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# Crystallographic structure reveals phosphorylated pilin from *Neisseria*: phosphoserine sites modify type IV pilus surface chemistry and fibre morphology

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## Summary

**Understanding the structural biology of type IV pili, fibres responsible for the virulent attachment and motility of numerous bacterial pathogens, requires a detailed understanding of the three-dimensional structure and chemistry of the constituent pilin subunit. X-ray crystallographic refinement of *Neisseria gonorrhoeae* pilin against diffraction data to 2.6 Å resolution, coupled with mass spectrometry of peptide fragments, reveals phosphoserine at residue 68. Phosphoserine is exposed on the surface of the modelled type IV pilus at the interface of neighbouring pilin molecules. The site-specific mutation of serine 68 to alanine showed that the loss of the phosphorylation alters the morphology of fibres examined by electron microscopy without a notable effect on adhesion, transformation, piliation or twitching motility. The structural and chemical characterization of protein phosphoserine in type IV pilin subunits is an important indication that this modification, key to numerous regulatory aspects of eukaryotic cell biology, exists in the virulence factor proteins of bacterial pathogens. These O-linked phosphate modifications, unusual in prokaryotes, thus merit study for possible roles in pilus biogenesis and modulation of pilin chemistry for optimal *in vivo* function.**

## Introduction

The infection of animals by pathogenic bacteria requires the attachment of microbes to host cell surfaces. Among the virulence factors required for this process are type IV pili (or fimbriae), the adhesive cell surface organelles that mediate attachment of numerous Gram-negative bacteria including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and pathogenic *E. coli* to their eukaryotic hosts (Buchanan and Pierce, 1976; Doig *et al*., 1988; Nassif *et al*., 1993; Jonsson *et al*., 1994; Rhine and Taylor, 1994; Taniguchi *et al*., 1995). Pili also mediate twitching motility, the mechanism by which piliated bacteria move across solid surfaces (Bradley, 1980; Tennent and Mattick, 1994) and presumably across mucosal surfaces to colonize their hosts. Furthermore, in gonococci (Gc), type IV pili are associated with greatly enhanced transformation efficiency (Biswas *et al*., 1977). The regulation of pilin subunit expression, the assembly of these subunits into pilus fibres and the chemical biology of the assembled fibres are thus critically important for modulating the functions of this multifunctional organelle.

Numerous genes involved in the biogenesis of type IV pili have been identified and characterized, although our understanding of pilus assembly is nascent at best. Pilin expression is partially regulated in *P. aeruginosa* by a classic two-component regulatory system, with PilS the predicted sensor protein and PilR the transcriptional activator (Hobbs *et al*., 1993). In *N. gonorrhoeae*, PilB and PilA may play these roles (Taha *et al*., 1991). Assembly of pilin into pili requires numerous other gene products conserved across species including, for example, the PilD protease that cleaves a conserved hydrophobic leader peptide from pilin (Strom *et al*., 1994), the PilC assembly protein (Nassif *et al*., 1994; Rudel *et al*., 1995), the cytoplasmic membrane protein PilC (in *Pseudomonas*)/PilG (in Gc) (Nunn *et al*., 1990; Tonjum *et al*., 1995), the multimeric PilQ outer membrane assembly protein (Martin *et al*., 1993; Drake and Koomey, 1995) and presumed NTP binding proteins PilB (in *Pseudomonas*)/PilF (in Gc), PilT and PilU (for recent reviews of pilus assembly, see Alm and Mattick, 1997 or Tonjum and Koomey, 1997).

Covalent changes within the pilin molecule itself provide another novel level of regulation. In *N. gonorrhoeae*, pili undergo dramatic antigenic variation by a non-reciprocal recombination of silent copies of the pilin hypervariable region into the expression locus for the pilin structural gene *pilE* (Hagblom *et al*., 1985; Segal *et al*., 1985), which allows bacteria to escape immune recognition by the human host. This recombination has a frequency as high

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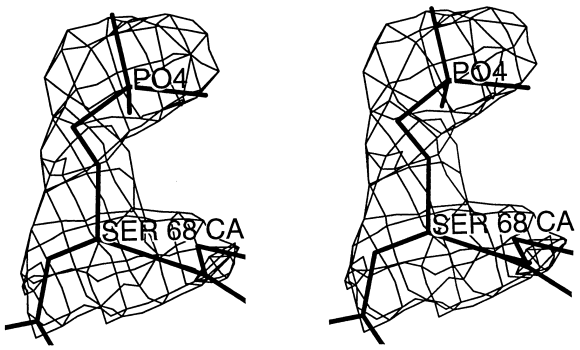


**Fig. 1.** The post-translational modification at Ser-68 is shown in a stereo pair of the refined atomic co-ordinates and $2F_o-F_c$ electron density (contoured at 1.0 standard deviation above the mean).

as 1% (Swanson *et al*., 1985; 1986). Pilin is cleaved to release soluble S-pilin (Koomey *et al*., 1991; Haas *et al*., 1987), the biological role of which is not well established but could be removal of excess or misassembled pilin or serving as a decoy for the human immune system. Other covalent modifications of the pilin subunit that may be involved in the regulation of its function have been characterized in detail recently, in particular saccharide (Parge *et al*., 1995; Stimson *et al*., 1995), α-glycerophosphate (Stimson *et al*., 1996) and phosphorylcholine (Weiser *et al*., 1998).

