

Cloning and Sequencing of a *Moraxella bovis* Pilin Gene

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Moraxella bovis pili have been shown to play a major role in both infectivity and protective immunity of bovine infectious keratoconjunctivitis. Sonicated *M. bovis* DNA from the piliated strain EPP63 was inserted into the vector λ gt11 with *Eco*RI linkers. Recombinant phage were screened with an oligonucleotide probe based on the amino-terminal portion of the DNA sequence of a *Neisseria gonorrhoeae* pilin gene. Two candidate phages produced a protein that comigrated with EPP63 β pilin in sodium dodecyl sulfate-polyacrylamide gels and bound anti-pilus antisera. The 1.9-kilobase insert from one of these, λ gt11M182, was subcloned in both orientations into pBR322, forming the plasmids pMxB7 and pMxB9, both of which produced β pilin, as did pMxB12, a *Hind*III deletion derivative of pMxB7. In HB101(pMxB12), the *M. bovis* pilin protein was shown to be primarily localized in the inner membrane. The entire 939-base-pair insert of pMxB12 was sequenced, revealing a ribosome binding site just upstream of the coding region and an AT-rich region further upstream containing some potential RNA polymerase recognition sites. The translation of the sequence predicts a six-amino-acid leader sequence preceding the phenylalanine that begins the mature protein. Codon usage analysis of the *M. bovis* β pilin gene revealed greater use of the CUA codon for leucine than usual for a well-expressed *Escherichia coli* gene. Comparisons of the *M. bovis* EPP63 β pilin protein sequence with other pilin gene sequences are presented.

Moraxella bovis is the primary cause of bovine infectious keratoconjunctivitis (16, 22, 24), a cattle disease causing temporary, and occasionally permanent, blindness (2, 24). Irradiation of the cow eye with UV radiation before inoculation greatly facilitates infection and disease with *M. bovis* (24, 25, 27). *M. bovis* produces a hemolysin that is required for virulence (39, 43). The other factor that appears to be involved in *M. bovis* virulence is its pili, since only piliated (P^+) strains are able to infect experimentally inoculated cattle (38).

The state of *M. bovis* piliation has a marked effect on its colony morphology in a manner analogous to that seen with *Neisseria gonorrhoeae* (7, 38). Electron microscopy studies have shown a correlation between the occurrence of *M. bovis* colonies that corrode agar and contain P^+ bacteria and the noncorroding colonies that contain nonpiliated (P^-) bacteria (7, 38). Based on these differences in colony morphology, it was observed that P^+ to P^- transitions occurred at a frequency of about 1 in 10^4 when 12- to 30-h-old P^+ colonies were subcultivated and sometimes at a higher frequency when older colonies were streaked (7). The reverse transition, from P^- to P^+ , was seen at various frequencies, from none observed to about 1 in 10^4 (7). The P^+ strains also are capable of twitching motility (20, 21) and are competent for DNA transformation (8), whereas P^- derivatives exhibit neither of these properties. The pili are adhesive and cause P^+ bacteria to autoagglutinate, hemagglutinate, and form a pellicle on the surface of broth cultures (44).

Different strains show serologically different pilus types (44), and a pilus vaccine induces protective immunity, but only against a homologous strain challenge exposure (40). Recently it has been discovered that different strains make

pilin with various molecular weights and that a single strain is capable of producing more than one type of pilin protein (G. Schoolnik, unpublished observation). For strain EPP63, we have named the two pilins so far observed α and β .

The *M. bovis* pilin proteins appear to be part of a conserved family of pilin proteins that share extensive amino-terminal amino acid sequence homology and all have the modified amino acid *N*-methyl-phenylalanine (MePhe) as the first residue (23, 30). These include the pilins of *Moraxella nonliquefaciens* (15), *N. gonorrhoeae* (23, 48), *Bacteroides nodosus* (30), and *Pseudomonas aeruginosa* (47). This paper describes, as far as we know, the first isolation and physical analysis of an *M. bovis* pilin gene.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and media. *M. bovis* EPP63 was isolated from a calf with bovine infectious keratoconjunctivitis and generously provided by G. W. Pugh, Jr., Agricultural Research Service, Ames, Iowa. The *E. coli* strains Y1088, Y1089, and Y1090 (55) were used for growing and screening λ gt11 and λ gt11 clones. *Escherichia coli* HB101 (9) was used for transformation studies, and *E. coli* JM101 (31) was used for transfection studies and as a host for producing single-stranded M13 template DNA for sequencing. The phage λ gt11 (56) was used as a vector for genomic cloning. The M13 phage mp8, mp9, and mp18 (32, 37) were used for both subcloning and sequencing. The plasmid pBR322 (6, 52) was used for subcloning.

M. bovis strains were grown on GC agar base (Difco Laboratories, Detroit, Mich.) with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Strains Y1088, Y1089, and Y1090 were grown on L agar containing 100 μ g of carbenicillin per ml (Sigma Chemical Co., St. Louis, Mo.), as were HB101 strains containing drug-resistant plasmids.

