JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1323–1331
0021-9193/94/$04.00+0

Vol. 176, No. 5

Type IV Prepilin Peptidase Gene of *Neisseria gonorrhoeae* MS11: Presence of a Related Gene in Other Piliated and Nonpiliated *Neisseria* Strains

BRUNO DUPUY[1]* AND ANTHONY P. PUGSLEY[2]

Unité des Neisseria,[1] and Unité de Génétique Moléculaire, [2] Centre National de la Recherche Scientifique URA 1149, Institut Pasteur, 75724 Paris Cedex 15, France

Received 20 September 1993/Accepted 17 December 1993

The assembly of type IV pili in *Neisseria gonorrhoeae* is a complex process likely to require the products of many genes. One of these is the enzyme prepilin peptidase, which cleaves and then N methylates the precursor pilin subunits prior to their assembly into pili. We have used a PCR amplification strategy to clone the *N. gonorrhoeae* prepilin peptidase gene, $pilD_{Ng}$. A single copy of the gene is shown to be present in the chromosome. Its product promotes correct cleavage of the gonococcal prepilin in *Escherichia coli* cells carrying both the prepilin peptidase gene and the pilin structural gene. $PilD_{Ng}$ also cleaves prePulG, a type IV pilin-like protein of *Klebsiella oxytoca*. Moreover, $PilD_{Ng}$ complements a mutation in the gene coding for the prepilin peptidase-like protein of *K. oxytoca*, *pulO*, partially restoring PulG-PulO-dependent extracellular secretion of the enzyme pullulanase. Finally, we show that genes homologous to $pilD_{Ng}$ are present and expressed in a variety of species in the genus *Neisseria*, including some commensal strains.

Pili are important virulence factors for certain types of human bacterial pathogens, such as *Pseudomonas aeruginosa* (9, 67), *Vibrio cholerae* (64), and *Neisseria gonorrhoeae* (43, 61). These three species all produce type IV pili composed of a single major protein subunit (pilin). These are produced as precursors which are processed at a highly conserved consensus cleavage site [G ↓ F(M)TLXE] located close to the amino terminus (12, 55, 58). Characteristically, the mature pilins have an N-methylated amino-terminal residue (usually phenylalanine or methionine) and retain a long continuous segment of hydrophobic amino acids close to their N terminus which is essential for their polymerization (19, 53, 58).

The assembly of type IV pili has been most extensively studied in *P. aeruginosa*, in which four assembly proteins (PilB, PilC, and PilD [37] and PilQ [29]) have been identified, and in *V. cholerae*, in which at least seven assembly proteins (TcpB, -C, -D, -E, -F, -G, and -J [23, 41, 44]) have been identified. Although the roles of most of these proteins remain obscure, PilD from *P. aeruginosa* ($PilD_{Pa}$) and TcpJ are known to correspond to the prepilin peptidase, which cleaves the pilin precursors (22, 38), and PilD has additionally been shown to N methylate the processed product (60). Intriguingly, several bacteria which are not known to have type IV pili also produce a protein with significant sequence homology to known prepilin peptidases and which is required for extracellular secretion (45). One of these proteins, PulO of *Klebsiella oxytoca*, can correctly cleave and N methylate the precursor of gonococcal type IV pilin (11) as well as the precursor of a type IV pilin-like protein (PulG) of *K. oxytoca* (46, 47). PulG is one of four type IV pilin-like proteins that are required, together with PulO, for extracellular secretion of pullulanase. A similar set of proteins exists in several other gram-negative bacteria (45), notably in *P. aeruginosa* (2, 3, 39, 40), in which the same prepilin peptidase, PilD (also called XcpA [2]) is required for both pilus assembly and protein secretion (3, 59).

* Corresponding author. Present address: Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: (1) 45 68 84 49. Fax: (1) 45 68 89 53.

Relatively little is known about the assembly of type IV pili in *N. gonorrhoeae*. Expression of the *N. gonorrhoeae*, *Dichelobacter nodosus*, or *Moraxella bovis* type IV pilin genes in *P. aeruginosa* results in their assembly into pili (13, 20, 31), suggesting that the mechanism of type IV pilus assembly is basically the same in all of these bacteria. One of the major difficulties encountered in attempts to use genetic techniques to identify factors necessary for pilus formation in *N. gonorrhoeae* is the high frequency of pilus phase variation caused by genome rearrangements (33). However, Jonsson et al. (21) have identified a gene (*N. gonorrhoeae pilC* [$pilC_{Ng}$]) which seems to be required for piliation, although this view has recently been challenged by Rudel et al. (51), who demonstrated that $PilC_{Ng}$ might be required for pilus-mediated adherence rather than for pilus assembly.

One of the aspects of *N. gonorrhoeae* piliation which seems to be readily amenable to genetic analysis is the processing of the pilin precursor. All type IV pilins and type IV pilin-like proteins have a glycine residue immediately upstream of the known or presumed cleavage site (45). Koomey et al. (25) have shown that substitution of this glycine residue in gonococcal prepilin abolishes processing and piliation. Substitution of the corresponding glycine in *P. aeruginosa* type IV prepilin also abolishes processing and piliation (58), while a similar change in the sequence of prePulG prevents processing and pullulanase secretion (46). We have shown that the prepilin peptidases of *K. oxytoca* and *P. aeruginosa* are both able to cleave gonococcal prepilin (11). These observations strengthen the idea that processing and N methylation of type IV pilins and related proteins occur by conserved mechanisms in widely different bacteria. We therefore attempted to identify and clone the gene coding for the prepilin peptidase of the well-characterized MS11 strain of *N. gonorrhoeae* and to determine whether it codes for an active enzyme. The results from this first part of the present study confirm and extend those of Lauer et al. (27), which were published while this paper was in preparation. We then extended our study to determine whether other pathogenic and commensal species of

Neisseria also possess and express the prepilin peptidase structural gene.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The *N. gonorrhoeae* strain used for most experiments was MS11 (*pilE1*$^+$ *pilE2*$^+$) and was described by Meyer et al. (32). The other *Neisseria* strains used in this study were obtained from the collection of J.-Y. Riou of the Institut Pasteur. Cultures were passaged every 18 to 22 h on G medium plates with G supplements (Sanofi Diagnostics Pasteur, Toulouse, France). The *Escherichia coli* K-12 strain used as the recipient of recombinant plasmids was DH5 (17). *E. coli* K-12 strain PAP7232 carries the entire *pul* gene cluster (including *pulO*) integrated into its chromosome (46). *E. coli* PAP7245 is identical to PAP7172 except that it carries the Δ*pulO1* mutation (48) instead of the wild-type *pulO* allele in the chromosome. Luria broth (35) was used with appropriate antibiotics for most experiments. Maltose (0.4%) and IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) were added when appropriate to induce the expression of maltose (*pulA* and *pulC-O*)- or lactose (*lacZ*)-regulated promoters.

The plasmids used were pNG300, a derivative of pGB2 (7) carrying the *pilE* gene; pJE4 (2); and pCHAP155, which is a pHSG575 derivative carrying the *pulO* gene under *lacZp* control (47).

Recombinant DNA techniques. All recombinant DNA protocols and other DNA manipulations were performed by standard procedures according to the methods of Maniatis et al. (28). pPNG100 and pPNG200 carry a 2.6-kb *Sau*3A fragment and a 0.9-kb *Sau*3A fragment, respectively, cloned into *Bam*HI-digested and dephosphorylated *E. coli* plasmid vector pBR322. pPNG101 carries a 1.6-kb *Hinc*II-*Sph*I fragment from pPNG100 cloned into the *Hinc*II-*Sph*I sites of pUC19. pPNG104 carries a 2.8-kb *Hin*dIII-*Sph*I fragment from pPNG100 cloned into the *Hin*dIII-*Sph*I sites of pUC19. pPNG107 was derived from pPNG104 by deletion of a 0.5-kb internal *Cla*I fragment of $pilD_{Ng}$ (Fig. 1).