The saccharide has been removed from meningococcal pili by site-directed mutation of the modified Ser-63 (Marceau *et al*., 1998) and by mutation in the locus encoding either a general galactosyl transferase (Stimson *et al*., 1995) or a pilin-specific glycosylase (Jennings *et al*., 1998). This loss causes no change or a very slight increase in adhesiveness for epithelial cells. Saccharide facilitates the solubilization of pilin (Marceau *et al*., 1998). Polysaccharide may contribute to antigenic variation and host immune escape, as the identity of the surface-exposed saccharide differs between *N. gonorrhoeae* and *N. meningitidis* and among meningococcal strains (Parge *et al*., 1995; Stimson *et al*., 1995; Jennings *et al*., 1998; Marceau *et al*., 1998) and the terminal Gal α1–3 Gal linkage found on many meningococcal pilins is a target for human anti-gal antibodies that block complement-mediated killing (Hamadeh *et al*., 1995). The roles of glycerophosphate and phosphorylcholine remain undefined. Over a decade ago, phosphate in the form of phosphoserine was reported as a possible constituent of gonococcal pilin (Robertson *et al*., 1977; Schoolnik *et al*., 1984), but this was difficult to establish unequivocally because of phospholipid contamination of pilin preparations. Yet, phosphate is a classic regulatory switch, not only in eukaryotes but also in prokaryotes in the case of two-component regulatory systems (Stock *et al*., 1990). Only recently have eukaryotic-like serine/threonine and tyrosine kinases been identified in prokaryotes, including *Anabaena*, *Yersinia* and *Streptomyces* (Zhang, 1996), and the sites that they may modify remain poorly characterized. These sites may ultimately prove to play regulatory roles as they do in eukaryotes, or they may have other functions, such as modifying surface chemistry of proteins. Here, we use X-ray crystallography, mass spectrometry, site-directed mutagenesis and electron microscopy to identify pilin phosphorylation sites, localize these phosphate modifications crystallographically to specific residues and three-dimensional positions, and examine the effect of the loss of phosphorylation on pilus morphology and functions.

## Results and discussion

### *Pilin is phosphorylated at Ser-68*

Further electron density map analysis and refinement of the originally reported MS11 pilin structure (Parge *et al*., 1995) revealed a tetrahedral electron density peak at the appropriate distance from Ser-68 Oγ to be a covalently linked phosphate group (Fig. 1). The Ser-68-phosphorylated pilin model was built and refined to an R-factor of 18.7% at 2.6 Å resolution.

**Table 1.** Peptides isolated by HPLC purification of protease-digested pilin.

| Enzyme | Pilin | 45 | Sequence[a] | 68 | 74 | Mass (Da) Obs/Exp[b] |
|---|---|---|---|---|---|---|
| LysC | WT | SAVTEYYLNHGKWPENN | TSAGV(ASPPSDIK) | | | 4250/4252 |
| Trypsin | WT | | WPENN TSAGV ASPPSDIK | | | 2314/2314 |
| Trypsin | S68A | | WPENN(TSAGV AAPPSDIK) | | | 1854/1853 |

**a.** Identities of residues in parentheses are assumed based on the expected sequence and the observed mass. The sequence of MS11 pilin used as the wild-type pilin in these experiments follows (residues 63, 68 and 94, modified by sugar, phosphate and glycerophosphate, respectively, are underlined).

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1   FTLIELMIVI AIVGILAAVA LPAYQDYTAR AQVSEAILLA EGQKSAVTEY YLNHGKWPEN
61  NTSAGVASPP SDIKGKYVKE VEVKNGVVTA TMLSSGVNNE IKGKKLSLWA RRENGSVKWF
121 CGQPVTRTDD DTVADAKDGK EIDTKHLPST CRDNFDAK
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**b.** Expected masses are calculated based on the molecular weight of the peptide shown with phosphoserine at position 68 and disaccharide at position 63, with the exception of the last peptide, which has a mass consistent with no modifications.

