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The *Bordetella avium BAV1965-1962* Fimbrial Locus Is Regulated by Temperature and Produces Fimbriae Involved in Adherence to Turkey Tracheal Tissue▽

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***Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* cause respiratory tract disease in mammals, whereas *Bordetella avium* causes respiratory tract disease in avian hosts. While there are striking similarities between the diseases caused by the mammalian- and avian-adapted bordetellae, differences at the genetic level may account for their different host tropisms. Bacterial pathogens utilize the chaperone-usher pathway to assemble extracellular multisubunit structures (fimbriae) that play a role in virulence. Fimbriae of the mammalian bordetellae mediate attachment to the host respiratory epithelium. They are assembled by a single chaperone/usher system encoded by the fimbrial biogenesis operon *fimA-D*. *B. avium* contains a homologous fimbrial operon (*BAV1965-1962*), and we report here the functionality of this locus. Reverse transcription (RT)-PCR and quantitative PCR analyses demonstrated that transcription of the locus is regulated by temperature. By immuno-transmission electron microscopy (TEM), BAV1965-containing fimbriae were observed on bacteria grown at 37°C but not those grown at 22°C. A mutant in which *BAV1965-1962* was deleted displayed significantly lower levels of adherence to turkey tracheal rings than the wild type. Thus, the *BAV1965-1962* fimbrial locus is functional, its expression is regulated in response to temperature, and it produces fimbriae involved in adherence to host respiratory tract tissue.**

Bordetella avium is the etiological agent of bordetellosis, a highly contagious upper respiratory tract disease of avian species. *B. avium* infects both wild and domesticated birds, but commercially raised turkeys are particularly susceptible (8, 18). In turkeys, bordetellosis is characterized by an onset of sneezing, accompanied by oculonasal discharge, mouth breathing, submandibular edema, altered voice, tracheal collapse, and stunted growth (2). Although mortality is typically low, morbidity in young turkeys is high due to a predisposition to secondary infections, such as *Escherichia coli* septicemia (20).

An early step in *Bordetella* pathogenesis is bacterial adherence to host structures. In particular, they have a strong tropism for the cilia of the respiratory tract epithelium. This binding results in ciliostasis and compromises the clearing action of the mucociliary escalator (1). A number of adhesins have been described for the mammalian-adapted bordetellae, but filamentous hemagglutinin (FHA) and fimbriae are regarded as key (5, 19, 28). For example, *Bordetella bronchiseptica* and *Bordetella pertussis fim* mutants are defective in colonization of mouse respiratory tracts and fimbriae are required for persistence in the trachea (7, 14, 15), demonstrating their importance *in vivo*.

In Gram-negative bacteria, fimbriae are synthesized via a protein secretion system termed the chaperone-usher pathway (21). The components of this pathway are typically encoded by individual gene clusters and include an outer-membrane usher, a periplasmic chaperone, a fimbrial tip adhesin, and a fimbrial subunit(s) (21). The mammalian-adapted bordetellae have a single fimbrial biogenesis operon (*fimA-D*) that encodes a fimbrial subunit (FimA), a chaperone (FimB), an usher (FimC), and a tip adhesin (FimD). Each species also contains additional fimbrial subunit genes outside this operon (11, 16, 17, 30). The expression of several different fimbrial types has been observed among these bordetellae, suggesting that a single chaperone-usher system is utilized to assemble multiple different fimbriae, although FimD is the only recognized tip adhesin (16, 30).

Transcription of many of the genes encoding *Bordetella* virulence determinants, including fimbriae, is regulated by the BvgAS two-component signal transduction system. BvgAS comprises a transmembrane sensor kinase (BvgS) and a DNA-binding response regulator (BvgA). Within this system, a multistep phosphorelay is responsible for the spectrum of phenotypic expression phases (reviewed in reference 3).

Very little is known about the bacterial factors involved in *B. avium* pathogenesis. Like *B. bronchiseptica*, *B. avium* has a specific tropism for the ciliated epithelium of the airways (2, 10), but of the adhesins described for *B. bronchiseptica* and *B. pertussis*, only FHA and fimbriae have been recognized in *B. avium*. Cell-associated and purified *B. avium* fimbriae have been examined using electron microscopy and, when purified, did not hemagglutinate guinea pig erythrocytes (9). Purified fimbriae adhered to the mucosal surface of turkey tracheal explants, and hyperimmune antisera against *B. avium* fimbriae blocked the adherence of whole-cell preparations of *B. avium* to explants (9). Furthermore, *B. avium fhaC* and *fimC* were identified by signature-tagged mutagenesis as being required

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TABLE 1. Sequences of primers used in this study

