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

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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 (*tfpQ*) from *Moraxella bovis* as a hybridization probe, we have cloned an 826 bp *Sau*3AI 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 septicaemic 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, 1972*a, b*), twitching motility (Henrichsen *et al.*, 1972), haemagglutination (Schoolnik *et al.*, 1982), surface pellicle formation in static broth (Bøvre *et al.*, 1970; Bøvre & Frøholm,

1972*a*) and competence for genetic transformation (Bøvre & Frøholm, 1972*b*). *M. nonliquefaciens* also undergoes phase variation of colony morphology (Bøvre & Frøholm, 1972*a*), 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, 1972*a, 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 (Bergstrøm *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* (Hermanson *et al.*, 1978), *Neisseria meningitidis* (Hermanson *et al.*, 1978), *Dichelobacter nodosus* [formerly *Bacteroides nodosus* (Dewhirst *et al.*, 1990)] (Elleman & Hoyne, 1984), *Pseudomonas aeruginosa* (Sastri *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

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M 59707.

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 *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 *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. *P. aeruginosa* strains have only a single copy of the pilin gene in each genome (Sastri *et al.*, 1985). Most serotypes of *D. nodosus* only have a single gene, but some have two (Mattick, 1989). *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 (Bergstrøm *et al.*, 1986; Swanson & Koomey, 1989; for a review, see Swanson *et al.*, 1987). *M. bovis* and *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 *M. nonliquefaciens* pilin gene, *tfpA*. Hybridization analysis of a panel of 10 *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 *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, 13385/62 (ATCC 19969), 3067/66, 3179/66, 3832/60 (ATCC 19966), and B2000/88. All (except NCTC 7784) had been isolated in our laboratories. B2000/88 originated from the blood of a leukaemic child with septicaemia. The strains were identified by genetic transformation (Bøvre, 1964; Bøvre & Frøholm, 1972; T. Tønjum & K. Bøvre, unpublished). *M. nonliquefaciens* as well as *M. bovis* ATCC 10900 (type strain) and *M. lacunata* ATCC 17967 (type strain) were grown on 5% (v/v) human blood agar (Difco; Bøvre & Frøholm, 1972). *Escherichia coli* PLK-F' [*recA*, *mcrAB*, *hsdR*, *hsdM*⁺, F'(*lacZ*Δ15 *lac*⁺)] (Stratagene) was used as a host for cloning, as it has the *mcrAB* negative background suitable for cloning methylated DNA (West & Clark, 1989). *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 Kan^R 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 *M. bovis* *tfpQ* 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 digestions 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; Tønjum *et al.*, 1989). The *tfpQ* 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 *Sau*3AI fragments of total *M. nonliquefaciens* NCTC 7784 DNA were obtained by electro-elution from 1% (w/v) agarose (SeaKem, FMC Bioproducts). Inserts were ligated into the *Bam*HI-cleaved pLG339, transformed into competent *E. coli* PLK-F' cells and selected on LBkan plates. Kan^r transformants were screened for hybridization to the *tfpQ* probe by a colony blot method (Sambrook *et al.*, 1989). The insert of one hybridization positive clone (pMxN7) was then subcloned into the vector pGEM7 (Promega) at the *Bam*HI 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 dideoxy-chain termination method of Sanger *et al.* (1977). The primers T7 (5'-TAATACGACTCACTATA-3') and SP6 (5'-TAAATCCACTGTGATAT-3') are in the pGEM7 vector flanking the insert. The primers Mnl (5'-CAACCGTGTCTGT-TACGTTGTC-3'), Mn3 (5'-GATACAGCTGCAGATGGCG-3'), and Mn4 (5'-CTAGCACTAGCTTACCTGC-3') were also used for 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 *M. nonliquefaciens* NCTC 7784 was kindly provided by Dr L. O. Frøholm, Folkehelsa, Oslo, Norway. The antiserum was diluted 1:1000 for reaction with the filters.