Construction of *N. gonorrhoeae* MS11 gene bank and screening of the DNA library for *pilD*-related sequences. DNA was isolated from *N. gonorrhoeae* as described by Segal et al. (54). Total genomic DNA was digested with *Sau*3A and cloned into *Bam*HI-digested and desphosphorylated *E. coli* plasmid vector pBR322. Recombinant *E. coli* clones were plated on Luria agar containing ampicillin. The resulting colonies were lifted off onto nitrocellulose filters (Hybond-C; Amersham) and then lysed. The DNA was denatured with NaOH before it was probed. Colony hybridization was carried out as described by Grunstein and Hogness (16). The probe was prepared by nick translation as recommended by the manufacturer (Nick Translation Kit; Amersham) with [α-^{32}-P]dCTP (3,000 Ci/mol; Amersham). The nitrocellulose filters were prehybridized for 2 h at 65°C and then hybridized overnight at 65°C with the probe. After hybridization and washing, the filters were autoradiographed with Kodak X-Omat film.

Southern hybridization. Chromosomal or plasmid DNA was digested to completion with appropriate restriction endonucleases and electrophoresed on 0.8% agarose gels. The DNA was transferred from the gels onto Hybond-N filters (Amersham) in 20× saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) essentially according to the method of Southern (56). Filters were prehybridized for 2 h at 65°C in 5× SSC standard–2× Denhart's solution–0.1% sodium dodecyl sulfate (SDS)–100 mg of denatured salmon sperm DNA per ml. Overnight hybridization was carried out in the same solution at 65°C with a nick-translated internal *pilD* fragment. The filters were washed 3 times in 0.1× SSC–0.1% SDS for 10 min per wash at 65°C before they were air dried and exposed to Kodak X-Omat film.

DNA sequence analysis. DNA sequencing was performed by the dideoxy chain termination method (52) with the Sequenase 2.0 kit (United States Biochemical Corp.). The complete sequence of both strands of $pilD_{Ng}$ was determined by using subcloned fragments and appropriate deletions of pPNG100 with M13 universal primer and additional, synthetic primers specific for vector (pBR322 or pUC19) or $pilD_{Ng}$ DNA. To sequence double-stranded DNA, samples (2 mg) of purified plasmid DNA were first denatured with a solution of 2 M NaOH and then neutralized with 2 M ammonium acetate (pH 4.5). The DNA was precipitated by addition of 100% ice-cold ethanol and washed in 70% ice-cold ethanol. The pellet was resuspended in 10 ml of sequencing buffer containing 0.5 pmol of the primer and then incubated for 3 min at 65°C. Samples were incubated at room temperature for 30 min prior to being sequenced. To sequence PCR products, DNA fragments were isolated by electroelution from agarose gels and recovered from the migration buffer by using an Elutip-d minicolumn (Schleicher and Schuell, Dassel, Germany). The DNA was precipitated overnight following addition of 100% ice-cold ethanol, dried, and resuspended in 20 μl of water. Six milliliters of the DNA preparation was used for one sequence reaction with 30 pmol of the primer. The samples were incubated for 3 min at 100°C and then immediately put on ice.

RNA dot blot experiments. Total RNA was isolated from *N. gonorrhoeae* cells as described by Taha et al. (63), and RNA dot blot analysis was performed as described by Taha and Marchal (62). The hybridization of *Neisseria* RNA was performed with the nick-translated probes corresponding to the *pilD* fragment amplified from each species of *Neisseria* tested.

PCR. PCRs were performed by using a Techne thermal cycler (Techne Corporation) for 35 cycles of denaturation (92°C, 1 min), annealing (37°C, 1 min), and elongation (72°C, 1 min). The primers used to amplify the DNA were a 21-bp degenerate oligomer, O21 (5′-IAITTTIAAITCICCITAICC-3′), and a 22-bp degenerate oligomer, O22 (5′-CAICGICT GCCGIIATGATGGA-3′), corresponding to highly conserved segments in the sequences of known type IV prepilin peptidase genes (*xcpA* and *pulO*; see Results and references 2 and 49 for details). The amplified DNA products were separated by electrophoresis on a 1% agarose gel in Tris-acetate-EDTA buffer.

Immunoblot analysis. Proteins were separated on gels containing 11.25% acrylamide, 0.3% bisacrylamide, and 8M urea as previously described (47). Electrophoresis was performed at 0.6 mA for 15 h. Samples for electrophoresis were prepared by precipitating proteins from 1.5 ml of cultures at an optical density at 600 nm of 1.0 to 1.2 in 1 ml with 10% trichloroacetic acid solution on ice. After centrifugation, the pellets were washed in 80% acetone, resuspended in 0.1 ml of 2.5% SDS–12.5% glycerol–10 mM dithiothreitol in 100 mM Tris HCl (pH 8), and heated at 100°C for 5 min before loading onto the gel. Proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electroblotted onto nitrocellulose (Hybond-C). Proteins on the filters were detected by using anti-PilE or anti-PulG serum and ^{32}S-protein A (Amersham).

Pullulanase assays. Pullulanase was assayed essentially as described by Michaelis et al. (34) except that the cells were lysed with 0.5% octylpolyoxylethylene in order to assay total enzyme activity. The proportion of the total activity (lysed cells) that could be detected in whole cells was taken as a measure of secretion efficiency.

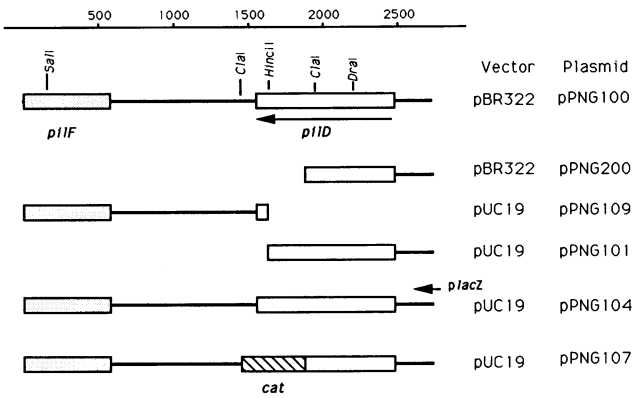

FIG. 1. Physical maps of pPNG100 and pPNG200 and organization of plasmids used in this study. The locations of cleavage sites are based on restriction digestion data obtained by agarose gel electrophoresis. pPNG100 and pPNG200 are *Sau*3A fragments cloned into *Bam*HI-digested and desphosphorylated *E. coli* plasmid vector pBR322. pPNG104 carries the DNA insert of pPNG100 cloned into *E. coli* plasmid vector pUC19 with $pilD_{Ng}$ in the same orientation as the *lacZ* promoter. pPNG101 and pPNG109 are subclones derived from pPNG100 and pPNG104, respectively, cloned in pUC19. pPNG107 carries a *cat* gene in place of the internal *Cla*I fragment of $pilD_{Ng}$ in pPNG104. Open boxes represent the *pilD* structural gene, and shaded boxes indicate the 3′ end of the *pilF* gene. The hatched box represents the *cat* gene, and p*lacZ* indicates the orientation of the *lacZ* promoter.

RESULTS

Cloning of the *N. gonorrhoeae* prepilin peptidase gene *pilD*. Initially, we attempted to identify and clone fragments from an *N. gonorrhoeae* chromosome gene bank which hybridized with probes containing sequences for the *K. oxytoca pulO* gene and from $pilD_{Pa}$ (11). None of the DNA fragments thus identified encoded an active prepilin peptidase, and a fragment which hybridized with both probes did not contain sequences encoding a prepilin peptidase-like polypeptide.

This strategy was therefore abandoned in favor of amplification of the *N. gonorrhoeae pilD* gene by PCR. For this purpose, we synthesized a pair of degenerate oligonucleotides corresponding to highly conserved segments in the sequences of the known type IV prepilin peptidase genes *pulO* (49) and $xcpA/pilD_{Pa}$ (2, 37) (see Materials and Methods). One or two DNA fragments were consistently amplified from *N. gonorrhoeae* chromosomal DNA in each of several experiments. In every case, the size of the major amplified DNA fragment was close to that predicted from the distance between the positions of the sequences corresponding to the two primers in *pulO* and in $xcpA/pilD_{Pa}$ (approximately 500 bp) (not shown). Partial sequencing of the DNA amplified from the *N. gonorrhoeae* chromosome showed that it was similar to *pulO* and $xcpA/pilD_{Pa}$, confirming that a fragment of the $pilD_{Ng}$ gene had been amplified.

