On the Role of Pili in Transformation of \textit{Neisseria gonorrhoeae}

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Transformation of competent transformable \textit{Neisseria gonorrhoeae} F62 to streptomycin resistance was unaffected by antibodies directed against the pilus protein (pilin) of this organism. The pilin component of either crude or purified pilus preparations, separated by SDS gel electrophoresis and transferred to nitrocellulose, failed to bind detectable amounts of DNA; DNA binding to other gonococcal polypeptides was observed under these conditions. These results suggest that gonococcal pilin does not play a direct role in gonococcal transformation.

INTRODUCTION

Pili are protein filaments which project from the surfaces of many bacterial cells (Swanson \textit{et al.}, 1971). Pili are classified into two groups; somatic pili or fimbriae, and conjugal or sex pili (Ottow, 1975). Pili appear to function as adhesive organelles in those bacteria possessing them (Beachey, 1981). Sex pili, such as F pili of \textit{Escherichia coli} (Brinton \textit{et al.}, 1964), mediate the cell contacts necessary for conjugation to occur. In many cases, the presence of pili allows bacteria to adhere to a suitable cellular substrate, and is thus a determinant of pathogenic potential (Beachey, 1981; Hagberg \textit{et al.}, 1981).

The somatic pili of \textit{Neisseria gonorrhoeae} are filaments about 8 nm in diameter (Swanson \textit{et al.}, 1971) extending up to 5 μm from the bacterial surface (Lambden \textit{et al.}, 1980); recent studies suggest that the pili form a lattice-like network connecting the cells in a colony (Todd \textit{et al.}, 1984). Gonococcal pili are composed of a single repeating protein, called pilin, with a subunit molecular weight of approximately 18000; this value varies slightly among different strains (Buchanan, 1977). Pili are present only on the virulent colonial types of \textit{N. gonorrhoeae}; the avirulent colonial types are not piliated (Kellogg \textit{et al.}, 1963, 1968; Jephcott \textit{et al.}, 1971; Swanson \textit{et al.}, 1971). Virulence and piliation are also associated with the capacity of \textit{N. gonorrhoeae} to function as recipients in genetic transformation. A number of possible functions have been ascribed to pili to account for the association of virulence and transformability with piliation (Brinton \textit{et al.}, 1978). These functions include attachment of gonococci to human cells (Buchanan, 1977; Buchanan \textit{et al.}, 1978); antiphagocytic function (Thongthai & Sawyer, 1973; Ofek \textit{et al.}, 1974; Rosenthal \textit{et al.}, 1977); binding iron and other cations (Payne & Finkelstein, 1975); and involvement in genetic transformation (Sparling, 1966; Sarubbi \textit{et al.}, 1974).

Since the F pili of \textit{E. coli} participate in conjugation and infection with filamentous phage (Brinton, 1965), it has been suggested that pilin might play an analogous role in genetic transformation in the gonococcus, by binding of DNA to pili, or by the transfer of DNA along pili into the recipient cell (Biswas \textit{et al.}, 1977). This proposal would account for the close correlation between piliation and transformability found among the colonial types of \textit{N. gonorrhoeae} (Sparling, 1966; Biswas \textit{et al.}, 1977). Binding of DNA to purified gonococcal pilin was not observed in sedimentation experiments on neutral sucrose gradients (Sparling \textit{et al.}, 1977). However, the disruption of pili by high concentrations of sucrose (Brinton \textit{et al.}, 1978) might well affect the interaction of DNA with these structures, making the interpretation of these experiments difficult.

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To assess the possible role of the gonococcal pilus protein, pilin, in DNA uptake during transformation, we studied the capacity of antibody prepared against purified pilin to affect the transformation efficiencies of competent *N. gonorrhoeae*. Binding of DNA to purified pilin and to other protein components of *N. gonorrhoeae* was examined by studies on the binding of radioactive DNA to electrophoretically separated pilin and pilin-containing preparations.

