Pilin expression and processing in pilus mutants of *Neisseria gonorrhoeae*: critical role of Gly $_{-1}$ in assembly

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Summary

**Spontaneous mutants of *Neisseria gonorrheae* failing
to express pili or having diminished levels of piliation
were studied with regard to pilin expression. All
mutants displayed altered pilin processing detectable
as the release of soluble, truncated pilin molecules
(S-pilin). Of particular interest was the finding, in one
mutant, that substitution of serine for glycine at
position −1 of propilin, a highly conserved residue
among *N*-metPhe and related pilins, abolished pilus
expression but not S-pilin release. The degree of
S-pilin processing and the levels of membrane-asso-
ciated pilin varied among the different classes of
mutants, suggesting that each was blocked at a
distinct step of pilus biogenesis. The data support a
model in which increased S-pilin processing is a result
of a decreased rate of pilus polymerization.**

Introduction

The pili of *Neisseria gonorrhoeae* are filamentous
appendages about 8 nm in diameter that belong to the
family of fimbriae found on *Neisseria meningitidis* (Potts
and Saunders, 1988; Hermodson *et al.*, 1978), *Pseudomo-
nas aeruginosa* (Pasloske *et al.*, 1985; Frost *et al.*, 1978;
Johnson *et al.*, 1986), *Moraxella bovis* (Marrs *et al.*, 1985),
Moraxella nonliquefaciens (Froholm and Sletten, 1977),
and *Bacteroides nodosus* (Elleman and Hoyne, 1984). The

major subunits of all of these organelles, termed pilin, have
short (6–7 amino acid) signal sequences, highly conserved
hydrophobic amino-terminal domains, and methylated
phenylalanine as their mature amino-terminal residues.

Of the many bacterial surface constitutents and extra-
cellular products that are thought to influence the patho-
genesis of gonococcal disease, pili are one component
that can be unambiguously correlated with virulence. All
primary clinical isolates are piliated (Swanson *et al.*,
1987b; Kellog *et al.*, 1968) and non-piliated mutants are
avirulent in male volunteers challenged intraurethrally
(Kellog *et al.*, 1968; Swanson and Koomey, 1989). Indirect
evidence of the import of this colonization factor in the
gonococcal–human host relationship can be found in their
capacity to undergo antigenic variation such that a single
strain can give rise to a broad spectrum of pilus variants at
an apparently high rate (10^{-4} per cell per generation)
(Swanson *et al.*, 1987b, 1985; Koomey *et al.*, 1987; Virji *et
al.*, 1983).

Certain aspects of colonial morphology (size, shape,
and edge) are reliable and sensitive indicators of gono-
coccal piliation status and these characteristics have been
used extensively to isolate variants and mutants altered in
pilus expression. In wild-type strains, these changes in
colonial piliation phenotype occur coincidentally with
either the expression of antigenically distinct pilin (Swan-
son and Barrera, 1983) or the failure to express pilin
capable of being assembled (Swanson *et al.*, 1985;
Bergstrom *et al.*, 1986; Haas *et al.*, 1987). Both of these
outcomes can be correlated with changes within the gene
encoding pilin, the major subunit of pili, that arise as a
result of homologous recombination between multiple
partial pilin gene copies (silent loci) and a complete pilin
(expression locus) (Swanson *et al.*, 1986; Swanson 1987b;
Koomey *et al.*, 1987; Haas and Meyer, 1986). Two other
genes, *pilA* and *pilB*, have been identified whose gene
products influence pilin gene transcription and colonial
piliation phenotype (Taha *et al.*, 1988).

Haas and coworkers have described a phase of pilin
expression coupled with pilin gene conversion in certain
gonococcal pilus[+] variants displaying a non-autoaggluti-
nating piliation (T4) phenotype in which soluble pilin
molecules (S-pilin) lacking the first 39 residues present in
processed pilin were released or secreted (Haas *et al.*,
1987). They also noted that the amount of S-pilin elabo-
rated appeared to be inversely proportional to the degree
of piliation. Other work has shown that pilin molecules

analogous to S-pilin were made by pilus⁻ mutants expressing pilin (Swanson *et al.*, 1986; Koomey *et al.*, 1987) and in some pilus⁺ variants recovered from infected male volunteers (Swanson *et al.*, 1987b). Little is known about the structure–function relationships of gonococcal pili and pilin, the processes of pilus biogenesis and the nature of the strong correlation between piliation and certain phenotypes. Principally, this situation results from the relative intransigence of gonococci to genetic manipulation, the failure to express gonococcal pilin as pili in a heterologous host such as *Escherichia coli* or *Pseudomonas aeruginosa* and the genetic instability of pilin expression in wild-type gonococci. This later obstacle has been alleviated by the ability to construct strains that bear null alleles of the *recA* gene (Koomey and Falkow, 1987). In order to begin to address these concerns, mutants altered in pilus expression have been isolated from recombination-deficient strains and characterized with regard to piliation status, pilin localization/processing and antigenicity.

