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Evaluation of the Role of the Bvg Intermediate Phase in *Bordetella pertussis* during Experimental Respiratory Infection

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The BvgAS system of *Bordetella pertussis* was traditionally considered to mediate a transition between two phenotypic phases (Bvg^+ and Bvg^-) in response to environmental signals. We characterized a third state, the intermediate (Bvg^i) phase, which can be induced by introducing a 1-bp substitution into *bvgS* (the *bvgS*-I1 mutation) or by growing *B. pertussis* under conditions intermediate between those leading to the Bvg^+ and Bvg^- phases. Like *B. bronchiseptica*, *B. pertussis* displays in its Bvg^i phase a characteristic colony morphology and hemolytic activity and expresses a Bvg^i-phase-specific polypeptide called BipA, whose synthesis is regulated by *bvgAS* at the transcriptional level. Based on our results, we hypothesize that the Bvg^i phase of *B. pertussis* may be involved in facilitating transmission between hosts. Thus, a *B. pertussis* mutant carrying the *bvgS*-I1 mutation (GMT1i) persisted at wild-type levels only in the upper murine respiratory tract. Interestingly, a *bipA* deletion derivative of GMT1i displayed a reduced ability to colonize the nasal cavity of mice compared with GMT1i. However, in experimental mixed infections GMT1i expressing the Bvg^i phase could establish an initial colonization in the nose and trachea of mice as efficiently as GMT1, but the wild-type strain outcompeted GMT1i at a later time point at all sites of the respiratory tract, suggesting that the Bvg^i phase does not serve as a phenotypic phase specialized in colonization. Finally, even though *B. pertussis* expresses in vitro the Bvg^i phase at the human nasal temperature, anti-BipA antibodies were undetectable in a large collection of sera from pertussis patients.

In his 1960 landmark article, B. W. Lacey reported that the etiological agent of whooping cough, *Bordetella pertussis*, was able to undergo profound phenotypic and antigenic alterations in response to changes in growth temperature or as a result of the addition of certain ions to the culture medium. After a methodical and thorough analysis, he identified three antigenically distinct phases, which he designated X mode, I mode and C mode, and coined the term antigenic modulation to define the transition (entirely reversible) leading from X mode through I mode to C mode (21). In the same report, he accurately anticipated that "modulation in *B. pertussis* is not a saltative change but a process of continuous change leading at equilibrium to one of an infinite number of antigenic states."

Some 25 years later, the locus governing this sophisticated transition was identified, cloned (34, 37), and found to encode a two-component regulatory system which was designated BvgAS (formerly *vir*) (3, 35). The BvgAS signaling pathway uses a complex four-step His-Asp-His-Asp phosphotransfer mechanism involving three domains located in the sensor component of the system (BvgS) and a fourth domain, a second receiver, located in the response regulator of the system (BvgA; for a review, see reference 7). Once phosphorylated, BvgA has increased affinity for Bvg-activated promoters and can also function as a repressor depending on the location of the BvgA-P binding sites within the promoter. In addition, each Bvg-activated promoter requires a specific level of BvgA-P to achieve its maximum transcriptional activity.

Initially, BvgAS was considered to mediate a biphasic transition between a virulent phase, the Bvg^+ phase (equivalent to X mode in Lacey's terminology), and an avirulent phase, the Bvg^- phase (corresponding to Lacey's C mode). At the molecular level, the Bvg^+ phase is characterized by the expression of the vast majority of virulence factors including all the known adhesins and proteinaceous toxins which are encoded by the so-called *vir* activated genes (*vag*s). Conversely, the Bvg^- phase is defined by the lack of activation of the *vag*s, as well as by the cessation of repression (mediated by the product of the *bvgR* locus) (25, 26) of the so-called *vir*-repressed genes (*vrg*s) encoding outer membrane proteins of unknown function (20, 32).

All these molecular events translate into macroscopically detectable differences between *B. pertussis* cultures growing on solid medium supplemented with blood and thus, the Bvg^+ phase colonies are small, domed (hemispheric), and hemolytic and the Bvg^--phase colonies are large, flat, and nonhemolytic. While *B. pertussis* expresses the Bvg^+ phase when growing at 37°C in the absence of certain ions, including magnesium sulfate and nicotinic acid (modulators), incubation below 30°C or addition of an appropriate amount of modulators to the culture medium brings about the transition to the Bvg^- phase. In marked contrast with these two phenotypic phases, the third phenotypic state reported by Lacey in *Bordetella* remained virtually forgotten for almost 40 years.

Instrumental in reviving the interest on this intermediate

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phase was the discovery of a spontaneous mutant of a closely related member of the *Bordetella* genus (*B. bronchiseptica* RB53i) displaying in vivo and in vitro phenotypes (pathogenic potential and ability to survive under nutrient-limiting conditions, respectively) intermediate between those characteristic of the Bvg^+ and Bvg^- phases (11). Subsequent genetic analyses revealed that, besides the constitutive mutation (*bvgS*-C3) which was already present in the chromosome of RB53 and makes this strain insensitive to modulation, RB53i carried another point mutation in its *bvgS* gene. This second mutation was found to result in a single-amino-acid substitution of four residues C-terminal to the BvgS primary site of autophosphorylation. When this point mutation (called *bvgS*-I1) was introduced into wild-type *B. bronchiseptica*, the resultant strain (RB50i) displayed an antigenic profile as well as a colony morphology and hemolytic activity under nonmodulating conditions indistinguishable from that of a semimodulated *Bordetella* culture (11). Therefore, the phenotypic state stably expressed by RB53i or displayed by an unmodulated culture of RB50i was seemingly equivalent to that termed I mode by Lacey and was subsequently designated the Bvg^i phase.

At the molecular level, the Bvg^i phase was characterized by the absence of Bvg-repressed phenotypes and by the expression of a subset of *vag*s corresponding to those not requiring full (Bvg^+ phase) levels of BvgA-P for their transcription (known as class 2 genes; see reference 7). However, the most prominent feature of the Bvg^i-phase antigenic profile was the expression of Bvg^i-phase-specific polypeptides (Bips), whose existence was also deduced by Lacey. Further studies led to the identification of *bipA*, the first Bvg^i-phase-specific gene, the characterization of the *bipA* gene product, BipA (36), and the study of the mechanism by which BvgAS controls the expression of *bipA* (12).

Whereas the open reading frame of *B. bronchiseptica bipA* is predicted to encode a 1,578-amino-acid protein, the equivalent *B. pertussis* open reading frame (29) code for a 1,308-amino-acid protein. Both gene products exhibit homology to intimin and invasin at their N termini, and *B. bronchiseptica* BipA has been shown to be exposed on the cell surface (36). In addition, polypeptides cross-reacting with *B. bronchiseptica* BipA have been detected in lysates obtained from several *B. pertussis* strains (14, 36) but not from human strains of *B. parapertussis* (14). However, the role of the Bvg^i phase in the life cycle of *B. bronchiseptica* and the potential contribution of BipA to its pathogenicity are still unclear. Thus, a *B. bronchiseptica bipA* deletion mutant did not show any detectable colonization defect in the context of a natural model of pathogen-host interaction (36), although additional unpublished evidence (8) supports the hypothesis that the Bvg^i phase of *B. bronchiseptica* may be necessary for transmission between hosts. In contrast, there is an almost complete void of information regarding the potential role of the Bvg^i phase of *B. pertussis* during the experimental respiratory infection. This fact prompted the investigation presented in this work.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All the *Bordetella* strains were grown on Bordet-Gengou (BBL) agar plates supplemented with either 15% or 7.5% defibrinated sheep blood (Oxoid, Basingstoke, United Kingdom) for *B. pertussis* and *B. bronchiseptica*, respectively. Plates were incubated at 37°C in loosely fitted jars for 96 h (for *B. pertussis*) or 48 h (for *B. bronchiseptica*). To ensure absence of Bvg^--phase mutants in cultures, an aliquot of each culture was plated on BG-blood agar, incubated for the appropriate time, and colonies were visually inspected for hemolytic activity.

When it was necessary to grow *Bordetella* under Bvg^--phase (modulating) conditions, nicotinic acid was added to the medium at a final concentration of 16 mM. Whole-cell lysates for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis were prepared from cells grown on BG-blood agar.

Escherichia coli strains were grown in LB medium. When necessary, culture media were supplemented with the appropriate combinations of the following antibiotics: nalidixic acid (50 μg/ml), kanamycin (50 μg/ml), ampicillin (100 μg/ml), gentamicin (20 μg/ml), streptomycin (20 μg/ml), and cephalexin (10 μg/ml).

Construction of mutant strains by allelic exchange. The plasmids used in this study are listed in Table 1. GMT1i and BPC1i were constructed via allelic exchange by introducing the *bvgS*-I1-mutation carried by the plasmid pGMT65 in a 988-bp EcoRI-BamHI fragment into the chromosomes of GMT1 and BPC1, respectively. The mutated fragment was generated by overlapping PCR (4, 38) with genomic DNA from *B. pertussis* GMT1 as the DNA template. The two DNA segments necessary for this reaction were generated in two independent PCRs with the following oligonucleotides: bvgi-1 (5′-GCT GGA ATT CAT GCG CGT GCT CA-3′) and bvgi-2 (5′-GCG TTC ATC GGC ATG CGG ATC TCG TG-3′) for the first PCR, and bvgi-3 (5′-CAC GAG ATC CGC ATG CCG ATG AAC GC-3′) and bvgi-4 (5′-CGG GAT CCT GCC ACC CAC TTG CGA GA-3′) for the second. These oligonucleotides (purchased from Life Technologies, Gibco BRL, Grand Island, N.Y.) were designed to anneal at positions 2557, 3003, 3523, and 3502, respectively, of the published *bvgAS* sequence of *B. pertussis* 165 (GenBank accession number M25401). Note both that primers bvgi-1 and bvgi-4 were engineered to include an EcoRI and a BamHI site, respectively, at their 5′ end and that primers bvgi-2 and bvgi-3 incorporate the *bvgS*-I1 point mutation in their sequence.

