A Novel Sensor Kinase Is Required for Bordetella bronchiseptica To Colonize the Lower Respiratory Tract

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Bacterial virulence is influenced by the activity of two-component regulator systems (TCSs), which consist of membrane-bound sensor kinases that allow bacteria to sense the external environment and cytoplasmic, DNA-binding response regulator proteins that control appropriate gene expression. Respiratory pathogens of the Bordetella genus require the well-studied TCS BvgAS to control the expression of many genes required for colonization of the mammalian respiratory tract. Here we describe the identification of a novel gene in Bordetella bronchiseptica, plrS, the product of which shares sequence homology to several NtrY-family sensor kinases and is required for B. bronchiseptica to colonize and persist in the lower, but not upper, respiratory tract in rats and mice. The plrS gene is located immediately 5' to and presumably cotranscribed with a gene encoding a putative response regulator, supporting the idea that PlrS and the product of the downstream gene may compose a TCS. Consistent with this hypothesis, the PlrS-dependent colonization phenotype requires a conserved histidine that serves as the site of autophosphorylation in other sensor kinases, and in strains lacking plrS, the production and/or cellular localization of several immune-recognized proteins is altered in comparison to that in the wild-type strain. Because plrS is required for colonization and persistence only in the lower respiratory tract, a site where innate and adaptive immune mechanisms actively target infectious agents, we hypothesize that its role may be to allow Bordetella to resist the host immune response.

The bordetellae comprise a group of Gram-negative respiratory tract pathogens. Bordetella bronchiseptica infects a wide range of mammals, while Bordetella pertussis and human-adapted Bordetella parapertussis, which presumably diverged from a B. bronchiseptica-like ancestor, are restricted to infecting humans and cause the disease known as whooping cough or pertussis (15, 16, 47, 64). Despite widespread vaccine coverage, the number of whooping cough cases has increased in recent years (8, 69), prompting the need for a better understanding of the mechanisms used by these pathogens to colonize the respiratory tract.

Two-component regulatory systems (TCSs) are used by prokaryotes to sense and respond to a particular environment by controlling the appropriate gene expression pattern (25, 38). They typically consist of a membrane-bound sensor histidine kinase (HK) that is sensitive to various stimuli, including temperature, pH, osmolarity, redox state, and nutrient concentration. Activation of the HK results in autophosphorylation of a conserved histidine residue, with subsequent phosphotransfer to a conserved aspartic acid residue present on a cytoplasmic, cytoplasmic, response regulator (RR) protein (66). Phosphorylation typically renders RRfs competent to bind DNA and control gene transcription, although some RRfs have other activities. For pathogenic bacteria, TCSs play key roles in modulating gene expression during infection (7, 18, 34, 48, 63). Despite differences in host range, B. pertussis and B. bronchiseptica produce a functionally interchangeable BvgAS TCS that is necessary for colonization of the respiratory tract (11, 12, 40) and that has been shown to control at least three phenotypic phases during growth in vitro. At 37°C and in the relative absence of sulfate ions and nicotinic acid, the BvgAS system is active (the Bvg° phase) and induces the expression of genes necessary for virulence, such as those required for adherence to host tissues and modulation of the immune response, while simultaneously repressing the expression of genes detrimental to virulence, such as those encoding flagella (1, 4, 40). When Bordetella cells are grown at 25°C or 37°C in the presence of mM concentrations of sulfate ions or nicotinic acid, BvgAS is inactive (the Bvg+ phase), resulting in the lack of virulence gene expression and concomitant expression of Bvg-repressed genes. A Bvg- intermediate (Bvg') phase, which occurs in the presence of relatively low sulfate ion or nicotinic acid concentrations, results in the expression of a subset of the virulence genes induced during the Bvg+ phase (10, 12, 30, 67). The BvgAS system and a more recently identified TCS termed RisAS, which has been reported to be required for intracellular survival of Bordetella (33), are the only TCSs implicated in Bordetella virulence.

Inspiration of the annotated B. bronchiseptica RB50 genome revealed the presence of over 20 loci with the potential to encode TCSs (47). Intriguingly, most are also present in the annotated genomes of the human-adapted B. pertussis and B. parapertussis strains. Given their ubiquitous role in regulating bacterial pathogenesis, we hypothesized that one or more...
additional TCSs play a role in *B. bronchiseptica* colonization of the mammalian respiratory tract. To test this hypothesis, we undertook a systematic mutational analysis of a majority of putative TCS-encoding genes in *B. bronchiseptica* and evaluated each mutant for its ability to colonize the respiratory tract. Of 19 mutants tested, 1 showed that *plrS* (sensor kinase). Further characterization showed that *plrS* harboring a *plrs* gene with codon 521 (histidine) changed to encode glutamine rescues the in-frame *plrs* deletion.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** All bacterial strains and plasmids used in this study are listed in Table 1. *B. bronchiseptica* strains were grown on Bordet-Gengou (BG) agar (BD Biosciences, San Jose, CA) supplemented with 7.5% defribinated sheep blood (Hardy Diagnostics, Santa Maria, CA) or Stainer-Scholte (SS) broth (60°C). *Escherichia coli* strains were grown on Luria-Bertani (LB) agar or broth at 37°C. When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; gentamicin, 20 μg/ml; streptomycin, 20 μg/ml.

