CHARACTERIZATION OF A TYPE VI SECRETION SYSTEM AND RELATED PROTEINS OF *Pseudomonas syringae*

A Dissertation

by

ANGELA R. RECORDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Dennis C. Gross
Committee Members,	Carlos F. Gonzalez
	Paul de Figueiredo
	James E. Samuel
Head of Department,	Dennis C. Gross

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ABSTRACT

Characterization of a Type VI Secretion System and

Related Proteins of *Pseudomonas syringae*. (December 2008)

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Chair of Advisory Committee: Dr. Dennis C. Gross

Pseudomonas syringae is a pathogen of numerous plant species, including several economically important crops. *P. syringae* pv. *syringae* B728a is a resident on leaves of common bean, where it utilizes several well-studied virulence factors, including secreted effectors and toxins, to develop a pathogenic interaction with its host. The B728a genome was recently sequenced, revealing the presence of 1,297 genes with unknown function. This dissertation demonstrates that a 29.9-kb cluster of genes in the B728a genome encodes a novel secretion pathway, the type VI secretion system (T6SS), that functions to deliver at least one protein outside of the bacterial cell. Western blot analyses show that this secretion is dependent on *clpV*, a gene that likely encodes an AAA^+ ATPase, and is repressed by *retS*, which apparently encodes a hybrid sensor kinase. RetS and a similar protein called LadS are shown to collectively modulate several virulence-related activities in addition to the T6SS. Plate assays demonstrate that RetS negatively controls mucoidy, while LadS negatively regulates swarming motility.

A mutation in *retS* affects B728a population levels on the surface of bean leaves. A model for the LadS and RetS control of B728a virulence activities is proposed, and possible roles for the B728a T6SS are addressed.

DEDICATION

To my husband, Stephen Records, and my patient daughter, Abigail Erica Records.

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CHAPTER I

INTRODUCTION

Pseudomonas syringae inhabits plants throughout the world, causing infections in hundreds of host species, with symptoms ranging from leaf spots to stem cankers. *P. syringae* pv. *syringae* is an especially important pathogen, because certain strains within this pathovar defoliate vegetative crop plants and severely damage fruit, nut, and ornamental species. The bean pathogen *P. syringae* pv. *syringae* strain B728a is a particularly interesting strain, because it exhibits exceptional fitness on its host, where it persists as an opportunistic pathogen.

B728a is a highly versatile foliar pathogen that maintains "chronic" resident associations with plants that often lead to "acute" invasion of leaf tissue, resulting in disease. Appropriate environmental conditions contribute to this change in lifestyle. For example, driving rains allow the bacteria to reach leaf surface populations sufficient for invasion (115). Although the specific molecular triggers that mediate the switch from epiphytic resident to endophytic infectious agent are not completely understood, several well-studied virulence mechanisms are known to influence disease development (5, 23, 37). The *P. syringae* Hrp type III secretion system (T3SS) has been the subject of intense study (1, 2, 31, 59, 68, 103), because it delivers effector proteins into host cells where they have determinative effects on the host-pathogen interaction. B728a also produces the necrosis-inducing lipopeptide toxins syringomycin and syringopeptin, which have

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This dissertation follows the style of Journal of Bacteriology.

been well characterized by the Gross lab (25, 32, 40, 44, 63, 64, 92-94, 105-107, 118). The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters lie in a 180-kb chromosomal region, where they collectively represent 2.2% of the B728a genome.

Sequencing of the 6.094 Mb B728a genome was recently completed, which provided a valuable resource for researchers studying *P. syringae* and related pathogens (23). Analysis of the genomic region adjacent to the *syr-syp* phytotoxin cluster led to the identification of a 16.2-kb group of genes, including Psyr_2628, which is conserved and associated with virulence in medically important bacterial pathogens (82). In *P. aeruginosa*, expression of the Psyr_2628 ortholog (PA0073) is activated by *ladS*, which encodes a "lost adherence sensor", and is repressed by *retS* (regulator of exopolysaccharide and type III secretion)(73, 101). In addition to regulating expression of PA0073, *P. aeruginosa* LadS and RetS also mediate expression of a novel virulence locus HSI-I (Hcp secretion island-I), which encodes a type VI secretion system (T6SS)(73). Analysis of the B728a genome revealed a cluster of genes (which I refer to as the T6 locus) orthologous to the HSI-I locus of *P. aeruginosa*. The 29.9-kb B728a T6 locus appears to contain a full complement of T6SS genes, including those that likely constitute "core components" of the type VI machine (11).

Mougous et al. reported the discovery of the *P. aeruginosa* T6SS in *Science*, where they noted that loci related to HSI-I are widely distributed among bacterial pathogens and are predicted to "play a general role in mediating host interactions" (73). Bacterial pathogens maintain intimate interactions with their hosts through direct contact and via chemical conversation involving toxins, phytohormones, and other virulence

factors. In order to deliver chemical messages into the environment or directly into host cells, bacteria have evolved a number of secretion systems. Until recently, only five secretion systems had been identified in Gram-negative bacteria (Fig. 1.1). The type I secretion system utilizes ATP-binding cassette proteins in the exportation of proteins from the cytoplasm into the environment (100). B728a relies on a type I secretion system for the transport of syringomycin and syringopeptin (44). The T3SS is critical for *P. syringae* virulence. At least 22 type III effectors are injected into host cells via this important pathway (103). Sec-dependent, type II secretion contributes to *P. syringae* pathogenicity by exporting additional proteins and virulence factors (9). Two other secretion systems are vital for certain bacteria-host interactions. The type IV pathway facilitates delivery of proteins and single-stranded-DNA-protein complexes into host cells (13, 18). The type V autotransporter pathway is one of the most widely-distributed Gram-negative bacterial secretion systems, and its substrates (mostly virulence factors) mediate their own transport across the outer membrane (36).

Very recently, the T6SS was described (19, 84), but critical findings that led to its discovery can be traced back over a decade. In 1997, Roest and colleagues identified a *Rhizobium leguminosarum* biovar *trifolii* genetic locus that inhibits the symbiont's ability to effectively nodulate pea (86). Sequencing of the "*imp*" locus (impaired in nodulation) revealed no significant homology to any genes known at the time of its discovery. It is clear now, however, that *R. leguminosarum* is one of many plant- and animal-associated bacterial species that carry such clusters in their genomes – the locus is found in at least 36 Gram-negative species (97). Clues to the function of the

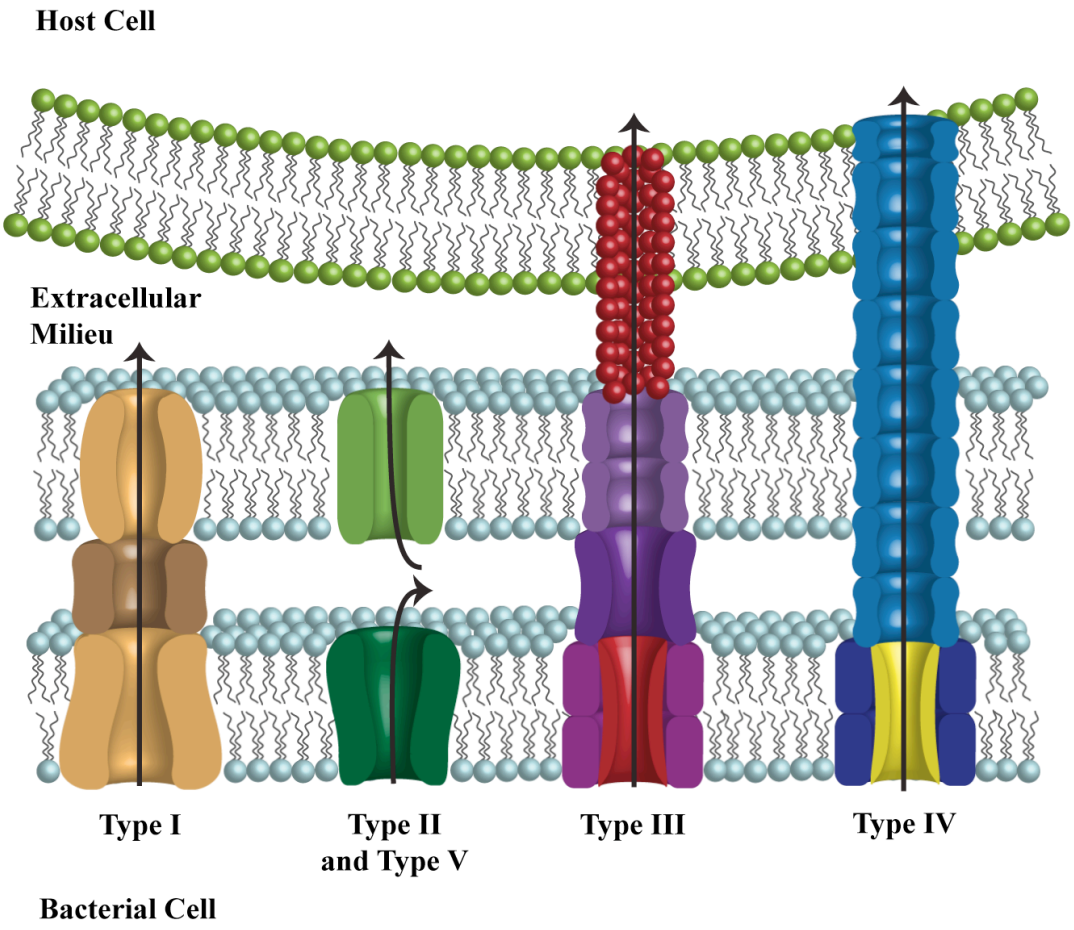

FIG. 1.1. An overview of Gram-negative protein secretion pathways. The type I pathway is a one-step secretion system that releases various molecules outside of the bacterial cell. Gram-negative bacteria may also use the two-step type II and type V pathways to secrete virulence proteins into the extracellular milieu. The type III and type IV secretion systems provide direct routes for virulence factors into host cells, where they are delivered through the host-cell plasma membrane.

imp locus came in 2002, when proteomic analyses of culture supernatants revealed that the *R. leguminosarum impJ* operon is required for the secretion of a 27-kDa protein (8). Similar observations were made for other organisms, including the fish pathogen *Edwardsiella tarda* (85), and the human pathogens *Vibrio cholerae* and *Pseudomonas aeruginosa* (73, 84). Each of these pathogens secretes at least one protein into culture, and this novel mechanism of protein secretion is linked to the *imp* locus and, importantly, to virulence.

The proteins encoded by the *imp* locus and its counterparts in other bacteria make up a bona fide "type VI secretion system", which appears to have an important role in pathogen/symbiont-host interactions. Several of the genes in the T6 locus have been implicated in virulence activities, including chronic lung infection in rats (82), replication in protozoa (16, 121), adherence to human intestinal cells (16, 84), and actin cross-linking in eukaryotic cells (84).

Most of our limited knowledge about regulation of the T6SS stems from whole-genome microarray studies of *P. aeruginosa*, which indicate that the HSI-I T6 locus is negatively-controlled by RetS (29, 52, 53, 101) and positively-regulated by LadS (101). Interestingly, RetS/LadS control of the *P. aeruginosa* T6SS is consistent with a switch between acute and chronic colonization of host lung tissue (101). RetS and LadS appear to be global regulators that mediate the switching-off of genes necessary for an acute infection of the lung (e.g., the T3SS) and a switching-on of genes required for long-term colonization (e.g., exopolysaccharide production). Based on this pattern of regulation,

and on the phenotypes described above, the *P. aeruginosa* HSI-I clearly has a role in chronic infection (73).

I have identified orthologs of both *P. aeruginosa retS* and *ladS* within the genome of *P. syringae* B728a. The predicted protein products for these genes, Psyr_4339 (*ladS*) and Psyr_4408 (*retS*), share relatively high identity with their *P. aeruginosa* counterparts, as well as conserved domain organization. I hypothesized that B728a RetS and LadS fill roles similar to those of their homologs in *P. aeruginosa* - as regulators of the T6SS and activities associated with a switch between "chronic" and "acute" infection of bean. In this dissertation, I demonstrate that the B728a T6SS is functional and that RetS and LadS oppositely regulate its expression. In addition, I show that RetS and LadS collectively modulate several B728a virulence-related activities, including swarming, mucoidy, and colonization of bean leaves.

CHAPTER II

IDENTIFICATION OF A FUNCTIONAL TYPE VI SECRETION SYSTEM IN *Pseudomonas syringae*

OVERVIEW

The widespread phytopathogenic bacterium *Pseudomonas syringae* relies on protein secretion systems to mediate interactions with the environment and with plant hosts. I have identified a novel type VI secretion system (T6SS) in *P. syringae* pv. *syringae* strain B728a. The B728a T6 locus features 22 genes, including those that are highly conserved among bacterial pathogens and are thought to make up the T6SS machinery. In this dissertation, I demonstrate that at least one protein, called Hcp, is secreted into culture by the B728a T6SS. The *clpV* gene, which encodes an AAA^+ ATPase, is required for Hcp secretion, presumably because it provides the energy necessary for the secretion process. The T6SS has been associated with virulence in several Gram-negative pathogens, where it apparently contributes to bacterial persistence in the eukaryotic host. A B728a *clpV* mutant multiplies *in planta* and produces disease symptoms on bean plants that are similar to those caused by wild-type B728a. Likewise, a mutation in the *clpV* gene does not compromise the pathogen's ability to adhere to poly(vinyl chloride) or polypropylene or to colonize the surface of bean leaves. Possible roles for the B728a T6SS are discussed, as are future strategies for elucidating its contribution to fitness.

INTRODUCTION

Pseudomonas syringae pv. *syringae* strain B728a is a pathogen of bean that is highly adapted to its host, where it resides as an epiphyte and is responsible for a leaf spot disease that ultimately defoliates the plant. Like other members of the *syringae* pathovar, B728a is particularly adept at enduring the environmental stressors associated with the leaf surface, including desiccation, sunlight, and limited access to nutrients (37, 41, 58). This survivability is significant because epiphytic B728a populations serve as inocula for subsequent initiation of disease (87). B728a has at its disposal an arsenal of virulence factors, including the pore-forming phytotoxins syringomycin and syringopeptin (92) and at least 22 effector proteins delivered into host cells by the type III secretion system (T3SS)(103). The T3SS is required for *P. syringae* to cause disease, and phytotoxins, secreted by the type I secretion pathway, significantly increase disease severity (6, 116). This underscores the critical role that secretion systems play in bacterial pathogenesis.

Until recently, only five distinct secretion systems had been identified in Gram-negative bacteria (Fig. 1.1). These secretion pathways, types I-V, facilitate the transport of proteins from the bacterial cytoplasm to the outside of the cell or, in some cases, directly into the cells of a eukaryotic host. Because of their critical roles in pathogenicity, bacterial secretion systems are widely studied and have been the subjects of several excellent reviews (4, 14, 26, 27, 31, 88, 109, 117). In 2006, a new secretion pathway was described and named the type VI secretion system (T6SS)(19, 84). The T6SS has since been associated with virulence in at least 13 Gram-negative pathogens of

both animals and plants (67, 79, 81, 82, 99, 119). I have identified a 29.9-kb region of the B728a genome that is homologous to the highly-conserved T6 locus (Fig. 2.1).

Genetic mutations within the T6 locus have various phenotypic consequences (Table 2.1). In many cases, the T6SS appears to facilitate processes associated with persistence in the host or in the environment. For example, the *Salmonella enterica* SCI T6SS curbs intracellular growth in macrophages at late stages of infection, limiting the toxic effect of the bacteria and resulting in decreased mortality rates in mice (79). In *P. aeruginosa*, mutations in the HSI-I T6 locus decrease virulence in the rat model of chronic pulmonary infection (82), and microarray studies indicate that genes in the HSI-I locus are expressed during persistent, chronic infection of the human lung (29). Although not involved in intracellular growth, bacterial persistence in the lung and in the outside world is largely dependent on biofilm formation (15). Both the *Vibrio parahaemolyticus* T6 locus and the *P. aeruginosa* HSI-I have been implicated in biofilm formation (22, 82, 98). These species are ubiquitous in the environment, where they rely on multicellular biofilm communities for survival (21).

