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An Improved Recombination-Based *In Vivo* Expression Technology-Like Reporter System Reveals Differential *cyaA* Gene Activation in *Bordetella* Species

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***Bordetella pertussis* and *Bordetella bronchiseptica* rely on the global two-component regulatory system BvgAS to control expression of distinct phenotypic phases. In the Bvg$^-$ phase, expression of *vrg* genes, including those required for motility in *B. bronchiseptica*, is activated and genes encoding virulence factors are not expressed. Conversely, in the Bvg$^+$ phase, genes encoding virulence factors are highly expressed while genes necessary for motility are repressed. Although several genetic analyses have demonstrated the importance of the Bvg$^+$ phase during respiratory infection, Bvg-regulated gene activation in *B. bronchiseptica* has not been investigated *in vivo*. To address this, we developed a plasmid, pGFLIP, that encodes a sensitive Flp recombinase-based fluorescent reporter system able to document gene activation both *in vitro* and *in vivo*. Using pGFLIP, we demonstrated that *cyaA*, considered to be a "late" Bvg$^+$ phase gene, is activated substantially earlier in *B. bronchiseptica* than *B. pertussis* following a switch from Bvg$^-$ to Bvg$^+$ phase conditions. We show that the altered activation of *cyaA* is not due to differences in the *cyaA* promoter or in the *bvgAS* alleles of *B. bronchiseptica* compared to *B. pertussis*, but appears to be species specific. Finally, we used pGFLIP to show that *flaA* remains repressed during infection, confirming that *B. bronchiseptica* does not modulate to the Bvg$^-$ phase *in vivo*.**

Bordetella species are Gram-negative bacterial respiratory pathogens. The genus includes *Bordetella pertussis*, an obligate human pathogen and the causative agent of whooping cough, and the closely related *Bordetella bronchiseptica*, which can infect a wide range of mammals, including several species that are commonly studied in the laboratory (1–3). These bacteria rely on the global two-component regulatory system BvgAS for virulence (1–3). The BvgAS phosphorelay regulates gene expression patterns according to environmental cues and controls at least three distinct phenotypic phases: Bvg minus (Bvg$^-$), Bvg plus (Bvg$^+$), and Bvg intermediate (Bvg^i) (4, 5). All evidence thus far suggests that the Bvg$^+$ phase is necessary and sufficient for infection and that modulation to the Bvg$^-$ or Bvg^i phase does not occur *in vivo* (6–8). Although it has been hypothesized that the Bvg^i phase and/or the Bvg$^-$ phase is required for transmission or survival outside a host (7, 9, 10), a recent study provided evidence that neither of these phenotypic phases is required for *B. bronchiseptica* transmission in swine (8).

Genes that define these three phenotypic phases have been divided into four classes based on their expression profile. Class 1 (late Bvg$^+$ phase) genes include *cyaA* (encoding the bifunctional hemolysin/adenylate cyclase toxin ACT) and *ptxA* (encoding the catalytic subunit of the AB_5-type pertussis toxin) (4). Class 2 (early Bvg$^+$ phase) genes include those encoding filamentous hemagglutinin (*fhaB*), fimbriae (*fim2* and *fim3*), and *bvgAS* itself (4). *bipA* is the only class 3 (Bvg^i phase) gene that has been characterized. Class 4 (Bvg$^-$ phase) genes include those encoding proteins involved in motility (*frlAB*) and chemotaxis in *B. bronchiseptica* (4).

Expression of genes that define the various Bvg-dependent phenotypic phases is determined mechanistically by the location and affinity of BvgA binding sites near the transcription start site (4). Class 1 genes contain multiple low-affinity BvgA binding sites 5′ distal to the start of transcription (4, 11), while class 2 genes contain high-affinity BvgA binding sites proximal to the transcription start site (12–14). The promoter region of the class 3 gene *bipA* contains high-affinity BvgA binding sites 5′ proximal to the transcription start site and low-affinity sites 3′ to the transcription start site (10, 15). Although it has been hypothesized that BvgAS directly represses transcription of *frlAB* in *B. bronchiseptica* (16), BvgA binding to the *frlAB* promoter has not been demonstrated and BvgAS-mediated repression of at least some genes in *B. pertussis* is indirect (17).

In vitro transcription and DNA binding studies indicate that the phosphorylated form of BvgA (BvgA∼P) is required to activate transcription of Bvg$^+$ phase genes and that a higher concentration of BvgA∼P is necessary to bind "late" Bvg$^+$ phase promoters than "early" Bvg$^+$ phase promoters (11, 18). Although the natural signals that activate the BvgAS system are unknown, it is possible to modulate *Bordetella* spp. to the Bvg$^-$ phase in the laboratory by adding a chemical modulator ($MgSO_4$ or nicotinic acid) to the growth medium or by growing bacteria at 25°C (15, 19–21). When bacteria are grown under Bvg$^-$ phase conditions, class 4 genes are expressed while class 1 to 3 genes are not expressed. Following a switch from Bvg$^-$ phase to Bvg$^+$ phase conditions, transcription of class 4 genes ceases and transcription of class 2 genes, along with the sole class 3 gene *bipA*, is immediately activated. After several hours, class 1 genes are expressed and class

Received 19 December 2012 **Returned for modification** 10 January 2013
Accepted 26 January 2013
Published ahead of print 4 February 2013
Editor: A. Camilli
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doi:10.1128/IAI.01445-12

3 (*bipA*) genes are repressed. These data are consistent with the model in which the concentration of BvgA~P within the cell is nearly zero in the Bvg^- phase, low in the Bvg^i phase, and high in the Bvg^+ phase (4, 5, 11, 15, 18, 22, 23).

In contrast with the extensive *in vitro* characterization of the steady-state expression patterns of BvgAS-regulated genes in both *B. pertussis* and *B. bronchiseptica*, as well as kinetic analyses of gene expression upon switching from modulating to nonmodulating conditions and vice versa (5, 10, 11, 15, 22–24), only one study has investigated BvgAS-dependent gene regulation *in vivo* (24). Veal-Carr et al. utilized recombination-based *in vivo* expression technology (RIVET) to analyze the kinetics of BvgAS-activated gene expression in *B. pertussis* both *in vitro* and following intranasal infection of mice (24, 25). They showed that the *in vivo* activation of Bvg^+ phase genes, including *ptxA*, *cyaA*, *fhaB*, and *prn*, temporally recapitulated the activation pattern of these genes upon switching *B. pertussis* from Bvg^- to Bvg^+ phase conditions *in vitro*; i.e., *fhaB* was activated early postinoculation (p.i.) (~1 h), followed by *prn* (~4 h), then later by *cyaA* (~12 h) (24). Significantly, the fact that the patterns of gene activation were nearly identical in bacteria switched from Bvg^- to Bvg^+ phase conditions *in vitro* and after inoculation of mice indicates that the mouse lung is a Bvg^+ phase environment (24).

We constructed a plasmid, pGFLIP, that encodes a Flp recombinase-based fluorescent reporter system to assess the activation kinetics of genes *in vivo* (26). The region of pGFLIP delivered to the chromosome contains *gfp* and *nptII* genes, encoding green fluorescent protein (GFP) and neomycin phosphotransferase (conferring kanamycin resistance [Km^r]), respectively, flanked by *F*lp *r*ecombinase *t*arget (*FRT*) sites. The plasmid also contains a promoterless *flp* recombinase gene with a multiple cloning site (MCS) immediately 5′ to the start codon. Upon expression of *flp* under the control of a promoter of interest, the *gfp* and *nptII* genes are permanently excised from the bacterial chromosome.

To test our system, we cloned the promoter regions of several BvgAS-controlled genes, including the Bvg-activated genes *cyaA*, *fhaB*, and *ptxA* and the Bvg-repressed gene *flaA* (encoding flagellin), into pGFLIP and evaluated transcription activation *in vitro* and *in vivo* in *B. bronchiseptica*. Among other results, we found unexpectedly that the *cyaA* gene is expressed differently in *B. pertussis* and *B. bronchiseptica*.

