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# Isolation and Characterization of *Bordetella avium* Phase Variants

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**Two spontaneous phase variants of *Bordetella avium* were isolated at a frequency of $2 \times 10^{-4}$ by colony immunoblot assay of *B. avium* with antibody against *B. avium* dermonecrotic toxin. The two phase variants, designated GOBL309 and GOBL312, lack dermonecrotic toxin and four outer membrane proteins with molecular masses of 93, 48, 38, and 27 kDa but retain the ability to agglutinate guinea pig erythrocytes. The proteins which are not expressed by GOBL309 and GOBL312 correspond to five proteins which are phenotypically modulated in *B. avium* by growth in the presence of nicotinic acid or $MgSO_4$. Growth of the phase variants in supplemented Stainer-Scholte media containing nicotinamide did not alter expression of these five proteins. Intranasal inoculation of the spontaneous phase variants into 3-day-old turkeys and reisolation of *B. avium* at 2 weeks postinoculation resulted in the recovery of *B. avium* which had the wild-type phenotype, colonized the turkey tracheas, and produced the four outer membrane proteins and dermonecrotic toxin. Hybridization of *B. avium* and *B. avium*-like chromosomal DNA with internal portions of the *Bordetella pertussis* virulence regulatory genes, *bvgA* and *bvgS*, revealed that *B. avium* and *B. avium*-like isolates contain 5.3- and 5.7-kb DNA fragments, respectively, which are homologous to *bvgS*. *B. avium* and *B. avium*-like chromosomal DNA failed to hybridize to *B. pertussis bvgA*.**

*Bordetella avium* causes an upper respiratory tract disease in birds which is characterized by a 5- to 14-day incubation period, coughing, mucus accumulation in the trachea and nares, depression, and weight loss (22, 28, 57). *B. avium* infection of birds mimics pertussis infection of humans since fowl and humans with bordetellosis exhibit similar clinical symptoms and histopathology (3, 4, 16, 18, 22, 33, 34, 44, 53, 54, 57, 59). *B. avium* produces two virulence factors, dermonecrotic toxin and tracheal cytotoxin, which are conserved among all of the *Bordetella* species, but lacks a pertussislike toxin, an extracytoplasmic adenylate cyclase, and a filamentous hemagglutinin (10, 14). In addition, *B. avium* produces a guinea pig hemagglutinin which may be functionally analogous to the *Bordetella pertussis* filamentous hemagglutinin (5, 26, 58). A closely related bacterium, designated *B. avium*-like, has also been isolated from commercial turkey flocks and wild birds (6, 26). These isolates lack dermonecrotic toxin, are nonpathogenic, and colonize the trachea for a shorter period of time than *B. avium*.

Two types of coordinate regulation of virulence factors and outer membrane proteins (OMP) occur in *Bordetella* species and have been designated phase variation and phenotypic modulation (25, 30–32, 46, 47). Phase variation in *Bordetella* species is due to a genetic change in the regulatory *bvg* locus, while phenotypic modulation is a reversible, phenotypic change due to the interaction of environmental factors such as temperature, nicotinic acid, or $MgSO_4$ with the proteins encoded by the *bvg* locus (1, 25, 30, 32, 42, 60). The result of either phase variation or phenotypic modulation is the simultaneous loss or acquisition of a number of virulence factors such as dermonecrotic toxin, adenylate cyclase, filamentous hemagglutinin, pertussis toxin, agglutinogens, and a number of OMP in *B. pertussis* (12, 25, 30–32, 37, 45–47, 56, 62). The production of one virulence factor, tracheal cytotoxin, is not altered by phase variation or phenotypic modulation (17).

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The molecular basis of phase variation and phenotypic modulation in *B. pertussis* and *Bordetella bronchiseptica* has recently been studied by several investigators (1, 29, 40, 42, 60, 63). The *bvg* locus of *B. pertussis* has been sequenced and is composed of two genes, *bvgA* and *bvgS*, which encode 23- and 134-kDa proteins, respectively (1, 55). The BvgA and BvgS proteins are homologous to two-component regulatory systems that respond to environmental factors and have been reported for other bacterial species (19, 41). Phase variation in *B. pertussis* Tohama III is due to a frameshift mutation in the *bvgS* gene (60), while phase variation in *B. bronchiseptica* is due to either a frameshift mutation or a 50- to 500-bp deletion in the *bvgS* locus (42). Phenotypic modulation of *B. pertussis* results in reduced transcription of the *bvg* locus, with some variation depending on the bacterial strain and the modulator (40). Additional analysis has indicated that there are four promoters for transcription of the *bvg* locus and that there is constitutive, low-level *bvgA* transcription via one of these promoters under modulating and nonmodulating conditions (55). A proposed model for phase change is that, in the absence of frameshift mutations and deletions in the *bvg* locus and environmental modulator, BvgS activates BvgA by phosphorylation (1, 51, 55). Activated BvgA then dimerizes and activates the other three promoters of the *bvg* locus, resulting in increased production of BvgA protein. The increased level of BvgA ultimately leads to activation of all *bvg*-regulated genes. In the presence of modulators, the BvgS protein is inactivated and subsequently the BvgA protein is not activated, and expression of numerous virulence genes is not induced. Thus, modulation by environmental factors or frameshift mutation or deletion in the *bvg* locus results in the inability to activate virulence protein genes, resulting in phase change from a virulent bacterium to an avirulent bacterium.

