INFECTION AND IMMUNITY, July 1988, p. 1698–1707
0019-9567/88/071698-10$02.00/0

Vol. 56, No. 7

Dermonecrotic Toxin and Tracheal Cytotoxin, Putative Virulence Factors of *Bordetella avium*

CLAUDIA R. GENTRY-WEEKS,[1] BRAD T. COOKSON,[2] WILLIAM E. GOLDMAN,[2] RICHARD B. RIMLER,[3] SUSAN B. PORTER,[1] AND ROY CURTISS III[1]*

Department of Biology, Washington University, St. Louis, Missouri 63130[1]; Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110[2]; and U.S. Department of Agriculture-ARS, National Animal Disease Center, Ames, Iowa 50010[3]

Received 2 November 1987/Accepted 10 April 1988

We examined *Bordetella avium* for virulence factors common to *Bordetella pertussis*, including pertussis toxin, filamentous hemagglutinin, adenylate cyclase, dermonecrotic toxin, and tracheal cytotoxin. *B. avium* produced a dermonecrotic toxin and a tracheal cytotoxin. The dermonecrotic toxin of *B. avium* is a 155,000-molecular-weight, heat-labile protein which was lethal for mice, guinea pigs, young chickens, and turkey poults and produced dermonecrosis when injected intradermally into guinea pigs, chickens, and turkey poults. High-pressure liquid chromatography of *B. avium* culture supernatant fluid revealed the presence of a tracheal cytotoxin chemically identical to that produced by *B. pertussis*. *B. avium* isolates were negative for *B. pertussis*-like filamentous hemagglutinin and pertussis toxin when assayed with antibody against *B. pertussis* filamentous hemagglutinin and pertussis toxin. Furthermore, *B. avium* failed to induce the clustered CHO cell morphology characteristic of pertussis toxin. Adenylate cyclase assays indicated that *B. avium* does not produce an extracytoplasmic adenylate cyclase, even after passage through embryonated turkey eggs. Since production of virulence proteins by *B. pertussis* is regulated by growth in media containing nicotinamide or $MgSO_4$ or by growth at reduced temperatures, we determined the effect of these supplements and growth conditions on production of dermonecrotic toxin by *B. avium*. Production of dermonecrotic toxin in *B. avium* was not altered by growth in media containing 100 μM $FeSO_4$ or 500 μg of nicotinamide per ml or by growth at 25 or 42°C, but production was significantly decreased by growth in media containing 20 mM $MgSO_4$ and slightly reduced by growth in media containing 500 μg of nicotinic acid per ml. These studies revealed that *B. avium* is similar to *B. pertussis* in that both species produce a dermonecrotic toxin and a tracheal cytotoxin and production of dermonecrotic toxin is regulated by nicotinamide and $MgSO_4$. The presence of dermonecrotic toxin and tracheal cytotoxin in all *Bordetella* species indicates that these products may be important virulence factors in bordetellosis.

Bordetella avium is the etiological agent of rhinotracheitis of birds, a disease of the upper respiratory tract which resembles pertussis infection in humans (23, 29, 52). Similarities between *B. avium* infection in birds and *Bordetella pertussis* infection in humans include a prolonged incubation period and similar clinical symptoms and histopathology. *B. avium* infection in birds initiates with an incubation period of 5 to 14 days, followed by clinical symptoms of depression, appetite loss, weight loss, coughing (snicking), mucous accumulation on the nares, and vocal alterations (6, 23, 38, 47, 51, 52). Bordetellosis in both humans and fowl is characterized by a primary localized infection of the ciliated tracheal epithelial cells, often followed by life-threatening secondary infections (4, 6, 23, 33, 34, 38, 39, 46, 47, 52, 54, 56). Histological examination of the trachea of diseased birds reveals pathological changes identical to those exhibited by tracheas from humans and hamster tracheal rings infected with *B. pertussis* (2, 6, 7, 16, 19, 38, 46, 47, 54). Microscopic examination of the trachea reveals an initial bacterial colonization of the ciliated tracheal epithelial cells, followed by loss of ciliated cells and mucous accumulation. As with *B. pertussis*, *B. avium* has a specific tropism for ciliated respiratory epithelial cells, and isolation of *B. avium* from nonciliated respiratory epithelial cells in the lung and air sacs rarely occurs (19, 47; C. R. Gentry-Weeks and R. Curtiss III, unpublished observations). In analogy with pertussis in humans, young birds are very susceptible to infection while older birds are refractory to infection (39, 51).

In view of these similarities, isolates of *B. avium* were examined for the presence of five virulence factors associated with *B. pertussis*, including an extracellular adenylate cyclase, dermonecrotic toxin, filamentous hemagglutinin, pertussis toxin, and tracheal cytotoxin.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study are listed in Table 1. The causative agent of turkey coryza was recently reclassified from *Alcaligenes faecalis* to *B. avium* on the basis of DNA-rRNA hybridization studies, protein electropherogram analyses, and serological studies (11, 29). Furthermore, *B. avium* isolates obtained from turkey flocks have been separated into two types, designated *B. avium* and *B. avium*-like on the basis of pathogenicity for turkey flocks, colony morphology, and fatty acid analysis (5, 27, 28, 35). For consistency, the virulent type I isolates will be designated *B. avium* while the avirulent type II isolates will be designated *B. avium*-like throughout this paper. *B. pertussis* BB114 and *B. pertussis* Tohama III were maintained on Bordet-Gengou agar supplemented with 10% sheep blood, while *B. avium* isolates were maintained on brain heart infusion agar (BHI). When necessary, *B. pertussis* strains were grown in modified Stainer-Scholte broth (SSM) (20)

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Virulence[a]	Source	Assay(s)[b]
B. bronchiseptica 469	NT[c]	E. Tuomanen, N.Y.	6
B. pertussis BB114	NT	W. E. Goldman, Mo.	1, 2, 4, 5, 6
B. pertussis Tohama III	NT	W. E. Goldman, Mo.	1, 2, 5, 6, 7
B. bronchiseptica 40-81	−	K.-H. Hinz, Federal Republic of Germany	6
B. bronchiseptica P-4448	−	R. Rimler, Iowa	6
B. bronchiseptica P-4446	−	R. Rimler, Iowa	6
B. avium F84-0107	+	J. K. Skeeles, Ark.	1
B. avium F84-0069.b	+	J. K. Skeeles, Ark.	1, 2, 3, 5, 8, 9
B. avium NCD	+	G. H. Luginbuhl, N.C.	1
B. avium NCD-1	−	G. H. Luginbuhl, N.C.	1
B. avium-like 044	−	Y. M. Saif, Ohio	1, 2
B. avium-like 008	−	Y. M. Saif, Ohio	1, 2, 3, 4, 5, 8, 9
B. avium-like 128	−	Y. M. Saif, Ohio	2
B. avium 178	+	Y. M. Saif, Ohio	1, 2
B. avium 193	+	Y. M. Saif, Ohio	1
B. avium 002 isolate B	+	Y. M. Saif, Ohio	1
B. avium 200	+	Y. M. Saif, Ohio	1, 2
B. avium-like 031	−	Y. M. Saif, Ohio	1, 2, 3, 5, 8, 9
B. avium 197	+	Y. M. Saif, Ohio	1, 2, 3, 4, 5, 7, 8
B. avium-like Hanks-C2	−	H. A. Berkhoff, N.C.	1
B. avium 27/83-T1	+	H. A. Berkhoff, N.C.	1, 6
B. avium L3-T1	+	H. A. Berkhoff, N.C.	2
B. avium 838	+	M. S. Hofstad, Iowa	1, 2, 3, 5, 7, 8, 9
B. avium 4671	+	D. G. Simmons, N.C.	1, 2, 3, 5, 6, 7, 8, 9
B. avium 450-78	+	K.-H. Hinz, Federal Republic of Germany	2
B. avium 270-80	+	K.-H. Hinz, Federal Republic of Germany	2
B. avium 105	+	J. K. Skeeles, Ark.	2, 6
B. avium 85-105.2	+	J. K. Skeeles, Ark.	1, 3, 5
B. avium 114-81	+	K.-H. Hinz, Federal Republic of Germany	1

[a] Virulence for turkey poults. Virulence for turkey poults was determined by source and confirmed by oxidative alkalinization of carbon substrates (5).

[b] Numbers designate the assay(s) performed with each isolate. 1, Colony immunoblots with antibodies against *B. pertussis* filamentous hemagglutinin and pertussis toxin; 2, hemagglutination of goose RBCs; 3, hemagglutination of guinea pig RBCs; 4, tracheal cell cytotoxin assay; 5, dermonecrotic toxin assay; 6, immunoblots with antibody against *B. avium* dermonecrotic toxin; 7, adenylate cyclase assay; 8, CHO cell assay; 9, egg passage.