MATERIALS AND METHODS

Strains, reagents, and growth conditions. *Escherichia coli* was grown in lysogeny broth (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) or on LB plates with 1.5% agar at 37°C. *Bordetella* was grown in Stainer-Scholte (SS) broth (27) or on Bordet-Gengou (BG) plates with 1.5% agar (BD Biosciences, San Jose, CA) supplemented with 7.5% (*B. bronchiseptica*) or 15.0% (*B. pertussis*) defibrinated sheep's blood (Colorado Serum Company, Denver, CO) at 37°C (28). As required, culture media were supplemented with kanamycin (Km; 50 or 100 μg/ml), ampicillin (100 μg/ml), streptomycin (Sm; 25 μg/ml), $MgSO_4$ (20 mM or 50 mM), heptakis (1 mg/ml; Sigma), and diaminopimelic acid (DAP; 400 μg/ml) for the Δ*asd* Δ*aphA* mobilizer strain RHO3 (29). Unless otherwise noted, all restriction enzymes and T4 DNA ligase were purchased from New England BioLabs.

Construction of pGFLIP and derivatives containing *Bordetella* promoters. The Tn*7* transposition plasmid pUC18T-mini-Tn7T-Km-*FRT* (30) was digested with BamHI, resulting in fragments of 3,636 and 1,299 bp in length containing the plasmid backbone and the *nptII* (Km^r) gene, respectively. Separately, a 797-bp fragment containing *gfp* driven by the constitutive *Burkholderia pseudomallei rpsL* promoter P_{S12} was amplified by PCR from mini-Tn7-*kan-gfp* (31) using *Pfu* Ultra II (Agilent) and primers GFP_UP and GFP_DN. This fragment was blunt-end ligated into the cloning vector pJET1.2/blunt (Fermentas) and was transformed into *E. coli* DH5α according to the manufacturer's instructions. Using restriction sites introduced by PCR, the P_{S12}-*gfp* fragment was digested from pJET using BamHI and was ligated together with the BamHI-digested pUC18T-mini-Tn7T-Km-*FRT* backbone and *nptII* fragment. As the *flp* gene would be sensitive to transcription readthrough from either the P_{S12} or the *nptII* promoter, primers specific to *gfp* (gfpseqR) and *nptII* (kanseqR) were used to confirm that both genes would be transcribed opposite the promoter of interest and would therefore not drive *flp* expression. Once the orientation of *gfp* and *nptII* was verified, the plasmid was digested with KpnI and StuI; a fragment containing promoterless *flp* amplified by PCR from pFLPe4 (30) using primers FLP_UP and FLP_DN was likewise digested with KpnI and StuI and was ligated into the digested vector. The resulting plasmid, pGFLIP, thus contained an MCS 5′ to *flp*, the *flp* recombinase gene, and constitutively expressed *nptII* and *gfp* genes flanked by *FRT* sites. pGFLIP was fully sequenced using a primer-walking approach with the primers listed in Table 1.

Promoters for five *Bordetella* genes (*cyaA*, $cyaA_{Bp}$, *fhaB*, *ptxA*, and *flaA*) were cloned into the MCS of pGFLIP as follows. For *cyaA* and $cyaA_{Bp}$, 605- and 604-bp fragments containing the *cyaA* promoter were amplified by PCR from *B. bronchiseptica* RB50 and *B. pertussis* BPSM, respectively, using primers cyaA_F and cyaA_R. These fragments were digested with SacI and KpnI and ligated into pGFLIP. For *fhaB*, a 426-bp fragment containing the *fhaB* promoter was amplified by PCR from RB50 using primers fhaprF2 and fhaprR2, digested with SacI and KpnI, and ligated into pGFLIP. For *ptxA*, a 454-bp fragment containing the *ptxA* promoter was amplified by PCR from BPSM using primers ptxprF and ptxprR, digested with SacI and ApaI, and ligated into pGFLIP. For *flaA*, a 514-bp fragment containing the *flaA* promoter was amplified by PCR from RB50 using primers flaA_F and flaA_R, digested with SacI and KpnI, and ligated into pGFLIP. These constructs were delivered to the chromosome by transposase-mediated insertion as described below.

Transposase-mediated delivery of pGFLIP to the *B. bronchiseptica att*Tn*7* site. The pGFLIP plasmid was delivered to *Bordetella* by triparental mating using a procedure modified from reference 28. *B. bronchiseptica* strains RB50 and RB52 were grown on BG agar for 48 h, and a portion of the cells was coincubated with conjugation-competent *E. coli* RHO3 cells (29) harboring pGFLIP and RHO3 cells containing the helper plasmid pTNS3 (30), which expresses *tnsABCD* from a constitutive promoter, on BG agar supplemented with DAP for 6 h at 37°C. Following incubation, cells were restreaked onto BG-Km agar containing 20 mM $MgSO_4$ (to maintain strains containing pGFLIP with Bvg^+ phase promoters in the Bvg^- phase) or without $MgSO_4$ (to maintain RB50*fla*AFLP in the Bvg^+ phase) and were incubated an additional 48 h at 37°C. Delivery of pGFLIP to BPSM followed the same procedure except that incubations required 4 days at 37°C and plates were supplemented with 50 mM $MgSO_4$. pGFLIP without a promoter driving *flp* was used as a positive control for GFP production and as a negative control for *flp* activation. The delivery of all constructs to the *att*Tn*7* site was confirmed by PCR using primers glmSF and gfpseqR (data not shown).

Evaluation of pGFLIP *in vitro*. We determined the functionality of pGFLIP using a plate-based assay in which RB50*cyaA*FLP and RB50*flaA*FLP were modulated between promoter-inactive and promoter-active conditions. BG agar containing 20 mM $MgSO_4$ was used to derepress P_{flaA} and to deactivate P_{cyaA}, while BG agar without $MgSO_4$ was used to activate P_{cyaA} and to repress P_{flaA} (7). The pGFLIP strains were grown under promoter-inactive conditions for 48 h at 37°C and were determined to be GFP positive (GFP^+) using a G:BOX Chemi imaging system with an UltraBright-LED blue transilluminator and an SW06 short-pass filter (495 to 600 nm; Syngene, Frederick, MD). A single GFP^+ colony was resuspended in PBS and was diluted and plated on BG agar under promoter-active and promoter-inactive conditions. To demonstrate the loss

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)[a]	Description
FLP_UP	ATCTACGGTACCATGAGCCAGTTCGATATCC	Forward and reverse primers to amplify *flp* from pFLPe4
FLP_DN	AGGTCCAGGCCTCTATATGCGTCTATTTATG	
GFP_UP	ATATATGGATCCCAGCTGTTGACTCGCTTG	Forward and reverse primers to amplify *gfp* from mini-Tn*7-kan-gfp*
GFP_DN	ACCTGGGGATCCTTATTTGTATAGTTCATCC	
Tn7seqF	GAGCGCTTTTGAAGCTGATGTGCT	Forward sequencing primer annealing within Tn*7R*
gfpseqR	GATGACGGGAACTACAAGACACGT	Reverse sequencing primer annealing within *gfp*
kanseqR	ATCGCCTTCTATCGCCTTCTTGAC	Reverse sequencing primer annealing within *nptII*
GFLseq1	ACGGTGAAAACCTCTGACACATGC	Forward sequencing primers for sequencing pGFLIP by primer walking
GFLseq2	CTGAAATCAGTCCAGTTATGCTGTG	
GFLseq3	AAATCCGCCGCTAGGAGCTT	
GFLseq4	GTCTGCCATGATGTATACATTGTGTG	
GFLseq5	GGGACAACTCCAGTGAAAAGTTCTTC	
GFLseq6	CTGATGCTCTTCGTCCAGATCATC	
GFLseq7	CCTGCGTGCAATCCATCTTGTTCA	
GFLseq8	CAGGGGATCTTGAAGTTCCTATTCCG	
GFLseq9	GCAACAATTCTGGAAGCCTCATT	
GFLseq10	TCTTTAGCGCAAGGGGTAGGATCG	
GFLseq11	TCCAATTGAGGAGTGGCAGCAT	
GFLseq12	TATCAGAGCTTATCGGCCAGCCT	
GFLseq13	ATAAAGATACCAGGCGTTTCCCCC	
GFLseq14	AAACAAACCACCGCTGGTAGC	
GFLseq15	CGCAGAAGTGGTCCTGCAACTTTA	
GFLseq16	CCGCGCCACATAGCAGAACTTTAA	
gflpmcsF	GTTGACAAAGGGAATCAGGGGATC	Forward sequencing primer for inserts in the MCS
gflpmcsR	GAACTGGGTGTAGCGTCGTAAGCT	Reverse sequencing primer for inserts in the MCS
glmSF	CAGCTGCTGTCGTACCACACGG	Forward primer to confirm Tn*7* insertion at *glmS* by PCR
cyaA_F	ATTATAGAGCTCTGCGAGCAGATGCAC	Forward and reverse primers to amplify P_{cyaA} from RB50 and BPSM
cyaA_R	TATAATGGTACCGTGGATCTGTCGATAAGTAG	
ptxprF	AGCTTCGAGCTCCAAGATAATCGTCCTGCTC	Forward and reverse primers to amplify P_{ptxA} from BPSM
ptxprR	ATATATGGGCCCTCCCGTCTTCCCCTCT	
fhaprF2	AGGCCTGAGCTCGATAAGAAGAATATGCTT	Forward and reverse primers to amplify P_{fhaB} from RB50
fhaprR2	ATATTCGGTACCATTCCGACCAGCGAAGTG	
flaA_F	AATGAGCTCGCCGTGCTCAACGTCA	Forward and reverse primers to amplify P_{flaA} from RB50
flaA_R	ATTATAGGTACCAGGCTCCCAAGAGAGAAA	

