Bordetella PlrSR regulatory system controls BvgAS activity and virulence in the lower respiratory tract

M. Ashley Bone[a,1], Aaron J. Wilk[b,1,2], Andrew I. Perault[a], Sara A. Marlatt[a,3], Erich V. Scheller[a], Rebecca Anthouard[a], Qing Chen[c], Scott Stibitz[c], Peggy A. Cotter[a,4], and Steven M. Julio[b,4]

[a]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [b]Department of Biology, Westmont College, Santa Barbara, CA 93108; and [c]Division of Bacterial, Parasitic, and Allergenic Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892

Edited by Scott J. Hultgren, Washington University School of Medicine, St. Louis, MO, and approved January 6, 2017 (received for review June 13, 2016)

Bacterial pathogens coordinate virulence using two-component regulatory systems (TCS). The *Bordetella* virulence gene (BvgAS) phosphorelay-type TCS controls expression of all known protein virulence factor-encoding genes and is considered the "master virulence regulator" in *Bordetella pertussis*, the causal agent of pertussis, and related organisms, including the broad host range pathogen *Bordetella bronchiseptica*. We recently discovered an additional sensor kinase, PlrS [for persistence in the lower respiratory tract (LRT) sensor], which is required for *B. bronchiseptica* persistence in the LRT. Here, we show that PlrS is required for BvgAS to become and remain fully active in mouse lungs but not the nasal cavity, demonstrating that PlrS coordinates virulence specifically in the LRT. PlrS is required for LRT persistence even when BvgAS is rendered constitutively active, suggesting the presence of BvgAS-independent, PlrS-dependent virulence factors that are critical for bacterial survival in the LRT. We show that PlrS is also required for persistence of the human pathogen *B. pertussis* in the murine LRT and we provide evidence that PlrS most likely functions via the putative cognate response regulator PlrR. These data support a model in which PlrS senses conditions present in the LRT and activates PlrR, which controls expression of genes required for the maintenance of BvgAS activity and for essential BvgAS-independent functions. In addition to providing a major advance in our understanding of virulence regulation in *Bordetella*, which has served as a paradigm for several decades, these results indicate the existence of previously unknown virulence factors that may serve as new vaccine components and therapeutic or diagnostic targets.

Bordetella | two-component system | virulence | respiratory infection | gene regulation

Caused by the human-specific, Gram-negative bacterium *Bordetella pertussis*, whooping cough (aka pertussis) is reemerging in the United States and other developed countries, despite high vaccine coverage (1, 2). Increased incidence in recent years coincides with the switch to acellular vaccines, which induce immunity that is less durable than that induced by whole-cell vaccines or by infection with *B. pertussis* (3–5). Although closely related to *B. pertussis*, *Bordetella bronchiseptica* infects nearly all mammals and typically causes more chronic, long-term respiratory infections (6). Despite these differences, *B. pertussis* and *B. bronchiseptica* produce a nearly identical set of virulence factors that includes adhesins, such as filamentous hemagglutinin (FHA) and fimbriae (FIM), and toxins, such as adenylate cyclase toxin (ACT), and a type III secretion system (T3SS) (2).

The *Bordetella* virulence gene (BvgAS) phosphorelay-type two-component regulatory system (TCS) is considered the "master virulence control system" in *Bordetella*. BvgAS differentially regulates (either directly or indirectly) hundreds of genes and at least three distinct phenotypic phases (7, 8). The Bvg^+ phase occurs when the bacteria are grown at 37 °C in Stainer–Scholte (SS) broth or on Bordet–Gengou (BG) blood agar and correlates with BvgAS activity. The Bvg^+ phase is characterized by expression of all currently known protein virulence factor-encoding genes (referred to collectively as *vag*s) and lack of expression of BvgAS-repressed genes (called *vrg*s), which includes those encoding flagella in *B. bronchiseptica*. The Bvg^- phase occurs when the bacteria are grown at ≤26 °C or when millimolar concentrations of $MgSO_4$ or nicotinic acid are added to the growth medium (referred to as "modulating conditions"). The Bvg^- phase is characterized by expression of *vrg* loci and lack of expression of *vag*s. The Bvg-intermediate (Bvg^i) phase occurs at intermediate temperatures or in the presence of low concentrations of $MgSO_4$ or nicotinic acid (9). It is characterized by expression of *vag*s that contain high-affinity BvgA binding sites at their promoters (such as *fhaB*, encoding FHA, *fimBCD*, encoding the FIM biogenesis proteins, and *bvgAS* itself), lack of expression of *vag*s with low-affinity BvgA binding sites (such as *cyaABDE*, encoding ACT and *ptxA-E*, encoding pertussis toxin), lack of expression of *vrg*s, and maximal expression of *bipA*, which encodes an outer-membrane protein of unknown function (9–11). Although BvgAS activity is altered by temperature, $MgSO_4$, and nicotinic acid in vitro, the true signals it senses in nature are unknown.

B. pertussis and *B. bronchiseptica* strains containing loss-of-function mutations in *bvgAS* are avirulent, whereas strains containing mutations that render BvgAS active—even under modulating conditions in vitro—are indistinguishable from WT bacteria

Significance

***Bordetella* spp. includes *Bordetella pertussis*, the causal agent of whooping cough. The *Bordetella* virulence gene (BvgAS) two-component regulatory system (TCS) is considered the "master virulence regulator" in *Bordetella*, as it controls expression of all known virulence factor-encoding genes. We show here that another TCS, PlrSR, is required for BvgAS activity in the lower respiratory tract (LRT) and for virulence even when BvgAS is rendered constitutively active, suggesting that it controls critical functions for bacterial survival in the LRT independently of BvgAS. Our data introduce a new layer of complexity to a paradigm of *Bordetella* virulence control that has held for more than 30 y, and they indicate the existence of previously unknown bacterial factors that may serve as vaccine components and therapeutic targets.**

Author contributions: M.A.B., A.J.W., S.S., P.A.C., and S.M.J. designed research; M.A.B., A.J.W., A.I.P., S.A.M., E.V.S., R.A., and Q.C. performed research; M.A.B., A.J.W., P.A.C., and S.M.J. analyzed data; and M.A.B., P.A.C., and S.M.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]M.A.B. and A.J.W. contributed equally to this work.

[2]Present address: Medical Scientist Training Program, Stanford University School of Medicine, Stanford, CA 94305.

[3]Present address: Department of Biology and Chemistry, Azusa Pacific University, Azusa, CA 91702.

[4]To whom correspondence may be addressed. Email: peggy_cotter@med.unc.edu or sjulio@westmont.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1609565114/-/DCSupplemental.

in their ability to cause respiratory tract infections (12–14). Characterization of antibody responses following infection and analyses using recombination-based reporters of gene expression indicate that modulation to the Bvg^- phase does not occur during infection (12, 15, 16). Studies with strains that produce Bvg^- phase factors ectopically in the Bvg^+ phase have demonstrated the importance of BvgAS-mediated repression of gene expression in vivo (14, 17). Taken together, these results have been interpreted to indicate that the Bvg^+ phase is both necessary and sufficient for the development of respiratory infection by *Bordetella*. Moreover, these data, together with the fact that the phenotypic profile of WT bacteria grown at 37 °C in SS medium or on BG agar (Bvg^+ phase conditions) is identical to that of mutants containing *bvgS* mutations that render BvgS insensitive to modulating conditions, have led to the conclusion that these in vitro growth conditions mimic, at least to some extent, those experienced by the bacteria in the respiratory tract.

In a survey of putative TCSs in *B. bronchiseptica*, we discovered a gene (BB0264) predicted to encode an NtrY-like sensor kinase that is essential for *B. bronchiseptica* to colonize the trachea of rats following low-dose, low-volume intranasal inoculation and to persist in the lungs of mice following high-dose, large-volume intranasal inoculation (18). We named BB0264 *plrS*, for persistence in the lower respiratory tract (LRT) sensor. Subsequently, Hester et al. reported the identification of a CO_2 responsive regulon in *Bordetella,* and hypothesized that CO_2 gradients may be sensed by the bacteria as a cue indicating their localization in the LRT (19). Here, we show that *plrS* is required for increased virulence factor production in response to CO_2, suggesting a functional link between PlrS and BvgAS. We investigated this link, showed that *plrS* is required for BvgAS to be fully active in the LRT, and showed that *plrS* is required for persistence in the LRT even when BvgAS is constitutively active, indicating that although BvgAS is necessary in vivo, it is not sufficient. Moreover, our data suggest the existence of genes that are expressed only in the LRT that encode previously unknown virulence factors, which may serve as new therapeutic or diagnostic targets and vaccine components.

