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Autoregulation Is Essential for Precise Temporal and Steady-State Regulation by the *Bordetella* BvgAS Phosphorelay[▽]

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The *Bordetella* BvgAS virulence control system is prototypical of phosphorelays that use a polydomain sensor and a response regulator to control gene expression in response to environmental cues. BvgAS controls the expression of at least three distinct phenotypic phases (Bvg$^-$, Bvg^i, and Bvg$^+$) by differentially regulating the expression of at least four classes of genes. Among the loci regulated by BvgAS is *bvgAS* itself. We investigated the role of autoregulation in the ability of BvgAS to control multiple gene expression patterns in a temporal and steady-state manner by constructing *Bordetella bronchiseptica* strains in which the *bvgAS* promoter was replaced with constitutively active promoters. Our results show that positive autoregulation of *bvgAS* transcription is required for the temporal expression of multiple phenotypic phases that occurs in response to a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions. Autoregulation was also shown to contribute to steady-state regulation; it influences the sensitivity of the system in response to subtle differences in signal intensity. In addition, considered in relation to BvgA and BvgS activities demonstrated in vitro, our results provide insight into how BvgA and BvgS function mechanistically.

Like all organisms, bacteria must be able to sense their environment and control their behavior appropriately to survive. They must also be able to change their behavior in a timely manner when they move from one environment to another or when the environment around them changes. Behavioral changes in bacteria usually require changes in gene expression, and therefore the transition from one behavior to another is neither instantaneous nor abrupt (i.e., without transient expression of alternate or intermediate behaviors). The sequence of events that occurs during adaptation from one set of environmental conditions to another can be considered temporal regulation. Once adaptation to the new environment occurs, the gene expression profile will, in general, remain constant as long as conditions remain constant. The ability to maintain a specific and appropriate gene expression profile depending on specific environmental conditions can be considered environmental or steady-state regulation. Although temporal and steady-state regulation probably occurs continuously and simultaneously in nature, there are circumstances in which temporal regulation may play a particularly important role. For example, the initial entry of a bacterium into a host or into a specific environment within a host may trigger a temporally defined program of gene expression that facilitates the ability of the bacterium to survive in that new environment and/or that temporarily prevents the bacterium from committing fully to the new environment so that it can readapt quickly if it is expelled back into the former environment. The inability to alter host environments in a controlled way precludes studying these processes in vivo. Laboratory media and growth conditions, however, can be controlled easily and precisely, allowing temporal and steady-state regulation to be separated and studied independently.

The most common mechanism used by bacteria to sense and respond to environmental conditions is the two-component regulatory system (TCS) (see reference 42 for a review). A subset of the TCS family, phosphorelays, uses a four-step His-Asp-His-Asp phosphorylation-phosphotransfer mechanism to convert environmental signals into behavioral changes, and a prototypical member of the subfamily of phosphorelays that contain polydomain sensors is BvgAS (see reference 10 for a review). BvgS, the sensor, and BvgA, the response regulator, control the expression of over 250 genes, including those encoding all known protein virulence factors, in *Bordetella* spp. (14). The *Bordetella* genus includes *Bordetella pertussis*, the causative agent of human whooping cough, and *Bordetella bronchiseptica*, which causes respiratory infections in a broad range of mammals (9, 11).

A variety of genetic and biochemical analyses have shown that Bvg-regulated genes fall into (at least) four classes. Class 1 genes include those encoding toxins, such as *cyaA-E*, which encode adenylyl cyclase, and *ptxA-E*, which encode pertussis toxin. Class 1 gene promoters contain low-affinity BvgA binding sites far upstream of the transcription start site, and relatively high levels of BvgA-phosphate (BvgA∼P) are required to activate their transcription (6, 21, 27, 41, 52). Class 2 genes include those encoding adhesins, such as *fhaB*, which encodes filamentous hemagglutinin (17, 24, 34). Class 2 gene promoters contain high-affinity BvgA binding sites close to the transcription start site, and low levels of BvgA∼P are sufficient to activate their transcription (5, 7, 8). The only class 3 gene characterized so far is *bipA*, whose product bears amino acid sequence similarity to intimins of enteropathogenic and enterohemorrhagic *Escherichia coli* and invasins of *Yersinia* species (43). The *bipA* promoter contains high-affinity BvgA bind-

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ing sites just upstream of the transcription start site and low-affinity BvgA binding sites downstream of the transcription start site (15, 50). *bipA* transcription is activated by low levels of BvgA~P and repressed by high levels of BvgA~P (15, 50). Class 4 genes are repressed by BvgAS. Although no class 4 gene promoter has been characterized with regard to BvgA binding, the *frlAB* promoter contains discernible BvgA binding sites overlapping the transcription start site (1), suggesting that relatively low levels of BvgA~P are sufficient to repress *frlAB* transcription. *frlAB* encodes orthologs of *E. coli* FlhDC and controls the expression of flagellum, motility, and chemotaxis genes in *B. bronchiseptica* (2). Together, these in vitro studies suggest that by controlling the intracellular concentration of BvgA~P, BvgAS can control three distinct gene expression profiles: when BvgA~P levels are at or near zero, class 4 gene expression will be maximal and class 1, 2, and 3 gene expression minimal; when BvgA~P levels are relatively low, class 2 and 3 gene expression will be maximal and class 1 and 4 gene expression minimal; and when BvgA~P levels are high, class 1 and 2 gene expression will be maximal, class 4 gene expression will be minimal, and class 3 gene expression will be low. Although intracellular BvgA~P levels have not been measured, these three gene expression profiles correspond to three phenotypic phases that have been characterized in vivo and are known as the Bvg^-, Bvg^i, and Bvg^+ phases, respectively (see reference 10 for a review).

