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Substitutions in the N-terminal alpha helical spine of *Neisseria gonorrhoeae* pilin affect Type IV pilus assembly, dynamics and associated functions

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Summary

Type IV pili (Tfp) are multifunctional surface appendages expressed by many Gram negative species of medical, environmental and industrial importance. The N-terminally localized, so called α-helical spine is the most conserved structural feature of pilin subunits in these organelles. Prevailing models of pilus assembly and structure invariably implicate its importance to membrane trafficking, organelle structure and related functions. Nonetheless, relatively few studies have examined the effects of missense substitutions within this domain. Using *Neisseria gonorrhoeae* as a model system, we constructed mutants with single and multiple amino acid substitutions localized to this region of the pilin subunit PilE and characterized them with regard to pilin stability, organelle expression and associated phenotypes. The consequences of simultaneous expression of the mutant and wild-type PilE forms were also examined. The findings document for the first time in a defined genetic background the phenomenon of pilin intermolecular complementation in which assembly defective pilin can be rescued into purifiable Tfp by coexpression of wild-type PilE. The results further demonstrate that pilin subunit composition can impact on organelle dynamics mediated by the PilT retraction protein via a process that appears to monitor the efficacy of subunit–subunit interactions. In addition to confirming and extending the evidence for PilE multimerization as an essential component for competence for natural genetic transformation, this work paves the way for detailed studies of Tfp subunit–subunit interactions including self-recognition within the membrane and packing within the pilus polymer.

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Introduction

Type IV pili (Tfp) are a unique class of proteinaceous surface appendages that play central roles in prokaryotic cell biology and disease pathogenesis. These organelles are implicated in motility, sensitivity to pilus-specific phage, natural genetic transformation, and dramatically influence multicellular behaviour, interactions with host tissue and virulence (Mattick, 2002). Tfp are comprised predominantly, if not exclusively, of relatively small protein subunits termed pilin that are arrayed as filamentous, helical polymers at the cytoplasmic membrane and extruded across the envelope of the outer membrane (Wolfgang *et al.*, 2000). Many of the ancillary proteins required for assembly of Tfp and their localization at the cell surface share significant sequence identity with components of both the type II protein secretion machinery (or secreton), that transports proteins across the outer membrane (Pugsley, 1993; Sandkvist, 2001) and of those involved in DNA uptake during natural genetic transformation (Chen and Dubnau, 2004). Likewise, proteins whose N-terminal domains share substantial sequence similarity with the corresponding regions of type IV pilins (referred to as pseudopilins in the secreton nomenclature) are necessary for protein secretion and DNA uptake in those systems. Collectively, these findings have led to the idea that polymerization of pilin-like proteins is involved in trafficking of macromolecules to and from the cell surface. Consistent with this hypothesis, PulG of *Klebsiella oxytoca* (Sauvonnet *et al.*, 2000) and XcpT of *Pseudomonas aeruginosa* secretons (the most abundant pseudopilins in both systems) (Durand *et al.*, 2003) have been

shown to be capable of being assembled into pseudopili, a Tfp-like filament, when overexpressed. Recently, the pilin-like ComGC protein has been demonstrated to form a high-molecular- weight homomultimer in competent *Bacillus subtilis* although Tfp-like organelles were not discernible (Chen *et al*., 2006).

Single residues within Tfp pilin subunits essential to Tfp expression or influencing levels of Tfp expression have been identified through forward and reverse genetics studies in a variety of systems (Koomey *et al*., 1991; Strom and Lory, 1991; Zhang *et al*., 1992; Horiuchi and Komano, 1998; Aas *et al*., 2002a). However, with the exception of mutants refractive to prepilin peptidase processing and export, the steps at which these alterations exert their effects are unknown as distinct events leading from pilin processing to Tfp expression remain undefined. Intuitively, blocks could be imposed at the levels of pilin translocation and membrane localization, folding and subunit–subunit interaction (polymerization). In addition, alterations at these steps could reflect aberrant interactions of pilin with other core components. To further complicate this situation, Tfp in many species undergo cycles of extension and retraction modelled as pilin subunit polymerization and depolymerization (Merz *et al*., 2000; Skerker and Berg, 2001; Merz and Forest, 2002). It is thus possible that some piliation defects associated with pilin subunit missense mutants are attributable to perturbations of normal Tfp dynamics rather than canonical defects in biogenesis.

Structural investigations of five Tfp pilins (Parge *et al*., 1995; Hazes *et al*., 2000; Keizer *et al*., 2001; Craig *et al*., 2003; Ramboarina *et al*., 2005) and the PulG pseudopilin (Kohler *et al*., 2004) have revealed remarkably conserved secondary structure consisting of an N-terminal, extended α-helical spine crowned by a C-terminal globular head domain comprised of a four-stranded anti-parallel β-sheet. These features appear to define the architecture for subunits to be exported, properly oriented in the membrane and assembled into filamentous polymers. Moreover, these composite structures can be readily reconciled with data derived from fibre diffraction and electron microscopy to build organelle models in which pilins are arranged in a helical manner, with the hydrophobic N-terminal alpha helix packed in the core of the pilus and the globular head domain making both lateral and longitudinal contacts (Craig *et al*., 2004). The N-terminal helical element, termed α1, has been further delineated into two subdomains: α1-N (residues 1–28) which is primarily hydrophobic and highly conserved and, α1-C (residues 29–53) which is amphipathic in character and is less conserved in primary structure (Craig *et al*., 2004). α1-N is predicted to mediate both membrane localization and crucial contacts between subunits involving parallel coiled-coil helix packing. Three classes of α1-N missense mutations have been documented: those with no effects on organelle expression and associated phenotypes, those that preclude assembly and those that perturb Tfp-associated autoagglutination while retaining Tfp expression (Chiang *et al*., 1995; Kirn *et al*., 2000; Park *et al*., 2001). The most thoroughly studied and perhaps most insightful α1-N mutants are substitutions at the most highly conserved and single charged residue glutamine plus 5 (E_5) that invariably preclude Tfp expression and associated functions (Pasloske *et al*., 1989; Strom and Lory, 1991; Macdonald *et al*., 1993; Horiuchi and Komano, 1998; Aas *et al*., 2002a). Together with structural data and fibre modelling, these findings are often invoked as evidence that a salt bridge between the N-terminal amine of F_1 of one pilin and the ε-oxygens of the E_5 residue of an interacting monomer plays a role in subunit registration and maintaining a neutral charge in the hydrophobic core of the pilus (Craig *et al*., 2004). Moreover, it has been shown that a *P. aeruginosa* $PilA^{PAK}$ E_5 mutant pilin, while defective on its own, was incorporated into heteropolymers together with the endogenous $PilA^{PAO}$ pilin (Pasloske *et al*., 1989). Interestingly, substitutions at residue E_5 of the PulG pseudopilin do not preclude its assembly into a pseudopilus but do abrogate secreton function (Vignon *et al*., 2003). With regard to α1-C, structural data and fibre modelling suggest its hydrophobic face interacts with packed globular head domains and the hydrophilic face is exposed on the surface of the protein but nonetheless buried in the pilus core. To date, only one study has addressed the influence of α 1-C substitutions on Tfp and pseudopilus biogenesis (Horiuchi and Komano, 1998).

The PilE protein subunit of *Neisseria gonorrhoeae* Tfp undergoes a number of post-translational modifications potentially relevant to its membrane trafficking, assembly/disassembly and associated phenotypes (Hegge *et al*., 2004). One of these involves its proteolysis into a soluble, truncated form lacking the first 39 residues of the mature polypeptide, termed S-pilin (Haas *et al*., 1987; Koomey *et al*., 1991). S-pilin is detectable in all mutants in which assembly is blocked as well as in piliated *pilE* missense mutants and antigenic variants where Tfp expression levels are diminished (Drake and Koomey, 1995; Freitag *et al*., 1995; Tonjum *et al*., 1995; Drake *et al*., 1997). In these backgrounds, it is found at relatively high levels in culture supernatants and it has been suggested that it might act as an 'antigenic decoy' that act at a distance from the bacterial cell to neutralize pilin-specific antibodies (Koomey *et al*., 1991). The precise signal that triggers S-pilin processing is not known but the data are most consistent with it being a consequence of a defect in stable fibre formation. However, it is formally possible that increased PilE turnover may contribute to Tfp expression defects or play a role in Tfp dynamics and associated phenotypes. For example, elaboration of S-pilin in some

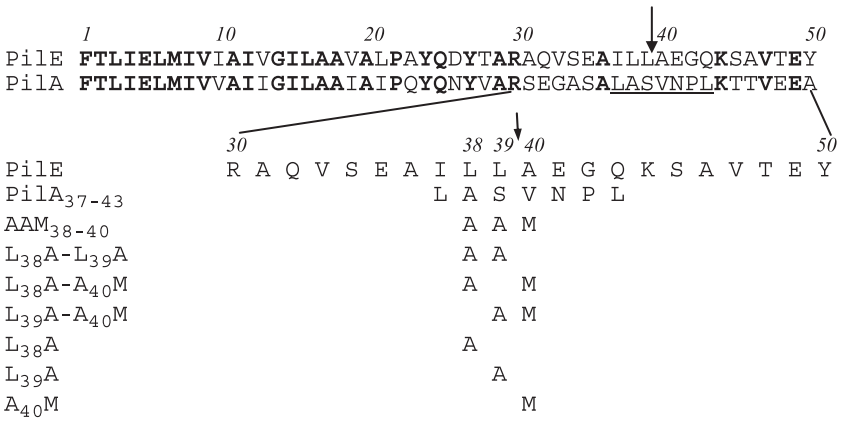

Fig. 1. PilE missense mutants altered in the putative S-pilin cleavage site.
Upper part. Alignment of the N-terminal sequences of the PilE and PilA pilin subunits. Identical residues are shown in bold. The PilA residues expressed in the $PilA_{37-43}$ pilin mutant are underlined.
Lower part. Altered residue(s) in each missense mutant aligned with the wild-type PilE amino acid sequence. The numbers above the wild-type sequence refer to the position in the mature protein. An arrow marks the putative S-pilin cleavage site.

biogenesis mutants is dependent on the PilT pilus retraction protein. When expression of functional PilT is abolished in these backgrounds, the restoration of Tfp expression is paralleled by the disappearance of S-pilin (Wolfgang *et al*., 1998a; 2000; Winther-Larsen *et al*., 2005). Thus, S-pilin in these instances may reflect subunits from depolymerizing Tfp that have re-entered the membrane pool and subsequently been degraded.