DNA isolation and manipulation. Total DNA was prepared

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by the method of Hull et al. (26), and plasmid DNA was isolated by the polyethylene glycol method of Humphreys et al. (27) and dialyzed exhaustively against DNA dialysis buffer (10 mM Tris [pH 7.9], 10 mM NaCl, 1 mM EDTA). Large-scale lysates of λ phage were prepared as described by Schumm et al. (49), and the DNA was extracted with formamide as described by Davis et al. (12). The oligonucleotide was synthesized with an Applied Biosystems Automated synthesizer, using solid-phase phosphoramidite chemistry as previously described (3, 10). The nucleotides were cleaved from the resin, purified by polyacrylamide gel electrophoresis, and eluted from the gel. The purified products were then concentrated by using DEAE-Sephacel and ^{32}P end-labeled by the use of T4 polynucleotide kinase (P-L Biochemicals, Inc., Milwaukee, Wis.) by the method of Maxam and Gilbert (29).

Restriction endonucleases were purchased from New England BioLabs, Inc., Beverly, Mass.; Bethesda Research Laboratories, Inc. (BRL), Gaithersburg, Md.; Boehringer Mannheim Corp., New York; or Promega Biotec, Madison, Wis. Restriction enzyme digests, agarose gel electrophoresis, hybridizations, and isolation of restriction enzyme-generated DNA fragments were carried out as described previously (28).

Antisera and Western blotting. Pili were purified by the method of Schoolnik et al. (48), emulsified in complete Freund adjuvant, and administered in 75- μg doses of pilin protein by intramuscular and subcutaneous injections to female New Zealand White rabbits. Eight weeks later, a booster dose, prepared with incomplete Freund adjuvant, was administered. After an additional 2 weeks, blood was obtained by cardiac puncture and the immune sera were filter-sterilized and stored at 4°C. Western blotting of whole-cell extracts was performed as described previously (11). For some experiments, antiserum raised against EPP63 β pili was preadsorbed against Y1090 and EPP63 P^- cells and diluted 1:1,000 or 1:10,000 before use.

Construction of phage libraries and subclones. *M. bovis* EPP63 DNA from colonies producing β pili was cleaved by sonication to an approximate size range of 0.2 to 6 kilobase pairs (kb). DNA polymerase I (Boehringer Mannheim) was used to fill in ends, and the DNA was methylated with *EcoRI* methylase (New England BioLabs) and then phosphorylated. *EcoRI* linkers (Collaborative Research, Inc.) were blunt-end ligated overnight at 15°C to the sonicated DNA, using T4 DNA ligase (BRL). The ligated material was then cleaved with *EcoRI*, ethanol precipitated, resuspended in ligation buffer (50 mM Tris [pH 7.4], 10 mM MgCl_2 , 10 mM dithiothreitol, 1 mM ATP), and ligated to *EcoRI*-cleaved $\lambda\text{gt}11$. The resulting recombinant phage were packaged using a λ in vitro packaging system (BRL) and were plated on Y1090 cells.

Screening recombinant $\lambda\text{gt}11$ phage by hybridization to the oligonucleotide probe was done by the method of Blattner et al. (5), except that hybridizations and washes were carried out at 37°C instead of 68°C. For rescreening, potential positives were picked, suspended in 0.1 M NaCl–10 mM Tris (pH 7.4) saturated with chloroform, and placed overnight at 4°C. The phage were then titrated, replated separately on small plates at a density of about 100 plaques per plate, and screened by the above procedures.

The plasmid subclones pMxB7 and pMxB9 were produced in the following manner. DNA from the recombinant phage $\lambda\text{gt}11\text{M}182$ was cleaved with *EcoRI*, and the resulting 1.9-kb *M. bovis* insert fragment was isolated from an agarose gel. DNA from pBR322 was cleaved with *EcoRI*, treated with

calf alkaline phosphatase (Boehringer Mannheim) at 37°C for 30 min, and then heat-killed by placing it into a heating block at 65°C for 10 min. After phenol-chloroform extraction and ethanol precipitation, the cleaved pBR322 DNA was resuspended in ligation buffer, and ligated to the 1.9-kb insert fragment with T4 DNA ligase overnight at 15°C. The ligated material was used to transform competent HB101 cells, which were then plated on LB agar containing 100 μg of carbenicillin per ml. Twelve colonies were picked and purified, and plasmid DNA was isolated from each. Restriction digest analysis showed that plasmids containing a single copy of the 1.9-kb insert had been obtained and that both orientations of the insert were obtained.

DNA sequencing. Restriction fragments from plasmids pMxB7, pMxB9, and pMxB12 were isolated and subcloned into the M13 phage mp8, mp9, and mp18. The M13 subclones were then sequenced by the dideoxy chain termination method of Sanger et al. (45, 46), using [α - ^{35}S]dATP for all sequencing reactions (4).

Fractionation of cellular components. Periplasmic proteins of strains HB101(pBR322) and HB101(pMxB12) were isolated by the mild osmotic shock method of Neu and Heppel (36). Periplasmic proteins were concentrated about 20-fold by evaporation in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, N.Y.). The pellets remaining after release of the periplasmic proteins were further separated into cytoplasmic, inner membrane, and outer membrane fractions by the method of Smit et al. (50), which involves breaking open the cells with a French press, pelleting the membranes away from the cytoplasmic fraction in an ultracentrifuge, and further separating the membranes into inner and outer membranes with a sucrose gradient. Proteins from the different fractions were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and screened with antiserum as described above.