Several studies have demonstrated the in-culture secretion of the Hcp protein, which is encoded by a gene in the T6 locus (11). The Hcp1 protein of *P. aeruginosa* was shown to form a hexameric ring structure that may create a channel associated with secretion activity (73). Hcp secretion is dependent on other genes in the T6 locus, and in a recent study of the *Edwardsiella tarda* Evp T6SS, systematic deletions revealed that 13 of the 16 *evp* genes are required for export of the Hcp-like EvpC protein (Table 2.1)(119).

FIG. 2.1. Gene organization of the type VI secretion system locus present in the genomes of various Gram-negative bacteria. Arrows of the same color represent homologous genes. Genes with no homologs in other T6SS loci are colored white. Each gene is marked with its name, if previously annotated. Genes from fully sequenced genomes are labeled with their NCBI locus tags. To conserve space, only two of *Burkholderia pseudomallei*'s six T6SS loci are shown. HSI, Hcp-secretion island (73); *ppk*, *Pseudomonas* protein kinase (104); *ppp*, *Pseudomonas* protein phosphatase; *icm*, intracellular multiplication (66); *fha*, forkhead associated; *hcp*, haemolysin co-regulated protein (112); *clp*, caseinolytic protease (45); *vgr*, valine glycine repeats (108); *stp*, serine/threonine phosphatase (75); *stk*, serine threonine kinase (75); *sci*, *Salmonella* centrisome island (24); *rhs*, recombination hot spot (108); *tss*, type six secretion (96); *tag*, *tss*-associated gene (96); *vas*, virulence-associated secretion (84); *evp*, *Edwardsiella* virulence protein (85); *imp*, impaired in nodulation (8).


P. aeruginosa
HSI-I
pppA
icmH1
hcp1
ppkA
icmF1
fha1
clpV1
vgrG1a
vgrG1b
PA0071 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95
HSI-II
hcp2
icmH2
stp1 stk1
vgrG2
clpV2
fha2
icmF2
PA1511 12 PA1656 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71
HSI-III
icmH3
hcp3
icmF3
clpV3
vgrG3
PA2359 60 61 62 63 64 65 66 67 68 69 70 71 72 73
P. syringae pv. tomato DC3000
PSPTO_2538 39 41 43 44 45 46 47 48 49 50 51 52 53 54 PSPTO_2874 75 76 77
PSPTO_5414 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
P. syringae pv. syringae B728a
Psyr_2626 27 28 29 Psyr_4953 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
Salmonella enterica
rhs2
sciA sciB sciC D E F sciG H sciI J K L M N sciO sciP Q R sciS T U V vgrS W rhs1 X Y
Burkholderia pseudomallei
tagJ tagK
TSS-2
La tagL B tssC D J tssK Lb tssM tssI rhs tssA tssG tssF E tssH
BPSS0095 096 097 098 099 100 101 102 103 104 105 106 108 109 110 111 112 113 114 115 116
tagF
tagI
TSS-6
tssI tssH tssG tssF E D tssC B tssA tagE tssM tssL tssK J tagH
BPSS2093 094 095 096 097 098 099 100 101 102 103 104 105 106 107 108 109
Vibrio cholerae
hcp2
hcp1
vgrG2 vasA B vasC D vasE F vasG vasH I vasJ vasK L vgrG3 vgrG1
VCA0017 18 VCA0107 08 09 10 11 12 13 14 15 16 17 18 19 20 21 22 VC1415 16
Edwardsiella tarda
P A evpB C evpD E evpF G evpH evpI J K L evpM N evpO
Rhizobium leguminosarum
impS R Q P impO A B impC impD E F impG H I J K impL impM N
Agrobacterium tumefaciens
impS R Q P clpB A B impC impD E F impG H I J K impL impM N
Atu4348 47 46 45 44 43 42 41 40 39 38 37 36 35 34 33 32 31 30
Pectobacterium atrosepticum
ECA3444 43 42 41 40 39 38 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21
1 kb

TABLE 2.1. Characteristics of type VI secretion-related proteins

Protein[a]	Homolog[b]	Assigned function	Phenotype(s)
TssA*	EvpK (*E.t.*)	inner membrane protein (69, 97)	↓ virulence in fish (119)
TssB*	EvpA (*E.t.*)	unknown	↓ virulence in fish (85, 119)
TssC*	EvpB (*E.t.*)	unknown	↓ virulence in fish (85, 119)
			↓ growth in phagocytes (85)
TssD*	TssD (*B.m.*)	required for type VI secretion (90)	ND
	EvpC (*E.t.*)	secreted protein (85, 119)	↓ virulence in fish (85)
			↓ growth in phagocytes (85)
	Hcp1 (*P.a.*)	secreted ring-shaped secreted protein (73)	ND
	Hcp1 (*P.at.*)	secreted protein (67)	↑ potato tuber rotting (67)
TssE*	TssE (*B.m.*)	required for type VI secretion (90)	ND
	EvpE (*E.t.*)	unknown	↓ virulence in fish (119)
TssF*	EvpF (*E.t.*)	unknown	↓ virulence in fish (119)
	VasA (*V.c.*)	unknown	↑ virulence in protozoa (84)
TssG*	EvpG (*E.t.*)	cytoplasmic protein (97)	↓ virulence in fish (119)
TssH*	EvpH (*E.t.*)	unknown	↓ virulence in fish (119)
	ClpV1 (*P.a.*)	AAA^+ ATPase (73)	↓ swarming (82)
		required for type VI secretion (73)	↓ chronic rat lung infection (82)
TssI*	EvpI (*E.t.*)	secreted protein (119)	↓ virulence in fish (119)
	VgrG (*V.c.*)	secreted phage tail spike-like protein (83)	actin cross-linking (84)
TssJ*	EvpL (*E.t.*)	outer membrane lipoprotein (97)	↓ virulence in fish (119)
TssK*	EvpM (*E.t.*)	unknown	↓ virulence in fish (119)
	ImpJ (*R.l.*)	unknown	nitrogen fixation in pea (8)
TssL*	EvpN (*E.t.*)	unknown	↓ virulence in fish (119)
	VasF (*V.c.*)	structural protein (97)	↓ virulence in protozoa (84)
TssM*	EvpO (*E.t.*)	unknown	↓ virulence in fish (119)
	VasK (*V.c.*)	involved in cell surface recognition (16)	↑ adherence to human intest. cells (16)
			↑ conjugation frequency (16)
			↓ growth/virulence protozoa (16, 84)
	IcmF1 (*P.a.*)	unknown	↓ biofilm formation (82)
	SciS (*S.e.*)	unknown	↑ growth in macrophages (79)
			↑ virulence in mice (79)
TagE	PpkA (*P.a.*)	serine-threonine kinase (74)	↓ virulence in neutropenic mice (104)
		post-translational regulator of T6SS (74)	
TagF		cytoplasmic (11)	ND
TagH	Fha1 (*P.a.*)	cytoplasmic (74)	ND
		involved in activation of T6SS machinery (74)	
TagJ		cytoplasmic (11)	ND
TagO	VasI (*V.c.*)	unknown	ND
--	PA0071 (*P.a.*)	unknown	ND
--	PA0072 (*P.a.*)	unknown	ND
--	PA0073 (*P.a.*)	ABC ATP-binding protein (82)	↓ biofilm formation, ↓ swarming (82)
			↓ chronic rat lung infection (82)
--	Stk1 (*P.a.*)	serine threonine kinase (75)	ND
--	Stp1 (*P.a.*)	serine threonine phosphatase (75)	ND
--	VasH (*V.c.*)	sigma 54 activator (84)	↓ virulence in protozoa (84)
		required for type VI secretion (84)	
--	EvpJ (*E.t.*)	unknown	↓ virulence in fish (119)
--	EvpP* (*E.t.*)	secreted protein (119)	↓ virulence in fish (119)

[a] Protein names according to universal nomenclature proposed by Shalom et al. (96). Proteins encoded by genes that are required for type VI secretion in *Edwardsiella tarda* are marked with an asterisk (119). --, no universal name assigned. [b] Only homologs with assigned functions and/or phenotypes are listed. Homolog names are followed by species names in parentheses. *E.t.*, *Edwardsiella tarda*; *B.m.*, *Burkholderia mallei*; *P.a.*, *Pseudomonas aeruginosa*; *P.at.*, *Pectobacterium atrosepticum*; *V.c.*, *Vibrio cholerae*; *R.l.*, *Rhizobium leguminosarum*; *S.e.*, *Salmonella enterica*.

One of the genes required for Hcp secretion, *clpV*, encodes a AAA^+ ATPase and is likely responsible for energizing the transport process (91). In this study, I demonstrate that the B728a T6 locus encodes a functional T6SS capable of Hcp secretion and that the secretion of Hcp is dependent on intact *clpV.* This is the first report of an active *P. syringae* T6SS. This dissertation addresses the potential role that type VI secretion plays in B728a pathogenicity and the hypothesis that the T6SS is involved in biofilm formation on the leaf surface and persistence in the phyllosphere environment.

RESULTS

Sequence analysis of the B728a T6 locus

In order to determine if the B728a genome carries a T6 locus, I obtained the sequence of the *P. aeruginosa* ClpV1 protein from NCBI (NP_248780) and used it in a BLAST search of the B728a genome (blastp, NCBI). Three B728a proteins showed strong homology (bit scores >200) to *P. aeruginosa* ClpV1. The genes Psyr_4958, Psyr_0728, and Psyr_3813, which correspond to the homologous proteins YP_238023 (bit score = 818, E value = 0.0), YP_233834 (bit score = 279, E value = 7e-76), and YP_236253 (bit score = 206, E value = 1e-53), respectively, were viewed in a genomic context via NCBI Genome Overview. Like *clpV1*, Psyr_0728 and Psyr_3813 are predicted to encode AAA^+ ATPases, but they do not appear to be associated with any other HSI-I homologs. Psyr_4958, however, is flanked by genes with homology to those in the *P. aeruginosa* HSI-I T6 locus (Fig. 2.1). Through systematic BLAST searches of the genomic sequence surrounding Psyr_4958, I was able to define the B728a T6 locus.

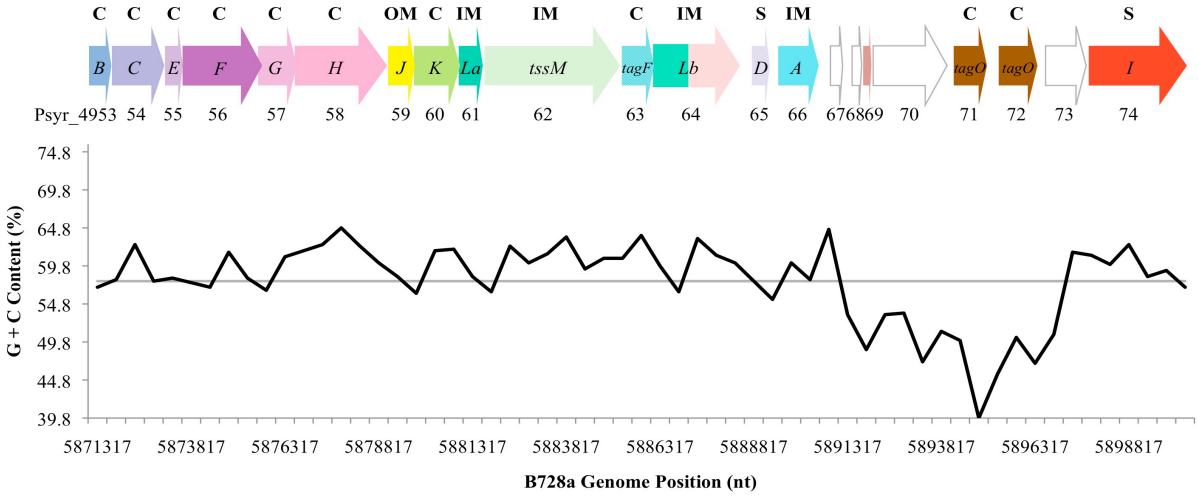

FIG 2.2. Schematic representation of the *P. syringae* pv. *syringae* B728a type VI locus and its corresponding G + C content. Arrows represent genes and are labeled according to the universal nomenclature proposed by Shalom et al. (96). The corresponding NCBI locus tag is listed below each gene. The predicted membrane localization for each gene product is shown (C, cytoplasm; OM, outer membrane; IM, inner membrane; S, secreted). Arrow colors are consistent with those used in Fig. 2.1. The G + C content for the region was analyzed via the CpG Ratio and GC Content Plotter (http://mwsross.bms.ed.ac.uk/public/cgi-bin/cpg.pl) with a window size of 500.

The B728a T6 locus is confined to a 29.876-kb region of the genome that includes 22 ORFs predicted to be transcribed in the same direction (Fig. 2.2) and likely as part of a single operon (19, 119). The G + C content of the T6 locus was analyzed via the CpG Ratio and GC Content Plotter (http://mwsross.bms.ed.ac.uk/public/cgi-bin/cpg.pl), with a window size of 500, and is depicted in graphical form in Fig. 2.2. The average G + C content for the T6 locus is 57.8%, which is similar to that of the rest of the B728a genome (58.5%), indicating that the cluster was not recently acquired by horizontal transfer. However, a 5-kb region of the locus, spanning from Psyr_4967 to Psyr_4972, maintains a strikingly low G + C content (49.1%), suggesting that the acquisition of these particular genes may have been recent. Only two of the six ORFs in the low-GC region have homology to previously identified T6SS-related genes.

Functions have been demonstrated for only a few of the proteins encoded by T6 loci (Table 2.1), although it is assumed that most of the T6 locus genes encode proteins that make up the T6SS machinery (11). Using three different web-based prediction programs (described in Materials and Methods), I determined the likely subcellular location for each of the T6 locus-encoded proteins (Fig. 2.2). The programs reached a consensus for most of the proteins, and in all cases, at least two of the three programs agreed on predicted localization.

An effective method for insertional mutagenesis in B728a

Because the *clpV* gene is predicted to encode an ATPase required for type VI secretion, it was selected for mutagenesis. Using the strategy outlined in Fig. 2.3, an

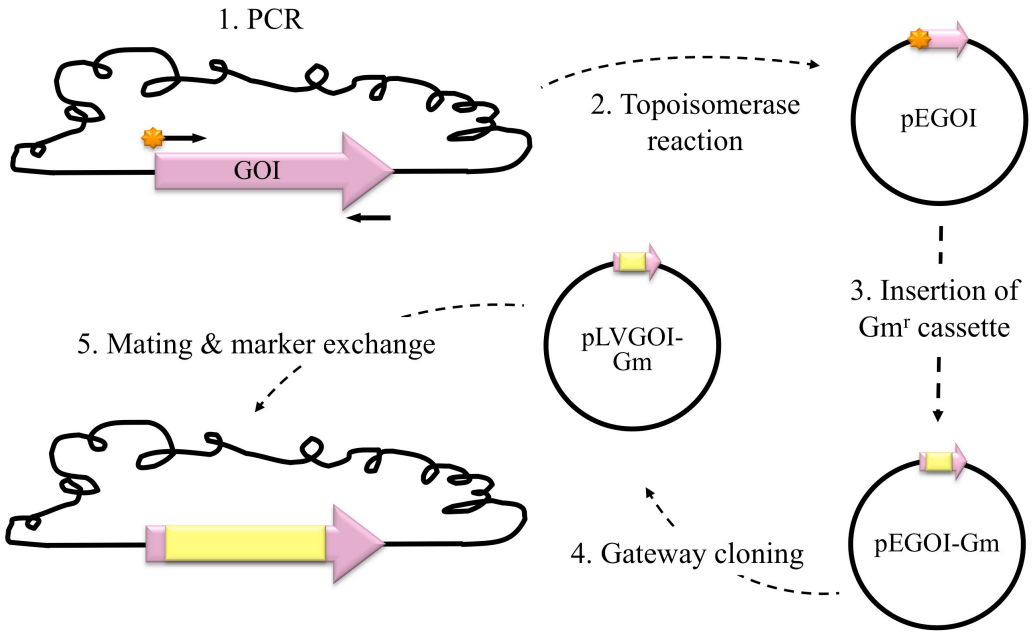

FIG. 2.3. Diagram of the strategy used for insertional mutagenesis of *P. syringae* genes. (1) The gene of interest (GOI, pink) is amplified from the B728a genome via PCR with a forward primer that contains the topoisomerase recognition sequence (orange star) on its 5' end. (2) The PCR product is moved into the pENTR/D-TOPO Gateway entry vector via a topoisomerase reaction and is then (3) disrupted by insertion of the *aacC1* cassette, which provides gentamycin resistance (Gm^r, yellow). (4) Gateway cloning is used to move the GOI::*aacC1* construct into the highly mobilizable pLVCD vector, resulting in pLVGOI-Gm. (5) Tri-parental mating is set up between *E. coli* carrying pLVGOI-Gm and B728a. The GOI within the B728a genome is replaced with GOI-Gm, via marker exchange.

insertion was made in the B728a *clpV* gene (Psyr_4958). Briefly, the 2.6-kb *clpV* gene was amplified by PCR and cloned into pENTR/D-TOPO by topoisomerase reaction, resulting in construct pEclpV. The *aacC1* gentamycin (Gm) resistance gene was used to disrupt *clpV*, and then the *clpV*-Gm entry construct was recombined into the pLVCD Gateway destination vector, resulting in pLVclpV-Gm. Mating between wild-type B728a and *E. coli* (pLVclpV-Gm) and selection for marker exchange resulted in a B728a *clpV* mutant. The *clpV* mutation was confirmed by colony PCR of two putative mutant colonies, which gave products 900 bp larger than that produced by PCR with a wild-type B728a colony as a template (Fig. 2.4). The *clpV* mutant, named B728aAR1, exhibited a normal growth rate in culture and colony morphology similar to that of wild-type B728a.

