PURIFICATION, CHARACTERIZATION, AND PATHOGENICITY OF *MORAXELLA BOVIS* PILI

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Moraxella bovis is the primary cause of Infectious Bovine Keratoconjunctivitis (IBK),[1] a highly contagious ocular disease of cattle that can result in temporary (or occasionally permanent) blindness (1–4). Hemolysin and pili have been proposed as *M. bovis* virulence factors (5–7); the latter are surface appendages of the bacterium composed of a repeating polypeptide subunit termed pilin.

Pili are associated with the ability of *M. bovis* to establish persistent ocular infection and cause clinical signs of IBK. Freshly isolated *M. bovis* strains are always piliated, a trait that may be lost during propagation in vitro (8). When inoculated into the eyes of calves, piliated *M. bovis* organisms establish infection and cause disease, but nonpiliated variants of the same strain do not (7).

Pili appear to confer pathogenicity by mediating the attachment of bacteria to epithelial cells (9–11). In addition, the pili of some bacterial species may prevent phagocytosis by leukocytes (12, 13). Pili prepared from different *M. bovis* strains exhibit antigenic diversity (14), a characteristic that might enable the species to evade the host immune response. Furthermore, vaccines composed of pili prepared from single *M. bovis* strains only conferred protection to homologous challenge (14, 15).

Recently we reported the cloning and sequence of the β pilin gene of *M. bovis* strain Epp63 (16). Comparison of the predicted amino acid sequence of this pilin with the pilin sequences of other bacterial species led to the identification of an NH_2-terminal region homologous with members of the *N*-methyl phenylalanine (type 4) family of pilins represented by *Moraxella nonliquefaciens* (17), *Neisseria gonorrhoeae* (18, 19), *Neisseria meningitidis* (18), *Pseudomonas aeruginosa* (20), *Bacteroides nodosus* (21, 22), and *Vibrio cholerae* (23). In the present report we describe the covalent structure and serologic properties of the α pilin of *M. bovis* strain Epp63 and the existence of a reproducible, oscillating mechanism of chromosomal rearrangement that limits the pilin expression by this strain to only the α and β pilin types. This observation led us to test the notion that, in addition to antigenic diversity, different pilins of the same strain might confer differences in infectivity or pathogenicity.

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[1] *Abbreviations used in this paper:* Eth, monoethanolamine; IBK, infectious Bovine Keratoconjunctivitis; Pth, phenylthiohydantoin; SC, spreading, corroding; SEC, steric exclusion column.

Materials and Methods

Bacterial Isolates and Propagation. *Moraxella bovis* strains Epp 63, Fla 64, and Mac were kindly provided by Dr. George Pugh, National Animal Disease Center, Ames, IA. The Tifton 1 isolate was a gift from Dr. Lisle George, School of Veterinary Medicine, University of California, Davis, California. All isolates were obtained from the eyes of cattle with keratoconjunctivitis, cultivated for two to six 24-h passages on bovine blood agar then preserved by lyophilization or freezing at −70°C in Greaves' solution (24). Aliquots of the stored organisms were thawed or reconstituted from the lyophilized state by the addition of 1 ml tryptic soy broth, inoculated onto 5% sheep blood agar, incubated in air at 37°C, and passed as single colonies to fresh bovine or ovine blood agar plates every 24 h.

Isolates were tentatively identified as *M. bovis* by the formation of characteristic spreading, corroding (SC) colonies that were hemolytic on blood agar. Identity was confirmed by a positive oxidase test result, the ability to liquify gelatin and digest casein, but the inability to grow on MacConkey agar, reduce nitrate, or attack glucose by either oxidation or fermentation (25, 26).

Colony Identification and Colonial Morphology. The colonial morphologies of organisms propagated as described above were examined with a 1–7× variable power dissecting microscope (model Stereozoom 7; Bausch & Lomb, Rochester, NY) or a 1–6× variable power dissecting microscope equipped with an auxilliary 6-V ring light source and a 35-mm camera with automatic exposure control (American Optical, Buffalo, NY). Photographs were exposed on Kodak Tri-X pan film, ASA 200 with the equipment set on maximum illumination, developed, and printed on glossy paper by Eastman Kodak Co. (Rochester, NY).

Electron Microscopy. Bacteria grown 20–24 h as described above were harvested from agar plates into PBS containing 1% (wt/vol) BSA (PBSA). Aliquots of the resulting suspension were placed on carbon, formvar-coated 300-mesh copper grids (Pelco, Tustin, CA) and incubated at room temperature for 2 min. Bacterial suspensions were removed carefully from grids with tissues, the grids were washed briefly in distilled water, then negatively stained for 2 min in 1% phosphotungstic acid (27). Excess stain was then removed with tissues and the grids were air dried. Specimens were examined using a Phillips model 201 transmission electron microscope and were photographed.

Immunoblots. Purified pili (1 μg) or intact *M. bovis* colonies grown on agar were boiled for 10 min in 30 μl of SDS-PAGE sample buffer (see below). The samples, which now contained reduced, denatured monomeric pilin subunits were loaded onto a vertical 10-cm 15% polyacrylamide/0.1% SDS gel and electrophoresed 3–4 h at constant current (60 mA). The separated proteins were transferred electrophoretically to a nitrocellulose membrane (BA 83, Schleicher & Schull, Keene, NH) by application of 60 mA for 3 h in transfer buffer, pH 8.0, according to Towbin et al. (28) and Burnette (29).

Unoccupied nitrocellulose binding sites were saturated by incubation overnight in 200 ml of PBS with 2 g gelatin. The membranes were incubated 1.5 h at 37°C with pilus antisera diluted 1:300 in PBSA-Brij, then washed five times (10 min each) in PBSA-Brij. The membranes were then incubated with ^{125}I staphylococcal protein A (50,000 cpm) (Amersham Corp., Arlington Heights, IL) in 20 ml PBSA-Brij for 1 h at 37°C, washed five times, and dried. Radiographic film (X-AR5; Eastman Kodak Co.) was exposed to the membranes for 15–72 h at −70°C, and the film was developed.

Hybridizations. Chromosomal DNAs were cleaved with the restriction endonuclease Hin fI and the fragments were separated by electrophoresis on 1% agarose gels. The DNA fragments were then denatured and transferred to nitrocellulose paper by the method of Southern (30). The Hin dIII–Eco RI fragment from plasmid pMxB12 (16) containing the β-pilin gene was radioactively labeled with [^{32}P]dCTP using a nick-translation kit (Bethesda Research Laboratories, Gaithersburg, MD). Hybridization of this β-pilin gene probe to the Southern filters was carried out essentially by the method of Blattner et al. (31), in 10 ml of 5×SSC, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml boiled salmon sperm DNA at 56°C for 16–24 h. The filters were then washed three times each using 100 ml of 2×SSC + 0.1% SDS at 56°C. After air drying, the filters were exposed to X-ray film (X-OMAT, Eastman Kodak Co.) at −70°C using intensifying screens (MCI Optonix Inc., Cedar Knolls, NJ) for varying lengths of time.

Purification of Pili. Cultures of organisms representing uniform colonial morphotypes were grown as confluent lawns on 5% sheep blood agar plates by incubation in air at 37°C for 24 h and harvested into ice-cold 0.15 M monoethanolamine buffer (Eth buffer) with the pH adjusted to 10.3–10.5 by the addition of HCl, as described by Brinton et al. (32).

