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Sensor Kinases RetS and LadS Regulate *Pseudomonas syringae* Type VI Secretion and Virulence Factors▽

Angela R. Records† and Dennis C. Gross*

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843

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Pseudomonas 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 study demonstrates that a 29.9-kb cluster of genes in the B728a genome shares homology to the novel type VI secretion system (T6SS) locus recently described for other Gram-negative bacteria. Western blot analyses showed that B728a secretes Hcp, a T6SS protein, in culture and that this secretion is dependent on *clpV*, a gene that likely encodes an AAA$^+$ ATPase. In addition, we have identified two B728a sensor kinases that have homology to the *P. aeruginosa* proteins RetS and LadS. We demonstrate that B728a RetS and LadS reciprocally regulate the T6SS and collectively modulate several virulence-related activities. Quantitative PCR analyses indicated that RetS and LadS regulate genes associated with the type III secretion system and that LadS controls the expression of genes involved in the production of the exopolysaccharides alginate and levan. These analyses also revealed that LadS and the hybrid sensor kinase GacS positively regulate the expression of a putative novel exopolysaccharide called Psl. Plate assays demonstrated that RetS negatively controls mucoidy, while LadS negatively regulates swarming motility. A mutation in *retS* affected B728a population levels on the surfaces of bean leaves. A model for the LadS and RetS control of B728a virulence activities is proposed.**

Pseudomonads have adapted to a remarkable range of environmental conditions, where they may exist as saprophytes (in water or soil), as benign residents (on a plant host), or as pathogens of animals or plants (18). *Pseudomonas syringae* pv. syringae is a widespread pathogen of economically significant 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 the invasion of leaf tissue and initiation of disease. The molecular basis for this switch is complex, requiring the interaction of multiple virulence factors and associated secretion systems (12, 26). Intricate global regulatory networks mediate the expression of these virulence traits, and in almost all cases, regulation begins with a sensor kinase or other surface receptor (57).

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 that detects an environmental signal and autophosphorylates a conserved histidine kinase residue within its transmitter domain (30). The phosphoryl group is then transferred to a cognate cytoplasmic response regulator. TCSs react to a wide range of stimuli, including nutrients, quorum signals, antibiotics, and more (19, 43). 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 TCSs (43). For example, bioinformatic analyses predict that the *Nostoc punctiforme* genome encodes 158 histidine kinases and 84 response regulators (17). Sometimes, a histidine phosphotransfer (Hpt) protein may act as a phosphorelay between a histidine kinase and a response regulator (54). The B728a genome is predicted to encode 68 histidine kinases, 93 response regulators, and one Hpt protein, which contribute to the adaptation of this bacterium to plant and nonplant environments (45).

The impact of the TCS regulation of virulence traits is exemplified by the RetS and LadS sensor kinases of the human pathogen *Pseudomonas aeruginosa*. RetS and LadS reciprocally regulate activities associated with biphasic *P. aeruginosa* lung infections in patients afflicted with the hereditary disease cystic fibrosis (20). 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). Cystic fibrosis patients usually develop chronic pulmonary *P. aeruginosa* infections, during which the bacteria express traits that contribute to long-term survival and protection in the lung, such as quorum sensing, biofilm formation, and the recently discovered type VI secretion system (T6SS) (20, 59). Microarray studies implicated RetS and LadS as global regulators that mediate a switch between the expression of genes necessary for an acute infection of the lung (e.g., the T3SS) and those required for long-term colonization (e.g., biofilm production; the T6SS) (75). Those studies also revealed that RetS and LadS signaling converge on the master virulence regulator GacA, influencing levels of the small regulatory

* Corresponding author. Mailing address: Dept. of Plant Pathology and Microbiology, Texas A&M University, 2132 TAMU, College Station, TX 77843-2132. Phone: (979) 458-0637. Fax: (979) 845-6483. E-mail: d-gross@tamu.edu.

† Present address: Dept. of Cell Biology and Molecular Genetics, University of Maryland, 3121 Biosciences Research Bldg., College Park, MD 20742.

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RNAs RsmZ and RsmY, which ultimately modulate gene expression by binding RsmA (75). The RsmA regulon includes over 500 genes, and a recent study showed that RsmA has the versatility to exert a posttranslational regulation of certain target genes—by binding the mRNA of their cognate regulatory proteins and the posttranscriptional regulation of other target genes—via directly binding their mRNA (5).

The *P. aeruginosa* RetS and LadS regulons control the expression of a wide range of virulence factors, including genes involved in motility (75, 87) and the production of the biofilm-associated exopolysaccharides (EPSs) Pel and Psl (16, 75). Secretion systems are also among the genes subject to regulation by RetS and LadS. These include the type II *xcp* system, responsible for the secretion of various toxins and enzymes into the extracellular environment (15); the T3SS, which delivers virulence factors directly into host cells via a syringe-like apparatus (24); and the T6SS, which was discovered recently and has since been implicated in the virulence of several bacterial pathogens (3, 7, 14, 66).

T6SS loci are widely prevalent among the genomes of bacteria that maintain pathogenic or symbiotic interactions with human, animal, or plant hosts. T6SS loci typically contain 15 to 25 genes, most of which are thought to encode structural components of the T6SS apparatus (66). Little is known about the individual functions of the T6SS genes. One feature of the prototypical T6SS locus is the presence of a *clpB* AAA^+ ATPase homologue (4, 13, 59). The ATPase (named *clpV* in the *P. aeruginosa* genome) is presumed to provide the energy for protein secretion via the hydrolysis of ATP (59). A second T6SS hallmark is the presence of an *icmF* homologue. IcmF confers structural stability upon the *Legionella pneumophila* type IV secretion apparatus (71), and its homologues likely perform a similar function for T6SSs. Ma et al. recently demonstrated that $ImpL_M$, an IcmF homologue in *Agrobacterium tumefaciens*, is an inner membrane protein featuring a Walker A motif required for type VI secretion activity (51). T6SS proteins do not contain signal peptides associated with other secretion systems, and thus far, T6SS-dependent secretion in culture has been demonstrated for only a few proteins (28, 59, 65, 69, 74, 84). All bacteria with a functional T6SS secrete Hcp, which is a hexameric, ring-shaped protein that may stack to form a conduit for protein delivery (59).

Like *P. aeruginosa*, *P. syringae* pv. syringae B728a utilizes protein secretion systems, exopolysaccharides, and other virulence factors during its interactions with its host. *P. syringae* is known to produce at least two EPSs: the polyfructan levan and the capsular polysaccharide alginate (44). Levan is a high-molecular-weight β-(2,6)-polyfructan that is thought to function as an extracellular storage compound metabolized by *P. syringae* during periods of nutrient deprivation (44). Levan synthesis is catalyzed by the periplasmic enzyme levansucrase, which is encoded by *lscC* (47). Alginate has been implicated in the virulence of *P. syringae* because it is involved in both epiphytic fitness and the dissemination of the bacterium *in planta* (36, 85). The B728a alginate biosynthetic cluster contains 11 genes, including *algA*, which was shown to encode a phosphomannose isomerase/guanosine 5′-diphospho-D-mannose pyrophosphorylase in *P. aeruginosa* (72) and is required for B728a alginate production (64).

