The Effect of Protein II and Pili on the Interaction of Neisseria gonorrhoeae with Human Polymorphonuclear Leucocytes

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Colonial variants of Neisseria gonorrhoeae strain P9 expressing different pili and/or outer membrane protein II (P.II) were investigated with respect to their interaction with human polymorphonuclear leucocytes (PMN). Two assay systems were used. A phagocytic killing assay measured the intracellular survival of gonococci, and PMN chemiluminescence (CL) was used to determine the initial surface interactions. All variants expressing P.II were killed effectively by PMN and also greatly stimulated PMN CL. The P.II- variants, on the other hand, were resistant to phagocytic killing and stimulated a much lower CL response. The presence of different P.II species was associated with different CL profiles and therefore different modes of interaction with the PMN membrane. A P.II-specific monoclonal IgG was opsonic and greatly increased PMN CL in contrast to F(ab')2 prepared from the same antibody, which inhibited it, thus confirming the role of P.II in the PMN interaction. Phagocytic killing assays revealed that with the loss of P.II, gonococcal variants acquired resistance to killing. Comparison of piliated and non-piliated pairs of variants with the same P.II profile showed that PMN–gonococcal interactions are dominated by the nature of the P.II species present whereas pili have little effect.

INTRODUCTION

A critical determinant of the virulence of gonococci is the possession of mechanisms for adhesion to mucosal cells of the genital tract which the organism invades. The surface protein structures which have been reported to facilitate adhesion include pili (Punsalang & Sawyer, 1973; Ward et al., 1974; Mardh & Westrom, 1976) and outer membrane protein II (P.II) (Lambden et al., 1979; James et al., 1980; Virji & Everson, 1981). Both these antigens may also influence the interaction of gonococci with human polymorphonuclear leucocytes (PMN). Several workers have implicated pili in resistance to phagocytosis (Gibbs & Roberts, 1975, Dilworth et al., 1975; Densen & Mandell, 1978) while others have suggested that small numbers of piliated organisms (Pil+) enter phagocytic cells and then resist intracellular killing by unknown mechanisms (Thomas et al., 1973; Witt et al., 1976). Swanson et al. (1975) on the other hand, ascribed the major role to outer membrane proteins in the initial interaction of gonococci with leucocytes. These workers observed that gonococci that exhibit high-level association (LA+) with human leucocytes in vitro have increased amounts of proteins of M, 28000 and 29000 on their surface (Swanson & King, 1978). These ‘leucocyte association’ proteins (LAP) belong to the P.II class which show characteristic heat modifiable properties on SDS-PAGE and are responsible for generating diversity in colonial opacity (Swanson & Heckels, 1980). Similarly Rest et al. (1982) showed that one Pil- opaque variant induced higher PMN chemiluminescence (CL) than its transparent counterpart.

Abbreviations: CL, chemiluminescence; P.II, outer membrane protein II; PMN, polymorphonuclear leucocytes.
In laboratory culture and during natural infection pili and P.II exhibit considerable antigenic diversity, not only between strains but within a strain (Swanson, 1978; Lambden & Heckels, 1979; Lambden, 1982; Zak et al., 1984; James & Swanson, 1978). The variant pili produced by a single strain show some structural homology, but variations occur in the immunodominant domain, generating antigenically distinct pili (Rothbard et al., 1984; Virji et al., 1983). With one strain, P9, we have identified variants which produce one of four pilus types, a, β, γ or δ, with subunit $M_r$ of 19500, 20500, 21000 and 18500 respectively (Lambden, 1982). Variants of the same strain have also been isolated which express zero, one or two species of P.II from a possible repertoire of at least six (McBride et al., 1981). Since pilus and P.II expression vary independently a large number of distinct variants can be generated from a single strain. The variant molecules differ not only antigenically but also in their biological properties. Consequently variants with differences in pili or P.II show alterations in specific adherence to different cell types (Lambden et al., 1979; Trust et al., 1980; Virji & Everson, 1981; Heckels, 1982). Several earlier studies which evaluated the interactions of Pil+ or P.II+ organisms with PMN used organisms which were undefined with regard to the molecular identity of the other variable antigens expressed. This may account for some of the apparent contradictions in the literature.

