The type 4 pilin of *Moraxella nonliquefaciens* exhibits unique similarities with the pilins of *Neisseria gonorrhoeae* and *Dichelobacter (Bacteroides) nodosus*

**TONE TØNJUM,**¹ **CARL F. MARRS,**² **FRANK ROZSA**² and **KJELL BØVRE**¹

¹Kaptein W. Wilhelmsen og Fries Bakteriologiske Institutt, University of Oslo, Rikshospitalet, N-0027 Oslo 1, Norway
²Department of Epidemiology, University of Michigan, Ann Arbor, Michigan 48109, USA

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*Moraxella nonliquefaciens* is a bacterium which is part of the normal flora of the human upper respiratory tract and is an occasional cause of disease. Using a previously cloned type 4 pilin gene (*tfpA*) from *Moraxella bovis* as a hybridization probe, we have cloned an 826 bp Sau3AI fragment which contains an *M. nonliquefaciens* type 4 pilin gene (*tfpA*) from strain NCTC 7784. The pilin gene is expressed in *Escherichia coli*. We have examined NCTC 7784 and nine other *M. nonliquefaciens* strains by genomic Southern hybridization using *tfpA* as a probe, and they all appeared to have more than one pilin gene. While the predicted amino acid sequence of the *M. nonliquefaciens* tfpA pilin has conserved regions as compared to pilins of *M. bovis* and *M. lacunata*, it also shows similarities to both the type 4 pilin of *Neisseria gonorrhoeae* and the type 4 pilin of *Dichelobacter nodosus* (formerly *Bacteroides nodosus*).

**Introduction**

*Moraxella nonliquefaciens* is part of the normal bacterial flora in the human upper respiratory tract, particularly the nasal cavity (Bøvre, 1984; Henriksen, 1973). It has also been isolated from blood, sometimes associated with septicemic disease, from cerebrospinal fluid, and from eye, lower respiratory tract, and other local infections (Brorson et al., 1983; Bøvre, 1984; Ebright et al., 1982; Graham et al., 1990; Lobue et al., 1985; Rosett et al., 1976; Sharma, 1974). Generally, the species is considered to be of low pathogenicity, depending on reduced host resistance for invasion and clinical manifestations. Together with *Moraxella bovis* and *Moraxella lacunata, M. nonliquefaciens* makes up the very closely related 'classical moraxellae' (Bøvre, 1984). As studied in *M. bovis*, in particular, *M. nonliquefaciens* has a wide variety of phenotypes associated with piliation (fimbriation), including spreading and/or corroding growth on agar (Bøvre et al., 1970; Bøvre & Frøholm, 1972a, b), twitching motility (Henrichsen et al., 1972), haemagglutination (Schoolnik et al., 1982), surface pellicle formation in static broth (Bøvre et al., 1970; Bøvre & Frøholm, 1972a) and competence for genetic transformation (Bøvre & Frøholm, 1972b). *M. nonliquefaciens* also undergoes phase variation of colony morphology (Bøvre & Frøholm, 1972a), switching between spreading-corroding (SC) growth and smooth, non-corroding (N) colonies. Piliation is found in SC, but generally not in N colonies (Bøvre & Frøholm, 1972a, b). Spontaneous change from SC to N may be observed easily in surface cultures on solid medium. Spontaneous N to SC variation is generally less easy to observe, sometimes only detectable through the occasional formation of a pellicle on the surface of static broth culture. Thus, it seems that *M. nonliquefaciens* undergoes the phase variation associated with type 4 pili that is seen in other species (Bergstrom et al., 1986).

Members of the type 4 (MePhe) class of bacterial pili are found on a variety of Gram-negative bacteria (Dalrymple & Mattick, 1987), including *M. bovis* (Marrs et al., 1985), *M. lacunata* (Marrs et al., 1990), *Neisseria gonorrhoeae* (Hermodson et al., 1978), *Neisseria meningitidis* (Hermodson et al., 1978), *Dichelobacter nodosus* (formerly *Bacteroides nodosus* (Dewhirst et al., 1990)) (Elleman & Hoyne, 1984), *Pseudomonas aeruginosa* (Sastry et al., 1985), and *Vibrio cholerae* (Taylor et al., 1987). The main structural subunits of these pili (pilins) share extensive amino-terminal amino acid sequence homology, and all except *V. cholerae* contain the modified amino acid *N*-methylphenylalanine as the first...
residue of the mature protein (McKern et al., 1983; Taylor et al., 1987). The amino acid sequence of the amino-terminal end of the pili in \textit{M. nonliquefaciens} NCTC 7784 was determined by Frøholm & Sletten (1977). Comparison of the amino acid sequence of type 4 pilin from different species to the original \textit{M. nonliquefaciens} sequence demonstrated that it also belonged to the type 4 pilins (Dalrymple & Mattick, 1987).