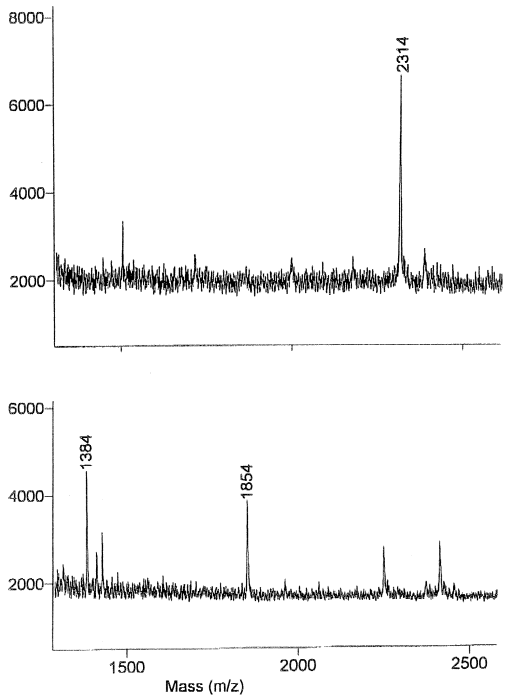


**Fig. 2.** The masses of trypsin-digested, partially purified peptides of pilin measured by MALDI mass spectrometry agree with the mass predicted for the wild-type peptide with disaccharide and phosphate (2314 D, top) and the Ser68Ala peptide with neither phosphate nor disaccharide (1854 D, bottom). The additional peak at the bottom (1384 D) corresponds to a second peptide (SAVTEYYLNHGK) in this fraction.

To verify the identity of the modification at Ser-68 independently, purified pilin was cleaved with proteases LysC or trypsin, and peptides purified by high-performance liquid chromatography (HPLC) were analysed by N-terminal sequencing and mass spectrometry. Several peptides were isolated that spanned the Ser-68 site, consistent with the known sequence variation within strains of *N. gonorrhoeae*. In each case, the peptide had the correct mass for the experimentally determined amino acid sequence, the galactose α1–3 GlcNAc disaccharide (Parge *et al*., 1995) and an additional phosphate (Table 1, Fig. 2). Given the unambiguous mass spectrometry data and clear electron density map interpretation, alternative modifications (such as the unidentified 61 D modification reported by Marceau *et al*., 1998) are extremely unlikely. Thus, multiple peaks observed upon isoelectric focusing of pilin (Parge *et al*., 1990) may be caused by variations in pilin phosphorylation state within a preparation, as well as by variations in sequence or glycosylation.

Virji and co-workers reported the novel modification of α-glycerophosphate covalently linked to Ser-93 in meningococcal pilin (Stimson *et al*., 1996). In the highly homologous gonococcal pilin (approximately 90% identical, depending on the exact strains compared), this modified serine residue corresponds to Ser-94. A phosphodiester linkage from Ser-94 Oγ was built into unmodelled electron density at this position in the *N. gonorrhoeae* electron density map and refined. The electron density for the glycerophosphate moiety is weaker than that for phosphoserine, which suggests that many pilin molecules in the crystal lack this modification or that the glycerophosphate is poorly ordered.

### *Effects on type IV pilin structure and chemistry*

The serine modification sites occur at pilin positions expected to modulate pilus fibre surface chemistry, as was predicted by the strong binding of an anti-peptide antibody raised against amino acids 58–78 to the sides of intact pilus fibres (Forest *et al*., 1995). Pilin adopts a highly asymmetrical three-dimensional fold (Fig. 3) comprising an 85 Å N-terminal α-helix, a 24-amino-acid 'sugar loop', a four-stranded anti-parallel β-sheet and a C-terminal β-sheet and extended loop, which contain the hypervariable domain. Phosphoserine 68 is found on the protruding sugar loop of pilin (Fig. 3), which is proposed to be

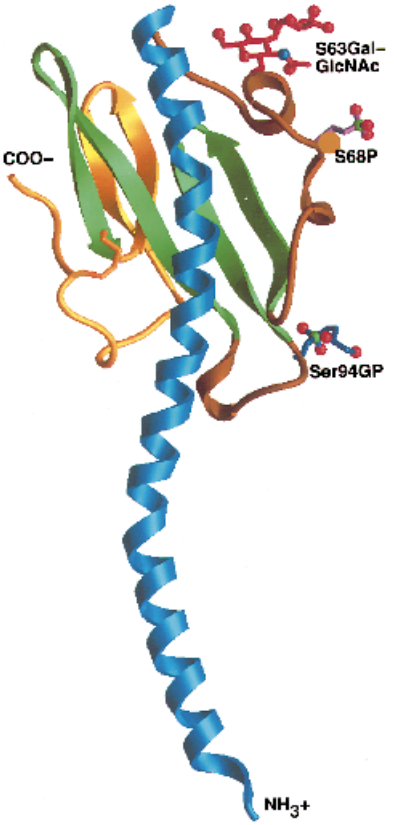


**Fig. 3.** Phosphoserine 68 (purple ball and stick) is located on the sugar loop of the pilin fold, shown here as a ribbon diagram. α-glycerophosphate (blue ball and stick) at Ser-94 is found within the connecting loop between $\beta_2$ and $\beta_3$.

recruited to hydrogen bond with the fourth strand of the β-sheet of the neighbouring molecule in the fibre to form a continuous β-sheet that wraps around the conserved, hydrophobic helical core (Forest and Tainer, 1997). Thus, Ser-68 would be positioned on the outer surface of the proposed helical fibre model (Fig. 4) near the lateral interface of two pilin monomers. Such a subunit interface location for Ser-68 within the fibre suggests possible roles for phosphorylation in the regulation of assembly or retraction of pili, in altering fibre assembly characteristics such as bending or bundling, or in changing the charge and chemistry at the fibre surface. From the top of the fivefold helical assembly, only four phosphoserine residues are accessible (Fig. 4B), which could influence fibre capping. The phosphoserine on monomer$_{n-4}$ is hidden from above by monomer$_n$. Conversely, only the bottom-most phosphoserine is accessible from the tail end of the fibre (Fig. 4C), presumed to be at the membrane.