[c] NT, Not tested for virulence for turkey poults. *B. pertussis* BB114 is virulent for humans, while *B. pertussis* Tohama III is avirulent for humans.

while *B. avium* strains were grown in modified Stainer-Scholte broth supplemented with 0.2% 2-ketoglutarate, 0.2% pyruvate, pantothenate (10 μg/ml), and L-phenylalanine (20 μg/ml; SSM-S).

Reagents and media. Dermonecrotic toxin antibody was prepared by injection of partially purified dermonecrotic toxin into New Zealand White rabbits (43). This antibody preparation neutralized dermonecrotic toxin from *B. avium* sonicates (43). Goat pertussis toxin antibody and goat filamentous hemagglutinin antibody were kindly provided by James Cowell (48). Bordet-Gengou agar and BHI agar were obtained from Difco Laboratories, Detroit, Mich. Sheep blood was obtained from Brown Laboratory, Topeka, Kans., while guinea pig blood and goose blood were obtained from Carolina Biological Supply Co., Burlington, N.C. Calmodulin (from bovine brain; activity, >40,000 U/mg), creatine phosphokinase (type I, from rabbit muscle, 100 to 150 U/mg), phosphocreatine (synthetic, Sigma grade 98 to 100%), lysozyme (grade I, from chicken egg white), deoxyribonuclease I (from bovine pancreas, 400 to 600 Kunitz units/mg), glutaraldehyde (grade II), DL-dithiothreitol, 4-chloro-1-naphthol, rabbit anti-goat immunoglobulin G (whole molecule) peroxidase conjugate, and Eagle minimal essential medium were obtained from Sigma Chemical Co., St. Louis, Mo. Fetal bovine serum was obtained from GIBCO Laboratories, Grand Island, N.Y. Horseradish peroxidase-conjugated, affinity-purified goat anti-rabbit immunoglobulin G antibody (heavy and light chains) was obtained from ICN Immunobiologicals, Lisle, Ill. [^{32}P]dATP (triethylammonium salt, >400 Ci/mmol) was from Amersham Corp., Arlington Heights, Ill. Nitrocellulose filter disks (82 mm, 0.45-μm pores, type BA85) were from Schleicher & Schuell, Keene, N.H. Nalgene syringe filters (0.45-μm pores, cellulose acetate) from Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y., were used for filtering culture supernatant fluid and cell homogenates.

Animals. Fertile Nicholas turkey eggs were obtained from Cargill, Inc., Elgin, Mo., or Cuddy Hatchery, Aurora, Mo. The eggs were incubated in a model no. 21 Humidaire incubator and were candled after 7 days to check for viability. After 8 days, the embryonated eggs were used for passage of *B. avium* isolates or hatched to obtain turkey poults for dermonecrotic toxin assays. Fertile White Leghorn chicken eggs were obtained from SPAFAS, Inc., Roanoke, Ill., and incubated in a Humidaire incubator until they hatched. BALB/c mice and White Hartley guinea pigs were obtained from SASCO, Inc., Omaha, Nebr.

Egg passage. Egg passages of *B. avium* isolates F84-0069.b, 197, 838, 4671, and *B. avium*-like isolates 008 and 031 were performed as described previously (9). Briefly, *B. avium* and *B. avium*-like isolates were grown to late-log phase, pelleted, and suspended in an equal volume of phosphate-buffered saline. Embryonated eggs (8 days old) were inoculated with 0.25 ml of the bacterial suspension into the allantoic cavity. Following 24 h of incubation, the egg yolks were harvested, plated on Bordet-Gengou medium containing 10% sheep blood, and incubated at 37°C overnight to obtain egg-passaged bacterial cultures.

Adenylate cyclase. Conversion of [^{32}P]dATP to [^{32}P]deoxycyclic AMP (d-cAMP) was used to measure the adenylate

cyclase activity of *B. avium* isolates 197, 838, and 4671 by using the method of Hewlett et al. (20). Cells were scraped from Bordet-Gengou plates (supplemented with 10% sheep blood), suspended in phosphate-buffered saline (pH 7.5) to contain 3.5×10^7 to 0.75×10^8 cells, and mixed with [^{32}P]dATP (1×10^6 cpm), calmodulin, and a regenerating system (creatine phosphate and creatine phosphokinase). [^{32}P]d-cAMP was separated from [^{32}P]dATP by ion-exchange chromatography, and the radioactivity was measured with an LS-3150 T scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). *B. pertussis* strains BB114 and Tohama III served as positive and negative controls, respectively. *B. avium* isolates 197, 838, and 4671 were passaged through 8-day-old embryonated turkey eggs prior to assay, as described above.

CHO cell assay for pertussis toxin. Chinese hamster ovary (CHO) cell assays were performed as described previously (21). Briefly, 250 μl of a CHO cell suspension (containing 5×10^3 cells per ml in Eagle modified essential medium with 1% fetal bovine serum) was added to each well of a 96-well, flat-bottom microtiter plate. After a stabilization period of 48 h, CHO cells were exposed to 25 μl of filtered culture supernatant fluid from late-log-phase *B. avium* isolates F84-0069.b, 197, 838, and 4671, *B. avium*-like isolates 008 and 031, and *B. pertussis* strains BB114 and Tohama III grown in SSM-S and SSM broth, respectively. Following incubation for 24 h, the CHO cells were air dried, fixed with methanol, and stained with Giemsa stain for 20 min. The cells were observed for the clustered morphology associated with the presence of pertussis toxin.

Dermonecrotic toxin assay. Cell homogenates of *B. avium* isolates F84-0069.b, 197, 838, 4671, 85-105.2 and *B. avium*-like isolates 008 and 031 were prepared by the method of Cowell et al. for assay of necrotic activity in guinea pig skin (10). Cell homogenates of *B. pertussis* strains BB114 and Tohama III served as positive and negative controls, respectively. Briefly, *B. avium* and *B. pertussis* strains were passaged twice in 25 ml of SSM-S and SSM media, respectively, prior to the skin test. The cells were grown to late-log phase (optical density at 540 nm, 1.1), pelleted, suspended in 1/12 the original volume of 10 mM Tris (pH 7.5), and exposed to lysozyme and EDTA to facilitate lysis. Cells were lysed by homogenization for 2.0 min by using 150- to 200-μm-diameter glass beads (Sigma, type III-W) in a cell homogenizer (B. Braun, Melsungen AG, Federal Republic of Germany), with a CO_2 cooling system. Deoxyribonuclease and $MgCl_2$ were added to the cell lysate, and the mixture was incubated for 15 min. Cell debris was removed by centrifugation of the cell homogenate, and the supernatant fluid was filtered through a Nalgene filter (pore size, 0.45 μm). Cell lysis was monitored by determining the titer of the cells before and after homogenization. White Hartley guinea pigs (weight, 400 g) were injected intradermally with 80-μl samples of the crude cell homogenates (from 0.5×10^9 to 1.0×10^9 lysed cells) and were observed for dermonecrosis after 24 h. Since the dermonecrotic toxin from *B. pertussis* is inactivated by heat treatment at 56°C for 10 min, *B. avium* cell homogenates were treated similarly to test for heat lability and were injected intradermally into depilated guinea pigs.

Cell homogenates were tested for lethality for BALB/c mice. *B. avium* 197 and *B. avium*-like 008 cell homogenates from 1×10^9 to 3×10^9 lysed cells were injected intraperitoneally into 4-week-old BALB/c mice. Mouse death within 24 h constituted a positive result.

Finally, cell homogenates containing 1×10^9 to 3×10^9 lysed cells of *B. avium* 197 and *B. avium*-like 008 were inoculated intradermally into 2-week-old White Leghorn chickens, 3-day-old Nicholas turkey poults (weight, 75 g), and 3-week-old Nicholas turkey poults (weight, 490 g). After 24 h, turkeys and chickens were observed for necrosis and death. In addition, the cell homogenates from *B. avium* 197 were treated with 0.5% (vol/vol) and 1% (vol/vol) Formalin at 37°C for 1 h and 4 h or incubated at 37°C for 1 h with glutaraldehyde (4 or 40 mM) or 100 μg of trypsin per ml (bovine pancreas, type XI, Sigma). Cell homogenates were also treated with dithiothreitol (final concentration, 100 mM) for 1 h at 37°C or heated at 37°C for 4 h. The treated cell homogenates were injected intradermally into 3-week-old turkey poults, and 24 h later, the poults were observed for necrotic skin lesions.

