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# Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and pilus-mediated adhesion

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## Summary

**Pili, which are assembled from protein subunits called pilin, are indispensable for the adhesion of capsulated *Neisseria meningitidis* (MC) to eukaryotic cells. Both MC and *Neisseria gonorrhoeae* (GC) pilins are glycosylated, but the effect of this modification is unknown. In GC, a galactose α-1,3-*N*-acetyl glucosamine is O-linked to Ser-63, whereas in MC, an O-linked trisaccharide is present between residues 45 and 73 of pilin. As Ser-63 was found to be conserved in pilin variants from different strains, it was replaced by Ala in two MC variants to test the possible role of this residue in pilin glycosylation and modulation of pili function. The mutated alleles were stably expressed in MC, and the proteins they encoded migrated more quickly than the normal protein during SDS–PAGE. As controls, neighbouring Asn-61 and Ser-62 were replaced by an Ala with no effect on electrophoretic mobility. Silver staining of purified pilin obtained from MC after oxidation with periodic acid confirmed the loss of glycosylation in the Ser-63→Ala pilin variants. Mass spectrometry of HPLC-purified trypsin-digested peptides of pilin and Ser-63→Ala pilin confirmed that peptide 45–73 has the molecular size of a glycopeptide in the wild type. In strains producing non-glycosylated pilin variants, we observed that (i) no truncated S pilin monomer was produced; (ii) piliation was slightly increased; and (iii) presumably as a consequence, adhesiveness for epithelial cells was increased 1.6- to twofold in these derivatives. In addition, pilin monomers and/or individual pilus fibres, obtained after solubilization of a crude pili preparation in a high pH buffer, were reassociated into insoluble aggregates of pili more completely with non-glycosylated variants than with the normal pilin. Taken together, these data eliminate a major role for pilin glycosylation in piliation and subsequent pilus-mediated adhesion, but they demonstrate that glycosylation facilitates solubilization of pilin monomers and/or individual pilus fibres.**

## Introduction

Pili are the only bacterial attribute that have been implicated to date in the adhesion of capsulated *Neisseria meningitidis* (MC) to eukaryotic cells (Virji *et al.*, 1993; Nassif *et al.*, 1994). MC produces two types of pilins, designated class I and class II (Perry *et al.*, 1988; Potts and Saunders, 1988; Virji *et al.*, 1989; Aho *et al.*, 1997). Unlike the latter, the former are highly related to gonococcal pilin. A single *Neisseria gonorrhoeae* (GC) or MC cell is genetically capable of producing antigenically different pilin variants, but some regions of the pilin monomer are conserved, whereas others are highly variable (Fig. 1). Conserved domains most probably play a role in pilus assembly and perhaps host cell recognition. Certain pilin variants are not completely assembled into pili but are processed to soluble pilin subunits. In this case, pilin lacks the first 39 amino acids of the N-terminal region (Haas *et al.*, 1987), and strains producing these variants are usually less piliated.

Recently, MC and GC pili have been found to be glycosylated. In solving the atomic resolution structure of a GC pilin, Parge *et al.* (1995) found electron density that matched galactose α-1,3-*N*-acetyl glucosamine O-linked to Ser-63. Stimson *et al.* (1995) have shown that an O-linked trisaccharide is present between amino acid residues 45 and 73 of MC pilin. This structure contains a terminal 1,4-linked digalactose covalently linked to a 2,4-diacetamido-2,4,5-trideoxyhexose. The difference in glycosylation pattern between these two species may reflect functional differences in the role of glycosylation. The mechanism and role of pilin glycosylation remain unknown.

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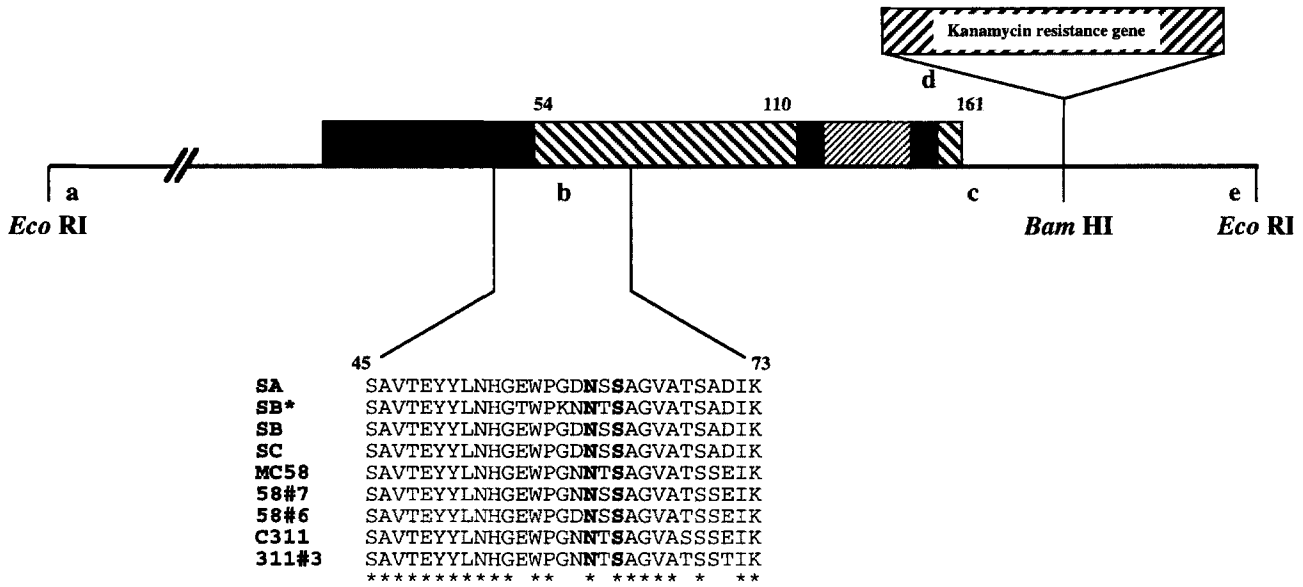


**Fig. 1.** Pilin expression locus of GC and MC class I strains. Residues 1–53 of the mature protein correspond to the constant region; residues 54–161 correspond to the variable domain. Solid boxes indicate the location of the conserved CYS1 and CYS2 regions. Class II pilins share extensive amino acid identity with the N-terminal conserved regions of class I pilin but lack several regions found within semi-variable and hypervariable regions present in class I pilin (Aho *et al.*, 1997). Constructions of pilE::Km fusions are as described previously (Nassif *et al.*, 1993; Marceau *et al.*, 1995). Regions a, b, c, d and e correspond to the location of the oligonucleotides described in Table 1. SA, SB, SB* and SC are pilin variants of strain 8013 and are described elsewhere (Nassif *et al.*, 1993). C311 and MC58 derivatives are from M. Virji (Virji *et al.*, 1993). Stars indicate conserved amino acid residues. Sequence alignment shows conservation of Asn-61 and Ser-63. Figure is not drawn to scale.

