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Neither the Bvg$^-$ Phase nor the *vrg6* Locus of *Bordetella pertussis* Is Required for Respiratory Infection in Mice†

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In *Bordetella* species, the BvgAS sensory transduction system mediates an alteration between the Bvg$^+$ phase, characterized by expression of adhesins and toxins, and the Bvg$^-$ phase, characterized by the expression of motility and coregulated phenotypes in *Bordetella bronchiseptica* and by the expression of *vrg* loci in *Bordetella pertussis*. Since there is no known environmental or animal reservoir for *B. pertussis*, the causative agent of whooping cough, it has been assumed that this phenotypic alteration must occur within the human host during infection. Consistent with this hypothesis was the observation that a *B. pertussis* mutant, SK6, containing a Tn*phoA* insertion mutation in a Bvg-repressed gene (*vrg6*) was defective for tracheal and lung colonization in a mouse model of respiratory infection (D. T. Beattie, R. Shahin, and J. Mekalanos, Infect. Immun. 60:571–577, 1992). This result was inconsistent, however, with the observation that a Bvg$^+$ phase-locked *B. bronchiseptica* mutant was indistinguishable from the wild type in its ability to establish a persistent respiratory infection in rabbits and rats (P. A. Cotter and J. F. Miller, Infect. Immun. 62:3381–3390, 1994; B. J. Akerley, P. A. Cotter, and J. F. Miller, Cell 80:611–620, 1995). To directly address the role of Bvg-mediated signal transduction in *B. pertussis* pathogenesis, we constructed Bvg$^+$ and Bvg$^-$ phase-locked mutants and compared them with the wild type for their ability to colonize the respiratory tracts of mice. Our results show that the Bvg$^+$ phase of *B. pertussis* is necessary and sufficient for respiratory infection. By constructing a strain with a deletion in the *bvgR* regulatory locus, we also show that ectopic expression of Bvg$^-$ phase phenotypes decreases the efficiency of colonization, underscoring the importance of Bvg-mediated repression of gene expression in vivo. Finally, we show that the virulence defect present in strain SK6 cannot be attributed to the *vrg6* mutation. These data contradict an in vivo role for the Bvg$^-$ phase of *B. pertussis*.

All of the known protein virulence factors expressed by *Bordetella pertussis*, the causative agent of whooping cough, are positively regulated by the BvgAS sensory transduction system (for reviews, see references 10, 32, and 33). When active, BvgAS also represses a class of genes (*vrg* genes) and outer membrane proteins (Vra proteins) of unknown function (16, 31). Hence, BvgAS mediates a phenotypic transition between the Bvg$^+$ phase, characterized by the expression of adhesins and toxins, and the Bvg$^-$ phase, characterized by the expression of *vrg* genes and Vra proteins. Transition from the Bvg$^+$ phase to the Bvg$^-$ phase has been termed phenotypic modulation. Since *B. pertussis* has no known environmental or animal reservoir, it has been assumed that phenotypic modulation must occur within the human host. Proposed roles for a switch to the Bvg$^-$ phase in vivo include evasion of antibodies directed primarily against Bvg$^+$ phase factors, tempering of damage to host tissues as a result of decreased toxin expression, increased transmission as a result of decreased adhesin expression, and a requirement for Bvg$^-$ phase factors for the initial interaction with the host or for surviving within host cells (6, 9, 17, 20, 25). Experimental evidence that *B. pertussis* switches to the Bvg$^-$ phase in vivo is limited to the observation that a *B. pertussis* mutant containing a transposon insertion in a *vrg* locus (*vrg6*) was defective for virulence in a mouse model (6).

Studies with *Bordetella bronchiseptica*, a very closely related member of the *Bordetella* genus, contradict the hypothesis that *Bordetella* switches to the Bvg$^-$ phase in vivo. *B. bronchiseptica* causes respiratory infections in a wide range of nonhuman mammals, including dogs, pigs, rabbits, rats, and mice. It contains a BvgAS sensory transduction system with 96% amino acid identity to that of *B. pertussis* and expresses a nearly identical set of Bvg-activated adhesins and toxins (4, 13). Studies with Bvg$^+$ and Bvg$^-$ phase-locked mutants showed that the Bvg$^+$ phase of *B. bronchiseptica* is necessary and sufficient for respiratory infection, while the Bvg$^-$ phase is required for surviving nutrient limitation (1, 11). Moreover, failure to repress a Bvg$^-$ phase phenotype (motility) was detrimental to the development of infection, demonstrating the importance of Bvg-mediated repression of gene expression in vivo (1). These results led to the hypothesis that the role of BvgAS is to sense whether the organism is within or outside a mammalian host.

A possible explanation for these apparently contradictory results is that BvgAS plays different roles for these two species, sensing whether the organism is within or outside a mammalian host in the case of *B. bronchiseptica* and sensing specific niches within the host in the case of *B. pertussis*. In support of this hypothesis, the Bvg$^-$ phases of these organisms appear to differ dramatically (2, 6), and their BvgAS virulence control

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TABLE 1. Strains used in this study

