

Evidence for phenotypic bistability resulting from transcriptional interference of *bvgAS* in *Bordetella bronchiseptica*

Eliza Mason, Michael W. Henderson,
Erich V. Scheller, Matthew S. Byrd and
Peggy A. Cotter*

Department of Microbiology and Immunology, School of
Medicine, University of North Carolina, Chapel Hill, NC
27599-7290, USA.

Summary

Bordetella species cause respiratory infections in mammals. Their master regulatory system BvgAS controls expression of at least three distinct phenotypic phases in response to environmental cues. The Bvg⁺ phase is necessary and sufficient for respiratory infection while the Bvg⁻ phase is required for survival *ex vivo*. We obtained large colony variants (LCVs) from the lungs of mice infected with *B. bronchiseptica* strain RBX9, which contains an in-frame deletion mutation in *fhaB*, encoding filamentous haemagglutinin. RBX9 also yielded LCVs when switched from Bvg⁻ phase conditions to Bvg⁺ phase conditions *in vitro*. We determined that LCVs are composed of both Bvg⁺ and Bvg⁻ phase bacteria and that they result from defective *bvgAS* positive autoregulation. The LCV phenotype was linked to the presence of a divergent promoter 5' to *bvgAS*, suggesting a previously undescribed mechanism of transcriptional interference that, in this case, leads to feedback-based bistability (FBM). Our results also indicate that a small proportion of RBX9 bacteria modulates to the Bvg⁻ phase *in vivo*. In addition to providing insight into transcriptional interference and FBM, our data provide an example of an in-frame deletion mutation exerting a 'polar' effect on nearby genes.

Introduction

The genus *Bordetella* includes Gram-negative bacteria that cause respiratory infections. *Bordetella pertussis*

and *Bordetella parapertussis*_{hu} are strictly human-specific pathogens that cause whooping cough, an acute disease that has experienced a recent resurgence despite widespread vaccination (Campos-Outcalt, 2005; Mattoo and Cherry, 2005; Marconi *et al.*, 2012). Phylogenetic analyses indicate that *B. pertussis* and *B. parapertussis*_{hu} have recently evolved from *Bordetella bronchiseptica*, which infects a wide range of mammals and can also survive naturally for long periods of time outside the host (Gerlach *et al.*, 2001; Diavatopoulos *et al.*, 2005; Gross *et al.*, 2010). Although the factors that determine host specificity remain unknown, the presence and regulation of virulence factor-encoding genes is highly conserved between these three subspecies (Tejada *et al.*, 1996; Cummings *et al.*, 2006).

Filamentous haemagglutinin (FHA), encoded by the *fhaB* gene, is a well-characterized virulence factor of *Bordetella* and is a primary component of acellular pertussis vaccines (Domenighini *et al.*, 1990; Sato and Sato, 1999; Mattoo and Cherry, 2005). A prototypical member of the Two Partner Secretion family of proteins, FHA is a large, surface-exposed protein that is produced and secreted at a high levels during growth *in vitro* (Domenighini *et al.*, 1990; Coutte *et al.*, 2001; Mazar and Cotter, 2006). In *B. bronchiseptica*, FHA mediates adherence to a wide range of cell lines and is required for colonization of the lower respiratory tract in both rats and mice (Cotter *et al.*, 1998; Mattoo *et al.*, 2000; Inatsuka *et al.*, 2005). Although FHA was first characterized as an adhesin, it has subsequently been reported to perform several other important functions. For example, exposure of lipopolysaccharide and IFN- γ -stimulated macrophages to purified *B. pertussis* FHA suppresses interleukin-12 (IL-12) production, and macrophages treated with FHA exhibit higher rates of apoptosis compared with untreated controls (McGuirk and Mills, 2000; Abramson *et al.*, 2001). FHA-deficient *B. bronchiseptica* causes an infection that is hyperinflammatory compared with infection caused by wild-type bacteria and is characterized by increased influx of interleukin-17 (IL-17)-positive neutrophils, macrophages, and CD4⁺ T cells, suggesting that FHA plays an immunomodulatory role *in vivo* (Inatsuka *et al.*, 2005; Henderson *et al.*, 2012). Additionally, there is strong evidence that FHA interacts with

Accepted 3 September, 2013. *For correspondence. E-mail peggy_cotter@med.unc.edu; Tel. (+1) 919 966 5612; Fax (+1) 919 962 8103.

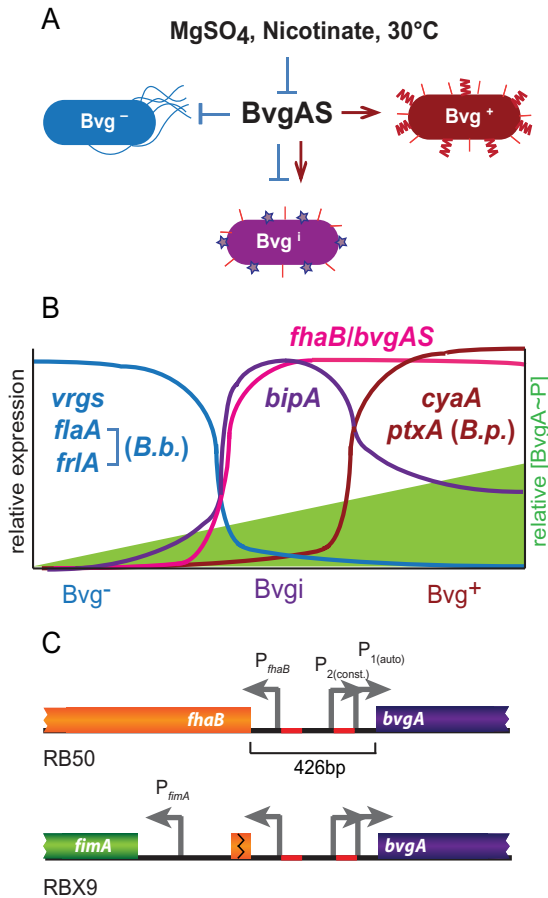


Fig. 1. The *Bordetella* BvgAS system controls at least four different classes of genes and three different phenotypic phases in response to environmental stimuli. A. BvgAS is responsible for the Bvg⁺, Bvgⁱ, and Bvg⁻ phases and is repressed by chemical modulators or low temperature. B. The three phenotypic phases are defined by unique patterns of gene expression as indicated, and rely on the intracellular concentration of BvgA-P. C. The chromosomal organization of the *fhaB* and *bvgAS* loci in RB50 (top) and RBX9 ($\Delta fhaB$, bottom). Red regions indicate relative BvgA binding regions.

another important virulence factor, adenylate cyclase toxin ACT (Zaretsky *et al.*, 2002; Perez Vidakovic *et al.*, 2006).

In *Bordetella*, the master regulator that controls the expression of all known virulence factor-encoding genes is called BvgAS (Aricò *et al.*, 1989). A two-component sensory transduction system, BvgAS controls at least three distinct phenotypic phases (Bvg⁺, Bvgⁱ, and Bvg⁻) by altering gene expression patterns in response to environmental stimuli (Fig. 1A) (Cotter and Jones, 2003). The Bvg⁺ phase is induced during standard laboratory growth conditions at 37°C on Bordet–Gengou (BG) blood agar or in Stainer–Scholte (SS) broth. Bvg⁺ phase bacteria are non-motile and form small, dome-shaped, haemolytic colonies on BG blood agar. Bacteria can be induced (or *modulated*) to the Bvg⁻ phase in the laboratory by growth at room

temperature or by supplementing media with millimolar concentrations of chemical modulators such as MgSO₄ or nicotinic acid. Bacteria in the Bvg⁻ phase are motile (*B. bronchiseptica* only) and form large, flat, non-haemolytic colonies. The Bvgⁱ phase can be induced in the laboratory with intermediate concentrations of chemical modulators and these bacteria form colonies that appear phenotypically intermediate compared with Bvg⁻ and Bvg⁺ phase colonies. Each phenotypic phase is defined by a unique pattern of gene expression (Fig. 1B) (Cotter and Miller, 1997; Cotter and Jones, 2003; Cummings *et al.*, 2006). For example, bacteria in the Bvg⁺ phase are characterized by maximal expression of virulence-activated genes (*vags*) such as *fhaB*, *cyaA-E* (encoding adenylate cyclase toxin ACT), *ptxA-E* (encoding pertussis toxin in *B. pertussis*), and *bvgAS* itself (which is positively autoregulated). In contrast, Bvg⁻ phase bacteria maximally express virulence-repressed genes (*vrgs*) including those required for motility (i.e. *flaA*, encoding flagellin and *fliA*, the *Bordetella* *flhDC* homologue) and chemotaxis (*B. bronchiseptica* only) but do not express *vags*. The *vags* fall into two classes: those expressed in the Bvgⁱ phase and the Bvg⁺ phase, and those expressed maximally only in the Bvg⁺ phase. Additionally, some genes, such as *bipA* (encoding Bvg-intermediate phase protein A), are expressed maximally only in the Bvgⁱ phase (Deora *et al.*, 2001; Williams *et al.*, 2005). Thus, Bvgⁱ phase bacteria are characterized by maximal expression of *bipA*, *bvgAS*, and *fhaB*, and minimal expression of *vrgs*, *cyaA*, and *ptxA* (*B. pertussis* only) (Fig. 1B) (Cotter and Miller, 1997; Deora *et al.*, 2001; Stockbauer *et al.*, 2001; Williams *et al.*, 2005; Cummings *et al.*, 2006).

Upon activation of the BvgAS phosphorelay in response to environmental signals, BvgS (the sensor kinase) autophosphorylates, becoming the substrate for BvgA (the response regulator). BvgA-phosphate (BvgA-P) binds DNA and activates or represses transcription (Boucher *et al.*, 2001a,b; 2003; Cotter and Jones, 2003; Jones *et al.*, 2005; Williams *et al.*, 2005). *In vitro* transcription and DNA binding experiments have identified both high and low affinity BvgA binding sites located at various positions relative to the transcription start sites of BvgAS-regulated genes (Steffen *et al.*, 1996; Boucher *et al.*, 2001b; Cotter and Jones, 2003; Jones *et al.*, 2005; Williams *et al.*, 2005). These data, together with a recent report describing a direct assessment of BvgA-P levels in *B. pertussis* cultures (Boulanger *et al.*, 2013), support a model in which BvgA-P levels are extremely low under Bvg⁻ phase conditions, moderate under Bvgⁱ phase conditions, and high under Bvg⁺ phase conditions (Cotter and Jones, 2003; Boulanger *et al.*, 2013). In addition to controlling distinct phenotypic phases in response to steady-state conditions, BvgAS can regulate gene expression in a temporal manner (Scarlato and Rappuoli, 1991; Prugnola *et al.*, 1995; Jones

et al., 2005; Williams and Cotter, 2007). Because *bvgAS* is positively autoregulated, both the concentration of BvgA and the proportion that is phosphorylated increase when bacteria sense activating signals. Therefore, gene expression patterns change temporally as the total concentration of BvgA–P gradually increases when bacteria are switched from Bvg[−] to Bvg⁺ phase conditions (Scarlatto and Rappuoli, 1991; Prugnola *et al.*, 1995; Jones *et al.*, 2005; Williams and Cotter, 2007).