Numerous studies using naturally occurring piliated ($P^+$) and non-piliated ($P^-$) variants have shown that pili play an important role in GC and MC adhesion to epithelial and endothelial cells in culture. $P^+$ bacteria adhere to eukaryotic cells much better than $P^-$ variants (Nassif *et al.*, 1994). Besides pilin, two additional proteins, PilC1 and PilC2, initially identified in GC, play a major role in pilus biogenesis (Jonsson *et al.*, 1991). These proteins have been localized in both the outer membrane and the pili (Jonsson *et al.*, 1991; Rahman *et al.*, 1997). One of the PilC proteins needs to be present for a strain to be piliated. In addition, both proteins have been shown to be pilus tip-located adhesins in GC (Rudel *et al.*, 1995). In MC, only variants producing PilC1 are adhesive (Nassif *et al.*, 1994), as $PilC2^+/PilC1^-$ isolates are piliated but non-adhesive. By analogy with the data obtained in GC (Rudel *et al.*, 1995), the current model for adhesion considers PilC1 as the MC pilus tip adhesin. Pilin antigenic variation has been shown to modulate MC adhesiveness to human cells (Rudel *et al.*, 1992; Nassif *et al.*, 1993; Virji *et al.*, 1993). High-adhesive pilin variants are responsible for the formation of large bundles of pili (Marceau *et al.*, 1995), thus increasing interbacterial interactions. Strains producing these variants grow as colonies on the surface of cells. On the other hand, low-adhesive pilin variants tend to form long flexible fibres, and strains producing these pili adhere to cells as isolated diplococci.

Glycosylation is less frequently described for bacterial proteins than for eukaryotic proteins, and the role of post-translational modifications remains largely unknown in the prokaryotic world. To assess the role of pilin glycosylation in pilus biogenesis and pilus-mediated adhesion, we have substituted the Ser-63 of a low- and a high-adhesive pilin variant of an MC class I strain with an Ala, confirmed that bacteria producing these mutated alleles do not have glycosylated pilin and assessed the consequences of these modifications on pilus biogenesis and pilus-mediated adhesion.

## Results

### *Replacement of Ser-63 by Ala in pilin reduced glycosylation*

To assess the role of pilin glycosylation in piliation and pilus-mediated adhesion, we first engineered isogenic pilin variants that were altered in their glycosylation site. Earlier data on GC clearly identified glycosylation of Ser-63 (Parge *et al.*, 1995). In MC, glycosylation was found to be linked to a peptide spanning residues 45–73 (Stimson *et al.*, 1995). Examination of several pilin variants from two MC strains confirmed that Ser-63 is highly conserved in MC class I strains (Fig. 1); furthermore, this residue is also conserved in MC class II pilin (Aho *et al.*, 1997). Therefore, we decided to replace this amino acid

**Table 1.** Site-directed mutations of *pilE*.

| Parental pilin variant[a] | Mutation | Name of the mutation | Oligonucleotides used for mutagenesis[b] |
|---|---|---|---|
| SA | Ser-62→Ala-62 | Ser-62→Ala | MS1A+ and MS1A− |
| SA | Ser-63→Ala-63 | Ser-63→Ala | MS2A+ and MS2A− |
| SA | Ser-62→Ala-62 and Ser-63→Ala-63 | Ser-62→Ala–Ser-63→Ala | M2S2A+ and M2S2A− |
| SA | Asn-61→Ala-61 | Asn-61→Ala | MN1A+ and MN1A− |
| SB | Ser-62→Ala-62 | Ser-62→Ala | MS1A+ and MS1A− |
| SB | Ser-63→Ala-63 | Ser-63→Ala | MS2A+ and MS2A− |
| SB | Ser-62→Ala-62 and Ser-63→Ala-63 | Ser-62→Ala–Ser-63→Ala | M2S2A+ and M2S2A− |
| SB | Asn-61→Ala-61 | Asn-61→Ala | MN1A+ and MN1A− |

**a.** SA and SB are two pilin variants of 8013, a MC class I strain. SA is responsible for low adhesiveness. SB is responsible for high adhesiveness.
**b.** See Table 3 and Fig. 1.

by an Ala in two pilin variants, SA and SB, isolated from MC strain 8013, which have a low- and a high-adhesive phenotype respectively. In addition to Ser-63 substitutions, neighbouring Asn-61 and Ser-62 were substituted, and double Ser-62–Ser-63 variants were obtained for both SA and SB pilins. These residues were replaced by Ala. As the only difference between an alanine and a serine is the substitution of the hydrogen by a hydroxyl, these changes, summarized in Table 1, are unlikely to modify the general structure of the protein. Wild-type and mutated alleles were transcriptionally fused to a kanamycin (Km) resistance gene before being introduced by transformation into clone 12, a capsulated, Opa$^-$, PilC1$^+$, PilC2$^+$ derivative of strain 8013. All experiments were then performed using these transformants.

To confirm the existence of a post-translational modification on our pilin variants, we first determined the electrophoretic mobility of each molecule. As for all type IV pilins, the product of the pilin-encoding gene is a precursor that is processed at a highly conserved consensus cleavage site, located close to the N-terminus, which removes seven amino acids (Schoolnik *et al.*, 1984). This cleavage requires the product of the *pilD* gene, a prepilin peptidase (Strom *et al.*, 1993). Some pilin variants are processed at an additional cleavage site, which removes 39 amino acids from the N-terminus (Haas *et al.*, 1987). These truncated forms of pilin, designated S pilin, are not assembled into pili and are secreted into the surrounding media. The SB pilin variant does not produce any S pilin, and a single band corresponding to the mature pilin is seen on Western blots (Fig. 2B, lanes 1 and 5). However, the SA pilin gives rise to mature and truncated S pilin. This latter form is better seen in culture supernatant (Fig. 7A, lane 1) than in total protein extracts (Fig. 2A, lanes 1 and 5). The electrophoretic mobility of the Ser-62→Ala variants (Fig. 2A and B, lanes 2) and the Asn-61→Ala variants (Fig. 2A and B, lanes 6) is identical to that of the normal protein. On the other hand, Ser-63→Ala and Ser-62→Ala–Ser-63→Ala variants (Fig. 2A and B, lanes 3 and 4) have molecular weights lower than those of the corresponding normal proteins (Fig. 2A and B, lanes 1 and 5). Considering that the replacement of Ser-62 by Ala does not modify the electrophoretic mobility of SA and SB pilins, the most likely explanation for the lower molecular weights observed when a