Ser-94 α-glycerophosphate lies within the connecting loop between $\beta_2$ and $\beta_3$ (Fig. 3). In this position, α-glycerophosphate is exposed towards one end of the assembled fibre (Fig. 4), consistent with the proposal that glycerophosphate may be the substrate for lipid attachment, which would help to anchor the pilus fibre in the outer membrane (Stimson *et al*., 1996). Assuming glycerophosphate is the substrate for lipidation, glycerophosphate then may define the cell-proximal end of the pilus. The low occupancy (or disorder) at this site could reflect a low population of pilin with this modification, at least in the crystals, which is consistent with a role for Ser-94 α-glycerophosphate in anchoring pilin to membrane components.

### *Effects of Ser68Ala mutation on pilus morphology and functions*

To probe possible functional roles of the phosphoserine at position 68, this residue was changed to alanine by site-directed mutagenesis of the pilin-encoding gene *pilE* in *N. gonorrhoeae*. Electron microscopy (EM) (Fig. 5) and immunoblotting (Fig. 6) indicated that the mutant pili are expressed at levels similar to wild-type pilin, and they were purified according to the identical purification procedure used for wild-type pili. However, Ser68Ala pili appear to form bundles more readily than wild-type pili, and both fibres and bundles appear to be more curly than the straight and often individual fibres seen on the wild-type N400 strain. These differences are visible in the appearance of pili still attached to whole cells (Fig. 5A and B), but are most readily apparent in purified pilus preparations (Fig. 5C and D). One explanation for this change

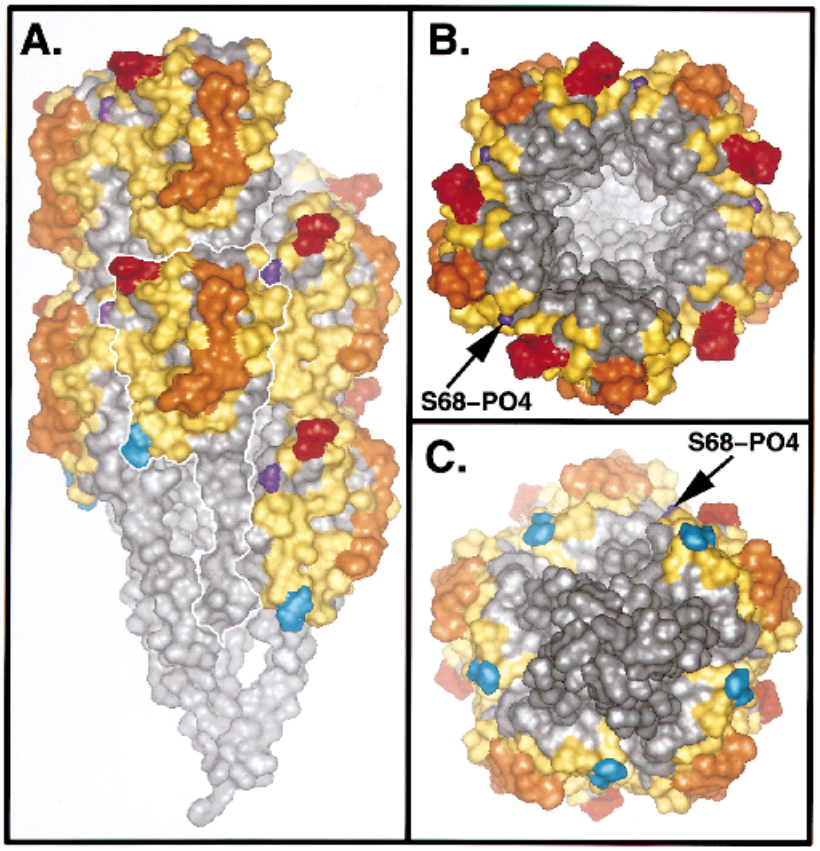


**Fig. 4.** Modifications and antigenic variation affect surface chemistry. Side view (A), top view (B) and bottom view (C) of the solvent-accessible surface of the proposed fivefold helical pilus fibre model showing the sites of glycosylation (red), phosphorylation (purple and also labelled) and modification by α-glycerophosphate (cyan) on the outer surface of the fibre. For clarification, a single pilin molecule including the associated modifications is outlined in (A). The glycero-phosphate is accessible from the end visible in (C) but not from the top view in (B). Sequence variation, as defined by Parge *et al*. (1990), is also colour coded (invariant residues grey, semi-variable residues yellow and hypervariable residues orange) and highlights the role of antigenic variation in modifying the fibre surface without affecting internal packing.