Species and strain	Characteristics	Source or reference
B. pertussis		
18323 (ATCC 9797)	Wild type, Sm^s	ATCC
SK6	*vrg6*::Tn*phoA* Sm^r; original mutant	6
NSK6	*vrg6*::Tn*phoA* Sm^r; 18323 transductant	This work
SC3	*bvgS*-C3 Sm^s	This work
D6	Δ*vrg6* Sm^s; in-frame deletion in *vrg6*	This work
DR	Δ*bvgR* Sm^s; in-frame deletion in *bvgR*	This work
DS1	*bvgS*::pGMT74 Sm^s; *bvgS* disruption	This work
SC3-SK6	*bvgS*-C3 *vrg6*::Tn*phoA* Sm^r; SC3 transductant	This work
DR-SK6	Δ*bvgR vrg6*::Tn*phoA* Sm^r; DR transductant	This work
DS1-SK6	*bvgS*::pGMT74 *vrg6*::Tn*phoA* Sm^r	This work
BP121	*vrg6*::Tn*phoA′-′tnpR-res-tet-res-′vrg6* Sm^r	This work
BP147	*vrg6*::Tn*phoA′-′tnpR-res-tet-res-vrg6* Sm^r	This work
NBP121	*vrg6*::Tn*phoA′-′tnpR-res-tet-res-′vrg6* Sm^r; 18323 transductant	This work
NBP147	*vrg6*::Tn*phoA′-′tnpR-res-tet-res-vrg6* Sm^r; 18323 transductant	This work
E. coli		
DH5α	F^- *hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*argF-lac*) *U169* ϕ80d*lacZ*Δ*M15*	Bethesda Research Laboratories
SM10	RP4-2-TcMu Km^r	28
DM1187(pCLB1)	Colicin B-expressing strain	Theresa Finn

systems were recently shown to differ in their sensing capabilities (19). Alternatively, we have proposed that BvgAS may have originally evolved to serve the same purpose in both species, sensing whether the organism is within or outside its host, but that survival outside the host is no longer a significant part of the life cycle for *B. pertussis* (19). Here we report an experimental assessment of the role of BvgAS-mediated signal transduction in *B. pertussis* pathogenesis as a means of testing these opposing hypotheses. We constructed *B. pertussis* phase-locked and ectopic expression mutants and compared them with the wild type in a mouse model of respiratory infection. We also addressed the role of *vrg6* expression during infection by using newly constructed *vrg6* mutants and strains containing reporter fusions designed to detect *vrg6* expression in vivo. Our results indicate that, like in *B. bronchiseptica*, the Bvg^+ phase of *B. pertussis* is necessary and sufficient for respiratory infection and that ectopic expression of Bvg-repressed phenotypes under Bvg^+ phase conditions is detrimental to the infection process. We also demonstrate that the virulence defect in the original *vrg6* mutant, SK6, cannot be attributed to the *vrg6* mutation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. pertussis* strains are described in Table 1 and in the figure legends. *B. pertussis* SK6 and its parental strain, 18323, were kindly provided by D. Beattie and R. Shahin, respectively. *B. pertussis* strains were grown on BG agar (BBL, Becton Dickinson, Cockeysville, Md.) supplemented with 15% defibrinated sheep blood (Mission Labs, Rosemead, Calif.). Plates were incubated at 37°C for 72 to 96 h in loosely fitted screw-top jars to provide a moist environment. When mid-log-phase cells were needed, *B. pertussis* strains were grown in Stainer-Scholte (SS) medium (29) supplemented with 1 g of heptakis (15) per liter at 37°C with constant shaking. To grow *B. pertussis* under modulating (Bvg^- phase) conditions, $MgSO_4$ (20 mM) and nicotinic acid (5 mM) were added to the culture medium. When appropriate, antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following final concentrations: cephalexin, 10 μg/ml; gentamicin, 20 μg/ml; ampicillin, 30 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; streptomycin, 20 μg/ml; and chloramphenicol, 50 μg/ml. *Escherichia coli* strains were grown on Luria-Bertani (LB) agar or in Luria-Bertani broth (26) supplemented, when appropriate, with ampicillin (100 μg/ml) or gentamicin (20 μg/ml). *E. coli* DH5α (Stratagene, La Jolla, Calif.) was used in all of the cloning steps, and *E. coli* SM10 (28) was used to mobilize plasmids into *B. pertussis.*

Electroporation. Cells were grown in SS medium to mid-exponential phase, chilled on ice for 10 min, and harvested by centrifugation (7,000 × *g*, 4°C). After two washes with ice-cold H_2O, cells were resuspended in ice-cold H_2O and the concentration was adjusted so that the optical density at 600 nm was 5.0. Forty microliters of this suspension was mixed with 1 μg of desalted plasmid DNA in a prechilled electroporation cuvette (Bio-Rad). After application of the electric pulse (25 μF, 2,500 V, 200 Ω; Bio-Rad Gene Pulser), cells were allowed to outgrow in 1 ml of SS medium for 60 min at 37°C, concentrated by centrifugation, and plated on selective BG-blood agar.

Conjugation and allelic exchange. Matings were performed as previously described (19). To counterselect against the donor strain, mating products were plated on BG blood agar supplemented with a colicin B-enriched bacterial lysate (approximately 5 mg of total protein per ml), which was prepared from the colicin-producing *E. coli* strain DM1187(pCLB1) (a gift of Theresa Finn) as previously described (7). For allelic exchange, the *sacBR*-based system was used (1). Bacteria that had undergone a second recombination event resulting in loss of plasmid sequences were selected on BG-blood agar containing 10% sucrose.

Construction of *B. pertussis* phase-locked and deletion mutants. A Bvg^+ phase-locked derivative of strain 18323 was constructed as follows. Suicide plasmid pJM503, which contains a 2.3-kb *Sfi*I fragment of *′bvgS′* containing the *bvgS-C3* mutation (23), was electroporated into strain 18323, and cointegrates were selected on BG-blood agar supplemented with gentamicin. As expected, cointegrates grew as large, flat, nonhemolytic colonies characteristic of the Bvg^- phase. Although pJM503 contains the *rpsL* gene, encoding streptomycin sensitivity (Sm^s), this selection was not used because the 18323 parental strain is Sm^s. Instead, two cointegrates were picked, grown without antibiotic selection, and plated on BG agar containing 40 mM nicotinic acid and 15 mM $MgSO_4$ to screen phenotypically for colonies in which a second recombination event resulting in loss of plasmid sequences had occurred. Small, domed, hemolytic colonies on this medium, indicative of the Bvg^c phenotype, were characterized further. These colonies were gentamicin sensitive (Gm^s), indicating that they had indeed lost plasmid pJM503. One was named SC3 and was used in all subsequent analyses.