Results

Isolation and characterization of pilus mutants

The presence of a functioning homologous recombination system mediated, in part, by the gene product of the *recA* locus has been shown to be required for the antigenic variation and high-frequency 'phase' mutation of gonococcal pilin. Thus strains that bear null alleles of the *recA* gene are stabilized with regard to pilin expression and in one such strain, VD302, it has been shown that a single class of spontaneous frameshift mutations within the pilin structure gene accounted for on/off pilus expression. In further studies of variants displaying altered colonial piliation phenotypes derived from VD302 P⁺⁺, two other classes of pilus mutants have been isolated and characterized.

One class of mutants consisted of many independently derived variants displaying the same colonial morphology and phenotypes. They failed to autoagglutinate in liquid culture and showed a unique colonial morphology that might at first sight be viewed as a pilus⁻ form. However, by using freshly poured (damp) plates they could be differentiated from isogenic pilus⁻ mutants by a slight degree of 'edge' character (Fig. 1). Electron microscopy of these variants revealed that they were piliated, albeit to a lesser degree than the pilus⁺ parent, and that the pili were morphologically distinct from those found on their progenitor. By scanning electron microscopy (SEM) the pili were seen to form the large rope-like bundles present on the pilus⁺, autoagglutinating strain. By transmission electron microscopy (TEM), the pilus filaments appeared to

have less tendency to laterally aggregate, a more disordered appearance and a wider diameter than that found on VD302 P⁺⁺ pili (Fig. 2). These represented the most frequently arising class of colonial piliation phenotype variants and gave rise to revertants of the P⁺⁺ parental phenotype at approximately the same rate at which they originally arose, i.e. approximately 10^{-6} per cell per generation. Based on their shared properties, these variants were presumed to represent the repeated occurrence of the same mutation. More-extensive analysis focused on two isolates, designated CV1 and CV2. Primer extension sequencing of the pilin mRNAs of these two revealed no nucleotide changes and Northern blotting showed no gross alterations in pilin transcript size or abundance (data not shown). This class of mutants did not, therefore, result from pilin gene mutation.

Only a single isolate of another class of mutant was recovered and it failed to express pili as determined by both SEM (Fig. 1) and TEM but unlike pilus⁻ mutants previously documented using strain VD302, it produced normal levels of immunoblot-detectable pilin. It could also be distinguished from those previous derivatives by its diminished colony size and increased generation time when propagated on solid media. Sequencing of its pilin mRNA revealed a single nucleotide alteration that changed the codon for amino acid residue −1 or propilin from specifying a glycine to a serine (GG*C* to A*G*C, Fig. 3) and this mutant was designated P⁻ ($G_{-1} \rightarrow S_{-1}$). The single base change found in the mRNA predicted the presence of a novel *Hin*dIII site within the pilin gene and the presence of that mutation within the genome was confirmed by Southern blotting of *Hin*dIII-digested genomic DNA and subsequently by similar restriction endonuclease digestion of the *pilE1* locus cloned in *E. coli* (data not shown). No revertants expressing pili or the parental colonial morphology were recovered using the selective conditions of static broth cultures (selection for pellicle formation).

Pilus/pilin expression in VD302 P⁺⁺, CV1 and P⁻ ($G_{-1} \rightarrow S_{-1}$)