Here, we addressed the potential relationships between Tfp expression, pilin stability and associated phenotypes by examining the effects of substitutions in the α1-C domain of PilE. We also assess the effects of the α1-C substitutions in the context of other PilE missense substitutions and the potential transdominance of α1 domain mutants when coexpressed with wild-type *pilE.*

Results

Characterization of PilE α1-C substitution mutants

Pilin missense substitution mutants altered at residues adjacent to the putative S-pilin cleavage site (Fig. 1, Fig. S1) were constructed by introducing altered alleles into the *iga* locus in a background in which the endogenous pilin locus was under the control of a derepressible promoter (Wolfgang *et al*., 2000). Mutants expressing pilin with single substitutions of either alanine for Leu_{38} ($L_{38}A$), alanine for Leu_{39} ($L_{39}A$), or methionine for Ala_{40} ($A_{40}M$) each produced wild-type levels of Tfp as seen by purification yields and were indistinguishable in transformation proficiency from the wild-type control (Fig. 2, Table 1). In addition, S-pilin was observed for all when examined in a *pilF* background [lacking PilF, a putative ATPase required for biogenesis (Freitag *et al*., 1995)] (data not shown). Double mutants representing all combinations of the single substitutions were then made and examined. The $L_{38}A$-$A_{40}M$ mutant expressed normal levels of Tfp, autoagglutinated and was highly transformable (Table 1) while the $L_{39}A$-$A_{40}M$ mutant had reduced levels of Tfp, did not autoagglutinate and retained high transformability (Fig. 2, Table 1). Tfp could not be recovered from the $L_{38}A$-$L_{39}A$ substitution mutant although it was highly transformable. In addition, S-pilin was observed in the $L_{38}A$-$L_{39}A$ mutant and was seen for the other two *pilE* alleles when examined in a *pilF* background (data not shown). Therefore, none of the single or double substitution mutants resulted in a discernible S-pilin processing defect. Next, a triple substitution mutant in which alterations at all three residues were combined (designated the AAM_{38-40} mutant) was constructed and examined. This mutant was very similar to the $L_{38}A$-$L_{39}A$ mutant in that it remained highly

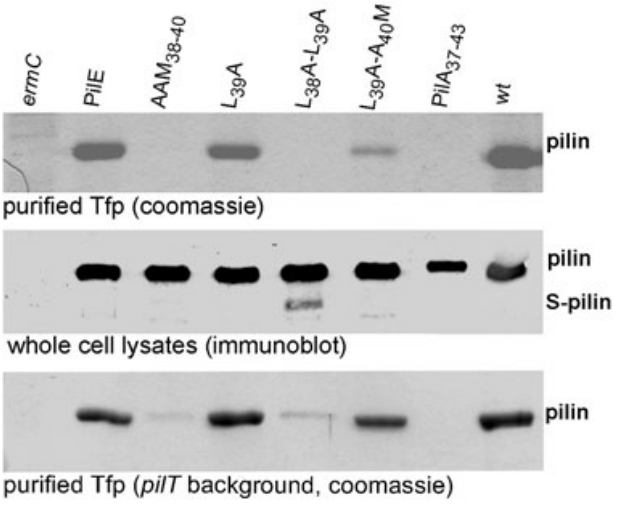

Fig. 2. Effects of PilE missense mutations on Tfp expression and S-pilin formation.
Upper panel. Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili.
Middle panel. Immunoblotting of whole cell lysates using rabbit antibodies specific for PilE. Strains: *ermC* (GE1), PilE (GE2), AAM_{38-40} (GE13), $L_{39}A$ (GE7), $L_{38}A$-$L_{39}A$ (GE10), $L_{39}A$-$A_{40}M$ (GE12), $PilA_{37-43}$ (GE14), wild type (wt) (N400).
Lower panel. Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili harvested from mutants expressing the same altered pilin alleles in a *pilT* background. Strains: *ermC* (GE30), PilE (GE31), AAM_{38-40} (GE37), $L_{39}A$ (GE34), $L_{38}A$-$L_{39}A$ (GE35), $L_{39}A$-$A_{40}M$ (GE36), $PilA_{37-43}$ (GE38), wild type (wt) (N400).

Table 1. *N. gonorrhoeae* strains used in this work and their relevant phenotypes.

Parent strain[a]	Relevant strain	Genotype	Pili[b]	Agg.[c]	S-pilin[d]	Transf. freq.[e]	Reference
VD300[f]	MS11		++	+	–		Koomey and Falkow (1987)
N400	VD300	*recA6(tetM)*[g]	++	+	–	ND	Tonjum *et al.* (1995)
N401	N400	*recA6(kan)*	++	+	–	ND	Wolfgang *et al.* (1998a)
GT104	VD300	$pilT_{ind}$	++	++	–	ND	Wolfgang *et al.* (1998b)
MW4	N401	$pilT_{ind}$	++	++	–	ND	Wolfgang *et al.* (1998a)
GT17	N400	*pilT::m*Tn*cm*17	++	++	–	NA	Park *et al.* (2002)
MW24	N401	$pilE_{ind}$	–	–	–	ND	Wolfgang *et al.* (2000)
KS101	VD300	$pilE_{ind}$	–	–	–	< 0.001	Wolfgang *et al.* (2000)
KS129/GE1	KS101/MW24	$pilE_{ind}$, *iga::ermC*	–	–	–	< 0.001	Aas *et al.* (2002a)
KS130/GE2	KS101/MW24	$pilE_{ind}$, *iga::pilE*	++	+	–	4.8 ± 2.5	Aas *et al.* (2002a)
KS131/GE3	KS101/MW24	$pilE_{ind}$, $iga::pilE_{G\text{-}1S}$	–	–	+	< 0.001	Aas *et al.* (2002a)
GE105/GE4	KS101/MW24	$pilE_{ind}$, $iga::pilE_{E5L}$	–	–	+	< 0.001	Aas *et al.* (2002a)
GE106/GE5	KS101/MW24	$pilE_{ind}$, $iga::pilE_{E5V}$	–	–	+	< 0.001	Aas *et al.* (2002a)
GE108/GE6	KS101/MW24	$pilE_{ind}$, $iga::pilE_{L38A}$	++	+	–	ND	This work
GE109/GE7	KS101/MW24	$pilE_{ind}$, $iga::pilE_{L39A}$	++	–	–	3.7 ± 0.8	This work
GE110/GE8	KS101/MW24	$pilE_{ind}$, $iga::pilE_{A40M}$	++	+	–	ND	This work
GE112/GE10	KS101/MW24	$pilE_{ind}$, $iga::pilE_{L38A\text{-}L39A}$	–	–	+	0.8 ± 0.2	This work
GE113/GE11	KS101/MW24	$pilE_{ind}$, $iga::pilE_{L38A\text{-}A40M}$	++	+	–	ND	This work
GE114/GE12	KS101/MW24	$pilE_{ind}$, $iga::pilE_{L39A\text{-}A40M}$	+	–	–	2.7 ± 0.2	This work
GE115/GE13	KS101/MW24	$pilE_{ind}$, $iga::pilE_{AAM38\text{–}40}$	(+)	–	–	1.4 ± 0.6	This work
GE116/GE14	KS101/MW24	$pilE_{ind}$, $iga::pilE_{pilA37\text{-}43}$	–	–	–	< 0.001	This work
GE117/GE15	KS101/MW24	$pilE_{ind}$, $iga::pilE_{W109S}$	–	–	++	< 0.001	This work
GE119/GE17	KS101/MW24	$pilE_{ind}$, $iga::pilE_{W109S\text{-}AAM38\text{–}40}$	–	–	+	0.003 ± 0.001	This work
GE120/GE18	KS101/MW24	$pilE_{ind}$, $iga::pilE_{E5L\text{-}AAM38\text{–}40}$	–	–	–	< 0.001	This work
GE121/GE19	KS101/MW24	$pilE_{ind}$, $iga::pilE_{E5V\text{-}AAM38\text{–}40}$	–	–	–	< 0.001	This work
GE122/GE20	VD300/N400	*iga::ermC*	++	+	–	6.7 ± 5.8	This work
GE123/GE21	VD300/N400	*iga::pilE*	+++	++	–	3.0 ± 0.8	This work
GE124/GE22	VD300/N400	$iga::pilE_{G\text{-}1S}$	–	–	+	< 0.001	This work
GE125/GE23	VD300/N400	$iga::pilE_{E5L}$	(+)	–	++	2.4 ± 1.3	This work
GE126/GE24	VD300/N400	$iga::pilE_{E5V}$	++	–/(+)	+	6.9 ± 6.4	This work
GE127/GE25	VD300/N400	$iga::pilE_{L39A}$	++	++	–	2.4 ± 1.3	This work
GE128/GE26	VD300/N400	$iga::pilE_{AAM38\text{–}40}$	++	++	+	7.1 ± 6.9	This work
GE129/GE27	VD300/N400	$iga::pilE_{pilA37\text{-}43}$	(+)	–	+	6.9 ± 2.5	This work
GE28	N400	$iga::pilE_{E5L\text{-}AAM38\text{–}40}$	(+)	–	+	ND	This work
GE29	N400	$iga::pilE_{E5V\text{-}AAM38\text{–}40}$	(+)	–	(+)	ND	This work
GE30	GE1	$pilE_{ind}$, *pilT::m*Tn*cm*17, *iga::ermC*	–	–	ND	NA	This work
GE31	GE2	$pilE_{ind}$, *pilT::m*Tn*cm*17, *iga::pilE*	++	++	ND	NA	This work
GE32	GE4	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{E5L}$	–	–	ND	NA	This work
GE33	GE5	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{E5V}$	–	–	ND	NA	This work
GE34	GE7	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{L39A}$	++	++	ND	NA	This work
GE35	GE10	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{L38A\text{-}L39A}$	(+)	+/–	ND	NA	This work
GE36	GE12	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{L39A\text{-}A40M}$	++	++	ND	NA	This work
GE37	GE13	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{AAM38\text{–}40}$	(+)	+	ND	NA	This work
GE38	GE14	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{pilA37\text{-}43}$	–	–	ND	NA	This work
GE39	GE15	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{W109S}$	–	–	ND	NA	This work
GE41	GE17	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{W109S\text{-}AAM38\text{–}40}$	–	–	ND	NA	This work
GE42	GE18	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{E5L\text{-}AAM38\text{–}40}$	–	+	ND	NA	This work
GE43	GE19	$pilE_{ind}$, *pilT::m*Tn*cm*17, $iga::pilE_{E5V\text{-}AAM38\text{–}40}$	–	+	ND	NA	This work
GE45	MW4	$pilT_{ind}$, *iga::pilE*	+++	+++	ND	ND	This work
GE46	MW4	$pilT_{ind}$, $iga::pilE_{G\text{-}1S}$	–	–	ND	ND	This work
GE47	MW4	$pilT_{ind}$, $iga::pilE_{E5L}$	+	+	ND	ND	This work
GE48	MW4	$pilT_{ind}$, $iga::pilE_{E5V}$	+	++	ND	ND	This work
GE49	MW4	$pilT_{ind}$, $iga::pilE_{L39A}$	++	++	ND	ND	This work
GE50	MW4	$pilT_{ind}$, $iga::pilE_{AAM38\text{–}40}$	+	++	ND	ND	This work
GE51	MW4	$pilT_{ind}$, $iga::pilE_{pilA37\text{–}43}$	+	+	ND	ND	This work
GE52	MW4	$pilT_{ind}$, $iga::pilE_{E5L\text{-}AAM38\text{–}40}$	+	+(+)	ND	ND	This work
GE53	MW4	$pilT_{ind}$, $iga::pilE_{E5V\text{-}AAM38\text{–}40}$	++	+	ND	ND	This work
GQ21	N400	*pilQ::mTnCm*	–	–	+	NA	Drake *et al.* (1997)