Although phase variation and phenotypic modulation in *B. pertussis* and *B. bronchiseptica* have been studied thoroughly, little information on phase change in *B. avium* is available. It has been suggested that *B. avium* has a regula-

tory system similar to that of *B. pertussis* since production of dermonecrotic toxin is phenotypically modulated by nicotinic acid and $MgSO_4$ in *B. avium*. In this article, we describe the isolation of spontaneous phase variants of *B. avium*, passage of these phase variants through turkeys, and characterization of the bird-passaged *B. avium* phase variants. Hybridization experiments using the *bvg* locus of *B. pertussis* as a probe were performed to determine whether *B. avium* and *B. avium*-like isolates contain a genetic locus homologous to the *bvg* locus of *B. pertussis*.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *B. avium* GOBL271, a spontaneous nalidixic acid-resistant mutant of *B. avium* 197, was used for isolation of *B. avium* spontaneous phase variants. *B. avium* GOBL271 was obtained by growing *B. avium* 197 at 37°C in brain heart infusion broth to an $A_{550}$ of 1.2, plating $10^8$ bacteria onto brain heart infusion agar containing 50 μg of nalidixic acid per ml of agar, and selecting individual colonies. *B. avium* 197 was obtained from Y. M. Saif and is virulent for turkeys (54).

The following *Bordetella* strains were used in Southern hybridization experiments and have been described previously (14): *B. avium* 002 isolate B, 27/83-T1, 838, 4671, and 105; *B. avium*-like 008 and 031; *B. pertussis* Tohama I and BB114; and *B. bronchiseptica* 469.

Plasmid pGB304 contains the *B. pertussis bvgA* and *bvgS* genes on a 14-kb *Bam*HI DNA fragment (29). Plasmid pGB304 was kindly provided by Knapp and Mekalanos (29), pUC8-2 was obtained from Hanna and coworkers (20), and pUC18 was supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md.

**Media and antibodies.** Supplemented, modified Stainer-Scholte (SSM-S) medium (14) containing 500 μg of nicotinamide per ml was used for growth of *B. avium* 197, GOBL271, GOBL309, GOBL312, and other *B. avium* isolates throughout these experiments unless specifically otherwise noted. All chemicals and supplements used in the SSM-S medium were obtained from Sigma Chemical Co., St. Louis, Mo. Rabbit antibody against *B. avium* dermonecrotic toxin was kindly supplied by Richard Rimler (49), while the antibody against *B. avium* OMP was prepared in this laboratory. *B. avium* 197 OMP were prepared according to the method of Rapp and coworkers (48), with minor modifications. *B. avium* 197 was incubated aerobically at 37°C to a titer of $9.9 \times 10^8$ CFU/ml in 1 liter of SSM-S medium containing 500 μg of nicotinamide per ml. The cells were pelleted, suspended in 50 ml of phosphate-buffered saline (PBS) (pH 7.5), and sonicated with a model W185D sonifier-cell disruptor (Heat Systems-Ultrasonics Inc., Farmingdale, N.Y.) for four bursts of 5 min each at a setting of 40 W with 50% output. The sonicate was incubated on ice for 5 min between each burst. The cell debris was pelleted at $2,000 \times g$ for 20 min at 4°C. The supernatant fluid was removed, and membranes were pelleted by centrifugation at $29,640 \times g$ for 1 h at 4°C. The membrane pellet was suspended in 5 ml of PBS, pH 7.5, and the protein concentration was determined to be 4 mg of protein per ml. One volume of 2% sodium lauryl sarcosinate in PBS, pH 7.5, was added to the suspended membranes to obtain a final concentration of 1% sodium lauryl sarcosinate. The solution was stirred at room temperature for 30 min, and the insoluble OMP were pelleted by centrifugation at $100,000 \times g$ for 1 h. The pelleted OMP were suspended in 2 ml of PBS, pH 7.5, and the protein concentration was determined to be 2.5 mg of protein per ml. The rabbit was immunized intradermally with 500 μg of the purified OMP preparation in an equal volume of Freund's complete adjuvant (Sigma Chemical Co.). Two weeks later, the rabbit was boosted intradermally with 500 μg of the OMP preparation in an equal volume of Freund's incomplete adjuvant (Sigma Chemical Co.). The rabbit was periodically boosted with 500 ng of OMP preparation in Freund's incomplete adjuvant and bled for larger volumes of blood (40 ml), and the serum was collected.

**Isolation of *B. avium* phase variants.** *B. avium* GOBL271 was grown at 37°C in SSM-S medium containing 500 μg of nicotinamide per ml to an $A_{550}$ of 1.2 and plated onto 20 SSM-S agar plates containing 50 μg of nalidixic acid and 500 μg of nicotinamide per ml to obtain 500 colonies per plate. *B. avium* GOBL271 colonies were tested for reactivity with antibody against *B. avium* dermonecrotic toxin by the colony immunoblot assay (14). Colonies were blotted onto nitrocellulose filters, lysed by exposure to chloroform fumes, and reacted for 4 h with a 1:500 dilution of rabbit antibody against *B. avium* dermonecrotic toxin. The filters were washed to remove residual primary antibody and reacted for 2 h with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (affinity purified) from ICN Biomedicals, Inc., Costa Mesa, Calif. The nitrocellulose filters were subsequently washed and developed with 4-chloro-1-naphthol (Sigma Chemical Co.). Colonies which failed to react with the antibody were isolated and stored on SSM-S agar with 500 μg of nicotinamide and 50 μg of nalidixic acid per ml and were designated GOBL309 and GOBL312. Colonies which reacted with the antibody were randomly selected and designated GOBL307, GOBL308, GOBL310, GOBL311, and GOBL313.