In this study, we report the identification of the *retS* and *ladS* genes in the B728a genome and demonstrate their collective roles in the modulation of several virulence activities, including swarming motility, the production of EPS, and the expression of T3SS genes. In addition, this study reveals for the first time the presence of a functional T6SS in *P. syringae* and shows that the expression of the B728a T6SS gene *icmF* is under RetS/LadS control. Interestingly, plant infection assays revealed a role for RetS in the B728a surface colonization of bean leaves.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH10B was used for general cloning (68) and was cultured at 37°C in Luria-Bertani (LB) liquid or agar medium. *E. coli* Mach1 T1 cells were used following topoisomerase reactions, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). *P. syringae* pv. syringae strains were routinely grown at 25°C in nutrient broth-yeast extract (NBY) liquid or agar medium (76) or on King's B (KB) agar medium (37). For quantitative reverse transcription-PCR (qRT-PCR) studies, bacteria were grown in modified Hrp minimal medium (HMM) [0.2 M KH_2PO_4, 1.2 M K_2HPO_4, 1.3 M $(NH_4)_2SO_4$, 5.9 M $MgCl_2$, 5.8 M NaCl, 0.2% fructose, 0.2% mannitol, 0.2% succinate, 10 μM acyl homoserine lactone in ethyl acetate, 10 mM glutamine, 10 μM $FeCl_3$] (32) or on potato dextrose agar (PDA) medium. Assays for mucoidy were conducted on PDA, mannitol glutamate-yeast extract (MGY) agar supplemented with 0.6 M sorbitol, or MGY supplemented with 5% sucrose (44). Assays for swarming activity were performed on NBY with 0.4% agar. Antibiotics were added at the following concentrations: rifampin at 100 μg/ml, kanamycin at 75 μg/ml, tetracycline at 20 μg/ml, chloramphenicol at 20 μg/ml, gentamicin at 5 μg/ml, and spectinomycin at 100 μg/ml.

General DNA manipulations. Restriction enzymes, T4 DNA ligase, and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were designed by using Lasergene expert analysis packages (DNAStar, Madison, WI) and purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences are available upon request. For cloning using Gateway technology (40), 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, according to the manufacturer's instructions. Plasmids were introduced into *E. coli* via chemical transformation or electroporation (68). Plasmids were introduced into *P. syringae* pv. syringae strains via triparental mating using helper plasmid pRK2073 (46). 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 by using primer P163 and primer P164, which contains a sequence encoding the vesicular stomatitis virus (VSV) glycoprotein epitope, and then cloned into the pENTR/D-TOPO vector via a 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. A 2.3-kb fragment containing the *ladS* gene along with its putative promoter was amplified by using primers P125 and P126 and cloned into the pENTR/D-TOPO vector, giving pEladS. The *hcp-vsv*, *retS*, and *ladS* entry constructs were each recombined into the pRH002 Gateway destination vector, resulting in pRH2::*hcp-vsv*, pRH2::*retS*, and pRH2::*ladS*, respectively. A 2.8-kb fragment containing the *gacS* gene along with 95 bp of upstream DNA was amplified by using primers P215 and P216, which contain HindIII and BamHI sites, respectively. The PCR product was digested and ligated into HindIII/BamHI-digested pPROBE-GT, giving pPGT::*gacS*. The 2.6-kb *clpV* gene was amplified from the B728a genome by using primers P160 and P161, which contain KpnI and SphI sites, respectively. The PCR product was digested and ligated into KpnI/SphI-digested pUCP26, resulting in pUCClpV.

Construction of markerless *retS* and *gacS* deletion mutations 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 by using primers P195 and P196. Because P194 and P195 feature the FLP recombinase recognition sequence (FLP recombination target [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 plasmid pKD4. The three PCR products were combined in a 1:1:1 molar ratio and subjected to fusion PCR (25), which joined the three products together at their mutual FRT sites. The fused product was cloned into pENTR/D-TOPO,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
Escherichia coli		
DB3.1	F$^-$ *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB mrr hsdS20*(r_B^- m_B^-) *ara14 galK2 lacY1 proA rpsL20*(Sm^r) *xyl5* Δ*leu mtl1*	2
DH10B	F$^-$ *mcrA* Δ*lacX74* (ϕ80*lacZ*ΔM15) Δ(*mrr-hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	22
Mach1 T1	F$^-$ Δ*recA1398 endA1 tonA* ϕ80(*lacZ*)ΔM15 Δ*lacX74 hsdR*(r_K^- m_K^+)	Invitrogen
P. syringae pv. syringae		
B728a	Wild type, bean pathogen; Rif^r	49
B728a *clpV* mutant	Rif^r Tc^r	This study
B728a Δ*gacS*	Rif^r	This study
B728a *gacS* mutant	Rif^r Km^r	83
B728a *ladS* mutant	Rif^r Tc^r	This study
B728a Δ*retS*	Rif^r	This study
Plasmids		
pBH474	*flp* constitutively expressed; Gm^r Suc^s	29
pENTR/D-TOPO	Gateway entry vector; Km^r	Invitrogen
pEclpV	pENTR/D-TOPO carrying *clpV*; Km^r	This study
pEclpV-Gm	pENTR/D-TOPO carrying *clpV*::*aacC1*; Km^r Gm^r	This study
pEgacS-FP	pENTR/D-TOPO carrying 1.0 kb upstream of *gacS* fused to *nptII* fused to 1.1 kb downstream of *gacS*	This study
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 *ladS* with its putative promoter region; Km^r	This study
pEladS′	pENTR/D-TOPO carrying a 650-bp fragment of *ladS*; 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*; Km^r	This study
pKD4	Template plasmid containing FRT-flanked *nptII*	9
pLVCD	Gateway destination vector for mating with *P. syringae*; pBR322 derivative with *mob* genes from RSF1010; Tc^r Ap^r Cm^r	52
pLVclpV-Gm	pLVCD carrying *clpV*::*aacC1*; Tc^r Ap^r Gm^r	This study
pLVgacS-FP	pLVCD carrying 1.0 kb upstream of *gacS* fused to *nptII* fused to 1.1 kb downstream of *gacS*; Tc^r Ap^r Km^r	This study
pLVladS’	pLVCD carrying a 650-bp fragment of *ladS*; 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*; Tc^r Ap^r Km^r	This study
pPROBE-GT	Promoter-probe vector with pVS1/p15a replicon and *gfp* reporter; Gm^r	56
pPGT::*gacS*	pPROBE-GT carrying *gacS* along with 95 bp upstream; Gm^r	This study
pRH002	Gateway destination vector, pBBR1MCS1 derivative; Cm^r	23
pRH2::*hcp-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
pRH2::*ladS*	pRH002 carrying *ladS* with its putative promoter region cloned in frame with the vector *lacZ* promoter; Cm^r	This study
pRH2::*retS*	pRH002 carrying *retS* with its putative promoter region cloned in frame with the vector *lacZ* promoter; Cm^r	This study
pRK2073	Helper plasmid; Sp^r Trm^r	46
pUCclpV	pUCP26 carrying *clpV* in frame with the vector *lacZ* promoter; Tc^r Ap^r	This study
pUCGm	Cloning vector; Gm^r Ap^r	70
pUCP26	Cloning vector; Tc^r Ap^r	79

resulting in pEretS-FP. The *retS*-FP entry construct was recombined into the pLVCD Gateway destination vector, resulting in pLVretS-FP. A triparental mating was set up between *E. coli* DH10B(pLVretS-FP), wild-type B728a, and *E. coli* DB3.1(pRK2073). Marker exchange resulted in a B728a *retS* deletion. pBH474, a plasmid that expresses FLP recombinase, was introduced into the cells via electroporation. FLP recombination resulted in the loss of the *nptII* marker, giving the markerless mutant. The Suc^s pBH474 plasmid was cured from the B728aΔ*retS* cells by plating onto NBY plus 5% sucrose.

A similar approach was used for the construction of the B728a Δ*gacS* strain. Primers P207 and P208 were used to amplify a 1.0-kb region upstream of *gacS*, and primers P209 and P210 amplified the 1.1-kb downstream region. Mating between DH10B(pLVgacS-FP), B728a, and DB3.1(pRK2073) resulted in a B728a *gacS* deletion. The *nptII* marker was removed from the B728a Δ*gacS* genome as described above.

Construction of B728a *ladS* and *clpV* insertion mutations. A mutation in the B728a *ladS* gene was made as follows. A 650-bp fragment of *ladS* was amplified by using primers P121 and P122, and the PCR product was cloned into pENTR/D-TOPO, resulting in pEladS′. The *ladS*′ entry construct was recombined into the pLVCD Gateway destination vector, resulting in pLVladS′. A triparental mating was set up between *E. coli* DH10B(pLVladS′), wild-type B728a, and *E. coli* DB3.1(pRK2073). The integration of pLVladS′ into the B728a genome resulted in the B728a *ladS* mutant.

