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Porin protein of *Neisseria gonorrhoeae*: Cloning and gene structure

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ABSTRACT **The outer membrane porin molecule of *Neisseria gonorrhoeae* is known as protein I (PI). Among different strains of gonococci there is variability of PI, and two main classes, PIA and PIB, have been recognized. A λgt11 bank of gonococcal DNA was screened using monoclonal antibodies directed to a PIB-type porin molecule of *N. gonorrhoeae*, and three immunoreactive clones were isolated. DNA sequence analysis indicated that each contained only portions of the PI structural gene, but that together they contained the complete gene, and its structure was determined. The DNA sequence predicts a protein of 348 amino acids with a typical 19 amino acid signal peptide. The PI protein resembles *Escherichia coli* porins in size, lack of long hydrophobic sequences, and absence of cysteine residues. Sequence homologies between PI and the *E. coli* porins were found, particularly in the 100 N-terminal and the 110 C-terminal amino acids. In addition to the coding sequence of PI, the complementary strand contains a large open reading frame. At the 3′ end of the PI gene, immediately following an inverted repeat (probably the transcription terminator), the clone contains an unusual sequence consisting of 31 perfect repeats of the heptamer CTGTTTT. Hybridization analysis suggests that there is a single structural gene for PI and that it is homologous to the gene found in a PIA-bearing strain of gonococcus.**

The outer membrane of *Neisseria gonorrhoeae* bears several antigens that have been studied in considerable detail (1). Three proteins are present in large amounts and these have been named proteins I, II, and III (PI, PII, and PIII). Expression of PII proteins (2) by a gonococcal strain is variable, being subject to phase variation with a frequency of about 10^{-3} per cell division (3). The structural gene of PIII has been cloned and according to its sequence is a homolog of the enterobacterial OmpA proteins (4). PI is invariably expressed and usually is quantitatively the predominant protein. Methods for the purification of PI have been developed (5, 6) and it has been demonstrated that the pure protein is a porin that is voltage-dependent and somewhat anion-selective (7, 8). PI resembles the porins OmpC, OmpF, and PhoE of *Escherichia coli* in molecular weight, trimeric configuration, and estimated pore size (9) and readily translocates from living gonococci to foreign membranes, artificial (10) or natural (11). Purified PI also has profound effects on the physiology of human polymorphonuclear leukocytes, markedly interfering with degranulation but having no significant effect on superoxide generation (12).

The surface topology of the gonococcal outer membrane has been studied using cleavable crosslinking reagents. PI appears to be trimeric in the native state, and PIII appears to be closely associated with PI (13). While the expression of a particular PI is a stable characteristic of a gonococcal strain, there is heterogeneity of PI proteins between strains. Biochemical and immunological criteria separate PI into two main classes, referred to as PIA and PIB (14). As a rule, only strains bearing a PIA protein are able to invade the human bloodstream and cause systemic gonococcal infection. PIB proteins exhibit, on the surface of living gonococci, sites susceptible to proteolysis. For instance, chymotrypsin cleaves at a single site, splitting the 36-kDa molecule into two fragments of 22 kDa and 14 kDa (15). The N-terminus of PI is on the 22-kDa fragment, and the new N-terminus on the smaller fragment has been sequenced.

Ganss *et al.* (16) reported the isolation of plasmid clones producing a truncated product reactive with a monoclonal antibody to gonococcal PI; however, these clones have not been analyzed further. We expended considerable effort in attempting to clone PI in plasmid vectors but were not successful. We report the cloning of the structural gene of PI from gonococcal strain R10,* using bacteriophage λgt11 as a vector (17).

MATERIALS AND METHODS

Reagents and Chemicals. Most restriction enzymes and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Boehringer Mannheim; bacteriophage T4 ligase and additional restriction enzymes were obtained from New England Biolabs. Nitrocellulose BA-85 was obtained from Schleicher & Schuell, and other reagents and chemicals were purchased from Sigma.