**METHODS**

*Bacteria.* *N. gonorrhoeae* strains FA19 (wild-type), FA130 (streptomycin resistant), and F62 (highly piliated) were kindly provided by P. F. Sparling, University of North Carolina, Chapel Hill, USA. Stock cultures were stored at −70 °C in Trypticase Soy Broth with 20% (v/v) glycerol.

**Enzymes and reagents.** *EcoRI* restriction endonuclease (EC 3.1.23.13) was purchased from New England Biolabs, Beverly, Mass., USA; alkaline phosphatase from *E. coli* (EC 3.1.3.1) was purchased from BRL; polynucleotide kinase (T4-infected *E. coli*, EC 2.7.1.78) was purchased from PL Biochemicals, Milwaukee, Wis., USA; DNAase I (EC 3.1.21.1) and ribonuclease A (EC 3.1.27.5) were from Worthington-Millipore, Freehold, NJ, USA. Goat anti-rabbit IgG conjugated to alkaline phosphatase was purchased from Miles-Yeda, Elkhart, Indiana, USA. [γ-32P]ATP (crude) was purchased from ICN, Irvine, Calif., USA. GC Medium Base, Trypticase Soy Broth, and brain heart infusion were purchased from Difco, IsoVitaleX from BBL. Microbiology Systems (Cockeysville, Md), and p-nitrophenyl phosphate from Sigma. Protein standards for SDS-PAGE, purchased from Bio-Rad, contained the following: lysozyme (*M*, 14400), soybean trypsin inhibitor (*M*, 21500), carbonic anhydrase (*M*, 31000), ovalbumin (*M*, 45000), bovine serum albumin (*M*, 66200), and phosphorylase B (*M*, 92500).

**Growth of bacteria.** *N. gonorrhoeae* was grown on plates of GC Medium Base (without haemoglobin) containing 1% (v/v) IsoVitaleX at 37 °C in a humid atmosphere of 8% (v/v) CO2 in air. This level of CO2 appeared to stabilize colonial type in culture considerably better than did 5% CO2. Colonial types which were pilated and transparent (P+**O**− or type 2) were propagated for these experiments. Cells were harvested by scraping after 18 h of growth.

**Preparation of DNA.** Bacteria were lysed by the method of Sox et al. (1978) and DNA was prepared as described by Scocca et al. (1974). For radiolabelling, the DNA was further purified by centrifugation to equilibrium in a CsCl/ethidium bromide gradient. The DNA band was recovered, ethidium bromide was removed by extraction with isobutanol, and the DNA recovered by precipitation with ethanol. The DNA was digested with *EcoRI* restriction endonuclease, deproteinized by extraction with phenol, dephosphorylated by treatment with alkaline phosphatase, and then phosphorylated by incubation with polynucleotide kinase and [γ-32P]ATP in 50 mM-Tris/HCl, 10 mM-MgCl2, 0.02% (w/v) NaN3. These methods were based on those described by Maniatis et al. (1982).

**Purification of pilin.** The method was based on the procedure of Hermodsen et al. (1978). *N. gonorrhoeae* strain F62 (*P+*, type 2) was suspended in ice-cold 10 mM-Tris/HCl pH 7.5 and blended in a VirTis homogenizer (VirTis Corporation, Gardiner, NY, USA) at low speed for 2 min to shear pili. Cells were sedimented at 12000 g for 10 min at 4 °C and the supernatant centrifuged at 50000 g for 1 h at 4°C. Solid ammonium sulphate was added to the supernatant to 90%, saturation, and the suspension was left at 4 °C for 18 h. Pilin was sedimented at 30000 g for 10 min at 4 °C and resuspended in deionized water. Further purification was by repeated cycles of precipitation with 10% saturated ammonium sulphate.