[a] The underlined sequences represent restriction enzyme cut sites.

of Km^r upon promoter activation, Km (100 μg/ml) was added to some plates. Plates were incubated for 48 h at 37°C, and white-light and fluorescent images were obtained using the G:BOX Chemi system.

For kinetic assays, strains containing pGFLIP were grown overnight with Km selection in SS under promoter-inactive conditions (20 mM $MgSO_4$ for RB50*cyaA*FLP, RB50*cyaA*$_{Bp}$FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP; no added $MgSO_4$ for RB50*flaA*FLP). Cells were washed twice in PBS with or without 5 mM $MgSO_4$ to prevent the premature activation of Bvg$^+$ phase and *flaA* promoters, respectively, and were added to fresh SS medium under promoter-active conditions (20 mM $MgSO_4$ for RB50*flaA*FLP; no added $MgSO_4$ for RB50*cyaA*FLP, RB50*cyaA*$_{Bp}$FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP) at an optical density at 600 nm (OD_{600}) that was equivalent to 1×10^9 CFU/ml. Tubes were incubated on a roller at 37°C for 8 h. At each time point, an aliquot of cells was removed, diluted in phosphate-buffered saline (PBS) under promoter-inactive conditions, and plated on BG agar under promoter-inactive conditions. Plates were incubated for 48 h at 37°C, and total CFU were enumerated. For kinetic assays with *B. pertussis*, cultures were inoculated at the CFU/ml corresponding to an OD_{600} of ~0.1, and cells were grown overnight in SS containing 50 mM $MgSO_4$, 100 μg/ml Km, and 1 mg/ml heptakis. Cells were washed in PBS containing 25 mM $MgSO_4$ to prevent premature *flp* activation. All other aspects of the assay were the same as for *B. bronchiseptica*, except that dilutions were plated on BG agar containing 50 mM $MgSO_4$ and plates were incubated 4 days at 37°C. GFP fluorescence was quantified using the G:BOX Chemi imaging system as described above. The percent resolution (the ratio of GFP$^-$ colonies to the total number of colonies × 100%) was calculated for each strain at each time point.

Evaluation of pGFLIP in a murine model of infection. Five- to 7-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intranasally with 1×10^5 CFU of *B. bronchiseptica* pGFLIP strains in 50 μl of PBS. For infections with RB50*cyaA*FLP, RB50*cyaA*$_{Bp}$FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP, bacteria were grown overnight in SS medium containing Km (100 μg/ml) and 20 mM $MgSO_4$ to maintain cells in the Bvg$^-$ phase. For infections with RB50*flaA*FLP, bacteria were grown overnight in SS media containing Km (100 μg/ml) to maintain cells in the Bvg$^+$ phase. Lungs were harvested from infected mice at 1 h and 30 h postinoculation (p.i.). For RB50*flaA*FLP, right lungs were homogenized in 1 ml of PBS, diluted in PBS, and plated in duplicate on BG agar. For strains containing Bvg$^+$ phase promoters in pGFLIP, homogenization, dilution, and plating were carried out in the presence of 20 mM $MgSO_4$. Percent resolution was calculated for each strain at each time point. This study was done in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Our protocol was approved by the University of North Carolina IACUC (protocol 12-307.0). All animals were properly anesthetized for inoculations, monitored regularly, and euthanized when moribund, and efforts were made to minimize suffering.

Statistical analyses. Statistical analyses were performed using Prism 5 (GraphPad Software, Inc.). Statistical significance was determined using the unpaired Student's *t* test or analysis of variance (ANOVA). Images

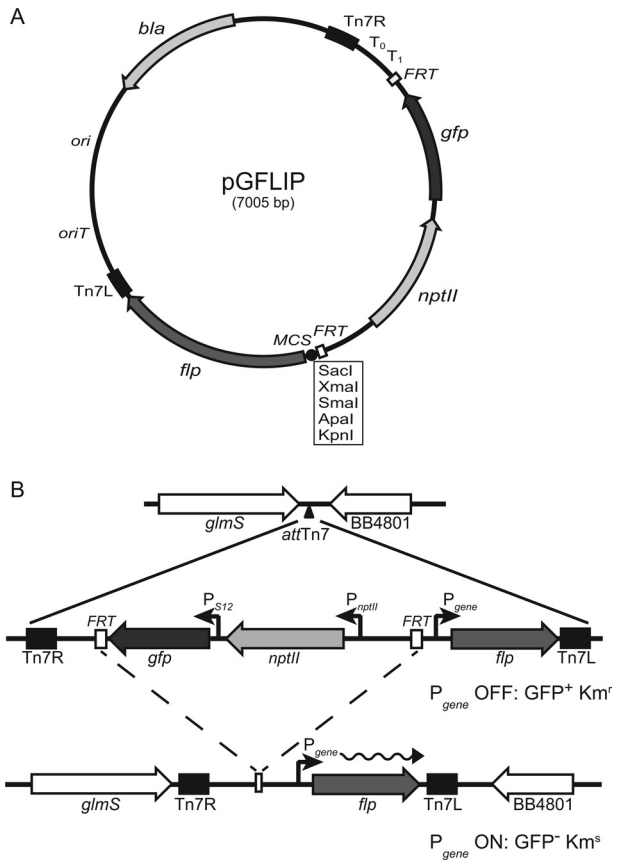

FIG 1 Design and mechanism of pGFLIP. (A) Diagram of pGFLIP. Tn*7R* and Tn*7L*, left and right ends of the Tn*7* transposon, respectively; T_0 and T_1, bacteriophage λ and *E. coli rrnB* transcriptional terminators, respectively; *FRT*, Flp recombinase target; *gfp*, green fluorescent protein gene; *nptII*, neomycin phosphotransferase gene; MCS, multiple cloning site with restriction sites indicated; *flp*, Flp recombinase gene; *oriT*, origin of conjugative transfer; *ori*, ColE1 origin of replication; and *bla*, β-lactamase gene. (B) Schematic illustration of Tn*7*-mediated delivery and Flp-mediated excision of pGFLIP in RB50. The region of pGFLIP flanked by Tn*7L* and Tn*7R* sequences is delivered to the *att*Tn*7* site located between *glmS* and BB4801. While the promoter driving expression of the gene of interest (P_{gene}) remains inactive, *gfp* and *nptII* are expressed constitutively, resulting in fluorescent and Km^r bacteria. When P_{gene} is activated, *flp* is expressed and Flp recombinase mediates site-specific recombination between *FRT* sites, permanently excising *gfp* and *nptII* and yielding bacteria that are nonfluorescent and sensitive to Km.

were formatted using Adobe Photoshop CS5, and figures were generated using Adobe Illustrator CS5 (Adobe Systems, Inc.).