**Hemagglutination assays.** *B. avium* 197, GOBL271, GOBL309, and GOBL312 were assayed for the ability to agglutinate guinea pig erythrocytes as previously described (14).

**Western blot (immunoblot) of proteins produced by *B. avium* phase variants.** *B. avium* 197, GOBL307, GOBL308, GOBL309, GOBL310, GOBL311, and GOBL312 and bird-passaged *B. avium* GOBL271, GOBL309, and GOBL312 were grown at 37°C to an $A_{550}$ of 1.2 in SSM-S medium supplemented with 500 μg of nicotinamide per ml. Proteins from cultures of the *B. avium* strains listed above were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of boiled whole cells, electrotransfer of proteins to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.), and Western immunoblot analysis as described previously (14, 61). The primary antibody was either anti-*B. avium* dermonecrotic toxin or anti-*B. avium* OMP antibody, while the secondary antibody was horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (affinity purified) from ICN Biomedicals, Inc., or Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.

For comparison of expression of OMP by phase variants and phenotypically modulated wild-type *B. avium* and for determination of the effect of phenotypic modulation with $MgSO_4$ on *B. avium* phase variants, *B. avium* 197 and GOBL309 were grown at 37°C to an $A_{550}$ of 1.2 in SSM-S medium with either 500 μg of nicotinamide per ml or 500 μg of nicotinamide per ml and 20 mM $MgSO_4$. Proteins from *B. avium* 197 and GOBL309 were analyzed by Western blot analysis with antibody against *B. avium* 197 OMP by the procedures described above.

**Passage of *B. avium* GOBL309 and GOBL312 in birds.** *B. avium* GOBL271, GOBL309, and GOBL312 were grown at 37°C to $A_{550}$ of 1.2 in SSM-S medium containing 500 μg of

nicotinamide per ml. Three groups of six 3-day-old Nicholas turkey poults (Cargill, Inc., Elgin, Mo.) were inoculated intranasally and intraocularly with $1.1 \times 10^8$ *B. avium* GOBL309, GOBL312, or GOBL271 cells per inoculation site. At 7 and 14 days postinoculation, three birds from each group were euthanized by $CO_2$ asphyxiation, and a 1-in. (1 in. = 2.54 cm) section from the middle of the trachea was removed from each bird. The tracheal sections were added to 3 ml of BSG buffer (0.15 M NaCl, 0.002 M $KH_2PO_4$, 0.004 M $Na_2HPO_4$, 1% gelatin) and disrupted for 2 min on setting 6 with a tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). The suspension was serially diluted and plated on SSM-S agar containing 50 μg of nalidixic acid per ml and 500 μg of nicotinamide per ml to quantitate the number of adherent *B. avium* cells. After incubation, the colonies were reacted with antibody against *B. avium* dermonecrotic toxin by using the colony immunoblot assay as described above. In addition, several colonies were randomly selected and grown in SSM-S medium with 500 μg of nicotinamide per ml and 50 μg of nalidixic acid per ml, and whole cellular proteins were subjected to Western blot analysis with anti-*B. avium* dermonecrotic toxin and anti-*B. avium* OMP antibody as described above.

Two bird passage experiments were undertaken, and a maximum of 18 turkey poults were used in each experiment because of cost and space limitations.

**Southern hybridization of *B. avium* chromosomal DNA with the *B. pertussis bvg* locus.** To construct a DNA probe specific for *B. pertussis bvgA*, plasmid pGB304 was digested with *Ava*II and *Pst*I, and the 509-bp *Ava*II-*Pst*I internal DNA fragment was isolated from an agarose gel by using reagents from the GENECLEAN kit (BIO 101, La Jolla, Calif.). The 509-bp *Ava*II-*Pst*I fragment was treated with the Klenow fragment of DNA polymerase I and ligated to *Sma*I-digested pUC8-2 to obtain pWDC101. All restriction enzymes and DNA-modifying enzymes were from Bethesda Research Laboratories, Inc.; Promega, Inc., Madison, Wis.; or New England Biolabs, Inc., Beverly, Mass.

A DNA probe specific for *B. pertussis bvgS* was constructed by first subcloning the 2.7-kb *Eco*RI-digested DNA fragment containing the *bvgS* gene from pGB304 into *Eco*RI-digested pUC18. This construct was subsequently digested with *Sna*BI and *Hin*dIII, treated with T4 DNA polymerase, and religated to obtain pYA3028, which contained a 1.89-kb internal DNA fragment encoding *bvgS*.