**Antiserum preparation and purification of immunoglobulins.** Preimmune and immune sera were prepared from a New Zealand White rabbit (3.2 kg) by the method of Robertson et al. (1977). Pilin preparations used for immunizations showed one major band (M, 18500) on SDS-PAGE, and had an estimated purity of greater than 95%. Purified pilin (50 μg in Freund’s incomplete adjuvant) was injected subcutaneously in the flank; injections were repeated at 2-week intervals for 10 weeks. Serum was collected at 2-week intervals, beginning on the eleventh week.

The immunoglobulin fraction was precipitated with ammonium sulphate (Hudson & Hay, 1976), and dialysed overnight against 10 mM-sodium phosphate buffer, pH 7.2, 50 mM-NaCl at 4 °C. To remove DNA-binding proteins, the retentate was passed over a salmon sperm DNA-Sepharose column and eluted with the dialysis buffer. The unretarded immunoglobulin peak was collected and used in transformation experiments.

**Direct immunoprecipitation.** Preimmune serum or anti-pilin serum (1 μg) and 1 μl pilin (260 μg ml−1) were placed in capillary tubes, the tubes were sealed, the contents mixed, and flocculation was observed immediately or after incubation for 1 h at 4°C.

**Immunoelctrophoresis.** This was done in 1% (w/v) agarose gels on microscope slides in 50 mM-sodium barbital buffer, pH 8.7 (Garvey et al., 1977). The antigen (2 to 5 μg) was separated by electrophoresis at 60 V and 3 mA for 5 h. After electrophoresis, antiserum (30 μl) was placed in the gel trough, and the slide was incubated at room temperature for 18 h in a humid chamber. The slides were washed twice with 0.3 M-NaCl, twice with 0.15 M-NaCl, covered with wet Whatman no. 4 filter paper and dried at 37 °C. Precipitin arcs were stained with acid fuchsin.
Enzyme-linked immunosorbent assay (ELISA). This procedure was based on that of Grant (1978). The wells of Cooke microtitre plates were filled with 200 µl purified pilin at 2 µg ml⁻¹ in coating buffer (0.05 M-sodium bicarbonate buffer, pH 9.6, 0.02% NaN₃) and left at 4 °C overnight. The wells were washed twice with 200 µl PBS-Tween (0.01 M-sodium phosphate buffer pH 7.0, 0.05% Tween 20, 0.8% NaCl, 0.02% NaN₃); BSA (1%, w/v) in coating buffer was added to the wells, incubated for 2 h at 4 °C, and removed by washing with PBS-Tween. Antiserum at a dilution of 2 × 10⁻⁵ was then added to the wells and incubated for 2 h at 37 °C. In some experiments, the antiserum was preincubated with competing protein or cell preparations for 15 min at 37 °C before addition to wells. After the incubation with antiserum, the plates were washed with PBS-Tween, and goat anti-rabbit IgG conjugated to alkaline phosphatase (diluted 300-fold in PBS-Tween containing 0.67% foetal bovine serum) was added, incubated for 2 h at 37 °C, and removed by washing with PBS-Tween. Bound alkaline phosphatase was assayed by adding 200 µl p-nitrophenyl phosphate (1 mg ml⁻¹ in 0.05 M-glycine/NaOH pH 9.8, 1 mM-MgCl₂, 0.02% NaN₃) and incubated for 30 min at 37 °C. The reaction was stopped by adding 50 µl 10 M-NaOH, water was added to give a final volume of 1 ml, and the absorbance at 400 nm was determined. Blank values were obtained by omitting antigen or antiserum. The assay detected binding of antiserum at dilutions of 1 × 10⁻⁵.

To measure the binding of antipilin immunoglobulin to cells, competent cells (6 × 10⁸ c.f.u. ml⁻¹ in supplemented brain heart infusion) were incubated with several concentrations of purified immunoglobulin prepared from preimmune serum or anti-pilin serum for 15 min at 37 °C. Cells were sedimented, washed in 1 ml PBS (10 mM-sodium phosphate buffer, pH 7.0), resedimented and resuspended in coating buffer. Portions of the cell suspension (200 µl, 1.2 × 10⁸ c.f.u.) were placed in the wells of a microtitre dish and incubated at 4 °C for 18 h. The remaining steps in the ELISA were as described above.