B728a secretes Hcp in a T6SS-dependent manner

To determine whether Hcp is secreted in culture by the B728a T6SS, a plasmid construct was made, pRHhcp-vsv, which expresses Hcp with a C-terminal fusion to the vesicular stomatitis virus glycoprotein (VSV) epitope. In order to ensure expression of *hcp-vsv*, the sequence was placed in frame with a *lac* promoter on the broad host-range vector pRH002 (33). Proper orientation and tagging of *hcp* were confirmed by sequencing of pRHhcp-vsv, and the construct was introduced into wild-type B728a and derivative strains.

Secretion assays were performed with these strains, and Hcp-VSV was localized to supernatant fractions from wild-type B728a cultures, indicating that Hcp is secreted in

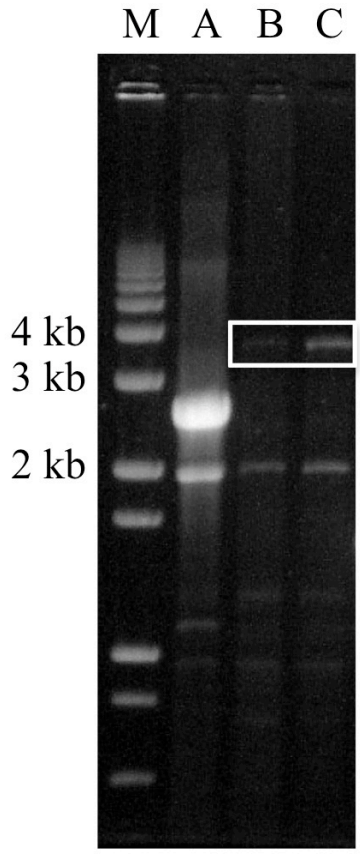

FIG. 2.4. Confirmation of a mutation in the *clpV* gene of *P. syringae* pv. *syringae* B728a. Colony PCR was set up using primers specific to DNA flanking the *clpV* gene in the B728a genome. PCR templates were (A) a wild-type B728a colony and (B,C) two putative B728a *clpV* mutant colonies. A 2.6-kb PCR product was expected for wild-type *clpV*, while a 3.5-kb product was expected for *clpV*::*aacC1* (marked by a white square). M, 1 kb Plus DNA ladder (Invitrogen, Carlsbad, Calif.).

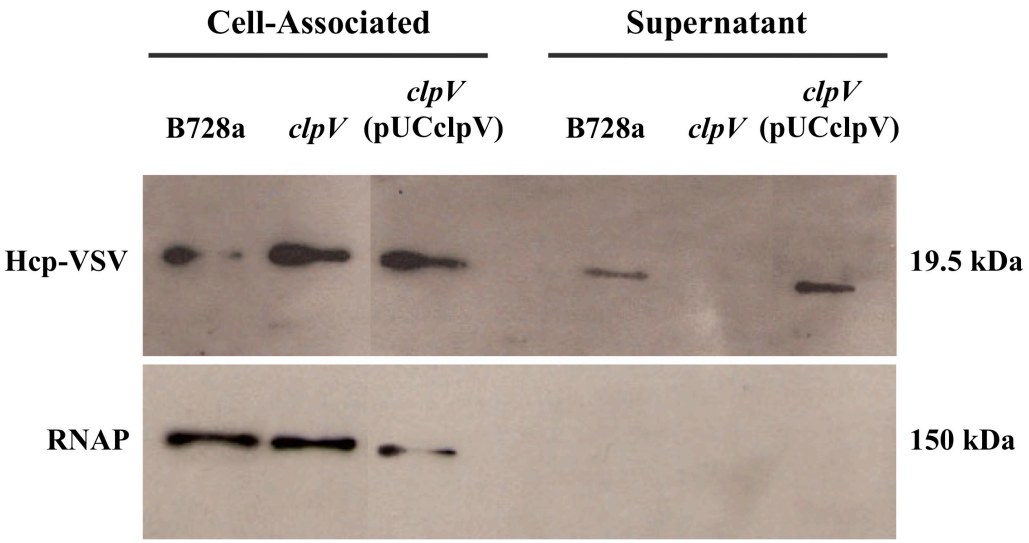

FIG. 2.5. Extracellular secretion of Hcp-VSV as observed by Western blot analyses. Strains carrying plasmid-borne *hcp-vsv* were grown to mid-log phase at 25°C in NBY. Cultures were separated into cell-associated and supernatant fractions as described in Materials and Methods. Equal sample quantities were run on SDS-PAGE gels, and Western blots were probed with antibodies to the vesicular stomatitis virus glycoprotein (VSV-G) epitope. In order to assess the quality of cell-associated and supernatant fractions, blots were also probed with antibodies to the β-subunit of RNA polymerase (RNAP), an abundant protein that is not secreted. Hcp-VSV was expected to have a molecular weight of 19.5 kDa (18.2 kDa Hcp + 1.3 kDa VSV-G epitope). The RNAP protein size is 150 kDa.

culture. Fig. 2.5 shows a representative Western Blot. Hcp-VSV was undetectable in culture supernatants from the B728a *clpV* mutant, suggesting that a functional T6SS is required for secretion of Hcp. The presence of extracellular Hcp was restored when pClpV, which carries an intact copy of the *clpV* gene, was introduced into the B728a *clpV* mutant. The ImageJ program (http://rsbweb.nih.gov/ij/) was used to estimate protein concentrations on Western blots by measuring band intensity. The experiment was repeated three times. On average, approximately 26% of the Hcp-VSV present in the wild-type B728a cultures was located in the supernatant fractions. No Hcp-VSV was present in the supernatant fractions of B728aAR1. Approximately 22% of the Hcp-VSV present in the B728aAR1(pClpV) cultures was located in the supernatant, indicating that intact *clpV in trans* is able to fully complement the B728aAR1 secretion phenotype.

The B728a *clpV* mutant does not exhibit reduced adhesion ability

Because genes within the *P. aeruginosa* T6 locus are reportedly involved in biofilm formation (16, 82), assays were performed to determine if *clpV* contributes to B728a adhesion ability. Standard adhesion assays were conducted, based on the procedures described by Koutsoudis et al. (50) and by O'Toole and Kolter (77). Because glucose enhances biofilm formation by *Pantoea stewartii* (50), B728a and B728aAR1 cultures were grown in LB + 0.2% glucose (LBG) to an OD_{600} of 0.1. *Pseudomonas fluorescens* Pf-5, which readily forms biofilms on various surfaces, was used as a positive control for this assay (77). Aliquots of culture were added to the wells of poly(vinyl chloride) 96-well plates. These plates provide excellent substrate for biofilm formation (50). The plates were incubated at 25°C for 12, 24, or 48 h with or without

shaking. Cell density was measured, and the wells were then vigorously washed under running tap water. A 1% crystal violet solution was added to each well. The plates were incubated at room temperature, washed again, and then the crystal violet was solubilized by the addition of 95% ethanol to each well. A_{590} (the maximum absorbance wavelength for crystal violet) was determined via plate reader. The A_{590} values for all of the sample wells were averaged for each strain. The average A_{590} values for Pf-5, B728a, and B728aAR1 were 1.31 ± 0.49, 0.16 ± 0.41, and 0.18 ± 0.19, respectively (mean ± SD). The results of this assay were highly variable, and no statistically valid differences were observed. A possible explanation for this variability is that it was difficult to wash all of the wells evenly. It is clear that, under the conditions tested, Pf-5 forms biofilms much better than B728a does. However, it was difficult to identify any differences between B728aAR1 and wild-type B728a.

In an alternative approach, we used a strategy that employed polypropylene tubes (77). Overnight NBY cultures were diluted to an OD_{600} of 0.05 in LBG or syringomycin minimal medium containing 100 μM arbutin and 0.1% fructose (SRM_{AF}), and then aliquots were placed in sterile 2-ml polypropylene microcentrifuge tubes. The tubes were incubated at 25°C for 10 h with or without shaking. After incubation, 1% crystal violet was added to each tube. The tubes were incubated at room temperature for 15 min, rinsed several times with tap water, and allowed to air dry. 95% ethanol was added to each tube. The tubes were incubated for 5 min, and then they were emptied and allowed to air dry. The tubes were inspected for the presence of a blue ring at the air-surface interface, which is indicative of a biofilm (77). Very thick blue rings were visible in all

of the tubes that had been inoculated with Pf-5 (data not shown). No rings were present in tubes that had contained B728a or B728aAR1 cultured in LBG. However, both B728a and B728aAR1 grown in SRM_{AF} (shaking and static) produced heavy biofilm rings that appeared identical to each other and to those present in the Pf-5 tubes. The experiment was performed three times with consistent results. This demonstrates that B728aAR1 is not impaired in its ability to form biofilms on polypropylene. These results also show that SRM_{AF} is conducive to biofilm formation by B728a, while LBG is not a good medium to use for adhesion assays with this bacterium.

The B728a *clpV* mutant reaches wild-type phyllosphere population levels

To address the possibility that the T6SS contributes to B728a colonization of the leaf surface, three two-week-old bean plants were each dipped in suspensions containing 10^5 CFU/ml of either wild-type B728a or the *clpV* mutant B728aAR1. Some of the plants were placed in a 25°C humid chamber, while the others were maintained under low relative humidity (RH) at 25°C for 24 h. Five leaves were removed from each plant. The bacteria were dislodged from the leaves by sonication, and populations were enumerated by dilution plating. The experiment was repeated three times. At both low and high RH, B728aAR1 exhibited population numbers similar to those of wild-type B728a. The average bacterial numbers recovered from leaves inoculated with B728a and incubated at low and high RH were $3.5 \pm 1.7 \times 10^5$ CFU/g and $2.0 \times 10^6 \pm 6.6 \times 10^5$ CFU/g of leaf tissue (mean ± SD), respectively. Average populations recovered from leaves inoculated with B728aAR1 and maintained at low and high RH were $4.1 \pm 1.3 \times 10^5$ CFU/g and $1.8 \times 10^6 \pm 2.6 \times 10^5$ CFU/g of leaf tissue, respectively. The differences

between B728a and B728aAR1 populations were not statistically significant (two-tailed *t*-test, $P = 0.64$), which suggests that *clpV* is not required for B728a colonization of leaf surfaces.

The B728a *clpV* mutant multiplies *in planta* and produces disease symptoms similar to those caused by wild-type B728a

To determine if *clpV* contributes to plant-microbe interactions beyond leaf colonization, pathogenicity assays were carried out by vacuum infiltration of bean plants with 10^6 CFU/ml suspensions of wild-type B728a, the B728a *clpV* mutant B728aAR1, or a B728a *gacS* mutant, which is unable to cause disease (49). Each bacterial strain was tested on three individual bean plants, and the experiment was repeated three times. B728aAR1 showed no reduction in its ability to produce foliar disease symptoms, as compared to wild-type B728a (Fig. 2.6A).

Bacterial populations in infected plants were monitored over a 3-day period. At three days post-inoculation, the B728aAR1 and wild-type B728a bacterial titers were $1.4 \times 10^8 \pm 6.7 \times 10^7$ CFU/cm^2 and $1.8 \times 10^8 \pm 1.5 \times 10^7$ CFU/cm^2 (mean ± SD), respectively. These differences were not statistically significant (two-tailed *t*-test, $P = 0.49$), indicating that *clpV* is not required for multiplication *in planta* (Fig. 2.6B). As expected, the B728a *gacS* mutant was reduced in its ability to grow in bean leaves, as indicated by a population of $1.4 \times 10^4 \pm 5.3 \times 10^3$ CFU/cm^2 at three days post-inoculation.

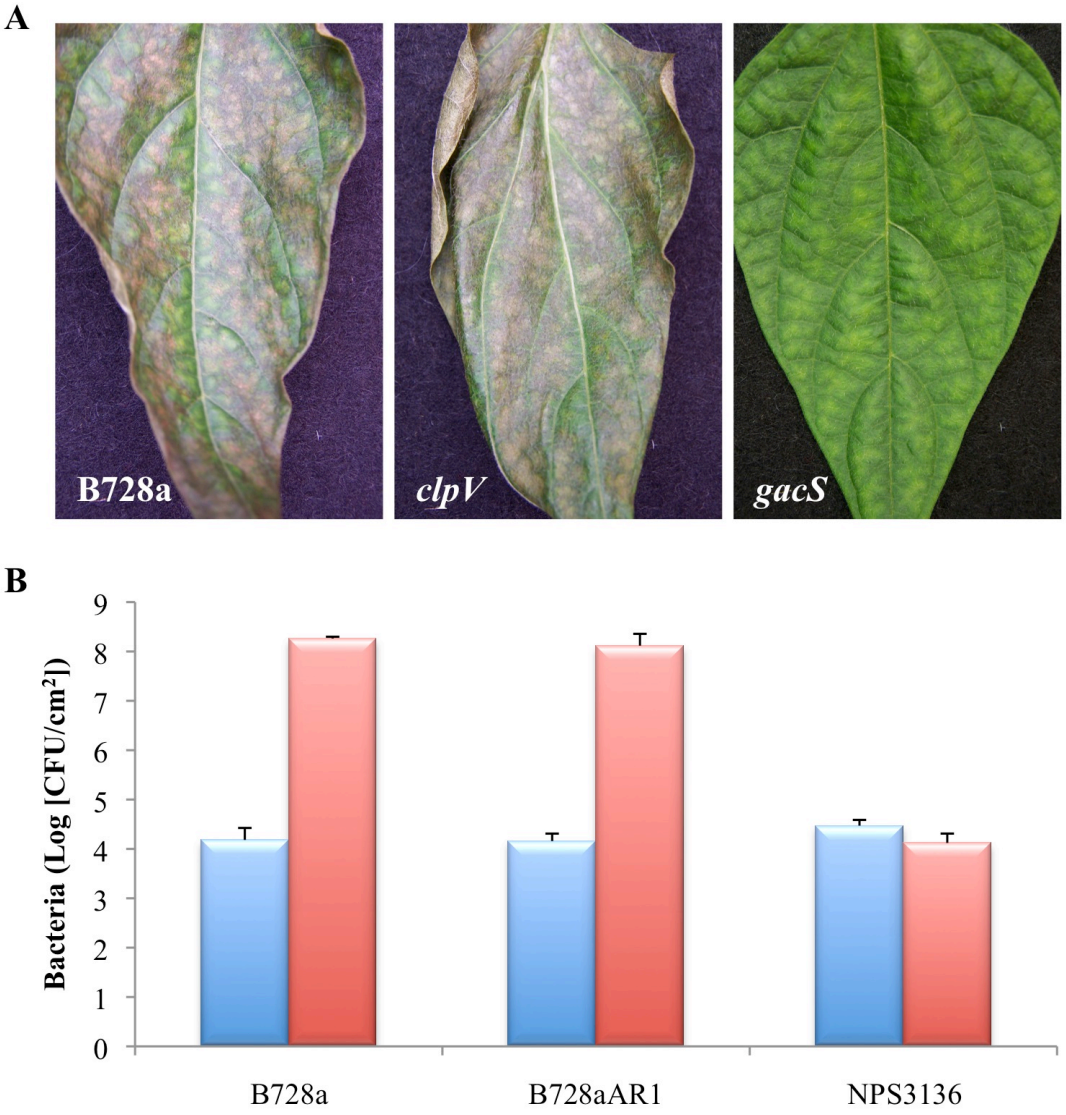

FIG. 2.6. Assay of *clpV* contribution to *P. syringae* pv. *syringae* B728a symptom production and growth in bean. (A) Bean leaves were inoculated via vacuum infiltration with suspensions containing 10^6 CFU/ml of either B728a, the B728a *clpV* mutant B728aAR1, or the B728a *gacS* mutant NPS3136 in water. Plants were maintained at 25°C in a growth chamber for 72 h. The experiment was performed in triplicate; representative results are shown. (B) *In planta* populations of the bacterial strains shown in panel A were monitored over a 3-day period. Blue and red bars represent Day 0 and Day 3 populations, respectively.