The 988-bp fragment containing the *bvgS*-I1 mutation was generated in a new PCR with oligonucleotides bvgi-1 and bvgi-4 in the presence of a 1:1 molar ratio of the two previously amplified fragments. The resultant PCR product was cut with EcoRI and BamHI and ligated to EcoRI- and BamHI-digested pEGBR to generate pGMT65. DNA sequencing of the PCR-generated segment confirmed that no other alterations besides the *bvgS*-I1 mutation were present in this plasmid. pGMT65 was mobilized from *E. coli* SM10 into *B. pertussis* GMT1 and BPC1 by conjugation. Exconjugants were selected on BG-blood agar supplemented with Gm and then grown on BG-blood agar without antibiotic selection to allow for the loss of the plasmid. Plasmid-cured clones were selected on BG-blood agar supplemented with 5% sucrose. Clones in which the allelic replacement had occurred as intended were readily detected by their phenotypic characteristics (see below).

Attempts to construct derivatives of *B. pertussis* carrying in-frame deletions in *bipA* by allelic exchange with the sucrose counterselection method were repeatedly unsuccessful since, for unknown reasons, all the selected exconjugants unexpectedly displayed a total insensitivity to sucrose. Mutant strains GMT1-DBA, GMT1i-DBA, and GG2-DBA were finally constructed by allelic exchange with pSS1129-based plasmids and an alternative counterselection method already described (33). As parental strains for these constructions, spontaneous nalidixic acid-resistant mutants of GMT1, GMT1i, and GG2 (GMT1-Nal, GMT1i-Nal, and GG2-Nal, respectively) were used.

The ≈0.6-kb DNA fragment carrying the *bipA* deletion allele was generated by overlapping PCR (4, 38) with genomic DNA from *B. pertussis* GMT1 as the DNA template. In this fragment, the deletion junction is flanked by two ≈0.3-kb segments; the one 5′ to the deletion encompasses the first 37 codons of the *bipA* ORF as well as 162 bp upstream the BipA start codon, whereas the portion 3′ to the deletion includes the last 55 codons of the *bipA* open reading frame and 210-bp downstream of the BipA stop codon. This mutation results in the loss of the 93% of the *bipA* open reading frame. The fragments upstream and downstream of the deletion junction were generated in two independent PCRs with oligonucleotides bipA-1 (5′-CCT GTG GCC GGA GAG ATG TA-3′) and bipA-2 (5′-GGT TCT GGC GAC GCG GGC T-3′) to generate the *bipA* 5′ end-containing fragment, and bipA-3 (5′-AGC CCG CGT CGC CAG AAC CCG CCG GGC CGT GAT TGC-3′) and bipA-4 (5′-CGC CGC ATC CAT CGC CGT-3′) to generate the corresponding 3′ portion. These oligonucleotides (purchased from Genset Oligos, Paris, France) were designed to anneal at positions 1164465, 1164738, 1168389, and 1168764, respectively, of the published *B. pertussis* Tohama I chromosome sequence (29) (GenBank accession number NC_002929). Note the presence of the deletion allele in the sequence of primer

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features[a]	Source or reference
B. bronchiseptica		
RB50	Wild type, Sm^r	10
RB53	*bvgS*-C3, Bvg$^+$-phase-locked derivative of RB50	10
RB50i	*bvgS*-I1-carrying derivative of RB50, unable to express the Bvg$^+$ phase	11; this work
RB50i-BAL	RB50i::pNur2; chromosomal *bipA-lacZ* fusion	This work
RB53i	*bvgS*-C3, *bvgS*-I1, Bvg^i-phase-locked derivative of RB50	11; this work
B. pertussis		
GG	Wild type, clinical isolate	This work
GMT1	Spontaneous Sm^r derivative of GG	24
GMT1 Nal^r	Spontaneous Nal^r derivative of GMT1	This work
GMT1i	*bvgS*-I1-carrying derivative of GMT1, unable to express the Bvg$^+$ phase, Sm^r	This work
GMT1i Nal^r	Spontaneous Nal^r derivative of GMT1i	This work
BPC1	*bvgS*-C3, Bvg$^+$-phase-locked derivative of GMT1, Sm^r	This work
BPC1i	*bvgS*-C3, *bvgS*-I1, Bvg^i-phase-locked derivative of GMT1, Sm^r	This work
GMT1-BAL	GMT1::pNur2; chromosomal *bipA-lacZ* fusion, Gm^r	This work
GMT1i-BAL	GMT1i::pNur2; chromosomal *bipA-lacZ* fusion, Gm^r	This work
GG2-BAL	GG2::pNur2; chromosomal *bipA-lacZ* fusion, Gm^r	This work
GG2	Δ*bvgA*, Bvg$^-$-phase-locked derivative of GMT1	This work
GG2 Nal^r	Spontaneous Nal^r mutant derivative of GG2	This work
GMT1-DBA	GMT1 Nal^r Δ*bipA*, Sm^r	This work
GMT1i-DBA	GMT1i Nal^r Δ*bipA*, Sm^r	This work
GG2-DBA	GG2 Nal^r Δ*bipA*, Sm^r	This work
Tohama 1	Wild type	19
E. coli		
SM10 (λ pir)	*thi-1 thr leu tonA lacY supE rec*::*RP4-2-Tet*::*Mu*, Kn^r λ pir	31
DH5α	F$^-$ *hsdR17 supE44 thi-1 recA1 gyrA* Δ(*argF-lac*) U169 ϕ 80d*lac*ZΔM15 RP4-2-TcMu Kn^r	Stratagene
Top 10 F′	F′*lacI*^{q} Tn *10* (Tet^r) mcrA Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lac*ZΔM15 Δ*lacX74 recA1alaD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
Plasmids		
pBRK2013	Helper plasmid for triparental matings, *oriT*$_{IncP}$ tra oriE1 Kn^r	13
pEGZ	Suicide plasmid for the construction of *lacZ* transcripcional fusions, Gm^r, Ap^r, *oriT*$_{IncP}$	24
pEGBR	*sac*BR-carrying derivative of pSS1129; Kn^r, Ap^r, Suc^s, *oriT*$_{IncP}$	1
pEG129	pSS1129 derivative carrying a 0.9-kb EcoRl-BbrPl fragment from RB53i *bvgS*	11
pNur2	pEGZ derivative carrying a 595-bp internal fragment of *B. pertussis bipA* cloned into EcoRI-BamHI site	This work
pNur5	630-bp *B. pertussis bipA* deletion allele generated by PCR and cloned into pCR2.1	This work
pNur8	BamHI-Xbal fragment from pNur5 cloned into the BamHI and NheI sites of pSS1129	This work
pGMT65	988-bp EcoRI-BamHI fragment from *B. pertussis* GMT1 containing the *bvgS*-I1 mutation generated by overlapping PCR and cloned into the EcoRI-BamHI sites of pEGBR	This work
pSS1129	Allelic exchange vector, Gm^r Ap^r, *rpsL oriT*$_{Incp}$	35
pCR 2.1	Cloning vector	Invitrogen

[a] Sm, streptomycin; Nal, nalidixic acid; Gm, gentamicin; Kn, kanamycin; Ap, ampicillin; Suc, sucrose.

bipA-3 as well as the complementarity exhibited by primers bipA-2 and bipA-3 over a long stretch of nucleotides.

The ≈0.6-kb fragment containing the deletion allele was generated in a new PCR with oligonucleotides bvgi-1 and bvgi-4 in the presence of a 1:1 molar ratio of the two previously amplified fragments. The Taq-amplified PCR product was directly ligated into 3′-T overhangs of pCR2.1 to construct pNur5 and then the ≈0.6-kb BamHI-XbaI fragment of this plasmid containing the *bipA* deletion allele was ligated to BamHI- and NheI-digested pSS1129 to generate pNur8. The ≈0.6-kb PCR-generated DNA fragment was sequenced to confirm that the *bipA* open reading frame had been maintained. pNur8 was mobilized from *E. coli* SM10 into *B. pertussis* GMT1, GMT1i, and GG2 by conjugation. Exconjugants were selected on BG-blood agar supplemented with nalidixic acid and gentamicin (streptomycin cannot be used as exconjugants become streptomycin sensitive) and then grown on BG-blood agar without antibiotic selection to allow for the loss of the plasmid. Plasmid-cured clones were selected on BG-blood agar supplemented with streptomycin, and clones in which the genetic rearrangement had occurred as intended were identified by PCR with primers bipA-1 and bipA-4.

BPC1 was constructed by allelic exchange with plasmid pJM503 (28) and the method described for the construction of GMT1-DBA. Likewise, the Bvg$^-$-phase-locked mutant GG2 was constructed with the same allelic exchange procedure, resulting in an in-frame deletion of a 233-bp XcmI fragment of *bvgA* in *B. pertussis* strain GMT1 (Gordon and Cotter, unpublished results).

To use as controls throughout the study, strains essentially identical to *B. bronchiseptica* RB50i and RB53i were constructed via allelic exchange by introducing the *bvgS*-I1 mutation carried by the plasmid pEG129 into the chromosomes of RB50 and RB53, respectively, as described by Cotter and Miller (11).

Construction of strains carrying *lacZ* fusions to *B. pertussis bipA* and quantitation of β-galactosidase activity. A 595-bp DNA fragment internal to *bipA* was generated by PCR with genomic DNA from *B. pertussis* GMT1 as template and oligonucleotides (Genset Oligos) bipA-5 (5′-GCG AAT TCT GGT GGG CGG CAG CAT CC-3′) and bipA-6 (5′-GCG GAT CCA GCG GCG CAT ACA CAT T-3′), engineered to incorporate EcoRI and BamHI sites, respectively, at their 5′ ends. These oligonucleotides were designed to anneal at positions 1164768 and 1165363, respectively, of the published *B. pertussis* Tohama I chromosome sequence (29) (GenBank accession number NC_002929). The PCR product was digested with EcoRI and BamHI and cloned into EcoRI- and BamHI-digested pEGZ (i.e., upstream of the promoterless *lacZ* gene) to generate pNur2. This plasmid was transformed into competent *E. coli* DH5α cells and mobilized into *B. pertussis* GMT1, GMT1i, and GG2 by triparental conjugation with the *tra* functions provided by plasmid pBRK2013. Exconjugants were selected on BG-blood agar supplemented with gentamicin. β-Galactosidase activity was measured as already described (24) in cells grown in BG-blood agar for 72 h. To detect the occurrence of spontaneous *bvgAS* mutants, aliquots of each test suspension were plated onto BG-blood agar. The statistical significance of differences in expression was determined by two-tailed Student's *t* test comparisons.