The bacteria were homogenized in a Sorvall Omni-mixer (DuPont Instruments-Sorvall, DuPont Co., Newton, CT) for 10 min at 2,500 rpm. Bacterial cells were removed by centrifugation at 10,000 *g* for 30 min and additional debris was cleared from the supernatant by centrifugation at 12,000 *g* for 1 h. Pilus filaments were precipitated from the supernatant by the addition of solid ammonium sulfate to 10% saturation. The precipitated pili were collected by centrifugation at 12,000 *g* for 1 h, the pellet was dissolved in the Eth buffer, and insoluble contaminants were removed by centrifugation at 12,000 *g* for 1 h. The supernatant was dialyzed against 0.15 M NaCl containing 0.05 M Tris-HCl, pH 8.0 (Tris-buffered saline). The pilus aggregates that formed were separated from soluble contaminants by centrifugation at 12,000 *g* for 1 h and the pellet dissolved in a small amount of the Eth buffer. Purity was ascertained by SDS-PAGE and Coomassie blue or silver staining as described below; if necessary, further purification was achieved by successive cycles of solubilization and aggregation of the pilus filaments via exposure to Eth buffer and Tris-buffered saline, respectively.

SDS-PAGE. Proteins were separated by electrophoresis through either 15% or 12.5% polyacrylamide gels containing 1% SDS, in accordance with the method of Laemmli (33). One or a few bacterial colonies were picked from agar plates, suspended in 30 μl of standard Laemmli sample buffer, then boiled for 10 min. Purified pili were diluted in Eth buffer, suspended in 30 μl of sample buffer and boiled for 10 min. Electrophoresis was conducted under conditions of constant current for 3–4 h. Gels were stained with Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, Mo.) or were silver stained (Eastman Kodak Co.).

Cyanogen Bromide Cleavage. The α-pilin sequence contains methionine residues at positions 7 and 86 (see below). Therefore, cyanogen bromide (CNBr) cleavage of lyophilized pili was conducted by the method of Gross and Witkop (34) to produce three CNBr fragments: CNBr-1 (residues 1–7), CNBr-2 (residues 8–86), and CNBr-3 (residues 87–155, the COOH terminus). 20 mg of lyophilized pili were dissolved in 75% TFA and 100 mg of CNBr (Pierce Chemical Co., Rockford, IL) were added. After 24 h at RT a 20-fold volume of distilled water was added and the reaction mixture lyophilized. CNBr-2 and CNBr-3 were separated by reverse-phase high-pressure liquid chromatography (HPLC) as described below.

Arginine-specific Hydrolysis. The α-pilin sequence contains three arginine residues at positions 35, 82, and 123. Therefore, arginine-specific tryptic cleavage (TC) was perfomred to produce four fragments: TC-1 (residues 1–35), TC-2 (residues 36–82), TC-3 (residues 83–123), and TC-4 (residues 124–155). Pili were citraconylated by the method of Atassi and Habeeb (35), as modified by Schoolnik et al. (19). A 15-fold molar excess of citraconic anhydride over total amino groups was added to a stirred solution of pili in 0.05 M sodium phosphate buffer, pH 9.0, in five aliquots over 60 min. The pH of the reaction was maintained at 9.0 by the addition of 2 M MaOH. The citraconylated protein was freed from salt by gel filtration with Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1 M ammonium bicarbonate, pH 8.0, and the arginyl bonds were cleaved by the addition of TPCK-treated trypsin (Millipore Corp., Bedford, MA). After 4 h at 37°C the insoluble peptides were removed by centrifugation and the supernatant was injected directly onto an HPLC TSK-type SW steric exclusion column (SEC) equilibrated with 0.1 M ammonium bicarbonate, pH 8.1 (see below).

Reduction and Alkylation. The presence of free sulfhydryl groups was determined with Ellman's reagent (36), 5,5'-dithiobis (2-nitrobenzoic acid). 1 mg of pili was dissolved in 0.3 ml of 6 M guanidine-HCl, pH 7.8, and incubated for 30 min at 50°C. 10 μl of Ellman's reagent (Pierce Chemical Co.) was added (10 mM in 0.1 M sodium phosphate buffer, pH 7.0) and the absorbance was measured at 412 nm.

Reduction and alkylation of pili preceded arginine-specific hydrolysis in some experiments. Pili (0.25 μmol) and 2.5 μmol DTT were dissolved in 2.5 ml of 0.1 M ammonium bicarbonate buffer, pH 8.2, containing 6 M guanidine-HCl and incubated under N_2 at 50°C for 1 h (37). Iodoacetic acid (3 μmol) was added and the reaction allowed to proceed for 1 h at 25°C in the dark.

Isolation of Peptides. Cyanogen bromide fragments were separated by reverse-phase HPLC. They were eluted from a Whatman Partisil-10 ODS-2 column in a linear gradient of acetonitrile in water containing 0.05% (vol/vol) TFA at a flow rate of 1.0 ml/min. Tryptic peptides TC-2, TC-3, and TC-4 of citraconylated pili were resolved by HPLC SEC in which one TSK G3000SW and two TSK G2000SW 7.5-mm × 50-cm columns (Varian Associates, Inc., Walnut Creek, CA) were connected in series and the peptides were eluted in 0.1 M ammonium bicarbonate buffer, pH 8.1, at a flow rate of 0.3 ml/min. Eluates were monitored by their absorbance at 218 nm and 280 nm; peptides were subsequently identified by amino acid analysis and their purity was assessed by TLC and analytical reverse-phase HPLC.

Amino Acid Analysis. The amino acid compositions of *M.bovis* pili and pilus peptides were determined by hydrolysis in 4 N methane-sulfonic acid (38) in evacuated, sealed tubes at 115°C for 22, 48, 72, and 108 h. The values for serine and theonine were corrected for destruction during hydrolysis by extrapolation to time zero. The values for leucine, isoleucine, and valine were corrected for slow hydrolysis of the peptide bond by extrapolation to infinite time. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation of the methane-sulfonic acid hydrolysate. Homoserine was identified by the method of Adelstein and Kuehl (39). The hydrolysates were analyzed on a Durrum D-500 amino acid analyzer using a one-column system.

Sequence Analysis. Automated Edman degradation was performed with a sequencer (model 890C; Beckman Instruments, Palo Alto, CA), using a modified Quadral program (No. 0011576, Beckman Instruments) in combination with polybrene. Thiazolinone derivatives of amino acids were converted to their corresponding phenylthiohydantoin (Pth) derivatives with aqueous 1.0 N HCl at 80°C for 10 min. Pth-amino acids were identified by HPLC with a model 1084B HPLC (Hewlett Packard Co., Cupertino, CA) using Zorbax ODS columns (DuPont Co.) and confirmed by gas chromatography (GLC) and/or TLC. Sequenator stepwise yields were 92–96%, and amino acids were assigned only when peak-to-background ratios were >2:1.

The α-pilin amino acid sequence was determined (except for positions 117 and 120) by Edman degradation of CNBr and TC fragments as follows. The NH_2-terminal region was sequenced through residue 39. CNBr-2 (residues 8–86) was sequenced through residue 46. TC-2 (residues 36–82) was sequenced completely (47 steps). TC-3 (residues 83–123) was sequenced through residue 104. CNBr-3 (residues 87–155) was sequenced through position 127; however, the residues at positions 117 and 120 could not be determined. TC-4 (residues 124–155) was sequenced completely, through the COOH terminus (32 steps). The alignment of these fragments is described in Results.