DISCUSSION

I have shown that the B728a T6 locus encodes a functional secretion system. At least one protein, Hcp, travels this secretion pathway and may be found in the supernatant of B728a cultures (Fig. 2.5). The crystal structure of Hcp1, encoded by the *P. aeruginosa* HSI-I T6 locus, has been determined. Mougous et al. showed that Hcp1 is a hexameric ring with an internal diameter of 40 Å, leading them propose that, upon secretion, the donut-shaped Hcp protein may stack to form a T6SS pilus through which other proteins are secreted (73). This hypothesis is supported by the fact that *hcp* is required for the secretion of another T6 locus-encoded protein, VgrG (84, 119). It is also possible that Hcp is delivered into host cells, much like the effector proteins of the T3SS (14). Indeed, Suarez et al. demonstrated that Hcp from *Aeromonas hydrophila* is translocated into human colonic epithelial cells (99). My results confirm that, unlike the type III pathway, the T6SS does not require host cell contact or precise culture conditions for secretion.

B728a secretion of Hcp is dependent on Psyr_4958 (Fig. 2.5), which is predicted to encode the AAA^+ ATPase ClpV. AAA^+ proteins (ATPases associated with diverse cellular activities) are widely represented in all living organisms, where they convert chemical energy into biological activity (91). ClpV represents a novel class of AAA^+ ATPases associated specifically with the T6 locus. ClpV is likely to be the driving force required for translocation through the T6SS.

Inoculation of bean plants via vacuum infiltration did not reveal a virulence defect in the B728a *clpV* mutant (Fig. 2.6). It is possible that the T6SS plays a subtle role

in the B728a-plant interaction requiring more sensitive experimental methods for detection. For example, co-inoculation of bean plants with B728aAR1 and wild-type B728a may reveal a subtle advantage to having a functional T6SS. Competitive indexing through co-inoculation is a virulence assay that has been used in the research of animal pathogens for many years, and this strategy was recently optimized for study of *P. syringae*-plant interactions (65).

For several pathogens, the T6SS contributes to persistence in the environment or within a eukaryotic host (22, 79, 82, 98). It is plausible, then, that a disabling of the B728a T6SS would diminish the epiphyte's capacity for chronic survival on the leaf surface. The colonization experiment described herein did not support this hypothesis. Leaf surface populations of the B728a *clpV* mutant were similar to those of wild-type B728a. In addition, adhesion assays showed that B728aAR1 is as efficient as wild-type B728a at adhering to poly(vinyl chloride) and polypropylene, substances routinely used in biofilm studies (77). Under the conditions tested, *clpV* is not required for biofilm formation in B728a.

In addition to plants, other "hosts" may provide insight into the function of the B728a T6SS. The phyllosphere is a heterogeneous environment where bacteria encounter other microbes that may serve as competition or as predators. Pseudomonads have evolved means to deal with predation and competition in the environment. For example, *Pseudomonas fluorescens* utilizes secondary metabolites to escape protozoan grazing (43). Recently, Lindow's group showed that a novel B728a protein induces programmed cell death in *Neurospora*, which B728a is able to use as a sole nutrient

source (111). It would be interesting to explore possible interactions between B728a and other phyllosphere residents and any role that the T6SS might play in these encounters. The social amoeba *Dictyostelium discoideum* is used as a model system for the study of *Vibrio cholerae* virulence, and it was through the *Vibrio-Dictyostelium* interaction that the T6SS was first discovered (84).

This research confirms the functionality of a T6SS in a plant pathogenic bacterium. While numerous bacterial species carry T6 loci in their genomes (97), secretion activity has been demonstrated for only 13 (11). Of the 13 species with a T6SS previously proven functional, only one is a plant pathogen (67). The demonstration of an active T6SS in B728a opens the door for future analyses of this pathway and the contribution that it makes to B728a fitness and that of other important plant pathogens.

MATERIALS AND METHODS

Bioinformatic analyses

Database searches were done with the blastp program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). G + C content was analyzed for nucleotides 5871317 to 5901193 of the B728a genome (NC_007005) via the CpG Ratio and GC Content Plotter program (http://mwsross.bms.ed.ac.uk/public/cgi-bin/cpg/pl), with a window size of 500. A graph of the G + C values was prepared in Microsoft Excel. Protein subcellular location predictions were made using LOCTree (http://cubi.bioc.columbia.edu/services/loctree/), PSLpred (http://www.imtech.res.in/raghava/pslpred/), and PSORTb (http://www.psort.org/psortb/).

TABLE 2.2. Strains and plasmids

Designation	Relevant Characteristics	Source
Bacterial strains		
Escherichia coli		
DB3.1	F- *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB* mrr *hsdS20*(r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) *xyl5* Δ*leu mtl1*	(7)
DH10B	F^- *mcrA* Δ*lacX74* (ϕ80d*lacZ*Δ*M15*) Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara*, *leu*)7697 *galU galK* λ^- *rpsL nupG*	(30)
Mach1 T1	Δ*recA1398 endA1 tonA* ϕ*80*Δ*lacM15* Δ*lacX74 hsdR*(r_K^- m_K^+)	Invitrogen
P. syringae pv. *syringae*		
B728a	Wild type, bean pathogen, Rif^r	(62)
B728aAR1	*clpV*::*aacC1* derivative of B728a, Rif^r Gm^r	This study
NPS3136	*gacS*::Tn5 derivative of B728a, Rif^r Km^r	(114)
Plasmids		
pUCclpV	pUCP26 carrying *clpV* in-frame with the vector *lacZ* promoter, Tc^r Ap^r	This study
pEclpV	pENTR/D-TOPO carrying *clpV*, Km^r	This study
pEclpV-Gm	pENTR/D-TOPO carrying *clpV*::*aacC1*, Km^r Gm^r	This study
pEhcp-vsv	pENTR/D-TOPO carrying *hcp* with its putative promoter region and a 3' *vsv* tag, Km^r	This study
pENTR/D-TOPO	Gateway entry vector, Km^r	Invitrogen
pLVCD	Gateway destination vector for mating with *P. syringae*, pBR322 derivative with *mob* genes from RSF1010, Tc^r Ap^r Cm^r	Steve Lindow
pLVclpV-Gm	pLVCD carrying *clpV*::*aacC1*, Tc^r Ap^r Gm^r	This study
pRH002	Gateway destination vector, pBBR1MCS1 derivative, Cm^r	(33)
pRHhcp-vsv	pRH002 carrying *hcp* with its putative promoter region and a 3' *vsv* tag cloned in-frame with the vector *lacZ* promoter, Cm^r	This study
pRK2073	Helper plasmid, Sp^r Trm^r	(57)
pUCGm	Cloning vector, Gm^r Ap^r	(95)
pUCP26	Cloning vector, Tc^r Ap^r	(110)

Bacterial strains and media

The bacterial strains used in this study are listed in Table 2.2. *Escherichia coli* strain DH10B was used for general cloning (89) and was cultured at 37°C in Luria-Bertani (LB) liquid or agar medium. *E. coli* Mach1 T1 cells were used following topoisomerase reactions, per manufacturer's instructions (Invitrogen, Carlsbad, Calif.). *P. syringae* pv. *syringae* strains were routinely grown at 25°C in nutrient broth-yeast extract (NBY) liquid or agar medium (102), in syringomycin minimal medium containing 100 μM arbutin and 0.1% fructose (SRM_{AF}) (70), or on King's B agar medium (KB) (47). Antibiotics were added at the following concentrations (μg ml^{-1}): rifampin, 100; kanamycin 75; tetracycline 20; chloramphenicol 20; gentamycin, 5; and spectinomycin 100.

General DNA manipulations

Restriction enzymes, T4 DNA ligase, and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa). For cloning using Gateway technology (51), target genes were amplified by PCR and cloned into the pENTR/D-TOPO vector (Invitrogen). LR clonase (Invitrogen) was used for recombination between pENTR constructs and Gateway destination vectors, per manufacturer's instructions. Plasmids were introduced into *E. coli* via chemical transformation or electroporation (89). Plasmids were introduced into *P. syringae* pv. *syringae* strains via tri-parental mating with helper plasmid pRK2073 (57). Standard cycling conditions were used for PCR and primer sequences are listed in Table 2.3.

TABLE 2.3. Primers used for PCR amplification

Name	Sequence
P131	CACCGTGAACCTGAAGTCC
P132	ACTAGGTCAGGGTGTAAGC
P160	ATGTGGTACCGTGAACCTGAAGTCCC
P161	ATGAGCATGCTCAGGTCAGGGTGTAA
P163	CACCCGCAGTCGTTGAAGGCTATC
P164	TCATTTTCCTAATCTATTCATTTCAATATCTGTATATGCGAATACTTTGTTCGC

Construction of plasmids

hcp was amplified using primer P163 and primer P164, which contains a sequence encoding the vesicular stomatitis glycoprotein (VSV) epitope (42) and then cloned into pENTR/D-TOPO vector (Invitrogen) via topoisomerase reaction, resulting in pEhcp-vsv. The *hcp-vsv* entry construct was recombined into the pRH002 Gateway destination vector, resulting in pRHhcp-vsv. The 2.6-kb *clpV* gene was amplified from the B728a genome using primers P160 and P161, which contain KpnI and SphI sites, respectively. The PCR product was digested and ligated to KpnI/SphI-digested pUCP26, resulting in pUCClpV.

Construction of the B728a *clpV* mutant

The 2.6-kb *clpV* gene was amplified by PCR using primers P131 and P132 and cloned into pENTR/D-TOPO by topoisomerase reaction, resulting in construct pEclpV. The *aacC1* gentamycin (Gm) resistance gene was isolated from pUCGm by digestion with HindIII and was ligated to HindIII-digested pEclpV, resulting in pEclpV-Gm. The *clpV*-Gm entry construct was recombined into the pLVCD Gateway destination vector, resulting in pLVclpV-Gm. Tri-parental mating was set up between wild-type B728a, *E. coli* DH10B(pLVclpV-Gm) and *E. coli* DB3.1(pRK2073), and marker exchange resulted in B728aAR1.

Secretion assays

P. syringae pv. *syringae* strains carrying pRHhcp-vsv were shaken overnight at 25°C in 2 ml of NBY liquid supplemented with chloramphenicol. The cells were pelleted and washed, and then 3 μl were inoculated into 2 ml of fresh NBY or SRM_{AF}

with chloramphenicol. The cultures were grown at 25°C with shaking to an optical density at 600 nm (OD_{600}) of 0.3. Cultures were separated into cell-associated and supernatant fractions via centrifugation, and the proteins in the supernatant fractions were precipitated with 12.5% trichloroacetic acid. Proteins in whole-cell lysates and supernatant fractions were separated on 15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to Hybond-P PVDF membranes (GE Healthcare, Piscataway, N.J.). Western blots were probed with antibodies to the vesicular stomatitis virus glycoprotein (VSV-G) epitope (Sigma Chemical Co., St. Louis, Mo.) or to the β-subunit of RNA polymerase (RNAP, Neoclone, Madison, Wis.). Primary antibodies were recognized by anti-mouse and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate secondary antibodies (Sigma Chemical Co.) and visualized via the Amersham ECL chemiluminescence system (GE Healthcare). Relative protein concentrations in culture fractions were estimated as follows. The Gel Analysis option of the ImageJ program (http://rsbweb.nih.gov/ij/) was used to measure signal intensity for each protein band on scanned images of the blots. For each bacterial strain, intensity values for cellular and supernatant fraction proteins were combined. The relative amount of Hcp-VSV present in each supernatant fraction was calculated by dividing the supernatant intensity value by the combined intensity value. The experiment was repeated three times with consistent results.

Adhesion assays

To determine if *clpV* contributes to B728a adhesion ability, assays were conducted as follows. First, cultures were grown overnight in NBY liquid medium and

then diluted to an OD_{600} of 0.05 in LB + 0.2% glucose (LBG). The cultures were grown to an OD_{600} of 0.1, and then 200-μl aliquots were added to the wells of a poly(vinyl chloride) 96-well plate (BD Falcon, Franklin Lakes, N.J.). Plates were incubated at 25°C for 12, 24, or 48 h with or without shaking. Cell density was measured using an Emax Precision Microplate Reader (Molecular Devices Co., Sunnyvale, Calif.). Wells were then vigorously washed under running tap water. In order to visualize adherent cells, 200 μl of 1% crystal violet were added to each well, and the plates were incubated at room temperature for 30 min. Plates were washed again under tap water before the crystal violet was solubilized by the addition 200 μl of 95% ethanol to each well. Plates were shaken gently for 2 min at room temperature, and A_{590} was determined via plate reader.

As an alternative strategy, overnight NBY cultures were diluted to an OD_{600} of 0.05 in LBG or SRM_{AF}, and then 100-μl aliquots were placed in sterile 2-ml polypropylene microcentrifuge tubes (USA Scientific. Inc., Ocala, Fla.) and incubated at 25°C for 10 h, with or without shaking. After incubation, 25 μl of 1% crystal violet were added to each tube, and the tubes were incubated at room temperature for 15 min. The tubes were rinsed several times with tap water and allowed to air dry. 200 μl of 95% ethanol were added to each tube, the tubes were allowed to air dry, and then they were inspected for the presence of a blue ring at the air-surface interface.

Leaf colonization assays

The B728a *clpV* mutant was tested for its ability to colonize bean leaves, using a protocol based on methods described by Monier and Lindow (72). B728a and B728aAR1 were grown overnight in 2 ml of NBY at 25°C with appropriate antibiotics.

2-ml cultures were used to seed fresh 100-ml NBY cultures, which were grown at 25°C to an OD_{600} of 0.6. Cultures were pelleted, washed, and diluted in sterile distilled, de-ionized water (ddH_2O) to 10^5 CFU/ml. Two-week-old Blue Lake 274 (*Phaseolus vulgaris*) bean plants were inverted and submerged in the bacterial suspensions for 3 sec. Plants were rinsed with distilled water and allowed to air dry. Plants were maintained at 25°C in a growth chamber with 45% relative humidity (low RH) for 48 h. Prior to placement in the growth chamber, some of the plants were covered with large plastic bags, which created conditions of high RH. Each bacterial strain was tested on three individual bean plants, and the experiment was repeated three times.