Hemagglutination assays. *B. avium* and *B. avium*-like isolates (Table 1) were examined for the ability to agglutinate goose and guinea pig erythrocytes (RBCs). *B. avium* and *B. avium*-like isolates were grown on either BHI agar, Bordet-Gengou agar containing 10% sheep blood, or colonization factor antigen agar (14) or were passaged twice in SSM-S medium prior to the hemagglutination assay. *B. pertussis* BB114, which served as a positive control for the goose blood agglutination assay, was passaged twice in SSM medium prior to the assay. In addition, *B. avium* isolates 197, 838, 4671 and *B. avium*-like 008 were passaged through embryonated eggs and plated on Bordet-Gengou agar containing 10% sheep blood prior to the assay. *B. avium* and *B. pertussis* cells were scraped from agar plates and suspended in phosphate-buffered saline (pH 7.5). A 50-μl sample of the bacterial cell suspension was mixed with 50 μl of the RBC suspension in a 96-well, V-bottom microtiter plate, incubated overnight at either 4 or 25°C, and observed for hemagglutination. RBC suspensions were prepared by washing the RBCs in phosphate-buffered saline (pH 7.5) and suspending them to a final concentration of 1.5% (goose RBC) or 3% (guinea pig RBC) in phosphate-buffered saline.

Tracheal cytotoxin. Tracheal cytotoxin was purified from *B. avium*-like 008, *B. avium* 197, and *B. pertussis* BB114. Culture supernatant fluids were first concentrated by reversed-phase and ion-exchange solid-phase extractions. The extracts were then subjected to reversed-phase high-pressure liquid chromatography (HPLC) with a C_8 column (Rainin Instrument Co., Inc., Woburn, Mass.) eluted with a triethylamine- or trifluoroacetic acid-containing solvent system. Details of this method will be described elsewhere (B. T. Cookson and W. E. Goldman, manuscript in preparation).

Colony immunoblots. *B. avium* isolates (Table 1) were examined for the ability to cross-react with antibodies to pertussis toxin and filamentous hemagglutinin of *B. pertussis* by using the procedure of Galan and Timoney (15). Cells were scraped from Bordet-Gengou agar plates containing 10% sheep blood and suspended in 50 μl of phosphate-buffered saline (pH 7.5), and 3-μl samples were spotted onto nitrocellulose filter disks. The filters were exposed for 20 min to chloroform fumes, washed with blocking buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin [wt/vol], 0.05% Triton X-100 [vol/vol], pH 7.4), and incubated with goat antibody to pertussis toxin or filamentous hemagglutinin for 2 h at room temperature. The filter was then incubated with affinity-purified rabbit anti-goat immunoglobulin G peroxidase conjugate for 3 h and developed with 4-chloro-1-naphthol.

Western immunoblots (immunoblots). Whole cells of *B. avium* 197, *B. avium*-like 008, *B. pertussis* BB114, *B. per-*

tussis Tohama III, and *Bordetella bronchiseptica* 40-81 were denatured by boiling in sample buffer and were electrophoresed through sodium dodecyl sulfate–7.5% polyacrylamide gels, and proteins were transferred to nitrocellulose by using established methods (55). Nitrocellulose filters were incubated with rabbit antibody against *B. avium* dermonecrotic toxin, washed with blocking buffer (described above), incubated with horseradish peroxidase-conjugated, affinity-purified goat anti-rabbit immunoglobulin G antibody, and developed as described in the previous section.

Regulation of production of dermonecrotic toxin. *B. avium* cultures were grown overnight at 37°C in SSM-S medium supplemented with 100 μM $FeSO_4$ and containing either 500 μg of nicotinamide per ml or 500 μg of nicotinic acid per ml. In addition, *B. avium* 197 was grown at 37°C in SSM-S medium which lacked NaCl, was supplemented with 20 mM $MgSO_4$, and contained either 500 μg of nicotinic acid per ml or 500 μg of nicotinamide per ml. Finally, *B. avium* 197 was grown at 25, 37, or 42°C in SSM-S medium containing either 500 μg of nicotinamide per ml or 500 μg of nicotinic acid per ml. All overnight cultures of *B. avium* 197 were grown to stationary phase (optical density at 540 nm, 1.5), and the titers were determined. Each culture (100 μl, containing between 5×10^8 and 10×10^8 cells) was denatured by boiling in sample buffer, electrophoresed through a sodium dodecyl sulfate-7.5% polyacrylamide gel, and transferred to a nitrocellulose filter. The nitrocellulose filter was incubated with rabbit antibody against *B. avium* dermonecrotic toxin, reacted with goat anti-rabbit peroxidase conjugate, and developed with 4-chloro-1-naphthol as described above.

RESULTS

Dermonecrotic toxin assay. *B. avium* isolates were tested for dermonecrotic toxin by intradermal injection of cell homogenates containing 0.5×10^9 to 1×10^9 lysed cells into depilated guinea pigs. Cell homogenates from virulent, late-log-phase *B. avium* produced a nonulcerating dermonecrosis in guinea pigs (Fig. 1) and caused death of guinea pigs within 16 h following injection. Dermonecrotic lesions produced by cell homogenates of virulent *B. avium* isolates F84-0069.b, 197, 838, 4671, and 85-105.2 were identical to lesions produced by cell homogenates of *B. pertussis* BB114. Cell homogenates of avirulent *B. avium*-like isolates 008 and 031 produced minimal necrosis when injected into guinea pigs, comparable to the level of dermonecrosis from injection of cell homogenates of *B. pertussis* Tohama III. Dermonecrosis was also obtained when the cell homogenates of virulent *B. avium* 197 containing 1×10^9 to 3×10^9 lysed cells were injected intradermally into turkey and chicken poults, while injection of *B. avium*-like 008 cell homogenates had no effect. The dermonecrotic lesion produced by injection of cell homogenates into turkeys and chickens was much more extensive and comprised an area two to five times larger than the lesions produced in guinea pigs.

Since dermonecrotic toxin of *B. pertussis* causes lethality in mice (34), BALB/c mice were injected intraperitoneally with 100 μl of 1×10^9 to 3×10^9 homogenized *B. avium* or *B. avium*-like cells to confirm the presence of a mouse lethal toxin. Mice injected intraperitoneally with cell homogenates from virulent *B. avium* 197 died within 24 h postinjection, while mice injected with cell homogenates of *B. avium*-like 008 survived with no ill effects.

Intradermal injection of cell homogenates from virulent *B. avium* 197 was lethal for 3-day-old turkey poults and 2-week-old chickens. However, injection of an equivalent amount of the *B. avium* 197 cell homogenate into 3-week-old turkey poults did not result in poult death, indicating that the weight or age of the bird was important in determining the susceptibility to the lethal effects of the toxin.

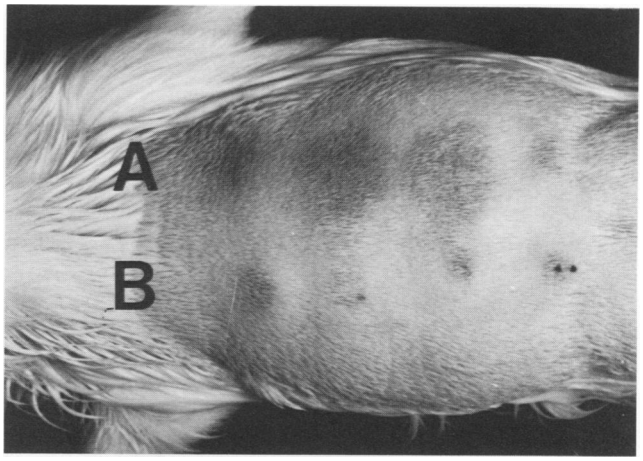

FIG. 1. Photograph of guinea pig skin 24 h after intradermal injection with cell homogenates from *B. avium* and *B. avium*-like isolates and *B. pertussis* BB114. Row A compares dermonecrosis obtained after injection of 100 μl of 0.5×10^9 to 1×10^9 lysed cells of *B. pertussis* BB114, *B. avium* 197, *B. avium* 4671, and *B. avium*-like 008, respectively (from left to right). Row B indicates inactivation of the dermonecrotic toxin following incubation at 56°C for 15 min. The order of injections in row B is identical to the order of injections in row A.