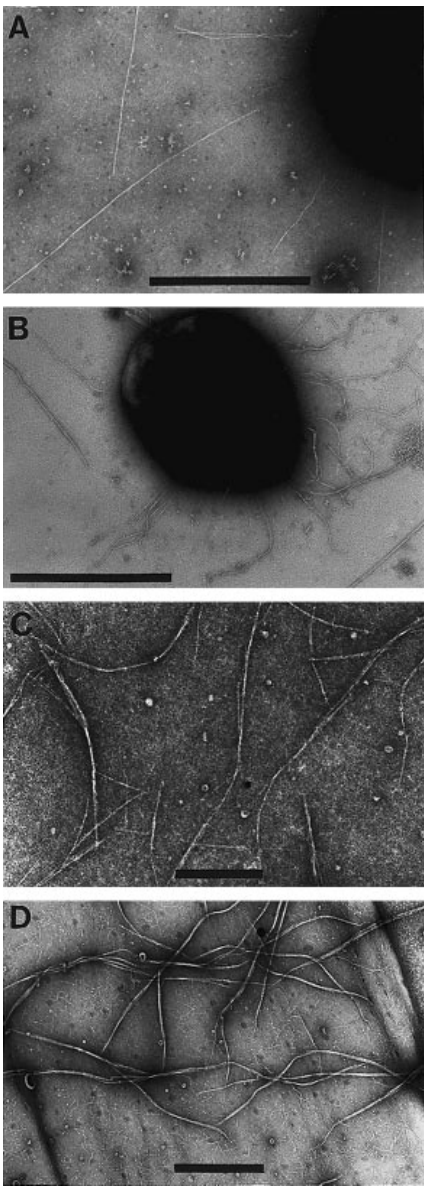


**Fig. 5.** Phosphoserine 68 alters the appearance of pili by electron microscopy. Comparison of electron micrographs of piliated whole cells from wild-type (A) or mutant (B) strains reveals subtly thicker, curlier bundles of pili on strains expressing Ser68Ala pilin. Similar comparison of purified pili from wild-type GC strain N400 (C) and the $pilE_{Ser68Ala}$ strain (D) shows that mutant pili are less straight. Micrographs were recorded at 11 000–30 000× and printed so that the scale bars shown represent 1 μm.

in morphology is that the presence of the negatively charged phosphate groups on the pilus surface provides an electrostatic repulsion that forces the greatest possible distance between phosphate moieties. This would give pili their straight appearance, much as it may keep B-form DNA extended in the absence of phosphate neutralization (Strauss and Maher, 1994).

Immunoblotting of gonococcal strains expressing either wild-type or Ser68Ala pili showed no difference in the migration pattern of pilin (Fig. 6), indicating that the loss of phosphorylation does not affect pilin maturation. Likewise, immunoblotting of *E. coli* strains expressing either pilin reveals a previously reported doublet of unprocessed and clipped pilins, which supports the conclusion that pilin subunit protein chemistry is not substantially different with the Ser68Ala change. Likewise, when purified gonococcally expressed pilin is subjected to SDS–PAGE and Coomassie staining (Fig. 6), there is no observed change in the migration of pilin. Mass spectrometry and N-terminal sequencing of peptides were used again to verify that the Ser68Ala pilin is no longer phosphorylated (Table 1, Fig. 2). Indeed, the purified peptide is neither phosphorylated nor glycosylated, although no change was made to the glycosylation site Ser-63. Our data thus suggest that glycosylation and phosphorylation may be coupled in some way, although further work is required to verify this, particularly because our analysis of peptides was not exhaustive, and it is likely that there are other peptides that were not purified and remain glycosylated in the absence of phosphorylation.

Despite differences in pilus morphology caused by the lack of phosphorylation, Ser68Ala pili continue to promote adhesion to epithelial cells, twitching motility and transformation at levels indistinguishable from wild-type pili under the conditions tested (Table 2). A negative control strain with a nonsense codon introduced at amino acid 69 does not make pili and does not promote any of these functions. Our data do not allow for a second serine or threonine within amino acids 57–74 (the extent of the tryptic peptide) to be modified. However, we have not addressed whether distant sites are newly phosphorylated in the strain expressing Ser68Ala pilin. Thus, removal of the phosphoserine at position 68 altered the morphology of pili on cells and of purified pili without noticeable effects on pili function.

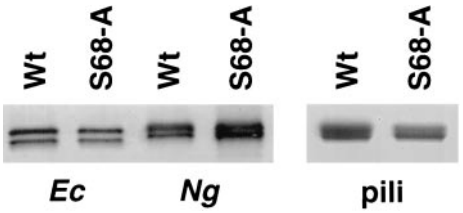


**Fig. 6.** Wild-type and Ser68Ala pili are indistinguishable by immunoblotting and SDS–PAGE. Pilin antigen detected by immunoblotting of *E. coli* and *N. gonorrhoeae* (Ng) strains expressing the wild-type and Ser68Ala *pilE* alleles is shown on the left. The two species seen in the *E. coli*-derived samples represent unprocessed prepilin (top) and a proteolytic form processed by an endogenous prepilin peptidase (bottom) (Koomey *et al*., 1991). The migration of pilin in purified pilus preparations from *N. gonorrhoeae* strains was detected by Coomassie staining after SDS–PAGE as shown on the right. The basis for the two species detected in the gonococcal whole-cell lysates and in purified pili is unknown, but both have an N-terminal sequence consistent with full-length mature pilin (as opposed to prepilin or S-pilin) (Tonjum *et al*., 1995).