The ca. 500-bp amplified DNA fragment was next used as a homologous DNA probe to screen 2,000 clones in the same *N. gonorrhoeae* chromosomal DNA gene bank as that screened with the *pulO* probe (see above). Two clones (pPNG100 and pPNG200) reacted very strongly with the probe. Restriction analysis of the DNA insert in these two plasmids indicated that they contained overlapping DNA fragments (Fig. 1). The restriction maps of the two plasmids are similar to those reported recently by Lauer et al. (27) for the *pilD* gene cloned from another strain of *N. gonorrhoeae* (strain MSO1-1X) and further indicate that pPNG100 also carries the 3′ end of the *pilF* gene.

```
1
CGT GCG ACG GAA CTG TTC CCC ATA TGA TGT TGC AGA TGT CCT CCA TCG GCG AGG AAT CGG
61
GTT CTT TGG ACG ATA TGC TCA ACA AAG CCG CCG AAT TTT ACG AAG ACG AGG TGG ACA ATG

121   -24    PIII     -12                    -10                    -24
CGG TCG GCA GGC TGT CCG CTA TGA TGG AGC CGA TTA TTA TTG TGA TTT TGG GCT TGG TCA

181 PII -12                                                  -24     PI      -12
TCG GTA CGC TTC TGG TCG CTA TGT ATC TGC CGC TGT TCA ACT TGG GCA ACG TGG TCG CCT

241
GAT TTG CCG CAC AGA TCC GGC GCG GAT TGG TTC TGC GCC GGT TTG TTT TTG CTT TGA ATA

301    SD
TAT CAA GGA CAA AAT ATG TCT GAT TTG TCT GTA TTG TCG CCG TTT GCC GTG CCT TTG GCA
                    M   S   D   L   S   V   L   S   P   F   A   V   P   L   A
361
GCA GTT TTG GGG CTG CTG GTC GGC AGC TTC CTG AAT GTC GTC ATT TAC CGC GTA CCC GTT
 A   V   L   G   L   L   V   G   S   F   L   N   V   V   I   Y   R   V   P   V
421                                                  DraI
ATG ATG GAA CGC GGC TGG ACG GTA TTT GCC AAA GAA CAT TTA AAC CTG CCG CTG ACC GAC
 M   M   E   R   G   W   T   V   F   A   K   E   H   L   N   L   P   L   T   D
481
GAT GAA AGC CGT ACC TTC AAC CTG ATG AAG CCG GAT TCC TGC TGT CCC AAA TGC CGT GTG
 D   E   S   R   T   F   N   L   M   K   P   D   S   C   C   P   K   C   R   V
541
CCG ATA CGC GCG TGG CAG AAC ATC CCG ATT GTC AGT TAC CTG CTC CTG CGC GGC AAA TGC
 P   I   R   A   W   Q   N   I   P   I   V   S   Y   L   L   L   R   G   K   C
601
GCT TCC TGC CAA ACC AAA ATC AGC ATA CGT TAT CCC TTA ATC GAG CTG CTG ACC GGC GTA
 A   S   C   Q   T   K   I   S   I   R   Y   P   L   I   E   L   L   T   G   V
661
TTG TTC GGG CTG GTC GCC TGG CAA TAC GGC TGG TCT TGG ATT ACG CTG GGC GGT TTG ATA
 L   F   G   L   V   A   W   Q   Y   G   W   S   W   I   T   L   G   G   L   I
721                                      ClaI
CTG ACC GCG TTT CTG ATT TCC CTG ACC TTT ATC GAT GAG GAC ACC CAA TAC CTG CCC GAC
 L   T   A   F   L   I   S   L   T   F   I   D   E   D   T   Q   Y   L   P   D
781
TCG ATG ACA TTA CCC TTG ATC TGG CTG GGG CTG ATA TTT AAT TTG GAC GGC GGC TTC GTG
 S   M   T   L   P   L   I   W   L   G   L   I   F   N   L   D   G   G   F   V
841
CCT TTG CAG TCT GCC GTT TTA GGT GCG GTT GCC GGC TAT AGT TCA TTA TGG CTC TTA TGT
 P   L   Q   S   A   V   L   G   A   V   A   G   Y   S   S   L   W   L   L   C
901
GCA GTG TAT AAA CTG CTC ACA GGA AAA ACC GGT ATG GGC AAC GGA GAT TTC AAA CTG ATT
 A   V   Y   K   L   L   T   G   K   T   G   M   G   N   G   D   F   K   L   I
961
GCC GCA TTG GGC GCG TGG CTC GGC ATA TCC GCA TTG CCC GTG CTG ATT TTT GTT TCC TCT
 A   A   L   G   A   W   L   G   I   S   A   L   P   V   L   I   F   V   S   S
1021
CTG ATC GGT TTG GTC GCG GCA ATC GTT ATG CGC GTC GCC AAG GGG CGG CAT TTT GCC TTC
 L   I   G   L   V   A   A   I   V   M   R   V   A   K   G   R   H   F   A   F
1081
GGC CCC GCA CTG ACA GTT TCG GGC TGG ATA ATT TTT ACG GCA AAC GAT TCC GTA TGG CGG
 G   P   A   L   T   V   S   G   W   I   I   F   T   A   N   D   S   V   W   R
1141  HincII
GCG GTC AAC TGG TGG CTG ACC CAT CCG GTG AGA TGA CAG CAT GGG TCG GAC TGA CCG GCG
 A   V   N   W   W   L   T   H   P   V   R   *
1201
GAA TCG GCA GCG GCA AAT CGG CAG CCG CGC AAT ATT TTG CCG ATT TGG CGG TGC CGC GCA
 ClaI
TCG ATG CGA CCG GCG GGC CAC TCG CTG ACG GCT TCA GAC GGC ATC GCC TGC CGG AAT CAG
1321
GCG GCT GTT CGG CGA CAC CGT TTT CGA CAC ACA GGG TTT GTT GCG GCC GAC ATA TTG CGT
1381
AAA GAA ATC TTT GCC TCC CCA TCG CGC AAA GCC TTG CTC GAA TCC GTG ATG TTG CCG CTG
1441
ATT TTC TCA GAA TCA AAA ACA GCA AGA AAC CTT TAC CGA TGC GTT T
```

FIG. 2. DNA sequence of $pilD_{Ng}$. The deduced amino acid sequence of the $PilD_{Ng}$ protein is given below the coding sequence. A putative Shine-Dalgarno (SD) sequence is indicated in boldface, and some restriction sites are shown. Sequences similar to the consensus −10 and −24/−12 sequences (65) are indicated in boldface. Potential promoters (−24/−12 type) are labeled PI, PII, and PIII (see text).

DNA sequence of the $pilD_{Ng}$ gene and flanking DNA. DNA fragments obtained by restriction endonuclease digestion of pPNG100 were subcloned and sequenced. The sequenced DNA (Fig. 2) contains a single open reading frame which is almost identical to that reported recently for the *pilD* gene of another *N. gonorrhoeae* strain by Lauer et al. (27). This sequence is preceded by sequences which could correspond to a Shine-Dalgano ribosome binding site (AGGA) and, further upstream, to a potential promoter with the consensus for a Pribnow (−10) box (18) and for three potential promoters with the consensus for a −24/−12 promoter, called PI, PII, and PIII (65) (Fig. 2). The $pilD_{Ng}$ gene is not immediately followed by a sequence which might form a stem-loop structure typical of a transcription terminator.

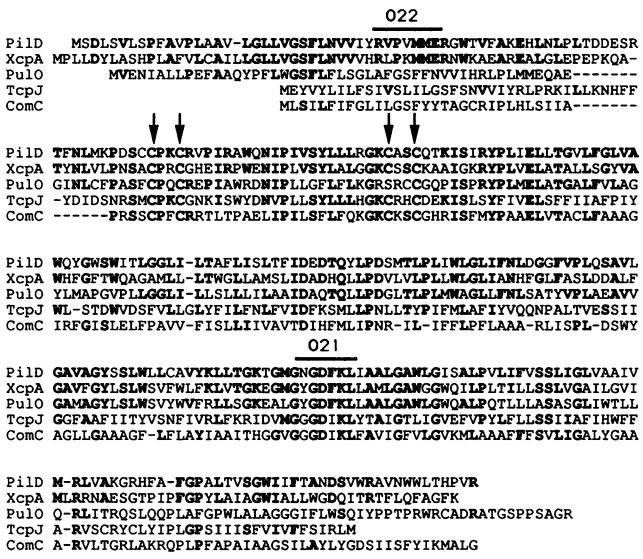

FIG. 3. Alignment of the predicted amino acid sequence of the *N. gonorrhoeae* MS11 *pilD* gene product with the predicted sequences of the XcpA/$PilD_{Pa}$ (2, 37), PulO (49), TcpJ (23), and ComC (36) prepilin peptidases. Residues conserved in $PilD_{Ng}$ and at least one other protein are shown in boldface. Dashes indicate gaps inserted to optimize alignment, and the locations of the conserved Cys residues are marked by vertical arrows. The overlined segments labeled O22 and O21 indicate the positions corresponding to the oligonucleotides used to amplify the initial $pilD_{Ng}$ fragment. Note that the PulO sequence presented here is longer than that published previously (49) because of the presence of a previously undetected sequencing error near the 3′ end of the gene.