A Bvg^- derivative of 18323 was constructed by creating a disruption in *bvgS* by using plasmid pGMT74. pGMT74 is a suicide plasmid containing a 1.9-kb *Eco*RI-*Sna*BI internal *bvgS* fragment. Integration of this plasmid into strain 18323 disrupts *bvgS*, rendering the strain phenotypically Bvg^- under all growth conditions. Integration of pGMT74 is not expected to have polar effects, as the gene 3′ to *bvgAS*, *bvgR*, is transcribed in the orientation opposite to that for *bvgAS* (reference 21 and our unpublished data). A Bvg^- derivative of SK6 (DS1-SK6) was constructed by mobilization of pGMT74 into SK6.

Strain D6, containing an in-frame deletion in *vrg6*, was constructed as follows. An 818-bp *Eco*RI fragment containing *vrg6* was amplified from the 18323 chromosome by PCR with oligonucleotides predicted to anneal at positions 1 (5′-GAATTCCGTCTGCTGAACCAGA-3′) and 792 (3′) (5′-GAATTCGCATAACGGCTGGTGGAAGG-3′) of the published sequence (6). The PCR product was digested with *Eco*RI and cloned into *Eco*RI-digested pUC19 to create pGMT42. To generate an in-frame deletion in *vrg6*, pGMT42 was digested with *Eco*RV and *Nco*I, filled in with Klenow fragment, and religated, resulting in the deletion of 0.2 kb of DNA corresponding to approximately 70% of *vrg6*. The deletion junction was confirmed to have occurred as intended by DNA sequence analysis. Sequences at the 5′ end of *vrg6* reported to be essential for maintaining Bvg-dependent regulation were left intact to avoid polar effects on downstream genes. The 0.6-kb *Eco*RI fragment was then cloned into plasmid pEG25 (19), and the resulting plasmid, containing the *sacBR* cassette, was used to transfer the *vrg6* deletion to the chromosome of strain 18323. PCR and Southern blot analyses confirmed that D6 was constructed as intended.

To construct an 18323 derivative containing an in-frame deletion in *bvgR*, a 2.5-kb *Bcl*I-*Eco*RV fragment containing the *bvgR* locus from *B. pertussis* 338 was cloned into *Fsp*I-*Bam*HI-digested pACYC177. The resulting plasmid was digest-

ed with *Fsp*I and *Sca*I and religated, resulting in the deletion of 425 bp of DNA containing approximately 66% of *bvgR*. The deletion junction was confirmed to result in an in-frame deletion by DNA sequence analysis. The resulting Δ*bvgR* allele, contained on a 0.9-kb *Sal*I-*Xho*I fragment, was then cloned into plasmid pEG25 (19) and used to transfer the Δ*bvgR* allele to the chromosome of strain 18323. Southern blot analysis confirmed that DR was constructed as intended.

Construction of *B. pertussis* strains containing resolvase reporters of *vrg6* expression. Strains BP121 and BP147, containing *tnpR-res-tet-res* cassettes for assessing *vrg6* transcription, were constructed as follows. A plasmid derivative of pSS1129 containing a *′tnpR-res-tet-res-neo* cassette (8) flanked by *phoA* sequences at the 5′ end and *vrg6* 3′ sequences at the 3′ end was used to transfer the *′tnpR-res-tet-res-neo* cassette to the chromosome of SK6 by allelic exchange, resulting in the construction of BP121. In this strain, transcription of *vrg6* drives expression of the promoterless *′tnpR*. The *tnpR* gene product mediates site-specific recombination between the *res* sequences, resulting in the excision of the *tet* gene. *vrg6* expression therefore results in the loss of tetracycline resistance (Tc^s). Strain BP147 is identical to BP121 except that a complete copy of *vrg6* was provided at the 3′ end of the construct such that this strain contains a wild-type copy of *vrg6*. Southern blots confirmed that BP121 and BP147 were constructed as intended.

Construction of *B. pertussis* strains by generalized transduction. A *Bordetella*-specific bacteriophage capable of mediating generalized transduction was recently discovered in our laboratory (18a). This phage, designated BP3c, was used to transduce the *vrg6-phoA* fusion and linked sequences from *B. pertussis* SK6, BP121, and BP147 to the wild-type strain 18323 to create NSK6, NBP121, and NBP147, respectively. SK6, BP121, and BP147 are kanamycin resistant (Km^r) due to the Km^r gene contained on the Tn*phoA* element. They are also Sm^r. The basis for their being Sm^r is unknown, as they are derivatives of 18323, which is Sm^s. Transductants were selected on BG-blood agar containing kanamycin. Unexpectedly, all transductants were found to be Sm^r, suggesting that the Km^r gene contained on Tn*phoA* also confers Sm^r to *B. pertussis*. Southern blot analysis indicated that the genomic organizations of the *vrg6-phoA* regions were indistinguishable between SK6, BP121, and BP147 and NSK6, NBP121, and NBP147, respectively. This transduction protocol was also used to transfer the *vrg6-phoA* fusion from SK6 to SC3 and DR, creating SC3-SK6 and DR-SK6, respectively. Details of BP3c-mediated generalized transduction will be described elsewhere.

Western immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18). *B. pertussis* whole-cell lysates solubilized in sample buffer (60 mM Tris, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 0.1 M dithiothreitol, pH 6.8) were stacked in a 5% polyacrylamide gel and separated in a 4 to 12% acrylamide-bisacrylamide (29:1) linear gradient gel. Proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) and probed with a 1:2,500 dilution of serum from a patient recovering from whooping cough (a gift of James Cherry, UCLA Department of Pediatrics) or a 1:10 dilution of anti-VraB monoclonal antibody (a gift of Mark Peppler [31]). Sheep anti-human or sheep anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham International, Little Chalfont, United Kingdom) were used at a dilution of 1:5,000. The immunocomplexes were detected by using an enhanced chemiluminescence assay (Amersham) according to the manufacturer's directions.