To construct 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 a

topoisomerase reaction, resulting in the construct pEclpV. The *aacC1* gentamicin (Gm) resistance gene was isolated from pUCGm by digestion with HindIII and was ligated into HindIII-digested pEclpV, resulting in pEclpV-Gm. The *clpV*-Gm entry construct was recombined into pLVCD. Triparental mating between wild-type B728a, *E. coli* DH10B(pLVclpV-Gm), and *E. coli* DB3.1(pRK2073) and marker exchange resulted in the B728a *clpV* mutant.

RNA isolation for qRT-PCR studies. For analyses of T3SS gene expression, bacterial strains were first cultured for 24 h with shaking at 25°C in 5 ml HMM. Ten microliters of initial cultures was transferred into fresh HMM and grown for 16 h at 25°C with shaking. Subcultures were prepared by transferring 300 μl of cultures grown overnight into 30 ml HMM. The cultures were grown at 25°C with shaking to an optical density at 600 nm (OD_{600}) of 0.6 (approximately 5×10^8 CFU/ml). RNAprotect stabilizing reagent (Qiagen Inc., Valencia, CA) was added to each culture according to the manufacturer's instructions. Cells were pelleted (5×10^8 cells/pellet) and resuspended in 200 μl of Tris-EDTA (TE) prior to the isolation of total RNA. For analyses of EPS and T6SS gene expression, bacterial strains were cultured in PDB as described above. Upon reaching an OD_{600} of 0.6, PDB cultures were concentrated, and 0.2 ml (5×10^8 cells) was then spotted onto the center of a PDA plate. Plates were incubated for 40 h at 25°C. RNAprotect was added to the plates after incubation, according to the manufacturer's instructions. Total RNA was purified by using the RNeasy mini-kit according to the manufacturer's protocol. RNA samples were treated with on-column RNase-free DNase I (Qiagen) to remove any residual DNA in the samples. The SuperScript VILO cDNA synthesis kit (Invitrogen) was used to prepare cDNA from RNA samples.

qRT-PCR analysis. To determine the effects of *retS*, *ladS*, and *gacS* mutations on the expression of representative EPS, T6SS, and T3SS genes, qRT-PCR was performed by using the Express two-step SYBR green ER kit (Invitrogen). Total RNA was prepared as described above. Primers specific for *hcp*, *icmF*, *lscC*, *algA*, *pslB*, *hrpL*, and *hrpR* were used for qRT-PCR (data not shown). Primers specific for 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 5-fold dilution curve with cDNA isolated from bacterial cells. qRT-PCR was performed in 40 cycles (95°C for 3 s and 58°C for 30 s), followed by melting curve analysis.

Swarming motility assays. Swarming motility was evaluated on semisolid NBY containing 0.4% agar (38). Initially, bacteria were grown for 48 h at 25°C on KB agar containing appropriate antibiotics. Cells were scraped from plates, washed, and adjusted to the desired OD_{600} in sterile double-distilled water (ddH_2O). Sterile filter discs (grade P8-creped; Fisherbrand) sized to 6 mm with a standard 1-hole punch were placed into the center of each plate and inoculated with a drop containing 1×10^8 cells. Plates were incubated at 25°C for 24 h in a moist chamber. The experiment was repeated three times.

Secretion assays. *P. syringae* pv. syringae strains carrying pRH2::*hcp-vsv* were shaken overnight at 25°C in 2 ml of NBY liquid supplemented with appropriate antibiotics. The cells were pelleted and washed, and 3 μl was then inoculated into fresh NBY with appropriate antibiotics. 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 gel electrophoresis (SDS-PAGE) gels and transferred onto Hybond-P polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ). Western blots were probed with antibodies to the VSV-G epitope (Sigma Chemical Co., St. Louis, MO) or to the β-subunit of RNA polymerase (RNAP) (Neoclone, Madison, WI). Primary antibodies were recognized by anti-mouse or anti-rabbit immunoglobulin G-alkaline phosphatase-conjugated 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 the signal intensity for each protein band on scanned images of the blots. For each bacterial strain, intensity values for cellular and supernatant 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.

Leaf colonization assays. B728a strains were tested for their abilities to colonize bean leaves by using a protocol based on methods described previously by Monier and Lindow (58). B728a and derivative strains were grown overnight in 2 ml of NBY at 25°C with appropriate antibiotics. Two-milliliter 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 into the bacterial suspensions for 3 s. 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 (RH) (low RH) for 48 h. Prior to placement into 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 into 20 ml of washing buffer (0.1 M 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 onto KB plates with appropriate antibiotics. Colonies were enumerated after plates were incubated for 48 h at 25°C.

RESULTS

Psyr_4408 and Psyr_4339 of *P. syringae* pv. syringae strain B728a are orthologues of the *P. aeruginosa retS* and *ladS* genes, respectively. BLAST searches against the *P. syringae* pv. syringae strain B728a genome (GenBank accession number CP000075) with the amino acid sequences of *P. aeruginosa* strain PAO1 RetS (GenBank accession number NP_253543) and LadS (GenBank accession number NP_252663) revealed that the predicted proteins encoded by Psyr_4408 (GenBank accession number YP_237476) and Psyr_4339 (GenBank accession number YP_237407) have homology to RetS and LadS, respectively. Pairwise amino acid sequence alignments using the SIM alignment tool (http://www.expasy.ch/tools/sim.html) showed that Psyr_4408 and RetS share 58.6% identity, while Psyr_4339 and LadS share 56.3% identity at the amino acid level. The *Pseudomonas* Genome Database GBrowse tool (www.pseudomonas.com) was used to view the B728a and PAO1 *retS* and *ladS* genes in a genomic context. Comparisons revealed that *retS* (PA4856) and *ladS* (PA3974) lie in regions of the *P. aeruginosa* PAO1 genome that are highly conserved among all sequenced *Pseudomonas* strains, including *P. syringae* pv. syringae B728a (data not shown).

Conserved domains within the Psyr_4408 and Psyr_4339 protein sequences were identified via comparison to the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The conserved domains of Psyr_4408, Psyr_4339, and their PAO1 counterparts RetS and LadS are depicted in Fig. 1. Both RetS and LadS contain a histidine kinase domain, an HATPase_C kinase domain, and one (LadS) or two (RetS) response regulator receiver domains. These domains are secured to the inner membrane via seven transmembrane segments linked to a periplasm-exposed signal-binding domain. Unlike its LadS orthologue, Psyr_4339 does not contain a response regulator receiver domain. It is likely that Psyr_4339 transmits signals through a receiver encoded elsewhere in the genome. 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 within a protein is unusual, making RetS unique (42).

B728a RetS and LadS regulate the expression of genes involved in EPS production and protein secretion. RetS and LadS regulate the expression of several genes involved in *P.*

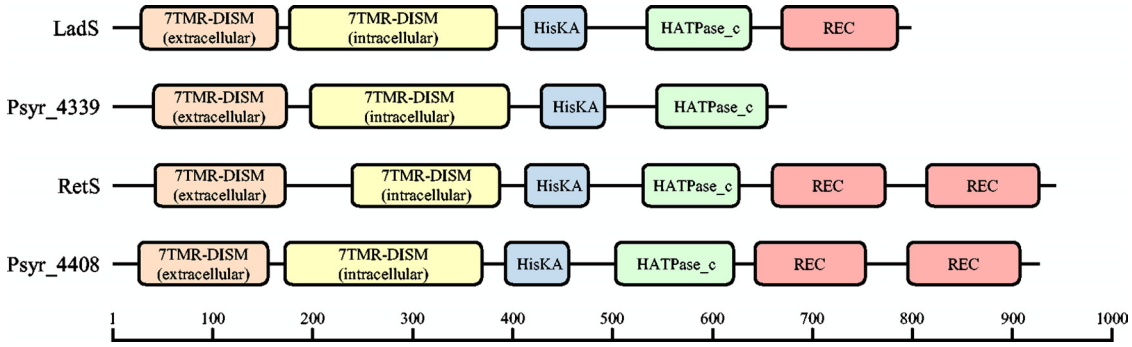

FIG. 1. Domain organizations of *P. aeruginosa* PAO1 LadS and RetS proteins and *P. syringae* pv. syringae B728a orthologues. PAO1 LadS is a 795-amino-acid hybrid sensor protein featuring a histidine kinase domain (HisKA), an HATPase_C kinase domain, a response regulator receiver domain (REC), and a transmembrane receptor (7TMR-DISM) (75). The 677-amino-acid protein product predicted for B728a Psyr_4339 shares an architecture similar to that of PAO1 LadS. PAO1 RetS is a 942-amino-acid protein with an architecture identical to that of LadS but with one additional receiver domain (75). The domain organization predicted for the 929-amino-acid B728a Psyr_4408 protein is identical to that of PAO1 RetS. Predictions were made by an NCBI conserved-domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The scale bar indicates the protein length in numbers of amino acid residues.