Results

PlrS Is Required for Enhanced BvgAS-Dependent Virulence-Associated Phenotypes in Response to Elevated CO_2 Concentrations. BvgAS-regulated virulence-associated phenotypes include adherence to epithelial cells and macrophages, which is mediated by FHA (20, 21), hemolysis on blood-containing agar, which is mediated by ACT (22), and toxicity to various eukaryotic cell types in culture, which is mediated, at least in part, by the T3SS (23). All of these phenotypes were enhanced when WT *B. bronchiseptica* was grown at 37 °C in 5% CO_2 compared with growth at 37 °C in ambient air (Fig. 1 and Fig. S1) (19). These virulence-associated phenotypes did not increase in response to 5% CO_2 in the Δ*plrS* mutant, indicating that PlrS is required for this effect. The effect was not a result of acidification of the growth medium because acidification alone, without increased CO_2, did not result in increased adherence (Fig. S2). These results suggest the possibility of a functional interaction between PlrS and BvgAS.

PlrS Likely Affects BvgAS-Dependent Phenotypes via PlrR. A putative response regulator, which we are naming PlrR, is encoded immediately 3′ to *plrS*. Multiple attempts using various approaches to disrupt or delete *plrR* (BB0265) were unsuccessful, suggesting *plrR* is essential for cell viability under the growth conditions tested. As an alternate approach to determine if PlrS and PlrR function as a cognate TCS, we delivered a *plrR* allele ($plrR^{D52E}$) encoding a PlrR protein in which the predicted site of phosphorylation, Asp52, was replaced with Glu, a predicted phosphomimetic (24), to the *att*Tn7 site in WT, Δ*plrS*, and Δ*bvgS* (which harbors a deletion in *bvgS*) *B. bronchiseptica*. When grown in 5% CO_2, the level of adherence of the Δ*plrS* strain expressing $plrR^{D52E}$ to L2 cells was as high as that of WT *B. bronchiseptica* (and WT-expressing $plrR^{D52E}$), demonstrating that the $plrR^{D52E}$ allele could

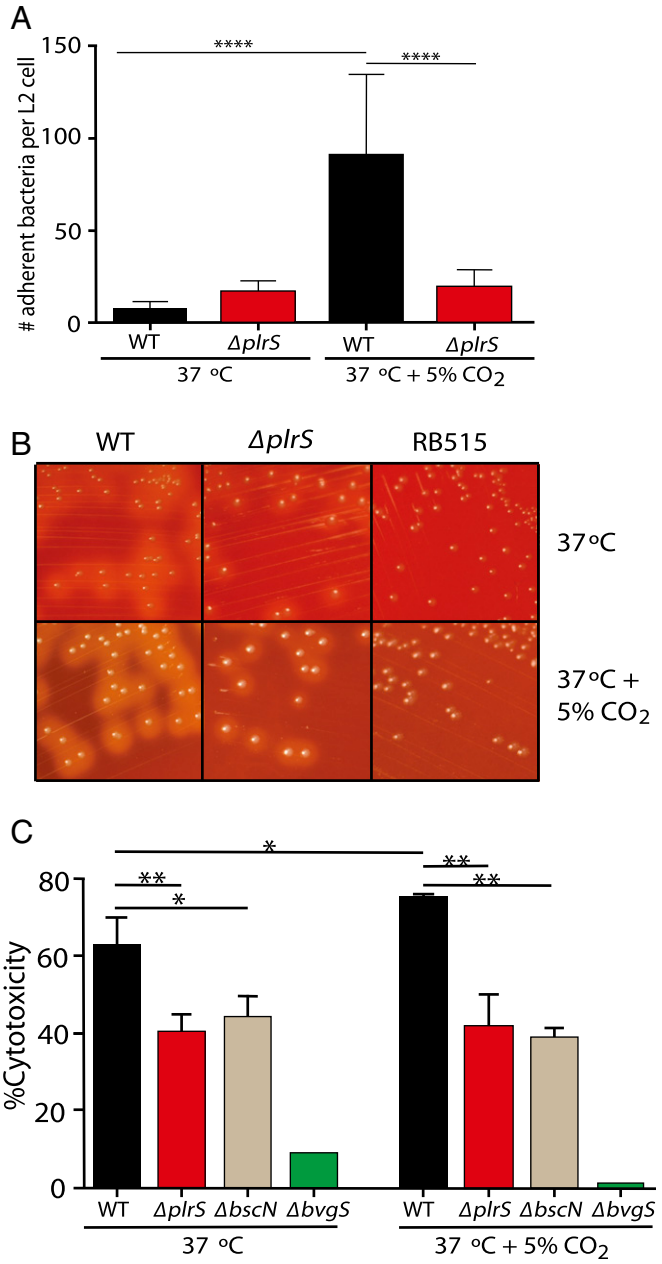

Fig. 1. PlrS is required for Bvg-dependent phenotypes in 5% CO_2 growth conditions. (*A*) Bacterial adherence to L2 lung epithelial cells. WT and Δ*plrS* bacteria were cultured in ambient (37 °C) or 5% CO_2 (37 °C + 5% CO_2) conditions before inoculation at an MOI of 150 onto an L2 monolayer. The number of adherent bacteria per L2 cell was enumerated by determining the average number of bacteria and L2 cells visible from four visual fields of the microscope. Error bars represent SD of the mean. (*B*) Hemolysis on blood agar media produced from WT, Δ*plrS*, and RB515 (an ACT-null mutant) colonies under the indicated growth condition. (Magnification, 1,000×.) (*C*) Cytotoxicity of macrophage-like J774 cells induced by WT, Δ*plrS*, Δ*bscN* (containing an insertional disruption in the gene encoding the ATPase of the T3SS), and Δ*bvgS* (containing a deletion in *bvgS*) strains, following bacterial growth under the indicated condition. Percent cytotoxicity was calculated as the ratio of lysed J774 cells resulting from bacterial inoculation to fully lysed J774 cells, and error bars represent SD. Statistical significance for *A* (*t* test with Welch's correction) and *C* (ANOVA) is indicated as $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$.

complement a Δ*plrS* mutation and, therefore, that PlrS likely affects BvgAS-dependent virulence-associated phenotypes via PlrR (Fig. 2). Increased adherence in the WT and Δ*plrS* strains expressing *plrR*^{D52E} in bacteria grown in ambient air is also consistent with the D52E substitution functioning as a phosphomimetic, rendering PlrR constitutively active (Fig. 2). Lack of adherence by the Δ*bvgS* strain with and without the *plrR*^{D52E} allele in the presence or absence of 5% CO_2 confirms that *bvgS* is required for adherence to host cells and that PlrR^{D52E} does not induce a BvgAS-independent adherence activity (Fig. 2). These data provide evidence (but do not prove) that PlrS and PlrR function as a canonical TCS, and that increased adherence in response to CO_2 is mediated by PlrS via PlrR. However, these data do not rule out the possibility that BvgA and PlrR interact synergistically to affect expression of some genes or that PlrS directly interacts with BvgS, while also affecting the phosphorylation state of PlrR.

Positive autoregulation of the *bvgAS* operon is well-documented (25–28). Nonetheless, to determine if PlrS affects BvgAS activity by controlling *bvgAS* transcription, we introduced a P_{bvgA}-*gfp* transcriptional reporter into the *att*Tn7 site of WT and Δ*plrS B. bronchiseptica*, and measured GFP activity in bacteria grown at 37 °C with and without 5% CO_2. Under all conditions tested the levels of GFP—and therefore the expression of *bvgAS*—was not significantly different between WT and the Δ*plrS* strains (Fig. S3). GFP levels in the Δ*bvgS* strain reflect activity from the *bvgA* P2 promoter, which is expressed at a low level under Bvg$^-$ phase conditions (providing the cell with a low amount of BvgAS so that it can respond, via positive autoregulation, when Bvg$^+$ phase conditions are encountered) (29). This low-level expression of P_{bvgA} was not affected by the Δ*plrS* mutation. Taken together, these data suggest that PlrS, via PlrR, exerts its effects on BvgAS activity posttranscriptionally.

***B. bronchiseptica* Lacking *plrS* Modulate to the Bvg$^-$ Phase Within the LRT.** Based on the functional link between PlrS and BvgAS (Fig. 1) and the similarly rapid clearance of *B. bronchiseptica* Δ*plrS* and Δ*bvgS* mutants from the LRT of mice (13, 18), we hypothesized that PlrS may influence BvgAS activity within the host. To test this hypothesis, we used two reporter systems developed in our laboratory. pGFLIP contains a recombinase-based reporter system, similar to recombinase-based in vivo technology (15). Expression of *flp*, encoding FLP recombinase, results in excision of *gfp* and *nptII* (encoding kanamycin resistance, Km^r) genes located between FLP recombinase target (FRT) sites and conversion of the bacteria from GFP$^+$ and Km^r to GFP$^-$ and Km^s. In pGFLIP-*flaA*, the Bvg$^-$ phase-specific P_{flaA} promoter is located 5′ to *flp* and conversion to GFP$^-$ (and Km^s) indicates that the bacteria have expressed the Bvg$^-$ phase at some point during the experiment.