SDS-PAGE and Western immunoblotting. SDS-PAGE and Western immunoblotting were performed as previously described (22, 24). Briefly, bacterial cells suspended in phosphate-buffered saline (PBS) were lysed by addition of 2× SDS-PAGE sample buffer (30). Bacterial lysates were boiled for 5 min, stacked in an SDS–4% polyacrylamide gel and separated in an SDS–7.5% polyacrylamide gel. Proteins were transferred to either polyvinylidene difluoride or nitrocellulose membranes for immunoblotting. Membranes were incubated with a 1:1,000 dilution of one of the following primary antibodies: serum from a rat infected with *B. bronchiseptica* RB53i (antiserum S3), serum from a rat hyperimmunized with killed whole *B. pertussis* GMT1i cells (antiserum S219), or sera from patients convalescing from pertussis. Antigen-antibody complexes were detected with a 1:5,000 dilution of horseradish peroxidase-conjugated antibody of the appropriate specificity (Amersham International, Little Chalfont, United Kingdom) and visualized by an enhanced chemiluminescence technique (Amersham) according to the manufacturer's instructions.

Experimental animals. Experimental intranasal inoculations of mice with *B. pertussis* were performed as previously described (23). Briefly, female BALB/c mice were obtained from Harlan Spain (Harlan Interfauna Iberica S.A., Barcelona, Spain) at 3 weeks of age. To confirm that they were *Bordetella*-free, two animals of each lot were euthanized, and samples of nasal, tracheal, and lung tissue were removed and cultured on BG-blood agar. Inocula were prepared from *B. pertussis* cells grown on BG-blood agar for 3 days and consisted of 10^6 CFU administered intranasally in 50 μl of PBS while the animals were slightly anesthetized by halothane inhalation. Each experimental group consisted of at least three mice (for time point zero) or at least five mice (for time point 8 days).

At each experimental time point, mice were anesthetized by halothane inhalation, a sample of blood was obtained, and then the animals were sacrificed by cervical dislocation. The chest cavity was opened, and approximately 0.5 cm of trachea and the right lung lobe were removed and placed in PBS for homogenization. The nose was dissected, and the entire nasal septum and adjacent tissues were removed and placed in PBS. Tissues were homogenized with tissue grinders, and aliquots of the suspensions were plated on BG-blood agar for viable count. The lower limits of detection for these counts were 4 CFU for both the nasal and tracheal samples and 50 CFU (5,000 in some experiments) for the lung samples. Viable counts below the lower limit of detection were arbitrarily assigned to half the lower limit of detection for each assay.

In all the animal experiments except for the respiratory mixed infections, statistical significance was determined by two-way analysis of variance, and when interaction was found to be significant, the means were compared with contrasts. For the respiratory mixed infection of mice with GMT1 and GMT1i, an identical experimental protocol was followed, although, in this case, the inoculum consisted of 10^6 CFU of each bacterial strain suspended in a final volume of 50 μl of PBS. To ensure that equal numbers of CFU for each strain had been delivered to the animals, appropriate dilutions of the inocula were plated on BG-blood agar for viable count. The two colonial types recovered from the animals were easily identified as formed by GMT1 or GMT1i by visually inspecting their distinct morphology and hemolytic activity. For these mixed infections, the differences in colonization both in the trachea and in the lungs were analyzed by the Wilcoxon signed rank test (due to the existence of a significant number of values falling below the lower limit of detection), whereas for the nasal septum a mixed factorial analysis of variance, with a between-subjects factor (time) and a within-subjects factor (strains), was used. All the statistical analyses were performed with SPSS software (SPSS 11.0 for Windows; SPSS Inc., Chicago, Ill.).

Intranasal inoculation of a *Bordetella*-free 3-week-old female Wistar rat (Harlan Interfauna Iberica S.A., Barcelona, Spain) with *B. bronchiseptica* RB53i was performed by placing a 10-μl drop of PBS containing 200 CFU of RB53i into one nostril as already described (24). The rat immune serum (antiserum S3) was obtained 3 weeks after the inoculation.

To obtain an antiserum against the Bvg^i phase of *B. pertussis* GMT1i (antiserum S219), a 4-week-old *Bordetella*-free Wistar rat was inoculated intraperitoneally with 10^9 killed whole cells of *B. pertussis* GMT1i suspended in 200 μl of PBS, essentially as previously described (32). Briefly, GMT1i cells grown on BG-blood agar for 3 days were washed once with saline, and the optical density at 540 nm of the suspension was then adjusted with saline to 0.12 (10^9 CFU/ml, approximately). Bacterial cells were killed by adding merthiolate and erythromycin to the suspensions, both at a final concentration of 0.01% (wt/vol), and by incubating the resultant mixture at 37°C for 3 h. This treatment killed 100% of the sampled cells as assessed by viable counting of the treated suspension on BG plates. Prior to each inoculation, whole cells were washed twice with saline. Three weeks after the primary immunization, the animal received by the same route a second dose of freshly prepared inoculum containing this time 10^7 killed cells of GMT1i. The immune antiserum was collected 2 weeks after the secondary immunization.

All the animal protocols used in this study were approved by the University of Navarra Animal Research Committee (protocol number 039/00).

PCR, cloning, and sequencing. PCRs were performed with the following conditions: 3 mM $MgCl_2$, 5% dimethyl sulfoxide, 1 U of Taq polymerase (PerkinElmer, Wellesley, Mass.), 250 μM each of the four deoxynucleoside triphosphates, and 20 pmol of each primer were combined and brought to a total volume of 25 μl. As the source of template DNA, a small portion of a colony was resuspended in the solution. A Perkin-Elmer GeneAmp 2400 thermal cycler was used for the reactions. The cycling parameters were incubation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final incubation at 72°C for 5 min. PCR products were cloned into the pCR2.1 vector with the TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. Plasmids containing the cloned PCR products were submitted for sequencing to Sistemas Genomicos, S.L. (Paterna, Spain).

RESULTS

Construction and phenotypic characterization of *B. pertussis* Bvg^i-phase mutants. In *B. bronchiseptica* it was previously shown that the presence of the *bvgS*-I1 mutation leads to the expression of the Bvg^i phase under growth conditions that would otherwise promote growth in the Bvg^+ phase (11). However, the *bvgS*-I1-carrying derivative of strain RB50, designated RB50i, was not totally locked in the Bvg^i phase but still retained its capacity to modulate to the Bvg^- phase when grown under fully modulating (Bvg^--phase) conditions. To determine whether *B. pertussis* can stably display an equivalent phenotype, we introduced by allelic exchange the *bvgS*-I1 point mutation into the chromosome of wild-type *B. pertussis* GMT1, generating strain GMT1i.

Unlike the wild-type strain, though similar to the behavior described for RB50i, when GMT1i was grown under nonmodulating (Bvg^+-phase) conditions, it gave rise to colonies whose morphology and hemolysis were intermediate between those of the Bvg^+ and Bvg^- phases (i.e., Bvg^i-phase-like colonies). However, GMT1i behaved like the wild-type strain when grown under other conditions, rendering Bvg^i-phase-like colonies and Bvg^--phase-like colonies when grown under semimodulating (3 mM nicotinic acid) or fully modulating (16 mM nicotinic acid) conditions, respectively (data not shown). This result suggests that, as shown in the equivalent *B. bronchiseptica* mutant, the single-amino-acid substitution carried by the BvgAS system of GMT1i alters the phosphorylation cascade in a way such that only intermediate amounts of BvgA-P are produced even under nonmodulating (Bvg^+-phase) conditions.

To construct a *B. pertussis* mutant expressing the Bvg^i phase under any growth conditions, we introduced the *bvgS*-I1 mutation into strain BPC1, a *B. pertussis* GMT1 derivative carrying the *bvgS*-C3 mutation. The resultant mutant strain was designated BPC1i and, as expected, gave rise to Bvg^i-phase-like colonies when grown under Bvg^+ and Bvg^--phase conditions (data not shown). GMT1i and BPC1i retained their phenotype after repeated passes on BG-blood agar, and neither of them were prone to revert to the wild-type phenotype or showed an increase in the rate of phase variation (irreversible transition to the Bvg^- phase) compared to the corresponding isogenic parental strain.