For population analyses, five leaves were arbitrarily collected from each inoculated plant, weighed, and placed in 20 ml of washing buffer (0.1M K_2HPO_4/KH_2PO_4, 0.1% Bacto-Peptone, pH 7.0) in a sterile Falcon tube. In order to remove bacteria from the leaves, the tubes were sonicated for 7 min in an ultrasonic water bath. Serial dilutions were made in sterile ddH_2O, and spread on KB plates with appropriate antibiotics. Colonies were enumerated after plates were incubated for 48 h at 25°C.

Pathogenicity assays

The B728a *clpV* mutant was tested for its ability to cause disease and multiply *in planta*. B728a and derivative strains were grown overnight in 5 ml of NBY at 25°C with appropriate antibiotics. 5-ml cultures were used to seed fresh 200 ml NBY cultures, which were grown at 25°C to an OD_{600} of 0.6. Cultures were pelleted, washed, and resuspended in ddH_2O to an OD_{600} of 0.3 (equivalent to 5×10^8 CFU/ml). Bacterial

suspensions were diluted to doses ranging from 10^4 to 10^7 CFU/ml and supplemented with 0.004% Silwet L-77 surfactant (Lehle Seeds, Round Rock, Tex.). Two-week-old Blue Lake 274 (*Phaseolus vulgaris*) bean plants were inoculated via vacuum infiltration of the bacterial suspensions. Vacuum was held at 20 in. Hg. for 3 x 1 min. Plants were rinsed with distilled water and allowed to air dry. The plants were maintained at 25°C in a growth chamber for 72 h. Each bacterial strain was tested on three individual bean plants, and the experiment was repeated three times.

For population analyses, a trifoliate leaf was removed from each inoculated plant, and the bottom of a sterile 2-ml screw cap microcentrifuge tube (Bio Plas Inc., San Francisco, Calif.) was used to punch 20 leaf discs from each detached leaf. The leaf discs were briefly rinsed in sterile ddH_2O and then homogenized via mortar and pestle in Silwet Phosphate Magnesium Buffer (SPM, 0.7% K_2HPO_4, 0.4% KH_2PO_4, 0.025% $MgSO_4$•7 H_2O, 0.004% Silwet L-77). Serial dilutions were made in SPM buffer, and spread on KB plates with appropriate antibiotics. Colonies were enumerated after plates were incubated for 48 h at 25°C.

CHAPTER III

LadS AND RetS ARE *Pseudomonas syringae* HYBRID SENSOR KINASES THAT COLLECTIVELY CONTROL MUCOIDY, REGULATE EXPRESSION OF THE TYPE VI SECRETION SYSTEM, CONTROL SWARMING MOTILITY, AND CONTRIBUTE TO COLONIZATION OF BEAN LEAVES

OVERVIEW

Bacterial pathogens utilize two-component systems (TCSs) to respond to environmental changes and to regulate the expression of virulence traits. A typical bacterial TCS is composed of a histidine kinase (HK) and a response regulator (RR), which is phosphorylated by the HK in response to an environmental signal. *Pseudomonas syringae* pv. *syringae* strain B728a is a cosmopolitan pathogen that is highly adapted to life on the surface of bean leaves but has the versatility to invade stomata under appropriate environmental conditions. The B728a genome is predicted to encode 68 HKs and 93 RRs, which contribute to the adaptation of this bacterium to plant and non-plant environments. LadS and RetS are unique hybrid sensor kinases with modular structures consisting of an HK followed by RR domains. LadS and RetS are known to regulate critical virulence pathways for the human pathogen *Pseudomonas aeruginosa*, including the type III secretion system (T3SS), the newly-discovered type VI secretion system (T6SS), biofilm formation, and swarming motility. I have identified *ladS* and *retS* homologs in the genome of B728a. Like its *P. aeruginosa* counterpart, B728a RetS also controls the T6SS and production of mucoidy. In addition, I have found

that B728a RetS influences colonization of bean leaves. LadS regulates expression of T6SS genes and controls swarming motility in B728a.

INTRODUCTION

Pseudomonads have adapted to a remarkable range of environmental conditions, where they may exist as saprophytes (in water or soil), as benign or beneficial residents (on a plant host), or as pathogens of animals or plants (28). *Pseudomonas syringae* pv. *syringae* is a widespread, economically significant pathogen of crop plants, fruit and nut trees, and ornamental species. *P. syringae* pv. *syringae* strain B728a is an especially versatile representative of this species. It exhibits a distinct epiphytic phase of growth, residing on the surfaces of bean leaves, where it persists until environmental conditions trigger invasion of leaf tissue and initiation of disease. The molecular basis for this switch from a resident epiphytic existence to an acute infectious agent is complex, requiring the interaction of multiple virulence factors and associated secretion systems (23, 37). Intricate global regulatory networks mediate expression of these virulence traits, and in almost all cases, regulation begins with a sensor kinase or other surface receptor (71).

Within the last few years, our understanding of *P. syringae* biology has been significantly advanced by the advent of genomics. Access to three fully sequenced *P. syringae* genomes is now available, including that of B728a (60). Using the B728a genome sequence as a reference, I have identified two important regulators of *P. syringae* virulence traits: the hybrid sensor kinases LadS and RetS.

Bacteria commonly use two-component systems (TCSs) to sense and respond to

signals in the environment. The prototypical TCS features a membrane-bound sensor histidine kinase (HK) that detects an environmental signal and auto-phosphorylates a conserved histidine residue within its transmitter domain (39). The phosphoryl group is then transferred to a cognate cytoplasmic response regulator (RR). TCSs react to a wide range of stimuli, including nutrients, quorum signals, antibiotics, and more (54). TCSs play critical roles in bacterial fitness, and this is underscored by their prevalence. TCSs are found in nearly every sequenced bacterial genome, with some genomes containing as many as 200 (54). The B728a genome is predicted to encode 68 HKs and 93 RRs, which contribute to the adaptation of this bacterium to plant and non-plant environments (56).

The impact of TCS regulation of virulence traits is exemplified by the RetS and LadS hybrid sensor kinases of the human pathogen *P. aeruginosa.* RetS and LadS reciprocally regulate biphasic *P. aeruginosa* lung infections in Cystic Fibrosis patients (29). Lung infection by *P. aeruginosa* begins as an acute colonization, which is mediated by factors important for invasion, such as motility and toxin delivery by the type III secretion system (T3SS). Virulence traits associated with acute infection are lost when the bacteria transition to a chronic state of infection. During chronic infection, the bacteria express traits that contribute to long-term survival and protection in the lung, such as quorum sensing, cell surface components that promote aggregation, and the recently discovered type VI secretion system (T6SS)(73). RetS and LadS appear to be global regulators that mediate the switching-off of genes necessary for an acute infection of the lung (e.g., the T3SS) and a switching-on of genes required for long-term colonization (e.g., biofilm production, the T6SS). The *P. aeruginosa* RetS/LadS

regulons are complex, involving elements of the GacS/GacA global regulatory pathway and the newly described HptB protein (Fig. 3.1)(39). I have identified orthologs of *retS* and *ladS* within the genome of B728a. The predicted protein products for both genes, Psyr_4339 (*ladS*) and Psyr_4408 (*retS*), share relatively high identity with their *P. aeruginosa* counterparts, as well as conserved domain organization.

Several studies of pathogenic bacteria have demonstrated the in-culture secretion of a small protein called "Hcp" (11). Hcp1 of *P. aeruginosa* was recently shown to form a hexameric ring structure that may create a channel associated with T6SS activity (73). In all of the bacteria studied thus far, Hcp secretion is dependent on T6SS genes. I have evidence that B728a also secretes Hcp in a T6SS-dependent manner (Fig. 2.4) and that this secretion is negatively regulated by RetS. In addition, I demonstrate that RetS has a negative effect on mucoidy and contributes to colonization of bean leaves by B728a. This study also shows that B728a LadS positively controls expression of a T6SS gene and swarming motility.

RESULTS

B728a genes Psyr_4339 and Psyr_4408 are homologs of *P. aeruginosa ladS* and *retS* genes, respectively

Using the blastp program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), I have identified orthologs of both *P. aeruginosa retS* and *ladS* within the genome of B728a. The predicted products for both genes, Psyr_4339 (*ladS*) and Psyr_4408 (*retS*), share 56% and 57% a.a. identity, respectively, with their *P. aeruginosa* counterparts, as

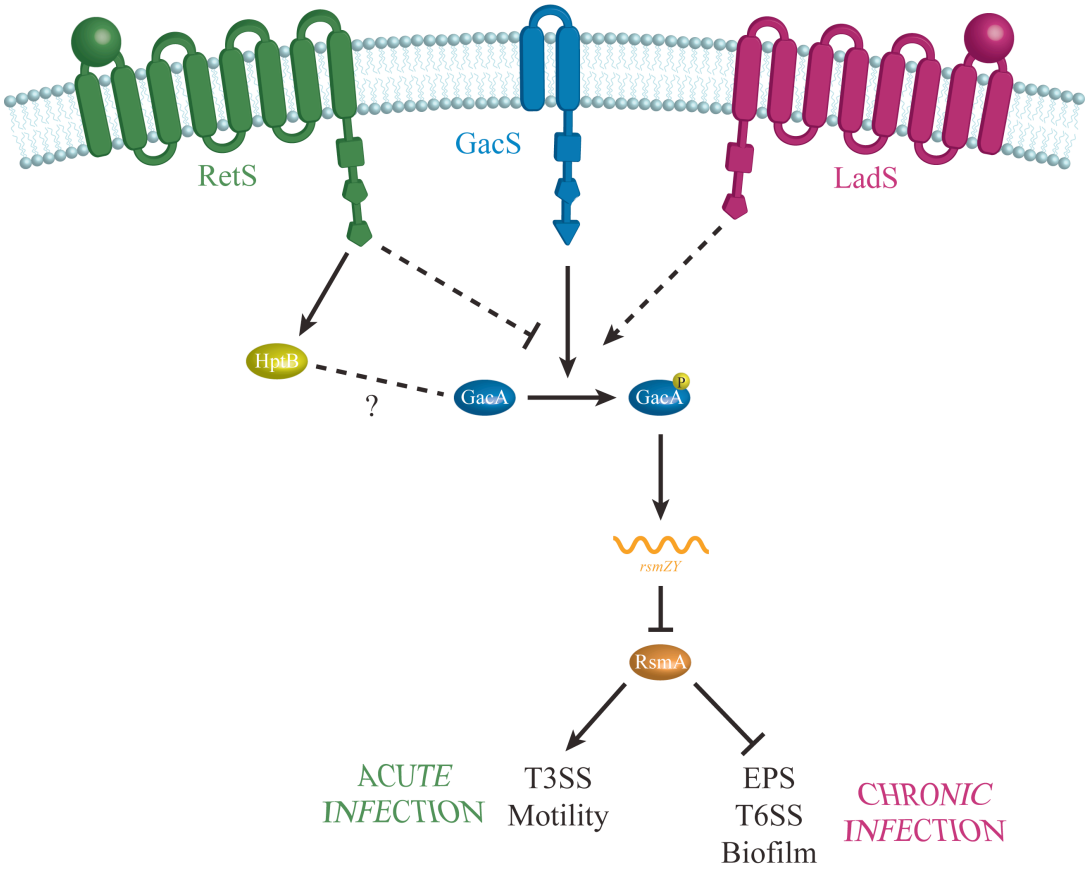

FIG. 3.1. Model of the *P. aeruginosa* LadS/RetS regulons. LadS (pink) is a hybrid sensor protein featuring a histidine kinase (HK) domain (depicted as a square) and a response regulator (RR) receiver domain (pentagon). These domains are anchored to the inner membrane via transmembrane segments linked to a periplasm-exposed signal-binding domain. RetS (green) protein architecture is identical to that of LadS, except that it contains an additional RR domain. A third global regulator, GacS (blue), features an HK followed by an RR domain and a histidine-containing phosphotransfer (Hpt) domain (triangle). RetS and LadS reciprocally regulate expression of virulence factors, and this regulation is modulated through the GacS/GacA network. Phosphorylated GacA activates the transcription of the small RNAs *rsmZ* and *rsmY*, which have high affinity for RsmA (46). RNA-bound RsmA is unable to inhibit expression of type VI secretion system (T6SS) genes or other factors associated with chronic infections, such as exopolysaccharide (EPS) and biofilm genes (10). Free RsmA positively regulates the Type III secretion system (T3SS), which is highly expressed during acute infection by *P. aeruginosa* (29). LadS and RetS promote and inhibit, respectively, phosphorylation of GacA. Hybrid sensors often mediate signal transduction through an Hpt domain or protein (101). RetS is capable of phosphorylating the HptB protein, which may be acting upstream of GacA (39). LadS does not appear to phosphorylate HptB and is, therefore, likely carrying out regulation through a distinct pathway.

well as conserved domain organization (Fig. 3.2). The proteins were analyzed via the SMART program (http://smart.embl-heidelberg.de/). Both LadS and RetS contain a histidine kinase domain, an HATPase_C kinase domain, and one (LadS) or two (RetS) response regulator receiver domain(s). These domains are secured to the inner membrane via seven transmembrane segments linked to a periplasm-exposed signal-binding domain. LadS and RetS are characterized as hybrid sensor kinases because they feature both sensor kinase and response regulator receiver domains. Hybrid sensor kinases are common among bacteria, but the presence of two tandem response regulator receiver domains is unusual, making RetS a unique protein (52).

A B728a *retS* mutant exhibits mucoid growth on certain media

Insertional mutations in the B728a *ladS* and *retS* genes were made by amplifying ~600-bp fragments of the genes and cloning them into a Gateway entry vector. The fragments were then recombined into the mobilizable Gateway destination vector pLVCD, and the constructs were introduced into B728a via tri-parental mating with the pRK2073 helper plasmid (57). Integration of pLVladS' or pLVretS' into the B728a genome resulted in B728aAR2 and B728aAR3, respectively.

When inoculated on potato dextrose agar (PDA), the B728a *retS* mutant exhibited a mucoid growth phenotype (Fig. 3.3A). In an effort to determine the nature of this mucoidy, wild-type B728a and the B728a *retS* mutant were inoculated on mannitol glutamate-yeast extract (MGY) agar, a medium that induces exopolysaccharide (EPS) production by *P. syringae* (55, 80), and incubated for 24 h at 25°C. On MGY, growth of the B728a *retS* mutant B728aAR3 appeared similar to that of wild-type B728a (Fig.

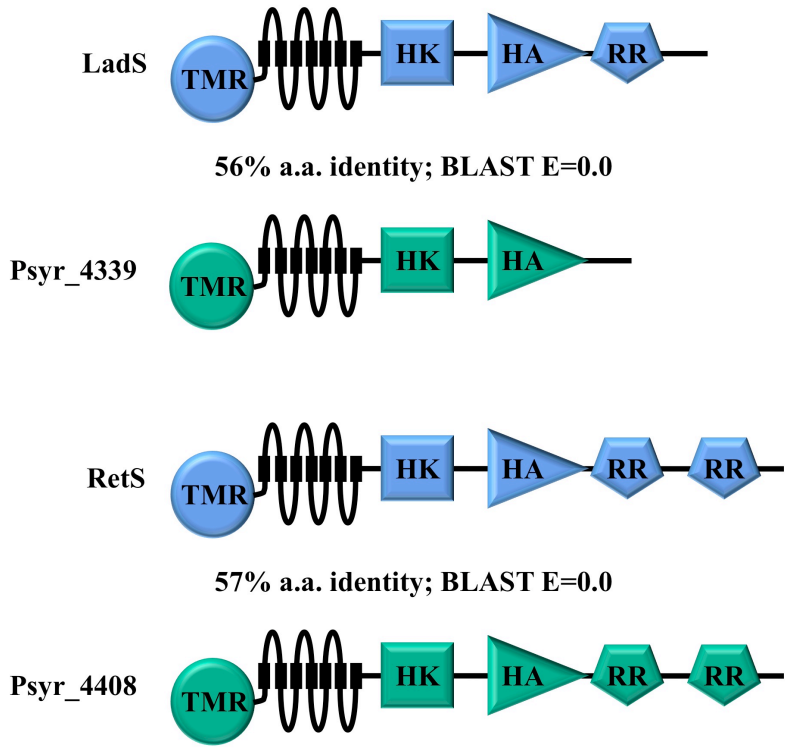

FIG. 3.2. Domain organizations of *P. aeruginosa* LadS and RetS proteins (blue) and *P. syringae* B728a orthologs (green). **A.** LadS is a hybrid sensor protein featuring a histidine kinase domain (depicted as a square), an HATPase_C kinase domain (triangle), and a response regulator receiver domain (pentagon). These domains are anchored to the inner membrane via transmembrane segments linked to a periplasm-exposed signal-binding domain (circle). The protein product predicted for B728a Psyr_4339 shares a similar structure and 56% identity at the amino acid level. Psyr_4339 does not contain a response regulator domain; this protein is likely transmitting signals through a response regulator located elsewhere in the genome. **B.** RetS protein architecture is identical to that of LadS, but with one additional receiver domain. The B728a Psyr_4408 predicted protein shares the same architecture and 57% a.a. identity.