In contrast to pGFLIP, which reports on the history of the bacteria, the pBAM plasmid reports on the status of the bacteria at the time of plating (29). The pBAM plasmid integrates within the *bvgAS* promoter region and causes the P2 promoter to be expressed at a lower-than-normal level such that, when bacteria are growing under Bvg$^-$ phase conditions, the amount of BvgAS in a small proportion of cells in the population (~5%) is below the threshold required for positive autoregulation, "trapping" these cells in the Bvg$^-$ phase (29). When WT *B. bronchiseptica* containing the pBAM plasmid are grown under Bvg$^-$ phase conditions and then plated onto BG-blood agar and incubated at 37 °C (Bvg$^+$ phase conditions), the trapped bacteria yield colonies that are larger, flatter, and less hemolytic than colonies formed by Bvg$^+$ phase bacteria because these colonies contain ~5% phenotypically Bvg$^-$ phase bacteria (as the colony grows, ~95% of the daughter cells produce enough BvgAS to convert to the Bvg+ phase, whereas ~5% remain phenotypically Bvg$^-$ phase). We previously referred to these colonies, which are easily scored by simple visual inspection, as large colony variants (29). However, because these colonies result from phenotypic bistability and not a genetic change, it is more appropriate to refer to them as large colony phenotypes (LCPs), which we will do henceforth. Although all LCPs result from a founder bacterium that was Bvg$^-$ phase at the time of plating, ~95% of bacteria that

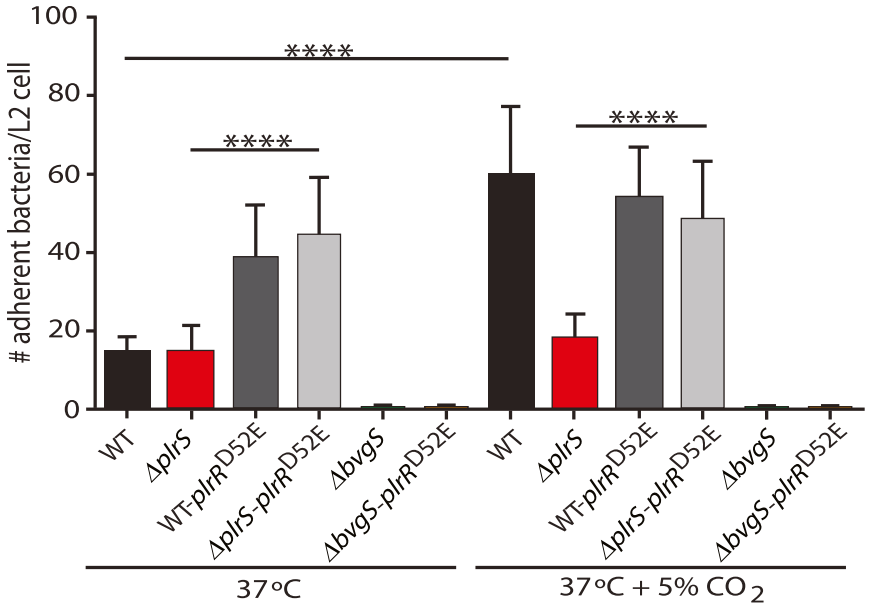

Fig. 2. Overexpression of a phosphomimetic *plrR* allele rescues the Δ*plrS* L2 cell adherence defect. L2 lung epithelial cell adherence displayed by WT, Δ*plrS*, WT-*plrR*^{D52E} (a strain isogenic to WT that produces PlrR with the phosphomimetic D52E substitution), Δ*plrS*-*plrR*^{D52E} (Δ*plrS* that produces PlrR with the phosphomimetic D52E substitution), Δ*bvgS*, and Δ*bvgS*-*plrR*^{D52E} (Δ*bvgS* that produces PlrR with the phosphomimetic D52E substitution) following bacterial growth under the indicated condition. Error bars represent SD of the mean. Statistical significance (*t* test with Welch's correction) is indicated as ****$P < 0.0001$.

contain pBAM and are Bvg⁻ phase at the time of plating form typical Bvg⁺ phase-appearing (non-LCP) colonies (29). Hence, although the presence of LCPs indicates that bacteria have modulated to the Bvg⁻ phase, it vastly underestimates the number of bacteria that are Bvg⁻ phase at the time of plating.

When grown at 37 °C without addition of $MgSO_4$ to the medium (Bvg⁺ phase conditions), WT and Δ*plrS* strains containing both reporter systems maintained GFP fluorescence and no LCPs formed (Fig. S4), indicating that neither strain modulated to the Bvg⁻ phase. When switched from Bvg⁺ phase growth conditions to Bvg⁻ phase growth conditions, both strains lost GFP fluorescence and a small proportion of LCPs formed after 24 h (Fig. S4). Both reporters, therefore, can accurately report that the bacteria modulated to the Bvg⁻ phase and Δ*plrS* mutants modulate to a similar extent as WT bacteria when grown in vitro.

Following intranasal inoculation of BALB/cJ mice, the numbers of cfu recovered from the nasal cavities and lungs of WT and Δ*plrS* strains containing the reporters were similar to cfu recovered of the parental strains (without reporters), indicating that the reporters do not influence virulence (Fig. 3*A*). The proportion of GFP⁻ and LCP cfu recovered from the nasal cavities was extremely low for both strains, indicating that the bacteria did not modulate to the Bvg⁻ phase at this site (Fig. 3 *B* and *C*). The proportion of GFP⁻ and LCP cfu recovered from the lungs of

