Sensor kinases RetS and LadS regulate *Pseudomonas syringae* type VI secretion and virulence factors

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ABSTRACT

*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 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 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 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.
INTRODUCTION

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 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 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 (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 non-plant environments (45).

The impact of TCS regulation of virulence traits is exemplified by the RetS and LadS sensor kinases of the human pathogen *P. 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 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). These studies also revealed that RetS and LadS signalling converge on the master virulence regulator GacA, influencing levels of the small regulatory 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 post-translational regulation of certain target genes – by binding the mRNA of their cognate regulatory proteins, and post-transcriptional 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 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 15-25 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 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 ImpLₘ, 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 handful of
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 \( \beta-(2,6)- \) 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 in 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 *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 expression of the B728a T6SS gene *icmF* is under RetS/LadS control. Interestingly, plant infection assays revealed a role for RetS in 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. *E. 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, 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 (76) or on King’s B (KB) agar medium (37). For qRT-PCR studies, bacteria were grown in a modified Hrp Minimal Medium (HMM; 0.2 M KH₂PO₄, 1.2 M K₂HPO₄, 1.3 M (NH₄)₂SO₄, 5.9 M MgCl₂, 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 FeCl₃) (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 (µg/ml): 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 Packages (DNAStar, Madison, Wisc.) and purchased from Integrated DNA Technologies (Coralville, Iowa). The 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, per 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 tri-parental 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 using primer P163 and primer P164, which contains a sequence encoding the vesicular stomatitis glycoprotein (VSV) epitope, and then cloned into pENTR/D-TOPO vector 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. A 2.3-kb fragment containing the *ladS* gene along with its putative promoter was amplified 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 using primers P215 and P216, which contain HindIII and BamHI sites, respectively. The PCR product was digested and ligated to HindIII/BamHI-digested pPROBE-GT, giving pPGT::gacS. 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 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 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 (25), 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 in 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 a B728a 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 mutant. The Suc⁺ pBH474 plasmid was cured from the B728aΔretS cells by plating on NBY + 5% sucrose.

A similar approach was used for the construction of B728aΔgacS. 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 B728aΔgacS genome as described above.
Construction of $B728a$ lad$S$ and clp$V$ insertion mutations. A mutation in the $B728a$ lad$S$ gene was made as follows. A 650-bp fragment of lad$S$ was amplified using primers P121 and P122, and the PCR product was cloned into pENTR/D-TOPO, resulting in pElad$S'$. The lad$S'$ entry construct was recombined into the pLVCD Gateway destination vector, resulting in pLVlad$S'$. A tri-parental mating was set up between $E. coli$ DH10B(pLVlad$S'$), wild-type $B728a$ and $E. coli$ DB3.1(pRK2073). Integration of pLVlad$S'$ into the $B728a$ genome resulted in $B728a$ lad$S$.

To construct $B728a$ clp$V$, the 2.6-kb clp$V$ gene was amplified by PCR using primers P131 and P132 and cloned into pENTR/D-TOPO by topoisomerase reaction, resulting in construct pEclp$V$. The aac$C1$ gentamycin (Gm) resistance gene was isolated from pUCGm by digestion with HindIII and was ligated to HindIII-digested pEclp$V$, resulting in pEclp$V$-Gm. The clp$V$-Gm entry construct was recombined into pLVCD. Tri-parental mating between wild-type $B728a$, $E. coli$ DH10B(pLVclpV-Gm), and $E. coli$ DB3.1(pRK2073), and marker exchange resulted in $B728a$ clp$V$.