**DNA methods.** Restriction enzymes and DNA ligase were obtained from New England BioLabs (Beverly, MA). GoTaq DNA polymerase was purchased from Promega Corp. (Fitchberg, WI), and high-fidelity iProof DNA polymerase was purchased from Bio-Rad (Hercules, CA). All enzymes were used according to the manufacturer’s instructions. Standard techniques were used for DNA isolation, manipulation, and cloning (56).

**Construction of plasmid insertion mutations in *B. bronchiseptica* two-component regulator genes and BB0264 mutants.** To construct a plasmid insertion mutation in selected genes that belong to putative two-component regulatory systems, the PCR was used to amplify an approximately 0.5-kb DNA fragment downstream of the predicted 5′ end of each ORF (ORFs targeted for disruption and primers are listed in Table 2). Primers were designed such that EcoRI and BamHI restriction sites were introduced at the 5′ and 3′ ends of the PCR product, respectively. The PCR products were digested with EcoRI and BamHI and cloned into pEGZH3 (40) that had been digested with the same restriction enzymes, to generate a series of plasmids used to disrupt a specific ORF. Plasmids were introduced into *E. coli* SM10pir, conjugated into RB50, and recombined into the chromosome; and cointegrates (strains in which the plasmid had recombined into the chromosome at the locus of homology) were selected on BG agar containing gentamicin and streptomycin as described previously (31).

To create an in-frame deletion in BB0264, we utilized a recently described allelic exchange system based on plasmid pSS4245 (27). A derivative of this plasmid, pMD11, was constructed as follows: primers 0264.380fwd (5′-GATTAGGCTTCTATTITCACCGGCGACCTGC-3′) and 0264.12rev (5′-GACCTCTAATTTCACCGCCGACCTGC-3′) and primers are listed in Table 2). Primers were designed such that EcoRI and BamHI restriction sites were introduced at the 5′ and 3′ ends of the PCR product, respectively. The PCR products were digested with EcoRI and BamHI and cloned into pEGZH3 (40) that had been digested with the same restriction enzymes, to generate a series of plasmids used to disrupt a specific ORF. Plasmids were introduced into *E. coli* SM10pir, conjugated into RB50, and recombined into the chromosome; and cointegrates (strains in which the plasmid had recombined into the chromosome at the locus of homology) were selected on BG agar containing gentamicin and streptomycin as described previously (31).

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GAAAACATTCTCATGAGGGGTCC-3') were used to amplify a 0.4-kb DNA fragment corresponding to the 5' upstream region and containing the first four codons of BB0264. Primers were designed such that HindIII and XbaI restriction sites were introduced at the 5' and 3' ends of the PCR product. Most primers were designed to encode glutamine (CAA). These primers were used to amplify the entire 3.6-kb DNA template, and the resulting digest was transformed into pMD10 by digestion with EcoRI and BglII and ligated into pSS4245 that had been digested with HindIII and XbaI to generate a 0.4-kb DNA fragment of the BB0264 gene (5'-GTCTGATTTCTAGAACCTTGCTGAACCAGCTG-3') and 0264-Srev (5'-GATAGTTGGATCCCTATCCCTCCAGCGCCCAGCC-3'). These primers were designed such that the codon for His521 was approximately in the center of the fragment.

A strain containing a point mutation in codon 521 of the BB0264 reading frame that results in a glutamine residue being encoded in place of histidine was constructed as follows: primers 0264-Sfwd (5'-GAGATCGTTTGCTATGCAAAGGACGCCGTC-3') and 0264-Srev (5'-GATAGTTGGATCCCTATCCCTCCAGCGCCCAGCC-3') were used to amplify a 0.6-kb DNA fragment of the BB0264 gene (5'-GTCTGATTTCTAGAACCTTGCTGAACCAGCTG-3') and 0264-Srev (5'-GATAGTTGGATCCCTATCCCTCCAGCGCCCAGCC-3'). These primers were designed such that the latter primer contains the reverse complement sequence of the former, and each contains a 1-nucleotide substitution (underlined) that changes the BB0264 codon for His521 (CAC) to GCCAG-3'.

**TABLE 2. B. bronchiseptica putative TCSs evaluated in this study**

<table>
<thead>
<tr>
<th>Sensor kinase gene</th>
<th>Response regulator gene</th>
<th>Primer used to amplify gene fragment</th>
<th>Primer orientation</th>
</tr>
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<tr>
<td>BB1281*</td>
<td>BB1282</td>
<td>5'-GATTGATACCGTTGCGGAGGGTACC-3'</td>
<td>Fwd</td>
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<tr>
<td></td>
<td></td>
<td>5'-GATTGATACCGTTGCGGAGGGTACC-3'</td>
<td>Rev</td>
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<tr>
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<td></td>
<td></td>
<td>5'-GATTGATACCGTTGCGGAGGGTACC-3'</td>
<td>Rev</td>
</tr>
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<td>BB5007</td>
<td>5'-GATTGATACCGTTGCGGAGGGTACC-3'</td>
<td>Rev</td>
</tr>
</tbody>
</table>

* Nineteen putative B. bronchiseptica TCS genes were analyzed in this study; one disruption (BB3182) was unable to be recovered, suggesting that this gene is not necessary for cell viability, and one disruption (BB2535) produced an extremely slow growth phenotype on solid medium and was not characterized further. Five TCS genes (BB8836, BB1384, BB2075, BB2664, and BB4542) were not yet successfully amplified by PCR at the time of this study. The well-studied TCS was not analyzed: the gene that was targeted for disruption in each sensor kinase/response regulator pair. Forward (Fwd) and reverse (Rev) primers that were used to amplify the 5' end which include the restriction site for EcoRI, and the reverse primer has 10 extra nucleotides at the 5' end which include the restriction site for BamHI, NP, genome annotation suggests cognate TCS gene not present. The well-studied TCS was not analyzed: * the gene that was targeted for disruption in each sensor kinase/response regulator pair.