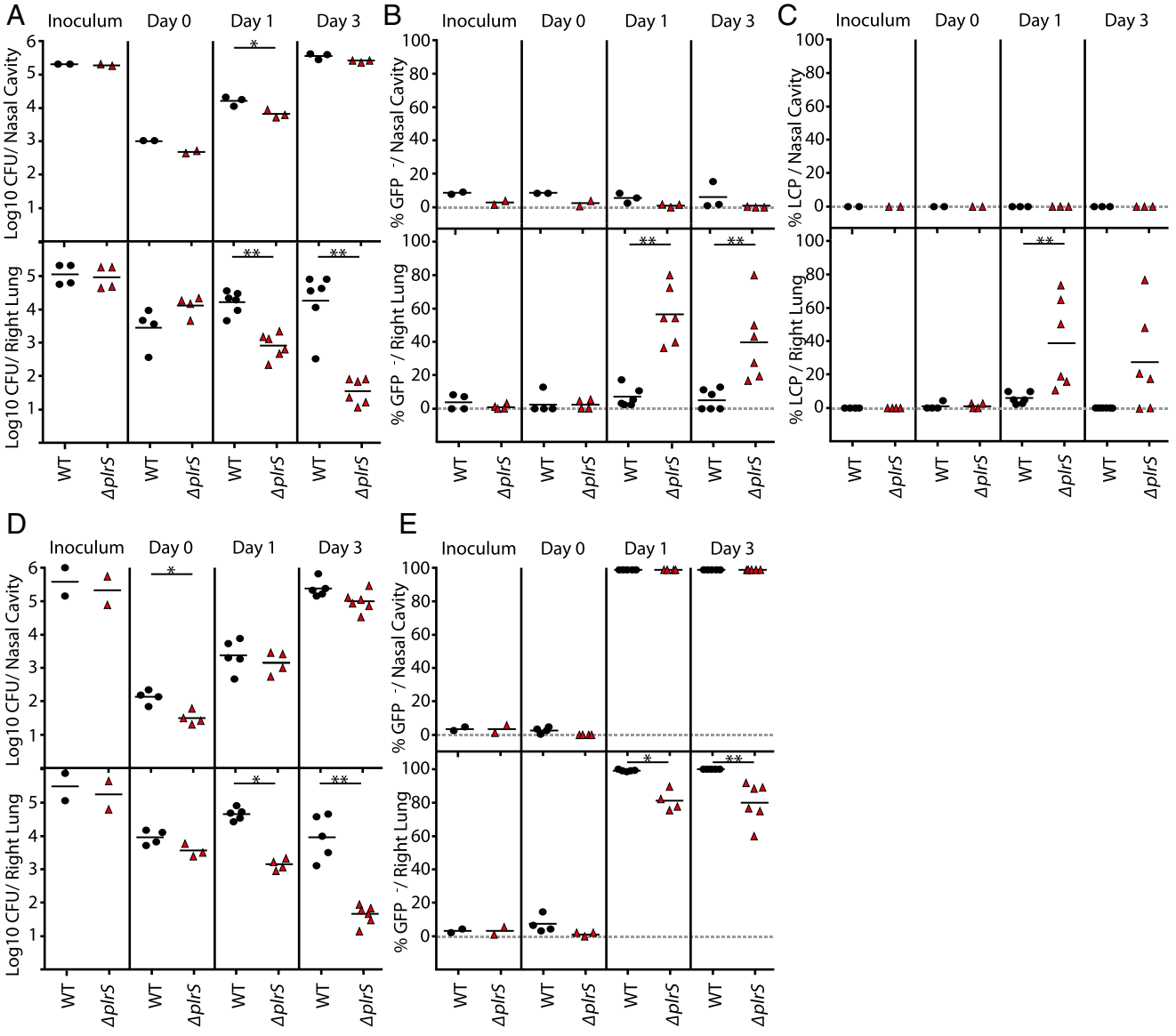

Fig. 3. Without PlrS, *B. bronchiseptica* modulates to the Bvg⁻ phase in the LRT. (A) Colonization of the nasal cavity (*Upper*) and right lung (*Lower*) for WT (filled circles) and Δ*plrS* (red triangles) on days 0, 1, and 3 postinoculation. Both strains contain the plasmid reporters pGFLIP-P_{flaA} and pBam. Female BALB/cJ mice were inoculated with 7.5×10^4 cfu via the external nares. Each symbol represents a single animal, with the mean colonization depicted as short horizontal bars. Homogenate from each organ was assessed to determine in vivo bacterial modulation shown in graphs in *B* and *C*. (*B*) Percentage of GFP⁻ bacteria recovered from the nasal cavity and right lung for WT and Δ*plrS* containing pGFLIP-P_{flaA} and pBam. GFP negativity indicates bacterial modulation. (*C*) Percentage of LCP-producing bacteria recovered from the nasal cavity and right lung for WT and Δ*plrS* containing pGFLIP-P_{flaA} and pBam. LCPs indicate bacteria present in the Bvg⁻ phase when plated. (*D*) Colonization of the nasal cavity (*Upper*) and right lung (*Lower*) for WT (filled circles) and Δ*plrS* (red triangles) containing the plasmid reporter, pGFLIP-P_{ptxA}. Homogenate from each organ was assessed to determine BvgAS activation in vivo, shown in graph in *E*. (*E*) Percentage of GFP⁻ bacteria recovered from the nasal cavity (*Upper*) and right lung (*Lower*) for WT and Δ*plrS* containing pGFLIP-P_{ptxA}. GFP negativity indicates BvgAS activation. Statistical significance (Mann–Whitney) is indicated as $*P < 0.05$ and $**P < 0.01$.

mice inoculated with WT bacteria was also extremely low. In contrast, 40–80% of the cfu recovered from the lungs of mice inoculated with the Δ*plrS* strain had lost GFP fluorescence and 10–80% formed LCPs by day 1 postinoculation (Fig. 3 *B* and *C*), indicating that a majority of these bacteria had modulated to the Bvg^- phase and that a majority of these bacteria were in the Bvg^- phase at the time of recovery from the lungs. These findings demonstrate that in strains lacking *plrS*, the BvgAS phosphorelay fails to remain active specifically within the LRT. Moreover, the fact that 10–80% of the bacteria recovered from the lungs formed LCPs (i.e., much more than 5%) indicates that the physiology of Δ*plrS* bacteria in the LRT is substantially different from the physiology of Δ*plrS* bacteria that have modulated to the Bvg^- phase in vitro by chemical modulators such as $MgSO_4$ (*Discussion*).

***B. bronchiseptica* Lacking *plrS* Fail to Activate BvgAS Within the LRT.** To determine if BvgAS can transition from an inactive (Bvg^- phase) to an active (Bvg^+ phase) state in the absence of PlrS activity within the LRT, we inoculated mice with WT and Δ*plrS* mutants containing a pGFLIP reporter in which the Bvg^+ phase-specific *ptxA* promoter from *B. pertussis* was cloned 5′ to *flp*. Both WT and Δ*plrS* strains activated the *ptxA* promoter when switched from Bvg^- phase to Bvg^+ phase growth conditions in vitro (Fig. S5). Similar to what has been shown in *B. bronchiseptica* and *B. pertussis* previously (15, 16), 100% of the bacteria recovered from both the nasal cavity and the lungs of mice inoculated with Bvg^- phase WT bacteria activated P_{ptxA}, and therefore transitioned to a Bvg^+ phase within 24 h postinoculation (Fig. 3 *D* and *E*). In contrast, although all of the cfu recovered from the nasal cavities of mice inoculated with the Δ*plrS* strain were GFP^- by 24 h postinoculation, only ∼80% of those recovered from the lungs were GFP^- (Fig. 3 *D* and *E*). Although, based on this reporter, only a seemingly small proportion of the Δ*plrS* mutants failed to switch to the Bvg^+ phase in vivo, it is important to note that the numbers of cfu of the Δ*plrS* mutant recovered from the lungs at days 1 and 3 postinoculation were ∼0.5 and 2 logs lower than the numbers initially present (day 0). It is impossible to determine if the bacteria cleared from the lungs at these time points had lost GFP. However, if they remained GFP^+ (indicating that they did not switch to the Bvg^+ phase), then the proportion of Δ*plrS* bacteria that had transitioned to the Bvg^+ phase in the LRT would in fact be far less than 1%. Taken together, our data indicate that full activation and maintenance of BvgAS activity in the LRT requires PlrS.

PlrS Is Required for *B. bronchiseptica* Persistence in the LRT, Independent of Its Effects on BvgAS Activity. Lack of production of BvgAS-dependent virulence factors could be the reason that *plrS* mutants are cleared rapidly from the LRT. To test this hypothesis, we introduced the *bvgS-C3* mutation, which encodes a BvgS protein that is active even under modulating conditions in vitro (12), into the *plrS* mutant, and compared this Δ*plrS-bvgS*^{c} strain with the *bvgS*^{c} mutant in vitro and in vivo. Like the *bvgS*^{c} strain, the Δ*plrS-bvgS*^{c} strain formed small, domed, hemolytic colonies characteristic of the Bvg^+ phase on BG-blood agar containing 50 mM $MgSO_4$ (i.e., Bvg^- phase conditions), indicating that BvgAS was constitutively active in the absence of *plrS* in vitro. In vivo, the Δ*plrS-bvgS*^{c} strain colonized and persisted in the nasal cavity similarly to the *bvgS*^{c} and Δ*plrS* strains (Fig. S6). However, the Δ*plrS-bvgS*^{c} strain was cleared from the lungs as rapidly as the Δ*plrS* mutant (Fig. S6), indicating that the Δ*plrS* mutation is epistatic to the *bvgS-C3* mutation with regard to persistence in the LRT.