The relative contributions of pili and P.II to gonococcal–PMN interactions thus remain unclear and the effect of their antigenic variation remains to be defined. We have therefore used a panel of 15 isogenic variants of strain P9 expressing one of three different pilus types either alone or in combination with one of four different P.II types. The initial interactions with human PMN at the membrane level were monitored by a CL assay and the subsequent fate of the organism was investigated by a phagocytic killing assay. The role of P.II has also been investigated using a monoclonal anti-P.II antibody and F(ab')$_2$ fragments derived from it.

**METHODS**

*Gonococci.* Variants of *Neisseria gonorrhoeae* strain P9 have been isolated previously by selection of colonial opacity variants on clear typing media (Lambden & Heckels, 1979) and following an *in vivo* selection pressure by growth in subcutaneous chambers implanted in guinea-pigs (Lambden et al., 1981; McBride et al., 1981). The variants used in this work expressed one of three different pilus types (α, γ or δ) and/or one of four different P.II types (a, b, d or e). The prototype P9-1 lacking both these antigens (Pil- P.II-) was also included. The surface antigens expressed by the variants used are summarized in Table 1.

**Growth conditions.** Pure cultures of variants were obtained on clear typing medium (Diaz & Heckels, 1982) by single colony isolation as described by Lambden & Heckels (1979). For each experiment, pure colonies were reisolated from stock cultures stored in liquid nitrogen; the cultures used always contained ≥99% of the relevant colony type. Exponential-phase gonococci were obtained by suspending 18–20 h agar-grown organisms in RPMI-1640 medium (Flow Laboratories) supplemented with 1% (v/v) foetal calf serum. Cultures were incubated at 37°C in an atmosphere of 5% (v/v) CO$_2$ in air for approximately 2 h and growth was monitored by estimation of numbers as described below.

**Preparation of gonococcal suspensions and enumeration.** Suspensions of gonococci were centrifuged at 100 g for 2 min to remove aggregates. A sample of the resultant suspension was centrifuged at 10000 g for 2 min to sediment all organisms, which were then solubilized in 1% (w/v) SDS in 0.1 M-NaOH, prior to determination of absorbance at 260 nm. Gonococcal numbers were obtained by using a standard curve relating DNA content as measured by $A_{260}$ to c.f.u. The original suspensions were each diluted to give the required final number of c.f.u. per ml. No significant deviations from the standard graph were observed with any of the variants, demonstrating that only minor differences in aggregation occurred.

**Confirmation of antigenic composition of organisms.** A sample of gonococcal suspension used in each experiment was stored at −20°C for later confirmation of the identity of the pili and P.II expressed. All antigens were identified on the basis of their different migration on SDS-PAGE (McBride et al., 1981; Lambden, 1982). In addition, where specific monoclonal antibodies were available, as in the case of pili (Virji et al., 1983) and P.IIb (unpublished studies) the identifications were confirmed by dot blots as described previously (Virji & Heckels, 1983).

**Preparation of PMN.** Human peripheral blood was collected in heparin and PMN were obtained either by a combined Ficoll and dextran sedimentation method as described previously (Virji & Heckels, 1985) or by the use of monocyte-polymorph resolving medium (M-PRM, Flow Laboratories) containing Ficoll 400 and Hypaque with a density of 1.114 g ml$^{-1}$. Approximately 15–18 ml blood was layered on 10 ml M-PRM in 30 ml universal bottles (22 mm diam.) and centrifuged at room temperature for 40 min at 300 g. PMN were obtained in a band which
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Table 1. Pilus and P.II expression in variants of N. gonorrhoeae strain P9