RESULTS

$\lambda\text{gt}11$ cloning and screening with an oligonucleotide probe.

The amino-terminal regions of *M. bovis* EPP63 α and β pilins are identical in amino acid sequence to *N. gonorrhoeae* pilin proteins (see Fig. 6). Before the publication of the DNA sequence of an *N. gonorrhoeae* pilin gene from strain MS11 by Meyer et al. (33), the sequence was determined in our laboratory (M. Koomey, J. Hardy, J. Rothbard, and S. Falkow, manuscript in preparation). Therefore, it was possible to produce an oligonucleotide representing the first eight amino acids. The oligonucleotide made, 5'-AATCATCAGCTCGATAAGGGTAAA-3', corresponds to the strand that would hybridize to mRNA. Since the *M. bovis* pilin DNA sequence coding for the first eight amino acids could differ from the above sequence while retaining identical amino acid sequence, we empirically determined the hybridization stringency conditions necessary for use of the oligonucleotide as a probe for the *M. bovis* pilin gene. Figure 1 shows the patterns of hybridization seen when the oligonucleotide 5' end labeled with ^{32}P (29) was hybridized to a genomic Southern blot containing *HaeII*-digested DNAs from a variety of *M. bovis* strains. After a low-stringency 25°C wash with 0.5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate), most of the lanes showed two strong bands of hybridization after a 15-h exposure, and at least three additional bands appeared after longer exposure. When the wash temperature, and thus the stringency, was increased, overall hybridization was decreased. At 37°C there was predominantly one band of hybridization in most

of the lanes, and after a 42°C wash no hybridization could be detected.

Random fragments of chromosomal DNA from β -pilated *M. bovis* EPP63 were ligated into the *EcoRI* site of λ gt11. After phage growth, the λ gt11 particles were plated on *E. coli* Y1090 and plaques were screened for hybridization to the oligonucleotide probe at 37°C as described above. Approximately 100,000 plaques were screened, and 29 potential positives were picked, resuspended in 0.1 M NaCl–10 mM Tris (pH 7.4) saturated with chloroform, and rescreened. Six were clearly positive upon rescreening, and *E. coli* Y1089 lysogens of these λ gt11 clones were prepared. The lysogens were heat induced, isopropyl thio- β -D-galactopyranoside (IPTG) induced, pelleted, boiled in loading buffer, and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose and probed with rabbit anti-pilus antisera and ¹²⁵I-labeled protein A. Lysogens of two phage, λ gt11M161 and λ gt11M182, were seen to produce a protein that comigrated with *M. bovis* EPP63 β pilin and bound anti-pilus antisera (Fig. 2). Production of this protein was later shown to be independent of IPTG (Fig. 2), indicating that transcription of the protein did not depend upon the β -galactosidase promoter present in λ gt11.

Restriction analysis of λ gt11M182 DNA revealed an *EcoRI* insert of about 1.9 kb. This insert was subcloned in both orientations into pBR322 at the *EcoRI* site (plasmids pMxB7 and pMxB9). Both plasmids produced pilin. Thus, pilin production in the λ gt11 and in the pBR322 clones was presumably from an *M. bovis* DNA promoter sequence.

Restriction maps of pMxB7 and pMxB9 were determined

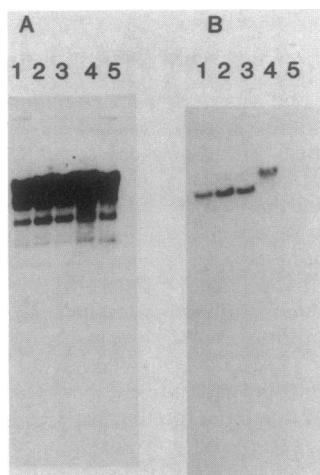


FIG. 1. Oligonucleotide probe hybridized to genomic digests of *M. bovis* DNAs. Total DNA from each of the listed *M. bovis* strains was digested to completion with *HaeII*, run on a 1% agarose gel, and transferred to nitrocellulose paper. Hybridizations were carried out at 25°C in 10 ml of hybridization solution (0.75 M NaCl, 0.075 M sodium citrate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% sodium PP_i, 0.5% sodium dodecyl sulfate, 100 μ g of boiled calf thymus DNA per ml) containing about 10⁷ cpm of 5' ³²P-end-labeled *N. gonorrhoeae* oligonucleotide. Lanes 1, EPP63, β , P⁺; lanes 2, EPP63, α , P⁺; lanes 3, EPP63, α , P⁻; lanes 4, MAC, P⁺; lanes 5, IBH64, P⁺. (A) Filter washed at 25°C in 100 ml of 0.075 M NaCl–7.5 mM sodium citrate and exposed to X-ray film with screens at –70°C for 15 h. (B) Filter washed again at 37°C in 100 ml of 0.075 M NaCl–7.5 mM sodium citrate and exposed to film for 3 days.