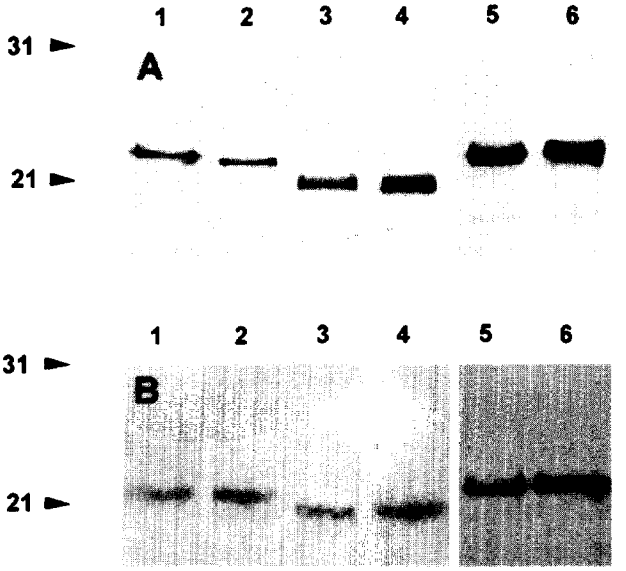


**Fig. 2.** Western blot of total bacterial extracts showing electrophoretic migration of SA pilin and its derivatives (A) or SB pilin and its derivatives (B). Total bacterial extracts were run in SDS–PAGE, 10–20% acrylamide, 4 M urea and transferred to nitrocellulose. The 5C5 monoclonal antibody, which recognizes an epitope located in the constant region, was used for pilin detection. Lanes A1 and B1, lanes A5 and B5: wild-type SA and SB pilins expressed by *N. meningitidis* clone 12 respectively. Lane A5, owing to the large quantity of proteins in this well 5C5 antibody detects, besides full-length mature pilin, a minor band around 14 kDa., which corresponds to S pilin. Lanes A2 and B2, Ser-62→Ala mutant of SA and SB pilin expressed by *N. meningitidis* clone 12 respectively. Lanes A3 and B3, Ser-63→Ala mutants of SA and SB pilins expressed by *N. meningitidis* clone 12 respectively. Lanes A4 and B4, Ser-62→Ala–Ser-63→Ala double mutants of SA and SB pilins expressed by *N. meningitidis* clone 12 respectively. Lanes A6 and B6, Asn-61→Ala mutant of SA and SB pilins expressed by *N. meningitidis* clone 12 respectively.

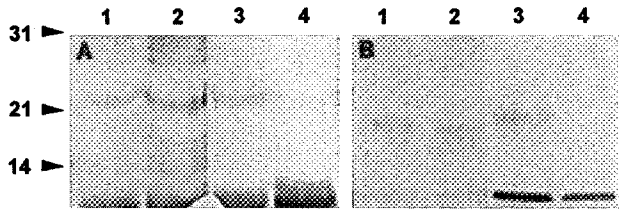


**Fig. 3.** Semi-purified SA (A) or SB (B) pilin preparations were electrophoresed in SDS–PAGE. Lanes 3 and 4 were treated with periodic acid before being silver stained, while lanes 1 and 2 were kept in distilled water. A1, A3 and B1, B3 wells were loaded with the same amount of semi-purified preparation of wild-type SA and SB pilins respectively. A2, A4 and B2, B4 were loaded with the same amount of semi-purified preparations of Ser-63 mutant of SA and SB pilins respectively. Pretreatment with periodic acid enhances the staining of glycosylated proteins (Hitchcock and Brown, 1983). The band seen at the bottom in lanes 3 and 4 corresponds to LOS.

similar replacement is performed at the neighbouring Ser-63 is that a post-translational modification is linked to Ser-63.

To confirm that this post-translational modification was a glycosylation, purified pilin preparations were stained with silver after oxidation with periodic acid. Such a treatment enhances the silver staining of carbohydrate and, subsequently, of glycoproteins (Hitchcock and Brown, 1983). Oxidation with periodic acid enhanced the staining of purified SB and SA pilins obtained from MC, whereas similar preparations of Ser-63 mutants showed dramatically reduced staining (Fig. 3). In addition, no change in the staining of the Ser-62 variant was observed when compared with the corresponding normal protein (data not shown). Taken together, these data demonstrate that the post-translational modification removed by the Ser-63→ Ala mutation is glycosylation. In addition, the fact that pilins with an Asn-61→Ala substitution have an electrophoretic mobility identical to the normal proteins (Fig. 2A and B, lanes 6) eliminates the possibility of an N-glycosylation on this Asn.

### *Structural studies on SB pilin and its Ser-63-Ala mutant*

To confirm that Ser-63→Ala pilin mutants were not glycosylated and gain insight into the saccharide involved in this modification, gel-purified pilin from clone 12 expressing SB or SB Ser-63→Ala was subjected to protease digestion, and high-performance liquid chromatography (HPLC) peak fractions were analysed by matrix-assisted laser desorption spectrometry (MALDI). The predicted mass of the tryptic 45–73 peptide without modification for wild-type SB pilin (Fig. 1) is 3040 Da. For wild-type SB (Fig. 4, top), the tryptic peptide 45–73 had an observed mass of 3467 Da, thus confirming a post-translational modification on this fragment in mature pilin. For the Ser-63→Ala pilin variant, the 45–73 peptide had a mass of 3085 Da (Fig. 4, middle and bottom) compared with a predicted mass of 3024. The N-terminal sequence confirmed the identity of both peptides and, in the case of the Ser-63→Ala mutant, the Ser to Ala substitution.

The mass difference between the 45–73 peptide of the SB pilin and its Ser-63→Ala mutant is therefore 382 Da, which is exactly as predicted for the loss of an O-linked galactose alpha-1,3-GlcNAc (366 Da; Parge *et al.*, 1995) and the substitution of Ala for Ser (16 Da). Surprisingly, mass is not consistent with the trisaccharide observed in MC strain C311 (Stimson *et al.*, 1995). In addition, each 45–73 peptide has a mass of 61 Da higher than predicted. There is, thus, apparently an additional uncharacterized modification of 61 Da in the 45–73 peptide that is not linked to Ser-63. This mass measurement is reproducible (Fig. 4, middle and bottom) and accurate to within ± 0.1% of the mass of the peptides (± 3 Da).