The *bvgAS* and *fhaB* genes are adjacent and transcribed divergently. Experiments with *B. pertussis* indicate that the 426 bp intergenic region contains at least three promoters (with at least two that control *bvgAS*, called P₂ and P₁) and multiple high-affinity BvgA binding sites (Scarlatto *et al.*, 1990; Roy and Falkow, 1991; Boucher *et al.*, 2001a,b) (Fig. 1C). In the Bvg[−] phase, *bvgAS* transcription is driven by the BvgAS-independent promoter P₂ that is responsible for basal levels of BvgA (which likely remain unphosphorylated) (Scarlatto *et al.*, 1990; Roy and Falkow, 1991). When switched to the Bvg⁺ phase, BvgA becomes phosphorylated and activates *fhaB* and *bvgAS* via binding to high-affinity sites near P_{fhaB} and P₁ (Roy and Falkow, 1991; Zu *et al.*, 1996). Once a relatively high concentration of BvgA–P is achieved, genes with low-affinity BvgA binding sites at their promoters, such as *cyaA* and *ptxA* in *B. pertussis*, are activated and the bacteria transition into the Bvg⁺ phase (Karimova *et al.*, 1996; Jones *et al.*, 2005; Williams and Cotter, 2007). The Bvg⁺ phase, and therefore high levels of BvgA–P, is maintained as long as bacteria sense a Bvg⁺ phase environment. Without *bvgAS* positive autoregulation, the ability of *B. bronchiseptica* to transition between and maintain each phenotypic phase is compromised (Williams and Cotter, 2007).

Data obtained thus far indicate that the Bvg⁺ phase is necessary and sufficient to cause respiratory infection, the Bvg[−] phase facilitates survival outside of the host, and BvgAS modulation to the Bvgⁱ or Bvg[−] phase does not occur during infection (Cotter and Miller, 1994; Akerley *et al.*, 1995; Merkel *et al.*, 1998; Tejada *et al.*, 1998; Vergara-Irigaray *et al.*, 2005; Nicholson *et al.*, 2012). For example, several groups have shown that Bvg⁺ phase-locked bacteria behave identically to wild-type bacteria in colonization, persistence, and contribution to lung pathology (Cotter and Miller, 1994; Akerley *et al.*, 1995; Tejada *et al.*, 1998; Nicholson *et al.*, 2012). In contrast, Bvg[−] phase-locked bacteria cannot establish an infection and Bvgⁱ phase-locked bacteria are severely limited in colonization and persistence (Cotter and Miller, 1994; 1997; Tejada *et al.*, 1998; Vergara-Irigaray *et al.*, 2005; Nicholson *et al.*, 2012). Additionally, we recently demonstrated that *flaA* is not expressed at a detectable level when mice are infected with the *B. bronchiseptica* wild-type strain RB50 (Byrd *et al.*, 2013) and Akerley *et al.* showed that production of flagella in the Bvg⁺ phase is detrimental

Table 1. LCV recovery frequency.

Condition	% ^a LCVs recovered on BG blood agar
Plating murine lung homogenate after 30 h	0.39 ± 0.13
Replating any RBX9 Bvg [−] phase colony	5.43 ± 0.98
Replating an LCV from BG agar (derived <i>in vitro</i>)	3.90 ± 0.80
Replating an LCV from BG agar (derived <i>in vivo</i>)	6.03 ± 1.26
Replating any RBX9 Bvg ⁺ phase colony	0

a. Values are means ± standard errors for experiments performed at least in triplicate.

to infection (Akerley *et al.*, 1995). Although the natural signals that affect BvgAS activity and the role of modulation in nature remain unknown, all of these data suggest that wild-type *Bordetella* do not modulate to the Bvg[−] phase within the mammalian host.

Bordetella bronchiseptica strain RBX9, which contains an in-frame deletion mutation of *fhaB* (Fig. 1C), has been used extensively to characterize the function of FHA *in vitro* and *in vivo* (Cotter *et al.*, 1998; Irie *et al.*, 2004; Inatsuka *et al.*, 2005; Mazar and Cotter, 2006; Julio *et al.*, 2009; Henderson *et al.*, 2012). RBX9 is defective in adherence to multiple cell lines, is unable to autoaggregate in liquid culture, and causes hyperinflammation in the murine lung infection model (Cotter *et al.*, 1998; Mattoo *et al.*, 2000; Inatsuka *et al.*, 2005; Julio *et al.*, 2009; Henderson *et al.*, 2012). We isolated large colony variants (LCVs) from mice infected with RBX9 and also by modulating RBX9 to the Bvg[−] phase *in vitro*. We determined that the LCVs were a product of transcriptional interference that influenced *bvgAS* and produced an unusual bistable phenotype. Despite evidence suggesting that *Bordetella* do not modulate during infection, the discovery of LCVs indicates that a subpopulation of RBX9 bacteria modulates *in vivo*.

Results

Isolation and characterization of LCVs

While comparing wild-type *B. bronchiseptica* strain RB50 with its Δ *fhaB* derivative RBX9 in a murine lung infection model, we noticed that at early time points post-inoculation (12 and 24 h), a small proportion (~1%) of colony-forming units (cfu) recovered from the lungs of RBX9-inoculated mice formed colonies on BG blood agar (Bvg⁺ phase conditions) that were larger, flatter, and less haemolytic than colonies typically formed by RBX9 and RB50 (Table 1) (Fig. 2D). These LCVs were not recovered from RB50-infected mice. When LCVs were picked, diluted, and replated on BG blood agar, approximately 95% of the resulting colonies were phenotypically Bvg⁺ phase, and approximately 5% were LCVs (Table 1)

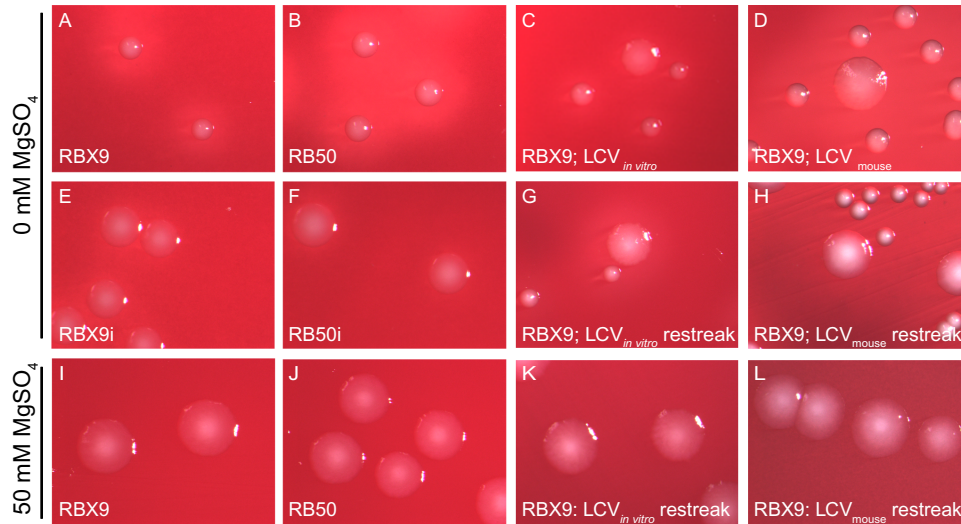


Fig. 2. RB50 and RBX9 colony morphology. Bacteria were plated on either BG agar or BG agar + 50 mM MgSO₄ and were imaged after 48 h. (A) RB50; (B) RBX9; (C) RBX9 LCV produced after *in vitro* modulation; (D) RBX9 LCV recovered from mouse lung homogenate; (E) RB50i (a Bvg-intermediate phase-locked strain in the RB50 background); (F) RBX9i (a Bvg-intermediate phase-locked strain in the RBX9 background); (G) RBX9 restreak of an LCV produced after modulation; (H) RBX9 restreak of an LCV recovered from the mouse lung; (I) RB50; (J) RBX9; (K) RBX9 restreak of an LCV produced after modulation; (L) RBX9 restreak of an LCV recovered from the mouse lung.

(Fig. 2H). When replated again, LCVs continued to yield 95% Bvg⁺ phase colonies and 5% LCVs. All phenotypically Bvg⁺ phase colonies yielded only phenotypically Bvg⁺ phase colonies after replating onto BG agar.

We found serendipitously that LCVs were also induced *in vitro* under certain conditions. Specifically, when RBX9 was grown on BG blood agar + 50 mM MgSO₄ (Bvg⁻ phase conditions) and then replated onto BG blood agar – effectively switching the bacteria from Bvg⁻ to Bvg⁺ phase conditions – approximately 95% of the colonies were phenotypically Bvg⁺ phase and 5% were LCVs (Table 1) (Fig. 2C). When these LCVs were picked, diluted, and replated onto BG blood agar, approximately 95% of colonies displayed the Bvg⁺ phase morphology and approximately 5% of colonies were LCVs (Table 1) (Fig. 2G). Again, all phenotypically Bvg⁺ phase colonies yielded only phenotypically Bvg⁺ phase colonies after replating onto BG agar. When RBX9 was streaked onto BG agar supplemented with 50 mM MgSO₄, or passaged continuously under Bvg⁻ phase conditions, all colonies displayed typical Bvg⁻ phase morphology.

To determine if the generation of LCVs in RBX9 was due to the $\Delta fhaB$ mutation and not an unknown secondary mutation, we reconstructed strain RBX9 by allelic exchange. The newly constructed strain behaved identically to RBX9, producing LCVs following BvgAS modulation and generating a similar proportion of LCVs upon restreaking an LCV onto BG blood agar. Although LCVs appear morphologically similar to Bvgⁱ phase colonies (Fig. 2), the fact that restreaking LCVs yielded a heterogeneous population of morphologically different colonies

indicates that the $\Delta fhaB$ mutation does not lock bacteria into one particular phenotypic phase (such as the Bvgⁱ phase).