Results

Cloning of the *M. nonliquefaciens* pilin gene

Since *M. bovis* and *M. nonliquefaciens* are closely related, we decided to use the previously cloned *M. bovis* *tfpQ* pilin gene as a hybridization probe to detect the equivalent *M. nonliquefaciens* pilin gene. To determine hybridization conditions and optimal restriction enzymes for cloning the *M. nonliquefaciens* pilin gene, we performed genomic Southern hybridization analysis on restriction endonuclease digested *M. nonliquefaciens* total DNA using the *tfpQ* pilin gene as a probe (Fig. 2). Based on these results, we decided to clone the approximately 800 bp hybridizing *Sau*3AI fragment into the high copy number plasmid vector pGEM7 in *E. coli*. After several unsuccessful attempts to do this directly, we first cloned the fragment into the low copy number plasmid vector

Fig. 1. Restriction map of the insert region of pMxN11, encoding the *M. nonliquefaciens* NCTC 7784 *tfpA* gene, and strategy for determining the sequence of the insert region. The insert region is represented by the bold line between the *Bam*HI sites. A few sites in the linker regions of the pGEM7 vector portions of pMxN11 are also listed. The positions of the oligonucleotide primers used to determine the DNA sequence (T7, Mn1, Mn3, Mn4 and SP6) and for PCR and oligo-hybridization studies (Mn5 and Mn6) are shown, as is the relative position of the gene within the insert. The arrows beneath the map show the direction of sequencing and the length of the sequence determined by using the listed oligonucleotide primers.

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 *M. nonliquefaciens* pilin gene clone

The strategy used for sequencing the *Bam*HI 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 *Bam*HI sites is presented in Fig. 3, together with the predicted amino acid sequence of the pilin. We have named this pilin gene *tfpA*. 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* *tfpQ* pilin gene (Marrs *et al.*, 1985).

Southern blot analysis using a *tfpA* 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* *tfpA* probe, as compared to hybridization of *M. nonliquefaciens* NCTC 7784 genomic DNA to both *tfpA* and the *M. bovis* *tfpQ* 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 *Bam*HI 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 pili. 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 &

Fig. 2. Genomic Southern hybridization of *M. nonliquefaciens*, *M. lacunata* and *M. bovis* DNAs using inserts of (a) the *M. bovis* pMxB12 (*tfpQ*) and (b) the *M. nonliquefaciens* pMxN11 (*tfpA*) as probes at a sodium salt concentration of 0.1 M and a temperature of 65 °C. Lanes: 1 and 5, λ /HindIII marker DNA visualized with a small amount of labelled λ DNA; 2, *M. nonliquefaciens* NCTC 7784/Sau3AI; 3, *M. lacunata* ATCC 17967/Sau3AI; 4, *M. bovis* ATCC 10900/Sau3AI.

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 (Tønjum *et al.*, 1989). The *M. bovis* pilin gene probe