aeruginosa virulence, including exopolysaccharide (EPS) genes in the *pel* and *psl* operons and genes encoding the type III and type VI protein secretion systems (20, 75). In order to analyze the influence of RetS and LadS on the expression of genes involved in EPS production and protein secretion by B728a, deletion or insertion mutants were constructed as described in Materials and Methods. Mutations were made in B728a Psyr_4408 (*retS*), Psyr_4339 (*ladS*), and Psyr_3698 (*gacS*). GacS was included in this study because it is a global regulator of *P. syringae* virulence (8), and its *P. aeruginosa* orthologue is a critical component of the RetS/LadS regulon (6, 75). cDNA was obtained from wild-type B728a or the Δ*retS*, *ladS* mutant, or Δ*gacS* strain grown either in HMM liquid medium (for analysis of type III gene expression) or on PDA plates (for analysis of type VI and EPS genes) as described in Materials and Methods. qRT-PCR was performed by using primers specific for the type III genes *hrpL* (Psyr_1217) and *hrpR* (Psyr_1190), the EPS genes *algA* (Psyr_1052) and *lscC* (Psyr_0754), the putative EPS gene *pslB* (Psyr_3302), and the putative type VI genes *hcp* (Psyr_4965) and *icmF* (Psyr_4962). Results of the qRT-PCR studies are summarized in Fig. 2. The B728a Δ*retS* strain exhibited a 2.8-fold increase in *icmF* transcript levels compared to those of wild-type B728a and greater-than-2.0-fold decreases in transcript levels of *hrpL* and *hrpR*. In contrast, *icmF* transcript levels were 3.3-fold lower in the B728a *ladS* mutant than in wild-type B728a, while *hrpL* transcript levels were 2.6-fold higher. Taken together, these results suggest that RetS is a negative regulator of the T6SS and a positive regulator of the T3SS. LadS appears to act in the opposite manner. In addition to its function as a regulator of protein secretion, the B728a *ladS* mutant produced lower levels of *lscC*, *algA*, and *pslB* transcripts (3.6-, 2.2-, and 2.0-fold, respectively) than did wild-type B728a, indicating that LadS may play a positive role in EPS production. Transcript levels of *hcp*, *icmF*, *algA*, and *pslB* were significantly lower in the B728a Δ*gacS* strain (5.3-, 3.1-, 15.4-, and 7.7-fold, respectively) than in wild-type B728a, which indicates that the GacS global regulator controls the expression of the T6SS and EPS production in B728a. A mutation in *gacS* did not have a measurable effect on the expression of the T3SS gene *hrpL* or *hrpR*.

Mutations in B728a *retS*, *ladS*, and *gacS* genes result in mucoidy phenotypes on various media. When inoculated onto PDA, the B728a Δ*retS* strain exhibited highly mucoid growth compared to that of wild-type B728a (Fig. 3A). Wild-type colony morphology was restored to the B728a Δ*retS* strain when a functional *retS* allele was expressed in *trans* on plasmid pRH002. In an effort to determine the nature of this mucoidy, B728a strains were inoculated onto MGY agar, a medium that induces EPS production by *P. syringae* (44, 64), and incubated for 24 h at 25°C. On MGY agar supplemented with 0.6 M sorbitol, which is known to induce the expression of alginate-related genes (64), the B728a Δ*retS* strain appeared much more mucoid than wild-type B728a (Fig. 3B). The inoculation of MGY supplemented with 5% sucrose, which stimulates the production of levan (44), did not reveal any observable differences between wild-type B728a and the B728a Δ*retS* strains (Fig. 3C). These results suggest that B728a RetS may negatively regulate alginate production. In contrast, the B728a *gacS* mutant exhibited a decrease in mucoidy on both PDA and MGY plus sorbitol (Fig. 3A and B). The colony morphology of the B728a *ladS* mutant appeared similar to that of wild-type B728a on all media tested except that it was slightly less mucoid on MGY plus sucrose (Fig. 3C). Taken together, these results suggest that GacS and LadS positively control the production of alginate and levan, respectively.

B728a swarming motility is enhanced by mutation of *ladS*. A low-agar medium was used to determine whether a mutation in *ladS* or *retS* has an effect 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. 4). The B728a Δ*retS* strain displayed a similar movement pattern. The B728a *ladS* mutant swarmed 18.6 ± 1 mm farther (mean of the difference measured in three independent assays ± standard deviation [SD]) from the point of inoculation than wild-type B728a, which suggests that LadS negatively controls B728a swarming ability. The B728a *gacS* mutant showed no movement on semisolid agar, as demonstrated previously (38).

The B728a T6SS locus. The fact that *P. aeruginosa* RetS and LadS modulate the expression of T6SS genes led us to inves-

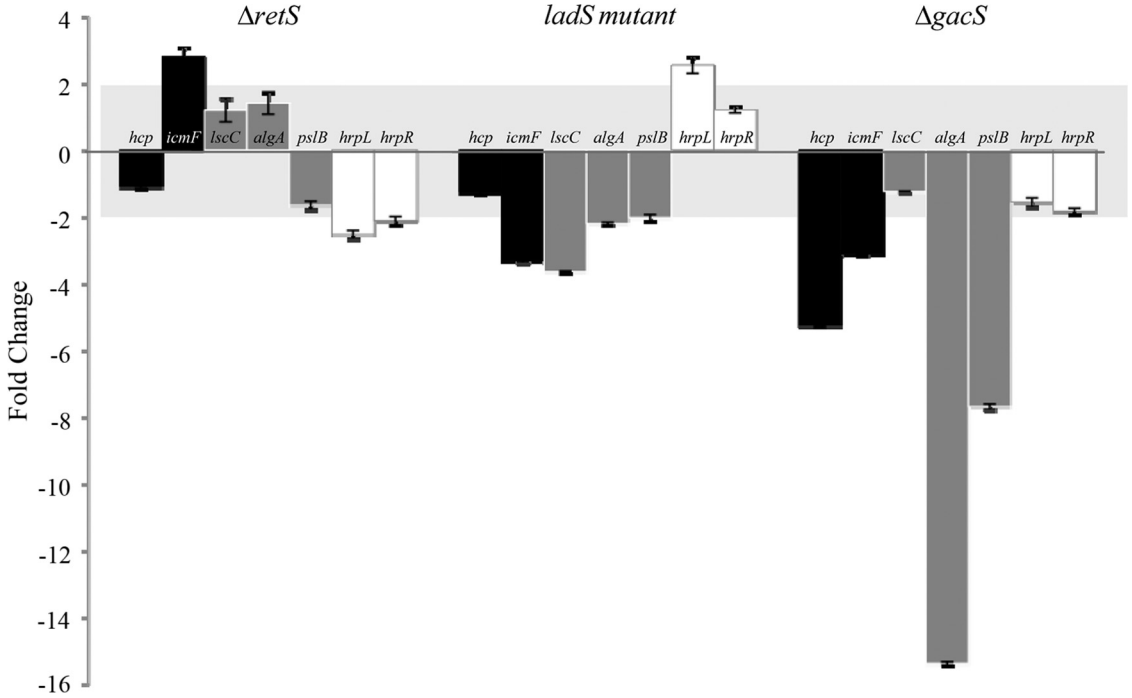

FIG. 2. Quantitative real-time PCR analysis of gene expression as influenced by *retS*, *ladS*, and *gacS* in *P. syringae* pv. syringae B728a. Bacterial cDNA was obtained from wild-type B728a and mutant strains after growth either in HMM liquid (for analysis of type III gene expression) or on PDA plates (for analysis of type VI and EPS genes) as described in Materials and Methods. The differential expression of representative type VI (represented by black bars), type III (white bars), and EPS (gray bars) genes is expressed as fold change values for the Δ*retS*, *ladS* mutant, or Δ*gacS* strain versus wild-type B728a. Error bars indicate standard deviations from the mean obtained from three independent biological samples. Expression for all samples was normalized to that of the housekeeping gene *recA*.