**Table 2.** Phenotypes of wild-type, S68A and P69fs strains.

| Strain | Pili | Autoagglutination | Competence[a] | Twitching motility | Epithelial cell adherence[b] |
|---|---|---|---|---|---|
| N400 | + | ++ | 100% | ++ | ++ |
| S68A | + | ++ | 100% | ++ | ++ |
| P69fs | − | − | <0.01% | − | − |

**a.** Competence for transformation is expressed as the percentage of the transformation frequency found using the wild-type strain N400. The frequency of N400 cells acquiring resistance to nalidixic acid was 2–4% of cells exposed to pSY6 transforming DNA.
**b.** ++, More than 50 cfu per epithelial cell; −, less than 1 cfu per 10 epithelial cells.

### *Implications*

O-linked phosphorylation of bacterial proteins is rare, and the identification of phosphoserine in gonococcal pilin is an important demonstration that this modification, important in the regulation of events in eukaryotic cells, also exists in prokaryotes. Moreover, the identification of covalent phosphoserine on pilin implies that there is at least one kinase responsible for the addition of the modification. Given that we have shown that the removal of the phosphorylation site does not greatly alter the assembly or function of pili, this enzyme is either a previously unidentified protein in the pilus biogenesis pathway or a protein whose loss leads to other more drastic phenotypes in addition to the loss of phosphoserine, so that this more subtle change has not been recognized yet.

While the site of saccharide attachment does vary, the Ser-68 phosphorylation site is invariant among published *N. gonorrhoeae* pilin sequences, and the Ser-94 α-glycerophosphate site is serine in all but the single unpublished sequence of the Pgh3-2 variant pilin (Tramont *et al*., 1981; Parge *et al*., 1995). This sequence conservation suggests a conserved functional role for these surface-accessible serines. The covalent addition of phosphate and glycerophosphate may have subtle but significant modulating effects on pili assembly, retraction, adhesion, transformation or twitching motility, which might be uncovered by future analysis of a broader range of assay conditions. By modulating the surface properties of pilin or pili, phosphate does appear to affect bundling or solubilization of pili on cells. This was indeed also the case when the surface chemistry of meningococcal pili was changed by a site-directed mutation that removed the glycosylated serine (Marceau *et al*., 1998). Alternatively these modifications may have other as yet untested roles *in vivo*, including effects on pathogenicity. Pilin is a remarkably plastic protein, which folds properly and maintains all of its functions despite changes to its primary sequence within a hypervariable C-terminal region (Hagblom *et al*., 1985). Antigenic variation via phosphorylation could be an additional immune escape mechanism. Taken together, our results indicate that the charged phosphoserine at residue 68 exists and may promote straighter and less bundled fibres by electrostatic repulsion of negative charges on this surface-exposed region. This is an example of a defined change in a structural subunit leading to a morphological change in a supramolecular assembly. The effects this evident structural change may have on significant aspects of pilus function merit further study.

## Experimental procedures

### *Crystal growth and data collection*

Pilin crystals were grown by the vapour diffusion method from a 26 mg ml$^{-1}$ protein solution of purified pilin containing β-octyl glucoside and 1,2,3-heptanetriol, with polyethylene glycol 400 as a precipitating agent (Parge *et al*., 1990; 1995). X-ray data used for this refinement were collected on a MAR image plate from a single $K_2Pt(NO_2)$-soaked crystal using 1.07 Å wavelength radiation. The data set had 6565 reflections and an overall $R_{sym}$ of 5.4%. These data were used by Parge *et al*. (1995) for the original pilin structure determination.

### *Crystallographic refinement*

The starting structure (PDB code 1AY2) was determined previously by multiple isomorphous replacement and anomalous scattering, using data from platinum, palladium and lead derivative crystals (Parge *et al*., 1995). Phosphate was built into the map unambiguously using the program XFIT (McRee, 1992). Simulated annealed 'omit maps' (in which the model was refined and phases calculated without the nine residues surrounding the phosphate) confirmed its position. Positional refinement against the observed structure factors was performed using XPLOR, including a correction for the modelled diffraction of the bulk solvent in the C222 unit cell (a = 127.6, b = 121.1, c = 26.9 Å) and an overall anisotropic B-factor applied to the diffraction data. The final model with 1207 protein, 25 sugar, 1 platinum, 5 phosphate, 10 heptanetriol and 127 water atoms has good geometry for the phosphate and a final R-factor of 18.7% for 6430 reflections, representing 95% of the data from 20 Å to 2.6 Å with $F \geq (\sigma F)$. The root mean square deviation on bond lengths is 0.017 Å and on bond angles is 3.504°. The average B-factor for phosphate atoms is 97.1 Å$^2$, for protein atoms 40.6 Å$^2$, and for water molecules 61.4 Å$^2$. As no water molecules clashed with the newly modelled phosphate, none were added or removed for the refinement.