Results obtained from immunoblotting of whole-cell lysates of VD302 P⁺⁺, CV1 and P⁻ ($G_{-1} \rightarrow S_{-1}$) harvested from plates showed that the pilins expressed by the mutants were altered in mobility relative to that found in the pilus⁺ parent (Fig. 4). Pilin from VD302 P⁺⁺appeared as two species of M_r 18.8 kDa and 19.5 kDa, with the lower M_r form co-migrating with the major species found in purified pili from that strain. CV1 lysates contained a major species co-migrating with the M_r 19.5 kDa form found for VD302 P⁺⁺ while the major species of pilin expressed by P⁻ ($G_{-1} > S_{-1}$) migrated with an M_r of 21 kDa. Additionally, minor species of pilin antigen migrating as a doublet of M_r 16.5 kDa were detected in lysates of both pili mutants.
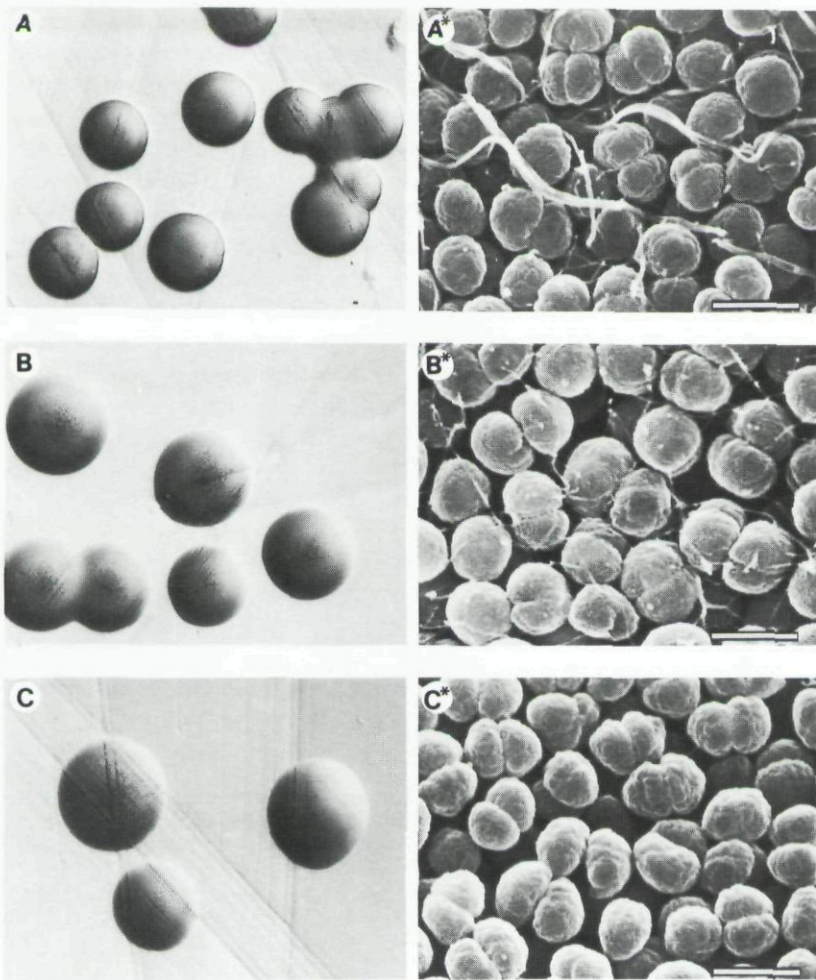
Fig. 1. Colonial morphologies and microscopic appearances (*SEM) of (A) VD302 P^{++}, (B) CV1, and (C) P$^-$ (G$_{-1}$ → S$_{-1}$).

Immunoblotting of HB101 expressing the cloned pilin genes showed that in all these cases, the relative mobility of pilin expressed in *E. coli* was different from that observed in gonococcal lysates (Fig. 4). The cloned genes of VD302 P^{++} and CV1 expressed identical doublet patterns of pilin migrating at M_r 18.5 kDa and 18 kDa while the mutant pilin of P$^-$ (G$_{-1}$ → S$_{-1}$) was detected as a single species of M_r 18.5 kDa.

Pili were purified from CV1 to determine the relationships between the pilin species detected in whole-cell lysates and assembled pili. The yields of CV1 pili were 2–4% of that found for VD302 P^{++} cultures. Immunoblotting showed that the major pilin species of P^{++} and CV1 purified pili had an identical relative mobility of 18.8 kDa but that the CV1 pili lacked a minor pilin species of M_r 21.5 kDa present in P^{++} pili (Fig. 5). Also, CV1 pili did not contain the M_r 16.5 kDa species detected in the whole-cell lysate. Amino-terminal sequence analysis of the purified pili confirmed in both preparations that pilin was properly cleaved and methylated at the N-terminal phenylalanine.

