Pili (fimbriae) of *Branhamella* Species

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PURPOSE: **Pili (fimbriae) have frequently been found to be involved in the attachment of bacteria to mucosal epithelial cells, an important initial step in the disease process. The purpose of this study was to determine if *Branhamella catarrhalis* expresses type 4 pili.**

MATERIALS AND METHODS: **Piliated *B. catarrhalis* phenotypic characteristics of colony morphology, agar corrosion, twitching motility, competence for deoxyribonucleic acid (DNA) transformation, autoagglutination, and pellical formation were observed. DNA was isolated from *Branhamella* spp. and used in genomic Southern hybridizations with a *Moraxella bovis* pilin gene as a probe. Electron microscopy of negatively stained bacteria was carried out to visualize pili.**

RESULTS: ***B. catarrhalis* has several (but not all) of the phenotypic characteristics that are related to the presence of type 4 (MePhe) pili in closely related *Moraxella* spp., including competence for DNA transformation, autoagglutination, pellicle formation, colony morphology, and pitting of agar. The one phenotype we have not found that is generally characteristic of type 4 piliated bacteria is twitching motility. Genomic Southern hybridization analysis using a cloned *M. bovis* Q pilin gene as a probe reveals DNA homologous to the Q pilin gene in *B. catarrhalis, Branhamella ovis, Branhamella caviae,* and *Branhamella cuniculi.* Examination of *B. catarrhalis* strain ATCC25240 by electron microscopy reveals two different kinds of pili. One kind appears similar to other type 4 pili, whereas a second class is short pili extending outward from all portions of the bacteria.**

CONCLUSION: **Phenotypic, electron-microscopic, and hybridization data are all consistent with type 4 pili being present on some *B. catarrhalis* strains.**

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Bacterial colonization of mucosal surfaces depends upon attachment of bacteria to mucosal epithelial cells, often mediated by means of pili or fimbriae [1], which are protein filaments that extend from the surface of the bacteria. Pili are composed of up to 10,000 polymerized protein subunits called pilins. There are many different types of pili as defined by morphology and by functions such as pellicle formation in broth, and there are many types of pilins as defined by sequence comparisons [2]. In addition to pilin, some pili have one or more minor structural components [3,4], and in the case of the *Escherichia coli* P and type 1 pili, one of these minor subunits is the actual adhesin responsible for the binding of the pilus to the receptor molecule present on the eukaryotic cell surface [4–6]. Not only can a single bacterial species have strains that express structurally different classes of pili [2,7], but a single bacterium may have the capacity to express more than one class of pili, either sequentially or simultaneously [7,8].

Type 4 (MePhe) pili are present on a wide variety of pathogenic bacteria [9], including *Moraxella bovis* [10,11], *Moraxella nonliquefaciens* [12], *Neisseria gonorrhoeae* [13,14], *Neisseria meningitidis* [12], *Bacteroides nodosus* [15], *Pseudomonas aeruginosa* [16], and *Vibrio cholerae* [17]. As currently defined by *Bergey's Manual of Systematic Bacteriology,* the family *Neisseriaceae* contains the genera *Neisseria, Moraxella, Acinetobacter,* and *Kingella* [18]. A newly named genus, *Psychrobacter* [19], is also related to these genera. The genus *Moraxella* is subdivided into two subgenera, *Moraxella* and *Branhamella* [18]. *B. catarrhalis* was previously classed as *Neisseria catarrhalis* [18].

Our laboratory has been studying the type 4 (MePhe) class of pili present on *Moraxella* sp. and those of related genera. These studies include the cloning of the pilin gene from *M. bovis*, which causes bovine keratoconjunctivitis, and the use of this pilin gene to examine the presence and genetic structure of type IV pilin genes in other members of the *Neiseriaciae* [10] (Marrs CF, Stevens SP, Weir S, and Patel P, manuscript in preparation). This article discusses what we know about the pili of *Branhamella* spp.

TYPE 4 PILI IN *MORAXELLA* SPP.

