December, 1997

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***Infect. Immun.*** **1997, 65(12):5295.**

INFECTION AND IMMUNITY, Dec. 1997, p. 5295–5300 Vol. 65, No. 12
0019-9567/97/$04.00+0


# A Highly Adherent Phenotype Associated with Virulent Bvg$^+$-Phase Swine Isolates of *Bordetella bronchiseptica* Grown under Modulating Conditions

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Received 9 June 1997/Returned for modification 1 August 1997/Accepted 17 September 1997

**The ability of Bvg$^-$-phase and Bvg$^+$-phase *Bordetella bronchiseptica* swine isolates, grown under modulating or nonmodulating conditions, to adhere to swine ciliated nasal epithelial cells was determined. When virulent strains were cultivated at 37°C in the Bvg$^+$ phase, numerous adherent bacteria (approximately eight per cell, depending on the strain used) were observed. However, when such strains were grown under modulating conditions (23°C), a significant increase in the level of attachment was seen, suggesting that *B. bronchiseptica* produces a Bvg-repressed adhesin under these conditions. *bvg* mutant strains, including an isogenic *bvgS* mutant, adhered minimally. Western blots indicated that two putative *B. bronchiseptica* adhesins, filamentous hemagglutinin and pertactin, were not detectable in cultures displaying the highly adherent phenotype. Several proteins apparent in Western blots obtained by using bacterial extracts enriched in outer membrane proteins derived from *B. bronchiseptica* grown at 23°C were not present in similar extracts prepared from an isogenic *bvgS* mutant grown at 23°C or from the parent strain grown at 37°C. Adherence of bacteria cultivated at 23°C was almost completely abolished by pretreatment of organisms at 60°C; adherence was reduced by 57% when bacteria were pretreated with pronase E. Temperature shift experiments revealed that the heightened level of adhesion that occurs following growth at 23°C was maintained for up to 18 h when bacteria were subsequently incubated at 37°C. We propose that a Bvg-repressed adhesin, expressed only by modulated *bvg*$^+$ strains of *B. bronchiseptica*, may play a key role in the initial colonization of naturally infected swine.**

*Bordetella bronchiseptica* is a primary etiologic agent of atrophic rhinitis and pneumonia in swine (15, 20). These diseases are extremely widespread and costly to swine production (21, 42). *B. bronchiseptica* also causes respiratory diseases in a variety of other mammals, including tracheobronchitis in dogs and rhinitis in rabbits (14, 21).

The locus in *Bordetella* species designated *bvg* positively regulates the expression of most virulence factors in response to environmental signals (36, 38). The two genes in this locus, *bvgA* and *bvgS*, are members of a broad family of two-component regulatory systems (5, 41). In the Bvg$^+$ phase, colonies on blood agar appear small, domed, and hemolytic; production of the BvgAS proteins, as well as toxins, adhesins, and other virulence factors, is maximal. Growth at or below 25°C, or the presence of sulfate ions or nicotinic acid, induces modulation to the Bvg$^-$ phase, which is characterized by large, flat, nonhemolytic colonies and the absence of *bvgAS* and virulence gene expression. A class of genes that is repressed by BvgAS has also been identified in *B. pertussis* (7, 24); one of these genes appears to be required for maximal colonization of trachea and lung in a mouse model (7). Expression of flagellin genes in *B. bronchiseptica* is also negatively regulated by BvgAS (4).

Like *B. pertussis*, *B. bronchiseptica* initiates infection by colonization of the ciliated nasal epithelium. Several studies have demonstrated that nonmodulated *bvg*$^+$ strains, but not *bvg* mutants, adhere to swine ciliated epithelial cells in vitro (10, 23, 39, 40, 43) and in vivo (18, 31). A correlation between adhesion and the ability to agglutinate calf erythrocytes has also been reported (10, 23, 31, 40). For *B. pertussis*, several BvgAS-induced proteins have been proposed as adhesins for various eukaryotic cell types, including filamentous hemagglutinin (FHA), pertactin, and, possibly, fimbriae (reviewed in reference 36). *B. bronchiseptica* synthesizes similar, but not identical, versions of these proteins. However, their role in adhesion of *B. bronchiseptica* to eukaryotic cells has not been fully examined.