Distribution and localization of pilin antigen in pili mutants

Pilin species analogous to the M_r 16.5 kDa forms have been detected in MS11 pilus$^-$ mutants expressing missense pilins (Swanson *et al.*, 1986), MS11 variants expressing reduced levels of piliation (Haas *et al.*, 1987), and some pilus$^+$ variants recovered from males infected with that same strain (Swanson *et al.*, 1987b). While those pili mutants/variants were generated by pilin gene recombination, this was not the case for the mutants CV1 and P$^-$ (G$_{-1}$ → S$_{-1}$). Protein samples of whole-cell lysates, total membranes and concentrated supernatants were prepared from fluid cultures of the latter strains as well as from VD302 P^{++}, VD302 P$^-$ no. 1 (frameshift amino acids 68–70), VD301 P$^-$ no. 31 (missense) and VD301 P$^-$ (nonsense amino acid 119) (Koomey *et al.*, 1987). These fractions were analysed by immunoblotting using a monoclonal antibody reactive with pilin (Fig. 6). In contrast to the results using plate-derived organisms, whole-cell lysates of VD302 P^{++} showed only a single species of M_r 18.8 kDa.
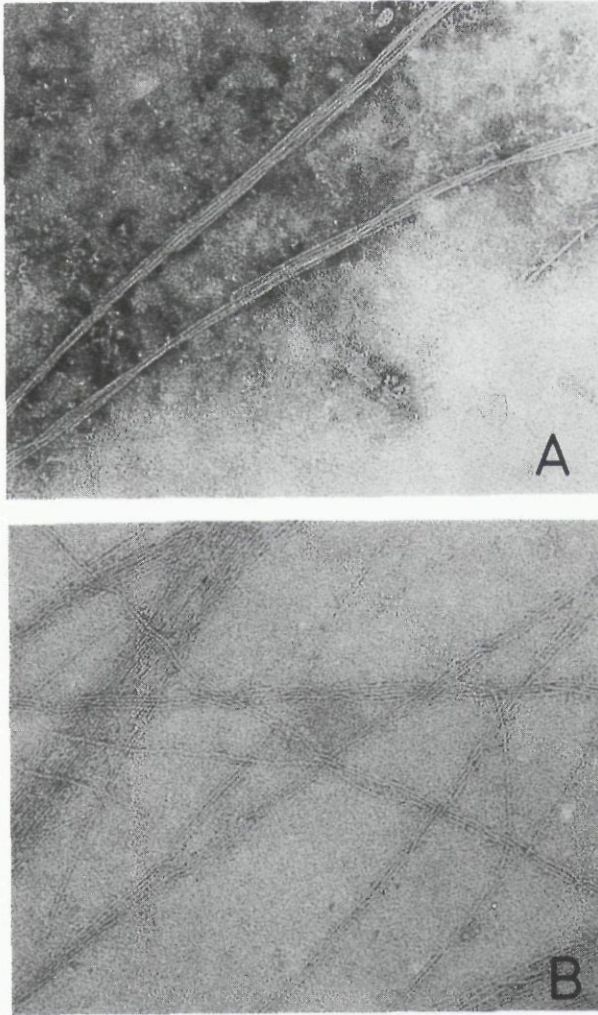
Fig. 2. Pili of VD302 P^{++} and CV1 observed by TEM (90000×).

species. Total membrane preparations of these strain revealed the distinct presence of the slower migrating forms present in whole cells and that the amounts of membrane-associated pilin found were higher in P$^-$ (G$_{-1}$ → S$_{-1}$) than CV1 which in turn were greater than that seen in VD302 P^{++}. Pilin was not detected in either whole cells or membranes of VD301 P$^-$ no. 3. Whole cells of VD301 P$^-$ no. 1 contained M_r 16.5 kDa pilin and no pilin was detected in membranes of this strain. Preliminary results suggest that the M_r 16.5 kDa pilin species associated with whole cells were periplasmically located but these experiments were complicated by the significant degree of lysis occurring during spheroplast formation.
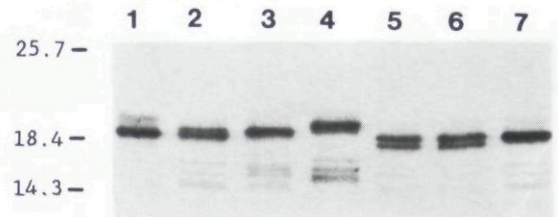

Fig. 4. Immunoblotting of pilin antigen in whole-cell lysates of (2) VD302 P^{++}, (3) CV1, (4) P$^-$ (G$_{-1}$ → S$_{-1}$) harvested from plate cultures, and *E. coli* HB101 expressing the cloned genes of (5) VD302 P^{++}, (6) CV1, and (7) P$^-$ (G$_{-1}$ → S$_{-1}$). Lane 1 was loaded with 100 ng of purified VD302 P^{++} pili. The blot was developed using monoclonal antibody O2 at a dilution of 1:1000.

Total membranes from this strain contained a small amount of this same-size pilin present in whole cells, while no pilin was detected in culture supernatants. Pilin was not detected in the fractions of VD302 P$^-$ no. 1. Whole cells of CV1 and P$^-$ (G$_{-1}$ → S$_{-1}$) displayed M_r 19.5 kDa and 21 kDa pilin species, respectively, along with an M_r 16.5 kDa