Mn1 →

GCTCCCATCATCCCAATCTGCCATAAAGTTGGCACAGAGCATGCAACGGTATCAGTGTA 60

AGTTAGCAAGATTAGCAGTACAGTCATCAGACTGCTTTGTTAATCGTTGCAAATAACATC 120

tfpA → Mn5

TAATAACTGGGCAATTCTGCCACCTAACTAAGGAGTTCATTATGAACGCTCAAAAAGGT 180

MetAsnAlaGlnLysGly

→

TTTACCTTAATTGAATTGATGATTGTTATCGCCATTATTGGTATCCTAGCTGCCATCGCA 240

PheThrLeuIleGluLeuMetIleValIleAlaIleIleGlyIleLeuAlaAlaIleAla

← Mn6

TTGCCTGCGTATCAGGACTATATCGCCCGTGACAGGTATCAGAAGCCTTACTTTGGCT 300

LeuProAlaTyrGlnAspTyrIleAlaArgAlaGlnValSerGluAlaPheThrLeuAla

GATGGACTAAAGACCAGCATCAGCACCAACCGTCAAAATGGGAGATGTTTGTCTGATGGT 360

AspGlyLeuLysThrSerIleSerThrAsnArgGlnAsnGlyArgCysPheAlaAspGly

Mn3 →

AAAGATACAGCTGCAGATGGCGTAGATATAATAACAGGTAAGTATGGTAAAGCCACTATT 420

LysAspThrAlaAlaAspGlyValAspIleIleThrGlyLysTyrGlyLysAlaThrIle

CTCGAAGAAAACCCATAACTGCTGATGGTTTAAATTTGGTATTTATTATGAATTTAAC 480

LeuGluGluAsnProAsnThrAlaAspGlyLeuIleCysGlyIleTyrTyrGluPheAsn

ACGACTGGCGTATCTGATAAGCTGATTGGAAAAACGATTGCTTTAAAGCAGATGAGAAA 540

ThrThrGlyValSerAspLysLeuIleGlyLysThrIleAlaLeuLysAlaAspGlyLys

← Mn4

GCAGGTAAGCTAGTGCTAGAACTGTTAATTCTAAACTACAAATGTTGAGAATAAATAT 600

AlaGlyLysLeuValLeuGluThrValAsnSerLysThrThrAsnValGluAsnLysTyr

CTCCCAAGTGCTTTTAAAAAGCCTTAATAAAAAACAACAGTCTCAAAAAACAAGCTC 660

LeuProSerAlaPheLysLysPro

TCATGAGAACTTGTTTTTTTGGACATTACGTTGTATGGAAGTTGATTATTATCTAAAAGC 720

ACTTGGGAGATATTTACTTGAACATTTGTACTTTTAGATTAAACAGTTTCCAATTTTAA 780

TACCGAATCTTCAGTTACTTTTAGAACATCTCTTTGCCTTTTAAAGC 826

Fig. 3. Nucleotide sequence and translation of the pMxN11 insert containing the *M. nonliquefaciens* NCTC 7784 *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 Mn1 (sequence listed in Methods) was constructed based on the sequence of the equivalent regions of sequence in *M. bovis* and *M. lacunata*, and is not the perfect complement of the sequence shown in this figure.

Fig. 4. Genomic Southern hybridization of a panel of *M. nonliquefaciens* strains using the *M. nonliquefaciens* *tfpA* pilin gene pMxN11 as probe at a sodium salt concentration of 0.1 M and a temperature of 65 °C. Genomic DNA of all strains was cut with *Sau3A*I. Lanes: 1, λ /HindIII marker DNA visualized with a small amount of labelled λ DNA; 2, NCTC 7784 (parent strain); 3, ATCC 19975 (type strain); 4, B2000/88; 5, B2000/88; 6, 3179/66; 7, 3179/66; 8, 178/62; 9, 13385/62; 10, ATCC 19968 (836/61); 11, 4378/62; 12, 3067/66; 13, 3832/60.

Fig. 5. Immunoblot analysis of crude pili and whole-cell extracts from *M. nonliquefaciens* NCTC 7784 and whole-cell extracts from *E. coli* containing cloned *M. nonliquefaciens* DNA. Lanes: 1, crude preparation of purified pili from *M. nonliquefaciens* NCTC 7784; 2, *M. nonliquefaciens* NCTC 7784; 3, *E. coli* PLK-F' with the plasmid pMxN11 (pGEM7 containing the *tfpA* pilin gene); 4, PLK-F' (pGEM7 without insert). Rabbit antiserum 130D raised against purified pili of *M. nonliquefaciens* NCTC 7784 was used at a dilution of 1:1000. Molecular mass markers are noted on the left.

pMxB12 contains parts of the region upstream of the gene in addition to the entire pilin gene *tfpQ* (Marrs *et al.*, 1985), while the *M. nonliquefaciens* clone pMxN11 contains little sequence outside of the pilin gene region *tfpA* (Fig. 1). The incomplete reciprocity of the hybridization results, i.e. the relatively strong heterologous signal given by pMxB12, is considered to be due to sequence similarity between pMxB12 and *M. nonliquefaciens* DNA flanking the pilin gene. Greater sequence