The type 4 pilins all share extensive amino-terminal amino acid sequence homology and, with the exception of *V. cholerae,* they all contain the modified amino acid N-methylphenylalanine (MePhe) as the first residue of the mature protein [9,13,20]. That these conservations are important in a functional sense has been demonstrated by experiments in which *P. aeruginosa* bacteria processed and assembled *B. nodosus* pilins into pili structurally and immunologically indistinguishable from authentic *B. nodosus* pili [21,22]. Recently, similar experiments with our *M. bovis* pilin gene expressed in *P. aeruginosa* produced bacteria

that actually had pili made up of both *M. bovis* pilin and *P. aeruginosa* pilin (Beard MKMG, Mattick JS, Moore LJ, *et al*, manuscript submitted). Functionally, the pili of *M. bovis* attach to the corneal epithelium and only piliated strains are able to infect experimentally exposed cattle [11,23]. Similarly, pili appear to promote the pathogenicity of *N. gonorrhoeae* by mediating their attachment to human host mucosal surfaces [24].

MATERIALS AND METHODS

Bacterial Strains

B. catarrhalis strains ATCC8176, ATCC8193, ATCC25238, ATCC25239, and ATCC25240 were obtained from the American Type Culture Collection. *B. catarrhalis* strain B10 and *Moraxella lacunata* ATCC17956 were obtained from Dr. E. Juni, University of Michigan. *M. bovis* strain Epp63 was obtained from G.W. Pugh, Jr., Agricultural Research Service, Ames, Iowa. *N. meningitidis* strain M1080X was obtained from J.M. Koomey, University of Michigan.

Laboratory Techniques

Deoxyribonucleic acid (DNA) isolations and genomic Southern hybridizations were carried out as previously described [25]. All strains were grown on GC agar base (Difco Laboratories, Detroit, Michigan) with 1 percent IsoVitaleX (BBL Microbiology Systems, Cockeysville, Maryland). Two methods were used to assay for twitching motility, a hanging drop method of Depiazzi and Richards [26], and a plate method described by Henrichsen [27]. Electron microscopy of negatively stained bacteria was performed with a Zeiss EM-10CA transmission electron microscope using the procedures described by Hayat [28].

PHENOTYPES OF PILIATED BACTERIA

For *N. gonorrhoeae*, *M. bovis*, *M. nonliquefaciens*, and other bacteria [9], type 4 piliation is associated with a variety of phenotypes. These include being located on the poles of the bacteria [9], colony morphology and agar corrosion [23,29], twitching motility [30,31], competence for DNA transformation [32], autoagglutination, and pellicle formation on the surface of broth cultures [33]. Of these phenotypes, autoagglutination and pellicle formation are also associated with other classes of pili [2], and bacteria like *Haemophilus influenzae* can be competent for DNA transformation in the absence of pili [34].

We have observed several of these phenotypes usually associated with type 4 piliated bacteria in our studies of *B. catarrhalis*. The colonies are similar in appearance to P^+ *M. bovis* or *N. gonorrhoeae*. However, unlike *M. bovis* or *N. gonorrhoeae*, which show a readily detectable switch to a distinct colony type when they become non-piliated, we have yet to observe a *B. catarrhalis* with the normal nonpiliated colony morphology. Because we have not yet isolated a *B. catarrhalis* that lacks pili, it is not clear how many of the following phenotypes we observe are specifically associated with being piliated. For all strains we have examined, *B. catarrhalis* colonies pit the agar, and the bacteria autoagglutinate and form pellicles in broth cultures. Most *B. catarrhalis* strains are capable of undergoing DNA transformation with related DNA [35,36] and in fact the ability of *B. catarrhalis* to be transformed by *M. nonliquefaciens* DNA was the first evidence that it was related to *Moraxella* sp. [37]. Strain B10 is an exception, being non-competent for transformation despite forming a pellicle and having colonies that pit the agar and have P^+ morphology. One characteristic type 4 piliation phenotype appears absent since two different twitching motility assays (described in Materials and Methods) were negative for every *B. catarrhalis* strain we tested, whereas the piliated controls from *M. bovis*, *M. lacunata*, and *N. meningitidis* all twitched. The presence of several phenotypes commonly correlated with type 4 piliation, coupled with the relatively close relationship between *B. catarrhalis* and several *Moraxella* spp. known to possess type 4 pili raised the question of whether *B. catarrhalis* might express type 4 pili.

