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Q pili enhance the attachment of *Moraxella bovis* to bovine corneas *in vitro*

W. W. Ruehl,[1,2]* C. Marrs,[3] M. K. Beard,[4] V. Shokooki,[2] J. R. Hinojoza,[2] S. Banks,[1] D. Bieber[2] and J. S. Mattick[5]

Departments of [1]Pathology and [2]Comparative Medicine, Stanford University School of Medicine, Stanford, California USA.

[3]Department of Epidemiology, School of Epidemiology and Public Health, University of Michigan, Ann Arbor, Michigan, USA.

[4]Department of Animal Health, The University of Sydney, Camden, New South Wales, Australia.

[5]Centre for Molecular Biology and Biotechnology, University of Queensland, St Lucia, Queensland, Australia.

Summary

Moraxella bovis*, the causative agent of infectious bovine keratoconjunctivitis, exhibits several virulence factors, including pili, haemolysin, leukotoxin, and proteases. The pili are filamentous appendages which mediate bacterial adherence. Prior studies have shown that Q-piliated *M. bovis* Epp63 are more infectious and more pathogenic than I-piliated and non-piliated isogenic variants, suggesting that Q pili *per se*, or traits associated with Q-pilin expression, promote the early association of Q-piliated bacteria with bovine corneal tissue. In order to better evaluate the role of Q pili in *M. bovis* attachment, several *M. bovis* strains and a recombinant *P. aeruginosa* strain which elaborates *M. bovis* Q pili but not *P. aeruginosa* PAK pili, were evaluated using an *in vitro* corneal attachment assay. For each strain tested, piliated organisms attached better than non-piliated bacteria. *M. bovis* Epp63 Q-piliated bacteria adhered better than either the I-piliated or non-piliated isogenic variants. Finally, recombinant *P. aeruginosa* organisms elaborating *M. bovis* Q pili adhered better than the parent *P. aeruginosa* strain which did not produce *M. bovis* pili. These results indicate that the presence of pili, especially Q pili, enhances the attachment of bacteria to bovine cornea *in vitro*.

Received 18 November 1991; revised 16 July 1992; accepted 16 September 1992. *For correspondence: Deprenyl Animal Health Inc., 8000 West 110th Street, Suite 115, Overland Park, Kansas 66210, USA. Tel. (913) 338 2120; Fax (913) 338 3804.

Introduction

Moraxella bovis is the primary cause of infectious bovine keratoconjunctivitis (IBK), a highly contagious ocular disease of cattle that can result in temporary (or occasionally permananent) blindness (Hughes and Pugh, 1970). Haemolysin and pili have been established as *M. bovis* virulence factors (Pugh and Hughes, 1968; Pedersen *et al.*, 1972). The pili are surface appendages of the bacterium composed of repeating, homologous polypeptide subunits termed pilin. The pili of *M. bovis* are classified as type 4, and the pilin molecule contains a methylated phenylalanine (mPhe) residue at the amino terminus (Mattick *et al.*, 1987). We have previously described, characterized, and sequenced two distinct pilin molecules, termed I and Q, from *M. bovis* strain Epp63 (Ruehl *et al.*, 1988) and have also cloned and sequenced both pilin genes (Marrs *et al.*, 1985; Fulks *et al.*, 1990).

Type 4 pili are also found on *Moraxella nonliquefaciens* (Froholm and Sletten, 1977), *Neisseria gonorrhoeae* (Hermodson *et al.*, 1978; Schoolnik *et al.*, 1984), *Neisseria meningitidis* (Hermodson *et al.*, 1978), *Bacteriodes nodosus* (McKern *et al.*, 1983; 1985), and *Pseudomonas aeruginosa* (Sastry *et al.*, 1983). The pilin subunits of these species range in size from 145 to 160 amino acids and are translated as prepilin. Prepilin has a six or seven residue leader sequence which when cleaved leaves the modified amino acid, methylphenylalanine, as the first reside of the mature protein (Bradley, 1980; Mattick *et al.*, 1987; Ruehl *et al.*, 1988). The 32 residue amino-terminal regions of all type 4 mPhe pilins exhibit at least 90% homology and are very hydrophobic. The carboxy-terminal two-thirds of these subunits, however, is relatively hydrophilic and has many variable and hypervariable domains. As such, this region is the source of both structural and antigenic variations between species (Mattick *et al.*, 1987).