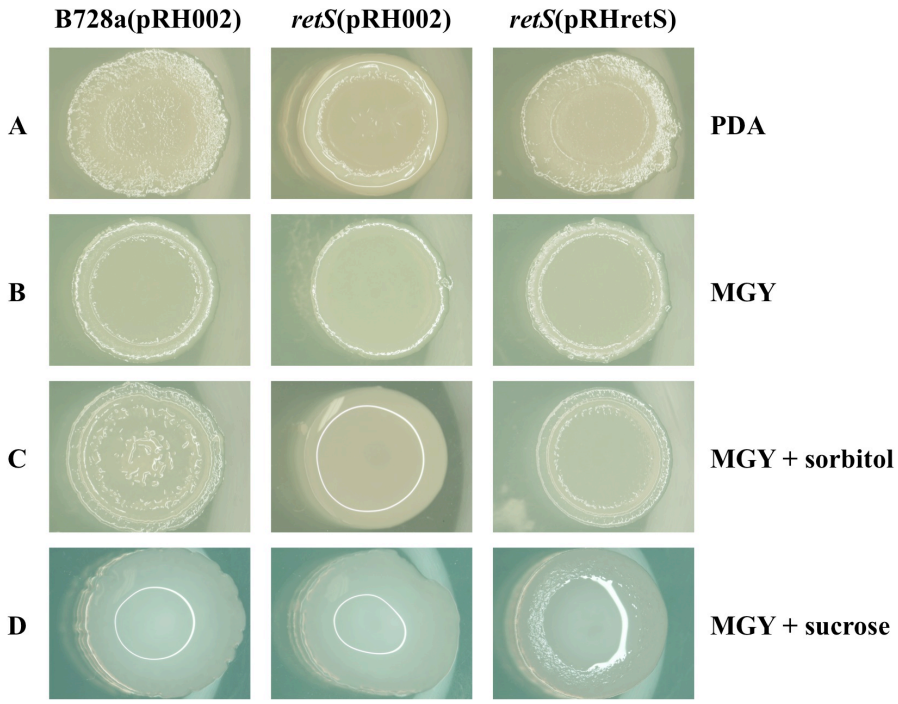

FIG. 3.3. Assay for mucoidy by *P. syringae* pv. *syringae* strains on various media. A drop of inoculum containing 10^5 CFU of either B728a carrying empty vector pRH002, the B728a *retS* mutant B728aAR3(pRH002), or B728aAR3(pRHretS) was placed in the center of either (A) PDA, (B) mannitol glutamate-yeast extract (MGY) agar, (C) MGY supplemented with 0.6M sorbitol, or (D) MGY supplemented with 5% sucrose. Plates were incubated at 25°C for 24 h and photographed under a dissecting microscope.

3.3B). However, when grown on MGY supplemented with 0.6M sorbitol, which is known to induce expression of alginate-related genes (80), B728aAR3 appeared much more mucoid than wild-type B728a (Fig. 3.3C). This phenotype was complemented *in trans* by the presence of the intact *retS* gene on plasmid pRH002. Inoculation of MGY supplemented with 5% sucrose, which stimulates production of levan (55), did not reveal any observable differences between wild-type B728a and B728aAR3 (Fig. 3.3D). The colony morphology of B728aAR2, a *ladS* mutant, appeared similar to wild-type on all media tested (data not shown).

The B728a LadS and RetS regulons include genes associated with the Type VI Secretion System

Because LadS and RetS regulate expression of genes in the *P. aeruginosa* T6 locus as well as those in the T3SS locus, quantitative real-time PCR (qRT-PCR) studies were performed to determine if the B728a LadS and RetS homologs control expression of representative T6SS and T3SS genes. RNA was extracted from bacteria grown in liquid NBY and subjected to qRT-PCR using primers specific to *hcp* and the gene encoding HrpZ, a protein copiously secreted by the T3SS (34). In addition, expression of Psyr_2628 was analyzed because, although it is not a conserved component of the T6 locus, its homolog is adjacent to the HSI-I T6 locus in the *P. aeruginosa* genome and is regulated by LadS and RetS (73). The B728a *ladS* mutant, B728aAR2, exhibited a 10-fold decrease in *hcp* transcript levels, as compared to those of wild-type B728a (Table 3.1). In contrast, *hcp* transcript levels were more than 200-fold higher in the B728a *retS* mutant, B728aAR3, as compared to wild-type B728a. Neither a *retS* nor a *ladS* mutation

TABLE 3.1. Quantitative real-time PCR analysis of expression of representative type VI and type III genes as influenced by *ladS* and *retS* in B728a

Gene	Change in transcriptional level (± SE) compared to wild-type B728a[a]	
	B728aAR2	B728aAR3
hcp	0.1 ± 0	237.5 ± 43.7
Psyr_2628	1.4 ± 0.1	1.9 ± 0.3
hrpZ	1.0 ± 0.1	0.5 ± 0

[a] Values represent the relative transcriptional level for mutant strains compared to wild-type B728a. Each value represents the average of three independent assays followed by the standard error (SE) of the mean.

had an apparent affect on Psyr_2628 or *hrpZ* expression. These results indicate that RetS and LadS are negative and positive regulators of type VI gene expression, respectively. RetS and LadS do not control expression of the type III gene *hrpZ* or the type VI-related gene Psyr_2628.

A mutation in *retS* results in increased Hcp secretion in culture

To determine whether a mutation in *ladS* or *retS* has an affect on Hcp secretion in culture by B728a, a plasmid construct was made, pRHhcp-vsv, which expresses Hcp with a C-terminal fusion to the vesicular stomatitis virus glycoprotein (VSV) epitope. In order to ensure expression of *hcp-vsv*, the sequence was placed in frame with a *lac* promoter on the broad host-range vector pRH002 (33). Proper orientation and tagging of *hcp* were confirmed by sequencing of pRHhcp-vsv, and the construct was introduced into wild-type B728a and derivative strains.

Three independent secretion assays were performed with these strains, giving consistent results. A representative Western blot is shown in Fig. 3.4. The ImageJ program (http://rsbweb.nih.gov/ij/) was used to estimate protein concentrations on Western blots by measuring band intensity. Approximately 34% of the Hcp-VSV present in the wild-type B728a cultures was localized to supernatant fractions. The levels of Hcp-VSV present in the supernatants of the B728a *ladS* mutant were similar to those of wild-type B728a; approximately 33% of the Hcp-VSV in the B728aAR2 cultures was localized to supernatant fractions. Approximately 54% of the Hcp-VSV present in the

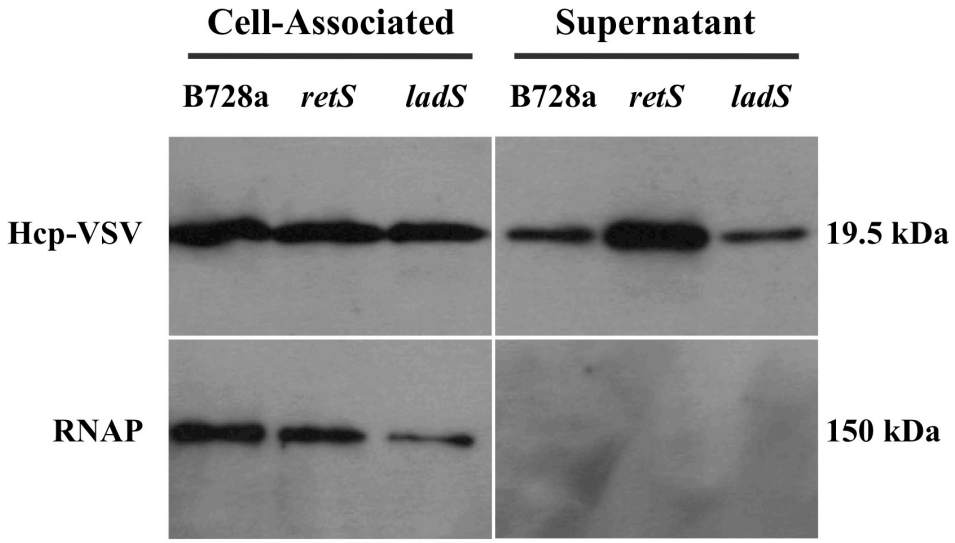

FIG. 3.4. Extracellular secretion of Hcp-VSV as observed by Western blot analyses. Strains carrying plasmid-borne *hcp-vsv* were grown to mid-log phase at 25°C in NBY. Cultures were separated into cell-associated and supernatant fractions as described in Materials and Methods. Equal sample quantities were run on SDS-PAGE gels, and Western blots were probed with antibodies to the VSV-G epitope or to the β-subunit of RNA polymerase (RNAP). Hcp-VSV was expected to have a molecular weight of 19.5 kDa (18.2 kDa Hcp + 1.3 kDa VSV-G epitope). The RNAP protein size is 150 kDa.

B728aAR3 cultures was located in the supernatant, suggesting that *retS* functions as a negative regulator for secretion of Hcp.

Swarming motility is enhanced by a mutation in the B728a *ladS* ortholog

A low-agar medium was used to determine whether a mutation in *ladS* or *retS* has an affect on B728a swarming motility. When inoculated onto filter discs in the center of semisolid NBY, wild-type B728a growth spread away from the disc, indicating an ability to swarm (Fig. 3.5). The B728a *retS* mutant displayed a similar movement pattern. The B728a *ladS* mutant swarmed 18.6 ± 1 mm farther (mean of the difference measured in three independent assays ± SD) from the point of inoculation than wild-type B728a, which suggests that *ladS* negatively controls B728a swarming ability. NPS3136, a B728a *gacS* mutant with a previously demonstrated inability to swarm (48), showed no movement on the semisolid agar.

RetS contributes to leaf colonization

In order to study the possible contributions of *retS* and *ladS* to B728a colonization of the leaf surface, three two-week-old bean plants were each dipped in bacterial suspensions containing 10^5 CFU/ml of either wild-type B728a, the *ladS* mutant B728aAR2, or the *retS* mutant B728aAR3. Some of the plants were placed in a 25°C humid chamber, while the others were maintained under low relative humidity (RH) at 25°C for 24 h. Five leaves were removed from each plant. The bacteria were dislodged from the leaves by sonication, and populations were enumerated by dilution plating. The experiment was repeated three times. B728aAR2, B728aAR3, and wild-type B728a

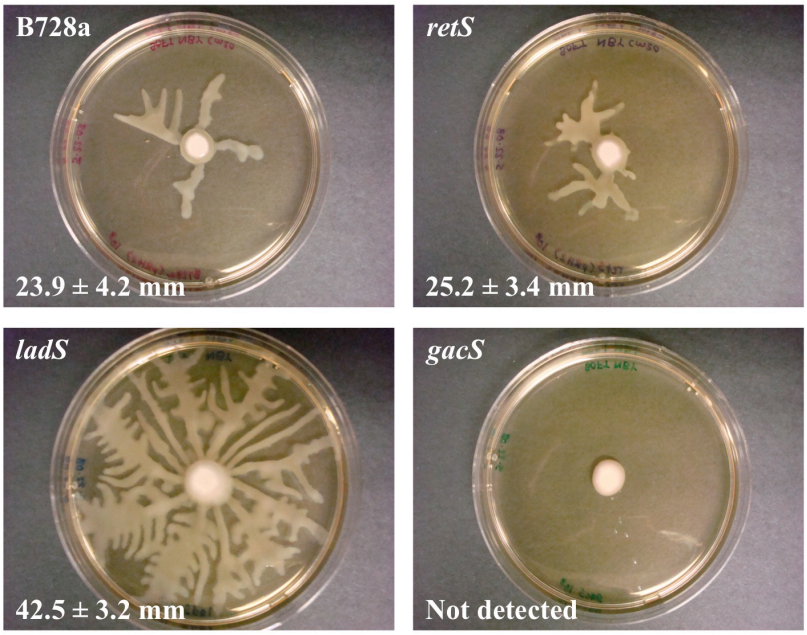

FIG. 3.5. Assay for swarming activity by *P. syringae* pv. *syringae* strains. Sterile filter disks were placed on semisolid NBY and inoculated with 10^8 CFU of either B728a, the B728a *retS* mutant B728aAR3, the B728a *ladS* mutant B728aAR2, or the swarm-deficient B728a *gacS* mutant NPS3136. Plates were incubated at 25°C for 24 h in a moist chamber. Measurements were made from the filter disk to the outer edge of the bacterial growth. Values are the mean and standard deviation from three experiments.

exhibited similar population numbers (around 10^6 CFU/g of leaf issue) when plants were placed under high RH (Fig. 3.6). The differences in bacterial numbers recovered from the plants incubated at high RH were not statistically significant. B728aAR2 reached phyllosphere populations similar those of wild-type B728a when inoculated plants were maintained under low RH (10^5 CFU/g leaf tissue, $P = 0.10$). However, the B728aAR3 population numbers under low RH were about 10-fold lower than those of wild-type B728a. The average B728a population recovered from plants incubated at low RH was $3.5 \pm 1.7 \times 10^5$ CFU/g (mean ± SD). The average population recovered from leaves inoculated with B728aAR3 and maintained at low RH was $1.5 \times 10^4 \pm 9.5 \times 10^3$ CFU/g of leaf tissue. The difference between these two strains is statistically significant (two-tailed *t*-test, $P = 0.01$), which suggests that *retS* contributes to B728a colonization of leaf surfaces.