The dermonecrosis and lethality caused by cell homogenates of *B. avium* isolates 197 and 4671 were eliminated by heating the cell homogenates for 15 min at 56°C (Fig. 1). Treatment of *B. pertussis* cell homogenates for 15 min at 56°C resulted in only partial elimination of dermonecrosis (Fig. 1, row B, first sample). However, there was a substantial reduction of dermonecrosis with this sample, and residual dermonecrosis may have been due to a greater quantity of dermonecrotic toxin in *B. pertussis* cell homogenates as compared with that in *B. avium* cell homogenates. Dermonecrosis was also eliminated by treating the cell homogenates with either trypsin at a final concentration of 100 μg/ml or 40 mM glutaraldehyde for 1 h at 37°C or with 1% Formalin for 4 h at 37°C. Treatment of *B. avium* 197 cell homogenates with 0.5 or 1% Formalin for 1 h at 37°C, incubation with 100 mM dithiothreitol for 1 h, and heat treatment at 37°C for 4 h did not eliminate the dermonecrotic activity. Furthermore, unconcentrated filtered culture supernatant fluid from *B. avium* 197 failed to produce dermonecrosis when injected into turkeys or guinea pigs.

Characterization of dermonecrotic toxin. Proteins from whole *B. avium* 197 cells were subjected to Western blot analysis by using antibody which neutralizes *B. avium* dermonecrotic toxin. Immunoblots revealed the presence of a protein with a molecular mass of 155,000 daltons in whole *B. avium* 197 cells (Fig. 2). To determine whether the dermonecrotic toxin of *B. avium* is immunologically cross-reactive with dermonecrotic toxins described for other *Bordetella* species, whole cells from *B. pertussis* BB114, *B. pertussis* Tohama III, *B. bronchiseptica* 469, and *B. avium*-like 008 were subjected to Western blot analysis by using antibody against *B. avium* dermonecrotic toxin (Fig. 2). *B. pertussis* BB114 contained a major cross-reactive protein with a molecular weight of 76,000, while *B. bronchiseptica* 469, *B. avium*-like 008, and *B. pertussis* Tohama III did not

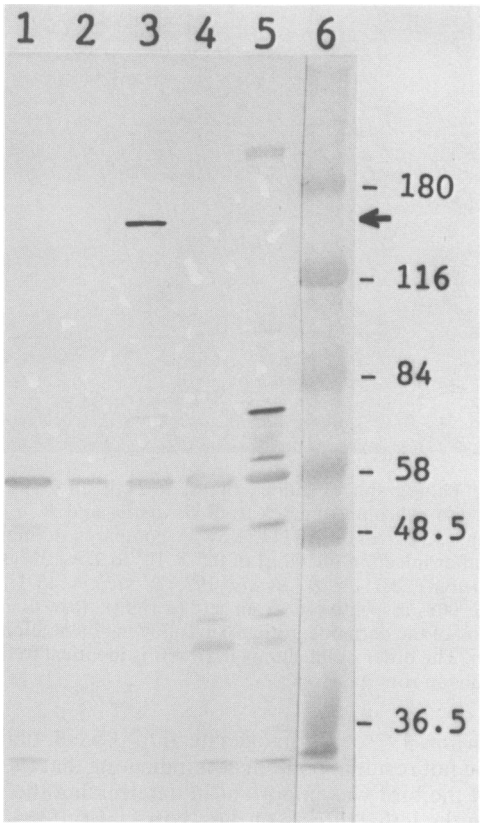

FIG. 2. Immunoblot of proteins from *Bordetella* species reacted with polyclonal antibody against *B. avium* dermonecrotic toxin. Whole cells were solubilized by boiling in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the following order. Lanes: 1, *B. bronchiseptica* 469; 2, *B. avium*-like 008; 3, *B. avium* 197; 4, *B. pertussis* Tohama III; 5, *B. pertussis* BB114; 6, prestained molecular weight markers (SDS-7B; Sigma).

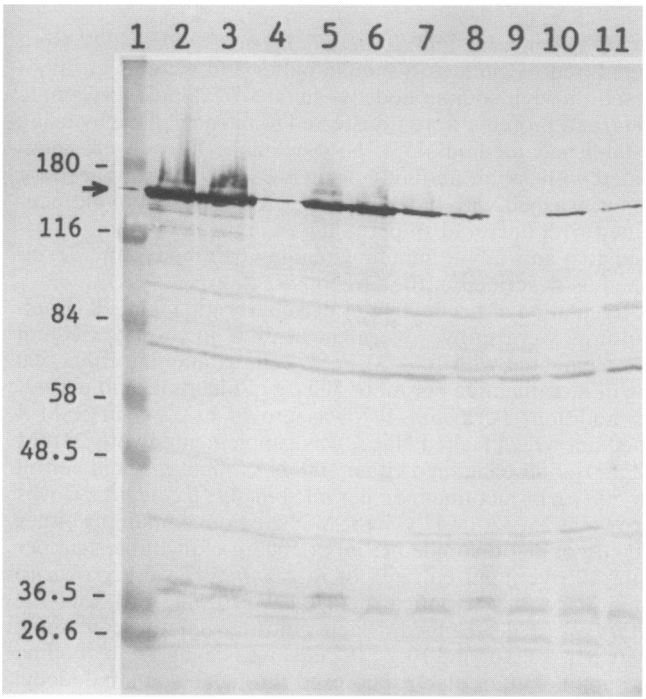

FIG. 3. Immunoblot analysis of the regulation of *B. avium* dermonecrotic toxin production by growth conditions with antibody against *B. avium* dermonecrotic toxin. *B. avium* 197 was grown at 37°C in SSM-S medium containing 500 μg of nicotinamide per ml (lane 2), 500 μg of nicotinamide per ml and 100 μM $FeSO_4$ (lane 3), or 500 μg of nicotinamide per ml and 20 mM $MgSO_4$ and lacking NaCl (lane 4). *B. avium* 197 cells were also grown at 25°C in SSM-S medium supplemented with 500 μg of nicotinamide per ml (lane 5) or at 42°C in SSM-S medium supplemented with 500 μg of nicotinamide per ml (lane 6). Lanes 7 through 11 contain *B. avium* 197 cells grown under identical conditions as specified for lanes 2 through 6, respectively, except that nicotinic acid (500 μg/ml) was substituted for nicotinamide. Lane 1 contains prestained molecular weight markers (SDS-7B; Sigma).

contain proteins which cross-reacted with significant intensity with the antiserum against dermonecrotic toxin. All *Bordetella* species were found to produce a 58,000-molecular-weight protein which cross-reacted with dermonecrotic toxin antibody. However, the 58,000-molecular-weight protein was also present in *Escherichia coli* cells (data not shown), suggesting that the 58,000-molecular-weight protein was a common bacterial protein and was unrelated to dermonecrotic toxin. Therefore, cross-reactivity of dermonecrotic toxin antibody with the 58,000-molecular-weight protein probably represented nonspecific, naturally acquired, cross-reactive antibody in the rabbit antiserum.

Regulation of dermonecrotic toxin production by *B. avium*. Previous investigators have studied the effect of different growth media or reduced growth temperatures on dermonecrotic toxin production by *B. pertussis* (32, 40, 49). Similarly, dermonecrotic toxin production by *B. avium* 197 was monitored by immunoblot analysis of *B. avium* cells grown under various conditions (Fig. 3) to determine whether regulation of toxin production in *B. avium* mimics regulation of *B. pertussis* dermonecrotic toxin production. Nicotinamide and nicotinic acid equally increased the growth rate of *B. avium* 197 but did not eliminate production of the dermonecrotic toxin. Growth with 500 μg of nicotinic acid per ml (Fig. 3, lane 7) caused a slight decrease in production of dermonecrotic toxin as compared with that when cells were grown with 500 μg of nicotinamide per ml (lane 2). Production of dermonecrotic toxin by *B. avium* 197 was significantly reduced by growth in medium supplemented with 20 mM $MgSO_4$ and lacking NaCl, as evidenced by reduced immunological cross-reactivity of cell homogenates with specific dermonecrotic toxin antibody in immunoblots (Fig. 3, lane 4). The addition of $FeSO_4$ (final concentration, 100 μM) to the growth medium did not significantly alter toxin production, and growth of *B. avium* 197 at 25, 37, or 42°C produced comparable results and did not affect toxin production.