It appears that the hydrophobic amino-terminal region may have a dual function. First, it has been proposed that it acts, perhaps in conjunction with the short leader sequence, as a signal sequence marking the peptide for transport to the cell membrane (Elleman and Hoyne, 1984). Second, it has been hypothesized that the region is involved in pili morphogenesis. There have been two mechanisms proposed for involvement of this region in morphogenesis: the region could be responsible for subunit–subunit interactions during pili assembly (Hermodson *et al.*, 1978; McKern *et al.*, 1983; Sastry *et al.*,

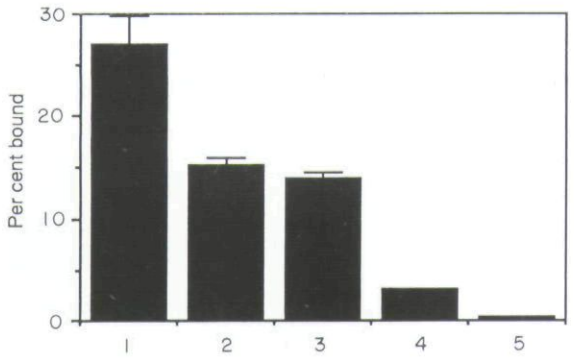

Fig. 1. Attachment *in vitro* of labelled *M. bovis* and *P. aeruginosa* to bovine corneal epithelium. Various piliated or non-piliated *M. bovis* and *P. aeruginosa* strains were incubated with intact bovine corneas. Details of the experimental methods and data analysis are in the text. 1, Q-piliated *M. bovis* Epp63; 2, recombinant *P. aeruginosa* which expresses *M. bovis* Epp63 Q pili; 3, I-piliated *M. bovis* Epp63; 4, non-piliated *M. bovis* Epp63; 5, PAK-piliated *P. aeruginosa*. The error bars indicate ±2 standard deviations.

1983); and/or the amino-terminal region might interact with other essential factors involved in the assembly system. The second hypothesis was suggested on the basis of the highly conserved sequences in this region between the various bacterial species expressing type 4 pili, and is supported by the fact that *B. nodosus* pili and *M. bovis* pili can be assembled from cloned pilin genes expressed by recombinant *P. aeruginosa* (Mattick *et al.*, 1987; Beard *et al.*, 1990).

Attachment of pathogenic *M. bovis* to the bovine corneal epithelial surface is an early (probably the first) event leading to colonization, then to infection and clinical disease. This attachment process appears to be mediated, at least in part, by the pili which are elaborated on the bacterial cell surface. It has been observed that piliated *M. bovis* attach better to bovine corneas *in vitro* than do non-piliated bacteria of the same strain (Jackman and Rosenbusch, 1984). Furthermore, piliated *M. bovis* cause disease in the experimentally inoculated eyes of calves, while non-piliated isogenic variants do not (Ruehl *et al.*, 1988). Additionally, the Q-piliated variant of strain Epp63 was found to be significantly more infectious for experimentally inoculated eyes than I-piliated or non-piliated variants of Epp63. These results indicated that Q pili *per se*, or traits associated with Q-pilin expression, promote the early association of Q-piliated bacteria with bovine corneal tissue.

We have constructed a recombinant *P. aeruginosa* strain which expresses *M. bovis* Epp63 Q pilin and elaborates Q-pili filaments to the exclusion of the normal indigenous *Pseudomonas* (PAK) pili (Beard *et al.*, 1990). We then compared this recombinant *P. aeruginosa* with the parent, non-piliated *P. aeruginosa* strain and various *M. bovis* strains for their ability to adhere to intact bovine corneas *in vitro*.

Results and Discussion

Data regarding attachment of *M. bovis*, parent *P. aeruginosa*, and recombinant *P. aeruginosa* bacteria to bovine corneas is presented in Fig. 1. The previous finding (Jackman and Rosenbusch, 1984) that piliated *M. bovis* 118F attached to corneas significantly better than non-piliated bacteria was confirmed for two additional strains, Epp63 and Tifton-1, suggesting that this is a general phenomenon for *M. bovis*.

It is apparent in Fig. 1 that *M. bovis* Q piliated Epp63 bacteria attached significantly better than I-piliated or non-piliated isogenic variants of the same strain. This greater adherence capability correlates well with the prior observation that Q-piliated organisms are significantly more infectious than I-piliated or non-piliated variants of the same strain when inoculated into the eyes of calves (Ruehl *et al.*, 1988; Lepper and Power, 1988), and suggests an attachment role for Q pili *per se*, or for some factor associated with Q-piliation.