The purpose of this study was to characterize the levels of adhesion of *bvg* mutants and modulated or nonmodulated *bvg*$^+$ swine isolates of *B. bronchiseptica* to swine ciliated nasal epithelial cells as a basis for subsequent evaluation of the role of specific bacterial proteins in this process.

## MATERIALS AND METHODS

**Bacterial strains and media.** The virulent, hemolytic *B. bronchiseptica* swine isolates 4609 and MBORD846 have been described elsewhere (2, 17). Strain 4607 is an avirulent, nonhemolytic porcine isolate (2). Strain PV6p15 is a nonhemolytic variant derived from a hemolytic swine isolate (30). Strain MBA4 is a phase-locked *bvg* mutant of MBORD846 containing a 1.4-kb deletion in *bvgS* (12, 19) (generously provided by David Dyer, University of Oklahoma, Oklahoma City, and Jeff Miller, University of California, Los Angeles). Bacteria were grown on Bordet-Gengou agar with 15% defibrinated sheep blood either at 37°C for 36 h or at 23°C for 60 h unless otherwise indicated.

**Attachment assay.** Swine ciliated nasal epithelial cells were isolated from 1-week-old piglets immediately following euthanasia. The ventral concha was sectioned near the ethmoid region, and the entire concha was removed. Epithelium from the caudal one-third of the ventral concha was removed by gently scraping with a scalpel blade. Cells were harvested in 45% Dulbecco modified Eagle medium–45% phosphate-buffered saline–10% CPSR-1 serum replacement (serum replacement medium; Sigma Chemical Co., St. Louis, Mo.) and were washed three times by differential centrifugation. Final cell pellets were resuspended in serum replacement medium. Cells were quantitated with a Nova

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CellTrak 6 cytometer (Nova Biomedical, Waltham, Mass.) and were examined by light microscopy before use to confirm that cilia were beating vigorously.

Bacteria used as inocula were suspended in phosphate-buffered saline, and the solutions were adjusted to an $A_{600}$ of 0.42. Aliquots containing approximately $8 \times 10^6$ CFU (determined in control experiments) were added to roughly $2 \times 10^5$ ciliated epithelial cells, and the total volume of each sample was adjusted to 200 μl. In some experiments, organisms were incubated at 60°C for 1 h or pretreated with pronase E (5 μg/ml; Sigma) for 30 min at 37°C before addition to ciliated cells.

Bacteria and epithelial cell mixtures were incubated at 37°C, with occasional mixing by inversion, for 1 h. Epithelial cells were washed three times in sterile normal saline by differential centrifugation. Smears made from the final pellets were stained with Diff-Quik (Baxter Scientific Products, McGaw Park, Ill.). At least three replicate slides were prepared for each sample; experiments were repeated a minimum of three times. The number of bacteria adherent to ciliary tufts of 25 cells from each slide was recorded and used to determine the average number of bacteria per cell.

**SDS-PAGE and immunoblotting.** *B. bronchiseptica* urea extracts enriched in outer membrane proteins were obtained as described previously (28). Twenty micrograms of protein from each extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, using the method of Doucet et al. (16). Following electrophoretic transfer to nitrocellulose, membranes were incubated with the FHA-specific monoclonal antibody X3C (27) or the pertactin-specific monoclonal antibody BPE3 (9) (both generously provided by Michael Brennan, Food and Drug Administration, Bethesda, Md.). Membranes were washed with 50 mM Tris (pH 7.5)–150 mM NaCl–0.05% Tween 20–0.25% fish gelatin and then incubated with a 1:1,000 dilution of either goat anti-mouse immunoglobulin G (IgG) (for X3C) or goat anti-mouse IgM (for BPE3) conjugated to alkaline phosphatase (both from Sigma). In some experiments, membranes were blotted with a 1:1,000 dilution of serum from *Bordetella*-free rabbits hyperimmunized with either *B. bronchiseptica* MBORD846 grown at 23°C or strain MBORD846 grown at 37°C, followed by a 1:1,000 dilution of goat anti-rabbit IgG-alkaline phosphatase. Blots were developed by using a commercially obtained alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, Calif.).