Experimental animals. Three-week-old, *Bordetella*-free, female BALB/cAnNCR mice obtained from Charles River Laboratories were used in this study. Inocula were prepared by growing *B. pertussis* strains on BG-blood agar for 3 days at 37°C and then suspending the harvested cells in sterile phosphate-buffered saline (PBS). Mice were inoculated intranasally with 50 μl of PBS containing 10^4 CFU while the animals were slightly anesthetized with halothane. At the indicated times postinoculation, mice were sacrificed by halothane inhalation, the chest cavity was opened, and blood was obtained by cardiac puncture. One centimeter of mid-trachea and the right lung lobes were removed aseptically, homogenized in PBS, diluted, and plated on BG-blood agar. The nasal cavity was opened, and the nasal septum and adjacent turbinates were removed, homogenized in PBS, diluted, and plated. Animal protocols were approved by the University of California, Los Angeles, Animal Research Committee (ARC protocol 94-043). Statistical significance was determined by using a paired *t* test ($P \leq 0.05$).

Alkaline phosphatase activity assays. Alkaline phosphatase activity was measured by a published method (22).

Resolution of *res-tet-res* sequences. To determine the resolution frequencies of *res-tet-res* sequences in NBP121 and NBP147 in vitro, NBP121 and NBP147 were grown on BG-blood agar with or without 5 mM nicotinic acid and 20 mM $MgSO_4$ for 72 h at 37°C, and cells were harvested and plated on BG-blood agar without tetracycline and then replica plated onto BG-blood agar with and without tetracycline. Percent resolution was defined as number of Tc^s colonies/total number of colonies. The percentage of Tc^s colonies was also determined by individually patching colonies onto BG-blood agar with and without tetracycline. To determine the resolution frequency following in vivo growth, colonies recovered from the respiratory tracts of mice at 12 and 20 days postinoculation were plated onto BG-blood agar without tetracycline and then replica plated onto BG-blood agar with and without tetracycline. Individual colonies were also patched onto agar with and without tetracycline to confirm these data. For both the in vitro and in vivo assays, the inocula were prepared by growing cells on BG-blood agar containing tetracycline.

RESULTS AND DISCUSSION

Construction and in vitro characterization of phase-locked *B. pertussis* strains. Strain 18323 (Table 1) is the American Type Culture Collection (ATCC) type strain for *B. pertussis*. Although it has been recognized for some time that this strain is not typical of clinical *B. pertussis* isolates (3, 14, 24), it was chosen for this study so that our results could be directly compared with those of Beattie et al., from which it was concluded that the Bvg^- phase gene, *vrg6*, was required for virulence (6). 18323 is also the strain used in the intracerebral challenge test to assess the potencies of whole-cell pertussis vaccines, and it has been used extensively in an aerosol model of *B. pertussis* respiratory infection (27). 18323 is phenotypically wild type for Bvg. It forms small, domed, hemolytic colonies on BG-blood agar after 4 days of incubation at 37°C (nonmodulating or Bvg^+ phase conditions) and large, flat, nonhemolytic colonies on BG-blood agar supplemented with 20 mM $MgSO_4$ and 5 mM nicotinic acid (modulating or Bvg^- phase conditions). When grown under nonmodulating conditions, 18323 expresses Bvg^+ phase-specific antigens which can be detected by Western blotting with sera from children recovering from pertussis (Fig. 1A). It is interesting that the vast majority of antigens detected by using convalescent-phase sera are Bvg^+ phase specific. When 18323 is grown under modulating conditions, these Bvg^+ phase antigens are not expressed (Fig. 1A), and instead Bvg-repressed factors, including VraB (Fig. 1B) and *vrg6* (Fig. 2), are expressed.

We constructed a Bvg^+ phase-locked derivative of 18323 by transferring the *bvgS*-C3 mutation to the chromosome by allelic exchange (1, 23) (see Materials and Methods). The *bvgS*-C3 allele, a single nucleotide change resulting in an arginine-to-histidine substitution at amino acid position 570 in the linker region of BvgS, was originally isolated from and characterized for *B. pertussis* BP370 (23). *bvgS*-C3 also confers a Bvg constitutive (Bvg^c) phenotype to *B. bronchiseptica* (11). Strain SC3 (18323 *bvgS*-C3 [Table 1]) formed small, domed, hemolytic colonies on BG-blood agar with or without the addition of 20 mM $MgSO_4$ and 5 mM nicotinic acid. Its antigenic profile was similarly insensitive to modulating conditions; it constitutively expressed Bvg^+ phase antigens recognized by convalescent-phase serum (Fig. 1A) as well as filamentous hemagglutinin, fimbriae, and pertactin (data not shown) and did not express Bvg-repressed factors such as VraB (Fig. 1B) or *vrg6* (Fig. 2) even under modulating conditions.

To create a Bvg^- phase-locked derivative of 18323, we disrupted the *bvgS* gene with plasmid pGMT74, a suicide plasmid containing an internal *bvgS* fragment (Table 1). DS1 (18323:: pGMT74 [Table 1]) forms large, flat, nonhemolytic colonies under both modulating and nonmodulating conditions, never expresses Bvg^+ phase factors (Fig. 1A), and constitutively expresses VraB (Fig. 1B) and *vrg6* (Fig. 2).