RESULTS

Design and construction of pGFLIP. To study transcription activation of genes both *in vitro* and *in vivo*, we engineered a reporter system, pGFLIP, that provides both fluorescent and selectable markers. We designed our system for simplicity and ease of use. Therefore, all of the components necessary for pGFLIP function are contained on a single plasmid. The region of pGFLIP delivered to the chromosome includes *gfp* and *nptII*, each under the control of a strong constitutive promoter and together flanked by *FRT* sequences (Fig. 1A). The Flp recombinase gene *flp* is present in the opposite orientation from *gfp* and *nptII* with an immediate 5′ MCS to facilitate the insertion of a promoter of interest. In the absence of promoter activity, *flp* is not transcribed and cells remain GFP^+ and Km^r. When the promoter of interest is activated, *flp* is transcribed and the gene product mediates recombination between *FRT* sites, permanently excising *gfp* and *nptII*.

To test the system, we cloned promoter regions from *B. bronchiseptica* and *B. pertussis* into the MCS to generate the strains described in Table 2. The region of pGFLIP between the Tn*7L* and Tn*7R* ends was delivered to the *att*Tn*7* site 3′ to the *glmS* gene in *Bordetella* spp. as described in Materials and Methods. Transcription terminators are present near the 5′ end of the transposon to prevent transcription readthrough from *glmS*. Delivery of promoterless pGFLIP to *B. bronchiseptica* RB50 and *B. pertussis* BPSM resulted in strains that were stably GFP^+ and Km^r. These strains did not lose GFP fluorescence or Km^r when passaged multiple times *in vitro* in the absence of selection (data not shown).

Functional evaluation of pGFLIP *in vitro*. To evaluate the functionality of pGFLIP, we used two *B. bronchiseptica* promoters for which activity can readily be induced *in vitro*: P_{cyaA} and P_{flaA}. In *B. bronchiseptica*, the *flaA* promoter is highly transcribed in the Bvg^- phase, while the *cyaA* promoter is transcribed strongly in the Bvg^+ phase (9, 10, 15, 22, 33). Therefore, RB50*flaA*FLP was constructed under Bvg^+ phase conditions to prevent expression of *flaA*, while RB50*cyaA*FLP was maintained under Bvg^- phase conditions to prevent expression of *cyaA*. Km was added to the media during construction of these strains to select against any bacteria that had activated *flp*, thus ensuring that the population only contained GFP^+ and Km^r cells. To test these strains for pGFLIP functionality, we suspended a single GFP^+ colony of each strain in PBS and plated on BG, BG-Km, BG-$MgSO_4$, and BG-Km-$MgSO_4$ agar and observed colony formation and GFP fluorescence after two (*B. bronchiseptica*) or four (*B. pertussis*) days of incubation at 37°C.

When plated under promoter-active conditions (20 mM $MgSO_4$ for RB50*flaA*FLP; no added $MgSO_4$ for RB50*cyaA*FLP) in the absence of Km, all RB50*flaA*FLP and RB50*cyaA*FLP colonies examined had lost fluorescence, and when tested subsequently for growth on agar containing Km, all colonies had lost Km^r (Fig. 2A and D). As expected, plating either strain under promoter-active conditions and in the presence of Km resulted in a lack of growth due to loss of *nptII* from the chromosome (Fig. 2E and H). Conversely, when plated under promoter-inactive conditions (no added $MgSO_4$ for RB50*flaA*FLP; 20 mM $MgSO_4$ for RB50*cyaA*FLP) in the presence of Km, all RB50*flaA*FLP and RB50*cyaA*FLP colonies maintained fluorescence (Fig. 2F and G). Likewise, under promoter-inactive conditions in the absence of Km, all RB50*flaA*FLP colonies maintained fluorescence (Fig. 2B), indicating that *flaA* is not expressed under these conditions. Unexpectedly, however, when RB50*cyaA*FLP was plated under promoter-inactive conditions in the absence of Km selection, approximately 14% of the colonies had lost fluorescence at the time of imaging (Fig. 2C). These colonies did not grow when restreaked onto BG-Km-$MgSO_4$, indicating that cells in these colonies were not *gfp* mutants but had lost fluorescence and Km^r due to Flp-mediated recombination. These results suggest that the transcription activity of P_{cyaA} under Bvg^- phase conditions *in vitro* is close to the threshold level of *flp* transcription required for Flp-mediated recombination between *FRT* sites. These plate-based assays for fluorescence and Km^r demonstrated that pGFLIP is indeed functional and that both markers (*gfp* and *nptII*) can be used to document promoter activation under active and inactive conditions.

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
DH5α	Molecular cloning strain	28
RHO3	Conjugation strain; Km^s Δ*asd* Δ*aphA*	29
Bordetella		
RB50	Wild-type *B. bronchiseptica* strain; Sm^r	7
RB52	RB50 containing *bvgAS* from BP338; Sm^r	22
BPSM	Sm^r Tohama I derivative	29, 32
RB50FLP	RB50 with promoterless pGFLIP integrated at *att*Tn*7*	This study
RB50*cyaA*FLP	RB50 with *flp* recombinase driven by P_{cyaA} integrated at *att*Tn*7*	This study
RB50$cyaA_{Bp}$FLP	RB50 with *flp* recombinase driven by P_{cyaA} from Tohama I integrated at *att*Tn*7*	This study
RB52$cyaA_{Bb}$FLP	RB52 with *flp* recombinase driven by P_{cyaA} from RB50 integrated at *att*Tn*7*	This study
RB52$cyaA_{Bp}$FLP	RB52 with *flp* recombinase driven by P_{cyaA} from Tohama I integrated at *att*Tn*7*	This study
RB50*fhaB*FLP	RB50 with *flp* recombinase driven by P_{fhaB} from RB50 integrated at *att*Tn*7*	This study
RB50*ptxA*FLP	RB50 with *flp* recombinase driven by P_{ptxA} from Tohama I integrated at *att*Tn*7*	This study
RB50*flaA*FLP	RB50 with *flp* recombinase driven by P_{flaA} integrated at *att*Tn*7*	This study
BPSMFLP	BPSM with promoterless pGFLIP integrated at *att*Tn7	This study
BPSM*cyaA*FLP	BPSM with *flp* recombinase driven by P_{cyaA} from RB50 integrated at *att*Tn7	This study
BPSM*fhaB*FLP	BPSM with *flp* recombinase driven by P_{fhaB} from RB50 integrated at *att*Tn7	This study
BPSM*ptxA*FLP	BPSM with *flp* recombinase driven by P_{ptxA} integrated at *att*Tn7	This study
Plasmids		
pUC18T-mini-Tn7T-Km-*FRT*	Mobilizable transposition vector; Ap^r Km^r	30
pFLPe4	Site-specific excision vector, source of Flp recombinase; Ap^r Km^r	30
mini-Tn7-*kan-gfp*	Mobilizable transposition vector, source of *gfp* driven by PS12; Km^r	31
pTNS3	Tn*7* transposase expression vector containing *tnsABCD*; Ap^r	30
pGFLIP	pUC18-based vector with P_{S12}-*gfp* and *nptII* flanked by *FRT* sequences and *flp* 3′ to the MCS; Ap^r Km^r	This study
pGFLIP-P_{cyaA}	pGFLIP with *flp* recombinase driven by the RB50 *cyaA* promoter; Ap^r Km^{ra}	This study
pGFLIP-P_{cyaABp}	pGFLIP with *flp* recombinase driven by the BPSM *cyaA* promoter; Ap^r Km^{ra}	This study
pGFLIP-P_{fhaB}	pGFLIP with *flp* recombinase driven by the RB50 *fhaB* promoter; Ap^r Km^{ra}	This study
pGFLIP-P_{ptxA}	pGFLIP with *flp* recombinase driven by the BPSM *ptxA* promoter; Ap^r Km^{ra}	This study
pGFLIP-P_{flaA}	pGFLIP with *flp* recombinase driven by the RB50 *flaA* promoter; Ap^r Km^{ra}	This study

[a] Km^r only under promoter-inactive conditions.