Figure 1 also shows that a recombinant *P. aeruginosa* bacteria which elaborates *M. bovis* Q pili, but which no longer expresses PAK pili, attached significantly better than the PAK-pili-expressing *P. aeruginosa* parent strain. The enhancement of attachment provided by Q pili was greater for *M. bovis* than for *P. aeruginosa* bacteria. The molecular basis for this is unknown but might be related to the fact that the recombinant organisms apparently express fewer pili per bacterial cell than the Q-piliated *M. bovis* (Fig. 2). Alternatively, other pilus-associated or co-regulated adhesins may be present in Q-piliated *M. bovis* but absent from the Q-piliated recombinant *P. aeruginosa* that contains only the Q-pilin subunit gene from *M. bovis* (Beard *et al.*, 1990). Yet another possible explanation is that the assembly or presentation of the Q pilus is slightly different in *P. aeruginosa* compared with the native Q pilus, and that this could result in the reduced adherence of the recombinant.

There are now several examples of *Escherichia coli* pili in which the adhesive molecule is a minor pilus component separate from the major structural subunit (Lindberg *et al.* 1984; Klemm and Christiansen, 1987). In contrast, the evidence to date for type 4 piliated bacteria has indicated that the major structural subunit is also the adhesin. Irvin *et al.* (1989) and Lee *et al.* (1989) have reported that the adherence of *P. aeruginosa* PAK-piliated bacteria to human epithelial cells is directly attributable to the pilin subunit molecule. There is also some evidence that the pili of *N. gonorrhoeae* is the adhesin (Schoolnik *et al.*, 1984; Rothbard *et al.*, 1985). In agreement with the above

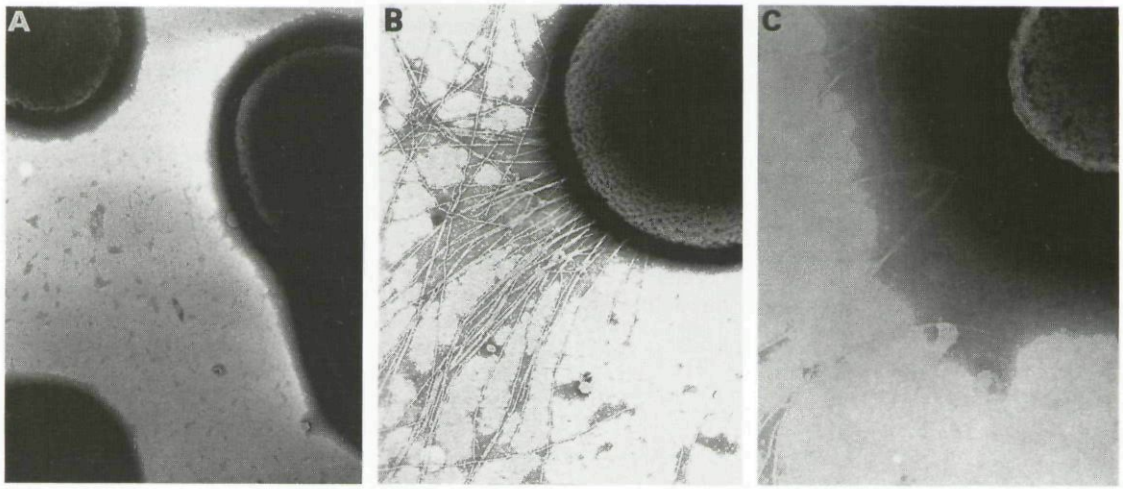

Fig. 2. Transmission electron micrographs of piliated and non-piliated bacteria. Organisms were grown 20–24 h at 37°C on GC or L-agar, then transferred to copper grids, negatively stained with 1% phosphotungstate and photographed using a transmission electron microscope. Strain: A, non-piliated *M. bovis* Epp63, ×12000; B, Q-piliated *M. bovis* Epp63, ×18000; C, recombinant *P. aeruginosa* which expresses *M. bovis* Epp63 Q pili, ×18000.

results, this paper provides evidence the *M. bovis* Q-pili-mediated adherence must be a direct result of the presence of the Q-pilin molecule.

Experimental procedures

Microorganisms

The bacterial strains used in this study were: *M. bovis* Epp63 (non-piliated, I-piliated, and Q-piliated isogenic variants), *M. bovis* Tifton-1 (piliated and non-piliated), *P. aeruginosa* recombinant PAK/2Pfs (MXB/Mxb6) which express *M. bovis* Epp63 Q pilin, but not PAK pilin, and exhibits plasmid-mediated resistance to carbenicillin (Beard *et al.*, 1990), and the parental *P. aeruginosa* strain PAK/2Pfs (ATCC 53308) (Bradley, 1974) which express PAK pilin and fails to grow in the presence of carbenicillin.