The α-pilin TC-4 fragment was found to contain a pair of half-cystine residues; since no sulfhydryl groups were detected after titration of intact pilin with 5,5'-dithiobis (2-nitrobenzoic acid) or after alkylation with iodoacetic acid in 6 M guanidine-HCl these residues were proposed to form a disulfide bond.

Antisera Production. 100 μg of purified pili in Eth buffer were added to 1.0 ml PBS, pH 7.4, then emulsified with CFA and injected subcutaneously and intramuscularly into female New Zealand White rabbits (40). 6 wk later each animal was boosted in an identical manner except that the pili were emulsified with IFA. After 10–14 d the rabbits were anesthetized with 5 mg/kg xylazine-HCl (Bayvet, Shawnee, KS) and 50 mg/kg ketamine-HCl (Bristol Laboratories, Syracuse, NY) intramuscularly and 30 ml of blood was harvested by aseptic cardiocentesis. The blood was allowed to clot for 30 min at RT and the clots were separated from the container walls using sterile wooden applicators. Specimens were kept overnight in the cold to allow clot retraction then centrifuged 10 min at 1,000 *g*. Sera were aspirated and dispensed as aliquots into sterile 1.5-ml tubes to which three drops of 2% sodium azide had been added.

Solid-phase Assay. A solid-phase assay was used to assess the binding of purified pili by pilus antisera. The wells of polyvinyl microtitre plates were coated with purified pili (0.1 μg) in 100 μl of 50 mM sodium carbonate, pH 9.5, and incubated overnight at RT. The wells were drained, then washed with 0.9% NaCl to which was added 1% Brij detergent. 100 μl of antisera, serially diluted in PBS pH 7.4, containing 1% BSA and Brij (PBSA-Brij) were added. After incubation for 1 h at RT the wells were washed, 50,000 cpm ^{125}I staphylococcal protein A/well was added, then they were incubated for 1 h at RT and washed. The radioac-

tive wells were cut from the plate and counted in a gamma counter (Beckman Instruments, Palo Alto, CA).

Infectivity and Pathogenicity Studies. Nonpiliated, and α- and β-piliated isogenic variants of *M. bovis* Epp 63 were evaluated for their ability to infect and cause clinical disease in the eyes of calves.

Inocula. *Moraxella bovis* Epp 63 bacteria were selectively subcultured on 5% sheep blood agar daily for three passages. Nonpiliated, α- and β-piliated clones were identified by observing the appropriate band on a Coomassie blue-stained gel after electrophoresis of boiled colonies. To provide sufficient organisms for ocular inoculation, one colony of each isogenic variant was subcultured to five plates; the following day the bacteria were harvested and subcultured to 30 plates. Pilin type was reconfirmed with SDS-PAGE and the bacteria harvested into 30 ml TSB broth, gently mixed, and 1-ml aliquots were placed into sterile tubes.

Experimental Protocol. Holstein and Holstein-cross calves ~3–5 mo old weighing 80–200 kg were obtained locally. Before proceeding, each calf was confirmed free of clinical ocular lesions. Eyes of all calves were cultured daily for 5 consecutive days in order to ensure that no calves were infected by *M. bovis*. Calves were randomly assigned to three groups and housed in fly-free, mechanically ventilated, windowless rooms equipped with insecticide strips. The entrance to each room was via a door sealed by a rubber gasket. Isolation procedures were enforced throughout the study, including separate protective cover-alls and disposable gloves for each room and disinfection of attendants' hands and arms before and after manipulation of calves in each room.

Ocular infections were established by a modification of a previously described procedure using sunlamp irradiation, followed by topical instillation of *M. bovis* bacteria (41, 42). Both eyes of each calf were irradiated for 10 min from a distance of 60 cm (250-W sunlamp) twice, 24 h apart. 24 h after the second irradiation each eye was inoculated once with 1 ml TSB suspension of either nonpiliated, α- or β-piliated *M. bovis* organisms.

Eyes were examined daily for 16 d for signs of IBK. An eye was considered affected when severe lacrimation, miosis, chemosis, scleral erythema, corneal edema, and ulceration were detected. Secretions from each eye were examined by culture daily for up to 16 d. Ocular secretions collected with sterile swabs were immediately inoculated onto 5% bovine blood agar plates, which were incubated aerobically for 24 h at 37°C. Colonies resembling *M. bovis* were subcultured until pure (one to three passages), and were identified positively by conventional morphologic and biochemical criteria and by immunoblotting. Pilin production was determined by immunoblotting.

Statistical Analysis. Infection rates were determined as the percentage of calves (or calf eyes) inoculated that developed infection. The incidence of disease (IBK) was determined as the percentage of calves (or calf eyes) inoculated that developed clinical signs. Fisher's exact test was used to test for statistical significance of differences of infection rates and disease incidence between groups (43).

Results

Colony Types. When *M. bovis* strain Epp 63 was propagated on 5% sheep blood agar, seven distinct colony types were observed; four of these are illustrated in Fig. 1. The nomenclature for colonial morphology initially proposed by Bovre et al. in their studies with *M. bovis* and other species of the *Moraxella* genus was used for this project (44, 45). All colonies were β hemolytic. Colonies designated as spreading and corroding (SC) (Fig. 1, *A-C*) varied somewhat in morphology, but all were small (1–2-mm diameter) and smooth, had well demarcated borders, and corroded the agar. The zones of corrosion, evident as grooves or depressions in the agar surface when SC colonies were scraped from the plate (Fig. 1 *A*), were equal in diameter to that of the corresponding colony. Some SC colonies were surrounded by peripheral projections (Fig. 1 *B*), which on higher magnification appeared to be composed