LCVs are composed of both Bvg⁻ and Bvg⁺ phase bacteria

To better understand the properties of LCVs, we investigated specific gene expression patterns within the bacteria that composed them. The fact that LCVs are haemolytic suggests that *cyaA*, a Bvg⁺ phase gene, is expressed because ACT is responsible for haemolysis on BG blood agar (Bellalou *et al.*, 1990). Additionally, electron micrographs of negatively stained LCVs revealed the presence of numerous flagella, which are only produced in the Bvg⁻ phase (data not shown). To determine if bacteria within LCVs were motile, we grew bacteria on SS plates with 0.3% agar (Bvg⁺ phase conditions). LCVs stab-inoculated into this agar produced a zone of migration that was smaller than that produced by Bvg⁻ phase-locked bacteria, but larger than Bvg⁺ and Bvgⁱ phase bacteria, which do not produce a zone of migration (data not shown), suggesting that at least some bacteria within LCVs are motile. Together, our observations indicate that both *cyaA* and *flaA* are expressed within each LCV. However, previous studies have shown that expression of *vags*, such as *cya*, and *vrgs*, such as *flaA*, is mutually exclusive (Cummings *et al.*, 2006). Therefore, we hypothesized that LCVs are composed of at least two phenotypically distinct populations of bacteria: a population in the Bvg⁺ phase and a population in the Bvg⁻ phase.

To determine the phenotypes of individual bacteria present in each LCV, we created RBX9BatB-HA*fla-gfp*, a strain that contains two unique tags that permit the distinction between Bvg⁺ and Bvg⁻ phase bacteria. RBX9BatB-HA*fla-gfp* contains an HA epitope-encoding sequence in *batB* (encoding the Bvg⁺ phase surface-exposed protein BatB) as well as *gfp* driven by the *flaA* promoter at a neutral site in the chromosome. The *batB* gene is expressed maximally in the Bvg⁺ phase and minimally in the Bvgⁱ and Bvg⁻ phases (Williams *et al.*, 2008). The *flaA* gene, as described previously, is a typical *vrg* and contains a strong promoter that is active only in the Bvg⁻ phase (Akerley *et al.*, 1992; Cotter and Jones, 2003). Therefore, Bvg⁺ phase bacteria should produce a surface-exposed HA-tagged BatB protein and be GFP⁻ and Bvg⁻ phase bacteria should be GFP⁺ and lack a surface-exposed HA epitope.

We used Alexa-Fluor 594-conjugated antibodies to indirectly recognize HA epitopes so that BatB-producing bacteria displayed red fluorescence. Bacteria expressing *fla-gfp* produced GFP and displayed green fluorescence. When Bvg⁺ phase colonies of RBX9BatB-HA*fla-gfp* were stained with anti-HA and an Alexa-Fluor 594-conjugated secondary antibody, only red-fluorescing bacteria were observed and no green fluorescence was detected (Fig. 3). When Bvg⁻ phase colonies were stained, only green-fluorescing bacteria were observed and no red fluorescence was detected. When Bvgⁱ phase colonies were stained, a small proportion of cells displayed red or green fluorescence, but the majority of cells were not fluorescent and no bacteria displayed both red and green fluorescence (Fig. 3). In contrast, when LCVs from RBX9BatB-HA*fla-gfp* were stained, approximately half of the bacteria fluoresced red and approximately half fluoresced green (Fig. 3). No colocalization of red and green fluorescence from either LCVs or Bvgⁱ phase colonies was observed, confirming that the expression of *vags* and *vrgs* is mutually exclusive under these conditions. These data demonstrate that LCVs are composed of both Bvg⁺ and Bvg⁻ phase bacteria and are not a homogeneous population of Bvgⁱ phase bacteria.

The ΔfhaB mutation in RBX9, but not lack of FHA protein, is responsible for the LCV phenotype

To determine if the generation of LCVs was due to lack of FHA protein production or the specific genetic architecture created by the $\Delta fhaB$ mutation in RBX9, we first determined if other *fhaB* mutants yielded LCVs. Strain RB50 ΔP_{fhaB} contains a deletion mutation of the *fhaB* promoter, strain RB50 ΔSP_{fhaB} contains a deletion mutation in *fhaB* such that the extended signal peptide of FHA is missing, and strain RB50 $\Delta\beta$ helix_{*fhaB*} contains a large deletion mutation in the region of *fhaB* encoding the β -helix

structure (Fig. S1). These strains were analysed for FHA production by Western blot, and either produced no FHA protein (RB50 ΔP_{fhaB} and RB50 ΔSP_{fhaB}) or a severely truncated FHA protein (RB50 $\Delta\beta$ -helix) (data not shown). We grew these strains under Bvg⁻ phase conditions and plated single colonies onto Bvg⁺ phase conditions to determine if they would produce LCVs similar to RBX9. No LCVs were observed, suggesting that a lack of wild-type FHA protein is not sufficient to produce the LCV phenotype.

To investigate the contribution of the genetic architecture created by the *fhaB* deletion to the LCV phenotype, we created RB50::pBam, a strain that produces FHA and contains an altered *fhaB-bvgAS* locus (Fig. S1). RB50::pBam was created by integrating pBam, a suicide plasmid containing the *fhaB-bvgAS* intergenic region, into the RB50 chromosome. When integrated in the chromosome, the genetic architecture 5' to *bvgAS* is similar in that *fhaB* coding sequences are replaced with non-native sequences (those from the suicide plasmid, in this case). By contrast with RBX9, however, the complete, intact *fhaB* gene, including its promoter region, is present in RB50::pBam – it is located 5' (relative to *bvgAS*) to the integrated plasmid sequences. After modulation, RB50::pBam produced LCVs similar to RBX9. These data suggest that the LCV phenotype can be produced by altering the genetic architecture 5' to the *fhaB-bvgAS* intergenic region and is independent of FHA production.

The LCV phenotype results from a defect in bvgAS positive autoregulation

In addition to activating all of the known virulence factor-encoding genes in *Bordetella*, BvgAS activates *bvgAS* expression through positive autoregulation. Williams *et al.* demonstrated that positive autoregulation is required for the precise transition between and maintenance of the Bvg⁺, Bvgⁱ, and Bvg⁻ phases (Williams and Cotter, 2007). Three observations suggested that LCVs resulted from defective *bvgAS* autoregulation in RBX9. First, the mutations that cause LCVs are genetically linked (immediately 5') to *bvgAS*; second, LCVs consist of bacteria in at least two separate BvgAS-controlled phenotypic phases; and third, LCVs were induced *in vitro* following a switch from Bvg⁻ to Bvg⁺ phase growth conditions.

We hypothesized that when RBX9 (or RB50::pBam) bacteria are switched from Bvg⁻ phase conditions to Bvg⁺ phase conditions, most, like all wild-type bacteria, are able to activate transcription at the *bvgAS* P₁ promoter, leading to increased BvgAS levels and resulting in the transition to and maintenance of the Bvg⁺ phase. According to our hypothesis, however, a small subset of RBX9 and RB50::pBam bacteria are unable to activate *bvgAS* transcription, possibly due to insufficient levels of BvgA and/or BvgS, and these bacteria are therefore 'trapped' in

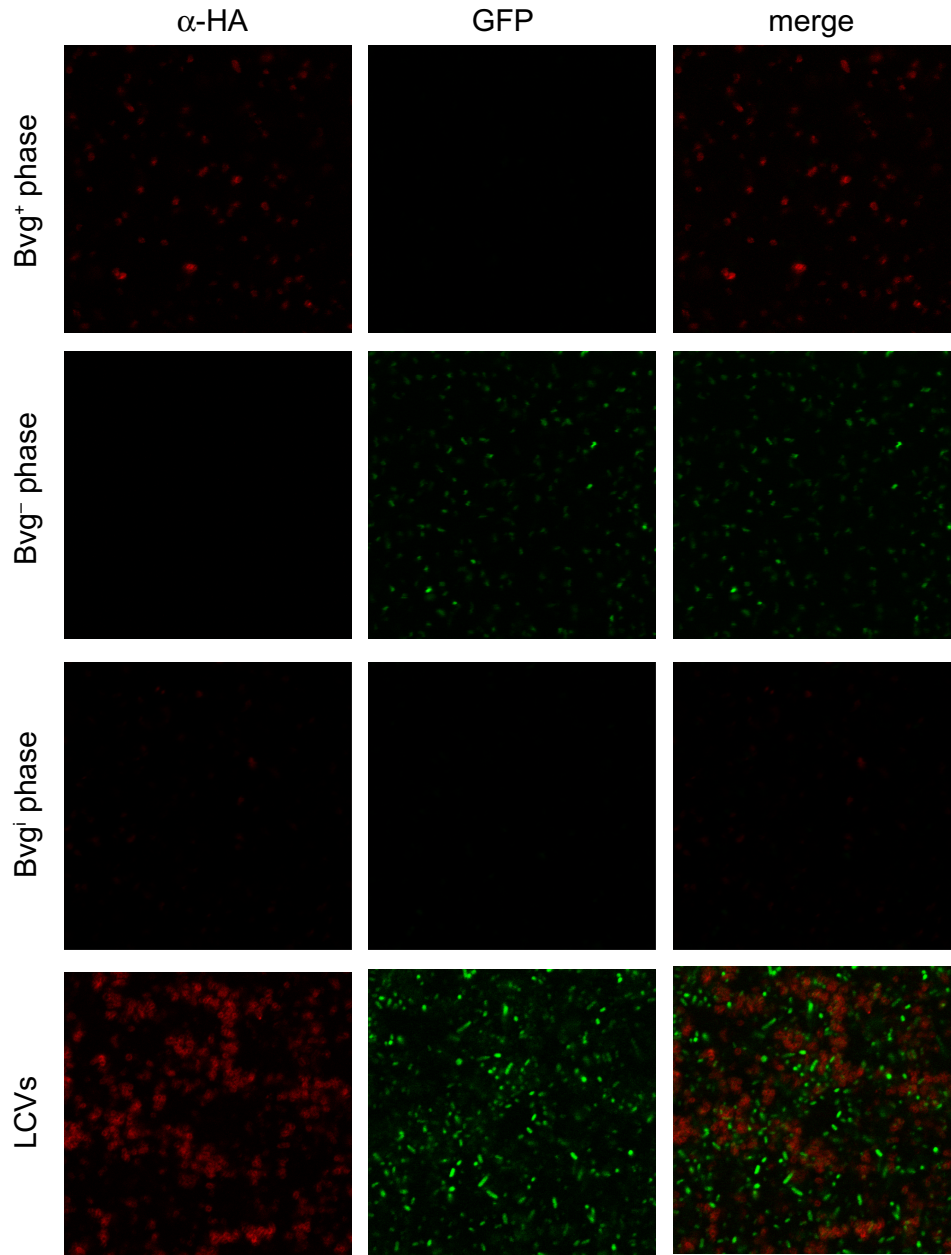


Fig. 3. Detection of Bvg⁺ (α -HA, red) and Bvg⁻ phase (*flaA-gfp*, green) bacteria in typical RBX9 colonies and LCVs. RBX9BatBN-HA*flaA-gfp* was grown on BG blood agar (Bvg⁺ phase conditions), BG blood agar + 50 mM MgSO₄ (Bvg⁻ phase conditions), or BG blood agar + 6 mM MgSO₄ (Bvg['] phase conditions). Several colonies of each phenotype were combined and stained with mouse monoclonal α -HA IgG followed by an Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody. Fluorescence was detected using a Zeiss LSM 710 confocal microscope.

the Bvg⁻ phase. We hypothesized further that although some descendants of these 'Bvg⁻ phase-trapped' bacteria will be able to activate *bvgAS* transcription and hence 'escape' to the Bvg⁺ phase, many will remain Bvg⁻ phase-trapped and thus a substantial Bvg⁻ phase population will be maintained in the LCV. An alternate hypothesis is that LCVs arise from spontaneous or transient shifting of bac-

teria between the Bvg⁺ and Bvg⁻ phases, which would also result in a mixture of Bvg⁺ and Bvg⁻ phase bacteria within a single colony.