**Polyclonal rabbit serum.** *Bordetella*-free New Zealand White rabbits were injected intravenously with approximately $10^9$ CFU of *B. bronchiseptica* MBORD846, grown at 23 or 37°C. Bacteria were rendered noninfectious prior to injection by overnight incubation in 0.3% formalin in saline and were then washed twice and resuspended in saline before administration to rabbits. The sterility of inocula was confirmed by culture. Two weeks after the initial injection, booster injections were administered intravenously once a week for 4 weeks. Two weeks after the final injection, rabbits were premedicated with xylazine and ketamine, bled by cardiac puncture, and euthanized by pentobarbitol overdose.

**Statistics.** Standard errors are indicated in the table and figures. Data were analyzed by using two-tailed Student's *t* test.

## RESULTS

**Effect of growth conditions on adhesion of *B. bronchiseptica* to swine ciliated nasal epithelial cells.** All known adhesins of *Bordetella* are expressed only when the bacteria are grown in the $Bvg^+$ phase (8, 36). When the virulent *B. bronchiseptica* strain 4609 was cultivated at 37°C in the $Bvg^+$ phase and tested for adherence to swine ciliated nasal epithelial cells, an average of 7.4 adherent organisms per cell were observed (Table 1). The avirulent *bvg* mutant strain 4607, cultivated similarly, displayed minimal adherence, with an average of only 1.6 bacteria attached per cell (Table 1).

Surprisingly, when strain 4609 was grown in the $Bvg^-$ phase by cultivation at 23°C, the average number of adherent bacteria per cell was almost three times greater than that of strain 4609 grown in the $Bvg^+$ phase (Table 1). Adhesion of strain 4607 was unaffected by growth at a lower temperature. Typical examples of ciliated nasal epithelial cells from these experiments are shown in Fig. 1. An additional *bvg* mutant, PV6p15, attached to ciliated cells at a level that was significantly less than that for strain 4609, regardless of the temperature used for cultivation (data not shown).

In the experiments described above, strain 4609 was cultured at 23°C for a single passage (approximately 60 h) prior to use in the attachment assay. Since this length of time may not have been sufficient for completely down-regulating the expression of BvgAS-induced adhesins, we repeated the experiment using strain 4609 or 4607 cultured at 23°C for three passages. The levels of adhesion were not significantly different from those found when the bacteria were grown at 23°C for only a single passage (data not shown).

TABLE 1. Effects of growth conditions on attachment of *B. bronchiseptica* to swine ciliated nasal epithelial cells

| Strain | Growth conditions | No. of adherent bacteria/cell (avg ± SE) | % of cells with adherent bacteria |
|---|---|---|---|
| 4609 | 37°C | 7.4 ± 4.6[a] | 95.6 |
| 4607 | 37°C | 1.6 ± 0.6 | 88.0 |
| MBORD846 | 37°C | 8.7 ± 1.9[a] | 99.0 |
| MBORD846 | 37°C, 50 mM $MgSO_4$ | 19.8 ± 1.5[b] | 100 |
| MBA4 | 37°C | 4.7 ± 2.1 | 96.7 |
| 4609 | 23°C | 20.3 ± 0.9[a,b] | 100 |
| 4607 | 23°C | 1.0 ± 0.4 | 75.0 |
| MBORD846 | 23°C | 18.8 ± 4.4[a,b] | 100 |
| MBA4 | 23°C | 3.4 ± 3.0 | 91.2 |
| 4609 | 23°C, treated with pronase | 8.7 ± 0.4[c] | 100 |
| 4609 | 23°C, heated to 60°C | 1.1 ± 0.5[c] | 67.6 |

[a] Statistically significant difference from results for 4607 and MBA4 grown at 37 or 23°C ($P \leq 0.005$).

[b] Statistically significant difference from results for homologous strain grown at 37°C ($P \leq 0.001$).

[c] Statistically significant difference from results for 4609 grown at 23°C with no pretreatment ($P \leq 0.001$).

To determine whether the attachment that was observed following growth of strain 4609 at 23°C is unique to this strain, another virulent swine isolate, MBORD846, was tested under similar conditions. A high level of adherence (18.8 bacteria/cell) occurred with this strain when it was grown at 23°C, and this number was significantly greater than when MBORD846 was grown at 37°C (Table 1).

