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BvgAS-Mediated Signal Transduction: Analysis of Phase-Locked Regulatory Mutants of *Bordetella bronchiseptica* in a Rabbit Model

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Members of the *Bordetella* genus alternate between two distinct phenotypic phases in response to changes in their environment. This switch, termed phenotypic modulation, is mediated by the BvgAS sensory transduction system. We developed an animal model based on the interaction of *Bordetella bronchiseptica* with one of its natural hosts, the rabbit. To investigate the importance of BvgAS signal transduction, we constructed constitutive (RB53) and Bvg$^-$ (RB54) phase-locked derivatives of a wild-type strain, RB50. RB50 and RB53, but not RB54, established respiratory infections in *B. bronchiseptica*-free rabbits with an intranasal 50% infective dose of less than 200 organisms, and the course of the infection closely resembled that observed with naturally infected rabbits. Bacteria were recovered from the nasal cavity, larynx, trachea, and lungs in similar numbers from RB50- and RB53-infected rabbits, yet no pathology was detected by histological examination of lung and tracheal sections. The antibody responses in rabbits inoculated with RB50 or RB53 were quantitatively and qualitatively indistinguishable; high titers of antibodies were generated primarily against Bvg$^+$-phase-specific antigens. No response against flagella, a Bvg$^-$ phase factor, was detected. Assessment of bacteria associated with alveolar macrophages indicated that only a small percentage of bacteria, if any, appear to be residing within lung macrophages. We also tested the ability of these strains to survive in a nutrient poor environment, conditions which may be encountered within certain niches in the host or in an environmental reservoir. The Bvg$^-$ phase was advantageous for growth under these conditions. Our results indicate the Bvg$^+$ phase is sufficient for establishment of respiratory tract infection in the rabbit and the normal BvgAS-mediated response to environmental signals is not required during initial colonization. The Bvg$^-$ phase may play a role at later stages of infection, including persistence, transmission, or survival in the environment.

Bordetella species cause respiratory infections in humans and other animals (17, 40). These gram-negative bacteria are capable of alternating between two distinct phenotypic phases in response to changes in their environment. This switch, termed phenotypic modulation, is mediated by the products of the *bvgAS* regulatory locus (5, 22, 25). Adhesins and toxins associated with virulence are synthesized in the Bvg$^+$ phase. In the Bvg$^-$ phase, most of these virulence factors are not expressed and in some species the phenotype of motility appears (2, 4). While the ability of *Bordetella* species to undergo phenotypic modulation has been recognized for many years (22, 39), the role of this signal transduction process in pathogenesis is still unknown.

The BvgAS sensory transduction proteins of *Bordetella pertussis*, the etiologic agent of whooping cough, and *Bordetella bronchiseptica*, which causes respiratory infections in a variety of lower animals, are 96% identical at the amino acid level (7). BvgA and BvgS are members of a family of bacterial proteins that regulate gene expression in response to changes in environmental conditions (5, 7, 37). The signaling pathway involves autophosphorylation of BvgS followed by phosphoryl-group transfer to BvgA (38). During growth on artificial media, the switch from Bvg$^+$ to Bvg$^-$ occurs in response to the presence of $MgSO_4$, nicotinic acid, or low temperature (modulating conditions). While the signals which are sensed in nature are unknown, the functional conservation of BvgAS within and across species suggests phenotypic modulation is an important adaptive response for these organisms. Bvg$^+$ phase factors include the adhesins filamentous hemagglutinin (FHA) (32), fimbriae (10, 42), and pertactin (11, 29) and the toxins adenylate cyclase-hemolysin (13, 16) and dermonecrotic toxin (23). *B. pertussis*, but not *B. bronchiseptica*, also synthesizes pertussis toxin (6, 26, 32, 41). Several of these factors have been shown to be required for virulence in mouse models (18, 41).

A prominent feature of the Bvg$^-$ phase is the phenotype of motility in *B. bronchiseptica*. Motility is regulated at the level of flagellar synthesis by a transcriptional cascade that is ultimately controlled by BvgAS (2, 4). Bvg$^+$-phase cells are nonmotile and do not synthesize flagella, while Bvg$^-$-phase cells produce peritrichous flagellar organelles and are highly motile. In addition, production of a hydroxamate siderophore, alcaligin, has recently been shown to be under negative control by BvgAS (1). Several Bvg-repressed loci, termed *vrg*s, have also been identified in *B. pertussis* (8, 9, 21); however, functional homologs of these genes have not yet been identified in *B. bronchiseptica*.

Our approach to investigate the roles of the Bvg$^+$ and Bvg$^-$ phases and the importance of BvgAS-mediated signal transduction in vivo is to study respiratory infection in an animal model using *Bordetella* strains that are isogenic except for characterized mutations that specifically affect signal transduction. We reasoned that if the ability to undergo phenotypic modulation was required at some stage during infection, then

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TABLE 1. Characteristics of strains used in this study

Strain	Genotype	Phenotype	Expression of characteristic[a]									
			FHA		Hemolytic activity		Colony morphology		Motility		Flagellin mol mass (kDa)	
			SS	SS mod	BG	BG mod	BG	BG mod	SSA	SSA mod	BG	BG mod
RB50		Bvg^{wt}	+	−	+	−	sd	lf	−	+	−	40
RB53	*bvgS-C3*	Bvg^{c}	+	+	+	+	sd	sd	−	−	−	−
RB54	*bvgSΔ54*	Bvg^{-}	−	−	−	−	lf	lf	+	+	40	40

[a] FHA expression was assayed with cultures grown in Stainer-Scholte medium (SS) by Western blot with anti-FHA antisera. Hemolytic activity and colony morphology were determined on BG agar (sd, small, domed; lf, large, flat). Motility was determined in Stainer-Scholte soft agar (SSA). Flagellin expression and protein molecular mass were determined with cells grown on BG agar by Western blot with antiflagellin specific antibody. mod, modulating conditions ($MgSO_4$) present during growth. +, present; −, not present.

mutant strains which are locked into either the Bvg^+ or Bvg^- phase would be altered in their ability to either establish an infection, cause disease, persist, or be transmitted to a new host.

Since *B. pertussis* exclusively infects the human respiratory tract, we chose to develop as a model the interaction between *B. bronchiseptica* and one of its natural hosts, the rabbit. *B. bronchiseptica* commonly colonizes the respiratory tract of rabbits, usually resulting in asymptomatic infections, although occasionally the upper respiratory tract disease known as snuffles and rarely bronchopneumonia occur (12, 17). Another advantage of this model is the fact that *B. bronchiseptica* is motile in the Bvg^- phase and therefore the flagellar filaments constitute a potential immunologic marker. Finally, since *B. bronchiseptica* efficiently colonizes its host while only rarely causing disease, we felt this model would allow us to identify subtle differences between strains, including both a decrease in colonization efficiency and an increase in virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are described in Table 1 and in the figure legends. *B. bronchiseptica* strains were grown either on Bordet-Gengou agar (BG; Difco Laboratories, Detroit, Mich.) containing 7.5% sheep blood, on L agar (33), or in Stainer-Scholte broth (35). *Escherichia coli* strains were grown on L agar or in L broth (33). The following additions to media were made as appropriate: 40 mM $MgSO_4$; 10 mM nicotinic acid; 20 μg of gentamicin (Gm), tetracycline, or streptomycin per ml; 40 μg of kanamycin per ml; 10 μg of cephalothin per ml; and 100 μg of ampicillin per ml. Motility assays were conducted in Stainer-Scholte medium with 0.35% agar.