Significant amounts of M_r 16.5 kDa pilin were specifically released into the culture supernatant of CV1, P$^-$ (G$_{-1}$ → S$_{-1}$) and VD301 P$^-$ no. 1 while the supernatant of VD301 P$^-$ no. 3 contained an M_r 8 kDa pilin species. The levels of pilin detected in these supernatants ranged from 0.2–1.2 mg l^{-1} in concentration. The M_r 16.5 kDa pilin was purified from culture supernatants of P$^-$ (G$_{-1}$ → S$_{-1}$) and amino-terminal sequence analysis showed that the first seven residues matched amino acids 40 to 46 of mature pilin. These results were identical to that found for the M_r 16 kDa S-pilin found in a pilus$^+$ variant (Haas *et al.*, 1987). The data obtained from liquid cultures of VD301 P$^-$ no. 1 and VD301 P$^-$ no. 3 were different from that found using plate-derived cells (Koomey *et al.*, 1987). To address this discrepancy, analysis of plate-derived whole-cell lysates of those two mutants was repeated and the results (Fig. 7) revealed that VD301 P$^-$ no. 3 cells contained an M_r 15 kDa and 8 kDa species while VD301 P$^-$ no. 1 cells contained equivalent amounts of M_r 18.5 kDa and 16.5 kDa forms. As found with the other pilin genes cloned in *E. coli*, there was no evidence of a truncated or faster-migrating form of pilin analogous to that seen in gonococci.
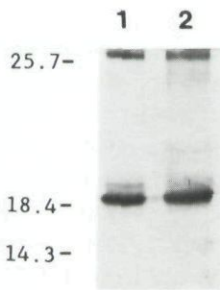
M. bovis	Met Asn		Ala Gln Lys Gly Phe Thr
P. aeruginosa	Met Lys		Ala Gln Lys Gly Phe Thr
B. nodosus	Met Lys Ser Leu Gln Lys Gly Phe Thr

N. gonorrhoeae	Met Asn Thr Leu Gln Lys Gly Phe Thr
VD302 P^{++}	ATG AAT ACC CTT CAA AAA GGC TTT ACC
VD302 P$^-$(G$_{-1}$-S$_{-1}$)	ATG AAT ACC CTT CAA AAA AGC TTT ACC
	Met Asn Thr Leu Gln Lys Ser Phe Thr

Fig. 3. Comparison of the *N*-terminal sequences of *N*-metPhe propilins and mutant pilin expressed by P$^-$ (G$_{-1}$ → S$_{-1}$).

Fig. 5. Immunoblotting of purified pili derived from (1) VD302 P^{++} and (2) CV1. The blot was developed using monoclonal antibody O2 at a dilution of 1:1000.

Studies of antigenic cross-reactivity between P. aeruginosa *PAK and gonococcal pili*

Watts and coworkers demonstrated that polyclonal rabbit antisera raised against PAK pili of *P. aeruginosa* would cross-react in an immunoblot with purified MS11 pili (Watts *et al.*, 1983a). They reasoned that since the only region of homology between PAK and gonococcal pilins is found at the amino terminus (residues 1–22), it was likely that epitopes within this common region were responsible for the observations. This possibility was tested by asking whether PAK pili antisera would react with the M_r 16.5 kDa S-pilin that lacked those amino-terminal residues. PAK
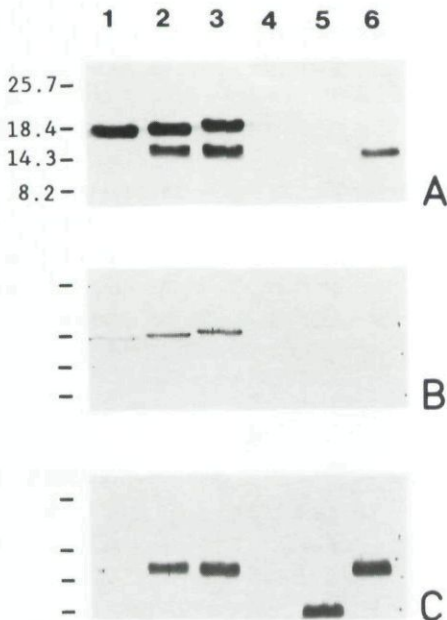

Fig. 6. Immunoblotting of whole-cell lysates (A), total membranes (B), and concentrated culture supernatants (C) of (1) VD302 P^{++}, (2) CV1, (3) P$^-$ (G$_{-1}$ → S$_{-1}$), (4) VD302 P$^-$ no. 1, (5) VD301 P$^-$ no. 3, and (6) VD301 P$^-$ no. 1. Samples were standardized according to total colony-forming units (5 × 10^8) (for A), total protein (for B), and volume of supernatant (for C). The blot was developed using monoclonal antibody O2 at a dilution of 1:1000.

antisera reacted with the M_r 18.5 kDa pilin of VD302 P^{++} and the M_r 19.5 kDa pilin of CV1 but failed to react with the faster-migrating species in whole cells of CV1 and P$^-$ (G$_{-1}$ → S$_{-1}$) (Fig. 8). Surprisingly, this serum did not react with the M_r 21 kDa pilin present in P$^-$ (G$_{-1}$ → S$_{-1}$). The PAK-specific antibodies also failed to detect the S-pilin in supernatants (data not shown).