The above findings suggest that the adhesin of swine isolates of *B. bronchiseptica* responsible for maximum attachment to ciliated nasal epithelial cells may be repressed by the *bvg* locus. Alternatively, the adhesin may be controlled by temperature independently of *bvg*. To distinguish between these two possibilities, we compared the level of attachment of *B. bronchiseptica* grown at 23°C (temperature-induced BvgAS repression) to the level of attachment of *B. bronchiseptica* grown at 37°C in the presence of 50 mM $MgSO_4$ (chemically induced BvgAS repression). Compared to nonmodulated $bvg^+$ MBORD846, adherence to cilia of epithelial cells was significantly increased when MBORD846 was grown in the $Bvg^-$ phase, regardless of the method used for repression (Table 1). Therefore, the adhesin responsible for attachment of organisms grown at 23°C may be a BvgAS-repressed virulence factor.

**Adherence of a defined *bvg* mutant.** If *B. bronchiseptica* expresses an adhesin that is repressed by the *bvg* locus, a *bvg*

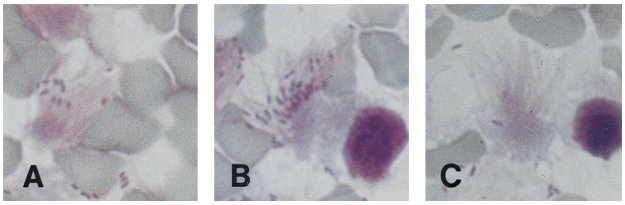


FIG. 1. Attachment of *B. bronchiseptica* to swine ciliated nasal epithelial cells. Strain 4609 grown at 37°C (A) or 23°C (B) or strain 4607 grown at 37°C (C) was incubated with swine ciliated nasal epithelial cells for 1 h. Epithelial cells were washed three times. Smears were prepared from the final pellets, stained, and examined by light microscopy. Magnification, ~×600.

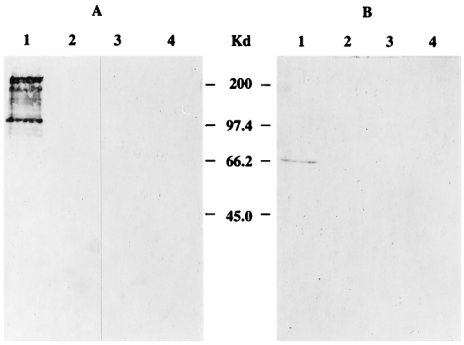


FIG. 2. Detection of FHA and pertactin by immunoblotting. Bacterial cell extracts enriched in outer membrane proteins were prepared from *B. bronchiseptica* 4609 (lanes 1 and 2) or 4607 (lanes 3 and 4) grown at 37°C (lanes 1 and 3) or 23°C (lanes 2 and 4). Proteins (20 μg per lane) were separated by SDS-PAGE and transferred to nitrocellulose. FHA was detected by incubation with monoclonal antibody X3C (A); pertactin was detected by using monoclonal antibody BPE3 (B). The locations of molecular mass markers are shown. Mature FHA has a molecular mass of 220 kDa; however, as reported by others (27), multiple bands were apparent. The molecular mass of pertactin is 68 kDa.

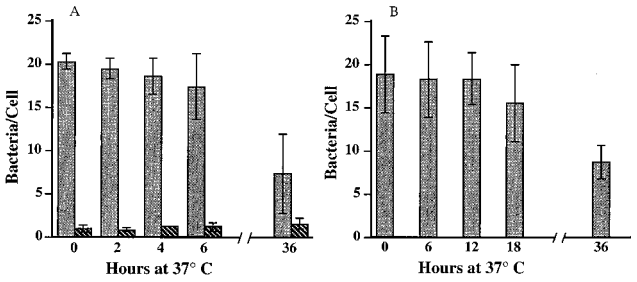


FIG. 3. Adherence of *B. bronchiseptica* to swine ciliated nasal epithelial cells. (A) Strain 4609 (▭) or 4607 (▨) was grown at 23°C for 60 h and then incubated at 37°C for 2, 4, or 6 h. The level of attachment for 4609 grown only at 37°C for 36 h (shown on the right) is significantly different from adherence of 4609 under the other conditions tested ($P \leq 0.001$). Adhesion of 4607 is significantly different from that of 4609 under all conditions tested ($P \leq 0.005$). (B) Strain MBORD846 was grown at 23°C for 60 h and then incubated at 37°C for 0, 6, 12, or 18 h. All values are significantly different from that of MBORD846 grown only at 37°C for 36 h ($P \leq 0.001$ for 0 to 12 h; $P \leq 0.01$ for 18 h). Data are expressed as mean ± standard error.