GENETICS OF TYPE IV PILI

There are both similarities and differences in the genetic organization of type 4 pilin genes in different bacterial species. One similarity is that *P. aeruginosa*, *N. gonorrhoeae*, and *M. bovis* pilin genes all appear to use *rpo*N (σ^{54})-dependent promoters [38,39]. In terms of numbers of pilin genes, *P. aeruginosa* strains only have a single copy of the pilin gene in each genome [40,41], and most serotypes of *B. nodosus* also only have a single gene, but some are organized with the genes *fim*A, *fim*C, *fim*Z in order in the same orientation, where *fim*A and *fim*Z are both pilin genes [42]. *M. bovis* produces serologically different pilus types, and each strain is capable of producing one or the other of two pilus types [33,43]. *M. bovis* strain Epp63 expresses either Q pilin (formerly called beta) or I pilin (formerly called alpha). We have cloned and sequenced the *M. bovis* Q pilin gene [10], and shown that expression of either Q or I pilin alternates via a 2-kilobase inversion of genomic DNA [25]. *N. gonorrhoeae*, by contrast, contains multiple pilin gene loci in every strain and transitions from P^+ to P^- and between different P^+ pilin types are often accompanied by chromosomal DNA rearrangements [44–46]. Strain MS11 can express at least seven different pilin genes [45], has two regions of its chromosome that act as pilin expression loci [45], and has many other sites that contain silent variant pilin sequences [45,47]. Most strains each have a single pilin expression locus and multiple silent variant pilin sequences [48]. All silent copies are only partial genes, lacking the common N-terminal coding sequence of pilin [49,50]. Antigenic variation from one P^+ type to another is a result of transformation of chromosomal DNA from other gonococci that have autolysed, followed by a *rec*A^+ dependent recombination event between silent copy donor DNA and the recipient expression locus [51]. Transitions from P^+ to P^- sometimes involve deletions at the expression loci [45,49], and sometimes involve sequences from silent copy loci recombining into the expression locus to produce pilin molecules, which are defective in assembly [52].

As part of a general survey of bacteria related to *M. bovis* that we are carrying out, we hybridized our *M. bovis* Q pilin gene probe to genomic Southern blots containing DNAs from a variety of organisms. **Figure 1** shows the hybridization patterns seen for four *Branhamella* spp. cleaved with *Eco*RI. Hybridization of the Q pilin probe to *Branhamella ovis* was moderately strong, about comparable with that seen with