Differences exist in the copy numbers of pilin genes among bacterial species containing type 4 pili. \textit{P. aeruginosa} strains have only a single copy of the pilin gene in each genome (Sastry et al., 1985). Most serotypes of \textit{D. nodosus} only have a single gene, but some have two (Mattick, 1989). \textit{N. gonorrhoeae} strains contain multiple pilin gene loci, and transitions between P+ and P- and between different P+ pilin types are often accompanied by chromosomal DNA rearrangements (Bergström et al., 1986; Swanson & Koomey, 1989; for a review, see Swanson et al., 1987). \textit{M. bovis} and \textit{M. lacunata} strains each express one or the other of two different pilin genes, and can switch between them via a 2-1 kb DNA inversion event (Fulks et al., 1990; Marrs et al., 1988, 1990).

In this report, we describe the cloning, sequencing and expression of an \textit{M. nonliquefaciens} pilin gene, \textit{tpfA}. Hybridization analysis of a panel of 10 \textit{M. nonliquefaciens} strains was performed. Comparison of the deduced amino acid sequence to other reported type 4 pilin protein sequences revealed new information on differences from and features shared with other species.

### Methods

**Bacterial strains, plasmids, and media.** Strains of \textit{M. nonliquefaciens} included in the study were NCTC 7784 (ATCC 17953), 4663/62 (ATCC 19975, type strain), ATCC 19968 (836/61), 178/62, 4378/62 (ATCC 19966), and B2000/88. All (except NCTC 7784) had been isolated in our laboratories. B2000/88 originated from the blood of a leukemic child with septicaemia. The strains were identified by genetic transformation (Bovre, 1964; Bøvre & Froholm, 1972; T. Tonjum & K. Bøvre, unpublished). \textit{M. nonliquefaciens} as well as \textit{M. bovis} ATCC 10900 (type strain) and \textit{M. lacunata} ATCC 17967 (type strain) were grown on 5% (v/v) human blood agar (Difco; Bøvre & Froholm, 1972). \textit{Escherichia coli} PLK-F' [recA, mcrAB, hsdR, hsdM+, F'(lacZA15 lacP)] was used as a host for cloning, as it has the mcrAB negative background suitable for cloning methylated DNA (West & Clark, 1989). \textit{E. coli} containing drug-resistant plasmids were grown on LB agar containing either 25 µg kanamycin (Sigma) ml⁻¹ (LBkan) or 100 µg carbenicillin (Beecham Pharmaceuticals) ml⁻¹ (Lbcb), as appropriate. The low copy number Kan⁸ vector pLG339 was obtained from Dr Bob Bender, University of Michigan, Ann Arbor, Michigan, USA (Stoker et al., 1982). The plasmid pMXB12, which contains the \textit{M. bovis} \textit{tpfQ} pilin gene, has been described previously (Fulks et al., 1990; Marrs et al., 1985).

**DNA isolation and manipulation.** Total DNA was prepared by the method of Hull et al. (1981). Plasmid DNA isolation was performed according to the method of Birnboim & Doly (1979). Restriction endonucleases were purchased from Bethesda Research Laboratories, Boehringer Mannheim or Promega. Restriction endonuclease digests and agarose gel electrophoresis were performed as previously described (Marrs et al., 1985). DNA transfer from agarose gels to nylon membrane (Hybond-N, Amersham) was done by a modification of the method of Southern (1975), using the Vacu-Gene System (Pharmacia-LKB). Hybridizations were performed at stringent conditions as previously described (Marrs et al., 1985; Tonjum et al., 1989). The \textit{tpfQ} pilin probe from plasmid pMXB12 was labelled with [α-³²P]dCTP (Amersham) by use of a random oligo-priming kit (Boehringer Mannheim).

Inserts of size-fractionated Sau3AI fragments of total \textit{M. nonliquefaciens} NCTC 7784 DNA were obtained by electro-elution from 1% (w/v) agarose (SeaKem, FMC Bioproducts). Inserts were ligated into the BamHI-cleaved pLG339, transformed into competent \textit{E. coli} PLK-F' cells and selected on LBkan plates. Kan' transformants were screened for hybridization to the \textit{tpfQ} probe by a colony blot method (Sambruk et al., 1989). The insert of one hybridization positive clone (pMXN7) was then subcloned into the vector pGEM7 (Promega) at the BamHI site. One of the resulting plasmids (pMXN11) was chosen for detailed analysis.