Tracheal cytotoxin assay. Culture supernatant fluids from *B. avium* and *B. avium*-like isolates were analyzed for the presence of a glycopeptide similar to the tracheal cytotoxin (TCT) produced by *B. pertussis* and *B. bronchiseptica* (8, 17, 45). HPLC analysis revealed the presence of a molecule in *B. avium* 197 and *B. avium*-like 008 culture supernatant fluids which was chemically indistinguishable from the tracheal cytotoxin produced by *B. pertussis* BB114. This was confirmed by identical retention times in two different reversed-phase HPLC systems (triethylamine and trifluoroacetic acid-buffered mobile phases); data for one of these analyses are shown in Fig. 4. Fast-atom bombardment mass spectrometry of this apparent TCT peak (A. Tyler, Department of Biological Chemistry, Washington University) confirmed its identity as an anhydropeptidoglycan monomer with a mass of 921

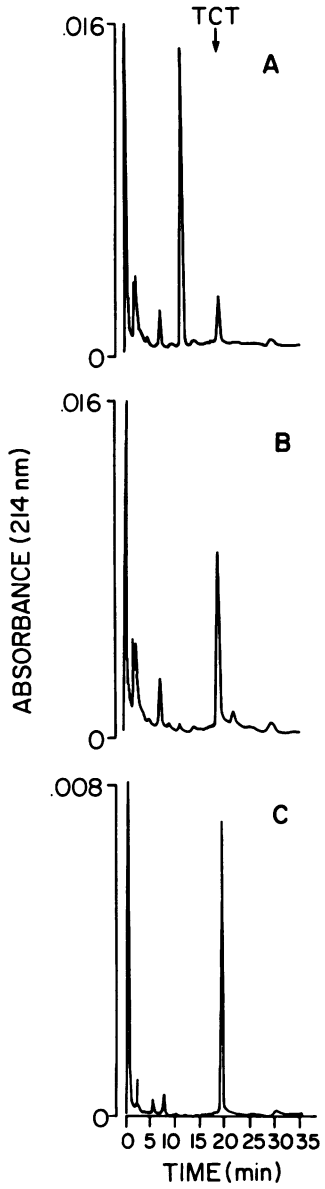

FIG. 4. HPLC analysis for tracheal cytotoxin. Each panel displays elution profiles from reversed-phase HPLC of bacterial culture supernatants by using a trifluoroacetic acid-buffered mobile phase and an acetonitrile gradient. Experimental samples from *B. avium*-like 008 (A), *B. avium* 197 (B), and *B. pertussis* BB114 (C) demonstrate the presence of tracheal cytotoxin with its characteristic retention time.

daltons, identical to *B. pertussis* TCT (B. T. Cookson, A. N. Tyler, and W. E. Goldman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, B70, p. 41). Although it appears that far less TCT is produced by *B. avium*-like 008 (compared with that of *B. avium* 197), we caution that optimized conditions for maximal TCT production have not been established for individual *B. avium* strains.

Adenylate cyclase assay. Although *B. pertussis* BB114 readily converted [^{32}P]dATP to d-cAMP, *B. avium* 197 and *B. pertussis* Tohama III failed to convert labeled dATP to d-cAMP (Table 2). The results were not altered by inclusion of an ATP-regenerating system (data not shown) or by substitution of [^{32}P]dATP for [^{32}P]ATP. However, since

TABLE 2. Adenylate cyclase assay

Strain	Virulence[a]	Adenylate cyclase activity (cpm)[b]	
		From cells grown on BHI agar	Following egg passage
B. pertussis BB114	+	689,000	520,000
B. pertussis Tohama III	−	660	990
B. avium 197	+	Not tested	630
B. avium 838	+	430	890
B. avium 4671	+	350	640

[a] Virulence for natural host species.

[b] Adenylate cyclase activity was measured by conversion of [^{32}P]dATP to [^{32}P]d-cAMP as described in Materials and Methods. The amount of [^{32}P]d-cAMP generated in the reaction mixture is measured as counts per minute per 3.5×10^7 to 0.75×10^8 cells.

duplicate assays were performed with [^{32}P]dATP as the substrate, only these results are shown in Table 2. To examine the possibility that the adenylate cyclase of *B. avium* might be induced by environmental conditions, *B. avium* isolates were passaged through 8-day-old embryonated eggs and assayed again for extracytoplasmic adenylate cyclase. Egg passage had no effect on the assay, i.e., egg-passaged *B. avium* did not produce detectable extracytoplasmic adenylate cyclase (Table 2).

Pertussis toxin assay. Since pertussis toxin is an important virulence factor for *B. pertussis* (34, 39, 58), *B. avium* and *B. avium*-like isolates were examined for pertussis toxin by using the CHO cell-clustering assay, colony immunoblot assay, and the ability to hemagglutinate goose RBCs, all of which are properties associated with pertussis toxin of *B. pertussis* (25, 48). CHO cells exposed to *B. avium* culture supernatant fluid did not exhibit the clustering effect induced by *B. pertussis* culture supernatant fluid which contained pertussis toxin (Fig. 5). *B. avium* isolates were unable to hemagglutinate goose RBCs under any of the conditions tested, i.e., growth medium, egg passage, or assay temperature did not alter the outcome of the hemagglutination reactions. To determine whether *B. avium* produced a protein which cross-reacted with pertussis toxin from *B. pertussis* but lacked hemagglutinating activity, *B. avium* isolates were reacted with pertussis toxin antibody (Fig. 6) in colony immunoblot assays. Although *B. pertussis* reacted intensely with the pertussis toxin antibody, the *B. avium* isolates failed to cross-react. It is possible, however, that the sensitivity of the colony immunoblot assay is too low to detect cross-reactive protein in strains which produce very low levels of the protein in vitro.

Filamentous hemagglutinin assay. The filamentous hemagglutinin of *B. pertussis* is responsible for adherence of *B. pertussis* to human ciliated tracheal epithelial cells (for reviews, see references 34 and 58) and agglutinates goose RBCs (25, 48). Goose RBCs were not agglutinated by *B. avium* isolates which were grown on BHI agar, Bordet-Gengou agar containing 10% sheep blood, or colonization factor antigen agar or which were egg passaged. In addition to the hemagglutination assays, colony immunoblots were performed by using specific polyclonal antibody (provided by J. Cowell) against *B. pertussis* filamentous hemagglutinin (Fig. 6) to determine whether *B. avium* produces a protein analogous to the filamentous hemagglutinin of *B. pertussis*. As expected, *B. pertussis* reacted intensely with the antiserum for *B. pertussis* filamentous hemagglutinin. However, there was no cross-reactivity between the *B. avium* isolates

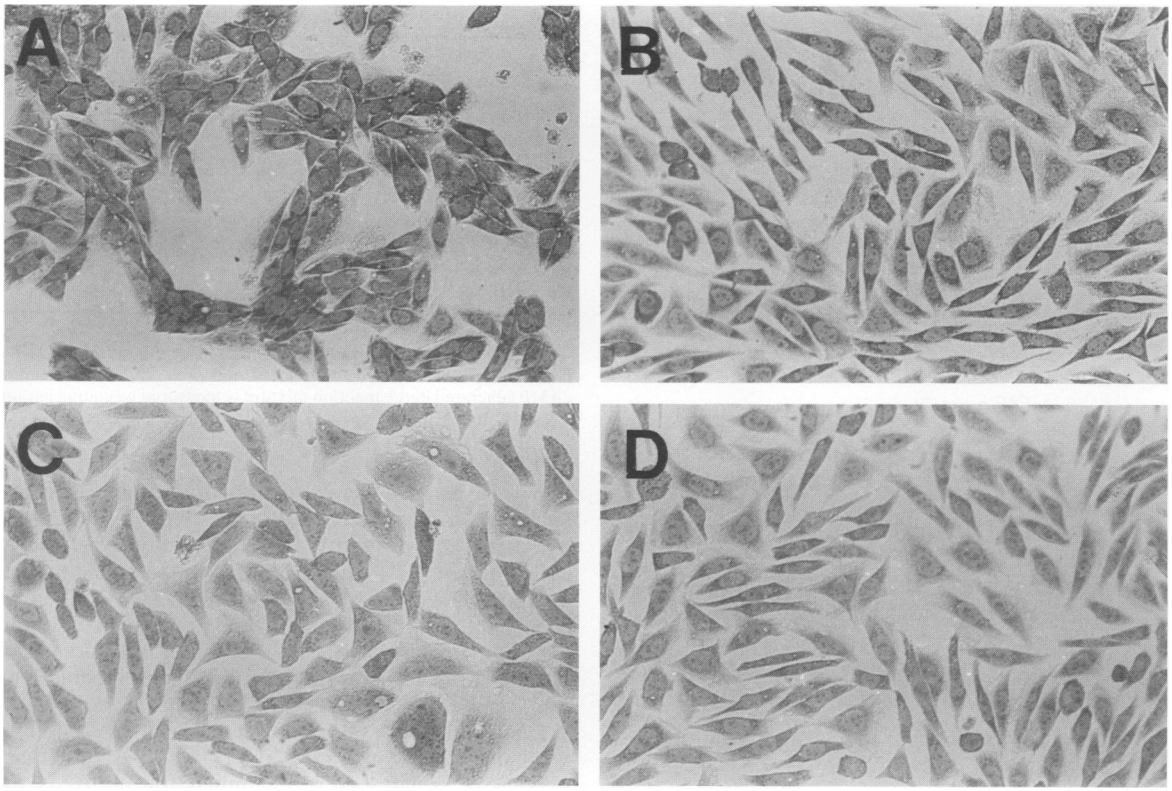

FIG. 5. Photomicrograph of CHO cells exposed to culture supernatant fluid from *B. avium* isolates or *B. pertussis* strains. CHO cells were exposed for 24 h to 25 μl of culture supernatant fluid from *B. pertussis* BB114 (A), *B. pertussis* Tohama III (B), *B. avium* 197 (C), and *B. avium* 838 (D).

and the filamentous hemagglutinin antibody. As mentioned above, this assay may not detect strains that produce low levels of protein which cross-reacts with the antibody.