tigate whether the B728a genome encodes a functional T6SS that may be regulated in a similar manner. In order to determine if the B728a genome carries a T6SS locus, the *P. aeruginosa* PAO1 ClpV1 protein sequence (GenBank accession number NP_248780) was used in a BLAST search of the B728a proteome. Three B728a proteins showed strong homology (bit scores of >200) to ClpV1. The Psyr_4958, Psyr_0728, and Psyr_3813 genes, which correspond to the homologous proteins reported under GenBank accession numbers 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 homologues. Psyr_4958, however, is flanked by genes with homology to those in the *P. aeruginosa* HSI-I T6SS locus (Fig. 5). Through systematic BLAST searches of the genomic sequence surrounding Psyr_4958, the B728a T6SS locus was defined. Schematic representations of the B728a T6SS locus and those present in the *P. aeruginosa* PAO1 genome are shown in Fig. 5. The B728a T6SS locus is confined to a 29.876-kb region of the genome that includes 22 open reading frames (ORFs) predicted to be transcribed in the same direction and likely as part of a single operon (10, 86).

To determine whether any other T6SS loci are present in the B728a genome, BLAST searches were also conducted by using the *P. aeruginosa* PAO1 Hcp1 (GenBank accession number NP_248775.1) and VgrG1 (accession number NP_248781.1) protein sequences. Many bacterial genomes carry multiple copies of *hcp* and *vgrG* in genomic locations distinct from the T6SS locus (66). Likewise, the B728a genome contains multiple ORFs with strong homology to *hcp1* (Psyr_0101 [bit score = 102; E value = 1e−23], Psyr_1935 [bit score = 98.2; E value = 3e−22], Psyr_4039 [bit score = 76.3; E value = 1e−15], and Psyr_4965 [bit score = 65.1; E value = 3e−12]) as well as multiple *vgrG1* paralogues (Psyr_4983 [bit score = 358; E value = 8e−100], Psyr_4080 [bit score = 290; E value = 4e−79], Psyr_4974 [bit score = 229; E value = 4e−61], Psyr_4382 [bit score = 190; E value = 3e−15], and Psyr_3092 [bit score = 72; E value = 1e−13]). Analysis of the genomic regions surrounding the various *hcp* and *vgrG* homologues revealed no additional T6SS gene clusters in the B728a genome.

B728a secretes the Hcp protein in a T6SS-dependent manner. Because Hcp secretion has been demonstrated for all known functional T6SSs, it was selected as an indicator of T6SS function. 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 (VSV) glycoprotein epitope. In order to ensure the expression of *hcp-vsv*, the sequence was placed in frame with a *lac* promoter on the broad-host-range vector pRH002 (23). The proper orientation and tagging of *hcp* were confirmed by the sequencing of pRH2::*hcp-vsv*, and the construct was introduced into wild-type B728a and derivative strains. An insertional mutation of the *clpV* (Psyr_4958) gene was constructed, as described in Materials and Methods.

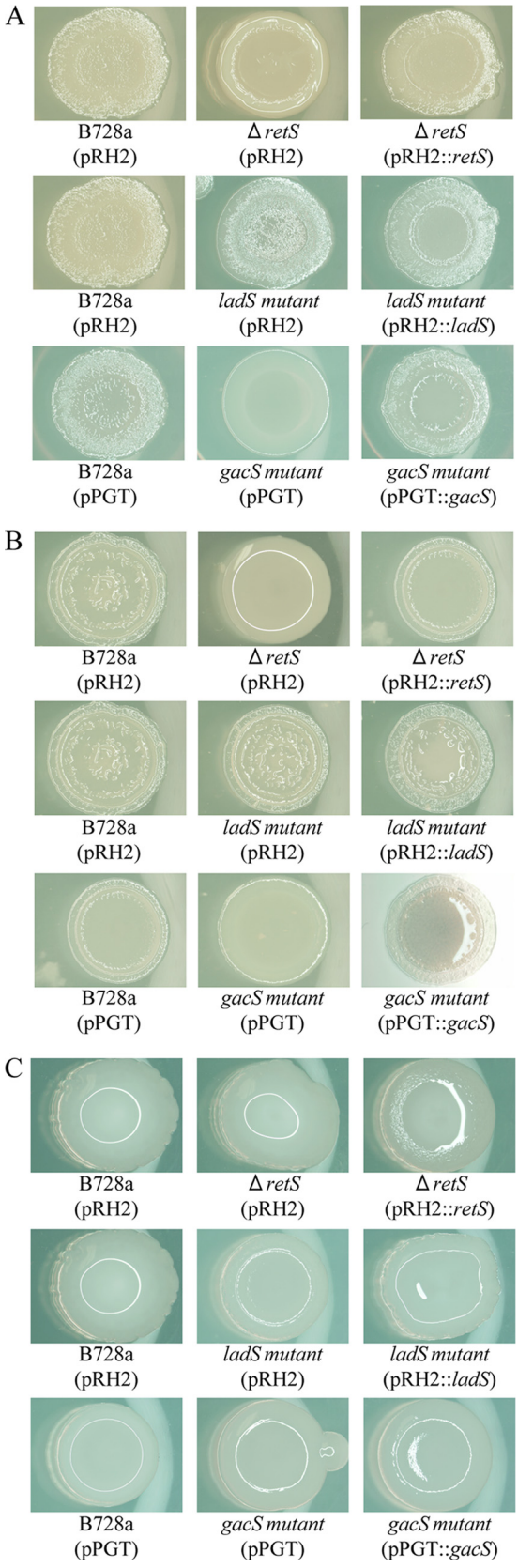

FIG. 3. Assay for mucoidy by *P. syringae* pv. syringae strains. A drop of inoculum containing 10^5 CFU of either B728a carrying an empty vector (pRH002 or pPROBE), the B728a Δ*retS* strain carrying pRH002, the B728a Δ*retS* strain carrying pRH002::*retS*, the B728a *ladS* mutant carrying pRH002, the *ladS* mutant carrying gpRH002::*ladS*, the B728a *gacS* mutant carrying pPROBE, or the B728a *gacS* mutant carrying pPROBE::*gacS* was placed onto PDA (A), MGY agar supplemented with 0.6 M sorbitol (B), or MGY supplemented with 5% sucrose (C). Plates were incubated at 25°C for 24 h and photographed under a dissecting microscope.

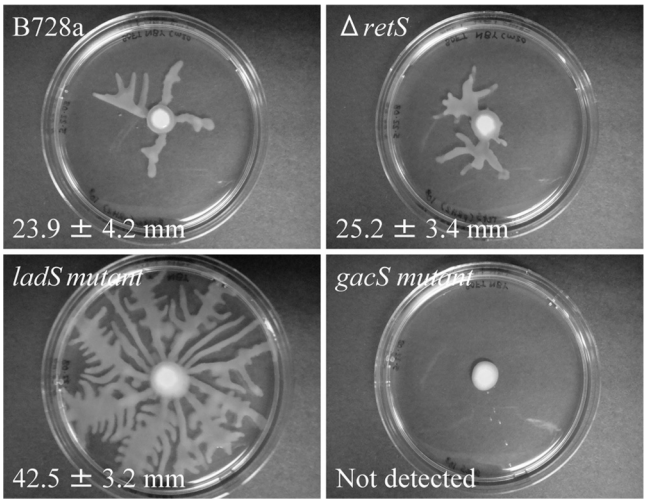

FIG. 4. Assay for swarming activity by *P. syringae* pv. syringae strains. Sterile, 6-mm filter disks were placed onto semisolid NBY agar and inoculated with 10^8 CFU of bacteria. Plates were incubated at 25°C for 24 h in a moist chamber. Measurements were made from the filter disk to the outer edge of the bacterial growth. Values are the means and standard deviations from three experiments.