<i>M. nonliquefaciens</i>	MNA	Q	K	G	F	T	L	I	E	L	M	I	V	I	A	I	I	G	I	L	A	A	I	A	L	P	A	Y	Q	D	Y	I	A	
<i>N. gonorrhoeae</i>	MNT	L	Q	K	G	F	T	L	I	E	L	M	I	V	I	A	I	V	G	I	L	A	A	V	A	L	P	A	Y	Q	D	Y	T	A
<i>M. bovis</i>	MNA	Q	K	G	F	T	L	I	E	L	M	I	V	I	A	I	I	G	I	L	A	A	I	A	L	P	A	Y	Q	D	Y	I	S	
<i>M. lacunata</i>	MNA	Q	K	G	F	T	L	I	E	L	M	I	V	I	A	I	I	G	I	L	A	A	I	A	L	P	A	Y	Q	D	Y	I	S	
<i>D. nodosus</i>	MKS	L	Q	K	G	F	T	L	I	E	L	M	I	V	I	A	I	I	G	I	L	A	A	F	A	I	P	A	Y	N	D	Y	I	A
<i>P. aeruginosa</i>	MKA	Q	K	G	F	T	L	I	E	L	M	I	V	V	A	I	I	G	I	L	A	A	I	A	I	P	Q	Y	Q	N	Y	V	A	

<i>M. nonliquefaciens</i>	R	A	Q	V	S	E	A	F	T		L	A	D	G	L	K	T	S	I	S	T	N	R	Q	N		G	R	C	F	A	D	G	K	D	T
<i>N. gonorrhoeae</i>	R	A	Q	V	S	E	A	I	L		L	A	E	G	Q	K	S	A	V	T	E	Y	Y	L	N	H	G	K	W	P	E	N	N	T	S	A
<i>M. bovis</i>	K	S	Q	T	T	R	V	V	G	E	L	A	A	G		K	T	A	V	D	A	A		L	F	E	G	K	T	P	K	L	G	K	A	A
<i>M. lacunata</i>	K	S	Q	T	T	R	V	V	G	E	L	A	A	G		K	T	A	V	D	A	A		L	F	E	G	K	E	P	V	L	D	A	G	N
<i>D. nodosus</i>	R	S	Q	A	A	E	G	L	T		L	A	D	G	L	K	V	R	I	S	D	H		L	E	S	G	E	C	K	G	D	A	N	P	A
<i>P. aeruginosa</i>	R	S	E	G	A	S	A	L	A		S	V	N	P	L	K	T	T	V	E	E	A		L	S	R	G	W	S	V	K	S	G	T	G	T

<i>M. nonliquefaciens</i>	A	A	D		G		V	D	I	I		T	G	K	Y	G	K	A	T	I		L	E			E	N	P	N	T	A	D	G	L	I
<i>N. gonorrhoeae</i>	G	V	A	S	P	P	S	D	I			K	G	K	Y	V	K	E	V	E		V	K			N	G	V	V	T	A	T	M	L	S
<i>M. bovis</i>	N	D	T		E		E	D	I	G	L	T	T	T	G	G	T	A	R	S	N	L	M			S	S	V	N	I	G	G	G	A	F
<i>M. lacunata</i>	T	A	T		S	K	E	D	I	G	L	T	S	T	G	G	K	A	R	S	N	L	L	K	E	T	G	G	V	V	L	A		G	F
<i>D. nodosus</i>	S	G	S	L	G		N	D	D			K	G	K	Y	A	L	A	T	I		D	G				D	Y	N	K	D	A	K	T	A
<i>P. aeruginosa</i>	E	D	A	T	K	K	E	V	P			L	G	V	A	A	D	A	N	K		L	G				T	I	A	L	K	P	D	P	A

<i>M. nonliquefaciens</i>	C	G	I	Y	Y	E	F	N	T		T	G	V	S	D	K	L	I	G	K	T	I	A	L	K	A	D	E	K	A	G	K	L	V	L	E
<i>N. gonorrhoeae</i>	S	G	V	N	N	E	I	K	G		K	K	L	S			L	L	W	A	R	R	E	N	G	S	V		K							
<i>M. bovis</i>		A	T		G	A	G	T	L		E	A	T	L	G	N	R	A	N	K	D	I	A		G	A	V	I	T	Q	S	R	D	A	E	G
<i>M. lacunata</i>	S	A	T	S	S	A	G	T	I		T	G	T	L	G	N	R	A	N	K	D	I	S		G	A	I	I	T	Q	K	R	A	N	D	G
<i>D. nodosus</i>	D	E	K	N	G	C	K	V	V	I	T	Y	G	Q	G	T		A	G	E	K	I	S	K	L	I	V	G	K		K	L	V	L	D	
<i>P. aeruginosa</i>	D	G	T	A	D	I	T	L	T	F	T	M	G	G	A	G	P	K	N	K	G	K	I	I	T	L	T	R	T	A	A	D	G		L	