The signals sensed by BvgAS in nature are unknown. In the laboratory, however, BvgAS activity varies in response to temperature and the concentration of $MgSO_4$ or nicotinic acid (NA) present in the growth medium (23, 31, 32, 37). These "modulators" have therefore been used to study Bvg-mediated gene regulation, and several analyses confirmed that BvgAS controls expression of the Bvg^-, Bvg^i, and Bvg^+ phases in both a steady-state and temporal manner. For example, it has been well established that *Bordetella* grown in Stainer-Scholte (SS) broth or on Bordet-Gengou (BG) agar at 37°C displays a gene expression profile indicative of the Bvg^+ phase, suggesting that the BvgAS phosphorelay is fully active under these conditions (14, 30, 38). Growth at 25°C or at 37°C in medium containing high concentrations of $MgSO_4$ or NA causes expression of the Bvg^- phase, suggesting that the BvgAS phosphorelay is inactive under these conditions (14, 30, 38). Growth at intermediate temperatures or in medium containing low levels of $MgSO_4$ or NA results in expression of the Bvg^i phase, suggesting that the BvgAS phosphorelay is semiactive under these conditions (14, 15, 43). Temporal regulation has been observed by growing *B. pertussis* under Bvg^--phase conditions and then switching it abruptly to Bvg^+-phase conditions and monitoring gene expression over time. This kind of experiment has shown that the Bvg^i phase is expressed for about 4 hours postshift before the Bvg^+ phase is expressed and maintained (14, 25, 36). BvgAS's ability to control three distinct phenotypic phases that are each characterized by a specific and distinct gene expression profile makes it extremely useful for investigating the mechanisms underlying steady-state and temporal regulation.

The expression of genes encoding many TCSs is positively autoregulated. Examples include *phoPQ* in *Salmonella*, *cpxAR* in *E. coli*, *misRS* in *Neisseria meningitidis*, and *bvgAS* (16, 37, 39, 44). What role autoregulation plays in the ability of these systems to control gene expression precisely and coordinately is unknown. We investigated the role of autoregulation in the ability of BvgAS to control gene expression in both a temporal and steady-state manner by constructing *B. bronchiseptica* mutants in which *bvgAS* was expressed constitutively. Our results show that positive autoregulation is essential for normal temporal regulation and is important, but less crucial, for steady-state regulation.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. *Bordetella* strains were cultured on BG agar (BD Biosciences, San Jose, CA) supplemented with 7.5% defibrinated sheep blood for 48 h at 37°C. For β-galactosidase assays and total RNA isolations, bacteria were grown in SS broth (40) at 37°C, with shaking. *E. coli* strains were cultured on LB agar or in LB broth. When appropriate, culture media were supplemented with gentamicin (Gm; 20 μg ml^{-1}), streptomycin (Sm; 25 μg ml^{-1}), or ampicillin (Ap; 100 μg ml^{-1}).

Molecular cloning and DNA sequence analysis. Standard cloning techniques were used for all DNA manipulations (35). Restriction enzymes, T4 DNA ligase, *Taq* polymerase, and high-fidelity Phusion polymerase were purchased from Promega Corp. (Madison, WI), New England Biolabs (Beverly, MA), and MJ Research (Waltham, MA) and were used according to the manufacturers' instructions. All plasmids used in this study are listed in Table 1. Plasmid pCW27 was constructed by PCR amplifying the −203-to-+45 region of the *ptxA* promoter from *B. pertussis* strain Tohama 1, using primers adding a 5′ EcoRI site (CWptxPF [5′-CC**GAATTC**GCATACGTGTTGGCAA-3′]) and a 3′ SalI site (CWptxPR [5′-CC**GTCGAC**AGTGCAACGCACC-3′]) (restriction sites are shown in bold), and ligating the digested PCR product into the EcoRI and SalI sites of pSS3110.

Construction of bacterial strains. The *bvgAS* promoter was replaced with two nonautoregulated promoters by using an allelic exchange system employing the *sacB* gene as previously described (1, 30). For replacement of the *bvgAS* promoter with the *recA* promoter, we amplified a DNA fragment containing 483 bp upstream of the *recA* translational start site, using primers that added a 5′ EcoRI site (CWrecAPF [5′-CC**GAATTC**CGGCGGCGGTGAGGATCAGCACCGGCA-3′]) and a 3′ EarI site (CWrecAPREar [5′-GCG**CTCTTCG**CATGTAAAGTCCTGTATTGAAGCGGCGCC-3′]), and cloned this fragment along with a 295-bp fragment from the *bvgA* locus beginning at the translational start site, obtained with primers that added a 5′ EarI site (CWbvgAPEarI [5′-CTA**CTCTTCG**ATGCGTTGCAGGATTTTTTTCTCGCC-3′]) and a 3′ HindIII site (CWbvgARHind [5′-GG**AAGCTT**AGCACCAGAACGCGTAGCGGCAACCCC-3′]), into the EcoRI and HindIII sites of pUC19 to create pCW46. The resulting 785-bp EcoRI/HindIII fragment was then cloned, along with a 269-bp BamHI/EcoRI fragment from the *fhaB* promoter (immediately upstream of *bvgAS*), including 15 bp downstream of the ATG, into BamHI/HindIII-cut pEG7S to create pCW54. The 269-bp BamHI/EcoRI fragment from the *fhaB* promoter was generated by PCR amplification using a primer that adds a 5′ BamHI site (CWfhaBFBam [5′-GG**GGATCC**ACAGGTTCGTGTTCATATTCC-3′]) and a primer that binds downstream of a natural EcoRI site (CWfhaBR [5′-GG**GAATTC**ACAGGTTCGTGTTCATATTCC-3′]). Plasmid pCW54 was used for allelic exchange with RB50 to create strain RBL. For replacement of the *bvgAS* promoter with the *aacC1* promoter, we amplified a DNA fragment containing 470 bp upstream of the translational start site of the *aacC1* gene in plasmid pSS3110, using primers that added a 5′ EcoRI site (CWaacC1FEcoRI [5′-**GAATTC**GCCGTTTCTGTAATGAAGGAG-3′]) and a 3′ EarI site (CWaacC1REar [5′-CCG**CTCTTCG**CATCGTTGCTGCTCCATAACATC-3′]), and ligated this fragment along with a 295-bp fragment from the *bvgA* locus beginning at the translational start site, obtained with primers that added a 5′ EarI site and a 3′ HindIII site (CWbvgAPEarI and CWbvgARHind), into the EcoRI and HindIII sites of pUC19 to create pCW57. We then ligated the 269-bp BamHI/HindIII fragment from pCW54 with the EcoRI/HindIII insert from pCW57 with BamHI/HindIII-cut pEG7S to create the allelic exchange vector pCW60. Plasmid pCW60 was used for allelic exchange with RB50 to create strain RBH.