The DNA sequence of the insert region of pMXN11 was determined using the dye-oxide-chain termination method of Sanger et al. (1977). The primers T7 (5’-TAAATCCACTGTGATAT-3') and SP6 (5’- TAAATCCACTGTGATAT-3') are in the pGEM7 vector flanking the insert. The primers Mn1 (5’-CAACCGTGTGTGTTACGTTGTC-3'), Mn3 (5’-GATACAGCTGCGATGCGG-3'), and Mn4 (5’-CTGACGTAGCTTACCTGCG-3') were also used, and sequencing and their positions are shown in Fig. 1. Sequence analysis was performed using computer software from DNASTAR Inc., Madison, Wisconsin, USA.

**Western blotting (immunoblotting).** Proteins of whole-cell extracts of bacteria were boiled in loading buffer, separated according to molecular mass by SDS-PAGE and transferred to a nitrocellulose filter (Immobilon-P, Millipore) (Towbin et al., 1979). Crudely purified pili and rabbit antiserum 130D raised against purified pili of \textit{M. nonliquefaciens} NCTC 7784 was kindly provided by Dr L. O. Froholm, Folkhelsa, Oslo, Norway. The antiserum was diluted 1:1000 for reaction with the filters.

### Results

**Cloning of the \textit{M. nonliquefaciens} pilin gene**

Since \textit{M. bovis} and \textit{M. nonliquefaciens} are closely related, we decided to use the previously cloned \textit{M. bovis} \textit{tpfQ} pilin gene as a hybridization probe to detect the equivalent \textit{M. nonliquefaciens} pilin gene. To determine hybridization conditions and optimal restriction enzymes for cloning the \textit{M. nonliquefaciens} pilin gene, we performed genomic Southern hybridization analysis on restriction endonuclease digested \textit{M. nonliquefaciens} total DNA using the \textit{tpfQ} pilin gene as a probe (Fig. 2). Based on these results, we decided to clone the approximately 800 bp hybridizing Sau3AI fragment into the high copy number plasmid vector pGEM7 in \textit{E. coli}. After several unsuccessful attempts to do this directly, we first cloned the fragment into the low copy number plasmid vector.
pLG339 in the mcrA−B− E. coli host strain PLK-F'. The insert region of the resultant recombinant plasmid (pMxN7) was then subcloned into pGEM7 producing the plasmid pMxN11. A restriction map of pMxN11 is presented in Fig. 1.

**Sequencing the *Moraxella nonliquefaciens* pilin gene clone**

The strategy used for sequencing the *BamHI* insert portion of pMxN11 is shown in Fig. 1. The sequence of the entire insert was determined in both orientations using five oligonucleotide primers whose positions are shown in Figs 1 and 3. The nucleotide sequence of 826 bp between, but not including, the *BamHI* sites is presented in Fig. 3, together with the predicted amino acid sequence of the pilin. We have named this pilin gene *tpfA*. The sequence AGGAG present on the 5' side of the starting ATG is homologous to known ribosome-binding sites in prokaryotes (Gold et al., 1981) and is identical to the ribosome-binding sequence of both an *N. gonorrhoeae* pilin gene (Meyer et al., 1984) and the *M. bovis* *tpfQ* pilin gene (Marrs et al., 1985).

**Southern blot analysis using a *tpfA* probe**

When stringent hybridization conditions were used, it became clear that genomic DNAs of the *M. bovis* and *M. lacunata* type strains hybridized relatively poorly to the *M. nonliquefaciens* *tpfA* probe, as compared to hybridization of *M. nonliquefaciens* NCTC 7784 genomic DNA to both *tpfA* and the *M. bovis* *tpfQ* probe (Fig. 2).

As described in the Introduction, the number of pilin genes or partial pilin genes varies greatly among the bacteria that express type 4 pili. Therefore, we wanted to determine the number and organization of the pilin gene(s) of *M. nonliquefaciens*. The *BamHI* insert fragment of pMxN11 was used as a probe in stringent genomic Southern hybridizations to both the parent strain NCTC 7784 and nine additional *M. nonliquefaciens* strains (Fig. 4). This analysis reveals several bands of strong and weaker hybridization with all of them. The parent strain deviates by having much smaller sized bands than the other strains.

**Western blot analysis**

As demonstrated in Fig. 5, the 130D antiserum reacted with a protein band from *M. nonliquefaciens* at 15 kDa. This band is equal in size to the band appearing with purified pilus. A similar band, only migrating slightly more slowly, was recognized when cell extracts of *E. coli* containing pMxN11 were reacted with the antiserum. The antiserum also reacted with higher molecular mass proteins that could be either pilin multimers or cross-reacting proteins (data not shown).