The Table shows the nomenclature for the variants of strain P9 used and M, values of pili and protein II antigens which they express. Thus variant P9-31 expresses α pili [M, 19500 (19.5K)] and protein IIA (M, 28.5K). All variants lacking P.II gave rise to transparent colonies. Those with different P.II produced colonies of varying opacities.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pili absent (Pil-)</th>
<th>α pili (19.5K)</th>
<th>γ pili (21.0K)</th>
<th>δ pili (18.5K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.II absent (P.II-)</td>
<td>P9-1</td>
<td>P9-2</td>
<td>P9-35</td>
<td>P9-37</td>
</tr>
<tr>
<td>P.Ila (28.5K)</td>
<td>P9-13</td>
<td>P9-31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P.Ilb (28.0K)</td>
<td>P9-16</td>
<td>P9-17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P.IId (28-9K)</td>
<td>P9-40</td>
<td>P9-33</td>
<td>-</td>
<td>P9-28</td>
</tr>
</tbody>
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separated from the monocyte layer and the erythrocyte pellet. PMN were washed twice in 0.15 M-phosphate buffer containing 0.15 M-NaCl and suspended in RPMI-1640 medium. The preparation was largely free from other leucocytes, and since little or no erythrocyte contamination was present, osmotic lysis was not required. The PMN were >97% viable as determined by trypan blue exclusion. After preparation, the PMN were incubated at 37 °C for 45–60 min before use.

**Phagocytic killing.** Assays were done in polypropylene tubes (100 x 14 mm; Gibco) rotating end over end at 4 r.p.m. at 37 °C, essentially as described by Virji & Heckels (1985). Briefly, 4 x 10⁶ each of gonococci and PMN were incubated in a final volume of 400 µl of HEPES-buffered RPMI-1640 medium containing 1% foetal calf serum. After various incubation periods, sample tubes were removed and the PMN were lysed by the addition of saponin to 5% (w/v) to release intracellular organisms. Total surviving gonococci were estimated by plating samples of the reaction mixture in quadruplicate. Each experiment was done on two to four occasions; results presented are from one typical experiment.

In some experiments, gonococci and PMN were mixed under similar conditions but at a ratio of 20:1 and placed on glass slides for microscopic examinations. After incubation in a moist chamber at 37 °C for up to 30 min, the attached PMN together with gonococci were fixed with absolute methanol, stained with 0.1% (w/v) safranin-O, and examined by light microscopy.

**CL assay.** Luminol-enhanced CL was measured in an LKB MiniBeta scintillation counter as previously described (Virji & Heckels, 1985). The measurements were made over short periods at ambient temperature (25 °C), which did not vary significantly during the experiment. Gonococcus:PMN ratios, and the Luminol concentrations used in each case are described in the figure legends. Experiments were done at least three times; results presented are from one typical experiment. Some measurements were also made using a luminometer (model 1251, LKB Wallac) at 37 °C with and without agitation to verify the data obtained using the scintillation counter at ambient temperature.

**Preparation of F(ab')₂ from anti-P.II monoclonal antibody.** IgG was purified on Protein A-Sepharose (Virji et al., 1983) from ascites containing monoclonal antibody reacting specifically with P.Ilb (K. Zak, M. Virji & J. E. Heckels, unpublished data). The F(ab')₂ fragment was prepared by the digestion of IgG with pepsin (Sigma) at pH 4 with a protein: enzyme ratio of 100:1. After overnight incubation at 37 °C, the pH was adjusted to 8.0 and the sample was applied to a Protein A-Sepharose column to remove all unreacted IgG. The column effluent was applied to an UltroPac TSK-G2000 SW column (LKB) and eluted with 0.1 M-NaCl in 0.1 M-sodium phosphate buffer pH 7.4. Fractions containing F(ab')₂ were pooled and used directly.

**RESULTS**

**Phagocytic killing assay**

The assay measured the total survival of gonococci after incubation with PMN since intracellular organisms were released by saponin lysis before enumeration (Virji & Heckels, 1985). All P.II⁻ variants were resistant to phagocytic killing by human PMN (Fig. 1). Both the
Pil− P.II− prototype (P9-1) as well as α and γ piliated variants (P-9-2 and P9-35) resisted phagocytic killing totally. The δ piliated variant (P9-37) showed some susceptibility to killing, but this was much less than that of the P.II+ variant (P9-40) used as a control in this experiment and the Pil+ P.II+ variants in the experiments described below. Little difference was seen in the pattern of survival up to a total incubation time of 3 h. When the resistant P.II− variants were examined microscopically few organisms were associated with PMN, whereas many internalized or membrane-bound organisms were seen when P.II+ variants were examined.