Kinetic analysis of *Bordetella* gene activation using pGFLIP. We next assessed the ability of this system to monitor gene activation over time in bacteria grown in liquid culture. Strains containing pGFLIP plasmids with *cyaA*, *fhaB*, *ptxA*, and *flaA* promoters were grown overnight under promoter-inactive conditions and switched to promoter-active conditions, and the percent resolution (i.e., loss of GFP fluorescence and Km^r) was calculated over 8 h. For the first 4 h after switching from Bvg^+ phase to Bvg^- phase conditions, the percentage of RB50*flaA*FLP colonies that had lost fluorescence remained below 20% (Fig. 3A), indicating that less than 20% of the bacteria had reached or surpassed the threshold of *flp* expression to result in recombination during this time. Approximately 75% of cells had lost fluorescence at 6 h, and this proportion was maintained at 8 h (Fig. 3A). When grown under Bvg^- phase conditions for 24 h, more than 85% of cells demonstrated a loss of fluorescence and Km^r (data not shown). The fact that resolution did not reach 100% suggests that the maximum level of *flaA* expression under *in vitro* Bvg^- phase conditions (SS medium containing 20 mM $MgSO_4$) is just at or above the threshold of *flp* expression needed for Flp-mediated recombination between *FRT* sites. The relatively long amount of time required for the majority of RB50*flaA*FLP cells to cross the threshold of *flp* expression is in agreement with a previous study that examined the expression kinetics of the BvgAS-regulated operon *frlAB*, which encodes the *E. coli* FlhDC homologs FrlAB that are at the top of the flagellum transcription cascade in *B. bronchiseptica* (16). Upon shifting from Bvg^+ phase to Bvg^- phase conditions, a *frlAB-lacZ* fusion strain exhibited a gradual increase in β-galactosidase activity over time, reaching approximately 40% of the maximum overnight expression level by 7.5 h (15).

In contrast, when RB50*fhaB*FLP cells were switched from Bvg^- phase to Bvg^+ phase conditions, the *fhaB* promoter was apparently activated as early as 1 h, at which time approximately 75% of cells had lost fluorescence (Fig. 3A). At 2 h and later time points, nearly 100% of RB50*fhaB*FLP cells had lost fluorescence, consistent with the classification of *fhaB* as a class 2 gene requiring a relatively low level of BvgA∼P for activation. As a canonical class 1 (or "late") gene, *ptxA* had only been activated in approximately 8% of cells at 1 h (Fig. 3A). Approximately 20% of cells had activated *ptxA* by 4 h, increasing to 90% of cells by 8 h. Surprisingly, *cyaA*, which is also considered to be a class 1 gene, was activated in over 85% of cells at 1 h, and in more than 95% of cells at 2 h and later time points (Fig. 3A). These data suggest that at early time points after switching to promoter-active conditions, the *cyaA*

20 mM $MgSO_4$	Promoter activity
+	*flaA* **ON**, *cyaA* **OFF**
–	*flaA* **OFF**, *cyaA* **ON**

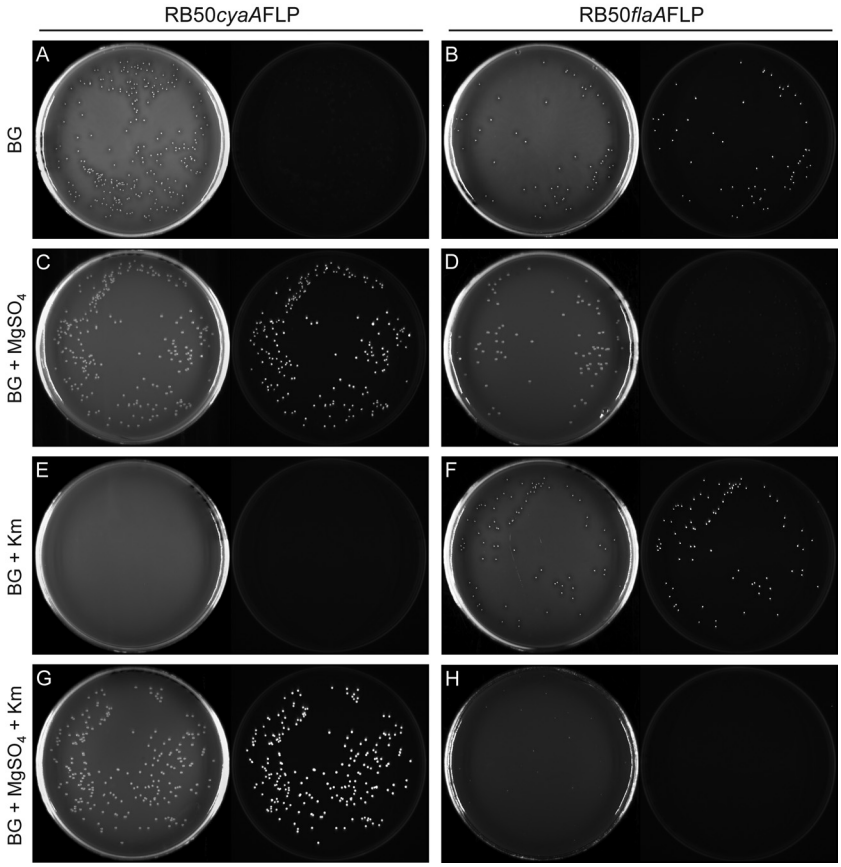

FIG 2 Plate-based validation of the pGFLIP reporter system using P_{cyaA} and P_{flaA}. Left column, images of RB50*cyaA*FLP plated under Bvg^+ phase (A) and Bvg^- (C) phase conditions (achieved by supplementing BG agar with 20 mM $MgSO_4$) in the presence (E) or absence (G) of 100 μg/ml Km. Right column, images of RB50*flaA*FLP plated under Bvg^+ phase (B) and Bvg^- (D) phase conditions in the presence (F) or absence (H) of 100 μg/ml Km. White light photographs are on the left and fluorescent images are on the right in each pair of images.

promoter is activated to a level sufficient to drive *flp*-mediated recombination. This result was unexpected based on *cyaA* transcription results in studies of *B. pertussis* (5, 11, 18, 23, 24).

At the initiation of each kinetic experiment, we observed a consistent background resolution (i.e., the percent resolution at 0 h) for each Bvg^+ phase promoter in pGFLIP. RB50*cyaA*FLP displayed the highest background resolution at 8.9%, while the background resolution for *fhaB* and that for *ptxA* were significantly lower at 1.54% and 1.99%, respectively ($P < 0.01$). The background resolution for RB50*flaA*FLP was the lowest of the pGFLIP constructs at 0.23% (Fig. 3A). The fact that a portion of RB50*cyaA*FLP cells had lost fluorescence at 0 h suggests that low-level expression of *cyaA* might be occurring in a BvgAS-independent manner, which could interfere with measuring BvgAS-dependent P_{cyaA} activation over time. Therefore, to test the sensitivity of pGFLIP to background resolution, we grew RB50*cyaA*FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP strains overnight as shown in Fig. 3A, washed the cells to remove Km, and maintained the cultures under Bvg^- conditions in the absence of selection for 8 h. At 0, 1, 4, and 8 h, the percent resolution was determined for each strain. We hypothesized that if P_{cyaA} was indeed active under Bvg^- conditions, we would observe a steady increase in resolution over time for RB50*cyaA*FLP but not for RB50*fhaB*FLP or RB50*ptxA*FLP. As expected, RB50*cyaA*FLP displayed the greatest background resolution at 11.9%, increasing to 18.1% at 8 h (Fig. 3B). Loss of fluorescence for RB50*fhaB*FLP and RB50*ptxA*FLP remained essentially unchanged over 8 h, averaging 0.22% and 5.4%, respectively (Fig. 3B). Although *cyaA* had been activated in approximately one-fifth of the population at 8 h, these data do not account for the >85% resolution observed for RB50*cyaA*FLP as early as 1 h following the switch to Bvg^+ phase conditions.