An effective method for markerless deletion mutagenesis in B728a

Mutagenesis is a relatively straightforward process for many *P. syringae* strains, but for B728a it is surprisingly challenging. To facilitate mutagenesis in B728a, I developed a novel deletion strategy that incorporates fusion PCR (35), FLP recombination (17), and a highly mobilizable mating vector. The mutagenesis strategy is outlined in Fig. 3.7 and described in detail in Materials and Methods. Using this strategy, the *retS* gene (Psyr_4408) was deleted from the B728a genome, and the deletion in the B728a *retS* mutant, B728aAR4, was confirmed by colony PCR. The Km^r marker was removed from B728aAR4 via FLP recombination, and the resulting strain was named B728aAR5. B728aAR5 colonies appear highly mucoid on PDA, as compared to wild-

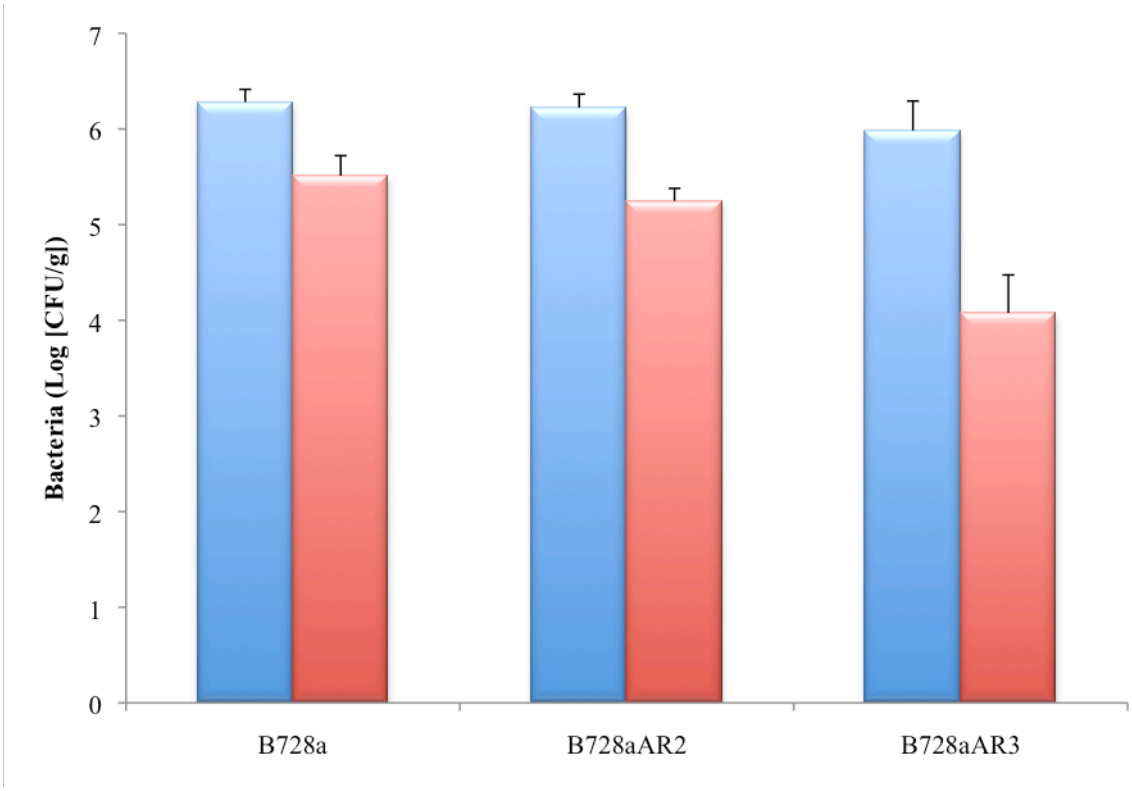

FIG. 3.6. Assay of *ladS* and *retS* contribution to *P. syringae* pv. *syringae* B728a colonization of bean leaves. Leaf surface populations from bean plants 24 h after dip inoculation with 10^5 CFU/ml of either wild-type B728a, the B728a *ladS* mutant B728aAR2, or the *retS* mutant B728aAR3. Blue bars represent surface populations per gram of leaf tissue from plants that were maintained under high relative humidity (RH) after inoculation. Red bars represent plants maintained under low RH. The experiment was repeated four times, and the vertical bars represent the standard deviation from the mean bacterial population.

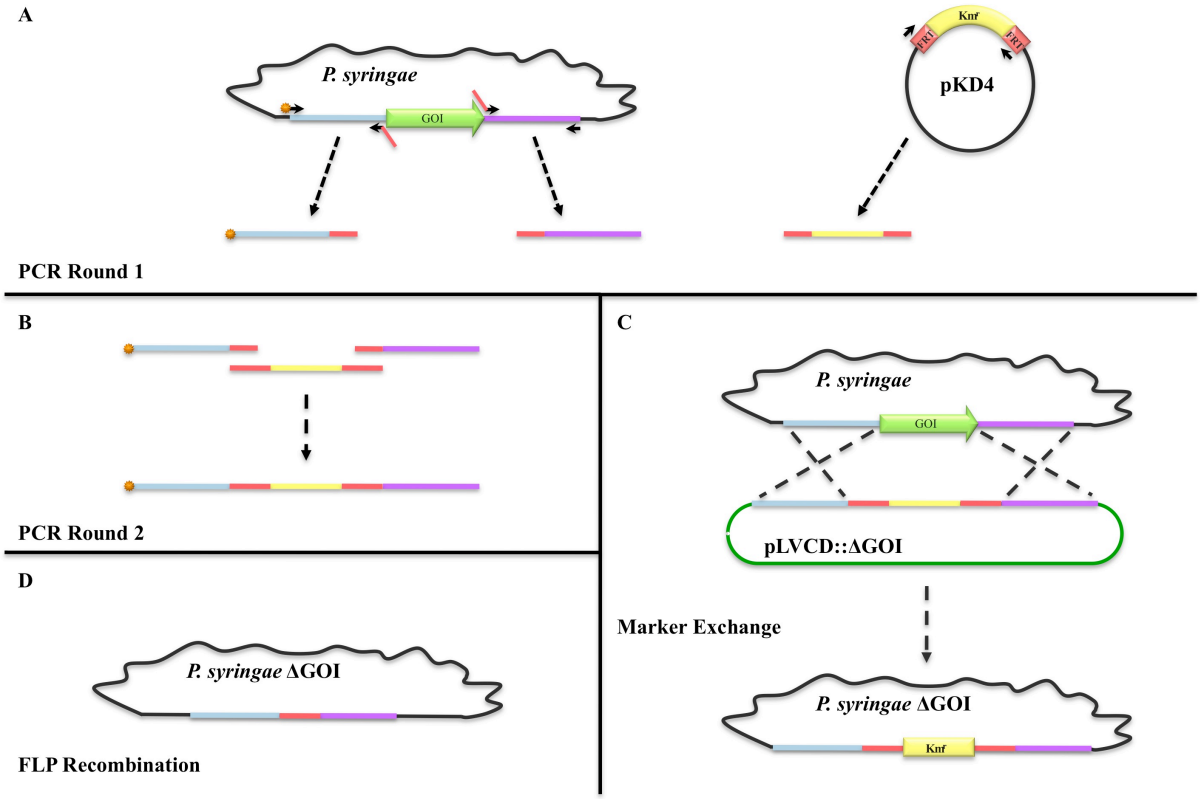

FIG. 3.7. Diagram of the strategy used for deletion mutagenesis of *P. syringae* genes. (A) The genomic regions upstream (blue) and downstream (violet) of a gene of interest (GOI, green) are amplified using primers that contain tails with FLP recombinase recognition sequences (FRT, pink). The upstream forward primer carries a topoisomerase recognition site (orange star), which is involved in a subsequent cloning step. In a separate PCR, a cassette containing an FRT-flanked *nptII* gene, which provides resistance to kanamycin (Km^r, yellow), is amplified from vector pKD4. (B) The three PCR products are combined, and another round of PCR is carried out to fuse the three fragments together. This is possible because of the overlap of the FRT sites. As described in Materials and Methods, the fused product is moved into a Gateway entry vector via TOPO cloning and then into the highly mobilizable pLVCD vector, resulting in pLVCD::ΔGOI. (C) The pLVCD::ΔGOI construct is introduced into *P. syringae* via mating and the GOI is replaced after selection for marker exchange. The resulting mutant carries a Km^r gene flanked by FRT sites in place of the GOI. (D) A plasmid expressing FLP recombinase, an enzyme that catalyzes deletion of DNA flanked by FRT sites, is introduced into the cells. FLP recombination results in a markerless deletion.

type B728a, which confirms that the B728a *retS* deletion mutant is phenotypically similar to the insertional mutant B728aAR3. This strategy for markerless deletion will be a valuable tool for researchers studying B728a molecular biology.

DISCUSSION

The B728a genome carries homologs of *ladS* and *retS*, which encode hybrid sensor kinases responsible for regulation of virulence-associated activities in *P. aeruginosa.* Bioinformatic analyses revealed that the protein products predicted for Psyr_4339 and Psyr_4408 feature domains similar to those of *P. aeruginosa* LadS and RetS, respectively (Fig. 3.2).

A B728a *retS* mutant exhibits mucoid growth on PDA (Fig. 3.3A), indicating that RetS negatively regulates EPS production in B728a, as its ortholog does in *P. aeruginosa* (101). B728a is known to produce at least two EPSs: the well-studied capsular polysaccharide alginate and the polyfructan levan (55). My experiments with MGY agar point to a RetS role in alginate synthesis because the addition of sorbitol, which stimulates alginate production, revealed a phenotype for the B728a *retS* mutant (Fig. 3.3C). Interestingly, alginate production is not regulated by RetS/LadS in *P. aeruginosa*. In addition to alginate, *P. aeruginosa* elaborates the Pel and Psl polysaccharides, which are involved in biofilm formation and are reciprocally regulated by LadS and RetS (101). It is possible that the mucoid phenotype exhibited by B728aAR3 is related to an uncharacterized EPS. A recent study of *P. syringae* pv. *glycinea* biofilm production uncovered the presence of a third *P. syringae* EPS, which

has not yet been studied in detail (55). It is interesting to note that the B728a genome carries orthologs of all of the *psl* genes (Psyr_3301-Psyr_3311).

In addition to mucoidy, RetS also negatively controls the B728a T6SS. qRT-PCR results indicated that a B728a *retS* mutant produces over 200 times more *hcp* transcript than wild-type B728a does (Table 3.1). Also, Hcp secretion in culture was enhanced by a mutation in *retS* (Fig. 3.4), which is consistent with its role as a negative regulator of the T6SS. LadS acts as a positive regulator of the B728a T6SS. Although LadS/RetS control of the T6SS in B728a is similar to that in *P. aeruginosa*, my qRT-PCR data suggests that control of the type VI-related gene Psyr_2628 and of the T3SS is different (Table 3.1). This is not totally surprising because another striking difference between the T3SS regulon of B728a and those of other *Pseudomonas* strains has been previously observed. The GacA/GacS TCS, which controls expression of the T3SS in *P. fluorescens* and *P. syringae* pv. *syringae* DC3000 (71), does not regulate T3SS expression in B728a (12). My qRT-PCR results further distinguish the B728a T3SS regulon from that of *P. aeruginosa,* where control of the T3SS is mediated by both RetS/LadS and GacA/GacS (Fig. 3.1).

Motility assays revealed that LadS negatively controls B728a swarming activity (Fig. 3.5). Similarly, microarray studies have shown that a *P. aeruginosa ladS* mutant exhibits down-regulation of *pilA*, the type IV pilus structural gene involved in adhesion and motility, and the flagellar biosynthesis genes *fliS'* and *fleP* (101). In *P. aeruginosa*, RetS positively controls twitching motility (120), but the B728a *retS* mutant was indistinguishable from wild-type B728a in my swarming motility assays (Fig. 3.5).

Several studies have shown that under low humidity conditions, fewer bacteria survive on the leaf surface (37, 76). This phenomenon is apparently exacerbated by a mutation in *retS*. In leaf colonization studies, epiphytic populations of the B728a *retS* mutant were consistently 10-fold lower than those of wild-type B728a when inoculated plants were maintained under low RH, conditions commonly present in the field (Fig. 3.6)(20). This difference in colonization is important because a reduction in cell numbers translates to a reduction in inoculum available for invasion of subdermal leaf tissue (87). Indeed, Lindemann et al. estimated the infection threshold for *P. syringae* pv. *syringae* on bean to be 10^4 CFU/g leaf tissue (61). In their study, no bacterial brown spot was detected in field plots where the epiphytic *P. syringae* populations were below the threshold.

This dissertation shows that regulation of several virulence-related activities in B728a is controlled, at least in part, by the hybrid sensor kinases RetS and LadS. These proteins likely act in concert with several other regulators, such as the GacA/GacS TCS and probably a homolog of the histidine-containing phosphotransfer protein HptB (39). In Fig. 3.8, I present a model for the LadS/RetS regulon in B728a. There are certainly similarities between the B728a and *P. aeruginosa* LadS/RetS regulons, but intriguing differences exist, including regulation of the T3SS and RetS control of swarming motility. Very recently, a RetS homolog named AtsR was identified in *Burkholderia cenocepacia*, where it is a negative regulator of both the T6SS and biofilm formation (3). RetS is clearly a conserved global regulator of virulence that is worthy of further investigation.

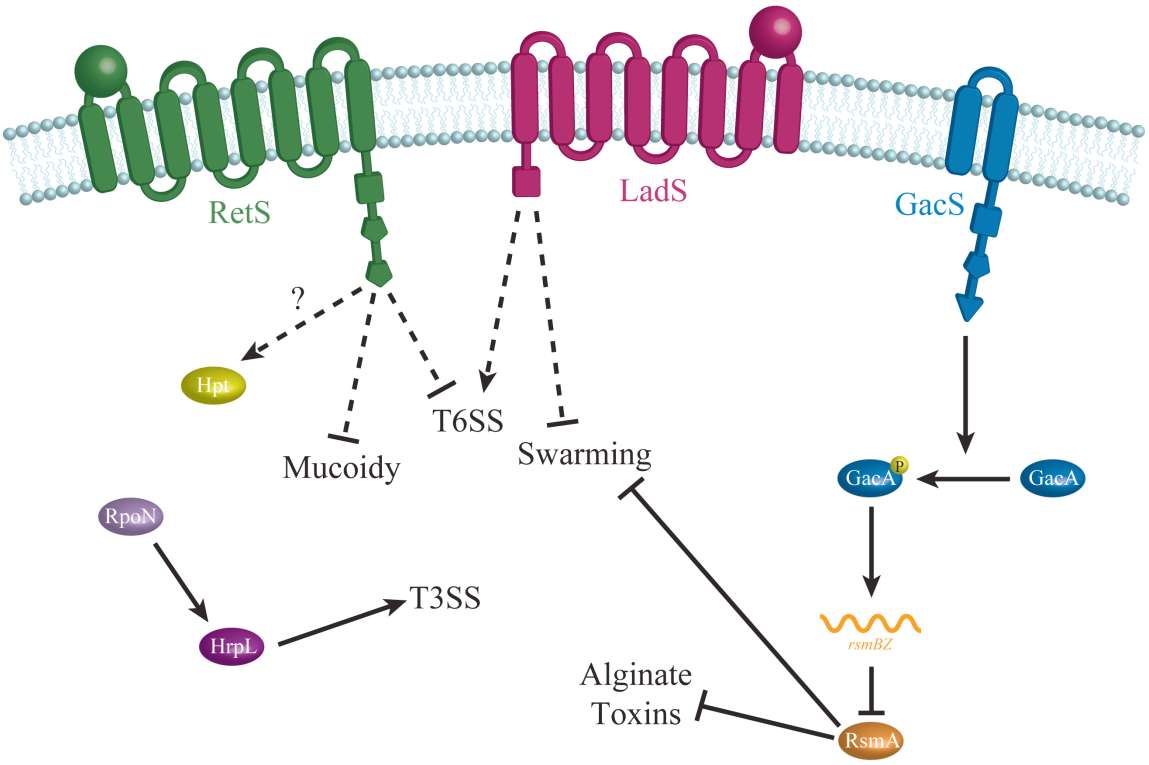

FIG. 3.8. Model of the *P. syringae* pv. *syringae* B728a LadS/RetS regulons. As is the case for *P. aeruginosa*, the B728a global regulators RetS, LadS, and GacS contribute to the control of several virulence factors. However, their activities in B728a are different from those in *P. aeruginosa*. The B728a GacS/GacA pathway is an important regulon, controlling virulence factors such as swarming (48), toxin production (49), and production of the EPS alginate (113). However, the GacS/GacA pathway does not control the T3SS in B728a (12, 114). Instead, a different receptor apparently signals T3SS expression through the alternative sigma factors RpoN and HrpL (59). RetS negatively regulates mucoidy in B728a, which suggests a role for RetS in EPS production. LadS negatively affects swarming motility, but it is not clear whether this control is mediated through the GacS pathway. The B728a genome contains a homolog of the *P. aeruginosa hptB* gene, which may possibly encode an Hpt that is phosphorylated by RetS.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in this study are listed in Table 3.2. *Escherichia coli* strain DH10B was used for general cloning (89) and was cultured at 37°C in Luria-Bertani (LB) liquid or agar medium. *E. coli* Mach1 T1 cells were used following topoisomerase reactions, per manufacturer's instructions (Invitrogen, Carlsbad, Calif.). *P. syringae* pv. *syringae* strains were routinely grown at 25°C in nutrient broth-yeast extract (NBY) liquid or agar medium (102), in syringomycin minimal medium containing 100 μM arbutin and 0.1% fructose (SRM_{AF}) (70), or on King's B agar medium (KB) (47). Assays for mucoidy were conducted on potato-dextrose agar (PDA) medium, mannitol glutamate-yeast extract (MGY) agar supplemented with 0.6M sorbitol, or MGY supplemented with 5% sucrose (55). Assays for swarming activity were performed on NBY with 0.4% agarose. Antibiotics were added at the following concentrations ($\mu g\ ml^{-1}$): rifampin, 100; kanamycin 75; tetracycline 20; chloramphenicol 20; gentamycin, 5; and spectinomycin 100.