Comparative analysis of *bipA* transcriptional activity in GMT1, GMT1i, and a Bvg^--phase-locked isogenic mutant. To study the role of the Bvg^i phase of *B. pertussis*, we first sought to characterize in detail the pattern of Bvg-dependent regula-

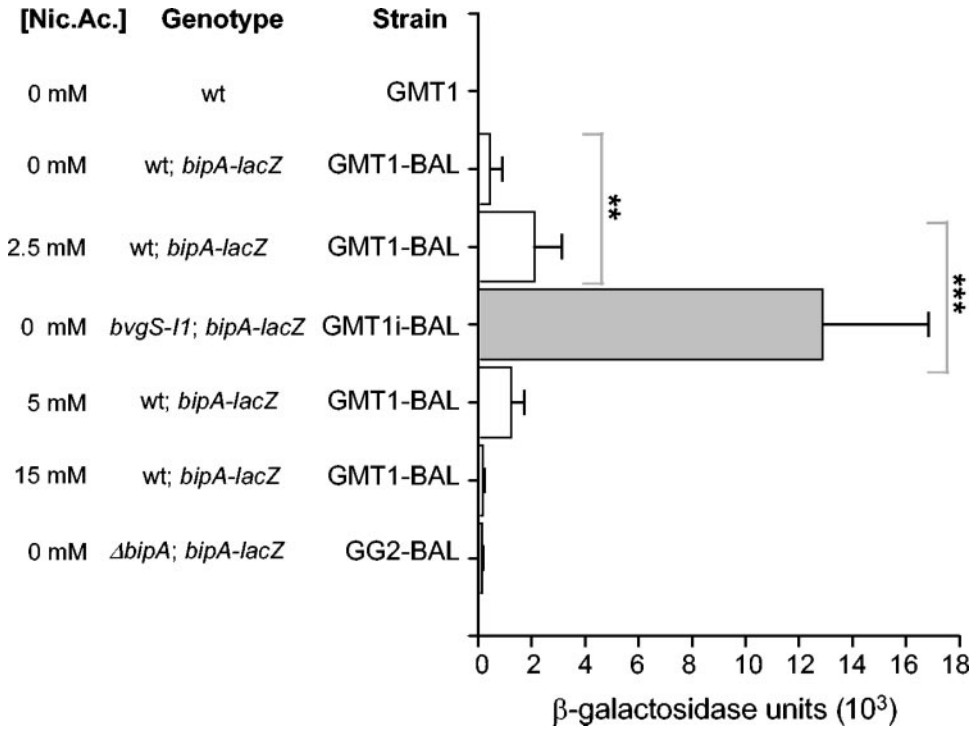

FIG. 1. Quantification of *B. pertussis bipA* transcriptional activity under various growth conditions. The *bipA-lacZ* fusion-carrying derivatives of *B. pertussis* GMT1, GMT1i, and GG2 (GMT1-BAL, GMT1i-BAL, and GG2-BAL, respectively) were grown under Bvg^+-phase conditions (0 mM nicotinic acid), and levels of β-galactosidase were quantitated as specified in the Materials and Methods section and expressed as β-galactosidase units. In other experiments, GMT1-BAL was grown in the presence of the indicated amount of nicotinic acid. In preliminary tests, these concentrations were found to induce the highest level of BipA expression. Results are the average of three independent experiments performed in triplicate. Error bars represent the means ± 1 standard deviation. The asterisks denote a statistically significant difference (**, $P < 0.01$; ***, $P < 0.001$) between the two specified groups.

tion of *bipA*, the only gene hitherto reported in a wild-type strain of a closely related member of the *Bordetella* genus, *B. bronchiseptica*, to be upregulated under semimodulating conditions (12). For this purpose, we constructed derivatives of GMT1, GMT1i, and GG2 (an isogenic Bvg^--phase-locked mutant of GMT1; see Table 1) carrying chromosomal *bipA-lacZ* transcriptional fusions and measured the production of β-galactosidase in the resultant strains, termed GMT1-BAL, GMT1i-BAL, and GG2-BAL, respectively.

As shown in Fig. 1, growth of GMT1-BAL under semimodulating conditions (2.5 to 5 mM nicotinic acid) resulted in a four- to fivefold increase in *bipA* transcription with respect to levels measured in cells from the same strain grown under Bvg^+ or Bvg^--phase conditions. These differences were found to be highly significant ($P = 0.001$). However, this physiological upregulation of *bipA* was dwarfed by that detected in the *bvgS*-I1-carrying derivative of GMT1-BAL, GMT1i-BAL, grown under nonmodulating conditions (≈25-fold with respect to the level measured in unmodulated cells of GMT1-BAL). The difference in *bipA* transcriptional activity (≈6-fold) between GMT1i and GMT1 grown under semimodulating conditions ($P < 0.001$) suggests that addition of submodulating amounts of nicotinic acid does not suffice to recreate a full Bvg^i-phase status in the wild-type strain, thus resembling the behavior reported to occur with a Bvg^--phase-specific phenotype (*frl* expression) in a *B. bronchiseptica* Bvg^--phase-locked mutant (11). In sharp contrast with the previous observation, transcriptional activity of *bipA* was almost undetectable in the isogenic Bvg^--phase-locked mutant of GMT1-BAL (GG2-BAL). These results complement and confirm those obtained by Fuchslocher and collaborators (14) with a similar experimental design and the same *B. pertussis* Bvg^i mutant whose construction we report in the present study (GMT1i).

Characterization of the expression profile of *B. pertussis* Bvg^i-phase-specific polypeptides cross-reacting with *B. bronchiseptica* Bvg^i-phase-specific polypeptides. With an antibody directed against the C terminus of *B. bronchiseptica* BipA, Stockbauer and collaborators identified in cell lysates of *B. pertussis* GMT1i two major cross-reacting bands of approximately 140 and 115 kDa that were absent in cells from Bvg^+ and Bvg^--phase-locked derivatives (36). To expand this analysis, we obtained whole-cell extracts of GMT1 and of its isogenic Bvg^i mutants (GMT1i and BPC1i) grown with various concentrations of nicotinic acid and analyzed them side by side by Western blot with serum from a rat infected with *B. bronchiseptica* RB53i (antiserum S3). As shown in Fig. 2A, antiserum S3 detected the presence of a type of polypeptides that was present in all the lysates irrespective of their modulation status and therefore can be classified as Bvg-independent polypeptides (stars in Fig. 2A). In contrast, a second set of bands were undetectable under both Bvg^+-phase and Bvg^--phase conditions (0 and 16 mM nicotinic acid, respectively), thus being exclusively expressed by *B. pertussis* GMT1 when

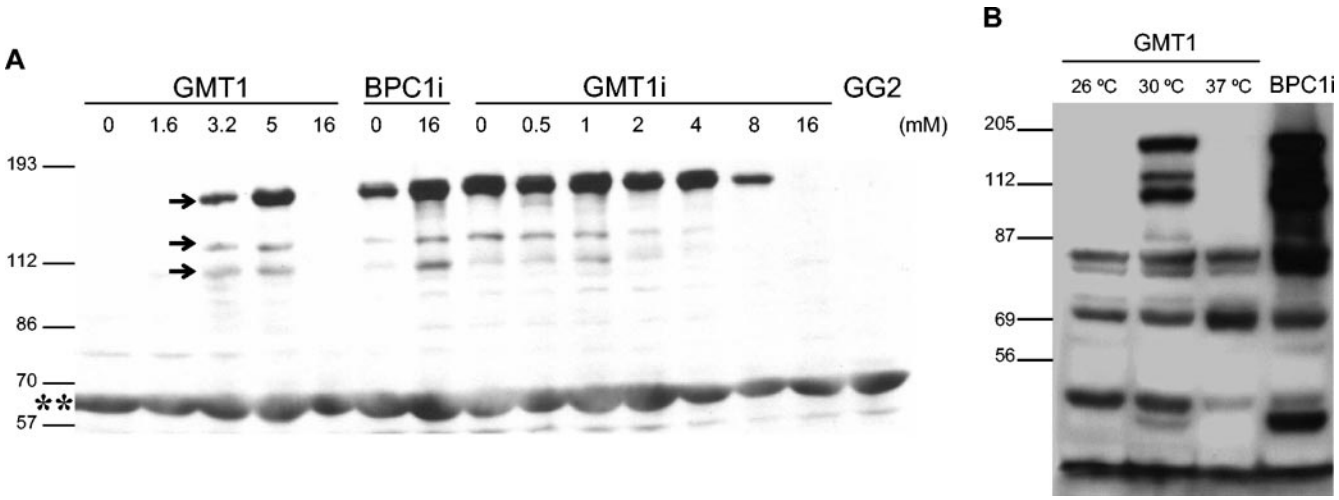

FIG. 2. Western blot analysis of *B. pertussis* Bvg^i-phase-specific polypeptides expressed under different modulating conditions. (A) Whole-cell extracts of *B. pertussis* GMT1, GMT1i, BPC1i, and GG2 grown with the indicated concentration of nicotinic acid were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed with antiserum S3. (B) Whole-cell extracts of *B. pertussis* GMT1 or BPC1i grown at various temperatures (26, 30, or 37°C for GMT1; 37°C for BPC1i) were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed with antiserum S3. Arrows and stars show Bvg^i-phase-specific polypeptides and Bvg-independent polypeptides, respectively. The positions of molecular size markers are indicated on the left (in kilodaltons).

growing under semimodulating conditions (i.e., 3.2 and 5 mM nicotinic acid; arrows in Fig. 2A).

These polypeptides consisted of a major band of ≈140 kDa and two minor bands of approximately 100 and 115 kDa. In view of the molecular mass of the deduced *B. pertussis* BipA gene product (137 kDa; GenBank accession number NC_002929-BP1112) (29) and of the aforementioned results by Stockbauer et al., it is likely that the slowest-running polypeptide could correspond to the *B. pertussis* BipA homolog. Consistent with this hypothesis, the range of expression of the ≈140-kDa polypeptide as a function of the concentration of nicotinic acid (Fig. 2A) nearly correlated with the range of *bipA* transcriptional activity shown in Fig. 1.

These results demonstrate that the expression of Bvg^i-phase polypeptides in *B. pertussis* (and presumably the rest of Bvg^i-phase-specific phenotypes) can be induced by growing the wild-type strain in the presence of levels of a chemical modulator somewhat intermediate (e.g., 3.2 mM and 5 mM nicotinic acid in Fig. 2A) between those leading to growth in the Bvg$^+$ phase (0 mM) or in the Bvg$^-$ phase (16 mM). Interestingly, Fig. 2 also shows that this conclusion holds strictly true when, instead of a chemical modulator, a physical modulator potentially having a more relevant physiological role (temperature) is used. Thus, production of Bvg^i-phase polypeptides could only be detected in GMT1 when this strain was incubated at temperatures intermediate (i.e., 30°C in Fig. 2B) between those leading to growth in the Bvg$^+$ and Bvg$^-$ phases (37°C and 26°C, respectively). In addition and regardless the type of modulator used, the profile of Bvg^i-phase polypeptides expressed by GMT1 matched that of its isogenic Bvg^i-phase mutant (BPC1i), as shown both in Fig. 2A and 2B.