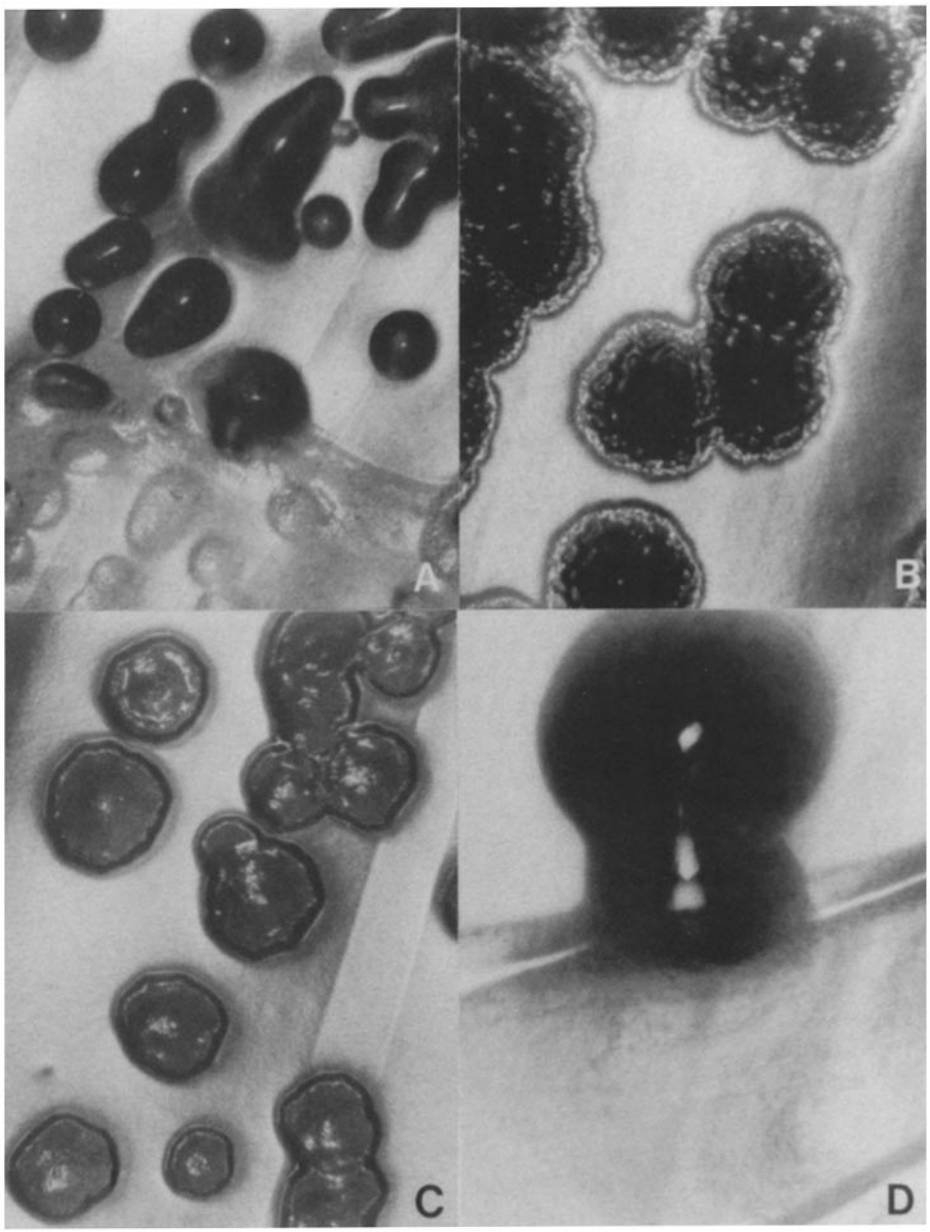

FIGURE 1. Appearance of *M. bovis* Epp63 colonies grown 20–24 h on 5% sheep blood agar at 37°C. Colonies were photographed with a dissecting microscope, magnification 2–3×. *A-C*, various morphotypes of spreading, corroding (SC) colonies, all of which expressed pilin detectable by immunoblotting and pili seen by transmission electron microscopy (TEM). Agar corrosion is evident in *A*, where some colonies were removed from the agar. *D*, nonspreading, noncorroding (N) colonies. These colonies did not express pilin detectable by immunoblotting or produce pili that could be seen by TEM.

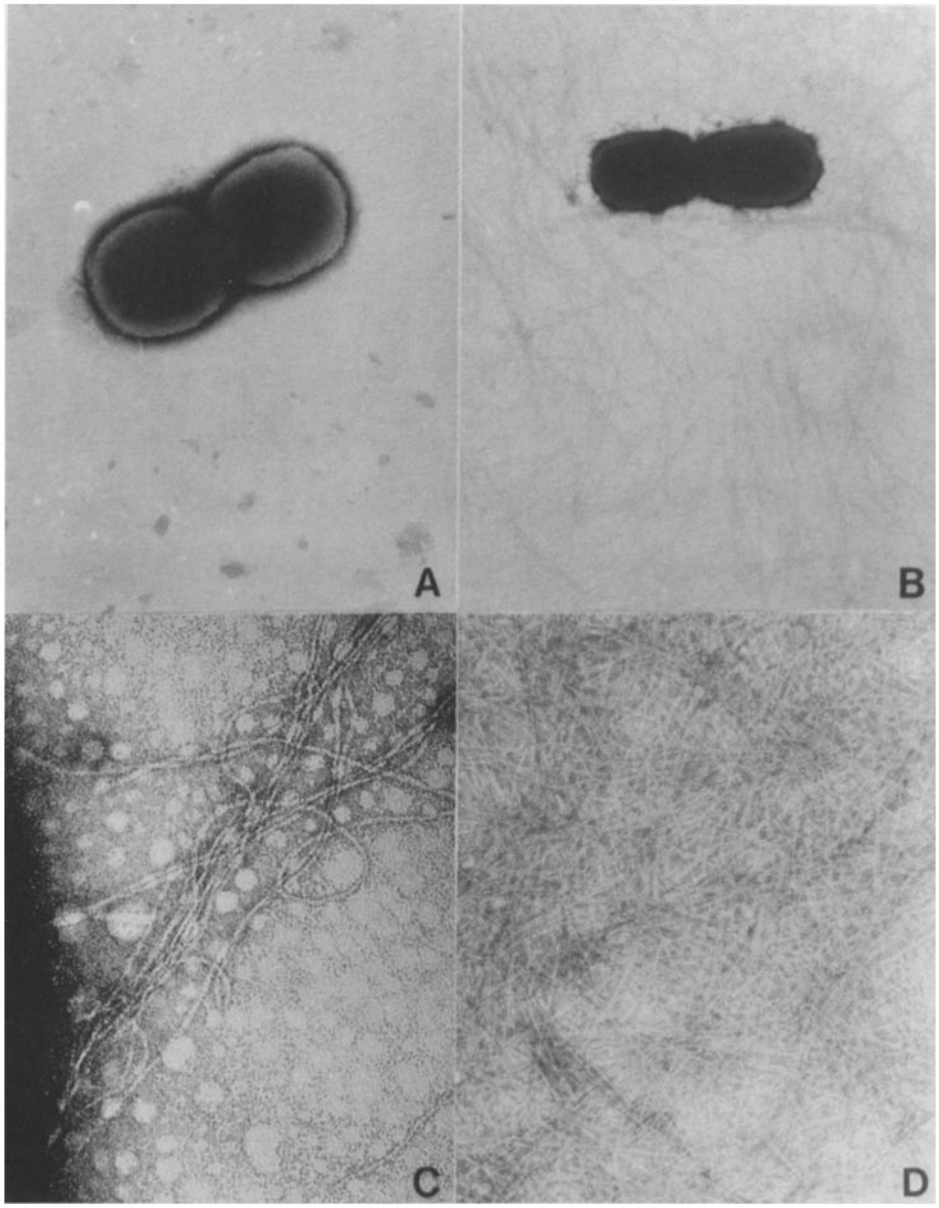

FIGURE 2. Transmission electron micrographs of *M. bovis* Epp63. Bacteria were harvested after incubation on 5% sheep blood agar plates at 37°C, stained with phosphotungstate, and examined by TEM. *A*, A nonpiliated diplobacillus originating from an N-type colony. 7,000×. *B*, A piliated diplobacillus originating from a SC type colony. 7,000×. *C*, Greater magnification (60,000×) showing detail of pilus filament attachment to an organism. *D*, Pilus filaments purified from *M. bovis* Epp63 organisms. 30,000×.