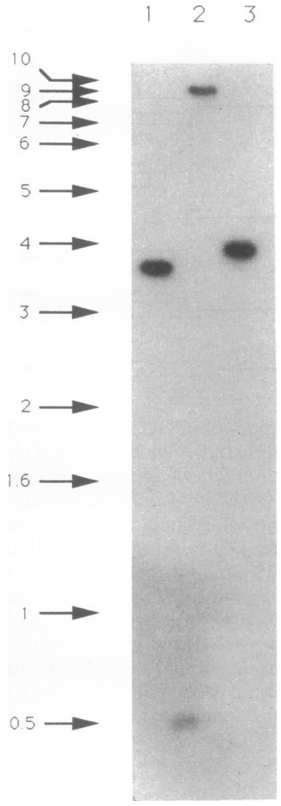

FIG. 4. Southern hybridization of $pilD_{Ng}$-specific probes to *N. gonorrhoeae* MS11 chromosomal DNA. Chromosomal DNA was digested with *Dra*I (lane 1), *Cla*I (lane 2), and *Mlu*I (lane 3) and hybridized with a nick-translated internal segment of $pilD_{Ng}$. DNA size markers (in kilobases; Bethesda Research Laboratories) are indicated to the left.

The molecular mass of $PilD_{Ng}$ predicted from the DNA sequence (Fig. 2) is 31,460 Da. The predicted translation product differs at eight amino acid positions (Glu-148, Val-247, Ala-248, Lys-249, Arg-251, His-252, Ala-254, and Phe-255) from the sequence reported by Lauer et al. (27) (Fig. 2). $PilD_{Ng}$ has 48 and 39% sequence identity with $PilD_{Pa}$ and PulO, respectively; 22% identity with TcpJ, the *V. cholerae* prepilin peptidase (22); and 20% identity with the putative prepilin peptidase (ComC) of *Bacillus subtilis* (36) (Fig. 3).

***N. gonorrhoeae* MS11 carries a single copy of the *pilD* gene.** The gonococcal chromosome is known to carry several copies of certain genes (e.g., the genes encoding PilE pilin, the pilin accessory protein $PilC_{Ng}$, the glycolipid-binding adhesin, and the Opa proteins [21, 32, 42, 57]). Previous studies using the *pulO*-specific probe suggested that *N. gonorrhoeae* might also carry several copies of the $pilD_{Ng}$ gene (11). This possibility was examined by hybridizing a DNA probe containing part of the cloned $pilD_{Ng}$ gene to *N. gonorrhoeae* MS11 chromosomal DNA that had been cleaved by several different restriction endonucleases (Fig. 4). The number of bands detected was compatible only with the presence of a single copy of the $pilD_{Ng}$ gene. In particular, it is worth noting that the 0.5-kb *Cla*I fragment which hybridized strongly with the $pilD_{Ng}$-specific probe corresponds to the 3′ end of the cloned $pilD_{Ng}$ gene (Fig. 2 and 4). Furthermore, none of the fragments which hybridized with the $pilD_{Ng}$ probe were identical to any of the fragments found previously to hybridize with the *pulO* and *xcpA*/$pilD_{Pa}$ probes (data not shown).

Prepilin peptidase activity in strains carrying the cloned $pilD_{Ng}$ gene. Plasmid pPNG100 and subclones derived therefrom (Fig. 1) were introduced into *E. coli* harboring pNG300 (*pilE*$^+$) to measure their effects on processing of prePilE. Plasmids carrying the known prepilin peptidase genes *pulO* (49) and *xcpA*/$pilD_{Pa}$ (2, 37), which were previously shown to promote correct cleavage of prePilE (11), were used as controls. As shown in Fig. 5, strains carrying pPNG100, pCHAP155 (*pulO*), and pJE4 (*xcpA*/$pilD_{Pa}$) all showed total processing of prePilE. As noted previously, incomplete pro-

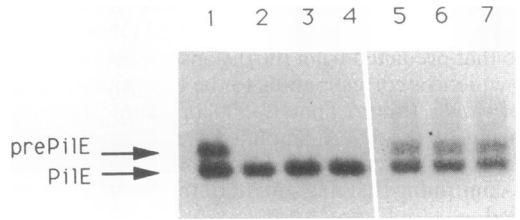

FIG. 5. Processing of gonococcal prepilin in *E. coli* strains expressing *N. gonorrhoeae* prepilin peptidase. The immunoblot shows results obtained with whole-cell lysates derived from *E. coli* DH5 derivatives. Lane 1, pNG300 (which carries the *pilE* gene encoding *N. gonorrhoeae* prepilin [11]); lane 2, pNG300 and pPNG100; lane 3, pNG300 and pCHAP155 (which carries the *pulO* gene [47]); lane 4, pNG300 and pJE4 (which carries the *xcpA* gene [2]); lane 5, pNG300 and pPNG200; lane 6, pNG300 and pPNG109; lane 7, pNG300 and pPNG107 (Fig. 1). Proteins were separated by SDS-PAGE before transfer onto nitrocellulose. Pilin was detected by immunoblotting with a polyclonal PilE antiserum.

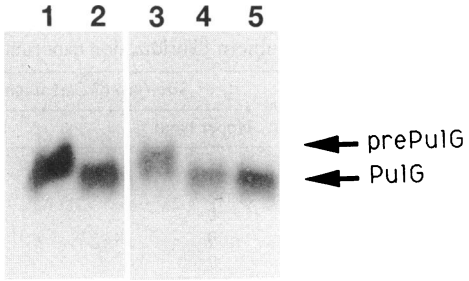

FIG. 6. Processing of prePulG protein by gonococcal prepilin peptidase. The immunoblot shows whole-cell lysates of *E. coli* PAP7245 (which has a complete set of functional *K. oxytoca*-derived pullulanase secretion genes except *pulO* in the chromosome [48]) (lane 1) or derivatives carrying pPNG100 (lane 2), pPNG200 (lane 3), pCHAP155 (lane 4), or pJE4 (lane 5). All cultures were grown in medium containing 0.4% maltose (to induce expression of the *pul* genes, including *pulG*) to an optical density at 600 nm of 1. Proteins were separated by SDS-PAGE before transfer onto nitrocellulose. The antiserum used was specific for PulG protein (46).

cessing occurs in strains devoid of exogenous prepilin peptidase genes, probably because of the action of an endogenous peptidase (11). Plasmids pPNG200 and pPNG109, which carry only the 5′ half of the $pilD_{Ng}$ gene and the DNA downstream of $pilD_{Ng}$, including the 3′ end of $pilD_{Ng}$, respectively (Fig. 1), and pPNG107, which carries a *cat* gene insertion in $pilD_{Ng}$, were all inactive. pPNG101, which lacks the last 10 codons of $pilD_{Ng}$, was also inactive (data not shown). These results provide evidence that the product of the cloned $pilD_{Ng}$ gene cleaves prePilE and therefore that it is a type IV prepilin peptidase.

We also examined the ability of the $pilD_{Ng}$ gene to promote cleavage of prePulG, one of the natural substrates for the PulO prepilin peptidase of *K. oxytoca* (46, 47). As shown in Fig. 6, the presence of a complete $pilD_{Ng}$ gene on pPNG100 promoted complete processing of prePulG, as did the cloned *pulO* gene (pCHAP155) and the cloned *xcpA*/$pilD_{Pa}$ gene (pJE4).