DNA methods. Isolation of plasmid and chromosomal DNA, restriction enzyme digestions, agarose gel electrophoresis and DNA ligations were performed by standard methods (33). Restriction enzymes, calf intestinal alkaline phosphatase, Klenow fragment, T4 DNA polymerase, and T4 DNA ligase were from Promega Corp. (Madison, Wis.), Boehringer Mannheim (Indianapolis, Ind.), New England Biolabs (Beverly, Mass.), or Bethesda Research Laboratories (Gaithersburg, Md.) and were used according to the manufacturers' directions. Radiolabeled nucleotides were from New England Nuclear. Plasmid constructions were performed with *E. coli* DH5α (33).

Isolation and construction of *B. bronchiseptica* strains. RB50 is a *B. bronchiseptica* strain isolated from the naris of a 3-month-old New Zealand White rabbit. In the Bvg^+ phase, RB50 expresses FHA and hemolytic activity (due to adenylate cyclase toxin) and produces small domed colonies on BG agar (Table 1; Fig. 1A). In the Bvg^- phase, resulting from modulation with $MgSO_4$, nicotinic acid, or growth at low temperature, FHA and hemolytic activity are no longer produced, colonies are large and flat, and the phenotype of motility appears. Bvg^--phase RB50 expresses a 40-kDa flagellin protein which corresponds to one of the two identified *B. bronchiseptica* flagellin isotypes that differ in electrophoretic mo-

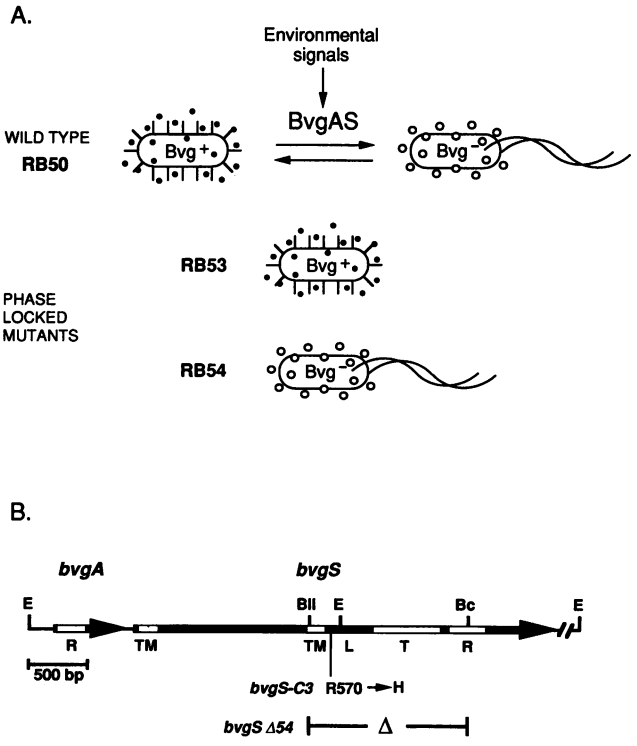

FIG. 1. Phase-locked *B. bronchiseptica* mutants. (A) Wild-type strain RB50 alternates between the Bvg^+ and Bvg^- phases in response to environmental signals by a process controlled by BvgAS. In the Bvg^+ phase several toxins (solid circles) and putative adhesins (rods) are expressed. Bvg^--phase characteristics include flagella, motility, and secretion of the siderophore alcaligin (open circles). RB53 is locked in the Bvg^+ phase (Bvg^c phenotype) because of the presence of the *bvgS-C3* allele, and RB54 is locked in the Bvg^- phase as a result of a deletion in *bvgS* (*bvgSΔ54*). (B) Structure of the *bvgAS* locus (5, 25). Sequences encoding the receiver (R), transmembrane (TM), linker (L), and transmitter (T) domains of BvgA and BvgS are indicated (5). The location of the *bvgS-C3* mutation resulting in an arginine-to-histidine substitution in BvgS at position 570 is shown (25). The *bvgSΔ54* allele consists of a 1.4-kb in-frame deletion from the unique *Bgl*II (BII) to *Bcl*I (Bc) sites in *bvgS*. E, *Eco*RI.

bility (2, 4). Additionally, RB50 is oxidase, catalase, urease, and citrate positive and does not ferment glucose or lactose, characteristics consistent with the identification of this strain as *B. bronchiseptica*.

The Bvg^--phase-locked mutant, RB54, was constructed as follows. The *bvgAS* locus from the *B. bronchiseptica* GP1SN strain (2) was cloned into a pBR322 derivative as a 5.2-kb *Eco*RI fragment. A 1.4-kb *Bgl*II-*Bcl*I fragment was removed, resulting in deletion of sequences encoding amino acids 541 to 1000, which span the second transmembrane domain, the linker, the transmitter, and most of the receiver domain (Fig. 1B). The resulting 3.8-kb *Eco*RI fragment was cloned into plasmid pSS1129 (36), and this plasmid was mobilized into *B. bronchiseptica* RB50. Exconjugants were selected on BG-Gm-streptomycin plates and were phenotypically Bvg^+. Colonies were then stabbed into Stainer-Scholte motility agar (without modulators) and incubated at 37°C. Motile bacteria which moved out from the site of inoculation were picked and struck onto BG agar, where they formed large, flat, nonhemolytic colonies in both the presence and absence of modulating conditions (Table 1). These isolates were Gm^s, indicating resolution of the plasmid from the chromosome. Southern blot analysis indicated that the plasmid had integrated and resolved from the chromosome at the expected location.