Refinement of the α-glycerophosphate was less unambiguous than that for the phosphoserine. The density was fitted best by glycerophosphate with an ester link through Ser-94 Oγ to C2 of glycerophosphate. However, fitting of glycerophosphate to an 'omit map' (an unbiased electron density map calculated without glycerophosphate or residues 90–98 contributing to the model) was equally plausible with either a phosphodiester link through Ser-94 Oγ to the phosphate or with an ester link through Ser-94 Oγ to C2 of glycerophosphate. The marginal fit to the omit map of this region suggests that either many of the molecules in the crystal lack this modification or the α-glycerophosphate is partly disordered in our crystals.

### *Protease digestion of pilin*

Wild-type pilin, purified as described previously (Parge *et al*., 1990; 1995), was digested with either LysC to cleave after lysines or trypsin to cleave after arginines and lysines. LysC and modified trypsin were purchased from Promega. Sample (100 pmol) in 67 μl of pili buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% β-octyl glucoside) was digested for 24 h at 37°C in a total volume of 500 μl of 100 mM Tris HCl, pH 7.7, 4 mM EDTA (for LysC) or 50 mM ammonium bicarbonate, pH 7.8 (for trypsin) with 1 μg of enzyme. These conditions were necessary to reach complete digestion for a control protein in the presence of 0.13% β-octyl glucoside. Digestion was terminated by injection onto the HPLC after the reaction mixture was reduced to a volume of less than 200 μl by freeze drying.

### *In-gel protease digestion*

Partially purified Ser68Ala pilin was run on a 12% denaturing SDS–polyacrylamide gel. After staining with Coomassie blue, the bands corresponding to pilin were excised, exhaustively destained and freeze dried. In-gel 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 rehydration in 2–5 μl of 0.2 M ammonium bicarbonate, 0.5 μg of modified sequence grade trypsin dissolved in buffer supplied by the manufacturer (Promega) was added. Additional ammonium bicarbonate/acetonitrile was added to cover the gel slices, and the slices were incubated overnight at 30°C. The supernatant was removed, and the peptides extracted further with 100 μl of 60% $CH_3CN$/0.01% TFA for 20 min. The volume of the combined supernatants was reduced to approximately 150 μl by freeze drying before HPLC.

### *Partial purification of peptides by HPLC*

Peptide fragments were partially purified by a single reverse-phase HPLC run on a Pharmacia Smart MicroBore instrument with a μRPC C2/C18, SC 2.1/10 column or on an analytical instrument with a 4.6×250 mM C18 Vydac column with 5 μm particles, in either case using a 1.0% or 0.5% gradient of acetonitrile in water in the presence of 0.1% TFA. Fractions were collected by peak (for MicroBore runs) or each minute (for analytical runs).

### *MALDI mass spectrometry and N-terminal sequencing*

Whole pilin as well as entire digest reactions and most 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 ($C_{10}H_7NO_3$), with a molecular weight of 189. The resulting masses are accurate to within 0.1%. Fractions chosen for sequencing had predominant peaks with masses in an appropriate range to be tryptic fragments encompassing amino acids 57–74 or 45–74 with or without post-translational modifications. Sequencing was performed on a Perkin-Elmer/Applied Biosystems 494 Procise sequencer using standard conditions.

### *Bacterial strains and plasmids*

All *pilE* alleles used in this study were cloned into pUP6, a derivative of pHSS6 (Seifert *et al*., 1986) containing the gonococcal uptake sequence adjacent to the polylinker region. pUP6 was created by annealing the oligonucleotides UP-link1 (5′-AATTCGCCGTCTGAATATCTTTCAGACGGC-3′) and UP-link2 (5′-AATTGCCGTCTGAAAGATATTCAGACGGCG-3′) to each other and ligating the synthetic linker into pHSS6 digested with *Eco*R1. A 950 bp *Cla*1/*Hpa*1 fragment containing the wild-type *pilE* allele of gonococcal strain N400 was subcloned into pUP6 [digested with *Cla*1 *and Xba*1 (blunted)] to create pPilE. $pPilE_{Ser68Ala}$ was created by replacing the 400 bp *Bgl*1/*Cla*1 fragment of pPilE with the polymerase chain reaction (PCR) product obtained using primers pHSS6-*Eco*R1 (5′-GATCCCCACCGGAATTGCG-3′) and $PilE_{Ser68Ala}$ (5′-CTTCTGCCGGCGTGGCA**G**CCCCCCCCT-3′) (bold = altered nucleotide, underlined = *Bgl*1 site). $pPilE_{Pro69fs}$ was created by replacing the 400 bp *Bgl*1/*Cla*1 fragment of pPilE with the PCR product obtained using primers pHSS6-*Eco*R1 and $PilE_{Ser68Ala}$ (5′-CTTCTGCCGGCGTGGCAT**GC**CCCCCCCCT-3′) (bold = altered/additional nucleotide, underlined = *Bgl*1 site). Restriction enzymes, Vent polymerase and T4 DNA ligase were all supplied by New England Biolabs. All plasmids were transformed into chemically competent HB101 and grown in LB media with kanamycin at 50 μg $ml^{-1}$.