### *Consequences of the loss of pilin glycosylation on piliation and pilus-mediated adhesion*

Monitoring of piliation of clone 12 producing SA or SB pilin or the corresponding non-glycosylated mutated alleles by transmission electron microscopy showed similar piliation (data not shown). Clone 12 producing SA or its Ser-63→ Ala variant has long flexible fibres made of a single pilus, as expected for a strain producing a low-adhesive pilin variant. In the case of clone 12 producing SB or its Ser-63→Ala variant, the fibres are made of large bundles of pili, this being consistent with the high-adhesive phenotype. These data ruled out a dramatic role for glycosylation in piliation. However, a careful examination suggested that pili may be slightly more abundant in clone 12 producing Ser-63→Ala mutants than in clone 12 producing the corresponding normal proteins. As quantification of piliation using electron microscopy is difficult because it does not allow visualization of a large number of bacteria, we used immunofluorescence staining of pili with monoclonal antibodies directed against pilin. Pili were present regardless of the glycosylation status of pilin (Fig. 5). For SB variants, the material stained by the antibody was thicker than with SA, consistent with the ability of SB pilin to form bundles of pili (Fig. 5, B1 and B2), whereas SA-producing strains make single-pilus fibre. A close examination confirmed that bacteria producing non-glycosylated derivatives were slightly more piliated than wild-type strains. This was especially noticeable in the case of SA pilin (Fig. 5, compare A1 and A2).

To test the role of glycosylation on pilus-mediated adhesion, the adhesiveness of clone 12 expressing different variants was assessed on epithelial cells. Adhesiveness was defined as the ratio of cell-associated CFUs to the total number of CFUs after 4 h (see *Experimental procedures*). Values obtained with clone 12 producing SA and

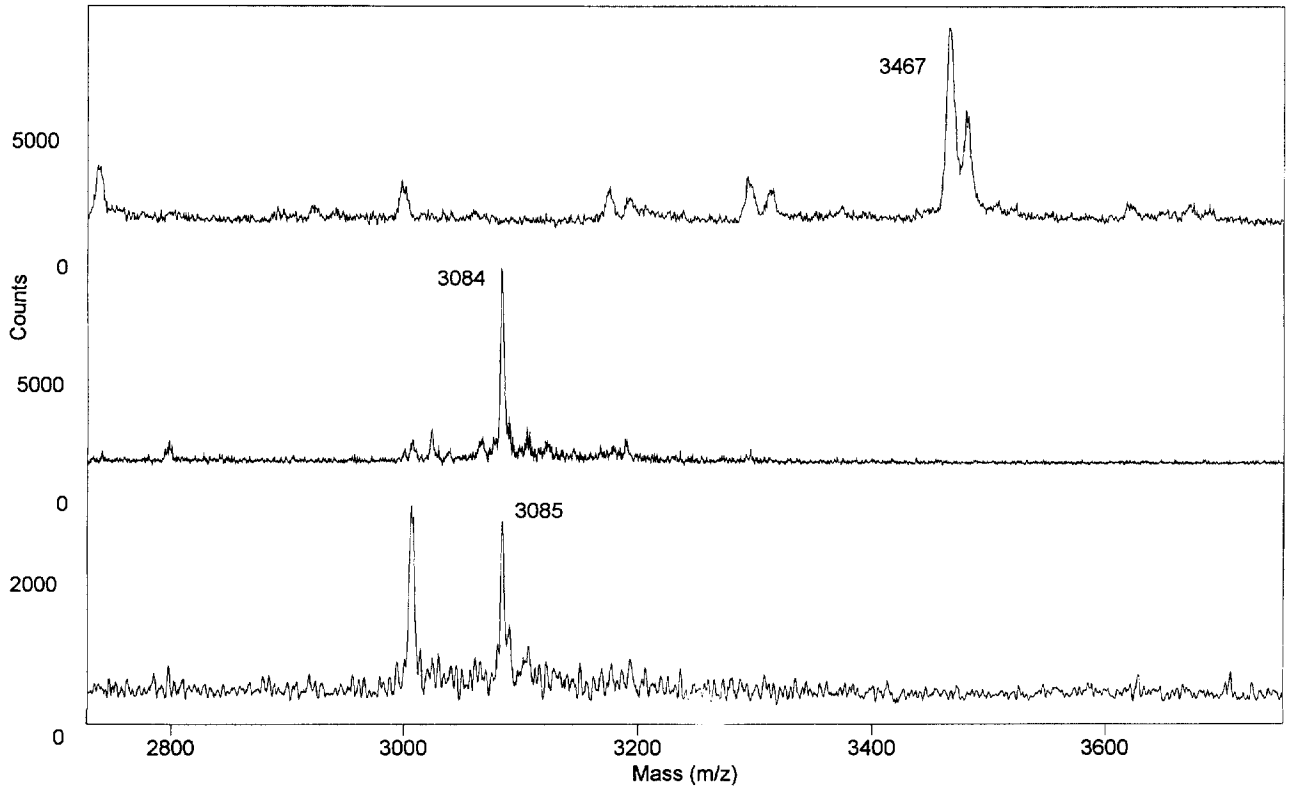


**Fig. 4.** MALDI mass spectrographs for trypsin-digested, partially purified peptides corresponding to the 45–73 trypsin fragment of pilin. Top, wild-type SB pilin. Middle and bottom, two independent fractions from SB Ser-63→Ala pilin, each of which contain the 45–73 peptide. In each case, the expected sequence was verified by N-terminal sequence analysis. The mass loss corresponds to a 366 Da saccharide and the 16 Da difference in mass for the Ser vs. Ala side-chain. Error is ± 0.1%.

SB pilins and the corresponding mutated pilins are shown in Table 2. As expected, SA pilin and all its derivatives were associated with a low-adhesive phenotype, whereas SB pilin and its derivatives were associated with a high-adhesive phenotype. Thus, the loss of glycosylation does not have a dramatic effect on MC adhesion onto cells. However, in order to detect small differences, adhesion assays were performed at the same time with clone 12 producing SA or SB pilins and the corresponding Ser-62 and Ser-63 variants. Adhesiveness was calculated as above, and the ratio (adhesiveness of clone 12 expressing Ser-62 or Ser-63 pilin variant to the adhesiveness of clone 12 expressing the corresponding wild-type allele in the same experiment) were determined (Fig. 6). With Ser-62 mutants, the ratio was very close to 1, thus confirming that this mutation did not affect pilus-mediated adhesion. On the other hand, clone 12 producing pilins with an Ala in place of Ser-63 was more adhesive than clone 12 producing the corresponding Ser-62 mutated alleles in all experiments. This variation was on average twofold for SA and 1.6-fold for SB and was constant in all experiments. In addition, statistical analysis confirmed that the difference observed between Ser-62 and Ser-63 for both variants was significant ($P<0.05$). The data obtained with pilus-mediated adhesion are consistent with those reported for piliation and suggest that the moderate increase in piliation is responsible for a moderate increase in adhesion.