Processing of prePulG is essential for efficient pullulanase secretion by *E. coli* strains carrying the complete pullulanase secretion machinery (46, 47). We therefore determined whether the cloned $pilD_{Ng}$ gene could complement the Δ*pulO1* mutation in *E. coli* PAP7245. This strain normally secretes <4% of the pullulanase it synthesizes, compared with >80% in the parent strain carrying the wild-type *pulO* gene (48) (Table 1). Secretion was partially restored by the introduction of pPNG100 and was increased even further when the higher-copy-number plasmid pPNG104 was used (Table 1). The latter plasmid carries $pilD_{Ng}$ in the same orientation as the *lacZ* promoter in the vector (pUC19) DNA. However, incubation in the presence of the *lacZp* inducer IPTG still did not restore pullulanase secretion to wild-type levels (Table 1). Whatever the explanation for the inability of the cloned $pilD_{Ng}$ gene to totally restore pullulanase secretion in the *pulO* mutant (see Discussion), these results show that the $pilD_{Ng}$ and *pulO* genes are at least partially interchangeable.

Presence of a $pilD_{Ng}$ gene homolog in other species of *Neisseria*. Genes encoding the major subunit of type IV pili have been observed in pathogenic *Neisseria* spp. (*N. gonorrhoeae* and *N. meningitidis*) but not in a majority of commensal species (1, 62). It was therefore pertinent to ask whether the prepilin peptidase gene was present only in pathogenic strains, as predicted. To answer this question, we screened pathogenic and commensal strains of *Neisseria* by Southern hybridization

TABLE 1. Complementation of *pulO* mutation in *E. coli* PAP7245 by plasmids carrying the cloned $pilE_{Ng}$ gene

Strain	*pulO* allele	Plasmid	IPTG	Pullulanase secretion (%)[a]
PAP7232	Wild type	None	−	88
PAP7245	Δ*pulO1*	None	−	<4
		pCHAP155 (*pulO*)	−	86
			+	81
		pJE4 (*xcpA*)	−	40
			+	97
		pNG100 ($pilD_{Ng}$)	−	18
		pNG104 ($pilD_{Ng}$)	−	30
			+	55

[a] Pullulanase secretion values are the proportion of the total amount of pullulanase activity (measured in lysed cells) that can be detected on the cell surface in unlysed cells. The values given are the means of the results from at least two independent experiments.

with the cloned $pilD_{Ng}$ gene as a probe under high-stringency conditions (Fig. 7). DNAs from two strains of *N. meningitidis* (LNP6930 and LNP6922) and from three strains of *N. gonorrhoeae* (MS11, LNP403, and LNP6934) showed strong hybridization with this probe. Surprisingly, the DNAs from one strain each of the commensal species *N. lactamica* (LNP411), *N. polysaccharea* (LNP462), and *N. cinerea* (LNP415) all showed significant hybridization. Furthermore, the profiles of the hybridizing fragments in *Cla*I-restricted DNAs from these strains were either identical or very similar to that of the corresponding DNA from *N. gonorrhoeae* MS11, most notably in the consistent presence of an approximately 0.5-kb *Cla*I fragment (Fig. 7).

Moreover, two fragments in *Cla*I-restricted DNAs from the other commensal strains tested (*N. subflava* LNP3260, *N. flava* LNP3264, *N. perflava* LNP407, *N. mucosa* LNP405, and *N. sicca* LNP3265) also hybridized with the $pilD_{Ng}$ probe, albeit much less strongly than the DNAs from the pathogenic strains (data not shown). The profiles of these hybridizing fragments were often similar to each other but were quite different from those detected in the restricted DNAs from the pathogenic strains (Table 2). Nevertheless, the hybridization seemed to be specific, since it was detected under high-stringency conditions (65°C). These results suggested that a *pilD*-type gene might be conserved among pathogenic and commensal strains of *Neisseria*.

As an alternative approach to detecting possible $pilD_{Ng}$ homologs in *Neisseria* spp. other than *N. gonorrhoeae*, we used the same PCR approach as that used to amplify the *N. gonorrhoeae* $pilD_{Ng}$ gene. As shown in Fig. 8, a 0.5-kb fragment was amplified from the DNAs of strains that hybridized with the $pilD_{Ng}$ probe. DNAs from the commensal strains *N. flavescens* LNP444 and *N. denitrificans* LNP412 did not hybridize with this probe. Nevertheless, when PCR was applied to these strains we were able to amplify a 0.5-kb fragment from the DNA of *N. flavescens* LNP444 and a DNA fragment from *N. denitrificans* LNP412 which was slightly smaller than that obtained with the DNAs of the other species tested (not shown).

We therefore electroeluted the 0.5-kb fragment amplified from several different strains which hybridized strongly or weakly with the $pilD_{Ng}$ probe and sequenced it by using the degenerate primer used for the amplification step. This analysis revealed the near identity of the sequences of the amplified DNAs. Extrapolation of this sequence analysis to the primary sequence of the putative encoded proteins revealed over 80% identity to the corresponding segment in the predicted se-

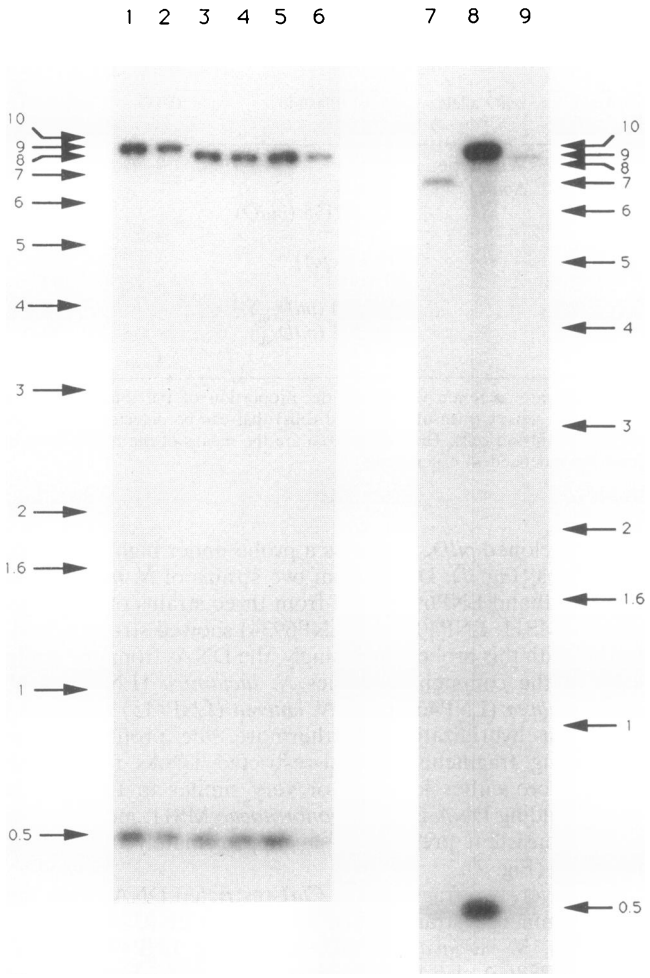

FIG. 7. Detection of *pilD* in *Neisseria* strains by Southern hybridization. Chromosomal DNA was digested with *Cla*I and probed with a nick-translated internal segment of $pilD_{Ng}$. The strains tested were *N. meningitidis* LNP6930 (lane 1), *N. meningitidis* LNP6922 (lane 2), *N. gonorrhoeae* MS11 (lane 3), *N. gonorrhoeae* 2D (a nonpiliated variant of *N. gonorrhoeae* MS11) (lane 4), *N. gonorrhoeae* LNP403 (lane 5), *N. gonorrhoeae* LNP6934 (lane 6), *N. cinerea* LNP415 (lane 7), *N. lactamica* LNP411 (lane 8), and *N. polysaccharea* LNP462 (lane 9). DNA size markers (in kilobases; Bethesda Research Laboratories) are indicated to the right and left.

quence of the $pilD_{Ng}$ gene product (Fig. 9). Thus, the prepilin peptidase gene appears to be highly conserved in all species of *Neisseria*, including commensal strains which apparently do not produce type IV pili.