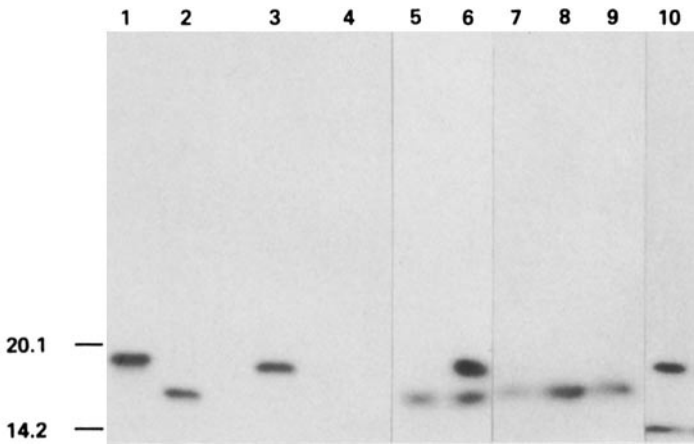

FIGURE 3. *M. bovis* pilin expression. Bacterial colonies or purified pili were analyzed by immunoblotting as described in Materials and Methods. Lanes: *1*, pure α pili; *2*, pure β pili; *3*, a single *M. bovis* Epp63 SC-type colony producing only α pilin; *4*, a single *M. bovis* Epp63 N-type colony demonstrating the absence of pilin production; *5*, a single *M. bovis* Epp63 SC-type colony producing only β pilin; *6*, a single *M. bovis* Epp63 SC-type colony producing both α and β pilins; *7–9*, single colonies of *M. bovis* strains Huc, UMC, and Fla producing pilins that comigrate with Epp63 β pilin; *10*, a single colony of *M. bovis* strain Mac 74 producing pilins with two different relative molecular masses.

of aggregated bacteria; these bacteria exhibited microscopic surface translocation by the mechanism of twitching motility (46, 47).

A distinct colonial type designated nonspreading and noncorroding (N) was also recognized (Fig. 1 *D*). Compared with the SC type, N colonies were larger (2–4-mm diameter), had poorly demarcated colonial borders, and did not corrode agar. When viewed on 6% bovine blood agar plates these colonies had a characteristic granular texture. Colonies of the N type did not exhibit projections or twitching motility.

Correlation of Colony Type With Piliation, Pilin Expression, and Type of Pilin Expressed. Using transmission electron microscopy (TEM), Bovre et al. observed pilus filaments on bacteria composing SC type colonies, but not on organisms from N-type colonies (44–46). The more recent observation by Marrs et al. (16) that a single *M. bovis* strain can express two distinct pilin types (α and β) differentiable by apparent molecular weight, led us to reexamine the relation between colony type as described above, pilus filament production as detected by TEM, and the production of either α or β pilin as detected by immunoblotting (see below).

Electron micrographs of negatively stained bacteria from SC- and N-type colonies are depicted in Fig. 2. Bacteria in the SC samples (Fig. 2 *B*) were usually present as diplobacilli or in large clusters, with single organisms encountered only occasionally. Individual bacteria were 1–1.2 × 0.6–0.8 μm. Most bacteria from SC type colonies were moderately or heavily piliated and many broken pilus filaments were present in the background (Fig. 2 *B*). Pilus filaments projected from all surfaces of the bacteria, but most were concentrated in a polar distribution. Individual pili were 6–8 nm in diameter and varied in length up to nearly 3 μm. No differences were detected between the pili of bacteria from different SC morphotypes. Greater detail

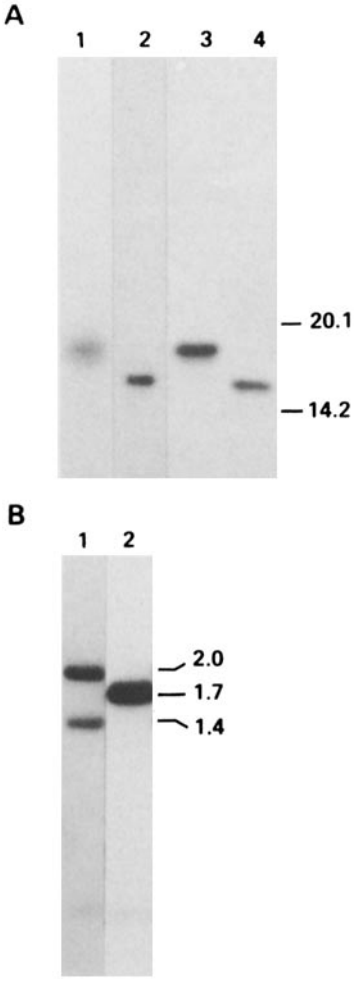

FIGURE 4. Pilin gene arrangements and protein expression of *M. bovis* Epp63 isolates procured from the eyes of calves inoculated with Epp63 β-piliated organisms. *A*, Immunoblot performed as described in Materials and Methods. Lanes: *1*, a single colony producing α pilin; *2*, a single colony producing β pilin; *3*, pure α pili; *4*, pure β pili. *B*, ^{32}P-labeled DNA probe containing the β pilin gene hybridized to genomic digests of *M. bovis* DNA from the same isolates depicted in *A*. Procedures were conducted as described in Materials and Methods. Lanes: *1*, a doublet, representing the α pilin gene, corresponds to α pilin expression (lane *1*, and panel *A*); *2*, a single band, representing the β pilin gene, corresponds to β pilin expression (lane *2*, and panel *A*).

of pilus attachment to a bacterium from a SC colony is shown in Fig. 2 *C*. Organisms from N-type colonies usually did not exhibit pili (Fig. 2 *A*), but in an occasional specimen a few very sparsely piliated bacteria were encountered. Otherwise, organisms from N-type colonies appeared morphologically similar to bacteria from SC colonies, except for a reduced tendency to form clusters.

Immunoblot analysis of single colonies was conducted using pilus-specific antisera in order to determine whether bacteria composing SC and N type colonies expressed pilin, and if so, whether they expressed the α or β type. The α pilin type was recognized as a band that comigrated with purified α pilin (M_r 20,000) and that reacted with antipilus sera (Fig. 3, lane *3*). The β-type pilin was recognized as a band that comigrated with purified β pilin (M_r 17,000) and which reacted with antipilus sera (Fig. 3, lane *5*). Results of this analysis confirmed that pilin expression was restricted to organisms of the SC-type colonies (Fig. 3, lanes *3–5*); moreover, all tested SC colonies expressed pilin. Individual colonies of each of the SC morphtypes could

express either α or β pilin. Thus, the SC morphotype was strongly associated with the expression of pilin per se, but not with the type of pilin expressed. These studies also revealed that, for the most part, different SC colonies of the same morphotype expressed either α pilin exclusively or β pilin exclusively. Rarely, immunoblot analysis of a single Epp63 SC colony revealed the presence of both α and β pilin types (Fig. 3, lane *6*). It is not known whether this was due to the simultaneous production of both pilins by some cells in the colony, or whether it resulted from the coexistence in the same colony of two populations of cells, one that produced α pilin while the other produced β pilin. No ultrastructural differences were noted in bacteria from SC colonies or their pili, regardless of the type of pilin expressed.

Transitions of α and β Pilin Expression In Vitro and Correlation with Chromosomal DNA Rearrangement. When *M. bovis* was passed nonselectively in vitro by Bovre et al., they observed spontaneous changes from the SC to the N colonial type, which corresponded to changes from the piliated (P^+) to the nonpiliated (P^-) state, as seen by TEM (44). We also observed P^+ to P^- transitions, and in addition used immunoblot analysis to evaluate the occurrence of spontaneous transitions between the production of α and β pilins by SC type colonies of *M. bovis* strain Epp63. Cultures were maintained by selective passage of single SC colonies every 24 h for periods as long as 3 mos. From these plates single SC colonis were removed each day and prepared for immunoblot analysis, and the expression of α or β pilin was determined as previously described. Bidirectional transitions between the α and β pilin types were observed. However, colonies that originally expressed one pilin type usually gave rise to daughter colonies that also expressed the same pilin type. Transitions to the alternate pilin type occurred rarely, and without change in colonial morphotype.