Chromosomal DNA was isolated from all *Bordetella* strains by the method of Hull and coworkers (24). Chromosomal DNA from the *B. avium*, *B. avium*-like, *B. pertussis*, and *B. bronchiseptica* strains was digested with *Eco*RI, electrophoresed through a 0.6% agarose gel, and transferred to GeneScreen Plus hybridization membranes (New England Nuclear Corp., Boston, Mass.) according to the manufacturer's instruction. The membranes were prehybridized in 1% SDS, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, and 300 μg of denatured salmon sperm DNA (Sigma Chemical Co.) per ml for 1 h at either 55°C (with the *bvgA* probe pWDC101) or 65°C (with the *bvgS* probe pYA3028). Either $^{32}$P-labeled pWDC101 or $^{32}$P-labeled pYA3028 (Lofstrand Laboratories Ltd., Gaithersburg, Md.) was added directly to the prehybridization solution at a concentration of $1 \times 10^5$ to $4 \times 10^5$ cpm/ml and incubated overnight at either 55 or 65°C, respectively. The membranes were washed according to the manufacturer's instructions, with the exception that the membrane exposed to radiolabeled pWDC101 was washed at 55°C, while the membrane exposed to radiolabeled pYA3028 was washed at 65°C. The membranes were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with two intensifying screens for 48 h at −70°C and developed with a Kodak M35A X-Omat processor (Eastman Kodak Co.).

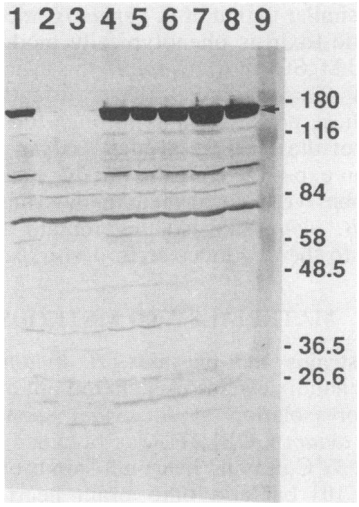


FIG. 1. Western blot analysis of the loss of production of dermonecrotic toxin by *B. avium* phase variants. Lanes: 1, *B. avium* GOBL311; 2, *B. avium* GOBL312; 3, *B. avium* GOBL309; 4, *B. avium* GOBL271; 5, *B. avium* GOBL310; 6, *B. avium* GOBL313; 7, *B. avium* GOBL308; 8, *B. avium* GOBL307; 9, molecular weight markers. The filter containing the transferred proteins was reacted with antibody against *B. avium* 197 dermonecrotic toxin. The arrow designates the dermonecrotic toxin.

## RESULTS

**Isolation and characterization of *B. avium* phase variants.** Since previous studies had revealed that expression of dermonecrotic toxin can be phenotypically modulated in *B. avium* 197 (14), we decided to examine *B. avium* 197 cultures for phase variation, a phenomenon common to *B. pertussis* and *B. bronchiseptica*. A spontaneous, nalidixic acid-resistant mutant, designated GOBL271, was plated on SSM-S medium containing nicotinamide, and approximately 10,000 colonies were examined for production of dermonecrotic toxin by using anti-*B. avium* dermonecrotic toxin antibody in the colony immunoblot assay. Examination of the colony immunoblots revealed that 2 of 10,000 colonies had lost the ability to produce dermonecrotic toxin. Western immunoblot analysis confirmed that the two putative phase variants, designated GOBL309 and GOBL312, had lost the ability to produce dermonecrotic toxin (Fig. 1). The two phase variants had a colony morphology that was dry and crusty and was distinct from the wild-type *B. avium* colony morphology. In addition, *B. avium* GOBL309 and GOBL312 agglutinated guinea pig erythrocytes, a property associated with wild-type *B. avium*. *B. avium* GOBL309 and GOBL312 were examined for the loss of OMP common to GOBL271. Western immunoblot analysis with anti-*B. avium* OMP antibody revealed that GOBL309 and GOBL312 had lost the ability to produce four OMP with molecular masses of 27, 38, 48, and 93 kDa (Fig. 2). Repeated passage of the two phase variants on SSM-S agar media supplemented with nicotinamide did not alter their ability to produce dermonecrotic toxin or OMP.

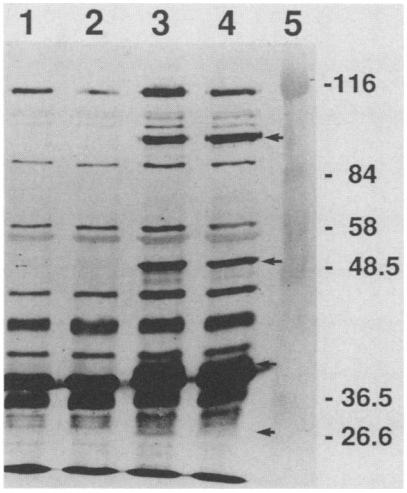


FIG. 2. Western blot analysis of the loss of production of four OMP by *B. avium* phase variants. Lanes: 1, *B. avium* GOBL309; 2, *B. avium* GOBL312; 3, *B. avium* 197; 4, *B. avium* GOBL311; 5, molecular weight markers. The primary antibody reacted with the filter was antibody against *B. avium* 197 OMP. The arrows designate the proteins lacking in *B. avium* phase variants.