mutant might be expected to display levels of adhesion similar to those of the parent strain grown at 23°C. The two *bvg* mutant strains used above, 4607 and PV6p15, are not appropriate for an analysis of this type since the genetic lesions responsible for their Bvg$^-$ phenotype have not been characterized and they may contain additional mutations in genes affecting adherence. Therefore, we used MBA4, an isogenic mutant of strain MBORD846 that contains a 1.4-kb deletion in the *bvgS* gene (12, 19). MBA4 produces large, flat, nonhemolytic colonies and is not responsive to modulation by growth at reduced temperatures or in the presence of $MgSO_4$ or nicotinic acid. When tested for its ability to attach to ciliated epithelial cells, this strain displayed only low levels of adhesion that were unaffected by reduced growth temperature (Table 1). Thus, it appears that only *bvg*$^+$ strains of *B. bronchiseptica*, cultivated in the Bvg$^-$ phase, express the highly adherent phenotype.

**Expression of *bvg*-induced adhesins.** FHA is believed to be a major eukaryotic cell adhesin for the genus *Bordetella* (36). Pertactin, another cell surface protein, has also been implicated in attachment (6, 26, 28). The expression of both adhesins is expected to be repressed when *B. bronchiseptica* is grown in the Bvg$^-$ phase (36).

When grown at 23°C, the colony morphology of all strains of *B. bronchiseptica* used in this study was consistent with the Bvg$^-$ phase (large, flat, and nonhemolytic). However, we determined whether expression of FHA and that of pertactin were down-regulated in these cultures, as expected. Bacterial cell extracts, enriched in outer membrane proteins, were prepared from strain 4609 and used for immunoblotting. Both FHA (Fig. 2A, lane 1) and pertactin (Fig. 2B, lane 1) were present in extracts from strain 4609 grown at 37°C. However, these proteins were not detectable when strain 4609 was cultivated at 23°C (Fig. 2A and B, lanes 2). Strain 4607 did not produce FHA or pertactin under either growth condition (Fig. 2A and B, lanes 3 and 4). Similar results were obtained with the strain MBORD846 (see Fig. 4, lanes labeled 0 and 36). Therefore, attachment of these strains in the Bvg$^-$ phase to ciliated epithelial cells is not mediated by inappropriate expression of FHA or pertactin.

**Effect of timed temperature shift on attachment of *B. bronchiseptica*.** Transmission of *B. bronchiseptica* among swine occurs through aerosol droplets or, possibly, through contact with environmental reservoirs (20, 35). In the course of transmission, it is likely that organisms are exposed to reduced temperatures. Furthermore, the temperature encountered during early stages of infection, when bacteria are present on the rostral nasal mucosa, is likely to be less than 37°C. If the adhesin expressed in vitro following growth at a reduced temperature plays a role in attachment in vivo, it must continue to function for at least a brief period following an increase in growth temperature. The effect of such a temperature shift on attachment was assessed by preparing inocula from cultures grown at 23°C for approximately 60 h, followed by incubation at 37°C for various lengths of time. Cultures grown exclusively at 23 or 37°C were included for comparison.

For strain 4609, significantly increased adhesion to epithelial cells persisted for at least 6 h following an increase in temperature (Fig. 3A). As observed previously, the low level of adherence of strain 4607 was not affected by changes in growth temperature.

Strain MBORD846 was also tested in a similar experiment, except that cultures were shifted to incubation at 37°C for longer periods of time. MBORD846 continued to maintain significantly increased levels of attachment for up to 18 h following the shift (Fig. 3B). After incubation at 37°C for 18 h, there was a slight reduction in the average number of adherent bacteria per cell. However, this average does not differ significantly from values of the other temperature-shifted cultures.