Non-piliated opaque variants with different P.11 were incubated with or without PMN for 2 h. All the variants grew well under these conditions in the absence of PMN (Fig. 2). However, in the presence of PMN each P.II+ variant was killed rapidly although some variations were seen in the rates of killing. Longer incubations of up to 4 h showed no increase in gonococcal numbers, but a further small decline. When the opacities of the surviving colonies were examined, it was apparent that the proportion of transparent colonies steadily increased during the course of the experiments. With variant P9-16, less than 1% of the organisms plated at zero time produced transparent colonies but 54% of the population which survived 2 h incubation were of this phenotype. The transparent variants arising from P9-16 and P9-39 were subcultured once and were found by SDS-PAGE to lack any P.11.

The role of pili in gonococcal survival was investigated by using a group of variants containing the same P.II type (P.IId, M, 28900) but with different pili (variants P9-40, 33 and 28; Table 1). All the opaque variants were killed effectively by PMN whether or not pili were present (Fig. 3). This was true of all the P.II variants with different pili which were tested. In order to investigate whether killing was an intracellular event, cytochalasin B (10 μg ml⁻¹) was included in some cases. Killing of a susceptible variant, P9-40, was virtually abolished in the presence of this drug (Fig. 3), which inhibits uptake and subsequent intracellular killing (Okuda, 1975; Densen & Mandell, 1978).
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Fig. 3. Influence of pili on gonococcal resistance to phagocytic killing. Opaque variants expressing P.1Id without pili (P9-40, ■) or with α (P9-33, ◊) or δ (P9-28, □) pili were compared in the phagocytic killing assay. ———. Controls without PMN. ————. Non-piliated variant P9-40 incubated in the presence of 10 μg cytochalasin B ml⁻¹ to inhibit phagocytosis. Mean values of quadruplicate estimations are shown (SD < 20% of the mean).

Fig. 4. Chemiluminescence of PMN in the presence of the Pil⁻ P.11⁻ prototype P9-1 (○) and different Pil⁺ P.11⁺ variants expressing P.11a (P9-13, ◆), P.11b (P9-16, ▲), P.11d (P9-40, □) or P.11e (P9-39, ♦). ▲, ♦. Transparent survivors from cultures of opaque variants P9-16 and P9-39, respectively, which had escaped phagocytic killing (see text). Both gonococci and PMN were present at \(2 \times 10^6\) in a volume of 250 μl reaction mixture containing 85 μM Luminol.

**CL of human PMN in the presence of opacity variants of N. gonorrhoeae strain P9**

Initial CL responses of human PMN were examined in a Luminol-enhanced CL assay with gonococci and PMN each present at \(8 \times 10^6\) ml⁻¹ (Fig. 4). These conditions were comparable to those used in the phagocytic killing assay. The opaque variants induced a rapid increase in CL compared with the transparent prototype P9-1, which gave a small increase and only after a prolonged lag. The overall CL patterns remained the same when gonococci:PMN ratios of up to 50:1 were examined with lower PMN numbers and Luminol concentrations. The mode of interaction varied between the variants since each reproducibly gave a characteristically different shaped graph (Fig. 4). In these experiments the transparent (P.11⁻) survivors from cultures of opaque (P.11⁺) variants (P9-16 and P9-39) which had escaped phagocytic killing by PMN (see above) were also examined after a single subculture. The organisms interacted with PMN in an identical manner to the prototype transparent variant and did not stimulate CL above this control value. Hence the reduction of CL signal was concurrent with the loss of P.11. Relative CL responses of PMN in the presence of different opacity variants were identical when experiments were done at 37°C in a luminometer.

**Role of pili in the interaction of gonococci with PMN**

All the piliated variants examined stimulated CL responses broadly similar to those of their non-piliated counterparts. Two examples are shown in Fig. 5. In addition, comparison of variants expressing different P.11 with the corresponding α-piliated variants showed that the CL response was unaffected by the presence of the pili (Fig. 6). Two δ piliated variants did show a marginal increase in CL (Fig. 5) but the response was dominated by the type of P.11 present.