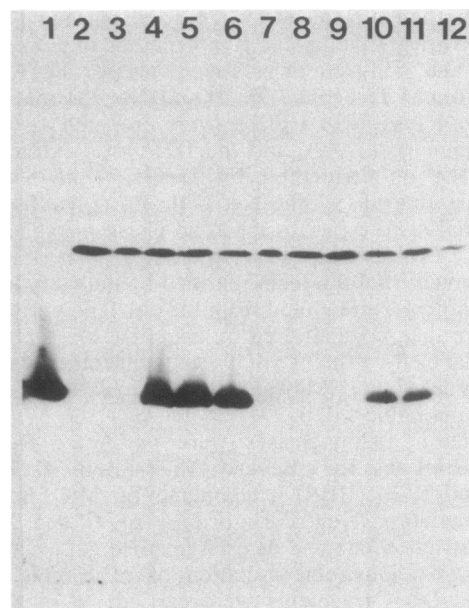


FIG. 2. Western transfer analysis of proteins expressed from cloned *M. bovis* DNA homologous to the *N. gonorrhoeae* pilin-specific oligonucleotide probe. Samples were electrophoresed on a 12.5% sodium dodecyl sulfate-acrylamide gel and electrophoretically transferred to nitrocellulose paper. The resulting filter was incubated with antisera and ¹²⁵I-labeled protein A as described in the text. Lane 1, EPP63, β , P⁺; lane 2, HB101(pMxB4); lane 3, HB101(pMxB5); lane 4, HB101(pMxB7); lane 5, HB101(pMxB8); lane 6, HB101(pMxB9); lane 7, HB101(pMxB10); lane 8, HB101(pMxB11); lane 9, HB101(pBR322); lane 10, Y1089(λ gt11M182), heat induced plus 10 μ g of IPTG per ml; lane 11, Y1089(λ gt11M182), heat induced, no IPTG; lane 12, Y1089(λ gt11M182), 10 μ g of IPTG per ml, no heat induction.

(Fig. 3). The position of the pilin amino terminus was ascertained by hybridization of the amino-terminal oligonucleotide to Southern transferred restriction fragments of pMxB7 and pMxB9 (data not shown). This analysis localized the amino terminus of the pilin gene between the *HaeIII* site and the leftmost *HincII* site of the pMxB7 insert. A deletion derivative of pMxB7 was generated by cleavage with *HindIII* and introduced into *E. coli* HB101. A map of the resultant plasmid, pMxB12, is shown in Fig. 3. This *E. coli* HB101(pMxB12) strain produced pilin.

Subcellular localization of *M. bovis* pilin protein in *E. coli*. For a determination of where the *M. bovis* pilin protein was localized when produced within an *E. coli* cell, HB101(pMxB12) was fractionated into periplasmic, cytoplasmic, inner membrane, and outer membrane. *E. coli* HB101(pBR322) was treated similarly as a negative control. Western blot analysis revealed that the majority of the pilin antigen appeared within the inner membrane fraction (Fig. 4). Since the lesser amount present in the outer membrane fraction may reflect the presence of inner membrane contamination, the pilin may have resided entirely within the inner membrane.

Sequencing the *M. bovis* β pilin gene clone. The strategy used for sequencing the *EcoRI*-*HindIII* insert fragment of pMxB12 is shown in Fig. 3. The sequence of the entire insert was determined in both orientations from recombinant M13 phage generated by cloning specific pMxB12 restriction fragments. The nucleotide sequence of 939 base pairs starting from the *HindIII* site is shown in Fig. 5, together with the