Primer	Left sequence	Right sequence
Fimbrial locus knockout		
BAV1962-1965 upstream	5′ATCAGGAAGTTCTGAATAGAGCA3′	5′GCAAGCTTGTAAGGAATCAAAG3′
BAV1962-1965 downstream	5′CCTGGGATTTCTCTCAACG3′	5′ATGGCCTTCCTCTTTCAAAT3′
RT-PCR		
tyrB cDNA	5′GCTTCAAAGTCGAAACCTACAC3′	5′GGTTAGATTGCGTGTTGTAGCTG3′
BAV1965 cDNA	5′GATCACTATTCGCGGTGAAATC3′	5′GCCAGGCTCAAAATAAATATGG3′
BAV1964 cDNA	5′CTCTCTCGGCCTGGTATTTCTT3′	5′AAAGGGCACCTCCATTTGAT3′
BAV1963 cDNA	5′TGTCTTATAGCAAGACCCTGGA3′	5′GAGAGATAGAGGCTGCCGTAAC3′
BAV1962 cDNA	5′GGGAGTTGTTCAGTCATGTCAC3′	5′GAATCCGTAGTTCTCCAGTTGC3′
BAV1965 expression construct	5′TTCATATGACCATGCATACGATCAAAAAA3′	5′TTGAATTCTCAGGGGTACATGACAGAGAA3′

for virulence in turkey poults (25). However, the direct role of *fimC* was not elucidated.

The generation and analysis of a *B. avium* genome sequence (23) greatly facilitate the study of the genetic basis for *B. avium* pathogenesis, including the role of fimbriae. *B. avium* has 11 putative fimbrial subunit genes, a gene cluster (*BAV1965-1962*) that is homologous to the mammalian-adapted *Bordetella fimA-D* operon, and a second locus that appears to encode a second chaperone/usher system (*BAV1777-1773*) and two fimbrial subunits (23).

Although there is striking similarity at the disease level between the mammalian and avian bordetellae, differences at the genetic level may account for the various host specificities of these species. As there is little known about the molecular basis of *B. avium* pathogenesis, a genetic investigation of virulence factors such as fimbriae can be used to increase understanding of its infection biology. The homology between the *BAV1965-1962* locus and the *B. bronchiseptica fimA-D* locus suggests a common functionality. However, the occurrence of a second fimbrial biogenesis operon and a larger subset of fimbrial subunits provides initial evidence that *B. avium* may synthesize fimbriae that differ from those of the mammalian bordetellae. Presented here is a characterization of the *B. avium BAV1965-1962* operon, the first step toward characterizing the complexity of *B. avium* fimbriae.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. avium* Gobl-124 (also designated strain 197 [6]) was propagated at 22°C or 37°C on Bordet-Gengou (BG) agar containing 15% defibrinated sheep blood (Med-Ox Diagnostics Inc., Ottawa, Ontario, Canada) and 1% glycerol or in tryptic soy broth (TSB). *Escherichia coli* strains DH5α and SM10 were propagated at 37°C in Luria-Bertani (LB) broth or on LB agar plates (solidified with 1.5% granulated agar). DH5α was used as a general cloning strain. SM10 was used as a donor strain in conjugations (24). Growth media were supplemented with antibiotics when appropriate at the following concentrations: ampicillin (Amp) at 100 μg per ml, kanamycin (Kan) at 50 μg per ml, and streptomycin (Strep) at 200 μg per ml.

DNA manipulations and genetic techniques. Plasmid DNA was isolated using the GenElute plasmid miniprep kit (Sigma Aldrich Canada Ltd.). Restriction endonucleases were purchased from Invitrogen (Burlington, Ontario, Canada) or New England BioLabs (Pickering, Ontario, Canada). Gel purification of DNA fragments was performed using the PureLink quick gel extraction kit (Invitrogen). Ligation reactions were performed using the Fast-Link DNA ligation kit (Epicentre Biotechnologies, Madison, WI). PCRs were performed using Platinum *Taq* polymerase (Invitrogen) using conditions of 95°C for 5 min followed by 30 cycles of 95°C for 1 min 15 s, 60°C for 1 min 15 s, and 72°C for 1 min 30 s and a final step of 72°C for 5 min. Primer sequences can be found in Table 1. PCR products were resolved by agarose electrophoresis and gel purified. All methods were performed according to the manufacturer's instructions.

Bacterial conjugation. Bacterial conjugation was carried out by mixing different ratios of the donor and recipient strains in phosphate-buffered saline (PBS). The subsequent mixture was spotted onto BG agar supplemented with 10 mM magnesium chloride for at least 6 h at 37°C. After incubation, cells were removed and resuspended in PBS. Serial dilutions (10-fold) were plated on appropriate agar.

Construction of a *B. avium* fimbrial locus deletion mutant (*B. avium* Δ*1965-1962*). Briefly, fragments containing the upstream and downstream regions of the *BAV1965-1962* fimbrial locus were amplified by standard PCR, using *B. avium* Gobl-124 genomic DNA as a template. The upstream region was ligated into the pGem-T Easy vector system (Promega, Madison, WI). The resulting plasmid and the "downstream" PCR product were digested with HindIII and SpeI and ligated together. The resulting plasmid was digested with HindIII, which cuts between the upstream and downstream elements. A kanamycin cassette with HindIII cohesive ends was ligated into this site. The resulting plasmid was digested with SpeI and EcoRI to release the upstream/downstream-Kan element. This element was ligated into the allelic exchange vector pEX100T (22), and this construct was introduced into *B. avium* via conjugation using LB agar without salt but supplemented with 15% (wt/vol) sucrose to counterselect against merodiploid recombinants. The expected genomic rearrangements in deletion mutants were confirmed by Southern hybridization analyses.