a. The pMD11 plasmid was transformed into E. coli DH5α (CA) and the resulting digest was transformed into E. coli DH5α by electroporation.
(BRL, Gaithersburg, MD). A plasmid (pCK22) which contained the altered His\textsubscript{521} codon (CAC\textrightarrow\text{CAA}; confirmed by DNA sequencing) was recovered. The 0.6-kb chromosomal DNA fragment containing this mutation was excised from pCK22 by digestion with HindIII and BamHI and subcloned into the allelic exchange vector pEG7S (13) that had been digested with the same enzymes, to generate pCK23. DNA sequencing was used to confirm the correct sequence of all PCR-amplified DNA. pCK23 was transformed into E. coli SM10pir and then conjugated into B. bronchiseptica RB50 as described previously (31). Strains that had excised pCK23 from the chromosome are expected to contain a chromosomal BB0264 ORF containing the CAC\textrightarrow\text{CAA} mutation 50% of the time (the other half will contain a wild-type copy of the ORF) (31). DNA sequencing was used to screen through several strains to identify those which contained the desired mutation, and one was designated strain CK5.

**Construction of an RB50aplS rescue strain.** In order to provide a functional copy of BB0264 to the deletion mutation present in RB50aplS, strain RB50aplS::pJK1 was constructed. The putative native promoter for BB0264 (determined by identifying a 120-bp sequence between BB0261 and BB0262, which are divergently transcribed) was amplified using primers 0264prom-fwd (5′-GATTAGCCTTGCGGACTCCGCGCGGATG-3′) and 0264prom-rev (5′-GATTTCGCGGGAATTCGACAGGGAAGGTCATG-3′), which amplified the 120-bp DNA fragment intergenic region between BB0262 and BB0265. The DNA fragment encompassing the 70 nucleotides upstream of the predicted start codon of BB0264 to 22 nucleotides downstream of the predicted stop codon. The DNA fragments containing the BB0264 promoter and coding sequence were cloned into pEG7 digested with HindIII and EcoRI, which released a 2.4-kb DNA fragment encompassing the 70 nucleotides upstream of the predicted start codon of BB0264 to 22 nucleotides downstream of the predicted stop codon. The DNA fragments containing the BB0264 promoter and coding sequence were cloned into pEG7 digested with HindIII and EcoRI, generating plasmid pJK1, placing the putative promoter sequence 70 bp upstream of the predicted BB0264 start codon. Plasmid pJK1 was transformed into SM10pir and conjugated into B. bronchiseptica RB50aplS, as described previously (31), generating strain RB50aplS::pJK1, which contains a functional BB0264 gene driven by its putative promoter at the native BB0264 locus.

**\(\beta\)-Galactosidase assays.** Bacteria harboring chromosomal lacZ gene fusions were grown for 16 h in SS broth. Cells were permeabilized by the addition of 0.1% sodium dodecyl sulfate (SDS) and chloroform, and \(\beta\)-galactosidase activity was measured as previously described (40). Optical density (OD) measurements were taken using a Multiskan EX plate reader (ThermoFisher Scientific, Waltham, MA).

**In vitro adherence assays.** Bacterial adherence to rat lung epithelial (L2) cells was evaluated as described previously (13). Briefly, bacterial cells were grown overnight in SS broth and added to a monolayer of L2 cells (at approximately 80% confluence) at a multiplicity of infection of 200:1. Adherence was visualized by Giemsa staining and light microscopy at 80% confluence using a Zeiss Axioscope microscope and Olympus Q Color5 camera.

**RESULTS**

**In vivo survey of two-component regulatory systems.** The B. bronchiseptica genome contains at least 25 genes predicted to encode proteins with significant similarity to known sensor kinase or response regulator proteins (47). We hypothesized that one or more B. bronchiseptica TCS-like ORFs might be required for colonization of the respiratory tract, since a majority (22) of these putative TCS-encoding genes are present as orthologues in B. pertussis, an organism whose only known reservoir is the human respiratory tract. Since B. pertussis presumably evolved from a B. bronchiseptica-like ancestor (16), a TCS might be predicted to play similar roles in both the derived and ancestral strains. To test this hypothesis, we used a plasmid insertion mutagenesis approach to attempt to disrupt 19 ORFs in B. bronchiseptica (Table 2) to encode sensor kinase or response regulator proteins. Disruptions of 17 of the 19 targeted ORFs produced viable colonies with normal growth rates on solid medium (Table 2). These mutants were then tested in a natural host rat infection model. Of these, one (CK18) was similar to the wild-type strain in its ability to colonize the nasal cavity at 14 days postinoculation but was completely unable to colonize the trachea (Fig. 1A). Strain CK18 has a plasmid insertion mutation in predicted ORF BB0264, which encodes a putative NtrY-like sensor kinase. Notably, BB0265, the ORF predicted to start 13 nucleotides 3′ to BB0264, encodes a putative response regulator protein, suggesting the potential for BB0264 and BB0265 to compose a two-component regulatory pair. The local DNA context of these genes suggests that they are part of a multigene operon and are cotranscribed with four other ORFs, with two located 5′ to and two located 3′ to BB0264 and BB0265 (53) (Fig. 1B). BB0262 is predicted to encode a polypeptide with homology to 16S rRNA methyltransferases; BB0263 is predicted to encode an exported polypeptide of unknown function; and the BB0266 and BB0267 ORFs are similar to trkA and trkH, respectively, from several Gram-negative bacteria (3). In E. coli, trkA and trkH are involved with low-affinity transport of potassium into the cell (6, 57). The expression of this transcript presumably is controlled by a promoter immediately 5′ to BB0262, on the basis of the fact that the ORF that resides 5′ to BB0262 is located 120 bp away and is divergently transcribed (47).