### *Solubilization of both pilin monomers and individual pilus fibres is facilitated by glycosylation*

As bacteria producing deglycosylated variants are slightly more piliated than those with normal pilin, pilin glycosylation might modulate the solubilization of pilin and/or individual pilus fibres. To address this question, we tested

**Table 2.** Adhesiveness of clone 12 expressing wild-type or mjutated pilin.

| Pilin variant | Adhesion[a] |
|---|---|
| SA wild type | 0.007 (0.004) |
| SA Ser-62→Ala | 0.006 (0.003) |
| SA Ser-63→Ala | 0.015 (0.008) |
| SB wild type | 0.084 (0.057) |
| SB Sera-62→Ala | 0.084 (0.051) |
| SB Ser-63→Ala | 0.125 (0.078) |

**a.** Adhesiveness is defined as the ratio of cell-associated CFU/CFU in the supernatant after 4 h of incubation.

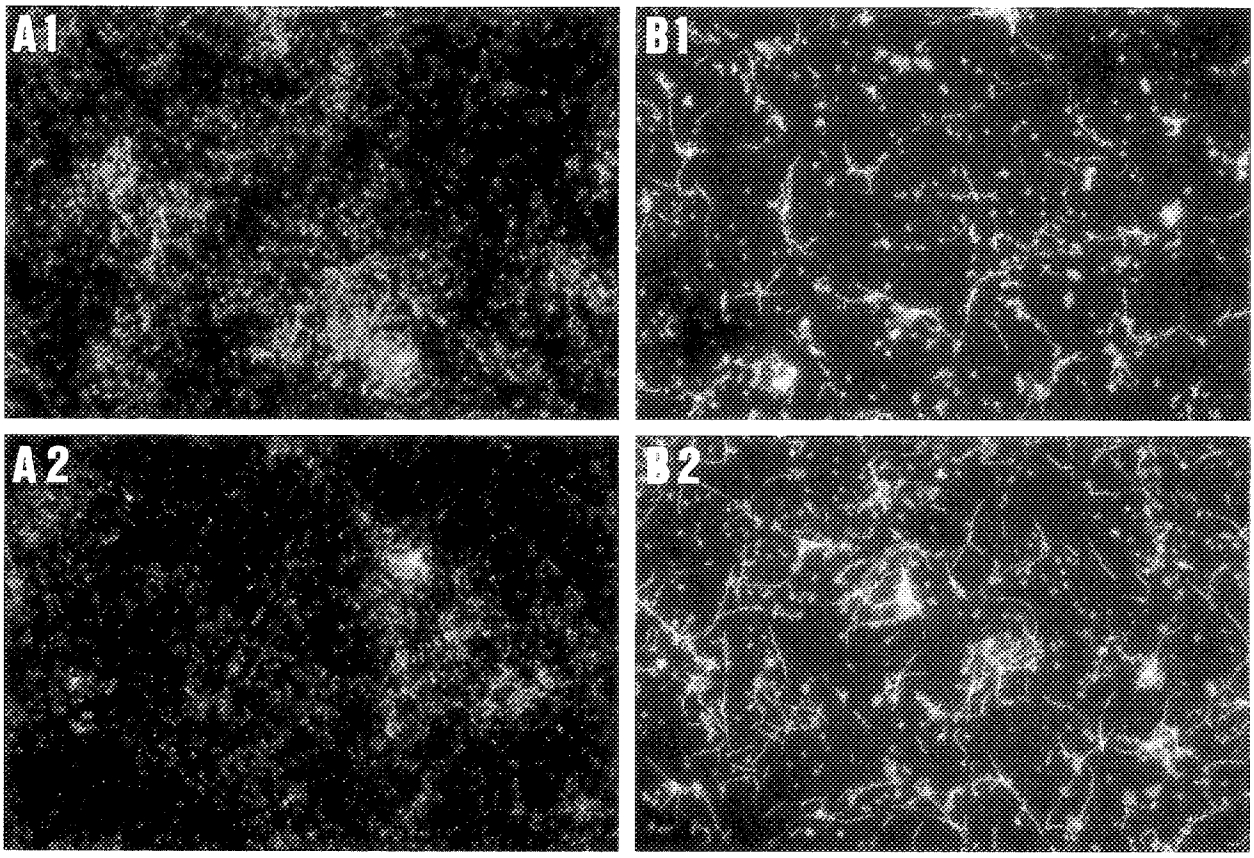


**Fig. 5.** Immunofluorescence staining of pili of GCB-grown bacterial cultures.
A. Corresponds to clone 12 expressing SA (A1) or its Ser-63→Ala mutant (A2).
B. Corresponds to clone 12 expressing SB (B1) or its Ser-63→Ala mutant (B2). Bacteria were stained with ethidium bromide. The primary monoclonal antibodies used to visualize pili are 5C5 (A) and 20D9 (B).

the ability of pilin and/or individual pilus fibres to be reassociated into insoluble aggregates of pili by monitoring the different steps of the pilin purification procedure. This technique relies on the ability of individual pilus fibres and/or pilin monomers to be solubilized at high pH in 20 mM ethanolamine. Lowering the pH favours the reassociation of these individual fibres and/or pilin monomers into insoluble aggregates of pili. These pili aggregates can then be pelleted by centrifugation, and the pellet resuspended in dissociation buffer (Parge *et al.*, 1990). To examine the ability of pilin monomers and/or individual soluble fibres to be reassociated, we monitored (i) the quantity of pilin present in the pellet, which therefore exists as pili aggregates; and (ii) the amount of pilin that remained in the supernatant, which therefore corresponds to pilin monomers and/or individual pilus fibres (see *Experimental procedures*). For these experiments, the number of bacteria harvested in the dissociation buffer was identical, and the volume in which the pellet was resuspended was identical in all experiments (see *Experimental procedures*). First, it should be pointed out that there is a correlation

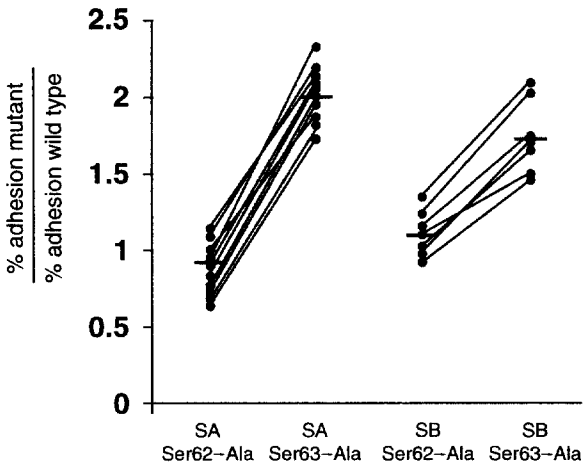


**Fig. 6.** Comparative analysis of adhesion associated with Ser-62→Ala and Ser-63→Ala mutations in the SA or SB pilin variants. Each dot corresponds to the ratio of adhesiveness of clone 12 expressing the mutated pilin over adhesiveness of clone 12 expressing the corresponding wild-type allele determined in the same experiment. Ratios that were determined with values obtained in the same experiment are linked. Horizontal bars correspond to the mean.