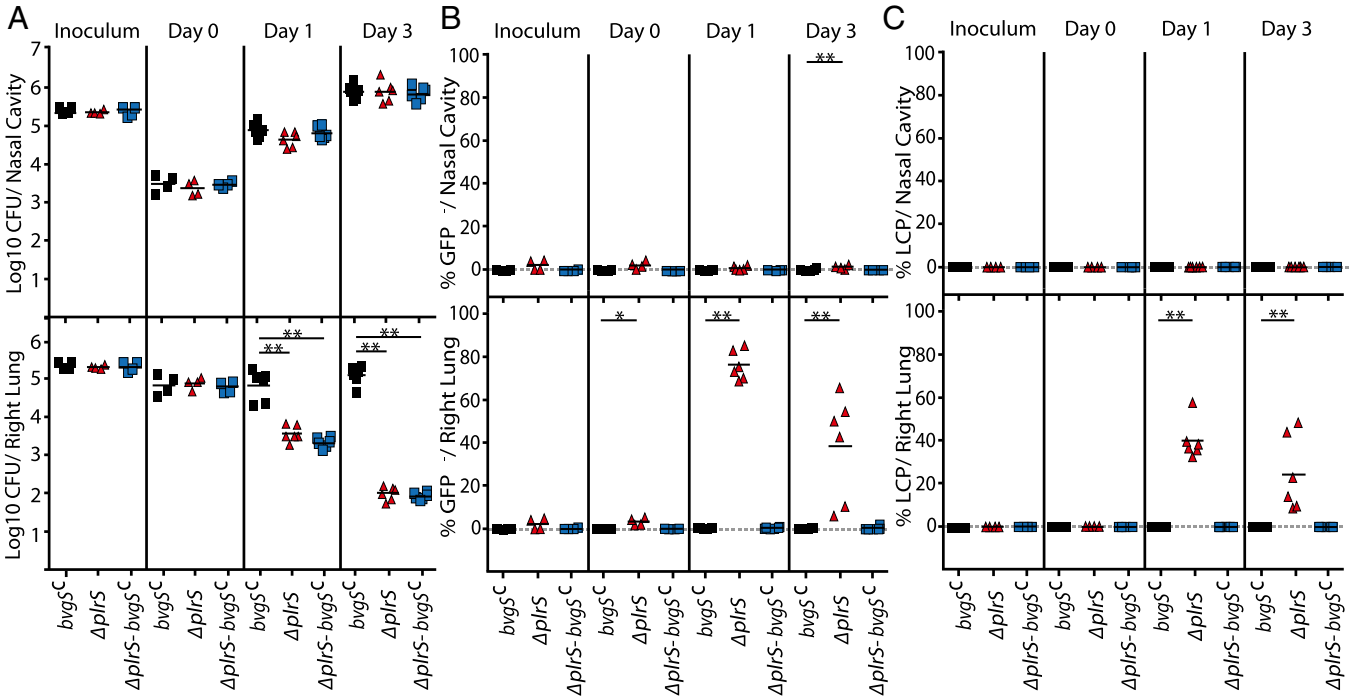

Fig. 4. PlrS is required for persistence of *B. bronchiseptica* in the LRT independent of BvgAS activity. (*A*) Colonization of the nasal cavity (*Upper*) and right lung (*Lower*) *B. bronchiseptica* containing constitutively active BvgS (*bvgS*^{c}; black squares), Δ*plrS* (red triangles), and Δ*plrS-bvgS*^{c} (blue squares) on days 0, 1, and 3 postinoculation. All strains contain the plasmid reporters pGFLIP-P_{flaA} and pBam. Female BALB/c mice were inoculated with 7.5×10^4 cfu via the external nares. Each symbol represents a single animal, with the mean colonization depicted as short horizontal bars. Homogenate from each organ was assessed to determine in vivo bacterial modulation shown in graphs in *B* and *C*. (*B*) Percentage of GFP^- bacteria recovered from the nasal cavity and right lung for *bvgS*^{c}, Δ*plrS*, and Δ*plrS-bvgS*^{c} containing pGFLIP-*flaA* and pBam. GFP negativity indicates bacterial modulation. (*C*) Percentage of LCP producing bacteria recovered from the nasal cavity and right lung for *bvgS*^{c}, Δ*plrS*, and Δ*plrS-bvgS*^{c} containing pGFLIP-P_{flaA} and pBam. LCPs indicate bacteria present in the Bvg^- phase when plated. Statistical significance (Mann–Whitney) is indicated as $*P < 0.05$ and $**P < 0.01$.

To investigate BvgAS activity in the Δ*plrS-bvgS*^{c} strain, we used our pGFLIP-*flaA* and pBAM reporters. None of the *bvgS*^{c} or Δ*plrS-bvgS*^{c} colonies containing these reporters were GFP^{-} or displayed the LCP phenotype after growth in medium containing 50 mM $MgSO_4$ (Fig. S7), indicating their insensitivity to modulating conditions in vitro. Numbers of cfu of each strain recovered from the nasal cavities and lungs of BALB/cJ mice were similar to those of the strains lacking the pGFLIP-*fla*A and pBAM reporters (Fig. 4*A*). Additionally, very few, if any, GFP^{-} or LCP colonies were recovered from the nasal cavity for any strain, indicating that no bacteria had modulated to the Bvg^{-} phase in the nasal cavity (Fig. 4 *B* and *C*). As expected, a significant proportion of GFP^{-} colonies and LCPs were recovered from the lungs of mice inoculated with the Δ*plrS* mutant on days 1 and 3, consistent with our previous results (Fig. 4 *B* and *C*). In contrast, no GFP^{-} or LCP colonies were recovered from the lungs of mice inoculated with the *bvgS*^{c} or Δ*plrS-bvgS*^{c} mutants (Fig. 4 *B* and *C*), indicating that neither strain modulated to the Bvg^{-} phase in the LRT. Therefore, modulation to the Bvg^{-} phase and lack of BvgAS-activated virulence factors is not the only reason that Δ*plrS* mutants fail to persist in the LRT. These data indicate that PlrS is required for bacterial persistence in the LRT, even when BvgAS remains active, likely because PlrR (presumably phosphorylated PlrR, PlrR~P) activates expression of one or more genes encoding proteins that are required in this environment or because PlrR~P represses expression of one or more genes that encode proteins that are detrimental to survival in this environment.

PlrS Is Required for Survival and Persistence of *B. pertussis* in the LRT. The *plrSR* locus is highly conserved (≥99% identical) among all strains of the classic bordetellae (*B. pertussis*, *B. bronchiseptica*, and *Bordatella parapertussis*). We constructed a derivative of *B. pertussis* strain BP536 with a large in-frame deletion mutation in *plrS* and compared it with WT BP536 in BALB/cJ mice. Both strains colonized the nasal cavity at similar levels, and were cleared from this site by day 3 postinoculation (Fig. 5). However, whereas ~10^3 cfu of BP536 were recovered from the lungs on days 1 and 3 postinoculation, significantly fewer BP536Δ*plrS* cfu were recovered from this site at these time points (Fig. 5). These data indicate that similar to *B. bronchiseptica*, *plrS* is required for the survival and persistence of *B. pertussis* specifically in the LRT.

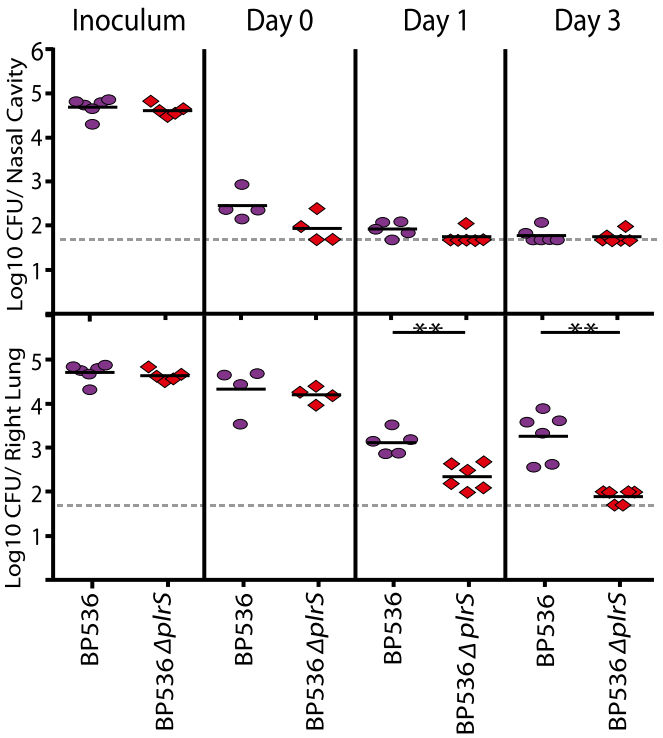

Fig. 5. PlrS is required for the persistence of *B. pertussis* in the LRT. Colonization of the nasal cavity (*Upper*) and right lung (*Lower*) for *B. pertussis* strains BP536 (filled circles) and BP536Δ*plrS* (red diamonds) on days 0, 1, and 3 postinoculation. Female BALB/c mice were inoculated with 1.0×10^5 cfu via the external nares. Each symbol represents a single animal, with the mean colonization depicted as short horizontal bars. Statistical significance (Mann–Whitney) is indicated as $^{**}P < 0.01$.

Discussion

BvgAS has been considered the master virulence regulator in *Bordetella* since its identification in 1983 (30) and demonstration of its penetrance by subsequent mutagenesis and genome-wide analyses (8, 31, 32). Our new data indicate, however, that in the LRT, BvgAS activity depends on PlrS, likely via the activity of PlrR. Moreover, PlrS(R) is required for bacterial survival in the LRT even when BvgS is rendered constitutively active, strongly suggesting that one or more PlrSR-dependent, BvgAS-independent genes (or genes that require both PlrSR and BvgAS) is required for bacterial survival at this site. PlrS(R) is therefore at least as important for *Bordetella* virulence as BvgAS.

Why is PlrS(R) required for bacterial persistence in the LRT? PlrSR belongs to the NtrYX family of TCS. NtrY family proteins, including PlrS, are predicted to contain three transmembrane domains at their N termini, followed by a periplasmically located PhoP-DcuS-CitA (PDC) domain, another transmembrane domain, then cytoplasmically located HAMP, Per-Arnt-Sim (PAS), and HisKA-type histidine kinase domains (Fig. 6.). NtrY of *Brucella abortus* has been shown to bind heme via its PAS domain and to function as a redox sensor, becoming active as a kinase in response to anaerobiosis (33). NtrX family response regulators contain N-terminal receiver and C-terminal DNA binding domains. In *Neisseria gonorrheae* and *Rhodobacter capsulatus*, the *ntrX* genes are required for induction of high-affinity cytochrome oxidases, which are required for bacterial growth under low oxygen conditions (34). Our data indicate that PlrS is required specifically in the LRT, an environment that is low in oxygen and high in CO_2. Although we have been unable to delete *plrR* in vitro, our experiments using PlrR^{D52E} indicate that PlrS affects BvgAS-dependent phenotypes in vitro via PlrR (providing evidence, but not proving, that PlrS and PlrR function as a TCS). It seems likely that PlrS functions through PlrR in vivo as well. By analogy with the few other NtrYX family members that have been studied so far, we hypothesize that PlrS senses low oxygen (and perhaps increased CO_2) in the LRT, phosphorylates PlrR, and that PlrR~P activates expression of one or more of the high-affinity cytochrome oxidase-encoding loci present in *B. bronchiseptica* (and *B. pertussis*) (35), allowing the bacteria to respire in this environment (Fig. 6). We note, however, that NtrY family members, including PlrS, contain HisKA-type DHp domains with ExxN motifs that suggest that these proteins possess both kinase and phosphatase activities (36–38). Although our data suggest that PlrS may function to phosphorylate PlrR in vitro, especially in 5% CO_2, the contributions of its predicted kinase and phosphatase activities in vivo cannot be predicted from our current data.