Finally, results shown in Fig. 2A confirm that, although GMT1i displays the most characteristic marker of the Bvg^i-phase (expression of Bvg^i-phase polypeptides) when grown under Bvg$^+$ or Bvg^i-phase conditions, it still retains its capacity to gradually modulate to the Bvg$^-$ phase when increasing amounts of nicotinic acid are added to the culture medium. Thus, expression of Bvg^i-phase polypeptides dropped to undetectable levels when GMT1i was grown in the presence of 16 mM nicotinic acid. On the contrary, these modulating conditions not only failed to downregulate Bvg^i-phase polypeptide expression in BPC1i (as expected of a mutant stably locked in the Bvg^i phase) but, for unknown reasons, induced the expression of levels of Bvg^i-phase polypeptides noticeably higher than those measured in cells of the same strain grown under Bvg$^+$-phase conditions.

Construction and phenotypic characterization of *bipA* strains of *B. pertussis*. Stockbauer and collaborators showed that all the Bvg^i-phase-specific polypeptides detected in *B. bronchiseptica* RB50i cell lysates by an antiserum of the same specificity as antiserum S3 are most probably BipA breakdown products since inactivation of *bipA* in RB50i brings about the loss of the entire Bvg^i-phase-specific reactivity (36). To study the contribution of BipA to the Bvg^i-phase-specific antigenic profile of *B. pertussis*, we analyzed BipA expression in a *B. pertussis bipA* mutant. For this purpose, we first made use of the *bipA-lacZ* fusion-carrying derivative of GMT1i (GMT1i-BAL) since the insertion of the DNA segment internal to *bipA* and transcriptionally fused to the *lacZ* gene should result in the inactivation of *bipA* in this mutant.

Lysates of GMT1i and GMT1i-BAL were separated by SDS-PAGE, transferred to polyvinylidene difluoride and probed with antiserum S3. As shown in Fig. 3A, none of the Bvg^i-phase-specific polypeptides expressed by GMT1i was detected in the lysate of the *B. pertussis bipA* mutant. As a control, the same *bipA-lacZ* fusion was introduced by homologous recombination into the chromosome of *B. bronchiseptica* RB50i to construct RB50i-BAL. In agreement with published observations (36) and similar to the results obtained with the equivalent *B. pertussis* strains (Fig. 3A), disruption of *bipA* in RB50i-

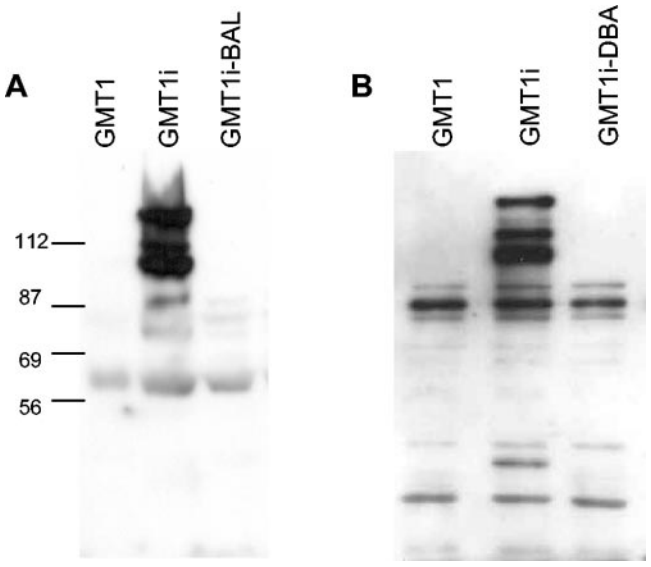

FIG. 3. Western blot-based assessment of the presence of Bvg^i-phase-specific polypeptides other than BipA in *B. pertussis*. Whole-cell extracts of the indicated strain of *B. pertussis* grown under Bvg$^+$-phase conditions were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed with antiserum S3. In each panel, the arrangement of the lanes is as follows: left lanes, wild-type strain; middle lanes, *bvgS*-I1-carrying derivative; right lanes, *bipA* mutant constructed either by insertional activation of *bipA* (A) or by introduction of an in-frame deletion in *bipA* (B). The positions of molecular size markers are indicated on the left (in kilodaltons).

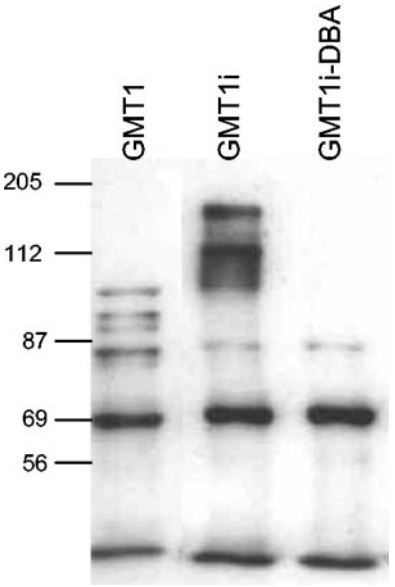

FIG. 4. Western blot-based assessment of the presence of Bvg^i-phase-specific polypeptides other than BipA and exclusive of *B. pertussis*. Whole-cell extracts of the indicated *B. pertussis* strains were grown under Bvg$^+$-phase conditions, separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed with serum from a rat immunized with killed whole cells of *B. pertussis* GMT1i (antiserum S219). The positions of molecular size markers are indicated on the left (in kilodaltons).

BAL abolished all the Bvg^i-phase-specific reactivity detected by antiserum S3 in its parental strain (RB50i; data not shown). However, since this phenomenon could also be due to unintended polar effects caused by the insertion of the plasmid on genes downstream of *bipA* and transcriptionally linked to it, we constructed by allelic exchange a GMT1i derivative carrying an in-frame deletion in *bipA* and designated it GMT1i-DBA.

As shown in Fig. 3B, Western blot analysis of GMT1i-DBA with S3 antiserum revealed that this mutant did not express any of the three major Bvg^i-phase-specific polypeptides and that its antigenic pattern was indistinguishable from that of the wild-type strain grown under Bvg$^+$-phase conditions. These results indicate that, similar to *B. bronchiseptica*, the fast-running (≈100-kDa and ≈115-kDa in *B. pertussis*) Bvg^i-phase-specific polypeptides are in all likelihood BipA breakdown products and therefore BipA seems to be the only *B. pertussis* Bvg^i-phase-specific antigen detectable by serum from a rat infected with a *B. bronchiseptica* Bvg^i-phase-locked mutant. Nevertheless, it cannot be excluded that the appropriate expression of the ≈100-kDa and ≈115-kDa polypeptides may depend on the presence of a wild-type *bipA* allele and/or a functional BipA protein.

Search for *B. pertussis* Bvg^i-phase-specific antigens other than BipA. To determine whether *B. pertussis* possesses an exclusive set of Bvg^i-phase-specific antigens not shared with *B. bronchiseptica*, we first compared the antigenic profile of a *B. pertussis* Bvg^i-phase-locked mutant (BPC1i) with that of its equivalent *B. bronchiseptica* mutant (RB53i) by Western immunoblot with sera from mice infected with *B. pertussis* GMT1i for 20 days. Not surprisingly, due to the poor antibody response elicited by *B. pertussis* when used to infect mice by the intranasal route (15), none of the tested sera showed a significant reactivity against any of the cell extracts (data not shown).

To circumvent this problem, we instead raised an antiserum against the Bvg^i phase of *B. pertussis* by immunizing a rat by the intraperitoneal route with suspensions containing killed whole cells of GMT1i. This immune serum was designated antiserum S219. To be able to differentiate the anti-BipA-specific antibody response from that potentially directed against other Bips, we analyzed side by side the antigenic profile of GMT1i and that of its isogenic BipA deletion mutant (GMT1i-DBA) by Western blot with antiserum S219. Intriguingly, although we expected to detect an antibody response directed against a rather wide variety of *B. pertussis* cell epitopes, a significant part of the detectable reactivity was found to be directed against Bvg^i-phase-specific polypeptides (Fig. 4) and strongly resembled that exhibited by antiserum S3 (see for example Fig. 3B). Similar to the results obtained with antiserum S3, inactivation of *bipA* in GMT1i-DBA sufficed to abolish all the Bvg^i-phase-specific reactivity detected by antiserum S219 in GMT1i. Our results demonstrate that, similar to *B. bronchiseptica* (11, 36), BipA behaves as the immunodominant Bvg^i-phase-specific antigen of *B. pertussis*, at least in rats immunized with killed whole cells by the intraperitoneal route.

Ability of GMT1i to colonize and persist in the respiratory tract of mice. To study whether expression of phases other than the Bvg$^+$ phase enables *B. pertussis* to colonize and persist in vivo, we performed a comparative analysis of the pathogenic potential of GMT1 and GMT1i in a mouse model of respiratory infection. To ensure delivery of large numbers of bacteria to the entire respiratory tract, we inoculated groups of eight

mice intranasally with a high-volume, high-dose inoculum (50 μl of PBS containing 10^6 CFU of the corresponding strain). Approximately 1 h later we sacrificed three mice per group to determine the initial level of colonization reached by GMT1 and GMT1i in the upper (nasal septum) and lower (trachea and lungs) respiratory tract. These levels of colonization were compared again in the remaining animals at day 8 postinoculation, approximately coinciding with the peak of infection in our experimental model. Since the virulence of GMT1 in a mouse model of respiratory infection had never been subjected to a side-to-side comparison with that of a well-characterized *B. pertussis* strain, we also included in this analysis the prototype *B. pertussis* strain, Tohama I.