The BvgS-constitutive strain was constructed as follows. Plasmid pIG1 contains approximately 19 kb of *B. bronchiseptica* DNA including the *bvgAS* locus on a *Bam*HI fragment carried in pBR322 (Fig. 1B [27]). The 2-kb *Stu*I-*Bbr*PI fragment which is internal to the *bvgS* coding region and which contains the region encoding the linker domain of *bvgS* was cloned into $pUC19RI^0$ (a pUC19 derivative in which the *Eco*RI site has been eliminated by Klenow treatment of *Eco*RI-digested pUC19) by use of *Bam*HI linkers to create plasmid pEG1. The *bvgS-C3* mutation, which was isolated and characterized in *B. pertussis* (25), is a single-base-pair change resulting in the substitution of a His for an Arg at position 570 of the BvgS protein. This point mutation is located on a 121-bp *Eco*RI-*Bgl*II fragment in which the DNA sequences of *B. pertussis* and *B. bronchiseptica* are identical. The 121-bp *Eco*RI-*Bgl*II fragment from pJMC21 (25), containing the *bvgS-C3* mutation, was swapped with that of pEG1, creating pEG3. The 2-kb *Bam*HI fragment from pEG3 was then cloned into pSS1129 for mobilization into RB50. Exconjugants were identified as Gm^r, flat, nonhemolytic colonies on BG-blood agar plates (Bvg^- phenotype). Individual colonies were grown without selection in Stainer-Scholte broth and then plated on BG-blood agar plates containing 20 mM nicotinic acid (modulating conditions). Three hemolytic colonies out of approximately 8,000 colonies were identified. Two of these isolates formed small, domed, hemolytic, nonmotile colonies when grown at 25°C in the presence of 20 mM $MgSO_4$ and 10 mM nicotinic acid (i.e., multiple modulating signals) as well as under nonmodulating conditions (Table 1) and thus had acquired the Bvg constitutive (Bvg^c) phenotype. Southern blot analysis confirmed that the plasmid had integrated and resolved from the chromosome at the intended chromosomal location.

Experimental animals. Rabbits were obtained from three commercial sources. A group of 12 New Zealand White rabbits obtained from Universal Farms (Bloomington, Calif.) were found to be colonized with *B. bronchiseptica* and were used to study naturally acquired infection. For the colonization experiment and for assessment of bacteria associated with alveolar macrophages, 4-month-old female, *B. bronchiseptica*-free New Zealand White rabbits were purchased from either Charles River Laboratories (Wilmington, Mass.) or Millbrook Farms (Amherst, Mass.). They were confirmed to be *B. bronchiseptica* free upon arrival by streaking nasal swabs onto BG agar with and without cephalothin (*Bordetella* species are naturally cephalothin resistant) and by the absence of serum anti-*Bordetella* antibodies as determined by Western blot (immunoblot) and enzyme-linked immunosorbent assay (ELISA) analyses. Rabbits were separated by an empty cage on all sides throughout the experiment to prevent cross-contamination. Rabbits were inoculated by gently delivering 5 μl of bacteria suspended in sterile phosphate-buffered saline (PBS) into each nostril while the rabbits were held in dorsal recumbency. The inoculum was absorbed immediately. Rabbits were monitored for signs of discomfort and respiratory distress daily, and nasal swabs and blood samples were obtained weekly. Blood was obtained by venous puncture of the marginal ear vein.

Animals were sacrificed 3 weeks postinoculation by intravenous injection of pentobarbital (1 ml of a 6-g/ml solution). Deep nasal swabs were obtained prior to opening of the chest cavity. Sterile techniques were used for removal of first the upper left and lower right lung lobes and then a 1-cm section of midtrachea and the larynx. These tissues were added to 5 ml of sterile PBS and homogenized in a Tekmar stomacher for 3 min. Aliquots were diluted and plated on BG agar to determine the CFU per gram of tissue. The paired differences of CFU $gram^{-1}$ between wild-type and mutant strains were compared by the two-tailed *t* test. The remaining portion of the respiratory system was removed and inflated with formalin, and lung and trachea sections were prepared for histological examination.

For determination of secretory immunoglobulin A (IgA) and association of bacteria with alveolar macrophages, animals were sacrificed 3 weeks postinoculation as described above but instead of dissecting the respiratory tract, the trachea and lungs were removed intact. A 2-cm section of trachea was washed with 2 ml of sterile PBS, and this fluid was used for determination of anti-*B. bronchiseptica* IgA. Bronchoalveolar lavage (BAL) was then performed as described previously (15). Approximately 10^7 cells were obtained per animal, of which >95% were determined to be macrophages by examination of Wright-Giemsa-stained samples and >90% were viable as determined by trypan blue exclusion. Erythrocytes were lysed by mild hypotonic lysis, and macrophages were washed in sterile PBS. Cells were treated with or without 100 μg of Gm per ml for 6 h either free in suspension or adhered to tissue culture wells, washed, and lysed, with dilutions plated for determination of CFU.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting. *B. bronchiseptica* whole-cell extracts or purified proteins solubilized in sample buffer were stacked in an SDS–4.5% polyacrylamide stacking gel and separated in an SDS-polyacrylamide (4 to 12%) gradient gel (acrylamide-bisacrylamide, 29:1) (33). Proteins were stained with Coomassie brilliant blue R-250 (Sigma, St. Louis, Mo.) or transferred to Immobilon (Millipore) for immunoblotting. Transferred proteins were incubated with rabbit serum (1:4,000 dilution) or antiflagellin monoclonal antibody 15D8 at 1:1,000 (14). Antigen-antibody complexes were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit Ig or anti-mouse IgG antibodies at a dilution of 1:5,000 (Amersham). HRP-conjugated antibody-antigen complexes were visualized by an enhanced chemiluminescence technique (Amersham).

ELISA. Antibody titers were quantitated by ELISA essentially as described previously (20). For whole cells, an overnight culture of RB50 was diluted 1:10 in coating buffer (carbonate-bicarbonate buffer, pH 9) and sonicated and 100 μl

of suspension was added to each well. FHA and flagella (see the description below) were suspended in coating buffer at concentrations of 5 and 10 μg/ml, respectively, and 100 μl of suspension was added to each well. The plates were incubated at 37°C in a humidified chamber for 2 h, then held at 4°C overnight, and used the next day. The wells were washed with PBS plus 0.5% Tween 20 (PBS-T); then 150 μl of 3% bovine serum albumin in PBS-T was added to block unbound sites, and the plates were incubated for 2 h. For detection of serum IgG, wells were washed again and then serum was added to the first well at a 1:100 dilution and twofold serial dilutions were performed across 11 wells. HRP-conjugated donkey anti-rabbit IgG was used as the secondary antibody at a dilution of 1:4,000. For detection of IgA, tracheal washings were diluted 1:10 and diluted serially across 11 wells. Goat anti-rabbit IgA was used as the secondary antibody at a dilution of 1:5,000. HRP-conjugated rabbit anti-goat IgG antibody was then used as the tertiary antibody at a dilution of 1:5,000. A_{402} was read after a 30-min incubation with the substrate 2,2′-azinobis(3′ ethylbenzthiazoline sulfonic acid) (ABTS) purchased from Sigma. Absorbance was plotted against dilution, and titers are expressed as the reciprocal of the dilution at the *y* intercept as extrapolated from the linear part of the curve. Samples from which the optical density at 402 nm was less than 0.4 at the lowest dilution were arbitrarily assigned a titer of <100 for serum IgG or <10 for secretory IgA.

Purified antigens. Flagellar filaments were purified by CsCl equilibrium density centrifugation as previously described (4). Purified FHA was obtained from Lederle-Praxis Biologicals (West Henrietta, N.Y.).