Why is PlrS(R) required for BvgAS activity in the LRT? Positive autoregulation by BvgAS has been well-characterized (25–28, 39) and we showed here that *bvgAS* expression in both ambient air and 5% CO_2 is not dependent on *plrS*, by evaluating activity of the *bvgAS* promoter from a single-copy chromosomal reporter (to avoid issues that arise when multicopy plasmids are used) while also leaving the native *bvg* locus intact, thereby preserving the positive autoregulation capability of BvgAS during the experiment. Our data demonstrate that although *bvgAS* autoregulation (the only known mechanism that affects *bvgAS* expression) remains intact, *plrS* does not influence *bvgAS* expression. Moreover, our experiments with the *bvgS*^{c} mutant further

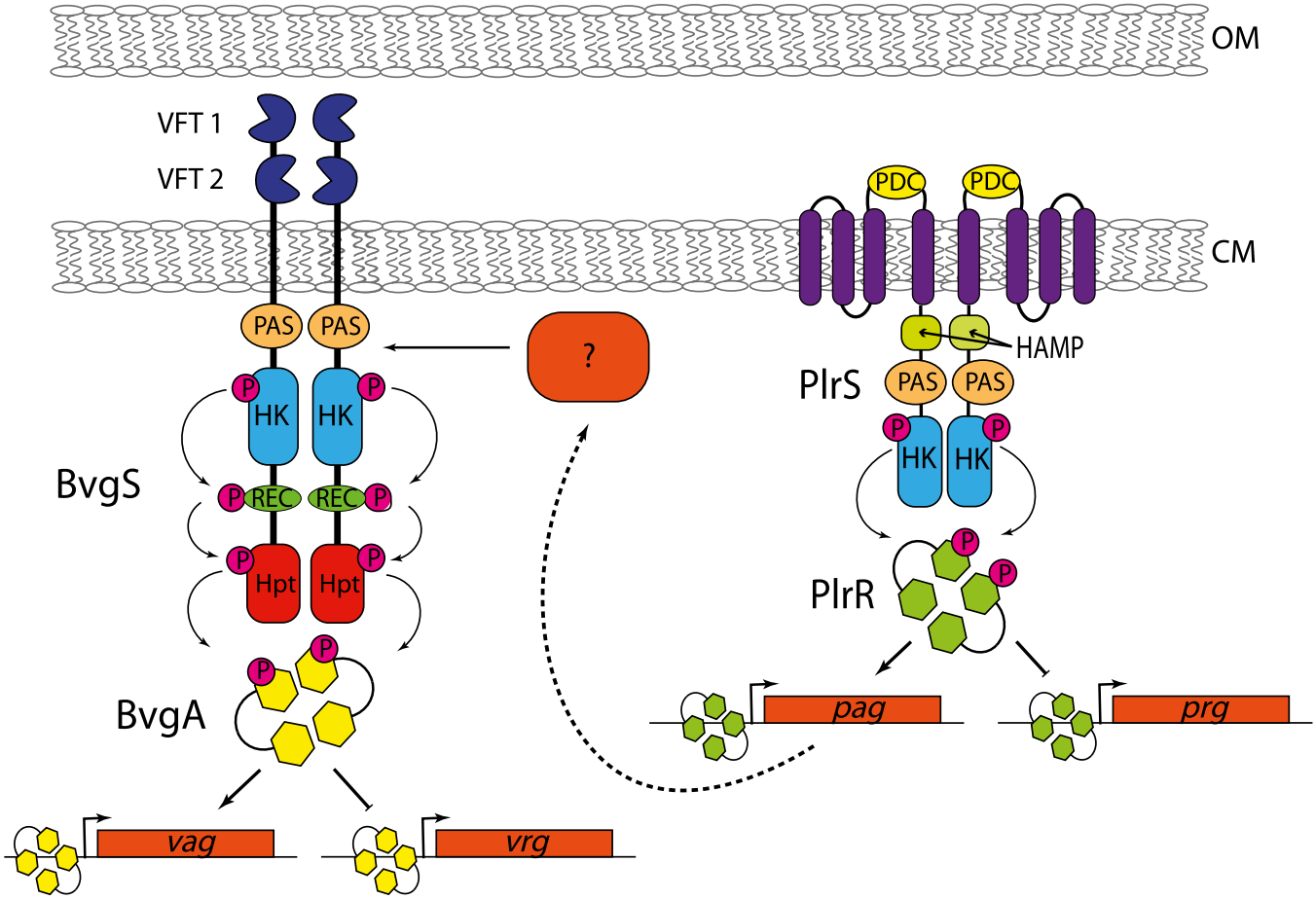

Fig. 6. PlrSR and BvgAS work in tandem to regulate gene expression required for *Bordetella* persistence in the LRT. Model of putative PlrSR and BvgAS regulatory networks in *Bordetella*. The sensor kinase PlrS may be sensitive to CO_2 or oxygen tension, directly or indirectly, within the LRT environment, transmitting a signal via autophosphorylation and phosphate transfer to its putative response regulator PlrR. PlrSR activates (*pags*) or represses (*prgs*), a set of genes independent of BvgAS that are required for survival of the bacteria specifically within the LRT. PlrSR may also regulate expression of a gene encoding a protein required for sustained activity of BvgAS in the LRT. If BvgS receives such a signal the sensor kinase is capable of phosphorelay transmission, activating transcription of essential virulence factors (*vags*) and repressing transcription of Bvg$^-$ phase genes (*vrgs*).

indicate that *bvgAS* expression is not dependent on PlrS(R) in vivo because BvgAS in the Δ*plrS-bvgS*^{c} strain—which contains a single nucleotide substitution in *bvgS*, rendering the BvgS protein constitutively active—was active in the LRT. If *bvgAS* transcription required PlrSR, the Δ*plrS* mutation would be epistatic to the *bvgS-C3* mutation with regard to BvgAS activity. Our data therefore rule out the possibility that PlrS(R) controls BvgAS activity by controlling *bvgAS* transcription. We hypothesize that instead, PlrSR controls expression of one or more genes that encode proteins required for BvgS activity specifically in the LRT. BvgS contains three predicted signal-sensing domains: two periplasmically located Venus flytrap domains (VFT) and a cytoplasmically located PAS domain (40, 41). Although the VFT appear to convert BvgS into a phosphatase that dephosphorylates BvgA in response to modulating conditions (i.e., $MgSO_4$ or nicotinic acid) (42–44), a role for the PAS domain has not been established. However, biochemical analyses using the cytoplasmic portion of BvgS suggested that the redox state of ubiquinone could affect BvgS kinase activity (45). PlrSR-dependent production of high-affinity cytochrome oxidases would allow electron transport-coupled oxidative phosphorylation to occur in the LRT, which is required for ATP production (and hence cell viability), and for preventing the accumulation of reduced ubiquinone, which could inactivate BvgS. This model is consistent with that proposed for the ArcB protein of *Escherichia coli*, which is activated by low-oxygen conditions (46, 47). If true, this model would predict that a role for the PAS domain in sensing signals in vitro may have been missed because the activity of low-affinity (high-efficiency) cytochrome oxidases present under all of the conditions tested would keep reduced ubiquinone levels at a minimum. These low-affinity cytochrome oxidases are also likely present and sufficient for respiration in the upper respiratory tract, making BvgS activity and bacterial survival at this site independent of PlrS.

Why was the proportion of LCPs recovered from the lungs of mice inoculated with the Δ*plrS* strain so much higher than the proportion obtained after switching the bacteria from Bvg$^-$ phase conditions to Bvg$^+$ phase conditions in vitro? Our previous studies showed that the reason that ∼5% of bacteria containing the pBAM plasmid integrated at the *bvgAS* promoter region form LCPs when shifted from Bvg$^-$ phase conditions to Bvg$^+$ phase conditions in vitro, is that activity of the P2 promoter in this strain is decreased such that ∼5% of the bacteria have BvgAS levels below the threshold required to respond to Bvg$^+$ phase conditions when they are encountered. It appeared, therefore, that the maximum proportion of LCPs that would form from a population of pBAM-containing bacteria in which 100% of the bacteria had modulated to the Bvg$^-$ phase was about 5%. We were surprised, therefore, to find that the proportion of LCPs formed after recovery of the Δ*plrS* mutant from the lungs of mice was up to 80%. Given that the activity of the P2 promoter is already

extremely low, it seems unlikely that P2 promoter activity in the Δ*plrS* mutant is substantially lower in the LRT than under Bvg^- phase conditions in vitro. A more likely possibility is that the physiology of the Δ*plrS* mutant in the LRT is substantially different from the physiology of the Δ*plrS* mutant growing under Bvg^- phase conditions in vitro (and from WT bacteria in the LRT, which do not modulate to the Bvg^- phase), and that this altered physiology prevents or delays BvgS from reactivating upon exposure to Bvg^+ phase conditions in vitro. If PlrS(R) controls expression of cytochrome oxidases in the LRT, and if BvgS is sensitive to the redox state of ubiquinone, then BvgS would be inactive in a *plrS* mutant in the LRT and would not become active again until cytochrome oxidase activity reached levels sufficient to reoxidize ubiquinone pools, which, in the case of a *plrS* mutant, could require expression of genes encoding low-affinity cytochrome oxidases, translation of proteins, and assembly of enzymatic complexes in the membrane after the bacteria are shifted to Bvg^+ phase conditions in ambient air. This delay could account for the substantially higher proportions of LCPs formed by the *plrS* mutant recovered from the LRT compared with the proportion formed after growth in vitro.