**Effect of temperature shift on expression of FHA and pertactin.** Portions of the cultures used in the temperature shift experiments described above were used to prepare extracts for immunoblotting to detect expression of FHA and pertactin. FHA was detectable 4 h after the shift to 37°C; pertactin expression was barely evident at 6 h (Fig. 4). Therefore, it is unlikely that either of these proteins plays a major role in attachment to ciliated epithelium for at least the first several hours following an increase in temperature. From 8 to 18 h postshift, the levels of FHA and pertactin are sufficient to suggest that at least some of the binding events may be the result of interaction of these proteins with ciliated epithelial cells.

**Preliminary characterization of the Bvg-repressed adhesin.** The effect of pretreatment with pronase E or heat on the ability of strain 4609, grown at 23°C, to attach to ciliated cells was determined. Pretreatment of bacteria with pronase E (5 μg/ml) resulted in a 57% reduction in the average number of adherent bacteria per cell (Table 1). Incubation of bacteria at

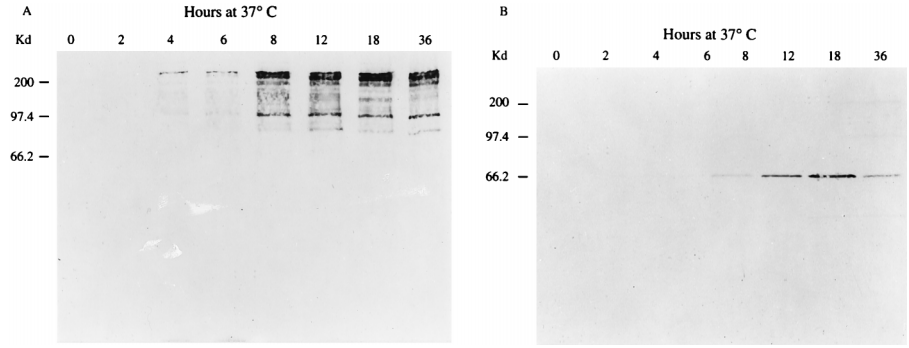


FIG. 4. Detection of FHA and pertactin by immunoblotting. Bacterial cell extracts enriched in outer membrane proteins were prepared from strain MBORD846 incubated for 60 h at 23°C and then incubated at 37°C for the time indicated. Extracts used in the lanes labeled 36 were derived from cultures grown only at 37°C for 36 h. Blots were incubated with the FHA-specific monoclonal antibody X3C (A) or the pertactin monoclonal antibody BPE3 (B). The locations of molecular mass markers are indicated.

60°C for 1 h prior to addition of ciliated epithelial cells caused a reduction of over 94% in attachment on a per-cell basis, and the percentage of epithelial cells with adherent bacteria also declined (Table 1).

In an attempt to identify potential outer membrane proteins that might function as Bvg-repressed adhesins, SDS-PAGE was used to compare the profiles of extracts enriched in outer membrane proteins from *B. bronchiseptica* grown at 23 or 37°C. Although we compared gels of several different acrylamide concentrations, ranging from 6 to 15%, Coomassie staining did not reveal any major proteins expressed exclusively by strain MBORD846 grown at 23°C compared to MBA4 grown at 23°C or MBORD846 grown at 37°C (unpublished results). However, when similar gels were used for immunoblotting with serum from a rabbit hyperimmunized with strain MBORD846 grown at 23°C, we detected in lanes containing extract from temperature-modulated MBORD846 several proteins that were either absent or present in reduced quantities in extracts derived from MBA4 grown at 23°C or MBORD846 grown at 37°C (Fig. 5A). These proteins were not apparent when the membrane was blotted with antiserum to MBORD846 cultivated at 37°C (Fig. 5B). One or more of these proteins may function as an adhesin for ciliated epithelial cells.

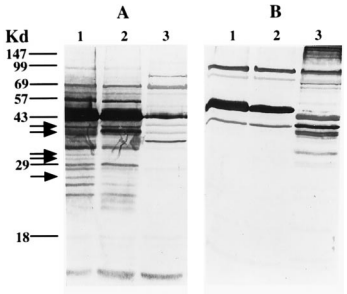


FIG. 5. Differentially expressed proteins of *B. bronchiseptica* grown at 23 or 37°C. Proteins in 20 μg of bacterial cell extract from strain MBORD846 grown at 23°C (lane 1), strain MBA4 grown at 23°C (lane 2), or strain MBORD846 grown at 37°C (lane 3) were separated by SDS-PAGE (15% gel), transferred to nitrocellulose, and blotted with sera from rabbits hyperimmunized with MBORD846 grown at 23°C (A) or 37°C (B). Arrows indicate some of the novel proteins, or those present in increased amounts, in strain MBORD846 cultivated at 23°C. The locations of molecular mass markers are indicated.