Evidence for gonococcal pilin processing in E. coli

On the basis of the specific reactivity pattern seen with the PAK antisera, we inferred that it might be a useful reagent for addressing the status of gonococcal pilin expressed in *E. coli*. Duplicate immunoblots of whole-cell lysates of *E. coli* expressing the cloned pilin genes of strains VD302 P^{++}, CV1 and P$^-$ (G$_{-1}$ → S$_{-1}$) were probed with PAK
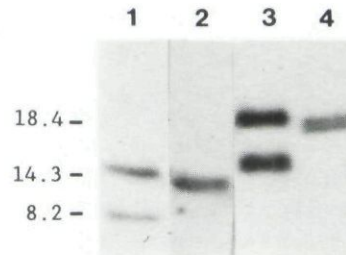

Fig. 7. Immunoblotting of whole-cell lysates of (1) VD301 P$^-$ no. 3 and (3) VS301 P$^-$ no. 1 harvested from plates, and HB101 expressing the pilin genes from (2) VD301 P$^-$ no. 3 and (4) VD301 P$^-$ no. 1. The blot was developed using monoclonal antibody at a dilution of 1:1000.

antisera and VD302 P^{++} pili antisera (Fig. 9). As an internal control, each blot also contained whole-cell lysate of VD302 P^{++} cells grown on plates, and, under the conditions employed, the two antisera reacted to a similar degree with the pilin in this sample. In contrast to the VD302 pilin antisera that reacted equally well with the various pilins expressed in *E. coli*, the PAK antibodies reacted only with the M_r 18 kDa form found in clones of the VD302 P^{++} and CV1 pilin genes and the signal detected was weak.

Discussion

The non-piliated state of P$^-$ (G$_{-1}$ → S$_{-1}$) clearly indicates a critical role for the amino acid at position −1 of pilin in the assembly of gonococcal pili. This idea is supported by the extreme degree of conservation of this residue in other *N*-metPhe pilins, the closely related *tcpA* gene product of *Vibrio cholerae* (Faast *et al.*, 1989), and the pilin-like *comG* open reading frames 3, 4, and 5 of *Bacillus subtilis* (Albano *et al.*, 1989). On the basis of the retarded migration of the mutant polypeptide, it appears that this pilin is incapable
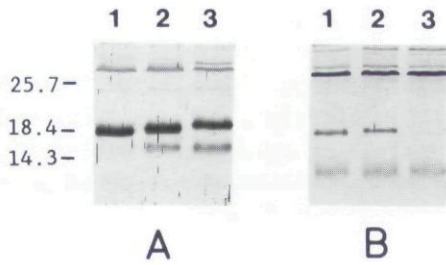
Fig. 8. Immunoblotting of whole-cell lysates of (1) VD302 P^{++}, (2) CV1, (3) P$^-$ (G$_{-1}$ → S$_{-1}$) from liquid cultures using rabbit antisera raised against VD302 P^{++} pili (Panel A, 1:1000 dilution) and PAK pilus antisera (Panel B, 1:500 dilution).

of being proteolytically processed at its amino terminus and that this accounts for the non-piliated phenotype. This conclusion is further supported by the failure of the mutant pilin to react with PAK antisera in immunoblotting since it is extremely difficult to envisage how an amino acid substitution in a part of the molecule not present in processed pilin could abolish the antigenicity of that polypeptide.

Because of the nature of processing that takes place during pilus biogenesis, the highly conserved amino-terminal domain of propilin must function both as a signal sequence (Strom and Lory, 1987) and in pilus assembly (Watts *et al.*, 1983b). The presence of (G$_{-1}$ → S$_{-1}$) pilin in total membranes along with the elaboration of S-pilin derived from mutant pilin implies that the polypeptide is defective for assembly and not membrane translocation. Studies of localization of PAK pilin mutants in *P. auerugi-nosa* have also shown that proteolytic processing at the Gly–Phe junction does not appear to be required for pilin to reach the bacterial surface (Pasloske and Paranchych, 1988b).

The class of mutants exemplified by CV1 yielded greatly reduced amounts of purified pili and the pili that were present appeared aberrant by both electron microscopic techniques. Using sodium dodecyl sulphate/polyacryl-amide gel electrophoresis (SDS–PAGE) and immunoblot-ting, CV1 purified pili lacked a minor slower-migrating pilin species found in VD302 P^{++} pili and the major form seen in the purified pili did not co-migrate with pilin found in whole cells or membranes. CV1 pilin in whole cells migrated slower than that seen in VD302 P^{++} whole cells but a similar form could be detected in VD302 P^{++} whole cells grown on plates. It appears that some post-trans-lational modification of pilin required for efficient assembly is diminished or missing in these mutants. Attempts to begin to identify the genetic lesion(s) responsible for this phenotype by genetic complementation have been com-plicated by the relatively high rate of spontaneous reversion.