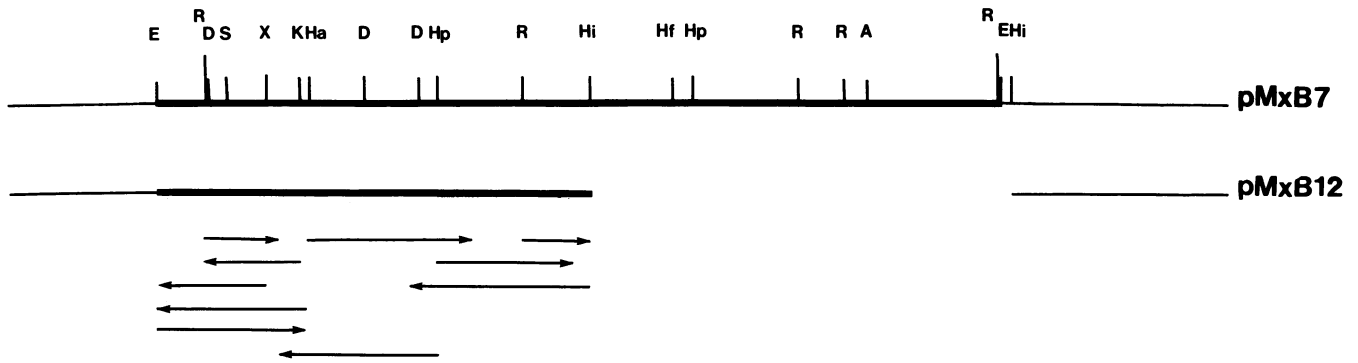


FIG. 3. Restriction maps of insert regions of pMxB7 and pMxB12, and strategy for determining the sequence of the pMxB12 insert. Restriction enzyme sites for *AccI* (A), *DdeI* (D), *EcoRI* (E), *HaeIII* (Ha), *HinfI* (Hf), *HindIII* (Hi), *HpaI* (Hp), *KpnI* (K), *RsaI* (R), *SinI* (S), and *XbaI* (X) are shown; the *KpnI* site is also an *RsaI* site, and the *HpaI* sites are also *HincII* sites. In the map of pMxB7, the thick line represents the region of *M. bovis* insert DNA and the thin lines represent parts of pBR322. The plasmid pMxB12 is a derivative of pMxB7 lacking the *HindIII* fragment represented by the central missing portion of the lines. The arrows beneath the map show the direction of sequencing and the length of the sequence determined by using restriction fragments subcloned into the M13 phages mp8, mp9, and mp18.

predicted amino acid sequence of the pilin gene. The sequence AGGAG present on the 5' side of the starting ATG is homologous to known ribosome binding sites in procaryotes (17) and is identical to the ribosome binding sequence of an *N. gonorrhoeae* pilin gene (33). Which sequences in the 5' flanking region of the insert of pMxB12 functioned as a promoter was unclear. The sequence from the *HindIII* site to the starting ATG is A/T rich (67.5% A+T), and one can

identify at least eight regions which could conform to a Pribnow box (41). Three of these have weak homology in the appropriate position to -35 regions of known procaryotic promoters (41); however, none has sufficient homology to known promoters to allow prediction of where the transcription starts.

The inferred amino acid sequence of the β pilin protein based on the DNA sequence shows a six-amino-acid leader

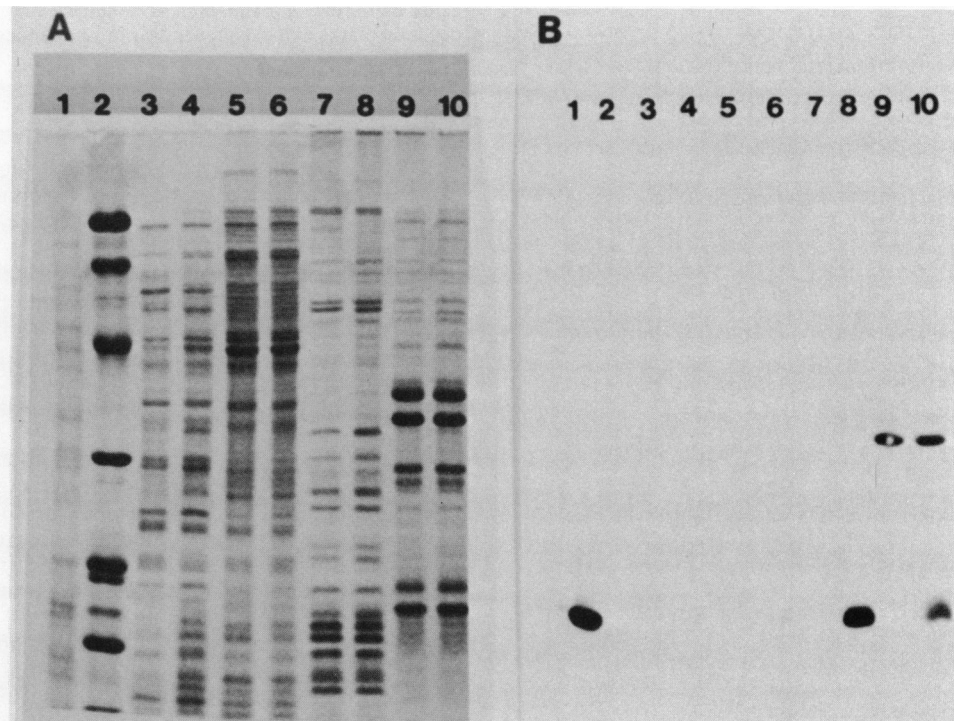


FIG. 4. Subcellular localization of *M. bovis* pilin protein in *E. coli* HB101 containing pMxB12 by Western analysis. Samples were electrophoresed on 12.5% sodium dodecyl sulfate-polyacrylamide gels. (A) Coomassie-blue stained gel. Lane 1, EPP63, β , P⁺; lane 2, molecular weight standards, phosphorylase *b* (94,000 [94K]), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20K), and lysozyme (14.3K); lane 3, HB101(pBR322) periplasmic proteins released by mild osmotic shock; lane 4, HB101(pMxB12) periplasmic proteins; lane 5, HB101(pBR322) cytoplasmic proteins; lane 6, HB101(pMxB12) cytoplasmic proteins; lane 7, HB101(pBR322) inner membrane proteins; lane 8, HB101(pMxB12) inner membrane proteins; lane 9, HB101(pBR322) outer membrane proteins; lane 10, HB101(pMxB12) outer membrane proteins. (B) Western transfer analysis of gel containing identical samples and amounts listed in A, using antisera against EPP63 β pilin as described in the text.

sequence preceding the phenylalanine which begins the mature protein. This leader is remarkably similar to the leader sequences reported for the *N. gonorrhoeae*, *P. aeruginosa*, and *B. nodosus* pilin genes (Fig. 6).