## DISCUSSION

Adhesion of a pathogen to mucosal surfaces is essential for colonization and subsequent infection. Various groups have demonstrated that during infection in swine, *B. bronchiseptica* preferentially colonizes ciliated nasal epithelium (1, 33, 43). An understanding of the molecular basis for this event is lacking but would greatly aid the development of more efficacious vaccines. For *B. pertussis*, several proteins, including FHA, pertactin, and, possibly, fimbriae, have been proposed as adhesins for various eukaryotic cell types (reviewed in reference 36). Since *B. bronchiseptica* produces similar versions of these proteins, it is widely assumed that they are likewise important for attachment. While this hypothesis is reasonable, no studies to date have directly addressed this question by using a primary host target cell. It is important to keep in mind that humans are the only natural host for *B. pertussis* and that *B. bronchiseptica* does not naturally infect humans. It follows, therefore, that the specific mechanisms used for adherence by these two pathogens may differ. Additionally, strains of *B. bronchiseptica* isolated from a specific host often display a reduced ability to colonize or induce disease in heterologous species (11, 25, 37), suggesting there may be host-specific differences in colonization factors of *B. bronchiseptica* strains as well. Thus, studies on adhesion of *B. bronchiseptica* in a particular host species are most meaningful when performed with relevant target cells from that species.

Our original goal was to compare the levels of adhesion of *bvg* mutants and modulated or nonmodulated *bvg*$^+$ swine isolates of *B. bronchiseptica* as a basis for subsequent evaluation of the role of specific bacterial proteins. As expected, initial experiments using strain 4609 grown in the Bvg$^+$ phase resulted in numerous bacteria bound per epithelial cell. Various groups have demonstrated that *bvg* mutant strains adhere poorly to ciliated epithelial cells, both in vitro and in vivo (10, 22, 31, 40, 43). Our data from assays using strains 4607 and PV6p15 as negative controls are in agreement with this observation. It has been shown that *bvg*$^+$ *B. pertussis*, modulated to the Bvg$^-$ phase, is poorly adherent (6). Once again, it is assumed that *B. bronchiseptica* behaves similarly, though few studies using a relevant target cell have addressed this point. Ishikawa and Isayama (22) did test one swine isolate of *B. bronchiseptica* grown at 25°C and found that it was poorly adherent to porcine nasal epithelial cells. We have shown that rather than exhibiting a reduction in adhesion, the virulent strain 4609, grown at 23°C, attaches to swine ciliated epithelial

cells at a significantly greater level than the same strain grown at 37°C. We have further shown that this phenotype is evident in a second $bvg^+$ swine strain, MBORD846, when grown at a reduced temperature. High levels of adhesion to ciliated cells by a temperature-modulated $bvg^+$ *B. bronchiseptica* swine isolate have also been observed by one other investigator (29). Therefore, this phenotype may be common, at least among porcine isolates.

To determine whether expression of adhesion at 23°C is controlled by temperature independently of Bvg, organisms were modulated chemically by growth at 37°C in the presence of 50 mM $MgSO_4$. Since increased attachment, comparable to that seen when bacteria were grown at 23°C, was observed, the adhesin(s) involved may be regulated by the *bvg* locus. However, this phenotype does not appear to be Bvg repressed in the strict sense. A Bvg-repressed adhesin would be expected to function not only in modulated organisms but also in strains locked permanently in the $Bvg^-$ phase. Yet our results suggest that adhesion of organisms cultivated at 23°C may be dependent on the presence of a responsive *bvg* locus, since the phase-locked mutant MBA4 adheres to ciliated cells at only low levels. The expression pattern of this adhesive phenotype bears some resemblance to the expression pattern of recently described $Bvg^{intermediate}$ ($Bvg^i$)-phase factors that are produced under semimodulating conditions (13). Production of $Bvg^i$-phase factors is dependent on the presence of a functional *bvg* locus, though some $Bvg^i$ factors are not expressed under $Bvg^+$ conditions. However, the Bvg-repressed adhesive phenotype that we report here is unique in that no other $Bvg^-$-phase factors that require an intact *bvg* locus have been identified. Whether the highly adhesive phenotype is also expressed under $Bvg^i$-phase conditions is not clear, since we did not determine the level of adhesion of semimodulated $bvg^+$ strains.