As shown in Fig. 5, while mice inoculated with GMT1i had numbers of CFU at the beginning of the experiment (day 0) comparable to those of mice inoculated with GMT1 at all the sites of the respiratory tract, GMT1i showed a marked defect in colonization in the lower respiratory tract of mice compared to the wild-type strain at day 8 postinoculation. Specifically, the numbers of CFU recovered from the trachea and the lungs of animals infected with the wild-type strain were 100 and 10,000 times higher, respectively, than those obtained from mice infected with its isogenic mutant lacking the capacity to express the Bvg^+ phase. These results, which resemble those obtained with RB50i in a natural host of *B. bronchiseptica*, the rat (11), demonstrate that Bvg phases of *B. pertussis* other than the Bvg^+ phase are not sufficient for the colonization of the lower respiratory tract of mice.

Interestingly, the ability of GMT1i to colonize the nasal septum of mice was indistinguishable from that of its parental strain at day 8 postinoculation (Fig. 5). It is important to note that, as we have previously shown with an identical animal model (23), a Bvg^--phase-locked mutant of *B. pertussis* is fully impaired for the colonization of the entire respiratory tract of mice and cannot even be recovered from the animal as early as day 6 postinoculation. Therefore, these results indicate that the Bvg^+ phase of *B. pertussis* is not strictly necessary for the colonization of the upper respiratory tract of mice, since a strain devoid of this phase can colonize the nasal cavity at wild-type levels. Finally, our results demonstrate that GMT1 exhibits an ability to colonize and persist in the mouse respiratory tract similar to that of the *B. pertussis* prototype strain Tohama I (Fig. 5).

Experimental respiratory mixed infection with GMT1 and GMT1i. If the colonization defect exhibited by GMT1i in the lower murine respiratory tract was due to an inability to express one or several Bvg^+-phase-specific factors at wild-type levels, coinoculation of the mutant along with the wild-type strain in a mixed respiratory infection could potentially restore that defect. To test this hypothesis, we followed the experimental design outlined in the previous section, but this time the animals were inoculated with a mixed suspension of GMT1 and GMT1i (10^6 and 10^6 CFU) suspended in 50 μl of PBS. As shown in Fig. 6, the ability of GMT1 to colonize the murine respiratory tract was not affected by concomitant infection with GMT1i at any experimental time point since the wild-type strain colonized all sites of the coinfected animals at levels indistinguishable from those reached when administered unmixed (see Fig. 5 and two independent experiments [not shown]).

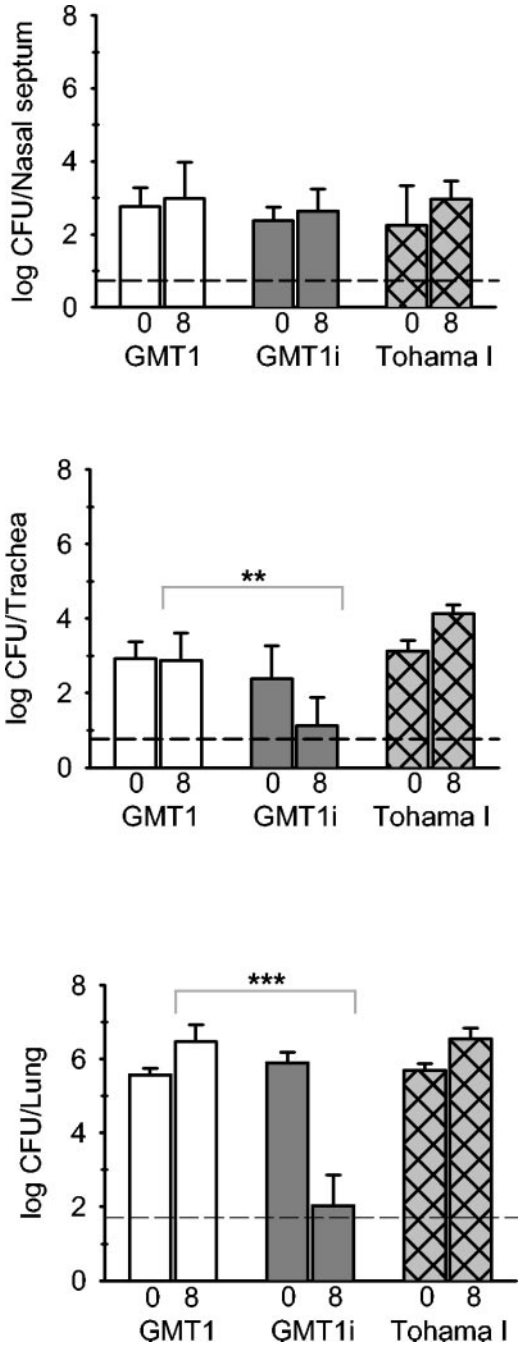

FIG. 5. Colonization of the mouse respiratory tract by wild-type and mutant (*bvgS*-I1 carrying) *B. pertussis* strains. Groups of mice were inoculated intranasally with 50 μl of PBS containing 10^6 CFU of one of the experimental strains (GMT1, GMT1i, and Tohama I). At days 0 (three mice at least) and 8 (five mice at least), the animals were sacrificed, and colonization at various sites of the respiratory tract was determined. Error bars represent 1 standard deviation. The dashed line indicates the lower limit of detection. The asterisks denote a statistically significant difference (**, $P < 0.01$; ***, $P < 0.001$) between the two specified groups.

In contrast, the presence of the wild-type strain not only proved to be insufficient to restore the colonization defect displayed by GMT1i but contributed to make it more pronounced. Thus, unlike the behavior described in single-infec-

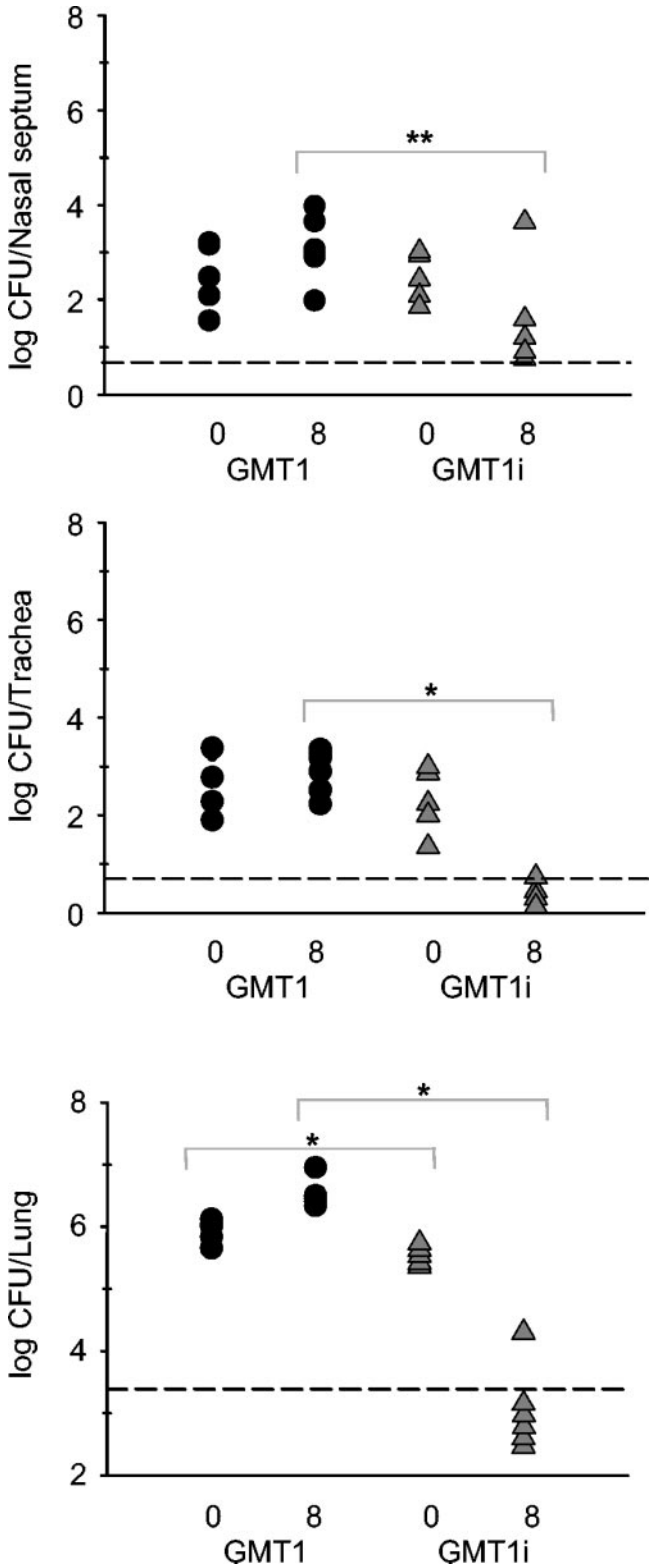

FIG. 6. Colonization of the mouse respiratory tract by two coinoculated *B. pertussis* strains, GMT1 and GMT1i. Groups of mice were intranasally administered 50 μl of PBS containing a mixed inoculum of GMT1 and GMT1i (10^6 CFU and 10^6 CFU, respectively). At days 0 (four mice at least) and 8 (five mice at least), the animals were sacrificed, and colonization at various sites in the respiratory tract was determined and represented as a scatter diagram. Solid circles, GMT1; shaded triangles, GMT1i. The dashed line indicates the lower limit of detection. Asterisks denote a statistically significant difference (*, $P < 0.05$; **, $P < 0.01$) between the two specified groups. Note that the sensitivity of the statistical tests used to analyze data from the upper and lower respiratory tract varies (see Materials and Methods).

tion experiments, GMT1i was recovered in lower numbers than GMT1 ($P < 0.05$) from the lungs of the animals at day 0. These differences became much more prominent at day 8 postinoculation, when GMT1 outcompeted GMT1i not only, as expected, in the lower respiratory tract ($P < 0.05$), but also in the upper respiratory tract ($P < 0.01$). These results demonstrate that the Bvg^+ phase is better endowed than the Bvg^i phase for colonization of the upper murine respiratory tract, although the latter phase can make up for the former in the absence of competition between the two. In addition, our results confirm that the expression of the Bvg^+ phase is absolutely required, at least temporarily, for the colonization of the lower respiratory tract. Finally, our observations indicate that the virulence factors that enable GMT1 to reach wild-type levels of colonization in the murine respiratory tract cannot be exploited by its isogenic *B. pertussis* Bvg^i mutant to restore its colonization defect.