A comparison was made to determine whether the OMP which were no longer produced by *B. avium* phase variants corresponded to OMP which were absent when *B. avium* 197 was modulated by $MgSO_4$. Western immunoblots of *B. avium* 197 and GOBL309 grown in SSM-S agar with nicotinamide and in the presence and absence of $MgSO_4$ indicated that the 38-, 48-, and 93-kDa OMP lacking in *B. avium* GOBL309 correlated with those proteins which were modulated by $MgSO_4$ in wild-type *B. avium* 197 (Fig. 3). The 27-kDa OMP was not evident in this blot, and its absence

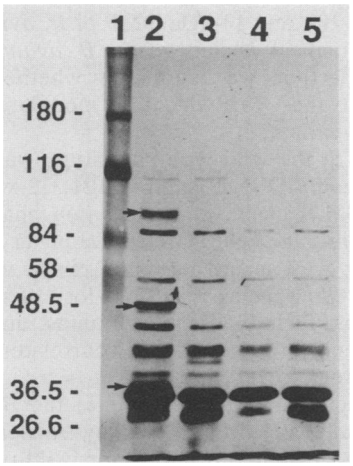


FIG. 3. Western blot analysis of OMP expressed by *B. avium* in response to phenotypic modulation and the correlation to OMP lacking in *B. avium* phase variants. Lanes: 2 and 4, *B. avium* 197 and GOBL309, respectively, grown in SSM-S media with 500 μg of nicotinamide per ml; 3 and 5, *B. avium* 197 and GOBL309, respectively, grown in SSM-S media with 500 μg of nicotinamide per ml and 20 mM $MgSO_4$; 1, molecular weight markers. Antibody against *B. avium* 197 OMP served as the primary antibody. The arrows designate proteins which are lacking in *B. avium* phase variants or proteins which are modulated in wild-type *B. avium*.

was probably due to poor resolution in this particular SDS-polyacrylamide gel.

**Characterization of bird-passaged *B. avium* phase variants.** *B. avium* GOBL309 and GOBL312 were passaged through birds and examined for production of dermonecrotic toxin and the 27-, 38-, 48-, and 93-kDa OMP to determine whether reversion to the wild-type phenotype would occur upon passage in the natural host. Passage of *B. avium* GOBL309 and GOBL312 through turkey poults restored their ability to express dermonecrotic toxin and the 38-, 48-, and 93-kDa OMP which were previously lacking in the nonpassaged phase variants (Fig. 4A and B). The 27-kDa protein was absent in the Western blot, and lack of the 27-kDa protein probably reflects poor resolution in the SDS-polyacrylamide gel in this region.

Although the number of infected birds was limited, there appeared to be a general reduction in the number of *B. avium* cells recovered from GOBL309- and GOBL312-infected birds compared with the number of *B. avium* cells recovered from GOBL271-infected birds (Table 1). Colony immunoblots were performed with at least 500 colonies (when present) from each infected bird (data not shown), and results from the colony blots indicated that all *B. avium* isolated from the tracheas had reverted to the wild-type phenotype. By 14 days postinoculation, the number of *B. avium* GOBL309 and GOBL312 cells recovered from the birds was comparable to the number of parent GOBL271 cells that was recovered from the birds. Bird passage experiments were performed twice; however, bacteria recovered from the tracheas were quantitated only in the second experiment.

**Hybridization of *B. avium* chromosomal DNA with the *B. pertussis bvg* locus.** In order to determine whether *B. avium* and *B. avium*-like strains contain a genetic locus homologous to the *B. pertussis bvg* locus, chromosomal DNA from various isolates were hybridized with the *B. pertussis bvgA* and *bvgS* genes. *Eco*RI-digested chromosomal DNA from *B. avium* 197, 105, 002 isolate B, 27/83-T1, 838, and 4671 and from *B. avium*-like strain 031 failed to hybridize at 55°C with an internal 509-bp DNA fragment of the *B. pertussis bvgA* gene (Fig. 5A). Under the same hybridization conditions, the *B. pertussis bvgA* gene hybridized with a 2.5-kb DNA fragment from *Eco*RI-digested *B. pertussis* Tohama I, *B. pertussis* BB114, and *B. bronchiseptica* 469 chromosomal DNA (Fig. 5A).

Chromosomal DNA from *B. avium* 197, 105, 002 isolate B, 27/83-T1, 83, and 4671 and from *B. avium*-like strains 008 and 031 was digested with *Eco*RI and hybridized at 65°C with a 1.89-kb DNA fragment internal to the *bvgS* gene (Fig. 5B). The *B. pertussis bvgS* gene hybridized to a 5.3-kb chromosomal DNA fragment from all *B. avium* strains tested and to a 5.7-kb chromosomal DNA fragment from the *B. avium*-like strains tested.