Construction of a complementing *BAV1965-1962* operon plasmid (pBBRfim). A chromosomal fragment (BamHI to HindIII) approximately 9 kb long was obtained from plasmid pLAFR5-4a (25) and relocated into plasmid pBBR1MCS (12) that would replicate in *B. avium* and provide a selectable antibiotic resistance marker (chloramphenicol). This recombinant plasmid (pBBRfim) or the empty vector (pBBR1) was moved by conjugation into *B. avium* Δ*1965-1962*, as described above.

Reverse transcription (RT)-PCR. RNA was extracted from overnight cultures of *B. avium*. Cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and were treated with RNAprotect (Qiagen) at a ratio of 1:2 (vol/vol) and incubated at room temperature for 5 min. Total RNA was extracted from protected cultures using the RNeasy minikit (Qiagen) according to the manufacturer's instructions.

Purified RNA was treated with RNase-free DNase (Epicentre, Madison, WI) and repurified using the RNeasy minikit. The purity and quantity of each RNA sample were evaluated by determining the A_{260} and A_{280}. Purified total RNA was converted to cDNA using SuperScriptII (Invitrogen) reverse transcriptase and random primers (Invitrogen). PCR was performed at 95°C for 5 min followed by 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s and a final step of 72°C for 5 min. A negative control using RNA sample as a template was used to confirm the absence of contaminating genomic DNA.

Quantitative RT-PCR. Quantitative RT-PCR was performed using the Corbett Rotor-Gene 6000 Q-PCR machine (Corbett Life Sciences, Sydney, Australia) and the QuantiTect SYBR green PCR kit (Qiagen). Cycling conditions were 1 cycle of 94°C for 15 min followed by 50 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The melt curve was 72°C to 99°C, with an increment of 1°C every 5 s. Primer sequences can be found in Table 1. The relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method (13).

Transmission electron microscopy (TEM) analysis of *B. avium*. The presence of *B. avium* fimbriae was examined by TEM. *B. avium* was grown at 22°C or 37°C

TABLE 2. Homologues of the *B. avium* putative fimbrial proteins[a]

Protein	Closest homologue(s)	Amino acid identity (%)	Conserved domain(s)
BAV1965	BB3425/$FimN_{BB}$	48	pfam00419
	BB2992/$FimA_{BB}$	47	Fimbrial protein
BAV1964	BB2991/$FimB_{BB}$	52	pfam00345, pfam02753, Gram-negative pilus assembly
BAV1963	BB2990/$FimC_{BB}$	46	pfam00577, fimbrial usher
BAV1962	BB2989/$FimD_{BB}$	25	pfam00419, fimbrial protein
BAV1773	BB2354	28	COG3539
	BB0338	21	FimA pilin protein
BAV1774	BB2990/$FimC_{BB}$	30	pfam00577, fimbrial usher
BAV1775	BB2991/$FimB_{BB}$	34	pfam00345, Gram-negative pilus assembly
BAV1776	STM0200/fimbrial protein—*Salmonella typhimurium*, LT2	28	pfam00419, fimbrial protein
BAV1777	BB3426/$FimX_{BB}$, BB3425/$FimN_{BB}$, BB2991/$Fim2_{BB}$, BB1658/$Fim3_{BB}$	27	pfam00419, fimbrial protein

[a] *B. avium* fimbrial biogenesis proteins were subject to BLASTP analysis and conserved domain analysis. BB, *B. bronchiseptica*.

on BG agar for 48 h. Colonies were resuspended in PBS. Formvar-coated copper grids (200-mesh) were placed on a 10-μl drop of bacterial suspension for 30 min and then negatively stained with 1% ammonium molybdate. Samples were examined using a Phillips CM10 electron microscope operating at 80 kV, and images were captured with an SIS Morada charge-coupled-device (CCD) camera equipped with iTEM software (Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada).

Construction of His_6-BAV1965 expression vector. The *BAV1965* coding sequence was amplified by PCR using *B. avium* genomic DNA as a template. Purified PCR product and plasmid pET28a DNA were digested with NdeI and EcoRI and ligated together.

Overexpression of His_6-BAV1965. pET28a encoding N-terminal histidine-tagged BAV1965 was used to express protein for antigen production. The construct was transformed into *E. coli* BL21(DE3) using 50 μg/ml kanamycin for selection. Expression of BAV1965 was carried out in 50 ml of LB-Kan. Cells were induced at an OD_{600} of ≈0.6 by the addition of isopropylthio-β-D-galactopyranoside (IPTG) (Invitrogen) to a final concentration of 0.1 mM and incubated for 3 h at 37°C.