To determine if LCVs contain Bvg⁻ phase-trapped bacteria, we used the recombinase-based reporter system pGFLIP, which creates a permanent genetic change in response to gene activation (Byrd *et al.*, 2013). In this

system, a promoter of interest drives expression of the site-specific recombinase-encoding gene *flp*, which when activated, results in recombination between Flp recombinase target (*FRT*) sites that flank *gfp* and the kanamycin (Km) resistance gene *nptII*. Therefore, any activation (even transient, low-level expression) of *flp* results in a permanent loss of Km resistance and GFP fluorescence. This system targets the reporter construct to the neutral *atfTn7* site 3' to *glmS*. We created strain RBX9*cyaAFLP* by mating the plasmid pGFLIP- P_{cyaA} , in which the *B. bronchiseptica* *cyaA* promoter drives *flp* expression, into RBX9. The *cyaA* gene is exclusively controlled by BvgAS, is highly expressed in the Bvg⁺ phase, and is expressed minimally in the Bvg⁻ phase (Cotter and Jones, 2003). In RBX9*cyaAFLP*, bacteria that have never expressed *cyaA* should remain GFP⁺ and Km^r, whereas bacteria that have expressed *cyaA* should convert to GFP⁻ and Km^s. If *gfp* is lost due to P_{cyaA} activation, all descendent cells will also be GFP⁻ and Km^s.

Previously, we demonstrated that RB50*cyaAFLP* bacteria remain GFP⁺ under Bvg⁻ phase conditions with Km selection and that they reach 100% resolution (GFP⁻ cfu/total cfu) when grown under Bvg⁺ phase conditions (Byrd *et al.*, 2013). When RBX9*cyaAFLP* was plated under Bvg⁻ phase conditions with Km selection, each colony was morphologically identical and fluorescent, indicating that *cyaA* had not been activated to a level required for sufficient *flp* expression to lead to recombination between *FRT* sites (Fig. 4A). In contrast, when a colony of RBX9*cyaAFLP* that was grown under Bvg⁻ phase conditions was plated and grown under Bvg⁺ phase conditions, approximately 15% of the colonies were LCVs and approximately 80% of those LCVs were GFP⁺ (Fig. 4B). None of the Bvg⁺ phase colonies were GFP⁺. When a GFP⁺ LCV was replated and grown under Bvg⁺ phase conditions, approximately 5% of the colonies were LCVs and approximately 80% of those were GFP⁺ (Fig. 4C). We serially replated GFP⁺ LCVs eight times and in all cases, additional GFP⁺ LCVs were generated (data not shown). These data indicate that a significant proportion of bacteria within a GFP⁺ LCV had never activated *cyaA* and had therefore failed to switch to Bvg⁺ phase in response to a change in conditions; i.e. they were Bvg⁻ phase-trapped. Moreover, our data suggest that all LCVs arise from a Bvg⁻ phase-trapped bacterium and that upon subsequent multiplication, most descendants have 'escaped' to the Bvg⁺ phase but a small proportion remain trapped in the Bvg⁻ phase. These data do not support a model in which LCVs consist of bacteria that transiently fluctuate between Bvg⁺ and Bvg⁻ phase, because if this was true, LCVs would not be GFP⁺.

Approximately 20% of the LCV colonies were GFP⁻, indicating that P_{cyaA} -*flp* expression was sufficient to mediate recombination in the bacterium that founded the

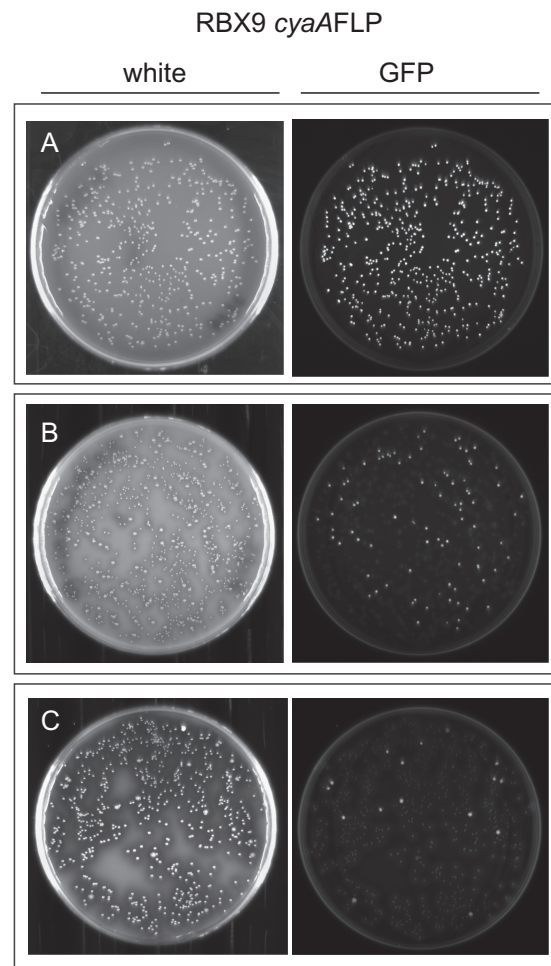


Fig. 4. LCVs from the strain RBX9*PcyaAFLP* are GFP⁺, indicating that *cyaA* has not been activated in a substantial proportion of these colonies. (A) RBX9*PcyaAFLP* on BG blood agar + 50 mM MgSO₄ (Bvg⁻ phase conditions); (B) a GFP⁺ colony from A plated onto BG blood agar (Bvg⁺ phase conditions); (C) a GFP⁺ LCV from B plated onto BG blood agar (Bvg⁺ phase conditions). Colonies were visualized after 48 h of growth.

LCV or in its early descendants. Although this result appears inconsistent with our model, we have observed previously that when RB50*cyaAFLP* is grown under Bvg⁻ phase conditions (when *cyaA* expression is minimal) and without Km selection, P_{cyaA} -*flp* is expressed sufficiently in approximately 15% of bacteria such that they convert to GFP⁻ and Km^s (Byrd *et al.*, 2013). These data indicate that the *cyaA* promoter activity under Bvg⁻ phase conditions is near the threshold level required for *flp* expression and subsequent recombination. Therefore, GFP⁻ LCVs are most likely due to the activity level of the *cyaA* promoter under Bvg⁻ phase conditions and not due to bacteria switching to the Bvg⁺ phase and then back to the Bvg⁻ phase. Nonetheless, our data indicate that in approximately 80% of LCVs, there are a substantial number of

bacteria that appear to have a defect in *bvgAS* positive autoregulation, leading to the observed Bvg⁻ phase-trapped population.

Sequences upstream of the fhaB-bvgAS intergenic region affect the efficiency of bvgAS activation in the Bvg⁻ phase

Our data indicate that *bvgAS* autoregulation is defective in RBX9 and specifically, which LCVs are composed of a subpopulation of Bvg⁻ phase-trapped bacteria. As stated in the introduction, *bvgAS* expression is controlled primarily by two promoters. Studies with *B. pertussis* have shown that under Bvg⁻ phase conditions, P₂ is transcribed at a low basal level (Scarlato *et al.*, 1990; Roy and Falkow, 1991). This level of transcription results in BvgS levels that are sufficient to respond to Bvg⁺ phase conditions by autophosphorylating and mediating phosphorylation of BvgA. The resulting BvgA~P levels are sufficient to bind at the *bvgAS* P₁ promoter, recruit RNAP, and activate transcription (Zu *et al.*, 1996). (Although similar transcriptional analyses have not been conducted with *B. bronchiseptica*, the nucleotide sequence of the *fhaB-bvgAS* intergenic region in *B. bronchiseptica* is 91.1% identical and most of those differences are located in regions that, based on *B. pertussis* studies, are not bound by either BvgAS or RNAP.) We considered two hypotheses: (1) the level of transcription from P₂ in RBX9 is lower than in RB50 such that in some bacteria the levels of BvgAS are too low to activate transcription at P₁ in response to Bvg⁺ phase conditions, and (2) transcription activation at P₁ by BvgA~P is somehow defective in RBX9 compared with RB50. Our data suggest that defective autoregulation in RBX9 is due to the lack of native sequences or presence of non-native sequences 5' to the *fhaB-bvgAS* intergenic region.

To test our hypotheses, we constructed RB50P_{short}*bvgAFLP* and RB50P_{long}*bvgAFLP*, where RB50P_{short}*bvgAFLP* contains only the *fhaB-bvgAS* intergenic region driving *flp* and RB50P_{long}*bvgAFLP* contains this region plus an additional 1200 bp of *fhaB* sequences driving *flp* (Fig. 5A). These strains were constructed under Bvg⁻ phase conditions in the presence of Km. To investigate P₂ expression, we determined the percent resolution (the percentage of cfu that had activated *flp*) of each strain grown under Bvg⁻ phase conditions by counting the ratio of GFP⁻ cfu to total cfu when one colony was plated from Bvg⁻ phase conditions with Km selection to Bvg⁻ phase conditions without Km selection. The average resolution under Bvg⁻ phase conditions in RB50P_{short}*bvgAFLP* was 68% whereas the average resolution in RB50P_{long}*bvgAFLP* was 97%. Plates are shown from one representative experiment (Fig. 5B and C). Our data indicate that the per-cell activation of RB50P_{short}*bvgAFLP* is lower than the per-cell activation of RB50P_{long}*bvgAFLP* under Bvg⁻ phase condi-

tions, and demonstrate that either a lack of native *fhaB* sequences or the presence of non-native sequences has a direct effect on the efficiency of *bvgAS* transcription from the P₂ promoter in the Bvg⁻ phase.

When a colony from each strain was grown on Bvg⁻ phase conditions with Km selection and then plated onto Bvg⁺ phase conditions without Km selection, maximum resolution was achieved and there were no GFP⁺ colonies in either strain (Fig. 5D and E). These data suggest that RBX9 does not have a defect in transcription activation at P₁.