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 culture. Figure 6 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 the secretion of Hcp (Fig. 6A). The presence of extracellular Hcp was restored when pUCclpV, 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 the band intensity. The experiment was repeated three times. On average, approximately 26% of the Hcp-VSV present in wild-type B728a cultures was located in the supernatant fractions. No Hcp-VSV was present in the supernatant fractions of the B728a *clpV* mutant carrying empty vector pUCP26 (Fig. 6A). Approximately 22% of the Hcp-VSV present in the cultures of the B728a *clpV* mutant carrying pUCclpV was located in the supernatant, indicating that intact *clpV in trans* is able to fully complement the B728a *clpV* mutant secretion phenotype.

A mutation in *retS* results in increased secretion of the Hcp protein in culture. In order to assess the role of RetS as a regulator of the T6SS, secretion assays were set up as described above for the B728a Δ*retS* strain. A representative Western blot is shown in Fig. 6B. No Hcp-VSV was visible in the supernatant fraction of the wild-type B728a culture. However,

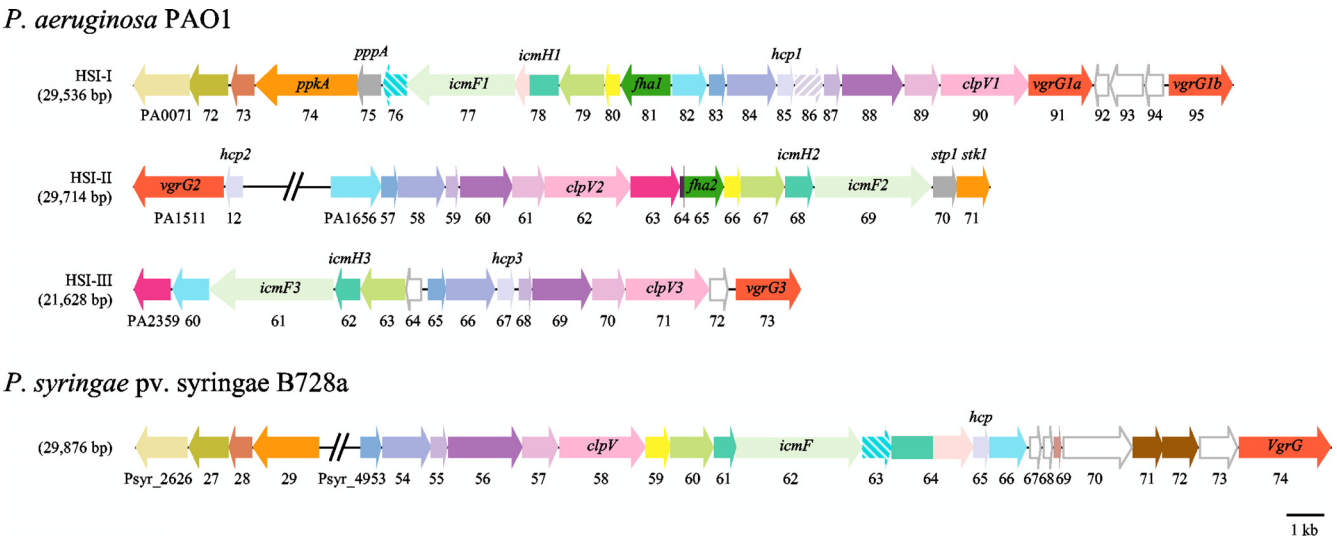

FIG. 5. Gene organization of the T6SS loci present in the genomes of *P. aeruginosa* PAO1 and *P. syringae* pv. syringae B728a. Arrows of the same color represent homologous genes. Genes with no homologues in other T6SS loci are in white. Each gene is marked with its name, if previously annotated, and its NCBI locus tag. HSI, Hcp secretion island (59); *ppk*, *Pseudomonas* protein kinase (77); *ppp*, *Pseudomonas* protein phosphatase (60); *icm*, intracellular multiplication (53); *fha*, forkhead associated (27); *hcp*, hemolysin-coregulated protein (81); *clp*, caseinolytic protease (35); *vgr*, valine glycine repeats (78); *stp*, serine/threonine phosphatase (61); *stk*, serine/threonine kinase (61).

approximately 23% of the Hcp-VSV present in the B728a Δ*retS* culture was located in the supernatant, suggesting that *retS* functions as a negative regulator for the secretion of Hcp. The secretion of Hcp-VSV by the B728a Δ*retS* strain was reduced to nearly wild-type levels (2.5% of total Hcp-VSV in the culture) when intact *retS* was present *in trans*. Protein concentrations were estimated by using the ImageJ program as described in Materials and Methods. Secretion assays were also conducted to determine if *ladS* is required for B728a secretion of Hcp. Western results for the B728a *ladS* mutant were identical to those of wild-type B728a (data not shown).

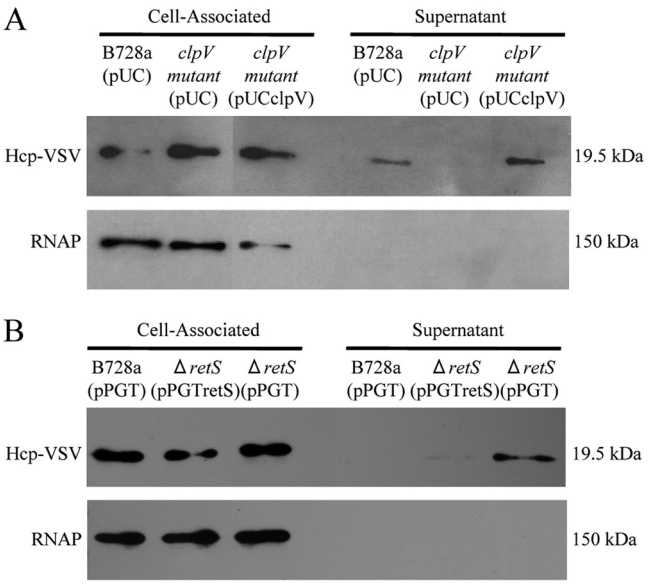

FIG. 6. Extracellular secretion of Hcp-VSV observed by Western blot analyses. *Pseudomonas syringae* pv. syringae strains carrying plasmid-borne *hcp-vsv* were grown to the mid-log phase at 25°C in NBY. Cultures were separated into cell-associated and supernatant fractions as described in Materials and 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 RNAP. Hcp-VSV was expected to have a molecular mass of 19.5 kDa (18.2-kDa Hcp plus 1.3-kDa VSV-G epitope). The RNAP protein size is 150 kDa. (A) The B728a *clpV* mutant and associated strains. (B) The B728a Δ*retS* strain and associated strains.

RetS contributes to leaf colonization. In order to study the possible contributions of *retS* and *ladS* to B728a colonization of the leaf surface, three 2-week-old bean plants were each dipped in bacterial suspensions containing 10^5 CFU/ml of either wild-type B728a, the B728a *ladS* mutant, or the B728a Δ*retS* strain. Some of the plants were placed into 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. The B728a *ladS* mutant, the B728a Δ*retS* strain, and wild-type B728a exhibited similar population numbers (around 10^6 CFU/g of leaf issue) when plants were placed under high RH (Fig. 7). The differences in bacterial numbers recovered from the plants incubated at high RH were not statistically significant. The B728a *ladS* mutant reached phyllosphere populations similar to those of wild-type B728a when inoculated plants were maintained under low RH (10^5 CFU/g leaf tissue; $P = 0.10$). However, the B728a Δ*retS* 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 $3.5 \times 10^5 \pm 1.7 \times 10^5$ CFU/g (mean ± SD). The average population recovered from leaves inoculated with the B728a Δ*retS* strain 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 ($P = 0.01$ by two-tailed *t*

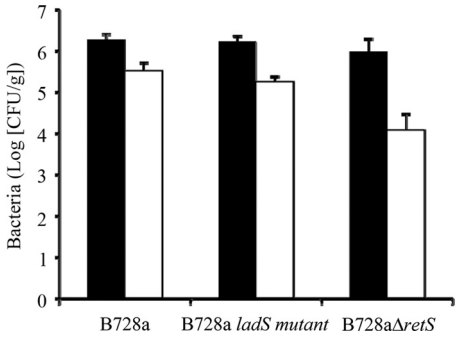

FIG. 7. Assay of *ladS* and *retS* contribution to *P. syringae* pv. syringae B728a colonization of bean leaves. Leaf surface populations from bean plants 24 h after dip inoculation with 10^5 CFU ml^{-1} of either wild-type B728a, the B728a *ladS* mutant, or the B728a Δ*retS* strain. Black columns represent plants that were maintained under high RH after inoculation. White columns represent plants maintained under low RH. Vertical bars represent the standard deviations from the mean bacterial populations present during four runs of the experiment.

test), which suggests that *retS* contributes to the B728a colonization of leaf surfaces.