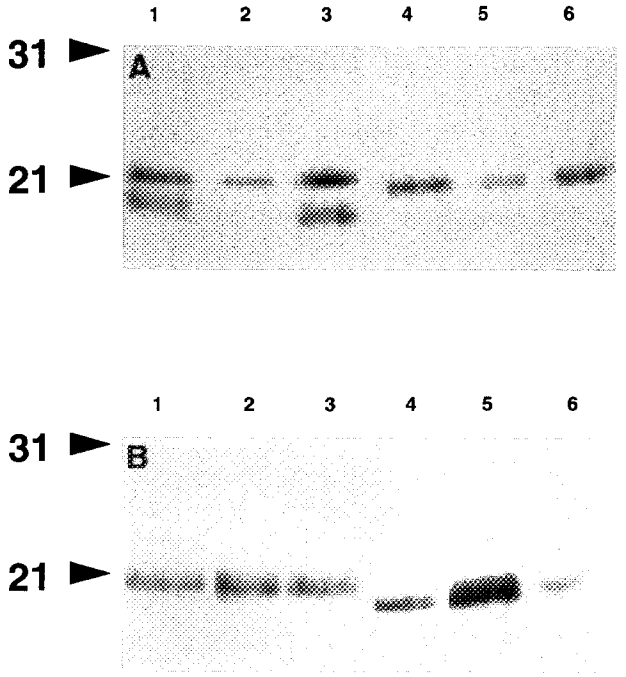


**Fig. 7.** Western blots performed on culture supernatants of clone 12 expressing SA (A) and SB (B) pilin variants. 15% polyacrylamide gels were run in the presence of β-mercaptoethanol and transferred onto nitrocellulose. The 5C5 monoclonal antibody was used as previously described to detect pilin. Lanes A1–A3 and B1–B3, SA and SB pilin variants respectively. Lanes A4–A6 and B4–B6, Ser-63→Ala mutant of SA and SB pilins respectively. Lanes 1 and 4, SUSP1, i.e. initial pilin suspension in ethanolamine (pH 10.5) obtained after harvesting and vortexing of bacteria before reassociation. Lanes 2 and 5, SUSP3, i.e. pilin solution obtained after solubilization of the pellet; this fraction corresponds to the amount of pilin that was reassociated as insoluble aggregates of pili. Lanes 3 and 6, SUSP2, i.e. supernatants of the centrifugation corresponding to the above pellet. This fraction corresponds to the amount of pilin that has not been reassociated into insoluble aggregates of pili and that was, therefore, as a pilin monomer and/or individual fibres. The lower band observed on lanes A1 and A3 corresponds to the S pilin.

between the ability of a pilin variant to form aggregates *in vitro*, on the one hand, and the presence of bundles of pili surrounding the bacteria expressing this variant, on the other hand. The high-adhesive SB pilin, which is responsible for the formation of bundles of pili, is found in a significant proportion in the pellet after reassociation (Fig. 7B, compare lanes 2 and 3). On the other hand, the low-adhesive SA variant was not responsible for the formation of bundles of pili, and only a small proportion of this pilin was found in the pellet after reassociation (Fig. 7A, compare lanes 2 and 3). In addition, this low-adhesive SA variant produced S-truncated pilins (Fig. 7A, lane 1). As expected, these S pilins, which cannot be assembled into pili, were not found in the pellet (Fig. 7A, lane 2) but were present in the supernatant (Fig. 7A, lane 3).

Similar experiments were performed with clone 12 producing Ser-63→Ala variants of SA and SB. In the case of the Ser-63→Ala variants of SB, very few pilin were present in the supernatant, and a much higher proportion of pilin was found in the pellet than with the wild-type SB pilin (Fig. 7B, compare lanes 2 and 3 with lanes 5 and 6). This suggests that the formation of aggregates of pili was facilitated by the loss of glycosylation. With the Ser-63→Ala variant of SA, the fraction of pilin present in the pellet was not significantly different to that observed with the normal SA variant. However, a major difference was that no S pilin was present in the initial pilin suspension (Fig. 7A lane 4). Taken together, these data demonstrate that glycosylation increases the amount of soluble pilin monomers and/or individual pili fibres.

## Discussion

Post-translational modifications have been reported recently for pathogenic *Neisseria* type IV pilin (Virji *et al.*, 1993). The first substitution reported for both MC and GC pilin was glycosylation (Parge *et al.*, 1995; Stimson *et al.*, 1995). In GC, characterization of pilin structure localized this modification on Ser-63, and the electron density matches that of a covalently O-linked, N-acetyl glucosamine-alpha1,3-galactose (Parge *et al.*, 1995). In MC, the sugar is a trisaccharide, a digalactosyl-2,4-diacetamido-2,4,6-trideoxyhexose (Stimson *et al.*, 1995). This trisaccharide was localized between residues 45 and 73. In addition, a serine-linked glycerophosphate has been reported on MC pilin (Stimson *et al.*, 1996). This modification has been localized to Ser-93. However, the role of these post-translational modifications remained unknown. To evaluate the role of pilin glycosylation, we have engineered a mutation in two pilin variants produced by an MC class I strain by site-directed mutagenesis. In both cases, the replacement of Ser-63 by Ala increased the electrophoretic mobility of the pilin, whereas the replacement of the neighbouring Asn-61 and Ser-62 by Ala was without any effect on the electrophoretic mobility. In addition, reduced staining of the Ser-63 mutant after periodic acid oxidization revealed that the post-translational modification that was removed is glycosylation. These data confirmed that glycosylation was O-linked onto Ser-63. The mass difference between SB and its Ser-63→Ala mutant is 382 Da, which is exactly as predicted for the loss of an O-linked galactose alpha-1,3 GlcNAc (366 Da) and the substitution of Ala for Ser (16 Da). This glycosylation is similar to the one reported by Parge *et al.* (1995) for pilin glycosylation in GC. A mass of 366 Da is not consistent with the trisaccharide observed in MC strain C311, which would result in a higher molecular weight (Stimson *et al.*, 1995). Thus, the different glycosylation pattern is not intrinsically a difference between *N. meningitidis* and *N. gonorrhoeae*; apparently, a strain to strain difference exists among MC. Therefore, the biological role of glycosylation may not be specific to