Both of the mutants isolated here have increased levels of membrane-associated pilin compared with VD302 P^{++}

and their presence in P$^-$ (G$_{-1}$ → S$_{-1}$) argues against the possibility that the pilin detected represented contamin-ation with assembled pili. By analogy with filamentous phage assembly (Smilowitz *et al.*, 1972) as well as PAK and F-pilus assembly (Watts *et al.*, 1982; Moore *et al.*, 1981), the enriched pools of pilin in these assembly mutants provided evidence that membrane-bound pilin represents an intermediate step in pilus assembly. The failure to find membrane pilin pools in VD301 P$^-$ no. 1 and VD301 P$^-$ no. 3 that have mutations altering the carboxy-terminal domains of pilin suggest that they are deficient in a different, and presumably subsequent, step of pilus assembly.

The reactivity of PAK pili antisera with gonococcal pilin found in these studies confirms the findings of Watts *et al.* The failure of S-pilin and (G$_{-1}$ → S$_{-1}$) pilin to react shows that the cross-reactive epitope(s) are localized to the amino terminus and that proper proteolytic processing is necessary to create or expose the epitope(s). The ability of the sera to detect the slower-migrating pilin in CV1 implies that this pilin is proteolytically clipped and that its altered mobility from P^{++} pilin is due to differences in some other form of post-translational modification. When gonococcal pilin expressed in *E. coli* was examined, the PAK antisera reacted only with the faster-moving species of the two forms seen with P^{++} and CV1 pilin and since (G$_{-1}$ → S$_{-1}$) pilin failed to display this faster species, no reactivity was found for that sample. Therefore, the G$_{-1}$ → S$_{-1}$ mutation inhibited processing of pilin in *E. coli* just as it did in gonococci, suggesting that *E. coli* may possess a pro-tease related to that operating in gonococci. Similar findings have been made for wild-type and mutant PAK pilins expressed in *E. coli* (Pasloske and Paranchych, 1988a).

The precise signals that trigger the alternate processing of pilin and expression of S-pilin are not known. On the basis of previous work and the results presented here, it is clear that S-pilin production occurs coincidentally with reduced levels of piliation or the absence of pilus
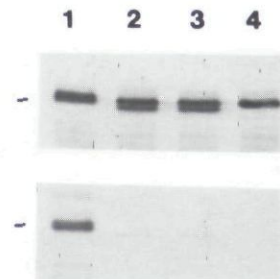

Fig. 9. Immunoblotting of whole-cell lysates of (1) VD302 P^{++} (from plates), and HB101 expressing the pilin genes from (2) VD302 P^{++}, (3) CV1, (4) P$^-$ (G$_{-1}$ → S$_{-1}$) using rabbit antisera raised against VD302 P^{++} pili (top Panel) and PAK pilus antisera (bottom Panel).

assembly. The elaboration of S-pilin by such a diverse group of mutants favours the notion that it is the result rather than the cause of non-assembly or reduced piliation. In such a scheme, it would seem that the signal that activates S-pilin processing is a reduced rate of pilin polymerizaton. Assuming that the protease responsible is present at all times, pilin being actively assembled remains in a protease-susceptible state for only a short time. A decrease in the rate of polymerization resulting from intrinsic structural features of variant and mutant pilins or from the occurrence of mutations in other genes involved in assembly thus increases the time in which pilin remains in a susceptible state. Differences in the degree of processing then would result from variability in the rate of polymerization and, in the case of non-piliated pilin mutants, the degree of accessibility or susceptibility of that particular pilin to the peptidase. The results also indicate that S-pilin can be significantly modified by the conditions of cultivation, with the process being most efficient in liquid cultures.

Many roles can be envisaged for S-pilin processing in pathogenesis (such as in adsorbing pilus-specific antibodies at a distance from the cell surface) or in the modulation of pilus extrusion and retraction. Alternatively, it may be that this is a mechanism by which cells alleviate the detrimental effects of pilin retained in the membrane under conditions where it is not being efficiently translocated and polymerized since the pilin gene is constitutively transcribed (Swanson et al., 1985). This situation might be analogous to that occurring in E. coli expressing high levels of hybrid proteins that accumulate in the membrane (Ito et al., 1981). Evidence for such a role for S-pilin processing is found in the genetic instability and small colony phenotype of P^- ($G_{-1} \rightarrow S_{-1}$) grown on plates. This mutant throws off large colony derivatives with shorter generation times and these variants have mutations in *pilE1* that preclude synthesis of full-size pilin (our unpublished data), suggesting that poor growth on plates is due to pilin expression in the absence of assembly. When grown in liquid cultures under conditions in which S-pilin processing is more efficient, the generation time is equivalent to that of the other pilus⁻ mutants. Although the significance of this form of pilin processing remains obscure, alterations in the levels of S-pilin production appears to be a sensitive marker for concurrent changes in gonococcal pilus expression and assembly.