The Bvg^+ phase of *B. pertussis* 18323 is necessary and sufficient for respiratory infection in mice. To determine if modulation to the Bvg^- phase is important for *B. pertussis* respiratory infection, we compared isogenic wild-type and phase-locked strains in a mouse model. Groups of 3-week-old BALB/c mice were inoculated intranasally with 50 μl of PBS containing 5×10^4 CFU of either 18323 or its mutant derivatives. Animals were sacrificed at day 0 (to estimate the number of CFU delivered to each site in the respiratory tract) and days 11, 26, and 35 postinoculation. For both 18323 and SC3, the numbers of CFU recovered from the nasal cavity, trachea, and lungs were increased at day 11 compared to day 0 and then decreased at day 26 and further still at day 35 (Fig. 3). There was no significant difference between the number of CFU recovered from

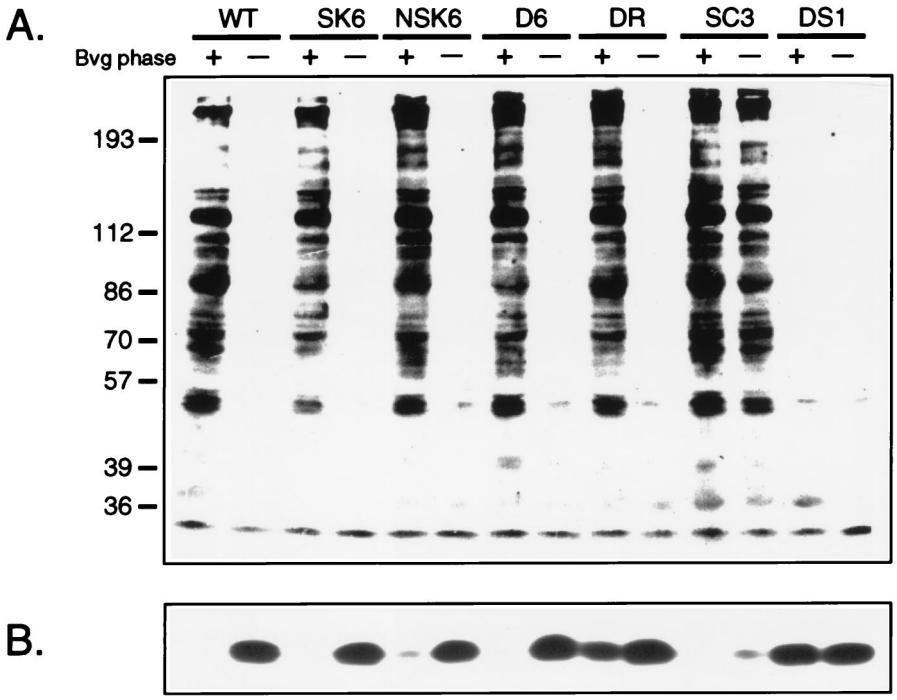

FIG. 1. Western blot analysis of the antigenic profiles of wild-type and mutant *B. pertussis* strains. Whole-cell lysates of the indicated strains grown under nonmodulating (Bvg^+ phase) conditions or modulating (Bvg^- phase) conditions were separated on an SDS–4 to 12% gradient polyacrylamide gel, transferred to polyvinylidene difluoride, and probed with serum from a child recovering from pertussis (A) or anti-VraB antibody (B). The positions of the molecular weight markers (in thousands) are shown at the left. WT, wild type.

18323- and SC3-inoculated mice at any site at any time point. In all cases, bacteria recovered from each site were phenotypically identical to the inoculum. In contrast, DS1 was not recovered from any site from any animal at day 6 postinoculation, confirming previous reports that Bvg^- mutants are unable to colonize the respiratory tract (34, 35).

Since strains 18323 and SC3 were indistinguishable in their ability to colonize the nasal cavity, trachea, and lungs, we conclude that the Bvg^+ phase is necessary and sufficient for respiratory infection by *B. pertussis*. We cannot conclude from these data, however, that wild-type *B. pertussis* does not modulate to the Bvg^- phase in vivo. To address this issue, we examined the antibody profile generated in response to *B. pertussis* infection. Sera from both 18323- and SC3-infected mice showed weak reactivity against Bvg^+ phase antigens and antigens common to both the Bvg^+ and Bvg^- phase but showed no reactivity against Bvg^- phase-specific antigens (data not shown). Similarly, sera from children recovering from pertussis contain high titers of antibody against Bvg^+ phase factors and factors common to both phases but not Bvg^- phase factors (Fig. 1A). These results indicate that either the transition to the Bvg^- phase does not occur in vivo or Bvg^- phase factors are nonantigenic. These results are exactly the same as those obtained with wild-type and phase-locked *B. bronchiseptica* strains in rabbits and rats (1, 11).

Bvg-mediated repression of gene expression is required for efficient tracheal colonization. The *bvgR* locus is involved in Bvg^+ phase repression of at least two *vrg* genes, *vrg6* and *vrg73* (21), as well as the VraB antigen (30). To further characterize the contribution of BvgR to regulation of *vrg* gene expression, we constructed a mutant derivative of strain 18323 containing an in-frame deletion in *bvgR* (see Materials and Methods) (Table 1). The colony morphologies displayed by DR (18323 Δ*bvgR*) under modulating and nonmodulating conditions were indistinguishable from those of 18323. Similarly, the Δ*bvgR* strain was unaltered in its ability to express Bvg^+ phase antigens, as determined by Western blot analysis with convalescent-phase serum (Fig. 1A). VraB expression, however, was