To construct *B. bronchiseptica* strains containing chromosomal *lacZ* reporter plasmids, strains RB50, RBL, and RBH were mated with *E. coli* SM10λpir containing pEG110, pEG112, pTEN34, or pCW27, and exconjugants were selected on BG agar containing streptomycin and gentamicin. Plasmids pEG110, pEG112, and pTEN34 contain internal fragments of *fhaB*, *frl*, and *bipA*, respectively, upstream of and in the same orientation as a promoterless *lacZ* gene. Integration of these plasmids into the *B. bronchiseptica* chromosome results in

TABLE 1. Strains and plasmids used in this analysis

Strain or plasmid	Description	Reference or source
Strains		
DH5α	*E. coli* strain used for molecular cloning	BRL, Gaithersburg, MD
SM10λpir	*E. coli* strain used for conjugation with *Bordetella*	33
RB50	Wild-type *B. bronchiseptica* strain	12
RBL	RB50 with the *bvgAS* promoter replaced with the *recA* promoter	This study
RBH	RB50 with the *bvgAS* promoter replaced with the *aacC1* promoter	This study
RB50::pEG111	RB50 containing a chromosomal *fhaB*::*lacZ* fusion	26
RB50::pEG112	RB50 containing a chromosomal *frl*::*lacZ* fusion	30
RB50::pTEN34	RB50 containing a chromosomal *bipA*::*lacZ* fusion	18
RB50::pCW27	RB50 containing *ptxA* promoter-*lacZYA* fusion	This study
RBL::pEG111	RBL containing a chromosomal *fhaB*::*lacZ* fusion	This study
RBL::pEG112	RBL containing a chromosomal *frl*::*lacZ* fusion	This study
RBL::pTEN34	RBL containing a chromosomal *bipA*::*lacZ* fusion	This study
RBL::pCW27	RBL containing *ptxA* promoter-*lacZYA* fusion	This study
RBH::pEG111	RBH containing a chromosomal *fhaB*::*lacZ* fusion	This study
RBH::pEG112	RBH containing a chromosomal *frl*::*lacZ* fusion	This study
RBH::pTEN34	RBH containing a chromosomal *bipA*::*lacZ* fusion	This study
RBH::pCW27	RBH containing *ptxA* promoter-*lacZYA* fusion	This study
TohamaI	Clinical *B. pertussis* isolate	28
Plasmids		
pUC19	Cloning vector; Ap^r	51
pSS3110	Promoterless *lacZYA* fusion vector, integrates at an untranscribed region of the chromosome; Ap^r Gm^r	49
pEG7S	Allelic exchange vector; Ap^r Gm^r	26
pEG111	Chromosomal *fhaB-lacZ* fusion plasmid; Ap^r Gm^r	26
pEG112	Chromosomal *frl-lacZ* fusion plasmid; Ap^r Gm^r	30
pTEN34	Chromosomal *bipA-lacZ* fusion plasmid; Ap^r Gm^r	18
pCW27	*ptxA* promoter-*lacZYA* fusion in pSS3110	This study
pCW46	pUC19 backbone with the *recA* promoter fused to *bvgA* homology region	This study
pCW54	pEG7S backbone plasmid for replacing the *bvgAS* promoter with the *recA* promoter	This study
pCW57	pUC19 backbone with the *aacC1* promoter fused to *bvgA* homology region	This study
pCW60	pEG7S backbone plasmid for replacing the *bvgAS* promoter with the *aacC1* promoter	This study

the formation of *fhaB-lacZ*, *frl-lacZ*, and *bipA-lacZ* transcriptional fusions, respectively. pCW27 contains the *ptxA* promoter from *B. pertussis* upstream of and in the same orientation as a promoterless *lacZ* gene. This plasmid also contains sequences homologous to a nonessential region of the chromosome, and integration of this plasmid at this location results in the formation of a strain containing a *ptxA-lacZ* fusion. PCR was used to verify the integration of all plasmids at the proper chromosomal location.

Bacterial conjugations. Matings between *B. bronchiseptica* strains and *E. coli* strain SM10λpir were achieved by mixing stationary-phase cultures of the strains on BG agar plus 7.5% sheep blood at a 10:1 (*B. bronchiseptica* to *E. coli*) ratio. The mating mixture was incubated at 37°C for 5 h and then plated onto BG agar plus 7.5% sheep blood containing Gm and Sm to select for cointegrates.

Total RNA isolation and cDNA synthesis. Total RNAs were isolated from RB50, RBL, and RBH grown for 16 to 18 h, to an optical density at 600 nm (OD_{600}) of about 1.0 (mid-log phase for *B. bronchiseptica* grown in SS broth), under Bvg^+-phase conditions (0 mM $MgSO_4$), Bvg^i-phase conditions (2 mM $MgSO_4$ for RB50 and RBL, 6 mM $MgSO_4$ for RBH), or Bvg^--phase conditions (12 mM $MgSO_4$), following the protocol of an RNAqueous-4PCR kit from Ambion Inc. (Austin, TX). For the reverse transcription step, 10 ng of total RNA was transcribed using oligo(dT) and random priming following the protocol supplied with Super Script II reverse transcriptase (Invitrogen Inc., Carlsbad, CA).

mRNA quantification. Relative levels of *bvgA* and *recA* transcripts were determined using quantitative real-time PCR using the following primers: RTrecAF (5′-GCCAGGGCAAGGACAATGT-3′), RTrecAR (5′-CTTCGCTG GCGGGAAG-3′), RTbvgAF (5′-TCCGGGTCCTGATGGAAAA-3′), and RTbvgAR (5′-CTTTTCCTCGCGACGATTATTG-3′). Quantitative real-time PCRs were performed in SYBR green super mix (Bio-Rad Laboratories, Hercules, CA), using a Bio-Rad iCycler PCR machine and software (Bio-Rad Laboratories, Hercules, CA). All samples were run in triplicate, and *bvgA* transcription was normalized to *recA* transcription for each sample.