Hemagglutination of guinea pig RBCs. *B. avium* was tested for the ability to agglutinate guinea pig RBCs since agglutination of guinea pig RBCs has been reported to be associated with virulence for turkey poults (28). All virulent *B. avium* isolates tested were positive for agglutination of guinea pig RBCs, while avirulent *B. avium*-like isolates failed to agglutinate guinea pig RBCs. Virulent *B. avium* isolates grown at 25 or at 37°C on BHI agar or Bordet-Gengou agar containing 10% sheep blood or without egg passage still agglutinated guinea pig RBCs.

DISCUSSION

B. avium produces a dermonecrotic toxin and a tracheal cytotoxin, two virulence factors common to all *Bordetella* species. Virulent *B. avium* isolates produced a cell-associated, 155,000-molecular-weight protein which was similar in biological activity to the dermonecrotic toxin of *B. pertussis* and *B. bronchiseptica*. The dermonecrotic toxin of *B. avium* was heat labile and trypsin, formaldehyde, and glutaraldehyde sensitive, and it produced nonulcerating, dermonecrotic lesions in guinea pigs and turkeys which were identical to lesions produced by *B. bronchiseptica* and *B. pertussis*. These results are similar to those obtained by Rimler and Rhoades (41, 42, 43). *B. bronchiseptica*, *B. pertussis*, and *Bordetella parapertussis* dermonecrotic toxins are reported to be serologically related (30), and molecular weights of 102,000 (36) and 190,000 (30) for the dermonecrotic toxin of *B. bronchiseptica* have been reported by different investigators. In addition, treatment of the 190,000-molecular-weight dermonecrotic toxin of *B. bronchiseptica* with trypsin, dithiothreitol, and urea results in the formation of two peptides with molecular weights of approximately 118,000 and 75,000 (30). Antibody against the dermonecrotic toxin of *B. avium* cross-reacted intensely with a 76,000-molecular-weight protein of *B. pertussis*. These results suggest that the dermonecrotic toxin of *B. avium* and *B. pertussis* share a common antigenic determinant(s). However, antibody against the dermonecrotic toxin of *B. avium* failed to cross-react with *B. bronchiseptica* 469. In addition, of four *B. bronchiseptica* isolates tested, only one isolate (*B. bronchiseptica* 40-81) was found to produce a 190,000-molecular-weight protein which cross-reacted with antibody against the *B. avium* dermonecrotic toxin (data not shown), and this isolate had been isolated from the trachea of a turkey (29). Lack of cross-reactivity between *B. avium* and *B. bronchiseptica* dermonecrotic toxins is supported by reports that antibody against *B. avium* dermonecrotic toxin is unable to neutralize the dermonecrotic toxin of one *B. bronchiseptica* isolate (43). Further assays must be performed in order to determine the relatedness between the *B. avium* and *B. bronchiseptica* dermonecrotic toxins.

Growth of *B. pertussis* in growth medium supplemented with different ions and nutrients or incubated at lower temperatures (25°C) results in a reversible phenotypic change, designated antigenic modulation, which is characterized by a decrease in virulence (31). There have been numerous reports concerning the regulation of virulence factors in *B. pertussis* (31; for reviews, see references 44 and 57); however, the effects of nicotinamide and nicotinic acid

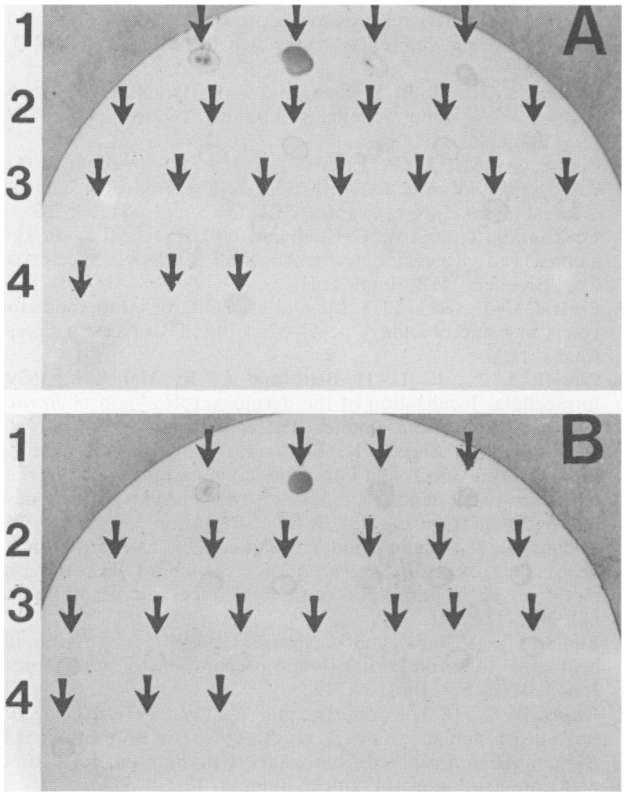

FIG. 6. Colony immunoblot of *B. avium* isolates reacted with antibody to *B. pertussis* filamentous hemagglutinin (A) or pertussis toxin (B). Cell suspensions were spotted, from left to right, onto the nitrocellulose filter in the following order. The first two spots in row 1 contain *B. pertussis* Tohama III and *B. pertussis* BB114, respectively; the remaining 18 spots in rows 1, 2, 3, and 4 are *B. avium* and *B. avium*-like isolates. Spots of cell suspensions are designated by arrows.

on the production of dermonecrotic toxin by *B. pertussis* are controversial (40, 49). Production of dermonecrotic toxin by *B. avium* was slightly suppressed but not eliminated by growth in nicotinic acid, while growth with nicotinamide at 37°C was optimum for dermonecrotic toxin production. These results parallel those of Schneider and Parker (49), who reported that *B. pertussis* cells grown in nicotinic acid produce very low levels of dermonecrotic toxin activity. In analogy with growth of *B. pertussis* (32), growth of *B. avium* in $MgSO_4$ resulted in a substantial decrease in dermonecrotic toxin production, while addition of $FeSO_4$ to the growth medium (57) did not alter dermonecrotic toxin production. These results indicate that regulation of dermonecrotic toxin in *B. avium* is similar to regulation of dermonecrotic toxin in *B. pertussis*. The effect of reduced growth temperature on the production of *B. pertussis* dermonecrotic toxin has not been reported. Growth of *B. avium* at 25 or 42°C did not alter dermonecrotic toxin production, and the amount of dermonecrotic toxin produced at these temperatures was comparable to the amount of dermonecrotic toxin produced at 37°C.

The exact role of the dermonecrotic toxin in the pathogenicities of *B. avium* and *B. pertussis* is unknown. It has been reported that the dermonecrotic toxin of *B. pertussis* inhibits a Na^+-K^+-ATPase in in vitro assays (36) and causes contraction of the smooth muscles surrounding peripheral blood vessels, resulting in vasoconstriction and increased perfusion pressure in perfused lungs from guinea pigs (12, 13). Furthermore, it has been proposed that the vasoconstriction induced by the toxin is responsible for the splenic atrophy of mice injected with *B. pertussis* dermonecrotic toxin (24, 36). In addition, it has been reported that the dermonecrotic toxins of *B. avium* and *B. pertussis* have no effect on the ciliary activity of cultured tracheal rings from turkeys and hamsters, respectively (13, 17, 43). However, conservation of a functional dermonecrotic toxin in all *Bordetella* species suggests that this toxin may play a role in the virulence of the organisms (for a review, see reference 30).