Screening for His_6-BAV1965. A 1-ml aliquot of induced cell culture was saved for analysis by SDS-PAGE and Western immunoblotting with anti-His antibodies. Cell lysis was accomplished by ultrasonication on ice using a model 500 sonicator (Fisher Scientific, Nepean, Ontario, Canada) equipped with a microtip with a 5-s pulse time, followed by a 10-s cooling period, for a total of 2 min at an amplitude of 30%. Aliquots (500 μl each) of total lysate and soluble and insoluble lysate fractions were saved for analysis by SDS-PAGE and Western immunoblotting. SDS-PAGE gels were stained with Coomassie brilliant blue-R250 to visualize overexpressed proteins. Western blots were probed using monoclonal penta-histidine primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-mouse $F(ab')_2$-alkaline phosphatase-conjugated (Santa Cruz Biotechnology) secondary antibody and were used to confirm the presence of the His_6 tag prior to protein purification.

Preparation of antigen. The insoluble protein fraction was run on a 7.5% SDS-PAGE gel. A portion of the gel that corresponded with the location of the BAV1965 band on a stained second gel was excised with an ethanol-cleaned scalpel. The gel fragments were chopped into 1-mm^3 pieces and loaded into an Electro-Eluter (model 422; Bio-Rad Laboratories, Mississauga, Ontario, Canada). Membrane caps (Bio-Rad Laboratories) with a molecular size cutoff of 6,000 kDa were used to seal the column. Proteins were electroeluted in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 50 mA for approximately 3 h. Buffer from the lower tank was replaced with buffer lacking SDS and run for approximately 3 h. Eluted proteins were removed from the membrane caps and stored in microcentrifuge tubes at 4°C.

Immunization. Antibody production was carried out according to the Canadian Council on Animal Care (CCAC) guidelines. Handling, immunization, and euthanasia of BALB/c mice were done by the Campus Animal Facilities (CAF) (University of Guelph, Guelph, Ontario, Canada). The protein sample was emulsified with an equal volume of Freund's incomplete adjuvant before immunization. On day 1, mice were injected with 100 μl of protein-adjuvant mixture subcutaneously (approximately 10 μg of recombinant protein). Mice were boosted on days 29, 53, and 80 with 50 μl of the protein-adjuvant mixture subcutaneously. Prior to boosting, mice were bled and serum tested on days 23, 49, and 73 by enzyme-linked immunosorbent assay (ELISA). Mice were euthanized and serum was collected on day 90. Serum was stored at −20°C.

Immuno-TEM procedure. *B. avium* was grown at 22°C or 37°C on BG agar plates for 48 h. Bacteria were resuspended in 500 μl PBS and fixed for 30 min in 0.5% glutaraldehyde. For immunogold labeling, Formvar-coated nickel grids were placed on the bacterial suspension. Subsequently, the grids were incubated for 30 min at room temperature, followed by blocking with 0.01 M PBS containing 0.3% skim milk for 20 min. Grids were incubated with anti-BAV1965 polyclonal serum for 2 h, washed three times with 0.01 M PBS, and then incubated with gold (12-nm diameter)-conjugated goat anti-mouse immunoglobulin (diluted 1:20; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 60 min. The grids were washed as described above and then negatively stained with 1% ammonium molybdate. Samples were examined using a Philips CM10 electron microscope operating at 80 kV, and images were captured with an SIS Morada CCD camera equipped with iTEM software (Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada).

Adherence of bacterial strains to turkey tracheal rings. Bacterial strains were grown overnight on Bordet Gengou agar (Fisher Scientific) with sheep blood (Cocalico, Reamstown, PA), suspended in Earle's basic salt solution (EBSS) (Sigma Chemicals, St. Louis, MO) to a density of 0.5 OD_{600} unit, and then diluted to 1×10^7 cells/ml in EBSS. Attachment to embryonic tracheal tissue was accomplished as described previously (27). Briefly, bacteria were applied in 0.5-ml portions to 5 rings/well in a 24-well tissue culture plate and incubated with rocking at 42°C for 1 h. After three 2-min washes, bound bacteria were loosened with 1% Triton X100 in phosphate-buffered saline at 4°C for 1 h, vortexed for 2 min, diluted, and spread on MacConkey agar plates for quantification. Results were expressed as a fraction of the inoculum, which was also quantified by diluting and plating the inoculum. Statistical significance was assessed using two sample *t* tests between the wild type (WT) and *B. avium* Δ*1965-1962*, the WT and the complemented mutant, and the WT and *B. avium* Δ*1965-1962* carrying empty vector. The numbers of repetitions (individual rings) were as follows: WT, 11; *B. avium* Δ*1965-1962*, 27; *B. avium* Δ*1965-196*(pBBR1-fim), 17; and *B. avium* Δ*1965-1962*(pBBR1), 8, representing two to five independent experiments with each strain.