Codon usage. Codon usage in the β pilin gene is shown in Table 1. The level of *M. bovis* pilin protein production, using *E. coli* transcription and translation machinery, may be influenced by differences in codon usage frequencies. In *E. coli*, genes of highly expressed proteins rarely contain codons recognized by minor or weakly interacting tRNA species (19). Of these rarely used codons (AUA, CUA, CGA, CGG, AGA, GGA, GGG), only CUA (Leu) is used in the β pilin sequence. Of other codons used only sparingly in the highly expressed genes of *E. coli* (UCA, UCG, ACG, CCC) (18), only one use of CCC (Pro) occurs in the β pilin sequence. The use of the CUA codon may be significant since it is the major codon used for Leu in the pilin gene, occurring in 5 of 11 Leu codons. In this it differs greatly from the codon usage of the *N. gonorrhoeae* pilin gene (33), the *B. nodosus* pilin gene (13), and the *P. aeruginosa* pilin gene (B. L. Pasloske, B. B. Finlay, and W. Paranchych, FEBS Lett., in press), which do not use the CUA codon at all. Since the pilin gene is apparently expressed at a high rate in *M. bovis*, it will be interesting to find out whether the tRNA species recognizing the CUA (Leu) codon is more prevalent in *Moraxella* species than in the other microbial species.

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HindIII
AAGCTTTTCCGCTCTTAATGCGTTCACCAATTTGGTAATACCTGCTAACTACCAATAT60
TATTATCCCAACGTTGTTGGTGATTGCAATAACCGTTCAGACATCAAAATTTAAAAAAT120
                RsaI
TTTTGGTAAAAAAGAAAAAATTTGACATATTTAATCAATTTAAAAAATAGCAAAAA180
TATGCTATTTTTAGACAAAATCTTAAAAATTTCTCAATTTTGGCTATTTTTCCAAA240
AACAGCCCAATCCGCTAAAAAGTTGGCACAGACAATGCAACAGTATGAGTGTAAAGTTA300
                HpaI
GCAAAATATGTTTTAAAATGCTAATCAGTTAACTTAAACAACTGGGCAATATCGCCCTTA360
DdeI
TGAACCTAAGGAGTTTCATTATGAAAGCTCAAAAAGGTTTCCACCTTATCGAATTGATGATT420
MetAsnAlaGlnLysGlyPheThrLeuIleGluLeuMetIle
GTTATCGCCATTATTGGTATCCTAGCTGCAATCGCTCTACCTGCTTACCAAGACTATATC480
ValIleAlaIleIleGlyIleLeuAlaAlaIleAlaLeuProAlaTyrGlnAspTyrIle
DdeI
TCTAAGTCTCAAACTACTCGTGTAGTTGGCGAACTAGCTGCTGGTAAAACTGCTGTGGAT540
SerLysSerGlnThrThrAlaValValGlyIleLeuAlaAlaGlyLysThrAlaValAsp
GCTGCTTTGTTTGGGGTAAAACTCCCAAACTAGGTAAGCTGCTAACGACACTGAAGAA600
AlaAlaLeuPheGluGlyLysThrProLysLeuGlyLysAlaAlaAsnAspThrGluGlu
                KpnI
                RsaI
HaeIII
GACATCGGCTTACCCTACTGGCGGTACCGCTGTTCTAAGTGTAGTGTAGTGTAAAT660
AspIleGlyLeuThrThrThrGlyGlyThrAlaArgSerAsnLeuMetSerSerValAsn
                XbaI
ATCGGTGGTGGTGCATTTGCAACTGGTGGCTGGCACTCTAGAAGCTACTTTGGGTAACCGT720
IleGlyGlyGlyAlaPheAlaThrGlyAlaGlyThrLeuGluAlaThrLeuGlyAsnArg
GCCAATAAAGACATCGCTGGTGTCTTACTCAATCTCGTGATCGGGAAGGTGTATGG780
AlaAsnLysAspIleAlaGlyAlaValIleThrGlnSerArgAspAlaGluGlyValTrp
SniI
ACTTGCACATTAACGGTTCTGCCGCTCCAGGTTGGAAATCTAAGTTCCTACTGCTGGT840
ThrCysThrIleAsnGlySerAlaAlaProGlyTrpLysSerLysPheValProThrGly
TGTAAAGATAATCATCTGATTGCAAAACACAACAACACTCCCTTGGAGTGGTGTTT900
CysLysGlu
                EcoRI
TTAACATAAAGACATAAATTTACAATAAATCCGAATTC939
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FIG. 5. Nucleotide sequence and translation of pMxB12 insert encoding β pilin of *M. bovis* EPP63. The predicted amino acid sequence of the open reading frame containing the coding sequence of pilin is shown; the amino acids present in the mature protein are in bold type. The AGGAG sequence underlined upstream of the starting Met is the putative ribosome binding site. Selected restriction endonuclease sites are underlined and designated by their names above the coding sequence.