Experimental procedures

Bacterial strains and cultivation conditions

N. gonorrhoeae strains VD302 P^{++}, VD302 P^- no. 1, VD302 P^- no. 1 and VD301 P^- no. 3 and conditions of growth on solid media

have been described (Koomey et al., 1987). VD302 P^- no. 1 expresses truncated pilin resulting from a frameshift mutation in the *pilE1* locus within the codons for amino acid residues 68–70, VD301 P^- no. 1 expresses a full-size variant pilin incapable of being assembled (analogous to the P-rp+ phenotype (Swanson et al., 1985) or the B1 variant of Haas (Haas et al., 1987)) and VD301 P^- no. 3 expresses truncated pilin resulting from a pilin gene nonsense mutation within the codon for aa residue 119. For detection of S-pilin, strains were grown to mid-log phase in Catlins Defined media (Gibco). Conditions of static broth cultures and selection for pellicle formation have been published (Koomey et al., 1987).

Nucleic acid manipulations

Methods of chromosomal and plasmid DNA isolation, Southern blotting, and recombinant DNA techniques have been detailed previously (Koomey and Falkow, 1987). The 2.7 kb EcoRI–ClaI fragments encompassing the *pilE1* locus were cloned into pBR325 and propagated in E. coli HB101. Sequencing of pilin mRNAs by primer extension/dideoxy chain termination and Northern blotting were performed according to published protocols (Bergstrom et al., 1986; Swanson et al., 1985).

Pilus purification and cell fractionation

Pili were purified from organisms harvested from plates using a modification of the method described by Brinton et al., (1978) utilizing repeated cycles of solubilzation (0.15 M ethanolamine-HCl buffer pH 10.4) and isoelectric precipitation (dialysis versus 0.05 M Tris-HCl pH 7.5/0.15 M NaCl (Tris-saline)) and all steps were performed at 4°C. Pilus protein concentrations were measured using the Coomassie Brilliant Blue Method (Spector, 1978) using bovine serum albumin as a standard. Purity of samples was determined by SDS–PAGE and Coomassie staining. Total gonococcal membranes were isolated by spheroplast formation followed by sucrose gradients as described (Johnston and Gotschlich, 1974).

Purification of S-pilin

Supernatants of broth cultures were cooled to 4°C and brought to 10% trichloroacetic acid. The precipitate was removed by centrifugation at 17 000 × g for 10 min and resuspended in Tris-saline buffer. The suspension was clarified by an additional centrifugation at 48 000 × g for 30 min. The clarified supernate was then adjusted to 10% trifluoroacetic acid (TFA) and the precipitate was collected by centrifugation. The precipitated material was solubilized in 8 M guanidine-HCl with 0.05% TFA and loaded onto a Whatman Partisal-10 ODS-2 column. Proteins and peptides were eluted with a 20–85% gradient of acetonitrile in water containing 0.05 TFA at a flow rate of 1.0 ml min⁻¹. One millilitre samples were collected, concentrated on a Savant Speedvac concentrator centrifuge, and analysed by SDS–PAGE and immunoblotting.

Determination of amino-terminal amino acids

Purified pili or S-pilin (50 nM) was precipitated with 10% ice-cold TFA and the precipitate washed seqentially with ethanol and

acetone and air-dried. The precipitate was then dissolved in 50% (v/v) acetic acid and 2.5 nM was introduced into a model 470A/477A protein/peptide pulse-liquid sequencer with an on-line PTH analyser (Applied Biosystems, Inc.).

SDS–PAGE and immunoblotting

SDS–PAGE was a variation of Laemmli's method (Laemmli, 1970) as described previously (Blake and Gotschlich, 1984). Procedures for immunoblotting (Western blotting) have been described previously (Blake et al., 1984). Whole-cell lysates were prepared from organisms swabbed from plates or liquid cultures and standardized by optical density at 600 nm and total protein concentration. Proteins in culture supernatants of defined media were concentrated by trichloroacetic acid precipitation followed by ethanol- and acetone washes and resuspension in sample buffer. Rabbit polyclonal antiserum 2-66 was raised by immunization with purifed VD302 P^{++} pili, and rabbit PAK pilus antisera was a gift of Emil Gotschlich. The pilin-specific monoclonal antibody O2 has been described and reacts with an invariant epitope of gonococcal pilin (Swanson et al., 1987a). Prestained low-range molecular weight protein electrophoresis markers (Diversified Biotech and BRL-Gibco) were used to standardize gels.

Transmission- and scanning electron microscopy

Transmission electron microscopy (using 0.5% phosphotungstic acid (PTA) as a negative stain) and scanning electron microscopy of whole colonies were performed as detailed (Swanson et al., 1971; 1985).

Acknowledgements

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