The LCV phenotype is caused by a (divergent) promoter in proximity to bvgAS

Based on our results, we hypothesized that sequences within *fhaB* (1–1200 nucleotides of the coding region) 5' to the *fhaB-bvgAS* intergenic region effect the low level of transcription from P₂ that occurs under Bvg⁻ phase conditions. To test this hypothesis, we constructed a strain containing a deletion mutation from nt 8 to 1256 of *fhaB* (Fig. S1C). This mutant did not produce LCVs when switched from the Bvg⁻ to the Bvg⁺ phase. We conclude that the lack of specific sequences within the first 1200 bp of *fhaB* does not cause the LCV phenotype.

These data led us to closely reexamine the genetic architecture of each strain that produced LCVs (RBX9 and RB50::pBam) as well as the strains that showed a difference in *bvgAS-flp* reporter activation (RB50P_{short}*bvgAFLP* and RB50P_{long}*bvgAFLP*). A comparison of these strains revealed the presence of a divergent promoter 5' to the *fhaB-bvgAS* intergenic region in RBX9, RB50::pBam, and RB50P_{short}*bvgAFLP* (Fig. 6). In RBX9, the *fimA* promoter is very close to the *fhaB-bvgAS* intergenic region, in contrast to RB50 in which it is separated by the entire (>12 kb) *fhaB* gene. In RB50::pBam, the *bla* promoter (driving expression of the ampicillin resistance gene on the plasmid) is adjacent to the *fhaB-bvgAS* intergenic region. In the strain RB50P_{short}*bvgAFLP*, the *npt* promoter (driving expression of the kanamycin resistance gene on the plasmid) is proximal to P_{bvgA-flp}, whereas the RB50P_{long}*bvgAFLP* reporter is 'buffered' from the same *npt* promoter by an additional 1200 bp of *fhaB* (Fig. 6). We hypothesized that the presence of a promoter 5' to the *bvgAS-fhaB* intergenic region was interfering with *bvgAS* P₂ transcription, possibly by sequestering RNA polymerase away from P₂. To test the hypothesis that a nearby promoter could affect P₂ transcription, we reversed the orientation of the insert in the plasmid pBam. In the resulting plasmid, pBamR, the *bla* promoter is no longer proximal to the *bvgAS* homology region. Instead, the closest promoter 5' to *bvgAS* on the plasmid is the *aaC1* promoter (driving expression of the gentamicin resistance gene), which is more than 1.5 kb away (Fig. 6). We created RB50::pBamR by integrating the

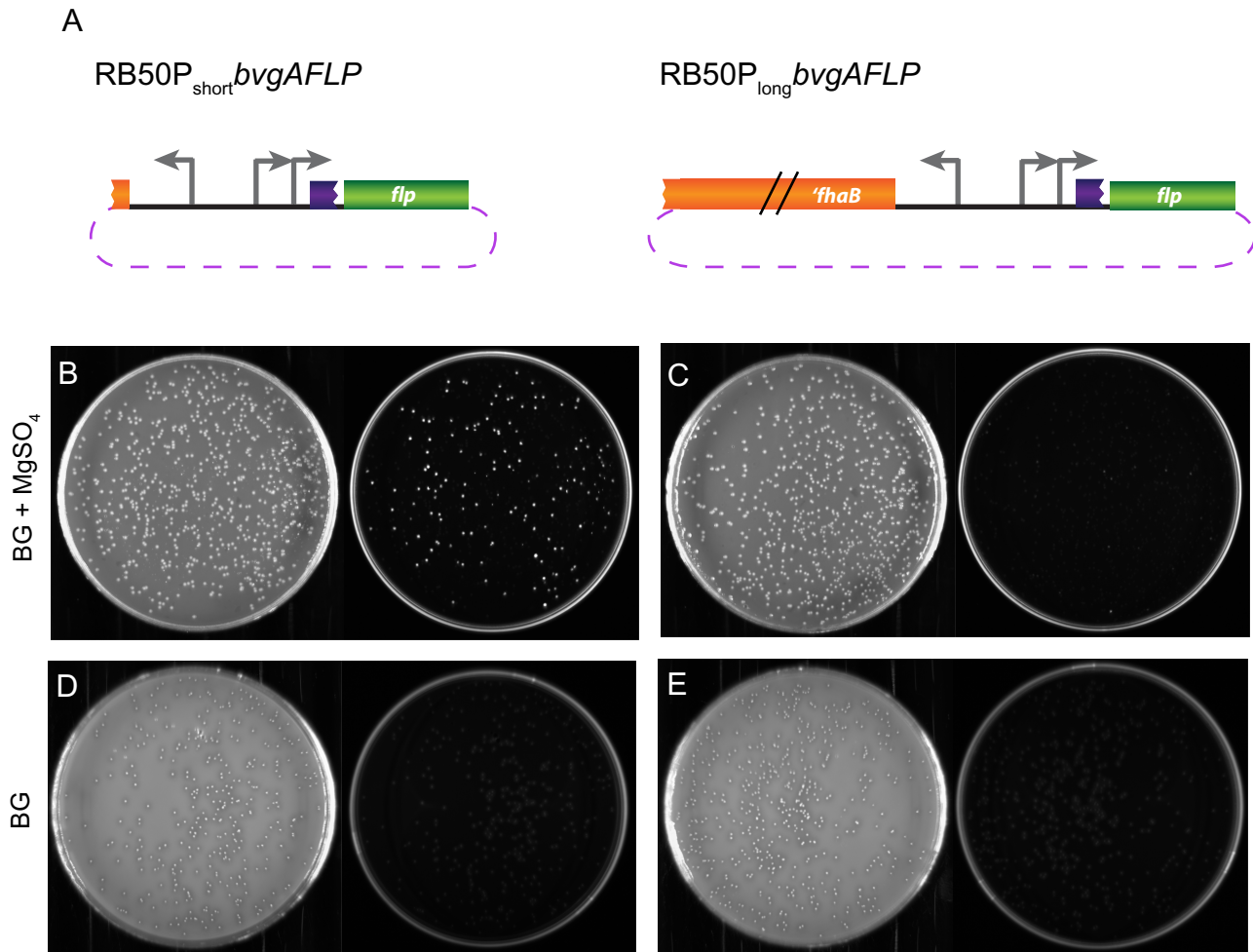


Fig. 5. Sequences upstream of *bvgAS* affect transcription efficiency under Bvg⁻ phase conditions.

A. Schematics of RB50P_{short}*bvgAFLP* and RB50P_{long}*bvgAFLP* showing the sequences 5' to *flp* inserted at the *attTn7* site (not drawn to scale). **B** and **C.** Strains were first grown on BG blood agar + 50 mM MgSO₄ + Km and one colony was plated onto BG blood agar + 50 mM MgSO₄ (Bvg⁻ phase conditions) without Km selection.

D and **E.** Strains were grown on BG blood agar + 50 mM MgSO₄ + Km selection and then one colony of each was plated onto BG blood agar (Bvg⁻ phase conditions) without selection.

Representative white light (left) and fluorescent (right) images are shown for panels **B**, **C**, **D** and **E**.

pBamR plasmid into the RB50 chromosome. Modulating RB50::pBamR and plating bacteria onto Bvg⁺ phase conditions resulted in all colonies having the typical Bvg⁺ phase morphology, and no LCVs were observed. The LCV phenotype was therefore abolished by changing the sequences upstream of the *bvgAS-fhaB* region, presumably by increasing the distance between a promoter and *bvgAS*. Additionally, we deleted the intergenic region between *fhaB* and *fimA* (which includes the *fimA* promoter) in RBX9. The resulting strain RBX9F did not produce LCVs after modulation. These data strongly support a model in which a promoter upstream of *bvgAS* interferes with normal P₂ transcription efficiency, resulting in some cells having an insufficient quantity of BvgAS to activate transcription at P₁.

Each Bvg⁻ phase-trapped bacterium within an LCV initiates the formation of a new LCV and the proportion of Bvg⁻ phase-trapped bacteria within an LCV decreases over time

Our data indicate that LCVs are founded by a single Bvg⁻ phase-trapped bacterium and that each LCV harbours Bvg⁻ phase-trapped bacteria that can propagate additional LCVs. However, it is unclear whether all Bvg⁻ phase-trapped bacteria, or only a subset of these cells, yield LCVs upon replating. To address this question, we needed two pieces of information: the proportion of Bvg⁻ phase-trapped bacteria within one LCV and the frequency of new LCV formation from the same parent colony when replated. We used RBX9*cyaAFLP* to

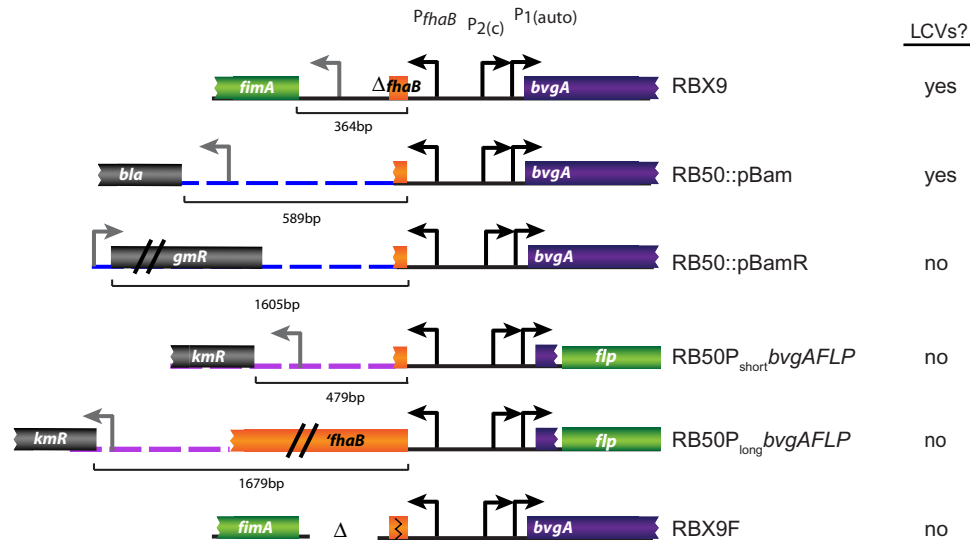


Fig. 6. The genetic architecture of the *bvgAS-fhaB* intergenic region of strains that do and do not produce LCVs or demonstrate a defect in *bvgAS-flp* activation have divergent promoters 5' to the *bvgAS-fhaB* intergenic region. Schematics for RB50P_{short}bvgAFLP and RB50P_{long}bvgAFLP represent sequences inserted at the *atfI*n7 site. Dotted lines represent non-coding plasmid DNA. Sequence lengths from the ATG of *fhaB* to the nearest 5' ATG are indicated.

evaluate the composition and LCV-forming capacity of single LCVs.