Table 1. *cont.*

Parent strain[a]	Relevant strain	Genotype	Pili[b]	Agg.[c]	S-pilin[d]	Transf. freq.[e]	Reference
GD101	GE2	$pilE_{ind}$, *iga*::*pilE*, $pilD_{cat}$	–	–	+	NA	This work
GF101	GE2	$pilE_{ind}$, *iga*::*pilE*, $pilF_{cat}$	–	–	+	NA	This work
GQ101	GE2	$pilE_{ind}$, *iga*::*pilE*, *pilQ*::mTn*Cm*	–	–	+	NA	This work
GD102	GE13	$pilE_{ind}$, *iga*::$pilE_{AAM38-40}$, $pilD_{cat}$	–	–	–	NA	This work
GF102	GE13	$pilE_{ind}$, *iga*::$pilE_{AAM38-40}$, $pilF_{cat}$	–	–	–	NA	This work
GQ102	GE13	$pilE_{ind}$, *iga*::$pilE_{AAM38-40}$, *pilQ*::mTn*Cm*	–	–	–	NA	This work
GP109	VD300	$comP_{oe}$[h]	++	++	–	ND	Wolfgang *et al.* (1999)
GP111	GP109	$pilE_{ind}$, $comP_{oe}$	–	–	ND	ND	Aas *et al.* (2002a)
GP121	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::*ermC*	–	–	ND	<0.001	This work
GP122	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::*pilE*	++	+(+)	ND	64.3 ± 24.0	This work
GP123	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::$pilE_{L39A}$	++	–/+	ND	70.1 ± 10.7	This work
GP124	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::$pilE_{AAM38-40}$	(+)	–	ND	56.3 ± 18.1	This work
GP125	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::$pilE_{pilA37-43}$	–	–	ND	< 0.001	This work
GP126	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::$pilE_{G-1S}$	–	–	ND	< 0.001	This work
GP127	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::$pilE_{E5L}$	–	–	ND	< 0.001	This work
GP128	GP111	$pilE_{ind}$, $comP_{oe}$, *iga*::$pilE_{E5V}$	–	–	ND	< 0.001	This work

a. Strains KS129 to KS131 and GE105 to GE129 were made from the wild-type recombination parent strain KS101 or VD300, while the isogenic mutants GE1 to GE29 were made in the *recA*-inducible parent strain MW24 or N400.
b. Piliation status was examined by immunoblotting of purified pili and immunofluorescence under non-inducible conditions.
c. Agglutination phenotype is based on colony morphology and clumping in liquid media under non-inducible conditions.
d. Levels of S-pilin in whole cell extracts under non-inducible conditions. ND, denotes not determined.
e. Transformation frequency as percentage of recipient cells, performed under non-inducible conditions. NA denotes not applicable. ND denotes not determined or that the assay was performed fewer than three times.
f. VD300 is an Opa$^-$ derivative of MS11.
g. *recA6* is an IPTG-inducible allele of *recA*.
h. $comP_{oe}$ is the new designation of the former *pilE*::*comP* allele (Wolfgang *et al.*, 1999).

transformable although Tfp could not be recovered (Fig. 2). Unlike the $L_{38}A$-$L_{39}A$ mutant, however, S-pilin was not detectable for the AAM_{38-40} mutant nor was it seen when this pilin was expressed in either *pilD*, *pilF* or *pilQ* (lacking the Tfp-associated secretin protein) backgrounds (Fig. 3). A mutant expressing a pilin in which residues 37–43 of PilE were replaced by the corresponding residues of *P. aeruginosa* PilA ($PilA_{37-43}$) was also constructed and examined. PilA pilin was previously shown to function in competence (Aas *et al.*, 2002a) and to be expressed as Tfp in gonococci although the steady state levels of Tfp found are relatively low (H.C. Winther-Larsen *et al.*, unpublished data). As PilE and PilA share conserved residues flanking this region (Fig. 1, Fig. S1) and the spacing of the flanking sequence was conserved, we reasoned that this hybrid might retain the capacity to form Tfp. However, the chimera failed to form purifiable Tfp (Fig. 2) and did not function in supporting transformation competence (Table 1).

Detection of pilin in shear fractions has a somewhat limited sensitivity as a readout of Tfp expression. In particular, short pilus filaments might be more shear resistant. Therefore, we used immunofluorescence microscopy (IFM) employing secondary antibodies coupled to a fluorescent label and confirmed the piliation defects in the AAM_{38-40} mutant, the $PilA_{37-43}$ mutant and in PilE missense mutants defective in supporting Tfp expression and competence (Fig. S2, data not shown). Using transmission electron microscopy (TEM), rare, short surface projections of Tfp-like filaments were seen in the AAM_{38-40} mutant but not in the $PilA_{37-43}$, $G_{-1}S$, E_5L, or E_5V mutants (Fig. 4A, data not shown). Immuno-TEM (using Tfp-specific antibodies with secondary antibodies coupled to gold beads) confirmed that the structures unique to the AAM_{38-40} mutant contained PilE (Fig. 4B). These results confirm and extend previous studies demonstrating a strict correlation between competence for natural transformation and *N. gonorrhoeae* Tfp expression (Zhang *et al.*, 1992; Drake

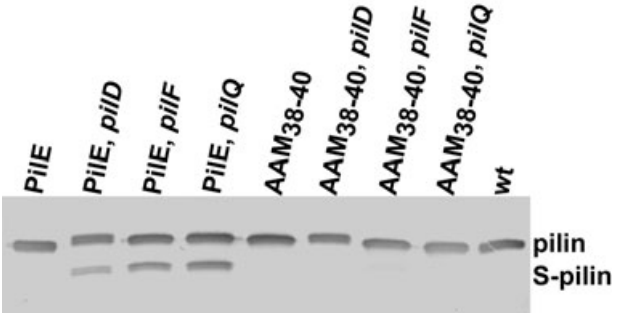

Fig. 3. The AAM_{38-40} substitution blocks S-pilin production. Immunoblotting of whole cell lysates using rabbit antibodies specific for PilE. Strains: PilE (GE2); PilE, *pilD* (GD101); PilE, *pilF* (GF101); PilE, *pilQ* (GQ101); AAM_{38-40} (GE13); AAM_{38-40}, *pilD* (GD102); AAM_{38-40}, *pilF* (GF102); AAM_{38-40}, *pilQ* (GQ102); wild type (wt) (N400).

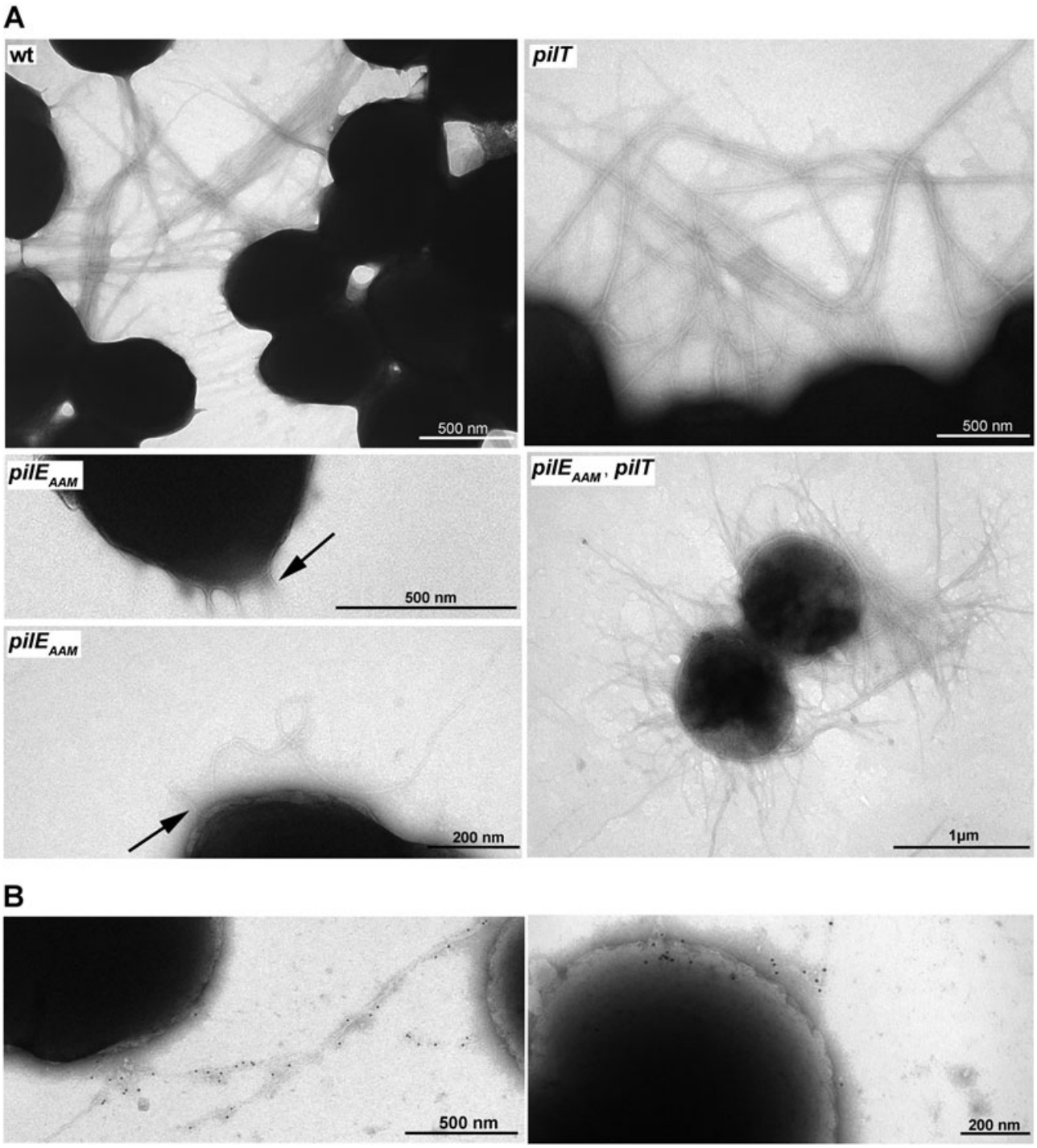

Fig. 4. Piliation defect in the AAM_{38-40} pilin mutant analysed by electron microscopy and immunogold labelling.
A. Transmission electron microscopy. Strains: wild type (wt) (N400), *pilT* (GT17), $pilE_{AAM}$ (GE13), $pilE_{AAM}$, *pilT* (GE37). Arrows indicate short truncated pili.
B. Immunogoldlabelling of pilus structures protruding from the AAM_{38-40} mutant (GE13) using rabbit antibodies specific for PilE.