RESULTS

***BAV1965-1962* is a putative fimbrial locus.** The fimbrial genes of *B. avium* were compared to the gene repertoire of *B. bronchiseptica*, as it has the largest intact genome within the genus (16, 29). BLASTP analysis revealed that BAV1965-1962 is homologous to and shares general fimbrial conserved domains with *B. bronchiseptica* FimA-D, suggesting that *BAV1965-1962* is a putative fimbrial locus (Table 2). In *B. bronchiseptica*, *fimA-D* is located immediately downstream of *fhaB*, which encodes FHA, and immediately upstream of *fhaC*, which encodes a protein involved in FHA secretion. The expression of *fimA-D* and *fhaB* is regulated in response to tem-

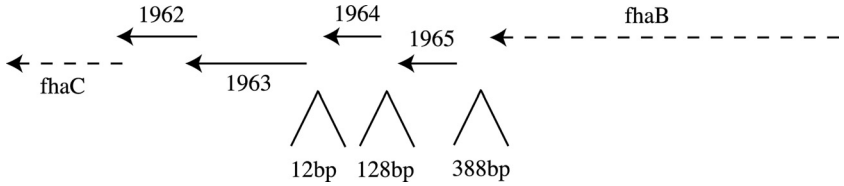

FIG. 1. Schematic diagram of the genetic organization of the *B. avium* chromosome spanning *fhaC-BAV1966*, and the sizes (in base pairs) of the DNA regions in between the predicted coding sequences of each gene. Where no size is given, the adjacent coding sequences have overlapping start and stop codons.

perature by *bvgAS* such that they are expressed at 37°C but not at 22°C (4). The 5′ end of the *fhaC* coding sequence overlaps the 3′ end of the *fimD* coding sequence, strongly suggesting that *fimA-D* and *fhaC* are transcribed on a polycistronic transcript. It is possible that the operon also includes the upstream *fhaB*, but that has not been demonstrated. *BAV1965-1962* shares the same genetic organization; *BAVfhaC* overlaps the 3′ end of *BAV1962*, and *BAVfhaB* is immediately upstream of *BAV1965* (Fig. 1). However, significant differences within *B. avium*, including a second putative fimbrial locus (*BAV1777-1773*) and the presence of a number of other putative fimbrial subunit genes, suggest that *B. avium* produces species-specific fimbriae, perhaps with different host ligands. To begin to characterize the functionality of the *BAV1965-1962* locus and its role in *B. avium* biology, the conditions under which the genes are transcribed and expressed were examined.

Transcription of *BAV1965-1962* is regulated by temperature. RT-PCR analyses were used to identify growth conditions under which *BAV1965-1962* transcripts could be detected (Fig. 2). RT-PCR products corresponding to transcription of *BAV1963*, *BAV1964*, and *BAV1965* were detected only from bacteria grown at 37°C; however, *BAV1962* was transcribed at both 37°C and 22°C (Fig. 2). The amount of *BAV1962* RT-PCR product from 22°C-grown bacteria is less than that from bacteria grown at 37°C. A housekeeping gene, *tyrB* (aromatic amino acid aminotransferase), was expressed to the same level at both temperatures (data not shown). The amount of *BAV1962*-derived PCR product was greater than that derived from the other genes (equal amounts of DNA marker are loaded on each gel), suggesting either that *BAV1962* is transcribed at higher levels than the other genes or that the RT-PCR efficiency of amplification of *BAV1962* was greater than for the other genes. Thus, while the data might suggest that *BAV1965-1963* is transcribed at 37°C but not 22°C, it could be that they are expressed predominantly at 37°C but at low levels at 22°C, with the amplification efficiency of product being too low to detect transcripts from 22°C-grown bacteria.

The level of BAV1962 transcript is greater at 37°C than at 22°C. In order to confirm the difference in the quantity of *BAV1962* transcript produced by *B. avium* when grown at 37°C or 22°C, relative gene expression was analyzed by quantitative RT-PCR. Expression was analyzed using the $2^{-\Delta\Delta CT}$ method (13), and the lower temperature was arbitrarily chosen as the calibrator in this study. The level of *BAV1962* transcript was normalized to *tyrB* and quantified relative to the data collected at 22°C as a baseline. The mean fold change in gene expression of *BAV1962* was 71 times greater at 37°C than at 22°C (Table 3). Thus, based on the increased level of *BAV1962* transcript at 37°C, it is likely that the transcription of *BAV1965-1962* is regulated such that the level of transcript is greater at 37°C than at 22°C.