Bacterial Strains. The *E. coli* strains Y1089 and Y1090 have been described (18). R10 is a gonococcal strain isolated from a genitourinary infection and expresses a PIB protein of serovar PIB-9; 120176-2 was isolated from a patient with disseminated gonorrhea and bears a PIA protein of serovar PIA-4.

Immunological Methods. The λgt11 bank, created by shearing gonococcal DNA of strain R10 by sonication, digestion with mung bean nuclease, and addition of *Eco*RI linkers, has been described (19). Immunological screening was performed by an immunoenzymatic method described previously (19). After $\approx 5 \times 10^5$ plaques were allowed to grow on *E. coli* strain Y1090 for 2.5 hr at 42°C, they were screened by overlaying with nitrocellulose filters, either untreated or previously impregnated with 10 mM IPTG, and incubating for 2 hr at 37°C. A mixture of five monoclonal antibodies to the PI of strain R10 was used. The phage from plaques identified by immunological activity were purified and used to infect *E. coli* Y1089 in order to produce lysogens. These were induced for phage production by shifting the incubation temperature to 43°C and were grown with or without IPTG to induce antigen production, as described by Young and Davis (18). Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis (PAGE) was performed on cells lysed in the $NaDodSO_4$-containing loading buffer (20), proteins were electrophoretically transferred to nitrocellulose membranes

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; PI, gonococcal protein I; PIA, PI of A class; PIB, PI of B class; PIII, gonococcal protein III.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) under accession no. J03017.

(21), and the blots were developed by an immunoenzymatic method (22). A variation (19) of the method of Weinberger *et al.* (23) was used to affinity-purify antibodies from rabbit serum by binding to the products of plaques fixed to nitrocellulose. Dense lawns of plaques (≈50,000) were grown on *E. coli* Y1090 at 42°C for 2.5 hr, overlaid with a nitrocellulose filter, and incubated at 37°C for 2 hr. The filter was washed, incubated with 5 ml of serum (diluted 1:2000) for 4 hr, and washed extensively, and antibodies were eluted with 5 ml of 150 mM glycine·HCl (pH 2.3) for 15 min. The eluate was promptly neutralized and used for immunoblots.

Growth of Lysogens. Lysogens were grown at 30°C, induced by heating to 43°C, and incubated at 37°C for 2 hr. The cells were harvested and lysed by addition of chloroform, and the phage were purified by sedimentation followed by flotation in solutions of CsCl (24).

DNA Hybridization Analysis. DNA hybridization analyses were generally performed under stringent conditions (68°C) (25).

DNA Sequencing. The gonococcal DNA inserts were excised by digestion with *Eco*RI restriction endonuclease and then purified by agarose gel electrophoresis and electroelution. The chain-termination method of DNA sequencing was employed (26). Fragments for subcloning were obtained by digestion of the purified insert with restriction enzymes, separated on SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, ME), and ligated to the M13 phage vector mp10, mp11, mp18, or mp19 (27). Sequential ordered deletions of the clone were produced by the method of Dale *et al.* (28) using the Cyclone kit produced by International Biotechnologies (New Haven, CT).

RESULTS

The construction of the λgt11 bank of genomic DNA from strain R10 has been described (19). Plaques were screened using monoclonal antibodies directed to PI of strain R10, with or without IPTG induction. Three immunologically reactive plaques were plaque-purified, and lysogens were created and named 63, 64, and 68. Two of these, 63 and 64, were selected without IPTG induction. To further characterize these clones, densely seeded plates were overlaid with nitrocellulose filters while the plaques were developing. The filters were then used to absorb antibodies from rabbit antiserum raised against PI, the filters were washed, and the antibodies were eluted at pH 2.3 and used in immunoblots (23). The filters were able to specifically absorb antibodies to PI from a polyclonal antiserum. Fig. 1 shows that antibodies eluted from plaques of clone 63 and 64 reacted with intact PI and the 22-kDa chymotryptic N-terminal fragment of PI, whereas antibodies affinity-purified with clone 68 reacted with intact PI, the 22-kDa fragment, and also the 14-kDa chymotryptic C-terminal fragment of PI.