and Koomey, 1995; Freitag *et al*., 1995; Tonjum *et al*., 1995; Drake *et al*., 1997; Wolfgang *et al*., 1999; Aas *et al*., 2002a) and that low levels of Tfp can suffice in the process (Rudel *et al*., 1995; Long *et al*., 2003).

Dramatic reductions in Tfp expression in certain mutants can be suppressed by the absence of functional PilT protein (Wolfgang *et al*., 1998a; 2000; Winther-Larsen *et al*., 2005). We introduced the *pilE* alleles

associated with diminished Tfp expression into a *pilT* background and examined Tfp levels in shear-released fractions (Fig. 2, bottom panel). While purifiable Tfp were not seen for the $PilA_{37–43}$ mutant in this background, the absence of PilT led to readily detectable but still low levels of Tfp recovery in both the $AAM_{38–40}$ and $L_{38}A$-$L_{39}A$ mutants and to an increase in Tfp recovery in the $L_{39}A$-$A_{40}M$ mutant (Fig. 2). IFM and EM findings were consistent with these results (Fig. S2, data not shown).

Effects of the $AAM_{38–40}$ substitutions in cis

The ability of the $AAM_{38–40}$ mutant to retain efficient transformability combined with its lack of S-pilin and dramatic reduction in Tfp expression might suggest that the competence defect in Tfp biogenesis mutants is related to increased PilE turnover or instability rather than a lack of pilus formation *per se*. If this were the case, the $AAM_{38–40}$ alteration might suppress biogenesis and competence defects in biogenesis mutants. However, neither phenotype was rescued in *pilD*, *pilF* or *pilQ* null mutants expressing the $AAM_{38–40}$ mutant pilin (Table 1). We next assessed if the $AAM_{38–40}$ alteration might restore function to PilE null mutants by increasing pilin stability or otherwise influencing subunit structure and function. To this end, strains expressing PilE bearing both the $AAM_{38–40}$ alteration and substitutions at either Glu_5 (E_5) or Trp_{109} (W_{109}) were examined. These latter single substitutions have been documented to preclude both Tfp expression and function in competence (Zhang *et al*., 1992; Aas *et al*., 2002a). As shown in Fig. 5, the $AAM_{38–40}$ substitutions restored neither competence nor the ability to form purifiable Tfp when combined with the E_5 substitutions although they did lead to PilE stabilization in these instances. The lack of Tfp expression was confirmed by IFM and EM (data not shown). In the case of the $W_{109}S$ substitution, the addition of the $AAM_{38–40}$ substitution did not overcome the defect in Tfp expression (Fig. 5A). Here, the levels of S-pilin seen were decreased but not abolished. Thus, the $AAM_{38–40}$ substitutions do not universally preclude S-pilin processing of PilE.

Effects of ComP overexpression in pilin mutants

The type IV prepilin-like ComP protein acts in a dose-dependent manner to promote DNA binding and uptake in conjunction with an intact Tfp biogenesis machinery. In order to understand better the basis for the transformation phenotypes of the PilE missense mutants, the effects of ComP overexpression were examined. As shown in Fig. 6, overexpression of ComP had a nearly identical positive effect on transformability but only in combination with those pilins that were functional vis-à-vis transformation in a wild-type background. As such, non-functional pilin missense mutants are devoid of any residual competence supporting function. Remarkably, dramatic enhancement in transformability afforded by ComP overexpression in the $pilE_{AAM38–40}$ background was not associated with an increase in levels of recoverable Tfp (Fig. 6). Loss-off-function *pilV* mutations generate phenocopies of ComP overexpressing strains in conjunction with increased levels of ComP recoverable in shear fractions (Aas *et al*., 2002b). When transformability of the pilin mutants was examined in a *pilV* background, virtually identical results to those seen with ComP overexpression were seen (data not shown).

Effects of the $AAM_{38–40}$ substitutions in trans

The non-transformability of strains expressing certain PilE missense mutants is presumed to result from lack of subunit multimerization and thus defective subunit–subunit interactions. Therefore, such PilE mutants might exert dominant-negative effects when coexpressed with wild-type PilE. These possibilities were first addressed by exploiting the derepressibility of wild-type *pilE* in the backgrounds ectopically expressing mutant PilE forms. In these experiments, derepression of the wild-type allele was carried out by addition of gratuitous inducer at the same time as transforming DNA was added. Here, substitutions at both Gly_{-1} ($G_{-1}S$) and E_5 had significant dominant-negative effects on competence under these conditions when compared with strains coexpressing no PilE, wild-type PilE, the $AAM_{38–40}$ mutant and the $W_{109}S$ mutant (Fig. 5B, white bars). In contrast, the combined E_5-$AAM_{38–40}$ mutant pilins were less interfering with the E_5L version showing virtually no effect and the E_5V version interfering moderately. Thus, in both these instances the $AAM_{38–40}$ substitutions rendered PilE less effective in exerting a dominant-negative effect. An opposite effect was seen with the $AAM_{38–40}$-$W_{109}S$ mutant which interfered better than the mutant carrying only the $W_{109}S$ substitution.

An alternative method to assessing dominant-negative effects of mutant forms was to have wild-type PilE expressed from its native promoter in conjunction with the ectopic gene copy such that the two PilE forms were expressed at the same high level. This approach also made it possible to access potential dominant-negative effects on levels of Tfp expression. For comparison, a strain diploid for the wild-type *pilE* allele was constructed and levels of recoverable Tfp were approximately doubled relative to the equivalent strain expressing only a single *pilE* copy (Fig. 7A). In contrast to the findings made with the derepressible *pilE* background, dominant-negative effects on competence were only observed for the $G_{-1}S$ mutant heterodiploid (Table 1). Tfp were not

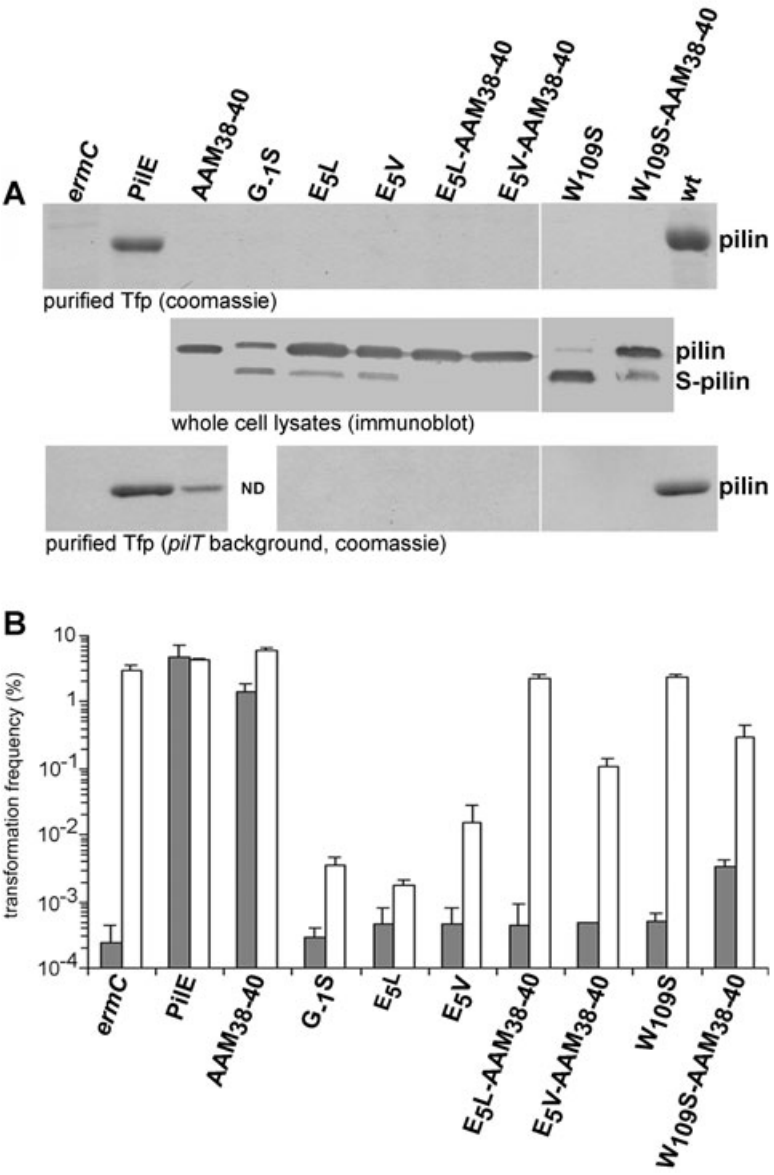

Fig. 5. Effects of the $AAM_{38–40}$ substitutions in combination with other mutations on Tfp expression, S-pilin formation and competence.
A. (Upper panel) Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili. (Middle panel) Immunoblotting of whole cell lysates using rabbit antibodies specific for PilE. Strains: *ermC* (GE1), PilE (GE2), $AAM_{38–40}$ (GE13), $G_{-1}S$ (GE3), E_5L (GE4), E_5V (GE5), E_5L-$AAM_{38–40}$ (GE18), E_5V-$AAM_{38–40}$ (GE19), $W_{109}S$ (GE15), $W_{109}S$-$AAM_{38–40}$ (GE17), wild type (wt) (N400). (Lower panel) Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili harvested from mutants expressing the same altered pilin alleles in a *pilT* background. Strains: *ermC* (GE30), PilE (GE31), $AAM_{38–40}$ (GE37), E_5L (GE32), E_5V (GE33), E_5L-$AAM_{38–40}$ (GE42), E_5V-$AAM_{38–40}$ (GE43), $W_{109}S$ (GE39), $W_{109}S$-$AAM_{38–40}$ (GE41), wild type (wt) (N400). ND, not determined.
B. Bar graphs show transformability as a percentage of recipient cells (values used are mean ± standard error of the mean, $n = 3$). White and grey bars represent transformation assays performed with and without induction of *pilE* respectively. The transformation assay was performed with strains: *ermC* (KS129), PilE (KS130), $AAM_{38–40}$ (GE115), $G_{-1}S$ (KS131), E_5L (GE105), E_5V (GE106), E_5L-$AAM_{38–40}$ (GE120), E_5V-$AAM_{38–40}$ (GE121), $W_{109}S$ (GE117), $W_{109}S$-$AAM_{38–40}$ (GE119).