A tracheal cytotoxin which is chemically identical to the tracheal cytotoxin of *B. pertussis* was produced by *B. avium* isolates. Since in vitro studies with *B. pertussis* tracheal cytotoxin have demonstrated specific damage to ciliated epithelial cells (16, 17), it has been proposed that the tracheal cytotoxin of *B. pertussis* is responsible for poor mucous clearance and increased susceptibility to infection with secondary bacterial pathogens. Similarly, tracheas from *B. avium*-infected turkeys exhibit loss of ciliated tracheal epithelial cells (2, 6, 18, 38, 46, 47), and death of infected turkeys is commonly due to infections with opportunistic pathogens (4, 46) or by suffocation due to the inability to clear the airways (6, 19). The similarity of histopathology and symptoms of *Bordetella* infection in humans and birds and the existence of the tracheal cytotoxin in all *Bordetella* species (8) strengthens the hypothesis that the tracheal cytotoxin is an important virulence factor in bordetelloses. Although an avirulent *B. avium*-like isolate produced tracheal cytotoxin, so did the avirulent *B. pertussis* strain Tohama III (16); in the latter case, however, toxin delivery cannot be achieved because the organism lacks the ability to colonize the respiratory tract. Similarly, *B. avium*-like isolates can rarely be recovered from infected birds after 12 days postinfection (26; C. R. Gentry-Weeks and R. Curtiss III, unpublished observations), well before major loss of ciliated cells is observed (3). Therefore, it is likely that the *B. avium*-like isolates are cleared from the trachea before damage due to tracheal cytotoxin can occur.

Results of CHO cell assays and immunoblot assays by using specific antibody against pertussis toxin and lack of hemagglutination of goose RBCs indicated that *B. avium* does not produce a protein which functions as a pertussislike toxin and does not share epitopes with the pertussis toxin produced by *B. pertussis*. These results confirmed studies by Arico and Rappuoli which demonstrated that *B. avium* does not contain the structural gene for pertussis toxin (1). Similar results were obtained with immunoblot assays for the *B. pertussis* filamentous hemagglutinin. Virulent *B. avium* lacked cross-reactivity with antibody against *B. pertussis* filamentous hemagglutinin and, as mentioned above, failed to agglutinate goose RBCs. Although *B. avium* lacks a protein which is immunologically related to *B. pertussis* filamentous hemagglutinin, this organism does produce a hemagglutinin which specifically agglutinates guinea pig RBCs and correlates with pathogenicity of the organism for turkey poults (28; this study). Although guinea pig hemagglutinin may play a role in species specificity or colonization, it is doubtful that guinea pig hemagglutinin is functionally analogous to the filamentous hemagglutinin of *B. pertussis*, since recent data by Jackwood and Saif indicated that pili are the primary adhesins of *B. avium* (26). Furthermore, *B. avium* pili do not have hemagglutinating activity (26, 53), and therefore, it is unlikely that guinea pig hemagglutinin is involved directly in adherence.

In contrast with the other *Bordetella* species (22, 37), *B. avium* did not produce an extracytoplasmic adenylate cy-

clase when grown in vitro. Passage of *B. avium* through embryonated eggs before the assay was done did not increase the expression or activity of adenylate cyclase of *B. avium* grown in vitro. Since the other *Bordetella* species produce adenylate cyclase and cause disease in mammals and *B. avium* produces disease in birds, it is possible that in adaptation to the bird host, *B. avium* lost the genes or the ability to express adenylate cyclase.

The results of this study indicated that in analogy with *B. pertussis*, virulent *B. avium* produces two putative virulence factors, a dermonecrotic toxin and a tracheal cytotoxin. Furthermore, all species of *Bordetella* which cause disease in animals produce dermonecrotic toxin and tracheal cytotoxin. In contrast with *B. pertussis*, *B. avium* did not express proteins which are functionally or immunologically common to *B. pertussis*, such as the pertussis toxin, the filamentous hemagglutinin, or the adenylate cyclase. While *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* produce adenylate cyclase, *B. pertussis* is the sole producer of pertussis toxin. Lack of virulence factors in *B. avium* common to *B. pertussis* may merely reflect differences in physiology, receptors, and immune networks of the host species. However, since in both birds and humans, the disease process includes prolonged incubation periods, an infection localized to the upper respiratory tract, loss of tracheal epithelial cells, and life-threatening secondary infections, further investigations into the roles and biological activities of the dermonecrotic toxin and the tracheal cytotoxin are merited. It is not unreasonable to expect that evolutionarily conserved toxins are likely to be responsible for the common pathology in infections by all *Bordetella* species. Finally, in addition to the dermonecrotic toxin and tracheal cytotoxin, a heat-stable toxin produced by *B. avium* has been described which is lethal when injected intraperitoneally into mice (50). Examination of other *Bordetella* strains may reveal the existence of a similar toxin. Therefore, further study of *B. avium* virulence factors may provide insight into the pathogenicity of other *Bordetella* species.

ACKNOWLEDGMENTS

We thank James Cowell for kindly providing antibody and H. A. Berkhoff, M. S. Hofstad, K.-H. Hinz, G. H. Luginbuhl, Y. M. Saif, D. G. Simmons, J. K. Skeeles, and E. Tuomanen for providing bacterial strains. In addition, we thank Jack Diani and Dan Piatchek for excellent animal care assistance.

This work was supported by U.S. Department of Agriculture grant 86-CRSR-2-2854; Public Health Service grant R01 AI-22243 from the National Institutes of Health; Council for Tobacco Research-U.S.A., Inc., grant 1708; Public Health Service training grants T32-GM-07200 and T32-AI-07172 from the National Institutes of Health; National Research Service award 1 F32 AI07628; and a grant from Molecular Engineering Associates, Inc., Birmingham, Ala.

LITERATURE CITED

1. **Arico, B., and R. Rappuoli.** 1987. *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. **169**:2847–2853.
2. **Arp, L. H., and N. F. Cheville.** 1984. Tracheal lesions in young turkeys infected with *Bordetella avium*. Am. J. Vet. Res. **45**:2196–2200.
3. **Arp, L. H., and J. A. Fagerland.** 1987. Ultrastructural pathology of *Bordetella avium* infection in turkeys. Vet. Pathol. **24**:411–418.
4. **Barnes, H. J., and M. S. Hofstad.** 1978. Factors involved in respiratory diseases of turkeys in Iowa. J. Am. Vet. Assoc. **173**:889.
5. **Berkhoff, H. A., and G. D. Riddle.** 1984. Differentiation of *Alcaligenes*-like bacteria of avian origin and comparison with *Alcaligenes* spp. reference strains. J. Clin. Microbiol. **19**:477–481.
6. **Boycott, B. R., H. R. Wyman, and F. C. Wong.** 1984. *Alcaligenes faecalis* rhinotracheitis in Manitoba turkeys. Avian Dis. **28**:1110–1114.
7. **Collier, A. M., L. P. Peterson, and J. B. Baseman.** 1977. Pathogenesis of infection with *Bordetella pertussis* in hamster tracheal organ culture. J. Infect. Dis. **36**(Suppl.):S196–S203.
8. **Cookson, B. T., and W. E. Goldman.** 1987. Tracheal cytotoxin: a conserved virulence determinant of all *Bordetella* species. J. Cell. Biochem. **11B**(Suppl.):124.
9. **Cottral, G. E. (ed.).** 1978. Manual of standardized methods for veterinary microbiology, p. 47–52. Cornell University Press, Ithaca, N.Y.
10. **Cowell, J. L., E. L. Hewlett, and C. R. Manclark.** 1979. Intracellular localization of the dermonecrotic toxin of *Bordetella pertussis*. Infect. Immun. **25**:896–901.
11. **De Ley, J., P. Segers, K. Kersters, W. Mannheim, and A. Lievens.** 1986. Intra- and intergeneric similarities of the *Bordetella* ribosomal ribonucleic acid cistrons: proposal for a new family, *Alcaligenaceae*. Int. J. Syst. Bacteriol. **36**:405–414.
12. **Endoh, M., M. Amitani, and Y. Nakase.** 1986. Effect of purified heat-labile toxin of *Bordetella bronchiseptica* on the peripheral blood vessels in guinea pigs or suckling mice. Microbiol. Immunol. **30**:1327–1330.
13. **Endoh, M., M. Nagai, and Y. Nakase.** 1986. Effect of *Bordetella* heat-labile toxin on perfused lung preparations of guinea pigs. Jpn. J. Med. Sci. Biol. **39**:249.
14. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
15. **Galan, J., and J. F. Timoney.** 1987. Molecular analysis of the M protein of *Streptococcus equi* and cloning and expression of the M protein gene in *Escherichia coli*. Infect. Immun. **55**:3181–3187.
16. **Goldman, W. E.** 1986. *Bordetella pertussis* tracheal cytotoxin: damage to the respiratory epithelium, p. 65–69. *In* D. Schlessinger (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
17. **Goldman, W. E., D. G. Klapper, and J. B. Baseman.** 1982. Detection, isolation, and analysis of a released *Bordetella pertussis* product toxic to cultured tracheal cells. Infect. Immun. **36**:782–794.
18. **Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons.** 1981. Cytotoxic activity of pathogenic *Alcaligenes faecalis* in turkey tracheal organ cultures. Am. J. Vet. Res. **42**:2184–2186.
19. **Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons.** 1983. Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an *Alcaligenes faecalis* isolate. Infect. Immun. **42**:350–355.
20. **Hewlett, E., and J. Wolff.** 1976. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: purification and characterization. J. Bacteriol. **127**:890–898.
21. **Hewlett, E. L., K. T. Sauer, G. A. Myers, J. L. Cowell, and R. L. Guerrant.** 1983. Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. Infect. Immun. **40**:1198–1203.
22. **Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff.** 1976. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **73**:1926–1930.
23. **Hinz, K.-H., G. Glunder, and H. Luders.** 1978. Acute respiratory disease in turkey poults caused by *Bordetella bronchiseptica*-like bacteria. Vet. Rec. **103**:262–263.
24. **Iida, T. T., and T. Okonogi.** 1971. Lienotoxicity of *Bordetella pertussis* in mice. J. Med. Microbiol. **4**:51–61.
25. **Irons, L. I., and A. P. MacLennan.** 1979. Substrate specificity and purification by affinity combination methods of the two *Bordetella pertussis* hemagglutinins, p. 338–349. *In* C. R. Manclark and J. C. Hill (ed.), International symposium on pertussis. U.S. Department of Health, Education, and Welfare publication no. 79-1830. U.S. Government Printing Office, Washington,