The lysogens, induced by heating to 43°C, were grown with or without IPTG for 60 min and analyzed by immunoblots. Clone 63 produces an immunoreactive product of about 20 kDa and its expression is not enhanced by IPTG (Fig. 2). Clone 68 produces a product of 36 kDa and the expression is enhanced by IPTG. Growth of the lysogen of clone 64 was poor and no immunoreactive product was seen on blots (data not shown).

The DNA of λgt11 clones 63, 64, and 68 was purified and the gonococcal DNA inserts were isolated by *Eco*RI digestion, agarose gel electrophoresis, and electroelution. Clones 63 and 64 contained single *Eco*RI fragments, of 1820 and 1400 base pairs (bp), respectively, whereas clone 68 yielded three *Eco*RI fragments, of 1160, 620, and 280 bp. DNA hybridization studies to determine the relationship of the clones indicated that the 1160-bp *Eco*RI fragment of clone 68 reacted with clones 63 and 64. The 620- and 280-bp *Eco*RI inserts

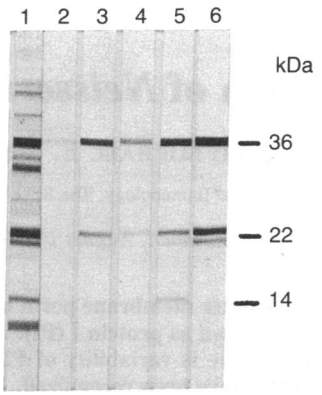

FIG. 1. Ability of products of λgt11 clone plaques to selectively absorb PI antibodies. The strips are from an immunoblot of chymotrypsin-treated whole-cell lysates of strain R10 gonococci. The strips were incubated with affinity-purified antibodies from an antiserum prepared against purified PI of strain R10. The affinity-purification was performed by letting dense lawns of plaques of the desired clone grow with an overlying nitrocellulose filter. The filter was allowed to react with the antiserum and then extensively washed, and the adherent antibodies were eluted with glycine·HCl (pH 2.3) and neutralized. Strip 1: PI antiserum diluted 1:2000. Strips 2–5: Antibodies eluted from plaques, respectively, of λgt11 vector, clone 63, clone 64, and clone 68. Strip 6: PI monoclonal antibody. Note that antibodies eluted from clones 63 and 64 react with PI and the 22-kDa chymotryptic fragment, whereas antibodies eluted from clone 68 react with PI and both chymotryptic fragments.

were unrelated *Eco*RI fragments ligated into a single vector molecule in the generation of the bank (data not shown).

DNA sequencing was performed as summarized in Fig. 3. The large (1160-bp) insert in clone 68 contained the coding frame for 274 amino acids of the C-terminus of PI, whereas clones 63 and 64 contained the information for the N-terminal 156 amino acids. This accords with the ability of the products of clone 68 to absorb antibodies reactive with the 14-kDa chymotryptic fragment of PI, while products of clones 63 and 64 only absorb antibodies reactive with the 22-kDa fragment (see Fig. 1). It was found that 70 bp at the 5′ end of the large insert of clone 68 deviated from the sequences found in clones 63 and 64. This artifact probably resulted from the blunt