recovered in the shear fraction of this strain (Fig. 7B) nor were they detectable in this background using IFM (Fig. 8). Remarkably, although the E_5L heterodiploid background retained high levels of transformability, Tfp were not detectable in the shear fraction of this strain (Fig. 7B). When examined by IFM and EM, rare, thin filaments were nonetheless seen (Fig. 8, data not shown). In the cases of the E_5V and $PilA_{37–43}$ heterodiploid strains, levels of recoverable Tfp were below that seen for the control strains expressing a single wild-type *pilE* gene (Figs 7 and 8, data not shown). In all four instances, S-pilin was detected in whole cell samples with the levels being most abundant in the E_5L heterodiploid background (Fig. 7A, middle panel). The $AAM_{38–40}$ substitutions appeared to have little transdominant effect with the levels of recoverable Tfp being nearly equivalent to that seen in the wild-type strain (Figs 7 and 8) while pilins with both these substitutions and the E_5 changes interfered with Tfp expression (Figs 7 and 8). In the case of the E_5V substitution, these alterations appeared to have a synergistic effect as the pilin with the combined alterations exhibited more interference than those bearing the single substitutions alone. IFM substantiated these findings and further revealed that Tfp in the E_5L-$AAM_{38–40}$ heterodiploid had a unique morphology consisting of long, thin filaments rather than short bundles (Fig. 8). Finally, in both instances, the levels of S-pilin were reduced relative to that seen for the heterodiploids with the E_5 substitutions mutations alone (Fig. 7A, middle panel).

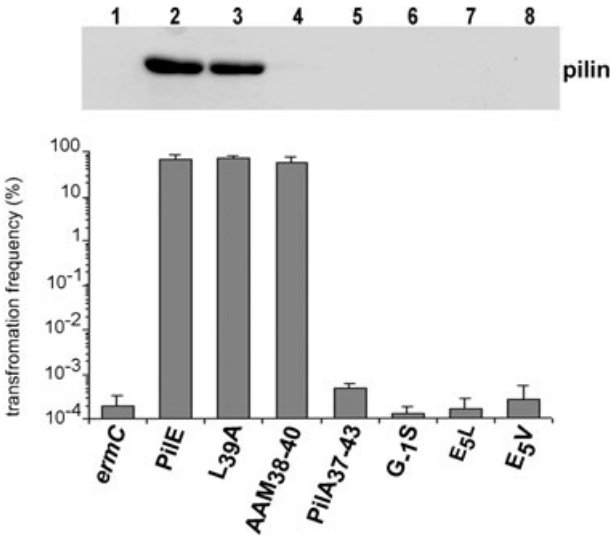

Fig. 6. Effects of ComP overexpression in pilin mutants. Upper part of figure is a Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili. All strains express high levels of ComP from the *pilE*::*comP* translational fusion allele. Lanes: 1, *ermC* (GP121); 2, PilE (GP122); 3, $L_{39}A$ (GP123); 4, $AAM38_{-40}$ (GP124); 5, $PilA_{37-43}$ (GP125); 6, $G_{-1}S$ (GP126); 7, E_5L (GP127); 8, E_5V (GP128). Graph shows transformability of the strains above as a percentage of recipient cells (values used are mean ± standard error of the mean, $n = 3$). Transformation data on the isogenic strains made in a wild-type *comP* background are shown in Table 1 and in bar graph of Fig. 5.

Pilin intermolecular complementation: rescue of E_5 mutant PilE into purifiable pili

When reconstructed in a *pilT* background, high and equivalent levels of Tfp were seen in all the *pilE* heterodiploids involving E_5 substitutions as well as that of the $PilA_{37-43}$ chimera heterodiploid (Fig. 7A, bottom panel). Thus, the dominant-negative character of these mutant pilins is PilT-dependent. In contrast, Tfp was not recoverable from the $G_{-1}S$ heterodiploid in a *pilT* background (Fig. 7B). This mutant also differed from the others (and that of its wild-type *pilT* congenic partner) in that it exhibited a poor growth phenotype. To examine if the E_5 substitution pilins were being incorporated into Tfp in the *pilT* background, N-terminal sequencing of PilE from purified Tfp was carried out. The E_5 heterodiploid Tfp preparations each contained levels of either Leu or Val in the fifth cycle consistent with the respective mutant pilin representing between 25% and 35% of total recoverable pilin (Fig. 9). In addition, Tfp preparations from these heterodiploids (but not those from the wild-type homodiploid) yielded elevated levels of unmethylated Phe in the first cycle consistent with the stoichiometry of the wild type to mutant pilin ratios derived from the data from the fifth cycles of sequence analysis. These data are consistent with findings made in *P. aeruginosa* showing that conserved E_5 of pilin is required for efficient *N*-methylation of F_1 (Pasloske and Paranchych, 1988; Strom and Lory, 1991; Macdonald *et al.*, 1993) by PilD prepilin peptidase (Strom *et al.*, 1993).

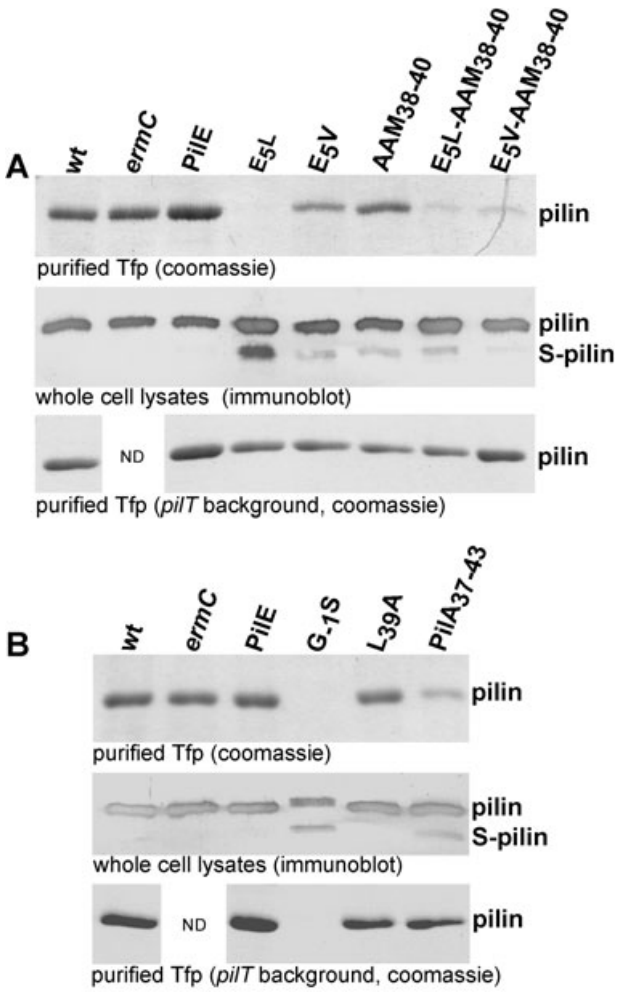

Fig. 7. Analyses of *pilE* heterodiploids with regard to Tfp expression and S-pilin formation.
A. (Upper panel) Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili. (Middle panel) Immunoblotting of whole cell lysates using rabbit antibodies specific for PilE. Strains: wild type (wt) (N400), *ermC* (GE20), PilE (GE21), E_5L (GE23), E_5V (GE24), AAM_{38-40} (GE26), E_5L-AAM_{38-40} (GE28), E_5V-AAM_{38-40} (GE29). (Lower panel) Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili harvested from mutants expressing the altered pilin alleles in a *pilT* background. Strains: wild type (wt) (N400), PilE (GE45), E_5L (GE47), E_5V (GE48), AAM_{38-40} (GE50), E_5L-AAM_{38-40} (GE52), E_5V-AAM_{38-40} (GE53). ND, not determined.
B. (Upper panel) Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili. (Middle panel) Immunoblotting of whole cell lysates using rabbit antibodies specific for PilE. Strains: wild type (wt) (N400), *ermC* (GE20), PilE (GE21), $G_{-1}S$ (GE22), $L_{39}A$ (GE25), $PilA_{37-43}$ (GE27). (Lower panel) Coomassie-stained SDS-PAGE gel showing the relative amounts of PilE in purified pili harvested from mutants expressing the altered pilin alleles in a *pilT* background. Strains: wild type (wt) (N400), PilE (GE45), $G_{-1}S$ (GE46), $L_{39}A$ (GE49), $PilA_{37-43}$ (GE51). ND, not determined.