The B728a *clpV* mutant multiplies *in planta* and produces disease symptoms similar to those caused by wild-type B728a. In an effort to assess the possible role that the T6SS may play in the B782a interaction with its host, leaf colonization assays similar to those described above were conducted by using the B728a *clpV* mutant. The results indicated that the B728a *clpV* mutant reaches wild-type phyllosphere population levels (data not shown). 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, or a B728a *gacS* mutant, which is unable to cause disease (39). Each bacterial strain was tested on three individual bean plants, and the experiment was repeated three times. The B728a *clpV* mutant showed no reduction in its ability to produce foliar disease symptoms compared to wild-type B728a (Fig. 8A). Bacterial populations in infected plants were monitored over a 3-day period. At 3 days postinoculation, B728a *clpV* mutant 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 ($P = 0.49$ by two-tailed *t* test), indicating that *clpV* is not required for multiplication *in planta* (Fig. 8B). 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 3 days postinoculation.

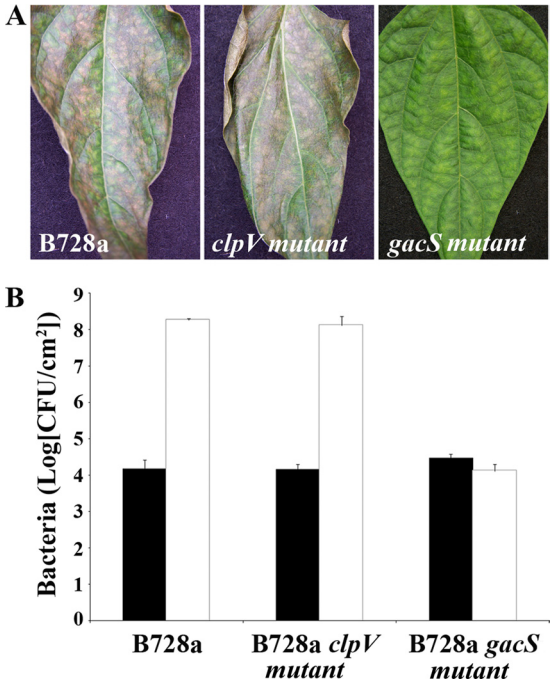

FIG. 8. Assay of the contribution of *clpV* to *P. syringae* pv. syringae B728a symptom production and growth in bean. (A) Bean leaves were inoculated via vacuum infiltration with suspensions containing 10^6 CFU ml^{-1} of either B728a or the B728a *clpV* or B728a *gacS* mutant in water. Plants were 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 A were monitored over a 3-day period. Black and white bars represent day 0 and day 3 populations, respectively.

DISCUSSION

In this study, we have identified two novel regulators of *P. syringae* pv. syringae B728a virulence. The putative sensor kinases Psyr_4408 and Psyr_4339 exhibit homology to RetS and LadS of *P. aeruginosa*, and we have shown that, like their counterparts in *P. aeruginosa*, B728a RetS and LadS collectively regulate the expression of the T3SS and the T6SS, EPS production, and swarming motility. While this study illuminates the similarities between the B728a and *P. aeruginosa* RetS and LadS regulons, we have uncovered striking differences between these two organisms in terms of virulence factor regulation. Models of the *P. aeruginosa* and B728a RetS/LadS regulons are depicted in Fig. 9 and will be discussed below.

Our qRT-PCR studies indicated that B728a RetS upregulates the expression of two genes associated with the T3SS: the alternative sigma factor gene *hrpL* and the gene that encodes the enhancer-binding protein HrpR (31). In contrast, qRT-PCR data showed that LadS negatively regulates *hrpL* transcript levels. These findings suggest that RetS and LadS reciprocally regulate the T3SS in B728a, as their homologues do in *P. aeruginosa*. This is significant because the T3SS is a critical virulence factor for *P. syringae*. The possibility that RetS and LadS regulate the T3SS is worthy of further investigation.

Our qRT-PCR data also implicated RetS and LadS as regulators of a putative B728a T6SS gene. The B728a genome carries a full complement of T6SS genes, including *icmF*, which is thought to encode a structural component of the T6SS (7, 51). Compared to wild-type B728a, the B728a Δ*retS* strain exhibited a 3.0-fold increase in *icmF* transcript levels, while the B728a *ladS* mutant exhibited a 3.8-fold decrease. This antagonistic RetS/LadS regulation of T6SS gene expression was observed for *P. aeruginosa* as well (59). Both *icmF* and *hcp* transcript levels were lower in the B782a Δ*gacS* strain, indicating that GacS is required for the expression of at least two T6SS locus genes (Fig. 2). While the control of *icmF* gene expression in B728a was consistent with that observed for *P. aeruginosa*, our qRT-PCR results showed that *hcp* expression is