<i>M. nonliquefaciens</i>																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
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Fig. 6. Comparison of the predicted amino acid sequence of pilin from *M. nonliquefaciens* NCTC 7784 to the pilin sequences from *N. gonorrhoeae* MS11 (Meyer *et al.*, 1984), *M. bovis* EPP63 *tfpQ* (Marrs *et al.*, 1985), *M. lacunata* ATCC 17956 *tfpQ* (Rozsa & Marrs, 1991), *D. nodosus* 198 (Ellemann & Hoyne, 1984; McKern *et al.*, 1983), and *P. aeruginosa* PAK (Sastry *et al.*, 1985). Shaded residues are identical to those from *M. nonliquefaciens* pilin.

differences within the pilin genes than in the surrounding region have been observed between *M. bovis* and *M. lacunata*, where the *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 *tfpB* and *piv* genes (Rozsa & Marrs, 1991). Comparison of the nucleotide sequence of the *M. nonliquefaciens* *tfpA* pilin gene (Fig. 3) to that of the *tfpQ* genes of *M. bovis* (Marrs *et al.*, 1985) and *M. lacunata* (Rozsa & Marrs, 1991), shows only 54% similarity in the DNA sequence.

As demonstrated in Fig. 2, the 10 strains of *M. nonliquefaciens* examined by hybridization analysis with the *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 *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, *tfpA* is present in all the *M. nonliquefaciens* strains examined so far (Fig. 4). Since this pilin gene is detectably different in hybridization analysis from that of *M. lacunata*, this may be a means by which to distinguish clinical isolates of *M. nonliquefaciens* from those of *M. lacunata*.

Western blot analysis showed that the *M. nonliquefaciens* pilin gene is expressed in *E. coli* as a 15 kDa protein, as compared to the *M. nonliquefaciens* purified pilin protein (Fig. 5). The pilin protein from the *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 *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* with 11 gaps, 39.6% identity with *M. bovis* with 12 gaps, 38.3% 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 (Pasloske *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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References

- BERGSTROM, S., ROBBINS, K., KOOMEY, J. M. & SWANSON, J. (1986). Piliation control mechanisms in *Neisseria gonorrhoeae*. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 3890–3894.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BRORSON, J. E., FALSEN, E., EHLE-NILSSON, H., RODJER, S. & WESTIN, J. (1983). Septicemia due to *Moraxella nonliquefaciens* in a patient with multiple myeloma. *Scandinavian Journal of Infectious Diseases* **15**, 221–223.
- BØVRE, K. (1984). Genus II. *Moraxella* Lwoff 1939, 173 emend. Henriksen and Bøvre 1968, 391^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 296–303. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- BØVRE, K. & FRØHOLM, L. O. (1972a). Variation of colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M. nonliquefaciens*. *Acta Pathologica et Microbiologica Scandinavica* **B80**, 629–640.
- BØVRE, K. & FRØHOLM, L. O. (1972b). Competence in genetic transformation related to colony type and fimbriation in three species of *Moraxella*. *Acta Pathologica et Microbiologica Scandinavica* **B80**, 649–659.
- BØVRE, K., BERGAN, T. & FRØHOLM, L. O. (1970). Electron microscopical and serological characteristics associated with colony type in *Moraxella nonliquefaciens*. *Acta Pathologica et Microbiologica Scandinavica* **B78**, 765–779.
- DALRYMPLE, B. & MATTICK, J. S. (1987). An analysis of the organization and evolution of type 4 fimbrial (MePhe) subunit proteins. *Journal of Molecular Evolution* **25**, 261–269.
- DEWHIRST, F. E., PASTER, B. J., LA FONTAINE, S. & ROOD, J. I. (1990). Transfer of *Kingella indologenes* (Snell and La Page, 1976) to the genus *Suttonella* gen. nov. as *Suttonella indologenes* comb. nov.; transfer of *Bacteroides nodosus* (Beveridge, 1941) to the *Dichelobacter* gen. nov., as *Dichelobacter nodosus* comb. nov.; and assignment of the genera *Cardiobacterium*, *Dichelobacter* and *Suttonella* to *Cardiobacteriaceae* fam. nov. in the gamma division of *Proteobacteria* based on 16S ribosomal ribonucleic acid sequence comparisons. *International Journal of Systematic Bacteriology* **40**, 426–433.
- EBRIGHT, J. R., LENTINO, J. R. & JUNI, E. (1982). Endophthalmitis caused by *Moraxella nonliquefaciens*. *American Journal of Clinical Pathology* **77**, 362–363.