## DISCUSSION

Two phase variants of *B. avium* 197, designated GOBL309 and GOBL312, were isolated at a frequency of $2 \times 10^{-4}$ and were found to have lost the ability to produce dermonecrotic toxin and four OMP with molecular masses of 27, 38, 48, and 93 kDa. Phase variants have similarly been isolated from *B. pertussis* and *B. bronchiseptica* at frequencies of $10^{-3}$ to $10^{-6}$ (46, 63) and $10^{-2}$ to $10^{-3}$ (47), respectively. The molecular masses for the proteins lacking in *B. pertussis* phase variants have been reported to be 90, 86, 81, 33, 31, and 30 kDa (50); 91, 32, and 30 kDa (2); 98, 88, 30, and 28

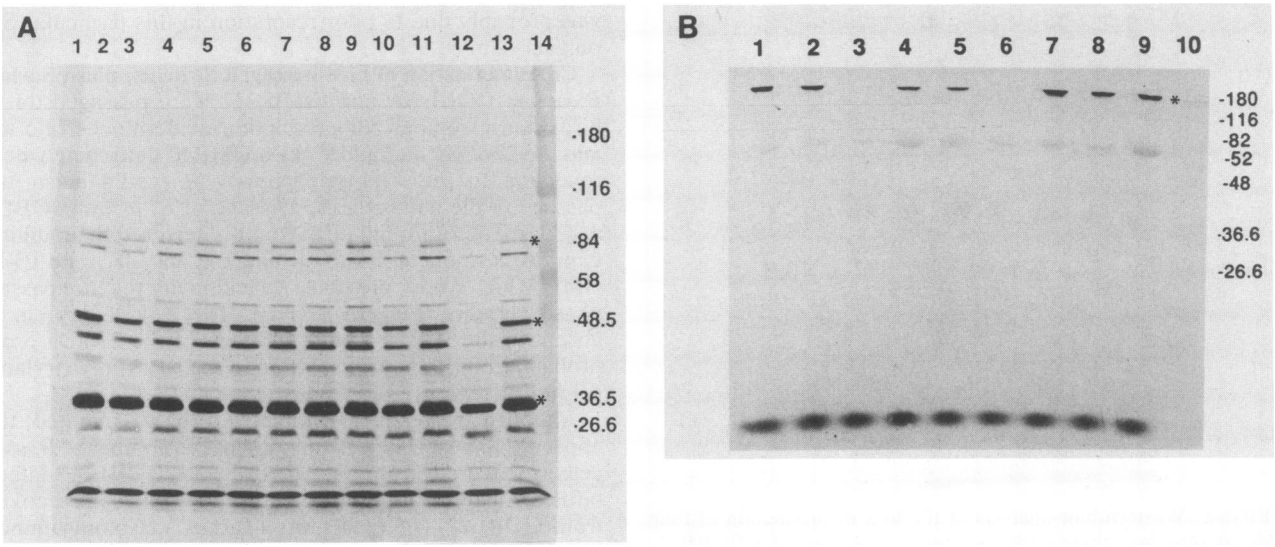


FIG. 4. Western blot analysis of OMP and dermonecrotic toxin produced by bird-passaged and nonpassaged *B. avium* phase variants GOBL309 and GOBL312. (A) Filter was reacted with antibody against *B. avium* 197 OMP. Lanes: 2 to 5, independent isolates of bird-passaged *B. avium* GOBL309; 6 to 9, independent isolates of bird-passaged *B. avium* GOBL312; 10 and 13, independent isolates of bird-passaged *B. avium* GOBL271; 11, nonpassaged *B. avium* GOBL124; 12, nonpassaged *B. avium* GOBL309; 1 and 14, molecular weight markers. (B) Filter was reacted with antibody against *B. avium* dermonecrotic toxin. Lanes: 1 and 2, independent isolates of bird-passaged *B. avium* GOBL312; 3, nonpassaged *B. avium* GOBL312; 4 and 5, independent isolates of bird-passaged *B. avium* GOBL309; 6, nonpassaged *B. avium* GOBL309; 7 and 8, bird-passaged *B. avium* GOBL271; 9, nonpassaged *B. avium* GOBL271; 10, molecular weight markers. Asterisks designate the proteins which are lacking in *B. avium* phase variants.

kDa (12); 30.5 and 25.5 kDa (46); and 30 and 33.5 kDa (45, 62). *Bordetella parapertussis* phase variants fail to produce 112-, 95-, 78-, 81-, 47-, 29-, and 26-kDa envelope proteins, while *B. bronchiseptica* loses 28- and 30-kDa proteins upon phase variation (12). As with the other *Bordetella* species, the proteins which were not expressed by *B. avium* phase variants corresponded with the proteins which were not expressed by the *B. avium* grown under modulating conditions with $MgSO_4$. Additional OMP may be produced by wild-type *B. avium* phase variants and may have been undetected by the Western blot analysis, since some proteins may be produced in such small quantities that the injection dose was inadequate for production of antibody, or the protein(s) may lack immunogenicity in the rabbit. Since *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* were not screened with antibody against *B. avium* OMP, it is not known whether the OMP which were lacking in *B. avium* phase variants are antigenically or functionally related to those which are lacking in phase variants of other *Bordetella* species. However, it is obvious from these results that *B. avium* undergoes phase variation and phenotypic modulation, and, despite differences in host range, these two methods for alteration of protein expression are a common theme among the *Bordetella* species. Further genetic analysis of the regulatory locus of *B. avium* is necessary to confirm that the genetic basis of phase variation in *B. avium* is similar to that of other *Bordetella* species and is due to an alteration in a central regulatory locus.