β-Galactosidase assays. For steady-state β-galactosidase activity assays, a single colony of each RB50, RBL, and RBH *lacZ* fusion strain was inoculated into 3 ml of SS broth containing 0, 2, 4, 6, 8, or 12 mM $MgSO_4$ and grown for 16 to 18 h, to an OD_{600} of between 1.0 and 2.0. These cells were in mid-log phase because the final density of RB50 grown in SS broth is ~4.7 (13). Cells were permeabilized by the addition of sodium dodecyl sulfate and $CHCl_3$, and β-galactosidase activity was determined as described previously (30), except that measurements were taken using a Victor^3 1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). For Bvg^+-phase-to-Bvg^--phase time courses, RB50, RBL, and RBH *lacZ* fusion strains were grown overnight in SS broth containing 25 mM $MgSO_4$, as described above, an aliquot of each strain was taken at time zero, and the remaining cells were harvested, washed twice with SS broth without $MgSO_4$, and resuspended to an OD_{600} of 0.3 in 2 ml fresh medium. A 1-ml aliquot was taken at 1.5, 3, 4.5, 6, 7.5, and 9 h postshift for β-galactosidase measurement, and 1 ml fresh medium was added to the remaining cells. The doubling time for *B. bronchiseptica* growing in SS broth is ~90 min. Removal of 1 ml of culture and replacement of 1 ml of fresh medium every 90 min therefore maintained the culture at a relatively constant density in mid-log phase. For the reverse time courses, RB50, RBL, and RBH strains were grown overnight in SS broth without $MgSO_4$, as described above, an aliquot of each strain was taken at time zero, and the remaining cells were harvested, resuspended in SS broth containing 25 mM $MgSO_4$, and adjusted to an OD_{600} of 0.3 in 2 ml fresh medium. A 1-ml aliquot was taken at 1.5, 3, 4.5, 6, and 7.5 h postshift, and 1 ml fresh medium was added to keep cells in mid-log phase as described above. β-Galactosidase assays were performed on each sample as described above. For steady-state and β-galactosidase assays, relative transcription was calculated by

setting the maximum β-galactosidase activity value for each gene transcribed in RB50 to 100%. For temporal (Bvg^--to-Bvg^+ time courses and Bvg^+-to-Bvg^- time courses) β-galactosidase assays, relative transcription was calculated by setting the maximum β-galactosidase activity value for each gene transcribed in RB50 during the Bvg^--to-Bvg^+ time course to 100%.

RESULTS

Steady-state regulation of gene expression in wild-type *B. bronchiseptica*. We used *B. bronchiseptica* RB50 derivatives containing *lacZ* fusions to representative class 1, 2, 3, and 4 genes to determine Bvg-regulated gene expression patterns in bacteria grown under different environmental conditions, i.e., to characterize steady-state regulation in wild-type *B. bronchiseptica*. Since the best-characterized class 1 gene promoter, that of *ptx*, is not expressed in *B. bronchiseptica* due to nucleotide differences at the primary BvgA binding site (3), we fused the *B. pertussis ptx* promoter to *lacZ* in plasmid pSS3110, which integrates into a nonessential region of the chromosome (49), and used the resulting cointegrate strain to measure *ptx* (class 1) gene expression in RB50. Bacteria grown in SS broth containing 6 to 12 mM $MgSO_4$ displayed a gene expression pattern characteristic of the Bvg^- phase: the class 4 gene *frl* was expressed at maximal levels, while expression of the class 1, 2, and 3 genes was barely detectable (Fig. 1A). Bacteria grown in SS broth without added $MgSO_4$ displayed a gene expression pattern characteristic of the Bvg^+ phase: *ptx* and *fha* (class 1 and 2 genes, respectively) were expressed at high levels, and *frl* and the class 3 gene *bipA* were expressed at low levels (Fig. 1A). Bacteria grown in medium containing 2 mM $MgSO_4$ displayed a gene expression pattern characteristic of the Bvg^i phase: expression of *fha* and *bipA* was maximal, and expression of *frl* and *ptx* was minimal (Fig. 1A). Bacteria grown in medium containing 4 mM $MgSO_4$ expressed high levels of *frl*, moderate levels of *fha* and *bipA*, and low levels of *ptx* (Fig. 1A). We refer to this phase as $Bvg^{-/i}$ since it displays a gene expression pattern intermediate between those of the Bvg^- and Bvg^i phases.

Temporal regulation in wild-type *B. bronchiseptica* in response to a shift from Bvg^--phase conditions to Bvg^+-phase conditions. To characterize Bvg-mediated gene regulation in response to a shift from Bvg^--phase conditions to Bvg^+-phase conditions, we grew bacteria overnight in SS broth containing 25 mM $MgSO_4$, washed the cells twice in SS broth without added $MgSO_4$, resuspended the cells in SS broth without $MgSO_4$ to an OD_{600} of 0.3, incubated the cultures at 37°C with shaking, removed samples every 1.5 h, and measured β-galactosidase activity. The gene expression pattern in the overnight cultures was characteristic of the Bvg^- phase, as expected (Fig. 2A). By 1.5 h postshift, β-galactosidase activity was maximal in the strains containing *bipA-lacZ* and *fha-lacZ* fusions, minimal in the strain containing the *ptx-lacZ* fusion, and half-maximal in the strain containing the *frl-lacZ* fusion (Fig. 2A). Because of the stability of the β-galactosidase enzyme, it is likely that *frl* transcription was actually minimal at this time point and that the β-galactosidase activity detected was due to enzyme that was produced preshift that had not yet degraded. At 3 and 4.5 h postshift, β-galactosidase activity remained high in the *fha-lacZ*- and *bipA-lacZ*-containing strains and low in the *frl-lacZ*- and *ptx-lacZ*-containing strains (Fig. 2A). At 6 h postshift and beyond, *fha-lacZ* and *ptx-lacZ* expression was high, *frl-lacZ* expression was nearly undetectable, and *bipA-lacZ* expression was low (Fig. 2A). These results indicate that when wild-type *B. bronchiseptica* is shifted abruptly from Bvg^--phase conditions to Bvg^+-phase conditions, the Bvg^i phase is expressed for approximately 3 hours before the transition to the Bvg^+ phase is complete. Expression of the Bvg^+ phase was maintained for the remainder of the time course. These results are consistent with observations for *B. pertussis* (14, 25, 36).