- ELLEMANN, T. C. (1988). Pilins of *Bacteroides nodosus*: molecular basis of serotypic variation and relationships to other bacterial pilins. *Microbiological Reviews* **52**, 233–247.
- ELLEMAN, T. C. & HOYNE, P. A. (1984). Nucleotide sequence of the gene encoding pilin of *Bacteroides nodosus*, the causal organism of ovine footrot. *Journal of Bacteriology* **160**, 1184–1187.
- FRØHOLM, L. O. & SLETTEN, K. (1977). Purification and N-terminal sequencing of a fimbrial protein from *Moraxella nonliquefaciens*. *FEBS Letters* **73**, 29–32.
- FULKS, K. A., STEPHENS, S. P., GREEN, M. R. & MARRS, C. F. (1990). Sequence analysis of the inversion region containing the pilin genes of *Moraxella bovis*. *Journal of Bacteriology* **172**, 310–316.
- GOLD, L., PRIENOW, D., SCHNEIDER, T., SHINEDLING, S., SWEBILIUS-SINGER, B. & STORMO, G. (1981). Translational initiation in prokaryotes. *Annual Review of Microbiology* **35**, 365–403.
- GRAHAM, D. R., BAND, J. D., THORNSBERRY, C., HOLLIS, D. G. & WEAVER, R. E. (1990). Infections caused by *Moraxella*, *Moraxella urethralis*, *Moraxella*-like groups M-5 and M-6, and *Kingella kingae* in the United States, 1953–1980. *Reviews of Infectious Diseases* **12**, 423–431.
- HENRICHSEN, J., FRØHOLM, L. O. & BØVRE, K. (1972). Studies on bacterial surface translocation. 2. Correlation of twitching motility and fimbriation in colony variants of *Moraxella nonliquefaciens*, *M. bovis*, and *M. kingii*. *Acta Pathologica et Microbiologica Scandinavica* **B80**, 445–452.
- HERMODSON, M. A., CHEN, K. C. S. & BUCHANAN, T. M. (1978). *Neisseria* pilin proteins; amino-terminal amino-acid sequences and identification of an unusual amino acid. *Biochemistry* **17**, 442–445.
- HULL, R., GILL, R. E., HSU, P., MINSHEW, B. H. & FALKOW, S. (1981). Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. *Infection and Immunity* **33**, 933–938.
- LOBUE, T. D., DEUTSCH, T. A. & STEIN, R. M. (1985). *Moraxella nonliquefaciens* endophthalmitis after trabeculectomy. *American Journal of Ophthalmology* **99**, 343–345.
- MARRS, C. F., SCHOOLNIK, G., KOOMEY, J. M., HARDY, J., ROTHBARD, J. & FALKOW, S. (1985). Cloning and sequencing of a *Moraxella bovis* pilin gene. *Journal of Bacteriology* **163**, 132–139.
- MARRS, C. F., RUEHL, W. W., SCHOOLNIK, G. K. & FALKOW, S. (1988). Pilin gene phase variation of *Moraxella bovis* is caused by an inversion of the pilin genes. *Journal of Bacteriology* **170**, 3032–3039.
- MARRS, C. F., ROZSA, F. W., HACKEL, M., STEPHENS, S. P. & GLASGOW, A. C. (1990). Identification, cloning, and sequencing of *piv*, a new gene involved in inverting the pilin genes of *Moraxella lacunata*. *Journal of Bacteriology* **172**, 4370–4377.
- MATTICK, J. S. (1989). The molecular biology of the fimbriae of *Bacteroides nodosus* and the development of a recombinant DNA-based vaccine against footrot. In *Footrot and Foot Abscess of Ruminants*, pp. 195–218. Edited by J. R. Egerton, W. K. Yang & G. G. Rifkin. Boca Raton, Florida: CRC Press.