When the expression of BB0264 was evaluated using a lacZ transcriptional fusion incorporated at the BB0264 locus, it was 2-fold higher when the bacteria were grown at 37°C (representing the approximate temperature of the respiratory tract) than at 25°C (representing temperatures found outside the...
FIG. 1. (A) A plasmid insertion mutation in *B. bronchiseptica* ORF BB0264 (strain CK18) prevents colonization of the trachea in rats. RB50, wild-type *B. bronchiseptica*. Each circle represents the colonization level in an individual rat, and the short horizontal bar indicates the average for each experimental group. The horizontal dashed line indicates the lower limit of detection. (B) Genetic organization of the putative operon containing BB0264. ORFs BB0262 through BB0267 are predicted to be monocistronic using an operon prediction algorithm (53), with the two members of the TCS shown in darker shading. The inverted triangle denotes the location of the plasmid insertion mutation (which also incorporates a promoterless *lacZ* gene) that generated strain CK18. The bent arrow denotes the location of the predicted promoter sequence. (C) Gene expression in the operon containing BB0264, as measured by a *lacZ* transcriptional fusion in BB0264, is elevated at higher temperature, and temperature dependence does not require BvgAS. Measurements were done in the presence (BB0264::*lacZ* [strain CK18]) and absence (BB0264::*lacZ* Δ*bvgS* [strain RB54::CK18]) of the gene encoding the sensor kinase protein of the BvgAS TCS. *, *P* < 0.05; ***, *P* < 0.001.
respiratory tract) (Fig. 1C). These data suggest that the expression of BB0264 may respond to conditions present in the respiratory tract (e.g., increased temperature). Since the BvgAS TCS is known to regulate the expression of virulence genes in response to temperature (54), we evaluated the expression of BB0264 in a strain containing a deletion of bvgAS, which encodes the sensor kinase of the BvgAS TCS (Fig. 1C). No difference in expression was observed compared to that in the bvgAS+ background, indicating that under these experimental conditions, the temperature-dependent expression of BB0264 does not require the presence of BvgAS. Because of its requirement for colonization of the trachea, but not nasal cavity, for the remainder of the study we refer to BB0264 by its new designation, plrS, for persistence in the lower respiratory tract, sensor kinase.

An in-frame deletion mutation in plrS confirms its requirement for tracheal colonization. We constructed an in-frame deletion mutation in plrS by deleting codons 5 through 198, which removed a majority of a putative signal sequence and two of three transmembrane regions, resulting in strain RB50ΔplrS (Fig. 2). RB50ΔplrS produces colonies of normal size and appearance on solid medium, and there is no difference in growth rate compared to that of the wild-type strain when RB50ΔplrS is grown in liquid culture (data not shown). When it was tested in our rat colonization model, RB50ΔplrS was found at levels similar to the wild-type strain RB50 in the nasal cavity for at least 28 days postinoculation (Fig. 3). However, RB50ΔplrS was significantly defective in colonizing the trachea at 7 days postinoculation and remained defective in establishing infection in this tissue through the remaining 28 days of the experiment (Fig. 3). Importantly, a strain (RB50ΔplrS::pIK1) in which the deletion in plrS was rescued with a copy of the intact plrS gene that was integrated into the chromosome at the plrS locus exhibited wild-type levels of colonization in the trachea. These data demonstrate that there is a specific requirement for plrS for B. bronchiseptica colonization of the trachea. Moreover, plrS is dispensable for bacterial colonization of the upper respiratory tract.

Although several B. bronchiseptica mutants that are defective in colonization of the rat trachea have been identified (42, 70), only strain RBX9, which contains a deletion of the gene encoding filamentous hemagglutinin (FHA), shows a complete lack of colonization by 10 days postinoculation, similar to the defect observed with RB50ΔplrS by day 7 postinoculation (13). FHA is an adhesin and immunomodulatory protein that has been shown to be indispensable for B. bronchiseptica colonization of the trachea, but not the nasal cavity (13, 26). We therefore evaluated expression levels of fhaB, the gene encoding FHA, in RB50ΔplrS and also examined FHA function by observing bacterial adherence to rat lung epithelial cells, a phenotype entirely dependent on the surface expression of FHA (13, 26). Figure 4A shows that the expression of fhaB in RB50ΔplrS is similar to that in the wild-type strain. RB50ΔplrS was also able to adhere to L2 cells in a manner similar to RB50 (Fig. 4B), indicating that, in cells lacking an intact plrS gene, FHA can mediate adherence to L2 cells. Taken together, these data demonstrate that FHA function does not appear to be regulated by plrS (at least for cells grown in Stainer-Scholte broth), and since adherence to L2 cells requires surface-localized FHA, these results also indicate that sec-mediated export of FHA is functional (9).

plrS is required for persistence in lung inflammation infection model. The observation that RB50ΔplrS was unable to establish colonization in the rat trachea by day 7 postinoculation suggests that plrS may be required in part to withstand the host innate immune responses that function at early stages of infection. To test this hypothesis, we evaluated the growth of RB50ΔplrS in a mouse lung inflammation model of infection. In this model, a large dose of B. bronchiseptica is deposited directly into the trachea and lungs to induce an inflammatory response, which represents the first line of immune defense to infection. Inflammation has been documented by recruitment of macrophages and neutrophils via observation of stained lung sections of infected mice (26). In this model, wild-type B. bronchiseptica induces a significant inflammatory response and is able to persist in the lungs for several weeks (26, 27, 32, 68).