Neither the *B. bronchiseptica bvgAS* allele nor P_{cyaA} accounts for the unexpectedly early activation of *cyaA*. As *cyaA* expression in RB50 was activated unexpectedly early compared to what has been observed for *B. pertussis* (5, 11, 18, 23, 24), we sought to determine whether differences in the *cyaA* promoter or in the *bvgAS* allele between *B. bronchiseptica* and *B. pertussis* accounted for this difference. There are five single nucleotide changes and one nucleotide insertion in the sequence 5′ to *cyaA* in *B. bron-*

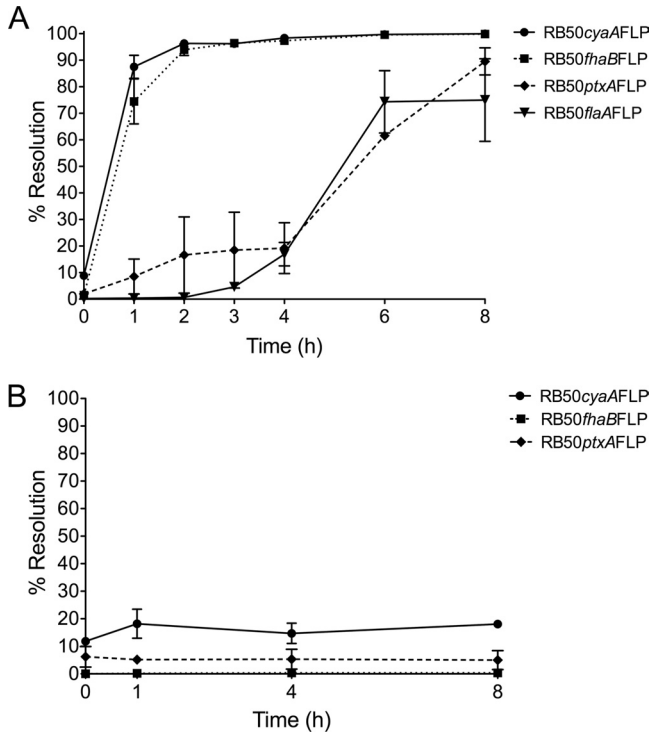

FIG 3 Kinetic analysis of *Bordetella* gene activation *in vitro*. *B. bronchiseptica* strains grown under promoter-off conditions and in the presence of 100 μg/ml Km were washed and placed in fresh SS medium under promoter-on conditions (A) or promoter-off conditions (B). Colonies arising from aliquots plated over 8 h were monitored for loss of fluorescence, and the percent resolution was calculated. Results are the means ± standard errors of the means (SEM) for experiments performed in duplicate or triplicate.

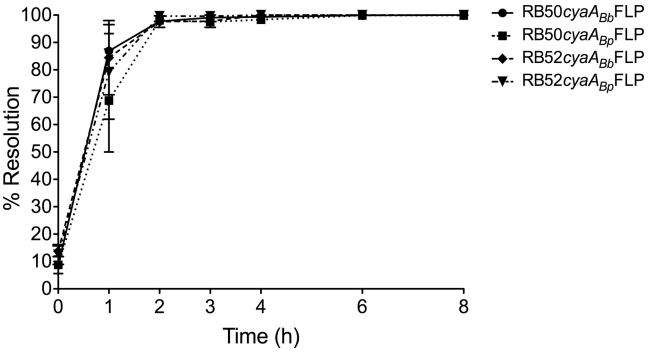

FIG 4 Effect of P_{cyaA} and *bvgAS* alleles on *cyaA* activation in *B. bronchiseptica*. RB50 and RB52, an RB50 derivative carrying the *bvgAS* allele from *B. pertussis* BP338 in place of the native *bvgAS* allele, each with pGFLIP containing RB50 and BPSM P_{cyaA}, were grown as shown in Fig. 3 and were switched to promoter-on conditions for 8 h. Loss of fluorescence was calculated for each strain as described in Materials and Methods. Results are the means ± SEM for experiments performed in duplicate.

chiseptica RB50 compared to *B. pertussis* BPSM. We hypothesized that replacing the *cyaA* promoter of RB50 with that of BPSM would delay the activation of P_{cyaA} relative to P_{fhaB}, similar to what was observed by Veal-Carr et al. (24). We cloned the *cyaA* promoter from *B. pertussis* BPSM into pGFLIP and introduced this plasmid into RB50, generating strain RB50*cyaA*$_{Bp}$FLP. When evaluated in the kinetic assay, there was no difference in the rate of resolution between RB50*cyaA*FLP and RB50*cyaA*$_{Bp}$FLP, suggesting that differences in the *cyaA* promoter do not account for the rapid resolution observed in *B. bronchiseptica* (Fig. 4).

Some strains of *B. pertussis*, such as BP338 and BPSM, exhibit decreased sensitivity to chemical modulation compared to *B. bronchiseptica* RB50 due to amino acid differences in BvgS, which causes these strains to remain in the Bvg^+ phase at a lower concentration of modulator than RB50 (22). We hypothesized, therefore, that the *bvgAS* allele from BP338 would permit activation of *cyaA* more quickly and in a greater percentage of cells than the *B. bronchiseptica* RB50 *bvgAS* allele. To assess this, we utilized strain RB52, which contains the entire *bvgAS* locus and *bvgA* promoter from *B. pertussis* BP338 in place of *bvgAS* in RB50 (22). RB52 recapitulates the decreased sensitivity to modulation characteristic of both BP338 and BPSM, requiring ≥40 mM $MgSO_4$ to modulate to the Bvg^- phase, in contrast to RB50, which requires ≥10 mM $MgSO_4$ to fully modulate (15, 22). We introduced pGFLIP containing the RB50 *cyaA* promoter driving *flp* expression into RB52, producing strain RB52*cyaA*$_{Bb}$FLP. We evaluated the kinetics of *cyaA* activation in RB52*cyaA*$_{Bb}$FLP compared to RB50*cyaA*FLP and did not observe any difference. Likewise, when we evaluated an RB52 derivative containing the BPSM *cyaA* promoter driving *flp* expression (RB52*cyaA*$_{Bp}$FLP) in the kinetic assay, there was no impact on *cyaA* activation compared to RB50*cyaA*FLP. Together, these data suggest that neither the *cyaA* promoter nor the *bvgAS* allele significantly affects the kinetics of *cyaA* activation in *B. bronchiseptica* as reported by the P_{cyaA}-*flp* promoter fusion in pGFLIP (Fig. 4).

Evaluation of *Bordetella* promoter activation *in vivo*. Although the kinetics of Bvg^+ phase gene activation *in vivo* have been examined for *B. pertussis* (24), these experiments have not been done in *B. bronchiseptica* or in the context of a natural bacterium-host interaction. To address BvgAS-regulated gene activation in *B. bronchiseptica in vivo*, we infected BALB/c mice intranasally with 1×10^5 CFU of the RB50 pGFLIP strains shown in Fig. 3A, grown under promoter-inactive conditions (100 μg/ml Km, 20 mM $MgSO_4$). Mice were sacrificed and lungs were harvested at 1 h and 30 h p.i. Lungs were homogenized and dilutions were plated on BG agar containing 20 mM $MgSO_4$ to prevent further recombination. Total CFU were enumerated, and the percentage of colonies that had lost fluorescence (percent resolution) was calculated for each strain and time point. The percentage of RB50*fhaB*FLP that had lost fluorescence at 1 h was 95.3% ± 0.60%, while only 5.4% ± 0.69% of RB50*ptxA*FLP had lost fluorescence at this time (Fig. 5). Similar to the *in vitro* kinetic assay (Fig. 3A), the majority (85.3% ± 3.3%) of RB50*cyaA*FLP cells had lost fluorescence at 1 h. At 30 h p.i., essentially all cells had lost fluorescence, indicating that the environment in the mouse induces the expression of Bvg^+ phase genes to a level at or above that required to activate *flp* expression (Fig. 5). The fact that the pGFLIP system functions *in vivo* much the same as *in vitro* when switched from Bvg^- to Bvg^+ phase conditions suggests that the pattern of Bvg^+ phase gene activation is similar between these two conditions and that the unexpectedly early expression from the *cyaA* promoter *in vitro* was not due to an artifact of that assay.