**Amino acid analysis**

The inferred amino acid sequence of the *M. nonliquefaciens* pilin protein based on the nucleotide sequence (Fig. 3) shows a six amino acid leader sequence preceding the phenylalanine which starts the mature protein. This leader is identical to the leader sequences of *M. bovis* and *M. lacunata* pilins (Fig. 6). The predicted amino acid sequence matches almost perfectly with the amino-terminal amino acid sequence reported by Frøholm &
Sletten (1977). The only difference is at position 46 of the mature pilin where they had tentatively identified the residue as Gly, whilst in our predicted sequence this would be a Ser residue.

**Discussion**

Dalrymple & Mattick (1987) classified the *M. nonliquefaciens* NCTC 7784 pilin as a type 4 pilin with strong amino acid sequence conservation compared at the amino terminus to all other type 4 pilins, including that of *M. bovis*. The data presented here describe the successful use of an *M. bovis* tfpQ pilin gene probe to isolate a pilin gene from *M. nonliquefaciens*, tfpA. When stringent hybridization conditions were used, it became clear that the tfpA pilin gene probe hybridized relatively poorly to total genomic DNA of *M. bovis* as well as *M. lacunata*. This was surprising, considering that the *M. bovis* tfpQ pilin gene probe hybridized clearly to genomic DNA of *M. nonliquefaciens* (Fig. 2a) and that distinction between these very closely related species by hybridization of total DNA under the same conditions is difficult (Tonjum et al., 1989). The *M. bovis* pilin gene probe
Fig. 3. Nucleotide sequence and translation of the pMxN11 insert containing the \textit{M. nonliquefaciens} NCTC 7784 \textit{tfpA} gene. 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. The positions to which the internal sequence primers bound are represented by the lines above the sequence with the primer name above that. Primer \textit{Mnl} (sequence listed in Methods) was constructed based on the sequence of the equivalent regions of sequence in \textit{M. bocis} and \textit{M. lucunutu}, and is not the perfect complement of the sequence shown in this figure.

Fig. 5. Immunoblot analysis of crude pili and whole-cell extracts from \textit{M. nonliquefaciens} NCTC 7784 and whole-cell extracts from \textit{E. coli} containing cloned \textit{M. nonliquefaciens} DNA. Lanes: 1, crude preparation of purified pili from \textit{M. nonliquefaciens} NCTC 7784; 2, \textit{M. nonliquefaciens} NCTC 7784; 3, \textit{E. coli} PLK-F with the plasmid pMxN11 (pGEM7 containing the \textit{tfpA} pilin gene); 4, PLK-F' (pGEM7 without insert). Rabbit antiserum 130D raised against purified pili of \textit{M. nonliquefaciens} NCTC 7784 was used at a dilution of 1:1000. Molecular mass markers are noted on the left.
Fig. 6. Comparison of the predicted amino acid sequence of pilin from \textit{M. nonliquefaciens} NCTC 7784 to the pilin sequences from \textit{N. gonorrhoeae} MS11 (Meyer et al., 1984), \textit{M. bovis} EPP63 tfpQ (Marrs et al., 1985), \textit{M. lacunata} ATCC 17956 rjpQ (Rozsa & Marrs, 1991), \textit{D. nodosus} 198 (Ellemann & Hoyne, 1984; McKern et al., 1983), and \textit{P. aeruginosa} PAK (Sastry et al., 1985). Shaded residues are identical to those from \textit{M. nonliquefaciens} pilin.

differences within the pilin genes than in the surrounding region have been observed between \textit{M. bovis} and \textit{M. lacunata}, where the \textit{tfpQ} genes are only 71\% similar to each other as compared to 87\% similarity over the entire pilin gene inversion region. This larger region includes the region 5′ of the pilin structural genes as well as the \textit{tfpB} and \textit{pic} genes (Rozsa & Marrs, 1991). Comparison of the nucleotide sequence of the \textit{M. nonliquefaciens} \textit{tfpA} pilin gene (Fig. 3) to that of the \textit{tfpQ} genes of \textit{M. bovis} (Marrs et al., 1985) and \textit{M. lacunata} (Rozsa & Marrs, 1991), shows only 54\% similarity in the DNA sequence.