To determine if the transition between α and β pilin production by SC colonies was accompanied by rearrangement of genomic DNA, Southern blot hybridization was performed with 24 α and β SC colonies of strain Epp63. Total DNA was isolated, digested with *Hinf* 1, and probed with a ^{32}P-labeled *Eco* RI insert fragment of plasmid pMxB12 (16), which contains the β pilin gene. Only two patterns were observed (Fig. 4). The SC colonies that expressed α pilin invariably produced a doublet pattern on Southern blot hybridization (Fig. 4, *A* and *B*, lanes *1*), while colonies that expressed β pilin were in all instances associated with a single strongly hybridized band (Fig. 4, *A* and *B*, lanes *2*). Similar experiments using a variety of other restriction enzymes (data not shown) in each case also revealed one hybridzation pattern associated with α pilin expression and a different pattern associated with β pilin expression. Therefore, apparently only two patterns of genomic organization exist, one associated with α pilin expression and the other with β pilin expression. Transitions between α and β pilin expression are accompanied by rearrangement of the chromosome to the alternate pattern.

Production of Pilin by Other M. bovis Strains. Five additional *M. bovis* strains (Fla, Mac, UMC, Huc, and Tifton-1) were cultivated, individual colonies harvested, and pilin expression sought by immunoblotting with a polyclonal serum capable of recognizing heterogeneous pilin types. All SC colonies from every strain produced pilin, whereas no N-type colonies expressed detectable amounts of pilin. Examples of some of these are depicted in Fig. 3, lanes *7–9*. When the immunoblot data from multiple SC colonies for these five strains were analyzed, it was apparent that each strain produced two distinct pilins of different relative molecular mass in a manner analo-

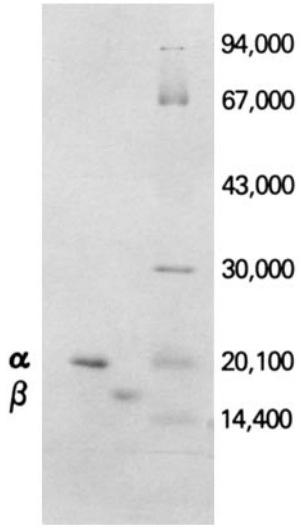

FIGURE 5. Analysis of *M. bovis* strain Epp63 α and β purified pili by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Molecular mass standards are shown on the right. *Moraxella bovis* Epp63 α pilin migrates with a relative molecular mass of 20,000; *M. bovis* Epp63 pilin migrates with a relative molecular mass of 17,000.

gous to the production of α and β pilins by strain Epp63, i.e., single colonies usually independently expressed an α equivalent or a β equivalent pilin, but not both simultaneously. However, on occasion single colonies of strain Mac74 (Fig. 3, lane *10*) and strain Fla (data not shown) each expressed two pilins of different relative molecular mass simultaneously. The subunit relative molecular masses of these pilins appeared to be stable between sister colonies on the same plate, and between colonies of the same strain picked on different days. However, the two pilins produced by these strains did not comigrate exactly with the two pilins produced by Epp63.

Purification of Pili. Pili were purified from several batches of SC colonies from strain Epp63; each batch was composed of colonies of a single morphotype. The purified pilus preparations were evaluated by TEM and SDS-PAGE. As shown in Fig. 2 *D*, the purified pilus filaments were ultrastructurally indistinguishable from pili attached to intact SC organisms. No contaminating material was observed in the specimens, and no ultrastructural differences were detected between pili composed of α or β pilins.

When analyzed by SDS-PAGE (Fig. 5) the purified pili were seen to consist of homopolymers composed of identical α or β pilins, with subunits of M_r 20,000 and 17,000, respectively. Thus, the α and β pilins not only are expressed by colonies of the SC type, but exist as polymerized pilus filaments. No purified α pilus preparations contained β pilin; however, some pilus preparations that contained primarily β pilin also contained small amounts of α pilin.

Amino Acid Composition and Sequence Analysis of M. bovis Epp63 α and β Pilins. Southern blot analysis of genomic DNA from colonies producing α and β pilins (Fig. 4) suggested that the proteins shown in Fig. 5 represent two distinct gene products and are not the result of posttranslational modification of one gene product. This question was further explored by performing amino acid compositional and sequence analyses of purified *M. bovis* pili preparations composed of either α or β pilins.

The α and β pilin amino acid compositions, depicted in Table I, are quite similar.

TABLE I

Amino Acid Compositions of M. Bovis Epp63 α and β Pilins

Amino acid	α Pilin		β Pilin
	Sequence*	Analysis‡	Sequence*
Alanine	18	18	24
Arginine	3	3	4
Aspartate§	12	12	12
Cysteine‖	2	2	2
Glycine	12	12	19
Glutamate¶	14	15	11
Histidine	0	ND**	0
Isoleucine	13	12	13
Leucine	11	11	11
Lysine	15	14	9
Methionine#	2	2	2
Phenylalanine	4	3	4
Proline	4	5	4
Serine	15	15	7
Threonine	18	19	17
Tryptophan	1	1	2
Tyrosine	3	3	2
Valine	8	8	8
Total residues	155	155	151

Residues per subunit shown.

* Number of residues derived from the sequences shown in Fig. 6.

‡ Integral number of α-pilin residues based on amino acid compositional analysis and mol wt of 16,554 calculated from the amino acid sequence shown in Fig. 6.

§ Total number residues of aspartic acid and asparagine.

‖ Cysteine was analyzed as cysteic acid.

¶ Total number residues of glutamic acid and glutamine.

** ND, no amino acid was detected.

Methionine was analyzed as methionine sulfone.

Both pilins are characterized by a high content of nonpolar, hydrophobic residues (38% of all α and of all β pilin residues) and each pilin contains two cysteines. However, significant differences in the content of alanyl, glycyl, glutamyl, lysyl, and seryl residues indicated that the α and β pilins may contain regions of sequence heterogeneity. This possibility was explored by elucidating a substantial portion of the α and β pilin amino acid sequences, using automated Edman degradation of cyanogen bromide and arginine-specific tryptic cleavage fragments (see Materials and Methods). The peptide fragments derived by cleavage of β pilin were aligned according to the sequence deduced from the genomic DNA sequence of β pilin (Fig. 6) (16). Amino acid sequence data were obtained for residues 1–80 and for residues 87–116. These sequences were found to be identical to the amino acid sequences deduced from the corresponding segments of the β pilin gene DNA sequence. The sequences of the peptide fragments generated by α pilin cleavage were aligned by reference to homologous regions of the β pilin sequence (Fig. 6). 153 of the 155 α pilin residues were identified; however, residues at positions 117 and 120 could not be determined. Recently, the α pilin gene DNA sequence was determined by Marrs et al. (unpublished observation). By reference to this sequence, residues 117 and 120 were both identified as threonine. The α pilin amino acid sequence derived by chemical methods