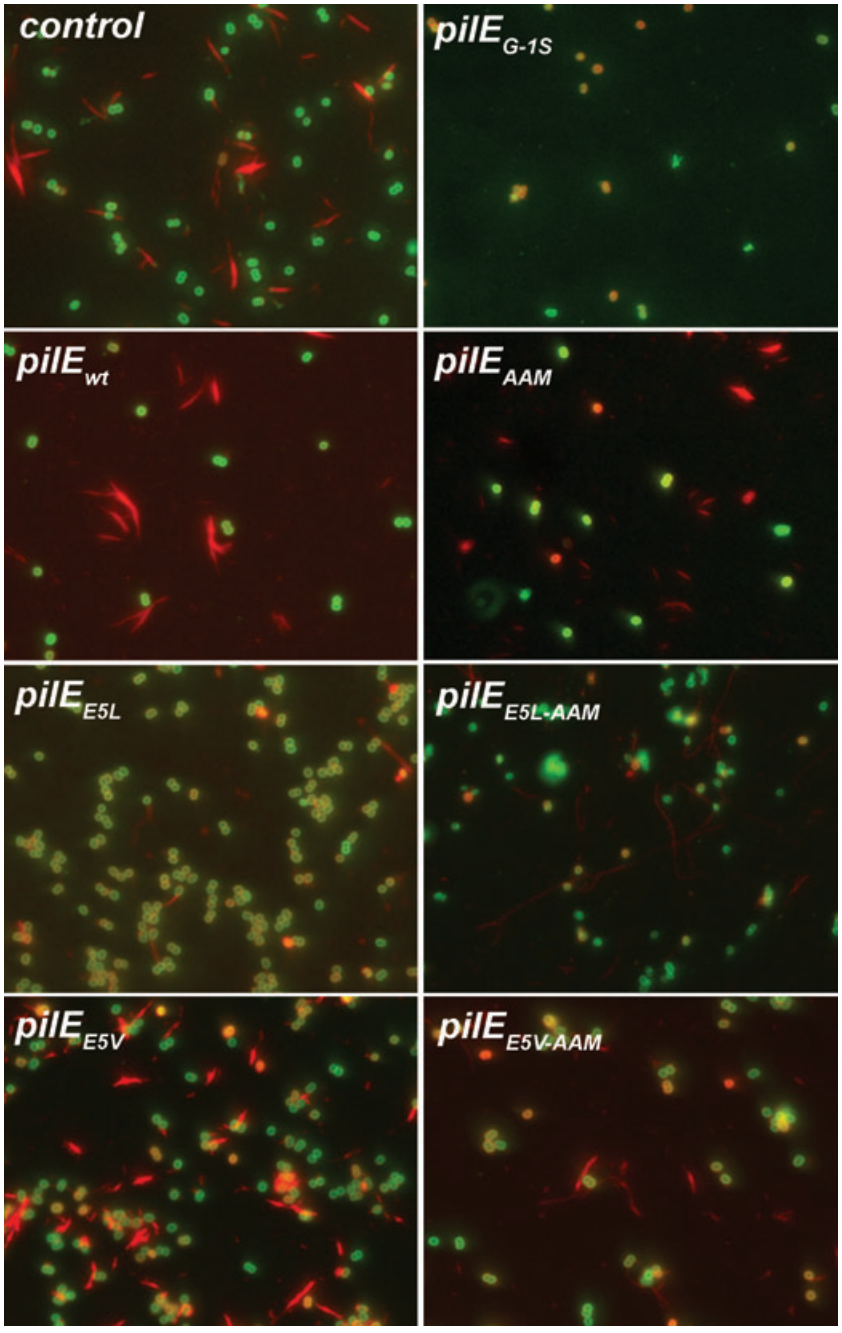

Fig. 8. Tfp expression in *pilE* heterodiploids analysed by IFM. Tfp were detected using first rabbit antibodies specific for PilE followed by Alexa red (594 nm) conjugated goat anti-rabbit IgG antibodies (red). Gonococci were detected using fluorescein-labelled monoclonal antibodies (green). Strains: *control* (wt; N400), $pilE_{G\text{-}1S}$ (GE22), $pilE_{wt}$ (GE21), $pilE_{AAM}$ (GE26), $pilE_{E5L}$ (GE23), $pilE_{E5L\text{-}AAM}$ (GE28), $pilE_{E5V}$ (GE24), $pilE_{E5V\text{-}AAM}$ (GE29).

PilE substitutions influence human corneal epithelial cell adherence

The PilE mutants here provided unique reagents to examine the relationship between Tfp expression and adherence to human epithelial cells as well as the influence of Tfp levels and dynamics on this phenotype. Without exception, mutants defined as being devoid of Tfp by purification, IFM and TEM failed to bind to the human cells (Fig. 10, data not shown). The $AAM_{38–40}$ mutant also failed to adhere while this defect was suppressed in a *pilT* background. Surprisingly, the heterodiploid coexpressing wild-type PilE and the E_5L allele and having very low steady state Tfp levels also adhered. In both this case and that of the $AAM_{38–40}$ mutant in a *pilT* background, the adherence pattern was diffuse rather than the localized pattern seen for wild-type strains. The diffuse adherence pattern correlated with the non-aggregating phenotype exhibited by these strains when grown or suspended in liquid culture (Table 1).

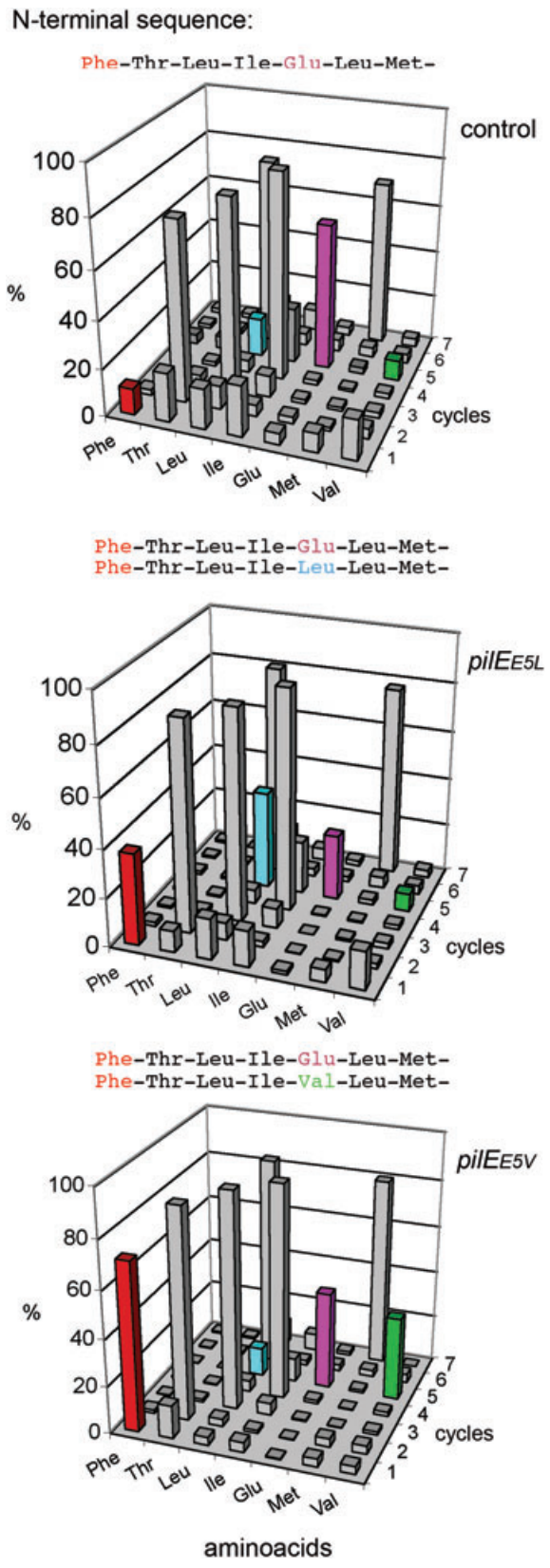

Fig. 9. Analysis of *pilE* heterodiploids by N-terminal amino acid sequencing. The N-terminal amino acid sequence was determined for the following strains: (upper panel) $pilT_{ind}$, *iga::pilE* (GE45), (middle panel) $pilT_{ind}$, $iga::pilE_{E5L}$ (GE47), and (lower panel) $pilT_{ind}$, $iga::pilE_{E5V}$ (GE48). The bars indicate the percentage (*y*-axis) of each amino acid (*x*-axis) from total recovered protein where the background has been subtracted. Only the amino acids from the first seven cycles (*z*-axis) are shown. The amino acids of interest are shown in colour; phenylalanine in red, leucine in blue, glutamic acid in magenta and valine in green.

Discussion

The influence of conserved residues in PilE pilin subunit on *N. gonorrhoeae* Tfp expression and associated phenotypes was evaluated through site-directed mutagenesis of *pilE*. We focused first on residues 38–40 in α1-C with the goal of investigating their potential influence on PilE stability. Single substitutions here were well tolerated with regard to Tfp expression and associated phenotypes (Table 1) with the exception of the $L_{39}A$ substitution that led to a loss of autoagglutination although Tfp levels were not reduced. The basis for Tfp-mediated autoagglutination is poorly understood but is proposed to involve pilus–pilus interactions mediating lateral aggregation (or bundling) of pili between cells (Craig *et al.*, 2004). The results here indicate that structural information in α1-C contributes to this phenotype. Combinations of the L_{39} substitution with those at its flanking sites differentially reduced Tfp expression. Although Tfp levels were increased for all these mutants in a *pilT* background, the effects were quite modest for the L_{38}-L_{39} and $AAM_{38–40}$ mutants. Thus the Tfp expression defect seen in the latter mutants do not appear to reflect altered Tfp extrusion/retraction dynamics but rather an intrinsic but incomplete defect in assembly or polymerization. In contrast, a mutant in which residues 37–43 of PilE were swapped with the corresponding region of *P. aeruginosa* PilA resulted in an absolute defect in Tfp expression. This result is remarkable because intact $PilA^{PAK}$ is assembled in *N. gonorrhoeae* (H.C. Winther-Larsen *et al.*, unpublished data) and because the spacing of flanking conserved residues in both $PilA^{PAK}$ and PilE is not disrupted by these changes. These results are reminiscent of those found for chimeras in which the first two-thirds of the α1-N domain of Tfp pilin subunits and the corresponding region of the PulG pseudopilin were exchanged with varying effects on organelle assembly and functionality (Kohler *et al.*, 2004). We assume as did Kohler and colleagues that these alterations disrupt structural compatibility between α1-N and other domains required for self-complementarity in polymerization or interaction with other biogenesis components.