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. 10 µl of initial cultures were transferred to fresh HMM and grown for 16 h at 25°C with shaking. Subcultures were prepared by transferring 300 µl of overnight cultures to 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 $5 \times 10^8$ CFU/ml). RNAProtec stabilizing reagent (Qiagen Inc., Valencia, Calif.) was added to each culture, per manufacturer’s instructions. Cells were pelleted ($5 \times 10^8$ cells/pellet) and resuspended in 200 µl TE prior to isolation of total RNA. For analyses of EPS and T6SS gene expression, bacterial strains were cultured in
PDB as described above. Upon reaching OD$_{600}$ = 0.6, PDB cultures were concentrated and then 0.2 ml (5 x 10$^8$ cells) were spotted on the center of a PDA plate. Plates were incubated for 40 h at 25°C. RNAprotect was added to the plates after incubation, per manufacturer’s instructions. Total RNA was purified using the RNeasy Mini Kit following 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 expression of representative EPS, T6SS, and T3SS genes, qRT-PCR was performed using the EXPRESS Two-Step SYBR GreenER kit (Invitrogen). Total RNA was prepared as described above. Primers specific for *hcp*, *icmF*, *lscC*, *algA*, *psIB*, *hrpL* and *hrpR* were used for qRT-PCR, and their sequences are listed in Table S1. Primers 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 five-fold dilution curve with cDNA isolated from bacterial cells. qRT-PCR was performed in 40 cycles (95°C for 3 s, 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 ddH$_2$O. 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 $1 \times 10^8$ 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 then 3 µl were 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 (SDS-PAGE) gels and
transferred to Hybond-P PVDF membranes (GE Healthcare, Piscataway, N.J.). 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, Wis.). Primary
antibodies were recognized by anti-mouse or 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 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, using a protocol based on the methods of Monier and Lindow (58). 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$_2$O 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 (0.1 M K$_2$HPO$_4$/KH$_2$PO$_4$, 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$_2$O, and spread on 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 orthologs of *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 NP_253543) and LadS (GenBank NP_252663) revealed that the predicted proteins encoded by Psyr_4408 (GenBank YP_237476) and Psyr_4339 (GenBank YP_237407) have homology to RetS and LadS, respectively. Pair-wise 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 of the 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 domain(s). These domains are secured to the inner membrane via seven transmembrane segments linked to a periplasm-exposed signal-binding domain. Unlike its LadS ortholog, 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
1 tandem response regulator receiver domains within a protein is unusual, making RetS
2 unique (42).

3 **B728a RetS and LadS regulate the expression of genes involved in EPS production and protein secretion.** RetS and LadS regulate the expression of several
4 genes involved in *P. aeruginosa* virulence, including exopolysaccharide (EPS) genes in
5 the *pel* and *psl* operons and genes encoding the type III and type VI protein secretion
6 systems (20, 75). In order to analyze the influence that RetS and LadS have on expression
7 of genes involved in EPS production and protein secretion by B728a, deletion or insertion
8 mutants were constructed as described in Materials and Methods. Mutations were made
9 in B728a Psyr_4408 (*retS*), Psyr_4339 (*ladS*), and Psyr_3698 (*gacS*). GacS was included
10 in this study because it is a global regulator of *P. syringae* virulence (8) and its *P.*
11 aeruginosa ortholog is a critical component of the RetS/LadS regulon (6, 75). cDNA was
12 obtained from wild-type B728a, ∆*retS*, *ladS*-, or ∆*gacS* grown in either HMM liquid
13 medium (for analysis of type III gene expression) or on PDA plates (for analysis of type
14 VI and EPS genes) as described in Materials and Methods. qRT-PCR was performed
15 using primers specific to the type III genes *hrpL* (Psyr_1217) and *hrpR* (Psyr_1190), the
16 EPS genes *algA* (Psyr_1052) and *lscC* (Psyr_0754), the putative EPS gene *pslB*
17 (Psyr_3302), and the putative type VI genes *hcp* (Psyr_4965) and *icmF* (Psyr_4962).
18 Results of the qRT-PCR studies are summarized in Fig. 2. The B728aΔ*retS* mutant
19 exhibited a 2.8-fold increase in *icmF* transcript levels, as compared to those of wild-type
20 B728a and greater than 2.0-fold decreases in transcript levels of *hrpL* and *hrpR*. In
21 contrast, *icmF* transcript levels were 3.3-fold lower in B728a *ladS*-, as compared to 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, B728a ladS produced lower levels of lscC, algA, and pslB transcripts (3.6-, 2.2-, and 2.0-fold, respectively), as compared to 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 B728aΔgacS (5.3-, 3.1-, 15.4-, and 7.7-fold, respectively), as compared to wild-type B728a, which indicates that the GacS global regulator controls expression of the T6SS and EPS production in B728a. A mutation in gacS did not have a measurable affect on expression of the T3SS genes hrpL or hrpR.

Mutations in B728a retS, ladS, and gacS genes result in mucoidy phenotypes on various media. When inoculated on PDA, B728aΔretS exhibited highly mucoid growth, as compared to wild-type B728a (Fig. 3A). Wild-type colony morphology was restored to B728aΔretS 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 on MGY agar, a medium that induces EPS production by P. syringae (44, 64), and incubated for 24 h at 25°C. On MGY supplemented with 0.6 M sorbitol, which is known to induce expression of alginate-related genes (64), B728aΔretS appeared much more mucoid than wild-type B728a (Fig. 3B). Inoculation of MGY supplemented with 5% sucrose, which stimulates production of levan (44), did not reveal any observable differences between wild-type B728a and B728aΔretS (Fig. 3C). These results suggest that B728a RetS may negatively regulate alginate production. In contrast, B728a gacS exhibited a decrease in mucoidy on both PDA and MGY + sorbitol (Fig. 3A and B). The
colony morphology of B728a ladS− appeared similar to wild-type B728a on all media tested, except that it was slightly less mucoid on MGY + sucrose (Fig. 3C). Taken together, these results suggest that GacS and LadS positively control 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 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. 4). B728aΔretS displayed a similar movement pattern. B728a ladS− swarmed 18.6 ± 1.9 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. B728a gacS− showed no movement on the semisolid agar, as demonstrated previously (38).