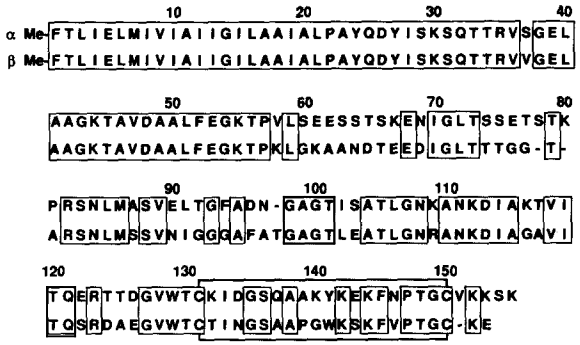

FIGURE 6. *M. bovis* α and β pilin amino acid sequences and homologies. The α protein was cleaved by chemical and enzymatic methods, the fragments purified by HPLC and sequenced by automated Edman degradation. The β sequence was predicted from the β pilin gene DNA sequence by Marrs et al. (16). The numbers designate α pilin residues. The β pilin sequence has been aligned to demonstrate maximal homology to the α pilin sequence by the introduction of gaps. Identical residues are enclosed in boxes. The brackets denote the intramolecular disulfide bonds that exist between α residues 131 and 150 and β residues 130 and 149. Positions 117 and 120 of the α pilin sequence were not determined by amino acid sequencing, but were deduced from the α pilin gene DNA sequence to be threonine residues (Marrs, unpublished observation). Position 112 of the α pilin sequence was found to be glycine by amino acid sequence analysis, but lysine by deduction from the α pilin gene DNA sequence; also, the α pilin gene DNA sequence did not indicate the presence of codons specifying residues 154 and 155 (Marrs, unpublished observation).

agreed with the amino acid sequence deduced from the α pilin gene DNA sequence except that residue 112 was identified as glycine by the former method and as lysine by the latter, and that the α pilin gene DNA sequence did not indicate the presence of codons specifying residues 154 and 155.

The α and β pilin sequences are identical through the first 57 residues, with the exception of a substitution of valine for serine at position 37 of the β molecule. The NH_2-terminal 25 amino acids comprise a segment of significant hydrophobicity. The NH_2-terminus of ~50% of the α and β pilin molecules was shown to be *N*-methyl phenylalanine; the remaining 50% of the α and β pilin molecules began with the second residue, threonine, a phenomenon previously described in some pilins of *N.gonorrhoeae* (18, 19). Both sequences contain a disulfide loop 20 residues in length, near the COOH terminus, a feature also found in the pilins of *N.gonorrhoeae* (19) and *Pseudomonas aeruginosa* (48).

After residue 57, homology between the α and β pilins decreases to ~60%, resulting in an overall sequence identity of ~70%. The unconserved substitutions in this region are concentrated, although not exclusively, in two segments. The first, a stretch between residues 58 and 80 contains 15 nonidentical residues, of which 6 represent a single- and 1 a double-charge change. In addition, the β pilin sequence in this region has a two-amino-acid deletion relative to the α pilin sequence. The second unconserved stretch occurs in the disulfide loop, where eight substitutions exist, four of which represent single-charge changes.

Immunologic Characteristics of Purified Pili. Prior studies have shown that pili purified from different *M. bovis* strains exhibit antigenic heterogeneity (14). In this study we examined the antigenic relatedness between purified pili composed only of α pilin and purified pili composed only of β pilin, prepared from two isogenic variants of strain Epp63. Competitive immunoassays were conducted with rabbit antisera to pure α and β pili and the corresponding pilus proteins to determine whether the differences in primary structure (Fig. 6) might be manifested by antigenic differences between pilus proteins of the same strain. The α and β pili exhibit ~50% shared antigenicity (data not shown), indicating that they are antigenically related but not

TABLE II

M. bovis Isolation and Occurrence of IBK in the Eyes of Calves Inoculated With 10^8 Colony-forming Units of α or β Piliated M. bovis Epp63 Isogenic Variants

Inoculum*	Infectivity (%)‡	Pathogenicity (%)§
α-piliated	2/16 (13)	2/2 (100)
β-piliated	7/12 (58)	6/7 (86)
Nonpiliated	0/6 (0)	0/0 (0)

* Refers to type of pilin expressed by the *M. bovis* used for ocular inoculation.

‡ Infectivity is defined as the number of eyes infected/the total number of eyes challenged by that variant. α vs. β, $p = 0.015$; β vs. nonpiliated, $p = 0.025$; α vs. nonpiliated, NS at $p < 0.05$, by Fisher's exact test.

§ Pathogenicity is defined as the number of eyes affected/the total number of eyes infected by that variant. α vs. β, was not significant at $p < 0.05$ by Fisher's exact test.

identical, and that both crossreacting and type-specific epitopes probably exist. Therefore, *M. bovis* pili exhibit not only interstrain, but also intrastrain, antigenic heterogeneity.

Infectivity of α- or β-piliated Isogenic Variants of Strain Epp63 for Experimentally Inoculated Calves Eyes. Earlier studies have demonstrated that piliated *M. bovis* Epp63 bacteria can produce infection and disease in the eyes of experimentally inoculated calves, whereas nonpiliated variants do not (7). To assess the association of α vs. β piliation with infectivity and pathogenicity the eyes of separate groups of calves were inoculated with isogenic variants of *M. bovis* Epp63 that expressed either α pili or β pili; a nonpiliated variant of strain Epp 63 was also studied.

Each eye of each calf was inoculated with $\sim 10^8$ bacteria, a challenge dose used by other investigators (14, 15, 42). For each inoculum, the production of α or β pilin was confirmed by immunoblot analysis; the production of pilus filaments was ascertained by electron microscopy. Ultrastructural examination revealed that the bacteria composing both the α and β inocula were piliated; no pili were seen in samples of the inoculum containing the nonpiliated variant. Electron microscopy also indicated that the number of pilus filaments per bacterium in the inocula containing the α- or β-piliated variants was similar, indicating that differences in the infectivity or pathogenicity of each variant could not be attributed to differences in the number of pilus filaments per organism. Group A calves received α-piliated organisms, while the calves of groups B and C were inoculated with β-piliated and nonpiliated organisms, respectively. Each eye was examined daily and specimens were procured for bacterial culture. Infection was considered established in eyes from which *M. bovis* organisms were recovered on at least two occasions by culture after postinoculation day 1. Pathogenicity was evaluated by daily examination of each eye for photophobia, excessive lacrimation, blepharospasm, conjunctival erythema, scleral injection, corneal edema, and ulceration.

Experimental results are summarized in Table II. Ocular inoculation with β-piliated bacteria was associated with significantly higher rates of infection, and the development of corneal ulceration was compared with inoculation of α-piliated or nonpiliated organisms. Infection was established in 7 of 12 (58%) group B eyes, significantly more than group A (2 of 16, 13%) ($p = 0.015$) or group C (0 of 6, 0%)

($p = 0.025$) eyes. Of the infected eyes, six of seven eyes infected with the β-piliated variant developed IBK, and both of the eyes infected with the α-piliated variant developed IBK. Therefore, β pilation appears to be associated with enhanced infectivity relative to α piliation, but not with enhanced pathogenicity once infection is established.

Discussion

Moraxella bovis is the etiologic agent of IBK. A hemolysin and pili have both been proposed as *M. bovis* virulence determinants; the latter are homopolymers of a polypeptide subunit termed pilin which forms filaments that project from the bacterial surface. Persuasive evidence exists that pili are associated with the ability of *M. bovis* to establish persistent ocular infection and cause clinial signs of IBK (7).