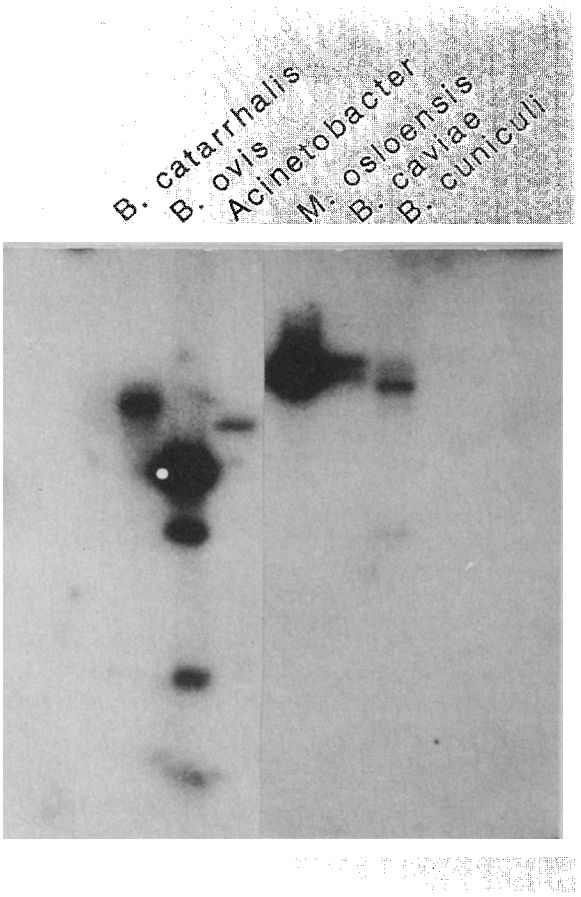

Figure 1. Hybridization using a *M. bovis* Q pilin gene probe to the following genomic DNAs cleaved with *Eco*RI: *B. catarrhalis* ATCC25238; *B. ovis* ATCC19575; *Acinetobacter calcoaceticus* ATCC15304; *M. osloensis* ATCC19962; *B. caviae* ATCC14659; *B. cuniculi* ATCC14688. Hybridization conditions were as described previously [44].

Moraxella osloensis (Figure 1), whereas *B. catarrhalis*, *Branhamella caviae*, and *Branhamella cuniculi* all hybridized at lesser, but clearly detectable levels. It is important to note that this hybridization data just tells us that DNA sequences similar to the *M. bovis* Q pilin gene are present in these bacteria. It does not tell us that type 4 pili are being expressed on the bacterial surface, or even guarantee that a complete pilin gene is present.

The observation that there are three bands of hybridization seen with *Eco*RI cleaved *B. ovis* DNA may mean that it contains more than one pilin gene. The fact that *B. catarrhalis* only has one band that hybridizes after *Eco*RI cleavage does not rule out it having more than one gene or partial gene. In *M. bovis* Epp63, the complete and partial pilin gene sequences are close enough that they appear on the same *Eco*RI fragment. It does, however, appear to rule out multiple, distinct pilin gene loci as is found in *N. gonorrhoeae* [48].

ELECTRON MICROSCOPIC EXAMINATION OF *B. CATARRHALIS*

In electron-microscopic studies, type 4 pili can be observed as long filaments extending from the pole of the cell [9,11]. An example of normal type 4 piliated bacteria is shown in **Figure 2**, *top left*.

The only report of pili being observed on *Branhamella* spp. was that of Wistreich and Baker [53], who reported that they failed to see any pili on two American Type Culture Collection strains of *N. catarrhalis* (ATCC8176,ATCC8193). In contrast, they reported that one *N. catarrhalis* strain from the culture collection of the University of Southern California had two different pili types. Some organisms had numerous short pili emerging from their outer borders, with an occasional long pilus extending beyond them, whereas with other bacteria the longer type predominated. Our observations of *B. catarrhalis* strain using the electron microscope match these descriptions quite well. Figure 2, *top right* and *lower left* and *right*, shows our electron micrographs of *B. catarrhalis* strain ATCC25240. In Figure 2, *top right* and *lower left* (at different magnification), can be seen examples of long, thin pili that appear very similar to the type 4 pili shown in Figure 2, *top left*. Figure 2, *bottom right*, shows the other class of short, thick pili with knobby ends that can be seen extending outward from all portions of the bacteria. Thus both phenotypic, electron-microscopic, and hybridization data all are in agreement that type 4 pili might be present on *B. catarrhalis*. The electron-microscopic data show that an additional, non-type 4 class of pili is also present. The relative roles of these two different classes of pili on the adherence properties and development of *B. catarrhalis* are exciting questions remaining to be explored.