Fimbria-like appendages are evident at 37°C and 22°C. To correlate the transcription of *BAV1965-1962* with the production of fimbriae, *B. avium* was examined by transmission electron microscopy (TEM). To investigate the role of the *BAV1962-1965* in the production of fimbrial structures, a nonpolar insertion/deletion mutant (*B. avium* Δ*1965-1962*) was constructed in which the four genes were replaced with a kanamycin resistance cassette. Fimbria-like structures were visible on the cell surface when grown at 37°C or 22°C (Fig. 3A and B). Flagella were also observed but only on bacteria grown at 22°C (Fig. 3C). Growth in liquid versus on solid

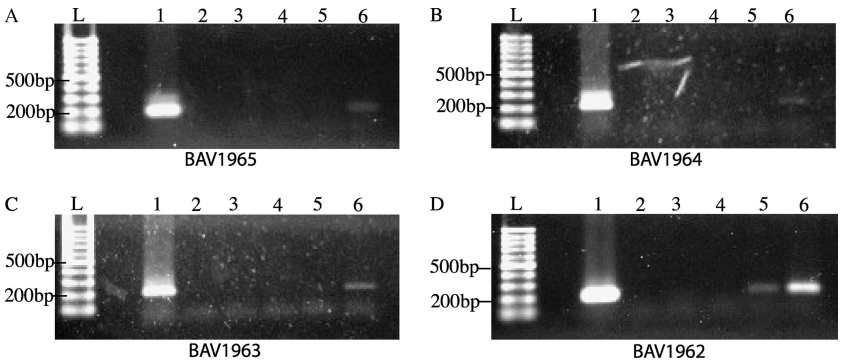

FIG. 2. RT-PCR analysis of transcription of the *B. avium BAV1962-1965* fimbrial locus. RT-PCR products were separated by agarose electrophoresis on a 1.5% gel and stained with ethidium bromide. L, 100-bp DNA ladder. Templates: lane 1, *B. avium* genomic DNA positive control; lane 2, negative control. No template: RNA from bacteria grown at 22°C (lane 3) or 37°C (lane 4), without reverse transcription, and cDNA reverse transcribed from RNA from bacteria grown at 22°C (lane 5) or 37°C (lane 6).

TABLE 3. Relative quantification of *BAV1965* expression at 22°C and 37°C using the $2^{-\Delta\Delta CT}$ method

Temp	C_T		ΔC_T[a]	$\Delta\Delta C_T$[b]	Normalized *BAV1965* amt[c]
	BAV1965	*tyrB*			
22°C	22.06	14.11			
	21.88	14.15			
	22.08	13.93			
Avg	22.01 ± 0.11	14.06 ± 0.12	7.94 ± 0.21	0.00 ± 0.21	1.00 (0.87–1.16)
37°C	17.48	15.87			
	17.92	15.88			
	17.62	15.90			
Avg	17.67 ± 0.23	15.88 ± 0.02	1.79 ± 0.22	−6.15 ± 0.22	71.18 (60.967–83.097)

[a] ΔC_T = (avg $C_{T,\ BAV1965}$ − avg $C_{T,\ tyrB}$).
[b] $\Delta\Delta C_T$ = (avg $\Delta C_{T,\ \text{target}}$ − avg $\Delta C_{T,\ 22°C}$).
[c] *BAV1965* amount ($2^{-\Delta\Delta CT}$) was normalized relative to the amount at 22°C. Values in parentheses indicate 95% confidence intervals.

media did not make a difference to the presence of fimbria-like structures. In contrast to the transcriptional analyses, initial TEM analysis showed no temperature regulation of fimbria-like structures. However, it is possible that the cell surface appendages observed are *BAV1965-1962*-independent fimbriae or other surface appendage structures, such as type IV pili or FHA.

***B. avium* expresses BAV1965-containing fimbriae.** To enable identification of BAV1965-containing fimbriae, antibodies were raised in mice against recombinant BAV1965, expressed in *E. coli* as an N-terminal His tag fusion (see Materials and Methods). ELISA analysis confirmed that the resultant polyclonal mouse sera recognized recombinant BAV1965 (data not shown). The anti-BAV1965 polyclonal sera were used in immuno-TEM experiments to identify fimbriae on the surface of *B. avium* grown at 37°C or 22°C. Colloidal gold particle-conjugated anti-mouse immunoglobulin secondary antibodies were used to stain fimbriae recognized by the polyclonal serum. Numerous colloidal gold particles were observed on the cell surface of wild-type *B. avium* grown at 37°C (Fig. 3D and E) but not at 22°C (data not shown). Gold particles were not observed associated with the mutant (Fig. 3F). These data are consistent with *B. avium*-expressing BAV1965 containing fimbriae at 37°C but not 22°C, and this is dependent on the presence of the *BAV1962-1965* locus.

The *BAV1965-1962* locus is required for adherence to turkey tracheae. Fimbriae are often involved in adherence of bacterial pathogens to host cells and tissues. To investigate the involvement of the *BAV1965-1962* locus in adherence of *B. avium* to host respiratory tract tissue, the adherence of the WT and *B. avium* Δ*1965-1962* to turkey embryonic tracheal tissue was assayed (Table 4). *B. avium* Δ*1965-1962* adhered significantly less than WT bacteria. Carriage of the plasmid, *BAV1965-1962*(pBBRfim), restored the adherence of the deletion mutant to the level of WT bacteria, whereas introducing vector alone into the deletion mutant did not affect its level of adherence. Thus, the *BAV1965-1962* locus is required for adherence of *B. avium* to turkey tracheal tissue, supporting the hypothesis that this locus codes for the production of fimbriae that are involved in adherence of the bacteria to host tissues.