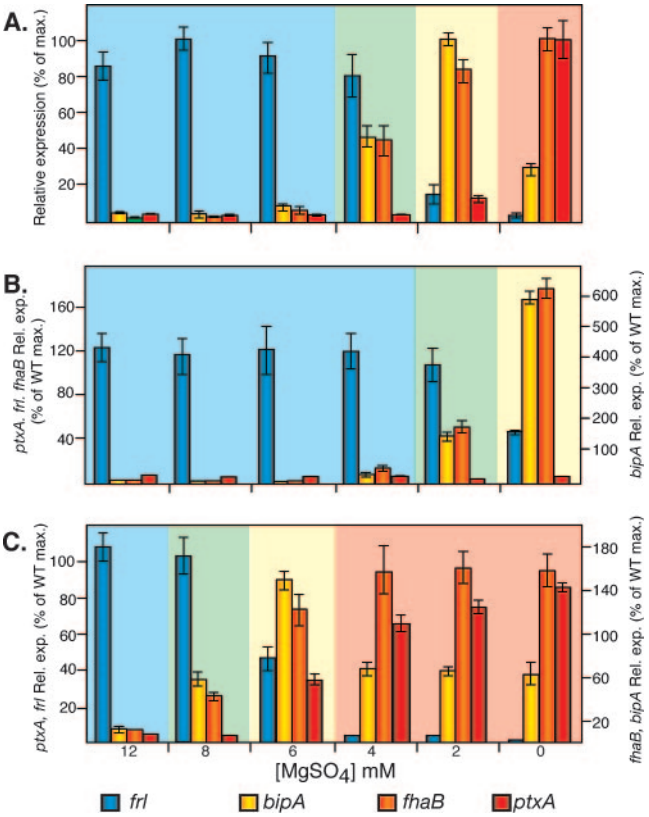

FIG. 1. Steady-state gene expression in RB50 (A), RBL (B), and RBH (C). *lacZ* fusion strains were grown overnight in SS broth containing 0 mM, 2 mM, 4 mM, 6 mM, 8 mM, or 12 mM $MgSO_4$. Relative expression of *frlAB* (blue bars), *bipA* (yellow bars), *fhaB* (orange bars), and *ptxA* (red bars) was calculated by setting the maximum β-galactosidase activity of each gene in RB50 (wild type [WT]) to 100%. The light blue background indicates Bvg^--phase expression, the yellow background indicates the Bvg^i phase, the light green background indicates the $Bvg^{-/i}$ phase, and the light red background indicates the Bvg^+ phase.

Temporal regulation in wild-type *B. bronchiseptica* in response to a shift from Bvg^+-phase conditions to Bvg^--phase conditions. To characterize Bvg-mediated gene regulation in response to a shift from Bvg^+-phase conditions to Bvg^--phase conditions, we grew bacteria overnight in SS broth without added $MgSO_4$, harvested the cells, resuspended them in SS broth containing 25 mM $MgSO_4$ to an OD_{600} of 0.3, incubated the cultures at 37°C with shaking, removed samples every 1.5 h, and measured β-galactosidase activity. *fha-lacZ*, *ptx-lacZ*, and *bipA-lacZ* levels were approximately 80% of their preshift levels at 1.5 h postshift, approximately 40% of preshift levels by 3 h postshift, and ≤20% of preshift levels at 4.5 h postshift (Fig. 3A). *bipA-lacZ* expression was not higher than the pre-

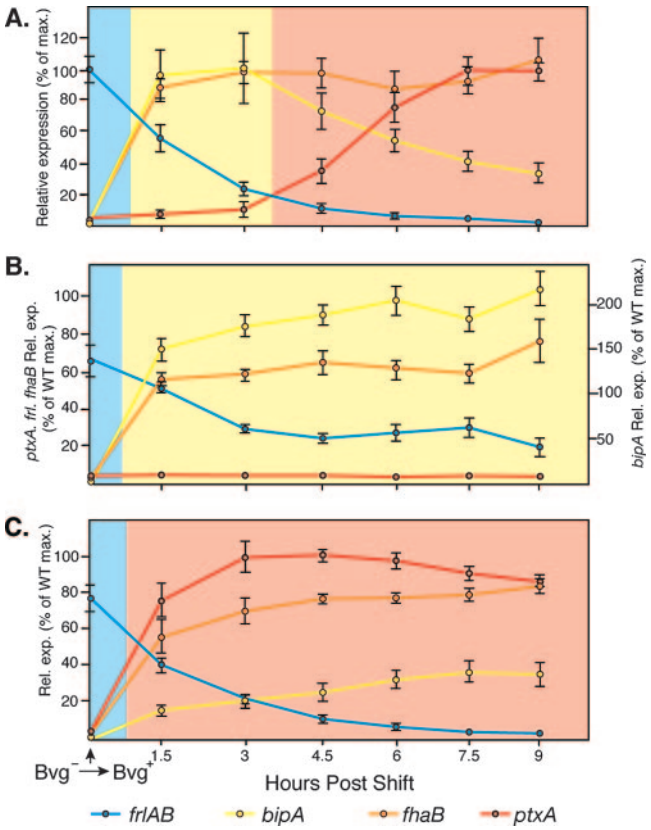

FIG. 2. Temporal gene expression in cells shifted from Bvg^--phase conditions to Bvg^+-phase conditions in RB50 (A), RBL (B), and RBH (C). *lacZ* fusion strains were grown overnight in SS broth containing 25 mM $MgSO_4$ and then washed and resuspended in SS broth without added $MgSO_4$ at time zero, and samples were taken every 1.5 h postshift. Relative expression of *frlAB* (blue lines), *bipA* (yellow lines), *fhaB* (orange lines), and *ptxA* (red lines) was calculated by setting the maximum β-galactosidase activity of each gene in RB50 (wild type [WT]) to 100%. The light blue background indicates Bvg^--phase expression, the yellow background indicates the Bvg^i phase, and the light red background indicates the Bvg^+ phase.