1. <i>M. bovis</i> α		MeFTLLIEELMIVVIA
2. <i>M. bovis</i> β	MN	AQKG MeFTLLIEELMIVVIA
3. <i>M. nonliquefaciens</i>		MeFTLLIEELMIVVIA
4. <i>N. gonorrhoeae</i>	MNT	LQKG MeFTLLIEELMIVVIA
5. <i>B. nodosus</i>	MKS	LQKG MeFTLLIEELMIVVIA
6. <i>P. aeruginosa</i>	MK	AQKG MeFTLLIEELMIVVIA

1.	IIGILAAIALPAYQDYISKSQTTTRVSGEELA
2.	IIGILAAIALPAYQDYISKSQTTTRVSGEELA
3.	IIGILAAIALPAYQDYIARAQVSEAFLLADE
4.	IVGILAAVALPAYQDYTARAQVSEAFLLADE
5.	IIGILAAIPAYNDYIARSEQAAEGLTLLADE
6.	IIGILAAIATPQYQNYVARSEGGASALLASVN

1.	AGKTAVDAAALFEGKTPVLSSEESSTKKNENIG
2.	AGKTAVDAAALFEGKTPKLGKAANDSTENEDIG
3.	GLKGTGIST-----
4.	GQKSAVTTEYLYLNHGKWPENNTSAGVASPPS
5.	GLKVRISDHLSESGECKGDANPASGSLGND
6.	PLKTTTVEEALSRGWSVKSSTGTEDATKKEV

1.	LTSSETSTKPRSNLMAASVELTGFAADN	GAG
2.	LTTTGGTARSNLMSBVNIGGAFATGAG	
4.	DIKGKYVKEVEVKNQVDTATMLKNSG	VNNEI
5.	KGKYALATIDGDYINKDADLEKNGCK	VVI
6.	PLGVAADANKLGTIALKPTDPA	DTTLT

1.	TISATLGNKANGDIAX?VI?QEBT	TDCVWT
2.	LEATLGNRRAMKDELAGAVITQSRDAE	VVWT
4.	KGKLSLWARRENGSVKWF	CGQPVTRTDD
5.	TYGQGTAGEKISKLIVKLLVLDQF	VNGSY
6.	FTMGGAAGPKNKKGKIIITLRT	ADGLWKCT

1.	CKIDGSAAKYKKEKFNPTGCVKKS	K.
2.	CTINSSAAPGWKSKFVPTGCKE.	
4.	TVADAKDKKEIDTKHLPSTCRDKASDAK.	
5.	KYNEGETDLELKFIPNAVKN.	
6.	DQDEQFIPKGC	S.R.

FIG. 6. Comparison of the amino acid sequences of pilins α and β from *M. bovis* (this work and G. Schoolnik, manuscript in preparation) to pilin sequences from *M. nonliquefaciens* NCTC 7784 SC-c (15), *N. gonorrhoeae* MS11 (33, 48), *B. nodosus* 198 (13, 30), and *P. aeruginosa* PAK (Pasloske et al., in press). Shaded residues are identical to those from *M. bovis* β pilin. Only the first 49 amino acids of the *M. nonliquefaciens* sequence are reported. The two question marks in the α sequence represent amino acids of unknown identity.

DISCUSSION

The data presented here describe the successful use of an oligonucleotide probe based on the amino-terminal DNA sequence of an *N. gonorrhoeae* pilin gene to isolate a pilin gene from a λ gt11 genomic library of the β -pilated *M. bovis* EPP63. Some of the λ gt11 recombinant phage isolated produced a protein that comigrated with the β pilin protein of EPP63 and reacted strongly with antisera generated against EPP63 β pili. This protein appears to be produced from a promoter present in the *M. bovis* DNA since inserts present in both orientations in the *EcoRI* site of pBR322 produce the protein.

The *M. bovis* β pilin protein is shown to be present primarily within the inner membrane fraction when present in an *E. coli* cell. This is consistent with the findings of two groups that the *B. nodosus* pilin protein produced in *E. coli* was located primarily in the membrane fraction, although they did not report which of the membrane fractions contained the majority of the pilin (1, 14).

The sequence of the 939-base-pair insert present in pMxB12 shows that the β pilin structural gene encodes a protein with a six-amino-acid leader and 151 amino acids of the mature protein. Figure 6 shows a comparison of the translated β pilin sequence with the amino acid sequence of the EPP63 α pilin, the amino acid sequencing of which will be described elsewhere (G. Schoolnik, manuscript in preparation). The α and β pilins are about 70% homologous at the amino acid level. Most of the homology is localized to the amino-terminal third of the molecules, with only one amino acid difference occurring in the first 57 amino acids. The