DISCUSSION

B. bronchiseptica contains a single fimbrial biogenesis operon, *fimA-D*, although its genome contains several fimbrial subunit genes. The *fimA-D* locus is flanked by *fhaC* and *fhaB* and is likely coexpressed with this other adhesin system. Expression of *fimA-D* is regulated in response to temperature by the Bvg two-component regulator such that FimA containing fimbriae are expressed at 37°C but not at temperatures below 25°C. Both FHA and fimbriae are involved in adherence of *B. bronchiseptica* to mammalian cells and its pathogenesis.

B. avium contains homologues of *B. bronchiseptica fimA-D*. The components of *BAV1965-1962* are arranged in the same order as those of *fimA-D* and are flanked by *B. avium fhaC* and *fhaB* homologues (23). This suggests a common functionality of the two loci. However, *B. avium* contains a second putative fimbrial biogenesis operon and a large number of fimbrial subunit genes. Thus, *B. avium* may express a complex repertoire of fimbrial types via two distinct chaperone-usher systems and containing two different tip adhesins. Here we sought to begin characterization of *B. avium* fimbrial expression.

We demonstrated that transcription of *BAV1965-1962* is greater at 37°C than at 22°C. Initial RT-PCR experiments suggested that several genes in the locus might be expressed only at 37°C, but this was likely due to limitations in detecting RT-PCR amplicons from these genes. A more likely scenario, supported by quantitative PCR estimation of transcription of *BAV1962*, is that the locus is transcribed at low levels at 22°C but significantly greater levels at 37°C.

Using TEM, we observed putative fimbrial structures on the surface of *B. avium* grown at either 22°C or 37°C. However, genome sequence data suggest that it is likely that these bacteria can express several different cell surface appendages, either alternative fimbriae, possibly via the second fimbrial locus, or others, such as FHA or type IV pili. To aid in the identification of fimbriae containing the fimbrial protein BAV1965, antisera were raised against recombinant BAV1965. iTEM analyses identified immunoreactive cell surface staining on bacteria grown at 37°C but not 22°C. Furthermore, this immunoreactivity was dependent on the functional *BAV1965-1962* locus, as deletion of this locus resulted in loss of reactivity