The significance of modulation of *Bordetella* by $MgSO_4$ and nicotinic acid in vitro is unclear, since no in vivo chemical modulators have been identified. However, it is highly likely that *B. bronchiseptica* undergoes temperature-induced Bvg repression during the infectious cycle. Unlike *B. pertussis*, *B. bronchiseptica* is able to survive, and even grow, in a nutrient-poor environment at temperatures as low as 10°C (34, 35). *B. bronchiseptica* is also able to survive in soil for extended periods (32). These observations suggest that reservoirs for infection may exist in water or soil environments. In addition, the temperature encountered during the initial hours of infection, when bacteria are located in anterior portions of the upper respiratory tract, may be significantly lower than 37°C. Thus, we propose there is ample opportunity during transmission and early infection for *B. bronchiseptica* to express the adhesive phenotype that we have observed in vitro. Since this phenotype persists for several hours following an increase in temperature, there is sufficient time for the adhesin(s) involved to play a role in the early stages of infection.

Beattie et al. (7) suggested that in *B. pertussis*, at least one Bvg-repressed gene product plays a role in colonization of mice. Others have demonstrated that the $Bvg^-$ phase is not required for colonization of rabbits (12) or of rats (3) by *B. bronchiseptica*. The role of Bvg-repressed genes in infection of swine has not been addressed. However, it is clear that organisms grown exclusively at 37°C can attach to swine ciliated epithelial cells in vitro and colonize and induce disease in swine under experimental conditions (1). The question arises as to the significance of an adhesin expressed only at 23°C. We propose that during the infectious cycle, reduced temperature, encountered either during persistence of *B. bronchiseptica* in an environmental reservoir or during transmission and initial stages of infection, triggers the expression of a novel adhesin(s) that possesses heightened ability to adhere to ciliated epithelial cells. Such an adhesin may be required for maximal colonization since the protective mucosal barrier is still intact at this early stage. As the infection progresses and bacteria move deeper into the respiratory tract, other adhesins induced by growth at increased temperatures may be operative. By this time, the mucosal barrier may be compromised and bacterial adhesins with somewhat reduced capabilities might be sufficient to sustain the infection. While organisms grown exclusively at 37°C may be able to initiate infection under experimental conditions, the degree of colonization and resulting disease may not be an accurate reflection of the natural disease. This hypothesis would provide an explanation for the unreliability of currently available *B. bronchiseptica* swine vaccines (20). All are derived from organisms grown at 37°C and would not be expected to contain an adhesin expressed only at lower temperatures. The absence of such an adhesin would permit initial colonization of vaccinated swine by *B. bronchiseptica*, while the presence of other bacterial components in these vaccines might reduce the progression of disease, a clinical outcome that is frequently observed.

In summary, we have demonstrated that swine isolates of *B. bronchiseptica* express an adhesin(s) for swine ciliated nasal epithelial cells that is repressed under conditions in which high-level expression of *bvgAS* is expected to occur. However, induction of this phenotype appears to require a functional *bvg* locus, since it is not observed in *bvg* mutant strains. While the use of *bvg* mutants is extremely informative for studies of Bvg-regulated gene expression, our findings suggest they may not be appropriate for studies addressing the pathogenesis of natural infection. Our data suggest that a heat-sensitive moiety, possibly an outer membrane protein, may be responsible for the adhesive phenotype described in this report. Future studies will address the identity of the adhesin and its role in vivo during natural infection of swine.

## ACKNOWLEDGMENTS

We thank David Dyer and Jeff Miller for providing strains and Michael Brennan for monoclonal antibodies used in this study. We are indebted to Tibor Magyar for the gift of strains and for providing unpublished data. The technical assistance of Kim Driftmier is also greatly appreciated.

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*Editor:* P. E. Orndorff