General DNA manipulations

Restriction enzymes, T4 DNA ligase, and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). Oligonucleotides were designed using the Lasergene Expert Analysis Package (DNAStar, Madison, Wis.) and purchased from Integrated DNA Technologies (Coralville, Iowa). The primer sequences are listed in Table 3.3. For cloning using Gateway technology (51), target genes were amplified by PCR and cloned into the pENTR/D-TOPO vector (Invitrogen). LR clonase

TABLE 3.2. Strains and plasmids

Designation	Relevant Characteristics	Source
Bacterial strains		
Escherichia coli		
DB3.1	F- *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB* mrr *hsdS20*(r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) *xyl5* Δ*leu mtl1*	(7)
DH10B	F^- *mcrA* Δ*lacX74* (ϕ80d*lacZ*Δ*M15)* Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara*, *leu*)7697 *galU galK* λ^- *rpsL nupG*	(30)
Mach1 T1	Δ*recA1398 endA1 tonA* ϕ*80*Δ*lacM15* Δ*lacX74 hsdR*(r_K^- m_K^+)	Invitrogen
P. syringae pv. *syringae*		
B728a	wild type, bean pathogen, Rif^r	(62)
B728aAR2	*ladS*$^-$ derivative of B728a, Rif^r Km^r	This study
B728aAR3	*retS*$^-$ derivative of B728a, Rif^r Km^r	This study
B728aAR4	B728aΔ*retS*, Rif^r Km^r	This study
B728aAR5	Markerless derivative of B728aAR4, Rif^r	This study
NPS3136	*gacS*::Tn5 derivative of B728a, Rif^r Km^r	(114)
Plasmids		
pBH474	*flp* constitutively expressed, Gm^r Suc^S	(38)
pENTR/D-TOPO	Gateway entry vector, Km^r	Invitrogen
pEhcp-vsv	pENTR/D-TOPO carrying *hcp* with its putative promoter region and a 3' *vsv* tag, Km^r	This study
pEladS'	pENTR/D-TOPO carrying a 650-bp fragment of *ladS*, Km^r	This study
pEretS'	pENTR/D-TOPO carrying a 600-bp fragment of *retS*, Km^r	This study
pEretS	pENTR/D-TOPO carrying *retS* with its putative promoter region, Km^r	This study
pEretS-FP	pENTR/D-TOPO carrying 1.8 kb upstream of *retS* fused to *nptII* fused to 1.9 kb downstream of *retS*	This study
pKD4	Template plasmid containing FRT-flanked *nptII*	(17)
pLVCD	Gateway destination vector for mating with *P. syringae*, pBR322 derivative with *mob* genes from RSF1010, Tc^r Ap^r Cm^r	Steve Lindow
pLVladS'	pLVCD carrying a 650 bp fragment of *ladS*, Tc^r Ap^r	This study
pLVretS'	pLVCD carrying a 600 bp fragment of *retS*, Tc^r Ap^r	This study
pLVretS-FP	pLVCD carrying 1.8 kb upstream of *retS* fused to *nptII* fused to 1.9 kb downstream of *retS*	This study
pRH002	Gateway destination vector, pBBR1MCS1 derivative, Cm^r	(33)
pRH2hcp-vsv	pRH002 carrying *hcp* with its putative promoter region and a 3' *vsv* tag cloned in-frame with the vector *lacZ* promoter, Cm^r	This study
pRHretS	pRH002 carrying *retS* with its putative promoter region and a cloned in-frame with the vector *lacZ* promoter, Cm^r	This study
pRK2073	Helper plasmid, Sp^r Trm^r	(57)

(Invitrogen) was used for recombination between pENTR constructs and Gateway destination vectors, per manufacturer's instructions. Plasmids were introduced into *E. coli* via chemical transformation or electroporation (89). Plasmids were introduced into *P. syringae* pv. *syringae* strains via tri-parental mating using helper plasmid pRK2073 (57). Standard cycling conditions were used for PCR. The annealing temperature for fusion PCR was 56°C.

Construction of plasmids

A 1.1-kb fragment containing the *hcp* gene and its putative promoter region was amplified using primer P163 and primer P164, which contains a sequence encoding the vesicular stomatitis glycoprotein (VSV) epitope (42), and then cloned into pENTR/D-TOPO vector (Invitrogen) via topoisomerase reaction, resulting in pEhcp-vsv. A 2.9-kb fragment including *retS* and its putative promoter region was amplified from the B728a genome using primers P183 and P184 and cloned into pENTR/D-TOPO, resulting in pERetS. The *hcp-vsv* and *retS* entry constructs were each recombined into the pRH002 Gateway destination vector, resulting in pRHhcp-vsv and pRHretS, respectively.

Construction of B728a *ladS* and *retS* insertion mutations

Insertional mutations in the B728a *ladS* and *retS* genes were made as follows. A 650-bp fragment of *ladS* was amplified using primers P121 and P122, and a 600-bp fragment of *retS* was amplified using primers P119 and P129. The PCR products were cloned into pENTR/D-TOPO, resulting in pEladS' and pEretS', respectively. The *ladS'* and *retS'* entry constructs were each recombined into the pLVCD Gateway destination vector, resulting in pLVladS' and pLVretS', respectively. Tri-parental matings were set

TABLE 3.3. Primers used for PCR amplification and quantitative real-time PCR analysis

Name	Sequence
P033	TCTCAACGCCCGGGACAACATCAA
P034	GCTCAGCGACCAGGCCCAATAACA
P119	CACCAACCGTCGGACTGCTGACTAA
P121	CACCGACCATCAGCATCGGGACTAA
P122	GATCGGGTCGCTGGAAGTCAT
P129	GATCGGGCGCAGCAGGTTGAGGAG
P143	CCTGGAAGAAGTGGTTGTGTC
P144	ATCGGAACGGCTCTGCTGGGTGTA
P147	CTTCGGTACGCCTGGACA
P148	AACTCGGCCTGACGGAAC
P163	CACCCGCAGTCGTTGAAGGCTATC
P164	TCATTTTCCTAATCTATTCATTTCAATATCTGTATATGCGAATACTTTGTTCGC
P183	GTCATAAGCTTCGCTGTCAGTTTTGCC
P184	CACCATAATCGATCGGACCAG
P193	CACCGTCGGTGCAGGCCAGATGAG
P194	GAAGCAGCTCCAGCCTACACTGAGTGAATGCCTGGTAAAA
P195	TAAGGAGGATATTCATATGGTGGCCTGATAGACTGGCGA
P196	TGATCCGAATGAAGCCCTGTTGAC
P197	GTGTAGGCTGGAGCTGCTTC
P198	CATATGAATATCCTCCTTAGTTCC
hrpZF	TCCTGAAACCGAGACGACTGG
hrpZR	GACCGTTGCGCATCAGTTCCTC

up between *E. coli* DH10B(pLVladS') or DH10B(pLVretS'), wild-type B728a, and *E. coli* DB3.1(pRK2073). Integration of pLVladS' or pLVretS' into the B728a genome resulted in B728aAR2, a *ladS*⁻ derivative, or B728aAR3, a *retS*⁻ derivative of B728a.

RNA isolation for qRT-PCR studies

Bacterial strains were cultured overnight with shaking at 25°C in 2 ml of liquid NBY medium with appropriate antibiotics. Cells were harvested by centrifugation, washed, and then 3 μl were inoculated into 2 ml of fresh NBY with appropriate antibiotics. The cultures were grown at 25°C with shaking to an optical density at 600 nm (OD_{600}) of 0.3 (approximately 5 x 10^8 CFU/ml). Total RNA was purified using the RNeasy Mini Kit in conjunction with RNAprotect reagent (Qiagen Inc., Valencia, Calif.) following the manufacturer's protocol. RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, Wis.) to remove any residual DNA in the samples.

qRT-PCR analysis

To determine the effects of *retS* and *ladS* mutations on expression of representative type VI and type III genes, qRT-PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen Inc.). Total RNA was prepared as described above. Primers used for qRT-PCR included *hcp*-specific primers P143 and P144, Psyr_2628-specific primers P033 and P034, and *hrpZ*-specific primers hrpZF and hrpZR. Primer sequences are listed in Table 3.2. Primers P147 and P148, which are specific to the *recA* housekeeping gene, were used for normalization. For each primer pair, the linearity of detection was confirmed to have a correlation coefficient of at least 0.98 ($r^2 > 0.98$) over the detection area by measuring a fivefold dilution curve with RNA isolated from

bacterial cells. Reverse transcription was conducted at 50°C for 30 min, followed by initial activation of DNA polymerase (95°C for 15 min). qRT-PCR was performed in 35 cycles (94°C for 15 s, 55°C for 30 s, 72°C for 30 s), followed by melting curve analysis.

Secretion assays

P. syringae pv. *syringae* strains carrying pRHhcp-vsv were shaken overnight at 25°C in 2 ml of NBY liquid supplemented with chloramphenicol. The cells were pelleted and washed, and then 3 μl were inoculated into 2 ml of fresh NBY or SRM_{AF} with chloramphenicol. The cultures were grown at 25°C with shaking to an OD_{600} of 0.3. Cultures were separated into cell-associated and supernatant fractions via centrifugation, and the proteins in the supernatant fractions were precipitated with 12.5% trichloroacetic acid. Proteins in whole-cell lysates and supernatant fractions were separated on 15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to Hybond-P PVDF membranes (GE Healthcare, Piscataway, N.J.). Western blots were probed with antibodies to the vesicular stomatitis virus glycoprotein (VSV-G) epitope (Sigma Chemical Co., St. Louis, Mo.) or to the β-subunit of RNA polymerase (RNAP, Neoclone, Madison, Wis.). Primary antibodies were recognized by anti-mouse and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate secondary antibodies (Sigma Chemical Co.) and visualized via the Amersham ECL chemiluminescence system (GE Healthcare). Relative protein concentrations in culture fractions were estimated as follows. The Gel Analysis option of the ImageJ program (http://rsbweb.nih.gov/ij/) was used to measure signal intensity for each protein band on scanned images of the blots. For each bacterial strain, intensity values for cellular and supernatant fraction proteins

were combined. The relative amount of Hcp-VSV present in each supernatant fraction was calculated by dividing the supernatant intensity value by the combined intensity value. The experiment was repeated three times with consistent results.

Swarming motility assays

Swarming motility was evaluated on semisolid NBY containing 0.4% agar (48). Initially, bacteria were grown for 48 h at 25°C on KB agar containing appropriate antibiotics. Cells were scraped from the plates, washed, and adjusted to the desired OD_{600} in sterile ddH_2O. Sterile filter discs (Fisherbrand, Grade P8-Creped) sized to 6 mm with a standard 1-hole punch, were placed in the center of each plate and inoculated with a drop containing 1 x 10^8 cells. Plates were incubated at 25°C for 24 h in a moist chamber. The experiment was repeated three times.

Leaf colonization assays

The B728a *ladS* and *retS* mutants were tested for their ability to colonize bean leaves, using a protocol based on methods described by Monier and Lindow (72). B728a and derivative strains were grown overnight in 2 ml of NBY at 25°C with appropriate antibiotics. 2-ml cultures were used to seed fresh 100-ml NBY cultures, which were grown at 25°C to an OD_{600} of 0.6. Cultures were pelleted, washed, and diluted in sterile ddH_2O to 10^5 CFU/ml. Two-week-old Blue Lake 274 (*Phaseolus vulgaris*) bean plants were inverted and submerged in the bacterial suspensions for 3 sec. Plants were rinsed with distilled water and allowed to air dry. Plants were maintained at 22°C in a growth chamber with 45% relative humidity (low RH) for 48 h. Prior to placement in the growth chamber, some of the plants were covered with large plastic bags, which created

conditions of high RH. Each bacterial strain was tested on three individual bean plants, and the experiment was repeated three times.

For population analyses, five leaves were arbitrarily collected from each inoculated plant, weighed, and placed in 20 ml of washing buffer (0.1M K_2HPO_4/KH_2PO_4, 0.1% Bacto-Peptone, pH 7.0) in a sterile Falcon tube. In order to remove bacteria from the leaves, the tubes were sonicated for 7 min in an ultrasonic water bath. Serial dilutions were made in sterile ddH_2O, and spread on KB plates with appropriate antibiotics. Colonies were enumerated after plates were incubated for 48 h at 25°C.

Construction of markerless *retS* deletion in B728a

PCR with primers P193 and P194 was used to amplify a 1.8-kb portion of the B728a genome upstream of the *retS* gene. Likewise, a 2.0-kb region downstream of *retS* was amplified using primers P195 and P196. Because P194 and P195 feature the FLP recombinase recognition sequence (FRT), the PCR products contained FRT sites. A third PCR, using primers P197 and P198, was set up to amplify a cassette containing an FRT-flanked *nptII* gene from the plasmid pKD4. The three PCR products were combined in a 1:1:1 molar ratio and subjected to fusion PCR, which joined the three products together at their mutual FRT sites. The fused product was cloned into pENTR/D-TOPO, resulting in pEretS-FP. The *retS*-FP entry construct was recombined into the pLVCD Gateway destination vector, resulting pLVretS-FP. A tri-parental mating was set up between *E. coli* DH10B(pLVretS-FP), wild-type B728a, and *E. coli* DB3.1(pRK2073). Marker exchange resulted in B728aAR4, a B728a derivative with a *retS* deletion. pBH474, a

plasmid that expresses FLP recombinase, was introduced into the cells via electroporation. FLP recombination resulted in loss of the *nptII* marker, giving the markerless B728a *retS* mutant B728aAR5. The Suc^{s} pBH474 plasmid was cured from the B728aAR5 cells by plating on NBY + 5% sucrose.

CHAPTER IV

CONCLUSIONS

The relationship between *P. syringae* and its plant host is complex. *P. syringae* pathogenicity involves an array of secretion systems, toxins, and effector proteins dedicated to virulence in the plant. In addition to survival within the plant host, the lifestyle of epiphytic strains, like B728a, requires that they are adapted to an existence on the leaf surface. Intricate signaling networks manage the epiphyte's delicate balance between surface resident and tissue invader. In this study, I addressed the hypothesis that the T6SS and its regulators RetS and LadS are involved in the B728a-plant interaction.

The B728a genome encodes a functional T6SS. This is an exciting discovery because type VI secretion is a newly discovered phenomenon that has not yet been studied in great detail. T6 loci are present in the genomes of several Gram-negative bacteria, including pathogens of both plants and animals. This dissertation confirms that at least two of the phytopathogen T6 loci are functional – the one present in the *P. atrosepticum* genome (67) and the B728a T6 locus, described here (Fig. 2.4). The benefit that the T6SS confers upon B728a is not yet clear, but this study lays the groundwork for investigations into its biological function.

RetS and LadS are at the top of important regulatory pathways in *P. aeruginosa* (Fig. 3.1). Likewise, this dissertation shows that RetS and LadS are regulators that control activities associated with virulence in B728a. B728a LadS is a positive regulator of swarming motility (Fig. 3.5), which has been linked to virulence in *P. aeruginosa*

(78). B728a RetS negatively controls production of an EPS (Fig. 3.3), which may potentially be identified as alginate or as a novel B728a EPS. It is very possible that a third EPS is involved in B728a virulence, because neither alginate nor levan are required for *P. syringae* biofilm formation (55). LadS and RetS up-regulate and down-regulate expression of the B728a T6SS, respectively (Table 3.1). Although a direct correlation between the B728a T6SS and virulence has not yet been established, it likely plays a role in the pathogen-host interaction as do its counterparts in other bacteria (11).

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