Challenging the long-held paradigm that the Bvg^+ phase is sufficient for respiratory infection and evidence that virulence factors that require PlrS(R) independently of (or codependently with) BvgAS exist is important from a translational medicine perspective. *B. bronchiseptica* and *B. pertussis* are sufficiently closely related to be considered members of the same species (35), and several *vag*s have been shown to be functionally interchangeable (48–51). As in *B. bronchiseptica*, a *B. pertussis plrS* mutant was defective in persistence in the LRT. Our model for the role of PlrSR during infection based on studies with *B. bronchiseptica* is therefore likely to apply to *B. pertussis* as well. In addition to mounting evidence that acellular and whole-cell vaccines provide suboptimal protection that is less durable than that induced by infection with *B. pertussis*, recent data from studies with baboons indicate that although both vaccines protect against disease, neither protects against colonization (52). Moreover, baboons vaccinated with an acellular vaccine and then challenged with *B. pertussis* were able to transmit the disease to naïve animals, even though they exhibited no signs of illness (52). The fact that infection induces a mixed Th1/Th17-type immune response and acellular vaccines induce a strong Th2-type immune response (4, 53–56) suggests that reformulation of acellular vaccines with adjuvants to induce a Th1/Th17 response is a reasonable approach to controlling the reemergence of pertussis. However, the phenomenon of epitope-linked suppression would likely render such vaccines ineffective in individuals previously vaccinated with acellular vaccines and perhaps also in those vaccinated with whole-cell vaccines, which are prepared from in vitro grown bacteria. Inclusion of antigens not present in previous vaccines would avoid this problem. The existence of virulence factors that are produced only in the host during infection, as suggested by our data, is significant from this translational medicine perspective. Antigens that are produced only during infection could also be important diagnostically as a means to distinguish individuals who have been vaccinated from those who have been infected. Finally, PlrSR itself, and factors it controls, may be exploitable as therapeutic targets.

Materials and Methods

In Vitro Adherence Assays. Bacterial adherence to rat lung epithelial (L2) cells was evaluated as described previously (21, 48). Bacteria were cultured in either ambient air or 5% CO_2 at 37° to an OD_{600} of ∼1.0. Bacteria were added to a monolayer of L2 cells (∼80% confluency) at a multiplicity of infection (MOI) of 150. Adherence was visualized by Giemsa staining and light microscopy at 1,000× magnification using a Zeiss Axiostar microscope, and quantified by counting the number of adherent bacteria and total L2 cells in at least four microscopic fields.

Macrophage Cytotoxicity Assays. J774 macrophage-like cells were grown to ∼60% confluency (∼1.5×10^4 cells per well) in a 96-well microtiter dish. Bacteria were cultured in either ambient air or 5% CO_2 to an OD_{600} of ∼1.0, and were then added to J774 cells at an MOI of 150. The plate was spun at $1,200 \times g$ and then allowed to incubate in a tissue culture incubator for 3 h. Macrophage cytotoxicity was quantified by measuring lactate dehydrogenase release using Promega's Cytotox96 Nonreactive Cytotoxicity Assay kit and a MultiskanEX plate reader (ThermoFisher Scientific) according to the manufacturer's instructions.

Bacterial Colonization of the Mouse Respiratory Tract. Six-week-old female BALB/cJ mice from Jackson Laboratories were inoculated intranasally with 7.5×10^4 cfu *B. bronchiseptica* or 1.0×10^5 cfu *B. pertussis* in 50 μL of PBS. For all time points, right lung lobes and nasal cavities were harvested, tissues were homogenized, and the number of cfu was determined by plating dilutions of tissue homogenates on BG blood agar.

Evaluation of *B. bronchiseptica* Phenotypic Phase Transition in Vivo. Six-week-old female BALB/cJ mice (Jackson Laboratories) were inoculated intranasally with 7.5×10^4 cfu of *B. bronchiseptica* pGFLIP strains in 50 μL of PBS. For experiments using the pGFLIP-P*ptxA* reporter, bacteria were cultured in media containing 50 mM $MgSO_4$ to maintain the bacteria in the Bvg^- phase before mouse inoculation (15). For infections using the pGFLIP-P*flaA* or pBam systems, bacteria were cultured at 37 °C in media without the addition of $MgSO_4$ to maintain bacteria in the Bvg^+ phase before mouse inoculation (15, 29). On days 0, 1, and 3 postinoculation, right lungs were harvested, homogenized in PBS, and plated in duplicate on BG-blood agar. For strains containing the pGFLIP-P*ptxA* system, homogenization, dilution, and plating were carried out in the presence of 50 mM $MgSO_4$. The percent GFP^- colonies and percent LCP colonies were calculated by determining the ratio of GFP^- or LCP colonies to the total number of colonies isolated. See Table S1 for strains and plasmids used in this study.

Ethics Statement. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (57). Our protocol was approved by the University of North Carolina Institutional Animal Care and Use Committee (Protocol ID: 13-238). All animals were properly anesthetized for inoculations, monitored regularly, and killed when moribund. Efforts were made to minimize suffering.

Statistical Analysis. Statistical analysis was performed using Prism 6.0 software from GraphPad Software. Statistical significance was determined using unpaired Student's *t* test with Welch's correction, a Mann–Whitney test, or ANOVA test. Figures were generated using Adobe Illustrator CS6 (Adobe Systems). $P < 0.05$ was considered significant.

ACKNOWLEDGMENTS. We thank members of the P.A.C. and S.M.J. laboratories for critical discussions and technical assistance. This work was supported by NIH Grant R01 AI AI094991 (to P.A.C.), and institutional funds (S.M.J.). S.A.M. was supported by Grant K12GM000678 from the Training, Workforce Development & Diversity division of the National Institute of General Medical Sciences, NIH.

1. Clark TA (2014) Changing pertussis epidemiology: Everything old is new again. *J Infect Dis* 209(7):978–981.
2. Melvin JA, Scheller EV, Miller JF, Cotter PA (2014) *Bordetella pertussis* pathogenesis: Current and future challenges. *Nat Rev Microbiol* 12(4):274–288.
3. Plotkin SA (2013) Complex correlates of protection after vaccination. *Clin Infect Dis* 56(10):1458–1465.
4. Edwards KM, Berbers GAM (2014) Immune responses to pertussis vaccines and disease. *J Infect Dis* 209(Suppl 1):S10–S15.
5. Acosta AM, et al. (2015) Tdap vaccine effectiveness in adolescents during the 2012 Washington state pertussis epidemic. *Pediatrics* 135(6):981–989.
6. Mattoo S, Cherry JD (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other Bordetella subspecies. *Clin Microbiol Rev* 18(2):326–382.
7. Cotter PA, Jones AM (2003) Phosphorelay control of virulence gene expression in *Bordetella*. *Trends Microbiol* 11(8):367–373.
8. Cummings CA, Bootsma HJ, Relman DA, Miller JF (2006) Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. *J Bacteriol* 188(5):1775–1785.
9. Cotter PA, Miller JF (1997) A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol Microbiol* 24(4):671–685.