Transformation assays. Transformation of N. gonorrhoeae was determined by the method of Dougherty et al. (1979) as described by Mathis & Scocca (1982). The concentration of competent F62 type 2 cells was 6×10⁸ c.f.u. ml⁻¹; the concentration of transforming DNA, prepared from strain FA130 (streptomycin resistant) was 2 µg ml⁻¹. To test the effect of anti-pilin immunoglobulin on transformation, the competent recipient cells were preincubated with preimmune or anti-pilin immunoglobulin for 15 min at 37 °C. The DNA was then added and the incubation was continued for 30 min at 37 °C. DNAase I was added to a final concentration of 50 µg ml⁻¹ and the mixture incubated for 5 min at 37 °C. The cells were then diluted 10-fold in liquid GC media (same composition as solid GC media base, without agar), spread onto plates of solid media, and assayed for transformation. The number of colony-forming units in each incubation mixture was also determined.

Transfer of polypeptides from SDS-polyacrylamide gels to nitrocellulose and detection of DNA-binding activity. Samples taken during pilin purifications were separated by electrophoresis in 12% polyacrylamide gels in the presence of SDS (Laemmli, 1970). The separated polypeptides were transferred to nitrocellulose paper as described by Bowen et al. (1980) except that all buffers used were at pH 8.5; this pH favoured dissociation of pilin and yielded the highest transfer of pilin. After transfer, the original gel was stained to verify the transfer of proteins. One nitrocellulose blot was stained with amido black to assay retention of protein on the filters. A second nitrocellulose blot was incubated with [³²P]DNA from N. gonorrhoeae (sp. act. 1 × 10⁹ c.p.m. µg⁻¹) for 1 h. The nitrocellulose sheet was then washed and air-dried. DNA binding was visualized by autoradiography at −70 °C for 24 h, using Kodak XAR-5 film and a DuPont Cronex intensifying screen.

Protein concentrations. These were determined by the Lowry method with BSA as the standard.

RESULTS AND DISCUSSION

The protein constituent of gonococcal pili, termed pilin, was purified from extracts of N. gonorrhoeae F62, a highly piliated strain. Pilin had an apparent monomer molecular weight of 18,500, determined by SDS-PAGE. In a typical preparation, 5-2 g (wet wt) of N. gonorrhoeae F62 cells were harvested. Pili and other shear-sensitive components were released from the cell surface by mechanical shearing; electron microscopy and electrophoretic analysis showed that the shear fraction was extensively contaminated with vesicles and debris. This shear fraction yielded 96 mg protein. Pilin was solubilized by treatment at pH 9.5, and precipitated from 10% saturated ammonium sulphate; this precipitate contained 3 mg protein. Repeated precipitation with ammonium sulphate at 10% saturation yielded a preparation of pilin (0.75 mg) that exhibited a single band on gel electrophoresis. The fractionation and purification of pilin was routinely monitored by SDS-PAGE. The course of a typical preparation of pilin is shown in Fig. 1; the minor contaminants of higher molecular weights could be removed by additional precipitation steps. Our preparations of pilin were estimated to be 95% homogeneous, and were used as the antigen preparation for the production of rabbit antisera against this protein.
Fig. 1. Fractionation and purification of pilin from *N. gonorrhoeae* F62. Fractions were separated by electrophoresis on 15% polyacrylamide gels in the presence of SDS. Lane 1, homogenized cell suspension; lane 2, 12000 g supernatant fraction; lane 3, 50000 g supernatant fraction; lane 4, fraction soluble in 10% ammonium sulphate; lanes 5 and 6, redissolved material insoluble in 10% ammonium sulphate; lane 7, molecular weight standards. Equal volumes of all fractions were loaded on the gel. In this preparation, which is representative of 15, the volumes of the homogenate and supernatants were 100 ml; the material precipitated by 10% ammonium sulphate was resuspended in 1-0 ml.