We wondered if plrS might contribute to the bacteria’s ability to survive under these experimental conditions. As shown in Fig. 5, 1 h after inoculation of BALB/c mice with either wild-type or RB50ΔplrS B. bronchiseptica, the levels of bacteria in the nasal cavity, trachea, and lungs were similar when the levels in each of these tissues were compared between the two experimental groups, indicating that similar amounts of bacteria were initially delivered to each of these sites. The wild-type strain was recovered from the trachea and lungs for at least 11 days postinoculation before beginning to be cleared (Fig. 5).
contrast, at as early as 4 days postinoculation, RB50ΔplrS was present at significantly lower levels in the trachea and lungs. By day 11 postinoculation, RB50ΔplrS was no longer recovered from the trachea, and by day 28 postinoculation, RB50ΔplrS was no longer recovered from the lungs. In contrast, in the nasal cavity, no significant differences in persistence between the wild-type and mutant strains were observed at any time point. These data demonstrate that, unlike the wild-type strain, RB50ΔplrS is rapidly cleared from the trachea and lungs, two sites where inflammatory responses are induced. From these data, we infer that plrS contributes to the ability of *B. bronchiseptica* to resist clearance by activated cells that compose the innate immune response during early stages of infection.

A conserved histidine is required for PlrS function in vivo. The predicted PlrS polypeptide shares conserved domains with other histidine kinases (Fig. 2) that compose the sensor protein module of TCSs. A critical component of these sensor proteins is a conserved histidine residue that serves as the site of autophosphorylation (38). Figure 6A shows an alignment of several NtrY-like histidine kinases, including PlrS, centered around this conserved histidine residue; in PlrS, this histidine residue is at position 521 of the predicted polypeptide (His521) (Fig. 6A). We hypothesized that if PlrS functions as a histidine kinase sensor protein in *B. bronchiseptica*, then His521 should be critical to its function. To test this hypothesis, we generated a strain, CK5, in which His521 of PlrS was replaced with a glutamine residue and evaluated this strain in a rat colonization model. Figure 6B shows that CK5 exhibited significantly reduced colonization of the trachea at all time points tested, similar to what was observed for RB50ΔplrS. These data indicate that His521 is critical for PlrS-dependent colonization of the rat trachea and suggest that the ability of PlrS to function as a sensor protein, via phosphorylation of His521, is required for *B. bronchiseptica* to colonize this site.

The proper production and cellular localization of several immunoreactive proteins are dependent on PlrS. We reasoned that the production or localization of several proteins, including those important for infection, might be dysregulated in a plrS mutant background. To investigate this possibility, we isolated whole-cell and supernatant proteins from wild-type and plrS mutant strains that had been grown overnight in Stainer-Scholte broth and separated them using SDS-PAGE. In order to focus specifically on proteins that might be important during infection, we transferred the proteins to nitrocellulose and probed the membrane with serum collected from a rat infected with wild-type *B. bronchiseptica* as the source of primary antibodies. Figure 7A shows a Western blot in which whole-cell proteins, collected from RB50, RB50ΔplrS, and CK5 strains grown overnight in SS broth, were used as antigens. Three polypeptides, of approximately 300, 75, and 65 kDa, were present more substantially in the mutant strains than in the wild-type strain, indicating that, at least during *in vitro* growth, these immune-recognized proteins are present in relatively larger quantities in a plrS background. Figure 7B shows a Western blot using proteins collected from culture supernatants as antigens. Proteins of approximately 280, 30, and 28 kDa were more abundant in culture supernatants prepared from the wild-type strain, suggesting that the secretion

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**FIG. 3.** RB50ΔplrS is defective for colonization of the trachea in Sprague-Dawley rats. Rats were inoculated intranasally with 1,000 CFU of either the wild-type (RB50), mutant (RB50ΔplrS), or plrS-rescued (RB50ΔplrS::pJK1) strain, and bacterial levels in the nasal septum and trachea were determined at the indicated time points postinoculation. Each circle represents the colonization level in an individual rat, and the short horizontal bar indicates the average for each experimental group. The horizontal dashed line indicates the lower limit of detection. *, statistically significant difference ($P < 0.05$) between RB50 and RB50ΔplrS.
of these proteins (or the expression of the genes encoding them) is downregulated in a \( \text{plrS} \) background. Conversely, proteins of approximately 60, 35, and 27 kDa were more abundant in culture supernatants prepared from one or both \( \text{plrS} \) strains. Together, these data indicate that the production and/or localization to the supernatant of several proteins that are recognized by the immune system is dysregulated when \( \text{plrS} \) is nonfunctional. Additionally, in both blots, there are several proteins whose relative recognition by immune sera appears to differ between the two mutant strains (for example, the 75-kDa and 300-kDa antigens collected from whole-cell preparations and the 28-kDa and 27-kDa antigens collected from supernatant preparations). We surmise that these differences may reflect the genetic composition of the particular \( \text{plrS} \) mutation being analyzed. For example, CK5 contains a \( \text{plrS} \) gene encoding a polypeptide with a single amino acid difference in the putative histidine kinase domain, and the resulting polypeptide may still be competent for transport and inser-
tion into the cytoplasmic membrane, albeit with severely reduced signaling capabilities. In contrast, RB50\textsuperscript{/H9004} plrS expresses a polypeptide predicted to be missing the signal sequence and two of three transmembrane domains, suggesting that it may be nearly or completely deficient for transport and/or membrane localization. Functional differences in the PlrS polypeptides encoded by RB50\textsuperscript{/H9004} plrS and CK5, including potential cellular localization differences, might be sufficient to explain subtle differences in antigen recognition in RB50\textsuperscript{/H9004} plrS and CK5 strains.