Immunoelectron microscopy. *Bordetella* strains grown on BG-agar plates were resuspended in SCM buffer (0.15 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$) and transferred to glow-discharged, Formvar-coated copper grids. Immunoelectron microscopy was performed as described previously (19), with rabbit sera diluted 1:20 as the primary antibody and colloidal gold-labeled goat anti-rabbit IgG as the secondary antibody diluted 1:20. Grids were examined in an electron microscope (JEOL 100CX) at 80 kV of accelerating voltage.

Immunofluorescent staining of cytological tissue. To visualize bacteria associated with alveolar macrophages, cells from BAL fluid which adhered to glass coverslips were examined by immunofluorescence as described previously (19), with postinfection rabbit sera diluted 1:100 as the primary antibody and fluorescein isothiocyanate-labeled anti-rabbit IgG as the secondary antibody.

RESULTS

Observations of naturally acquired *B. bronchiseptica* infection in rabbits. A population of 3-month-old female New Zealand White rabbits was obtained from a commercial rabbitry for intended use in respiratory infection studies. Contrary to expectations, culture of the distal nares indicated that all animals were already colonized with *B. bronchiseptica*. High titers of serum antibody reacting with whole cells and purified FHA were detected by ELISA and Western blots (data not shown). Our wild-type strain RB50 was isolated from the naris of one of these rabbits, passaged once on BG-blood agar and frozen, and bacteria from this stock were used as the source for all subsequent genetic manipulations and for growth of cultures for inoculation.

We took advantage of the opportunity to study the clinical course of naturally acquired *B. bronchiseptica* infections in a rabbit population. Upon necropsy at 3 or 5 weeks after arrival, *B. bronchiseptica* was recovered from the nasal cavities and larynxes of all animals (n = 12), and in most cases *B. bronchiseptica* was the predominant isolate from deep cultures of the nasal turbinates (data not shown). Five of the twelve animals were colonized to detectable levels in the trachea and 6 of 12 were colonized in the upper left or lower right lung lobe. Despite the presence of *B. bronchiseptica* in the upper and often the lower respiratory tract, at no time were clinical signs of respiratory disease (i.e., labored breathing, hyperpnea, cyanosis, or serous or purulent nasal discharge) observed and by all other criteria the animals remained healthy until sacrifice. Larynx, trachea, and lung specimens showed no gross pathology, and histological examination of lung and tracheal sections showed no indications of inflammation or abnormal tissue structure. Naturally acquired *B. bronchiseptica* infection in our population of New Zealand White rabbits was exclusively asymptomatic.

TABLE 2. Inoculation and colonization schedule[a]

Group and no. of rabbits	Inoculum dose (CFU)	No. of rabbits colonized	
		Day 3	Day 7
Group 1			
2	PBS	0	0
4	2×10^6 RB54	0	0
6	2×10^2 RB50	2	4
6	2×10^3 RB50	4	6
6	2×10^4 RB50	6	6
6	2×10^2 RB53	2	3
6	2×10^3 RB53	5	6
6	2×10^4 RB53	6	6
Group 2			
2	PBS	0	0
4	1×10^3 RB50	2	4
4	1×10^3 RB53	1	4

[a] The number of rabbits from which *B. bronchiseptica* was recovered from nasal swabs on days 3 and 7 is indicated. After day 7, rabbits remained colonized or not colonized for the duration of the experiment. Group 1 rabbits were used for the colonization experiment, and group 2 rabbits were used for detection of IgA and association of bacteria with alveolar macrophages.

Wild-type and phase-locked mutant *B. bronchiseptica* strains. With respect to the Bvg-regulated characteristics examined (Table 1), RB50 is phenotypically Bvg^{wt} (Bvg wild type) and responds to environmental signals that modulate BvgAS activity in a manner that is characteristic of *B. bronchiseptica* strains isolated from a variety of mammalian hosts (4, 27).

Bvg^+ (RB53) and Bvg^- (RB54) phase-locked derivatives of RB50 were constructed by allelic exchange. RB53 (*bvgS-C3*) constitutively expresses Bvg^+-phase characteristics and is nonmotile in the presence or absence of modulating signals due to a single-amino-acid substitution in the linker region of BvgS (Arg-570→His, Fig. 1; Table 1). RB53 is therefore locked in the Bvg^+ phase and has a Bvg^c (Bvg constitutive) phenotype. RB54 (*bvgSΔ54*) contains a 1.4-kb in-frame deletion that removes *bvgS* sequences encoding the second transmembrane domain, the linker region, the transmitter domain, and part of the receiver domain (amino acids 541 to 1000; Fig. 1B). RB54 is motile in the presence or absence of modulating conditions, is unable to express Bvg^+-phase phenotypes (Table 1), and is therefore locked in the Bvg^- phase.

Infection of New Zealand White rabbits with wild-type and mutant *B. bronchiseptica* strains. To investigate the role of BvgAS-mediated signal transduction in establishment of infection and short-term colonization, we inoculated female, 4-month-old, *B. bronchiseptica*-free New Zealand White rab-