Assessment of the role of BipA in the pathogenesis of *B. pertussis*. To determine whether BipA plays a role in the pathogenicity of *B. pertussis*, we first constructed a mutant equivalent to GMT1i-DBA (i.e., carrying an in-frame deletion in *bipA*) in the wild-type strain (GMT1) background and designated it GMT1-DBA. We then compared the ability of GMT1-DBA to colonize and persist in the respiratory tract of mice with that of GMT1 with the same experimental design of respiratory infection described above. As shown in Fig. 7A, GMT1-DBA was found to be as capable as the wild-type strain to colonize all the sites of the murine respiratory tract during the entire course of the experiment. Our results are consistent with previous observations reporting that a *B. bronchiseptica* mutant carrying an in-frame deletion in *bipA* was not defective for the colonization of any site of the respiratory tract of the rabbit, one of its natural hosts (36).

Nevertheless, since the experiment shown in Fig. 7A was performed with *bipA* mutants constructed in the wild-type strain background, it cannot be ruled out that the bacteria never underwent a transition to the Bvg^i phase in vivo in their experimental host. To determine whether actual expression of BipA in vivo provides *B. pertussis* with an adaptive advantage when this pathogen is genetically forced to express its Bvg^i phase inside its host, we compared the ability of GMT1i to colonize the murine respiratory tract with that of its isogenic mutant carrying an in-frame deletion in *bipA* (GMT1i-DBA). At the same time, this experiment potentially allowed us to investigate whether BipA facilitates the initial interaction between *B. pertussis* and the respiratory epithelium by comparing the level of colonization reached by the unmodulated GMT1i and GMT1i-DBA cells (i.e., expressing and lacking BipA, respectively) shortly after the inoculation.

As shown in Fig. 7B, although GMT1i-DBA showed a tendency to colonize the murine respiratory tract at levels slightly lower than GMT1i, this difference was not found to be significant at the beginning of the experiment. Interestingly, this

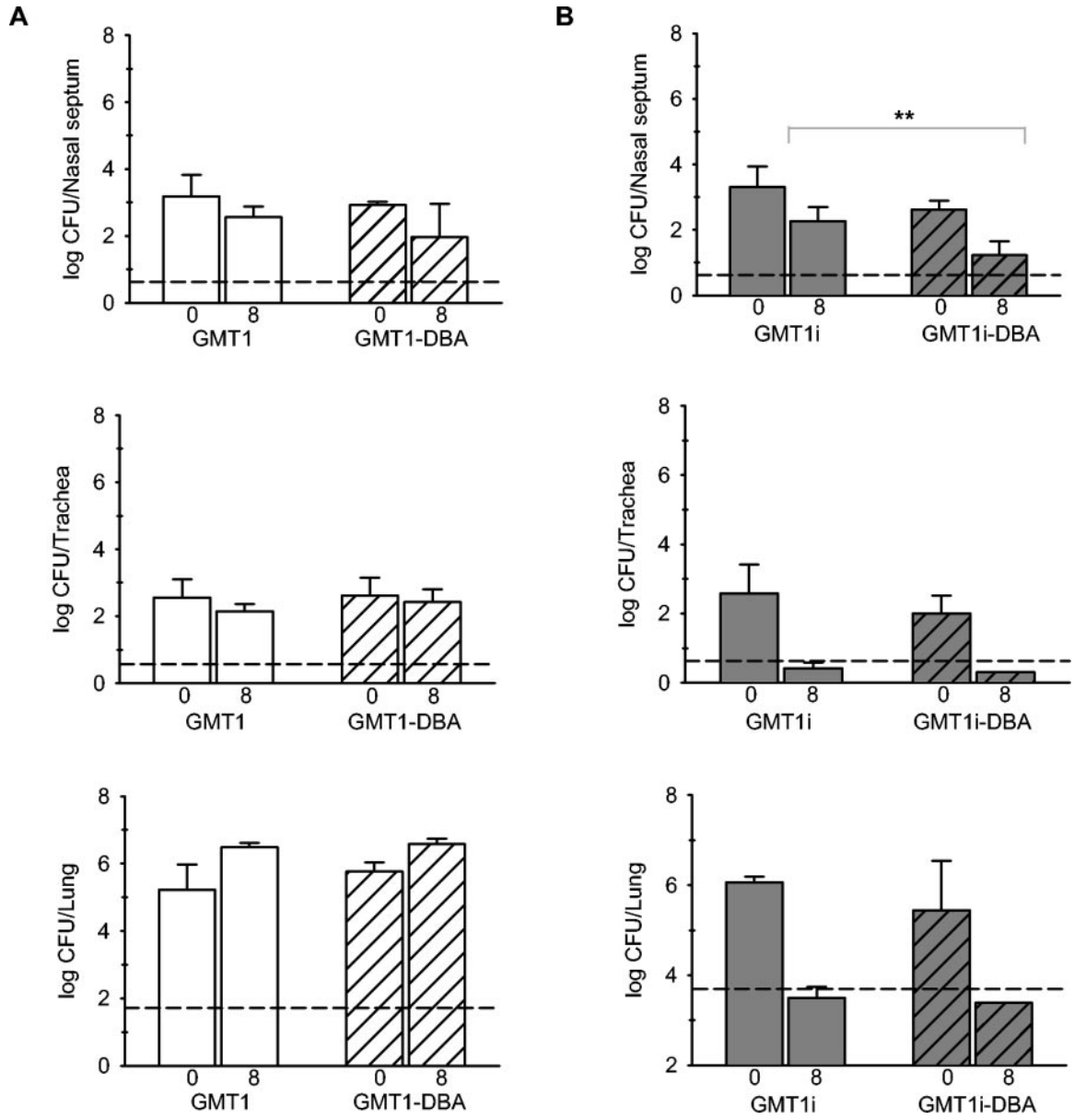

FIG. 7. Colonization of the mouse respiratory tract by *bipA* deletion mutants derived either from wild-type *B. pertussis* GMT1 (A) or from GMT1i (B). Groups of mice were inoculated intranasally with 50 μl of PBS containing 10^6 CFU of one of the experimental strains (A, GMT1 and GMT1-DBA; B, GMT1i and GMT1i-DBA). At days 0 (three mice at least) and 8 (five mice at least), the animals were sacrificed, and colonization at various sites of the respiratory tract was determined. Error bars represent 1 standard deviation. The dashed line indicates the lower limit of detection. The asterisks denote a statistically significant difference (**, $P < 0.01$) between the two specified groups.

deficiency became more pronounced after 8 days of infection, and thus the level of colonization reached by the *bipA* mutant in the nasal septum at the end of the experiment was markedly lower ($P = 0.002$; i.e., $P < 0.01$) than that of its parental strain. It is important to point out that the actual inocula used in these assays (as in all the animal experiments presented in this work) were plated on BG-blood agar for viable counts to ensure that equal numbers of CFU of the corresponding strain had been delivered to the animals ($n = 8$ in this particular experiment).

Search for serological markers indicating that *B. pertussis* may modulate to the Bvg^i phase in vivo. To indirectly assess whether wild-type *B. pertussis* undergoes a transition to the Bvg^i phase in vivo, we screened sera from 100 patients (80 children and 20 adults) convalescent from pertussis for anti-Bvg^i-phase-specific antibodies. For that purpose, whole-cell extracts of *B. pertussis* GMT1 and GMT1i were separated by SDS-PAGE in adjacent gel lanes, transferred to polyvinylidene difluoride, and probed with the corresponding serum sample. As a positive control, duplicate lysates were probed with antiserum S3. Whereas the presence of an antibody response of varying intensity against Bvg$^+$-phase-specific polypeptides and Bvg-independent polypeptides (stars and arrows, respectively, in Fig. 8) was often detectable in the sera, antibodies against Bvg^i-phase-specific polypeptides were not detected in any sam-

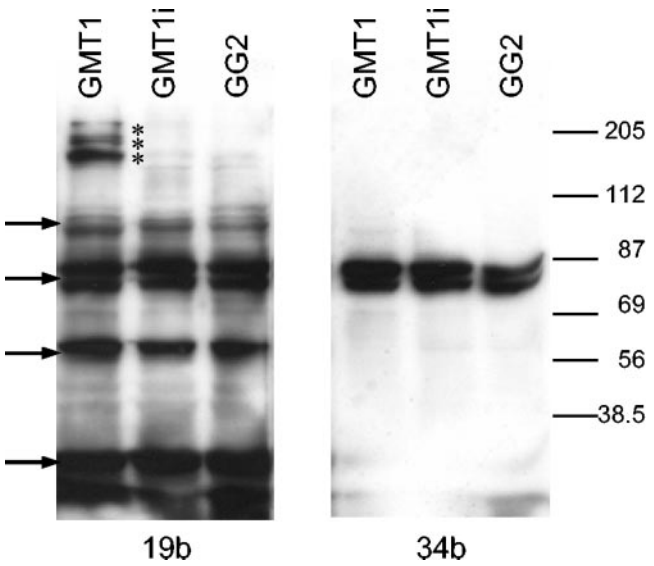

FIG. 8. Assessment of the presence of anti-Bvg^i-phase-specific antibodies in sera from patients recovering from whooping cough. Whole-cell extracts of GMT1, GMT1i, and GG2 were separated by SDS-PAGE in adjacent gel lanes, transferred to polyvinylidene difluoride, and probed with the corresponding serum. Results are representative of the two main profiles obtained with the 100 sera tested. Arrows and small stars show Bvg-independent and Bvg$^+$-phase-specific polypeptides, respectively. The positions of molecular size markers are indicated on the right (in kilodaltons).

ple. In light of these results, it was not surprising to likewise be unable to detect this type of antibodies in sera from a nonnatural host of *B. pertussis* (the mouse) infected with GMT1 for at least 20 days (data not shown). In contrast, positive detection of BipA by antiserum S3 in all the assays demonstrated that the protein was present in the GMT1i extract. These results suggest that if *B. pertussis* undergoes a transition to the Bvg^i phase in vivo, this phenomenon (temporally and/or spatially) passes unnoticed by the immune system of its host.