**DISCUSSION**

Here we demonstrate the requirement of plrS, whose predicted protein product shares homology to known bacterial sensor kinases, for *B. bronchiseptica* colonization of the lower respiratory tract in natural infection models. We propose that PlrS functions as a sensor kinase that is required for *Bordetella* virulence. Importantly, a conserved histidine residue (His\textsuperscript{521}) in the putative histidine kinase domain of PlrS is absolutely required for *B. bronchiseptica* to colonize the respiratory tract. This histidine, present in the kinase domain of various bacterial sensor kinases, has been shown to serve as the site of autophosphorylation (10, 38). Additionally, a BLAST search of the polypeptide encoded by the predicted ORF immediately 3' to plrS reveals extensive homology to bacterial response regulator proteins (data not shown). Taken together, our data are consistent with a scenario where, in response to an unknown environmental signal(s) present during infection, PlrS becomes activated, resulting in phosphorylation of His\textsuperscript{521}, with subsequent phosphotransfer to a cognate response regulator, which is likely to be encoded by the ORF immediately 3' to plrS. These events are required for *Bordetella* to colonize the lower respiratory tract, such that loss of PlrS activity results in the colonization/persistence defects observed in this study. Our data also suggest that plrS mutants are more susceptible to innate immune mechanisms that function at early stages of infection, perhaps by enhanced clearance by phagocytic cells. *Bordetella* can interact directly with macrophages (26, 28), and previous work using *B. bronchiseptica* infection of murine lungs has shown that bacterial clearance involves activation of Toll-like receptor 4 and CD11b immune cell signaling pathways (39, 50). An important next step in characterization of plrS function will include an evaluation of its contribution to immune cell activity, and toward this end, we have observed that the levels of adherence of plrS mutants to J774 macrophage-like cells in culture are similar to those of wild-type *B. bronchiseptica* (K. T. Cochran and S. M. Julio, unpublished observations), suggesting that the defect present in plrS strains may exist at a level other than direct interactions with immune cells. Whatever its specific function might be, given the global role that TCSs play in controlling gene expression, it is likely that the activity of PlrS influences the expression of a set of genes that contribute to *Bordetella* survival in the lower respiratory tract. Further work will need to be done to establish the specific contribution that plrS makes to *B. bronchiseptica* virulence, as well as the

![FIG. 5. RB50ΔplrS is unable to withstand the inflammatory response in the mouse lower respiratory tract. BALB/c mice were inoculated intranasally with 500,000 CFU of either the wild-type (RB50) or mutant (RB50ΔplrS) strain, and bacterial levels in the nasal septum, trachea, and lungs were determined at the indicated time points postinoculation. Each data point represents the average of at least four mice, and error bars represent standard errors of the means. The lower limit of detection was 10 bacteria. Statistically significant differences between RB50 and RB50ΔplrS in the corresponding tissue are indicated: *, *P < 0.05; **, *P < 0.005; ***, *P < 0.001.](http://iai.asm.org/.../2017)
function of the product encoded by the ORF immediately 3′ to
plrS, and whether together it and PlrS compose a TCS.

Our experiments demonstrating the requirement of PlrS for
respiratory tract colonization extend the number of TCS-like
loci that have been reported to regulate Bordetella virulence to
three. This is perhaps not surprising, since coordinating the
appropriate patterns of gene expression during the course of
infection often requires the activity of multiple TCSs that re-
spond to different signals encountered within the host environ-
ment (18–20, 22, 48). In Bordetella, the BvgAS TCS has been
extensively characterized as the master regulator of virulence,
as it controls the expression of virtually all known genes that
contribute to the ability of the bacteria to colonize and persist
in the host, including genes encoding adhesins (e.g., FHA,
fimbriae, and pertactin) and toxins (e.g., pertussis toxin, CyaA,
and type III secretion effectors) (10, 40, 41, 43, 45). More
recently, a second TCS, risAS, that contributes to the ability of
B. bronchiseptica to resist oxidative stress and colonize the
mouse respiratory tract was identified (33, 61, 71). RisAS has
been suggested to be required specifically for intracellular sur-