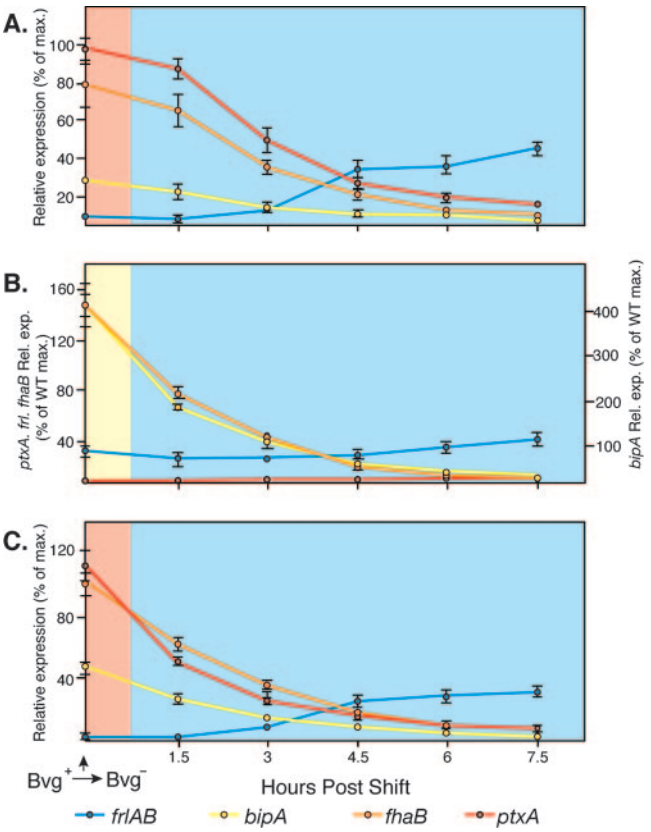

FIG. 3. Temporal gene expression in cells shifted from Bvg^+-phase conditions to Bvg^--phase conditions in RB50 (A), RBL (B), and RBH (C). *lacZ* fusion strains were grown overnight in SS broth without added $MgSO_4$ and then resuspended in SS broth containing 25 mM $MgSO_4$ at time zero, and samples were taken every 1.5 h postshift. Relative expression of *frlAB* (blue lines), *bipA* (yellow lines), *fhaB* (orange lines), and *ptxA* (red lines) was calculated by setting the maximum β-galactosidase activity of each gene in RB50 (wild type [WT]) shifted from Bvg^--phase conditions to Bvg^+-phase conditions (i.e., the values from Fig. 2A) to 100%. The light blue background indicates Bvg^--phase expression, the yellow background indicates the Bvg^i phase, and the light red background indicates the Bvg^+ phase.

shift level at any time point. *frl-lacZ* expression increased gradually over the course of the experiment (Fig. 3A). Therefore, when wild-type *B. bronchiseptica* is shifted from Bvg^+-phase conditions to Bvg^--phase conditions, the temporal pattern of gene expression that occurs is not the reverse of that which occurs when cells are shifted from Bvg^--phase conditions to Bvg^+-phase conditions. Instead, the transition from the Bvg^+ phase to the Bvg^- phase occurs directly, without even transient expression of the Bvg^i phase.

Construction of *bvgAS* autoregulation mutants. Because *bvgAS* is autoregulated, we could not measure its expression using *bvg-lacZ* fusions and therefore used quantitative reverse transcription-PCR (RT-PCR). Consistent with previous reports, *bvgAS* transcripts were of very low abundance in bacteria grown under Bvg^--phase conditions, of moderate abundance in bacteria grown under Bvg^i-phase conditions, and of high abundance in bacteria grown under Bvg^+-phase conditions (Fig. 4). To investigate the role of autoregulation in the ability of BvgAS to control multiple patterns of gene expression in a steady-state and temporal manner, we constructed strains in which *bvgAS* was not autoregulated. We sought to construct one strain in which *bvgAS* was expressed constitutively at a high level, similar to the level at which wild-type *bvgAS* is expressed in the Bvg^+ phase, and one strain in which *bvgAS* was ex-

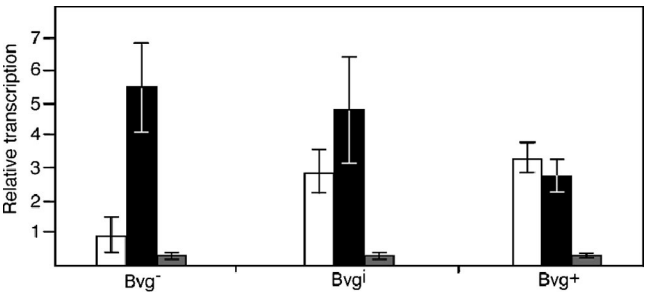

FIG. 4. Relative *bvgA* mRNA levels in RB50 (white bars), RBH (black bars), and RBL (gray bars). Quantitative PCR was used to determine relative levels of the *bvgA* transcript in RB50, RBL, and RBH grown under Bvg^--, Bvg^i-, and Bvg^+-phase conditions.

pressed constitutively at a low level, similar to that at which wild-type *bvgAS* is expressed under Bvg$^-$-phase conditions. We tested the expression of several promoters for constitutive expression in RB50, and although we were unable to find promoters that met either condition exactly, the *recA* and *aacC1* promoters proved useful and adequate for our analyses. We constructed strain RBL by replacing the native *bvgAS* promoter with the *B. bronchiseptica recA* promoter. Quantitative RT-PCR indicated that in this strain, *bvgAS* expression under all growth conditions was about half that of *bvgAS* in wild-type bacteria grown under Bvg$^-$-phase conditions (Fig. 4). We constructed strain RBH by replacing the native *bvgAS* promoter with that of the *aacC1* gene, which encodes gentamicin acetyltransferase. Quantitative RT-PCR indicated that in this strain, *bvgAS* expression was slightly higher in cells grown under Bvg$^-$- and Bvg^i-phase conditions and the same in cells grown under Bvg$^+$-phase conditions as that of *bvgAS* in wild-type bacteria grown under Bvg$^+$-phase conditions (Fig. 4). Although *bvgAS* expression in these strains is not exactly the same as that in wild-type *B. bronchiseptica* grown under either Bvg$^+$- or Bvg$^-$-phase conditions, it is not autoregulated, and therefore the role of autoregulation in steady-state and temporal gene regulation can be determined using these strains.

Steady-state gene expression profiles of *bvgAS* autoregulation mutants. We measured *frl-lacZ*, *bipA-lacZ*, *fha-lacZ*, and *ptx-lacZ* expression in RBL and RBH after growing the bacteria in SS broth containing various concentrations of $MgSO_4$, as described above for RB50. The gene expression patterns displayed by RBL indicated that this strain expressed the Bvg$^-$ phase when grown in medium containing 4 to 12 mM $MgSO_4$, the Bvg$^{-/i}$ phase when grown in medium containing 2 mM $MgSO_4$, and the Bvg^i phase when grown in medium without added $MgSO_4$ (Fig. 1B). *bipA-lacZ* levels in this strain grown without added $MgSO_4$ were approximately sixfold greater than the level in RB50 grown in 2 mM $MgSO_4$ (Fig. 1B). (The reason for this high level of *bipA* expression is addressed in Discussion.) The low level of *bvgAS* expressed in RBL is therefore sufficient to induce a Bvg^i-phase pattern of gene expression but not a Bvg$^+$-phase pattern of gene expression. The gene expression patterns displayed by RBH indicated that this strain was able to control all of the various Bvg-regulated gene expression patterns, but with a slightly different sensitivity to $MgSO_4$; the Bvg$^{-/i}$ phase was expressed in cells grown in medium containing 8 mM $MgSO_4$, and the Bvg^i phase was expressed in cells grown in medium containing 6 mM $MgSO_4$ (Fig. 1C). Together, these data indicate that autoregulation is not required for BvgAS to control multiple gene expression patterns if it is expressed at a high level. However, positive autoregulation appears to influence the sensitivity of the system, since more $MgSO_4$ was required to modulate the activity of BvgAS in RBH than in RB50.