**The B728a T6SS locus.** The fact that *P. aeruginosa* RetS and LadS modulate expression of T6SS genes led us to investigate 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 (NP_248780) was used in a BLAST search of the B728a proteome. Three B728a proteins showed strong homology (bit scores >200) to 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 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 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 using the *P. aeruginosa* PAO1 Hcp1 (NP_248775.1) and VgrG1 (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 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 (23). Proper orientation and tagging of hcp were confirmed by 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.

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. Fig. 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 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 B728a clpV. 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 B728a clpV carrying the empty vector pUCP26 (Fig. 6A). Approximately 22% of the Hcp-VSV present in the B728a clpV (pUCclpV) cultures was located in the supernatant, indicating that intact clpV in trans is able to fully complement the B728a clpV 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 B728aΔretS. 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, approximately 23% of the Hcp-VSV present in the B728aΔretS culture was located in the supernatant, suggesting that retS functions as a negative regulator for secretion of Hcp. Secretion of Hcp-VSV by B728aΔretS was reduced nearly to wild-type levels (2.5% of total Hcp-VSV in the culture) when intact retS was present in trans.

Protein concentrations were estimated 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 B728a ladS were identical to those of wild-type B728a (data not shown).

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, B728a ladS or B728aΔretS. 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. B728a ladS, B728aΔretS 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. B728a ladS 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
B728aΔretS 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 B728aΔretS 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.

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 using B728a clpV. The results indicated that B728a clpV 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, B728a clpV 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. B728a clpV showed no reduction in its ability to produce foliar disease symptoms, as compared to wild-type B728a (Fig. 8A). Bacterial populations in infected plants were monitored over a 3-day period. At three days post-inoculation, the B728a clpV 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. 8B). As expected, B728a gacS
was reduced in its ability to grow in bean leaves, as indicated by a population of $1.4 \times 10^4 \pm 5.3 \times 10^3 \text{ CFU cm}^{-2}$ at three days post-inoculation.

**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 expression of the T3SS, 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 up-regulates 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). As compared to wild-type B728a, B728aΔretS exhibited a 3.0-fold increase in *icmF*.
transcript levels, while B728a ladS- exhibited a 3.8-fold decrease. This antagonistic RetS/LadS regulation of T6SS gene expression is observed in \textit{P. aeruginosa}, as well (59). Both icmF and hcp transcript levels were lower in B728aΔgacS, indicating that GacS is required for expression of at least two T6SS locus genes (Fig. 2). While control of icmF gene expression in B728a was consistent with that observed in \textit{P. aeruginosa}, our qRT-PCR results showed that hcp expression is controlled neither by LadS nor RetS (Fig. 2, <2.0-fold change in hcp transcript levels). It is surprising that hcp and icmF are not co-regulated by RetS and LadS in B728a, but it is certainly feasible. The \textit{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 (7). Of the species with a T6SS previously proven 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 \textit{clpV} mutant. It is possible that the T6SS plays a subtle role in the B728a-plant interactions, which warrants further investigation.
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, 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 (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) comes from a recent study by Hood et al. in which the T6SS effector Tse2 was identified as a toxin targeted to bacteria (28).

B728aΔretS exhibited mucoid growth on PDA (Fig. 3A), indicating that RetS negatively regulates EPS production in B728a, as its ortholog 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 RetS role 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 B728aΔretS 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 have been studied in *P. aeruginosa* (50), but Psl has never been observed in *P. syringae*. The B728a genome carries orthologs of all of the psl genes (Psyr_3301-Psyr_3311), and our qRT-PCR studies showed that expression of the B728a ortholog of *pslB*, a gene required for Psl production in *P. aeruginosa* (33), was down-regulated in B728a ladS. In addition to Psl, LadS apparently up-regulates 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 up-regulation 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, 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 inoculum available for invasion of subdermal leaf tissue (67). Indeed,
Lindemann et al. 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 B728aΔretS 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 B728aΔretS and B728aΔladS were able to
multiply *in planta* and produce disease symptoms comparable to 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). These 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 in *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 this observation and suggest that RetS and LadS regulate expression of the T3SS in a manner independent of GacS. Secondly, alginate production is not regulated by GacA in \textit{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 \textit{ladS/gacA} or \textit{retS/gacA} double-mutations on the 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 \textit{P. aeruginosa}. The B728a genome contains homologues of \textit{rsmY} and \textit{rsmZ} (41), but no published studies have examined their functions in this \textit{P. syringae} strain. A better understanding of the probable roles that GacA and small RNAs play downstream of RetS and LadS in control of B728a fitness would enhance our model of this complex regulatory network and provide further insights into the similarities and differences in global regulation of virulence among pseudomonads. Future studies should also be aimed at identification of the environmental signals responsible for triggering these regulatory cascades.