- McKERN, N. M., O'DONNELL, I. J., INGLIS, A. S., STEWART, D. J. & CLARK, B. L. (1983). Amino acid sequence of pilin from *Bacteroides nodosus* (strain 198), the causative organism of ovine footrot. *FEBS Letters* **164**, 149–153.
- MEYER, T. F., BILLYARD, E., HAAS, R., STORZBACH, S. & SO, M. (1984). Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 6110–6114.
- NICOLSON, I. J., PERRY, A. C. F., VIRJI, M., HECKELS, J. E. & SAUNDERS, J. R. (1987). Localization of antibody-binding sites by sequence analysis of cloned pilin genes from *Neisseria gonorrhoeae*. *Journal of General Microbiology* **133**, 825–833.
- PASLOSKE, B. L., FINLAY, B. B. & PARANCHYCH, W. (1985). Cloning and sequencing of the *Pseudomonas aeruginosa* PAK pilin gene. *FEBS Letters* **183**, 408–412.
- ROSETT, W., HECK, D. M. & HODGES, G. R. (1976). Pneumonitis and pulmonary abscess associated with *Moraxella nonliquefaciens*. *Chest* **70**, 664–665.
- ROZSA, F. W. & MARRS, C. F. (1991). Interesting sequence differences between the pilin gene inversion regions of *Moraxella lacunata* ATCC 17956 and *Moraxella bovis* EPP63. *Journal of Bacteriology* **173** (in the Press).
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn, pp. 1.90–1.104. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SASTRY, P. A., FINLAY, B. B., PASLOSKE, B. L., PARANCHYCH, W., PEARLSTONE, J. R. & SMILLIE, L. B. (1985). Comparative studies of the amino acid and nucleotide sequences of pilin derived from *Pseudomonas aeruginosa* PAK and PAO. *Journal of Bacteriology* **164**, 571–577.
- SCHOOLNIK, G. K., TAI, J. Y. & GOTSCHLICH, E. C. (1982). The human erythrocyte binding domain of gonococcal pili. *Seminars of Infectious Diseases* **4**, 172–180.
- SHARMA, D. L. B. (1974). Fatal septicaemia due to *Moraxella nonliquefaciens*. *Archives of Diseases in Children* **49**, 966–967.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- STOKER, N. G., FAIRWEATHER, N. C. & SPRATT, B. G. (1982). Versatile low-copy number vectors for cloning in *Escherichia coli*. *Gene* **18**, 335–341.
- SWANSON, J. & KOOMEY, J. M. (1989). Mechanisms for variation of pili and outer membrane protein II in *Neisseria gonorrhoeae*. In *Mobile DNA*, pp. 743–761. Edited by D. Berg & M. Howe. Washington, DC: American Society for Microbiology.
- SWANSON, J., ROBBINS, K., BARRERA, O., CORWIN, D., BOSLEGO, J., CIAK, J., BLAKE, M. & KOOMEY, J. M. (1987). Gonococcal pilin variants in experimental gonorrhoeae. *Journal of Experimental Medicine* **165**, 1344–1357.
- TAYLOR, R. K., MILLER, V. L., FURLONG, D. B. & MEKALANOS, J. J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 2833–2837.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- TØNJUM, T., BUKHOLM, G. & BØVRE, K. (1989). Differentiation of some species of *Neisseriaceae* and other bacterial groups by DNA–DNA hybridization. *APMIS* **97**, 395–405.
- WEST, S. E. H. & CLARK, V. L. (1989). Genetic loci and linkage associations in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Clinical Microbiological Reviews* **2**, S92–S103.