Plating a GFP⁺ LCV onto Bvg⁻ phase conditions, in which *cyaA* expression is minimal, will minimize further Flp-mediated recombination due to *cyaA* activation during colony formation, permitting us to determine the proportion of GFP⁺ bacteria (and hence Bvg⁻ phase-trapped) that existed within the original LCV at that time. Plating the same GFP⁺ LCV onto Bvg⁺ phase conditions allows us to determine the number of new LCVs generated from the subpopulation of Bvg⁻ phase-trapped bacteria in the parent LCV. Comparing the frequency of newly generated LCVs under Bvg⁺ phase conditions to the frequency of GFP⁺ cfu under Bvg⁻ phase conditions will reveal the proportion of Bvg⁻ phase bacteria that form LCVs when replated.

We first grew RBX9*cyaAFLP* on Bvg⁻ phase conditions with Km selection to maintain the *gfp* and Km^r markers (Fig. 7A I). Then we took single GFP⁺ (Bvg⁻ phase) colonies and plated them onto Bvg⁺ and Bvg⁻ phase conditions (Fig. 7A II). GFP⁺ LCVs that were recovered from Bvg⁺ phase plates were then plated again onto Bvg⁺ and Bvg⁻ phase conditions (Fig. 7A III). The frequencies of GFP⁺ cfu, LCVs, and Bvg⁺ phase colonies from each plate were recorded, and the results from one representative experiment are shown in Fig. 7A. When the GFP⁺ LCVs were plated onto Bvg⁺ and Bvg⁻ phase conditions, there was no significant difference in the average number of GFP⁺ cfu under Bvg⁻ phase conditions compared with the average number of GFP⁺ LCVs on the corresponding Bvg⁺ phase BG blood agar plate (Fig. 7B). These data suggest that

each Bvg⁻ phase bacterium within an LCV is Bvg⁻ phase-trapped and forms an LCV when replated onto Bvg⁺ phase conditions.

Additionally, we asked if the composition of LCVs (i.e. the ratio of Bvg⁻ to Bvg⁺ phase bacteria) changed over time. We hypothesized that this ratio would change due to bacterial division as well as the rate of conversion of Bvg⁻ phase-trapped bacteria to Bvg⁺ phase bacteria. Because we expected a unidirectional conversion of phenotypes (Bvg⁻ to Bvg⁺ phase only) under Bvg⁺ phase conditions, we predicted that the ratio of Bvg⁻ to Bvg⁺ phase bacteria would decrease as the bacterial population increased. To determine if the compositions changed after an additional day of growth, we compared GFP⁺ LCVs plated after our standard incubation time (48 h) (Fig. 7A III) to GFP⁺ LCVs plated after 72 h (Fig. 7A IV). When GFP⁺ LCVs were plated after 48 h of incubation, we obtained an average of $12 \pm 1.2\%$ GFP⁺ cfu under Bvg⁻ phase conditions, whereas after 72 h, we obtained an average of $5.75 \pm 1\%$ GFP⁺ cfu under the same conditions ($P = 0.005$) (Fig. 7C). These data indicate that the frequency of GFP⁺ (and therefore Bvg⁻ phase-trapped) bacteria in an LCV decreases over time.

These data also strongly suggest (as discussed in a previous result) that GFP⁻ LCVs are a result of the background activation of *cyaA* in RBX9P*cyaAFLP*, as the background *cyaA-flp* activation under conditions of inactivity (Bvg⁻ phase conditions) was the same as the frequency of GFP⁻ LCVs (to total LCVs) under Bvg⁺ phase conditions (Fig. 7A II).

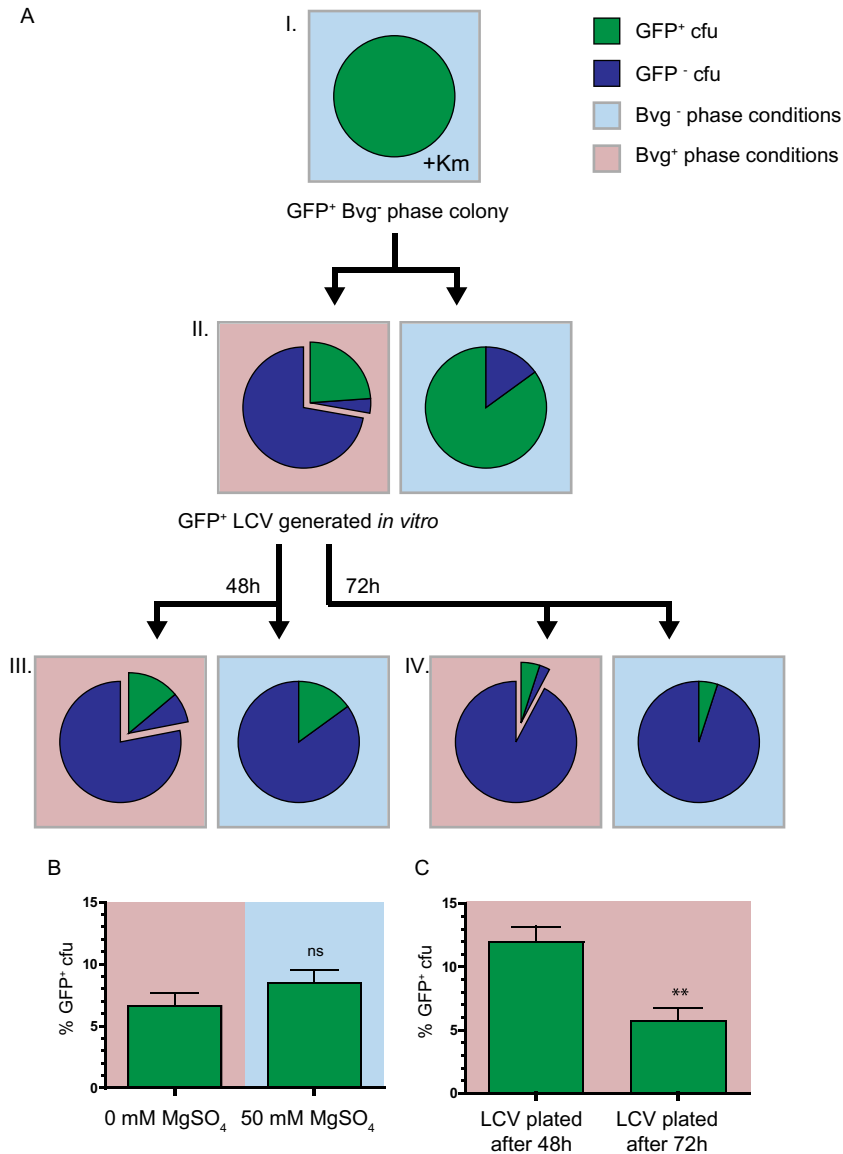


Fig. 7. A. Schematic of RBX9cyaAFLP experimental design, including a data set from one replicate. Each pie chart represents the population obtained from plating a single GFP⁺ colony from the previous plate (see text for details). Blue sectors in pie charts represent the frequency of GFP⁺ cfu; Green sectors in pie charts represent the frequency of GFP⁻ cfu; offset regions of pie charts represent the frequency of LCVs; Frequencies were determined by counting at least 500 cfu per condition.

B. Comparison of GFP⁺ cfu frequencies obtained from plating a single GFP⁺ LCV onto Bvg⁺ and Bvg⁻ phase conditions.

C. Comparison of GFP⁺ cfu frequencies obtained from plating a GFP⁺ LCV grown after 48 h and 72 h. Background colour represents BvgAS conditions, where red is Bvg⁺ phase conditions and blue is Bvg⁻ phase conditions.

** $P = 0.005$ by Student's unpaired t -test.

Modulation of RBX9 *in vivo* occurs at a very low frequency

All data published thus far strongly suggest that wild-type *Bordetella* do not modulate to the Bvg⁺ or Bvg⁻ phase *in vivo* and that the Bvg⁺ phase is necessary and sufficient for infection (Cotter and Miller, 1994; Akerley *et al.*, 1995; Merkel *et al.*, 1998; Tejada *et al.*, 1998; Vergara-Irigaray *et al.*, 2005; Nicholson *et al.*, 2012). The recovery of LCVs from mouse lung homogenates and the fact that LCVs were recovered *in vitro* only following modulation, however, supports the hypothesis that RBX9 modulates during infection. To test this hypothesis, we constructed strain RBX9flaAFLP, a strain containing the pGFLIP cassette in which the *flaA* promoter drives expression of

flp (Byrd *et al.*, 2013). Previously, using the same P_{flaA} -*flp*-containing cassette in wild-type RB50, we showed that *flaA* was not activated to a detectable level in RB50 during murine infection (Byrd *et al.*, 2013). RB50flaAFLP and RBX9flaAFLP bacteria were grown under Bvg⁺ phase conditions with Km selection to minimize background resolution prior to inoculation. Mice were inoculated intranasally with 7.5×10^4 – 1×10^5 cfu and lungs were harvested at 3, 24, 30 and 72 h post-inoculation. We conducted this experiment several times. In all experiments, a low proportion ($\leq 1\%$) of GFP⁻ bacteria was recovered from the lungs of both RB50flaAFLP- and RBX9flaAFLP-inoculated animals (data not shown). This low proportion was not significantly different, however, from the proportion of GFP⁻ bacteria present in the

samples used for inoculation (plated after inoculating the animals). Data from previous work with RB50/*flaAFLP* (Byrd *et al.*, 2013) and our experiments with RBX9/*flaAFLP* indicate that resolution of the P_{flaA^-flp} cassette is BvgAS-dependent, and GFP⁻ bacteria were not recovered from strains containing the *bvgS-C3* mutation, which locks the bacteria in the Bvg⁺ phase (data not shown). For the RBX9/*flaAFLP*-inoculated animals, most of the GFP⁻ colonies recovered from the mouse lungs were LCVs and no GFP⁺ LCVs were recovered, indicating that formation of LCVs *in vivo* correlates with, and is most likely caused by, BvgAS modulation. Together, these data suggest that a very small proportion of RBX9 modulates to the Bvg⁻ phase during infection. However, our data neither support nor refute the possibility that a small number of RB50 bacteria modulates as well.

To determine if bacteria modulate to the Bvgⁱ phase during infection, we attempted to construct strains with Bvgⁱ phase promoters, including the *bipA* promoter, driving *flp*. However, we were unable to construct these strains, presumably because the level of expression of these genes in Bvg⁺ phase conditions was above the threshold of *flp* activation required for recombination and loss of GFP and Km^R.

