
Syringae	Pss61					
Tabaci						
Tagetis	53534					
Tomato	PT23					
Tomato	Pto 2811 Cit7					

P. syringae strains isolated from diverse geographical locations were analyzed for the presence of the EEL signature. EEL probes were designed from conserved regions and were used in hybridization experiments to establish strain relatedness. Probes from *Pst* DC3000 failed to consistently hybridize or detect (e.g. strain PT23) seven strains of pv. tomato including PT23, 4325, Pto 483, Pto 2811, Pto 119, 483, DC-84 (Table 16). EEL probes from pv. syringae failed to consistently hybridize or detect six strains of pv. syringae including strains HS191, 1680, 6006, Pss 61, 3476, and 3910 (Table 17). All

five pv. syringae probes failed to hybridize with four strains including B301D, FF5, 5076, and 1391 (Table 17). Although the probes were designed from conserved regions of the *Pst* DC3000 and B728a EEL region, they did not consistently hybridize to all strains of pvs. tomato and syringae, respectively.

Additionally, probes from *Pst* DC3000 consistently cross-hybridized to pv. maculicola 4981 (Table 16), while EEL probes from pv. syringae consistently cross-hybridized to strains in several pathovars including pv. tomato strains DC3000, 3357, 4325, pv. maculicola 4981, pv. phaseolicola strains F2, 1448A, 482, 501, 524, pv. glycinea strains 5562, 36a, 4180, 4182, and pv. morsprunorum strains 567, 239, 5795 (Table 17). Furthermore, probes from both pathovars cross-reacted with *E. coli* (Tables 16, 17). These results show that EEL probes from both pv. tomato and pv. syringae cross-react with other pathovars and do not clearly differentiate unrelated strains.

VITA

Christina M. Baker

Candidate for the Degree of

Doctor of Philosophy

Dissertation: STRAIN TYPING AND CHARACTERIZATION OF σ^{54}-DEPENDENT TRANSCRIPTIONAL ACTIVATOR MUTANTS IN *PSEUDOMONAS SYRINGAE* PV. TOMATO

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Name: Christina Baker Date of Degree: December, 2008

Institution: Oklahoma State University Location, Stillwater, Oklahoma

Title of Study: STRAIN TYPING AND CHARACTERIZATION OF σ^{54}-DEPENDENT TRANSCRIPTIONAL ACTIVATOR MUTANTS IN *PSEUDOMONAS SYRINGAE* PV. TOMATO

Pages in Study: 161 Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Sciences

Scope and Method of Study: *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000) is a gram-negative plant pathogenic bacterium that is virulent on tomato, *Brassica spp*. and Arabidopsis. One aspect of this study was the utilization of variable number tandem repeat loci (VNTR) by multiple locus variable number tandem repeat analysis (MLVA) for typing of *P. syringae* pv. tomato. The specific objectives of this project were to: (i) design and identify VNTR primer sets useful for strain typing; and (ii) use these to type a collection of *P. syringae* pv. tomato strains. A second aspect of this study focused on the characterization of σ^{54}-dependent transcriptional activator mutants of *Pst* DC3000. The main objectives of the study were to (iii) verify and complement transcriptional activator mutants in *Pst* DC3000 and determine the phenotype on host and nonhost plants; and (iv) analyze σ^{54}-dependent transcriptional activator mutants for traits relevant to specific activator (motility, nutrient utilization, alginate production).

Findings and Conclusions: Objective I. 34 VNTR primer sets were designed and five of these were identified for strain typing. These primer sets were used to type a collection of 58 *P. syringae* pv. tomato strains, and 23 different MLVA sequence types were identified (Objective II). This is the first report using MLVA to type a large collection of *P. syringae* pv. tomato strains of diverse origin. Objectives III and IV: The DC3000-*fleQ* mutant was non-motile, non-flagellated, and defective in biofilm formation, traits that were restored with a complementing clone; whereas the DC3000-*dctD2* mutant was defective in the ability to transport and/or utilize succinic acid as a carbon source. All of the mutants were able to elicit the HR on tobacco, and DC3000-*algB* showed a reduction in symptom production on Arabidopsis, suggesting a role for alginate in *Pst* DC3000 virulence. In summary, this study shows that σ^{54}-dependent activator genes in *Pst* DC3000 are important for pathogenicity, nutrient assimilation, and various physiological processes.

ADVISOR'S APPROVAL: _____

Dr. Carol L. Bender