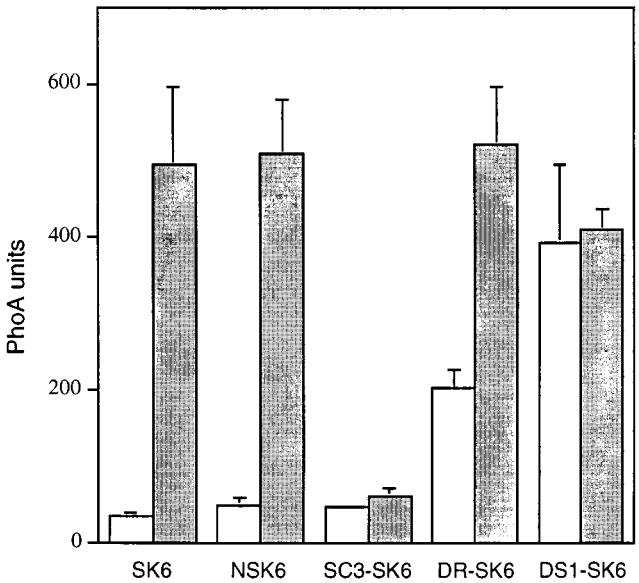

FIG. 2. Effect of BvgAS and BvgR on *vrg6* expression. Wild-type and mutant *B. pertussis* strains containing *vrg6-phoA* fusions were grown on BG-blood agar under nonmodulating (open bars) or modulating (shaded bars) conditions and suspended in assay buffer, and alkaline phosphatase activity was measured. Values are the means and standard errors for at least three independent assays performed in duplicate.

partially derepressed in the *bvgR* mutant grown under nonmodulating conditions (Fig. 1B), as was *vrg6* expression as determined by measuring alkaline phosphatase activity in a strain containing a *vrg6*::Tn*phoA* fusion (Fig. 2; also see below). These results are consistent with previous reports in which the involvement of BvgR in repression of *vrg6* and VraB was first described (21, 30) and suggest that an additional form of Bvg-dependent negative regulation exists. Since deletion of *bvgR* results in the inappropriate Bvg^+ phase expression of genes that are normally not expressed in this phase, the Δ*bvgR* strain can be classified as an ectopic expression mutant.

To assess the importance of BvgR-mediated repression of gene expression during infection, we compared the Δ*bvgR* strain with strain 18323 in our mouse model. The numbers of DR CFU recovered from the nasal cavity, trachea, and lungs at day 11 postinoculation were decreased compared to those for 18323. This moderate difference was statistically significant in the trachea and lungs (Fig. 3). Ectopic Bvg^+ phase expression of at least one class of *vrg* genes therefore inhibits tracheal and lung colonization, demonstrating the importance of BvgR-mediated repression, and hence BvgAS-mediated repression, of gene expression in vivo. This result is analogous to our previous report showing that ectopic expression of motility in the Bvg^+ phase of *B. bronchiseptica* is detrimental to the development of respiratory infection in rats (1).

Construction and in vitro characterization of *vrg6* mutants. *B. pertussis* SK6 is a derivative of strain 18323 containing a Tn*phoA* insertion in the *vrg6* locus (16). This strain is Sm^r and Km^r due to the transposon (see Materials and Methods). SK6 has been shown to be defective for tracheal and lung colonization in mice (6). To determine if the virulence defect of SK6 was in fact due to the *vrg6* transposon insertion, we constructed two new mutants. First, we used a recently identified *Bordetella*-specific bacteriophage (18a) to transduce the *vrg6*::Tn*phoA* mutation into wild-type 18323. The resulting strain, NSK6 (Table 1), is isogenic with 18323 except for the transposon insertion into *vrg6*. Like SK6, NSK6 is Sm^r and Km^r due to the transposon. To specifically examine *vrg6* gene function in the absence of polar effects, we also constructed an 18323 derivative containing an in-frame deletion in *vrg6* (Table 1) (see Materials and Methods). Like 18323, this strain (D6) is Sm^s.

The mini-Tn*phoA* element in SK6 inserted into *vrg6* such that a *vrg6-phoA* translational fusion was created, allowing alkaline phosphatase activity to serve as an indicator of *vrg6* expression (16). In both SK6 and NSK6, alkaline phosphatase activity was about 10-fold higher when the strains were grown under modulating conditions compared to nonmodulating conditions (Fig. 2), consistent with previous reports (5, 16). To confirm the roles of BvgAS and BvgR in *vrg6* repression, we transduced the *vrg6*::Tn*phoA* allele into SC3, the Bvg^+ phase-locked strain, and DR, the Δ*bvgR* strain, creating SC3-SK6 and DR-SK6, respectively. Integration of pGMT74 into NSK6 created DS1-NSK6, a Bvg^- mutant containing the *vrg6-phoA* fusion. *vrg6-phoA* expression was constitutively low in SC3-SK6 and high in DS1-NSK6 regardless of growth conditions (Fig. 2), confirming that BvgAS mediates repression, either directly or indirectly, of *vrg6* gene expression under Bvg^+ phase conditions. *vrg6-phoA* expression was partially derepressed in DR-SK6 grown under nonmodulating conditions compared to in DS1-NSK6 and compared to in DR-SK6 grown under modulating conditions. This result confirms that of Merkel and Stibitz (21) and suggests that while BvgR plays a role in BvgAS-mediated repression of *vrg6*, it may not account for full repression of *vrg6* under nonmodulating conditions.

***vrg6* is not required for respiratory infection.** We compared the various *vrg6* mutant strains with strain 18323 in our murine model of respiratory infection. Consistent with the results of Beattie et al. (6), recovery of SK6 from all sites in the respiratory tract was dramatically reduced compared to that of 18323 at day 11 postinoculation, and SK6 was not recovered from the trachea or lungs on day 26 or 35 postinoculation (Fig. 4). In contrast, the numbers of CFU of NSK6 and D6 were statistically indistinguishable from those of 18323 at all sites and time points (Fig. 4). These results demonstrate first and