**Table 3.** Oligonucleotides.

| Location on Fig. 1 | Name | Strand | Sequence | Restriction site incorporated at the 5′ end |
|---|---|---|---|---|
| a | PILEM3ECO | + | 5′-GCGAATTCACCGACCCAATCAACACACCCG-3′ | *Eco*RI |
| b | MS1A+ | + | 5′-TGGCCCGGCGACAACGCCTCTGCCGGCGTGGCA-3′ | |
| b | MS1A− | − | 5′-TGCCACGCCGGCAGAGGCGTTGTCGCCGGGCCA-3′ | |
| b | MS2A+ | + | 5′-TGGCCCGGCGACAACAGTGCCGCCGGCGTGGCA-3′ | |
| b | MS2A− | − | 5′-TGCCACGCCGGCGGCACTGTTGTCGCCGGGCCA-3′ | |
| b | M2S2A+ | + | 5′-TGGCCCGGCGACAACGCCGCCGCCGGCGTGGCA-3′ | |
| b | M2S2A− | − | 5′-TGCCACGCCGGCGGCGGCGTTGTCGCCGGGCCA-3′ | |
| b | MN1A+ | + | 5′-TGGCCCGGCGACGCCAGTTCTGCCGGCGTGGCA-3′ | |
| b | MN1A− | − | 5′-TGCCACGCCGGCAGAACTGGCGTCGCCGGGCCA-3′ | |
| c | 6 | − | 5′-CGGGATCCTTACCTTAGCTGGCAGATGAAT-3′ | *Bam*HI |
| d | KM5 | − | 5′-GGAGACATTCCTTCCGTATC-3′ | |
| e | OLIGO2ECO | − | 5′-CGGAATTCAGCCAAAACGGACGACCCC-3′ | *Eco*RI |

the precise nature of this modification, but may be caused by a general property conferred by glycosylation independently of the sugar chemistry. However, each tryptic 45–73 peptide had a mass of 61 Da higher than that predicted for the observed peptide sequences. This mass measurement is reproducible and accurate to within ± 0.1% of the mass of the peptides (± 3 Da). Thus, there is, apparently, an additional uncharacterized modification of 61 Da in the 45–73 peptide. The identity of the additional 61 Da modification has not been established. This mass is compatible with -CH2-CH2-COHH2 (58 Da) or -COHH-COHH2 (60 Da). There is a double peak on the mass chromatogram for the wild-type SB peptide (Fig. 4A). The mass of the second peak would be consistent with the presence of an additional OH group. As this disappears in the Ser-63 variant, the saccharide may be mixed in some bacteria or on some pilin molecules, so that it occasionally has an extra hydroxyl group.

The role of pili in mediating bacteria–cell interaction has been well characterized, especially in capsulated bacteria. Our data demonstrate that bacteria producing non-glycosylated variants are slightly more piliated and presumably, as a consequence, more adhesive than isogenic strains producing normal pilin variants. However, these changes are not dramatic. Similar results have been reported by Stimson *et al.* (1995), who demonstrated that the removal of the terminal digalactose residues of the modification in *galE* mutants also had no significant effect on the adhesive abilities of MC. The fact that pilin glycosylation modulated the solubilization of pilin monomer and/or individual pili fibres is consistent with the more abundant piliation observed with strains producing non-glycosylated variants. The non-glycosylated high-adhesive variant formed more aggregates than glycosylated pilin *in vitro*. Considering the correlation that exists between *in vitro* aggregation and bundling, the slight increase in piliation may be a consequence of the increase in the formation of bundles of pili. With the non-glycosylated low-adhesive variant, *in vitro* aggregation of pili was not enhanced. This is consistent with the fact that bacteria producing these non-glycosylated variants were not forming bundles of pili. On the other hand, the most striking effect of the loss of glycosylation was that bacteria did not produce S pilin. Therefore, low-adhesive pilins altered in their glycosylation site are produced only as full-length monomers. It seems that the increase in piliation observed with a non-glycosylated low-adhesive pilin is the result of an increased production of full-length monomer. No function has been yet found for S pilin. It has been shown recently that there is a cell-binding domain in pilin within the first 77 residues of the mature pilin (Marceau *et al.*, 1995). The structural data suggest that this potential pilin cell-binding domain is hidden in assembled pili. One hypothesis is that it is available only in the context of the soluble monomer and could play a role in meningococcal pathogenesis by interacting with some components of the host.

## Experimental procedure

### *Bacterial strains, growth conditions and oligonucleotides*

Strain 8013 is an MC class I strain belonging to the C serogroup. This strain possesses only one copy of the *pilE* locus (Nassif *et al.*, 1991). Clone12 is a spontaneously occurring piliated, Opa$^-$, PilC1$^+$/PilC2$^+$ variant of 8013. SB (accession number S34940) and SA (accession number S34939) pilins are two variants isolated from 8013, responsible for a high- and a low-adhesive phenotype respectively (Nassif *et al.*, 1993; 1994). MC were grown and transformed using previously described standard techniques (Nassif *et al.*, 1991). Kanamycin was used at a concentration of 100 μg ml$^{-1}$ for the selection of MC and 25 mg ml$^{-1}$ for the selection of *Escherichia coli* strain MC1061. Oligonucleotides used in this study are described in Table 3 and Fig. 1. Standard molecular biology techniques were performed according to Sambrook *et al.* (1989).

### *Constructions of* pilE*::Km transcriptional fusions and site-directed mutagenesis*

*pilE*::Km fusions correspond to pilin genes that are transcriptionally fused with a Km resistance gene lacking its own

promoter (Fig. 1). The construction of the SA::Km and SB::Km pilin fusions have been described elsewhere (Nassif *et al.*, 1993). The SA::Km and SB::Km fusions were cloned into the *Eco*RI site of pBR325 after amplification using oligonucleotides PILEM3ECO and OLIGO2ECO. The SA::Km and SB::Km fusions have been introduced into clone 12 by transformation. Pools of 1000 Km-resistant transformants were realized as previously described (Marceau *et al.*, 1995). All biological assays were performed with these pools to avoid the possibility that spontaneous variation of other bacterial components beside pilin was responsible for the modification of a phenotype. The pools express the same PilC phenotype as the parental clone 12.