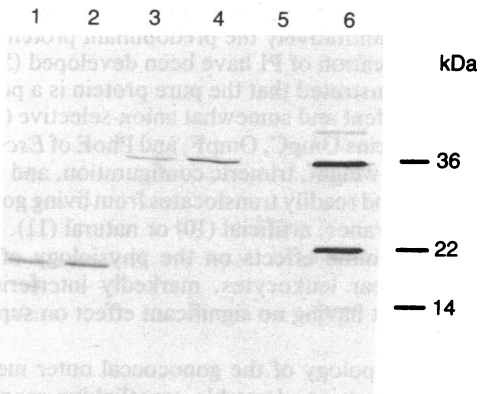

FIG. 2. Immunoblot of lysogens of PI clones. Lysogens were induced by heating to 43°C and IPTG was added to half the culture as an inducer of β-galactosidase. Lanes: 1 and 2, clone 63; 3 and 4, clone 68; 5, λgt11 vector; 6, chymotrypsin-treated gonococcus. The cultures used for lanes 2, 4, and 5 had IPTG added. The proteins were separated by $NaDodSO_4$/PAGE and electrophoretically transferred to nitrocellulose. The blot was reacted with a rabbit antiserum to PI. Markers at right indicate the positions of the intact PI and of its two chymotryptic fragments. IPTG increases the production of product by clone 68 (lane 4) but has no effect on expression by clone 63 (lane 2).

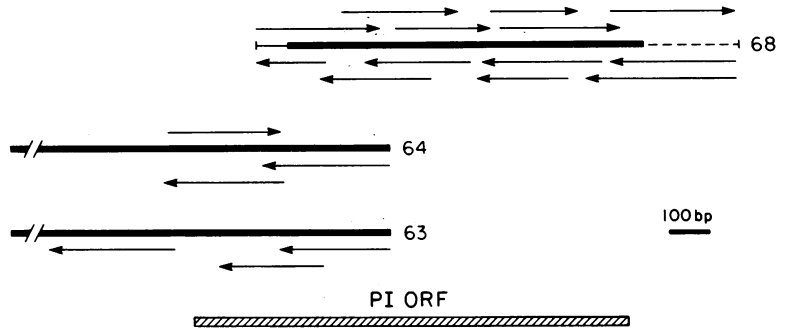

FIG. 3. DNA sequencing strategy. DNA sequencing was performed on M13 subclones of clones 63, 64, and 68. The last clone was completely sequenced. Pertinent portions of clones 63 and 64 were also sequenced, and the overlap of the three clones is indicated. The location of the open reading frame (ORF) coding for PI is shown. The first 70 bp of clone 68 (indicated by the thin line) were not present in clones 63 and 64 (see *Results* for details). The dashed portion at the 3′ end of clone 68 consists of 31 perfect repeats of the heptameric sequence CTGTTTT.

ligation of two unrelated genomic fragments in the step where *Eco*RI linkers were being added in the construction of the bank. This assumption was supported by demonstrating that a 21-bp synthetic oligonucleotide identical to the 5′ sequence immediately following the *Eco*RI linker yielded hybridization patterns that were different from those obtained with the full 68 insert, when used to probe parent genomic DNA (data not shown).

The composite DNA sequence obtained by analysis of the three clones is shown in Fig. 4. An open reading frame coding for a protein of 348 amino acids was found. The first 19 amino acids are typical of a type I signal-peptide sequence and are followed by the known N-terminus of PI (11). The sequence matched two additional landmarks obtained by protein sequencing: at amino acid 207 the N-terminus of the 14-kDa chymotryptic fragment, and at 142 the sequence of a cyanogen bromide fragment. The predicted amino acid composition of PI is in excellent agreement with the one obtained by amino acid analysis. The calculated molecular weight of the entire protein and of the chymotryptic fragments agree with data obtained by $NaDodSO_4$/PAGE. Twenty-one base pairs downstream from the termination codon, a 12-bp inverted repeat with a 4-bp loop was found; this structure is followed by a T-rich region and may serve as the transcription terminator. The T-rich region is highly unusual and consists of 31 perfect repeats of the heptameric sequence CTGTTTT. Note that this clone was generated by random cleavage (sonication) of the genome and that the heptamer may repeat more than 31 times.