Temporal gene expression profiles of *bvgAS* autoregulation mutants in response to a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions. When shifted from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions, RBL and RBH switched within 1.5 h to gene expression patterns indicative of the Bvg^i and Bvg$^+$ phases, respectively, and these gene expression profiles were maintained for the duration of the experiment (Fig. 2B and C). Notably, the Bvg^i phase was not expressed even briefly in RBH (Fig. 2C). Positive autoregulation is therefore essential for BvgAS to control multiple gene expression patterns in a temporal manner in response to a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions.

Temporal gene expression profiles of *bvgAS* autoregulation mutants in response to a shift from Bvg$^+$-phase conditions to Bvg$^-$-phase conditions. When shifted from Bvg$^+$-phase conditions to Bvg$^-$-phase conditions, the expression of Bvg-activated genes in RBL and RBH was significantly decreased by the first time point (1.5 h) (except that of *ptx* in RBL, which was never activated in this strain even under Bvg$^+$-phase conditions) (Fig. 3B and C). *frl-lacZ* expression increased gradually in all strains over the course of the experiment. These gene expression patterns are similar to those measured for RB50 and therefore indicate that positive autoregulation does not play a significant role in the ability of BvgAS to control gene expression temporally in response to a shift from Bvg$^+$-phase conditions to Bvg$^-$-phase conditions.

DISCUSSION

A variety of pathogens regulate virulence gene expression in response to the surrounding environment through the use of two-component regulatory systems (13, 32, 34). Although many of these systems are positively autoregulated, how autoregulation affects their functionality is not understood. Using *Bordetella* BvgAS as a model, we have shown that autoregulation of *bvgAS* transcription is critical for temporal expression of multiple phenotypic phases in response to a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions. We also showed that *bvgAS* autoregulation contributes to steady-state regulation, as it influences the sensitivity of the system in response to subtle differences in signal intensity. As discussed below, our results also provide insight into how BvgAS functions mechanistically.

A model for how BvgA and BvgS respond to a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions, based primarily on experiments done with *B. pertussis*, is as follows. Both protein quantification and gene expression data indicate that BvgA and BvgS levels are very low in Bvg$^-$-phase cells (14, 25, 37, 38). The fact that mutations in *bvgA* or *bvgS* that prevent phosphorylation of BvgA in vitro result in constitutive Bvg$^-$-phase expression in vivo suggests that the amount of BvgA that is phosphorylated under Bvg$^-$-phase conditions is at or near zero (12, 45, 47). When cells are shifted to Bvg$^+$-phase conditions, BvgS autophosphorylates, and the phosphoryl group is transferred to BvgA. The amount of BvgA~P that is present immediately postshift (which must be very low) is sufficient to activate transcription of class 2 and 3 genes as well as *bvgAS* itself but not to activate transcription of class 1 genes or to repress *bipA*, and therefore the Bvg^i phase is expressed. After 3 to 4 h of positive autoregulation, BvgS and BvgA (and, most importantly, BvgA~P) reach levels sufficient to activate the expression of class 1 genes and to repress the expression of *bipA*. Cells then express the Bvg$^+$ phase as long as they are exposed to Bvg$^+$-phase conditions.

Consistent with the results of studies done with *B. pertussis* (14, 25, 36), our studies showed that when wild-type *B. bronchiseptica* was shifted from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions, the Bvg^i phase was expressed for approximately 3 hours before the Bvg$^+$ phase was expressed. However, when

bvgAS was expressed constitutively at a low level, the bacteria switched from the Bvg$^-$ phase to the Bvg^i phase and never expressed the Bvg$^+$ phase, and when *bvgAS* was expressed constitutively at a high level, the bacteria switched from the Bvg$^-$ phase to the Bvg$^+$ phase without ever expressing the Bvg^i phase. Positive autoregulation is therefore essential for the bacteria to express multiple phenotypic phases in a temporal manner in response to a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions. Consistent with the model, therefore, positive autoregulation appears to be responsible for the relatively slow accumulation of BvgA (and hence BvgA~P) postshift, which is responsible for the transient (2 to 3 h) expression of the Bvg^i phase. These data also provide insight into BvgS activation kinetics: the lack of Bvg^i-phase expression in RBH postshift indicates that the kinase activity of BvgS can be activated rapidly upon exposure to Bvg$^+$-phase conditions.

When wild-type *B. bronchiseptica* was shifted from Bvg$^+$-phase conditions to Bvg$^-$-phase conditions, the cells switched directly to the Bvg$^-$ phase without transient Bvg^i-phase expression. This result concurs with previous studies with *B. pertussis* in which mRNA transcripts for *fhaB*, *cyaA*, and *ptxA* decreased rapidly postshift (Bvg$^+$-phase conditions to Bvg$^-$-phase conditions) (38). Scarlato et al. hypothesized that BvgA was rapidly inactivated under noninducing conditions and that BvgA levels remained high, at least temporarily, to prime the cells for detection of a new signal (37). This study was done prior to the demonstration that BvgS and BvgA communicate via phosphorylation and prior to the identification of the Bvg^i-phase-specific gene *bipA* (43, 45, 47). Our experiments showed that in *B. bronchiseptica*, class 1 and 2 gene expression decreased significantly by the first time point following a shift from Bvg$^+$-phase conditions to Bvg$^-$-phase conditions and that *bipA* expression never increased, demonstrating conclusively that the Bvg^i phase was not expressed even transiently postshift. Like wild-type *B. bronchiseptica*, both RBL and RBH switched directly to the Bvg$^-$ phase postshift, indicating that autoregulation is not required for temporal regulation in response to a shift to Bvg$^-$-phase conditions. The fact that RBH switched directly to the Bvg$^-$ phase indicates that, consistent with the hypothesis put forth by Scarlato et al., BvgA is inactivated rapidly postshift. Because in vitro studies indicate that BvgA~P is stable in the absence of BvgS (48), these data suggest that the phosphatase activity of BvgS is activated immediately upon exposure to Bvg$^-$-phase conditions.