FIG. 6. (A) ClustalW alignment of a portion of the HisKA domains from several NtrY-like sensor kinase proteins, with inclusive amino acid
numbers relative to the full-length protein. PlrS_BB, PlrS from B. bronchiseptica; NtrY_ER, NtrY from Erlichia chaffeensis; NtrY_AC, NtrY from
Azorhizobium caulinodans; NtrY_BA, NtrY-like protein from Brucella abortus; NtrY_NM, NtrY-like protein from Neisseria meningitidis. con,
consensus sequence, with amino acid identities marked with an asterisk. The histidine residue that serves as the site of autophosphorylation is
boxed; this histidine was changed to a glutamine residue in strain CK5 as part of this study. (B) CK5 mutants are unable to colonize the lower
respiratory tract in rats. Animals were inoculated with 1,000 CFU of either the wild type (RB50) or CK5, and bacterial colonization levels in the
nasal cavity and trachea were determined at the indicated time points postinoculation. Each symbol represents the bacterial numbers recovered
from an individual rat, and the dashed line indicates the lower limit of detection. *, statistically significant difference (P < 0.05) between RB50
and CK5 in the corresponding tissue.

required for intracellular survival and which are activated by
Ris, are also part of the BvgAS regulon, but their expression is
repressed by Bvg (14, 61). This might suggest that these two
systems control gene expression in response to different envi-
enrons encountered during the infectious process (perhaps
intracellular versus extracellular environments); however, a
common theme is that each system must ensure the appropri-
ate timing and levels of expression of the genes contained
within its regulon and that failure to do so results in an attenu-
ation of virulence for the organism (10, 12, 33). If, indeed,
PlrS is a sensor kinase that controls the expression of one or
more genes, two lines of evidence suggest that it and BvgAS
function independently of one another. First, we show that the
transcription of plrS itself appears to be regulated independ-
ently of BvgAS activity, even though transcription of plrS is
elevated under conditions where BvgAS is active (specifically,
increased temperature). This indicates that, under the in vitro
experimental conditions used in this study, plrS is not a Bvg-
activated gene (vag). Second, plrS does not appear to be re-
quired for the expression of fhaB (the gene encoding FHA),
which is known to be under Bvg control (62). On the basis of
these data, it could be inferred that genes whose expression is

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of immune clearance mechanisms, and their inappropriate expression in the absence of \(\text{plrS}\) might compromise bacterial survival. Such a scenario has indeed been shown to occur in \textit{Bordetella}: when flagella, which are normally produced only during the Bvg\textsuperscript{+} phase (2), are expressed ectopically during infection, the bacteria are rendered avirulent (1). Ectopic expression of PlrS-regulated antigens may provide a mechanism by which immune recognition of mutant strains either prohibits their establishment or rapidly clears them at sites where active immunity exists. Clearly, an important next step is to identify these PlrS-dependent, immune-recognized proteins in order to provide additional clues as to how PlrS-controlled gene expression allows persistence of \textit{B. bronchiseptica} in the lower respiratory tract.

Sequence comparisons reveal that PlrS shares the most extensive homology to several NtrY-like sensor kinases, with some 50\% identity over nearly the entire predicted polypeptide sequence (Fig. 6 and data not shown). NtrY (and its cognate response regulator, NtrX) was first identified in the plant symbiont \textit{Azorhizobium caulinodans} and was shown to be involved in the control of gene expression required for nitrogen fixation (49). This raises the possibility that \(\text{plrS}\) might control the expression of a subset of genes that are responsible for either nitrogen sensing or assimilation in \textit{B. bronchiseptica}. Consistent with this idea, as well as with our data revealing a role for \(\text{plrS}\) in virulence, other respiratory tract pathogens have been shown to respond to nitrogen levels \textit{in vivo}: \textit{Pseudomonas aeruginosa} upregulates the expression of at least 15 genes during infection that are involved in nitrogen metabolism (59), and genes involved in nitrogen assimilation are potentially required for \textit{Mycobacterium tuberculosis} pathogenesis (25). And despite a dissimilar tissue tropism from \textit{Bordetella}, \textit{Salmonella} strains defective in the initial steps of nitrogen assimilation are attenuated for virulence and show a reduced ability to survive in macrophages, further demonstrating that nitrogen-limiting host environments are one constraint with which pathogens must contend (35). Thus, given the relatively high degree of sequence homology between PlrS in \textit{Bordetella} and NtrY-like sensor kinases, a straightforward interpretation of our data would be that PlrS responds to \textit{B. bronchiseptica}'s} nitrogenous requirements during infection.

However, in addition to NtrYX, another TCS known as NtrBC has been described that also responds to the nitrogen status of the cell and controls gene expression related to nitrogen fixation and assimilation (65). Interestingly, in bacteria that possess both NtrXY and NtrBC, it has been shown that mutations in \textit{ntrBC} can be compensated for at least partially by the presence of NtrYX (17, 29, 36, 46, 49), and in at least one case the sensor kinases NtrY and NtrB are functionally interchangeable for phosphotransfer to the response regulator NtrC (17). This raises an intriguing question with respect to the idea that the role of \textit{B. bronchiseptica} \(\text{plrS}\) involves nitrogen metabolism: since \textit{B. bronchiseptica} presumably encodes the NtrBC two-component system (47), why was it not able to compensate for the lack of \(\text{plrS}\) during infection? At least three possibilities exist: (i) the \(\text{ntrBC}\) genes are not functional in \textit{B. bronchiseptica}, (ii) \(\text{ntrBC}\) is unable to compensate for the specific or unique role that \(\text{plrS}\) plays in controlling the response to nitrogen levels during infection, or (iii) \(\text{plrS}\) is not involved in controlling gene expression related to nitrogen metabolism.

controlled by \textit{bvgAS} are not subject to \(\text{plrS}\)-based regulation. This idea is supported by the fact that the expression of \textit{cyA} is also not influenced by \(\text{plrS}\) (S. Roberts and S. M. Julio, unpublished observations). \textit{cyA} encodes a cytotoxin required for \textit{Bordetella} virulence (21, 24), and its expression requires relatively high levels of phosphorylated BvgA (10).