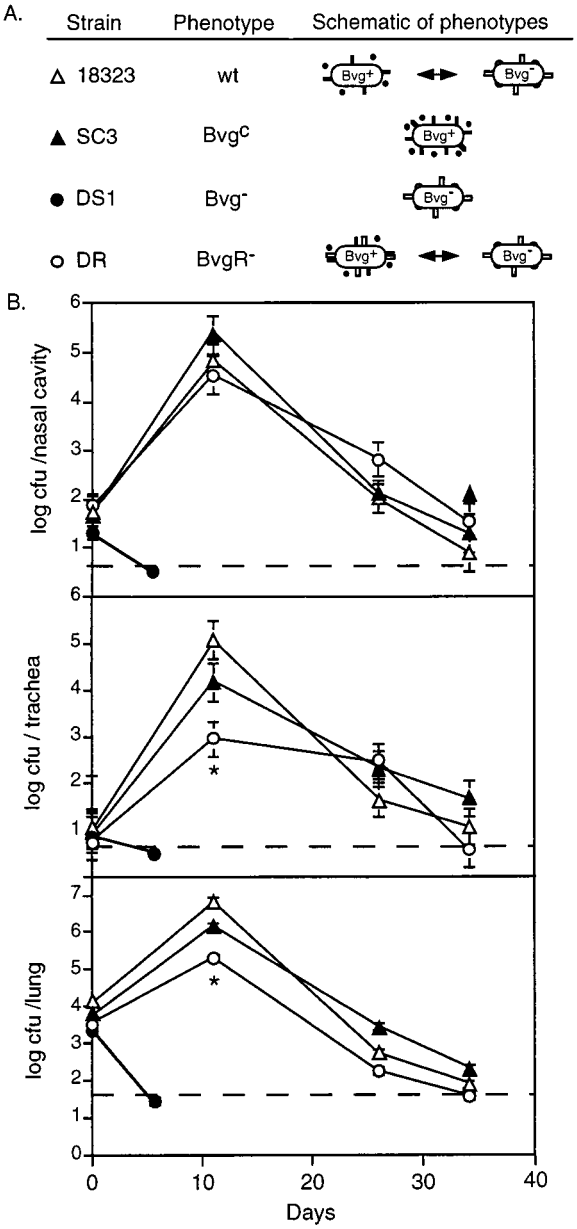

FIG. 3. Time course of respiratory tract colonization by wild-type and mutant *B. pertussis* strains. (A) Schematic of strains used in this experiment. Bvg^+ phase bacteria normally express adhesins (solid bars) and toxins (solid circles), while Bvg^- phase bacteria express *vrg6* (open bars) and other BvgR-regulated factors (open circles) and possibly other, non-BvgR-regulated *vrg* genes (not shown). wt, wild type. (B) Respiratory tract colonization. Mice were inoculated intranasally with 50 μl of PBS containing 5×10^4 CFU of the indicated strains, and the numbers of CFU present in the nasal cavity, trachea, and lungs were determined at the indicated times postinoculation. Each symbol represents the mean number of CFU recovered from three animals. Error bars represent ±1 standard error. Dashed lines indicate the lower limit of detection. *, $P < 0.05$.

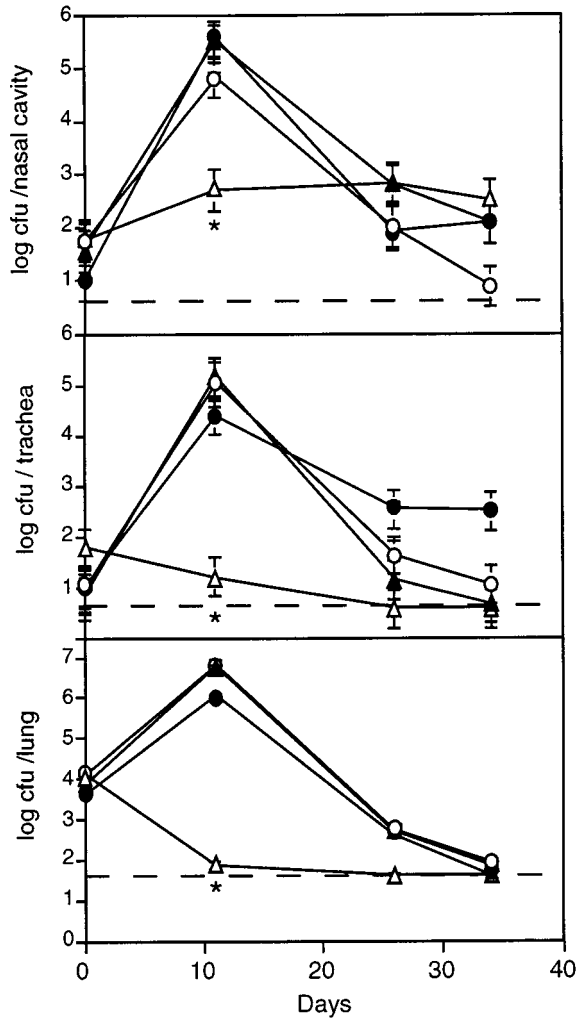

FIG. 4. Time course of respiratory tract colonization by the wild-type strain and *vrg6* mutants. Mice were inoculated intranasally with 50 μl of PBS containing 5×10^4 CFU of strain 18323 (○), SK6 (△), NSK6 (▲), or D6 (●) (Table 1), and the numbers of CFU present in the nasal cavity, trachea, and lungs were determined at the indicated times postinoculation. Each symbol represents the mean number of CFU recovered from three animals. Error bars represent ±1 standard error. Dashed lines indicate the lower limit of detection. *, $P < 0.05$.

foremost that *vrg6* is not required for respiratory infection by *B. pertussis*. Since polar effects present in SK6 would also be present in NSK6, the colonization defect of SK6 is also not due to polar effects on genes downstream of *vrg6*. Additionally, since NSK6, which is Sm^r, is not defective for colonization, the colonization defect of SK6 is not due to the fact that it is Sm^r. Taken together, these results indicate that the SK6 virulence defect observed here and by Beattie et al. (6) must be due to a mutation in a locus other than *vrg6*. In vitro comparison of SK6 with 18323 revealed no obvious differences in colony morphology or antigenic profile, suggesting that SK6 may contain a mutation in a previously unrecognized virulence factor. Whatever the mutation, it has a profound effect on virulence.