To determine the number of PI genes present in the gonococcal genome, DNA hybridization studies were performed (Fig. 5). The parent PIB strain R10 and the PIA strain

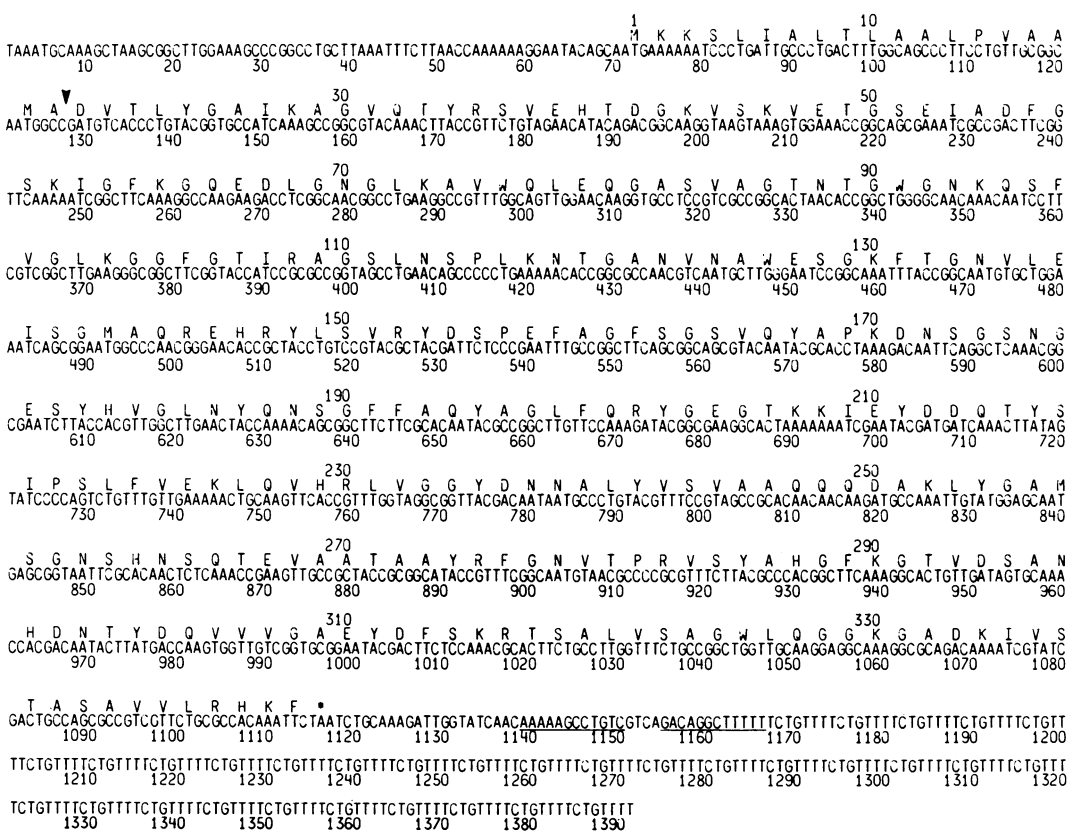

FIG. 4. Nucleotide sequence and predicted amino acid sequence (standard one-letter amino acid symbols) of PI. Arrowhead indicates the site cleaved to produce the mature protein. The underlined portion of the DNA sequence represents a 12-bp inverted repeat with a 4-bp loop and followed by a T-rich region. This structure may serve as the transcription terminator.