Attempts to understand the potential influence of stereotypic PilE degradation on Tfp phenotypes were confounded by the fact that the only mutant for which S-pilin could not be readily detected was also severely perturbed

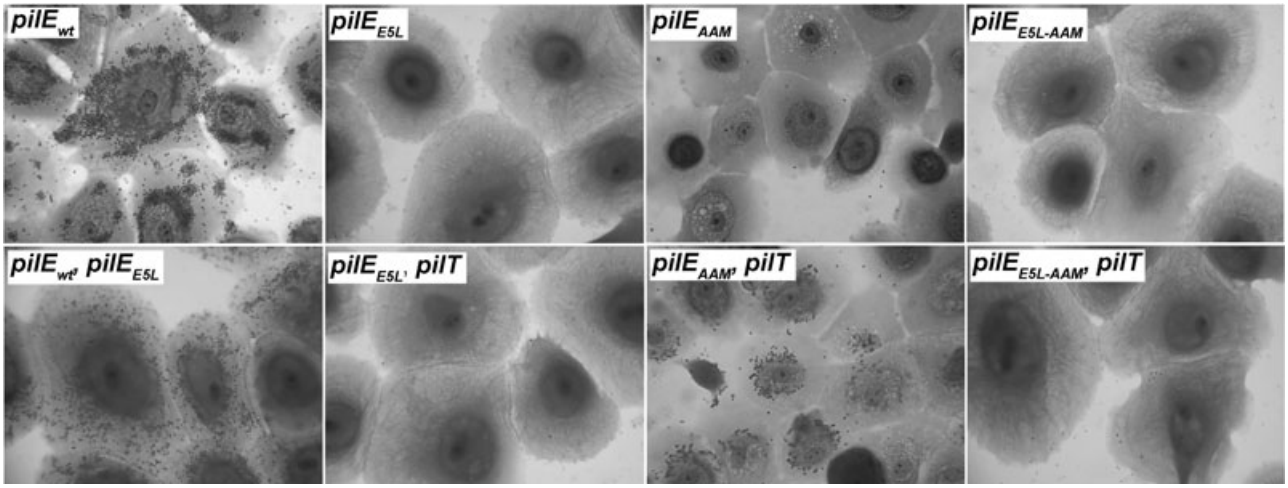

Fig. 10. The ability of pilin mutants to adhere to human corneal epithelial cells correlates with their ability to assemble pili. Adherent cells are stained with crystal violet. Strains: *pilE$_{wt}$* (GE2), *pilE$_{E5L}$* (GE4), *pilE$_{AAM}$* (GE13), *pilE$_{E5L-AAM}$* (GE18), *pilE$_{wt}$*, *pilE$_{E5L}$* (GE23), *pilE$_{E5L}$*, *pilT* (GE32), *pilE$_{AAM}$*, *pilT* (GE37), *pilE$_{E5L-AAM}$*, *pilT* (GE42). All panels are shown at the same level of magnification.

in Tfp expression. As an alternate approach, we have constructed *N. gonorrhoeae* mutants lacking orthologues of many of the well-characterized periplasmic proteases found in other species but found no alterations in PilE degradation in those backgrounds (data not shown). It is of interest to note here that mutants expressing the *pilE*$_{AAM38–40}$ allele and carrying loss-of-function mutations in canonical biogenesis components did not exhibit growth defects or other distinct phenotypes when compared with analogous mutants expressing wild-type *pilE*. As such, accumulation of intact PilE in the absence of Tfp expression was not overtly detrimental to the bacteria. We went on then to examine the potential influence of the AAM$_{38–40}$ substitutions (and its associated defect in S-pilin processing) on Tfp phenotypes when combined with mutations precluding Tfp assembly. However, the combination of these substitutions with mutations in either ancillary biogenesis components or loss-of-function missense mutations in *pilE* failed to correct the defects in Tfp expression and associated phenotypes. This was even true when transformation competence, which is a sensitive, surrogate marker for Tfp expression, was measured. Thus with the appropriate caveats, increased PilE turnover manifest as S-pilin production does not appear to contribute significantly to Tfp assembly defects or dynamics. This finding is consistent with the model that this form of stereotypic PilE degradation is a consequence of subunits being driven 'off pathway' using the nomenclature established in the P pilus system (Jones *et al*., 1997).

Studies here of substitutions at E_5, the sole charged residue within the first 25 residues of α1 and the most conserved in Tfp pilins and related molecules, were particularly insightful. In combination with structural information and fibre modelling, the prevailing dogma is that electrostatic attractive forces mediated through a salt bridge between E_5 ε-oxygens and the N-terminal amine of F_1 in an adjacent monomer promote helical contacts/bundling within the membrane crucial to assembly (Craig *et al*., 2004). This view has been predicated on observations reported for *P. aeruginosa* in which a PilA^{PAK} E_5K mutant pilin, which, while defective for assembly on its own, could be incorporated into composite fibres when coexpressed with wild-type PilA^{PAO} pilin (Pasloske *et al*., 1989). It is frequently overlooked, however, that this observation was made in a genetically uncharacterized, hyperpiliated background and that when these two pilins were coexpressed in a wild-type background, it was stated that the 'majority of cells were unpiliated, although some cells (~5%) produced one or two very short pili' (Pasloske *et al*., 1989). Our study clearly demonstrates that E_5 substitutions exert dominant-negative effects on Tfp expression in a wild-type background but are recessive in a *pilT* background. This study here differs primarily from that of Pasloske and colleagues in that here (i) pilins differing only at the E_5 residue were used, (ii) the pilin genes were expressed at identical levels (versus the relative overexpression of the mutant allele in the *P. aeruginosa* work) and (iii) a genetically defined *pilT* mutant background was employed. Furthermore, we found that the E_5V allele exerted less interference than the E_5L allele. To our knowledge, this is the first time in either the Tfp or secreton pseudopilus systems that different substitutions at E_5 have distinguishing organelle-related phenotypes. With deference to previous work, we are unable, however, to show that the mutant pilins are incorporated into a composite polymer with wild-type pilin in a *pilT* background,

although they are clearly abundant in purified Tfp. It is also important to note two other discrepancies between observations made here and in *P. aeruginosa*. First, in the $PilA^{PAK/PAO}$ work the composite pili had a wavy, braided appearance while we found no evidence for altered Tfp morphology in PilE E_5 heterodiploids (data not shown). We surmise therefore that the aberrant appearance of composite Tfp in *P. aeruginosa* is attributable primarily to the presence of pilins with structurally differing globular head domains rather than the E_5 substitution. Second, the ratio of mutant pilin to wild-type pilin in composite pili was reported to be 3:1 in the *P. aeruginosa* studies whereas here it was closer to 1:1. More precise determinations of the relative stoichiometries of mutant and wild-type pilin in composite Tfp should be critical to understanding how the E_5 residue might contribute to, or influence, subunit registration and assembly. Whatever the case, these data together with that for other pilins exerting transdominance in a PilT-dependent fashion demonstrate for the first time the influence of pilin subunit composition on Tfp dynamics.

In addition to the effects seen on steady state Tfp levels, some PilE α1 domain missense mutants also displayed strong dominant-negative effects on transformability when wild-type PilE expression was induced *de novo* but not when wild-type PilE was expressed constitutively from the endogenous promoter. This latter finding is not surprising given that very low levels of assembly proficient PilE (together with an intact Tfp biogenesis pathway) are sufficient for high levels of transformability (Rudel *et al*., 1995; Aas *et al*., 2002b; Long *et al*., 2003). Many factors could account for the findings when wild-type PilE expression was induced at the time of DNA addition. Based on the manner in which the inducible background was used, wild-type PilE is entering into a pool of pre-existing mutant pilin and levels of PilE expression achieved from the inducible construct are two- to threefold lower than that from the mutant (but wild-type promoter) allele. Thus, under these conditions, wild-type PilE may not traffic properly to its site of action or may undergo non-productive interactions with mutant PilE that lead to PilT-mediated disassembly of a PilE heteropolymer (see below). The finding that E_5L allele simultaneously exerts greater dominant negativity both on transformability and steady state Tfp levels than does the E_5V allele favours the latter hypothesis. Likewise, the former hypothesis is somewhat incompatible with the finding that combining the AAM_{38-40} substitutions with the E_5 substitutions renders mutant PilE more resistant to S-pilin processing (and thus perhaps more likely to occupy a PilE trafficking pathway) but at the same time less interfering in transformation. However, in the case where the E_5V substitution is combined with the AAM_{38-40} substitutions, the heterodiploid strain becomes more transformable while having reduced steady state Tfp levels when compared with the heterodiploid strain expressing the mutant PilE with only the E_5V substitution. While the results strongly argue that PilE multimerization is an integral part of the DNA binding and uptake machinery, more studies are required to rule out formally the possibility that the dominant effects of mutant PilE involve perturbed interactions between wild-type PilE and other components such as ComP (Aas *et al*., 2002a,b) or any one of the other PilH-L pilin-like molecules required for transformability (Winther-Larsen *et al*., 2005).

One surprising finding of this work was the epithelial cell adherence capability of the $PilE_{wt}/PilE_{E5L}$ heterodiploid strain despite it having very low steady state levels of Tfp. Although it is possible that this property has been dissociated from Tfp expression in this background, a more likely scenario is that Tfp are still displayed at the cell surface but have a shorter half-life outside the cell with the frequency of retraction events being increased. Thus, an extended pilus would still be able to bind to an epithelial cell receptor. Alternately, the dynamics of Tfp expression in this background might be influenced differentially by the conditions experienced during the adherence assay versus those encountered during preparation for Tfp purification and IFM. By way of example, Bradley noted during his studies of *P. aeruginosa* retractile pili that growth conditions and treatment with pilus ligands such as phage and antibodies could have profound effects on the percentage of piliated cells detected and average number of pili seen per bacterium (Bradley, 1972a,b,c). Further work is needed before clear conclusions can be drawn in the *N. gonorrhoeae* system.

Steady state levels of Tfp are in part dictated by the relative frequencies of extension event initiation versus the frequencies with which such events are terminated by retraction and disassembly. E_5 mutant pilins therefore impact on Tfp dynamics by either inhibiting extension events or promoting retraction events. However, as E_5 pilin transdominance is PilT-dependent and mutant subunits efficiently enter the purifiable Tfp fraction in a *pilT* background, it appears that the presence of mixed multimers favours the activity of PilT over that of PilF at the site of assembly. This finding is in line with earlier works indicating that PilT-driven retraction/disassembly has aspects of a quality control mechanism that senses when the integrity or stability of the extension complex is compromised (Wolfgang *et al*., 1998a; 2000; Winther-Larsen *et al*., 2005). It is possible that transdominance results from the mutant pilin titrating out or otherwise failing to interact properly with effectors of Tfp homeostasis (such as PilC1/2, PilH-L and PilQ) that are defined by their requirement for Tfp expression only when PilT is active (Wolfgang *et al*., 1998a; 2000; Winther-Larsen

et al., 2005). However, E_5 mutant pilins are clearly export-compatible and assembly-proficient in the presence of the wild-type subunit and presumably must make contacts with the wild-type subunit to be incorporated into Tfp. This type of intermolecular complementation is not limited to just the E_5 mutants as this behaviour has also been seen with other PilE missense mutants incapable of self-assembly (M. Aspholm *et al*., unpublished data). We favour the hypothesis that the trigger that shifts equilibrium dynamics towards retraction (and PilT activity or occupancy/residency at the base of the pilus) is mixed multimers and concomitant, aberrant contacts between wild type and mutant pilin. This model further suggests that PilT (and possibly PilF) might make direct contact with the assembling subunits although pilin multimer status could also be sensed or transduced through other membrane constituents such as the assembly component PilG (Tonjum *et al*., 1995). Further studies of these types of mutants will likely have significant implications for modelling of pilin assembly and organelle dynamics. Additionally, composite pili are likely to be useful tools for probing Tfp biology, organelle composition and architecture, as well as elucidating the molecular bases for associated functions.