TABLE 1. Codon usage of the *M. bovis* pilin gene^a

Amino acid	Codon	No. of codons in gene	Amino acid	Codon	No. of codons in gene	Amino acid	Codon	No. of codons in gene	Amino acid	Codon	No. of codons in gene		
Phe	UUU	2	Ser	UCU	7	Tyr	UAU	1	Cys	UGU	1		
	UUC	2		UCC	2		UAC	1		UGC	1		
Leu	UUA	0		UCA	0	Term	UAA	1	Term	UGA	0		
	UUG	4		UCG	0		UAG	0		Trp	UGG	2	
Leu	CUU	2	Pro	CCU	2	His	CAU	0	Arg	CGU	4		
	CUC	0		CCC	1		CAC	0		CGC	0		
	<u>CUA</u>	5		CCA	1		CAA	4		<u>CGA</u>	0		
	<u>CUG</u>	0		CCG	0		CAG	0		<u>CGG</u>	0		
Ile	AUU	5	Thr	ACU	12	Asn	AAU	2	Ser	<u>AGU</u>	1		
	AUC	8		ACC	5		AAC	5		AGC	0		
	<u>AUA</u>	0		ACA	0		AAA	8		Arg	<u>AGA</u>	0	
Met	AUG	3	Ala	ACG	0	Lys	AAG	2	Arg	<u>AGG</u>	0		
Val	GUU	3		GCU	17		Asp	GAU		2	Fly	<u>GGU</u>	16
	GUC	1			GCC			3		GAC		4	GGC
	GUA	3	GCA		3	GAA		6	<u>GGA</u>	0			
	GUG	1	GCG		1	GAG		2	<u>GGG</u>	0			

^a Codons corresponding to minor or weakly interacting tRNA species in *E. coli* (19) are underlined.

remaining two-thirds of the pilin proteins are about 50% homologous, with most of the differences occurring in blocks of three to eight residues.

In addition to the N-terminal amino acid sequence homology, members of the MePhe family of pilin proteins all have identical cysteine contents. Each possesses two cysteine residues per subunit, and these have been proven for *N. gonorrhoeae* to form a disulfide loop that encompasses segments of sequence heterogeneity that encode strain-specific antigenic determinants (42). The *M. bovis* α and β pilin sequences also exhibit this structural feature. Furthermore, a synthetic peptide corresponding to the β pilin cysteine loop is recognized only by the β pilus antisera, indicating that it contains a β -specific epitope (G. Schoolnik, unpublished observation).

Hybridization studies in which a cloned *N. gonorrhoeae* pilin gene was used as a probe demonstrated that a single *N. gonorrhoeae* genome contains several pilin-related sequences (34). The phenomenon of multiple bands seen after hybridization of the *N. gonorrhoeae* oligonucleotide to *Hae*II-digested *M. bovis* DNAs at low stringency, followed by a single band being present at high stringency (Fig. 1), is very similar to the situation seen when this same *N. gonorrhoeae* oligonucleotide is used to probe *N. gonorrhoeae* genomes (J. Rothbard, J. M. Koomey, S. Falkow, and G. Schoolnik, submitted for publication). It therefore may be that the multiple bands shown in Fig. 1 represent multiple pilin genes. We are currently using the cloned β pilin gene described in this paper as a probe to investigate this possibility. It is interesting that this pattern does not hold up for the *P. aeruginosa* pilin gene, which appears in only a single copy in the *P. aeruginosa* genome (Pasloske et al., in press).

In both *M. bovis* and *N. gonorrhoeae*, piliation is required for DNA transformation (8, 51). Perhaps this correlation exists also with *B. nodosus* and *P. aeruginosa*.

The six-amino-acid leader sequence of the *M. bovis* β pilin gene predicted by the DNA sequence presented here is much shorter than previously described signal sequences (54). Meyer et al. have proposed that the seven-amino-acid leader sequence of a pilin protein in *N. gonorrhoeae* MS11 is the signal sequence needed for transport of the pilin protein across the bacterial membrane (33). Although both leaders contain a positively charged residue (Lys), a feature charac-

teristic of the start of signal peptides (35, 54), they do not contain all of the usual features seen in other procaryotic signal sequences (35). McKern et al. proposed that the highly conserved, hydrophobic, amino-terminal region of the MePhe family of mature pilin proteins might be involved in the transport of the proteins through cell membranes (30), and Watson noted that the amino-terminal regions of these pilin proteins resemble normal signal peptides but differ in not being cleaved between the Ala-Ala bond at residues 17 and 18 of the mature proteins (54; Fig. 6). Elleman and Hoyne proposed that the seven-amino-acid leader of the *B. nodosus* pilin, together with the amino-terminal sequence of mature pilin, might constitute a signal sequence (13). Thus, we believe that the distinctive feature of the MePhe class of pilin proteins is not an unusual length or amino acid composition of the signal sequence, but rather that the mature protein is cleaved at an unusual position, leaving much of the signal present in the mature protein, and that an N-methylation of the first Phe also occurs. Whether the methylation of the Phe occurs before, during, or after cleavage of the leader peptide is not known. Interestingly, about half of the pilin molecules of *N. gonorrhoeae* (23), *B. nodosus* (30), and *M. bovis* (G. Schoolnik, unpublished observation) lack the N-MePhe residue and start with a Thr, the next amino acid. The N-terminal hydrophobic region of the MePhe pilins have been proposed to be involved in subunit-subunit interactions to form functional pili (23, 30, 47). It may be that the high level of amino acid conservation seen in the amino-terminal regions of pilin proteins from genera as divergent as *Moraxella*, *Neisseria*, *Bacteroides*, and *Pseudomonas* is due to the necessity of maintaining the dual functions of membrane transport and subunit interactions.

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