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FIGURE LEGENDS

FIG. 1. Domain organizations of *P. aeruginosa* PAO1 LadS and RetS proteins and *P. syringae* pv. syringae B728a orthologs. 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 (7TM-R-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 made by NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Scale bar indicates protein length in number of amino acid residues.

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 in either 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. Differential expression of representative type VI (represented by black bars), type III (white bars), and EPS (grey bars) genes is expressed as fold-change values for ΔretS, ladS, or ΔgacS versus wild-type B728a. Error bars indicate standard deviation from the mean obtained from three independent biological samples. Expression for all samples was normalized to the housekeeping gene recA.

FIG. 3. Assay for mucoidy by P. syringae pv. syringae strains. A drop of inoculum containing $10^5$ CFU of either B728a carrying empty vector (pRH002 or pPROBE), B728aΔretS(pRH002), the ΔretS strain carrying pRH002::retS, B728a ladS carrying pRH002, ladS(pRH002::ladS), B728a gacS carrying pPROBE, or B728a gacS (pPROBE::gacS) was placed on (A) PDA, (B) mannitol glutamate-yeast extract agar (MGY) supplemented with 0.6M sorbitol, or (C) MGY supplemented with 5% sucrose. 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 on semisolid NBY 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 mean and standard deviation from three experiments.

**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 homologs in other T6SS loci are colored 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*, haemolysin co-regulated protein (81); *clp*, caseinolytic protease (35); *vgr*, valine glycine repeats (78); *stp*, serine/threonine phosphatase (61); *stk*, serine threonine kinase (61).

**FIG. 6.** Extracellular secretion of Hcp-VSV as observed by Western blot analyses. *Pseudomonas syringae* pv. syringae 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. (A) B728a *clpV* and associated strains. (B) B728aΔ*retS* and associated strains.

**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 10⁵ CFU ml⁻¹ of either wild-type B728a, B728a *ladS*, or B728aΔ*retS*.
Black columns represent plants that were maintained under high relative humidity (RH) after inoculation. White columns represent plants maintained under low RH. Vertical bars represent the standard deviation from the mean bacterial populations present during four runs of the experiment.

FIG. 8. 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$^{-1}$ of either B728a, B728a clpV, or B728a gacS in water. Plants were maintained at 25°C in a growth chamber of 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. Black and white bars represent Day 0 and Day 3 populations, respectively.

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 HK domain followed by an RR domain and a histidine-containing phosphotransfer domain (triangle). (A) P. aeruginosa RetS and LadS reciprocally regulate expression of virulence factors, and this regulation is modulated through the GacS/GacA network. RetS directly interacts 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

1. *rsmY*, which have high affinity for RsmA. RNA-bound RsmA is unable to inhibit
2. expression of T6SS genes or other factors associated with chronic infections, such as EPS
3. and biofilm genes. Free RsmA positively regulates the T3SS, which is highly expressed
4. during acute infection by *P. aeruginosa*. LadS and RetS promote and inhibit,
5. respectively, phosphorylation of GacA. (B) As is the case for *P. aeruginosa*, B728a RetS,
6. LadS and GacS collectively contribute to the control of several virulence factors,
7. including the T3SS, the T6SS, swarming motility, and EPS production. However, the
8. interplay between the B728a RetS, LadS and GacS regulons differs from that of the *P.
9. aeruginosa* regulons. Dotted lines indicate RetS or LadS regulatory pathways that do not
10. appear to overlap with the GacS regulon in B728a.
# TABLE 1. Strains and plasmids used in this study