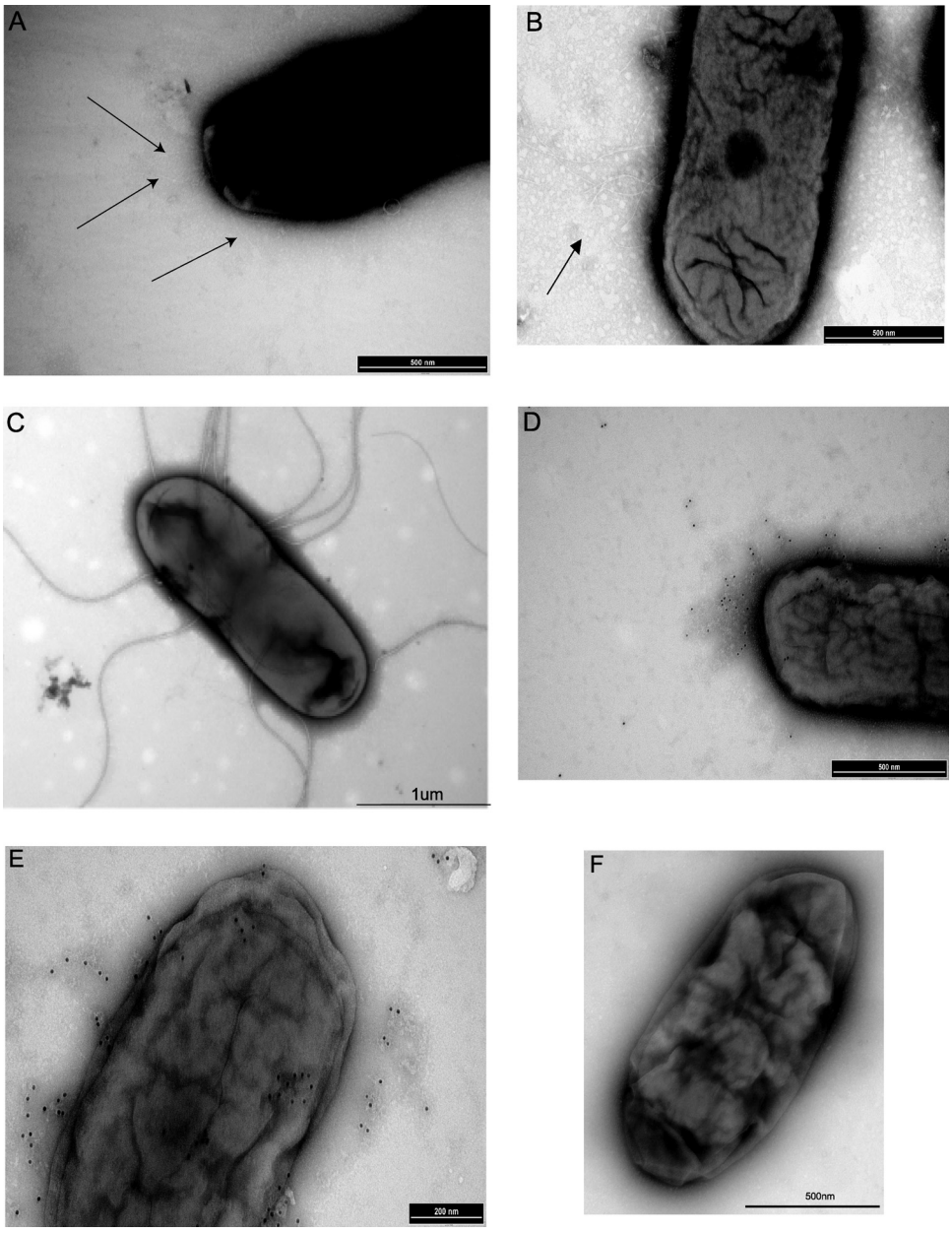

FIG. 3. Transmission electron micrographs of *B. avium* cells negatively stained with ammonium molybdate. Putative fimbria-like cell surface appendages (highlighted by arrows) were observed on WT bacteria grown at 37°C (A) or 22°C (B). Flagella were observed only on WT bacteria grown at 22°C (C). Immunostaining of bacteria with anti-BAV1965 antiserum and colloidal gold particles revealed immunoreactive material on WT bacteria grown at 37°C (D and E) but not on a *BAV1965-1962* deletion mutant (F) or WT bacteria grown at 22°C (data not shown). Due to space constraints, images representative of many are shown.

TABLE 4. Adherence of WT and *B. avium* Δ*1965-1962* to turkey tracheal ring cultures

Strain	Fraction of inoculum adhered	SD
WT	0.0365	0.0251
B. avium Δ*1965-1962*	0.0083	0.0049[a]
B. avium Δ*1965-1962*(pBBR1fim)	0.0345	0.0212
B. avium Δ*1965-1962*(pBBR1)	0.0050	0.0016[a]

[a] Significantly different ($P < 0.05$) from values for either WT or the deletion mutant complemented by carriage of *BAV1965-1962* on a plasmid (pBBR1fim) as calculated by Student's *t* test.

with the antisera. The presence of fimbriae containing BAV1965 at 37°C but not at 22°C suggests that although *BAV1965-1962* may be transcribed at low levels at 22°C, these fimbriae are not produced.

In the mammalian host-adapted *Bordetella* species, temperature regulation of gene expression is mediated by the Bvg two-component system. Slip-strand mispairing in a poly(C) tract in *bvgS* between the 2nd and 3rd phosphorelay sites that are essential for BvgS activity causes phase-variable expression of BvgS and attenuation of virulence in phase variants that do not express the protein (26). *B. avium* also contains a Bvg

locus, but its function has not been characterized. The genome sequence of *B. avium* 197N revealed that *BAVbvgS* contained a frameshift mutation within the poly(C) tract, suggesting that the sequenced isolate is a phase variant in which *bvgS* is not functional (23). However, experimental studies using the same isolate identified *bvgS* as required for virulence, suggesting that the BvgS protein is active (25). A survey of *bvgS* in a number of *B. avium* strains revealed heterogeneity in the length of this poly(C) tract (23), and the role of Bvg and the impact of mutations within this tract on BvgS activity remain unclear. Thus, the role of Bvg in expression of fimbriae was not studied here.

To confirm that the fimbrial locus produced functional fimbriae, *in vitro* adherence assays were performed. A *B. avium* Δ*1965-1962* mutant was significantly less adherent to embryonic turkey tracheal tissue than the WT strain, a defect that was relieved by carriage of the *BAV1965-1962* locus on a plasmid. This is in agreement with a previous study in which a mini-transposon insertion mutant in *BAV1963* was shown to be defective in adherence assays (25). Here, the deletion mutant adhered at approximately 25% of the level of WT, suggesting that the *BAV1965-1962* locus plays a major role in the adherence of the bacteria to the tissue under these conditions. This is of interest given that *B. avium* possesses a second fimbrial locus and other putative adhesin genes (including *fhaB*, type IV pili) and suggests that if produced, these other structures play relatively minor roles in adherence of bacteria to the tracheal tissue or are functional under conditions other than those used here.

This work has characterized the production of fimbriae by *B. avium* at the genetic and functional levels, the first definitive study of this type in this bacterium. The *BAV1965-1962* locus is functional and is regulated by temperature. This was confirmed through the identification of fimbrial structures that were dependent on the *BAV1965-1962* locus. The identification of fimbria-like structures at both temperatures in the initial TEM experiments suggests that a different type of fimbriae may be produced at 22°C than at 37°C, possibly involving the second putative fimbrial locus. This might suggest that *B. avium* produces fimbriae that are pathogenesis (37°C) and nonpathogenesis (environmental) (22°C) specific. These are important first steps toward understanding the role of fimbriae in *B. avium* biology.

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