**Opsonic activity of anti-P.11 IgG and blocking of P.11-mediated interactions with F(ab')₂**

Purified IgG or F(ab')₂, prepared from a monoclonal antibody (SM40, isotype \(\gamma_3\)) directed against P.11b were used in the CL assay to assess their effect on gonococcal–PMN interactions.
Fig. 5. Effect of different pilus types on CL response. ---, CL induced by P. II− variants P9-2 with α (△), P9-35 with γ (▲), P9-37 with δ (▼) pilus, or P9-1 (Pil−, ○). ---, CL induced by variants expressing P. IIα with α (P9-33, ■) or δ (P9-28, △) pilus, or Pil− (P9-40, □). A gonococcus:PMN ratio of 4:1 was used with $5 \times 10^5$ PMN in a total volume of 1 ml containing 100 μM-Luminol.

Fig. 6. Effect of P. II on CL response of α-piliated variants in the presence of four Pil− P. II+ variants (---) and their α-piliated counterparts (-----). The variants expressed P. IIα (P9-13, ●), P. IIb (P9-16, ▲), P. IIε (P9-39, ▼), or were P. II− (P9-1, ○). Gonococci and PMN were present at $2 \times 10^9$ ml$^{-1}$ in 100 μM-Luminol.

Fig. 7. Effect of anti-P. IIb IgG and F(ab')$_2$ on CL. Variant P9-17 (α piliated, expressing P. IIb) was incubated with PMN (○) and in the presence of ascites containing monoclonal antibody SM40 at a dilution of 1:500 (●), purified IgG (5 μg ml$^{-1}$) (■) or F(ab')$_2$ at 1 μg ml$^{-1}$ (▲). PMN were present at $10^6$ and gonococci at $4 \times 10^8$ in 1 ml reaction mixture containing 100 μM-Luminol.

Purified IgG was opsonic as shown by a further increase in CL (Fig. 7). In contrast F(ab')$_2$, had a blocking effect which was reflected in a significant decrease in CL compared with the control without antibody.

DISCUSSION

The association of gonococci with human PMN is a cellular interaction with important consequences for the eventual outcome of an infection. Several previous studies have suggested that pili are antiphagocytic. Densen & Mandell (1978) reported that piliated gonococci attach to phagocytes but are not killed, due to inhibition of phagosome formation, by a mechanism which
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might involve direct interaction of pili with the PMN membrane. In contrast, other workers have reported that internalized piliated gonococci are relatively resistant to intracellular killing by human phagocytes (Thomas et al., 1973; Witt et al., 1976). Many studies have compared a single piliated colony type with a non-piliated variant without recording possible differences in P.11 expression. Such differences are crucial since at least some P.11 species are associated with a considerable increase in interaction of PMN with non-piliated gonococci (Swanson & King, 1978).

In this study we used variants with defined differences in both pili and P.11 to determine the contribution of each to gonococcal–PMN interactions in two different assay systems. A phagocytic killing assay was used to determine the eventual fate of the variants. Killing was virtually abolished by cytochalasin B, which inhibits uptake with little effect on particle attachment (Davies et al., 1971; Densen & Mandell, 1978), suggesting that killing was an intracellular event. A CL assay was also employed. CL results from the activation of oxidative bactericidal mechanisms of PMN (Allen et al., 1972; Allen & Loose, 1976) and has been correlated with phagocytic and bactericidal activity of PMN by several authors (Greber et al., 1977; Rest et al., 1982; Repine et al., 1984); we have previously reported a similar correlation using gonococci opsonized with antipilus monoclonal antibodies (Virji & Heckels, 1985). However, intracellular killing of gonococci may be due to nonoxidative mechanisms as suggested by Rest et al. (1982). Nevertheless, since activation of oxidative bactericidal mechanisms of normal PMN results from the initial interactions at the PMN membrane (Goldstein et al., 1975) the CL assay was used to study the mode of interaction of different variants with PMN. Indeed Robinson et al. (1984) used variation in the CL response of PMN with different bacteria to show the influence of specific nature of the surface of the organism on the kinetics of CL increase.

Results from the current study show firstly that the prototype (Pil− P.11−) variant is resistant to phagocytosis and that in addition, Pil+ P.11+ variants exhibit similar interactions with PMN. Most variants induced a low-level, delayed CL response that was not associated with detectable phagocytic killing of the organisms, which remained viable for up to 3 h in the presence of human PMN. The resistance to phagocytic killing was indeed due to a low level of interaction since microscopic examination of stained preparations also showed very few intracellular organisms. One piliated variant, expressing 6 pili, did increase CL marginally and this was reflected in a similar marginal increase in susceptibility to phagocytic killing. This effect was reproducible and in addition killing of this variant was reduced when cytochalasin B was present, suggesting that 6 pili may cause some increased interaction of the variant, which is phagocytosed to a small extent and killed intracellularly.