Table 1. *Purification of pilin monitored by competition with purified pilin for antipilin serum binding in ELISA*

The assay procedure is described in Methods. The concentration of competing protein was 10 µg ml⁻¹ in all cases. Binding was measured as the absorbance at 400 nm of the contents of a well. The absorbance measured in incubations without competing protein was typically 0-245. Results varied by no more than 15% in different experiments.

<table>
<thead>
<tr>
<th>Competing fraction</th>
<th>Binding (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Homogenization step</td>
<td>92</td>
</tr>
<tr>
<td>12000 g supernatant</td>
<td>89</td>
</tr>
<tr>
<td>50000 g supernatant</td>
<td>80</td>
</tr>
<tr>
<td>10% ammonium sulphate supernatant</td>
<td>102</td>
</tr>
<tr>
<td>10% ammonium sulphate pellet</td>
<td>51</td>
</tr>
</tbody>
</table>

These antisera gave positive reactions in direct immunoprecipitation tests in which purified pilin preparations were used as antigens. Immunelectrophoresis of the non-sedimentable fraction from sheared bacteria produced a single uniform arc of precipitation when developed against the antiserum; a single arc was also produced when this same fraction and purified pilin were analysed together. The specificity of the antiserum for pilin was also demonstrated by competition experiments using ELISA as a test system. In this assay, antiserum was pre-
incubated with the various fractions from the purification procedure. The reaction mixtures were then placed in the wells of a microtitre dish which had been coated with purified pilin, and residual binding of antibody to the wells was measured. As shown by the data in Table 1, the fractions from the purification increased in the capacity to compete with purified pilin for antipilin antibody when tested at a constant competing protein concentration of 10 \( \mu \text{g ml}^{-1} \). Purified pilin competed to a much greater extent (almost twofold under these conditions) than did any other fraction in the purification. The supernatant fraction from the 10\% ammonium sulphate precipitation had no effect on the binding of antibody to pilin-coated wells in the assay, indicating that the precipitation of pilin was complete.

To study the effects of anti-pilin antibody on gonococcal transformation, the immunoglobulin fraction was prepared from both preimmune serum and anti-pilin serum by ammonium sulphate precipitation and chromatography on DNA-Sepharose as described in Methods. The DNA-Sepharose column was used to remove any contaminating DNA binding proteins from the immunoglobulin fraction. To show that the antibody bound to competent cells under the conditions of the transformation assay, an ELISA assay was used. Purified anti-pilin immunoglobulin or preimmune immunoglobulin was incubated at concentrations of 0 to 60 \( \mu \text{g ml}^{-1} \) with FA62 cells for 15 min at 37 \( ^\circ \text{C} \) in the transformation medium. The cells were sedimented, washed, and used to coat the wells of the microtitre dish. Binding of the purified immunoglobulins to the cells was measured by determining the binding of alkaline phosphatase-conjugated goat anti-rabbit IgG to the cell-coated wells in an ELISA assay. As shown in Fig. 2, cells that had been incubated with anti-pilin immunoglobulin bound the anti-rabbit IgG, and the extent of this binding was dependent on the concentration of antipilin immunoglobulin in the original incubation mixture. Cells incubated with immunoglobulin from preimmune serum did not bind anti-rabbit IgG, indicating that the reaction was specific. Incubation mixtures from which the cells had been omitted also failed to bind anti-rabbit IgG.