If BvgAS modulation occurs in vivo, it does not alter the outcome of infection

Our data suggest that a small proportion RBX9 (and possibly RB50) bacteria may modulate to the Bvg⁻ phase during infection. Although several previous experiments have shown that wild-type and Bvg⁺ phase-locked *B. bronchiseptica* strains are indistinguishable in animal models (Cotter and Miller, 1994; Merkel *et al.*, 1998; Tejada *et al.*, 1998; Vergara-Irigaray *et al.*, 2005; Nicholson *et al.*, 2012), we considered the possibility that the proportion of RBX9 bacteria that modulate *in vivo* could actually be greater than that of RB50, but not apparent from the P_{flaA^-flp} data because modulated RBX9 bacteria are killed in the host (i.e. that modulated bacteria, and perhaps specifically modulated RBX9 bacteria, are more susceptible to host-mediated clearance than modulated RB50 bacteria). To test this hypothesis, we compared RBX9, RBX9c (the Bvg⁺ phase-locked derivative of RBX9), RBX9F (the ΔP_{fimA} derivative of RBX9 which is not defective for *bvgAS* autoregulation), and RBX9cF (a Bvg⁺ phase-locked derivative of RBX9F) in mice. The results of three independent experiments are shown in Fig. 8. In no case did a statistically significant difference in bacterial burden occur among the various strains. These data negate our hypothesis and provide strong evidence that the low level of BvgAS modulation that occurs *in vivo* (based on the recovery of LCVs) does not impact the outcome of infection.

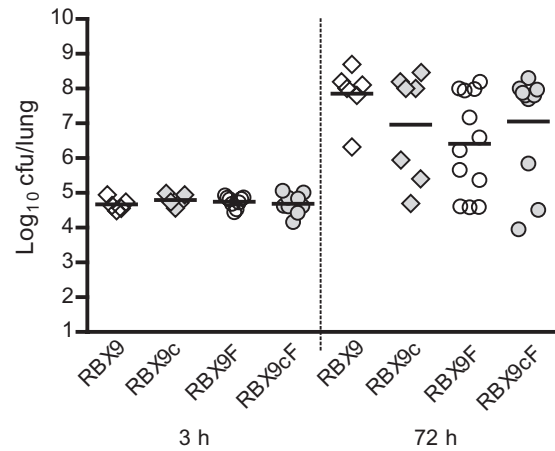


Fig. 8. Comparison of RBX9, RBX9c, RBX9F, and RBX9cF burdens in the mouse lung after 3 h and 72 h p.i. RBX9c and RBX9cF are Bvg⁺ phase-locked derivatives of RBX9 and RBX9F, respectively; 4–8-week-old BALB/C mice were intranasally infected with 1×10^5 cfu in 50 μ l and lungs were harvested at each time point; each diamond or circle indicates the number of cfu recovered from a single animal and each horizontal line indicates the geometric mean for each group; these data represent three independent experiments with at least two mice per strain per time point.

Discussion

The discovery and characterization of LCVs in *B. bronchiseptica* yielded several interesting findings, the most significant being evidence that transcriptional interference can result from activity at a promoter located several hundred nucleotides 5' to the affected promoter. Given this relatively large distance between promoters, the mechanism of interference likely does not involve direct blocking of transcription; therefore, we suggest the name 'passive transcriptional interference' for this phenomenon. Our data indicate that the *bvgAS* P_2 promoter is sensitive to passive transcriptional interference and that it results in the emergence of a bistable phenotype, apparent as LCVs, when bacteria are switched from Bvg⁻ phase conditions to Bvg⁺ phase conditions. The fact that LCVs, which contain Bvg⁻ phase-trapped bacteria, were recovered from the lungs of infected mice, provided evidence that BvgAS modulation occurs *in vivo*. Our experiments indicate, however, that although a small proportion of bacteria apparently do modulate during infection, this level of modulation does not alter the outcome of infection.

Bacterial populations often exhibit phenotypic heterogeneity. A common mechanism by which bacteria can generate this heterogeneity is phase variation, a reversible and heritable change in phenotype (due to either genetic or epigenetic modifications) often manifested as different colony morphologies (Hallet, 2001; van der Woude and Bäumlér, 2004). Phase variation frequently alters the production of surface-exposed epitopes such as pili, capsule,

flagella, lipopolysaccharide (LPS), and adhesins (van der Woude and Bäuml, 2004). Coincidentally, phase variation is often associated with virulence and is an important strategy used by pathogens to avoid immune selection. Some well-characterized examples of phase variation include the *opa* operon encoding adhesin proteins in *Neisseria* species, the *pap* operon encoding fimbriae in *Escherichia coli*, and the flagella subunits encoded by *fljBA* and *fliC* in *Salmonella enterica* serotype Typhimurium (Simon et al., 1980; Stern and Meyer, 1987; Hernday et al., 2002). In *Bordetella*, phase variation in both *fim3* and *bvgAS* has been described (Stibitz et al., 1989; Willems et al., 1990).

More recently, a phenomenon that generates bistable populations at the single-cell level has been discovered, called feedback-based multistability (FBM) (Smits et al., 2006). FBM is distinct from phase variation in that it is not based on genetic mutations but is instead based on feedback loops of regulatory networks (Smits et al., 2006). In isogenic populations, these feedback networks can result in bistability, which occurs when individuals in a population exhibit either one of two alternative stable steady-states (but not intermediate states) (Ferrell, 2002). A well-characterized example of FBM is in *Bacillus subtilis*, in the regulation of competence orchestrated by the transcription factor ComK (van Sinderen et al., 1995). Competence is a cellular state induced by nutrient depletion, but only occurs in a fraction of the *B. subtilis* population due to oscillating levels of ComK at the single-cell level (Veening et al., 2008; Espinar et al., 2013). In one study, Smits et al. removed the external regulation of *comK*, leaving only positive autoregulation, and showed that ComK levels continued to exhibit bistability. Therefore, the authors argue that ComK bistability can be reduced to a positive autoregulatory loop in concert with random transcriptional and translational fluctuations or 'noise' (Smits et al., 2005). This claim is supported by other examples, in which feedback regulation and a non-linear input are the only required components for a bistable system (Ferrell, 2002; Maamar and Dubnau, 2005).

We discovered LCVs of *B. bronchiseptica* after plating lung homogenates of mice infected with strain RBX9 and found that they yielded a heterogeneous population upon restreaking onto BG blood agar. We did not find evidence of classical phase variation in RBX9. Instead, the mechanism by which LCVs are generated appears more similar to FBM, in which the concentration of BvgA under Bvg⁻ phase conditions varies in the population and results in some, but not all, bacteria committing to a positive feedback loop when switched to Bvg⁺ phase conditions. In support of this hypothesis, we were able to label bacteria within LCVs with tags unique to the Bvg⁺ and Bvg⁻ phases and demonstrate the existence of two phenotypically distinct populations within LCVs (Fig. 3). The dual-tagged RBX9 strain also provided the first direct evidence that

Bvg⁻ phase cultures are not simply a mixture of Bvg⁺ and Bvg⁻ phase bacteria.

Use of the recombinase-based reporter system pGFLIP (Byrd et al., 2013) showed that the $\Delta fhaB$ mutation in RBX9 causes a decrease in the efficiency of *bvgAS* positive autoregulation and results in Bvg⁻ phase-trapped bacteria that decline in proportion over time and can initiate the formation of new LCVs (Figs 4, 5 and 7). Based on these results and previous data, we postulate a model of LCV formation and propagation (Fig. 9). According to this model, the concentration of BvgA varies in a population and also in individual cells as they grow and divide. In RB50, the average concentration of BvgA under Bvg⁻ phase conditions is such that 100% of the bacteria are able to respond to Bvg⁺ phase conditions and transition to the Bvg⁺ phase phenotype (Fig. 9A and B). In RBX9, however, the average concentration of BvgA is decreased under Bvg⁻ phase conditions compared with wild-type bacteria (curve shifted to the left in Fig. 9A), such that a subpopulation is below the threshold level required to respond to Bvg⁺ phase conditions (Fig. 9B). These bacteria are thus Bvg⁻ phase trapped and remain phenotypically Bvg⁻ phase even under Bvg⁺ phase conditions. Under Bvg⁺ phase conditions, these bacteria form LCVs, which continue to harbour Bvg⁻ phase-trapped bacteria. The Bvg⁻ phase-trapped bacteria within LCVs occasionally escape to become Bvg⁺ phase descendants, possibly through unequal distribution of BvgA upon cell division or by stochastic accumulation of BvgA (Fig. 9C). Therefore, in this system, a mutation that decreases the basal concentration of the positively autoregulated factor (BvgA) results in an FBM-like phenotype, whereas in other systems, FBM is the natural mechanism by which bacteria reach a bistable state.

Our pGFLIP data indicated that the *bvgAS* positive autoregulation defect is due to decreased activity of P₂. This result explains why RBX9 has lower levels of BvgA: it has decreased transcription of *bvgAS*. Our data suggest that the reason for decreased transcription is the presence of a promoter located 5' to the P₂ promoter. This upstream divergent promoter exerts its negative effects on *bvgAS* from relatively far away (~ 800 bp) and this phenomenon appears to represent a previously undescribed form of transcriptional interference (the suppressive influence of one transcriptional process on another) (Shearwin et al., 2005). It is unclear whether this promoter must be highly active or divergently transcribed. However, as with other examples of transcription interference, we predict that increasing this promoter's strength would also increase the degree of interference (Sneppen et al., 2005). Additionally, we predict that the orientation of the promoter may not be important and that reversing its orientation would not abolish interference if transcription read through was prevented. We do not understand mechanistically how this