TABLE 1. Bacterial titers recovered from turkey tracheas[a]

| Wk post-infection | Titer of: | | |
|---|---|---|---|
| | GOBL271 | GOBL309 | GOBL312 |
| 1 | $5.1 \times 10^6$ | $9.3 \times 10^3$ | $3.0 \times 10^2$ |
| | $1.9 \times 10^6$ | <30 | $8.1 \times 10^2$ |
| | <30 | $8.7 \times 10^6$ | $1.7 \times 10^3$ |
| 2 | $1.4 \times 10^6$ | $1.3 \times 10^7$ | $6.6 \times 10^6$ |
| | $1.4 \times 10^5$ | $5.1 \times 10^6$ | $2.1 \times 10^6$ |
| | $1.1 \times 10^7$ | | $8.7 \times 10^6$ |

[a] Numbers of bacteria obtained from 1-in. sections of tracheas from individual birds.

It has been reported that antibodies against 100-, 97-, 55-, 36-, 31-, 21-, 18-, and 14-kDa OMP of *B. avium* are present in sera and tracheal washings from *B. avium*-infected birds (15, 21). At this time, we do not know whether these proteins correspond to the OMP which are modulated or lost in *B. avium* phase variants.

Reversion to the wild-type phenotype was not observed when *B. avium* GOBL309 and GOBL312 were repeatedly grown in SSM-S media, and *B. avium* phase variants retained the ability to agglutinate guinea pig erythrocytes. The latter finding was surprising since phase variation in the other *Bordetella* species results in loss of numerous virulence proteins, including hemagglutinins, and agglutination of guinea pig erythrocytes strongly correlates with virulence of *B. avium* for birds (26, 43).

When infected into turkey poults, the *B. avium* phase variants initially colonized the turkey tracheas at a quantity that was lower than observed for wild-type *B. avium*. However, by 2 weeks postinoculation, the number of phase-variant *B. avium* isolated from the tracheas was equivalent to the number of wild-type *B. avium* cells that were isolated from the tracheas of infected birds. Furthermore, *B. avium* isolated from birds at 1 and 2 weeks after infection with *B. avium* GOBL309 or GOBL312 exhibited the wild-type phenotype and produced dermonecrotic toxin and the 93-, 48-, 38-, and 27-kDa OMP that were absent from nonpassaged *B. avium* phase variants. These results suggest that in the

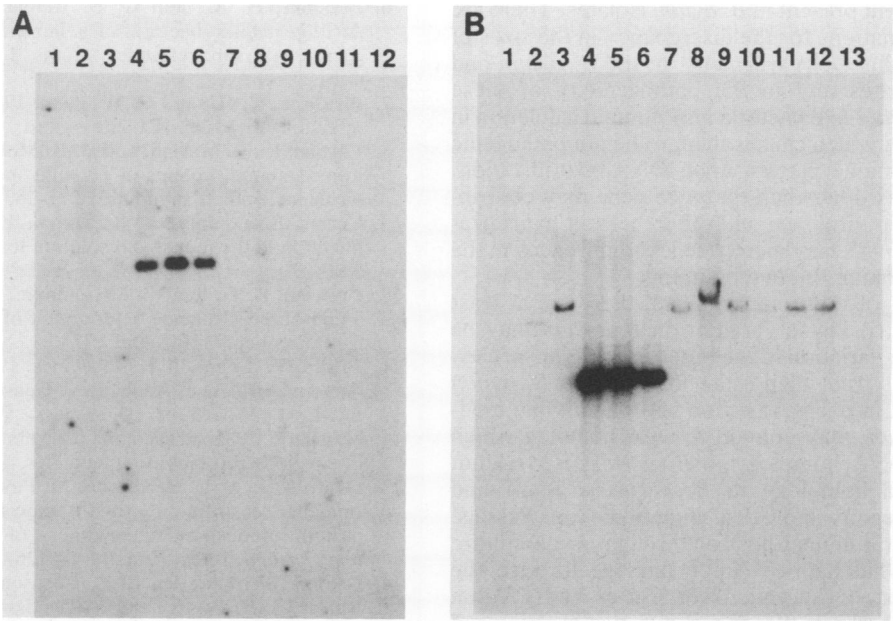


FIG. 5. Southern hybridization analysis of chromosomal DNA from *B. avium* strains. (A) Chromosomal DNA was digested with *Eco*RI and probed with pWDC101. Lanes: 2, *B. avium* 197; 3, *B. avium* 105; 4, *B. pertussis* Tohama I; 5, *B. pertussis* BB114; 6, *B. bronchiseptica* 469; 7, *B. avium* 002 isolate B; 8, *B. avium*-like 031; 9, *B. avium* 27/83-T1; 10, *B. avium* 838; 11, *B. avium* 4671; 1 and 12, molecular weight markers. (B) Chromosomal DNA was digested with *Eco*RI and probed with pYA3028. Lanes: 2, *B. avium* 197; 3, *B. avium*-like 008; 4, *B. pertussis* Tohama I; 5, *B. pertussis* BB114; 6, *B. bronchiseptica* 469; 7, *B. avium* 002 isolate B; 8, *B. avium*-like 031; 9, *B. avium* 27/83-T1; 10, *B. avium* 838; 11, *B. avium* 4671; 12, *B. avium* 105; 1 and 13, molecular weight markers.

inoculum, there was a low number of *B. avium* phase variants which had reverted to the wild-type phenotype and that during colonization of the bird, there was selection for the wild-type *B. avium*. If this is true, then proteins which are specific for the virulent-phase *B. avium* need to be expressed for prolonged colonization of the trachea. The reversibility of the spontaneous mutants indicates that phase variation in *B. avium* is not caused by a deletion in a regulatory gene and is probably due to a single point mutation. This finding agrees with phase conversion in *B. pertussis* in which addition of a cytosine residue in the *bvgS* gene results in a frameshift mutation and is responsible for conversion from wild-type to phase-variant *B. pertussis* (60).