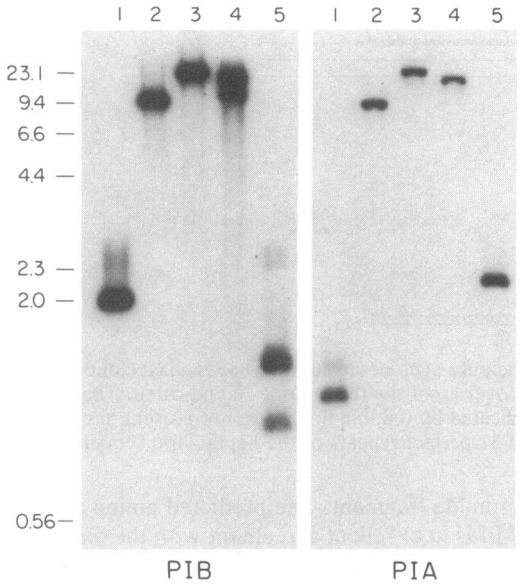

FIG. 5. Hybridization analysis of PI-associated sequences from strains R10 (PIB) and 120176-2 (PIA). Digests in both blots are *Alu* I, *Eco*RI, *Hin*dIII, *Kpn* I, and *Sau*3A in lanes 1–5, respectively. The probe is a 0.7-kilobase (kb) fragment containing most of the PI structural gene. Size markers (kb, at left) are λ DNA cleaved with *Hin*dIII. In this experiment the R10 DNA *Kpn* I digest was incomplete; other experiments have shown the smaller 10-kb fragment of the doublet to be the correct fragment.

120176-2 were studied. The probe employed was a 0.7-kb fragment (nucleotides 387–1075, Fig. 4) and represents the coding frame for 227 amino acids of PI. In the PIB strain, single *Alu* I, *Eco*RI, *Hin*dIII, and *Kpn* I fragments were detected (Fig. 5), in agreement with the absence of these restriction sites in the probe. Two *Sau*3A fragments were detected, in accord with the presence of a *Sau*3A site (nucleotide 707, Fig. 4). The probe also reacted under stringent conditions with restriction fragments of DNA from the PIA strain. Single *Eco*RI, *Hin*dIII, *Kpn* I, and *Sau*3A fragments, but perhaps two *Alu* I fragments, were detected, suggesting that the PIA homologous sequence lacks the *Sau*3A site but may have an *Alu* I site. Additional hybridization studies under less stringent conditions yielded similar results (data not shown), arguing for the presence of a single structural gene for PI in the gonococcal genome.

DISCUSSION

Availability of the primary structure of PI is a prerequisite for a deeper understanding of the voltage-dependent, anion-preferring channel behavior of this molecule, of its ability to translocate from the gonococcal outer membrane to foreign membranes, and of the physiologic effects that PI may have once it is present in the eukaryotic cell membrane. In addition, this class of antigen has been considered for the development of vaccines. No selective method has been found to obtain mutants lacking PI, to determine the effect this might have on the gonococcus. Thus, there has been an impetus to clone the structural gene of PI in order to elucidate the structure and to provide a vehicle for obtaining the