Given its \textit{in vivo} phenotype and its probable role as part of a TCS, it is likely that PlrS regulates the expression of one or more genes required for persistence in the lower respiratory tract. Consistent with this idea, we observed the aberrant production or localization of several proteins in \(\text{plrS}\) mutants that are recognized by immune sera. Compared to the wild-type strain, several antigens are present more abundantly in \(\text{plrS}\) mutants, suggesting that \(\text{plrS}\) might repress the production or localization of these proteins, while other antigens are present less abundantly, suggesting that \(\text{plrS}\) may function simultaneously to promote the production or localization of other proteins. These \(\text{plrS}\)-repressed or \(\text{plrS}\)-activated antigens (or others not identified in this study) might play an important role in the establishment of colonization and/or successful avoidance

![FIG. 7. Expression profile of PlrS-dependent proteins recognized by immune serum. (A) Western blot analysis of whole-cell proteins. Proteins from wild-type (RB50), RB50\(\Delta\text{plrS}\), and CK5 strains grown overnight in SS medium were separated by 6\% SDS-PAGE, transferred to nitrocellulose, and probed with sera collected from rats infected with wild-type \textit{B. bronchiseptica}. Antigens recognized more heavily in the wild-type strains than the wild type are marked with an asterisk. Molecular mass standards (in kDa) are shown on the left. (B) Western blot analysis of supernatant proteins. Supernatant proteins from wild-type (RB50), RB50\(\Delta\text{plrS}\), and CK5 strains grown overnight in SS medium were separated by 12\% SDS-PAGE, transferred to nitrocellulose, and probed with sera collected from rats infected with wild-type \textit{B. bronchiseptica}. Antigens recognized more heavily in the wild-type strain than \(\text{plrS}\) mutant strains are marked with a dagger, and antigens recognized more heavily in the \(\text{plrS}\) mutant strains than the wild-type strain are marked with an asterisk. Molecular mass standards (in kDa) are shown on the left.](http://iai.asm.org/Downloaded_from_host http://iai.asm.org/ on October 16, 2017 by guest)
and regulates some other critical component of infection. To address these scenarios, we are currently evaluating the expression of glnA (which is repressed by ntrBC in E. coli [55]) in plrS and ntrB mutant backgrounds. A role for plrS in controlling glnA expression would be consistent with a scenario where plrS is required for proper responses to nitrogen levels in the mammalian respiratory tract during Bordetella infection.

A more general question that can be raised from our studies centers on the genomic survey of TCSs that are present in Bordetella. The fact that there are at least 25 predicted (and presumably functional) TCSs in B. bronchiseptica RB50 (47) could be explained by the observation that B. bronchiseptica not only is found in multiple animal reservoirs but also can survive outside the host organism (51), suggesting the need to control gene expression in response to widely different environmental signals. Somewhat surprising, then, is the fact that orthologues of a majority of these TCSs are found in the human-restricted strain B. pertussis. Since gene loss is hypothesized to be a mechanism by which B. pertussis restricted its host range to the human respiratory tract (15, 47, 52), it is reasonable to assume that regulatory systems providing no selective advantage in this niche would be lost as well. Our data suggest that, in B. bronchiseptica, a majority of TCSs are not required for colonization of the respiratory tract, at least in a rat natural infection model, as all mutant B. bronchiseptica strains tested in this study (except the plrS mutant) that harbor a single mutation in a predicted TCS were as efficient as the wild-type strain in colonizing the respiratory tract (Table 2 and data not shown). This result could be explained by functional redundancy among various TCSs or, more likely, due to TCS specialization for use in a particular (nonrat) mammalian host or even nonmammalian environment. The presence of a majority of these orthologous TCSs in B. pertussis, then, suggests either that sufficient time has not elapsed since its divergence from a B. bronchiseptica-like ancestor (16) for adequate gene loss or drift or that perhaps these regulators have somehow been co-opted for use in one particular (e.g., human-adapted) host environment. Indeed, these two hypotheses are not mutually exclusive: in B. pertussis, the gene for risS is a pseudogene, but the gene for its cognate risA is intact and RisA is likely involved in cross talk within an as-yet-unidentified regulatory system (61). It will be informative to compare the profiles of TCSs present in the genomes of many bordetellae, including both broad-host-range and human-associated B. bronchiseptica strains (16) and human-adapted B. pertussis and B. parapertussis strains, to determine whether the presence of particular TCSs correlates with the in vivo niches available to a species.

The identification of specific regulatory networks responsible for controlling gene expression during infection is crucial to understanding the mechanisms of bacterial disease. The results of our study highlight the fact that Bordetella requires the activities of several TCSs that presumably respond to specific environmental cues encountered during the course of infection in the respiratory tract. We have identified a novel sensor kinase-like gene, plrS, that is required for both bacterial persistence (specifically at a site where active immunity is present) and the proper production and/or localization of several antigens recognized during infection. It is expected that work aimed at identifying the particular gene(s) whose expression is under the control of PlrS, under way in our laboratory, will provide a better understanding of the specific mechanisms employed by Bordetella to circumvent the immune response that allows it to persist in the mammalian respiratory tract.

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