The importance of P.11 in gonococcal–PMN interactions was first demonstrated by Swanson and co-workers, who reported that gonococci which exhibited high-level leucocyte association (LA+) contained additional outer membrane protein(s) (Swanson & King, 1978). These leucocyte association proteins (LAP) have subsequently been recognized as belonging to the P.11 family (Swanson & Heckels, 1980). They also suggested that LAP did not influence interaction with other cells, including buccal epithelial cells. However, studies with variants of strain P9 have shown clearly that the presence of different P.11 species influences attachment to epithelial cells in vitro (Virji & Everson, 1981; Heckels, 1982). Similar variations have now been demonstrated in their interactions with PMN. Possession of P.11 always caused increased killing of gonococci and this was associated with increased PMN interactions as measured by CL. Moreover, the CL response varied between P.11 variants, suggesting different modes of interaction with receptors on the PMN surface. However, the eventual outcome for each variant appeared similar in the phagocytic killing assay. This suggests that the latter assay is less sensitive in discriminating between variants in their modes of interaction.

Similar differences in CL response were also seen when Pil+ variants expressing different P.11 species were compared. The dominant role of P.11 in PMN interaction was confirmed by comparison of Pil+ and Pil− pairs of variants expressing the same P.11. In each case the shape of the CL response was determined by the particular species of P.11 present, with pili having an insignificant effect. Moreover, there was no evidence that pili inhibit either uptake or intra-
cellular killing of Pil+ P.II+ variants since increased stimulation of CL was paralleled by increased phagocytic killing.

The influence of P.II on phagocytic killing was also revealed by the large proportion of P.II− variants surviving incubation with PMN. Concurrent with the loss of P.II there was decreased CL and acquired resistance to phagocytic killing. Hence P.II− variants survived in the presence of PMN, with the consequence that in these assays 100% killing could never be achieved. This survival was clearly related to loss of P.II and hence lack of interaction with phagocytes. This is in contrast with the intracellular resistance of in vivo-grown gonococci reported by Witt et al. (1976).

In view of the influence of P.II on PMN interaction it is interesting that the P.II variants used exhibit little difference in surface hydrophobicity (Lambden et al., 1979), and other workers have also reported that P.II does not affect the hydrophobic nature or surface charge of gonococci (Magnusson et al., 1979). These observations suggest that the increased gonococcal–PMN interactions seen with all P.II-containing variants result from specific interactions rather than simply from a change in gross physicochemical properties of the bacterial surface. Moreover, P.II species are not equal in their effect since the differing mode of PMN interaction revealed by the CL response curve is dependent on the particular P.II present. The importance of P.II is also revealed by the ability of monoclonal F(ab′)2 directed against P.IIb to inhibit the CL of the piliated variant containing P.IIb. In contrast IgG increases CL, reflecting an increased interaction via Fc receptors on the PMN membrane.

It is clear that the possession of P.II renders gonococci susceptible to phagocytosis in vitro both due to increased direct interaction with PMN and also since at least some antibodies to P.II are opsonic. Nevertheless, the vast majority of fresh clinical isolates possess P.II (Zak et al., 1984), which must clearly impart other essential properties such as effective colonization of epithelial surfaces. The organism may avoid the specific immune responses via antigenic shift in the expression of this antigen (Zak et al., 1984). But, in addition, the fact that the loss of P.II gives the organism ability to resist phagocytic killing implies that in vivo, transparent variants naturally arising would escape phagocytosis and in time re-colonize other niches. In this context it is interesting that gonococci isolated from synovial fluid of a patient with gonococcal arthritis were predominantly Pil− P.II− (unpublished observations), in contrast to the Pil+ P.II+ variants typical of genital isolates (Zak et al., 1984). Such possibilities illustrate the potential of antigenic shift in enabling gonococci to adapt to different environments encountered during natural infection.

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