Site-directed mutagenesis was performed by polymerase chain reaction (PCR) overlap extension (Ho *et al.*, 1989) using PILEM3ECO, OLIGO2ECO and a set of two complementary mutagenizing oligonucleotides. Table 1 indicates the oligonucleotides used to generate each mutation. The template was total DNA of clone 12 containing either the SA::Km or the SB::Km fusion. All mutated alleles linked to the Km resistance gene were transformed back into clone 12. Km-resistant transformants were reisolated twice, and sequencing of *pilE* was done using, as template, total DNA of the transformants and, as primers, oligonucleotides 1 and Km5. PCR products were then directly sequenced by dye-dideoxy chain termination using a Perkin-Elmer ABI 310 sequencing apparatus. For each mutagenesis, a clone was selected for further study, and the total DNA of this clone was transformed back into clone 12. As above, a pool of 1000 Km-resistant transformants was obtained. Mutation stability of Ser-63→Ala mutants and Ser-62→Ala–Ser-63→Ala double mutants was assayed by reisolating 12 colonies from each variant from the frozen pool. The electrophoretic mobility of these pilin variants was checked to detect reversion of mutations, which would be indicated by an increase in the molecular weight.

### *Pili preparation, antibodies and immunoblots*

Semi-purified pili preparations were obtained as already described by Parge *et al.* (1990). Briefly, bacteria from batches of 50 plates were harvested into 120 ml of pili denaturation buffer (20 mM ethanolamine, pH 10.5) and vortexed to shear pili. Bacteria and membrane aggregates were removed by centrifugation. Pili were obtained by reassociation at neutral pH (50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% $NaN_3$) and centrifugation.

To examine the ability of pilin monomers and individual pili fibres to form aggregates, a similar technique was performed, but the amount of full-length mature pilin was monitored carefully in all steps. Pili were sheared from $10^9$ CFU of MC resuspended into 2 ml of ethanolamine buffer, pH 10.5. This initial pilin suspension was designated SUSP1. After reassociation, these 2 ml were centrifuged and the pellet resuspended in 200 μl of dissociation buffer. The supernatant obtained from this step was designated SUSP2 and the resuspended pellet SUSP3. The presence in each of these fractions of mature pilin and/or S pilin was appreciated by loading a 15% SDS–PAGE gel with 10 μl of SUSP1, SUSP2 and SUSP3.

The 20D9 monoclonal antibody, which has been described previously (Pujol *et al.*, 1997), is specific for the hypervariable region of the SB pilin and does not react with the SA pilin. The 5C5 monoclonal antibody was obtained by injection into Balb/c mice of purified SB pilin and was found to recognize the peptide VTEYYLNHGEW, which corresponds to the SM1 peptide (Virji and Heckels, 1983), located in the constant region.

Pilin supernatants or total protein extracts of MC were prepared using standard techniques (Laemmli, 1970). Samples were electrophoresed either in 15% acrylamide or in 10–20% gradient acrylamide, 4 M urea gels. Gels were either silver stained or proteins were transferred from SDS–PAGE to a nitrocellulose filter and incubated with the appropriate antiserum.

### *Comparative analysis of pilin glycosylation status by silver staining*

Semi-purified pili preparations were obtained as described above. Aliquots of 50 μg of each sample were run on a 15% acrylamide gel in the presence of β-mercaptoethanol. Silver staining was performed according to a technique developed by Hitchcock and Brown, (1983). Gels were fixed for at least 1 h in 25% isopropanol, 7% acetic acid in water, shaken in 30 mM periodic acid solution for 10 min, then washed for 8 × 15 min in water and kept for 12 min in silver-staining solution. Silver-staining solution was prepared by adding 1 g of silver nitrate to 200 ml of 75 mM NaOH, 75 mM ammonia in water. Precipitate was redissolved by adding 20% ammonia concentrate dropwise. Gels were then washed for 5 × 10 min in water and immersed in 200 ml of developing solution containing 10 mg of citric acid and 200 μl of 37% formaldehyde in water. The reaction was stopped with several washes of 1% (v/v) acetic acid. Oxidation with periodic acid before silver staining enhances the staining of carbohydrate and, subsequently, of glycoproteins (Hitchcock and Brown, 1983), whereas non-glycosylated proteins stain after a much longer developing period.

### *Protease digestion of pilin, mass spectrometry and N-terminal sequencing of peptides*

Partially purified pilin was run on a 12% denaturing SDS–polyacrylamide gel. After staining with Coomassie blue, the bands corresponding to pilin were excised, destained exhaustively and freeze dried. Trypsin digestion was adapted from the procedure of Helmann *et al.* (1995). Bands were washed twice for 30–60 min at 30°C in 0.2 M ammonium bicarbonate, 50% acetonitrile, then dried completely in a slow stream of nitrogen. After rehydrating in 2–5 μl of 0.2 M ammonium bicarbonate, 0.5 μg of modified sequence grade trypsin was added. An additional 0.2 M ammonium bicarbonate was added to cover the gel slices, and the slices were incubated overnight at 30°C. The supernatant was removed and the peptides further extracted with 100 μl of 60% CH3CN/0.01% TFA for 20 min. The volume of the combined supernatants was reduced to approximately 150 μl by freeze drying before HPLC. In each case, the peptide fragments were partially purified by reverse-phase HPLC on a Pharmacia Smart instrument with a uRPC C2/C18, SC 2.1/10 column, using a 1% or 0.5% gradient of acetonitrile in water in the presence of 0.1% TFA.

Entire digest reactions and HPLC fractions were characterized by matrix-assisted laser deabsorption (MALDI) mass spectrometry on a Perseptive Voyager Elite MALDI/TOF

instrument using the matrix alpha-cyano-4-hydroxy-cinnamic acid C10H7N. N-terminal sequencing of peptides was done on a Perkin-Elmer/Applied Biosystems 494 Procise sequencer.

### *Adhesion assays and monitoring of piliation status*

Adhesion assays were performed using Hec-1B cells as described previously (Nassif *et al.*, 1993). Adhesiveness was calculated as the ratio of cell-associated CFU to CFU present in the supernatant after 4 h of incubation.

Monitoring of piliation was first performed by transmission electron microscopy, as described previously (Nassif *et al.*, 1993). Evaluation of piliation, using immunofluorescence staining, was performed with bacteria grown overnight on GCB plates without antibiotics and resuspended in saline after 12 h incubation at a density of 106 CFU $ml^{-1}$. Bacteria were fixed for 1 min with methanol. Antibodies were incubated in 1% gelatin in phosphate-buffered saline (PBS) for 30 min each. 5C5 was used as primary antibody and Cy3-coupled goat anti-mouse IgG was used for the second labelling step. Observations were carried out using fluorescence microscopy. The bacteria were stained fluorescently using a 1:4000 dilution of a 10 mg $ml^{-1}$ ethidium bromide solution.

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