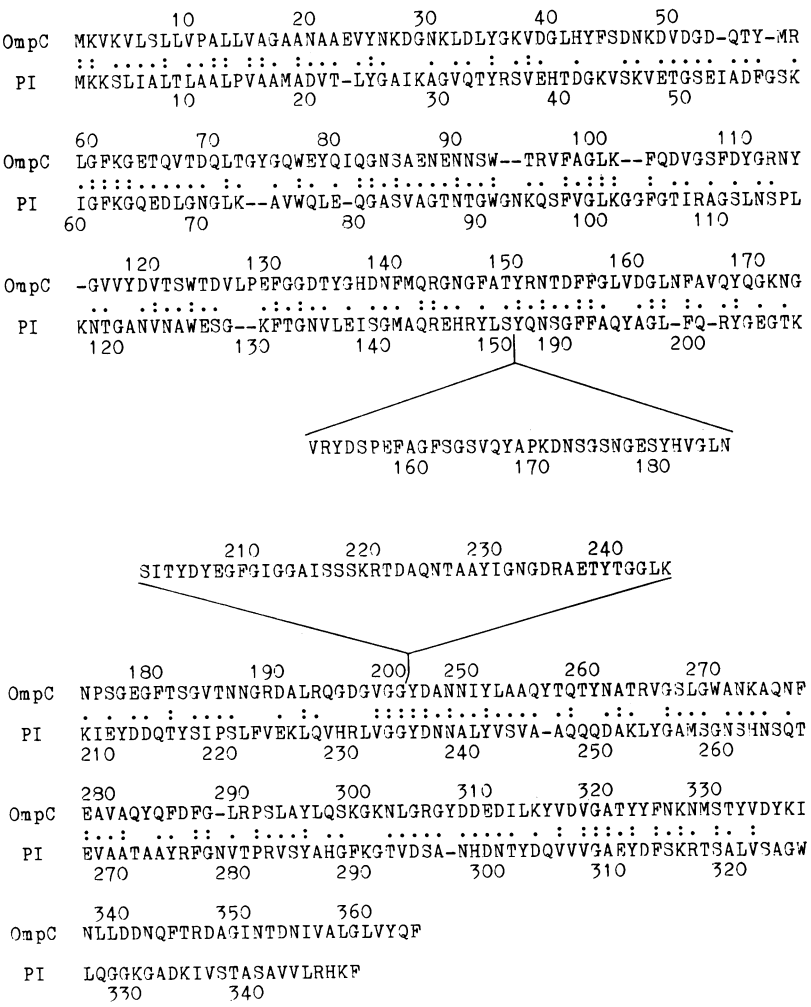

FIG. 6. Homology of predicted primary structure of PI to *E. coli* porins. The homology between OmpC and PI consists of 22.9% identity over a 296 amino acid overlap if the areas of each molecule indicated as inserts are not included in the comparison. The homology was analyzed using the FASTP algorithm (36). Colons indicate identity; periods indicate conservative substitutions.

desired mutants (29). However, the cloning of the complete gonococcal porin molecule in plasmids has not been possible; most likely the intact gene is deleterious in the *E. coli* host. We have constructed a λgt11 bank that served as the source of clones for several gonococcal genes—i.e., those encoding PIII (4), H.8 antigen (19), and gonococcal azurin (30). Using this bank, it has been possible to obtain immunoreactive clones consisting of fragments of the structural gene of the porin. Sequence analysis of three clones provided the information needed to define completely the structural gene. Stephens *et al.* (31) have cloned and fully characterized the porin gene of *Chlamydia trachomatis*. Initially, only a fragment of this gene was cloned in λgt11 and was then used to screen a conventional λ bank to obtain the complete gene (31, 32).

The predicted protein sequence of PI shows a resemblance to the porin proteins of *E. coli* that have been characterized (33), but not to the porin of *Chylamydia trachomatis* (31). The structural similarities of PI to OmpC (33), OmpF (34), and PhoE (35) include the same overall size, the lack of cysteine residues, the lack of any major hydrophobic sequences, and the homologies shown in Fig. 6. The same homologies are also evident in OmpF and PhoE (particularly in the N-terminal 100 residues and the 110 C-terminal residues of OmpF and PhoE), since these proteins are closely related to OmpC (33).

Since PI is one of the major products of gonococci, the codon usage was compared to that reported for the OmpC/OmpF proteins of *E. coli* (33). The same skew of codon usage was seen for Phe, Leu, Ile, Val, Thr, Tyr, Lys, Arg, and Gly. However, a different codon was markedly preferred in the case of Ala (GCC) and Gln (CAA). These two are also the preferred codons in the case of pilin, PIII, and azurin, suggesting that the distribution of major tRNA species may be different in gonococcus (37).

In addition to the open reading frame coding for PI, another large open reading frame was found on the opposite strand, beginning at the 3′ end of the sequence and extending to the stop codon, the complement of the nucleotides TCA at position 594 (see Fig. 4). This open reading frame is preceded by the repeated heptameric sequence and we have not determined whether it is expressed. Recently, a repeated pentameric sequence has been reported to be involved in the regulation of the expression of the PII proteins of the gonococcus (3).

Hybridization data (Fig. 5) suggest that the gonococcal genome contains a single structural gene for PI. This is in contrast with the *E. coli* genome, which encodes a number of homologous porins. The homology found between the genes coding for PIA and PIB is in accord with genetic data. Following DNA transformation, intragenic recombination can occur, as evidenced by the isolation of mutants bearing hybrid PI proteins with serological specificities of both parents' PIA and PIB (38).

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