10. Stockbauer KE, Fuchslocher B, Miller JF, Cotter PA (2001) Identification and characterization of BipA, a *Bordetella* Bvg-intermediate phase protein. *Mol Microbiol* 39(1): 65–78.
11. Deora R, Bootsma HJ, Miller JF, Cotter PA (2001) Diversity in the *Bordetella* virulence regulon: Transcriptional control of a Bvg-intermediate phase gene. *Mol Microbiol* 40(3):669–683.
12. Cotter PA, Miller JF (1994) BvgAS-mediated signal transduction: Analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. *Infect Immun* 62(8):3381–3390.
13. Martinez de Tejada G, et al. (1998) Neither the Bvg- phase nor the vrg6 locus of *Bordetella pertussis* is required for respiratory infection in mice. *Infect Immun* 66(6): 2762–2768.
14. Merkel TJ, Stibitz S, Keith JM, Leef M, Shahin R (1998) Contribution of regulation by the *bvg* locus to respiratory infection of mice by *Bordetella pertussis*. *Infect Immun* 66(9):4367–4373.
15. Byrd MS, Mason E, Henderson MW, Scheller EV, Cotter PA (2013) An improved recombination-based in vivo expression technology-like reporter system reveals differential *cyaA* gene activation in *Bordetella* species. *Infect Immun* 81(4):1295–1305.
16. Veal-Carr WL, Stibitz S (2005) Demonstration of differential virulence gene promoter activation in vivo in *Bordetella pertussis* using RIVET. *Mol Microbiol* 55(3):788–798.
17. Akerley BJ, Cotter PA, Miller JF (1995) Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. *Cell* 80(4):611–620.
18. Kaut CS, et al. (2011) A novel sensor kinase is required for *Bordetella bronchiseptica* to colonize the lower respiratory tract. *Infect Immun* 79(8):3216–3228.
19. Hester SE, Lui M, Nicholson T, Nowacki D, Harvill ET (2012) Identification of a CO2 responsive regulon in *Bordetella*. *PLoS One* 7(10):e47635.
20. Relman DA, Domenighini M, Tuomanen E, Rappuoli R, Falkow S (1989) Filamentous hemagglutinin of *Bordetella pertussis*: Nucleotide sequence and crucial role in adherence. *Proc Natl Acad Sci USA* 86(8):2637–2641.
21. Cotter PA, et al. (1998) Filamentous hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. *Infect Immun* 66(12): 5921–5929.
22. Vojtova J, Kamanova J, Sebo P (2006) *Bordetella* adenylate cyclase toxin: A swift saboteur of host defense. *Curr Opin Microbiol* 9(1):69–75.
23. Stockbauer KE, Foreman-Wykert AK, Miller JF (2003) *Bordetella* type III secretion induces caspase 1-independent necrosis. *Cell Microbiol* 5(2):123–132.
24. Smith JG, et al. (2004) A search for amino acid substitutions that universally activate response regulators. *Mol Microbiol* 51(3):887–901.
25. Scarlato V, Prugnola A, Aricó B, Rappuoli R (1990) Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis*. *Proc Natl Acad Sci USA* 87(17):6753–6757.
26. Roy CR, Miller JF, Falkow S (1990) Autogenous regulation of the *Bordetella pertussis bvgABC operon*. *Proc Natl Acad Sci USA* 87(10):3763–3767.
27. Boucher PE, Yang MS, Schmidt DM, Stibitz S (2001) Genetic and biochemical analyses of BvgA interaction with the secondary binding region of the *fha* promoter of *Bordetella pertussis*. *J Bacteriol* 183(2):536–544.
28. Boucher PE, Yang MS, Stibitz S (2001) Mutational analysis of the high-affinity BvgA binding site in the *fha* promoter of *Bordetella pertussis*. *Mol Microbiol* 40(4):991–999.
29. Mason E, Henderson MW, Scheller EV, Byrd MS, Cotter PA (2013) Evidence for phenotypic bistability resulting from transcriptional interference of *bvgAS* in *Bordetella bronchiseptica*. *Mol Microbiol* 90(4):716–733.
30. Weiss AA, Hewlett EL, Myers GA, Falkow S (1983) Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. *Infect Immun* 42(1):33–41.
31. Knapp S, Mekalanos JJ (1988) Two *trans*-acting regulatory genes (*vir* and *mod*) control antigenic modulation in *Bordetella pertussis*. *J Bacteriol* 170(11):5059–5066.
32. Hot D, et al. (2003) Differential modulation of *Bordetella pertussis* virulence genes as evidenced by DNA microarray analysis. *Mol Genet Genomics* 269(4):475–486.
33. Carrica MdelC, Fernandez I, Martí MA, Paris G, Goldbaum FA (2012) The NtrY/X two-component system of *Brucella* spp. acts as a redox sensor and regulates the expression of nitrogen respiration enzymes. *Mol Microbiol* 85(1):39–50.
34. Atack JM, et al. (2013) Characterization of an ntrX mutant of *Neisseria gonorrhoeae* reveals a response regulator that controls expression of respiratory enzymes in oxidase-positive proteobacteria. *J Bacteriol* 195(11):2632–2641.
35. Parkhill J, et al. (2003) Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat Genet* 35(1): 32–40.
36. Pazy Y, et al. (2010) Identical phosphatase mechanisms achieved through distinct modes of binding phosphoprotein substrate. *Proc Natl Acad Sci USA* 107(5):1924–1929.
37. Huynh TN, Stewart V (2011) Negative control in two-component signal transduction by transmitter phosphatase activity. *Mol Microbiol* 82(2):275–286.
38. Zhao R, Collins EJ, Bourret RB, Silversmith RE (2002) Structure and catalytic mechanism of the *E. coli* chemotaxis phosphatase CheZ. *Nat Struct Biol* 9(8):570–575.
39. Zu T, Manetti R, Rappuoli R, Scarlato V (1996) Differential binding of BvgA to two classes of virulence genes of *Bordetella pertussis* directs promoter selectivity by RNA polymerase. *Mol Microbiol* 21(3):557–565.
40. Dupré E, et al. (2015) Virulence regulation with Venus flytrap domains: Structure and function of the periplasmic moiety of the sensor-kinase BvgS. *PLoS Pathog* 11(3): e1004700.
41. Dupré E, et al. (2013) Characterization of the PAS domain in the sensor-kinase BvgS: Mechanical role in signal transmission. *BMC Microbiol* 13(1):172.
42. Melton AR, Weiss AA (1989) Environmental regulation of expression of virulence determinants in *Bordetella pertussis*. *J Bacteriol* 171(11):6206–6212.
43. Herrou J, et al. (2010) Periplasmic domain of the sensor-kinase BvgS reveals a new paradigm for the Venus flytrap mechanism. *Proc Natl Acad Sci USA* 107(40): 17351–17355.
44. Lesne E, et al. (2016) Balance between coiled-coil stability and dynamics regulates activity of BvgS sensor kinase in *Bordetella*. *mBio* 7(2):e02089–15.
45. Bock A, Gross R (2002) The unorthodox histidine kinases BvgS and EvgS are responsive to the oxidation status of a quinone electron carrier. *Eur J Biochem* 269(14): 3479–3484.
46. Alvarez AF, Rodriguez C, Georgellis D (2013) Ubiquinone and menaquinone electron carriers represent the yin and yang in the redox regulation of the ArcB sensor kinase. *J Bacteriol* 195(13):3054–3061.
47. Georgellis D, Kwon O, Lin EC (2001) Quinones as the redox signal for the arc two-component system of bacteria. *Science* 292(5525):2314–2316.
48. Julio SM, et al. (2009) Natural-host animal models indicate functional interchangeability between the filamentous haemagglutinins of *Bordetella pertussis* and *Bordetella bronchiseptica* and reveal a role for the mature C-terminal domain, but not the RGD motif, during infection. *Mol Microbiol* 71(6):1574–1590.
49. Martínez de Tejada G, Miller JF, Cotter PA (1996) Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Mol Microbiol* 22(5):895–908.
50. Inatsuka CS, et al. (2010) Pertactin is required for *Bordetella* species to resist neutrophil-mediated clearance. *Infect Immun* 78(7):2901–2909.
51. Henderson MW, et al. (2012) Contribution of *Bordetella* filamentous hemagglutinin and adenylate cyclase toxin to suppression and evasion of interleukin-17-mediated inflammation. *Infect Immun* 80(6):2061–2075.
52. Warfel JM, Zimmerman LI, Merkel TJ (2014) Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proc Natl Acad Sci USA* 111(2):787–792.
53. Zepp F, et al. (1996) Pertussis-specific cell-mediated immunity in infants after vaccination with a tricomponent acellular pertussis vaccine. *Infect Immun* 64(10):4078–4084.
54. Ausiello CM, et al. (1999) Cell-mediated immune responses in four-year-old children after primary immunization with acellular pertussis vaccines. *Infect Immun* 67(8): 4064–4071.
55. Ausiello CM, Urbani F, la Sala A, Lande R, Cassone A (1997) Vaccine- and antigen-dependent type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. *Infect Immun* 65(6):2168–2174.
56. Ryan M, McCarthy L, Rappuoli R, Mahon BP, Mills KH (1998) Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: Adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7-1, B7-2 and CD28. *Int Immunol* 10(5):651–662.
57. Committee on Care and Use of Laboratory Animals (1996) *Guide for the Care and Use of Laboratory Animals* (NIH, Bethesda), DHHS Publ No (NIH) 85-23.
58. Anderson MS, Garcia EC, Cotter PA (2012) The *Burkholderia* bcpAIOB genes define unique classes of two-partner secretion and contact dependent growth inhibition systems. *PLoS Genet* 8(8):e1002877.
59. Soane MC, et al. (2000) Interaction of *Bordetella pertussis* with human respiratory mucosa in vitro. *Respir Med* 94(8):791–799.