Solid media for cultivating microorganisms and performing colony counts consisted of blood agar (Remel), GC (gonococcal) agar base (Difco Laboratories) to which was added 2% (v/v) IsoVitaleX (BBL Microbiology Systems), and L-agar with or without 75 μg ml^{-1} carbenicillin added. The expression of pilin and the presence of pili were confirmed by immunoblotting (Beard *et al.*, 1990) and electron microscope inspection (Fig. 2) of phosphotungstate-stained organisms, respectively.

Preparation of radiolabelled bacteria

For each strain, bacterial growth from two plates was gently suspended in 750 μl of ice-cold Hanks Balanced Salt Solution (HBSS) (Sigma) and 250 μl of the following salt solution: 50 mM Tris-HCl, pH 8, 50 mM Na acetate, 140 mM NaCl, 5 mM $CaCl_2$, 4 mM KCl, 2 mM $MgCl_2$, 1% bovine serum albumin (BSA), and 1% glycerol. To determine the concentration of each of these bacterial suspensions, a 1 μl aliquot of each suspension was diluted $1:10^6$, inoculated onto a blood agar plate, incubated overnight at 37°C, and the colonies counted. Also, a single drop of each bacterial suspension was transferred to a microscope slide and Gram-stained. Ten fields were examined using ×1000 magnification to determine the number of bacterial aggregates present. If more than five bacterial aggregates were counted, the suspension was discarded.

The remainder of each bacterial suspension was labelled by incubation with 20 μCi ml^{-1} of L-[4′5-^{3}H]-proline (Amersham) for 2 min at 22°C, followed by incubation with 20 μg ml^{-1} unlabelled proline under the same conditions.

Preparation of corneas

Adult cattle eyes were obtained from a local slaughter house and were transported in ice-cold HBSS to which 100 μg ml^{-1} gentamicin had been added. Only corneas without visible opacity were used. Each entire cornea was placed on a flat surface with the epithelial cell layer facing upward. A second matching plastic surface containing holes was placed on top thereby forming wells: the floor of each well thus consisted exclusively of corneal epithelium. The wells were filled with HBSS until use in the adherence assay, at which time the HBSS was removed and the tissue was washed twice with phosphate-buffered saline (PBS).

Adherence assay

The adherence assay was a modification of one described by Jackman and Rosenbusch (1984). One-hundred microlitres of the radiolabelled bacterial suspension were added to each well and the apparatus was incubated 60 min at 37°C in a moist environment. The reaction was terminated by removal of the unattached bacteria and the wells washed with PBS. Using a biopsy skin punch a 9 mm diameter piece of exposed cornea was harvested from each well, transferred to 1 ml of 2% SDS and boiled for 10 min. The tubes were centrifuged 2 min; 100 μl of the supernatant from each tube (containing radiolabelled bacterial components) were added to 3 ml of scintillation fluid, and radioactivity determined as counts per minute (c.p.m.) using a Beckman 8000

beta counter. In each assay, the background radioactivity (c.p.m.) was determined for one well which contained buffer only. The percentage of bacteria attached was calculated by the following formula:

$$\frac{\text{(cfu attached to cornea)} - \text{(cfu on background cornea)}}{\text{(cfu added to experimental cornea)}} \times (100)$$

The results were derived from three to five experiments with each microorganism. The significance of the differnces in attachment between strains was determined using Students *t*-test with paired values and a two-tailed hypothesis.

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References

Beard, M.K., Mattick, J.S., Moore, L.J., Mott, M.R., Marrs, C.F., and Egerton, J.R. (1990) Morphogenic expression of *Moraxella bovis* fimbriae (pili) in *Pseudomonas aeruginosa. J Bacteriol* **172:** 2601–2607.

Bradley, D.E. (1974) The adsorption of *Pseudomonas aeruginosa* pilus-dependent bacteriophages to a host mutant with non-retractile pili. *Virology* **58:** 149–163.

Bradley, D.E. (1980) The function of *Pseudomonas aeruginosa* PAO pili: twitching motility. *Can J Microbiol* **26:** 146–154.

Elleman, T.C., and Hoyne, P.A. (1984) Nucleotide sequence of the gene encoding pilin of *Bacteroides nodosus,* the causal organism of ovine footrot. *J Bacteriol* **160:** 1184–1187.

Froholm, L.O., and Sletten, K. (1977) Purification and *N*-terminal sequence of a fimbrial protein from *Moraxella nonliquifaciens. FEBS Lett* **73:** 29–32.

Fulks, K.A., Marrs, C.F., Stevens, S.P., and Green, M.R. (1990) Sequence analysis of the inversion region containing the pilin genes of *Moraxella bovis. J Bacteriol* **172:** 310–316.