Analysis of purified pili from isogenic variants of strain Epp63 by SDS-PAGE indicate that they are composed of α or β pilins, which migrate with distinctive relative molecular masses. Immunoblot examination of Epp63 colonies with pili antisera reveal that the bacteria composing serially propagated daughter colonies produce either α or β pilin over many months of observation. The expression of α and β pilins also was noted to spontaneously alternate between daughter colonies. However, the organisms composing a single colony usually synchronously expressed only α or β pilin, but not both. Since hundreds of Epp63 colonies were examined by immunoblotting over a 3-yr period, and were shown to produce only pilins that comigrated precisely with purified α or β pilin, it seems likely that the repertoire of pilin expression by this strain is limited to only two polypeptides. The expression of two distinct pilins by other *M. bovis* strains suggests that this phenomenon may be characteristic of the species.

The α and β pilins of *M. bovis* strain Epp63 could be coded for by separate structural genes or be produced by the posttranslational modification of a single gene product. This ambiguity was resolved by the amino acid sequence analysis of each pilin type (Fig. 6). Although the polypeptides were found to contain substantial regions of conserved sequence, the presence of multiple amino acid substitutions indicate the existence of two separate pilin genes. Further evidence for the existence of two separate sets of pilin gene sequences comes from the Southern blot hybridization patterns seen in Fig. 4, which indicate the presence of at least two different sequences containing regions homologous to the β pilin gene. The differences in hybridization patterns observed between the DNAs from the α- and β-pilin variants of Epp63 indicate that the α and β pilin genes arise from a single genetic locus by a process of chromosomal rearrangement. Additional Southern hybridization studies have been performed that reveal this chromosomal rearrangement to be an inversion event in which the end parts are within the pilin structural genes (49). This mechanism provides for the alternative expression of one or the other pilin gene, but never both simultaneously. The limited pilin repertoire of *M. bovis* Epp63 observed in vitro therefore, is governed by a genetic mechanism that should restrict pilin diversity by this strain in vivo.

Transitions from the piliated to the nonpiliated state occur; the genetic mechanism responsible for this unknown. The genomic arrangement(s) of pilin-negative colonies is currently being investigated.

The amino acid sequence heterogeneity of the α and β pilins creates antigenic differences between the two pilus organelles. Although many of the amino acid sub-

stitutions are conservative, at least two separate regions contain amino acid substitutions that result in localized changes in charge or hydrophobicity. These regions may specify antigenic determinants that are unique to α or β pilin. Conserved regions between α and β pilin also exist and must be responsible for the ~50% shared antigenicity that exists between the two pilins. Because detailed sequence and immunologic data from the pilins of other *M. bovis* strains are not available, it is not known whether the structural and antigenic diversity of pilins from different strains exceeds the observed diversity of the α and β pilins from strain Epp63.

Studies were conducted in calves to determine if structural differences between α and β pilins could cause differences in how α- or β-piliated variants of the same strain interact with host tissues. The relatively large challenge dose of 10^8 bacteria used in this and other studies (14, 15, 42) may reflect the multifactorial nature of pathogenicity and the use of agar-grown organisms, which may not fully exhibit all necessary virulence determinants. Moreover, in nature, corneal trauma due to prolonged exposure to UV irradiation (especially in cattle grazing at high altitude), dust, and abrasions caused by grass and other foreign bodies enhances the clinical signs of IBK (4). Finally, under natural conditions an eye may be subjected to more than one challenge due to repeated ocular deposition of organisms by face flies.

A β-piliated variant of strain Epp63 was found to be significantly more infectious for experimentally inoculated eyes than an α-piliated or a nonpiliated variant of Epp63, indicating that β pili per se, or another trait associated with β pilin expression, promotes the early association of β-piliated bacteria with host tissue. It seems unlikely that this attribute can be ascribed to the antigenic differences between the α and β pilins, since differences in infectivity were apparent within 48 h of inoculation. Instead, these data imply that β pilin expression confers, or is associated with, a higher capacity to colonize ocular tissues than α pilin expression. If so, unique regions of the β pilin sequence could specify a higher-affinity binding domain for a putative pilin receptor on ocular conjunctival and corneal epithelia when compared with the corresponding regions of the α pilin sequence. In contrast, the pathogenic role of α pilin remains unclarified by the infectivity experiment. Conceivably, α pilin could serve the ecology of *M. bovis* by promoting the association of the organism with other sites containing complementary α pilin receptor compounds. Such α pilin receptor sites might include the structures that comprise the bovine nasolacrimal ducts or the nasopharyngeal epithelia, tissues known to be colonized during natural infection; α pilin receptor sites might also be located on the bodies of face flies (*Musca autumnalis*), which have been implicated in the transmission of *M. bovis* between animals (50, 51).

Moraxella bovis is one of several bacterial species that produce pilins with methylphenylalanine as the NH_2 terminus; others in this group include *Moraxella nonliquefaciens* (17), *Neisseria gonorrhoeae* (18, 19), *Neisseria meningitidis* (18), *Pseudomonas aeruginosa* (20), *Bacteroides nodosus* (21, 22), and *Vibrio cholerae* (23). In *Neisseria gonorrhoeae*, the most extensively studied member of this group, isogenic variants of a single strain produce multiple (>11) pilin types in vitro (52). Recently, Swanson et al., showed in human volunteers that a single gonococcal strain can also produce different pilins in vivo (53). The production of multiple pilin types greatly enhances the antigenic diversity of *N. gonorrhoeae*. In contrast, *M. bovis* Epp63 (and probably other *M. bovis* strains as well) appears to be able to produce only two pilin types in vitro and in vivo (Ruehl, unpublished results). These differences reflect different genetic mecha-

nisms of phase variation between pilin types, and could indicate differences between the pathogenic strategies of *M. bovis* and *N. gonorrhoeae*. It seems likely that studies which compare the virulence mechanisms of *M. bovis* and *N.gonorrhoeae* may provide additional general information about the pathogenesis of infections on mucosal surfaces, thus facilitating the development of effective vaccines for the prevention of both gonorrhea and Infectious Bovine Keratoconjunctivitis.

Summary

Pilins composed of the α or β pilins of *Moraxella bovis* strain Epp63 were purified, subjected to chemical or enzymatic cleavage, and the resulting fragments sequenced by automated Edman degradation. α Pilin was found to be a 155-amino-acid polypeptide with a single intramolecular disulfide bridge. The β pilin amino acid sequence substantiated the previously reported structure derived from the β pilin gene DNA sequence, and indicated that the α and β pilins of this strain are ~70% homologous. DNA hybridization studies of genomic DNA from the α- and β-piliated variants of strain Epp63 indicated that the expression of the two pilin types was governed by an oscillating mechanism of chromosomal rearrangement. The α and β pili were evaluated serologically and found to exhibit ~50% shared antigenicity, indicating that regions of conserved and heterologous sequence specify both type-specific and crossreacting epitopes. The pathogenicity of the α- and β-piliated variants was studied by ocular inoculation of calves eyes; β-piliated organisms were significantly more infectious than α-piliated organisms, indicating that β pili confer, or are associated with, a relative advantage during the first stages of ocular infection. Preliminary analysis of other *M. bovis* strains suggests that each strain produces two types of pilin, and that this property may be characteristic of the species.

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