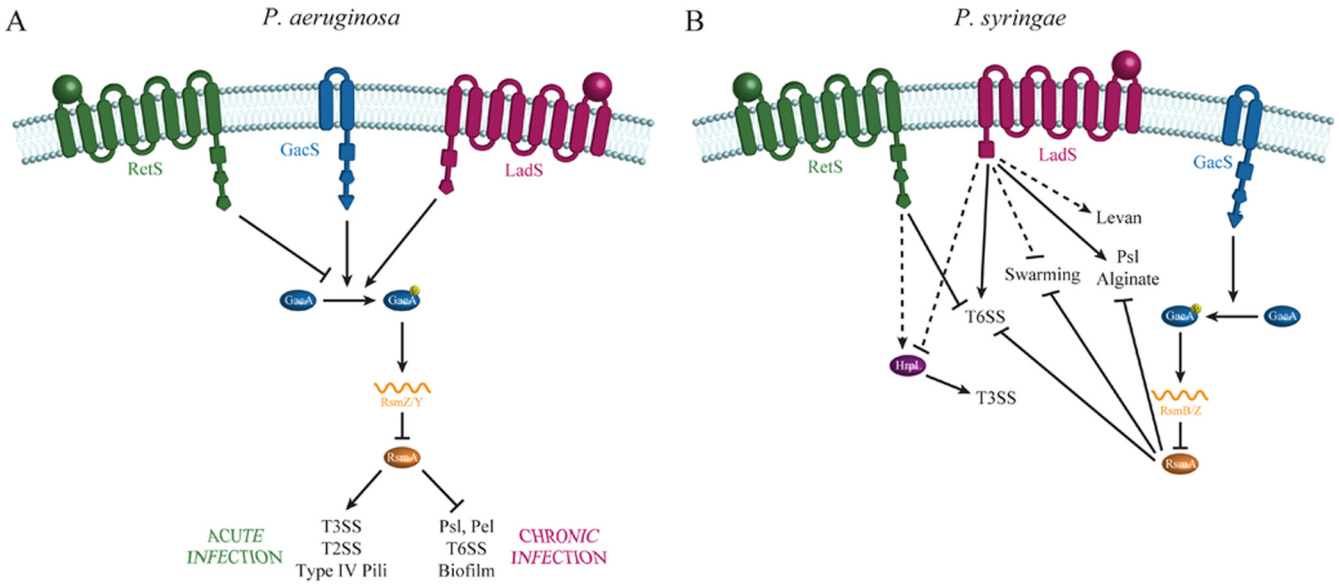

FIG. 9. Models of the *P. aeruginosa* and *P. syringae* RetS/LadS regulons. LadS (pink) is a hybrid sensor protein featuring a histidine kinase (HK) domain (depicted as a square) and a response regulator (RR) receiver domain (pentagon). These domains are anchored to the inner membrane via transmembrane segments linked to a periplasm-exposed signal-binding domain (sphere). 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 a histidine kinase domain followed by an RR domain and a histidine-containing phosphotransfer domain (triangle). (A) *P. aeruginosa* RetS and LadS reciprocally regulate the expression of virulence factors, and this regulation is modulated through the GacS/GacA network. RetS interacts directly with GacS, modulating its phosphorylation state and thereby affecting its rate of phosphotransfer with its cognate response regulator GacA (21). Phosphorylated GacA activates the transcription of the small RNAs *rsmZ* and *rsmY*, which have high affinity for RsmA. RNA-bound RsmA is unable to inhibit the expression of T6SS genes or other factors associated with chronic infections, such as EPS and biofilm genes. Free RsmA positively regulates the T3SS, which is highly expressed during acute infection by *P. aeruginosa*. LadS and RetS promote and inhibit, respectively, the phosphorylation of GacA. (B) As is the case for *P. aeruginosa*, B728a RetS, LadS, and GacS collectively contribute to the control of several virulence factors, including the T3SS, the T6SS, swarming motility, and EPS production. However, the interplay between the B728a RetS, LadS, and GacS regulons differs from that of the *P. aeruginosa* regulons. Dotted lines indicate RetS or LadS regulatory pathways that do not appear to overlap with the GacS regulon in B728a.

controlled by neither LadS nor RetS (<2.0-fold change in *hcp* transcript levels) (Fig. 2). It is surprising that *hcp* and *icmF* are not coregulated by RetS and LadS in B728a, but it is certainly feasible. The *P. aeruginosa* HSI-I *hcp* and *icmF* genes are members of separate operons (59), and it is likely that the B728a *hcp* gene (Psyr_4965), which is 156 bp downstream of Psyr_4964, is transcribed independently of the other T6SS genes.

Secretion studies corroborated the qRT-PCR findings and showed that the B728a T6SS 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. 6). This research confirms the functionality of a T6SS in a plant-pathogenic bacterium. While numerous bacterial species carry T6SS loci in their genomes (73), secretion activity has been demonstrated for relatively few of them (7). Of the species with a T6SS previously proven to be functional, only one is a plant pathogen (55). 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.

We are still only beginning to understand the role of the T6SS in bacterial fitness. Inoculation of bean plants via vacuum infiltration did not reveal a virulence defect in the B728a *clpV* mutant. It is possible that the T6SS plays a subtle role in the B728a-plant interaction, requiring more sensitive experimental methods for detection. 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 (34). Recently, Wichmann et al. showed that a novel B728a protein induces programmed cell death in *Neurospora*, which B728a is able to use as a sole nutrient source (80). 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 (65). Support for the hypothesis that the T6SS may play a role in environmental fitness (versus overt virulence) came from a recent study by Hood et al. in which the T6SS effector Tse2 was identified as a toxin targeted to bacteria (28).

The B728a Δ*retS* strain exhibited mucoid growth on PDA (Fig. 3A), indicating that RetS negatively regulates EPS production in B728a, as its orthologue does in *P. aeruginosa* (75). B728a is known to produce at least two EPSs: the well-studied capsular polysaccharide alginate and the polyfructan levan

(44). Our experiments with MGY agar point to a role of RetS in alginate synthesis because the addition of sorbitol, which stimulates alginate production, revealed a phenotype for the B728a *retS* mutant (Fig. 3B). Interestingly, alginate production is not regulated by RetS/LadS in *P. aeruginosa*. It is possible that the mucoid phenotype exhibited by the B728a Δ*retS* strain 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 (44). 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 (75). The production of Psl and its role in biofilm formation in *P. aeruginosa* have been studied (50), but Psl has never been observed for *P. syringae*. The B728a genome carries orthologues of all *psl* genes (Psyr_3301 to Psyr_3311), and our qRT-PCR studies showed that the expression of the B728a orthologue of *pslB*, a gene required for Psl production in *P. aeruginosa* (33), was downregulated in the B728a *ladS* mutant. In addition to Psl, LadS apparently upregulates the expression of the EPSs alginate and levan.

Motility assays revealed that LadS negatively controls B728a swarming activity (Fig. 4). Similarly, microarray studies have shown that a *P. aeruginosa ladS* mutant exhibits an upregulation of *pilA*, the type IV pilus structural gene involved in adhesion and motility, and the flagellar biosynthesis genes *fliS′* and *fleP* (75). In *P. aeruginosa*, RetS positively controls twitching motility (87), but the B728a *retS* mutant was indistinguishable from wild-type B728a in our swarming motility assays (Fig. 4).

This study demonstrated that RetS is involved in B728a leaf colonization. Several studies have shown that under low-humidity conditions, fewer bacteria survive on the leaf surface (26, 62). This phenomenon is apparently exacerbated by a mutation in *retS*. In our leaf colonization studies, levels of 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. 7) (11). This difference in colonization is important because a reduction in cell numbers translates to a reduction in the amount of inoculum available for the invasion of subdermal leaf tissue (67). Indeed, Lindemann et al. previously estimated the infection threshold for *P. syringae* pv. syringae on bean to be 10^4 CFU/g leaf tissue (48). In their study, no bacterial brown spot was detected in field plots where the epiphytic *P. syringae* populations were below the threshold. As our EPS and swarm assays showed that the *retS* mutant is both mucoid and motile, the basis for the B728a Δ*retS* leaf colonization phenotype is intriguing and warrants further study. Although the *retS* mutant exhibited limited colonization of the leaf surface, vacuum infiltration experiments showed that both the B728a Δ*retS* and B728a Δ*ladS* strains were able to multiply *in planta* and produce disease symptoms comparable to those of parental strain B728a (data not shown).

Models of the *P. aeruginosa* and B728 RetS/LadS regulons are depicted in Fig. 9. Our current understanding of RetS and LadS function in *P. aeruginosa* stems from whole-genome microarray studies aimed at identifying the collection of genes subject to RetS/LadS control (20, 75), from structural studies of RetS and other sensor kinases (1, 42), and from screens of suppressor transposon mutants focused on downstream components of the RetS/LadS regulons (20, 75). Those studies revealed that the GacS/GacA/RsmZ pathway plays an important role in the *P. aeruginosa* RetS and LadS regulatory network. The current model places the RetS, LadS, and GacS sensor kinases at the top of a regulatory cascade in which GacA controls a switch between acute and chronic *P. aeruginosa* infections (Fig. 9A) (19). While many of the same virulence factors are important for *P. syringae* infection, the model for their regulation by RetS, LadS, and GacS is distinct from that of *P. aeruginosa*. First, studies previous to this work have shown that the B728a T3SS is not under GacS control (8, 83). Our qRT-PCR data confirm that observation and suggest that RetS and LadS regulate the expression of the T3SS in a manner independent of GacS. Second, alginate production is not regulated by GacA in *P. aeruginosa* PA14 (63), but B728a GacS has been shown to regulate alginate production (82), as our qRT-PCR data confirm. It is possible that B728a RetS and LadS communicate at least a subset of their signals through the response regulator GacA in a manner independent of GacS (Fig. 9B). It would be interesting to determine the effects of *ladS gacA* or *retS gacA* double mutations on B728a T6SS, T3SS, and EPS production. Importantly, GacA activation of the small RNAs RsmZ and RsmY is central to T6SS, T3SS, and EPS regulation in *P. aeruginosa*. The B728a genome contains homologues of *rsmY* and *rsmZ* (41), but no published studies have examined their functions in this *P. syringae* strain. A better understanding of the probable roles that GacA and small RNAs play downstream of RetS and LadS in the control of B728a fitness would enhance our model of this complex regulatory network and provide further insights into the similarities and differences in the global regulation of virulence among pseudomonads. Future studies should also be aimed at the identification of the environmental signals responsible for triggering these regulatory cascades.

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