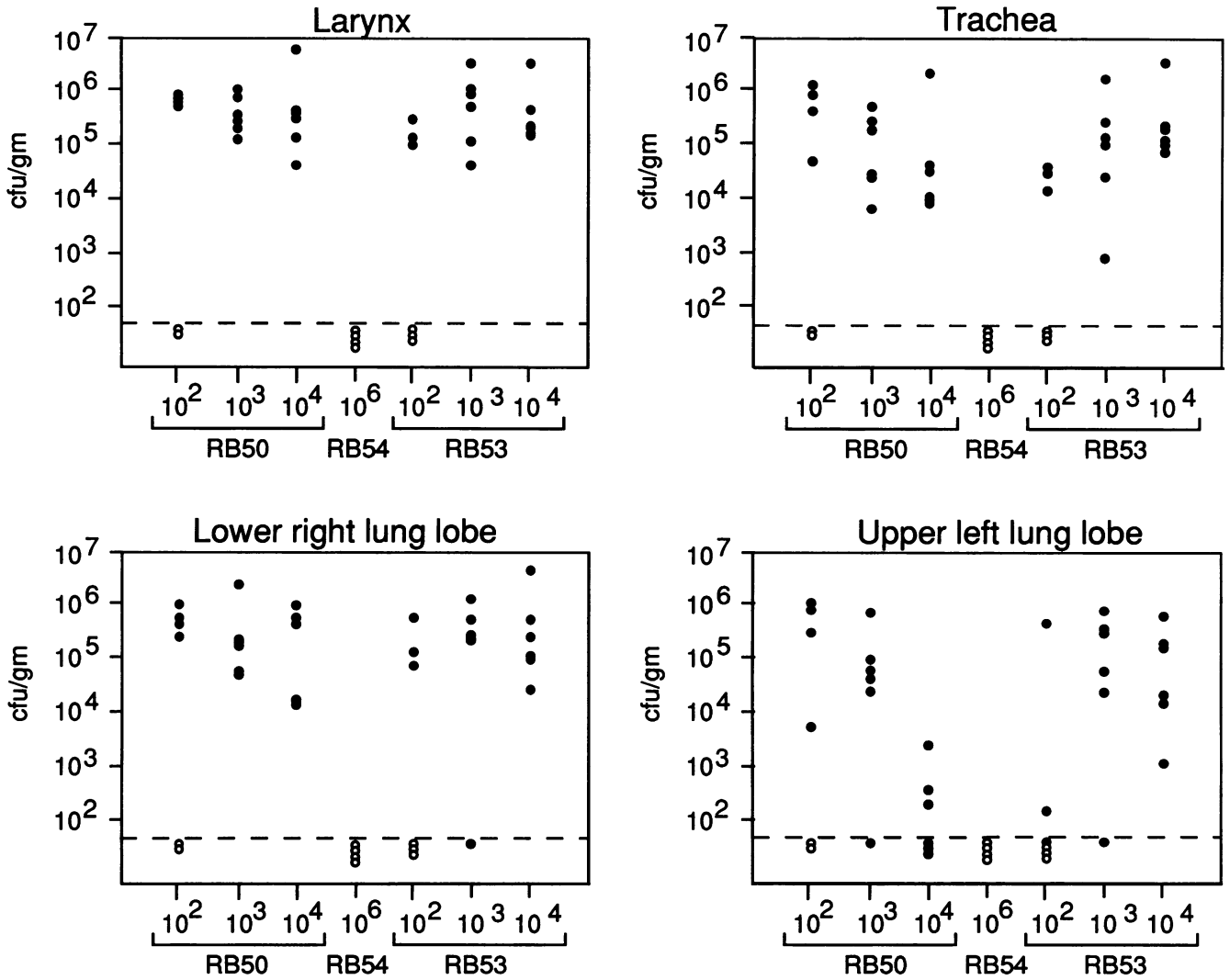

FIG. 2. Scatter plots showing colonization levels in the trachea, larynx, and upper left and lower right lung lobes. Rabbits were inoculated with RB50, RB54, or RB53 at the doses (in CFU) indicated and sacrificed 3 weeks later. Counts of CFU per gram of tissue are shown. Open circles represent uncolonized animals, and solid circles represent infected rabbits. The lower limit of detection was 50 bacteria g^{-1}, as represented by dashed lines.

bits with the wild-type strain RB50 or the phase-locked mutants RB53 (Bvg^c) or RB54 (Bvg^-). Five microliters of a bacterial suspension in PBS was delivered to each nostril according to the inoculation schedule shown in Table 2 (group 1). We had previously determined the 50% infective dose (ID_{50}) to be approximately 200 bacteria by the intranasal route.

Rabbits inoculated with PBS or RB54 remained *B. bronchiseptica* free throughout the course of the experiment (Table 2). *B. bronchiseptica* was recovered by nasal swab from some rabbits inoculated with 2×10^2 or 2×10^3 CFU and from all rabbits inoculated with 2×10^4 CFU of RB50 or RB53 after 3 days (Table 2). A few rabbits inoculated with 2×10^2 or 2×10^3 CFU of RB50 or RB53 were not positive by nasal swab until day 7. All rabbits that were positive by day 7 remained positive for the duration of the experiment, and all that were negative remained negative. Rabbits were sacrificed 3 weeks postinoculation, and CFU from the larynx, trachea, and upper left and lower right lung lobes were determined. *B. bronchiseptica* became the predominant organism in the deep nasal cavity and larynx (>90% of culturable organisms on BG agar) in all animals which became colonized, apparently displacing the normal flora. *B. bronchiseptica* was also isolated from the midtrachea and lungs of all infected animals, tissues which were sterile in PBS- and RB54-inoculated animals. As shown in Fig. 2, the patterns of colonization of the larynx, trachea, and lung lobes by RB50 or RB53 were nearly indistinguishable. The sole exception was a decrease in colonization of the left upper lung lobe in animals inoculated at the highest dose (2×10^4 CFU) with RB50 compared with RB53 colonization ($P < 0.05$). For animals in which infection was established, colonization levels in the respiratory tract appeared to be independent of inoculum size for both RB50 and RB53. Additionally, the numbers of bacteria isolated from the various tissues were similar to the number obtained from the naturally infected rabbits described above. In all cases, bacteria recovered from colonized animals were phenotypically identical to the inocula: Bvg^- mutants were not detected in RB50-infected rabbits, and Bvg^{wt} revertants were not found in RB53-infected animals.