Although all evidence thus far suggests that the Bvg^+ phase is necessary and sufficient for *Bordetella* spp. to cause respiratory infection in rats and mice (6, 7, 22, 34), it is possible that rare *in vivo* environments exist that induce modulation to the Bvg^- phase. Therefore, using the activation of the *flaA* promoter as an

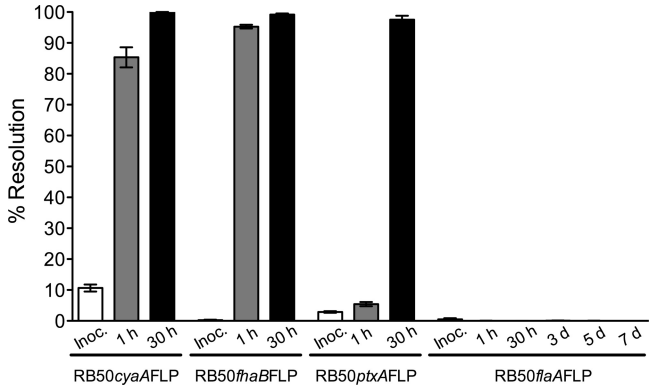

FIG 5 Analysis of *Bordetella* gene activation *in vivo* using pGFLIP. RB50*cyaA*FLP, RB50*fhaB*FLP, RB50*ptxA*FLP, and RB50*flaA*FLP were grown as shown in Fig. 3, and 1×10^5 CFU was inoculated intranasally into mice in a total volume of 50 μl. Mice were sacrificed at 0, 1, and 30 h p.i., with additional time points at 3, 5, and 7 days for RB50*flaA*FLP, and lungs were homogenized and plated on BG agar supplemented with 20 mM $MgSO_4$ and Sm (for RB50*cyaA*FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP) or on BG agar supplemented only with Sm (for RB50*flaA*FLP). Loss of fluorescence was calculated for each strain as described in Materials and Methods. Results are the means ± SEM for experiments performed in duplicate with three mice per time point.

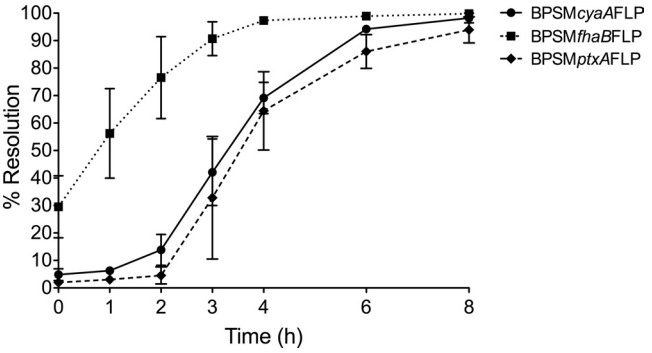

FIG 6 Kinetic analysis of *Bordetella* gene activation *in vitro* in *B. pertussis*. Strains grown under promoter-off conditions and in the presence of 100 μg/ml Km were washed and placed in fresh SS medium under promoter-on conditions. Colonies arising from aliquots plated over 8 h were monitored for loss of fluorescence, and the percent resolution was calculated. Results are the means ± SEM for experiments performed in triplicate.

indicator of the Bvg^- phase, we infected mice as described above and evaluated the loss of fluorescence at 1 h and 30 h p.i. We did not observe activation of *flaA* in any RB50*flaA*FLP cells at 1 h, 30 h, 3 days, 5 days, or 7 days (Fig. 5). These data suggest that *B. bronchiseptica* does not enter the Bvg^- phase in the mouse during the time period that we examined.

Kinetic analysis of *Bordetella* gene activation in *B. pertussis* reveals delayed activation of *cyaA* compared to *B. bronchiseptica*. Due to the observation that neither the *cyaA* promoter nor the *bvgAS* allele affects the kinetics of *cyaA* activation in *B. bronchiseptica*, we hypothesized that additional, species-specific factors account for the differences in the activation of *cyaA*. To evaluate this possibility, we delivered the same pGFLIP plasmids as those used in *B. bronchiseptica* to the chromosome of *B. pertussis* BPSM and evaluated loss of fluorescence in the kinetic assay. We reasoned that if the activation of *cyaA*, *fhaB*, and *ptxA* in *B. pertussis* resembled the pattern seen in *B. bronchiseptica*, then the discrepancy with the study by Veal-Carr et al. (24) was likely due to differences in recombinase expression sensitivity between pGFLIP and RIVET (i.e., Flp recombinase is activated at a lower threshold of P_{cyaA} activity than TnpR, making *cyaA* appear to be activated sooner in the Flp-*FRT* system). Alternatively, if activation of *cyaA*, *fhaB*, and *ptxA* was canonical as in Veal-Carr et al. (24), then the difference in *cyaA* activation would be attributed to species-specific gene regulation.

In contrast to what we observed in *B. bronchiseptica*, the proportion of BPSM*cyaA*FLP that had lost fluorescence at 1 h was less than 10%, essentially equal to the level of background resolution (Fig. 6). There was a steady loss of fluorescence in BPSM*cyaA*FLP beginning at 2 h postswitch and continuing until 6 h, at which time more than 90% of colonies had lost fluorescence. The pattern of P_{cyaA} activation in *B. pertussis* matched that of P_{ptxA}, which displayed similar but somewhat earlier activation in *B. pertussis* compared to *B. bronchiseptica* (Fig. 6). Interestingly, the loss of fluorescence in BPSM*fhaB*FLP occurred more slowly than in RB50*fhaB*FLP, with approximately 55% of cells having lost fluorescence at 1 h. At 2 h postswitch, the proportion of GFP^- BPSM*fhaB*FLP equaled that of RB50*fhaB*FLP at 1 h, indicating that the activation of P_{fhaB} may be delayed in *B. pertussis* compared to *B. bronchiseptica*; however, the background resolution for BPSM*fhaB*FLP (29.5% ± 11.3%) was substantially higher than RB50*fhaB*FLP. At 1.99%, the background resolution for BPSM*ptxA*FLP was identical to that seen for RB50*ptxA*FLP, while the background for BPSM*cyaA*FLP (4.8% ± 2.2%) was not significantly different compared to RB50*cyaA*FLP (Fig. 6).

DISCUSSION

Evaluating bacterial gene expression within the host is critical for understanding the complex host-pathogen interactions that result in disease, clearance, or asymptomatic colonization. One approach that has been developed to evaluate gene expression in the host is *in vivo* expression technology (IVET) (35). In IVET, promoter sequences are cloned 5′ to a gene that either confers resistance to an antibiotic or complements a specific auxotrophy (35–37). Under conditions in which the promoter is active, these genes are expressed and permit the survival of bacteria in hosts that have either been dosed with the relevant antibiotic (for antibiotic resistance-based selection) or naturally lack the ability to complement the auxotrophy (for auxotrophy-based selection) (35). A drawback of using IVET is that promoters that are activated transiently or at a low level may not be identified due to insufficient production of the missing survival factor (35). In RIVET, a modification of IVET, a promoter of interest drives the expression of a site-specific recombinase that irreversibly excises a genetic marker, often an antibiotic resistance gene (24, 35, 38). By selective plating, it is possible to determine if the promoter of interest was active at some time during infection. Unlike IVET, RIVET permits the detection of transiently or weakly expressed genes because the recombinase-mediated loss of a marker need only occur once to document promoter activation (35).

pGFLIP is a pUC18-based plasmid that, while conceptually similar to RIVET, possesses several advantages over this well-characterized genetic tool. The Tn*7* transposon system specifically delivers sequences to the *att*Tn*7* site located 3′ to the highly conserved *glmS* gene, which provides pGFLIP with an especially broad host range that includes many Gram-negative bacteria (39). As a result of this specific recombination at the *att*Tn*7* site, only one

integration event is required for all components of pGFLIP to be delivered in single copy to the bacterial chromosome. Single-copy delivery eliminates potential gene dosage issues inherent to multicopy plasmid systems, and integration at the *att*Tn*7* site does not disturb the native locus of the gene to be tested. Once delivered, a Tn*7* transposon is stable in the absence of selection, unlike suicide plasmids that have been integrated into the chromosome via single-crossover homologous recombination, which can spontaneously resolve in the absence of selection. In contrast with other published systems, pGFLIP also possesses two markers—*gfp* and *nptII*—allowing either the loss of fluorescence or Km^r to indicate promoter activation.