While the results of our temporal regulation experiments support the hypothesis that BvgS converts rapidly to a kinase or a phosphatase upon exposure to Bvg$^+$- or Bvg$^-$-phase conditions, respectively, the results of our steady-state regulation experiments support the hypothesis that the kinase and phosphatase activities of BvgS can be adjusted to intermediate levels, i.e., that BvgS functions like a rheostat rather than a switch. Although this hypothesis was proposed nearly a decade ago (13), the mechanistic basis for how BvgS activity is fine-tuned is still unknown. Moreover, the possibility that expression of the Bvg^i phase results from autoregulation rather than intermediate BvgS activity could not previously be ruled out. The fact that RBH was capable of expressing all of the same phenotypic phases as RB50, however, indicates that BvgA~P levels can be adjusted precisely and maintained indefinitely at specific levels in response to specific environmental cues, even when BvgA is maintained at a high level. Since there is considerable evidence that phosphorylation and dephosphorylation of BvgA are controlled only by BvgS (41, 45–48), these data provide strong evidence that the kinase and/or phosphatase activity of BvgS can be adjusted to and maintained at intermediate levels. Our data also suggest that it is the absolute amount of BvgA~P, rather than the ratio of BvgA~P to BvgA, that is important in controlling gene expression, because this ratio must be well below one in RBH cells grown under Bvg^i-phase conditions but one or nearly one in RBL cells grown under Bvg$^+$-phase conditions and in RB50 cells immediately after a shift from Bvg$^-$-phase conditions to Bvg$^+$-phase conditions, yet in all of these cases, the Bvg^i phase is expressed.

The fact that the sensitivity of RBH to environmental cues differs from that of RB50 demonstrates that although it is relatively subtle, autoregulation does contribute to the ability of BvgAS to control gene expression in a steady-state manner. This result suggests that BvgS activity is controlled primarily in response to environmental signals and that if a feedback mechanism that adjusts BvgS activity in response to the absolute amount of BvgA~P exists, it plays only a minor role. If such a feedback mechanism played a major role, then RB50 and RBH would control the same pattern of gene expression in response to the same environmental conditions. Instead, it appears that BvgS activity is controlled predominantly in response to environmental cues and that BvgA~P levels reflect BvgS activity and the amount of BvgA and BvgS present in the cell. Thus, the data suggest that BvgS activities in RB50 and RBH grown in the presence of 6 M $MgSO_4$, for example, are the same, but the higher absolute amount of BvgA and BvgS in RBH than in RB50 under these conditions results in higher levels of BvgA~P in RBH and therefore in expression of the Bvg^i phase instead of the Bvg$^-$ phase.

In the course of our experiments, we noted that *bipA* expression in RBL grown under Bvg$^+$-phase conditions was much higher than *bipA* expression in RB50 under any condition. This extremely high level of *bipA* expression is most likely due to binding of BvgA~P to the high-affinity BvgA binding sites present upstream of the *bipA* transcription start site and the lack of BvgA~P binding to the low-affinity BvgA binding sites present downstream of the *bipA* transcription start site (50). The concentration of BvgA~P produced in RBL under Bvg$^+$-phase conditions must therefore be greater than the binding constants of the high-affinity sites and less than the binding constants of the low-affinity sites. The failure of RB50 to express *bipA* at this high level indicates that the concentration of BvgA~P present in RBL cells grown under Bvg$^+$-phase conditions is never stably produced in RB50. This is most likely due to the fact that *bvgAS* transcription is activated by binding of BvgA~P to sites that are of similar high affinity to those present upstream of the *bipA* promoter. Together, these data indicate that although the concentration of BvgA~P in the cell can be adjusted over a broad range, autoregulation prevents the stable expression of some (low) concentrations. Thus, while BvgAS may function like a rheostat, autoregulation apparently makes it an imperfect one.

Our experiments have provided insight into the role of autoregulation in the ability of BvgAS to control multiple gene expression profiles in response to changes in environmental conditions and have also provided clues regarding how BvgA

and BvgS function mechanistically. Important questions begging to be addressed, however, relate to the role of autoregulation in survival of the organism in its natural environments. For both *B. pertussis* and *B. bronchiseptica*, there is considerable evidence that the Bvg^+ phase is necessary and sufficient for the bacteria to cause respiratory infection and that a failure to repress Bvg^--phase phenotypes is detrimental to the development of infection (1, 12, 29). Autoregulation therefore must not play an important role during respiratory infection (other than to maintain high levels of BvgA and BvgS), and the fact that Bvg^+-phase-locked strains are indistinguishable from wild-type *B. pertussis* and *B. bronchiseptica* in various animal models supports this conclusion (12, 29). It has been hypothesized that transition to the Bvg^i phase is important for aerosol transmission by both *B. pertussis* and *B. bronchiseptica* and that the Bvg^- phase is required for *B. bronchiseptica* to survive for extended periods of time outside the mammalian host, an ability that *B. pertussis* apparently lacks. We hypothesize that *bvgAS* autoregulation plays an important role in transmission between mammalian hosts, either directly or via an environmental reservoir (in the case of *B. bronchiseptica*). Testing this hypothesis, however, will require the use of models that encompass the entire *Bordetella* infectious cycle, which are not yet available.

Although this hypothesis has not been tested, it seems likely that autoregulation of two-component systems is important generally. In *Salmonella*, for example, the PhoPQ TCS, which is positively autoregulated, controls the expression of many virulence genes, including those required for survival within macrophages (see reference 20 for a review). It is possible that autoregulation of *phoPQ* and subsequent (at least partial) autoregulation of *ssrA* and *ssrB* control a temporal pattern of virulence gene expression that is important for survival of *Salmonella* cells after they enter macrophage phagosomes (see reference 4 for a review). In *Bacillus subtilis*, the Spo0A response regulator controls the expression of genes required for sporulation. Spo0A synthesis is controlled by a positive feedback loop in which Spo0A~P stimulates expression of the gene encoding the RNA polymerase sigma factor σ^H, which leads, in turn, to increased *spo0A* transcription (22). This feedback loop results in a gradual increase in Spo0A after cells are shifted to nutrient-limiting conditions (19). Fujita and Losick hypothesized that the gradual increase in Spo0A~P concentration allowed early-threshold genes to be expressed before late-threshold genes, and by ectopically expressing *spo0A* from an inducible promoter, they showed that a gradual increase in Spo0A was indeed required for cells to sporulate properly after they were shifted to starvation conditions (19). The role of autoregulation in other regulatory systems remains to be determined.

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