As demonstrated in Fig. 2, the 10 strains of \textit{M. nonliquefaciens} examined by hybridization analysis with the \textit{tfpA} probe revealed several hybridizing bands. This suggested the existence of more than one pilin gene. Oligonucleotide hybridizations using probes specific for the 5′ and 3′ fragments flanking a potential inversion site reveals more bands with the 3′ and \textit{tfpA} probes than the 5′ probe, suggesting the existence of partial pilin genes lacking parts of the 5′ end of the gene (data not shown).

As we have shown, \textit{tfpA} is present in all the \textit{M. nonliquefaciens} strains examined so far (Fig. 4). Since this pilin gene is detectably different in hybridization analysis from that of \textit{M. lacunata}, this may be a means by which to distinguish clinical isolates of \textit{M. nonliquefaciens} from those of \textit{M. lacunata}.

Western blot analysis showed that the \textit{M. nonliquefaciens} pilin gene is expressed in \textit{E. coli} as a 15 kDa protein, as compared to the \textit{M. nonliquefaciens} purified pilin protein (Fig. 5). The pilin protein from the \textit{E. coli} host strain migrates slightly more slowly than the pilin protein of the parent strain, as expected due to the leader sequence not being clipped off in the maturation process.

Comparison of the amino-terminal amino acid sequence of the mature \textit{M. nonliquefaciens} pilin to the pilin amino acid sequence deduced from the DNA sequence
of the 826 bp insert present in pMxN11 shows that tfpA encodes a protein with a six amino acid leader and 148 amino acids of the mature protein. Fig. 6 shows a comparison of the translated M. nonliquefaciens pilin sequence with the amino acid sequences of pilins from N. gonorrhoeae, M. bovis, M. lacunata, D. nodosus and P. aeruginosa. As aligned in Fig. 6, M. nonliquefaciens pilin matches with the others as follows: 41.6% identity with N. gonorrhoeae, 39.6% identity with M. bovis, 38.3% identity with D. nodosus and 44.2% identity with M. lacunata with 15 gaps, 44.2% identity with D. nodosus with 12 gaps, and 31.8% identity with P. aeruginosa with 10 gaps. The relatively large amount of gapping reflects the fact that the alignment was not done in a simple pairwise maximization of sequence similarity, but has been adjusted to include alignment at positions conserved across all six pilins being compared. It is also worth noting that comparisons have previously been carried out on different pilin sequences present within the same N. gonorrhoeae strain which required as many as 6 gaps for optimal alignment (Nicholson et al., 1987).

It is notable that while M. nonliquefaciens is phylogenetically much closer to M. bovis and M. lacunata than to N. gonorrhoeae or D. nodosus, the amino acid sequence of the pilin protein of M. nonliquefaciens is about as similar to N. gonorrhoeae and D. nodosus pilins as it is to either M. bovis or M. lacunata pilin. This is in great contrast to the much higher similarity found between M. bovis and M. lacunata pilins (Rozsa & Marrs, 1991). The M. nonliquefaciens pilin is identical in amino acid sequence to the pilins of M. bovis and M. lacunata starting with the six amino leader sequence and through the first 28 amino-terminal amino acids of the mature proteins. These regions are the most conserved among all of the type 4 pilins, but as can be seen in Fig. 6, pairwise comparisons of the other three pilins shown all differ from the Moraxella pilins in this region by at least five residues. However, amino acids 29–36 of the mature pilins are identical between M. nonliquefaciens and N. gonorrhoeae, while only one residue in this region matches the amino acids found in the other Moraxella pilins. In N. gonorrhoeae pilins, amino acids 29–36 are part of the completely conserved region that does not vary during antigenic variation of pilin types. In a third major feature, the two cysteine residues in M. nonliquefaciens pilin have the central location observed in class I D. nodosus pilins, occurring at amino acids 56 and 97 of the mature pilin. In contrast, all type 4 pilins sequenced to date from N. gonorrhoeae, P. aeruginosa, M. bovis, and M. lacunata have a cysteine loop region very near the carboxy terminus of the pilin molecule. Class II D. nodosus pilins (Ellemann, 1988; McKern et al., 1983) and one P. aeruginosa strain (Paslowske et al., 1985) have cysteine pairs both centrally located and in the carboxy-terminal regions. In the M. nonliquefaciens pilin the positioning of the cysteines is similar to that seen in the class I D. nodosus pilin, coming at amino acids 56 and 93 of the mature pilin.

The significance of differential homologies between the M. nonliquefaciens pilin and the others discussed above is not yet clear. They might be of importance for colonization, adherence, and invasion of microhabitats by these species, as well as for escape from the host’s immune defence. Further investigation of the pilin gene region will provide a basis for the study of adherence properties as well as other properties associated with piliation.

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