In this study, we used pGFLIP to analyze the transcription activation of Bvg-regulated genes in both *B. bronchiseptica* RB50 and *B. pertussis* BPSM. Our results showed that, *in vitro*, *fhaB* and *ptxA* promoters were activated early and late, respectively, following a switch from Bvg^- to Bvg^+ phase conditions, which is in agreement with previous reports for both *Bordetella* species (15, 23, 24). However, the *cyaA* promoter was activated unexpectedly early in our assay; these results appear to stand in contradiction to the established view that *cyaA* is transcribed solely as a late gene. Using *B. bronchiseptica* RB50 in a BALB/c mouse model, we found that the pattern of gene activation for *cyaA*, *fhaB*, and *ptxA* was nearly identical to that observed *in vitro* at early time points. Veal-Carr et al. reported similar agreement between *in vitro* and *in vivo* gene activation patterns using RIVET (24), although in that study, maximal activation of *fhaB*, *cyaA*, and *prn* occurred over a much greater time scale (approximately 24 h to full activation), likely a result of differences in sensitivity between the TnpR-*res* and Flp-*FRT* systems. Our use of pGFLIP to evaluate gene activation in *B. bronchiseptica* is both the first kinetic analysis of P_{cyaA} and the first *in vivo* kinetic analysis of any promoter to be reported for this organism.

Although we expected P_{cyaA} to behave like a class 1 promoter in *B. bronchiseptica*, based on gene activation and expression data obtained by us and others for *B. pertussis* (5, 11, 18, 23, 24), our data nevertheless suggest that P_{cyaA} acts more like a class 2 promoter in this organism. However, it is not necessarily the case that *cyaA* reaches maximal expression at 1 to 2 h following a switch from Bvg^- phase to Bvg^+ phase; it is possible that *cyaA* activation occurs in a stepwise manner, i.e., P_{cyaA} may rapidly reach a level of expression necessary to activate *flp* transcription in our system, but may not reach maximal expression until much later. This scenario would account for the apparently rapid activation of P_{cyaA} without requiring a bacterium to be producing and secreting a significant amount of ACT immediately upon switching to nonmodulating conditions. Evidence exists to support *cyaA* activation and ACT production, albeit at a reduced level, in the Bvg^i phase, as strains RB50i and RB53i are slightly hemolytic on BG agar (a consequence of the hemolysin activity of ACT), and RB53i produces measurable levels of *cyaA* transcript (9). We were able to determine that neither differences in the *cyaA* promoter nor differences in the *bvgAS* allele between the two species accounted for the difference in *cyaA* activation (Fig. 4), suggesting that other (potentially Bvg-independent) factors may be influencing *cyaA* activation in *B. bronchiseptica* compared to *B. pertussis*.

In *B. pertussis* BPSM, P_{cyaA} demonstrated an activation pattern consistent with both indirect (RIVET) and direct (RNA hybridization) assessments, reinforcing the conclusion that *cyaA* activation is indeed different between RB50 and BPSM (23, 24). Given the differences in host range and the ability of *B. bronchiseptica* to survive outside a host (7, 9, 10, 34), it is possible that the relatively early activation of *cyaA* in *B. bronchiseptica* is advantageous during the establishment of infection in a mammalian host or in transmission between hosts. Previous studies have shown that RB50 *cyaA* deletion mutants are more susceptible to clearance from the mouse respiratory tract, presumably as a result of neutrophil-mediated killing, and that ACT may interact with FHA (an "early" Bvg^+ gene) on the cell surface and modulate cytokine production by the host (40, 41). All strains of *B. bronchiseptica* that have been tested show the same modulation characteristics *in vitro* (i.e., the same relatively low concentration of modulator is required for inducing the Bvg^- phase), while *B. pertussis* isolates exhibit variable resistance to modulation at concentrations of modulator equal to or greater than for *B. bronchiseptica* (22). These observations suggest that early expression of *cyaA* may not be detrimental to human-adapted *B. pertussis* but may be necessary for *B. bronchiseptica* to establish an infection.

For both *B. bronchiseptica* and *B. pertussis*, it has been shown that the Bvg^+ phase is necessary and sufficient for infection (6, 7, 9, 16, 22). Our results do not contradict these observations for *B. bronchiseptica*, but they demonstrate that once inside the host, bacteria begin to transcribe Bvg^+ phase genes within 1 h p.i., with essentially every cell having activated *fhaB*, *cyaA*, and *ptxA* by 30 h p.i. The transition of *B. bronchiseptica* from Bvg^- phase to Bvg^+ phase upon inoculation also indicates that the mouse lung is a Bvg^+ phase environment. We likewise provided evidence that *B. bronchiseptica* does not modulate to the Bvg^- phase *in vivo*, supporting studies conducted using Bvg^- phase-locked mutants that were unable to establish an infection and were quickly cleared from the respiratory tract and those using Bvg^+ phase-locked mutants that displayed no colonization defect (7, 8). Moreover, ectopic expression of flagellin in Bvg^+ phase *B. bronchiseptica* results in impaired persistence in the rat respiratory tract, possibly due to an immune response to this antigen (16). Therefore, modulation to the Bvg^- phase likely does not occur *in vivo*, as it would be disadvantageous to bacterial survival. We cannot rule out the possibility, however, that some Bvg^+ RB50*flaA*FLP bacteria did modulate to the Bvg^- phase and were quickly eliminated by the immune response or were present in a niche other than the lung (such as the trachea or nasopharynx) and were not represented in the lung homogenates that we analyzed. It is also conceivable that *B. bronchiseptica* is able to partially modulate, perhaps to the Bvg^i phase, which would not be documented using RB50*flaA*FLP. We are currently constructing additional strains to test this possibility.

The pGFLIP plasmid has proven to be useful in understanding the regulation of gene activation in *Bordetella*. However, there remain caveats for the use of this system in other applications. As with other IVET and RIVET systems, pGFLIP requires that strains be manipulated under strict promoter-inactive conditions to prevent unwanted Flp-mediated resolution. This requirement poses problems for studying genes for which conditions of repression or lack of activation are unknown, genes that are essential for growth, and genes that may not be fully transcriptionally inactive *in vitro*. Additionally, like other systems that have been developed to monitor transcription, pGFLIP cannot provide information about posttranscriptional or posttranslational regulation of target genes. Finally, as was shown by the variable background resolution of Bvg-regulated promoters in our study, pGFLIP appears to be sensitive to low-level promoter activation even under "repressed"

conditions for certain genes. Lee et al. were able to modulate the sensitivity of RIVET by mutating the ribosome binding site (RBS) of *tnpR*, effectively raising the threshold of promoter activity required for resolution (42). pGFLIP does not possess an RBS 5′ to *flp*, instead relying on the RBS of the promoter of interest, though it would be feasible to develop a modified pGFLIP plasmid that contains an RBS with reduced sensitivity to permit the study of genes that are not fully inactive or are constitutively active at a low level.

In this study, we used pGFLIP to detect the activation of BvgAS-regulated genes in *Bordetella*, but there are additional uses for this system to measure transcription activation at the population or single cell level. Using pGFLIP, fluorescence can be used to quickly differentiate cells that have activated the promoter of interest from those that have not. Over time, stochastic and/or transient promoter activation can result in sectoring of fluorescent colonies, permitting spatiotemporal observation of gene activation within a single colony (M. S. Byrd and E. Mason, unpublished observation). The addition of a second, non-*gfp*-encoded fluorescent label (e.g., a constitutively expressed fluorescent protein or a fluorescently labeled antibody) to cells already containing pGFLIP would allow cells that had activated the promoter of interest to be differentiated from cells that had not. Cells labeled using such an approach could be visualized using fluorescence-activated cell sorting or by microscopy. We are currently developing an improved pGFLIP plasmid that contains a constitutively expressed fluorescent protein gene not flanked by *FRT* sites that will provide a two-color to one-color readout upon activation of *flp* by the promoter of interest. The development of pGFLIP has resulted in a sensitive genetic tool that can be used to document promoter activation in a broad range of Gram-negative bacteria both *in vitro* and *in vivo*. Our use of pGFLIP to document the activation of Bvg-regulated promoters revealed unexpectedly early activation of *cyaA* in *B. bronchiseptica*, suggesting a possible explanation for the less restrictive host range of this organism compared to *B. pertussis*, and is the first *in vivo* use of a recombination-based genetic reporter of *B. bronchiseptica* gene activation.

ACKNOWLEDGMENTS

We thank Megan Ray for technical assistance and members of the Cotter laboratory for helpful discussions of the manuscript.

This work was supported by grants from the National Institutes of Health (U54 AI065359, AI43986, and AI094991 to P.A.C.).

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