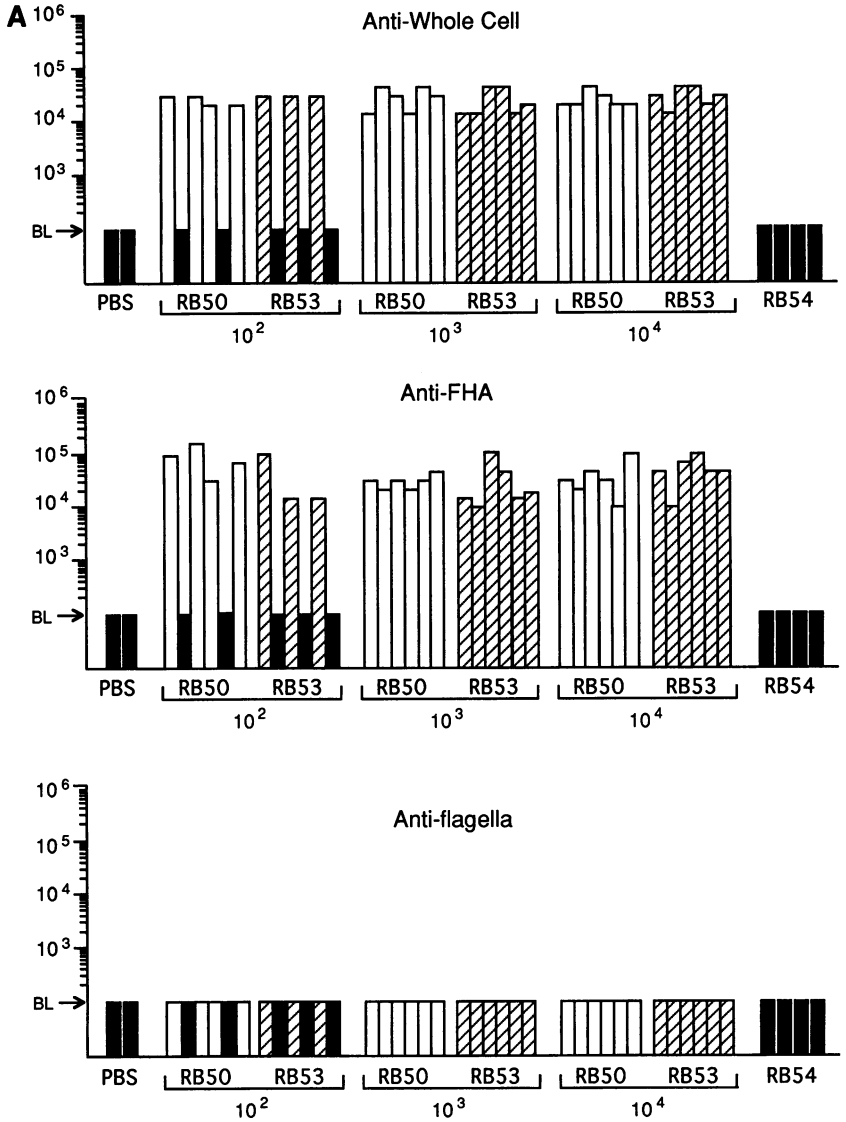

FIG. 3. (A) Antibody titers against a whole-cell preparation of strain RB50, purified FHA, or purified flagella determined by ELISA. The sera used were those obtained at the time of sacrifice for each animal. The strains and doses (in CFU) used to infect each rabbit are indicated below the graph. Solid bars represent animals that did not become infected with *B. bronchiseptica*, open bars designate animals infected with RB50, and striped bars indicate animals infected with RB53. Background levels (BL) of reactivity that were nonlinear upon dilution of the samples are indicated (arrows). (B) Time course of the appearance of serum antibodies against whole cells, FHA, and flagella as determined by ELISA is shown for a representative animal inoculated with 2×10^3 CFU of strain RB50. Preinoculation samples (P) and samples obtained 11 (line 1), 18 (line 2), and 26 (line 3) days after inoculation are represented. OD_{402}, optical density at 402 nm.

Clinical disease and histological examination of tracheal and lung tissue. Rabbits were monitored daily for signs of disease. All animals appeared healthy throughout the 3-week course of the experiment. Sections of trachea and lung tissue stained with hematoxylin and eosin revealed no microscopic abnormalities consistent with respiratory disease or inflammation (data not shown). Tissues of infected animals were indistinguishable from those of uninfected and control animals. Infection by both RB50 and RB53 was therefore asymptomatic, as previously observed in rabbits with naturally acquired infections. Infection of guinea pigs with wild-type *B. bronchiseptica* has also been shown to result in asymptomatic colonization of the respiratory tract (28).

Quantitation of anti-*Bordetella*, anti-FHA, and antiflagellar antibodies by ELISA. The production of serum antibody directed against whole cells as well as phase-specific factors was examined by ELISA (Fig. 3A). FHA was used as a marker for the Bvg^+ phase, and purified flagella were used as a marker for the Bvg^- phase. In all cases, infection with *B. bronchiseptica* was associated with a significant rise in serum IgG titers against whole cells and FHA by week 3 (Fig. 3A). Animals inoculated with PBS or RB54 and animals that failed to become colonized after inoculation with RB50 or RB53 had only background levels of anti-*Bordetella* antibody. There was no significant difference between antibody titers against whole cells and against FHA in rabbits infected with either RB50 or RB53. As observed with colonization levels, antibody titers at 3 weeks postinfection were independent of the infective dose (Fig. 3A).

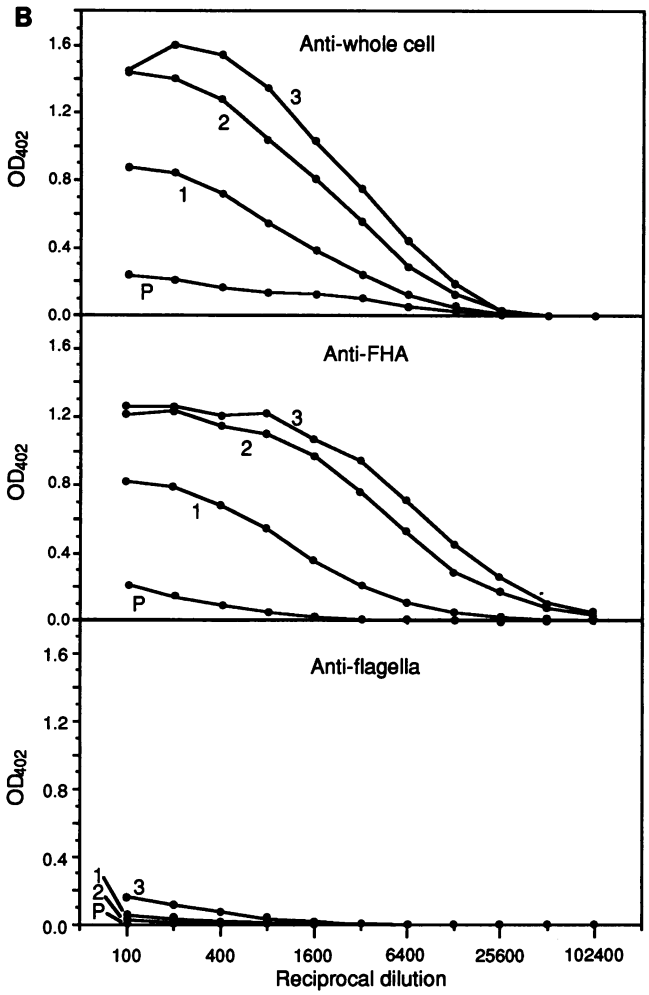

FIG. 3—*Continued.*

The kinetics of appearance of serum IgG against whole cells and FHA were similar in all infected rabbits, and results from a representative animal are shown in Fig. 3B.

Although there was a marked increase in circulating antibody directed against whole cells and FHA, no increase in antibody against flagella was observed in animals infected with RB50, RB53, or RB54 (Fig. 3). Antiflagellar antibodies were also not detected in serum from a small set ($n = 3$) of naturally infected rabbits which were colonized for at least 6 months (data not shown). In contrast, serum from rabbits infected with a derivative of RB50 in which flagella are expressed in the Bvg^+ phase generated high titers of antiflagellar antibody, indicating that *B. bronchiseptica* flagella are immunogenic and that antiflagellar antibodies can be detected by this assay (3).

Qualitative analysis of the antibody response against *B. bronchiseptica*. Western blots were used to obtain a broader assessment of the serum antibody response to antigens regulated by the BvgAS control system. Whole-cell extracts from RB53 (Bvg^+ phase) and RB54 (Bvg^- phase) separated by SDS-PAGE were probed with rabbit sera (Fig. 4). Preinoculation sera and sera obtained at the time of sacrifice from rabbits inoculated with RB54 were unreactive. Sera obtained at the time of sacrifice from rabbits infected with either RB50 or RB53 reacted with a variety of Bvg^+-phase-specific proteins. Figure 4 shows representative blots, with sera from rabbits