To see whether some of the newly identified *pilD* genes were expressed, we hybridized the DNA fragments amplified from *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. subflava*, and *N. sicca* to RNA extracts from the same strain. The results of this dot blot experiment (Fig. 10) indicate that RNAs from all five strains hybridized with their homologous probe, indicating that all five *pilD* genes, including those in the commensal strains, are probably expressed. We did not determine whether these strains possess prepilin peptidase activity.

DISCUSSION

In this study we used PCR to amplify a fragment of the *N. gonorrhoeae* prepilin peptidase structural gene, $pilD_{Ng}$, using degenerate primers corresponding to internal, highly conserved segments of known prepilin peptidase genes from other bacteria. The size of the major amplified product was close to those of products amplified from DNAs known to contain *pulO* or $xcpA/pilD_{Pa}$. Preliminary sequence analysis showed that the fragment amplified from *N. gonorrhoeae* DNA contained sequences homologous to those of these two genes. The amplified fragment was then cloned and used as a homologous probe to screen an *N. gonorrhoeae* chromosomal gene bank in which a clone containing the entire $pilD_{Ng}$ gene was identified.

A similar approach could be used to clone the prepilin peptidase gene from other species of bacteria that are known to have type IV pili, and this may be more widely applicable to the cloning of homologs of known genes in situations in which heterologous DNA probes produce false-positive clones. For example, several bands in restricted *N. gonorrhoeae* MS11 chromosomal DNA hybridized in an identical fashion with DNA probes containing part or all of the *pulO* or $xcpA/pilD_{Pa}$ gene but did not contain the prepilin peptidase gene (not shown). Interestingly, one of the clones which hybridized with the *pulO* and $pilD_{Pa}$ probes contained a large segment (1,215 bp) containing an open reading frame which, when translated, could code for a protein with 40% identity to a 405-amino-acid sequence present in RNA helicase PRP2 and 41.5% identity to pre-mRNA splicing factor PRP16 of the yeast *Saccharomyces*

TABLE 2. Sizes of *Cla*I fragments in restricted chromosomal DNAs from various strains of *Neisseria* which hybridized to the $pilD_{Ng}$ probe in Southern hybridization experiments

Species	Size (kb) of *Cla*I fragment	
	Upper band	Lower band
N. gonorrhoeae	8	0.5
N. meningitidis	9	0.5
N. lactamica	9	0.5
N. polysaccharea	9	0.5
N. cinerea	7	0.5
N. flava	7	0.7
N. subflava	6	0.7
N. perflava	2.5	0.7
N. mucosa	4	0.7
N. sicca	7	0.7

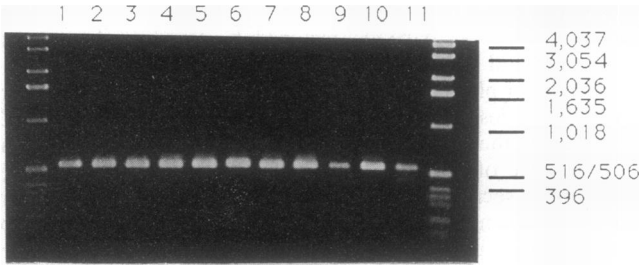

FIG. 8. PCR amplification of an approximately 0.5-kb DNA fragment from chromosomal DNAs from different species of *Neisseria*. Lane 1, *N. gonorrhoeae* MS11; lane 2, *N. meningitidis* LNP6930; lane 3, *N. polysaccharea* LNP462; lane 4, *N. lactamica* LNP411; lane 5, *N. cinerea* LNP415; lane 6, *N. flava* LNP3264; lane 7, *N. subflava* LNP3260; lane 8, *N. perflava* LNP407; lane 9, *N. mucosa* LNP405; lane 10, *N. sicca* LNP3265; and lane 11, *N. flavescens* LNP444. The PCR primers were the same as those used to amplify the *N. gonorrhoeae* MS11 *pilD* gene (Fig. 3). DNA size markers (in kilobases; Bethesda Research Laboratories) are indicated to the right.

```
N.gono ISIRYPLIELLTGVLFGLVAWQYGWSWITLGGLILTAFLISLTFIDEDTQYLPDSM
N.meni ISIRYPLIELLSGVLFGLVAWQYGWSWITLGGLILTAFLISLTFIDADTQYLPDSM
N.lact  QIRYPSIELLTGVLFGWSPWQYGWSWITLGGLVLTAFLISLTFIDADTQYLPDSM
N.subf       LIELLTGVLFGIVAWQYGWSWATIGGLILTAILIALTFIDADTQYLPDSL
N.sicc      PLIELLTGVLFGIVAWQYGWSWATIGGLILTAILIALTFIDADTQYLPDRM

N.gono TLPLIWLGLIFNLDGGFVPLQSAVLGAVAGYSSLWLLCAVYKLLTGK
N.meni TLPLIWLGLIFNLDGGFVPLQSAVLGAVAGYGSIWLLC
N.lact TLPLIWLGLIFNLDGGFVPLQSAVLGAVAGYGSLWLLCAVYKLLTGK
N.subf TQPLIWIGLLFNLNDTFVSLHSAVWGAIAGYMSLYTLCAVYKLLTGK
N.sicc TQPLIWIGLLFTLNDTFVPLHSAVWGAIAGYMSLYTLCAVYKLLTGK
```

FIG. 9. Comparison of the amino acid sequence of a segment of *N. gonorrhoeae* PilD with the extrapolated amino acid sequences encoded by the PCR-amplified products of *N. meningitidis* LNP6930, *N. lactamica* LNP411, *N. subflava* LNP3260, and *N. sicca* LNP3265. DNA sequences were directly determined on a single strand of the amplified DNA by using PCR primer O21. Amino acids in these sequences which differ from that in the corresponding segment of $PilD_{Ng}$ are indicated in boldface.

cerevisiae (5, 6). Toone et al. (66) have reported the presence in *E. coli* of a putative RNA helicase which has considerable sequence similarity with these proteins. Further investigation of this segment of the *N. gonorrhoeae* chromosome is currently under way.

The results presented here indicate that *N. gonorrhoeae* possesses a single copy of the $pilD_{Ng}$ gene, which codes for the enzyme, prepilin peptidase, which cleaves (and presumably also N methylates) type IV pilin precursors. In an attempt to study the effects of the absence of $pilD_{Ng}$ on piliation and other properties of *N. gonorrhoeae*, we tried to inactivate the chromosomal copy of $pilD_{Ng}$ by homologous recombination with the $pilD_{Ng}$::mTn*3*Cm-3 gene carried by pPNG107, which had been introduced by electroporation or by natural transformation (the *N. gonorrhoeae* DNA fragment cloned in pNG107 includes an uptake sequence necessary for transformation [27]). Although this procedure worked well in parallel experiments with a plasmid carrying the mTn*3*Cm-3 transposon inserted into cloned copies of the *pilA* and *pilB* genes (8, 63) (data not shown), leading to inactivation of the chromosomal *pilA* and *pilB* genes, we were unable to obtain viable colonies in which the $pilD_{Ng}$::mTn*3*Cm-3 gene had been integrated into the chromosome. This may mean that the $pilD_{Ng}$ gene is essential for *N. gonorrhoeae* viability. The possibility that accumulation of prepilin caused by the absence of $pilD_{Ng}$ might be toxic to the cells is currently under investigation, but it is intriguing that our results also suggest that commensal, nonpiliated species of *Neisseria* carry a *pilD*-like gene. The results of RNA-DNA hybridization experiments suggest that the *pilD* gene is expressed in at least some of these commensal strains and therefore that the prepilin peptidase performs another function, unrelated to piliation. We speculate that the $pilD_{Ng}$ gene product might also perform this function, in addition to processing of type IV prepilin, in *N. gonorrhoeae* and furthermore that this additional function is essential for viability. Interestingly, the corresponding gene in *P. aeruginosa* is required not only for piliation but also for extracellular secretion (2, 37). However, *Neisseria* spp. are not known to secrete extracellular proteins by a pathway similar to that in which the *P. aeruginosa* prepilin peptidase is involved, nor is it likely that extracellular secretion would be essential for viability. Indeed, mutants of *P. aeruginosa* lacking prepilin peptidase are perfectly viable (2, 37).