The results obtained from bird passage of *B. avium* phase variants contrast with those obtained from intraperitoneal infection of mice with *B. bronchiseptica* phase variants. A report by Nagano and coworkers indicated that 20 intraperitoneal passages of *B. bronchiseptica* phase variants in mice did not result in reversion of the phase variants to the wild-type phenotype, i.e., they had reduced lethality, splenotoxicity, and hemolysin activity and failed to produce dermonecrotic toxin (43). Their studies showed that guinea pigs were protected against intranasal challenge with wild-type *B. bronchiseptica* when injected intranasally or intramuscularly with the *B. bronchiseptica* mutants. They also reported that the *B. bronchiseptica* phase variants could adhere to and colonize the nasal cavity of intranasally inoculated guinea pigs without manifesting clinical signs. Although these reports implied that *B. bronchiseptica* phase variants did not regain the ability to produce dermonecrotic toxin or other virulence proteins, they did not indicate whether the *B. bronchiseptica* recovered from the guinea pigs were actually tested for the acquisition of dermonecrotic toxin and other OMP. Therefore, it is difficult to compare the results of Nagano and coworkers with those obtained in this laboratory.

Selective or increased expression of proteins which are important in virulence upon infection in the animal host is not a new concept. It has been demonstrated that passage of chemically induced, nontoxigenic mutants of *Vibrio cholerae* in infant rabbits (23) or rabbit ligated ileal loops (39) results in reversion of the mutant *V. cholerae* to toxigenicity. In addition, hypertoxigenic *V. cholerae* isolates are selected by passage of toxigenic *V. cholerae* through rabbit ligated intestinal loops. The hypertoxigenicity is due to amplification of the toxin genetic region during animal passage (38). There are numerous examples in the literature which indicate that growth of pathogens under conditions which mimic those in the natural host increases virulence (7, 8, 11, 13, 27, 35, 36, 52, 64). These studies support the hypothesis of differential expression of proteins in the animal host and in the external environment.

*B. avium* does not contain a genetic locus which is detectable by DNA hybridization to *B. pertussis bvgA*, but it does contain a genetic locus homologous to *B. pertussis bvgS*. However, since phase variation occurs in *B. avium* and phenotypic modulation occurs via nicotinic acid or $MgSO_4$, it is probable that *B. avium* contains a gene which is functionally homologous to the *B. pertussis bvgA* gene and interacts with the *bvgS* homolog. Alternatively, *B. avium* may contain a unique or additional system for phenotypic modulation and phase variation. Isolation of a *bvgA* functional homolog from *B. avium* or production of *B. avium* transposon insertions in a regulatory gene(s) which controls expression of dermonecrotic toxin and the four OMP will answer this question. The *B. avium*-like isolates contain a chromosomal DNA fragment that hybridizes to *B. pertussis bvgS*, and this DNA fragment is 5.7 kb, compared with the

5.3-kb DNA fragment present in *B. avium* isolates. There are two possible explanations for the discrepancy in the sizes of the *bvgS*-homologous DNA fragments of the *B. avium* and *B. avium*-like isolates and in the pathogenicity of these isolates. The *B. avium*-like bacteria may contain a deletion in their *bvgS* homolog which causes them to be nonpathogenic and to exhibit a unique pattern upon DNA hybridization. Alternatively, genes other than the *bvgS* gene may contribute to differences in virulence and differences in hybridization patterns with *bvgS* may merely reflect differences in the DNA sequence flanking the *bvg* homolog.

From the results of these experiments, there are at least three possibilities. (i) Phase variation in *B. avium* may be distinct from phase variation in the other *Bordetella* species. If this possibility is true, then other undescribed *B. avium* genes are involved in phase variation and phenotypic modulation. (ii) *B. avium* may contain a *bvgA* homolog which lacks significant DNA homology to *B. pertussis bvgA* but retains amino acid homology to *B. pertussis* BvgA and performs an analogous biological function. (iii) Passage through a nonnatural animal host or through tissue culture does not reflect what happens upon passage through the natural animal host. Observations of Carter and Preston support the last hypothesis (9). These investigators reported that phase variants rarely occur and never predominate in human infection. Mouse infection studies indicated that phase variants did not revert to the wild-type bacteria, indicating that *B. pertussis* infection of the mouse does not accurately represent human infection with *B. pertussis*.

This study supports findings that proteins present in wild-type *Bordetella* species and absent in phase-variant *Bordetella* species play an important role in bordetellosis. Since *B. avium* does not produce proteins which are functionally related to *B. pertussis* pertussis toxin and adenylate cyclase or immunologically related to filamentous hemagglutinin, additional studies are needed to determine which protein(s) of *B. avium* is necessary for colonization and virulence.

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