Experimental procedures

Strains, plasmids and mutants

The bacterial strains used in this study are described in Table 1. *Escherichia coli* and gonococcal strains were grown as described (Freitag *et al*., 1995). pPilE2 is a derivative of p2/16/1 that carries the entire *pilE* ORF and 5′ promoter sequences linked to the *erm*C gene (Wolfgang *et al*., 2000). pUP6 is a derivative of pHSS6 (Seifert *et al*., 1990) that carries two gonococcal DNA uptake sequences (Wolfgang *et al*., 2000). To express the $pilE_{W109S}$ allele ectopically from the *iga* locus the Bsu36I-BglI fragment from a pPilE derivative carrying the $pilE_{W109S}$ allele (Park *et al*., 2001) was cloned into pPilE2 digested with Bsu36I-BglI.

Ectopic expression of altered pilE *alleles*

pilE mutagenesis was performed by PCR-based splicing-by-overlap extension, and altered alleles were cloned into plasmid pPilE2 such that they could be expressed ectopically from the *iga* locus. Each pair of overlapping PCR fragments containing the *pilE* mutation(s) was spliced together by using the flanking primers PilE5′Sac (5′-CTA**GAGCTC**AAATTCCGACCCAATCAACACAC-3′) and PilE3′Sac (5′-GTC**GAGCTC**ATCGATATATTATTTCCACCGG-3′) (SacI sites in bold) and the Bsu36I-BglI fragments from these were cloned into pPilE2 digested with Bsu36I-BglI.

To generate the $L_{38}A$, $L_{39}A$, $A_{40}M$, $L_{38}A$-$L_{39}A$, $L_{38}A$-$A_{40}M$ and the $L_{39}A$-$A_{40}M$ mutations, the following primers pilE-38A-5′ (5′-CAAGTTTCCGAAGCCATC**GCC**TTGGCCGAAGGTCAAAAATC-3′), pilE-39A-5′ (5′-CAAGTTTCCGAAGCCATCCTT**GCG**GCCGAAGGTCAAAAATC-3′), pilE-40M-5′ (5′-CAAGTTTCCGAAGCCATCCTTTTG**ATG**GAAGGTCAAAAATCAG-3′), pilE-38A39A-5′ (5′-CAAGTTTCCGAAGCCATC**GCCG CG**GCCGAAGGTCAAAAATC-3′), pilE-38A40M-5′ (5′-CAAGTTTCCGAAGCCATC**GCC**TTG**ATG**GAAGGTCAAAAATCAG-3′) and pilE-39A40M-5′ (5′-CAAGTTTCCGAAGCCATCTT**GCGATG**GAAGGTCAAAAATCAG-3′) (altered codons in bold) respectively, in combination with the primer PilE3′Sac. The second overlapping fragment was made by using the primer PilE5′Sac in combination with primer pilE-oli132-108-3′ (5′-GATGGCTTCGGAAACTTGCGCGCGG-3′).

To generate the $AAM_{38–40}$ mutation, the primer PilE5′Sac was used in combination with primer pilE-AAM-3′ (5′-GACGGCTGATTTTTGACCTTC**CATCGCGGC**GATGGCTTCGGAAACTTGCG-3′) (altered codons in bold). The primer pilE-oli108-132-5′ (5′-CCGCGCGCAAGTTTCCGAAGCCATC-3′) in combination with the primer PilE3′Sac was used to make the second overlapping PCR fragment.

To change residues 37–43 of PilE into the corresponding residues of *P. aeruginosa* PilA, the primer PilA/E-43–37-5′ (5′-**CTTGCTAGCGTCAATCCGTTG**AAATCAGCCGTCACCGAG-3′) (altered codons in bold) in combination with the primer PilE3′Sac, and the primer PilE5′Sac in combination with primer 3′-PilA/E (43–37) (5′-**CAACGGATTGACGCTAGCAAG**GGCTTCGGAAACTTGCGC-3′) (altered codons in bold) were used.

A stop codon at position 109 in the mature protein was created by using the primer pilE-W109Stop-5′ (5′-GGCAAAAAACTCTCCCTGTG**A**GGCAGGCGTGAAAACGGTTC-3′) (alteration in bold) in combination with the primer PilE3′Sac. The second overlapping PCR fragment was generated by primers PilE5′Sac and pilE-oli345-328-3′ (5′-CAGGGAGAGTTTTTTGCC-3′).

Fragments containing the E_5L-$AAM_{38–40}$ and the E_5V-$AAM_{38–40}$ mutations were generated by PCR from plasmid pPilE2-AAM (the pPilE2 derivative containing the $AAM_{38–40}$ mutation) by using the primers E_5L (5′-CAAAAAGGCTTTACCCTTATC**CTG**CTGATGATTGTG-3′) and E_5V (5′-CAAAAAGGCTTTACCCTTATC**GTG**CTGATGATTGTG-3′) (altered codons in bold) respectively, in combination with the primer PilE3′Sac. Overlapping fragments were generated using the primers PilE5′Sac and pilE-oli8-33-3′ (5′-GATAAGGGTAAAGCCTTTTTGAAGGG-3′).

A fragment containing the W_{109}S-$AAM_{38–40}$ mutations were generated by PCR from plasmid pPilE2-W_{109}S (the pPilE2 derivative containing the W_{109}S missense substitution) by using the primer PilE5′Sac in combination with the primer pilE-AAM-3′. The second overlapping PCR fragment was generated by using the primers, pilE-oli108-132-5′ and PilE3′Sac.

Modified alleles were introduced into the *iga* locus of wild-type strains and strains in which endogenous *pilE* expression was placed under the control of an inducible promoter using transformation with the pPilE2 derivatives and selection on Gc agar plates containing 8 μg ml^{-1} erythromycin. Direct DNA sequencing of PCR products derived from the gonococcal transformants was done using custom primers at GATC Biotech AG (Konstanz, Germany) to insure the correct introduction of the alleles and the absence of any other alterations.

Construction of pilD *and* pilF *null alleles*

The *pilD* allele was amplified by PCR using primers pilD5′ (5′-GCGGGATCCTCACGGATTCGAGCAAG-3′) and pilD3′ (5′-CCGAATTCTACGAAGACGAGGTGGAC-3′), which are designed with a BamHI and an EcoRI restriction sites respectively (underlined). The PCR product was digested with EcoRI-BamHI and cloned into pUP6 digested with EcoRI-BamHI. The HindIII fragment of plasmid pCM7 (Close and Rodriguez, 1982), containing a chloramphenicol acetyltransferase gene cassette, was treated with Klenow fragment to create blunt ends and then cloned into a DraI site of the *pilD* clone in pUP6. Similarly, the blunted HindIII fragment of plasmid pCM7 was cloned into a blunted EcoRI site of the *pilF* gene situated in plasmid p12/7/1 (Lauer *et al*., 1993). The loss-of-function constructs were introduced into strains GE2 and GE13 (see Table 1) by using transformation with the resulting pUP6 and p12/7/1 derivatives and selection on Gc agar plates containing 10 μg ml^{-1} chloramphenicol.

Transformation assays

The transformation assays were carried using 0.5 μg of plasmid pSY6 DNA (Stein *et al*., 1991) mixed with 0.5 ml of cells (5×10^7 ml^{-1}), supplemented with 7 mM $MgCl_2$, and incubated for 30 min (37°C, 5% CO_2). After the incubation the samples were diluted 10× in Gc broth and grown for 3 h before appropriate dilutions were plated onto agar medium with and without 1 μg ml^{-1} of nalidixic acid. In the assays where *pilE* expression was induced this was performed by adding IPTG (250 μM final concentration) at the same time as the transforming DNA.

Tfp purification and quantification, immunofluorescence, electron microscopy, SDS-PAGE and immunoblotting

Pilus purification was carried out as described (Wolfgang *et al*., 1998b). Relative quantification was achieved by comparison of the PilE band intensity of the mutants to that seen for serial dilutions of Tfp preparations from the wild-type strain after samples were first standardized based on amounts of whole cells from which the preparations were made (Winther-Larsen *et al*., 2001). Procedures for immunofluorescence (Winther-Larsen *et al*., 2005), SDS-PAGE, Coomassie staining and immunoblotting have been described previously (Freitag *et al*., 1995). TEM was performed as previously described (Park *et al*., 2001), with the exceptions that formvar-coated grids was used and that cells were stained with uranylacetate. Immunogoldlabelling was carried out as described (Wolfgang *et al*., 2000) except that incubation with antibodies was reduced from 45 min to 10 min and cells were labelled with 10 nm gold particles. PilE and S-pilin was detected by immunoblotting of whole cell lysates using rabbit polyclonal antibodies, generated against a synthetic peptide (KSAVTEYYLNHGKWPENNTSA) corresponding to the amino acid residues 44–64 of the mature PilE protein (Research Genetics, Huntsville, AL), and alkaline phosphatase coupled goat anti-rabbit antibodies (Tago). The PilE-specific sera (2–66), used to identify Tfp in the EM and IFM experiments, have been described previously (Drake and Koomey, 1995).

PilE N-terminal sequencing

Purified pili were separated on a 15% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane and stained in a Ponceau S solution (0.1% Ponceau S diluted in 10% acetic acid and 45% methanol in water). The 18 kDa PilE protein band was cut out and the N-terminal amino acid sequence was determined by direct Edman degradation performed on an automatic protein sequencer (Model 477 A from Applied Biosystems or Model G 1000 A from Hewlett Packard Bioscience Products, Palo Alto, CA, USA).

Epithelial cell bacterial adherence

Primary cultures of human corneal epithelial cells were established (van Putten and Paul, 1995) and the adherence assays of gonococcal strains to the human corneal epithelial cells were performed as described (Winther-Larsen *et al*., 2001).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Comparison of the crystal structure of a PilE monomer from *N. gonorrhoeae* strain MS11 (pdb code 2PIL) with the known structure of the type IVa pilin PilA from *P. aeruginosa* strain PAK (pdb code 1OQW).

Fig. S2. Piliation defect in the AAM_{38-40} pilin mutant analysed by immunofluorescence microscopy.

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