D.C.
26. **Jackwood, M. W., and Y. M. Saif.** 1987. Pili of *Bordetella avium*: expression, characterization, and role in *in vitro* adherence. Avian Dis. **31**:277–286.
27. **Jackwood, M. W., M. Sasser, and Y. M. Saif.** 1986. Contribution to the taxonomy of the turkey coryza agent: cellular fatty acid analysis of the bacterium. Avian Dis. **30**:172–178.
28. **Jackwood, M. W., Y. M. Saif, P. D. Moorhead, and R. N. Dearth.** 1985. Further characterization of the agent causing coryza in turkeys. Avian Dis. **29**:690–705.
29. **Kersters, K., K.-H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. De Ley.** 1984. *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. Int. J. Syst. Bacteriol. **34**:56–70.
30. **Kume, K., T. Nakai, Y. Samejima, and C. Sugimoto.** 1986. Properties of dermonecrotic toxin prepared from sonic extracts of *Bordetella bronchiseptica*. Infect. Immun. **52**:370–377.
31. **Lacey, B. W.** 1960. Antigenic modulation of *Bordetella pertussis*. J. Hyg. **58**:57–93.
32. **Livey, I., R. Parton, and A. C. Wardlaw.** 1978. Loss of heat-labile toxin from *Bordetella pertussis* grown in modified Hornibrook medium. FEMS Microbiol. Lett. **3**:203–205.
33. **Mallory, F. B., and A. A. Hornor.** 1912. Pertussis: the histological lesion in the respiratory tract. J. Med. Res. **27**:115–123.
34. **Manclark, C. R., and J. L. Cowell.** 1984. Pertussis, p. 69–106. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
35. **Moore, C. J., H. MaWhinney, and P. J. Blackall.** 1987. Differentiation of *Bordetella avium* and related species by cellular fatty acid analysis. J. Clin. Microbiol. **25**:1059–1062.
36. **Nakase, Y., and M. Endoh.** 1985. *Bordetella* heat-labile toxin: further purification, characterization, and mode of action. Dev. Biol. Stand. **61**:93–102.
37. **Novotny, P., A. P. Chubb, K. Cownley, and J. A. Montaraz.** 1985. Adenylate cyclase activity of a 68,000-molecular-weight protein isolated from the outer membrane of *Bordetella bronchiseptica*. Infect. Immun. **50**:199–206.
38. **Panigraphy, B., L. C. Grumbles, R. J. Terry, D. L. Millar, and C. F. Hall.** 1981. Bacterial coryza in turkeys in Texas. Poultry Sci. **60**:107–113.
39. **Pittman, M.** 1979. Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough: a hypothesis. Rev. Infect. Dis. **1**:401–412.
40. **Pusztai, Z., and I. Joo.** 1967. Influence of nicotinic acid on the antigenic structure of *Bordetella pertussis*. Ann. Immunol. Hung. **10**:63–67.
41. **Rhoades, K. R., and R. B. Rimler.** 1987. The effects of heat-labile *Bordetella avium* toxin on turkey poults. Avian Dis. **31**:345–350.
42. **Rimler, R. B.** 1985. Turkey coryza: toxin production by *Bordetella avium*. Avian Dis. **29**:1043–1046.
43. **Rimler, R. B., and K. R. Rhoades.** 1986. Turkey coryza: selected tests for detection and neutralization of *Bordetella avium* heat-labile toxin. Avian Dis. **30**:808–812.
44. **Robinson, A., A. R. Gorringe, and C. W. Keevil.** Expression of virulence determinants in *Bordetella pertussis* and *Neisseria gonorrhoeae*, p. 22–37. *In* A. C. R. Dean, D. C. Ellwood, and C. G. T. Evans (ed.), Continuous culture 8: biotechnology, medicine, and the environment. Ellis Horwood, Ltd., Chichester, England.
45. **Rosenthal, R. S., W. Nogami, B. T. Cookson, W. E. Goldman, and W. J. Folkening.** 1987. Major fragment of soluble peptidoglycan released from *Bordetella pertussis* is tracheal cytotoxin. Infect. Immun. **55**:2117–2120.
46. **Saif, Y. M., and P. D. Moorhead.** 1981. Scanning electron microscopy of tracheas from turkey poults infected with *Alcaligenes faecalis*. Avian Dis. **25**:730–735.
47. **Saif, Y. M., P. D. Moorhead, R. N. Dearth, and D. J. Jackwood.** 1980. Observations on *Alcaligenes faecalis* infection in turkeys. Avian Dis. **24**:665–684.
48. **Sato, Y., J. L. Cowell, H. Sato, D. C. Burstyn, and C. R. Manclark.** 1983. Separation and purification of the hemagglutinins from *Bordetella pertussis*. Infect. Immun. **41**:313–320.
49. **Schneider, D. R., and C. D. Parker.** 1982. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. Infect. Immun. **38**:548–553.
50. **Simmons, D. G., C. Dees, and L. P. Rose.** 1986. A heat-stable toxin isolated from the turkey coryza agent, *Bordetella avium*. Avian Dis. **30**:761–765.
51. **Simmons, D. G., and J. G. Gray.** 1979. Transmission of acute respiratory disease (rhinotracheitis) of turkeys. Avian Dis. **23**:132–138.
52. **Simmons, D. G., J. G. Gray, L. P. Rose, R. C. Dillman, and S. E. Miller.** 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. Avian Dis. **23**:194–203.
53. **Simmons, D. G., L. P. Rose, K. A. Brogden, and R. B. Rimler.** 1984. Partial characterization of the hemagglutinin of *Alcaligenes faecalis*. Avian Dis. **28**:700–709.
54. **Slavik, M. F., J. K. Skeeles, J. N. Beasley, G. C. Harris, P. Roblee, and D. Hellwig.** 1981. Effect of humidity on infection of turkeys with *Alcaligenes faecalis*. Avian Dis. **25**:937–942.
55. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
56. **Tuomanen, E. I., and J. O. Hendley.** 1983. Adherence of *Bordetella pertussis* to human respiratory epithelial cells. J. Infect. Dis. **148**:125–130.
57. **Wardlaw, A. C., and R. Parton.** 1983. *Bordetella pertussis* toxins. Pharmacol. & Ther. **19**:1–53.
58. **Weiss, A. A., and E. L. Hewlett.** 1986. Virulence factors of *Bordetella pertussis*. Annu. Rev. Microbiol. **40**:661–686.