Another possible explanation for the second role of the prepilin peptidase in *N. gonorrhoeae* and for the primary role of this enzyme in the commensal *Neisseria* strains is that it processes proteins required for transformation. This possibility arises by analogy with the fact that a PilD homolog (ComC) is required for transformation competence in *B. subtilis* (36), in which it is probably required to process four type IV pilin-like proteins that form part of the machinery involved in the uptake of transforming DNA (10). However, very little is known about how DNA is transported into *N. gonorrhoeae* other than the fact that specific uptake sequences must be present in the DNA (15). Although pili seem to be required for DNA uptake (4), there is no evidence that pili bind DNA or otherwise directly promote DNA entry (30), and the involvement of other pilin-like proteins in transformation has not been investigated. Once again, however, the loss of transformation competence as a result of the inactivation of the $pilD_{Ng}$ gene is unlikely to lead to loss of viability in *N. gonorrhoeae*. A clearer picture of the role of the $pilD_{Ng}$ gene in *N. gonorrhoeae* should result from studies designed to modulate the level of $pilD_{Ng}$ expression.

It would be informative to study the conditions leading to *pilD* expression in *N. gonorrhoeae*. The *P. aeruginosa pilD* gene seems to be transcribed independently of all other piliation and secretion genes by RNA polymerase containing σ^{70} (24). The DNA sequence upstream from the $pilD_{Ng}$ gene of *N. gonorrhoeae* has the -10-type sequence which characterizes eubacterial promoters transcribed by σ^{70}-dependent RNA polymerase. However, this region also contains three $-24/-12$ sequences (PI, PII, and PIII in Fig. 2) similar to those found in σ^{54}-dependent promoters (see reference 26 for a review). Preliminary primer extension mapping shows that there are two potential transcription start sites, at -11 and -13 bp, respectively, in both *N. gonorrhoeae* and *E. coli* carrying the cloned $pilD_{Ng}$ gene, which would be most consistent with transcription initiation involving PI. It is worth noting that the promoter region of the pilin structural gene *pilE* also includes

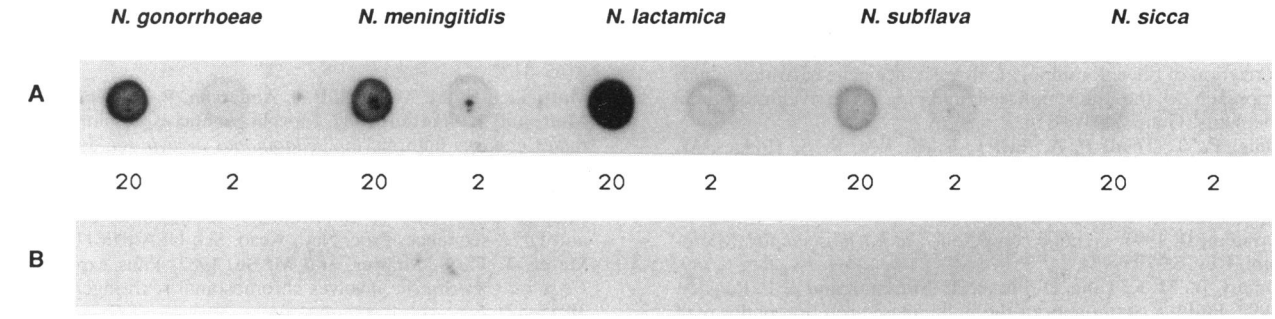

FIG. 10. Detection of *pilD* transcripts from *Neisseria* strains by dot blot analysis. (A) Dilutions of RNA were hybridized with the probes made by using the PCR fragment amplified from each of the *Neisseria* strains tested (*N. gonorrhoeae* MS11, *N. meningitidis* LNP 6930, *N. lactamica* LNP411, *N. subflava* LNP 3260, and *N. sicca* LNP3265). (B) Control RNA samples were treated with 0.4 N NaOH at 65°C for 45 min before treatment as for panel A (see Materials and Methods). The numbers indicate micrograms of total RNA spotted onto the filter.

−24/−12-type sequences, although it is not known whether σ^{54} is needed for its transcription (14, 32). This interesting aspect of $pilD_{Ng}$ and *pilE* expression would appear to warrant further investigation.

The cloned $pilD_{Ng}$ gene was found to complement a mutation in the *K. oxytoca* prepilin peptidase gene, *pulO*, leading to complete restoration of processing of the PulG precursor and to partial restoration of PulO- and PulG-dependent pullulanase secretion (Table 1 and Fig. 6). It is interesting that the $pilD_{Ng}$ gene carried by pPNG100 did not fully restore pullulanase secretion even though prePulG was fully processed, which is in contrast to the effects of the cloned $xcpA/pilD_{Pa}$ gene (Table 1). This may reflect the lower ability of the gonococcal prepilin peptidase to process and/or methylate the precursors of the three other type IV pilin-like proteins (PulH, PulI, and PulJ) that are required for pullulanase secretion (50). Indeed, the whole question of the recognition of the cleavage site by members of this family of peptidases remains to be fully investigated.

ACKNOWLEDGMENTS

We are grateful to M. K. Taha, M. Larribe, H. de Reuse, S. Lory, and O. Possot for helpful discussions, to D. Giorgini for technical help, to J.-Y. Riou for *Neisseria* strains and for generous support, and to A. Lazdunski for providing pJE4.

This work was supported by INSERM (CRE 930613) and the Institut Pasteur.

REFERENCES

1. **Aho, E. L., G. L. Murphy, and J. G. Cannon.** 1987. Distribution of specific DNA sequences among pathogenic and commensal *Neisseria* species. Infect. Immun. **55:**1009–1013.
2. **Bally, M., G. Ball, A. Badere, and A. Lazdunski.** 1991. Protein secretion by *Pseudomonas aeruginosa*: the *xcpA* gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function protein PulO. J. Bacteriol. **173:**479–486.
3. **Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen.** 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. **6:**1121–1131.
4. **Biswas, G. D., T. Sox, E. Blackman, and P. F. Sparling.** 1977. Factors affecting genetic transformation of *Neisseria gonorrhoeae*. J. Bacteriol. **129:**983–992.
5. **Burgess, S., J. R. Cauto, and C. Guthrie.** 1990. A putative ATP binding protein influences the fidelity of branchpoint recognition in yeast splicing. Cell **60:**705–717.
6. **Chen, J. H., and R. J. Lin.** 1990. The yeast PRP2 protein, a putative RNA-dependent ATPase, shares extensive sequence homology with two other pre-mRNA splicing factors. Nucleic Acids Res. **18:**6447.
7. **Churchward, G., D. Belin, and Y. Nagaamse.** 1984. A pSC101-derived plasmid which shows no sequence homology to other commonly used cloning vectors. Gene **31:**165–171.
8. **Close, T. J., and R. L. Rodriguez.** 1982. Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptional mapping of extrachromosomal elements. Gene **20:**305–316.
9. **Doig, P., T. Tood, P. A. Sastry, K. K. Lee, R. S. Hodges, W. Paranchych, and R. T. Irwin.** 1988. Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect. Immun. **56:**1641–1646.
10. **Dubnau, D.** 1991. Genetic competence in *Bacillus subtilis*. Microbiol. Rev. **55:**395–424.
11. **Dupuy, B., M. K. Taha, O. Possot, C. Marchal, and A. P. Pugsley.** 1992. PulO, a component of the pullulanase secretion pathway of *Klebsiella oxytoca*, correctly and efficiently processes gonococcal type IV prepilin in *Escherichia coli*. Mol. Microbiol. **6:**1887–1894.
12. **Dupuy, B., M. K. Taha, A. P. Pugsley, and C. Marchal.** 1991. *Neisseria gonorrhoeae* prepilin export studied in *Escherichia coli*. J. Bacteriol. **173:**7589–7598.
13. **Elleman, T. C., and J. E. Peterson.** 1987. Expression of multiple types of N-methyl-Phe pili in *Pseudomonas aeruginosa*. Mol. Microbiol. **1:**377–380.
14. **Fyfe, J. A. M., R. A. Strugnell, and J. K. Davies.** 1993. Control of gonococcal pilin-encoding gene expression in *Escherichia coli*. Gene **123:**45–50.
15. **Goodman, S. D., and J. J. Scocca.** 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **85:**6982–6986.
16. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:**3961–3965.
17. **Hanahan, D.** 1983. Studies on transformation in *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
18. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. **15:**2343–2361.
19. **Hermodson, M. A., K. C. S. Chen, and T. M. Buchanan.** 1978. *Neisseria* pili proteins: amino-terminal amino acid sequences and identification of an unusual amino acid. Biochemistry **17:**442–445.
20. **Hoyne, P. A., R. Hass, T. F. Meyer, J. K. Davies, and T. C. Elleman.** 1992. Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. J. Bacteriol. **174:**7321–7327.
21. **Jonsson, A. B., G. Nyberg, and S. Normark.** 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. EMBO J. **10:**477–488.
22. **Kaufman, M. R., J. M. Seyer, and R. K. Taylor.** 1991. Processing of TCP pilin by TCPJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by Gram-negative bacteria. Genes Dev. **5:**1834–1846.
23. **Kaufman, M. R., C. E. Shaw, I. D. Jones, and R. K. Taylor.** 1993. Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated-pilus: analogies to other virulence factor secretory systems and localization of the *toxT* virulence regulator gene to the TCP cluster. Gene **126:**43–49.
24. **Koga, T., K. Ishimoto, and S. Lory.** 1993. Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili. Infect. Immun. **61:**1371–1377.
25. **Koomey, M., S. Bergstrom, M. Blake, and J. Swanson.** 1991. Pilin expression and processing in mutants of *Neisseria gonorrhoeae*: critical role of Gly-1 in assembly. Mol. Microbiol. **5:**279–287.
26. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53:**367–376.
27. **Lauer, P., N. H. Albertson, and M. Koomey.** 1993. Conservation of genes encoding components of a type IV pilus assembly/two-step protein export pathway in *Neisseria gonorrhoeae*. Mol. Microbiol. **8:**357–368.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Martin, P. R., M. Hobbs, P. D. Free, Y. Jeske, and J. S. Mattick.** 1993. Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbriae of *Pseudomonas aeruginosa*. Mol. Microbiol. **9:**857–868.
30. **Mathis, L., and J. J. Scocca.** 1984. On the role of pili in transformation of *Neisseria gonorrhoeae*. J. Gen. Microbiol. **130:**3165–3173.
31. **Mattick, J. S., M. M. Bills, B. J. Anderson, B. Dolrymple, M. R. Mott, and J. Egerton.** 1987. Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. J. Bacteriol. **169:**33–41.
32. **Meyer, T. F., E. Billyard, R. Hass, S. Storzbach, and M. So.** 1984. Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. Proc. Natl. Acad. Sci. USA **81:**6110–6114.
33. **Meyer, T. F., N. Mlawer, and M. So.** 1982. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. Cell **30:**45–52.
34. **Michaelis, S., C. Chapon, C. d'Enfert, A. P. Pugsley, and M. Schwartz.** 1985. Characterization and expression of the structural gene for pullulanase, a maltose-inducible secreted protein of *Klebsiella pneumoniae*. J. Bacteriol. **164:**633–638.

35. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Mohan, S. A., J. Aghion, N. Guillen, and D. Dubnau.** 1989. Molecular cloning and characterization of *comC*, a late competence gene of *Bacillus subtilis*. J. Bacteriol. **171:**6043–6051.
37. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172:**2911–2919.
38. **Nunn, D. N., and S. Lory.** 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin-leader peptidase. Proc. Natl. Acad. Sci. USA **88:**3281–3285.
39. **Nunn, D. N., and S. Lory.** 1992. Components of the protein excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. USA **89:**47–51.
40. **Nunn, D. N., and S. Lory.** 1993. Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. J. Bacteriol. **175:**4375–4382.
41. **Ogierman, M. A., S. Zabihi, L. Mourtzios, and P. A. Manning.** 1993. Genetic organization and sequence of promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. Gene **126:**51–60.
42. **Paruchuri, D. K., H. S. Seifert, R. S. Ajioka, K. A. Karlsson, and M. So.** 1990. Identification and characterization of a *Neisseria gonorrhoeae* gene encoding a glycolipid binding adhesin. Proc. Natl. Acad. Sci. USA **87:**333–337.
43. **Pearce, W. A., and T. M. Buchanan.** 1978. Attachment role of gonococcal pili: optimum conditions and quantitation of adherence of isolated pili to human cells *in vitro*. Clin. Invest. **61:**931–943.
44. **Peck, J. A., and R. K. Taylor.** 1992. Characterization of a periplasmic thiol disulfide interchange protein required for the functional maturation of secreted virulence factors in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**1641–1645.
45. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
46. **Pugsley, A. P.** 1993. Processing and methylation of PulG, a pilin-like component of the general secretory pathway of *Klebsiella oxytoca*. Mol. Microbiol. **9:**295–308.
47. **Pugsley, A. P., and B. Dupuy.** 1992. An enzyme with type IV prepilin peptidase activity is required to process components of the general extracellular protein secretion pathway of *Klebsiella oxytoca*. Mol. Microbiol. **6:**751–760.
48. **Pugsley, A. P., and O. Possot.** 1993. The general secretory pathway of *Klebsiella oxytoca*: no evidence for relocalisation of assembly of pilin-like PulG protein into a multiprotein complex. Mol. Microbiol. **10:**665–674.
49. **Pugsley, A. P., and I. Reyss.** 1990. Five genes at the 3′ end of the *Klebsiella pneumoniae pulC* operon are required for pullulanase secretion. Mol. Microbiol. **4:**365–379.
50. **Reyss, I., and A. P. Pugsley.** 1990. Five additional genes in the *pulC-O* operon of the gram-negative bacterium *Klebsiella oxytoca* UNF5023 which are required for pullulanase secretion. Mol. Gen. Genet. **222:**176–184.
51. **Rudel, T., J. P. M. Van Putten, C. P. Gibbs, R. Haas, and T. F. Meyer.** 1992. Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. Mol. Microbiol. **6:**3439–3450.
52. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
53. **Schoolnik, G., J. Tai, and E. Gotschlich.** 1983. A pilus peptide vaccine for the prevention of gonorrhoeae. Prog. Allergy **33:**314–331.
54. **Segal, E., E. Billyard, M. So, S. Storzbach, and T. F. Meyer.** 1985. Role of chromosomal rearrangement in *Neisseria gonorrhoeae* pilus phase variation. Cell **40:**293–300.
55. **Shaw, C. E., and R. K. Taylor.** 1990. *Vibrio cholerae* O395 *tcpA* pilin gene sequence and comparison of predicted protein structural features with those of type 4 pilins. Infect. Immun. **58:**3042–3049.
56. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. J. Exp. Med. **127:**571–589.
57. **Stern, A., M. Brown, P. Nickel, and T. F. Meyer.** 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. Cell **47:**61–71.
58. **Strom, M. S., and S. Lory.** 1991. Amino acid substitutions in pilin of *Pseudomonas aeruginosa*. J. Biol. Chem. **266:**1656–1664.
59. **Strom, M. S., D. Nunn, and S. Lory.** 1991. Multiple roles of the pilus biogenesis protein PilD: involvement of PilD in excretion of enzymes from *Pseudomonas aeruginosa*. J. Bacteriol. **173:**1175–1180.
60. **Strom, M. S., D. Nunn, and S. Lory.** 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc. Natl. Acad. Sci. USA **90:**2404–2408.
61. **Swanson, J.** 1973. Studies on gonococcus infection. VI. Pili: their role in attachment of gonococci to tissue culture cells. J. Exp. Med. **137:**571–589.
62. **Tahar, M. K., and C. Marchal.** 1990. Conservation of *Neisseria gonorrhoeae* pilus expression regulatory genes *pilA* and *pilB* in the genus *Neisseria*. Infect. Immun. **58:**4145–4148.
63. **Tahar, M. K., M. So, H. S. Seifert, E. Billyard, and C. Marchal.** 1988. Pilus expression in *Neisseria gonorrhoeae* is under both positive and negative transcriptional control. EMBO J. **7:**4367–4378.
64. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. The use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
65. **Thony, B., and H. Hennecke.** 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. **63:**341–358.
66. **Toone, W. M., K. E. Rudd, and J. D. Friesen.** 1991. *deaD*, a new *Escherichia coli* gene encoding a presumed ATP-dependent RNA helicase, can suppress a mutation in *rpsB*, the gene encoding ribosomal protein S2. J. Bacteriol. **173:**3291–3302.
67. **Woods, D. E., D. C. Strauss, W. G. Johanson, V. K. Berry, and J. A. Bass.** 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29:**135–143.