Competent \textit{N. gonorrhoeae} F62 cells were preincubated with various concentrations of either
Fig. 4. The detection of DNA binding proteins in cell fractions from N. gonorrhoeae strain F62 type 2. Fractions were separated by SDS-PAGE, transferred to nitrocellulose sheets, and incubated with radioactive DNA as detailed in Methods. (a) SDS-polyacrylamide gel stained with Coomassie brilliant blue; (b) nitrocellulose blot of a gel identical to that in (a), stained with amido black; (c) autoradiograph of nitrocellulose blot incubated with \([^{32}P]DNA\) from N. gonorrhoeae. Lane 1, molecular weight standards; lane 2, lysate (100 μg); lane 3, soluble material from the lysate (100 μg); lane 4, shear fraction from a pilin preparation (30 μg); lane 5, purified pilin (30 μg); lane 6, purified pilin (10 μg).
Gonococcal pili in transformation

anti-pilin or preimmune immunoglobulin for 15 min before the addition of DNA to the transformation assay; the results are shown in Fig. 3. At concentrations of immunoglobulin between 0 and 100 µg ml⁻¹, the presence of either preimmune or anti-pilin immunoglobulin had no effect on the frequency of transformation. At a concentration of 600 µg ml⁻¹, both antipilin and preimmune immunoglobulins reduced the yield of transformants to between 40 and 50% of the control levels (not shown). This non-specific decrease in transformation frequency observed with both immunoglobulin preparations may be the result of the high protein concentrations in these experiments. We concluded that the antibody preparation bound to native pilin, that this binding occurred under the conditions of the transformation assay, and that the binding of the antibody to cell-associated pili did not affect the capacity of the cells to bind DNA and undergo genetic transformation. This result might be due to the antibody binding to sites on pilin different from those involved in transformation, or to the absence of a role for pilin in the transformation process.

The capacity of pilin to bind to DNA was investigated directly by the method of Bowen et al. (1980), in which polypeptides, separated by SDS-PAGE and transferred to nitrocellulose sheets by diffusion, were analysed for their DNA binding abilities. This method was applied to preparations of purified pilin, fractions from sheared cells and crude cell lysates. The polypeptides were first separated by SDS-PAGE and the polypeptides were then transferred to two sheets of nitrocellulose paper. The extent of polypeptide transfer in all experiments was monitored by staining one nitrocellulose blot with amido black. Initial trials showed that efficient transfer of pilin to nitrocellulose occurred only under conditions favouring dissociation of pilin to monomers. This observation implied that pilin retained the capacity to associate to form pili-like structures after purification and electrophoresis. DNA binding was assessed by incubating nitrocellulose blots with [³²P]DNA from N. gonorrhoeae, washing to remove unbound DNA, and autoradiography to visualize DNA binding proteins. The results are shown in Fig. 4. There was no detectable binding of DNA to any polypeptides of the fractions from sheared bacteria nor to purified pilin preparations. Pilin was transferred to the nitrocellulose paper, as shown by amido black staining, but did not bind appreciable quantities of DNA. However, several polypeptides capable of binding DNA were readily detected both in cell lysates and in the corresponding soluble fractions.

These studies showed that neither crude nor extensively purified pilin was capable of binding to DNA under conditions which allowed detection of other DNA binding proteins. It is possible that any DNA-binding capacity of pilin in vivo was eliminated by the isolation and separation processes. The lack of DNA binding by pilin was also observed in crude extracts, suggesting that denaturation of pilin during the course of purification was not the reason for lack of activity. Since efficient transfer of pilin from polyacrylamide gels to nitrocellulose required conditions such as alkaline pH which promoted the reversible dissociation of pilin multimers, it does seem that the purified protein retained at least some of its native properties during purification. DNA binding studies on cell lysates has detected a number of DNA binding proteins, one of which shares the specificity expected for the transforming DNA receptor of the gonococcus (unpublished results), indicating that the binding activity of interest could be assayed by our methods.

The results of our binding and immunological studies strongly suggest that pilin itself is not directly involved in transformation in N. gonorrhoeae. The association between piliation and transformability in this organism still remains to be explained. It is possible that components other than pilin, involved in the export or anchorage of pili, may participate in the transformation process. Alternatively, the association between piliation and transformability might not be structural, but reflect similar regulation of the two processes. It seems probable that components of piliated cells other than pilin participate in DNA binding and uptake during transformation in N. gonorrhoeae.

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