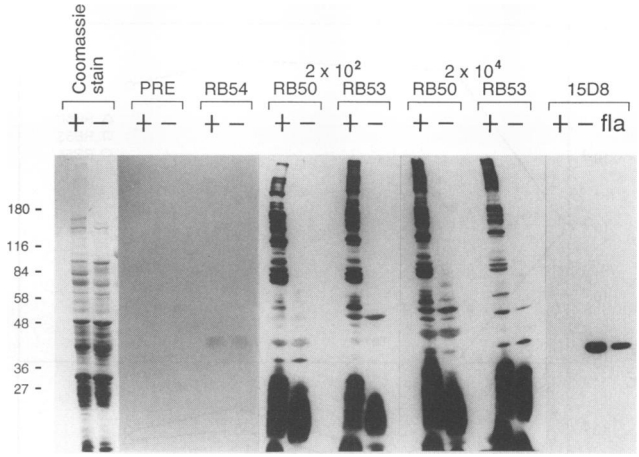

FIG. 4. Western blots comparing the antibody responses to infection with antigens regulated by BvgAS. Whole-cell extracts of RB53 (+) and RB54 (−) or purified flagellin (fla) were run on a 4 to 12% gradient polyacrylamide-SDS gel and transferred to polyvinylidene difluoride. Membrane strips were incubated with serum obtained at the time of sacrifice from animals infected with the strains and doses indicated or with pooled preinoculation sera (PRE) from the same rabbits. 15D8 is a monoclonal antibody against flagellin. The mobilities of protein molecular weight markers are indicated on the left in thousands.

inoculated with the lowest or highest dose of RB50 or RB53. All infected rabbits demonstrated similar patterns of antibody response. Relatively few proteins from Bvg^--phase cells were recognized by postinoculation sera, and those that were recognized appeared to be common to both Bvg^+-phase and Bvg^--phase organisms. No reactivity to the 40-kDa flagellin protein synthesized by RB54 was detected in Western blots of whole-cell extracts. Purified flagellin protein was also used for Western blot analysis, and again no increase in specific reactivity against flagellin during infection was detected in the various sera (data not shown). As a control, flagellin-specific monoclonal antibody 15D8 showed strong reactivity against purified flagellin as well as flagellin protein present in whole-cell extracts from Bvg^--phase cells (Fig. 4).

The antibody response against surface-exposed antigens was also examined by immunoelectron microscopy. Use of postin-

TABLE 3. Localization of *B. bronchiseptica* in BAL samples[a]

Inoculum	Expt. no.	Bacterial count (CFU)		Gm^r (%)
		−Gm	+Gm	
PBS	1	0	0	0
RB50	1	4.0×10^5	0	0
	2	1.0×10^5	0	0
	3	2.5×10^5	400	0.16
	4	1.3×10^5	5	<0.01
RB53	1	4.5×10^5	15	<0.01
	2	1.0×10^5	10	<0.01
	3	1.1×10^4	30	0.27
	4	2.5×10^5	40	0.02

[a] BAL samples were obtained from rabbits infected intranasally with 1×10^3 CFU of either RB50 or RB53 and sacrificed 3 weeks after inoculation. Bacterial counts obtained following 6 h of incubation in the absence (−Gm) or presence (+Gm) of 100 μg of Gm per ml are shown for each rabbit. A 6-h incubation with 100 μg of Gm per ml was sufficient to reduce bacterial viability by 6 orders of magnitude. A control animal inoculated with sterile PBS did not become colonized during the experiment.

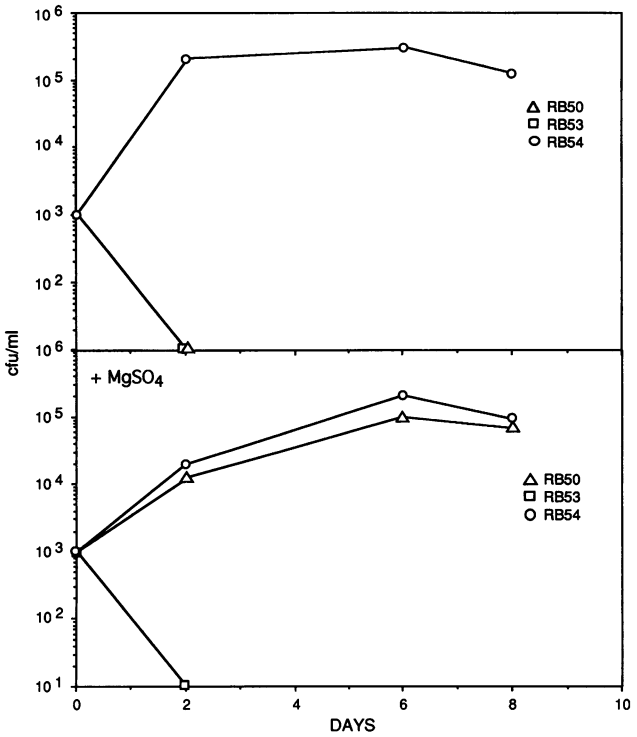

FIG. 5. Growth and/or survival of wild-type and mutant strains in PBS with or without 40 mM $MgSO_4$. All strains were inoculated at 10^3 CFU/ml and grown with shaking at 37°C. The graphs show data from one representative experiment that has been repeated several times with similar results.

oculation rabbit serum as the primary antibody was followed by use of colloidal gold-labeled anti-rabbit IgG. While many gold particles were observed decorating the bacterial cell surface, the number of gold particles associated with flagellar filaments was no greater than background (data not shown).

These results demonstrate that a major portion of the immunodominant antigens generated during respiratory tract infection are specific to the Bvg^+ phase. Antibodies to Bvg^--phase-specific antigens such as flagellin were not detected by ELISA, Western blot, or immunoelectron microscopy.

Distribution of *B. bronchiseptica* in samples obtained by BAL. It has previously been suggested that *B. pertussis* colonizes the lungs of rabbits as two approximately equal populations, one extracellular and the other within pulmonary macrophages (34). In those studies, rabbits were inoculated intratracheally with very large numbers of bacteria (10^8 CFU [34]). To examine the localization of *B. bronchiseptica* within the lungs of rabbits infected by the intranasal route, and to determine if phenotypic modulation affected this parameter, we inoculated female New Zealand White rabbits with 10^3 CFU of RB50, RB53, or sterile PBS (Table 2, group 2). After 3 weeks, rabbits were sacrificed and BAL was performed to recover alveolar macrophages. Approximately 10^7 alveolar cells were recovered per rabbit, >95% of which were macrophages as identified by microscopic examination of Wright-Giemsa-stained samples, and >90% of the cells were determined viable by trypan blue exclusion. Association of bacteria with macrophages was initially assessed by immunofluorescence. BAL cells were allowed to settle onto coverslips and then fixed in methanol. The coverslips were incubated first with rabbit serum and then with fluorescein isothiocyanate-labeled anti-rabbit Ig. Fluorescent bacteria were observed in association with approximately 1 to 5% of the macrophages (data not shown).

To determine whether viable bacteria in BAL samples were primarily extracellular or intracellular, aliquots were incubated with or without Gm for 6 h and then washed, lysed, diluted, and plated. As shown in Table 3, the vast majority of bacteria were susceptible to killing by Gm and no difference was observed between animals infected with RB50 and those infected with RB53. In control experiments, internalization of *B. bronchiseptica* by J774A.1 cells conferred Gm resistance. These results demonstrate that *B. bronchiseptica* colonizes the lungs of intranasally infected rabbits in a niche that is primarily extracellular.