Gonococcal strains were grown at 37°C in 5% $CO_2$ on standard Gc media supplemented with Isovitalex. *pilE* point mutations were introduced into N400 (Tonjum *et al*., 1995), a derivative of strain MS11 that carries the IPTG-inducible *recA*6 allele (Seifert, 1997), as follows. First, single colonies of strain N400 were streaked onto agar medium plates containing 250 μM IPTG. Plasmid DNA (2 μg; 0.5 μl) was then spotted onto sections of the plate where single colonies were predicted to arise. After 18 h of incubation, individual colonies from regions spotted with DNA were streaked for the isolation of single colonies. *pilE* was amplified by PCR from individual colonies, and those in which mutations were introduced were identified by restriction enzyme digestion patterns that were peculiar to each allele (a new *Fnu*4HI site for the Ser68Ala allele and a new *Sph*1 site for the Pro69fs allele). The complete nucleotide sequence of the *pilE* alleles was determined using a ThermoSequenase protocol for PCR products (Amersham) to ensure the introduction of the mutations and the lack of any other nucleotide changes within the genes.

### *Pilus purification and immunoblotting*

Pili used for immunoblotting were purified by the ammonium sulphate modified procedure described previously (Brinton *et al*., 1978). Cells from three to five heavily streaked agar media plates were suspended in 8 ml of 0.15 M ethanolamine, pH 10.5, and vortexed for 60 s. Cellular debris was removed by centrifugation at 17 000 *g* for 15 min. Pilus crystals were precipitated at room temperature for 30 min with a 1/10th volume of ammonium sulphate-saturated 0.15 M ethanolamine and collected by centrifugation at 17 000 *g* for 15 min. Pilus filaments were washed twice with 0.05 M Tris-buffered saline.

Procedures for SDS–PAGE and immunoblotting have been described previously (Koomey *et al*., 1991). The presence of PilE was detected by immunoblotting pilus preparations using rabbit polyclonal antibodies and alkaline phosphatase-coupled goat anti-rabbit antibodies (Tago). Prestained molecular weight markers (Gibco BRL) were used for immunoblotting, and unstained markers (Promega) were used for Coomassie staining according to the manufacturer's instructions.

### *Electron microscopy*

For microscopy of whole cells, cells were grown overnight on Gc agar as described above. Formvar-coated, carbon-coated nickel grids were placed gently directly on top of single colonies and then floated for 1 min on TBS (10 mM Tris, pH 7.5, 150 mM NaCl) followed by 1 min on 1% aqueous uranyl acetate (UA). These grids were then wicked and allowed to dry. For microscopy of purified pili, 5 μl drops of a 0.06 mg ml$^{-1}$ solution of pili in either 50 mM CHES, pH 9.0 or TBS (not shown) were applied to grids and wicked off after 5 min. These grids were stained for 3 min with 1% UA, followed directly by wicking and air drying. Micrographs were recorded on a Philips CM100 microscope operating at 100 kV.

### *Transformation assay*

Competence for natural transformation was evaluated as described previously with pSY6, a plasmid containing a single point mutation that confers low-level nalidixic acid resistance (Stein *et al*., 1991). Briefly, 0.5 ml of a slightly turbid suspension of Gc was incubated in $CO_2$-saturated transformation broth (Gc broth with Isovitalex, 250 μM IPTG and 7 mM $MgCl_2$) with 2 μg of pSY6 for 0.5 h at 37°C. Cultures were then diluted 10-fold and grown for 4 h to allow the expression of nalidixic acid resistance. Finally, serial dilutions of cultures were plated on plain and nalidixic acid (1 μg ml$^{-1}$)-containing plates and incubated in 5% $CO_2$ for 36 h. The experiment repeated with non-saturating levels of DNA also showed no difference in transformation efficiency between N400 and the strain carrying the Ser68Ala mutation.

### *Twitching motility*

Twitching motility was scored based on light microscopy of jerky movement of cells or clumps of cells at the periphery of colonies growing on solid agar media after 28 h of incubation.

### *Adherence assay*

The gonococcal adherence assay was performed using the human epithelial cell line ME-180 (ATCC) maintained in McCoy's 5A medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS). Epithelial cells were seeded into a 24-well tissue culture plate at a density of $5 \times 10^5$ cells on the day before infection. For assays, $2–5 \times 10^7$ bacteria in 1 ml of McCoy's 5A medium supplemented with 1% FBS (assay medium) were added to each well of monolayers and incubated at 37°C in 5% $CO_2$. After 1 h, non-adherent bacteria were removed by washing five times with assay media, and the monolayers and cell-associated bacteria were recovered by treatment with 0.25% trypsin for 5 min at 37°C. The recovered bacteria were plated on agar after dilution, and the number of cell-associated bacteria determined by the number of colony-forming units (cfu).

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