***vrg6* expression is not induced in vivo.** Comparison of SK6, NSK6, and D6 with 18323 indicates that *vrg6* expression is not required during infection in our murine model. These results do not, however, rule out the possibility that *vrg6* expression is induced in vivo. To investigate this possibility, we constructed strains containing recombinase gene fusions as reporters of *vrg6* expression (Fig. 5). This reporter system is based on site-

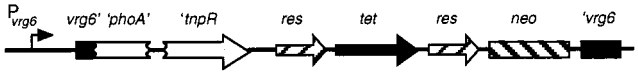

FIG. 5. Use of a resolvase reporter fusion to determine *vrg6* expression in vivo. A *'tnpR-res-tet-res-neo* cassette was inserted into the Tn*phoA* present in SK6. *tnpR* expression is under the control of the *vrg6* promoter, and therefore excision of *res-tet-res* sequences reflects *vrg6* expression. *neo* contains a Km^r gene.

specific recombination in response to *tnpR* (resolvase) gene expression (8). NBP121 and NBP147 are SK6 derivatives in which a promoterless resolvase gene (*'tnpR*) is inserted downstream of *vrg6*::*phoA* followed by *res-tet-res* sequences. The *res* sequences are the targets of the resolvase, and *tet* confers Tc^r. Expression of *vrg6* results in expression of the resolvase, excision of the *res-tet-res* cassette, and conversion of the strain from Tc^r to Tc^s. Approximately 7% of the colonies tested following 4 days of growth on BG-blood agar at 37°C (Bvg^+ phase conditions) were Tc^s, while almost 90% of the colonies tested following growth on BG-blood agar containing 5 mM nicotinic acid and 20 mM $MgSO_4$ (Bvg^- phase conditions) were Tc^s (Table 2). Since the levels of *vrg6* expression in NSK6 grown on BG-blood agar in the absence and presence of 5 mM nicotinic acid and 20 mM $MgSO_4$ are similar to those in SC3-SK6 and DS1-SK6, respectively (Fig. 2), we conclude that 7 and 90% resolutions reflect the levels of *vrg6* expression under nonmodulating and modulating conditions, respectively. To determine if *vrg6* expression is induced in vivo, we inoculated groups of 3-week-old BALB/c mice with 5×10^4 CFU of NBP121 and determined the ratio of Tc^s to Tc^r CFU recovered from the nasal cavity, trachea, and lungs on days 12 and 20 postinoculation. An average of 17% of all colonies recovered from all sites in the respiratory tract on any day were Tc^s (Table 2). These results indicate that *vrg6* expression is low in vivo, only slightly greater than levels observed following in vitro growth under nonmodulating conditions, suggesting that modulation to the Bvg^- phase may not occur in vivo.

Implications. We have shown, by using phase-locked and ectopic expression mutants, that the Bvg^+ phase of *B. pertussis* is necessary and sufficient for respiratory tract colonization and that inappropriate expression of Bvg^- phase factors in the Bvg^+ phase decreases colonization efficiency. We further demonstrated that *vrg6* is not required for virulence. These data repudiate an in vivo role for the Bvg^- phase of *B. pertussis* and suggest that BvgAS may in fact perform the same function(s)

TABLE 2. Resolution of *res-tet-res* sequences following growth in vitro and in vivo[a]

Strain or site	% Tc^s colonies (mean ± SE) after growth:			
	In vitro[b]		In vivo[c]	
	Bvg^+ phase conditions	Bvg^- phase conditions	Day 12	Day 20
NBP121	7.4 ± 4.5	89.6 ± 5.2		
NBP147	7.6 ± 3.4	86.9 ± 4.4		
Nasal cavity			14 ± 6.4	16 ± 5.8
Trachea			17 ± 4.3	18 ± 7.2
Lungs			19 ± 5.5	16 ± 6.7

[a] See Fig. 5.
[b] Strains were grown on BG-blood agar with or without the addition of 5 mM nicotinic acid and 5 mM $MgSO_4$ for 72 h. Cells were then harvested and plated, and the percentage of Tc^s resolvants was determined.
[c] Mice were inoculated with NBP121 grown on plates containing tetracycline. At 12 and 20 days postinoculation, the percentages of Tc^s colonies recovered from the nose, trachea, and lungs were determined.

in *B. pertussis* and *B. bronchiseptica*. If this suggestion is true, how do we account for differences in Bvg^- phase phenotypes and for the fact that *B. pertussis* is thought to be incapable of surviving outside its human host? We recently reported the discovery of Bvg intermediate (Bvg^i) phase antigens in *B. bronchiseptica* and put forth the hypothesis that the Bvg^i phase may be important for aerosol transmission while the Bvg^- phase may be important for transmission via an environmental reservoir (12). Characterization of the Bvg^i phase of *B. pertussis* has revealed cross-reacting Bvg^i phase antigens, and sera from children recovering from pertussis contain antibodies that recognize some of these antigens (19a). These data suggest that the Bvg^i phase of *B. pertussis* is expressed in vivo. We propose, therefore, that BvgAS may function to distinguish sites within and outside the mammalian respiratory tract in both *B. pertussis* and *B. bronchiseptica*. Since *B. pertussis* appears to be confined to transmission by the aerosol route, the role of BvgAS in this species may be primarily to mediate the transition between the Bvg^+ and Bvg^i phases, allowing *B. pertussis* to alternate between a virulent phase and a transmission-competent phase. In *B. bronchiseptica*, the role of BvgAS may be extended to include the transition to the Bvg^- phase, which may allow this organism to survive in environmental reservoirs. We are currently characterizing Bvg^i and Bvg^- phase phenotypes in *B. pertussis* and *B. bronchiseptica* and their potential roles in transmission to test these hypotheses.

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