DISCUSSION

Despite intense investigation since Lacey's pioneering observations in 1960, the role of phenotypic modulation in the *B. pertussis* life cycle remains an enigma. Results from two independent reports performed with different experimental models of respiratory infection showed that a Bvg$^+$-phase-locked mutant of *B. pertussis* colonized the respiratory tract as efficiently as the wild type (23, 27) whereas a Bvg$^-$-phase-locked mutant was cleared soon after the beginning of the experiment, even from the upper respiratory tract (23). In addition, both studies showed that *B. pertussis* mutants manipulated to ectopically express *vrg*s displayed a significant colonization defect in the lower respiratory tract of mice. These observations suggest that the Bvg$^+$ phase of *B. pertussis* is necessary and sufficient for the colonization of the murine respiratory tract. In contrast, the observation that *vrg6* was not required for full *B. pertussis* virulence in the mouse, as was initially reported, and that the transcriptional activity from the *vrg6* promoter was very low throughout the respiratory infection (23) argue against a role for the Bvg$^-$ phase during respiratory infection.

Paradoxically, the host seems to be the only niche where the BvgAS-mediated transition to the Bvg$^-$ phase in *B. pertussis* can be expected to occur, since this *Bordetella* species, unlike its close relative *B. bronchiseptica* (9), is not able to survive under the nutrient-limiting conditions prevailing in the environment. Alternatively, as other authors have proposed, the Bvg$^-$ phase could be a nonfunctional remnant that *B. pertussis* retained from its progenitor, *B. bronchiseptica* (9). However, even if the latter hypothesis is correct, the BvgAS-mediated phenotypic modulation may still be necessary in the life cycle of *B. pertussis* to facilitate its alteration at least between the Bvg$^+$ and the Bvg^i phases, as the present study indicates.

First, our results show that *B. pertussis* expresses the Bvg^i phase in vitro when growing at 30°C, thereby confirming early observations reported by Lacey, though in a different strain background (21). Although a number of environmental signals that *Bordetella* can sense may be important in vivo, temperature appears, in all likelihood, as a relevant modulator for *B. pertussis* inside its host. In fact, the temperature in the human nasal cavity ranges from 30.3 to 32.3°C when measured at an ambient temperature of 23°C (6, 18) and is notably similar to that of rabbits and small rodents (6, 17, 18). Therefore, it seems likely that *B. pertussis* may express its Bvg^i phase in that niche. Consistent with this hypothesis, the only site of the respiratory tract where GMT1i was able to persist at wild-type levels was the upper respiratory tract. These results demonstrate that the Bvg$^+$ phase of *B. pertussis* is not strictly necessary for the colonization of the upper respiratory tract of mice.

In conflict with a potential role for the Bvg^i phase in vivo, we failed to detect anti-BipA-specific antibodies in a large collection of sera from patients convalescent from pertussis. To reconcile these observations, we propose that the Bvg^i phase of *B. pertussis* may be necessary for transmission between hosts, as may be the case for *B. bronchiseptica* (8). According to this hypothesis, which has been previously postulated for the members of the *B. bronchiseptica* cluster (9), transition to the Bvg^i phase in response to low temperature in the distal upper respiratory tract might facilitate the release of *B. pertussis* from the nasal epithelium and/or the initial interaction with the new host. In line with our results, this hypothetical transition should be short-lasting enough to pass unnoticed to the immune system of the host, thereby precluding the elicitation of an anti-BipA-specific antibody response. Likewise, in its new host *B. pertussis* may need to undergo a transition back to the Bvg$^+$ phase to prevent immune recognition of BipA and subsequent production of anti-BipA antibodies.

Alternatively, although we have demonstrated by immunizing rats with GMT1i that *B. pertussis* BipA is highly immunogenic, the possibility remains that this protein may not be immunogenic in humans naturally infected with *B. pertussis*. If so, this feature of *B. pertussis* BipA would be exclusive of the human pathogen since *B. bronchiseptica* BipA has also been shown to be highly immunogenic in both naturally (Martínez de Tejada and Lorenzo-Pajuelo, unpublished results) and experimentally infected animals (36).

Based on our results, several lines of evidence support a hypothetical role of the Bvg^i phase in the transmission of *B. pertussis*. First, we have shown that a *B. pertussis* strain expressing the Bvg^i phase (GMT1i grown under nonmodulating conditions) can establish an initial colonization in the nasal sep-

tum and the trachea of mice at wild-type levels even when coinoculated with an isogenic strain expressing the Bvg^+ phase (GMT1 grown under nonmodulating conditions). More importantly, we demonstrated that in the absence of wild-type cells GMT1i can persist in the upper respiratory tract as efficiently as the wild-type strain. Taken together, these observations suggest that a wild-type *B. pertussis* strain expressing the Bvg^i phase would be sufficiently well endowed to initiate the colonization of the respiratory tract immediately after a transmission event.

Interestingly, our results show that the expression of BipA conferred on GMT1i a noticeably higher (although non-statistically significant) ability to establish colonization in both the upper and lower respiratory tracts of mice compared with a $BipA^-$ isogenic strain. In this context, it is tempting to speculate that the poor interaction of the *bipA* derivative of GMT1i with the murine respiratory tract at the initial stages of infection may have resulted at a later experimental time point in the marked defect in colonization displayed by this strain in the upper respiratory tract. On the other hand, results from the coinoculation experiments show that the Bvg^+ phase is also better endowed than the Bvg^i phase for the colonization of the upper murine respiratory tract. This fact indicates that the Bvg^i phase does not appear to be a phenotypic phase specialized in the colonization of any site of the respiratory tract, not even of the nasal cavity. On the contrary, our observations are consistent with the rapid transition from the Bvg^i phase to the Bvg^+ phase postulated by our hypothesis. Finally, the fact that loss of *bipA* by deletion in the wild-type strain background did not cause any detectable colonization defect supports our hypothesis that BipA expression is not strictly required for the colonization of the respiratory tract but rather necessary only immediately before and/or after a transmission event.

Nevertheless, a definitive conclusion cannot be formulated since the specific conditions encountered by *B. pertussis* in the mouse respiratory tract may not be adequate to promote a transition to the Bvg^i phase. Obviously, in this hypothetical scenario, our animal model would not allow us to detect a colonization defect caused by the absence of BipA. To address this specific point, we plan to monitor *bipA* transcription during the experimental respiratory infection with an in vivo reporter system. Furthermore, to more stringently test our hypothesis, we plan to carry out experiments with *B. bronchiseptica* (which possesses Bvg^i phenotypes very similar to those of the human pathogen [11 and results shown in the present work]) and a model mimicking natural transmission to investigate whether immunization with BipA confers on the animals a certain degree of protection from being naturally infected. Likewise, we will test whether BipA-immunized animals are less infectious than those not immunized but similarly infected with wild-type *B. bronchiseptica*.

The biochemical characterization of GMT1 and GMT1i revealed that, as shown in *B. bronchiseptica* (12, 36), BvgAS also controls the expression of BipA at the level of transcription in *B. pertussis*. This conclusion is in good agreement with recent results published by Fuschlocher and collaborators with the same mutant whose construction is reported in the present work (14). In addition, our results show that BipA expression is undetectable under fully modulating (Bvg^- phase) conditions (16 mM nicotinic acid in our experiments). Consistent with this, the *bipA-lacZ* fusion was found to be fully inactive when introduced in the chromosome of a Bvg^--phase-locked mutant (GG2) and this strain did not produce detectable amounts of BipA. Therefore, taken together, our results demonstrate that *bipA* is a Bvg^i-phase-specific gene. This conclusion confirms previous studies on *B. bronchiseptica bipA* (12, 36) and *B. pertussis bipA* (14) but contradicts conclusions from other authors reporting either upregulation of *bipA* under Bvg^--phase conditions, thus regarding *bipA* as a *vrg* (2) or irregular transcriptional behaviors (16).

At the protein level, comparison of the Bvg^i-phase-specific antigenic profile of GMT1i with that of its isogenic *bipA* mutant (GMT1i-DBA) indicates that BipA is the only *B. pertussis* Bip detectable with serum raised against the Bvg^i phase of either *B. bronchiseptica* or *B. pertussis*. Interestingly, DNA microarray analyses have revealed the existence of additional Bvg^i-phase-specific genes in *Bordetella* (5). To circumvent a potential interference caused by BipA (i.e., immunodominance) and to simultaneously complement at the protein level this search for Bvg^i-phase polypeptide-encoding genes, we plan to use an antiserum raised against GMT1i-DBA to identify Bvg^i-phase polypeptides other than BipA.

Finally, our results also show that the Bvg^i phases of *B. pertussis* and *B. bronchiseptica* differ markedly in the range of modulating conditions at which they are expressed. Thus, whereas the Bvg^i phase of *B. bronchiseptica* is detectable over a narrow range of concentrations of nicotinic acid (approximately 0 to 1.6 mM) (11), *B. pertussis* needs a larger amount of modulating agent (up to 2 mM nicotinic acid) to switch to the Bvg^i phase. In this regard, it is worth noting that, unlike strain Tohama I, GMT1 belongs to a group of *B. pertussis* strains characterized by their high sensitivity to modulation (24). Interestingly, with a *B. bronchiseptica* chimeric strain carrying the *bvgAS* locus of *B. pertussis* Tohama I, it has been shown that both upregulation and downregulation of *bipA* transcription require levels of nicotinic acid twice as high as those shown here for GMT1 (12). Whether these differences between the Bvg^i phases of *B. pertussis* and *B. bronchiseptica* are related to peculiarities in their life cycle and their pathogenicity remains to be elucidated.

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