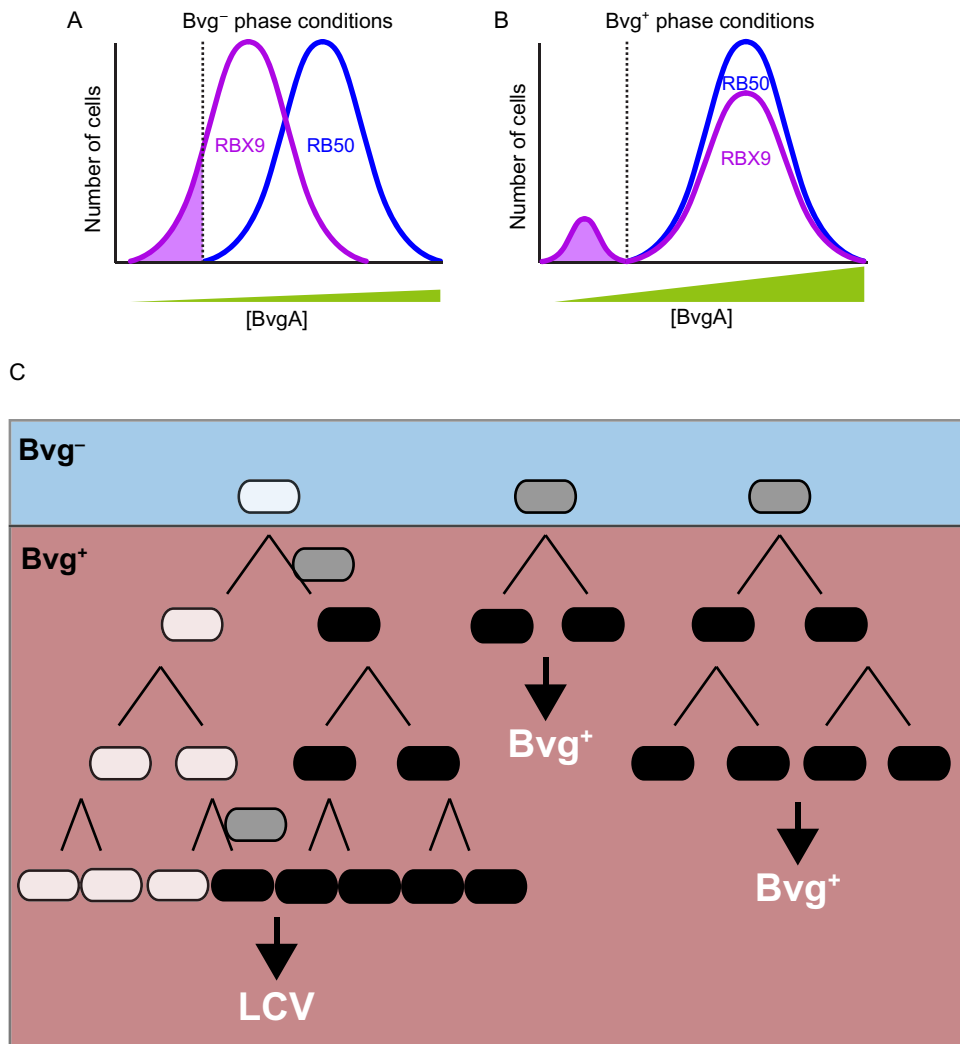


Fig. 9. Proposed distribution of BvgA concentration within populations of RBX9 and RB50.

A. In the Bvg⁻ phase, a proportion of RBX9 cells (shaded region) are Bvg⁻ phase-trapped [i.e. have a concentration of BvgA below the threshold (dotted line) necessary to stimulate positive autoregulation upon transition to Bvg⁺ phase conditions]. By contrast, all RB50 cells have a level of BvgA sufficient to initiate positive autoregulation upon transition to Bvg⁺ phase conditions.

B. In the Bvg⁺ phase, the RBX9 cells that were below the threshold BvgA concentration in the Bvg⁻ phase (shaded region as in A) maintain their low concentration of BvgA and are thus unable to switch to the Bvg⁺ phase. These cells are able to initiate LCV formation as described in C. Consistent with our *in vitro* data, all RB50 cells are able to switch to the Bvg⁺ phase.

C. Model of LCV formation and propagation illustrated as a lineage diagram (see text for details). RBX9 bacteria exist as a heterogeneous population under Bvg⁻ phase conditions, with some bacteria (white) being below the threshold of BvgA required to initiate positive autoregulation and others above this threshold (grey). When bacteria are switched to the Bvg⁺ phase, the Bvg⁻ phase trapped bacteria form LCVs, whereas the other bacteria transition into the Bvg⁺ phase (black) and form Bvg⁺ phase colonies. Occasionally, Bvg⁻ phase-trapped bacteria 'escape' and can transition into the Bvg⁺ phase (indicated by grey cells between white and black cells), resulting in LCV formation after 48 h.

'passive' transcriptional interference occurs. One possibility is that the divergent promoter sequesters RNA polymerase away from the sensitive promoter (P_2) or that transcription at this site influences DNA topology in a way that is prohibitive to P_2 activation. These data suggest that the level of expression from the *bvgAS* P_2 promoter is poised at the threshold of that required for all cells in the population to respond to Bvg⁺ phase conditions when they are encountered. Although the LCV phenotype appeared

as an artefact of genetic manipulation, our results are important as they demonstrate a form of transcriptional interference that, to our knowledge, has not been described previously, and also because they reveal a mechanism by which in-frame deletion mutations can have unanticipated polar effects on neighbouring genes. Furthermore, RBX9 and its derivatives constitute a genetically tractable system for studying additional mechanisms of transcriptional interference and details of FBM.

The LCVs also provided insight into the behaviour of BvgAS during infection. The role of BvgAS-dependent modulation in the *Bordetella* life cycle is not completely understood and remains an important area of investigation. Several studies have attempted to determine if Bvgⁱ or Bvg⁻ phase bacteria exist at any point during *Bordetella* infection, and so far none have yielded positive results (Cotter and Miller, 1994; Merkel *et al.*, 1998; Tejada *et al.*, 1998; Vergara-Irigaray *et al.*, 2005; Nicholson *et al.*, 2012). These data, together with those demonstrating that Bvg⁻ phase bacteria transition rapidly to the Bvg⁺ phase following intranasal inoculation (Veal-Carr and Stibitz, 2005; Byrd *et al.*, 2013), have led to the conclusion that not only is the Bvg⁺ phase necessary and sufficient for infection, but that bacteria switch to and remain in the Bvg⁺ phase *in vivo*. The Bvgⁱ and Bvg⁻ phases are hypothesized to be important for transmission and survival *ex vivo*, however, no role for these phenotypic phases in a natural setting has been demonstrated. For *B. pertussis* particularly, which appears to survive outside the host only briefly during transmission to a new host, the role of BvgAS modulation remains mysterious. The isolation of LCVs from mouse lungs provides strong evidence that at least some RBX9 bacteria modulate during infection. However, the proportion of bacteria that modulated and that could be recovered from the animals was very low. Because the *P_{fliA}-flp* system was unable to reliably distinguish this low proportion of modulated bacteria from background resolution, we could not determine if wild type bacteria modulate *in vivo*. If they do not, our data would suggest that only FHA-deficient bacteria modulate *in vivo*, which would suggest that FHA functions to prevent the bacteria from experiencing a Bvg⁻ phase environment during infection. In pilot experiments, we also recovered LCVs from mice infected with $\Delta fhaB$, $\Delta cyaA$ double mutants – in higher proportions, in some cases, than in mice infected with RBX9. These preliminary data suggest the intriguing possibility that FHA and ACT function together to prevent *Bordetella* from creating or entering a modulating environment in the host. Our future experiments will be aimed at testing this hypothesis.

Experimental procedures

Strains and growth conditions

Escherichia coli were grown in lysogeny broth (LB; 10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 2.5 g l⁻¹ NaCl) or on LB with agar (1.5%) at 37°C. *Bordetella* were grown in SS broth or on BG agar (1.5%) (BD Biosciences, San Jose, CA) supplemented with 7.5% defibrinated sheep's blood (Colorado Serum Company, Denver, CO) at 37°C. As required, culture media were supplemented with kanamycin (Km; 50 µg ml⁻¹), ampicillin (Ap; 100 µg ml⁻¹), streptomycin (Sm; 25 µg ml⁻¹), magnesium sulphate (MgSO₄; 50 mM in plates and 20 mM in

liquid), and diaminopimelic acid (DAP; 400 µg ml⁻¹) for the *E. coli* mobilizer strain RHO3 ($\Delta asd \Delta aphA$). Unless otherwise noted, all restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. For a complete list of the strains and plasmids used in this study, see Table S1.

Construction of bacterial strains

Allelic exchange and Campbell-type integrations were done by matings using parental *Bordetella* and *E. coli* strain RHO3 harbouring the appropriate suicide plasmid. The pGFLIP plasmid was delivered to the *atfTn7* site using tri-parental mating with the above strains and with RHO3 cells harbouring pTNS3, which encodes the transposase genes *tnsABCD*. Integration at the *atfTn7* locus was confirmed via PCR. For details on specific strain constructions see the Supplemental text.

Immunofluorescence

HA epitopes on the surface of RBX9BatB-HA*fliA-gfp* bacteria were stained and visualized using indirect immunofluorescence. After 72 h of growth at 37°C, five to 20 colonies of each morphology including Bvg⁺, Bvg⁻, Bvgⁱ, and LCV, were scraped off of BG blood agar plates and resuspended into 1 ml of 4% paraformaldehyde and were allowed to fix on ice for 30 min. Cells were pelleted and washed with 1% BSA in PBS in a microcentrifuge tube. Primary antibody (mouse monoclonal anti-HA IgG diluted 1:2000 in 1% BSA in PBS) was used to resuspend the pellet and this mixture was incubated for 1 h at room temperature (RT). The pellets were washed twice in 1% BSA for 5 min. Secondary antibody (Alexa Fluor 594-conjugated goat anti-mouse IgG, diluted 1:250 in 1% BSA in PBS; Invitrogen) was used to resuspend the pellet and this mixture was then incubated for 30 min at RT in the dark. The pellets were washed twice with 1% BSA for 5 min. Four microlitres of the leftover pellet and liquid was pipetted onto a slide for visualization.

Confocal microscopy

Immunofluorescent RBX9BatB-HA*fliA-gfp* bacteria were visualized using a Zeiss LSM 710 confocal microscope. Secondary antibody (Alexa Fluor 594-conjugated goat anti-mouse IgG) was detected using a 594 nm laser and GFP was detected at 488 nm. We used the 63× oil objective with 3× digital zoom. Images were viewed and saved with the Zen software from Carl Zeiss Microscopy.

pGFLIP assays

For RBX9*cyaA*FLP, RB50P*bvgA*_{short}FLP and RB50P*bvgA*_{long}FLP, strains were grown on BG blood agar plates under promoter-inactive conditions (Bvg⁻ phase) with Km 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). Single GFP⁺ colonies were then resuspended in PBS, diluted, and plated onto Bvg⁺ or Bvg⁻ phase conditions (promoter-active

and promoter-inactive conditions respectively) in the absence of Km selection. For RBX9*cya*AFLP, GFP⁺ LCVs were picked, diluted, and plated onto Bvg⁺ and Bvg⁻ phase conditions in the absence of Km selection. Percent resolution was determined by averaging the ratio of GFP⁻ cfu/total cfu for at least three plates.

Intranasal inoculation of mice

Four- to 8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intranasally with 1×10^5 cfu of *B. bronchiseptica* in 50 μ l of PBS. For all infections, bacteria were grown overnight in SS medium. Lungs were harvested at 1 h and 72 h p.i. Right lungs were homogenized in 1 ml of PBS, diluted in PBS, and plated in at least duplicate on BG agar. Figure 8 represents data from three independent experiments performed with at least two mice per strain per time point.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol was approved by the University of North Carolina IACUC (10-134, 12-307). All animals were properly anesthetized for inoculations, monitored regularly, euthanized when moribund, and efforts were made to minimize suffering.

Statistical analyses

Statistical analyses were performed using Prism 5.0 (GraphPad Software). Statistical significance was determined using the unpaired Student's *t*-test or analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Images were formatted using Adobe Photoshop CS5 and figures were generated using Adobe Illustrator CS5 (Adobe Systems).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.