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<th>Designation</th>
<th>Relevant Characteristics</th>
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<tr>
<td>B728a clpV</td>
<td>Rif&lt;sup&gt;r&lt;/sup&gt; Te&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>B728a ΔgacS</td>
<td>Rif&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>B728a gacS</td>
<td>Rif&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(83)</td>
</tr>
<tr>
<td>B728a ladS</td>
<td>Rif&lt;sup&gt;r&lt;/sup&gt; Te&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>B728a ΔretS</td>
<td>Rif&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBH474</td>
<td>flp constitutively expressed, Gm&lt;sup&gt;r&lt;/sup&gt; Suc&lt;sup&gt;5&lt;/sup&gt;</td>
<td>(29)</td>
</tr>
<tr>
<td>pENTR/D-TOPO</td>
<td>Gateway entry vector, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEclpV</td>
<td>pENTR/D-TOPO carrying clpV, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEclpV-Gm</td>
<td>pENTR/D-TOPO carrying clpV::aacC1, Km&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEgacS-FP</td>
<td>pENTR/D-TOPO carrying 1.0 kb upstream of gacS fused to nptII fused to 1.1 kb downstream of gacS</td>
<td>This study</td>
</tr>
<tr>
<td>pEhcp-vsv</td>
<td>pENTR/D-TOPO carrying hcp with its putative promoter region and a 3' vsv tag, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEladS</td>
<td>pENTR/D-TOPO carrying ladS with its putative promoter region, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEladS'</td>
<td>pENTR/D-TOPO carrying a 650-bp fragment of ladS, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEretS</td>
<td>pENTR/D-TOPO carrying retS with its putative promoter region, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pEretS-FP</td>
<td>pENTR/D-TOPO carrying 1.8 kb upstream of retS fused to nptII fused to 1.9 kb downstream of retS, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pKD4</td>
<td>Template plasmid containing FRT-flanked nptII</td>
<td>(9)</td>
</tr>
<tr>
<td>pLVCD</td>
<td>Gateway destination vector for mating with P. syringae, pBR322 derivative with mob genes from RSF1010, Te&lt;sup&gt;c&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(52)</td>
</tr>
<tr>
<td>pLVclpV-Gm</td>
<td>pLVCD carrying clpV::aacC1, Te&lt;sup&gt;c&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Plasmid</td>
<td>Description</td>
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<tr>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>1</td>
<td>pLVgacS-FP</td>
<td>pLVCD carrying 1.0 kb upstream of gacS fused to nptII fused to 1.1 kb downstream of gacS</td>
</tr>
<tr>
<td>2</td>
<td>pLVladS'</td>
<td>pLVCD carrying a 650-bp fragment of ladS, Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>pLVretS-FP</td>
<td>pLVCD carrying 1.8 kb upstream of retS fused to nptII fused to 1.9 kb downstream of retS, Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>4</td>
<td>pPROBE-GT</td>
<td>Promoter-probe vector with pVS1/p15a replicon and gfp reporter, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>pPGT::gacS</td>
<td>pPROBE-GT carrying gacS along with 95 bp upstream, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>6</td>
<td>pRH002</td>
<td>Gateway destination vector, pBBR1MCS1 derivative, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>pRH2hcp-vsv</td>
<td>pRH002 carrying hcp with its putative promoter region and a 3' vsv tag cloned in-frame with the vector lacZ promoter, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>8</td>
<td>pRH2ladS</td>
<td>pRH002 carrying ladS with its putative promoter region cloned in-frame with the vector lacZ promoter, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>pRH2retS</td>
<td>pRH002 carrying retS with its putative promoter region cloned in-frame with the vector lacZ promoter, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>pRK2073</td>
<td>Helper plasmid, Sp&lt;sup&gt;r&lt;/sup&gt; Trm&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>11</td>
<td>pUCclpV</td>
<td>pUCP26 carrying clpV in-frame with the vector lacZ promoter, Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>12</td>
<td>pUCGm</td>
<td>Cloning vector, Gm&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>pUCP26</td>
<td>Cloning vector, Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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</table>
LaS 7TMR-DISM (extracellular) HATPase REC REC
Psyr_4339 7TMR-DISM (extracellular) HATPase REC
ReCS 7TMR-DISM (extracellular) HATPase REC
Psyr_4408 7TMR-DISM (extracellular) HATPase REC
A

B728a(pRH2) ΔretS(pRH2) ΔretS(pRH2::retS)

B728a(pRH2) lads (pRH2) lads (pRH2::lads)

B728a(pPGT) gacS (pPGT) gacS (pPGT::gacS)

B

B728a(pRH2) ΔretS(pRH2) ΔretS(pRH2::retS)

B728a(pRH2) lads (pRH2) lads (pRH2::lads)

B728a(pPGT) gacS (pPGT) gacS (pPGT::gacS)

C

B728a(pRH2) ΔretS(pRH2) ΔretS(pRH2::retS)

B728a(pRH2) lads (pRH2) lads (pRH2::lads)

B728a(pPGT) gacS (pPGT) gacS (pPGT::gacS)