Growth of *B. bronchiseptica* strains under nutrient-limiting conditions. *B. bronchiseptica* has been shown to survive and grow in lake water and PBS, and it has been suggested that an environmental reservoir may exist for this organism (30, 31). To determine whether phenotypic modulation is required for growth and/or survival under nutrient-limiting conditions, we inoculated PBS with or without $MgSO_4$ (modulating or nonmodulating conditions, respectively) with RB50, RB53, or RB54. Aliquots were plated at 2, 6, and 8 days. Surprisingly, RB54 increased approximately 2 logs under these conditions, with or without the addition of $MgSO_4$, whereas RB50 and RB53 were unable to survive in the absence of $MgSO_4$ (Fig. 5). In the presence of $MgSO_4$, RB50 (which can switch to the Bvg^- phase) survived and grew as well as RB54, while RB53 was unable to survive. These results clearly indicate that the Bvg^- phase is advantageous under nutrient-limiting conditions, in contrast with the demonstrated necessity of the Bvg^+ phase for colonization in animals.

DISCUSSION

The ability of bacteria to sense and adapt to changes in their environment is often mediated by differential regulation of gene expression (26). The BvgAS sensory transduction system, common to *B. pertussis* and *B. bronchiseptica*, coordinately regulates expression of nearly all of the known virulence factors synthesized by these organisms and would be expected to play a pivotal role in their survival strategy. Our goal was to investigate the importance of BvgAS signal transduction in vivo with specific *bvgS* mutant derivatives of *B. bronchiseptica*.

We observed that *B. bronchiseptica* infection of rabbits, either naturally acquired or experimentally induced, is characterized by efficient establishment and colonization throughout the entire respiratory tract. The infection is typically asymptomatic and persistent despite the generation of high titers of serum anti-*Bordetella* antibodies. We anticipated that the inability to down-regulate virulence factor expression, and up-regulate expression of Bvg repressed genes, would alter the normal course of infection. We were surprised to find instead that RB53, a Bvg^+-phase-locked mutant, was indistinguishable from wild-type RB50 in our rabbit model. RB53 established infection with the same ID_{50} as RB50, and similar numbers of bacteria were recovered from sites throughout the respiratory tract. Additionally, RB50 and RB53 showed similar patterns of distribution in the lungs, with viable cells of both strains existing primarily in an extracellular location. Furthermore, RB53 infection was asymptomatic, indicating that down-regulation of adhesins and toxins is not required for limiting the extent of respiratory tissue damage.

Our results suggest that the Bvg^+ phase is both necessary and sufficient for efficient establishment of respiratory tract infection, since infection by the Bvg^+-phase-locked mutant,

ZRB53, was indistinguishable from that by the wild type. Although the Bvg^c mutant has a pronounced signal-independent phenotype in vitro, we cannot rule out the possibility that an unknown signal is modulatory in vivo. However, several lines of evidence indicate that this is not the case. RB53 displays a Bvg^+ phenotype even in the presence of multiple modulating signals which are used in vitro (i.e., growth at low temperature in the presence of both nicotinic acid and $MgSO_4$). Additionally, the *bvgS-C3* allele is dominant over in-frame deletions that remove sequences encoding nearly the entire periplasmic region of BvgS, which is assumed to be required for sensing modulators (25).

The antibody response to bacterial infection can be used to assess gene expression in vivo. All colonized rabbits generated a strong antibody response directed primarily against Bvg^+-phase factors. It was therefore surprising that the inability of RB53 to down-regulate expression of a large portion of the immunodominant antigens did not affect persistence. The lack of a response against Bvg^--phase-specific factors such as flagellin suggests that the Bvg^- phase may not even be expressed in vivo. Experiments using a strain which expresses flagella ectopically in the Bvg^+ phase indicate that *B. bronchiseptica* flagella are immunogenic and can stimulate high titers of specific antibody (3); however, we cannot rule out the possibility that wild-type *B. bronchiseptica* switches to the Bvg^- phase at a location within the host in which an antibody response is not efficiently generated.

We did not directly address whether BvgAS plays a role in transmission. It is possible that phenotypic modulation is required either for the organism to exit one host or for the initial interaction with the next. Since the RB50 inoculum was prepared by growing cells under nonmodulating (Bvg^+ phase) conditions, growth under modulating conditions may have led to a difference in the infectivities of RB50 and RB53, and we are currently testing this possibility. Alternatively, phenotypic modulation, resulting in down-regulation of adhesins, could be required for the organisms to be released from the infected host or to survive in the environment between hosts. Indeed, our results showing a growth advantage for the Bvg^- phase in PBS indicate that phenotypic modulation may be required for growth and/or survival under nutrient-limiting conditions, such as may be encountered in an environmental reservoir. It remains to be determined if an environmental reservoir does exist for *B. bronchiseptica* and, if so, whether it plays a role in transmission.

In contrast to our results with *B. bronchiseptica*, a role for the Bvg^- phase in *B. pertussis* virulence has been suggested by Beattie et al. (8). Strains with Tn*phoA* insertions in two Bvg-repressed genes, *vrg-6* and *vrg-18*, were tested in a mouse respiratory infection model. The *vrg-6* mutant was less able to proliferate in the trachea and lungs of infected mice than wild-type *B. pertussis*. This result suggests a role for at least one Bvg^--phase-specific factor in colonization and predicts that a Bvg^+-phase-locked mutant of *B. pertussis* would show a similar defect. We are currently investigating the effects of Bvg^+- and Bvg^--phase-locked derivatives of *B. pertussis* in a mouse model to test this prediction.

Resolution of the apparent contrast between our results and those of Beattie et al. (8) will require a careful comparison of the Bvg^- phases of *B. pertussis* and *B. bronchiseptica* and a thorough assessment of the roles of each in the biology of the respective organisms. Common Bvg^--specific factors have not been identified, and it appears that the Bvg^- phases of these two organisms may be quite different. *B. pertussis* contains DNA sequences which hybridize to the *flaA* gene of *B. bronchiseptica*, but flagellin is not synthesized and *B. pertussis* is nonmotile (3, 4). Conversely, *B. bronchiseptica vrg-6* hybridizing sequences have been identified, but this pseudogene is transcriptionally silent (8, 9). Thus, while the Bvg^+ phases of *B. pertussis* and *B. bronchiseptica* are quite similar, the Bvg^- phases may have diverged as these species evolved to adapt different survival strategies. The fact that BvgA and BvgS are almost identical in the two organisms and respond to the same set of environmental signals in the laboratory strongly suggests that BvgAS-mediated phenotypic modulation is important and serves similar functions for both organisms. A detailed comparison of the role of BvgAS signal transduction in *B. pertussis* and *B. bronchiseptica* will contribute to our understanding of these pathogens.

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