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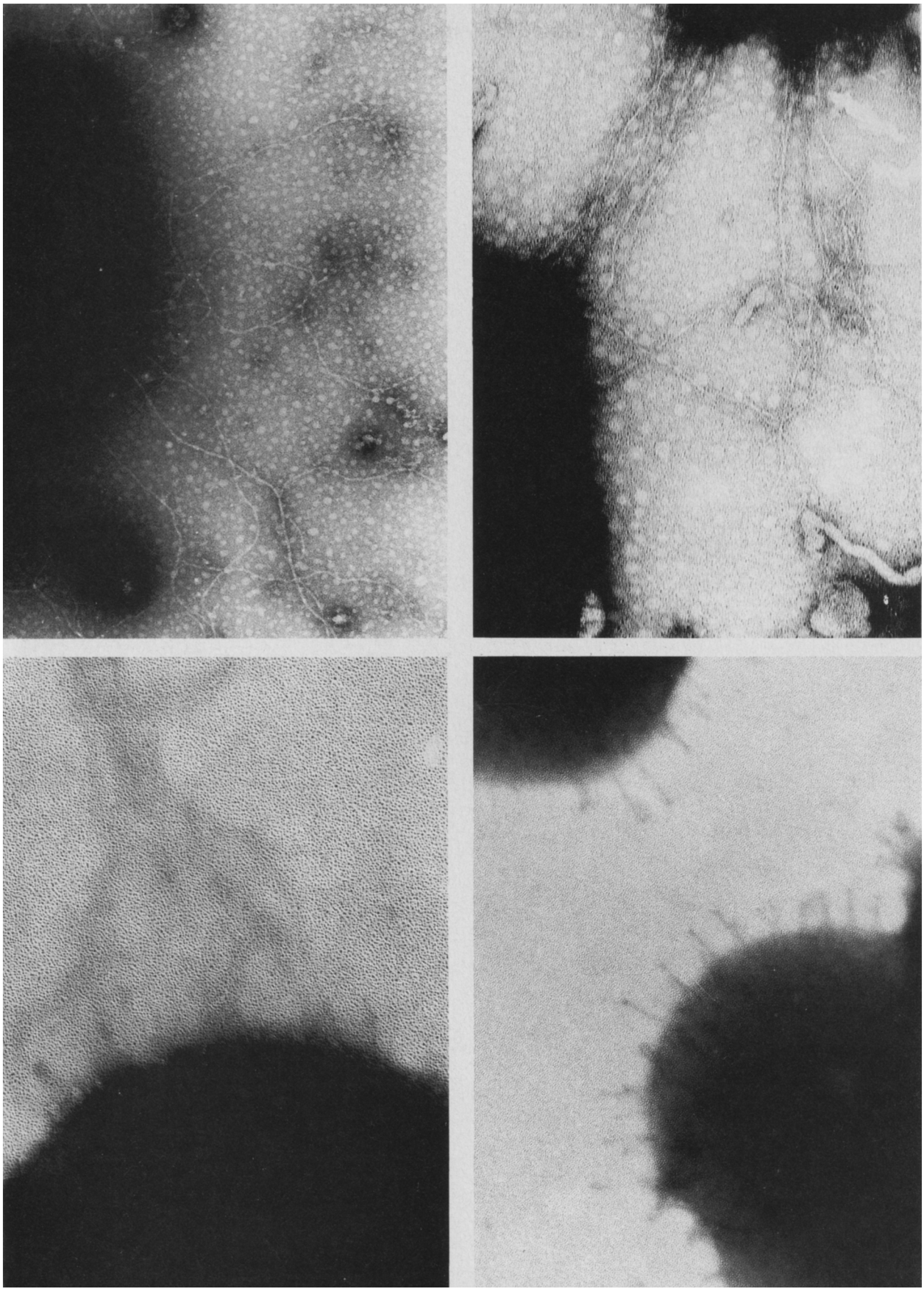

Figure 2. Top left, type 4 pili of *M. bovis* Epp63 P^+, magnification × 94,000; **top right**, long pili of *B. catarrhalis* ATCC25240, magnification × 140,000; **bottom left**, potential type 4 pili of *B. catarrhalis* ATCC25240, magnification × 110,000; **bottom right**, potential non-type 4 pili of *B. catarrhalis* ATCC25240, magnification × 91,500.

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