Hermodson, M.A., Chen, K.C.S., and Buchanan, T.M. (1978) *Neisseria* pili proteins: amino terminal amino acid sequences and identification of an unusual amino acid. *Biochemistry* **17:** 442–445.

Hughes, D.E., and Pugh, G.W. (1970) A five year study of IBK in a beef herd. *J Am Vet Med Assoc* **157:** 443–451.

Irvin, R.T., Doig, P., Lee, K.K., Sastry, P.A., Paranchych, W., Todd, T., and Hodges, R.S. (1989) Characterization of the *Pseudomonas aeruginosa* pilus adhesin: confirmation that the pilin structural protein subunit contains a human epithelial cell-binding domain. *Infect Immun* **57:** 3720–3726.

Jackman, S.H., and Rosenbusch, R.F. (1984) *In vitro* adherence of *Moraxella bovis* to intact corneal epithelium. *Curr Eye Res* **3:** 1107–1112.

Klemm, P., and Christiansen, G. (1987) Three *fim* genes required for the regulation of length and mediation of adhesion of *E. coli* type 1 fimbriae. *Mol Gen Genet* **208:** 439–445.

Lee, K.K., Doig, P., Irvin, R.T., Paranchych, W., and Hodges, R.S. (1989) Mapping the surface regions of *Pseudomonas aeruginosa* PAK pilin: the importance of the C-terminal region for adherence to human buccal epithelial cells. *Mol Microbiol* **3:** 1493–1499.

Lepper, A.W., and Power, B.E. (1988) Infectivity and virulence of Australian strains of *Moraxella bovis* for the murine and bovine eye in relation to pilus serogroup sub-unit size and degree of piliation. *Austr Vet J* **65:** 305–309.

Lindberg, F.P., Lund, B., and Normark, S. (1984) Genes of pyelonephritogenic *E. coli* required for digalactoside-specific agglutination of human cells. *EMBO J* **3:** 1167–1173.

Marrs, C.F., Schoolnik, G., Koomey, J.M., Hardy, J., Rothbard, J., and Falkow, S. (1985) Cloning and sequencing of a *Moraxella bovis* pilin gene. *J Bacteriol* **163:** 132–139.

Mattick, J.S., Bills, M.M., Anderson, B.J., Dalrymple, B., Mott, M.R., and Egerton, J.R. (1987) Morphogenic expression of *Bacteriodes nodosus* fimbriae in *Pseudomonas aeruginosa. J Bacteriol* **169:** 33–41.

McKern, N.M., O'Donnell, I.J., Inglis, A.S., Stewart, D.J., and Clark, B.L. (1983) Amino acid sequences of pilin from *Bacteroides nodosus* (Strain 198), the causative agent of ovine footrot. *FEBS Lett* **164:** 149–153.

McKern, N.M., O'Donnell, I.J., Stewart, D.J., and Clark, B.L. (1985) Primary structure of pilin protein from *Bacterioides nodosus* (strain 216): comparison with the corresponding protein from strain 198. *J Gen Microbiol* **131:** 1–6.

Pedersen, K.B., Froholm, L.O., and Bovre, K. (1972) Fimbriation and colony type of *Moraxella bovis* in relation to conjunctival colonization and development of keratoconjunctivitus in cattle. *Acta Pathol Microbiol Scand Sect B Microbiol* **80:** 911–918.

Pugh, G.W., and Hughes, D.E. (1968) Experimental bovine infectious keratoconjunctivitus caused by sunlamp irradiation and *Moraxella bovis* infection: Correlation of hemolytic ability and pathogenicity. *Am J Vet Res* **29:** 835–839.

Rothbard, J.B., Fernandez, R., Wang, L., Teng, N.N., and Schoolnik, G.K. (1985) Antibodies to peptides corresponding to a conserved sequence of gonococcal pilins block bacterial adhesin. *Proc Natl Acad Sci USA* **82:** 915–919.

Ruehl, W.W., Marrs, C.F., Fernandez, R., Falkow, S., and Schoolnik, G.K. (1988) Purification, characterization, and pathogenicity of *Moraxella bovis* pili. *J Exp Med* **168:** 983–1002.

Sastry, P.A., Pearlstone, J.R., Smillie, L.B., and Paranchych, W. (1983) Amino acid sequence of pilin isolated from *Pseudomonas aeruginosa* PAK. *FEBS Lett* **151:** 253–256.

Schoolnik, G.K., Fernandez, R., Tai, Y.Y., Rothbard, J., and Gotschlich, E.C. (1984) Gonococcal pili: primary structure and receptor binding domain. *J Exp Med* **159:** 1351–1370.